ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

# EFFECT OF A DIET RICH IN OLIVE OIL PHENOLICS ON AGE-RELATED CHANGES IN MOUSE MODEL OF AGEING AND HPLC ANALYSIS OF OLIVE OIL PHENOLICS WITH ELECTROCHEMICAL DETECTOR

**Ph.D. Thesis by**
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**Programme : Food Engineering**

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**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

# ZEYTİNYAĞI FENOLİKLERİNCE ZENGİN DİYETİN YAŞLANMASI HIZLANDIRILMIŞ FARE MODELİNDE YAŞLANMAYA BAĞLI DEĞİŞİKLİKLER ÜZERİNE ETKİSİ VE ZEYTİNYAĞINDAKİ FENOLİK MADDELERİN ELEKTROKİMYASAL DEDEKTÖR İLE HPLC ANALİZİ

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Biologist

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## ABBREVIATIONS

| | |
|---|---|
| **ABCA1** | : ATP binding cassette A1 |
| **CRP** | : C reactive protein |
| **CYP3A** | : Cytochrome P450 family 3A |
| **EVOO** | : Extra virgin olive oil |
| **GAPDH** | : Glyceraldehyde phosphate dehydrogenase |
| **GSH** | : Glutathion |
| **GSSG** | : Glutathion disulphite |
| **GST** | : Glutathion-S-transferase |
| **γ-GCS** | : γ-glutamyl-cysteinyl-synthetase |
| **HMGCoAR** | : 3-hydroxy-3-methyl-glutaryl-CoA reductase |
| **HO-1** | : Hemeoxygenase-1 |
| **HP** | : High phenolic |
| **LCAT** | : Lecithin Cholesterin Acyltransferase |
| **LDLrec** | : Low density lipoprotein receptor |
| **LLE** | : Liquid liquid extraction |
| **LLOQ** | : Lower limit of quantification |
| **LLOD** | : Lower limit of detection |
| **LP** | : Low phenolic |
| **LPL** | : Lipoprotein lipase |
| **LRP-1** | : Low density lipoprotein receptor related protein 1 |
| **MDR-1** | : Multidrug resistance transporter 1 |
| **NQO-1** | : NADPH quionone oxidoreductase |
| **Nrf2** | : Nuclear factor erythroid 2-related factor 2 |
| **oxo8dG** | : 8-oxo-2-deoxyguanosine |
| **PON-1** | : Paraoxonase-1 |
| **PON-2** | : Paraoxonase-2 |
| **SAMP8** | : Senescence Accelerated Mouse Prone 8 |
| **SAMR1** | : Senescence Accelerated Mouse Resistant 1 |
| **SAP** | : Serum amyloid P |
| **SIRT1** | : Sirtuin1 |
| **SPE** | : Solid phase extraction |
| **SRB1** | : Scavenger receptor B1 |
| **SVCT1** | : Na- dependent vitamin C transporter-1 |
| **SVCT2** | : Na- dependent vitamin C transporter-2 |
| **TBARS** | : Thiobarbituric acid reactive substances |
| **α-TTP** | : Alpha tocopherol transfer protein |
| **VOO** | : Virgin olive oil |

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**EFFECT OF A DIET RICH IN OLIVE OIL PHENOLICS ON AGE-RELATED CHANGES IN MOUSE MODEL OF AGEING AND HPLC ANALYSIS OF OLIVE OIL PHENOLICS WITH ELECTROCHEMICAL DETECTOR**

## SUMMARY

Olive oil is the primary source of fat in the Mediterranean diet, which has been associated with reduced risk of cardiovascular mortality, cancer incidence and mortality and incidence of Parkinson and Alzheimer diseases. Despite the scarcity of clinical studies investigating the underlying mechanisms, beneficial effects of the Mediterranean diet have partly been attributed to the use of olive oil. It is an important source of monounsaturated fatty acids (mainly oleic acid). However, it has been suggested that the beneficial effects of olive oil consumption in the prevention of chronic diseases may not only be attributed to its fatty acid composition but also to its high content of phenolics.

Tyrosol, hydroxytyrosol and oleuropein are the main hydrophilic phenolic compounds of olive oil, and their pharmacological activities reported in many in vivo and in vitro studies. They show beneficial effects either directly by their antioxidant properties or indirectly with their impact on cellular signaling pathways, expression of certain genes and acting as inhibitors or activators of regulatory enzymes. Epidemiological studies are performed in terms of their protective role on cardiovascular diseases, neurodegenerative diseases and cancer.

Olive oil phenolics are well known for its antioxidant activities. But their role in metabolic pathways, their effect on transcription factors, gene expressions need to be investigated. In this study it is aimed to study the effect of diet rich in olive oil phenolic compounds on age-related changes in heart and liver tissues of 9-10 weeks old female SAMP8 (Senescence Accelerated Mouse Prone 8) mice. We fed SAMP8 semisynthetic diets with 10% olive oil containing either high (HP) or low amounts of olive oil phenolics (LP) for 4.5 months. As a control SAMR1 (senescence-accelerated mouse-resistant 1) mice were used that have a normal ageing process. SAMP8 is a widely used human model of ageing due to their shortened lifespan and pathological disorders such as elevated lipid and protein oxidation, inflammation, mitochondrial dysfunction, early onset of atherogenesis. These properties render SAMP8 mice as a suitable rodent model for experimental ageing research.

Oxidative stress and inflammation play an important role in the pathogenesis of ageing and age-related disorders. Therefore in this study the parameters regarding oxidative stress and inflammation were investigated. As a biomarker of oxidative stress in both tissues, lipid oxidation and protein oxidation were determined by TBARS (Thiobarbituric acid reactive substances) assay and protein carbonyls, respectively. Also proteasomal activity of different subunits in the proteasomal system was measured. In serum of mice total cholesterol, total triglycerides, HDL cholesterol and paraoxonase-1 (PON-1) activity were determined. Nrf2-dependent gene expression may be impaired during the ageing process. In both tissues transcription factor Nrf2 and its target genes; glutathione-S-transferease (GST), γ-glutamyl-cysteinyl-synthetase (γ-GCS), NADPH quionone oxidoreductase (NOQ1),

hemeoxygenase (HO-1), paraoxonase-1 (PON-1 in liver) and PON2 in heart were the main focus. Their amounts were determined both in mRNA level and protein level. Furthermore the level of antioxidant vitamins (vitamin C & E), genes that play a role in the metabolism of these vitamins and the parameters of redox status were determined.

As a result different results in each parameter were obtained depending on the response of the tissue to the oxidative stress. Generally higher level of oxidative stress biomarkers was detected in both tissues of SAMP8 mice as compared to SAMR1 mice. Proteasomal activity and redox status remained unchanged in both tissues and mice types as well.

Nrf2 as well GST, γ-GCS, NOQ1 and PON2 mRNA levels were significantly higher in heart tissue of the HP as compared to the LP group. The mRNA expression of HO-1 did not differ in heart with HP treatment. The mRNA expression levels of Nrf2 and its target genes did not differ between groups in liver. Only HO-1 mRNA and protein level decreased in SAMP8 LP group as compared to SAMR1 LP group but no effect of HP treatment was observed.

The mRNA expression level of inflammation related genes, SAP (Serum Amyloid P), CRP (C Reactive Protein) significantly increased and HO-1 significantly decreased in the liver of SAMP8 mice as compared to SAMR1 mice. Interestingly the level of two well known antioxidant vitamins (vitamin C & E) significantly increased in SAMP8 LP group. The expression of genes regarding ascorbic acid uptake and synthesis (sodium-dependent vitamin C transporter type 1-SVCT1, sodium-dependent vitamin C transporter type2-SVCT2 and L-gulonolactone oxidase-Gulo) were also analysed. The mRNA expression level of SVCT1 expression was significantly increased in SAMP8 mice but no change was determined in terms of SVCT2 and Gulo level.

Our data suggests that oxidative stress and inflammation may play an important role in the ageing of liver and heart in SAMP8 mice. Depending on the results, a diet rich in olive oil phenolics may prevent oxidative stress in the heart of SAMP8 mice by modulating Nrf2-dependent gene expression. As the two olive oil that were used in the feeding study compared, it has been observed that they have similar phenolic profile. On the other hand the HP oil contains approximately 40 fold higher hydroxytyrosol as compared to LP oil. Therefore the beneficial effects of the diet my be attributed to its hydroxytyrosol content. Vitamin C level may be a response of the body to oxidative stress it increased to protect the body homeostasis. This increase has reflected to vitamin E level, which was higher in SAMP8 LP mice.

Another aim of that study is to develop and validate a method with the sensitive electrochemical detector for the quantification of olive oil phenolics in oils. Electrochemical detectors provide a new analytical tool for resolving and accurately detecting trace amounts of any electroactive compound in a wide range of samples. In our validated method it is possible to analyze selected phenolic compounds (tyrosol, hydroxytyrosol, oleuropein, pinoresinol, caffeic acid, vanillic acid, p-coumaric acid, ferulic acid) in 17 minutes. This method was validated in terms of selectivity, lower limit of quantification and detection, accuracy, precision, recovery, short term stability, freeze thaw stability and post preparative stability. The phenolics were quantified in ng level. The recoveries of these phenolic compounds ranged between 72-96%.

The liver and heart are the major organs responsible for a number of physiological processes that are crucial to sustaining life. Most of the studies dealing with SAMP8 mice have focused on brain aging. Therefore our study could be one of the pioneer studies in liver and heart ageing and associated pathophysiological processes in SAMP8 mice. On the other hand in the literature there is only one study that uses

electrochemical detector in the quantification of olive oil phenolics but it is not validated. In this respect our validated method with good recovery of phenolics and lower limit of detection (ng level) will be beneficial for the future analysis of olive oil phenolics in shorter time.

**ZEYTİNYAĞI FENOLİKLERİNCE ZENGİN DİYETİN YAŞLANDIRILMASI HIZLANDIRILMIŞ FARE MODELİNDE YAŞLANMAYA BAĞLI DEĞİŞİKLİKLER ÜZERİNE ETKİSİ VE ZEYTİNYAĞINDAKİ FENOLİK MADDELERİN ELEKTROKİMYASAL DEDEKTÖR İLE HPLC ANALİZİ**

## ÖZET

Zeytinyağı Akdeniz diyetinin temel yağ kaynağıdır. Zeytinyağı tüketimi kardiyovasküler hastalıklar, kanser, Parkinson ve Alzheimer gibi hastalıkların oluşum riskini ve bu hastalıklardan kaynaklanan ölüm riskini azaltmaktadır. Akdeniz diyetinin yararlı etkilerinin altında yatan mekanizmaları inceleyen klinik çalışmalar eksik olmasına rağmen, bu etkiler kısmen zeytinyağı tüketimine bağlanmaktadır. Zeytinyağı önemli bir doymamış yağ asidi (oleik asit) kaynağıdır. Ancak kronik hastalıkların önlenmesi ile ilişkili olarak zeytinyağı tüketiminin yararlı etkileri sadece yağ asidi bileşimine değil yüksek fenolik madde miktarına da bağlanmaktadır.

Tirozol, hidroksitirozol ve oleuropein zeytinyağında bulunan temel fenolik bileşenlerdir ve birçok in vivo ve in vitro çalışmada farmakolojik aktiviteleri belirtilmiştir. Bu bileşenler yararlı etkilerini direkt olarak antioksidan özellikleri ile veya indirekt olarak hücresel sinyal yolizlerini, bazı genlerin anlatımını etkileyerek, bazı düzenleyici enzimleri aktive ya da inhibe ederek göstermektedir. Bu bileşenlerin kardiyovasküler hastalıklar, nörodejeneratif hastalıklar ve kanser üzerine koruyucu etkileri ile ilgili olarak epidemiyolojik çalışmalar gerçekleştirilmektedir.

Zeytinyağı fenoliklerinin antioksidan aktivileri çok iyi bilinmektedir. Ancak metabolik yolizlerindeki görevleri, transkripsiyon faktörleri ve gen anlatımı üzerine etkilerinin araştırılması gerekmektedir. Bu çalışmada fenolik maddelerce zengin zeytinyağı içeren diyetin 9-10 haftalık dişi SAMP8 (Senescence Accelerated Mouse Prone 8) farelerinin kalp ve karaciğer dokularında yaşlanmaya bağlı oluşan değişiklikler üzerine etkisinin incelenmesi amaçlanmıştır. SAMP8 fareleri 4.5 hafta boyunca düşük (DF) ya da yüksek fenolik (YF) madde miktarına sahip %10 zeytinyağı içeren yarı sentetik diyetlerle beslenmiştir. Kontrol olarak normal yaşlanma prosesine sahip SAMR1 (senescence-accelerated mouse-resistant 1) fareleri kullanılmıştır. SAMP8 fareleri düşük yaşam döngüsüne ve yüksek lipit ve protein oksidasyonu, iltihaplanma, mitokondri bozuklukları, erken aterogenez başlangıcı gibi patolojik bozukluklara sahip olduğundan dolayı insan yaşlanma modeli olarak sıklıkla kullanılmaktadır. Bu özellikler SAMP8 fare grubunu yaşlanma ile ilgili çalışmalarda kullanımını uygun kılmaktadır.

Oksidatif stres ve iltihaplanma yaşlanma ve yaşlanmaya bağlı bozuklukların patogenezinde önemli rol oynamaktadır. Bu nedenle bu çalışmada oksidatif stres ve iltihaplanmaya bağlı parametreler incelenmiştir. Her iki dokuda da oksidatif stres biyomarkırı olarak lipit ve protein oksidayonu sırasıyla TBARS ve protein karbonil metotları ile belirlenmiştir. Ayrıca proteazomal sistemdeki farklı altbirimlerin proteazomal aktivitesi ölçülmüştür. Farelerin serumunda toplam kolesterol, toplam trigliserit, HDL kolesterol ve paraoksonaz-1 aktivitesi belirlenmiştir. Yaşlanmaya bağlı olarak Nrf2 (nuclear factor erythroid 2-related factor 2)' ye bağlı gen anlatımı bozulabildiğinden Nrf2 ve hedef genlerinin anlatımı; glutatyon-S-transferaz (GST), γ-glutamil-sisteinil sentetaz (γ-GCS), NADPH kuinon oksidoredüktaz (NOQ1),

hemoksijenaz (HO-1), paraoksonaz-1 (PON-1 karaciğerde) ve kalpte PON2, hedef olarak seçilmiştir. Bu genlerin miktarları mRNA ve protein seviyesinde belirlenmiştir. Bunun dışında antioksidan vitamin miktarları (E ve C vitamini), bu vitaminlerin metabolizmasında rol oynayan genler ve redoks durumu parametreleri belirlenmiştir.

Sonuç olarak her parametrede dokuların oksidatif strese karşı yanıtına bağlı olarak farklı sonuçlar elde edilmiştir. Genel olarak SAMP8 farelerinin her iki dokusunda da oksidatif stres biyomarkırları SAMR1 farelerine göre yüksek saptanmıştır. Proteazomal aktivite ve redoks durumu parametreleri her iki doku ve fare tiplerinde degişmemiştir.

Nrf2 ve buna bağlı GST, γ-GCS, NOQ1 ve PON2 genlerinin mRNA seviyeleri kalpte YF grup farelerde DF grup farelere göre önemli derecede yüksek saptanmıştır. YF uygulaması ile HO-1 mRNA seviyesinde bir değişim gözlenmemiştir. Karaciğerde Nrf2 ve hedef genlerinin anlatımında gruplar arasında fark bulunmamıştır. Sadece HO-1 mRNA ve protein seviyesi SAMP8 DF grubunda SAMR1 DF grubuna oranla düşük saptanmıştır. Ancak YF uygulamasının bir etkisi saptanmamıştır.

Karaciğerde itihaplanma ile ilişkili olan Serum amyloid P (SAP) ve C Reaktif Protein (CRP) genlerinin mRNA anlatım miktarları SAMP8 DF farelerinde SAMR1 DF farelerine göre önemli derece artmış, HO-1 geninin mRNA miktarı ise azalmıştır. Ilginç olarak iki iyi tanınan antioksidan vitaminlerin miktarı (E ve C vitamini) SAMP8 DF grubunda önemli derecede artmıştır. Sodyuma bağlı C vitamini taşıyıcısı-1 (SVCT1), sodyuma bağlı C vitamini taşıyıcısı-2 (SVCT2), L-gulonolakton oksidaz gibi askorbik asidin doku içine alımı ve sentezi ile ilişkili genlerin anlatımı analizlenmiştir. SVCT1 anlatım miktarı SAMP8 DF grubu farelerde önemli derece artmış ancak SVCT2 ve L-gulonolakton oksidaz açısından bir değişiklik görülmemiştir.

Elde edilen veriler SAMP8 farelerinin kalp ve karaciğer yaşlanmasında oksidatif stres ve iltihaplanmanın önemli rol oynayabileceğini göstermektedir. Sonuçlara bağlı olarak zeytinyağı fenolik maddelerince zengin diyetin SAMP8 farelerinde Nrf2 ve buna bağlı genlerin anlatımını düzenleyerek oksidatif stresi önleyebilecegi söylenebilir. Besleme çalışmalarında kullanılan iki yağ örneği karşılaştırıldığında her ikisinin benzer profillere sahip olduğu görülmektedir. Diğer yandan YF yağ örneği DF yağ örneğine göre yaklaşık 40 kat daha fazla hidroksitirozol içermektedir. Bu nedenle YF içeren diyetin yararlı etkileri hidroksitirozol içeriğine de bağlanabilir. Karaciğerde SAMP8 farelerinde görülen yüksek C vitamini miktarı oksidatif strese karşı vücut dengesini sağlamak için vücudun bir cevabı olarak düşünülebilir. SAMP8 DF farelerinde görülen C vitaminindeki bu artış C vitamini tarafından indirgenen E vitamini miktarına da yansımıştır.

Bu çalışmanın diğer bir amacı da hassas elektrokimyasal dedektör ile zeytinyağındaki fenolik maddelerin belirlenmesi için metot geliştirmek ve metodun validasyonunu sağlamaktır. Elektrokimyasal dedektörler pekçok üründe iz miktardaki elektroaktif bileşiklerin doğru bir şekilde belirlenmesinde yeni bir analitik araç olarak görev yapmaktadır. Çalışmada valide edilmiş metot ile zeytinyağında bulunan seçilmiş fenolik maddelerin analizi (tirozol, hidroksitirozol, oleuropein, piniresinol, kafeik asit, vanilik asit, p-koumarik asit, ferulik asit) 17 dakika içinde yapılması mümkün olmuştur. Bu metot seçicilik, en düşük belirleme limit değeri, geri kazanım, doğruluk, duyarlık, dondurma-çözündürme dayanıklılığı, kısa süreli dayanıklılık, örnekleyici içindeki dayanıklılık açısından valide edilmiştir. Fenolik maddeler ng seviyesinde belirlenmiştir. Incelenen fenolik maddelerin geri kazanımları %72-96 arasında saptanmıştır.

Kalp ve karaciğer hayatı sürdürmek için gerekli pekçok fizyolojik prosesten sorumlu organlardır. SAMP8 fareleri ile gerçekleştirilen pekçok çalışmada beyin yaşlanması üzerinde durulmuştur. Bu nedenle yapılan çalışma SAMP8 farelerinde kalp ve

karaciğer yaşlanması ve buna bağlı patofizyolojik prosesler açısından öncü bir çalışma olarak değerlendirilmektedir. Diğer yandan literatürde zeytinyağının fenolik maddelerinin elektrokimyasal dedektör ile belirlenmesi açısından sadece bir çalışma mevcuttur ancak metot valide edilmemiştir. Bu açıdan valide edilmiş, fenolik madddelerin geri kazanımı oldukça yüksek ve en düşük belirlenme limitinin ng seviyesinde olduğu metot gelecekte kısa sürede fenolik madde analizi açısından yararlı olacaktır.

# 1. INTRODUCTION

The 'Mediterranean Diet is rich in olive oil, fruits, vegetables, grains and legumes. A high degree of adherence to the traditional Mediterranean diet has been associated with a reduced risk of overall and cardiovascular mortality, cancer incidence and mortality, and incidence of Parkinson and Alzheimer disease (Trichopoulou et al., 2003; Sofi et al., 2008). Despite the scarcity of clinical studies investigating the underlying mechanisms, beneficial effects of the Mediterranean diet have partly been attributed to the use of olive oil (Alonso et al., 2004; Esposito et al., 2004; Estruch et al., 2006; Kontogianni et al., 2007). Olive oil is the primary source of fat in the Mediterranean diet.

Olive oil is obtained from the fruits of *Olea Europea L.* tree and it contributes ~4% of total vegetable oil production. More than 95% of world olive oil production is supplied by Mediterranean countries. Due to growing interest in Mediterranean diet and its health benefits, the production of olive oil is now expanding to non-traditional producers such as United States, Canada, Australia and Japan (Visioli et al., 2002).

Olive oil is composed of major and minor components. The major components represent more than 98% of the total oil weight and they include glycerols. High content of oleic acid is unique to olive oil. Compared to polyunsaturated fatty acids, oleic acid is monounsaturated, making it much less susceptible to oxidation and contributing to the antioxidant action, high stability and long shelf life of olive oil (Owen et al., 2000). The non-glycerol or unsaponifiable fraction consists of 0.4-2% including more than 230 chemical compounds such as sterols, aliphatic alcohols, triterpene alcohols, hydrocarbons, pigments, volatile compounds, and antioxidant compounds (Servili et al., 2004; Tripoli et al, 2005).

Olive oil is known to have antioxidant (Liu et al., 2007; Loru et al., 2009), anti-inflammatory (Bogani et al., 2007; Zhang et al., 2009), anti-atherogenic (Visioli and Galli, 2000), anti-ulcer (Serrano et al., 1997), anti-cancer (Della Regione et al., 2000; Quiles et al., 2002; Gill et al., 2005), anti-microbial (Medina et al., 2006), diabetic (Soriguer et al. 2004; Al-Azzawie et al., 2006; Rigacci et al., 2009), neuroprotective (Schaffer et al., 2007; Young et al., 2008), skin protective (Perugini et al., 2008) and anti-ageing (Hao et al., 2009) effects. The overall beneficial effects

of virgin olive oil have been linked to both oleic acid and its antioxidant components. The main antioxidants of olive oil are β-carotenes and phenolic compounds including lipophilic and hydrophilic phenols (Boskou, 1996). While the lipophilic phenols, among which tocopherols can be found in other vegetable oils, some hydrophilic phenols of olive oil (including phenolic acids, secoiridoid compounds and derivatives) are not generally present in other oils and fats. Moreover, the hydrophilic phenols of olive oil constitute a group of secondary plant metabolites which show sensory and health properties (Servili et al., 2004).

Tyrosol, hydroxytyrosol and oleuropein are the main phenolic compounds of olive oil, and their pharmacological activities reported in many in vivo and in vitro studies. They show beneficial effects either directly by their antioxidant properties or indirectly with their impact on cellular signaling pathways, expression of certain genes and acting as inhibitors or activators of regulatory enzymes. Epidemiological studies are performed in terms of their protective role on cardiovascular diseases, neurodegenerative diseases and cancer.

The susceptibility to these diseases increases with ageing. Aging is associated with several changes in structure and function of different organs and tissues and it is characterized as a progressive decline in biological functions with time (Farooqui and Farooqui, 2010). One of the most accepted theories of aging is the free radical theory, which postulates that aging is the result of the accumulation of cellular and tissue damage caused by the continuous and endogenous generation of reactive oxygen species (ROS) (Sohal, 2002; Harman, 2003; Barja, 2004). As the amount of ROS increases oxidative stress occurs, that contributes to the pathogenesis and progression of age related disorders (Kregel and Zhang, 2007).

Another theory for ageing is the molecular inflammation hypothesis suggesting that there is link between chronic, low grade inflammation and the pathogenesis of age-related diseases, it is based on dysregulation of the immune system with age and altered redox status during aging. Both processes lead to activation of a wide variety of inflammatory mediators through overproduced reactive species during aging (Chung et al., 2009).

There is a great interest in the development of anti-ageing therapies, protecting from the deleterious effects of ageing, and phytochemicals, dietary interventions containing antioxidants have been widely investigated in this respect. Olive oil, a food naturally containing high amounts of phenolic compounds, may be an alternative. To understand the effect of olive oil phenolics on ageing in vivo studies

are warranted. Researchers have demonstrated that several genes may modulate aging and influence longevity. Olive oil phenolic compounds are well known for their antioxidant activities. But their role in metabolic pathways, their effect on transcription factors, gene expressions needs to be investigated.

In our study it is aimed to study the effect of diet rich in olive oil phenolic compounds on age-related changes in heart and liver tissues of SAMP8 mice, a widely used rodent model for experimental ageing research, which have a shorter life span and pathological disorders. As oxidative stress and inflammation play an important role in the pathogenesis of ageing and age-related disorders, the parameters regarding these factors were investigated. In both tissues Nrf2 and the genes that are controlled under that transcription factor was the main focus. Furthermore the level of antioxidant vitamins (vitamin C and vitamin E), biomarkers of redox status were measured. As a biomarker of oxidative stress, lipid oxidation was determined by TBARS assay, protein oxidation was determined by measuring protein carbonyl levels. Also proteasomal activity of different subunits in the proteasomal system was measured.

Another aim of that study is to develop and validate a method with the sensitive electrochemical detector for the quantification of phenolics in olive oil.

## 2. LITERATURE

### 2.1 Production of Olive Oil

The most vegetable oils are extracted from seeds by solvents. On the other hand olive oil is produced by a cold-press mechanical process that preserves both the chemical nature of the oil and the natural antioxidants that are produced in response to environmental stress which is suggested as the uniqueness of olive oil (Visioli and Galli, 2000).

The first step in olive oil production is washing of the olives in order to remove any foreign material that could damage machinery or contaminate the oil. Then the olives are crushed to produce a paste with easily extracted oil droplets. A step called malaxation takes place, where olive paste is mixed and prepared for separation of the oil from the pomace. The next step is separating the oil from the paste and fruit water (water of vegetation). The oil can be extracted by pressing, centrifugation, percolation, or through combinations of the different methods. In the last step oil is separated from the water. The figure 2.1 shows olive oil production with classical, two phase and three phase systems.

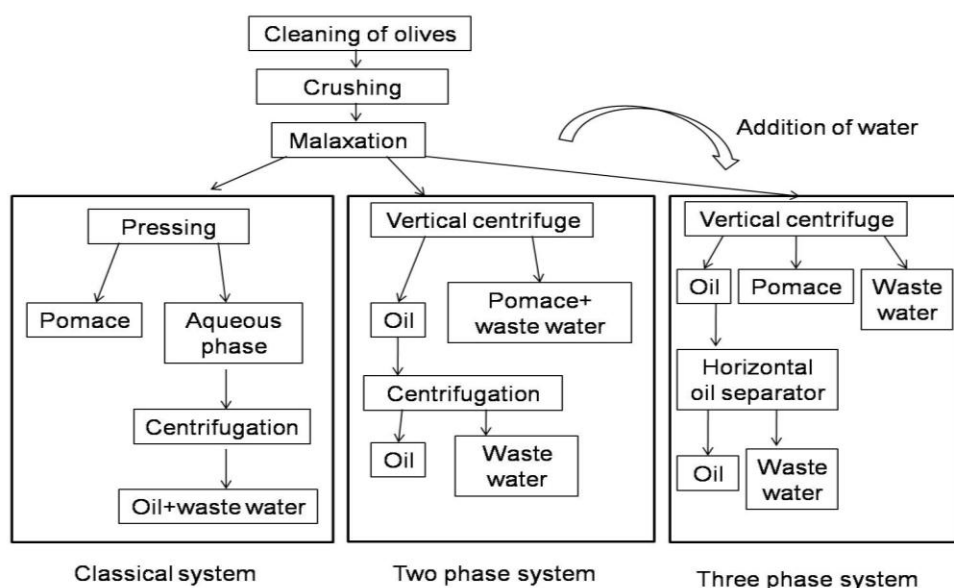


**Figure 2.1:** Olive oil production (Boskou, 2006).

## 2.2 Chemical Composition of Olive Oil

Olive oil is composed of major and minor components. The major components include glycerols and represent more than 98% of the total oil weight. The non-glycerol or unsaponifiable fraction consists of 0.4-2% (Servili et al., 2004; Tripoli et al, 2005). The chemical composition of olive oil is given in Table 2.1.

**Table 2.1:** Chemical composition of olive oil.

| Saponifiable fraction (98-99%) | Unsaponifiable fraction (~2%) |
|---|---|
| Main fatty acids present in triacylglycerols<br>Miristic acid (14:0)<br>Palmitic acid (16:0)<br><br>Palmitoleic acid (16:1)<br>Estearic acid (18:0)<br>Oleic acid (18:1)<br>Linoleic acid (18:2)<br>Linolenic acid (18:3) | Non glyceride esters (alcoholic and sterol compounds, waxes)<br>Aliphatic alcohols<br>Triterpene alcohols<br><br>Sterols (β-sitosterol, campesterol, estigmasterol)<br>Hydrocarbons (squalene, lycopene, β-carotene)<br>Pigments (β-carotene, chlorophylls)<br>Lipophilic phenolic (Vitamin E)<br>Hydrophilic phenolics (phenolic acids, phenolic alcohols, secoiridoids, lignans, flavones)<br>Volatile compounds |

Olive oil is graded in six categories: extra virgin olive oil (EVOO), virgin olive oil (VOO), refined olive oil, olive oil, refined residue oil, and olive residue oil. These oils differ in their acidity, way of production and micronutrients content. Among them VOO and EVOO are attributed to health effects of olive oil.

### 2.2.1 Major compounds

Triacylglycerols are constituted by most of the 12 identified fatty acids (C14-C24), although only 6 are major compounds. These are; oleic acid (55.23-86.64%), palmitic acid (6.30-20.93%), linoleic acid (2.7-20.24%), stearic acid (0.32-5.33%), palmitoleic (0.32-3.52%), and linolenic acid (0.11-1.52%) (Garcia-Gonzales et al., 2008). Virgin olive oil is unique with respect to the high oleic acid content because the majority of seed oils are composed primarily of polyunsaturated fatty acids, including the essential omega-6 fatty acid, linoleic acid. Compared to polyunsaturated fatty acids, oleic acid is monounsaturated, making it much less susceptible to oxidation and contributing to the antioxidant action, high stability and long shelf life of olive oil (Owen et al., 2000).

### 2.2.2 Minor compounds

Minor compounds of virgin olive oil are present in low amount and include more than 230 chemical compounds such as sterols, aliphatic alcohols, triterpene alcohols,

hydrocarbons, pigments, volatile compounds, lipophilic and hydrophilic phenolic compounds (Servili et al, 2004).

#### 2.2.2.1 Lipophilic phenolics (Vitamin E)

Vitamin E is the most abundant lipophilic phenolic. İt is a general name of four tocopherol (namely α-, β-, γ-, and δ-) and four tocotrienol isoforms (namely α-, β-, γ-, and δ-). Virgin olive oil contains high amount of α-tocopherol in range of 50-250 mg/kg followed by β- and γ-tocopherols (García-González et al., 2008).

#### 2.2.2.2 Sterols

β-sitosterol is represent the sterol content of virgin olive oil with 90-95% that is followed by campesterol and stigmasterol with 3% and 1% respectively (Gutierrez et al., 1999). The sterol amount of virgin olive oil is approximately 1600 mg/kg (Covas et al., 2006).

#### 2.2.2.3 Hydrocarbons

Squalene is the most important hydrocarbon of virgin olive oil which constitutes around 50% of the unsaponifiable matter (García-González et al., 2008). It is the metabolic precursor of cholesterol and other sterols and it has a role in the synthesis of steroid hormones. İt is found in olive oil (~0.7%) in higher amounts as compared to other vegetable oils (0.002-0.03%) (Reedy and Couvreur, 2009).

#### 2.2.2.4 Pigments

Chlorophyll and carotenoid pigments are responsible for the color of virgin olive oil, ranging from yellow-green to greenish gold. The chlorophyll pigments are responsible for the greenish hues of virgin olive oil, with pheophytin a being a major compound in this regard, while the major “yellow” pigments are lutein and β-carotene. Carotenoids are pigments whose content does not usually exceed 10 mg/kg, with lutein being the major carotenoid followed by β-carotene and other xantophylls. Xantophylls esterified with fatty acids play an important role in the stability of carotenoids, stabilizing the oil colors and ensuring a sufficient level of substances with antioxidant activity (García-González et al., 2008).

#### 2.2.2.5 Volatile compounds

Volatile are responsible for the olive oil flavor and aroma. They are the main compounds responsible for the “green” and fruity desirable aroma attributes of olive oil. On the contrary, the low-quality olive oil have complex profiles composed of a

large number of volatiles responsible for off flavors such as rancid, mustiness, fusty and muddy sediment (García-González et al., 2008).

#### 2.2.2.6 Triterpenes

Triterpenoic alcohols (erithrodiol and uvaol) and triterpenoic acids (maslinic, olanolic, ursolic ane betulinic acids) are the most relevant triterpene molecules in virgin olive oil. They are concentrated in the pericarp of olives. Therefore it is found in virgin olive oil in less amount (Covas et al., 2006).

### 2.3 Health Benefits of Olive Oil

Olive oil is characterized with its high content of monounsaturated fats and is also a good source of phytochemicals including phenolic compounds, squalene and vitamin E. It is also considered as a “natural functional food” or “liquid gold” with protective effects on oxidative stress and age-related disorders such as cancer, cardiovascular diseases, ulcer, diabetes, alzheimer and dementia. The health effects of olive oil is given in Figure 2.2. Many intervention studies (Tzonou et al., 1993; Braga et al., 1998; Stoneham et al., 2000; Fung et al., 2009; Perona et al., 2010; Martinez-Gonzalez et al., 2011) demonstrate that the lower incidence of cancer and cardiovascular diseases is attributed to high comsumption of olive oil in Mediterrenean countries.

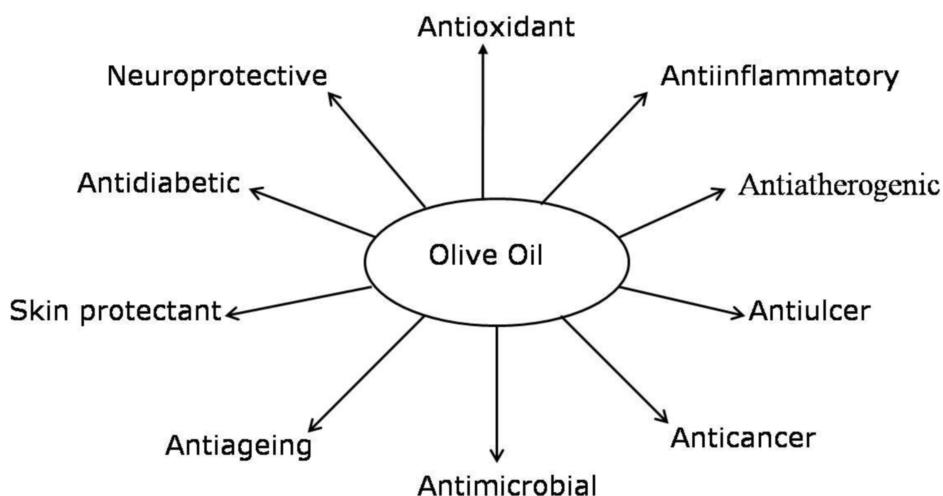


**Figure 2.2:** Health effects of olive oil.

The overall beneficial effects of virgin olive oil have been linked to both its monounsaturated fatty acids (oleic acid) and its antioxidant components. It cannot

be explained by the effect of a single component. These compounds show their effect by different mechanism of actions as summarized in Table 2.2.

In recent years many epidemiological and clinical studies have been conducted with phenolic compounds of virgin olive oil and more emphasis will be given to these compounds in the next sections including their health benefits, technological role in olive oil and their analysis techniques.

**Table 2.2:** Mechanism of actions of olive oil components in their health effects.

| Compound | Mechanism of action | Reference |
|---|---|---|
| Sterols | Protect against membrane lipid peroxidation, anti-inflammatory effect, improvement of endothelial dysfunction, decreases plaque formation, decreases LDL cholesterol, total cholesterol and triglycerides, anti-cancinogenic effect against lung, breast, stomach cancers via decrasing the growth of tumors, increasing the immune response and antioxidant enzyme activity | Mendilaharsu et al., 1998; De Stefani et al., 2000; van Rensburg et al., 2000; Devaraj et al., 2006; Raitakari et al., 2008; Hallikainen et al., 2006; Seppo et al., 2007 |
| Skualen | Protection against UV radiation and radioactivity | Reedy and Couvreur, 2009 |
| Carotenoids | Protective effect against cancer and cardiovascular diseases, antioxidant effect | Bernstein et al., 2002; Voutilainen et al., 2006 |
| Luteolin | Protective against cataract and macular degredation, antioxidat and anticarcinogenic effect, protection of DNA from oxidation | Trumbo and Ellwood 2006; Santocono et al., 2007; Kim et al., 2007 |
| Chlorophylls | Antioxidant effect, protective against cancer | Kamat et al., 2000; Lanfer-Marquez et al., 2005; Ferruzzi and Blakeslee, 2007 |
| α-Tocopherol | Antioxidant effect, prevent oxidation of lipoproteins and biological membranes, prevent atherosclerosis, reduce breast cancer risk, protection of DNA from oxidation, neuroprotection | Rimm et al., 1993; Saldeen et al., 1999; Kline et al., 2003 |
| Oleic acid | Decreases triacylglycerol and cholesterol, anti-inflammatory and antihypertensive effects, protects cell membrane and lipoproteins from oxidative stress, lowers the risk of colon, lung, breast, coleractal, and prostate cancers, improve insulin sensitivity, lowers plasma glucose and insulin levels | Yamaki et al., 2002; Lopez et al., 2008; Paniagua et al., 2007; Barberger-Gateau et al., 2007; Solfrizzi et al., 2006 |
| Triterpenes | Antioxidant, anti-inflammatory, vasodilatory, cardiotonic effects | Somova et al., 2003; Martinez-Gonzalez et al., 2008 |

## 2.4 Hydrophilic Phenolic Compounds

Virgin olive oil contains different classes of phenolic compounds such as phenolic acids, phenolic alcohols, flavonoids, hydroxy-isocromans, secoiridoids, and lignans as reported in Table 2.3. The most abundant phenols are the secoiridoids. These compounds, e.g., oleuropein and ligstroside, are derivatives of the secoiridoid glucosides of olive fruits. Breakdown products of two major phenolic constituents of the olive fruit, oleuropein and ligstroside, form the majority of the phenolic fraction. There are at least thirty-six structurally distinct virgin olive oil phenolics that have been identified (Cicerale et al., 2009). They can be grouped according to their similar chemical structure in the following groups:

Phenolic acids with the basic chemical structure of C6-C1 (benzoic acids) and C6-C3 (cinnamic acids), such as caffeic, vanillic, p-coumaric, o-coumaric, protocatechuic, sinapic, and p-hydroxybenzoic acid, are the first group of phenols observed in olive oil.

Phenolic alcohols are the compounds with hydroxyl group attached to an aromatic hydrocarbon group. Hydroxytyrosol and tyrosol are the main phenolic alcohols in virgin olive oil. Secoiridoids are characterized by the presence of either elenoic acid or elenoic acid derivatives in their molecular structure (Carrasco-Pancorbo et al., 2005). Hydroxyl-isocromans are 3,4-dihydroxy-1H-benzopyran derivatives (Bianco et al., 2001). Flavonoids are compounds containing two benzene rings joined by a linear three carbon chains, lignans are compounds which structure based on the condensation of aromatic aldehydes (Carrasco- Pancorbo et al., 2005). They are the second prevalent group in virgin olive oil.

**Table 2.3:** Phenolic compounds in virgin olive oil (Carrosco-Pancorbo et al., 2005; Servili et al., 2004).

| Phenolic acids and derivatives |
|---|
| Gentisic acid<br>Vanillic acid<br>p-coumaric acid<br>o-coumaric acid<br>Gallic acid<br>Caffeic acid<br>Protocatechuic acid<br>Ferulic acid<br>Cinnamic acid<br>Benzoic acid<br>p-hydroxybenzoic acid<br>Sinapinic acid<br>4-Acetoxyethyl-1,2-dihydroxybenzene<br>2-(4-hydroxyphenyl)ethyl acetate |
| **Phenolic alcohols** |
| (3,4-dihydroxyphenyl)ethanol (3,4 DHPEA)<br>(p-hydroxyphenyl)ethanol (p-HPEA)<br>(3,4-dihydroxyphenyl)ethanol glucoside |
| **Secoiridoids** |
| Dialdehydic form of decarboxymethyl elenoic acid linked to 3,4-DHPEA (3,4 DHPEA-EDA)<br>Dialdehydic form of decarboxymethyl elenoic acid linked to p-HPEA (p-HPEA-EDA)<br>Oleuropein aglycone (3,4 DHPEA-EA)<br>Oleuropein<br>p-HPEA derivative<br>Dialdehydic form of oleuropein aglycone<br>Dialdehydic form of ligstroside aglycone<br>Ligstroside aglycone<br>Oleochanthal |
| **Lignans** |
| (+)-1-Acetoxypinoresinol<br>(+)-Pinoresinol<br>(+)-1-Hydroxypinoresinol |
| **Flavonoids** |
| Apigenin<br>Luteolin<br>(+)-Taxifolin |
| **Hydroxyisochromans** |
| 1-phenyl-6,7-dihydroxy-isochroman<br>1-(3 -methoxy-4 -hydroxy)phenyl-6,7-dihydroxy-isochroman |

## 2.5 Factors that Affect the Phenolic Content of Olive Oil

The qualitative and quantitative composition of virgin olive oil hydrophilic phenols is strongly affected by the agronomic and technological conditions of production.

### 2.5.1 Agronomic conditions

Among agronomic parameters, the most studied aspects include cultivar, geographical origin, fruit ripening, climatic conditions of production and some agronomic techniques such as the irrigation.

As reported, phenolic composition of fruit is affected by the cultivar (Briante et al., 2002; Gomes-Alonso et al., 2002; Pinelli et al., 2003). Each olive cultivar has different phenolic content (Esti et al., 1998). For example Italian "coratina" cultivar is known to have higher phenolic content (Aparicio and Luna, 2002).

Geographical origin has a great influence on phenolic composition of virgin olive oils (Criado et al., 2004). Depending on the olive grove, climate and altitude change, thus climatic conditions (rainfall, temperature, humidity) differ.

In some aspects controversy results are obtained as there are many other factors that may affect the results. Several studies related to the relationships between irrigation during olive growing and phenolic concentration of olive oil. For instance Motilva et al. (1999) concluded that hydrophilic phenols in oil increase when the olives were grown under conditions of deficit of irrigation whereas Ismail et al. (1999) reported that the highest level of hydrophilic phenols in oil was obtained from regularly irrigated olives. Using a linear irrigation strategies Tovar et al. (2001), found a negative correlation between the level of water used and the concentration of secoiridoid derivatives of virgin olive oil, on the contrary the concentration of lignans was lower in the oil from the least irrigated treatment.

Olive ripening stage is another factor for the phenolic content. As the maturity increases, the amount of total phenols decreases in almost all samples (Beltran et al., 2005; Ayton et al., 2006). During maturation, the esterase activity causes degradation of oleuropein, the main phenolic compound present in unripened olive fruit. Therefore, it is important to establish the optimal harvest time, which should correspond to the optimal phenol content and the best quality of oils (Skevin et al., 2003).

### 2.5.2 Technological Conditions

Since the occurrence of hydrophilic phenols in virgin olive oil is strictly related to the activities of various endogenous enzymes of olive fruit, their concentration in the oil is strongly affected by the extraction conditions. Crushing and malaxation are the most important critical points of the oil mechanical extraction process (Servili et al, 2004). The amount of phenolics decreases as the time and the temperature of malaxation increases (Servili et al., 2003). Secoiridoid aglycons are originated during crushing from the hydrolysis of their glucosides, oleuropein, demethyloleuropein and ligstroside with the activity of endogenous glucosidases. It is reported that olives which were blanched before crushing to inactivate endogenous glycosidases the concentration of oleuropein and demethyloleuropein was not significantly modified (Servili et al., 2002).

Extraction systems, such as pressure and centrifugation, play an important role in the oil phenolic composition. Oil obtained by pressure or two phase centrifugation systems that do not require addition of water, shows higher phenolic concentration in comparison to the one obtained by the traditional three phase centrifugation system (Kaula et al., 2006). In general, the concentration of phenols in virgin olive oils may range between 40 and 900 mg/kg or up to 1000 mg/kg (Montedoro et al., 1992).

Apart from their nutritional value phenolic compounds play an important role in stability, and sensory properties of olive oil.

The phenolic constituents confer a bitter, astringent, throat-catching and pungent taste to the oil. Oils produced from greener olives, which contain high amount of phenolic compounds, have ''fruity'' and complex aroma. Conversely, ''sweet'' oils are almost devoid of phenols. On the other hand very high load of phenols may result in an excessive and unpleasant bitterness and is not synonymous with quality (Visioli and Galli, 2000). Oleuropein is the bitter principle of olives and is found in virgin olive oil as such and in its aglycone form. It undergoes hydrolysis and yields several simpler molecules (simple phenols) that build up the well-known olive oil complex taste. These modifications enhance the aroma and flavor of olive oils. Also strong correlation is reported between the bitter and pungent sensory notes and the ligstroside derivatives such as *p*-HPEA-EDA and 3,4-DHPEA-EDA (Gutíerrez-Rosales et al, 2001; Tovar et al., 2001).

Phenolic compounds also affect the stability of virgin olive oil. During storage, fatty acids in oils undergo oxidative degradation and odorless molecules, such as

hydroxyperoxides and secondary products, are produced. It is stated that virgin olive oil is highly stable to oxidation and has a longer shelf life as compared to other edible vegetable oils, due to its fatty acid composition, mainly high monounsaturated fatty acid content, and to the presence of minor compounds (lipophilic tocopherols and hydrophilic phenolic compounds) that have a major role in preventing oxidation. Particularly phenolic compounds contribute significantly to its prolonged shelf life and among them the secoiridoid derivatives show a large capacity to protect virgin olive oil from oxidative degradation (Gutierrez et al., 2001; Mateos et al., 2003; Bendini et al., 2006). Phenolic compounds can inhibit oxidation by a variety of mechanisms based on radical scavenging, hydrogen atom transfer, and metal-chelating attributes (Huang et al., 2005; Prior et al., 2005). Beside it is reported that virgin olive oil phenolics contribute to stability of olive oil under deep-frying conditions (Quiles et al., 2002; Napolitano et al., 2008; Casal et al., 2010).

### 2.6 Health Effects of Olive Oil Phenolics

Tyrosol, hydroxytyrosol and oleuropein are the main phenolic compounds of virgin olive oil, and their pharmacological activities reported in many studies.

Hydroxytyrosol is the principal phenolic compound found in olive oil and is known to possess strong antioxidant scavenging abilities. It has been reported that hydroxytyrosol inhibits low density lipoprotein (LDL) oxidation (Visioli, et al., 1998a), platelet aggregation (Petroni et al., 1995) and endothelial cell activation (Carluccio et al., 2003), accumulation of tromboxane and production of proinflammatory leukotrienes, cytokines as well as scavenge superoxide anion and hypochlorous acid (Visioli et al., 2002; Zhang et al., 2009). Hydroxytyrosol possesses a marked antioxidant activity, scavenging peroxyl radicals (Saija et al., 1998), other ROS (Gordon et al., 2001) and RNS (Deiana et al., 1999; de la Puerta et al., 2001), or breaking peroxidative chain reactions and preventing metal ion catalyzed production of reactive oxygen species (Gutierrez et al., 2001). Hydroxytyrosol is highly protective against the peroxynitrite-dependent nitration of tyrosine and DNA damage by peroxynitrite in vitro (Deiana et al., 1999).

Oleuropein has been shown to possess several pharmacological properties, including antioxidant (Visioli et al., 2002), anti-inflammatory (Visioli et al., 1998a), anti-atherogenic (Carluccio et al., 2003) and anti-cancer (Owen et al., 2000) activities. In addition, oleuropein has been shown to be cardioprotective against acute adriamycin cardiotoxicity (Andreadou et al., 2006) and to exhibit anti-ischemic and hypolipidemic activities (Andreadou et al., 2006). It has been suggested to

reduce atherosclerosis development, improve antioxidant status (González-Santiago et al., 2006), increase iNOS activity by scavenging superoxide anion (Visioli et al., 1998b), increase resistance of LDL to oxidation (Coni et al., 2000). Both oleuropein and hydroxytyrosol have been proven to be more potent antioxidants than vitamin E and BHT (Visioli et al., 1998b).

Tyrosol is the most abundant biophenol in olive oil. Tyrosol is reported to scavenge peroxynitrite (de la Puerta et al., 2001) and $O^{2-}$ (Bertelli et al., 2002). In addition to its antioxidative effects, it has been reported that tyrosol inhibits lipopolysaccharide (LPS)-induced cytokine release from human monocytes (Giovannini et al., 2002) and LPS-induced leukotriene B4 release in human mononuclear cells (de la Puerta et al., 1999). Although it has a weaker antioxidant capacity, it has been reported that tyrosol can restore antioxidant defense, modulate ROS and NO production, COX and lipoxygenase pathways (Moreno, 2003; di Benedetto et al., 2007). Tyrosol is able to inhibit LDL-cholesterol oxidation and to prevent the modification of the apoproteic moiety (Caruso et al., 1999). Tyrosol has also been effective in inhibiting leukocyte 5-lipooxygenase (de la Puerta et al., 1999), protecting the CaCo-2 intestinal mucosa cells against the cytostatic effects produced by oxidized LDL (Giovannini et al., 1999) and inhibiting lipopolysaccharide (LPS)-induced cytokine release from human monocytes (Giovannini et al., 2002). Moreover, it showed a neuroprotective effect against the peroxynitrite-induced neuronal oxidative damage (Vauzour et al., 2007) and in the focal cerebral ischemia/reperfusion rat model (Bu et al., 2007). Some other pharmacological effects of these main phenolics are summarized in Table 2.4.

**Table 2.4:** Health effect of the main olive oil phenolic compounds.

| Phenolic compound | Mechanism of action | Protective effect | Conditions (Cell/mouse/human) | Level (in vivo/ invitro) | Reference |
|---|---|---|---|---|---|
| Hydroxytyrosol | Antioxidant | Scavenge hydrogen peroxide | Human neutrophils | In vitro | O'Dowd et al., 2004 |
| | Antioxidant | Protect from acrolein-induced oxidative stress and mitochondrial dysfunction | Retinal pigment epithelial cells | In vitro | Liu et al., 2007 |
| | Antioxidant, antiageing | Induce Phase II enzymes, enhance mitochondrial biogenesis and improve mitochondrial function, strengthen the cell's antioxidant defenses | 3T3-L1 adipocytes | In vitro | Hao et al., 2009 |
| | Antioxidant | protect cells from H2O2-induced damage via inhibiting production of MDA, hydroperoxides | Renal proximal tubular cells | In vitro | Loru et al., 2009 |
| | Antidiabetic, antioxidant | inhibit hyperglycemia and oxidative stress induced by diabetes, increase antioxidant enzyme activities, decreases lipit peroxidation | Diabetes rat model | In vivo | Hamden et al., 2009 |
| | Anticancer | lower the levels of hydroxyperoxides, DNA damage and mRNA levels of classic glutathine peroxidase | Human prostate cells | In vitro | Quiles et al., 2002 |
| | Anticancer | antiproliferative activity, induce apoptosis | White blood cell from promyelocyticleukemia | In vitro | Della Regione et al., 2000 |
| | Antioxidant, skin protective | Protect against UV-B induced DNA damage | HaCaT cells | In vitro | Guo et al., 2010 |
| | Anti-inflammatory | Inhibit pro-inflammatory cytokines, iNOS and COX-2 expression | Human monocytic cells | In vitro | Zhang et al., 2009 |
| | Antioxidant, Neuroprotective | Protect against DNA damage, quench radicals | Murine-dissociated brain cells | In vitro | Young et al., 2008 |

**Table 2.4 (continued) :** Health effect of the main olive oil phenolic compounds.

| Phenolic compound | Mechanism of action | Protective effect | Conditions (Cell/mouse/human) | Level (in vivo/ invitro) | Reference |
|---|---|---|---|---|---|
| Hydroxytyrosol | Antioxidant, Neuroprotective | Attenuate Fe2+- and nitric oxide (NO)-induced cytotoxicity, prevents oxidative stress-induced loss of cellular ATP levels, decreases basal and iron-stimulated lipid peroxidation | PC-12 cells and mouse | In vitro and ex-vivo | Schaffer et al., 2007 |
| Oleuropein | Antidiabetic, antioxidant | Decrease lipit peroxidation, inhibiting hyperglycemia , increase antioxidant enzyme activities | Diabetic rabbits | In vivo | Al-Azzawie et al., 2006 |
| | Skin protective | Protect against UV-induced erythema | 20-30 yrs females | In vivo | Perugini et al., 2008 |
| | Neuroprotective | Stabilize amyloid-β structure and prevent the formation to a toxic β-sheet conformation | Model system | In vitro | Bazoti et al., 2008 |
| | Antidiabetic | Interfere with human islet amyloid polypeptide (hIAPP) and protect against aggregate cytotoxicity in type II diabetes | Rat RIN-5F insulinoma pancreatic β cells | In vitro | Rigacci et al., 2009 |
| Tyrosol | Neuroprotective | Protect against the sensory motor dysfunction, neuroprotection | Stroke rat model | In vivo | Bu et al., 2007 |
| Oleocanthal | Anti-inflammatory | Inhibit COX-1 and COX-2 | Model system | In vitro | Smith et al., 2005 |
| | Neuroprotective | Alter aggregation of amyloid-β protein via changing its structure, immnoreactivity | Model system | In vitro | Pitt et al, 2009 |
| | | Inhibit Tau fibrilization | Model system | In vitro | Li et al., 2009 |
| Pinoresinol rich olive oil | Anticanceronic | Decrease cell viability, induce apoptosis | Colon cancer cell line | In vitro | Fini et al., 2008 |

### 2.7 Analytical Determination of Phenolic Compounds In Olive Oil

The identification and quantification of phenolic compounds are of great interest due to its nutritional and technological significance. The first step is the extraction of phenolic compounds. The most prevalent methods are solid phase extraction (SPE) and liquid-liquid extraction (LLE). Different cartridges are used in SPE including C8, C18, NH2, and diol. But more promising results are obtained with diol cartridges being the most suitable cartridge for phenolic analysis. In terms of recovery comparable results are obtained in the literature. But SPE is more advantageous than LLE in terms of cleanliness, simplicity, time of analysis, less amounts of solvents and sample required.

For the quantitative determination of total phenols the most widely used method is Folin-Ciocalteu Method which is based on the reaction Folin-Ciocalteu reagent with the hydroxyl groups of phenolic compounds. After the colour reaction the absorbance is measured spectrophotometrically (Singleton et al., 1999). It is a simple and fast method but has low specificity as the colour reaction can occur with any oxidiziable phenolic hydroxyl group.

Apart from that various methods have been developed to analyze the qualitative and quantitative occurrence of phenolic compounds. The simple techniques include Thin Layer Chromatography (TLC) (Ragazzi et al, 1973), reversed phase High Performance Liquid Chromatography (HPLC) coupled with Ultraviolet (UV) (Tsimidou et al., 1992), Diode Array Detector (DAD) (Pirisi et al., 1997; Servili et al., 1999; Esti et al., 1998), ECD (Electrochemical Detector) (Brenes et al., 1999), Gas Chromatography (GC) alone (Solinas et al., 1982) or coupled with Mass Spectrometry (GC-MS) (Liberatore et al., 2001; Marsilio et al., 2001) and Capillary Electrophoresis (CE) (Bendini et al., 2003; Bonoli et al., 2004). Beside phenolic compounds can be determined by NMR (nuclear magnetic resonance) (Angerosa et al., 1995; Montedoro et al., 1993, Christophoridou et al., 2005) and IR (Infrared) (Montedoro et al., 1993) techniques.

Among them LC-MS determination of phenolic compounds is the most prevalent technique which can determine the compounds in ng range. However it is hard to interpret MS results, without having any additional information about the compound.

Another technique that is comparable with LC-MS in terms of sensitivity is coulometric electrode array detector (a type of Electrochemical Detector) coupled to HPLC. Because of a high selectivity and sensitivity, HPLC with electrochemical

detection has become increasingly popular for the analysis of natural antioxidants in food and beverages. It is frequently used in the analysis of phenolic compound in fruits (Aaby et al., 2005; Novak et al., 2008), vegetables (Xu et al., 2006), wort (Vanbeneden et al., 2006), beer (Řehov et al., 2004), beverages (Peng et al., 2005; Wu et al., 2007; Novak et al., 2010) and pharmaceutical analyses. Electrochemical detectors provide a new analytical tool for resolving and accurately detecting trace amounts of any electroactive compound in a wide range of samples. However in the literature there is only one study that uses that detector in quantification of olive oil phenolics.

## 2.8 Principle of the Electrochemical Detector

EC detectors apply a voltage at an electrode surface over which the HPLC eluent flows. Electroactive compounds eluting from the column either oxidize or reduce generating a current peak in real time. The amount of current generated depends on both the concentration of the analyte and the voltage applied, with each compound having a specific voltage at which it begins to oxidize (Svendsen 1993).

There are two types of electrochemical detectors that can be coupled to LC. These are amperometric and coulometric detectors. Both are based in the same principle of oxidation/ reduction of the analyte induced by an electrode held at a fixed potential. In an amperometric detector, mobile phase containing analytes flows over a planar electrode and only partial oxidation/reduction occurs, whereas in a coulometric detector the carrier passes through a porous electrode, resulting in an increased reactive surface and therefore almost complete oxidation/ reduction. Thereby the sensitivity of the detector increases (Penalvo and Nurmi, 2005).

The coulometric array detector simply consists of a series of coulometric electrode pairs placed in series performing a multichannel (or array) detection. The system is based on analytical cells containing a reference electrode that sets the electrochemical zero and four working electrodes that measure the redox reaction of interest. Each analytical cell therefore provides four channels and the systems available at present can bear up to four cells. It can be built up to 16 channels (Penalvo and Nurmi, 2005).

## 2.9 Advantages of Electrochemical Detector

-Enhanced Resolving Power: In HPLC with UV or DAD some contaminants in the sample co-elute with peaks of interest even after optimizing the chromatographic

conditions. Under, these conditions coulometric EC array detectors provide the separation and quantification of the co-eluting compounds have different oxidation potentials (Achilli et al., 1993).

-Simplified Sample Preparation: With the increased resolving power of EC array detectors it is possible to separate interfering compounds from the compound of interest. Hence, in many instances sample clean-up procedures can be simplified (Svendsen, 1993).

-The HPLC with ED is more sensitive, selective, and has a lower detection limit than that obtained by diode array detection.

-Confirmation of Compound Purity: When compounds pass through the EC array they are normally detected on three contiguous electrodes. The first electrode will oxidize a small portion of the compound, the second or dominant electrode oxidizes a large portion of the compound and the third electrode oxidizes the remainder of the compound. Pure standards eluting at a given retention time will give a predictable response at all three electrodes and the ratio across these three electrodes remains constant and is independent of concentration. In real samples, compounds eluting at a retention time matching that of the standard can also be 'ratioed' across three electrodes. If the ratio of the unknown matches the ratio of the standard, this can help to confirm the identity of the compound. Any difference in the ratio signifies that either there is some contamination of this compound with another unknown compound or that this compound is not the same as the standard compound (Svendsen, 1993).

### 2.10 Mechanism of Ageing and Ageing Related Theories

Aging is characterized as a progressive decline in biological functions with time. The most prominent characteristics of aging are a progressive decrease in physiological capacity, a reduced ability to respond adaptively to environmental stimuli, an increased susceptibility to diseases, and increased mortality (Farooqui and Farooqui, 2010). Ageing increases the susceptibility to the onset of age related diseases such as Alzheimer's, Parkinson's, diabetes, cancer, autoimmune, and cardiovascular diseases (Kirkwood and Austad, 2000). Aging is associated with several changes in structure and function of different organs and tissues. The liver is the major organ responsible for a number of physiological processes that are crucial to sustaining life. Liver ageing may disturb the liver functions.

In the aging of heart adverse metabolic alterations with complex changes in cardiovascular structure and function take place which can accelerate vascular degenerative diseases (Zhu et al. 2001; Ferrari et al., 2003). In addition, the incidence and prevalence of coronary disease, hypertension, heart failure and stroke increase exponentially with advanced age (Forman et al., 2010). Cardiovascular disease is the leading cause of death and illness in developed countries. It is estimated that by the year 2020 almost 40% of all deaths will be due to cardiovascular disease (Chung et al., 2009).

Many ageing theories were proposed including genome instability theory which postulates that genomic modifications in ribosomal RNA, mitochondrial DNA play an important role in ageing. Telomerase theory is based on the shortening of telomeres due to lack of telomerase activity with ageing. Also it is stated that cell death triggers ageing process (Johnson et al., 1999).

Among them the most accepted theories of aging is the free radical theory, which postulates that aging is the result of the accumulation of cellular and tissue damage caused by the continuous and endogenous generation of reactive oxygen species (ROS) (Sohal, 2002; Harman, 2003; Barja, 2004). Reactive species are produced continuously as a result of normal metabolic processes. At low levels, ROS function as signaling intermediates for the modulation of fundamental cell activities such as growth and adaptation responses.

But if they are produced in excessive amount, ROS contribute to damage in cells and the imbalance between the antioxidant defense system and reactive species is observed. As a result oxidative stress occurs, that contribute to the pathogenesis and progression of age related disorders (Kregel and Zhang, 2007).

Oxidative stress in a physiological setting can be defined as an excessive bioavailability of ROS, which is the net result of an imbalance between production and destruction of ROS (with the latter being influenced by antioxidant defenses) (Hagen, 2003). As a result of a rise in intracellular oxidant concentrations, various cell components are damaged and activation of specific signaling pathways is triggered. Both can influence numerous cellular processes linked to aging and the development of age-related diseases (Finkel and Holbrook, 2000; Valko et al., 2007).

The damage on cell components includes modification of molecules and other cellular components, and is called oxidative damage. Main targets for oxidative damage are proteins, lipids and nucleic acids of living organism as shown in Figure

2.3 and some of their oxidation products are often used as biomarkers of oxidative stress (Blumberg, 2004; Dalle-Donne et al, 2006).

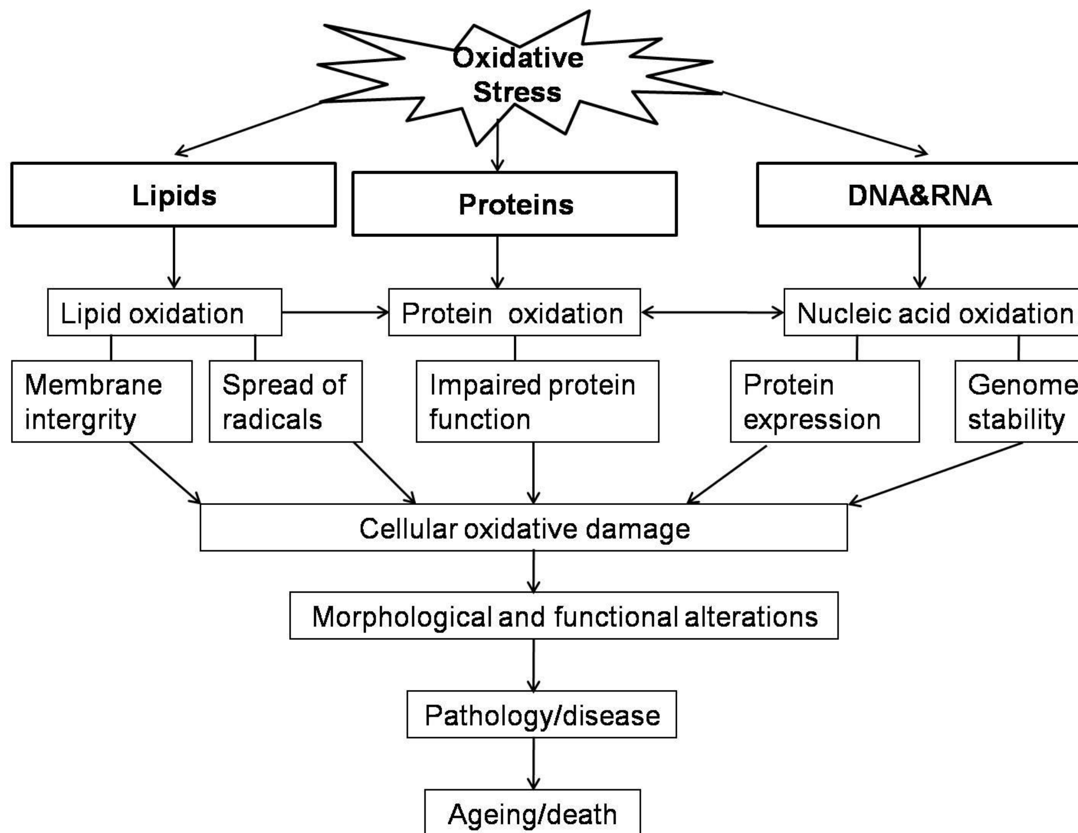


**Figure 2.3:** Main targets of oxidative stress.

Another theory for ageing is the molecular inflammation hypothesis suggesting that there is link between chronic, low grade inflammation and the pathogenesis of age-related diseases, it is based on dysregulation of the immune system with age and altered redox status during ageing. Both processes lead to activation of a wide variety of inflammatory mediators through overproduced reactive species during aging (Chung et al., 2009).

### 2.11 Biomarkers of Oxidative Stress

As a biomarker of oxidative stress there are several parameters. But in our study lipid peroxidation, protein oxidation, proteasomal activity, some biomarkers of inflammation and the mRNA expression level of some genes as a member of antioxidant defense system were investigated.

#### 2.11.1 Lipid peroxidation

Lipids are also important targets for oxidation by radical species. Oxidative stress induced peroxidation of membrane lipids leads to alterations in the biological properties of the membrane, such as fluidity, and can lead to inactivation of membrane bound receptors or enzymes, which in turn may impair normal cellular function and increase tissue permeability (Dalle-Donne et al., 2006).

Lipid peroxidation has been extensively studied biomarker of oxidative stress as polyunsaturated fatty acid components of phospholipids in cellular membranes are sensitive to free radical attack. The most prevalent method is the measurement of lipid peroxidation by the thiobarbituric acid reactive substances (TBARS) assay, in which a color is produced by the reaction of aldhydic groups on products e.g., malondialdehyde (MDA). Besides F2-isoprostanes, and 4-hydroxy-2-nonenol (4-HNE), which arose from free radical initiated oxidative damage of polyunsaturated fatty acids can be measured as a biomarker (Bokov et al., 2004).

#### 2.11.2 Nucleic acid oxidation

The hydroxyl radical is known to react with DNA molecule, damaging the purine and pyrimidine bases and also the deoxyribose backbone. The most extensively studied DNA lesion is the formation of 8-OH-G. Modification of genetic material due to oxidative damage is known to be the first step involved in mutagenesis, carcinogenesis, and ageing. The level of 8-oxo-2-deoxyguanosine (oxo8dG) in DNA has been consistently used as a measure of oxidative damage to DNA in ageing (Valko et al., 2007).

#### 2.11.3 Protein oxidation

As proteins are major components of biological systems and play an important role in many cellular functions such as signal transduction, cellular transport systems, age-related increase in oxidative damage to proteins could be physiologically important. Almost all amino acids in proteins are potential targets for oxidation by ROS (Bokov et al., 2004).

Exposure of proteins to ROS may alter every level of protein structure, causing physical changes in protein structure. Oxidative damage to proteins lead to peptide backbone cleavage, cross-linking, and/or modification of the side chain of amino acids (Stadtman and Berlett, 1997; Davies, 1999). These oxidative modifications, inhibit enzymatic and binding activities, increase susceptibility to aggregation and proteolysis. In addition, accumulation of the modified proteins disrupts cellular

function either by loss of catalytic and structural integrity or by interruption of regulatory pathways.

The most widely studied modification to proteins is the formation of carbonyl derivatives on lysine, arginine, proline, histidine, cysteine, and threonine residues. These biomarkers are useful indicators of redox status and have been shown to accumulate during ageing and age-related disease in variety of organisms (Stadtman and Berlett, 1997).

### 2.11.4 Decreased proteasomal activity

Proteasome is an intracellular proteolytic complex involved in a variety of cellular processes, including the removal of altered forms of protein, growth and differentiation, immune and stress response, signal transduction, aging and prevention of apoptosis (Zeng et al., 2005). The 20S proteasome is the proteolytic core of the proteasomal system. It is composed of two sets of 14 different subunits and has a molecular mass of 670-700 kDa. The subunits form a cylinder of four rings, each containing seven subunits. It has three proteolytic centers showing either peptidyl-glutamyl peptide hydrolyzing (β-1), trypsin-like (β-2), or chymotrypsin-like activity (β-5) based on the proteolysis of peptide substrates for cleavage after acidic basic and hydrophobic residues respectively (Breusing and Grune, 2008). It has been reported that proteasomal activity decreases with age (Viteri et al., 2004; Breusing et al., 2009).

### 2.11.5 Increased inflammation

During ageing reactive species are overproduced due to oxidative stress-induced redox imbalance. The main cause of age-related redox imbalance is the weakened anti-oxidative defense system which activates the immune systems (Brod, 2000). In human studies, circulating levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL- 1β, are well-recognized biomarkers of inflammation. Also inflammatory cell (neutrophils, monocytes) counts increase during aging (Bruunsgaard, 2006). Furthermore, aging is associated with increased levels of C-reactive protein (CRP) and serum amyloid P (SAP) proteins (Bruunsgaard et al., 1999). Chronic inflammation is a major underlying condition of many age-related diseases, such as atherosclerosis, cancer, diabetes, osteoporosis, dementia, vascular diseases, obesity and metabolic syndrome (Yu and Chung, 2006).

### 2.12 Protection from Detrimental Effects of Oxidative Stress

To protect the body from detrimental effects of oxidative stress, the members of antioxidant defense system, including both non-enzymatic (tocopherols, vitamin C, glutathione, etc.) and enzymatic antioxidants (superoxide dismutases SOD, glutathione peroxidases, catalase, etc.) play important roles. In our study vitamin C and vitamin E, glutathione, uric acid, paraoxonase family members (PON-1 and PON-2) and Phase II enzymes were investigated.

#### 2.12.1 Antioxidant defense system

##### 2.12.1.1 Vitamin C (Ascorbic acid)

Ascorbic acid is an essential micronutrient in primates which exhibits some of its biological activity due to its free radical scavenging activity. It is a water soluble chain breaking radical scavenger and recycles plasma membrane α-tocopherol via the reduction of the α-tocopheroxyl radical (May, 2000). Ascorbic acid is transported into mammalian cells either by the Sodium-Vitamin C Transporters, SVCT1 and SVCT2, which cause $Na^+$-dependent uptake of L-ascorbate or glucose transporter (GLUT). Unlike humans, primates and guinea pigs, rodents such as laboratory mice exhibit an intact endogenous ascorbic acid synthesis since they express the rate limiting enzyme of vitamin C synthesis which is gulonolactone oxidase, the last enzyme in ascorbic acid synthesis. It has been reported that primary role of SVCT1 is maintenance of the whole-body homeostasis, through dietary absorption and renal reabsorption, while SVCT2 is crucial for ascorbic acid uptake in metabolically active and specialized tissues, such as eye, brain, skeletal muscle and heart (Savini et al., 2008). Beside Kuo et al. (2004) showed that SVCTs appear to function independently of each other, as SVCT1 expression and the ascorbic acid concentration in SVCT1-predominant organs such as liver.

##### 2.12.1.2 Vitamin E (α-tocopherol)

Vitamin E is a generic name for all substances exerting the biological activity of α-Tocopherol. The eight natural existing Vitamin E-compounds (α-, β-, δ-, γ-Tocopherol and α, β, δ-, γ-Tocotrienol) are only generated in plants and therefore they must be supplied to the body. α-Tocopherol the most abundant form of dietary vitamin E, and the major lipid soluble chain-breaking antioxidant in vivo, protecting membranes from oxidative damage. It has been recognized as a potent inhibitor of lipid peroxidation in cellular membrane through the scavenging of peroxyl radicals

forming tocoperoxyl radicals (Burton et al., 1982). Apart from its antioxidant activity, it has anti-cancer, anti-atherosclerotic, and neuroprotective effects. Beside antioxidant gene-regulatory activities of α-tocopherol have also been described.

Lipoproteins take part in α-tocopherol transport and its metabolism is closely linked to lipid and lipoprotein metabolism and common proteins are responsible for vitamin E metabolism. Vitamin E is absorbed in the intestine and incorporated in chylomicrons. After entering the circulation remnant chylomicron-associated vitamin E is delivered to the liver, where the α-tocopherol form is selectively incorporated into very low-density lipoproteins (VLDL) by the hepatic α-tocopherol transfer protein (TTP) and resecreted into the blood stream. Subsequently, VLDL and low-density lipoproteins (LDL) exchange α-tocopherol with high-density lipoproteins (HDL) (Kayden et al., 1993; Traber and Arai, 1999). α-tocopherol transfer from plasma to cells occurs by lipid transport between lipoprotein and cells. Uptake is facilitated by a) lipid transfer proteins and lipases (lipoprotein lipase-LPL), b) receptor-mediated lipoprotein endocytosis (LDL Receptor-LDLR; LDL receptor-related protein 1-LRP1), c) selective lipid uptake (scavenger receptor class B type I- SR-BI). Intracellular α-tocopherol is subjected to microsomal degradation by the cytochrome P450 family 3A (CYP3A) enzyme subfamily. Delivery of α-tocopherol to liver is facilitated by the activity of the α-tocopherol transport protein (α-TTP) and ATP binding cassette A1 (ABCA1). Excessive amounts of α -tocopherol may be also excluded via the multidrug resistance transporter 1 (MDR1) (Huebbe et al., 2009).

Another compound in vitamin E metabolism is Vitamin C (ascorbate) which regenerate the membrane bound tocoperoxyl radical to tocopherol. In turn, the ascorbyl radical may be regenerated to ascorbate by thiol or polyphenol antioxidants within the so-called antioxidant network (Packer et al., 2001).

#### 2.12.1.3 Glutathione (GSH)

GSH is a tripeptide containing glutamic acid, cysteine and glycine and the predominant non-protein thiol. It has vital functions in the cell, including; the transport of amino acids, maintenance of proteins in a reduced state and protection of cells against reactive oxygen species, drugs or heavy metal ions, detoxifying electrophiles; maintaining of the cellular redox status, maintaining the essential thiol status of proteins by preventing oxidation of -SH groups or by reducing disulfide bonds induced by oxidant stress; scavenging free radicals; providing a reservoir for cysteine; and modulating critical cellular processes such as DNA synthesis, microtubular-related processes, and immune function (Lu, 1999). GSH exists

primarily in two redox forms, i.e., reduced GSH and glutathione disulfide (GSSG, the oxidized form). The GSSG/GSH ratio is considered to be the cellular redox buffer and it can serve as a good indicator of the cellular redox state of the cell. Redox state is a term that has historically been used to describe the ratio of the interconvertible oxidized and reduced form of a specific redox couple. It describes the redox environment of the cell. Redox couples in cells are, responsive to electron flow that changes in the reducing/oxidizing environment (Schafer and Buettner, 2001).

Also aminothiols such as methionine, cysteine, homocysteine take part in the synthesis of GSH, thus in redox state. The aminothiol metabolism that leads to GSH synthesis is given in Figure 2.4.

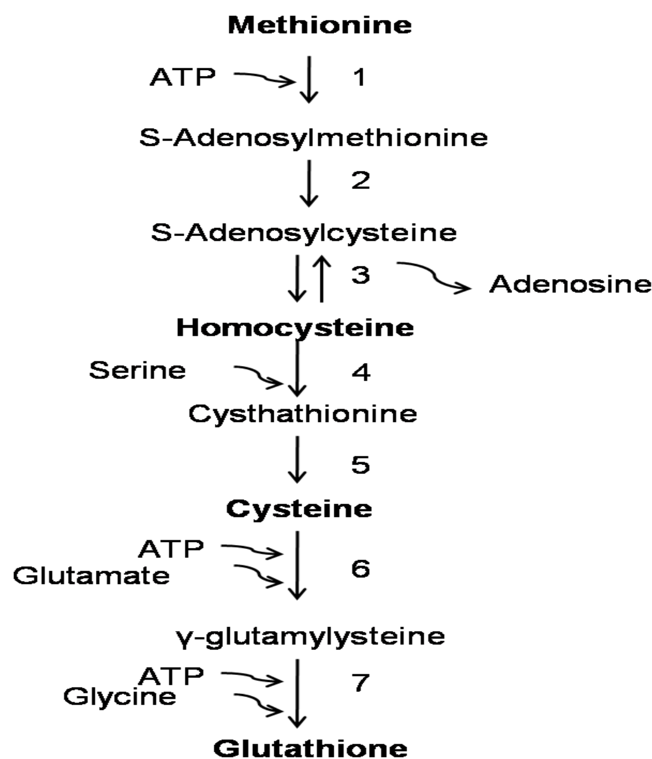


**Figure 2.4 :** Aminothiol metabolism and GSH synthesis 1) Methionine adenosyl transferase 2) transmethylation reactions 3) S- adenosyl homocystein hydrolase 4) cystathione β–synthase 5) γ-cystathionase 6) γ-glutamyl cysteine synthetase 7) GSH synthetase.

#### 2.12.1.4 Uric acid

Uric acid is the final product of purine metabolism in human. In blood, it is a powerful antioxidant and is a scavenger of singlet oxygen, hydroxyl radical, and peroxynitrite. In humans, approximately one half of the antioxidant capacity of plasma comes from uric acid (Becker et al., 1993). Recently it has been stated that uric acid increases

the cysteine uptake and GSH synthesis in brain (Aoyama et al., 2011) and in liver (Allameh et al., 2008).

On the other hand it has been shown that uric acid is an independent risk factor for cardiovascular disease and metabolic abnormalities (Bonora et al., 1996). Increased uric acid levels have been associated with the presence of the metabolic syndrome (Choi et al, 2007). Indeed, hyperuricemia often precedes the development of hyperinsulinemia, obesity, and diabetes (Johnson et al., 2009; Dehghan et al., 2008). It has been reported that uric acid may also induce inflammatory responses, elevate serum triglyceride and cholesterol concentrations, blood glucose levels (Bonora et al., 1996; Lin et al., 2007).

#### 2.12.1.5 Paraoxonase (PON)

Paraoxonases (PON1, PON2, and PON3) are a unique family of calcium-dependent hydrolases, with enzymatic activities toward a broad range of substrates (lactones, thiolactones, carbonates, esters, and phosphotriesters). The activity of PON1 and PON3 are HDL associated proteins that have been reported to contribute to the anti-atherogenic effects of HDL (Ng et al., 2006). In contrast to PON1 and PON3, PON2 does not associate with HDL in the circulation and it is an intracellular membrane-associated protein. PON1 is widely expressed in liver and PON3 in liver and kidney. Beside PON2 is widely expressed in a number of tissues with the highest expression in the heart, brain, liver, lungs, and testis (Devarajan et al., 2011).

PON1 is known as a longetivity gene (Lescai et al., 2009) and loss of PON1 activity with aging has been described in humans (Seres et al., 2004; Jaouad et al., 2006). PON-1 is an enzyme that first synthesized in the liver and subsequently released into the blood (Thomàs-Moyá et al., 2006). It protects LDL and HDL particles from oxidative stress (Aviram et al., 1998) and exerts anti-inflammatory properties (Dullaart et al., 2009). Low serum PON1 activity has been found in numerous pathological conditions, such as cardiovascular disease, type1 and 2 diabetes, hypercholesterolemia, metabolic syndrome and renal failure (Mackness et al., 2003; Lescai et al., 2009). It is suggested that PON2 may protect against oxidative stress (Ng et al., 2005; Reddy et al., 2008), atherosclerosis, and foam cell formation (Rosenblat and Aviram, 2009; Ng et al., 2006) it may act as a cellular antioxidant, play an antiatherogenic role (Rosenblat et al., 2006; Ng et al., 2006). It is reported that PON-2 deficient mice show atherosclerotic lesions with enhanced mitochondrial dysfunction (Devarajan et al. 2011), accumulation of triglycerides in macrophages, liver, aortas (Rosenblat et al., 2009; Meilin et al., 2010), increased atherosclerosis

(Ng et al., 2006). Decreased PON2 mRNA and protein levels were demonstrated in human atherosclerotic plaque areas as compared with plaque-adjacent region and normal artery (Fortunato et al., 2008).

### 2.12.2 Phase II metabolizing enzymes

Phase II metabolizing enzymes are the subgroup of drug metabolizing enzymes, which play central roles in the metabolism, elimination and/or detoxification of xenobiotics or exogenous compounds and drugs introduced into the human body. They protect the body against the potential harmful exposure to xenobiotics from the environment as well as certain endobiotics. In order to minimize the potential injury caused by these compounds, most of the tissues and organs are well equipped with these enzymes (Xu et al., 2005). Some of the Phase II metabolizing enzymes are GST, γ-GCS, NOQ-1, and HO-1.

#### 2.12.2.1 NADPH quionone oxidoreductase (NOQ-1)

NOQ-1 is an enzyme that detoxifies the cycling quinones via reduction thus, it protects cellular membranes against oxidative damage. As a result of reduction, stable hydroquinones are formed that can be readily conjugated and excreted. NOQ-1 is known to have antioxidant properties. It takes part in the regeneration of antioxidant forms of ubiquinone (coenzyme Q) and Vitamin E with the reduction mechanism. It effectively catalyzes the reduction of a-tocopherolquinone (a product of vitamin E) to α-tocopherolhydroquinone, a compound which has antioxidant properties (Ross et al., 2000).

#### 2.12.2.2 Glutathione-S-transferase (GST)

GST is one of the detoxification enzymes that catalyze the conjugation of both endogenous and exogenous compounds with electrophilic moiety, toxic or carcinogenic substrates that cause oxidative stress, with GSH. It has been postulated that GST activity might result in more efficient elimination of carcinogens and ultimately lead to cancer prevention GST catalyzes the addition of the glutathione moiety to a great variety of acceptor molecules. This conjugation is usually considered as a detoxification procedure because conjugate is degraded by the enzymes of the γ-glutamyl cycle (Wilkinson et al., 1997). .

#### 2.12.2.3 Gamma glutamyl cysteinyl synthetase (γ-GCS)

γ-GCS is the rate limiting enzyme in GSH synthesis and its activity is a major determinant of intracellular GSH homeostasis. It is composed of two subunits,

namely catalytic and regulatory subunits. It has been suggested that, without the regulatory subunit, the catalytic subunit may not have any activity under physiological.

#### 2.12.2.4 Hemeoxygenase-1 (HO-1)

HO-1 is the rate limiting enzyme in the oxidative degradation of heme to biliverdin, free iron and carbon monoxide. Free heme can be quite cytotoxic, it can rapidly intercalate with membranes and cause severe damage. Exposure of microsomal membrane fractions to free heme in the presence of $H_2O_2$ leads to a dose-dependent peroxidation of unsaturated lipids in vitro. Heme also catalyzes the protein peroxidation of susceptible groups in membranes (Morita et al., 2005). HO-1 is well known for its antioxidant, anti-inflammatory, antiapoptotic and cytoprotective functions (Ferris et al., 1999; Petrache et al., 2000; Idriss et al., 2008). It may have crucial roles in the pathogenesis of cardiovascular diseases, angiogenesis, diabetes mellitus and cerebrovascular diseases (Idriss et al., 2008).

All of these enzymes, GST, γ-GCS, NOQ-1, PON, HO-1 are regulated under the control of nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor. Nrf2 is an important molecular switch which orchestrates the gene expression of antioxidant and phase 2 enzymes, centrally involved in the ageing process (Lewis et al., 2010; Sykiotis et al., 2011). Nrf2 is a basic leucine zipper transcription factor that binds to the antioxidant response element (ARE) in the promoter region of many adaptive genes, such GST, γ-GCS, NQO1. Under basal conditions, Nrf2 is sequestered in the cytosol. Upon activation, Nrf2 is released, translocates into the nucleus where it heterodimerizes, binds to the ARE and subsequently increases Nrf2 target gene expression (Jaiswal, 2004).

Researchers have demonstrated the role of several genes may modulate aging and influence longevity. The aim is to find the ways for healthy longevity rather than enhancing human longevity (Farooqui and Farooqui, 2010). This may be mediated through modulating age-related genes via phytochemicals, dietary interventions. It has been recently shown that hydroxytyrosol induces antioxidant/detoxificant enzymes and Nrf2 translocation in hepatocytes (Martin et al., 2010). Furthermore hydroxytyrosol induced cytoprotection against oxidative injury in vascular endothelial cells via Nrf2 dependent signal transduction pathway (Zrelli et al., 2011). Little is known about the role of olive oil phenolics on Nrf2 dependent gene expression in vivo.

Apart from Nrf2 and antioxidant enzymes, in recent years many studies have focused on SIRT1 gene, a class III deacetylase, that can modulate numerous longevity promoting cellular functions, endothelial function, lipid metabolism, cell cycling, gene expression, cardioprotection and aging. It decreases glucose levels, improves insulin sensitivity, decreases production of ROS, modulates DNA repair, and apoptosis (Longo and Kennedy, 2006). Furthermore activation of SIRT1 by polyphenols may be beneficial for regulation of oxidative stress, ageing and metabolism (Chung et al., 2010).

There is a great interest in the development of anti-ageing therapies, protecting from the deleterious effects of ageing, and antioxidants have been widely investigated in this respect. Olive oil, a food naturally containing high amounts of phenolic compounds, may be an alternative treatment. To understand the effect of olive oil phenolics on ageing in vivo studies are needed in human ageing models.

### 2.13 Models of Human Ageing and the Senescence-Accelerated Mouse Prone (SAMP)

In order to study human ageing yeasts, worms, flies, cells, rodents (aged rats and mice) are used. Among these models, senescence-accelerated mouse (SAM) is an animal model of human aging that is widely used in aging research.

Rodent models may be used to understand the cellular and molecular mechanisms of age-dependent degeneration, and to develop dietary interventions for healthy ageing (Chiba et al., 2009). The senescence-accelerated mouse (SAM) is a model of accelerated senescence that was established through phenotypic selection from a common genetic pool of AKR/J strain of mice. In 1975, certain littermates of AKR/J mice were noticed to become senile at an early age and had a shorter life span. Five of these litters with early senescence were selected as the progenitors of the senescence-accelerated-prone mice (SAMP). Three litters with normal aging process were also selected as the progenitors of senescence-accelerated-resistant mice (SAMR). Thereafter, selective inbreeding was applied based on the degree of senescence, the lifespan, and the age-associated pathologic phenotypes (Butterfield and Poon, 2005).

Among the two strains of SAMs, namely senescence-accelerated prone mice (SAMP) and senescence-accelerated resistant mice (SAMR), SAMR strain serves as the control strain that ages normally. Compared with the SAMR strain, the SAMP strain has higher oxidative stress (Sato et al., 1996, Rebrin et al., 2005; Álvarez-García, et al., 2006), elevated biomarkers of lipid (Matsugo et al., 2000; Yasui et al.,

2003; Rodriguez et al., 2008) and protein oxidation (Nabeshi et al., 2006; Okatani et al., 2002), inflammation (Tha et al., 2000), mitochondrial dysfunction (Rodriguez et al., 2008; Carratero et al., 2009), deteriorated antioxidative defense systems (Boldyrev et al., 2001), extensive liver dysfunction (Ye et al., 2004), an early onset of atherogenesis (Fenton et al., 2004), ultimately leading to a shorter lifespan (Takeda et al., 1997). These properties render SAMP8 mice as a suitable rodent model for experimental ageing research (Satoh et al., 2004).

It has been reported that antioxidant compounds would have beneficial effects as anti-aging compounds, and several studies have been carried out to ascertain this in SAMP mice. Successful results were obtained by melatonin (Carratero et al. 2009, Garcia et al., 2010, Cuesta et al., 2010), growth hormone (Forman et al., 2010), Oligonol (Tomobe et al., 2007), Pycnogenol (Liu et al., 1998), green tea catechins (Unno et al, 2004; Kichido et al., 2007), mulberry extract (Shih et al., 2009), proanthocyanidins (Gong et al., 2008), lipoic acid and N-acetylcysteine (Farr et al., 2003) as well as caloric restriction (Komatsu et al., 2008; Tajes et al., 2010) and exercise in combination with resveratrol (Murase et al., 2009). Most of these studies focused on brain aging but there is a need to research cardiological ageing that lead to atherosclerosis and coroner heart diseases, liver aging and associated pathophysiological processes in SAMP mice.

## 2.14 In Vivo Studies Related to Effects of Olive Oil on Ageing

Studies related to effects of olive oil on ageing are scarce. In the literature there are a few studies that are performed with aged rats. To our knowledge there is no study investigating the effect of olive oil on aging related disorders in SAMP8 mice but studies are warranted in these mice. Pitozzi et al. (2010) treated the aged rats with high fat feed containing phenol rich extra virgin olive oil and examined the parameters related to oxidative stress. At the end of the study DNA damage, lipid peroxidation, GSH and GSSG levels, and glutathion peroxidase, superoxide dismutase (SOD) and xanthan oxidase (XO) activity in the cerebral cortex, were not different among the groups. Also extra virgin olive oil phenols did not exert protective actions on motor and cognitive functions of the ageing brain, but it is reported that they might have an anxiety-lowering effect associated with decreased glutathione reductase activity and expression in the brain. In the similar study the endogenous levels of DNA breaks, DNA oxidized bases and TBARS in the liver did not differ among the treatment groups of aged rats that fed corn oil, olive oil with high phenolic content and olive oil with low phenolic content. However, a significant

increase in SOD activity and decrease in XO activity were found in the rats fed with high phenolic olive oil. In that group a decrease in DNA oxidative damage in blood cells and plasma TBARS levels, lower incidence of hypophyseal tumors and ulcerative dermatitis were also observed that increased with aging (Jacomelli et al., 2010). In both studies it is reported that the high calorie diet containing large amounts of fat overwhelmed the possible beneficial effect of olive oil phenols. Quiles et al. (2004) have investigated the effects of lifelong olive oil feeding in rats, and found increased antioxidant capacity, reduced DNA damage and improved plasma lipid profile as compared to sunflower oil. Bello et al. (2006) reported a regulatory effect of olive oil on hepatic apoptotic pathways in aged rats with increased caspase-9 and caspase-3 activities as compared to rats fed with sunflower oil. Olive oil also decreased Bcl-2 levels, increased membrane bound Bax and decreased plasma membrane $Mg^{2+}$-dependent neutral sphingomyelinase. The higher CoQ levels found in membranes from the olive oil group in aged rats was also contributed to a better antioxidant protection.

### 2.15 Aim of the Study

Olive oil phenolics are well known for its antioxidant activities. But their role in metabolic pathways, their effect on transcription factors, gene expressions need to be investigated. In this study it is aimed to study the effect of diet rich in olive oil phenolic compounds on age-related changes in heart and liver tissues of SAMP mice. As oxidative stress and inflammation play an important role in the pathogenesis of ageing and age-related disorders, the parameters regarding these factors were investigated. In both tissues Nrf2 and the genes that are controlled under that transcription factor was the main focus. Their amounts were determined both in mRNA level and protein level. Furthermore the level of antioxidant vitamins (vitamin C and vitamin E) was measured. As a biomarker of oxidative stress, lipid oxidation was determined by TBARS assay, protein oxidation was determined by measuring protein carbonyl levels. Also proteasomal activity of different subunits in the proteasomal system was measured.

Another aim of that study is to develop and validate a method with the sensitiveelectrochemical detector for the quantification of olive oil phenolics in oils

# 3. MATERIALS AND METHOD

## 3.1 Chemicals and Reagents

HPLC grade methanol was obtained from J.T. Baker (Deventer, The Netherlands) and acetonitrile was obtained from Sigma Aldrich (Steinheim, Germany). Standards of tyrosol (CAS no. 501-94-0), vanillic acid (CAS no. 121-34-6), caffeic acid (CAS no. 331-39-5), p-coumaric acid (CAS no. 501-98-4), and ferulic acid (CAS no. 1135-24-6) were purchased from Sigma Aldrich (Schnelldorf, Germany). Oleuropein (CAS no. 32619-42-4) and hydroxytyrosol (CAS no. 10597-60-1) were supplied by Extrasynthese (Genay Cedex, France) and pinoresinol by Separation Research (Turku, Finland). Reference standards of all analyzed compounds were HPLC grade with purity higher than 98% (except oleuropein, >90%). Stock solutions were prepared in methanol and stored at -20°C. HPLC grade 60% perchloric acid was obtained from Fisher Scientific (Leicestershire, UK).

For the protein carbonyl quantification potassium chloride, mono potassium phosphate, sodium chloride, sodium phosphate dibasic dihydrate, disodium phosphate, monosodium phosphate and guanidine hydrochloride, citric acid, sulfuric acid, hydrogen chloride, sodium hydroxide, bovine serum albumin and Roti Quant© were purchased from Carl Roth GmbH (Karlsruhe, Germany). Tween 20, 2,4 dinitrophenylhydrazine, biotin-conjugated rabbit IgG polyclonal antibody raised against a DNP conjugate of keyhole limpet hemocyanin (anti-DNP), streptavidinbiotinylated horseradish peroxidase, and o-phenylenediamine were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Hydrogen peroxide was obtained from Merck KGaA (Darmstadt, Germany).

## 3.2 Sampling of Olive Oil

In total 57 olive oils from Italy, Spain, France, Turkey, Portugal, Australian, New Zealand, South Africa which were directly collected from producers or supermarkets were analysed in terms of their phenolic content. The oil with the lowest (Coopérative Oléicole du Canton d'Ollioules, France) and highest total phenolic content (Fratelli Ferrara s.a.s, Italy) was then chosen for the feeding study in mice.

### 3.3 Preparation of Olive Oil Extracts

The phenolic fraction of the oil was obtained by solid-phase extraction (SPE) using the diol-bonded SPE cartridges (Varian, Bond Elut Diol 500 mg/3 mL) according to the procedure of Mateos et al. (2001). As a modification oil sample of 1.5 g was mixed with 1.5 mL hexane. The phenolic extract was redissolved in 1 mL methanol. Samples were filtered through 0.2-μm PTFE Membrane filter (Phenomenex, USA) into the vial.

### 3.4 Spectrophotometric Determination of Total Phenolic Content

The total phenolic content of the extracts was determined according to the Folin-Ciocalteu spectrophotometric method (Singleton et al., 1999). Diluted Folin-Ciocalteu reagent and 20% sodium carbonate solution were added to the phenolic extract. The solution was left in the dark for 45 min. The absorbance of each sample as well as gallic acid standards was determined at 750 nm. The results were expressed as mg gallic acid/kg of oil.

### 3.5 HPLC Analysis of Olive Oil Phenolics

The HPLC analyses were carried out on a Jasco system (Jasco GmbH Deutschland, Gross-Umstadt, Germany) consisting of a pump (PU-2085), autosampler (XLC-3059AS) with integrated column heater, while detection was carried out on 8-channel ESA Model 5600A CoulArray Detector (ESA Inc., Chelmsford, MA, USA). For analyte detection, increasing potentials of +250, +400, +500 and +750 mV were applied on channels. Separation of compounds was carried out on a fused core type column (Kinetex C18, 2.6μm, 100×4.6mm, Phenomenex, USA). Column temperature was maintained at 40°C and flow rate was 1.25 mL/min. Mobile phase was composed of water (solvent A), acetonitrile (solvent B) and methanol (solvent C) each containing 60mM $LiClO_4$. The pH was adjusted to 3.1 with perchloric acid, and the eluent was vacuum-filtered through a 0.2 μm hydrophilic polypropylene membrane filter (Pall Corporation, Michigan, USA). Separation was achieved by elution gradient using an initial composition of 96% A 3% B 1% C. The composition changed to 85% A 10% B 5% C at 4 min, 82.5% A 11.5% B 6% C at 6 min, 65% A 24% B 11% C at 15 min and finally increased to 85% B 15% C at 21 min and maintained for 5 min. Initial conditions were reached in 5 min. The flow rate was 1.25 mL/min and injection volume was 10μL.

## 3.6 Method Validation

The analytical method for the determination of olive oil phenolics was validated according to the requirements of FDA (FDA, 2001) in terms of linearity, selectivity, lower limit of quantification and detection, accuracy, precision, recovery, stock solution stability, short term stability, freeze thaw stability and post preparative stability.

### 3.6.1 Selectivity

For evaluation of selectivity, phenolic compound free olive oil (Sigma Aldrich, Steinheim, Germany) extracted and analyzed to check for possible interference with the retention time of the analytes.

### 3.6.2 Linearity, lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

The linearity range of the detector response was performed by serial dilution of a stock solution of the studied compounds. Stock solutions were prepared in methanol and further diluted by $H_2O$/methanol (50:50). Regression parameters were calculated for calibration curves. All the calibrations curves were linear over the range of study and were calculated by using seven points at different concentrations.

The lower limit of quantification is defined as the concentration of the lowest standard in the analytical run which is quantified with a deviation of the actual concentration and a coefficient of variation of precision of less than 20%. The limit of detection (LOD) was the lowest detectable analyte concentration at the retention time where no peak of the analyte was observed.

### 3.6.3 Accuracy

The accuracy of the method was determined by five replicate analyses of quality control samples, which were obtained by spiking the phenolic free olive oil with known amounts of olive oil phenolic standards at three concentrations (low, medium, high). The olive oil was extracted and the calculated analyte concentrations of spiked oil samples were compared with the theoretical concentration values. The highest amount of phenolics added to the olive oil were determined according to the literature in order to study with realistic values. This was diluted by 4 for the middle concentration and by 20 for the low concentration.

### 3.6.4 Precision

The precision of the method was determined by five replicate analyses of quality control samples, which were obtained by spiking the phenolic free olive oil with known amounts of olive oil phenolic standards at three concentrations (low, medium, high) on three consecutive days. The olive oil was extracted and the calculated analyte concentrations of spiked oil samples were compared with the theoretical concentration values within the same day (inter-day) and from day-to-day (intra-day) for three consecutive days. Values should not deviate by more then 15 % (high and medium concentrations) or 20 % (low concentration). The highest amount of phenolics added to the olive oil were determined according to the literature in order to study with realistic values. This was diluted by 4 for the middle concentration and by 20 for the low concentration.

### 3.6.5 Recovery

The recovery of phenolics calculated by analyzing five replicates for each concentration (low, middle, high) and comparing the detector responses with those of calibration standards with identical concentrations.

### 3.6.6 Stock solution stability

Inject stock solution (if necessary prepare appropriate dilution) into HPLC and store aliquots of the stock at -20°C and room temperature for 24 h. Compare initial detector response to that after 24 h storage. The stability of stock solution were determined for three concentration levels (low, medium and high) after storage at room temperature, 4°C, -20°C, -80°C for 24 h.

### 3.6.7 Short term stability

Five aliquots of spiked oil samples were prepared at 3 concentrations (low, medium and high). The first aliquot was analysed at 0h and remaining samples were stored at room temperature, 4°C, -20°C, -80°C for 24 h. To determine stability initial values were compared to those obtained after storage at different temperatures.

### 3.6.8 Freeze thaw stability

Three aliquots of spiked oil samples were prepared at 2 concentrations (low and high). Oil samples were analysed at 0h and the remaining three aliquots were frozen at -80°C for 24 h, thawed at room temperature and frozen again for 12-24 h at -80°C. The freeze-thaw-cycle were repeated two additional times. The stability was

determined by comparing initial values to those obtained after repeated freezing-thawing.

### 3.6.9 Post preparative stability

Three samples of spiked olive oil with low and high concentrations were prepared in order to determine the stability of processed samples in the autosampler. Samples were injected from the same HPLC vial at 0h and compared with the injections at 6h intervals (6h, 12h and 18h). The cups of HPLC vial caps were replaced after each injection to minimize solvent loss.

## 3.7 FRAP and TEAC Assays

The FRAP assay was conducted according to Benzie and Strain (1996), using ascorbic acid as a reference. The TEAC assay was conducted as described by Miller et al. (1996) using Trolox as a reference.

## 3.8 Animals and Study Design

The animal experiment was performed according to German animal welfare laws and regulations and with permission of the appropriate authorities. Twenty two female SAMP8, aged 9-10 weeks were obtained from Harlan Winkelmann GmbH (Borchen, Germany). The mice were housed in groups in type II polypropylene cages equipped with softwood bedding, a water bottle, a mouse house and a table tennis ball in a climate-controlled room (temperature, 22 ± 2°C; humidity, 55 ± 5 %) with a 12 h light/dark cycle. One of the SAMP8 mouse died during the experiment. Mice were fed with pelletized Western type diet (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) with 0.15 % cholesterol and 20 % fat, in which 10% of fat was from olive oil. Mice were randomly divided into two groups. All animals had free access to feed and water. Food intake was controlled daily and body weights were controlled weekly during the 4.5 month feeding trial. At the end of the experiment the mice were starved before anaesthesia by $CO_2$ and decapitated. Blood samples were collected in tubes and serum was separated by centrifugation (Eppendorf 5804 R, Rotor F34-6-38, Wesseling-Berzdorf, Germany). Tissues were excised in liquid nitrogen and stored at -80°C until analysed.

### 3.9 PON1 Arylesterase Activity in Serum

Arylesterase activity was measured by using phenylacetate as an artificial substrate for PON1. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm. The assay mixture included 4 mmol/L of phenylacetate and 1 mmol/L of CaCl2 in 20 mmol/L of Tris HCl, pH 8.0. Non-enzymatic hydrolysis of phenylacetate was subtracted from the total rate of hydrolysis. One unit of arylesterase activity is equal to 1 µmol of phenylacetate hydrolyzed per minute per milliliter (Fuhrman et al., 2006).

### 3.10 Liver and Heart Tissue Preparation

A ratio of 1:10 homogenates of liver and 1:12 of heart were prepared with ice cold phosphate buffered saline (PBS) buffer (pH 7.4) at 26.000 rpm with a Miccra D-8 homogenizer (ART Prozess- & Labortechnik GmbH & Co. KG, Mullheim, Germany). Homogenates were centrifuged at 4,800 *g* at 4°C for 10 min (Eppendorf 5804 R, Rotor F34-6-38, Wesseling-Berzdorf, Germany) and the supernatant was stored at -80°C until further use.

### 3.11 Lipid Peroxidation

Lipid peroxidation was assayed fluorometrically with Tecan Infinite 200 microplate (Tecan Group Ltd., Crailsheim, Germany) as thiobarbituric acid reactive substances (TBARS) in heart homogenates after protein precipitation with TCA and extraction in 1-butanol. Excitation and emission wavelengths were 520 nm and 560 nm, respectively. Calibration curve was prepared with TEP (1,1,3,3-tetraexthoxypropane) as an external standard (Morel et al., 1983).

### 3.12 Quantification of Protein Carbonyls

Protein carbonyl content was determined in the homogenized heart tissue supernatant according to the ELISA method by Buss et al. (1997) with required modifications. The detection system used an anti-dinitrophenyl rabbit IgG-antiserum (Sigma Aldrich, Steinheim, Germany) as the primary antibody and a monoclonal antirabbit IgG antibody peroxidase conjugate (Sigma Aldrich, Steinheim, Germany) as the secondary antibody. Colour change was induced with *o*-phenylenediamine and $H_2O_2$.

### 3.13 Proteasomal Activity

Twenty to fourty mg tissue was homogenized in lysis buffer (250 mM sucrose, 25 mM HEPES, 10 mM magnesium chloride, 1 mM EDTA and 1.7 mM DTT) using a homogenizer (ultra-Turrax®) and afterwards centrifuged at 14,000 x g for 30 min. The supernatant was used for determination of protein content using the Bradford assay and for measurement of the proteasomal activity. For proteasomal activity, samples were incubated in 225 mM Tris buffer (pH 7.8), 45 mM potassium chloride, 7.5 mM magnesium acetate, 7.5 mM magnesium chloride and 1 mM DTT. For the peptidyl-glutamyl like-(β1), trypsin like-(β2) and chymotrypsin like-(β5) activity the substrates Z-Leu-Leu-Glu-MCA (Biochem, Boston, USA), Ac-Arg-Leu-Arg-MCA (Biochem, Boston, USA) and N-Succinyl-Leu-Leu-Val-Tyr-MCA (Sigma Aldrich, Steinheim, Germany) were used, respectively. MCA liberation of the substrates was measured with a fluorescence reader at 360 nm excitation and 460 nm emission. Free MCA was utilized as a standard (Breusing and Grune, 2006).

### 3.14 α-Tocopherol Analysis

Tissue samples (50 mg) were placed in a test tube with screw-cap and 2 mL ethanol (EtOH) containing 1% ascorbic acid (w/v) and 700 µL H2O. Three hundred microlitres of saturated potassium hydroxide (KOH) solution was added, closed, vortexed for 10 s and incubated for 30 min at 70°C in a shaking water bath. Thereafter samples were immediately chilled on ice and 50 µL butylated hydroxytoluene (BHT)-solution (0.1% BHT in EtOH, w/v) and 2 mL n-hexane were added. Test tubes were mixed per hand and centrifuged 5 min at 1500 rpm. 500 µl of the supernatant was transferred to a clean test tube and dried at room temperature (Savant SpeedVac; Thermo, Langenselbold, Germany). Samples were reconstituted with 150 µL MeOH: $H_2O$ (98:2, v/v), and stored at 20°C until analysis. For quantification of the tocopherols, 40 µl of the sample were injected into an HPLC system (Jasco, Gross-Umstadt, Germany; pump PU2080Plus, autosampler AS2057Plus, detector FP2020Plus) and separated on a Waters Spherisorb ODS-2 column (3µm, 100x4.6 mm) by isocratic elution at a flow rate of 1.2 mL/min with MeOH: $H_2O$ (98:2, v/v) as mobile phase. The fluorescence detector was set to an excitation wavelength of 290 nm and an emission wavelength of 325 nm. Peaks were recorded and integrated using the chromatography software Jasco ChromPass. The concentrations of α- and γ-tocopherol were quantified using an external standard curve with tocopherols (Tocopherol set, Calbiochem®,

Schwalbach, Germany). The same protocol was applied for the quantification of α-tocopherol in olive oils.

### 3.15 Quantification of Ascorbic Acid, GSH, GSSG, Uric Acid, Cysteine Homocysteine and Methionine by HPLC with Coulometric Electrochemical Detection

Heart and liver tissue homogenates were prepared as described by Rebrin et al. (2005). The chromatographic separation was carried out with an autosampler 851-AS (Jasco GmbH Deutschland, Gross-Umstadt, Germany) and a pump 510 (Waters, Milford, MA, USA) on a Trentec Reprosil-Pur 120 C18 AQ column (150x4.6 mm, 3 µm) from Trentec-Analysentechnik (Rutesheim, Germany) at a flow rate of 1 mL/min. The mobile phase for isocratic elution consisted of 50 mM sodium phosphate monobasic and 0.25 % (v/v) acetonitrile, the pH was adjusted to 2.5 with 85 % o-phosphoric acid, and the eluent was vacuum-filtered through a 0.2 um hydrophilic polypropylene membrane filter (Pall Corporation, Michigan, USA). The column temperature was maintained at 40°C and the analytes were quantified with an 8-channel ESA Model 5600A CoulArray Detector (ESA Inc., Chelmsford, MA, USA). For analyte detection, increasing potentials of +50, +175, +350, +600, +750 and +825 mV were applied on channels. Serial dilutions were prepared from stock solutions in PBS/10 % m-phosphoric acid. The injection volume of samples and calibration standards was 10 µL. Each sample was analysed in duplicate.

### 3.16 RNA Isolation and Real-Time Quantitative RT-PCR

RNA was isolated from liver samples (20-30 mg) using TRIsure lysis reagent (Bioline, Luckenwalde, Germany). Real-time quantitative PCR was performed as a one-step procedure (SensiMix One-step Kit; Quantace, Berlin, Germany) with SybrGreen detection, using the Rotorgene cycler (Corbett Life Science, Sydney, Australia). Relative mRNA concentrations of genes were quantified by the use of a standard curve. Target gene mRNA concentration was normalized to the mRNA concentration of the housekeeping gene GAPDH. Primers were designed by standard tools (Spidey, Primer3 and NCBI Blast) and purchased from MWG (Ebersberg, Germany). Primer sequences for analyzed genes are summarized in Table 3.1.

**Table 3.1:** Primers used in real time PCR experiments.

| Primers | Sequence | Annealing temperature (°C) |
|---|---|---|
| ABCA1 | F: CTGCCACCTCCTCAGAGAAA | 55 |
| | R: GCCATACCGAAACTCGTTCA | |
| CRP | F: AGATCCCAGCAGCATCCATA | 59 |
| | R: CAGTGGCTTCTTTGACTCTGC | |
| CYP3A | F: CAAGGAGATGTTCCCTGTCA | 55 |
| | R: CTGGTGATCACATCCATGCT | |
| GAPDH | F: GACAGGATGCAGAAGAGATTACT | 55 |
| | R: TGATCCACATCTGCTGGAAGGT | |
| γ-GCS | F: AACACAGACCCAACCCAGAG | 59 |
| | R: GTCTGGCTGAGAAGCCTTTG | |
| Gulo | F: CCAAGATCACACCAACAAGG | 57 |
| | R: GTCTTGGACATGCTGCTTGA | |
| GST | F: TACTTTGATGGCAGGGGAAG | 58 |
| | R: TCATCCCGTCGATCTCTACC | |
| HO-1 | F: GAGCCTGAATCGAGCAGAAC | 59 |
| | R: AGCCTTCTCTGGACACCTGA | |
| LCAT | F: TAGCAGGCTACCTGCACACA | 59 |
| | R: GCCAGCCAGCTTCTGTAGT | |
| LDLrec | F: AAGCTGTGACCGATGAGGAG | 55 |
| | R: CATTGTTCAGGCCCTGCTTA | |
| LPL | F: ATCAACTGGATGGAGGAGGA | 58 |
| | R: CAGCTGGATCCAAACCAGTA | |
| LRP-1 | F: AAGCTGTGACCGATGAGGAG | 55 |
| | R: CATTGTTCAGGCCCTGCTTA | |
| MDR-1 | F: GCCAGTATTCTGCCAAGCAT | 55 |
| | R: CTATGAGCACACCAGCACCA | |
| NQO-1 | F: TTCTTCTGGCCGATTCAGAGT | 55 |
| | R: TCCAGACGTTTCTTCCATCC | |
| Nrf2 | F: GGGGACAGAATCACCATTTG | 57 |
| | R: GATGCAGGCTGACATTCTGA | |
| PON1 | F: CAGCCTGTCCATCTGTCTCA | 59 |
| | R: CACCCGTCTCGATTCCTTTA | |
| PON2 | F: ATGGTGGCTCTGAGTTTGCT | 57 |
| | R: TCCTCAGCTCCAGTTTCGAT | |

**Table 3.1 (continued):** Primers used in real time PCR experiments.

| Primers | Sequence | Annealing temperature (°C) |
|---|---|---|
| SAP | F: AAGCTGCTGCTTTGGATGTT | 55 |
| | R: CATTGTCTCTGCCCTTGACA | |
| SIRT1 | F: GTCTCCTGTGGGATTCCTGA | 57 |
| | R: ACACAGAGACGGCTGGAACT | |
| SRB1 | F: GCAAATTTGGCCTGTTTGTT | 55 |
| | R: GATCTTGCTGAGTCCGTTCC | |
| SVCT-1 | F: GGGAAAGCCCTCTTCTTTTC | 57 |
| | R: TGAAGCACGTCAGGTAATGC | |
| SVCT-2 | F: TATTCCTGGGATTGCAGCAC | 57 |
| | R: AAAGCACTGGCCTGAAACAG | |
| α-TTP | F: GCCAAGAAGATTGCTGCTGT | 58 |
| | R: GCAGATGAATCCGGTCCTTA | |

### 3.17 Western Blot Analysis

Liver tissue (20 mg) was homogenized in radio immuno precipitation assay buffer (50mM-Tris-HCl, 150mM-NaCl, 0·5% deoxycholate, 0·1% SDS and 1% NP-40; pH 7·4 with protease-inhibitor cocktail, 1:100; Sigma, St Louis, MO, USA). Lysates were purified by centrifugation (12,000 rpm, 4°C, 20 min) after incubation on ice for 30 min. Total protein concentrations in each lysate were quantified using a BCA Protein Assay kit (Pierce, Rockford, USA). Total protein of the lysates (40 µg per lane) was mixed with loading buffer, denatured at 95°C for 5 min and separated by 12% SDS gel electrophoresis followed by transferring the proteins to a PVDF membrane, which was then blocked for 2h in blocking buffer (3% non-fat milk in Tris-buffered saline, pH 7.4, with 0.05% Tween-20 (TBS/T)). Primary antibodies were diluted in 3% non fat milk (GAPDH (Santa Cruz), 1:500; GST (Abcam), 1:3000; 1:200, NOQ-1 (Santa Cruz), 1:500, HO-1 (Stressgen), 1:1000; Tubulin (Abcam), 1:10000) and the blots were incubated overnight at 4°C. The blots were washed and incubated with the respective IgG secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) in blocking buffer with gentle agitation for 1 h at room temperature. The blots were washed and exposed to Immun-Star Western Chemiluminescent kit (Bio-Rad Laboratories, Hercules, CA, USA) and scanned with a ChemiDoc XRS system (BioRad, Munich, Germany). Digital images were captured and quantified by densitometry using the Quantity-One system (Bio-Rad). Relative concentration of

the proteins was quantified as the ratio between the amount of target proteins and the amount of the housekeeping protein α-tubulin.

### 3.18 Statistical Analysis

Results are expressed as mean values with SEM. Data were tested for normal distribution and analyzed by *t*-test. Statistical analysis was performed using PASW Statistics 18 (IBM, Chicago, IL, USA). Data were analysed for normality of distribution (Kolmogorow-Smirnov and Shapiro-Wik tests) and equality of variance (Levene's test) before the t test for independent samples and in the case of non-parametric data Mann-Whitney U test. Differences were considered significant when the $p$ value was $\leq 0.05$.

# 4. RESULTS AND DISSCUSSION

## 4.1 Method Validation

For the analysis of selected phenolic compounds of olive oil, the developed method was validated in terms of selectivity, lower limit of quantification and detection, accuracy, precision, recovery, short term stability, freeze thaw stability and post preparative stability. Although all the phenolics can be detected in 16 minutes, the total time of analysis for the proposed method is 30 minutes and equilibration time is 5 minutes. The duration could not be shortened due to the fluctiations caused by the fast gradients, which affect the sensitivity of the detector. As compared to the other methods in the literature, which are longer than 40 minutes, this method gives the opportunity to analyze phenolic compounds in shorter time.

### 4.1.1 Selectivity

The analytical method was found to be selective for all the analytes and no endogenous substances were found to interfere with the compound separation. Representative chromatogram of the blank olive oil extract is shown in Figure 3 (appendix A).

### 4.1.2 Linearity, lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

The linearity of the standard curves of the phenolic compounds was detected for ten points measurement. The regression coefficients of all phenolic compounds were over 0.99. The LLOQ and LLOD levels of the phenolics were in ng level. The linear range, LLOQ, LLOD of selected phenolic compounds were given in Table 4.1.

**Table 4.1:** Linear range, LLOQ, LLOD of selected phenolic compounds for the method validation.

| Compound | Linear range (µg/mL-ng/mL) | Regression coefficient | LLOD (ng/mL) | LLOQ (ng/mL) |
|---|---|---|---|---|
| Tyrosol | 16.67-0.3 | 0.999 | 0.065 | 0.3 |
| Hydroxytyrosol | 62.5-15.3 | 0.999 | 0.062 | 15.3 |
| Pinoresinol | 33.33-2 | 0.999 | 0.5 | 2.0 |
| Oleuropein | 222.22-3.4 | 0.999 | 0.11 | 3.4 |
| Caffeic acid | 22.22-5.4 | 0.999 | 0.3 | 5.4 |
| Ferulic acid | 27.78-3.4 | 0.999 | 1.7 | 3.4 |
| Vanillic acid | 40-0.6 | 0.999 | 0.15 | 0.6 |
| p-coumaric acid | 22.22-1.4 | 0.999 | 0.025 | 1.4 |

### 4.1.3 Accuracy, precision and recovery

The proposed method has good accuracies and reproducibility for all the analytes at all three level of concentrations. The within-day and day-to-day precision have coefficient of variation of <15%. The extraction method was able to produce high recoveries (>72%) for all the analytes as well as the internal standards except middle concentrations of ferulic- and caffeic acid which were 65% and 58% respectively. Further details of the accuracy, precision and extraction recovery are shown in Table 4.2. The highest amount of phenolics added to the olive oil were determined according to the literature in order to study with realistic values. But the recoveries of phenolic acids (caffeic acid, ferulic acid, p-coumaric acid) were very bad (130-160%). Therefore the concentration of these phenolics were increased 10 times.

**Table 4.2:** Intraday and inter-day recoveries, accuracies and precisions of selected phenolic compounds of olive oil.

| | Nominal concentration (mg/kg) | Calculated concentration (mg/kg) | Recovery [%] | Accuracy [%] | Precision [% CV] |
|---|---|---|---|---|---|
| | | **Tyrosol** | | | |
| Intraday (n=5) | 1.75 | 1.64 | 93.7 | 6.3 | 2.56 |
| | 8.75 | 6.50 | 74.3 | 25.7 | 10.15 |
| | 35 | 29.89 | 85.4 | 14.6 | 6.93 |
| Interday | 1.75 | 1.68 | 95.8 | 4.2 | 3.77 |
| | 8.75 | 7.24 | 82.7 | 17.3 | 5.73 |
| | 35 | 30.28 | 86.5 | 13.5 | 6.01 |
| | | **Hydroxytyrosol** | | | |
| Intraday (n=5) | 2.5 | 1.92 | 76.6 | 23.4 | 3.64 |
| | 12.5 | 8.05 | 64.4 | 35.6 | 10.34 |
| | 50 | 38.65 | 77.3 | 22.7 | 6.69 |
| Interday | 2.5 | 1.99 | 79.8 | 20.2 | 3.78 |
| | 12.5 | 9.14 | 73.1 | 26.9 | 5.40 |
| | 50 | 39.90 | 79.8 | 20.2 | 6.21 |
| | | **Oleuropein** | | | |
| Intraday (n=5) | 0.6 | 0.61 | 100.9 | -0.9 | 3.27 |
| | 3 | 2.17 | 72.2 | 27.8 | 13.71 |
| | 12 | 10.45 | 87.1 | 12.9 | 3.24 |
| Interday | 0.6 | 0.56 | 94.1 | 5.9 | 5.75 |
| | 3 | 2.19 | 72.9 | 27.1 | 9.45 |
| | 12 | 9.79 | 81.6 | 18.4 | 10.88 |
| | | **Pinoresinol** | | | |
| Intraday (n=5) | 4.75 | 4.12 | 86.8 | 13.2 | 3.57 |
| | 23.75 | 17.71 | 74.6 | 25.4 | 12.12 |
| | 95 | 84.55 | 89.0 | 11.0 | 7.43 |
| Interday | 4.75 | 4.30 | 90.6 | 9.4 | 4.24 |
| | 23.75 | 20.38 | 85.8 | 14.2 | 6.72 |
| | 95 | 89.58 | 94.3 | 5.7 | 6.51 |
| | | **Caffeic acid** | | | |
| Intraday (n=5) | 0.75 | 0.56 | 74.5 | 25.5 | 9 |
| | 3.75 | 2.18 | 58.1 | 41.9 | 11.7 |
| | 15 | 12.58 | 83.9 | 16.1 | 5.44 |
| Interday | 0.75 | 0.57 | 76.4 | 23.6 | 7.68 |
| | 3.75 | 2.76 | 73.5 | 26.5 | 6.16 |
| | 15 | 13.23 | 88.2 | 11.8 | 5.79 |
| | | **Ferulic acid** | | | |
| Intraday (n=5) | 0.75 | 0.67 | 89.9 | 10.1 | 3.85 |
| | 3.75 | 2.47 | 65.9 | 34.1 | 14.10 |
| | 15 | 13.94 | 92.9 | 7.1 | 4.74 |
| Interday | 0.75 | 0.67 | 89.8 | 10.2 | 4.46 |
| | 3.75 | 2.97 | 79.3 | 20.7 | 6.92 |
| | 15 | 13.75 | 91.7 | 8.3 | 5.55 |
| | | **Vanillic acid** | | | |
| Intraday (n=5) | 0.27 | 0.26 | 96.0 | 4.0 | 6.24 |
| | 1.33 | 0.97 | 73.3 | 26.7 | 12.43 |
| | 5.3 | 4.46 | 84.2 | 15.8 | 4.53 |
| Interday | 0.27 | 0.26 | 95.5 | 4.5 | 5.49 |
| | 1.33 | 1.05 | 78.9 | 21.1 | 6.55 |
| | 5.3 | 4.46 | 84.1 | 15.9 | 5.78 |
| | | **p-coumaric acid** | | | |
| Intraday (n=5) | 0.75 | 0.72 | 96.3 | 3.7 | 3.63 |
| | 3.75 | 2.98 | 79.6 | 20.4 | 11.72 |
| | 15 | 14.88 | 99.2 | 0.8 | 4.56 |
| Interday | 0.75 | 0.69 | 91.6 | 8.4 | 4.44 |
| | 3.75 | 3.19 | 85.2 | 14.8 | 6.10 |
| | 15 | 10.80 | 72.0 | 5.0 | 5.37 |

#### 4.1.4 Stability studies

Stability studies carried out to determine whether any sample deterioration occurred during storage and handling. In terms of stability of stock solutions, the values of high and middle concentrations of phenolic compounds at three different temperatures were slightly different from the freshly prepared standards. However great differences observed for degradation of the low concentrations of hydroxytyrosol at -20°C (-18.31), caffeic acid at room temperature (-23.72) and -20°C (-21.02), ferulic acid at 4°C (-12.36) (Table 4.3).

The analytes were found to be stable after three freeze–thaw cycles as well as in autosampler at room temperature (Table 4.4). Regarding the short term stability and freeze thaw stability, the recovered amount of compounds were >85% for all concentrations at tested four different temperatures. In autosampler stability, greater degradation was observed for the low concentration of caffeic acid and oleuropein with 18.18% and 11.09%, respectively (Table 4.5).

**Table 4.3**: Stock solution stability of standards after 24 h storage at room temperature (RT), 4°C, and -20°C.

| | Stock solution stability [Degradation %] | | |
|---|---|---|---|
| | **RT** | **4°C** | **-20°C** |
| **Tyrosol** | | | |
| Low | -6.77 | 3.52 | 5.48 |
| Middle | -2.48 | 1.64 | -0.45 |
| High | -1.68 | -0.89 | -0.74 |
| **Hydroxytyrosol** | | | |
| Low | -0.85 | -6.72 | -18.31 |
| Middle | 0.62 | 2.00 | 0.32 |
| High | -1.31 | -0.20 | 1.52 |
| **Oleuropein** | | | |
| Low | -0.57 | -4.96 | 4.04 |
| Middle | 0.71 | 2.43 | 0.11 |
| High | -1.21 | -0.52 | -0.40 |
| **Pinoresinol** | | | |
| Low | -0.64 | -4.02 | 2.37 |
| Middle | 2.99 | 1.83 | 0.01 |
| High | -1.00 | -0.63 | -1.20 |
| **Caffeic acid** | | | |
| Low | -23.72 | -8.06 | -21.02 |
| Middle | 2.59 | 8.84 | 3.77 |
| High | 1.78 | 1.53 | 2.0 |
| **Ferulic acid** | | | |
| Low | -8.10 | -12.36 | 3.60 |
| Middle | 3.28 | 2.15 | -0.14 |
| High | -1.92 | -1.27 | -0.94 |
| **Vanillic acid** | | | |
| Low | 1.40 | -0.57 | -1.60 |
| Middle | -0.14 | 1.96 | -0.14 |
| High | -0.56 | -0.04 | -1.19 |
| **p-coumaric acid** | | | |
| Low | 9.90 | 0.32 | 2.02 |
| Middle | 1.45 | 2.07 | 0.26 |
| High | -1.72 | -1.19 | 0.21 |

**Table 4.4:** Freeze thaw stability and short term stability of selected phenolic compounds in olive oil at room temperature (RT), 4°C, -20°C, and -80°C.

| | **Short term stability [Degradation %]** | | | | **Freeze thaw stability [Degradation %]** |
|---|---|---|---|---|---|
| **Nominal Concentration [mg/kg]** | **-80°C** | **-20°C** | **+4°C** | **RT** | |
| **Tyrosol** | | | | | |
| 1.75 | -3.21 | -3.70 | -4.05 | 6.12 | 10.08 |
| 35 | -11.49 | -10.15 | -9.10 | -7.46 | 3.95 |
| **Hydroxytyrosol** | | | | | |
| 2.5 | -2.34 | -3.07 | -1.02 | 4.39 | 10.33 |
| 50 | -12.46 | -11.01 | -9.0 | -5.51 | 4.21 |
| **Oleuropein** | | | | | |
| 0.6 | -3.29 | -5.54 | -9.65 | 9.03 | -1.56 |
| 12 | -11.71 | -11.07 | -9.15 | -6.21 | 7.91 |
| **Pinoresinol** | | | | | |
| 4.75 | -6.41 | -6.81 | -2.71 | 6.61 | 9.03 |
| 95 | -14.45 | -13.87 | -12.11 | -9.57 | 1.54 |
| **Caffeic acid** | | | | | |
| 0.75 | -5.79 | -5.47 | -5.14 | 6.82 | 8.05 |
| 15 | -13.73 | -12.05 | -10.11 | -6.15 | 2.25 |
| **Ferulic acid** | | | | | |
| 0.75 | -4.75 | -5.17 | -5.97 | 7.61 | 9.28 |
| 15 | -13.23 | -11.83 | -7.89 | -7.19 | 2.45 |
| **Vanillic acid** | | | | | |
| 0.27 | -5.06 | -4.10 | -5.63 | 7.44 | 11.33 |
| 5.3 | -14.99 | -13.95 | -12.40 | -9.56 | 0.10 |
| **p-coumaric acid** | | | | | |
| 0.75 | -4.48 | -4.89 | -4.89 | 7.54 | 8.90 |
| 15 | -11.64 | -10.34 | -9.10 | -6.26 | -0.21 |

**Table 4.5:** Post preparative stability of selected phenolic compounds in olive oil.

| | Post preparative stability [Degradation %] | | |
|---|---|---|---|
| **Nominal Concentration [mg/kg]** | **6h** | **12h** | **18h** |
| **Tyrosol** | | | |
| 1.75 | 0.00 | 0.24 | 0.63 |
| 35 | 0.28 | 0.70 | 0.70 |
| **Hydroxytyrosol** | | | |
| 2.5 | -2.03 | -3.93 | -0.14 |
| 50 | -0.42 | -0.25 | 1.38 |
| **Oleuropein** | | | |
| 0.6 | -5.64 | -11.09 | 1.32 |
| 12 | 0.05 | 0.21 | 1.43 |
| **Pinoresinol** | | | |
| 4.75 | -1.61 | -1.85 | -0.87 |
| 95 | 0.19 | 0.00 | 0.95 |
| **Caffeic acid** | | | |
| 0.75 | -5.87 | -18.18 | -5.28 |
| 15 | -0.51 | -1.20 | -1.20 |
| **Ferulic acid** | | | |
| 0.75 | -1.12 | -2.77 | -0.12 |
| 15 | 0.57 | 0.57 | 1.26 |
| **Vanillic acid** | | | |
| 0.27 | -0.80 | -6.97 | -0.45 |
| 5.3 | 0.00 | -0.26 | -0.26 |
| **p-coumaric acid** | | | |
| 0.75 | -1.04 | -2.25 | 0.69 |
| 15 | 0.59 | 0.54 | 1.78 |

## 4.2 Application of the Method for the Determination of Selected Phenolic Compounds in Olive Oils

In order to show the applicability of the developed method, the olive oil samples that were collected from different countries were analyzed in terms of selected phenolic compounds. Figure 2 in appendix shows the representative HPLC chromatogram of an olive oil showing the selected eight compounds.

Table 4.6 presents the quantitative results obtained for the analysis of olive oil by HPLC coupled with electrochemical detector. Analyte concentrations were quantified by calibration curves for the phenolic compounds.

**Table 4.6**: FRAP, TEAC, total phenolic, α- tocopherol, γ- tocopherol and phenolic contents of collected olive oil samples. Values are expressed as mean±SD.

| Sample | TP | TEAC | FRAP | α-Toc | γ-Toc | TY | HTY | OP | Pino | CA | VA | FA | p-COU |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 532±10.6 | 1.02±0.14 | 1.28±0.16 | 216±14 | 9.9±1.0 | 20.78± | 18.87± | 1.33±0.03 | 3.56±0.07 | 0.56±0.02 | 0.34±0.00 | 0.09±0.01 | 0.13±0.02 |
| 2 | 396±7.6 | 0.84±0.03 | 0.87±0.01 | 263±20 | 5.8±0.5 | 8.42±0.14 | 4.8±0.04 | 0.76±0.01 | 3.48±0.00 | 0.08±0.00 | 0.56±0.00 | 0.23±0.00 | 1.74±0.03 |
| 3 | 334±6.3 | 0.77±0.03 | 0.89±0.03 | 208±8.3 | 13.2±1.2 | 11.6±0.07 | 10.2±0.01 | 1.90±0.35 | 2.76±0.23 | 0.07±0.00 | 0.20±0.00 | 0.13±0.00 | 0.06±0.00 |
| 4 | 306±4.1 | 0.78±0.03 | 0.83±0.02 | 181±0.3 | 11.2±0.7 | 17.2±0.49 | 25.3±0.49 | 1.27±0.13 | 1.41±0.01 | 0.19±0.09 | 0.21±0.05 | 0.25±0.01 | 0.43±0.01 |
| 5 | 300±6.0 | 0.56±0.03 | 0.63±0.01 | 207±2.8 | 11.5±0.2 | 13.1±0.07 | 9.3±0.07 | 6.62±0.01 | 2.93±0.01 | 0.07±0.02 | 0.16±0.00 | 0.10±0.01 | 0.09±0.01 |
| 6 | 286±6.5 | 0.61±0.02 | 0.72±0.02 | 256±1.5 | 13.5±0.0 | 24.0±0.42 | 22.5±0.92 | 1.03±0.01 | 2.95±0.00 | 0.08±0.00 | 0.25±0.00 | 0.19±0.00 | 0.09±0.00 |
| 7 | 278±8.3 | 0.52±0.03 | 0.57±0.02 | 227±7.8 | 13.3±0.6 | 21.27±1.13 | 11.1±0.42 | 3.91±0.05 | 3.18±0.02 | 0.07±0.00 | 0.19±0.00 | 0.13±0.00 | 0.06±0.00 |
| 8 | 276±4.0 | 0.62±0.05 | 0.65±0.03 | 58±0.7 | 3.8±0.1 | 8.0±0.28 | 10.7±0.18 | 1.36±0.00 | 2.79±0.03 | 0.07±0.00 | 0.79±0.02 | 0.2±0.01 | 0.02±0.00 |
| 9 | 269±5.3 | 0.57±0.01 | 0.59±0.02 | 330±7.9 | 22.4±0.2 | 4.71±0.08 | 3.7±0.01 | 3.49±0.01 | 5.23±0.04 | 0.07±0.00 | 0.79±0.01 | 0.16±0.00 | 0.39±0.01 |
| 10 | 268±7.5 | 0.49±0.05 | 0.54±0.02 | 217±10 | 11.0±0.6 | 27±1.2 | 16.3±0.57 | 10.8±0.13 | 5.74±0.10 | 0.14±0.06 | 0.44±0.02 | 0.21±0.00 | 0.24±0.01 |
| 11 | 265±8.2 | 0.68±0.03 | 0.79±0.03 | 134±6.6 | 5.7±0.4 | 10.9±0.14 | 18.2±0.07 | 1,87±0.01 | 3.97±0.02 | 0.11±0.01 | 0.36±0.00 | 0.16±0.00 | 0.07±0.00 |
| 12 | 254±6.8 | 0.60±0.00 | 0.65±0.01 | 172±4.9 | 25.6±0.7 | 8.1±0.14 | 8.2±0.07 | 1.71±0.01 | 3.38±0.02 | 0.07±0.00 | 0.20±0.00 | 0.14±0.00 | 0.05±0.00 |
| 13 | 237± 6.0 | 0.51±0.02 | 0.59±0.02 | 252±9.5 | 11.9±0.3 | 30.75±0.99 | 16.6±0.35 | 1.39±0.01 | 3.6±0.04 | 0.09±0.00 | 0.34±0.01 | 0.18±0.00 | 0.19±0.01 |
| 14 | 228±5.6 | 0.50±0.03 | 0.61±0.03 | 209±2.0 | 11.3±0.1 | 17.7±0.49 | 22.3±0.49 | 1.46±0.03 | 4.74±0.07 | 0.07±0.00 | 0.25±0.00 | 0.23±0.00 | 0.09±0.01 |
| 15 | 224±6.9 | 0.53±0.01 | 0.61±0.04 | 266±10 | 7.3±1.7 | 22.1±0.3 | 25.7±0.1 | 1.81±0.02 | 4.6±0.01 | 0.11±0.02 | 0.16±0.00 | 0.14±0.00 | 0.12±0.00 |
| 16 | 205±5.9 | 0.48±0.03 | 0.52±0.02 | 182±5.2 | 6.3±0.5 | 11.1±0.03 | 14.1±0.7 | 1.2±0.01 | 3.33±0.06 | 0.09±0.00 | 0.01±0.02 | 0.03±0.02 | 0.4±0.09 |
| 17 | 205±0.7 | 0.50±0.00 | 0.40±0.01 | 280±4.7 | 21.2±0.4 | 4.26±0.30 | 4.1±0.10 | 0.56±0.06 | 6.62±0.18 | 0.17±0.08 | 0.39±0.09 | 0.15±0.01 | 0.01±0.00 |
| 18 | 197±12.4 | 0.42±0.01 | 0.54±0.02 | 232±9.7 | 7.0±0.4 | 15.5±0.00 | 11.8±0.01 | 0.53±0.01 | 3.07±0.01 | 0.19±0.09 | 0.27±0.00 | 0.12±0.00 | 0.02±0.00 |
| 19 | 195±9.2 | 0.41±0.02 | 0.46±0.00 | 259±13.6 | 7.8±0.3 | 20.1±0.21 | 17.2±0.14 | 11.3±0.04 | 4.35±0.04 | 0.07±0.00 | 0.14±0.00 | 0.15±0.00 | 0.03±0.00 |
| 20 | 194±8.7 | 0.35±0.03 | 0.40±0.01 | 256±3.3 | 12.2±0.6 | 29.3±0.07 | 15.2±0.00 | 3.51±0.01 | 3.82±0.00 | 0.09±0.00 | 0.56±0.01 | 0.24±0.00 | 0.21±0.00 |
| 21 | 191±3.9 | 0.44±0.01 | 0.48±0.00 | 218±0.6 | 6.6±0.1 | 12.7±0.14 | 8.9±0.05 | 1.90±0.00 | 3.62±0.01 | 0.09±0.00 | 0.41±0.00 | 0.13±0.00 | 0.06±0.00 |
| 22 | 179±2.7 | 0.41±0.06 | 0.52±0.03 | 199±11.2 | 6.7±0.1 | 16.7±0.21 | 18.7±0.21 | 2.14±0.01 | 13.92±0.06 | 0.08±0.00 | 0.36±0.00 | 0.18±0.00 | 0.05±0.00 |
| 23 | 177±3.7 | 0.45±0.02 | 0.52±0.02 | 120±3.4 | 10.5±0.5 | 5.38±0.19 | 6.6±0.07 | 1.47±0.01 | 7.51±0.09 | 0.07±0.00 | 0.17±0.00 | 0.12±0.00 | n.d |
| 24 | 166±3.0 | 0.37±0.03 | 0.43±0.01 | 192±21 | 6.3±0.6 | 38.36±0.49 | 25.4±0.14 | 2.0±0.01 | 3.89±0.01 | 0.08±0.00 | 0.37±0.00 | 0.17±0.00 | 0.06±0.00 |
| 25 | 163±6.7 | 0.35±0.02 | 0.40±0.01 | 256±4.7 | 8.3±0.5 | 7.38±0.99 | 4.6±0.29 | 1.75±0.04 | 2.86±0.14 | 0.08±0.00 | 0.34±0.16 | 0.11±0.00 | 0.01±0.00 |
| 26 | 162±2.0 | 0.43±0.02 | 0.46±0.02 | 177±1.8 | 4.4±0.3 | 4.05±0.54 | 5.6±0.34 | 0.50±0.00 | 5.34±0.03 | 0.08±0.01 | 0.34±0.06 | 0.14±0.00 | 0.42±0.06 |
| 27 | 159±12.6 | 0.40±0.01 | 0.43±0.03 | 173±11 | 13±0.7 | 9.7±0.42 | 11.2±0.24 | 1.78±0.02 | 1.11±0.02 | 0.08±0.00 | 0.16±0.00 | 0.14±0.00 | 0.06±0.00 |
| 28 | 157±6.6 | 0.39±0.01 | 0.44±0.01 | 147±6.6 | 13.5±0.2 | 8.7±0.42 | 8.2±0.16 | 0.74±0.00 | 2.77±0.00 | 0.09±0.01 | 0.31±0.08 | 0.16±0.00 | 0.17±0.01 |
| 29 | 156±9.0 | 0.40±0.03 | 0.47±0.02 | 133±2.3 | 9.3±0.2 | 19.3±0.01 | 25.3±0.14 | 0.65±0.00 | 3,3±0.00 | 0.09±0.00 | 0.21±0.00 | 0.14±0.00 | 0.09±0.00 |
| 30 | 156±1.8 | 0.36±0.01 | 0.40±0.00 | 239±6.4 | 9.5±1.6 | 14.9±0.78 | 13.3±0.42 | 0.96±0.01 | 3.79±0.08 | 0.08±0.00 | 0.37±0.03 | 0.16±0.00 | 0.08±0.00 |
| 31 | 153±3.5 | 0.33±0.03 | 0.35±0.01 | 215±6.1 | 7.3±0.2 | 13.5±0.14 | 9.6±0.04 | 0.76±0.01 | 2.19±0.01 | 0.08±0.00 | 0.34±0.00 | 0.16±0.00 | 0.03±0.00 |

**Table 4.6 (continued):** FRAP, TEAC, total phenolic, α- tocopherol, γ- tocopherol and phenolic contents of collected olive oil samples. Values are expressed as mean ± SD.

| Sample | TP | TEAC | FRAP | α-Toc | γ-Toc | TY | HTY | OP | Pino | CA | VA | FA | p-COU |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 32 | 153±9.9 | 0.41±0.01 | 0.47±0.01 | 160±3.7 | 9.3±0.6 | 9.19±0.35 | 12.4±0.28 | 0.52±0.00 | 3.06±0.06 | 0.08±0.00 | 0.25±0.00 | 0.13±0.00 | 0.10±0.01 |
| 33 | 147±1.8 | 0.32±0.04 | 0.39±0.02 | 202±6.8 | 7.9±0.4 | 12.1±0.28 | 6.3±0.28 | 0.59±0.01 | 3.85±0.00 | 0.07±0.00 | 0.61±0.00 | 0.16±0.00 | 0.08±0.01 |
| 34 | 145±3.4 | 0.38±0.02 | 0.40±0.01 | 193±12 | 15.1±0.3 | 10.9±0.49 | 17.3±0.42 | 0.59±0.00 | 2.38±0.04 | 0.08±0.00 | 0.25±0.00 | 0.17±0.00 | 0.14±0.01 |
| 35 | 145±2.7 | 0.37±0.02 | 0.42±0.01 | 193±4.9 | 14.5±1.0 | 30.1±0.92 | 19.3±0.42 | 0.49±0.01 | 2.56±0.04 | 0.12±0.00 | 0.30±0.02 | 0.14±0.00 | 0.12±0.01 |
| 36 | 144±2.5 | 0.34±0.00 | 0.37±0.01 | 164±1.1 | 13.7±0.5 | 10.8±0.00 | 9.9±0.03 | 1.99±0.01 | 1.29±0.02 | 0.08±0.00 | 0.19±0.00 | 0.16±0.00 | 0.09±0.00 |
| 37 | 138±2.4 | 0.33±0.03 | 0.42±0.01 | 240±27 | 15.7±1.9 | 10.6±0.28 | 12.3±0.14 | 1.11±0.01 | 10.78±0.12 | 0.08±0.00 | 0.39±0.02 | 0.19±0.00 | 0.30±0.01 |
| 38 | 129±5.8 | 0.28±0.01 | 0.30±0.01 | 154±2.5 | 4.0±1.1 | 3.11±0.07 | 3.4±0.04 | 0.51±0.00 | 4.87±0.03 | 0.07±0.00 | 0.26±0.01 | 0.14±0.00 | 0.29±0.01 |
| 39 | 128±1.4 | 0.19±0.02 | 0.24±0.01 | 289±9.3 | 16.3±0.4 | 23.5±0.21 | 3.7±0.01 | 0.37±0.00 | 4.15±0.02 | 0.09±0.00 | 1.45±0.01 | 0.24±0.00 | 0.13±0.01 |
| 40 | 126±11.1 | 0.28±0.02 | 0.34±0.01 | 120±0.2 | 4.2±0.3 | 19.8±0.07 | 6.8±0.01 | 2.09±0.00 | 3.10±0.01 | 0.07±0.00 | 0.20±0.00 | 0.14±0.00 | 0.10±0.00 |
| 41 | 124±0.9 | 0.22±0.02 | 0.29±0.01 | 264±17 | 6.3±0.4 | 7.3±0.14 | 3.9±0.04 | 0.34±0.02 | 3.5±0.03 | 0.08±0.00 | 0.48±0.01 | 0.16±0.00 | 0.07±0.00 |
| 42 | 122±3.3 | 0.33±0.01 | 0.43±0.01 | 209±4.4 | 6.5±0.2 | 3.96±0.11 | 4.6±0.10 | 0.41±0.00 | 4.57±0.01 | 0.09±0.00 | 0.60±0.00 | 0.18±0.00 | 0.16±0.00 |
| 43 | 121±3.4 | 0.29±0.01 | 0.34±0.00 | 202±0.3 | 14.7±0.7 | 28.9±1.06 | 3.0±0.04 | 0.42±0.00 | 2.27±0.04 | 0.10±0.00 | 0.23±0.01 | 0.13±0.00 | 0.11±0.00 |
| 44 | 118±2.8 | 0.15±0.02 | 0.16±0.00 | 97±2.6 | 4.8±0.1 | 20.4±1.56 | 1.1±0.01 | 0.68±0.06 | 1.43±0.04 | 0.17±0.08 | 0.20±0.02 | 0.15±0.01 | 0.16±0.01 |
| 45 | 116±1.9 | 0.25±0.00 | 0.30±0.00 | 240±9.3 | 8.0±0.4 | 7.32±0.14 | 3.5±0.04 | 1.57±0.02 | 3.11±0.01 | 0.09±0.01 | 0.20±0.00 | 0.12±0.00 | n.d |
| 46 | 114±5.7 | 0.25±0.01 | 0.32±0.01 | 281±8.5 | 12.0±0.3 | 8.64±0.35 | 3.6±0.07 | 0.38±0.01 | 2.94±0.04 | 0.08±0.00 | 0.20±0.00 | 0.15±0.00 | 0.50±0.01 |
| 47 | 112±2.3 | 0.26±0.02 | 0.32±0.01 | 171±1.5 | 6.3±0.2 | 8.2±0.07 | 6.6±0.09 | 0.45±0.00 | 6.02±0.00 | 0.08±0.00 | 0.51±0.00 | 0.20±0.00 | 0.26±0.00 |
| 48 | 109±2.5 | 0.14±0.02 | 0.22±0.00 | 38±0.2 | 4.0±0.1 | 3.61±0.05 | 0.2±0.04 | 0.49±0.00 | 4.32±0.04 | 0.07±0.00 | 0.26±0.01 | 0.13±0.00 | n.d |
| 49 | 107±1.3 | 0.33±0.03 | 0.24±0.01 | 167±6.7 | 9.3±0.3 | 16.2±0.49 | 6.7±0.14 | 2.64±0.55 | 2.17±0.02 | 0.64±0.09 | 0.30±0.17 | 0.19±0.08 | 0.42±0.02 |
| 50 | 104±3.8 | 0.27±0.02 | 0.30±0.01 | 152±5.1 | 3.7±0.2 | 7.1±0.00 | 7.7±0.01 | 2.7±0.01 | 3.5±0.01 | 0.08±0.00 | 0.16±0.00 | 0.14±0.00 | 0.14±0.01 |
| 51 | 101±1.1 | 0.23±0.00 | 0.27±0.01 | 181±7.0 | 8.0±0.3 | 4.60±0.04 | 3.5±0.01 | 2.0±0.00 | 1.87±0.00 | 0.08±0.01 | 0.16±0.00 | 0.12±0.00 | 0.01±0.00 |
| 52 | 100±0.4 | 0.27±0.01 | 0.36±0.00 | 164±1.1 | 13.7±0.5 | 4.1±0.16 | 4.1±0.04 | 0.38±0.01 | 4.49±0.06 | 0.08±0.00 | 0.62±0.01 | 0.19±0.00 | 0.16±0.01 |
| 53 | 91±1.3 | 0.16±0.01 | 0.21±0.00 | 201±4.5 | 5.9±0.1 | 6.7±0.57 | 2.8±0.12 | 0.59±0.04 | 2.17±0.08 | 0.07±0.00 | 0.17±0.00 | 0.13±0.00 | 0.04±0.00 |
| 54 | 88±0.9 | 0.18±0.02 | 0.26±0.00 | 138±2.1 | 5.7±1.2 | 7.9±0.28 | 2.7±0.05 | 0.42±0.00 | 2.13±00.04 | 0.07±0.00 | 0.20±0.02 | 0.17±0.00 | 0.63±0.03 |
| 55 | 83±2.6 | 0.18±0.01 | 0.22±0.00 | 209±1.0 | 14.8±0.2 | 11.7±0.42 | 3.0±0.04 | 0.39±0.01 | 1.50±0.02 | 0.09±0.00 | 0.46±0.00 | 0.16±0.01 | 0.30±0.02 |
| 56 | 69±1.1 | 0.18±0.01 | 0.22±0.01 | 188±1.0 | 7.3±0.1 | 4.51±0.07 | 3.8±0.03 | 0.49±0.00 | 4.26±0.02 | 0.07±0.00 | 0.15±0.00 | 0.12±0.00 | 0.08±0.00 |
| 57 | 45±3.2 | 0.11±0.01 | 0.18±0.00 | 197±15 | 13.2±1.3 | 7.03±0.05 | 0.54±0.01 | 1.19±0.03 | 1.86±0.02 | 0.91±0.03 | 0.15±0.01 | 0.08±0.02 | 0.19±0.00 |

Abbreviations: TP; total phenolic, α-Toc; alpha tocopherol, γ-Toc; gamma tocopherol, TY; tyrosol, HTY; hydroxytyrosol, OP; oleuropein, Pino; pinoresinol, FA; ferulic acid, CA; caffeic acid, VA; vanillic acid, p-COU; p-coumaric acid,n.d; not detected. Values are expressed as mg/kg

## 4.3 Animal Study

In this study, we have focused on investigating the effects of olive oil naturally rich in phenolic compounds on several oxidative stress and inflammation parameters in SAMP8 mice. It is important to take into account that in the present study, we used a Western type diet with 20% fat, in which 10% fat replaced by olive oil and 0.15% cholesterol to trigger the oxidative stress in mice. Western type diet is a human diet of many countries associated with increased risk of atherosclerosis and CHD (Fung et al., 2009; Perona et al., 2010). The content of antioxidant vitamins was kept low but adequate and synthetic antioxidants and flavonoids were completely excluded from the diet in order to minimize their impact on the outcome of the experiment. The two oils that are used in the study were identical for major and minor constituents (Table 4.7).

**Table 4.7:** Concentrations of phenols and TEAC and FRAP values of the high-polyphenol (HP) and low-polyphenol (LP) olive oils.

| | **High-polyphenol olive oil** | **Low-polyphenol olive oil** |
|---|---|---|
| **Phenolic compounds [mg/kg oil]** | | |
| Tyrosol | 20.78 | 7.03 |
| Hydroxytyrosol | 18.87 | 0.54 |
| Pinoresinol | 3.56 | 1.85 |
| Oleuropein | 1.33 | 1.19 |
| Caffeic acid | 0.56 | 0.91 |
| Vanillic acid | 0.34 | 0.16 |
| p-coumaric acid | 0.13 | 0.20 |
| Ferulic acid | 0.09 | 0.08 |
| α-Tocopherol | 217 | 197 |
| **Total phenolics [mg GA equivalent/kg oil]** | 532 | 44 |
| **FRAP [mmol AA equivalent/kg oil]** | 1.27 | 0.17 |
| **TEAC [mmol trolox equivalent/kg oil]** | 1.01 | 0.11 |

### 4.3.1 Body weight and feed intake

At the end of the 4.5-month feeding trial, no differences were observed in the final body weight and daily feed intake of mice fed the HP and LP diets, as shown in Table 4.8.

**Table 4.8:** Body weight and food intake of SAMR1 and SAMP8 mice fed either with high or low phenolic diet.

| | SAMR1 LP | SAMP8 LP | SAMP8 HP |
|---|---|---|---|
| Body Weight (g) | 31.15±0.45 | 29.12±1.31 | 27.19±1.43 |
| Food intake (g/day) | 2.42±0.01 | 3.13±0.07 | 3.26±0.48 |

Values are expressed as mean±SEM (n=10)

### 4.3.2 Effect of age and HP diet on serum parameters

In serum samples of mice PON1 activity was measured as a marker of the age-associated oxidative status. Arylesterase activity was measured, which is more closely related to the levels of PON1 protein (Costa et al., 2005) thus, more indicative of the status of the PON1 enzyme. Significant decrease was found in aged SAMP8 LP mice as compared to SAMR1 LP mice ($p<0.001$). Feeding the mice with a HP diet increased the PON-1 activity in serum of mice ($p<0.05$, Figure 4.1). The change in PON-1 activity may stem from either HDL levels or PON1 transcription in liver. But no significant difference was found either in serum HDL level between groups (Table 4.9) or PON-1 mRNA expression in liver (Figure 4.7).

According to our results the difference in arylesterase activity cannot be attributed to changes in these parameters due to the fact that no significant difference of HDL levels in serum and PON-1 mRNA expression levels were determined in liver. This decline in PON-1 activity may be related to increased oxidative stress with aging (Seres et al., 2004) and the increase in PON-1 activity may be related to effect of HP diet.

In serum, total triglyceride levels did not differ among groups on the other hand total cholesterol levels were significantly decreased in SAMP8 LP mice ($p<0.001$). The effect of age and diet on serum biochemical parameters (total cholesterol, total triglyceride and HDL) is given in Table 4.9.

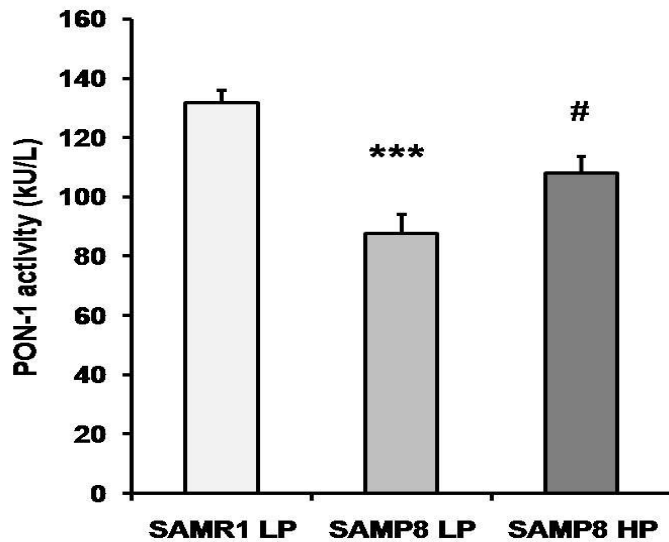


**Figure 4.1:** Mean (±SEM) PON1 activity in serum of SAMR1 and SAMP8 mice fed a Western type diet with 0.15% cholesterol and 20% fat, in which 10% of fat was from olive oil containing either LP or HP (n=9).

**Table 4.9 :** Serum parameters of SAMR1 and SAMP8 mice.

| | SAMR1 LP | SAMP8 LP | SAMP8 HP |
|---|---|---|---|
| HDL [mg/dl] | 92±8.1 | 63.3±15 | 59.5±11 |
| Total cholesterol [mg/dl] | 160±3.1 | 116±6.9*** | 105±2.9 |
| Total triglyceride [mg/dl] | 120±13 | 142±13 | 110±9.3 |

Values are expressed as mean± SEM (n=6-10) ***p<0.001 SAMR1-LP vs SAMP8-LP.

### 4.3.3 Effect of age and hp diet on oxidative stress biomarkers

In order to determine the effect of ageing on lipid peroxidation and protein oxidation, TBARS, protein carbonyl levels and proteasomal activity were determined in liver and heart tissues of mice. In liver significantly higher TBARS ($p<0.01$) and protein carbonyl levels ($p<0.05$) were obtained in SAMP8 LP mice as compared to SAMR1 LP mice (Table 4.10). No effect of HP diet was observed on TBARS levels but level of protein carbonyls decreased with HP treatment ($p<0.05$). In order to detect if the change in protein carbonyl level is related to proteasomal activity, we measured the activity of subunits of proteasomal system. Proteasomal activity of the β-1, β-2 and β-5 subunits in liver tissue remained unchanged (Table 4.10).

In heart TBARS levels were interestingly lower in SAMP8 LP mice as compared to SAMR1 mice ($p<0.05$). As observed in liver, treatment with phenolic rich olive oil significantly decreased TBARS levels of SAMP8 mice as compared to SAMP8 LP mice ($p<0.001$, Table). Protein carbonyl levels were higher in SAMP8 LP than that of SAMR1 LP ($p<0.05$, Table 4.11) and a significant decrease was observed in with

HP diet as compared to LP diet ($p<0.01$, Table 4.11). In terms of proteasomal activities, no significant difference was observed in terms of β-2 and β-5 subunits among groups, whereas activity of β-1 subunit was decreased in SAMP8 LP mice as compared to SAMR1 LP mice ($p<0.05$). No effect of HP treatment was observed in SAMP8 mice ($p>0.05$, Table 4.11).

**Table 4.10:** Concentrations of TBARS and protein carbonyls as well as proteasomal activities in liver homogenates from SAMR1 and SAMP8 mice fed a Western type diet with 0.15% cholesterol and 20% fat, in which 10% of fat was from olive oil containing either LP or HP for 4.5 months.

| | **SAMR1 LP** | **SAMP8 LP** | **SAMP8 HP** |
|---|---|---|---|
| TBARS [nmol/g tissue] | 442±35 | 700±78** | 663±48 |
| Protein carbonyls [nmol/g protein] | 775±36 | 874±36* | 730±46## |
| Proteasomal activity [µmol/mg*min] | | | |
| β-1 subunit | 32.8±2.1 | 31.1±2.9 | 31.2±6.7 |
| β-2 subunit | 6.2±0.2 | 6.1±0.1 | 5.9±0.2 |
| β-5 subunit | 56.7±5.7 | 40.9±3.1 | 49.7±4.2 |

Values are expressed as mean ± SEM (n=10) *p<0.05 SAMR1-LP vs SAMP8-LP, **p<0.01 SAMR1-LP vs SAMP8-LP ##p<0.01 SAMP8-LP vs SAMP8-HP.

**Table 4.11:** Concentrations of TBARS and protein carbonyls as well as proteasomal activities in heart homogenates from SAMR1 and SAMP8 mice fed diets with 10% olive oil for 4.5 months.

| | **SAMR1 LP** | **SAMP8 LP** | **SAMP8 HP** |
|---|---|---|---|
| TBARS [nmol/g tissue] | 563±46 | 428±27* | 274±22### |
| Protein carbonyls [nmol/g protein] | 763±33 | 959±70* | 665±37## |
| Proteasomal activity [µmol/mg*min] | | | |
| β-1 subunit | 29.6±1.0 | 26.1±1.0* | 31.7±3.4 |
| β-2 subunit | 6.9±0.4 | 8.2±0.5 | 6.8±0.5 |
| β-5 subunit | 72.2±6.9 | 93.0±9.2 | 79.3±11 |

Values are expressed as mean ± SEM (n=10) *p<0.05 SAMR1-LP vs SAMP8-LP, ##p<0.01 SAMP8-LP vs SAMP8-HP,### p<0.001 SAMP8-LP vs SAMP8-HP.

Depending on the age and analyzed organs different profiles can be observed in literature regarding TBARS and protein carbonyl levels of SAMP8 mice. Nabeshi et al. (2006) determined higher protein carbonyl levels in liver at 3 and 6 mouth of age however results did not differ at 9 month of age, and in brain higher levels were detected at 3 month of age but no difference was found at 6 and 9 month of age. Higher TBARS level was observed in brain of SAMP8 mice as compared to ddY mice at 11 month of age but no difference was found at 3 month of age. Matsugo et al. (2000) found increased TBARS level in heart at 3 and 6 month of age but the

results did not differ in SAMP8 mice of 9 month of age. In the study performed by Okatani et al. (2002) no difference was observed in TBARS level of SAMP8 brain at the age of 6 and 12 month but protein carbonyl levels significantly increased. In another study no age related difference was found in terms of TBARS and protein carbonyls in brain of SAMP8 mice (Sato et al., 1996). Similarly no effect of treatment and age on protein carbonyl levels was found in the heart of SAMP8 mice by Schiborr et al. (2010).

As a general consensus the decline in proteasomal activity is one of the parameters for oxidative stress, and aging but controversy results are obtained depending on the age, tissue, and different region of tissues. In addition three catalytic sites of proteasome can be differentially affected in aged tissues. It has been reported that proteasomal activity decreased significantly with age in lung and liver samples but not in heart, kidney and skeletal muscle (Breusing et al., 2009). El mohsen et al. (2004) reported that proteasomal enzyme activity was not affected by age in any of the brain regions (cortex, cerebellum and hippocampus) of rats. Also no difference between subunits were found in young and aged Wistar rats (Gavilan et al., 2009), Fisher 344 rats (Kavazis et al., 2007). Age-dependent inhibition of β-5 subunit activity in the retinal pigment epithelium of Fischer 344 rats were reported (Kapphahn et al., 2007). Lower expression of β-2 and β-5 catalytic subunits in peripheral blood T lymphocytes was detected during aging (Ponnapannan et al., 2007). Activities of β-1 and β-2 subunits were diminished with age, while β-5 activity did not exhibit significant alterations in different age groups of Fisher 344 rat hearts (Bulteau et al., 2002). Age-dependent protein modifications and declining proteasome activity in the human lens detected in all catalytic subunits of age group >60 but just β-1 subunit activity declined in age group of 25-55 years (Viteri et al., 2004). By contrast there was a 3-fold increase (Ferrington et al., 2005) and 4- fold increase in β-1 and β-5 subunits in aged muscle of Fischer 344 rats (Husom et al., 2004).

#### 4.3.4 Effect of age and HP diet on redox status

As indicators of the redox status, we measured concentrations of the GSH and oxidized GSH (GSSG), aminothiols (methionine, homocysteine and cysteine) and the antioxidant uric acid in liver and heart. Homocysteine level could not be measured in both tissues as the concentration was above the standard range. No difference was found in GSH, GSSG, GSH/GSSG, methionine and uric acid between groups in liver of mice (Table 4.12). Cysteine level was significantly higher in SAMP8 LP as compared to SAMR1 group ($p<0.05$).

**Table 4.12:** The concentration of GSH, GSSG, GSH/GSSG, cysteine, methionine and uric acid in liver of SAMR1 and SAMP8 mice at 7 month of age.

| | SAMR1 LP | SAMP8 LP | SAMP8 HP |
|---|---|---|---|
| GSH [nmol/mg protein] | 28.9±5.2 | 31.9±3.0 | 38.6±5.9 |
| GSSG [nmol/mg protein] | 0.63±0.11 | 0.94±0.14 | 1.05±0.13 |
| GSH/GSSG [nmol/mg protein] | 38.1±3.7 | 31.3±4.0 | 37.2±3.2 |
| Cysteine [nmol/mg protein] | 0.50±0.07 | 0.68±0.05* | 0.75±0.08 |
| Methionine [nmol/mg protein] | 1.68±0.14 | 1.61±0.15 | 1.74±0.36 |
| Uric Acid [nmol/mg protein] | 0.67±0.13 | 0.88±0.10 | 0.68±0.09 |

Values are expressed as mean± SEM (n=10). *p<0.05 SAMR1-LP vs SAMP8-LP.

In heart GSSG and thus GSH/GSSG could not be measured as the concentration was below the calibration range. No difference was found in GSH and methionine level between groups in heart of mice (Table 4.13). Cysteine and uric acid levels were significantly higher in SAMP8 LP as compared to SAMR1 group ($p<0.05$). Feeding the mice with HP diet decreased both parameters significantly ($p<0.05$) as compared to SAMP8 LP mice.

**Table 4.13:** The concentration of GSH, GSSG, GSH/GSSG, cysteine, methionine and uric acid in heart of SAMR1 and SAMP8 mice at 7 month of age.

| | SAMR1 LP | SAMP8 LP | SAMP8 HP |
|---|---|---|---|
| GSH [nmol/mg protein] | 3.97±0.57 | 4.80±0.57 | 3.66±0.74 |
| Cysteine [nmol/mg protein] | 0.14±0.03 | 0.22±0.04* | 0.14±0.03# |
| Methionine [nmol/mg protein] | 0.27±0.04 | 0.39±0.08 | 0.20±0.06 |
| Uric Acid [nmol/mg protein] | 0.25±0.04 | 0.55±0.13* | 0.23±0.04# |

Values are expressed as mean± SEM (n=10). No significant difference was found.

Cysteine is a sulfydryl-containing amino acid which shows cytotoxicity in vitro and promotes detachment of human arterial endothelial cells in culture (Dudman et al., 1991). It also exhibits autooxidation properties in the presence of metal ions resulting in the generation of free radicals and hydrogen peroxide (Hogg, 1999). It is indicated that cysteine could be atherogenic as it supports superoxide- mediated modification of LDL, thus facilitating foam cell formation (Verhoef et al., 1996; El-Khairy et al., 2001). Furthermore increased cysteine levels associate with cardiovascular diseases (Mills et al., 2000; Jacob et al., 1999). In addition to its antioxidant activity, uric acid is another cardiovascular risk factor which can promote LDL oxidation in vitro and liberate peroxide and superoxide free radical. Hyperuricemia is considered to be a risk factor in many clinical conditions including hypertension, cardiovascular diseases (Nakanishi et al., 2003; Strazzullo and Puig,

2007). In our study elevated levels of cysteine and uric acid was detected in heart of SAMP8 LP group as compared to SAMR1 LP group indicating a susceptibility of SAMP8 for cardiovascular diseases ($p<0.05$). Phenolic rich diet improved the both parameters significantly ($p<0.05$, Table 4.13). Schiborr et al. (2010) did not find any significant difference either between the hearts of SAMP8 and SAMR1 mice or treatment groups regarding cysteine and uric acid levels. Rebrin and Sohal (2004) reported significantly lower levels of cysteine in blood of SAMP mice as compared to C57BL/6 mice at 13 month of age.

#### 4.3.5 Effect of age and HP diet on antioxidant vitamins in liver

Age related alterations of two important endogenous vitamins, ascorbic acid and α-tocopherol, which are related to inflammation and oxidative stress were also measured. Ascorbic acid is an essential micronutrient in primates which exhibits some of its biological activity due to its free radical scavenging activity. It is a water soluble chain breaking radical scavenger and recycles plasma membrane α-tocopherol via the reduction of the α-tocopheroxyl radical (May, 2000). It exhibited a 2 fold increase in SAMP8 LP mice than those in SAMR1 LP. No change was observed in vitamin C level in heart of SAMP8 and SAMR1 mice (Figure 4.2 A-B).

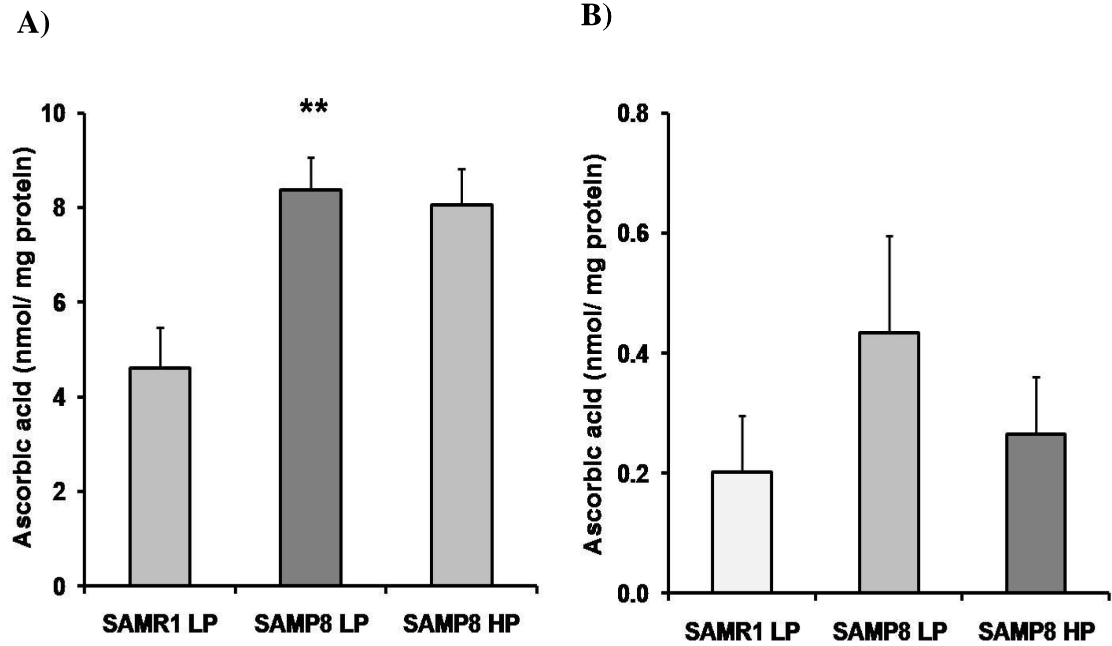


**Figure 4.2 A-B:** Mean (±SEM) vitamin C concentration (nmol/mg protein) in liver A) in heart B) of SAMR1 and SAMP8 mice fed a Western type diet with 0.15% cholesterol and 20% fat, in which 10% of fat was from olive oil containing either low or high amount of phenolics (n=10).

The mechanisms for increased ascorbic acid levels may include increased synthesis and/or transport of that vitamin (Siqueira et al., 2011). Ascorbic acid is transported into mammalian cells by two types of proteins: the sodium-ascorbate co-transporters, SVCT1 and SVCT2, which cause $Na^+$-dependent uptake of L-ascorbate. Gluconolactone oxidase (Gulo) is the last enzyme in ascorbic acid synthesis. Therefore the mRNA expression level of these genes was also measured. The level of SVCT1 mRNA expression was significantly increased in SAMP8 LP mice as compared to SAMR1 but no change was determined in terms of SVCT2 and Gulo (Table). Treatment with HP diet increased Gulo mRNA expression as compared to SAMP8 LP mice but SVCT1 and SVCT2 remained unchanged (Table 4.14).

**Table 4.14:** Relative mRNA expression (normalized for GAPDH) of SVCT1, SVCT2 and Gulonolactone oxidase in liver of SAMR1 and SAMP8 mice fed for 4.5 months a Western type diet with 0.15% cholesterol and 20% fat, in which 10% of fat was from olive oil containing either low or high amount of phenolics. Mice were killed at 7 months of age.

| | **SAMR1 LP** | **SAMP8 LP** | **SAMP8 HP** |
|---|---|---|---|
| SVCT1 | 0.62±0.05 | 0.84±0.09* | 0.80±0.08 |
| SVCT2 | 0.70±0.14 | 0.55±0.10 | 0.70±0.09 |
| Gulonolactone oxidase | 0.54±0.07 | 0.59±0.08 | 0.88±0.07$^{\#}$ |

Values are expressed as mean ± SEM (n=10). *p<0.05 SAMR1-LP vs SAMP8-LP, $^{\#}$p<0.05 SAMP8-LP vs SAMP8-HP.

As ascorbic acid recycles α-tocopherol, we determined the age- and ascorbic acid related change of that lipid soluble vitamin. In the same manner α-tocopherol exhibited a 2 fold increase in SAMP8 LP mice than those in SAMR1 LP mice (Figure 4.3). Also mice fed HP diet exhibited increased α-tocopherol levels as compared to SAMP8 LP mice (Figure 4.3).

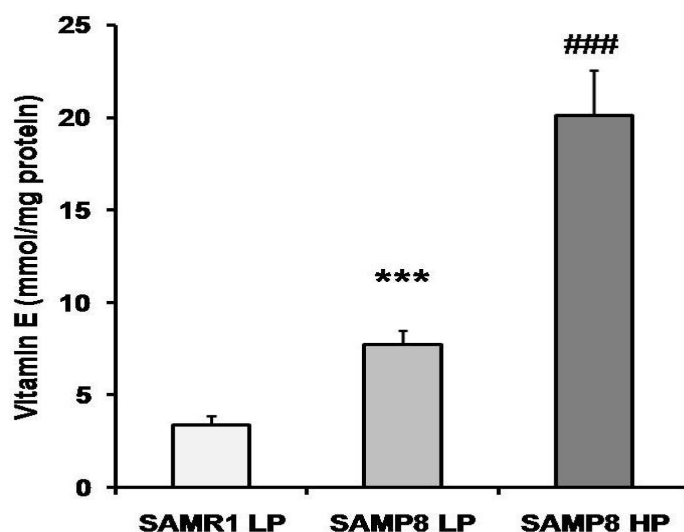


**Figure 4.3:** Mean (±SEM) vitamin E concentration (mmol/mg protein) in liver of SAMR1 and SAMP8 mice fed a Western type diet with 0.15% cholesterol and 20% fat, in which 10% of fat was from olive oil containing either low or high amount of phenolics (n=10).

As far as the antioxidant vitamins concerned ascorbic acid content significantly increased in SAMP8 LP mice. We measured the mRNA level of genes related to ascorbic acid transport or synthesis as an eventual mechanism of the increase. The mRNA expression of dominant ascorbic acid transporter in liver (SVCT1) increased in SAMP8 LP mice. However the expression of SVCT2 remained unchanged in aged mice. This result is in accordance with the study of Michels et al (2003) who

found no age related change in rat hepatocytes. It has been reported that primary role of SVCT1 is maintenance of the whole-body homeostasis, through dietary absorption and renal reabsorption, while SVCT2 is crucial for ascorbic acid uptake in metabolically active and specialized tissues, such as eye, brain, skeletal muscle and heart (Savini et al., 2008). Beside Kuo et al. (2004) showed that SVCTs appear to function independently of each other, as SVCT1 expression and the ascorbate concentration in SVCT1-predominant organs such as liver, was not affected by SVCT2 deficiency. Furthermore no difference was found in Gulo levels in our study suggesting that this increase may not be attributed to de novo ascorbic acid synthesis. Also Lykkesfeldt et al. (1998) reported no age associated alteration in rat hepatic ascorbic acid synthesis.

In addition increased level of α-tocopherol was also observed in our aged SAMP8 mice. This result is in agreement with other studies (Battisti et al., 1994; van der Loo et al., 2002; Petusottir et al., 2007). It is suggested that both ascorbic acid and α-tocopherol levels of SAMP8 mice may increase to counteract the negative effects of age related oxidative stress with their antioxidant activities. The increase in α-tocopherol levels in HP group could not be explained with increased vitamin C level. It is supposed that this increase could be due to difference in vitamin E metabolites.

The membrane bound tocoperoxyl radical may be reduced back to tocopherol by ascorbate (Vitamin C). In turn, the ascorbyl radical may be regenerated to ascorbate by thiol or polyphenol antioxidants within the so-called antioxidant network (Packer et al., 2001). Besides the increase in tocopherol levels may be due to the genes that take part in vitamin E metabolism. Therefore we measured the mRNA expression levels of genes in vitamin E metabolism. As shown in Figure 4.4 A-H no significant change was found for all the genes analysed except the expression of MDR-1 in SAMR1 LP and SAMP8 LP groups, which means the increase in vitamin E is may be due to the regeneration effect of vitamin C.

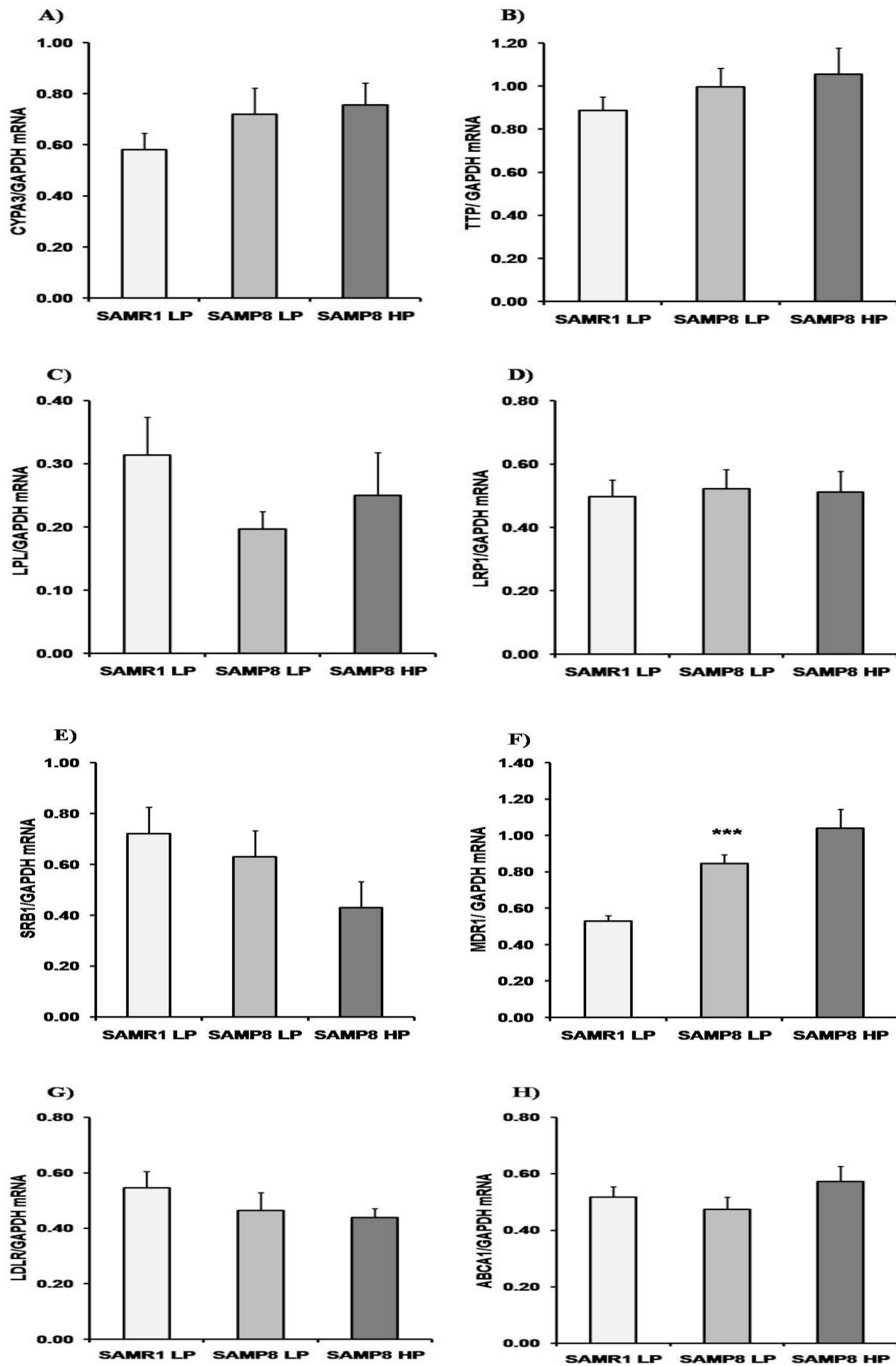


**Figure 4.4 A-H:** Relative mRNA expression of genes in vitamin E matabolism (normalized for GAPDH) of CYP3A (A), TTP (B), LPL (C), LRP1 (D), SRB1 (E), MDR-1 (F), LDLR (G), ABCA1(H) in liver of SAMP8 and SAMR1 mice.

Present data suggest that SAMP8 mice increase hepatic ascorbic acid uptake via SVCT1 as an adaptive response mechanisms. Increased tissue levels of ascorbic may in turn affect Nrf2 dependent gene expression thereby impairing stress response via enzymatic antioxidant defence mechanisms. Ristow et al. (2010) have recently demonstrated that ascorbic acid may interfere with physical exercise related induction of the Nrf2 target genes superoxide dismutase and glutathione peroxidase in humans.

Apart from their antioxidant properties, prooxidant activities of ascorbic acid and α-tocopherol have been described. Ascorbic acid promotes the Fenton reaction in vitro and may accelerate non enzymatic lipid peroxidation in various tissues (Halliwell, 1996). It is also reported that ascorbic acid increases inflammation in ascorbic acid deficient mice (Ki et al., 2010), down regulate key genes connected with free radical scavenging and repair (Selman et al., 2006) and dose dependently causes exacerbating stress contributing to liver injury (Abdul-Rezzak et al., 2010). α-tocopherol gives rise to the formation of tocopheroxyl radicals in increased oxidative stress conditions (Rietjens et al., 2002), it can catalyze hydroperoxide-induced hepatocyte lipid peroxidation and cytotoxicity (Tafazoli et al., 2005). Alternatively high ascorbic acid and α-tocopherol content in SAMP8 mice may also facilitate inflammation and oxidative stress acting as a prooxidant in our study. Further studies are required to measure ascorbic acid and α-tocopherol levels in other tissues of SAMP8 mice to see whether our findings in liver are in consistent with the other tissues regarding oxidative damage. Also the amount of tocopheroxyl radicals can be measured in liver to support our statement.

### 4.3.6 Effect of age and HP diet on antioxidant phase II enzymes

Increased attention has been given to induction of phase II enzymes as an important mechanism for chemoprevention against carcinogenesis, but the studies in terms of correlation between antioxidant enzymes and aging are scarce.

In the present study, we examined expression levels of γ-GCS, GST-α, NQO-1, HO-1, PON-1 (in liver) and PON-2 (in heart) genes as Phase II enzymes in heart and liver to evaluate the antioxidant capacity. These genes are modulated by ARE (antioxidant response element) via binding of Nrf2 transcription factor. Nrf2 is an important molecular switch, which orchestrates the gene expression of antioxidant and phase II enzymes (Lewis et al., 2010; Sykiotis et al., 2011). Little is known about the role of olive oil phenolics on Nrf2 dependent gene expression in vivo.

In our study no significant difference was found between SAMR1 LP and SAMP8 LP mice in terms of γ-GCS, GST-α, PON-2 and Nrf2 expression levels. On the contrary NQO-1 mRNA level was significantly lower ($p<0.001$) and HO-1 level was significantly higher ($p<0.05$) in SAMP8 LP group. Treatment with phenolic rich olive oil improved the levels of γ-GCS and GST-α ($p<0.01$), NOQ-1, PON-2 and Nrf2 ($p<0.05$) significantly in heart (Figure 4.5). No effect was observed in HO-1 level.

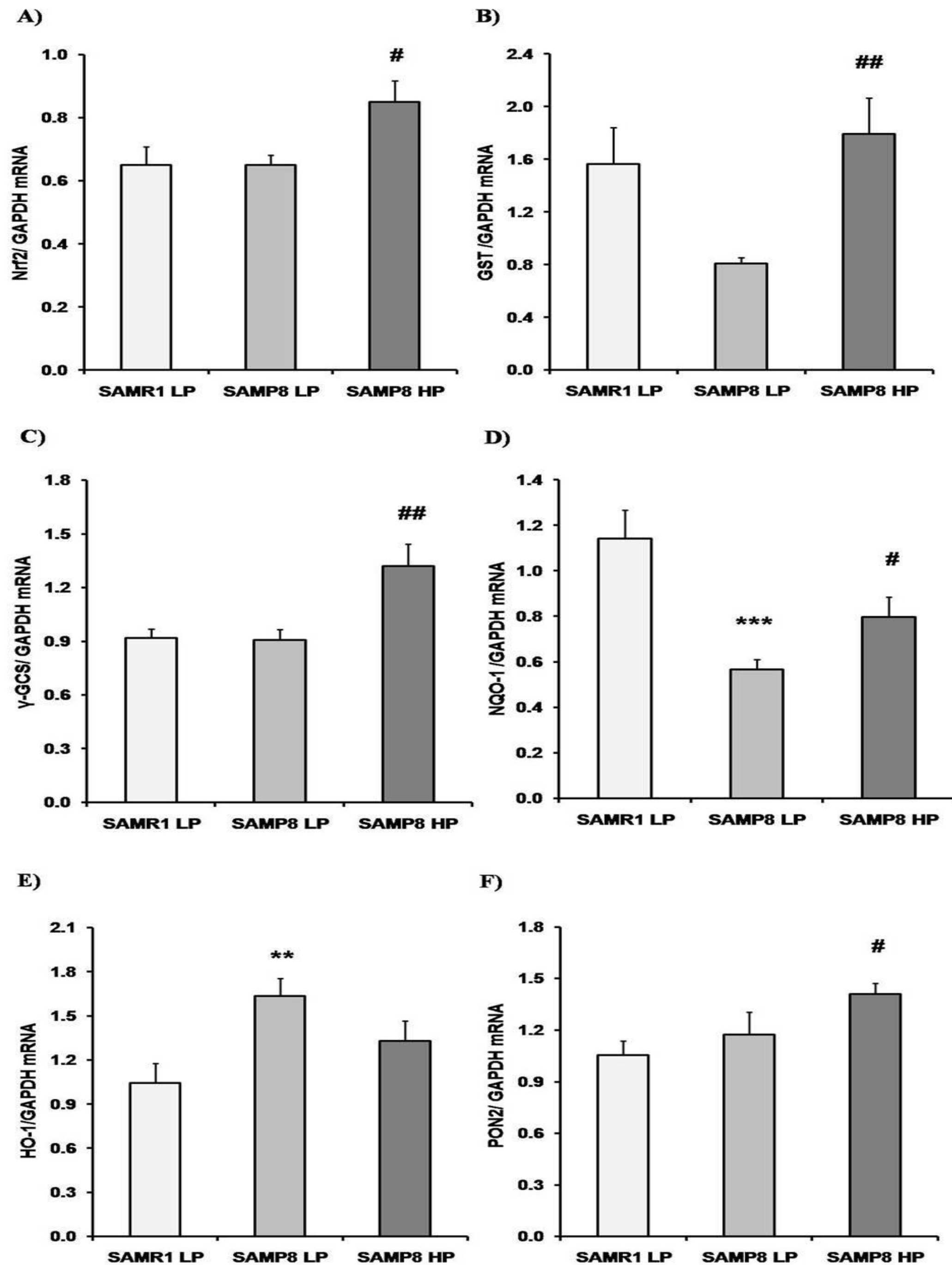


**Figure 4.5 A-F:** Relative mRNA expression (normalized for GAPDH) of Nrf2 (A), GST, (B), γ-GCS, (C) NQO-1, (D), HO-1 (E), and PON-2 (F) in hearts of SAMP8 mice fed for 4.5 months a Western type diet with 0.15% cholesterol and 20% fat, in which 10% of fat was from olive oil containing either low or high amount of phenolics. Values are expressed as mean ± SEM (n=10).

In liver no difference was detected between mice groups regarding Nrf2 modulated genes. Only HO-1 mRNA expression was significantly lower ($p<0.05$) in SAMP8 LP group as compared to SAMR1 LP group (Figure 4.7). This decrease was also shown in protein level by Western blot (Figure 4.6). We detected a tendency for γ-GCS and GST-α genes in mRNA level. Therefore we detected the protein level of both genes but similarly as observed in mRNA level, no difference was found for the protein levels of γ-GCS and GST-α (Figure 4.8).

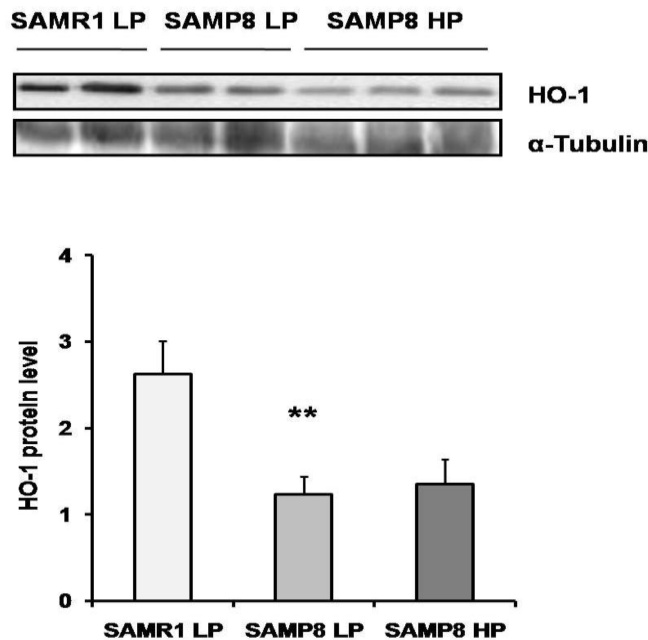


**Figure 4.6:** Protein levels of HO-1. Protein levels were determined via western blotting. Shown is a representative western blot. Values are expressed as the mean ± SEM (n=10) **p<0.01 SAMR1 vs SAMP8.

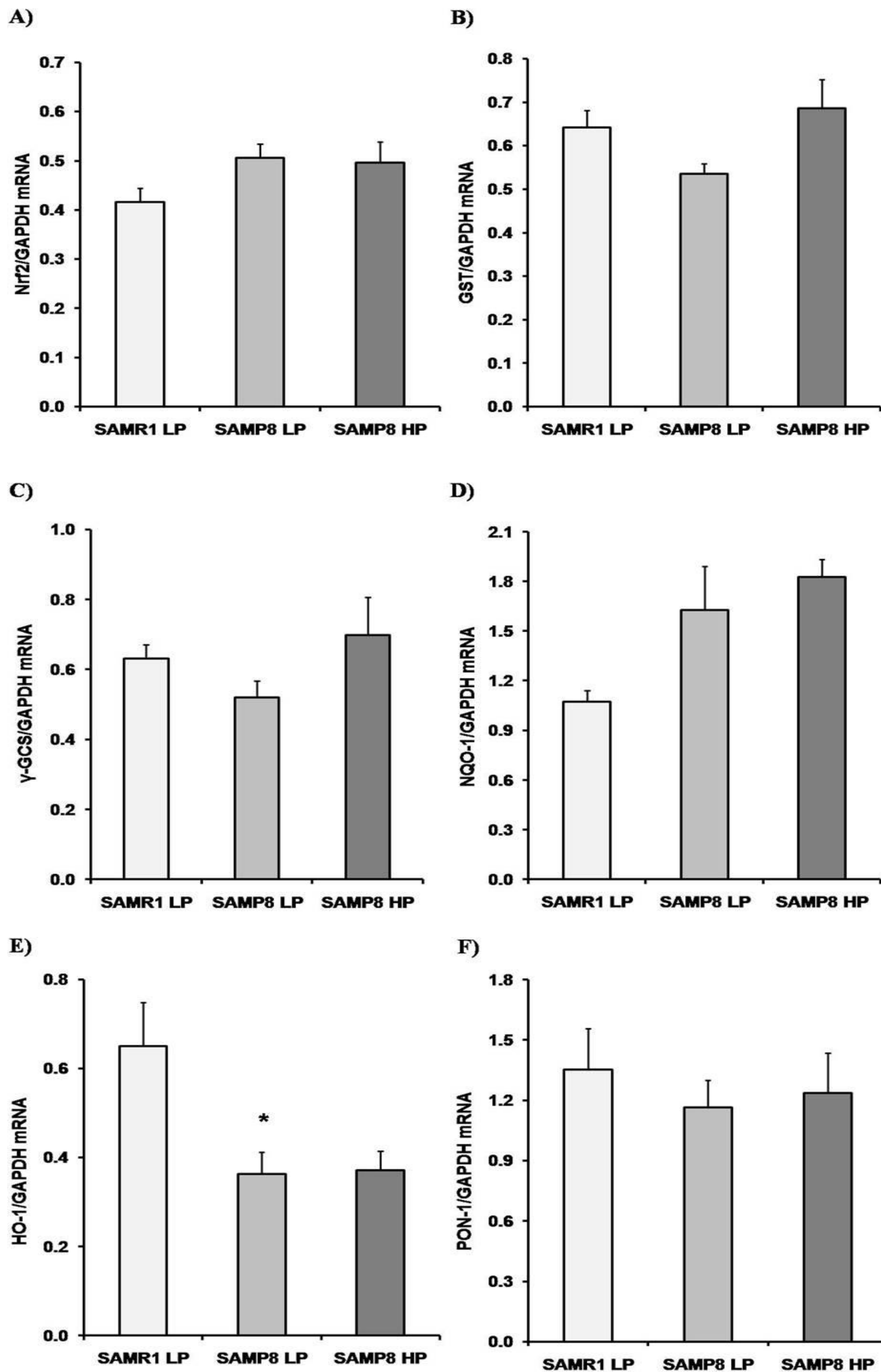


**Figure 4.7 A-F:** Relative mRNA expression (normalized for GAPDH) of Nrf2 (A), GST, (B), γ-GCS, (C), NQO- 1, (D), HO-1 (E), and PON-2 (F) in livers of SAMP8 mice fed for 4.5 months Western type diet with 0.15% cholesterol and 20% fat, in which 10% of fat was from olive oil containing either low or high amount of phenolics. Mice were killed at 7 months of age. Values are expressed as mean ± SEM (n=10).

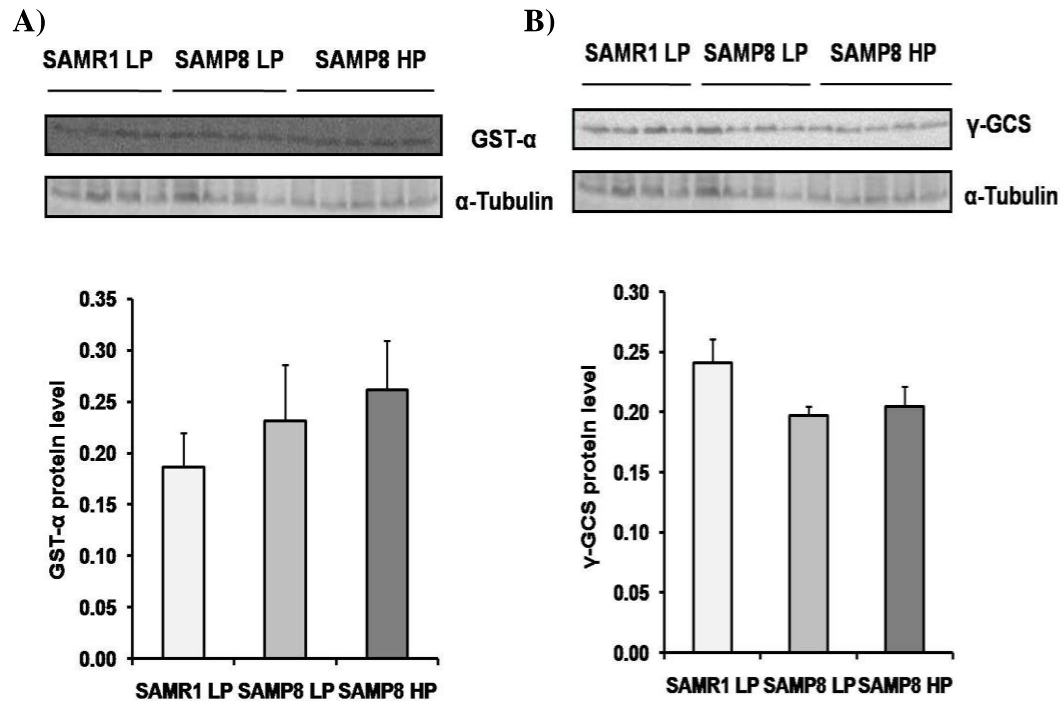


**Figure 4.8 A-B:** Protein levels of GST-α (A) and γ-GCS (B). Protein levels were determined via western blotting. Shown is a representative western blot. Values are expressed as the mean ± SEM (n=10).

Protective effect of Nrf2 mediated antioxidant enzyme activities on cardiovascular diseases and vascular health has been documented (Zhu et al., 2005; Mann et al., 2007; Mann et al., 2009; Hur et al., 2010). It is suggested that olive oil phenolics may exert their modulatory effects on aging, cardiovascular health through Nrf2/ARE pathway. It has been recently shown that hydroxytyrosol induces antioxidant/ detoxificant enzymes and Nrf2 translocation in hepatocytes (Martin et al., 2010). Furthermore hydroxytyrosol induced cytoprotection against oxidative injury in vascular endothelial cells via Nrf2 dependent signal transduction pathway (Zrelli et al., 2011).

In studies regarding Nrf2-related genes that were conducted with aged rodents, no modulation of Nrf2 was observed neither with mulberry nor bilberry extract in SAMP8 mice (Shih et al, 2009). Suh et al. (2004) showed reduced Nrf2/ARE binding and Nrf2 mediated gene expression and NQO-1 levels in aging rat liver.

### 4.3.7 Effect of age and HP diet on SIRT1 expression

SIRT1, is a class III deacetylase, that can modulate numerous longevity-promoting cellular functions, endothelial function, lipid metabolism, cell cycling, gene expression, cardioprotection and aging (Longo and Kennedy, 2006). Previously decreased level of SIRT1 expression was documented in SAMP8 mice as compared

to its control SAMR1 mice (Pallas et al., 2008; Gutierrez-Cuesta et al., 2008; Tajes et al., 2010). In accordance with these studies, the SIRT1 expression level was significantly decreased in SAMP8 LP group ($p<0.001$, Figure 4.9) but high phenolic olive oil treatment significantly increased mRNA expression levels in SAMP8 mice in our study ($p<0.05$). SIRT1 exerts its functions by deacetylating variety of metabolic transcription factors. A moderate induction of SIRT retarded ageing of the heart and induced resistance to oxidative stress (Alcendor et al., 2007). Thus, the decreased concentrations of biomarkers of oxidative stress in heart of mice fed the HP diet might be related to the moderate SIRT1 induction observed in these animals.

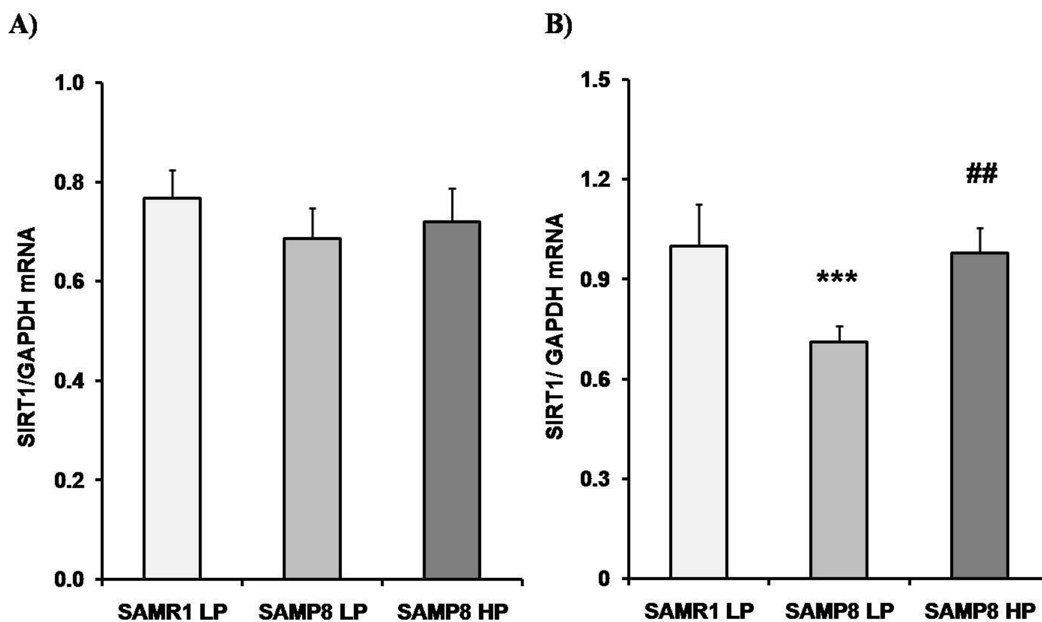


**Figure 4.9 A-B:** Relative mRNA expression (normalized for GAPDH) of SIRT1 in liver (A) and heart (B) of mice fed for 4.5 months a Western type diet with 0.15% cholesterol and 20% fat, in which 10% of fat was from olive oil containing either low or and high amount of phenolics. Mice were killed at 7 months of age. Values are expressed as mean ± SEM (n=10).

### 4.3.8 Effect of age and HP diet on inflammation markers

As aging is accompanied by an increase in circulating acute phase reactants we measured the CRP and SAP mRNA expression levels as a marker of inflammatory status and an increased risk of cardiovascular diseases. Increased levels of CRP and SAP together with decreased level of HO-1 indicate that SAMP8 mice have higher inflammatory state. These results may also affect PON1 activity, which reduced during acute phase response (Ayub et al., 1999). Dullaart et al. (2009) reported a lower PON-1 activity related to higher plasma CRP levels in type 2

diabetic patients. As shown in figure 4.10 both mRNA expression levels of CRP and SAP increased in SAMP8 LP mkice but no effect of HP treatment was observed.

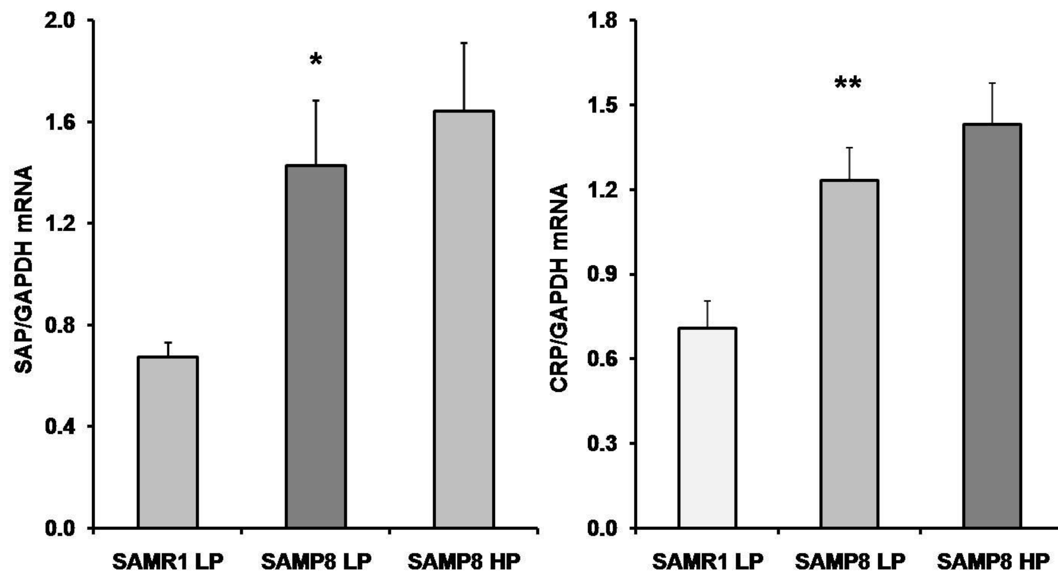


**Figure 4.10:** Relative mRNA expression (normalized for GAPDH) of SAP and CRP levels in livers of SAMP8 and SAMR1 mice fed for 4.5 months a Western type diet with 0.15% cholesterol and 20% fat, in which 10% of fat was from olive oil containing either low or high amount of phenolics. Mice were killed at 7 months of age. Values are expressed as mean ± SEM (n=10).

## 5. CONCLUSION

The aim of the present study was to investigate the effect of a diet rich in phenolic compounds on the ageing related changes in human model of ageing, SAMP8 mice. Most of the studies have focused on the brain ageing of SAMP8 mice and effective results are obtained but studies regarding the heart and liver ageing of this mice are scarce and needs to be studied. The liver and heart are the major organs responsible for a number of physiological processes that are crucial to sustaining life. Another aim was to develop and validate a method for the quantification of olive oil phenolics with sensitive electrochemical detector.

As a result the effect of ageing was remarkable in liver of SAMP8 mice as compared to SAMR1 mice. This study demonstrates that age dependent oxidative stress and inflammation are elevated in SAMP8 mice from a quite early age as compared to its control SAMR1 mice which may lead to a rise in free radical formation and thus age related diseases. The increase in the protein and lipid oxidation may contribute to age related changes as reflected in PON1 activity and expression of inflammation related genes (CRP, SAP, HO-1). The amount of vitamin E and vitamin C was also higher in SAMP8 LP mice. On the other hand redox status, Nrf2 and Nrf2-dependent gene expressions remained to be unchanged in liver. The effect of HP diet was not observed except in TBARS and protein carbonyl levels.

In heart the effect of HP diet was obvious in SAMP8 HP. Feeding a HP diet reduced biomarkers of oxidative stress, induced Nrf2 and Nrf2- dependent gene expression and increased SIRT1 gene expression in the heart of SAMP8 mice compared to animals fed a LP diet. In the literature the effect of Nrf2 dependent antioxidant enzyme activities on cardiovascular diseases and vascular health is well known. Overall, our results suggest that a diet rich in olive oil phenolics reduces oxidative stress in the heart of SAMP8 mice most likely by induction of Nrf2-dependent gene expression. Mice fed the HP diet had lower concentrations of TBARS and protein carbonyls in the heart than mice on the LP diet. However, proteasomal activity was not changed. Thus differences in heart protein carbonyl levels were independent of proteasomal activity. Regarding Nrf2 and Nrf2-dependent gene expression and redox status no effect was detected in SAMP8 LP mice as compared to SAMR1 LP mice.

The HP and LP olive oils used in this study had similar profile but they notably differed in their content of the major olive oil phenolics hydroxytyrosol, tyrosol, oleuropein and pinoresionol which were reflected in the corresponding FRAP and TEAC values of the two oils. Importantly, the HP olive oil contained 12-fold higher concentrations of total phenolics than the LP olive oil. As far as the main olive oil phenolic compound, hydroxytyrosol, is considered, HP oil contains approximately 40 fold higher hydroxytyrosol than the LP oil. It is suggested that the observed beneficial effects of HP diet may be due to high hydroxytyrosol content, which seems to be of importance for the potential health benefits of olive oils.

In conclusion differences in tissue responces to the oxidative stress, different profiles of investigated biomarkers were obtained in heart and liver tissue of the mice. Some tissues respond to the environmental changes quickly but some tissues needs more stressed conditions. We fed the mice with a diet containing 20% fat and 0.15% cholesterol in order to trigger the oxidative stress. The 9-10 weeks old female mice fed either HP or LP diet for 4.5 months and were killed at 7 months old. In the literature different results have been obtained depending on the age of SAMP8 mice. Besides the results differ depending on the experimental design, type of the bioactive compound and the amount of the compounds. According to the results of our study, it is suggested that the mice were not old enough to see clear effects of phenolic rich diet therefore they should be fed longer period of time or older mice should be used.

Another factor for that may be the daily consumption of phenolic compounds. The amount of total phenolic compound in HP oil is 532 mg/kg and the diet contains 10% olive oil. Thus the amount of phenolics in the diet is 53.2 mg/kg. If the daily feed intake of the mice is taken into account, ~2 grams, which means daily amount of phenolic consumption is 0.1 mg per mouse. It is a quite low amount to show the clear beneficial effects of olive oil phenolics. The differences in tissues of mice fed either LP or HP diet may be attributed to low amount of phenolic compound consumption.

In further studies it is suggested to use different ageing models to study the effect of olive oil phenolics on ageing and life span. Besides different tissues of SAMP8 mice should be considered in order to understand the pathological processes in ageing of other tissues.

In terms of our validated method it is possible to analyze selected phenolic compounds (tyrosol, hydroxytyrosol, oleuropein, pinoresinol, caffeic acid, vanillic

acid, p-coumaric acid, ferulic acid) in 17 minutes. This method was validated in terms of selectivity, lower limit of quantification and detection, accuracy, precision, recovery, short term stability, freeze thaw stability and post preparative stability. The phenolics were quantified in ng level. The recoveries of these phenolic compounds ranged between 72-96%. This method may also be used in biological samples (urine, blood, tissues) to analyse the phenolics and as well as their metabolites.

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## APPENDICES

**APPENDIX A:** Chromatograms

**APPENDIX B:** Calibration curves

**APPENDIX C:** List of olive oils that are analysed in the study

**APPENDIX D:** Statistical values

**APPENDIX A:** Chromatograms

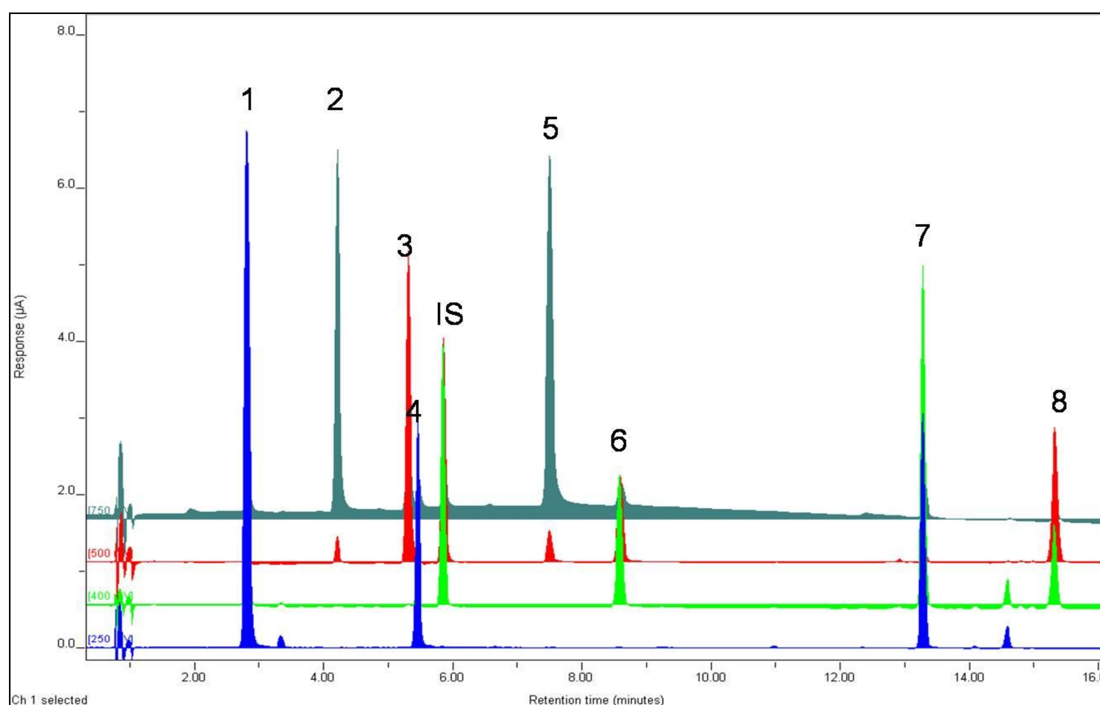


**Figure A.1:** Representative chromatogram of the standards 1) hydroxytyrosol 2) tyrosol 3) caffeic acid 4) vanillic acid 5) ferulic acid 6) p-coumaric acid 7)oleuropein 8) pinoresinol IS: internal standard.

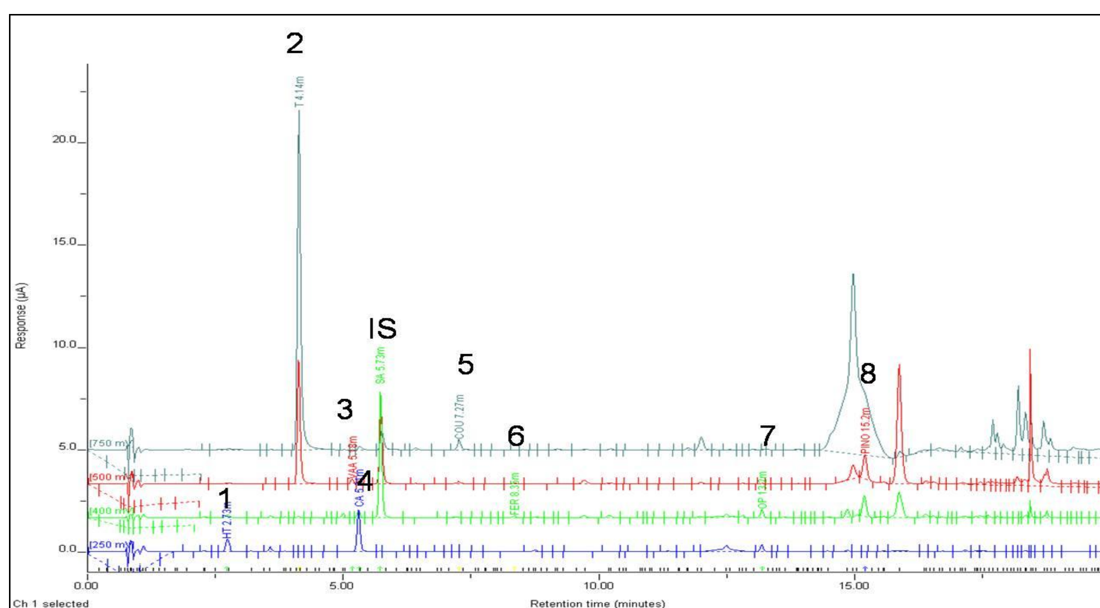


**Figure A.2:** Representative chromatogram of the olive oil extract1) hydroxytyrosol 2) tyrosol 3) caffeic acid 4) vanillic acid 5) ferulic acid 6) p-coumaric acid 7) oleuropein 8) pinoresinol IS: internal standard.

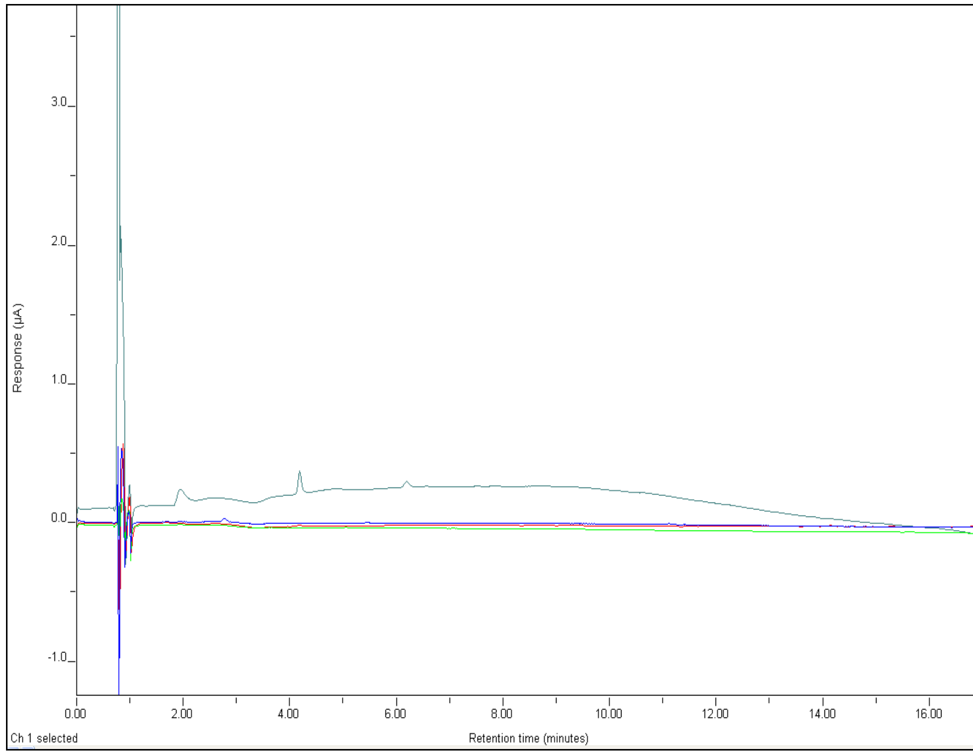


**Figure A.3:** Representative chromatogram of the blank olive oil extract.

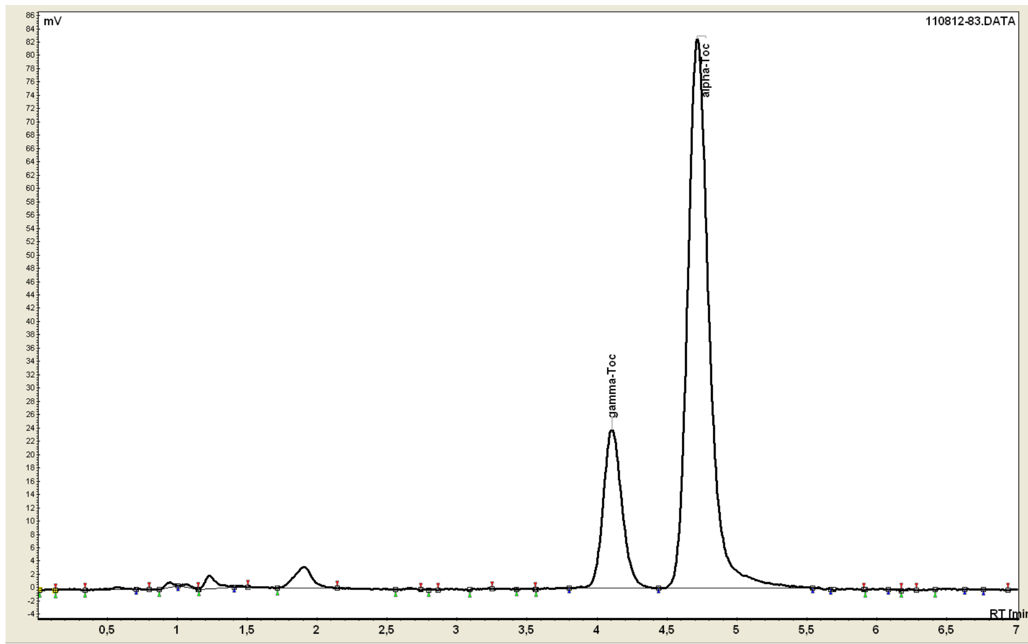


**Figure A.4:** Representative chromatogram of α- and γ-tocopherol.

**APPENDIX B:** Calibration Curves

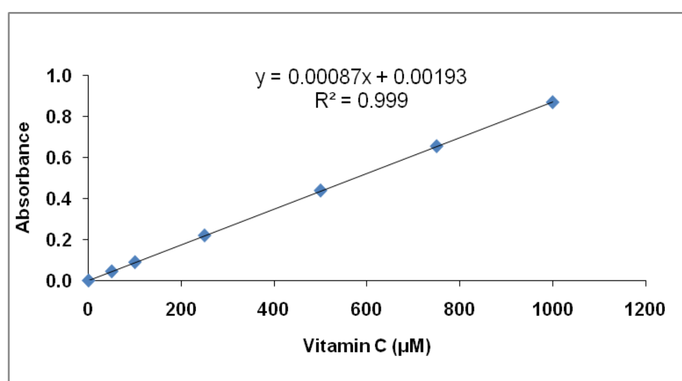


**Figure B.1:** Calibration curve for FRAP assay.

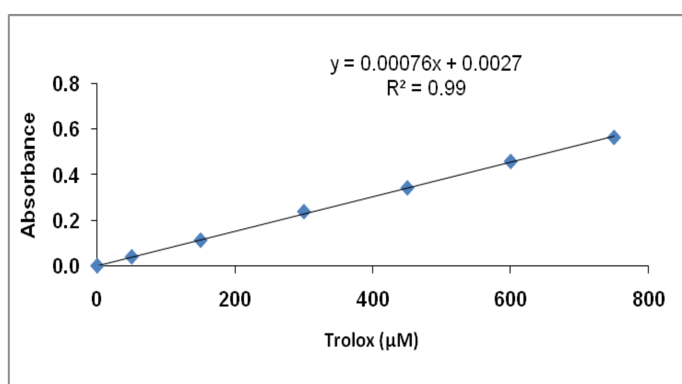


**Figure B.2:** Calibration curve for TEAC assay.

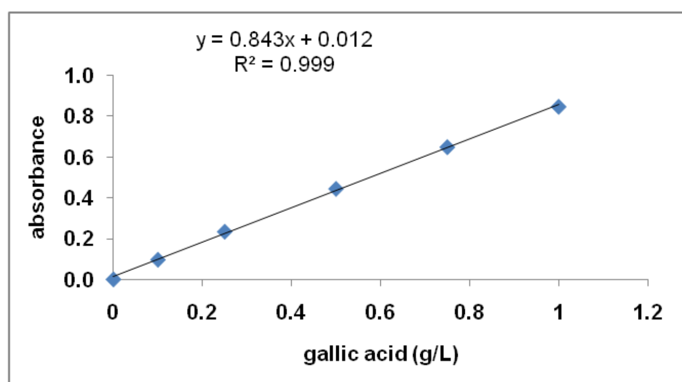


**Figure B.3:** Calibration curve for the determination of total phenolics.

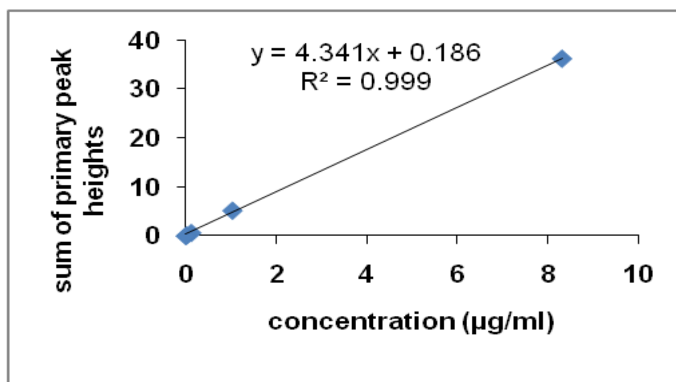


**Figure B.4:** Calibration curve for tyrosol.

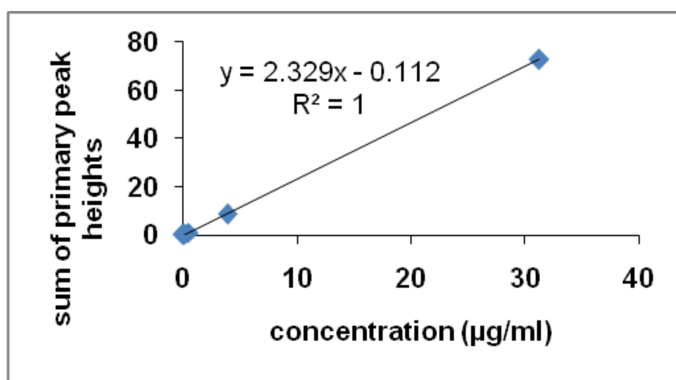


**Figure B.5:** Calibration curve for hydroxytyrosol.

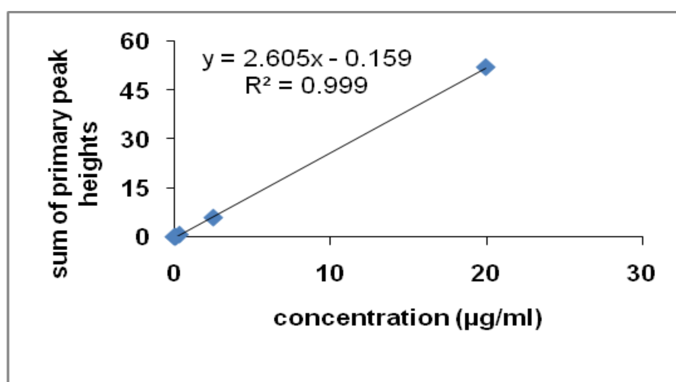


**Figure B.6:** Calibration curve for vanillic acid.

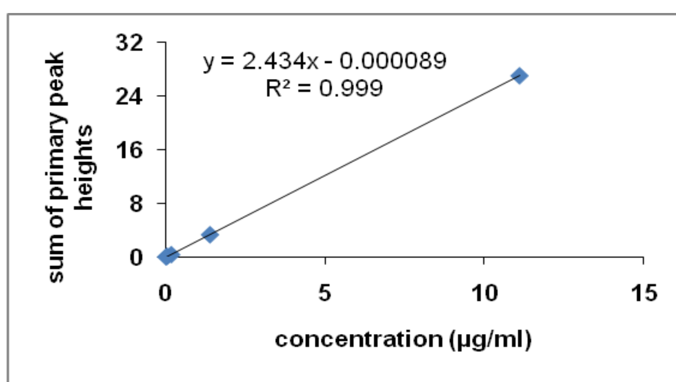


**Figure B.7:** Calibration curve for caffeic acid.

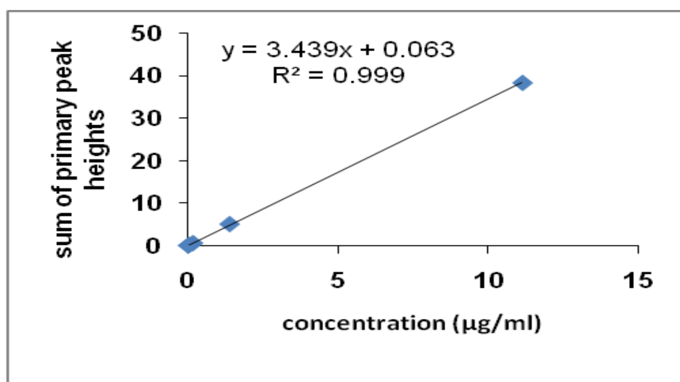


**Figure B.8:** Calibration curve for p-coumaric acid.

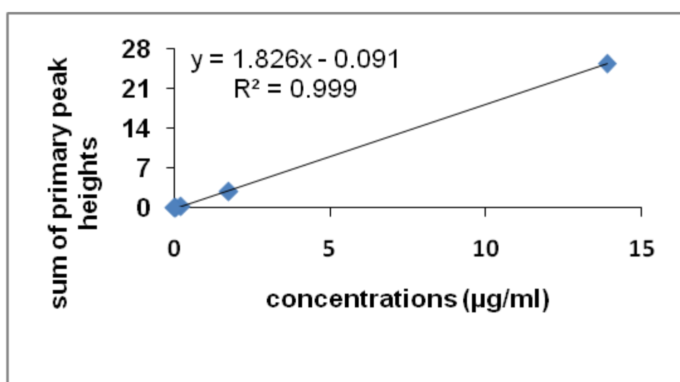


**Figure B.9:** Calibration curve for ferulic acid.

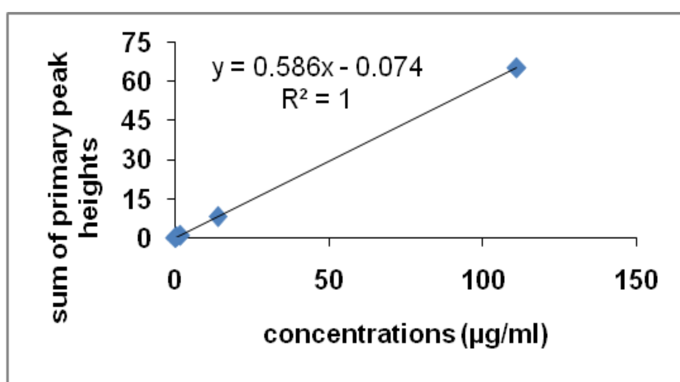


**Figure B.10:** Calibration curve for oleuropein.

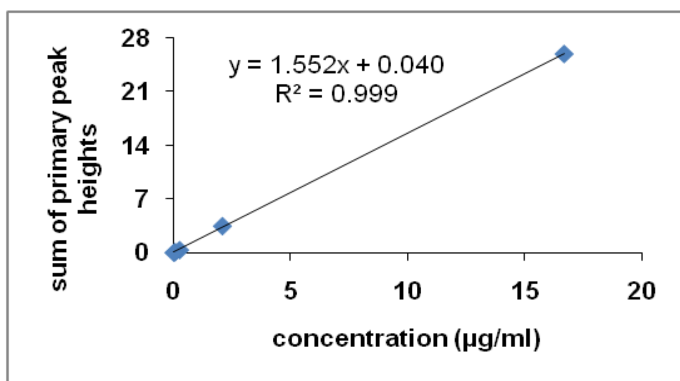


**Figure B.11:** Calibration curve for pinoresinol.

**APPENDIX C:**

**Table C.1:** The list of olive oil samples that are analyzed in the study.

| Sample | Country | Region | Olive variety | Harvest year | Extraction | Type |
|---|---|---|---|---|---|---|
| 1 | Spain | n.a. | Lechin | 2007 | Cold pressed | Extra virgin |
| 2 | Spain | n.a. | Picual | 2007 | Cold pressed | Extra virgin |
| 3 | Spain | n.a. | Hojiblanca | 2007 | Cold pressed | Extra virgin |
| 4 | Spain | n.a. | Arbequina | 2007 | Cold pressed | Extra virgin |
| 5 | Spain | Andalusia | Picual | 2008 | Cold pressed | Extra virgin |
| 6 | Spain | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 7 | Spain | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 8 | Spain | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 9 | Spain | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 10 | Spain | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 11 | Spain | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 12 | Spain | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 13 | Turkey | Ezine | n.a. | 2008 | Cold pressed | Extra virgin |
| 14 | Turkey | Zeytindağ | n.a. | 2008 | Cold pressed | Extra virgin |
| 15 | Turkey | Aydin | Memecik | 2007 | Cold pressed | Extra virgin |
| 16 | Turkey | Hatay | Saurani | n.a. | Cold pressed | Extra virgin |
| 17 | Turkey | Hatay | Hasebi | n.a. | Cold pressed | Extra virgin |
| 18 | Turkey | Hatay | Halhali | n.a. | Cold pressed | Extra virgin |
| 19 | Turkey | Hatay | Multivariety | n.a. | Cold pressed | Extra virgin |
| 20 | Turkey | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 21 | Turkey | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 22 | Turkey | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 23 | Turkey | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 24 | Turkey | Ayvalik | Ayvalık | 2007 | Cold pressed | Extra virgin |
| 25 | Italy | Sicily | Tonda iblea | 2008 | Cold pressed | Extra virgin |
| 26 | Italy | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 27 | Italy | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 28 | Italy | n.a. | n.a. | 2007 | Cold pressed | Extra virgin |
| 29 | Italy | Sicily | Cerasuola,Nocellara,Biancolilla | 2007 | Cold pressed | Extra virgin |

**Table C.1 (continued):** The list of olive oil samples that are analyzed in the study.

| Sample | Country | Region | Olive variety | Harvest year | Extraction | Type |
|---|---|---|---|---|---|---|
| 30 | Italy | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 31 | Italy | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 32 | Italy | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 33 | Italy | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 34 | Italy | n.a. | n.a. | n.a. | Cold pressed | Extra virgin |
| 35 | Greece | Lakonia | Koroneiki,Athinoleia | 2007 | Cold pressed | Extra virgin |
| 36 | Greece | Kalamata | Koroneiki | 2007 | Cold pressed | Extra virgin |
| 37 | Greece | Crete | Koroneiki | 2007 | Cold pressed | Extra virgin |
| 38 | Greece | Sitia, Crete | Koroneiki | 2007 | Cold pressed | Extra virgin |
| 39 | Greece | Crete | Koroneiki | 2007 | Cold pressed | Extra virgin |
| 40 | Greece | Crete | Koroneiki | 2007 | Cold pressed | Extra virgin |
| 41 | Greece | Mani | Koroneiki | 2007 | Cold pressed | Extra virgin |
| 42 | Greece | Kolymvari, Crete | Koroneiki | n.a. | Cold pressed | Extra virgin |
| 43 | France | Ollioules | Brun | 2008 | Cold pressed | Extra virgin |
| 44 | France | Ollioules | Cayon | 2008 | Cold pressed | Extra virgin |
| 45 | France | Ollioules | Multivariety | 2008 | Cold pressed | Extra virgin |
| 46 | France | n.a. | n.a. | 2008 | Cold pressed | Extra virgin |
| 47 | Portugal | Alentejo | 80% Arbequina, 20%Cobrançosa | 2007 | Cold pressed | Extra virgin |
| 48 | Portugal | Alentejo | 80% Arbequina, 20%Cobrançosa | 2007 | Cold pressed | Extra virgin |
| 49 | Portugal | n.a. | n.a. | 2007 | Cold pressed | Extra virgin |
| 50 | Portugal | n.a. | n.a. | 2007 | Cold pressed | Extra virgin |
| 51 | Australia | Kiewa River | Lechino | 2008 | Cold pressed | Extra virgin |
| 52 | Australia | Frankland River | Lechino | 2007 | Cold pressed | Extra virgin |
| 53 | Australia | Frankland River | Frantoio | 2007 | Cold pressed | Extra virgin |
| 54 | USA | California | Manzanillo, Mission | 2007 | Cold pressed | Extra virgin |
| 55 | USA | California | Lechino | 2007 | Cold pressed | Extra virgin |
| 56 | USA | California | Frantoio | 2007 | Cold pressed | Extra virgin |
| 57 | South Africa | Morgenster | Frantoio, leccino, coratina | 2007 | Cold pressed | Extra virgin |

Abbreviations: n.a; not available

**APPENDIX D:** Statistical values

**Table D.1:** p values of t-test for the analysed parameters in serum of mice

| ANALYSED PARAMETER | SAMR1 LP *vs* SAMP8 LP | SAMP8 LP *vs* SAMP8 HP |
|---|---|---|
| **Triglyceride** | 0.29 | 0.07 |
| **Total cholesterol** | 0.002 | 0.23 |
| **HDL** | 0.13 | 0.84 |
| **PON1 activity** | 0.0001 | 0.024 |

**Table D.2:** p values of t-test for the analysed parameters in heart of mice

| ANALYSED PARAMETER | SAMR1 LP *vs* SAMP8 LP | SAMP8 LP *vs* SAMP8 HP |
|---|---|---|
| **TBARS** | 0.013 | 0.0001 |
| **Protein carbonyl** | 0.031 | 0.004 |
| **Proteasomal activity** | | |
| β-1 subunit | 0.173 | 0.15 |
| β-2 subunit | 0.06 | 0.06 |
| β-5 subunit | 0.357 | 0.35 |
| **GSH** | 0.13 | 0.088 |
| **Cysteine** | 0.03 | 0.03 |
| **Methionine** | 0.17 | 0.10 |
| **Uric acid** | 0.021 | 0.027 |
| **Ascorbic acid** | 0.12 | 0.10 |
| **Nrf-2** | 0.76 | 0.036 |
| **GST** | 0.005 | 0.10 |
| **γ-GCS** | 0.66 | 0.06 |
| **NQO-1** | 0.001 | 0.028 |
| **HO-1** | 0.003 | 0.86 |
| **PON-2** | 1.00 | 0.004 |
| **SIRT-1** | 0.029 | 0.006 |

**Table D.3:** p values of t-test for the analysed parameters in liver of mice

| ANALYSED PARAMETER | SAMR1 LP *vs* SAMP8 LP | SAMP8 LP *vs* SAMP8 HP |
|---|---|---|
| **TBARS** | 0.002 | 0.66 |
| **Protein carbonyl** | 0.016 | 0.008 |
| **Proteasomal activity** | | |
| β-1 subunit | 0.63 | 0.98 |
| β-2 subunit | 0.87 | 0.26 |
| β-5 subunit | 0.02 | 0.16 |
| **GSH** | 0.63 | 0.33 |
| **GSSG** | 0.10 | 0.56 |
| **Cysteine** | 0.005 | 0.46 |
| **Methionine** | 0.86 | 0.51 |
| **Uric acid** | 0.20 | 0.16 |
| **Ascorbic acid** | 0.003 | 0.76 |
| **SVCT1** | 0.029 | 0.71 |
| **SVCT2** | 0.38 | 0.29 |
| **GLUOX** | 0.77 | 0.0001 |
| **Vitamin E** | 0.0001 | 0.0001 |
| **CYPA3** | 0.28 | 0.81 |
| **TTP** | 0.32 | 0.69 |
| **LPL** | 0.08 | 0.53 |
| **LRP1** | 0.77 | 0.92 |
| **SRB1** | 0.76 | 0.09 |
| **MDR-1** | 0.0001 | 0.11 |
| **LDLR** | 0.36 | 0.72 |
| **ABCA1** | 0.22 | 0.17 |
| **Nrf-2** | 0.09 | 0.71 |
| **GST** | 0.16 | 0.12 |
| **GST (protein level)** | 0.84 | 0.15 |
| **γ-GCS** | 0.22 | 0.33 |
| **γ-GCS (protein level)** | 0.49 | 0.40 |
| **NQO-1** | 0.069 | 0.53 |
| **HO-1** | 0.24 | 0.65 |
| **HO-1 (protein level)** | 0.01 | 0.87 |
| **PON-1** | 0.47 | 1.00 |
| **SIRT-1** | 0.35 | 0.33 |
| **SAP** | 0.016 | 0.57 |
| **CRP** | 0.004 | 0.30 |

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**Papers**

**Bayram, B.,** Ozcelik, B., Grimm, S., Roeder, T., Schrader, C., Ernst, I.M.A., Wagner, A.E., Grune, T., Frank, J., Rimbach, G. 2011. A diet rich in olive oil phenolics reduces oxidative stress in the heart of SAMP8 mice by induction of Nrf2-dependent gene expression. *Rejuvenation Research* (Accepted).

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