DEVELOPMENT OF BIOSENSORS FOR DETERMINATION OF THE TOTAL ANTIOXIDANT CAPACITY

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**by
Seçil ÇOBAN**

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We approve the thesis of **Seçil ÇOBAN**

_____
Assoc. Prof. Dr. Oğuz Bayraktar
Supervisor

_____
Assist. Prof Dr. Sait Sargın
Committee Member

_____
Assist. Prof. Dr. Ayşegül Batıgün
Committee Member

11 July 2008
     Date

_____
Prof. Dr. Devrim BALKÖSE
Head of the Chemical Engineering
Department

_____
Prof. Dr. Hasan BÖKE
Dean of the Graduate School of
Enginering and Sciences

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ABSTRACT

DEVELOPMENT OF BIOSENSORS FOR DETERMINATION OF THE TOTAL ANTIOXIDANT CAPACITY

In this study, an amperometric laccase biosensor was developed for determination of the oleuropein concentration that is the biological active component of olive leaf and contributes dominantly to the total antioxidant capacity. The biosensor was prepared by immobilization of laccase from *Trametes versicolor* by addition of cross-linking agent, glutaraldehyde, into the carbon paste electrode. Different biosensors were prepared by changing the amount of crosslinking agent and concentration of the enzyme solution. So, effect of these parameters on biosensor performance was investigated. The best biosensor performance was determined for the biosensor having glutaraldehyde amount of 12.03 % vol. of the biosensor bottom part and 5 mg/ml of laccase enzyme. The effect of scan rate and temperature on the biosensor performance was also investigated in this study. The scan rate of 10 mV/s was decided to be the optimum for the amperometric detection of oleuropein considering the fastest response and maximum reduction current. $25^{0}C$ was chosen as an optimum temperature value due to the maximum laccase activity and capability of oleuropein acting as an antioxidant.

Extraction of phenolics from olive leaf was also an important part of this study. The extract was divided into fractions varying in their oleuropein amounts such as polar fractions and relatively less polar fractions. Therefore, biosensor performance was investigated for fractions containing different type of phenolics. HPLC analyses of the fractions were also performed in this study. In addition total phenol content and antioxidant capacity of the fractions were determined by conventional methods.

ÖZET

TOPLAM ANTİOKSİDAN KAPASİTE TAYİNİ İÇİN BİYOSENSÖRLER GELİŞTİRİLMESİ

Bu çalışmada, zeytin yaprağı özütündeki toplam antioksidan madde miktarının önemli bir bölümünü oluşturan fenolik bir bileşik olan oleuropein'e karşı spesifik biyosensörler geliştirilmesi esas alınmıştır. Bu amaçla, lakkaz temelli amperometrik biyosensörler geliştirilmiştir. Bu biyosensörler, *Trametes versicolor* kaynaklı lakkaz enziminin glutaraldehit kullanılarak karbon pastasına immobilizasyonu ile hazırlanmıştır. Çapraz bağlayıcı miktarı ve lakkaz enzimi konsantrasyonu değiştirilerek çeşitli biyosensörler geliştiştirilmiştir. Böylelikle, bu parametrelerin biyosensör performansına etkisi incelenmiştir. En iyi performans, 5 mg/ml lakkaz konsantrasyonu ve alt tabakası hacimce %12.03 glutaraldehitten oluşan biyosensörden elde edilmiştir. Bunun yanında tarama hızı ve sıcaklığın da biyosensör performansına olan etkisi incelenmiştir. Oleuropeinin'in tayininde kullanılan amperometrik biyosensörler için, minimum yanıt zamanı ve ölçülebilen maksimum indirgenme akımı esas alınarak döngüsel voltametre ölçümlerinde optimum tarama hızı 10 mV/s olarak belirlenmiştir. Ayrıca, lakkaz enziminin en aktif olduğu ve oleuropeinin antioksidan özelliğini devam ettirebildiği en uygun çalışma sıcaklığı 25^0C olarak saptanmıştır.

Bu çalışmada zeytin yaprağı özütündeki fenolik bileşiklerin ekstraksiyon çalışmaları gerçekleştirilmiştir. Elde edilen özüt, oleuropein'ce zengin ve oleuropein'ce zengin olmayan farklı fraksiyonlara ayrılmıştır. Buradaki amaç, geliştirilen biyosensörün farklı fenolik bileşikler içeren fraksiyonlara karşı olan tepkisini incelemektir. Bu çalışmada fraksiyonların HPLC analizleri de yapılmıştır. Buna ek olarak, fraksiyonların toplam fenol ve toplam antioksidan kapasiteleri de bilinen metodlarla tayin edilmiştir.

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LIST OF ABBREVIATIONS

OLE	Olive leaf extract
FI	Fraction I (W1+W2)
FII	Fraction II (W3+W4)
FIII	Fraction III (W5+W6)
FIV	Fraction IV (W7+W8+W9)
FV	Fraction V (E1+E2)
FVI	Fraction VI (E3+E4)
HPLC	High performance liquid chromatography
SOD	Superoxide dismutase
IUPAC	International Union of Pure and Applied Chemistry
PCL	Photochemiluminescence
ACW	Water soluble antioxidant capacity
ACL	Lipid soluble antioxidant capacity
AOC	Antioxidant capacity

CHAPTER 1

INTRODUCTION

In recent years, there is a growing interest obtaining the biologically active compounds from natural sources. The protective effects of diets rich in fruit and vegetables against cardiovascular diseases and certain cancers have been attributed partly to the antioxidants contained therein. Olive tree is one of the potential natural antioxidant source because of its phenolic content. There are various methods used to determine the antioxidant capacity of biologically active components. Analytical methods used in the qualitative and quantitative determination of polyphenols in olive leaf involve techniques of chromatographic separation such as HPLC and GC-MS. However these techniques are expensive, reagent and time consuming.

Biosensors are attractive alternative techniques due to their unique characteristics such as selectivity, low cost, miniaturization, easy automation, time saving and simplicity of operation and manufacturing. Thus, the development of biosensors for the polyphenolic fraction of olive leaf is a great ongoing challenge. Biosensors can be defined as an analytical tool or system consisting of an immobilized biological material in intimate contact with a suitable transducer. Biological recognition elements are the major selective element in a biosensor system. They can be organisms, tissues, organelles, enzymes, antibodies and nucleic acids. The type of the biological recognition element determines the degree of selectivity or specificity of the biosensor. The transducer element in a biosensor system converts the biological signal into an electrical one. Biosensors can be classified in several types according to their transducer type such as amperometric, potentiometric, conductimetric, optical, acoustic, piezoelectric and thermal.

Enzymes are extremely specific and selective for the substrates which they interact with. Problems like selectivity and slow response characteristics of biosensors can be overcomed by the use of enzymes. Laccase is an object of intensive studies in the fields of basis research of oxidation of various phenols. It is an oxidoreductase able to catalyze the oxidation of various aromatic compounds, particularly phenols with the concomitant reduction of oxygen to water. It displays a broad specificity for the

reducing substrates including mono-phenols, di-phenols, polyphenols, amino phenols and aromatic diamines. The broad specificity for the phenolic substrates enables laccase to be developed as a biosensor for the determination of total phenols.

An amperometric biosensor may be more attractive among the other biosensor systems due to its promising properties such as high sensitivity and a wide linear range. Amperometric biosensors are based on the measurement of the current resulting from the oxidation or reduction of electroactive species. In amperometry, the resulting current is correlated to the bulk analyte concentration of the electroactive species.

In this study, an amperometric laccase biosensor was developed for determination of the oleuropein concentration which contributes dominantly to the total antioxidant capacity in olive leaf. This study is very important due to the fact that it shows how to detect oleuropein, the phenolic that determines the quality of olive leaf extract, in an easy and economic way. Also, this study will give an idea about how to determine the antioxidant capacities of different products in food industry.

CHAPTER 2

LITERATURE REVIEW

2.1. Free Radicals

Oxidative metabolism is essential for survival of cells and a side effect of this dependence is the production of free radicals (Antolovich, et al. 2002). Free radicals are unstable atom or molecules that have unpaired electrons. Free radicals are not only the products of oxidative metabolism; they can also be formed in response to light, radiation, smog, tobacco or alcohol. Formation of free radicals is shown in Figure 2.1.
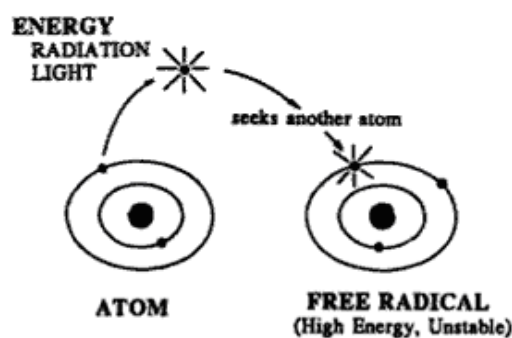
Figure 2.1. Free radical formation

(Source: Simone 1992)

Unstable free radicals try to become stable by transferring their high energy to nearby substances. When free radicals are formed in the body, in an attempt to stabilize, they attack other molecules in the body by blocking protective enzymes such as superoxide dismutase, catalase and peroxidase. Hence, they cause destructive and lethal cellular effects by oxidizing membrane lipids, cellular proteins, DNA and enzymes, thus shutting down cellular respiration. Cell damage by free radicals is schematically shown in Figure 2.2 (Antolovich, et al. 2002, Simone 1992).
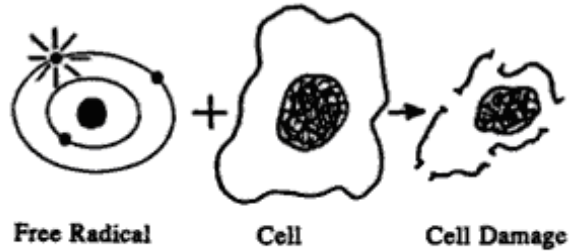
Figure 2.2. Cell damage by free radicals

(Source: Simone 1992)

The interaction of oxygen free radicals with molecules of a lipidic nature also produces new radicals; hydroperoxides and different peroxidases. This group of radicals may interact with biological systems and though studies indicate that they have cytotoxic, mutagenic, carcinogenic, atheragenic and antiotoxic effects (Benavente-Garcia, et al. 2000, Madhavi, et al. 1996).

The direct reaction of a lipid molecule with a molecule of oxygen, *autoxidation*, is a free radical chain reaction (Madhavi, et al. 1996). Lipid oxidation proceeds via three different pathways;

(1) non-enzymatic free radical-mediated chain reaction,

(2) non-enzymatic, non-radical photo-oxidation and

(3) enzymatic reaction.

Pathway (1) is the classical free radical route and this pathway is composed of initiation, propagation, branching and termination steps (Antolovich, et al. 2002).

Initiation:

$$LH + R^\bullet \longrightarrow L^\bullet + RH$$
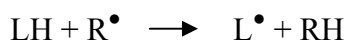
An unsaturated lipid gives rise to free radicals when in contact with oxygen. Initiation reactions take place when the substrate molecule (LH) reacts with the initiating oxidizing radical ($R^\bullet$), generating a highly reactive allyl radical ($L^\bullet$) that can rapidly react with oxygen to form a lipid peroxyl radical ($LOO^\bullet$).

4

Propagation:

$$L^\bullet + O_2 \longrightarrow LOO^\bullet$$
$$LOO^\bullet + LH \longrightarrow L^\bullet + LOOH$$
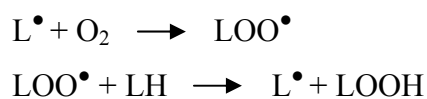
The peroxyl radicals are the chain carriers of the reaction that can further oxidize the lipid, producing lipid hydroperoxides (LOOH), which in turn break down to a wide range of compounds, including alcohols, aldehydes, alkyl formates, ketones, hydrocarbons and radicals including the alkoxyl radical ($LO^\bullet$). Thus, a general feature of the propagation reactions is that they tend to proceed as chain reactions, that is, one radical yields another and so on. Therefore, formation of one radical is responsible for the subsequent chemical transformations due to the chain events.

Branching:

$$LOOH \longrightarrow LO^\bullet + HO^\bullet$$
$$2\,LOOH \longrightarrow LOO^\bullet + LO^\bullet + H_2O$$
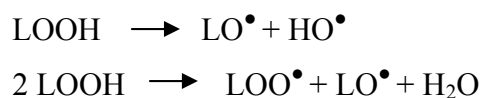
The breakdown of lipid hydroperoxides often involves transition metal ion catalysis, in reactions analogous to that with hydrogen peroxide, yielding lipid peroxyl and lipid alkoxyl radicals:

$$LOOH + M^{n+} + H^+ \longrightarrow LO^\bullet + M^{(n+1)+} + H_2O$$
$$LOOH + M^{(n+1)+} + OH^- \longrightarrow LOO^\bullet + M^{n+} + H_2O$$

Termination:

$$LO^\bullet + LO^\bullet \longrightarrow$$
$$LOO^\bullet + LOO^\bullet \longrightarrow \quad \Big\} \quad \text{non-radical products}$$
$$LO^\bullet + LOO^\bullet \longrightarrow$$
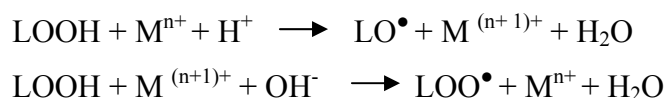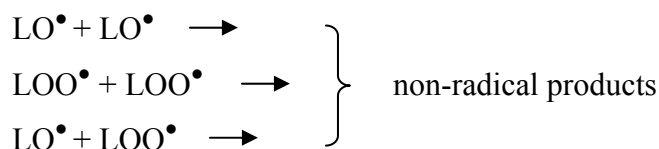
Free radicals are electrically neutral and their solvation effects are generally too small. They are considered to be bonding-deficient and structurally unstable. Therefore, they tend to react whenever possible and that is why a radical is highly reactive. When

there is a reduction in the amount of fatty acids, radicals bond to one another. Termination reactions involve the combination of radicals to form non-radical products (Madhavi, et al. 1996, Antolovich, et al. 2002).

Among the causes of the major chronic health problems, harmful free radicals and reactive oxygen species (ROS) have been found to play an important role. Radicals and ROS such as the superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and peroxy radical ($ROO^{\bullet}$) have been implicated as mediators of degenerative and chronic deteriorative, inflammatory, autoimmune diseases, diabetes, vascular disease, hypertension, cancer, hyperplastic diseases, cataract formation, emphysema, arthritis, malaria, multiple sclerosis, myocardial ischemia-reperfusion injury, immune system decline, brain dysfunction as well as the aging process (Tsao and Deng 2004).

To prevent free radical damage, the body has a defense system of antioxidants (Antolovich, et al. 2002). Antioxidants, as bioactive components, are capable to reduce the cell and tissue damages derived from the free radical mechanism (Simone 1992). Many plants have been identified as having potential antioxidant activities. Bioactive phenols, especially bioflavonoids, are very interesting as antioxidants because of their natural origin and the ability to act as efficient free radical scavengers (Katalinic, et al. 2006). Due to its high proportion of bioactive compounds such as vitamins, flavonoids and polyphenols, the Mediterranean diet that is rich in fresh fruits and vegetables has been known with a low incidence of cardiovascular disease and cancer (Benavente-Garcia, et al. 2000).

2.2. Phenolic Compounds

Phenolic compounds have been reported to have multiple biological effects, including antioxidant activity. Crude extracts of fruits, herbs, vegetables, cereals and other plant materials rich in phenolics are increasingly of interest because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Kahkönen, et al. 1999).

Phenolic compounds, or polyphenols, constitute one of the most numerous and widely-distributed groups of substances in the plant kingdom. Polyphenols are products of the secondary metabolism of plants. Phenolics are a group of organic compounds with one or more hydroxyl groups on the aromatic ring and/or rings. Most of the major

classes of plant polyphenol are listed in Table 2.1, according to the number of carbon atoms of the basic skeleton. The structure of natural polyphenols varies from simple molecules, such as phenolic acids, to highly polymerized compounds, such as condensed tannins (Urquiaga and Leighton 2000).

Table 2.1. Major classes of phenolic compounds in plants
(Source: Urquiaga and Leighton 2000)

Carbon no	Basic skeleton	Class	Examples
6	C_6	Simple phenols Benzoquinones	Catechol, hydroquinone 2,6-Dimethoxybenzoquinone
7	C_6-C_1	Phenolic acids	Gallic, salicylic
8	C_6-C_2	Acetophene Tyrosine derivatives Phenylacetic acids	3-Acetl-6-methoxybenzaldehyde Tyrosol p-Hydroxyphenylacetic
9	C_6-C_3	Hydroxycinnamic acids Phenylpropenes Coumarins Isocoumarins Chromones	Caffeic, ferulic Myristicin, eugenol Umbelliferone, aesculetin Bergenon Eugenin
10	C_6-C_4	Naphthoquinones	Juglone, plumbagin
13	C_6-C_1-C_6	Xanthones	Mangiferin
14	C_6-C_2-C_6	Stilbenes Anthraquinones	Resveratrol Emodin
15	C_6-C_3-C_6	Flavonoids Isoflavonoids	Quercetin, cyanidin Genistein
18	$(C_6$-$C_3)_2$	Lignans Neolignans	Pinoresinol Eusiderin
30	$(C_6$-C_3-$C_6)_2$	Biflavonoids	Amentoflavone
n	$(C_6$-$C_3)_n$ $(C_6)_n$ $(C_6$-C_3-$C_6)_n$	Lignins Catechol melanins Flavolans (Condensed Tannins)	

The three important groups of phenolics are flavonoids, phenolic acids and tannins (King and Young 1999).

Flavonoids

Flavonoids are the most common and widely distributed group of plant phenolics. Their common structure is that of diphenylpropanes (C_6-C_3-C_6) and consists of two benzene rings linked by an oxygen containing heterocycle as it is seen in Figure 2.3 (Urquiaga and Leighton 2000).
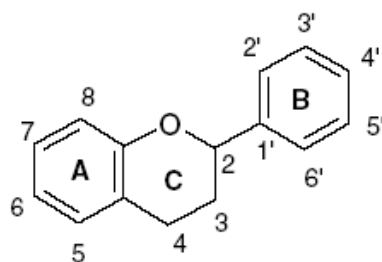
Figure 2.3. General structure of a flavonoid molecule
(Source: Urquiaga and Leighton 2000)

Flavonoids are divided into anthocyanins and anthoxanthins. Anthocyanins are the molecules that posses some color pigments such as red, blue and purple. Anthoxanthins are colorless or white to yellow molecules and they are subdivided into four groups. These are flavones, flavonols, flavanols and isoflavonoids (King and Young 1999). The basic structures of the flavonoid subgroups can be seen in Figure 2.4.
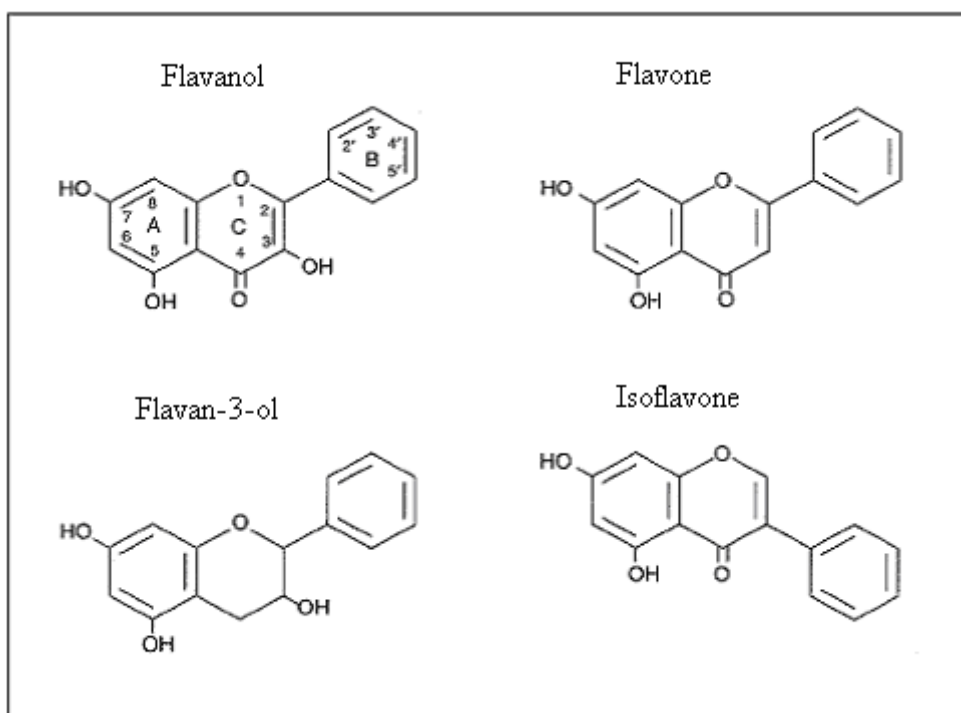
Figure 2.4. Structure of the flavonoid subgroups: flavonol, flavone, flavanol, and isoflavone (Source: Rice-Evans, et al.1997)

• Flavones:

Flavones are phenolic compounds that contain one carbonyl group (Cowan 1999). Apigenin and luteolin are the examples of flavones. Celery and olives are the foods having been known with their high flavones content (King and Young 1999).

• Flavonols:

Flavonols are the most common type of flavonoids. The addition of 3-hydroxyl group to the flavones yields a flavonol (Cowan 1999). Quercetin, kaempferol and myricetin are the three widely distributed flavonols. Onion, apple, kale and tea are the examples of foods which are high in their flavonol levels (King and Young 1999).

• Flavanols:

Flavanols are the flavonoids lacking the 2,3-double bond and the 4-one structure (Rice-Evans, et al.1997). Catechin and epicatechin are the most famous flavanols. These flavanols can be seen in combination with gallic acid as in tea or with condensed tannin polymers in the case of fruits, legumes and grains (King and Young 1999).

• Isoflavones:

In Isoflavones, the B ring is located in the 3 position of the C ring (Rice-Evans, et al.1997). Isoflavones are the phenolic constituents that are mostly specific for legume family, especially for soybeans. The most famous isoflavones are genistein and daidzein (King and Young 1999).

Phenolic Acids

Phenolic acids are grouped into hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic and hydroxycinnamic acids have a single-ring structure (Tsao and Deng 2004).

• Hydroxybenzoic Acid:

Ellagic and gallic acids can be evaluated under this group and hydroxybenzoic acids are commonly seen in berries and nuts.

• Hydroxycinnamic Acid:

Caffeic and ferulic acids are examples of hydroxycinnamic acids. These groups of phenolic acids have the property of heat sensitivity.

Tannins

Vegetable tannins (plant polyphenols) are one of the most numerous and widely distributed categories in the plant kingdom, with more than 8000 phenolic structures currently known (Liao, et al. 2003). Tannins are water-soluble phenolic compounds having a molecular weight between 500 and 3000 Da. These polyphenols contain a large number of hydroxyl or other functional groups. In addition, they are capable of forming cross-linkages with proteins and other macromolecules (Chung, et al. 1998). This group of phenolics can be investigated in two categories. Hydrolyzable tannins (polyesters of gallic acid and polysaccharides) and condensed tannins (polymerized products of flavan-3-ols and flavan-3,4-diols, or a mixture of the two). Other type of tannins may also be seen as a combination of these two basic structures (Liao, et al. 2003).

• Hydrolyzable Tannins:

Hydrolyzable tannins contain a central core of polyhydric alcohol such as glucose and hydroxyl groups which are esterified either partially or wholly by gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins). After hydrolysis by acids, bases or certain enzymes, gallotannins yield glucose and gallic acids. The hexahydroxydiphenic acid of ellagitannins undergoes lactonization to produce ellagic acid (Chung, et al. 1998).

• Condensed Tannins:

Condensed tannins are structurally more complex than hydrolyzable tannins. Their complete structures are yet to be determined. They are mainly the polymerized products of flavan-3-ols and flavan-3,4-diols or a mixture of the two. The polymers, referred to as "flavolans", are popularly called condensed tannins. Condensed tannins are widely distributed in fruits, vegetables, forage,

plants, cocoa, red wine and certain food grains such as sorghum, finger millets, and legume (Chung, et al. 1998).

Phenolic compounds are known with their high level of antioxidant activities and this property of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Kahkönen, et al. 1999). The antioxidant activity of the dietary polyphenolics is considered to be much greater than that of the essential vitamins, therefore contributing significantly to the health benefits of fruits (Tsao and Deng 2004). Before going into detail about the

antioxidant activity of phenolic compounds, it is important to learn what antioxidants are and what are their action towards free radical species.

2.3. Antioxidants

Organisms develop a series of defence mechanisms as they exposure to free radicals from a variety of sources. Defence mechanisms against free radicals include i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defences, and (iv) antioxidant defences (Kirschvink, et al. 2007, Valko, et al. 2007).

An antioxidant may be defined as 'any substance that when present at low concentrations, compared with those of the oxidizable substrate significantly delays or inhibits oxidation of that substrate' (Antolovich, et al. 2002, Prior and Cao 1999). The molecular structure of antioxidants is so suitable that they can safely react with free radicals and they are willing to give up their own electrons to free radicals. Antioxidants are not only donate a hydrogen atom, but also form a radical with low reactivity and in this state there is no further possibility of antioxidants to react with lipids (Madhavi, et al. 1996).

Antioxidants are of two types based on mechanism of action; primary or chain-breaking and secondary or preventative antioxidants. Primary antioxidants, such as Vitamin E and beta-carotene, may either delay or inhibit the initiation step by reacting with a lipid radical or inhibit the propagation step by reacting with peroxyl or alkoxyl radicals. Secondary antioxidants, usually enzymes, retard the rate of chain initiation by various mechanisms. Binding metal ions, scavenging oxygen, decomposing hydroperoxides to nonradical products, absorbing UV radiation and deactivating singlet oxygen are the processes that secondary antioxidants follow in the prevention of lipid oxidation (Antolovich, et al. 2002, Scheibmeir, et al. 2005, Madhavi, et al. 1996).

The antioxidant enzymes inside cells are an important defense against free radicals. Enzymatic antioxidant defences include superoxide dismutase, glutathione peroxidase and catalase. The catalytic activity of these enzymes allows the transformation of superoxide anion into hydrogen peroxide and water, thereby inactivating important amounts of oxidants. Trace-elements, such as selenium zinc, copper and manganese play an important catalytic role for the enzymatic activity (Kirschvink, et al. 2007). Non-enzymatic antioxidants are represented by ascorbic acid

(Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants (Valko, et al. 2007).

2.3.1. Reaction of Antioxidants

Lipid peroxidation is an important process that gives rise to many radicals such as lipid radical, peroxyl radical and alkoxyl radical. To understand the role of antioxidants in inhibiting lipid peroxidation, the reaction of antioxidants towards these radials should be taken into account.

Primary antioxidants may either delay or inhibit the initiation step by reacting with a free radical or they may inhibit the propagation step by reacting with the peroxyl or alkoxyl radicals. The reaction of antioxidant towards these radicals can be seen below (Madhavi, et al. 1996, Antolovich, et al. 2002).

$$L^{\bullet} + AH \longrightarrow LH + A^{\bullet}$$
$$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet}$$
$$LO^{\bullet} + AH \longrightarrow LOH + A^{\bullet}$$
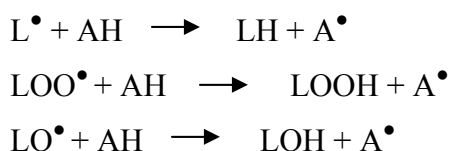
where,

AH: an antioxidant

$L^{\bullet}$: lipid radical

$LOO^{\bullet}$: peroxyl radical

$LO^{\bullet}$: alkoxyl radical

It is seen from the above reactions that after donating a H atom to the radicals, antioxidants form antioxidant free radicals ($A^{\bullet}$). Once antioxidant free radicals are formed they are neutralized by forming nonradical compounds.

$$A^{\bullet} + LOO^{\bullet} \longrightarrow LOOA$$
$$A^{\bullet} + LO^{\bullet} \longrightarrow LOA$$
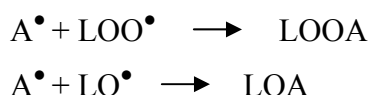
If there is an increment in the A-H and L-H bond dissociation energies, the activation energy of the antioxidant reactions increases. Hence, the efficiency of the

antioxidant increases with decreasing A-H bond strength. In other means, the weaker A-H bonds yields the more efficient antioxidants (Antolovich, et al. 2002).

2.4. Antioxidative Activity of Polyphenols

Antioxidant activity of polyphenols means first of all radical scavenging associated with their simultaneous oxidizing (Filipiak 2001). To be effective against free-radical-mediated cell disturbances, the antioxidants or free-radical scavengers must have several important characteristics. These criteria are as follows:

o The scavenger (antioxidant) must get to the right site within the cell of the relevant tissue in a concentration that is sufficient to allow effective competition with neighboring molecules.

o The scavenger (antioxidant) must get to the right site at the right time in order to interact with transient damaging free-radical species as they are formed.

o The scavenger (antioxidant) must be able to interact with the toxic species sufficiently rapidly to ensure successful competition with biologically sensitive loci in the immediate vicinity of free radical production.

o The scavenger (antioxidant) must have acceptable biological properties, that is, its inherent toxicity must be low.

o Finally in summary, the scavenger (antioxidant) must get to the right site at the right time and in the right concentration; it must have acceptable low intrinsic toxicity for use under conditions in vivo (Madhavi, et al. 1996).

Antioxidants are divided into two groups according to their origin as 'natural antioxidants' and 'synthetic antioxidants'. Most of the synthetic antioxidants are of the phenolic type. The differences in their antioxidant activities are related to their chemical structures, which also influence their physical properties such as volatility, solubility and thermal stability (Madhavi, et al. 1996). The commercially available and currently used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butyl hydroquinone (TBHQ). In recent years, there is an increasing interest in natural antioxidants and subsequently looking through the literature it is recognized that the replacement of synthetic antioxidants by natural ones may have several benefits and much of the research on natural antioxidants has focused on phenolic compounds, in particular flavonoids as potential sources of natural

antioxidants (Balasundram, et al. 2006, Moure, et al. 2001, Bonilla, et al. 2006). Structural features and nature of substitutions on rings B and C determine the antioxidant activity of flavonoids. These can be summarized as follows:

o The degree of hydroxylation and the positions of the –OH groups in the B ring, in particular an ortho-dihydroxyl structure of ring B (catechol group) results in higher activity as it confers higher stability to the aroxyl radical by electron delocalisation or acts as the preferred binding site for trace metals.

o The presence of hydroxyl groups at the 3'-, 4'-, and 5'-positions of ring B (a pyrogallol group) has been reported to enhance the antioxidant activity of flavonoids compared to those that have a single hydroxyl group. However, under some conditions, such compounds may act as pro-oxidants, thus counteracting the antioxidant effect. This is consistent with the observation of Seeram and Nair who reported that the conservation of the 3',4'-dihydroxyphenyl to 3',4',5'-trihydroxylphenyl increases the antioxidant activity for anthocyanidins but decreases the activity for catechins.

o A double bond between C-2 and C-3, conjugated with the 4-oxo group in ring C enhances the radical scavenging capacity of flavonoids.

o A double bond between C-2 and C-3, combined with a 3-OH, in ring C, also enhances the active radical scavenging capacity of flavonoids, as seen in the case of kaempferol. Substitution of the 3-OH results in increase in torsion angle and loss of coplanarity and subsequently reduced antioxidant activity.

o Substitution of hydroxyl groups in ring B by methoxyl groups alters the redox potential, which affects the radical scavenging capacity of flavonoids (Balasundram, et al. 2006).

2.5. Olive Leaf and its Antioxidative Properties

The principal letters of the oldest alphabets are symbols for an agricultural society. Alpha refers to an ox, beta to a house, gamma to a camel and zeta to an olive. In Hebrew, the name of olive is "Zait". Arabs call it "Zaitun", Cretans called it "Elaiwa" and the Greeks call it today "Elai". In French and English it is referred to as "olive", Romans first called it "olea" then "oliva". The word became "vivax oliva", which means "the one which has seven lives". Turks encountered the olive when they arrived

in Anatolia and they first called it "zeytun" and then "zeytin", which is the modern Turkish word for olive today.

There are two species of tree in the Garden of Eden, the fig tree and the olive tree. The Fig tree is the "tree of the truth" and the olive tree is the "tree of longevity". Since we began with the Garden of Eden, let's go to the beginning of everything. "The moment when having had eaten the forbidden fruit, Adam and Eve were banished from the Garden of Eden. Adam, after falling from the Garden of Eden, felt that the end was near and he decided to beg God for his forgiveness upon himself and therefore, upon the humanity as a whole. Meanwhile, he sent his son Seth to the Garden of Eden. The guardian angel of the Garden of Eden gave Seth three seeds that he had taken from the tree of the knowledge of the good and evil telling him to bury these three seeds into his father's mouth after his death. When Adam died, he was buried in the Mount Tabor. Three shoots sprouted from the ground on the site of his grave, an Olive Tree, a Cedar and a Cypress, three symbols of the Mediterranean flora. The first to get green among them is the Olive Tree. As the oldest statement referring to the Olive in the Old Testament as well other references in the New Testament and the Koran",

"olea prima arborum umnium est."
"The olive tree is the first tree of all." (sadecezeytin 2005)

In recent years, there is a growing interest obtaining the biologically active compounds from natural sources. The protective effects of diets rich in fruit and vegetables against cardiovascular diseases and certain cancers have been attributed partly to the antioxidants contained therein, particularly to phenolic compounds (Benavente-Garcia, et al. 2000).

Olive tree is botanically known as *Olea europaea* and its products have been recognized as important components of a healthy diet because of their phenolic content. Large body of epidemiological studies has shown that the incidence of coronary heart disease and certain cancers, e.g., breast and colon cancers, is lowest in the Mediterranean basin where the diet is rich in olives and olive products (Al-Azzawie, et al. 2006).

Many data have been reported on the polyphenols of olive fruits and olive oil, but only few studies have been published on olive leaves (Savournin, et al. 2001).

Olive leaf has known as a symbol of Mediterranean Region and peace since ancient times. Historically, olive leaf has been used as a folk remedy against fevers and other diseases such as malaria. Several reports have shown that olive leaf extract had the capacity to lower blood pressure in animals, to increase blood flow in the coronary arteries, to relieve arrhythmia and to prevent intestinal muscle spasms. These health benefits of olive leaf are probably due to its rich polyphenolic content (Benavente-Garcia, et al. 2000).

Three structural groups are important for determining the radical scavenging and/or antioxidative capacity of flavonoids.

- o The o-dihydroxy (catechol) structure in the B-ring, which confers greater stability to aroxyl radicals
- o The 2,3-double bond conjugated with a 4-oxo function, responsible for electron delocation from the B-ring
- o The presence of both 3- and 5-hydroxyl groups for maximal radical-scavenging capacity and strongest radical absorption.

From these structures, it is mainly the o-dihydroxy (catechol) structure, which confers the antioxidant properties to the olive leaf extract (Benavente-Garcia, et al. 2000).

The stability of the aroxyl radical formed is another factor that determines the antioxidant potential of phenolic compounds. However, the aroxyl radical species of the olive leaf mono and polyphenols have molecular structures capable of an extensive electron delocation, which is a prerequisite for radical stabilization, generating multiple mesomeric structures. The decay rate constants of flavonoid aroxyl radicals generated by interrelation with other radicals show that all the most stable aroxyl radicals, without exception, contain the 3',4'-catechol B-ring substitution pattern. All other polyphenolic compounds form far less stable aroxyl radicals (Benavente-Garcia, et al. 2000).

Olive leaf extract from *Olea europaea* leaves contain five groups of phenolic compounds. These are oleuropeosides, flavones, flavonols, flavan-3-ols and substituted phenols. The phenolic groups of olive leaf extract with their examples are given in Table 2.2 and the chemical structure of the most abundant phenolics from each of these groups is given in Figure 2.5.

Table 2.2. The phenolic groups of OLE with their examples
(Source: Benavente-Garcia, et al. 2000)

Group	Examples
oleuropeosides	oleuropein
	verbascoside
flavones	luteolin-7-glucoside
	apigenin-7-glucoside
	diosmetin-7-glucoside
	luteolin
	diosmetin
flavonols	rutin
flavan-3-ols	catechin
substituted phenols	tyrosol
	hydroxytyrosol
	vanillin
	vanillic acid
	caffeic acid
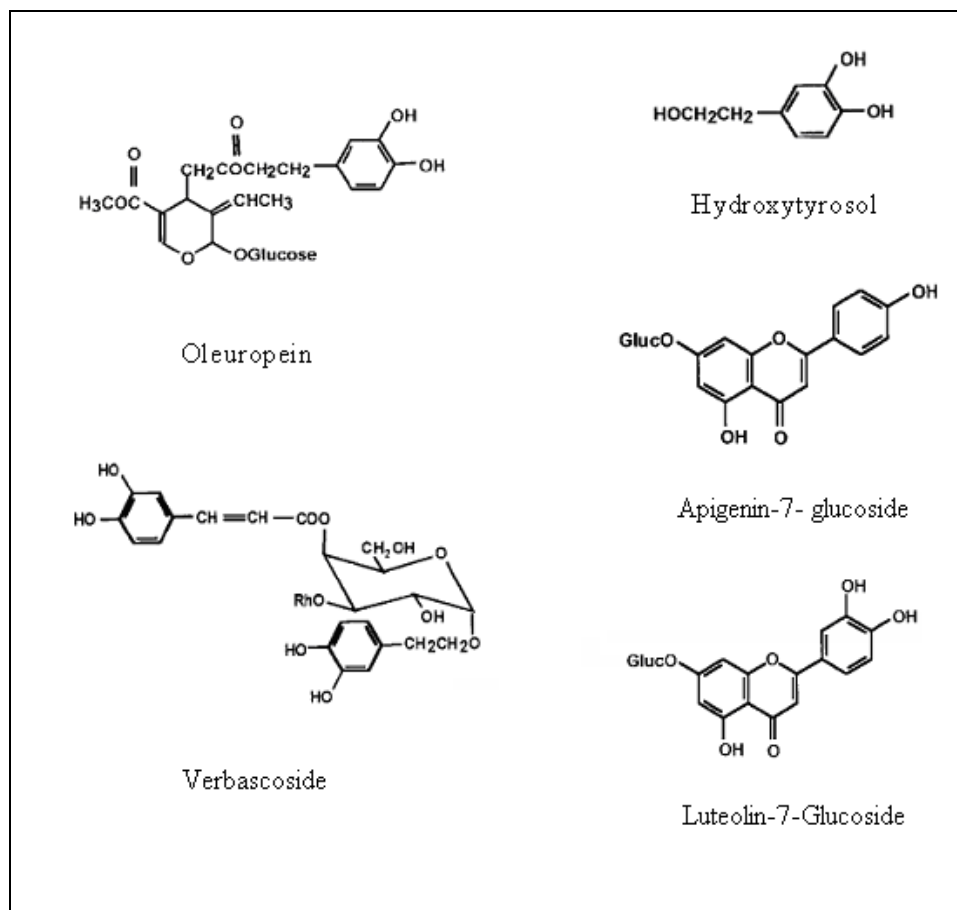
Figure 2.5. Groups of phenolics and chemical structures of the most abundant phenolics in olive leaf extract (Source: Benavente-Garcia, et al. 2000)

Garcia et al. (2000) reported the sequence of the antioxidant capacity of the flavanoids in olive leaf extract as;

rutin > catechin ≈ luteolin > OL ≈ hydroxytyrosol > diosmetin > caffeic acid > verbascoside > oleuropein > luteolin-7-glucoside ≈ vanillic acid ≈ diosmetin-7-glucoside > apigenin-7-glucoside > tyrosol > vanillin.

This sequence of relative radical scavenging abilities confirm the importance of the flavonoid B-ring catechol structure that present in rutin, catechin and luteolin, the presence of a 3-hydroxyl free or glycosylated group (catechin and rutin), and the 2,3-double bond conjugated with a 4-oxo function (rutin and luteolin). Although the 2,3-double bond conjugated with a 4-oxo function is absent in catechin, its antioxidant activity is closer to rutin, that confirms the importance of flavonoid B-ring catechol structure and the presence of a free 3-hydroxyl group when scavenging ABTS$^{\bullet+}$ radical. From this sequence, it is understood that flavonols, flavan-3-ols and flavones with catechol structures are the most efficient phenolic compounds of olive leaf when

scavenging ABTS$^{\bullet+}$ radical and this ability increases as more free hydroxyl groups are present in the flavonoid structure. In addition, the flavonoids, oleuropeosides and substituted phenols are realized to show synergic behaviour in mixed form, as it is a characteristic for olive leaf extract with a high content of oleuropein (Benavente-Garcia, et al. 2000).

2.5.1. Oleuropein

Oleuropein is the principle active component in olive leaf extract (*Olea europaea*) and it is also present in olive oil and olive fruit (Caturla, et al. 2005, Japon-Lujan and Castro 2006, Savournin, et al. 2001, Tuck and Hayball 2002).

Oleuropein was discovered in 1908 by Bourquelot and Vintiles (Benavente-Garcia, et al. 2000). The bitter compound oleuropein is the major constituent of the secoiridoid family in olive tree (Malik and Bradford 2006, Benavente-Garcia, et al. 2000). Oleuropein is an ester that consists of elenoic acid and 3,4-Dihydroxyphenylethanol. 3,4-Dihydroxyphenylethanol is more commonly known as hydroxytyrosol and it is the principal degradation product of oleuropein (Tuck and Hayball 2002, Tan, et al. 2003, Benavente-Garcia, et al. 2000). The structure of oleuropein and its metabolites are shown in Figure 2.6.
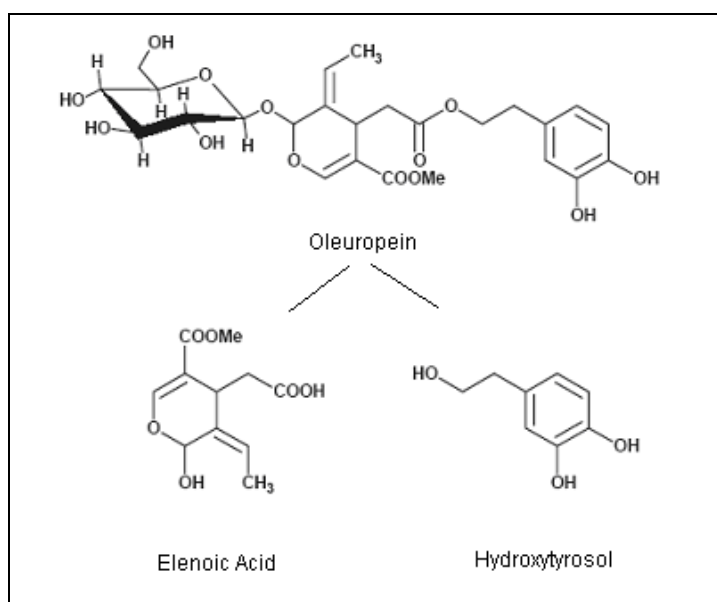
Figure 2.6. Structure of oleuropein and its metabolites elenoic acid and hydroxytyrosol

(Source: Al-Azzawie, et al. 2006)

Oleuropein is present in high amount in unprocessed olive fruit and leaves while hydroxytyrosol is more abundant in the processed olive fruit and olive oil (Tan, et al. 2003).

Oleuropein posses a wide range of pharmacologic and health promoting properties. It prevents cardiac diseases by protecting membrane lipid oxidation acting on coronary dilation and by antiarythmic action; improves the lipid metabolism to make the obesity problems better; protects enzymes and cell death in cancer patients. Many of these properties have been observed as a result of the antioxidant character of oleuropein (Japon-Lujan, et al. 2006, Japon-Lujan and Castro 2006, Al-Azzawie, et al. 2006). Antioxidant activity of oleuropein is mainly due to the hydroxytyrosol moiety in its structure (Benavente-Garcia, et al. 2000).

Oleuropein acts as an antioxidant at both prevention and intervention levels. Formation of free radicals is prevented by its ability to chelating metal ions such as Cu and Fe. Intervention of oleuropein with already present free radicals may come about through providing hydroxyl group to directly neutralize and quench free radicals (Al-Azzawie, et al. 2006).

Oleuropein and its metabolite hydroxytyrosol both posses the structural requirement needed for optimum antioxidant activity; it is the catechol structure in the B-ring. Oleuropein and hydroxytyrosol have been shown to be scavengers of superoxide ions and inhibitors of the respiratory burst of neutrophils and hypochlorous acid-derived radicals. Both compounds also scavenged hydroxyl radicals with oleuropein showing greater activity. These compounds are also reported to be effective scavengers of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Al-Azzawie, et al. 2006).

Oleuropein is also known with its antimicrobial activity against viruses, retroviruses, bacteria, yeasts, fungi, molds and other parasites (Benavente-Garcia, et al. 2000). Micol, et al. (2005) reported the antiviral activity of oleuropein against the viral haemorrhagic septicaemia virus (VHSV), which infects continental and sea farmed fish and a wide range of wild marine species in Europe, North America and Japan. Oleuropein is reported to show in vitro capacity to inhibit viral infectivity in a dose dependent manner when preincubated with the virus before infecting cells and also when administered post-infection. It is indicated that the olive leaf extract can be considered as a potential source of natural, selective, safe, low environmental impact and cost-effective antivirals with relevant interest in aquaculture. Furthermore,

oleuropein can be considered as a lead compound for the rational design of therapeutic agents for other rhabdovirus and/or enveloped virus (Micol, et al. 2005).

Due to the biological activities, it is quite important to determine the oleuropein amount in olive leaf. Analytical methods used in the qualitative and quantitative determination of polyphenols involve techniques of chromatographic separation such as HPLC and GC-MS. However, these techniques are expensive, reagent and time consuming. On the other hand, biosensors are attractive alternative techniques in polyphenols detection due to their unique characteristics such as selectivity, low cost, miniaturization, easy automation, time saving and simplicity of operation and manufacturing (Gomes, et al. 2004).

2.6. Biosensors - Definitions

The term sensor can be defined as a device or system including control and processing electronics, software and interconnection networks that responds to a physical or chemical quantity by producing an output which is a measure of that quantity (Patel 2002). Sensors can be divided into three categories, namely physical sensors, chemical sensors and biosensors. Physical sensors are used for measuring distance, mass, temperature, pH and etc (Eggins 2002). According to the common nomenclature proposal, chemical sensors are the compact devices that transform a chemical information into an analytically useful and measurable signal. Chemical sensors usually contain two basic components that are connected in series; a chemical recognition system and a physico-chemical transducer. The selective and reversible detection of the chemical sensors is accompanied by the electrical signal that is obtained from the physicochemical transducer (Thevenot, et al. 2001, Vastarella 2001).

Biosensors are special chemical sensors in which the recognition system utilises a biochemical mechanism (Thevenot, et al. 2001, Vastarella 2001).

A biosensor is an analytical tool or system consisting of a biological material that is in intimate contact with a suitable transducer which can convert a biochemical signal into a qualifiable electrical signal (Gronow 1991, Freire, et al. 2003b).

Biosensors can be also defined as analytical devices incorporating biological materials such as enzymes, tissues, micro-organisms, antibodies, cell receptors or biologically derived materials or a biomimic component in intimate contact with a

physico-chemical transducer or transducing microsystems (Mello and Kubota 2002, Malhotra, et al. 2005).

According to International Union of Pure and Applied Chemistry (IUPAC), a biosensor is precisely defined as a self-contained integrated device, capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element, which is retained in direct spatial contact with a transduction element (Vastarella 2001).

Schematical representation of a biosensor system is given in Figure 2.7.
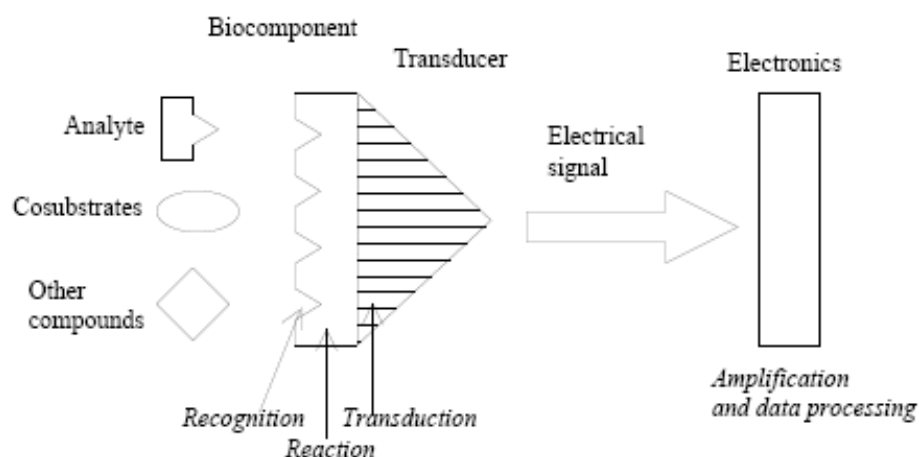
Figure 2.7. Biosensor system
(Source: Streffer 2002)

As it is seen from the above figure, biosensors are made up of three different but strictly connected elements, the selector (biocomponent), the transducer and the detector. The selector is the part of the biosensor that selectively binds and recognizes the compound to be detected; the transducer transfers the signal from the output domain of the recognition system to a physically measurable signal; the detector permits to display the chemico-physical signal into a suitable form (Vastarella 2001).

2.7. Classification of Biosensors

All biosensors rely on highly specific recognition events to detect the target analytes and suitable transducers to obtain measurable signal for the analyte of interest. Figure 2.8 shows some analytes that are possible to be analyzed in a biosensor system

(Mello and Kubota 2002). When this figure is investigated, it is understood that there are various combinations of the biological material and the transducer depending on each sample of interest and the type of physical magnitute to be measured. So, it is clear that the classification of the biosensors is made in agreement of their composition. Biosensors are classified based on their biological recognition elements or transducers or alternatively the combination of these two aspects (Vastarella 2001).
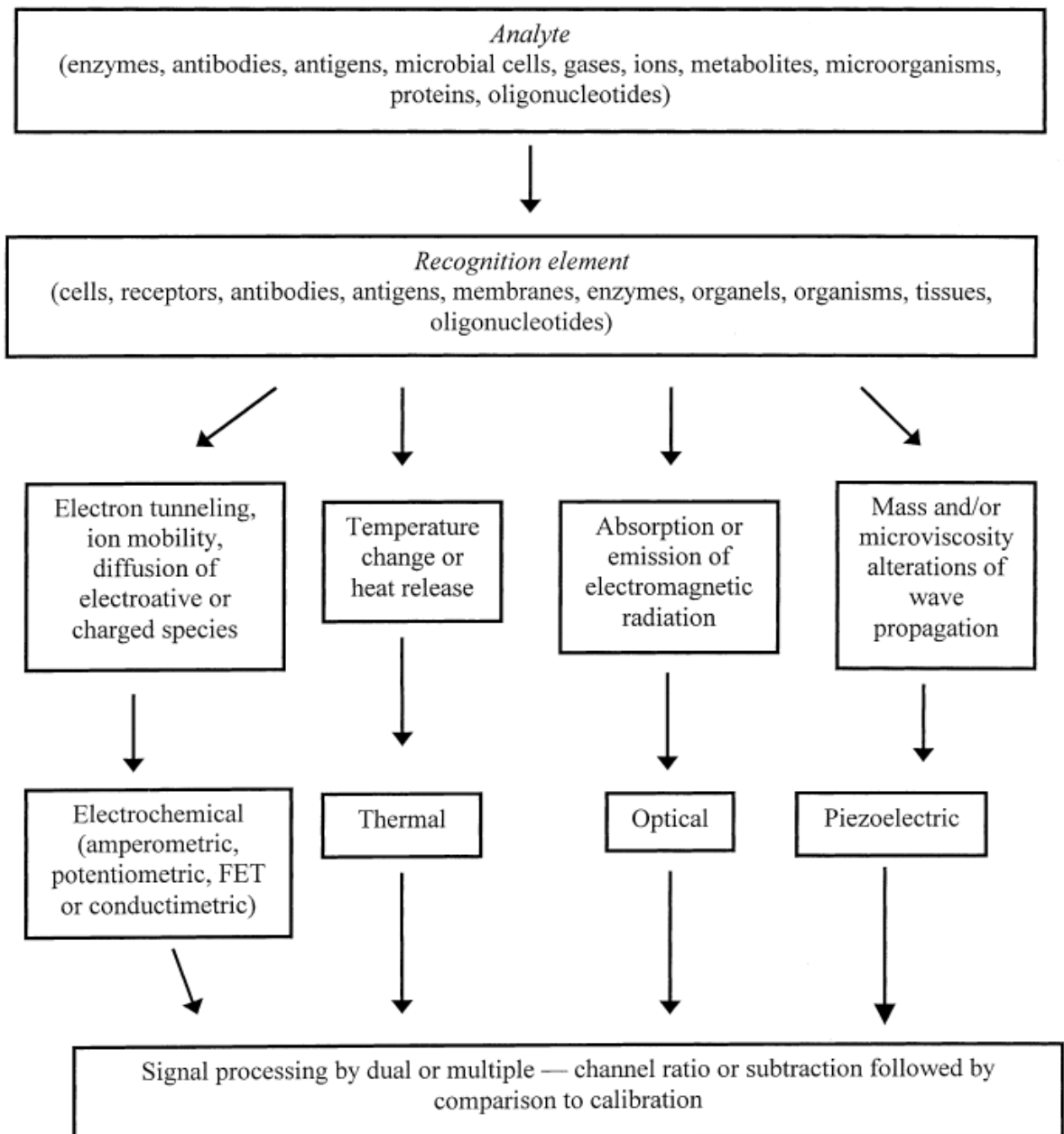
Figure 2.8. Biocomponent and transducers in a biosensor system

(Source: Mello and Kubota 2002)

Biological elements are the major selective elements in a biosensor system. The type of the biological recognition element determines the degree of selectivity or specificity of the biosensor. Biological recognizers in a biosensor system can be enzymes, tissues, antibodies, microorganisms, cells and organelles (Voort, et al. 2005). A transducer in a biosensor system converts the biological signal into an electrical one. The transducer element may be categorized into several groups. The major groups are electrochemical, optical, piezoelectric, acoustic, wave and thermal (Gooding 2006).

2.7.1. Biological Recognition Elements

The biorecognition molecule such as an enzyme, antibody, sequence of DNA, peptide or even a microorganism provides the biosensor with a high degree of selectivity for the target analyte so that the molecule of interest can be picked out by the biosensor from a matrix of many other molecules (Gooding 2006). The biological recognizers in a biosensor system are divided in three major groups; biocatalytic, bioaffinity and hybrid receptors (Mello and Kubota 2002).

Biocatalytic receptors:

Among the other types of biological recognition elements, the biocatalytic-based biosensors are the best known and studied and have been most frequently applied to biological matrices since pioneering work of Clark, et al. (1962). Three types of biocatalyst are commonly used. These are enzymes, whole cells and tissue slices. Among the enzymes used as biocatalyst, mono or multi enzymes take the attention. These are the most common and well developed recognition systems. Examples to whole cells are microorganisms and cell organelles or particles. Plant or animal tissue slices are taken into consideration under the type of tissue slices (Thevenot, et al. 2001).

In the case of biocatalytic receptors, enzymes, microorganisms or tissue slices are employed and a reaction is catalyzed with these birecognition molecules involving the analyte to give a product (Gooding 2006).

Biosensors which use microorganisms, plant or animal tissue as biocomponents have the advantage of the elimination of the unnecessary procedures such as extraction

and purification because of the fact that enzymes are used as active components (Mello and Kubota 2002).

Bioaffinity receptors:

Biorecognition molecules commonly used in affinity biosensors may be antibodies, DNA, peptides and lectins. Affinity biosensors are characterised by a binding event between the biorecognition molecule and the analyte often with no further reaction occurring (Gooding 2006). Affinity based biosensors provide selective interactions with a given ligand to form a thermodynamically stable complex (Mello and Kubota 2002). Hence, transducing the biorecognition element is a challenging task. As this class of biosensor is compatible with the detection of virtually all biological agents, researchers attempt to develop portable devices for detecting toxins, microbes and viruses. Transduction of affinity biosensors has been achieved using labelled species and using label free apprpoaches (Gooding 2006).

The potential use of immunosensors is due to their general applicability, specifity, selectivity of the antijen-antibody reaction. The antigen–antibody complex may be utilized in all types of sensors. The physichochemical change induced by antigen–antibody binding does not generate an electrochemically detectable signal. Therefore, enzymes, fluorescent compounds, electrochemically active substrates, radionuclides or avidin-biotin complexes are used to label either the antigen or the antibody. The most common transducers to immunosensors are acoustic and optical systems (Mello and Kubota 2002).

Hybrid receptors:

The principle of selective detection by hybrid receptors is based on the detection of a unique sequence of nucleic acid bases through hybridization. The nucleic acid structure is a double helix conformation of two polynucleotide strands. Each strand is constituted of a polymeric chain that contains Adenin, Thymine, Cytosine and Guanine bases. These bases are complemented by three hydrogen bonds in the C-G base pair and by two hydrogen bonds in the T-A base pair. This property of base pairing gives the ability of one single strand to recognize its complementary strand to form a duplex. DNA sensors composed of well defined sequences of single strands as biological

receptors in their immobilized form onto a solid matrix. A DNA probe is added to DNA or RNA from an unknown sample. If the probe combines with the unknown nucleic acid because of pairing of complementary base recognition, detection and identification are possible. DNA-based analytical method seems to be the only method for detecting genetic modifications and is the most sensitive approach for detecting microorganisms (Mello and Kubota 2002).

2.7.2. Transducers

The transduction element of a biosensor must be capable of converting a specific biological reaction (binding or catalytic) into a response which can be processed into a useable signal (Scott 1998). The transducer part of the sensor serves to transfer the signal from the output domain of the recognition system mostly to electrical domain. Bi-directional signal transfer which means the transfer of non-electrical signal to electrical one and vice versa is provided by the transducer. The synonyms of transducer are detector, sensor or electrode but the term transducer is preferred to avoid confusion (Thevenot, et al. 2001). The specifity of the biorecognition element for a substrate can be monitored by several ways such as oxygen consumption, hydrogen peroxide formation, changes in NADH concentration, fluorescence, absorption, pH change, conductivity, temperature or mass. Hence, the biosensor can be classified in several types according to its transduction element (Mello and Kubota 2002). The commonly used transducers are outlined in Table 2.3.

Table 2.3. Commonly used transducers

(Source: Scott 1998)

Transducer	Examples
Electrochemical	
Amperometric	Clark oxygen electrode, chemically modified electrodes
Potentiometric	Ion-selective electrodes, field effect transistors
Conductimetric	Platinum electrodes
Optical	Optical fibres, evanescent field devices
Acoustic	Surface acoustic wave devices
Thermal	Thermistor, thermopile
Piezoelectric	Piezoelectric crystals

Amperometry was the basis of the first biosensor, designed by Clark and Lyons in 1962, in which glucose oxidase was immobilized next to a Clark oxygen electrode. It has continued to be the most popular approach to biosensing largely due to its inherent simplicity, the ease of mass production, the low cost and availability of instrumentation. The technique involves the measurement of current at a fixed potential. The signal is dependent on the rate of mass transfer to the electrode surface and hence it is common to use a diffusion barrier to minimize the variations due to turbulance and to extend the linear range of the sensor. In the simplest mode of operation, the release of an electroactive product or consumption of reactant due to a biocatalytic reaction can be monitored directly at an inert working electrode in amperometry (Scott 1998).

Potentiometric sensors are also known as ion-selective electrodes. These sensors are a popular class of analytical sensors that generally posses long lifetimes and acceptable mechanical stability. The main appeal lies in the simple instrumentation, low cost and their suitability for continuous monitoring. However, these sensors recently undergone a revolution in terms of lowering the detection limits to ultratrace levels. Consequently they are being used to perform billions of measurements each year in virtually every hospital throughout the world. A major problem with potentiometric sensors is the leaching of the membrane components into the sample but this influence has been improved by incorporating minute amounts of electroactive ingredients (Pejcic and Marco 2006).

Conductiometric biosensors are based on the principle of the change of conductivity of the medium when microorganisms metabolize uncharged particles or

intermediates such as carbohydrates or lactic acid. The amount of the charged metabolites is directly proportional to the growth rate of the organism and it can be easily quantified. By ion conductometric transducers it is possible to monitor many biological membrane receptors. Conductimetric biosensors are usually nonspecific and have a poor signal noise ratio therefore, have been little used (Mello and Kubota 2002).

Optical biosensors comprise a rather heterogeneous group of sensors in which the interaction of light with an immobilized biologically active material is sensed. They often contain a light source in addition to the signal transducer. Optical sensors offer a wide range of advantages; they are not susceptible to disturbances by electric fields, they are suited for continuous indication and the shape remains chemically unchanged during the measurement. Although optical sensors offer a number of advantages there is a limitation in their usage. They should be operated only in the dark since the day light disturbs the measuring procedure (Scheller and Schubert 1992).

Even if the electrochemical and the optical sensors dominate, other forms of transducers such as thermal and acoustic transducers are also used. Thermal and acoustic transducers are sufficiently effective in analytical applications, they have the advantage of miniaturization and the possibility of construction of arrays of sensors for simultaneous determination of several compounds but a characteristic problem of these transducers are that they have a lack of selectivity (Mello and Kubota 2002). When a chemical reaction is catalyzed by enzymes or microorganisms, there is a change in energy and this change can be monitored by thermal transducers. However heat cannot be confined in an adiabatic system so, there is always a loss of information due to the fact that the produced heat is partly wasted by irradiation, conduction or convection (Mello and Kubota 2002).

The principle of piezoelectric sensors is that the frequency of vibration of an oscillating crystal is decreased by the adsorption of a foreign material on its surface. The crystal is sensitized by covering it with material binding or reacting with the analyte. Piezoelectric sensors are used for the measurement of ammonia, nitrous oxides, carbon monoxide, hydrocarbons, hydrogen, methane, sulfur dioxide and certain organophosphate compounds.

Up to this point, biosensor classes depending both on the biorecognition element and the transducer are explained. To sum up, enzymes are the biological components most commonly used in biosensors while electrochemical transduction is the most popular method often employing potentiometric or amperometric techniques. In

potentiometric devices the analytical information is obtained by converting the biorecognition process into a potential signal, whereas the amperometric types are based on monitoring the current associated with oxidation or reduction of an electroactive species involved in the recognition process (Freire, et al. 2003b). Amperometry is the most valuable tool in the electrochemical detection of polyphenols and due to this reason much attention will be given to amperometric biosensors.

2.8. Amperometric Biosensors

Amperometric biosensors are based on the measurement of the current resulting from the oxidation or reduction of electroactive species (Vastarella 2001). It is usually performed by maintaining a constant potential at a Pt, Au or C based working electrode or an array of electrodes with respect to a reference electrode, which may also serve as the auxiliary electrode, if currents are low (Thevenot, et al. 2001). The resulting current is correlated to the reaction of electroactive substances within the adjacent biocatalytic layer or it can be correlated to the bulk analyte concentration of electroactive species (Vastarella 2001). As biocatalytic reaction rates are often chosen to be first order dependent on the bulk analyte concentration, such steady-state currents are usually proportional to the bulk analyte concentration (Thevenot, et al. 2001).

An amperometric biosensor may be more attractive due to its high sensitivity and a wide linear range. It offers more precise and accurate results and it is also not necessary to wait until the thermodynamic equilibrium is obtained. Therefore, amperometric enzymatic electrodes hold a leading position among the presently available biosensor systems. These devices combine the selectivity of the enzyme for the recognition of a given target analyte with the direct transduction of the rate of the biocatalytic reaction into a current signal, allowing a rapid, simple and direct determination of various compounds. However, the selectivity of the amperometric devices is only governed by the redox potential of the electroactive species present (Mello and Kubota 2002, Freire, et al. 2003b).

2.8.1. Response Characteristics of Amperometric Sensors

Some criteria must be satisfied in the use of amperometric sensors for the best detection performance. Accordingly, it is useful to examine how standard protocols for performance criteria may be defined in accordance with the standard IUPAC protocols.

Response (slope) of the electrode:

One of the most important response characteristics of amperometric electrodes is the slope considered for the linear concentration range. The slope of the amperometric electrodes can be determined either by the graphical method or by the linear regression method. From the graphical method, the slope of the amperometric electrode is calculated as the tangent of the angle formed in the calibration line (Nejem 2004).

Limit of detection:

The limit of detection is defined by IUPAC as the concentration at which, under specified conditions, the intensity of the current I, deviates from the average value by a multiple of the standard error of a single measurement of the intensity of the current in this region. The amperometric electrode limit of detection can be considered as the concentration

i) where the limiting current intensity value is equal with the one obtained for the buffer solution,

ii) where the limiting current intensity value is double the one obtained for the buffer solution,

iii) below the one where the intensity of the current remains constant (Nejem 2004).

Linear concentration range:

The linear concentration range can be defined by the range of concentration of the substrate over which the sensitivity of the electrode is constant with a specific variation. This response characteristic is very important because the activity of concentration of all the solutions to be measured must lie within the linear concentration range (Nejem 2004).

pH range:

The pH value plays a critical role in the biochemical reaction that occurs at the electrode-solution interface. The enzymes are working in a specific pH range so, it is very important to optimize the pH to find the best catalytic activity of the enzyme (Nejem 2004).

Response time:

IUPAC defines the response time as the time which elapses between the instant when the electrodes of the amprometric cells are brought into contact with a sample solution or at which the activity of the ion of intrest in solution is changed and the first instant at which the slope becomes equal to a limiting value selected on the basis of the experimental conditions. Response time is a function of the kinetics of the reaction that takes place on the electrode surface and it increases as the concentration of the analyte decreases (Nejem 2004).

The influence of temperature:

Similar to pH, the response of the sensor is highly affected by the temperature. The increase of the temperature will favor the kinetics and the thermodynamics of the processes that occur at the sensor surface and as a result the slope will increase. It is also very important to optimize the temperature for the biochemical reaction and maintain it in its optimized value during the experimental work (Nejem 2004).

2.8.2. Selectivity of Amperometric Sensors

Selectivity is related to the accuracy and precision of the measurements in the presence of the inferring substances. Two classes inferring substances effects the response of amperometric sensors. These are the substances whose response is similar to the analyte and the substances which interact with the detected compound. Both mixed solution and separate solution methods can be used for the determination of amperometric selectivity coeffiecients (Nejem 2004). In the mixed solution method, selectivity is expressed as the as the ratio of the signal output with the analyte alone and with the interfering substance alone at the same concentration as that of the analyte. In the separate solution method, interfering substances are added, at their expected

concentration, into the measuring cell, already containing usual analyte concentrationat the mid-range of its expected value. Selectivity is then expressed as the percentage of variation of the biosensor response (Thevenot et a. 2001).

2.8.3. Enzymes for Amperometric Biosensors

Enzymes are proteins which catalyze the substrate specific reactions extremely rapidly. In an analytical process, enzymes are used for specific estimation of the corresponding substrates and they provide a significant amplification system for the sensitive detection of a substrate. Enzymatic biosensors utilize specific enzymes for the capture and catalytic generation of the product (Patel 2002). Generation of the product is directly determined by using a range of transducers however a majority of enzyme based biosensors employ the amperometric transduction method (Pejcic and Marco 2006).

Enzymes are divided into six major classes according to their function. These are oxido-reductases, transferases, hydrolases, isomerases and ligases (Streffer 2002). Among the enzymes commercially available, oxidases are the most often used in biosensor applications. This type of enzyme offers the advantages of being stable, and in some situations does not require coenzymes or cofactors (Mello and Kubota 2002). Oxido-reductases can be classified into four groups; oxidases, dehydrogenases peroxidases and oxygenases. Oxidases are the enzymes capable of transfering hydrogen from a substrate to molecular oxygen. Oxidases are also divided into two groups based on the product formed during catalysis. These are water producing (copper containing) and hydrogen peroxide producing oxidases (Streffer 2002). Copper containing oxidase enzyme laccase is the most commonly used enzyme in biosensor applications.

2.8.3.1. Laccases

Oxidation reactions are essential for biosensor applications but these reactions have some drawbacks such as having non-specific or undesirable side reactions and using of environmentally hazardous chemicals. However, enzymatic oxidation is known to be a promising technology with a lot of advantages over chemical oxidation. Enzymes are specific and biodegradable catalysts and enzyme catalyzed reactions are

carried out in mild conditions. Several enzymes are responsible for the enzymatic oxidation and enzymes recycling on molecular oxygen as an electron acceptor are the most interesting ones. Thus, laccase is a particularly promising enzyme for the oxidation recations (Couto and Herrera 2006).

Laccase (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) is an oxidoreductase able to catalyze the oxidation of various aromatic compounds, particularly phenols with the concomitant reduction of oxygen to water. Laccases exhibit four copper atoms, which play an important role in the enzyme catalytic mechanisms (Duran, et al. 2002). Laccase was first described by Yoshida in 1883 which makes it one of the oldest enzymes ever described and it was characterized as a metal containing oxidase by Bertrand in 1985 (Mayer and Staples 2002).

Until recently, laccase is known to be widely distributed in higher plants, in fungi and in insects but now there is a strong evidence for their widespread distribution in some bacterial strains (Claus 2004).

In a typical laccase reaction, a phenolic substrate is subjected to a one-electron oxidation giving rise to an aryloxyradical. This active species can be converted to a quinone in the second stage of the oxidation. The quinone as well as the free radical product, undergoes non-enzymatic coupling reactions leading to polymerization (Duran, et al. 2002, Minussi, et al. 2002). Laccase does not require hydrogen peroxide (H_2O_2) as co-substrate and any co-factors for the catalytic oxidation which makes the use of laccase very useful in oxidation reactions (Roy, et al. 2005, Gamella, et al. 2006).

Laccases are found in nature in different forms. Among the different laccases, the enzymes isolated from Trametes strains are generally more stable (Ncanana, et al. 2007). The structure of laccase from *Trametes versicolor* is given in Figure 2.9.

Figure 2.9. *Trametes versicolor* laccase

(Source: The Armstrong Research Group 2005)

The spectroscopic studies have shown that the catalytic unit of the laccase contains four copper atoms classified in three types,

Type I (T1): paramagnetic 'blue' copper,

Type II (T2): paramagnetic 'non-blue' copper and

Type III (T3): diamagnetic spin-coupled copper-copper pair (Duran, et al. 2002, Claus 2004).

The copper ions in the active site of the laccase, provide electron transfer mechanism by switching their oxidation states between Cu(II) and Cu(I). The function of the T1 center is to provide the long-range intramolecular electron transfer from the substrate to the T2/T3 redox copper center. The T2/T3 copper center plays a key role in the reduction of oxygen. Between the two T3 coppers there is an oxygen ligand, either OH^- or O^{-2}, which coordinates with the T2 and T3 copper ions. The solvent and oxygen have access to the T2/T3 center through two channels. The fully reduced trinuclear copper center reacts with dioxygen to generate a peroxide level intermediate and finally, molecular oxygen is reduced to water (Ivnitski and Atanassov 2007),

$$O_2 + 4H^+ + 4e^- \xrightarrow{4Cu^{+2}} 2H_2O$$
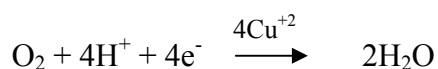
Laccases have wide substrate specificity and great potential for the determination of phenolic compounds (Timur, et al. 2004). The broad specificity of the phenolic substrates enables laccase to be developed as a biosensor for the determination of total phenols (Quan, et al. 2004).

In several studies it is reported that laccase in its immobilized form is more stable than laccase in solution (Fei, et al. 2007). Looking through the literatute, it is seen that there are various studies using immobilized laccase in biosensor applications. Laccase enzyme was immobilized on different supports in biosensor applications such as carbon fibres, redox hydrogel on glassy carbon, graphite, carbon paste, polyethersulphone membranes and platinum (Gomes, et al. 2004).

2.8.4. Enzyme Immobilization in Amperometric Biosensors

The success behind the enzyme biosensor relies on how well the enzyme bonds to the sensor surface and remains there during use (Vastarella 2001). Thus, the stabilization and storage of the enzyme is an important criterion in the biosensor development. Immobilization is the commonly used method for the bonding of the

enzyme to the sensor surface (Roy, et al. 2005). Depending on the nature of the support and properties and stability of the biomolecule, several methods can be used for immobilizing the enzyme onto the electrode surface including physical adsorption, covalent binding, encapsulation, entrapment and cross-linking (Freire, et al. 2003b). These methods can be schematically seen in Figure 2.10.
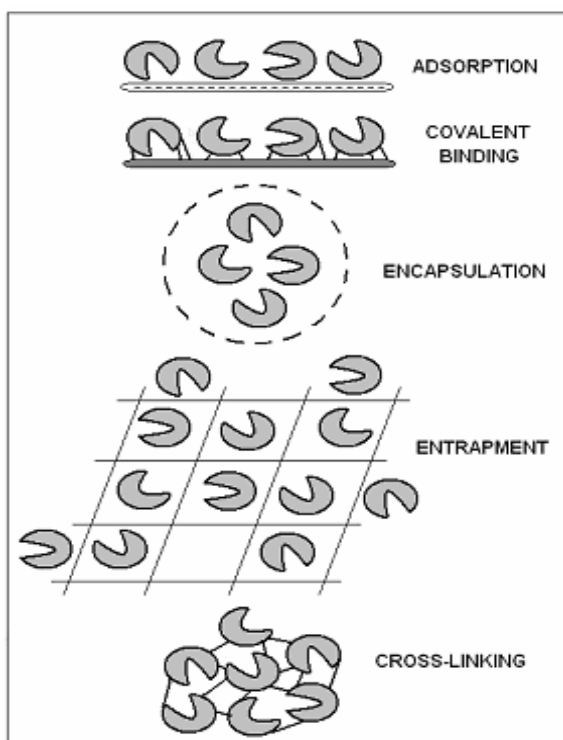
Figure 2.10. Principle methods of immobilization
(Source: Vastarella 2001).

The selection of an appropriate immobilization method depends on the nature of the biological element, type of transducer used, physicochemical properties of the analyte and operating conditions for the biosensor (Mello and Kubota 2002). A successful matrix should immobilize or integrate the biomolecules stably at a transducer surface and efficiently maintain the functionality of the biomolecules while providing accessibility towards the target analyte and an intimate contact with the transducer surface. The development of a good biocompatible matrix for immobilization of biomolecules is very crucial to improving the analytical performance of a biosensor (Xu, et al. 2006). Although, the most common methods for immobilization of biocomponents are adsorption and covalent bonding, the suitability of a method for a

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particular task is at present still being empirically elucidated. However, some generally valid aspects of immobilization techniques are outlined below (Scheller and Schubert 1992).

Physical Adsorption:

The pysical adsorption of the biocomponent is the simplest and oldest method of immobilization. It involves reversible surface interactions between enzyme and support material. The forces involved are mostly electrostatic as Van der Waals forces, ionic and hydrogen bonds. These forces are weak but they are sufficiently large in number in order to enable the necessary binding. Existing surface chemistry between enzyme and support is used in this method so, neither activation nor chemical modification are required. Thus, little damage is done to enzymes with this method. The simplest and cheap procedure consists of mixing together enzyme and support under suitable conditions of pH, ionic strength, incubation period and this is followed by the collection of the immobilized material and extensive washing step to remove the non-bound biological component (Vastarella 2001). Anionic and cationic ion exchange resins, active charcoal, silica gel, clay, aluminum oxide, porous glass and ceramics are being currently used as active material. The carrier should exhibit high affinity and capacity for the biomolecule and the latter must remain active in the adsorbed state. The carrier should also adsorb neither reaction products nor inhibitors of the biocatalyst. Since the adsorption of a protein to a surface is a reversible process, changes of pH, ionic strength, substrate concentration and temperature may detach the biomolecule from the carrier (Scheller and Schubert 1992). The most significant disadvantage is the leakage of biomolecules from the support with probable desorption and contamination of the solution. Physical factors as flow rate, bubble agitation, particle-particle abrasion can affect the desorption of the biocatalyst from the support. Therefore this method alone has not been used under flow conditions (Vastarella 2001). Despite having some limitations and disadvantages, immobilization by physical adsoption also offers an advantage additional to the simplicity of the procedure. It does not need nonphysiological coupling conditions or chemicals potentially impairing enzyme or cell functions. Hence, an activity loss is seldom observed in this method (Scheller and Schubert 1992).

Covalent Binding:

This method involves the formation of covalent bonds. Functional groups available in the enzymes or protein mainly originate from the side chain of the amino acids (Mello and Kubota 2002). Functional chemical groups belonging to amino acid residues on the surface of the enzyme that are not essential for activity may be attached covalently to chemically activated supports (Vastarella 2001). The immobilization by covalent binding is conducted in three steps; activation of the carrier, coupling of the biomolecule and removal of the adsorbed biomolecules. A disadvantage of covalent coupling is the frequently occuring loss of activity (Scheller and Schubert 1992).

Entrapment:

Immobilization by entrapment differs from adsorption and covalent binding in the case that enzymes are free in solution but they are restricted by the lattice structure of the entrapment system. Three general methods for entrapment are given below.

Entrapment behind a membrane: a solution of enzyme, a suspension of cells or a slice of tissue is simply confined by an analyte permeable membrane as a thin film covering the electrochemical detector,

Entrapment of biological receptors within a polymeric matrix: such as polyacrylonitrile, agar gel, polyurethane or polyvinyl-alcohol membranes, sol gels or redox hydrogels with redox centers,

Entrapment of biological receptors within self assembled monolayers or bilayer lipid membranes (Thevenot, et al. 2001).

The most used technique is the entrapment in polymeric film via casting or electropolymerization and in redox gel lattice. Gel porosity is an important parameter due to the reason that it gives an idea whether the structure is tight enough to prevent enzyme leakage and at the same time allow free movement of substrates and products. Inevitably, the support act as a barrier to mass transfer with serious implications for reaction kinetics, but it has also some useful advantages. For instance, interaction of the harmful biological compounds with the immobilized catalyst is prevented with the support matrix. In addition, some dangerous interference are avoided (Vastarella 2001).

Encapsulation:

Encapsulation of receptors can be achieved by enveloping the biological components within a semipermeable membrane. Encapsulation is very similar to entrapment method but it is restricted in a space. The driving force for the transfer of molecules out or into the membrane is the molecular weight. Large molecules cannot pass out or into the capsule but small substrates and products can pass freely across the semipermeable membrane. The problem associated with diffusion is acute and may result in rupture of the membrane if the reaction products accumulate rapidly. An additional problem is the similar density of the enzyme to that of the bulk solution. A distinct advantage of this method is the co-immobilization of enzyme/cell in any desired combination (Vastarella 2001).

Cross-linking:

This is a support free procedure and involves joining the receptor molecules with each other to form a large three dimensional complex structure. Crosslinking or cocrosslinking can be achieved by chemical or physical methods. Chemical method normally involves the formation of covalent bonds between the enzyme by means of bi- or multi functional reagents such as glutataldehyde and toluene diisocyanate. Both albumin and gelatine has been proved as good molecular spaces to minimize the close proximity problems that can be caused by crosslinking a single enzyme. Physical crosslinking of cells by flocculation is well known in biotechnology industry and does lead to high cell densities (Vastarella 2001).

Crosslinking method offers some advantages of being a simple method and having a strong chemical binding of the biomolecules. Furhermore, the choice of the degree of crosslinking permits the physical properties and the particle size to be influenced. The main drawback is the possibility of activity losses due to chemical alternations of the catalytically essential sites of the protein (Scheller and Schubert 1992).

2.9. Carbon Paste Electrodes

Carbon paste electrodes have been extensively used for electroanalytical applications since their introduction by Adams in 1958 (Mailley, et al. 2004). Carbon paste electrodes are produced by mixing graphite powder with a non-electrolytic binder. A non-electrolytic binder can be a hydrophobic material such as parafin oil or teflon or it can be a hydrophilic material such as polyacrylamide. Another constituent of the carbon paste electrode is a modifier. Modifying agent is usually one substance but the pastes can also be modified with two or even more components, which is in the case of carbon paste biosensors containing enzyme together with its appropriate mediator. The amount of modifier in the paste usually varies between 10-30 % (w/w) depending on the character of modifying agent and its capability of forming enough active sites in modified paste. Carbon paste based biosensors are developed by filling the mixture into a pipette tip. Electrical contact is supplied by inserting a silver wire into the carbon paste (Svancara, et al. 2001).

Carbon paste electrodes are suitable for various sensing and detection applications however, the use of a carbon paste electrodes in the electroanalytical applications exhibit some limitations. First, they have weak mechanical stability which results in leakage of the enzyme into the solution. Second, their fabrication reproducibility is very low. In addition, the lifetime of the carbon paste electrode is limited due the presence of the electrolytic binder. Moreover, the viscosity of the binder shows a significant effect on the electrode performance (Almeida and Giannetti 2002, Mailley, et al. 2004).

Despite having some advantages, carbon paste electrodes offer intristic advantages in the electroanalysis. Carbon paste electrodes are cheap, easy to handle. They offer a wide potential range and high electric conductivity. They have low background current and rich surface chemistry. Surface renewal and modification of carbon paste electrodes are also simple (Ghobadi, et al. 1996, Bolado, et al. 2007).

2.10. Electron Transfer Mechanism of Polyphenolic Compounds

Most of the antioxidants contain a phenolic group attached to the ring structures. The presence of a phenolic group and its relative ease of oxidation makes

electroanalytical methods suitable for the determination of such compounds. Electron transfer between the analyte and the electrode surface is the fundamental process of electroanalytical methods. In order to get the principles of the electroanalytical methods, first the electron transfer mechanisms should be understand in detail. The phenomenon of direct electron transfer in enzymes was first described for laccase (Freire, et al. 2003a).

Laccase, horseradish peroxidase and tyrosinase-based electrodes have shown good sensitivity and selectivity for determination of phenols. Laccases are o-diphenol and p-diphenol dioxygen oxidoreductases that catalyze the oxidation of diphenolic substrates in the presence of molecular oxygen. Schematical representation of the mechanism of the reactions on laccase biosensor is given in Figure 2.11. In these reactions, oxygen is reduced directly to water without the intermediate formation of hydrogen peroxide. The formed product can be electrochemically reduced to phenolic substances at low potential without any mediator. This kind of amperometric enzyme-based biosensors have been shown several advantages over direct electrochemical oxidation of phenolic compounds (Freire, et al. 2001, Freire, et al. 2003a).
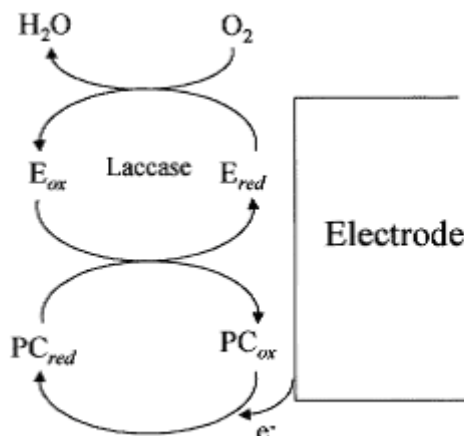
Figure 2.11. Mechanism of the reactions on the laccase biosensor; PC: phenolic compound, E: enzyme (Source: Freire, et al. 2001)

The oxidation of flavonoids is of great interest because of their action as antioxidants with the ability to scavenge radicals by electron transfer processes (Janeiro and Brett 2004). Flavonoids, particularly o-diphenols, can be oxidized to their corresponding semiquinones and quinones by oxidases such as polyphenol oxidases and

peroxidases. Oxidation of a flavonoid structute by laccase can be seen in Figure 2.12 (Pourcel, et al. 2007).
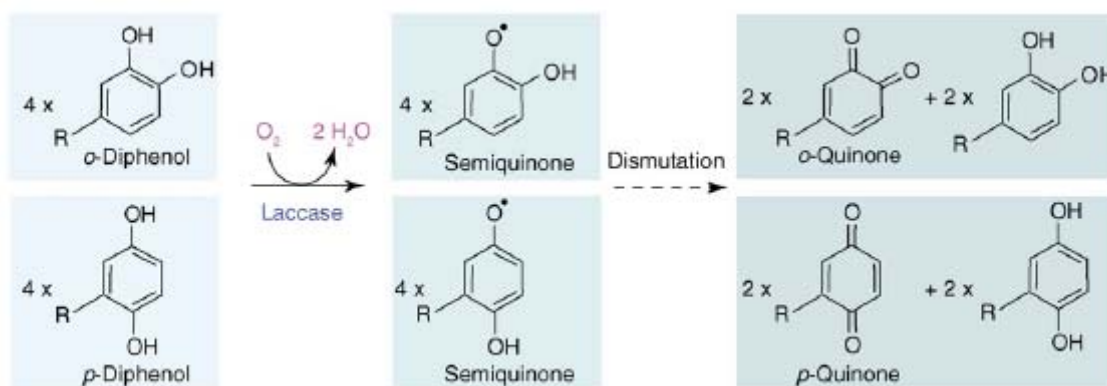
Figure 2.12. Oxidation of o- and p-diphenols by laccase

(Source: Pourcel, et al. 2007)

Figure 2.12 demonstrates that oxidation by laccase enzyme results in the formation of semiquinones and quinones which are highly reactive species and undergo undergo further non-enzymatic reactions. While this transformation occur, molecular oxygen is at the same time reduced at the electrode surface to water according to a direct four-electron mechanism as shown in the following,

$$O_2 + 4H^+ + 4e^- \xrightarrow{\text{laccase}} 2H_2O \text{ (Freire, et al. 2003b)}$$

After learning the general principles of the electron transfer mechanisms between the laccase enzyme and a polyphenolic compound, it is now necessary to learn the oxidation and reduction mechanism of the phenolic compound, oleuropein. Mechanism of oleuropein oxidation is given in Figure 2.13.
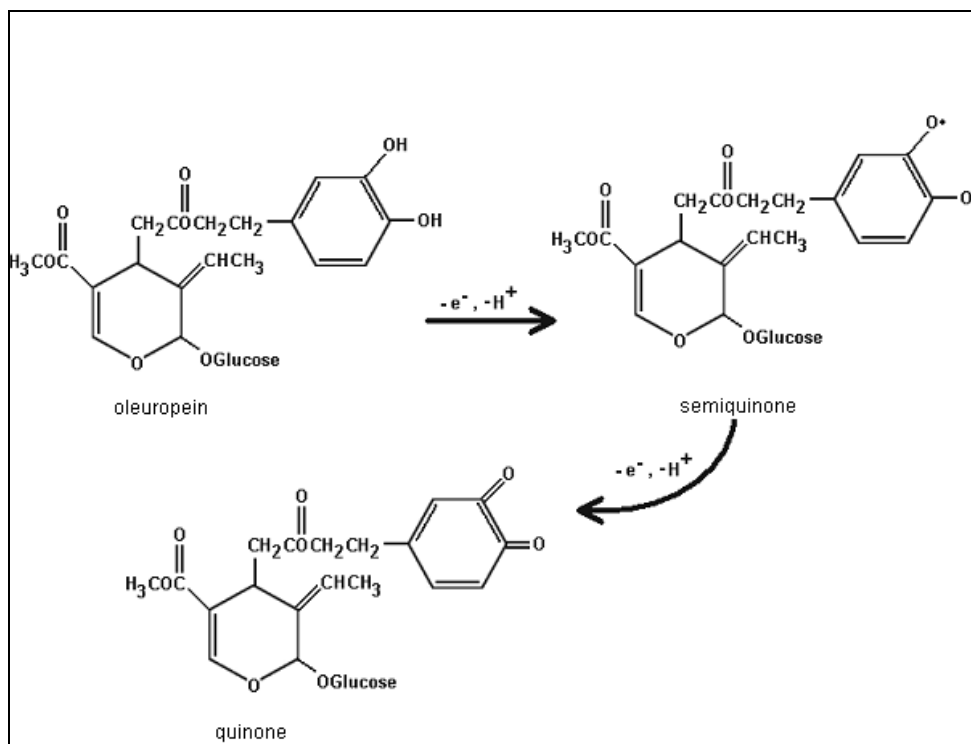
Figure 2.13. Mechanism of oleuropein oxidation

As shown in Figure 2.13, oleuropein gives its H atoms to the radical species and then it becomes a radical. However, polyphenols having antioxidant capabilities are able to stable themselves and does not posses any harmful effects for the living metabolism. Thus, it makes double bonds with its oxygen atoms and becomes a quinone.

2.11. Electrochemical Analysis of Polyphenolic Compounds

Qualitative and quantitative determination of polyhenols is usually done by HPLC or spectrometry. However these techniques are expensive reagent and time consuming. Electroanalytical methods are promising techniques to determine these substances with high accuracy in an extremely wide range of concentrations for separating and preconcentrating them (Climent, et al. 2001). There are many studies in the literature related with the electrochemical detection of polyphenolic compounds some of which are illustrated in Table 2.4.

Table 2.4. Some of the electrochemical studies of polyphenols from the literature

Detected Item	Sensor System	Reference
Phenolic content of extra virgin olive oil	Tyrosinase based sensor operating in organic solvent	Capannesi, et al. 2000
Polyphenolic compounds in red wine	Laccase biosensor immobilized on polyethersulphone membranes	Gomes, et al. 2004
Antioxidant capacity of different tea samples	SOD biosensor immobilized in kappa-carrageenan membrane	Campanella, et al. 2003
Antioxidant capacity of red and white wines		Campanella, et al. 2004a
Antioxidant capacity of several drug specialities		Campanella, et al. 2004b
Total antioxidant capacity of algae		Campanella, et al. 2005
Polyphenol content of different tea samples	Tyrosinase biosensor immobilized in kappa-carrageenan membrane	Campanella, et al. 2003
Polyphenol content of red and white wines		Campanella, et al. 2004a

Capannesi, et al. (2000) studied the phenolic content of extra virgin olive oil using two rapid procedures based on a disposable screen-printed sensor coupled with differential pulse voltammetry and an amperometric tyrosinase based biosensor operating in an organic solvent. The results obtained with these two innovative procedures were compared with a classical spectrophotometric assay using Folin-ciocalteau reagent and HPLC analysis. It was found that two proposed methods monitor the degradation reactions of oleuropein derivatives occurring in an extra virgin olive oil during storage, while the spectrophotometric analysis reveals the final products of these degradation reactions. Thus, the two proposed methods were more rapid and inexpensive in comparison with the classical methods for polyphenol analysis and could be considered promising systems for the evaluation of this class of compounds in oil samples (Capannesi, et al. 2000).

A biosensor was developed with laccase, immobilized on polyethersulphone membranes for the amperometric detection of polyphenolic compounds in red wine. Gomes, et al. (2004) could determine the amount of catechin and caffeic acid in red wines but the results were different from the HPLC results indicating that other polyphenols detected could have an important role in the deviation (Gomes, et al. 2004).

Campanella et al. (2003) developed superoxide dismutase (SOD) and tyrosinase biosensors for the determination of the polyphenol content and the antioxidant capacity of different tea samples. SOD biosensor was proved to be perfectly capable of determining the antioxidant capacity of all the different tea samples. Besides, the polyphenol content obtained using the tyrosinase biosensor displayed a trend that was very similar to that of antioxidant capacity (Campanella, et al. 2003).

Campanella et al (2004a) determined of the polyphenol content and the antioxidant capacity of red and white wines with SOD and tyrosinase biosensors. The results were compared with those of two traditional spectrophotometric methods and a spectrofluorimetric method. Good agreement was found between the results obtained using the latter methods and those obtained with the SOD biosensor. In addition, analysis of polyphenol content performed by a tyrosinase biosensor clearly revealed the good correlation in wine samples between polyphenol content and antioxidant capacity (Campanella, et al 2004a).

Antioxidant capacity of several drug specialities containing acetylsalicylic acid were studied by Campanella et al. (2004b) using SOD biosensor. The results were also compared with the traditional spectrofluorimetric method and by two other methods,

cyclic and pulsed voltammetry. The results obtained in this work showed a particularly good correlation between biosensor method and the ORAC spectrofluorimetric method and also a comparatively good correlation with the cyclic voltammetric method. So, SOD biosensor was found to be a reliable method also in the case of the antioxidant capacity of the drug specialities (Campanella, et al. 2004b).

Campanella et al. (2005) also studied the antioxidant capacity of three types of algea using different analytical methods both in their whole, homogenized and centrifugated forms. One of the analytical methods was based on the use of SOD biosensor which had already developed in the previous researches. From this study it could be confirmed that the SOD biosensor was a valid sensor type for measuring antioxidant capacity also in the case of algea.

2.12. Cyclic Voltammetry

Cyclic voltammetry is a potential-controlled reversible electrochemical experiment. In a typical cyclic voltammetry experiment, a voltage is applied to the working electrode in a triangle waveform from an initial value to a predetermined limit where the direction of scan is reversed and measuring the resulting current. Triangular waveform of the potential is given in Figure 2.14 (Lojou and Bianco 2006).
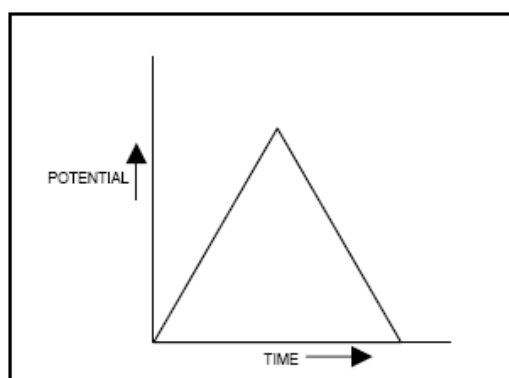
Figure 2.14. Traiangle waveform for the applied potential
(Source: Princeton Applied Research 2005)

As the potential is swept back and forth, the current on the working electrode is observed. Analysis of the current response can be used to study the thermodynamics and kinetics of electron transfer at the electrode-solution interface. Moreover it helps to

study the kinetics and mechanism of solution chemical reactions initiated by the heterogeneous electron transfer (Gosser 1993). Cyclic voltammetry can also be described as an analytical tool by which information about the analyte can be obtained by measuring the current flowing on the working electrode that either oxidizes or reduces the analyte. The magnitude of this current is proportional to the concentration of the analyte in solution, which allows cyclic voltammetry to be used in an analytical determination of the analyte concentration (Pine Instrument 1996).

The current at the working electrode is called faradaic current and this current is transduced to an output at a selected sensitivity. The output can be current-time curve or since the potential is linearly related with time, it is current-potential curve. The representation of the response in cyclic voltammetry experiment is usually the current-potential curve, which is called "cyclic votammogram". Voltage sweep and current response for the cyclic voltammetry experiment are given in Figure 2.15.
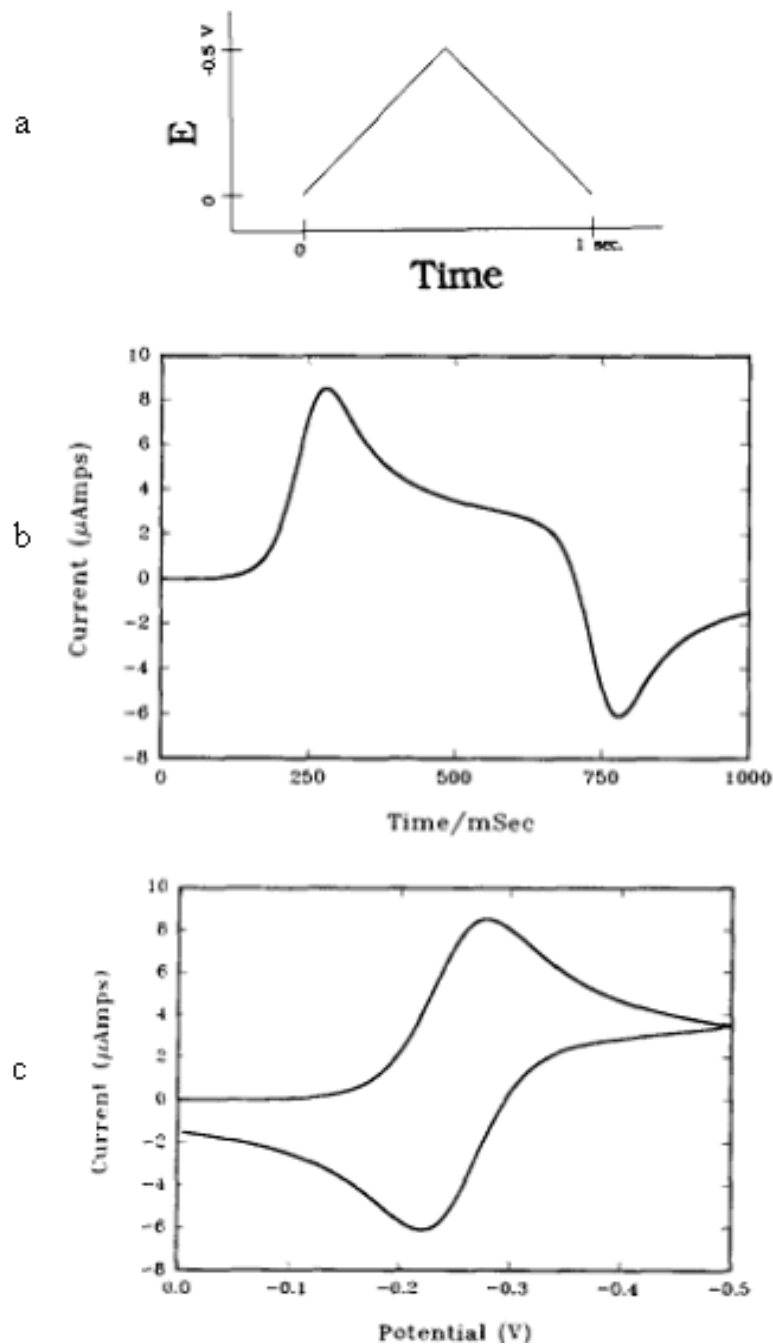
Figure 2.15. (a) potential waveform (b) current-time (c) current-potential representations in cyclic voltammetry experiment (Source: Gosser 1993)

Figure 2.15c shows a typical cyclic voltammogram for the case of a simple one electron transfer reaction. As the potential is swept in the forward direction, a cathodic peak is observed. Reduction occurs in this positive scan and the current resulting from the reduction is called cathodic peak current. The potential value in this point is called as cathodic peak potential. The representations of these terms are i_{pc} and E_{pc}

respectively. Note that the reduction current is taken as positive as the cathodic sweep goes from left to right. At the switching potential, the direction of the potential sweep is reversed. In the reverse scan, oxidation occurs and a peak resulting from the oxidation process is called anodic peak current. A negative anodic peak current is observed in the case of oxidation process. The representations for the anodic peak current and anodic peak potential are i_{pa} and E_{pa} respectively (Pine Instrument 1996, Gosser 1993). A typical cyclic voltammogram is given in Figure 2.16 with a clear representation of the defined characteristics such as i_{pc}, E_{pc}, i_{pa} and E_{pa}.
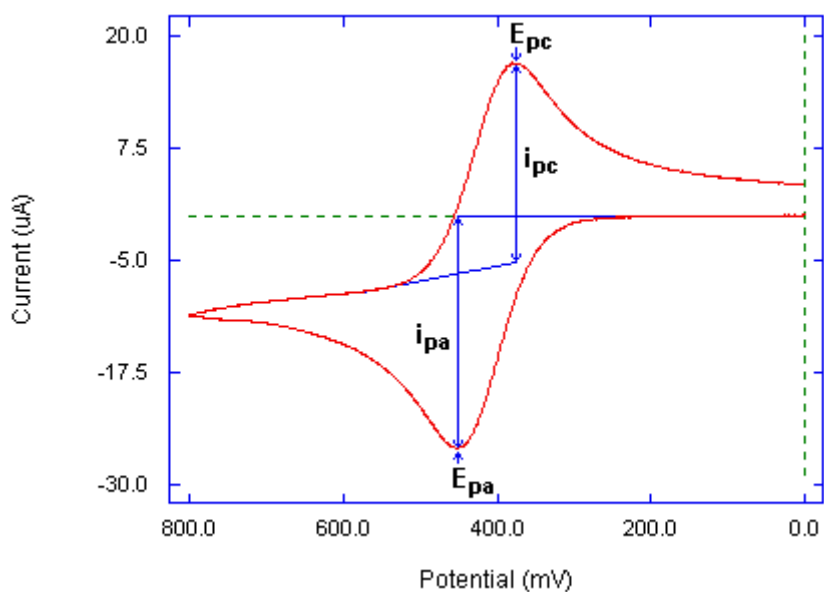
Figure 2.16. A typical cyclic voltammogram with defined characteristics
(Source: Bioanalytical Systems 2008)

As it is seen in Figure 2.16, during the positive scan, cathodic peak potential and cathodic peak currents are obtained. This is the direction of the scan where the electroactive species are reduced at the elecrode surface. As the scan is reversed, a negative anodic peak current and anodic peak potential are obtained. In this direction, reduced species are reoxidized on the electrode surface.

The equipment required to perform cyclic voltammetry experiment consists of a three electrode potentiostat that is connected to working, reference and auxiliary electrodes immersed in the test solution. The principle function of a potentiostat is to control the potential and measure the current. For the three electrode configuration, potential is applied to the working electrode with respect to the reference electrode and

an auxiliary electrode is used to complete the electrical circuit. A recording device such as computer or plotter is used to record the resulting cyclic

voltammogram as a graph of current versus potential (Pine Instrument 1996). Overall view of cyclic voltammetry experiment is given in Figure 2.17.
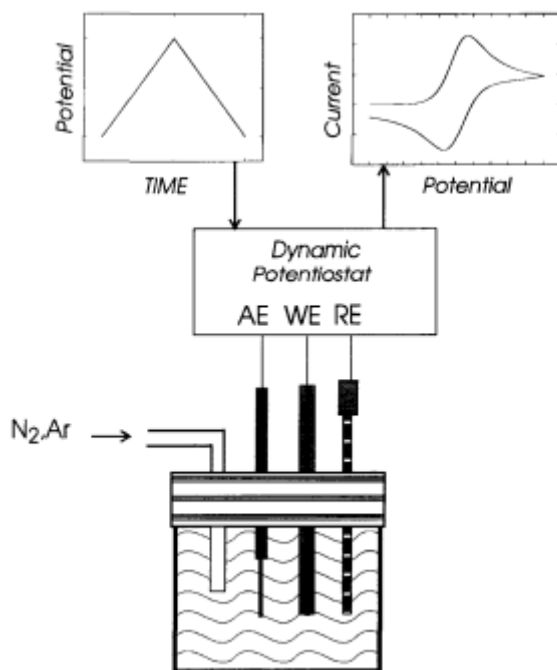
Figure 2.17. Overall view of cyclic voltammetry experiment; WE: working electrode, RE: reference electrode, AE: auxiliary electrode (Source: Gosser 1993)

The Electrochemical Cell:

Electrochemical cell, in its simplest form is a single piece of glassware. It provides to hold a necessary volume of analyte for electrochemical studies. Three electrodes (WE, RE and AE) are immersed in the test solution and electrical connection of this system to a potentiostat is achieved. The three neck round bottom flask is suitable piece of glassware that can be used in electrochemical studies. Having large enough diameters permits the electrodes to be fit through the neck openings. Each neck on the flask is used to mount one of the three electrodes. This configuration is quite suitable for the experiments where the test solution can be safely exposed to oxygen in the air and where isolation of each electrode in a separate compartment is not required. On the other hand, it is often necessary to eliminate dissolved oxygen from the test solution usually in the situations when quite negative potentials are applied to the

working electrode. At these potentials, dissolved oxygen can be reduced, resulting to an undesired cathodic current which may interfere with the measurement of interest. A solution to this problem is to use a cell which is airtight except for one or two gas inlets and a single small outlet. With this configuration, an inert gas such as nitrogen is bubbled through the solution to expel any dissolved oxygen. A four neck round bottom flask can also be used for the removal of oxygen. Three of the openings are used to mount the electrodes in an airtight fashion. The fourth opening is sealed by using a rubber septum. A small syringe needle is inserted through the septum near the outer edge of the septum. Then, a much longer needle is inserted through the centre of the septum and down into the test solution. Nitrogen gas is passed into the cell through the long needle while the small needle simply serves as the outlet. The long needle is first pushed down into the solution and nitrogen gas is allowed to bubble through the solution. Then, when it is time to perform an experiment, the long needle is pulled out of the solution (but not all the way out of the cell) and a blanket of nitrogen gas then covers the solution (Pine Instrument 1996).

Working Electrode (WE):

The working electrode is the electrode where the current is measured and at the same time the potential is controlled. Working electrode is the electrode in which the electrochemical reaction of interest takes place in its surface (Gamry Instruments 1997). The working electrode is usually an inert material with a well-defined geometry that is in direct contact with the analyte. The most widely used working electrodes are mercury, platinum, gold, and various forms of carbon (Pine Instrument 1996).

Reference Electrode (RE):

The potential of the working electrode in a voltammetry experiment is always controlled with respect to some standard and that standard is the reference electrode (Guide). A reference electrode may be considered a small battery whose voltage (potential) is determined by the chemistry taking place between a solid conductor (usually a metal salt) and the electrolytic solution around it (Bioanalytical Systems 2008). The most common reference electrodes are the Saturated Calomel Electrode (SCE) and the Silver/Silver Chloride (Ag/AgCl) electrodes (Gamry Instruments 1997).

Auxiliary (Counter) Electrode (AE):

In a traditional two electrode cells that have only a working and a reference electrode, current is necessarily forced to flow through the reference electrode. If current flows through a reference electrode, its internal chemical composition may be significantly altered, causing its potential to drift away from the expected standard value. Due to this reason it is desirable to make electrochemical measurements without current flowing through the reference electrode. Auxiliary or counter electrode provides an alternate route for the current to follow so that only a small current flows through the reference electrode (Pine Instrument 1996). The current that flows into the solution via the working electrode leaves the solution via the auxiliary electrode. The auxiliary electrode is generally an inert conductor like platinum or graphite (Gamry Instruments 1997).

Criterion for reversibility:

Reversibility is an important criterion for the cyclic voltammetry experiments. Several criteria can be used for the confirmation of the reversibility of one electron transfer process at the working electrode.

1) The difference in cathodic and anodic peak potentials is around 57-60 mV depending on the switching potential. Mathematically,

$$\Delta E_p = \text{abs}\ [E_{p,c} - E_{p,a}] \approx 58\ \text{mV}$$

But the expected 58 mV value may not be observed because of small distortions due to solution resistance effects and electronic or mathematical "smoothing" of the data. In most situations, the result is that ΔE_p is often 60-70 mV for reversible electron transfer.

2) The difference between the initial sweep peak and half-peak potentials of the forward sweep is 56 mV/n.

3) The shifted ratio of the cathodic to anodic currents is unity. Mathematically speaking,

$$i_{pc}\ /\ i_{pa}{}^* = 1$$

In the shifted ratio, the anodic peak current is measured from a baseline that is moved to a value that can be predicted from the decaying portion of the cathodic peak.

4) The forward scan peak current should be proportional to the square root of the scan rate. This criterion is used to distinguish "diffusion-controlled" processes from

processes featuring the adsorption of the electroactive species onto the electrode (in which case a linear current-scan rate relationship is observed). A plot of the log i_p versus log v is linear, with a slope of 0.5 for a diffusion peak and a slope of 1 for adsorption peak. Intermediate values of the slope are sometimes observed, suggesting a "mixed" diffusion-adsorption peak (Gosser 1993).

After learning some necessary concepts about cyclic voltammety, now it should be focused on how cyclic voltammetry can be used as an analytical tool for the evaluation of the antioxidant capacity of polyphenols. Redox properties are crucial for better understanding of the electron transfer process. Cyclic voltammetry is one of the most useful methods for the measurement of the electron transfer process (Chatterjee et. al 2007, Hotta, et al 2002). The phenolic groups of flavonoids can be electrochemically oxidized and show an oxidation or reduction peak in cyclic voltammetry measurements (Janeiro and Brett 2004). Thus, redox potentials of flavonoids determined by cyclic voltammetry have been utilised as a measure of the antioxidant capacity (Firuzi, et al. 2005). After a cyclic voltammogram is obtained from the cyclic voltammetry experiments, a number of parameters can be extracted from this voltammogram such as cathodic peak current, cathodic peak potential, anodic peak current and anodic peak potential. Evaluation of these cyclic voltammetry parameters is an important task to characterise the phenolics as reducing agents thus, gives an idea about the antioxidant capacity of phenolic compounds (Kilmartin and Hsu 2003).

CHAPTER 3

OBJECTIVES

The objective of this study was to develop an amperometric laccase biosensor for the detection of oleuropein that is the major component that contributes to the total antioxidant capacity in olive leaf. Since oleuropein is the biological active component of olive leaf, detection of this compound in an easy and cost effective way by biosensor method will be useful for food industry applications. The main goals of this study can be summarized as follows,

- o To investigate the behaviour of laccase enzyme for standard oleuropein solution,
- o To investigate the effect of glutaraldehyde amount and laccase concentration on the amperometric response,
- o To investigate the effect of scan rate and temperature on biosensor performance,
- o Extraction and fractionation of olive leaves,
- o Determination of the oleuropein amount in the fractions by biosensor and HPLC analysis,
- o To perform antioxidant capacity and total phenol analysis of the fractions for the determination of their biological activities.

CHAPTER 4

EXPERIMENTAL STUDY

4.1. Materials

Graphite powder (282863) was used as a conductive material for the construction of carbon paste biosensor. It is synthetic and < 20 microns and it was purchased from Aldrich (Steinheim, Germany). Mineral oil (69794), ultra for molecular biology, was used as a non-electroactive binder in the biosensor experiments and it was purchased from Fluka (Steinheim, Germany). Glutaraldehyde (49629) was the type of the crosslinking agent that was used in this study. It was obtained from Fluka in the form of solution. Laccase (38429) from *Trametes versicolor* (EC 1.10.3.2) was the selected biological recognition element and it was purchased from Fluka. Sodium phosphate dibasic and sodium phosphate monobasic dihydrate were used to prepare 50 mM phosphate buffer at pH: 6. Sodium phosphate dibasic (30427) and sodium phosphate monobasic dihydrate (04269) were both obtained from Riedel-de Haën (Seelze, Germany). Oleuropein was used as an analyte in the biosensor experiments. Oleuropein standard was purchased from Extrasynthese (Genay, France). Mobile phases for High Performance Liquid Chromatography experiments were acetonitrile (34851) and glacial acetic acid (1.0063). They were purchased from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany) respectively. Olive leaves used in the experiments were collected from the olive trees grown in the campus of Izmir Institute of Technology (Izmir, Turkey). Besides oleuropein, coumarin was also used as an internal standard and it was also obtained from Extrasynthese. HPLC grade ethanol (34870) was used in the extraction of olive leaves and it was purchased from Riedel-de Haën. Hydrophobic silk fibroin was used as an adsorbent for the adsorption of olive leaf phenols and it was obtained from Silk Biochemical Co., LTD. (China). Besides oleuropein standard, the response of the laccase biosensor to rutin, catechin, epicatechin and caffeic acid standards were investigated. Catechin (C-1251) and epicatechin (E-1753) were purchased from Sigma, caffeic acid (60020) was obtained from Fluka and rutin (5.00017)was purchased from Merck. Trolox (6-Hydroxy-2,5,7,8,-

tetramethylchromane-2-carboxylic acid) (56510) was used as a calibration standard in the lipid soluble antioxidant capacity (ACL) analysis and it was obtained from Fluka. Metanol (24229) was used as a reagent in the ACL analysis and it was purchased from Riedel-de Haën. Ascorbic acid (33034) was used as calibration standard in the water soluble antioxidant analysis (ACW) and purchased from Riedel-de Haën. Carbonate buffer was the reaction buffer in the antioxidant analysis and in order to prepare this buffer, sodium carbonate anhydrous, sodium bicarbonate and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) were used. Sodium carbonate anhydrous (71350) was purchased from Fluka, sodium bicarbonate (13433) was purchased from Riedel-de Haën, EDTA (03685) was purchased from Fluka. Luminol (09253) was the photosensitizer that was also used in the antioxidant analysis and it was obtained from Fluka. Dimethylsulphoxide (67-68-5) was the solvent used to dissolve the extracts and it was purchased from Carlo Erba (MI, Italy). For the total phenol analysis, gallic acid standard was used to construct the calibration curve. Gallic acid standard (1.59630) was obtained from Merck. Folin&Ciocalteu's reagent (F-9252) and sodium carbonate anhydrous (71350) were used in the total phenol analysis and they were obtained from Sigma and Fluka respectively.

Gamry Electrochemical Measurement Apparatus and Software was used in the cyclic voltammetry experiments. This system consists of three electrodes, a 7.5 cm long platinum electrode as an auxiliary electrode, saturated Ag/AgCl electrode in 3 M NaCl solution as a reference electrode and a working electrode which is enzyme immobilized carbon paste biosensor.

4.2. Pretreatment and Extraction of Olive Leaves

Extraction was used in order to recover phenolic compounds from olive leaves. For this purpose, firstly the leaves were pretreated and then the extraction procedure was followed.

4.2.1. Pretreatment of Olive Leaves

After the olive leaves were washed with deionized water, they were left into the oven (Membert-800, at 37^0C) for 3 consecutive days. 37^0C is a critical temperature for

drying due to the fact that there is a degradation risk of antioxidants over 40^0C. Then, grinding process was followed and the grinder was operated till the leaves became fine powder to about 90-150 μm. In this process, the grinder was stopped in 5 minute intervals to prevent frictional heating of the sample.

4.2.2. Extraction of Olive Leaves

Aqueous ethanol solution was used as extracting solvent due to its low cost and non-toxic nature. In the study of Hızal (2006), it was seen that the highest oleuropein and rutin yields was obtained by 70 % aqueous ethanol solution thus, this ratio was used in the extraction of olive leaves. After the suitable ethanol-water ratio was decided, olive leaf powder was processed with this solvent in 1/20 solid/liquid ratio. Extraction was performed in Comecta Thermoshaker for 2 hours at 180 rpm and 25^oC in order to ensure a complete homogenization of the solution. Centrifugation was the next step that enables to remove insoluble particles from the extraction medium. For this purpose, the solution was centrifuged in Beckman Coulter centrifuge at 5,000 rpm for 15 minutes. Then the liquid phase, separated in the centrifugation process, was transferred to the Heidolph rotary evaporator. Rotary evaporator was operated at 38^0C and 120 rpm rotation under vacuum. In the rotary evaporator, ethanol and partial water evaporation is achieved with a vacuum technique by reducing the interior pressure. The last step of the extraction was the lyophilization. Solvent free olive leaf extract was put in Telstar Cryodos freeze drier to remove the water content of the extract. Olive leaf extract was dried in a freeze drier system at -52^0C and below 0.2 mbar. After all these steps, olive leaf crude extract was obtained to be used in the next studies.

4.3. HPLC Analysis and Identification of Phenolic Compounds

High performance liquid chromatography (HPLC) analysis was performed for the analytical qualification and quantification of the phenolics in olive leaf extract. The operating conditions and properties of HPLC are given in Table 4.1.

Table 4.1. Operating conditions and properties of HPLC

Property	Value or Attribute
Column	Column C18 LiChrospher 100 analytical column
Column Length	250 mm
Column Diameter	4 mm
Particle Size	5 μm
Mobile Phase	Mobile Phase A: 2.5 % acetic acid in deionized water Mobile Phase B: 100 % acetonitrile
Flow Rate	1 ml/min
Temperature	30 °C
Detector	Diode Array Detector
Absorbance	280 nm

A linear gradient of the mobile phases was used in the HPLC analysis and this elution system is given in Table 4.2.

Table 4.2. HPLC elution program

Time (min)	Mobile phase A 2.5 % Acetic Acid in Deionized Water	Mobile phase B 100 % Acetonitrile
0	95 %	5 %
20	75 %	25 %
40	50 %	50 %
50	20 %	80 %
60	5 %	95 %

Identification of the phenolics in olive leaf extract was achieved by comparing their retention times with the corresponding standards. In this study, identification of the major phenolic of olive leaf extract, oleuropein, was taken into account. For this reason oleuropein in the olive leaf extract was firstly qualified by comparing its retention time with its standard and then its quantification was made by using the calibration curve. Calibration curve for this compound was constructed by using internal and external standard methods. In internal calibration, coumarin was used as a standard. Internal and external calibration curves of oleuropein were given in Appendix A.

4.4. Fractionation of Olive Leaf Antioxidants

Olive leaf extract contains various phenolic compounds in its structure and isolation of polyphenols from olive leaf extract is a very important task. It is quite important to concentrate on the fractionation of olive leaf phenols and obtain some olive leaf fractions to be used in biosensor studies. In this case, the aim is to achieve the adsoption of olive leaf phenols with a suitable adsorbent and then desorb them selectively in order to fractionate the olive leaf phenols.

An edible protein polymer silk fibroin is a favourable adsorbent used in adsorption studies. Due to its promising health effects, bounding mechanisms and hydrophobic character, Bayçın et al. (2007) studied the extraction and adsorption of the two most abundant polyphenols in olive leaf, oleuropein and rutin, on silk fibroin under different conditions. They also aimed to increase the purity of oleuropein and rutin therefore, desorption studies were performed after the adsorption to understand whether silk fibroin can be used as a purification material for these polyphenols. In addition, Altıok et al. (2008) used silk fibroin for the isolation of antioxidants from the olive leaf extract. From these studies, silk fibroin was understood to be a promising adsorbent for the purification of oleuropein in olive leaf extract and thus silk fibroin was used as an adsorbent for the fractionation of olive leaf phenols in this study.

Olive leaf crude extract which was prepared previously was dissolved in deionized water in 1/20 solid/liquid ratio. In order to remove the insoluble particles, this solution was centrifuged at 5000 rpm for 15 minutes. Then, a clear solution was obtained. A syringe column of 63 m in height and 10 mm internal diameter was filled with 7.8 g of silk fibroin powder. The column was preconditioned by washing with deionized water and ethanol. Then the clear solution of olive leaf crude extract was loaded to the column with GilsonTM ASPEC XL liquid handling system. The loading procedure was repeated for four times until silk fibroin was saturated with olive leaf phenols. The saturation of the adsorbent was checked by HPLC analysis of olive leaf extract solution at the column outlet after each loading procedure.

After the adsorption stage, desorption was followed. Proper eluting solvents should be selected for this goal. Deionized water and 70 % ethanol solution was respectively used as the eluting solvents in the desorption stage. The color of the eluting stream turned from yellow to white while nine batches of 100 ml deionized water were

passed through the column. In addition, desorption by four batches of 100 ml 70 % ethanol solution, caused the color change of the outlet stream from brown to white.

At the end of the desorption process, nine batches of polar and four batches of relatively less polar olive leaf extract fractions were analyzed in HPLC in order to determine the desorption efficiency. Depending on the HPLC results, some fractions were grouped together. These fractions were in the form of solution and they were processed in rotary evaporator and then freeze dryer. So, fractions were collected in their powder form and they were stored at $+4^0$ C to be used in biosensor experiments. The combined form of the fractions are given in Table 4.3.

Table 4.3. Fractions and their combinations

Fractions	Combination
Fraction I (FI)	W1+W2
Fraction II (FII)	W3+W4
Fraction III (FIII)	W5+W6
Fraction IV (FIV)	W7+W8+W9
Fraction V (FV)	E1+E2
Fraction VI (FVI)	E2+E3

After this work, olive leaf crude extract was fractionated and partial purification of phenolic compounds was achieved for biosensor studies.

4.5. Antioxidant Capacity Analysis

A number of assays have been developed for the detection of both general and specific antioxidant action. Of these, Trolox equivalent antioxidant capacity (TEAC) assay, first reported by Miller and Rice-Evans, is based on the scavenging ability of antioxidants on the $ABTS^{\bullet+}$ radical. The total radical-trapping antioxidant parameter (TRAP) assay is most often used for measurements of in vivo antioxidant capacity in serum or plasma as it measures the nonenzymatic antioxidants. The oxygen radical absorbance capacity (ORAC) assay has found broader application for measuring the antioxidant capacity of botanical and biological samples (Besco, et al. 2007, Prior, et al. 2005).

However most of these techniques require long experimental times and high costs for the detection of antioxidant capacity. Due to this reason, researchers are looking for a method that allow a quick analyse of antioxidant capacity (Besco, et al. 2007). A system for the measurement of the antioxidative capacity consists of a method that is first based on the generation of the radicals and then allows a detector for the quantification of the generated radicals. Generated radicals are partially eliminated by the antioxidants of the sample allowing the quantification of the antioxidative capacity (Rohe). This system is known as photochemiluminescence (PCL) assay and it is known to be a time and cost-effective system for the determination of the antioxidative capacity (Besco, et al. 2007).

This assay was described by Popov and Lewin and was commercialized by Analytik Jena AG (Jena, Germany). The PCL assay is sold as a complete system under the name PHOTOCHEM (Prior, et al. 2005). In the PCL assay, the photochemical generation of free radicals is combined with the sensitive detection by using chemiluminescence. The reaction is induced by optical excitation ($h\upsilon$) of the photosensitiser (S), which results in the generation of the superoxide radical $O_2^{\bullet-}$ (Vertuani, et al. 2004, Prior, et al. 2005).

$$S + h\upsilon + O_2 \rightarrow [\, S^* O_2 \,] \rightarrow S^{\bullet+} + O_2^{\bullet-}$$

Two basic kinds of radicals are present in the PLC measuring system; superoxide radical and luminol radical (Prior, et al.2005). Luminol acts as photosensitiser as well as oxygen radical detection reagent (Vertuani, et al. 2002). The PCL method can be conducted by two different propocols; ACW and ACL. The ACW protocol permits the measurement of the antioxidant capacity of water soluble components whereas; ACL permits the measurement of the antioxidant capacity of lipid soluble components (Vertuani, et al. 2004). In other means, antioxidant capacity of hydrophilic and lipophilic compounds can be separately determined by these protocols. On the other hand, integral antioxidant capacity (IAC) which represents the sum of the lipophilic antioxidant capacity and hydrophilic antioxidant capacity can also be determined by the summation of the antioxidant capacity obtained from these two different protocols. These two protocols have a different measurement mechanism. Calibration and measurements for ACW are based on the difference in lag time (L) between the sample and the blank (Harrison, et al. 2007).

$$L = L_0 - L_1$$

where L_0 and L_1 are the respective parameters in seconds for the blank and the sample.

The lipophilic antioxidant capacity is assayed by the degree of PCL inhibition (I), according to the calculation;

$$I = 1 - S/S_0$$

where S_0 is the integral under the blank curve and S is the integral under the sample curve.

Ascorbic acid and Trolox are typically used as calibration reagents for hydrophilic and lipophilic antioxidant capacity respectively (Prior, et al. 2005).

The PCL assay offers various advantages for the determination of the antioxidative capacity. First, as it is emphasized before, this system is marketed as a time and cost-effective system. Second, it does not require high temperatures to generate radicals and it is more sensitive (nanomolar range) to measure the scavenging activity of antioxidants against the superoxide radical which is one of the most dangerous reactive oxygen species (ROS) also occurring in human body (Prior, et al., 2005, Vertuani, et al. 2002, Vertuani, et al. 2004). Besides, it is the PCL method that provides the first ever possibility to measure the antioxidant capacity of water and lipid soluble substances with a single system (Killenberg-Jabs and Tirok).

In the present study PCL assay was used to determine the antioxidant capacity of olive leaf extract and its fractions. To determine the antioxidant capacity of olive leaf extract and its fractions by PCL assay, both ACW and ACL protocols were followed.

ACW Protocol:

In ACW protocol, olive leaf extract fractions were firstly dissolved in DMSO in a presolution ratio of 1g extract in 20 ml DMSO. Then the samples were diluted with deionized water. Next, ACW kit procedure was followed. In this procedure, 1.5 ml Reagent 1 (solvent), 1 ml Reagent 2 (buffer solution, pH: 10.5) and 25 µl Reagent 3 (photosensitiser) were mixed for blank measurement. Then appropriate amount of olive leaf extract fraction was added to this mixture and sample measurement was performed.

Measurements were conducted using 10-50 μl of the sample. Each measurement was repeated three times.

<u>ACL Protocol:</u>

After dissolving the olive leaf fractions in DMSO in a presolution ratio of 1g extract in 20 ml DMSO, the fractions were diluted with methanol. Then ACL kit procedure was applied. In this procedure, 2.3 ml Reagent 1 (solvent), 200 μl Reagent 2 (buffer solution, pH:10.5) and 25 μl Reagent 3 (photosensitiser) were mixed for blank measurement. Then the addition of the fractions was followed. Next, sample measurement was applied with that mixture. Each measurement was also repeated three times in the ACL protocol.

4.6. Total Phenol Analysis

Total phenol analysis of the olive leaf extract fractions were performed by Folin-ciocalteu method using gallic acid as a standard.

For the total phenol analysis, calibration curve was constructed for gallic acid standard. For the construction of calibration curve, 250 mg gallic acid was firstly dissolved in 10 ml ethanol and then it was diluted to 500 ml by distilled water. The prepared 0.5 mg/ml stock gallic acid standard was kept at +4 C^0. Next, working standards of 0.02, 0.03, 0.04, 0.05 and 0.06 mg/ml were prepared for calibration. Finally, absorbance values at 725 nm versus gallic acid concentrations were plotted and the calibration curve of gallic acid was obtained with an R^2 value of 0.9994.

After the construction of the calibration curve, olive leaf extract fractions were dissolved in DMSO in a ratio of 1 g extract in 20 ml solvent. Then, suitable dilutions were prepared for the samples. Next, 500 μl of the diluted sample solution were mixed with 2.5 ml folin-ciocalteu reagent that was previously diluted in 1:10 ratio. The mixture was left at room temperature for 2.5 minutes to allow folin-ciocalteu reagent to react completely with the oxidizable substances or phenolates. Then 2 ml of Na_2CO_3 (7.5 %) was added to destroy the residual reagent. After that, the mixture was incubated for 1 hour at room temperature in a dark place. Incubated samples can be seen in Figure 4.1.
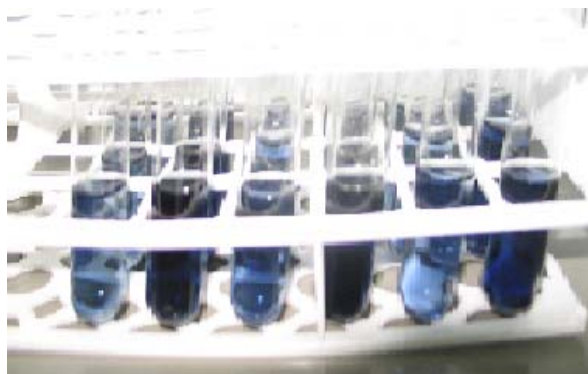
Figure 4.1. Samples after incubating 1 hour at room temperature in a dark place

After the incubation, absorbance values were measured at 725 nm by UV-Visible Spectrophotometer (Perkin Elmer-Lambda 25). All samples were analyzed at least three times.

4.7. Biosensor Construction

Carbon paste biosensors were constructed for this study. Top and bottom parts of the biosensor were prepared separately. The top part of the biosensor was prepared by mixing graphite powder and mineral oil in definite amounts; besides, preparation of the bottom part was achieved by the addition of glutaraldehyde in order to allow the cross-linking reaction and laccase enzyme solution which was the key component in this study. Laccase enzyme from *Trametes versicolor* (E.C. 1.10.3.2) was dissolved in phosphate buffer solution (0.05 M, pH: 6) and 5 % vol. of glutaraldehyde solution was prepared before using it for the construction of the bottom part. While constructing a biosensor, first the top mixture and then the bottom mixture were filled into a pipette tip. Finally, preparation of the biosensor was completed by immersing silver wire into the pipette tip. In addition, carbon paste biosensor was polished by using a polishing paper just before using and storage. Carbon paste biosensor was stored at about $+4^0$ C to avoid the deactivation of the laccase enzyme. An electrochemical sensor which did not contain any enzyme and crosslinking agent was also prepared in this study and it was used as a reference sensor.

Laccase enzyme concentration and crosslinking agent amount were the investigated parameters in this study. In order to observe the effect of these parameters on biosensor performance, carbon paste biosensors were prepared by changing the

values of these parameters. The content of the top and the bottom part of the biosensors are given in Table 4.4 and Table 4.5 respectively.

Table 4.4. The top part of the biosensor components

Biosensor No	G.P.* (mg)	M.O.*(ml)
Biosensor 00	325	0.18
Biosensor 01	300	0.17
Biosensor 02	300	0.17
Biosensor 03	300	0.17
Biosensor 04	300	0.17
Biosensor 05	300	0.17
Biosensor 06	300	0.17
Biosensor 07	300	0.17
Biosensor 08	300	0.17
Biosensor 09	300	0.17

G.P.*: Graphite powder
M.O.*: Mineral oil

Table 4.5. The bottom part of the biosensor components

Biosensor No	G.P.(mg)	M.O.(ml)	G.A. (% v)[*]	L.S. (% v)*	L.C. [*] (mg/ml)
Biosensor 00	-	-	-	-	-
Biosensor 01	25	0.01	6.40	63.97	1
Biosensor 02	25	0.01	12.03	60.13	1
Biosensor 03	25	0.01	17.01	56.72	1
Biosensor 04	25	0.01	6.40	63.97	5
Biosensor 05	25	0.01	12.03	60.13	5
Biosensor 06	25	0.01	17.01	56.72	5
Biosensor 07	25	0.01	6.40	63.97	10
Biosensor 08	25	0.01	12.03	60.13	10
Biosensor 09	25	0.01	17.01	56.72	10

G.A. (% v)[*]: % vol. of the glutaraldehyde solution in deionized water used in the bottom part of the biosensor
L.S. (% v)*: % vol. of the laccase solution in the bottom part of the biosensor
L.C.[*]: Laccase concentration

When the above tables are investigated, it is found that the top part of all biosensors contain the same amount of graphite powder and mineral oil. The variability of biosensors was due to the bottom part. As it is seen from Table 4.5, bottom parts of the biosensors were changed in three levels of glutaraldehyde amount as 6.40, 12.03 and 17.01 % vol. of the biosensor bottom part and in three levels of laccase concentration as 1, 5 and 10 mg/ml. Biosensor 00 is the electrochemical sensor without any enzyme or crosslinking agent.

4.8. Cyclic Voltammetry Experiments for Oleuropein

Standard oleuropein solution was used in cyclic voltammetry experiments. This phenolic compound was used as a substrate for laccase enzyme. Oleuropein was dissolved in 50 % vol. acetonitrile-water solution and 2 mg/ml oleuropein solution was prepared with that solvent. Biosensor 05 was used and cyclic voltammetry experiment was run. A cyclic voltammogram was obtained and this voltammogram was investigated in order to learn about the redox properties of oleuropein.

Oxidizibility of a compound has been used as a measure of antioxidant property and this can be determined by measuring an oxidation potential in cyclic voltammetry. Except oxidation potential, a huge number of parameters can be found from the cyclic voltammograms, such as reduction potential, switching potential, anodic peak current and cathodic peak current. A lot of information about the redox properties of polyphenols can be obtained from the investigation of these parameters. So, cyclic voltammograms of oleuropein were investigated in detail in order to characterize this phenolic compound as a reducing agent.

4.9. Effect of Laccase Enzyme on Biosensor Response

To investigate the effect of laccase enzyme on biosensor response, both laccase biosensors and the electrochemical sensor were used and cyclic voltammetry experiments were performed under the same experimental conditions. These experiments were useful to understand whether oleuropein showed a good substrate behavior for laccase enzyme or not. After obtaining a response with laccase biosensors, laccase concentration effect on biosensor performance was also investigated in this study. Thus, cyclic voltammetry experiments were conducted with biosensors having an enzyme concentration of 1, 5 and 10 mg/ml and laccase concentration effect was investigated when the other parameters were fixed.

4.10. Effect of Crosslinking Agent on Biosensor Response

The second factor in this experiment was the glutaraldehyde amount. Three levels of glutaraldehyde amount were used to check its effect on biosensor performance. Lowest amount was 6.40 % vol. of the bottom part, 12.03 % vol. of the bottom part was the medium level and the highest level was chosen as 17.01 % vol. of the biosensor bottom part. After cyclic voltammograms were obtained by biosensors differentiating in their glutaraldehyde amounts, they were investigated in detail to learn more about the effect of glutaraldehyde on the performance of the biosensor.

4.11. Cyclic Voltammetry Experiments with Different Biosensors

After nine different biosensors were constructed, the cyclic voltammetry experiments were conducted in the same conditions (25^0C and 10 mV/s scan rate) and cyclic voltammograms were obtained for all biosensors for the analyte concentration of 2 mg/ml. As all the parameters were fixed such as temperature, scan rate and analyte concentration, it was clear that the difference in the biosensor response was due to the change in enzyme concentration and crosslinking agent amount. This situation led us to investigate the effect of both enzyme concentration and crosslinking agent amount at the same time on biosensor performance. To sum up, the objective here was that, to determine the best biosensor performance in the same experimental conditions. The determination of the best biosensor performance was really important because, the following experiments would be performed with that biosensor.

4.12. Effect of Scan Rate on Biosensor Response

Scan rate is one of the most important parameters which influences the redox properties of the substrates. In order to investigate the effect of scan rate on biosensor response, cyclic voltammetry experiments of laccase biosensor in 2 mg/ml of oleuropein solution were conducted in the scan rate values of 1, 2, 3, 5, 10, 30, 50, 70, 90, 110, 130, 150, 200, 250 and 500 mV/s.

4.13. Effect of Temperature on Biosensor Response

Oxidation and reduction of oleuropein was catalyzed with laccase enzyme and investigation of the temperature effect is very important in the enzyme catalyzed reactions. After the investigation, the best working temperature should be found and this value should be set in the experiments for higher activity of the enzyme and higher response of the biosensor. To achieve this, cyclic voltammetry experiments were run in the temperatures of 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 ^{0}C and the temperature effect was investigated.

4.14. Calibration Curve for Oleuropein

Construction of a calibration curve is a necessary task that should be followed for the quantification of oleuropein. To construct a calibration curve, first, oleuropein solutions of 1.25, 2, 3.2, 5.18, 7.33, 9.18 mg/ml were prepared and then the experiment was conducted for each of these concentration values. The current responses were recorded at different oleuropein concentrations. Next, oleuropein solutions lower than 1.25 mg/ml were prepared and it was tried to find out at which concentration a cathodic peak current value could be recorded. At the end of the trials, it was found that a noticeable cathodic peak occurred at the oleuropein concentration of 1.05 mg/ml. This concentration value is the minimum detection limit of the amperometric laccase biosensor in oleuropein oxidation. Finally, oleuropein concentrations of 1.05, 1.25, 2, 3.2, 5.18, 7.33, 9.18 were plotted against the corresponding current responses and the calibration curve was obtained. Construction of a calibration curve and determination of its linear region are very significant parts of this study because the linear calibration curve should be used to determine the oleuropein amount in an unknown solution.

4.15. Response of Laccase Biosensor to OLE and Its Fractions

Up to this point, the performance of laccase biosensor to standard oleuropein solution was investigated in cyclic voltammetry experiments. Working with standard oleuropein solution was very crucial because it was helpful to understand the principles of the biosensor system. In addition, the effect of various parameters such as enzyme concentration, crosslinking agent amount, temperature and scan rate on biosensor response were studied and suitable values for these parameters were also determined in the former parts of the study. After studying the principles and determining the appropriate values for the parameters that had a significant effect on the performance of the laccase biosensor, it was now suitable to work with the olive leaf extract and its fractions which were obtained in the preceeding experiments.

Olive leaf extract and its fractions (FI, FII, FIII, FIV and FV) were prepared as 3 mg/ml with 50 % vol. acetonitrile-water solution. Due to the reason that there was not enough yield for the FVI fraction, it was not possible to work with this fraction. When the analytes of interest were prepared, cyclic voltammetry experiments were followed

with Biosensor 05 in suitable experimental conditions (25^0C temperature, 10 mV/s scan rate, in the potential range of -500 to 500 mV). At the end of the experiments, cyclic voltammograms were obtained for olive leaf extract and its fractions. Investigation of the voltammograms would give some important information about the performance of laccase immobilized carbon paste biosensor to olive leaf extract and its fractions.

4.16. Response of Laccase Biosensor to Catechin, Rutin and Caffeic Acid

The response of laccase biosensor to rutin, catechin and caffeic acid was also studied. For this purpose 2 mg/ml aqueous solutions were prepared with these standards. Cyclic voltammetry experiments were performed with these analytes in the same experimental conditions (25^0C temperature, 10 mV/s scan rate and in the potential range of -500 to 500 mV) and cyclic voltammograms were obtained.

4.17. Comparison of Biosensor Response with HPLC Response, Antioxidant Capacity and Total Phenol Analysis

After obtaining the response of laccase immobilized carbon paste biosensor to olive leaf extract and its fractions, biosensor response in terms of oleuropein amount was compared with the HPLC response, antioxidant capacity analysis (ACW and ACL) and total phenol analysis. This comparison was very significant for finding a relationship between these methods.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Pretreatment and Extraction of Olive Leaves

In the extraction of olive leaves, 70% aqueous ethanol solution and 1/20 solid/liquid ratio was used. At the end of the extraction process, olive leaf crude extract was obtained to be used in the next studies.

5.2. HPLC Analysis and Identification of Phenolic Compounds

Oleuropein is the most abundant polyphenol, contributes to total antioxidant capacity in olive leaf. So, detection of this compound is very important in olive leaf studies. Qualitative and quantitative determination of oleuropein can be performed by HPLC analysis. HPLC chromatogram of olive leaf crude extract and abundance of the main phenolic compounds with the retention times are given in Figure 5.1 and Table 5.1.
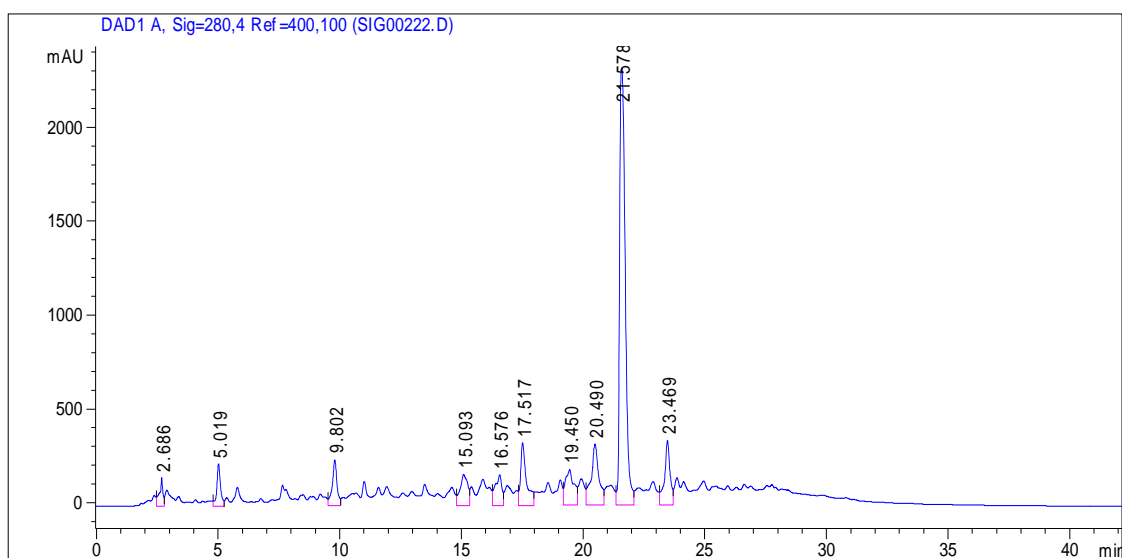

Figure 5.1. Chromatogram of olive leaf crude extract

Table 5.1. Retention time and abundance of the main phenolic compounds in OLE

Phenolics	Retention time (min)	Peak area (%)
Hydroxytyrosol	5.02	1.45
Catechin	9.80	2.01
Vanillic acid	15.09	2.09
Vanillin	16.58	1.88
Rutin	17.52	3.35
Luteolin-7-glucoside	19.45	2.74
Verbascoside	20.49	4.25
Oleuropein	21.58	22.31

Oleuropein, the major phenolic compound of olive leaf extract, was first qualitatively determined by comparing its retention time with its standard and then it was quantitatively determined by using external and internal calibration methods. These methods are explained in detail and external and internal calibration curves for oleuropein are given in Appendix A.

Retention time of oleuropein in olive leaf extract is 21.58 minutes. Retention time of a compound gives an idea about its polarity. Reversed phase HPLC is a combination of non-polar column with high polar mobile phase. High polarity solutes elute first in reversed phase HPLC.

Identification of oleuropein was followed by the quantitative determination of oleuropein. A detailed sample calculation for oleuropein quantification is given in Appendix B. When this calculation is investigated, it was found that, 1 gram of olive leaf extract contains 141.6 mg oleuropein. Hızal (2006) found the oleuropein amount in olive leaf extract as 9.25 %. In addition to her study, Savournin et al. (2001) calculated the oleuropein amount of 14 cultivated olive trees and their results show that oleuropein content changed from 9.04 % to 14.32 % depending on the variety of olive tree. The oleuropein amount of the olive trees that are found in our campus was calculated as 14.16 % in this study and the result show that the value is in the range that was given in the literature.

5.3. Fractionation of Olive Leaf Antioxidants

Isolation of olive leaf phenols is a necessary task for biosensor applications. For this purpose, fractionation of olive leaf extract was performed. After, the olive leaf phenols were adsorbed on silk fibroin, desorption process was followed for the partial fractionation of olive leaf phenols.

Four stage of adsorption process was applied in this work. In order to have an idea about adsorption efficiency, a volume of liquid olive leaf extract was injected to HPLC after each adsorption stage and area of oleuropein peak was recorded. HPLC results of olive leaf crude extract before adsorption and after each stage of adsorption are given in Table 5.2.

Table 5.2. HPLC results of olive leaf extract in the four stage adsorption process

	Retention time (min)	Area (mAU*s)	Area (%)
Crude Extract	21.58	37,558	22.24
After 1st loading	22.06	31,601	22.14
After 2nd loading	22.06	33,279	22.42
After 3rd loading	22.07	32,267	23.28
After 4th loading	22.07	32,755	23.60

For the determination of adsorbed amount of oleuropein, the HPLC response of crude extract (its chromatogram was previously given in Figure 5.1) and the results obtained from the chromatogram after the 4th loading (it is given in Figure 5.2) would be used. HPLC response in terms of area for oleuropein is 37,558 mAU*s before adsorption and 32,755 mAU*s after the adsorption stage.
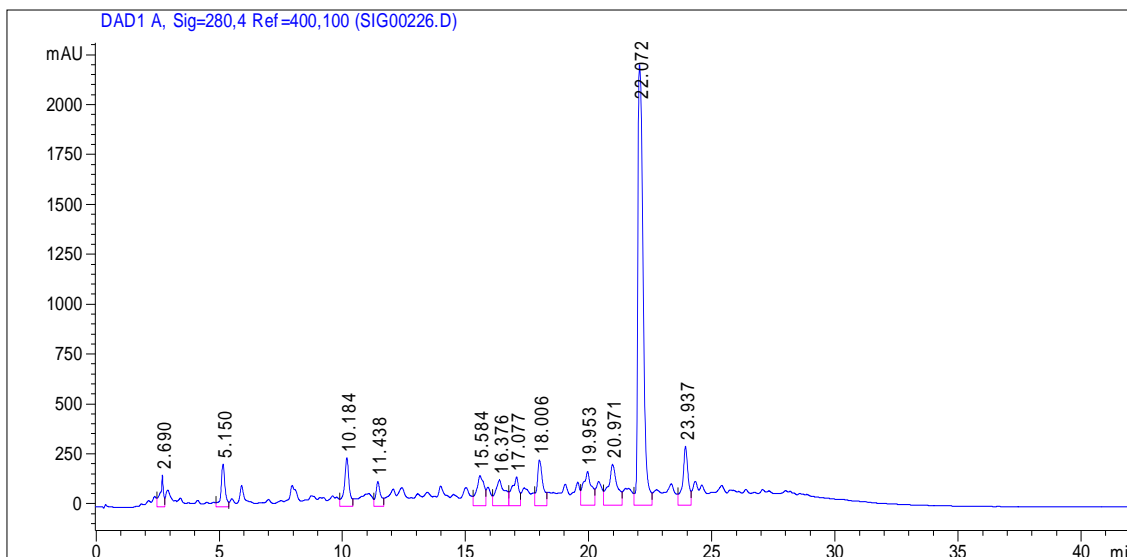
Figure 5.2. Chromatogram of olive leaf crude extract after the forth loading procedure

Adsorbed amount of oleuropein on silk fibroin was calculated and the calculation is given in Appendix B.

After adsorption, there were visible changes in the color of silk fibroin from white to brown. This change can be visually observed in Figure 5.3.

Figure 5.3. Color change in silk fibroin after adsorption

After adsorption, desorption process was followed for the removal of the olive leaf phenols. Selection of the solvent type is the most critical step in the desorption process. The type of the solvent is directly proportional to the desorption efficiency. Deionized water and 70% aqueous ethanol solution was respectively used as the eluting solvents in the desorption stage. First, polar and then relatively less polar phenolic compounds were eluted from the column by this selection. At the end of the desorption process, nine batches of polar and four batches of relatively less polar olive leaf extract

fractions were collected and they were analyzed in HPLC. Then the fractions were combined according to the HPLC response. Next, the fractions in their combined form were treated in rotary evaporator and then dried in freeze dryer. Fractions were obtained in powder form after this process. The combined form of the olive leaf fractions were also analyzed in HPLC. For the analysis, 10 mg/ml solutions of each fraction were prepared and then they were injected to HPLC. HPLC results, in terms of oleuropein peak, of the combined form of the fractions are given in Table 5.3.

Table 5.3. HPLC results for oleuropein peak in the combined olive leaf fractions

	Retention time (min)	Area (mAU*s)	Area (%)
FI	21.82	30,123	90.95
FII	22.04	32,168	78.15
FIII	22.11	13,677	54.70
FIV	22.21	5,861	49.43
FV	no oleuropein peak	--	--
FVI	no oleuropein peak	--	--

HPLC profiles of the two fractions, FI and FII, which were richer with their oleuropein contents are given in Figures 5.4 and 5.5.
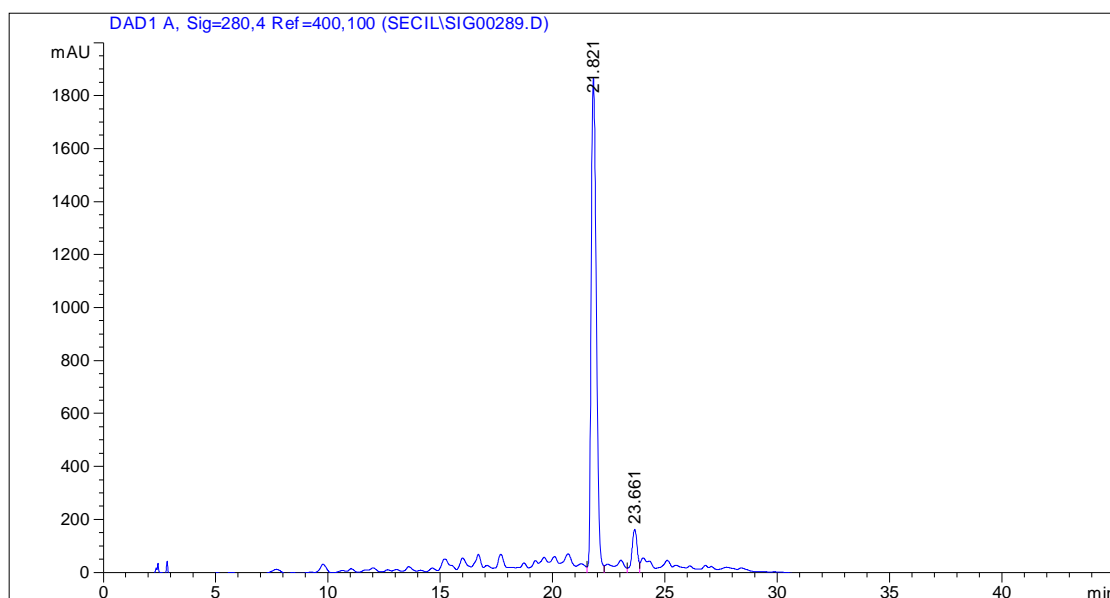

Figure 5.4. Chromatogram of FI (W1+W2)

According to analysis of FI, retention time for oleuropein was recorded as 21.82 minutes and HPLC response in terms of area was recorded as 30,123. From the external calibration curve, oleuropein concentration in this fraction was calculated as 5.66 mg/ml. As the concentration of the analyte was 10 mg/ml, 56.6 % of this fraction was composed of oleuropein.
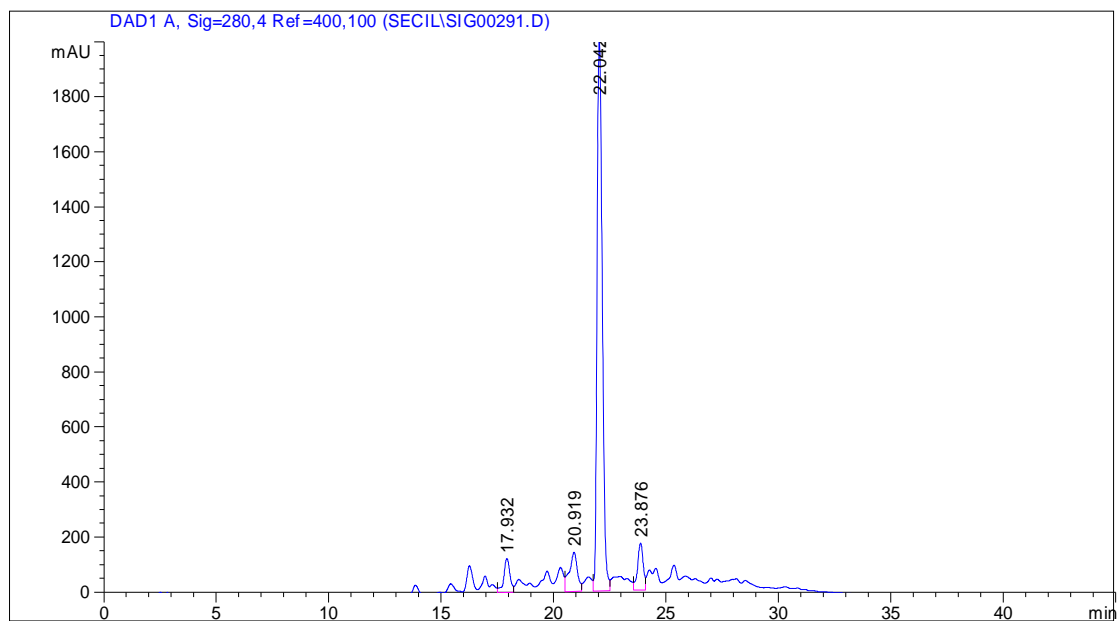

Figure 5.5. Chromatogram of FII (W3+W4)

Retention time for oleuropein in FII was found as 22.04 minutes and the area value was 32,168. Oleuropein concentration was calculated as 6.05 mg/ml from the calibration curve. Oleuropein content of this fraction is 60.5 % by weight. HPLC profiles of FIII and FIV are given in Figures 5.6 and 5.7. Oleuropein content in these fractions were also calculated and they were found as 25.3 % and 10.4 % by weight respectively.
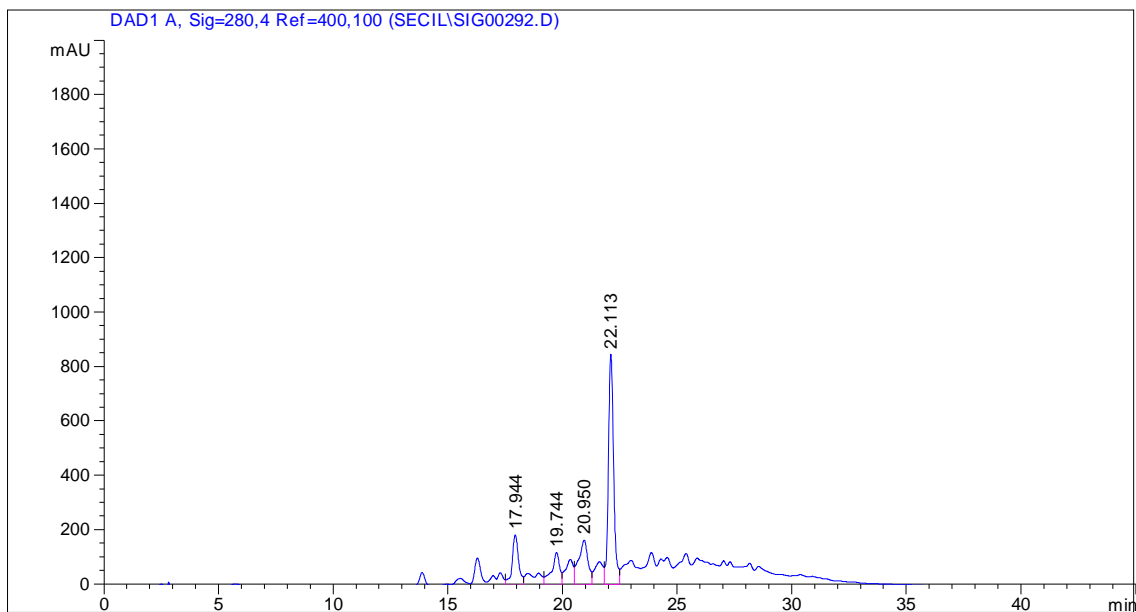
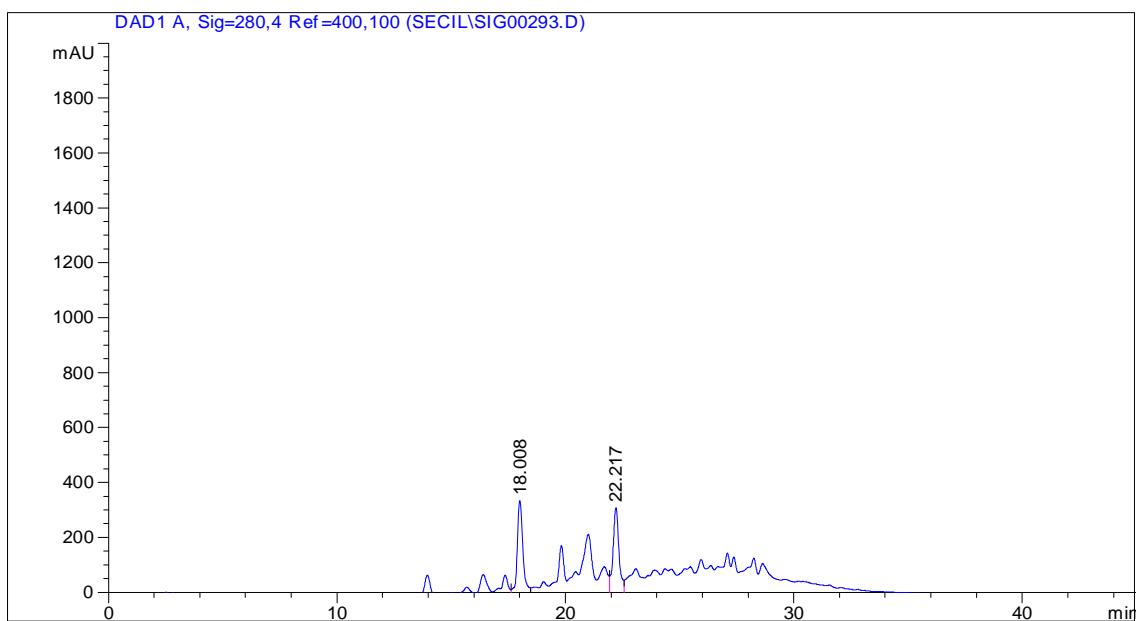Figure 5.6. Chromatogram of FIII (W5+W6)

Figure 5.7. Chromatogram of FIV (W7+W8+W9)

HPLC chromatograms of less polar fractions, obtained from the column outlet by eluting 70% aqueous ethanol, are given in Figures 5.8 and 5.9. When these chromatograms are investigated it is realized that there is no oleuropein peak in these fractions meaning that there is only a little (below the detection limit of HPLC) or no oleuropein in these fractions.
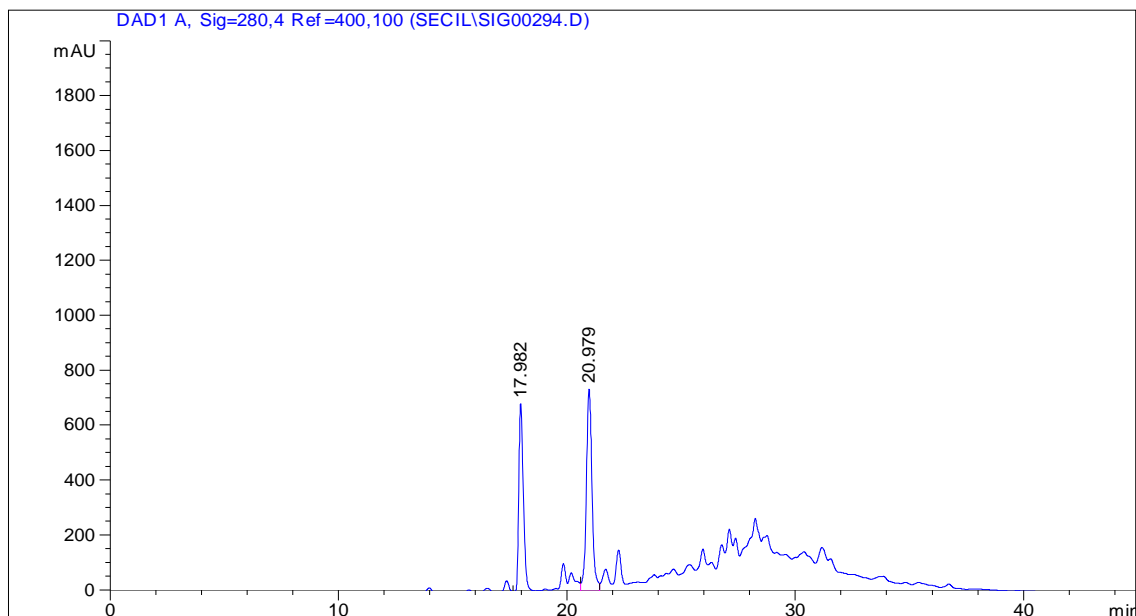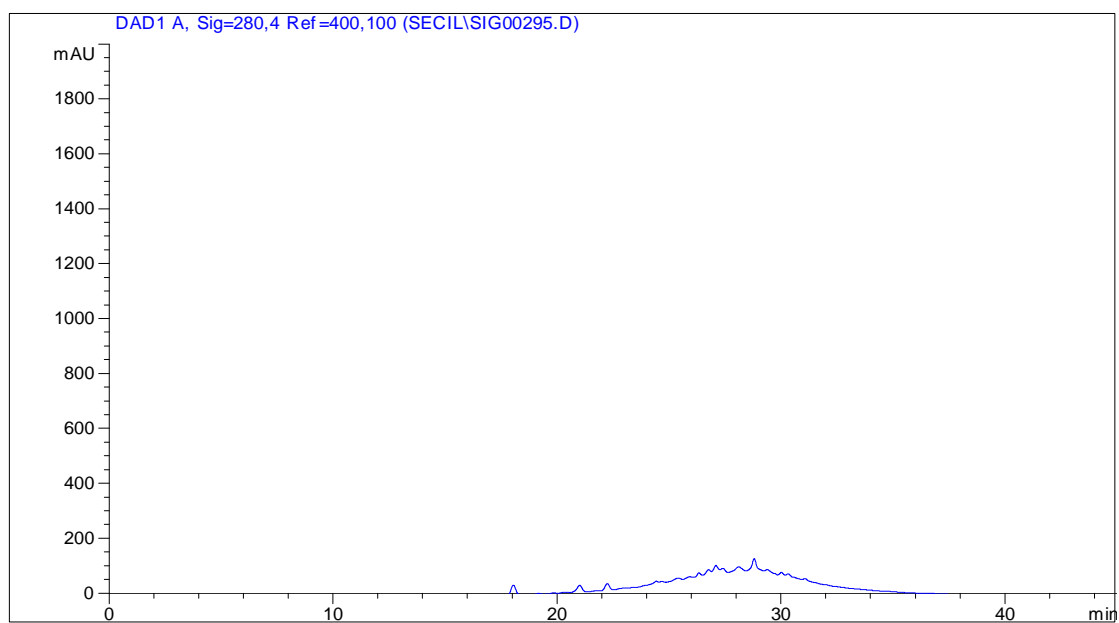
Figure 5.8. Chromatogram of FV (E1+E2)

Figure 5.9. Chromatogram of FVI (E3+E4)

The HPLC chromatograms of the fractions are also given in Appendix C with the identification of the other phenolics with respect to their retention times. Fractionation, in other means, partial purification of olive leaf extract was achieved in this work.

5.4. Antioxidant Capacity Analysis

In the present study, PCL assay was performed for determination of the both water soluble and lipid soluble antioxidant capacities of olive leaf crude extract and its fractions.

Antioxidant Capacity of Water Soluble Compounds:

ACW protocol was applied for the detection of the antioxidant capacity of water soluble compounds. Curves for the blank and the samples were constructed according to the protocol and they are given in Figure 5.10.
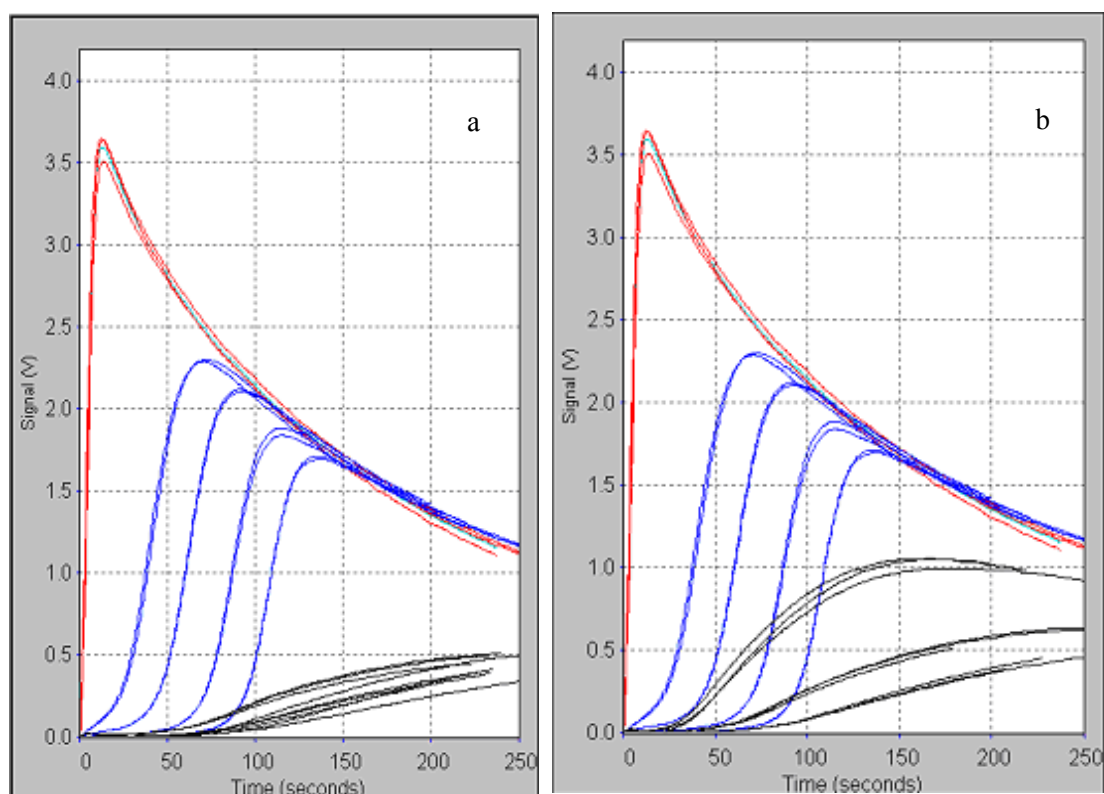

Figure 5.10. ACW curves for the olive leaf crude extract and the fractions (Figure 5.10.a. for the fractions FI, FII and FV, Figure 5.10.b. for the samples CE, FIII and FIV)

Antioxidant capacity (AOC) of olive leaf extract and its fractions, in terms of ascorbic acid concentration, were calculated and a sample calculation is given in Appendix D. The results are given in Figure 5.11.
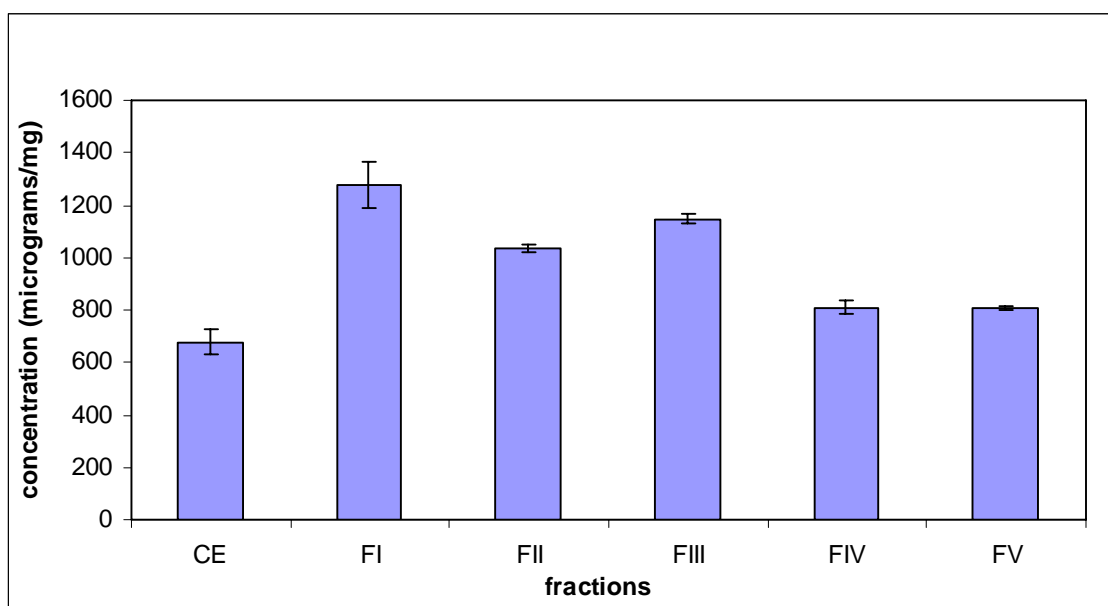
Figure 5.11. Ascorbic acid equivalent concentration values (µg/mg) for AOC of water
soluble compounds in olive leaf extract and its fractions

As it is seen in Figure 5.11, the highest water-soluble antioxidant capacity was observed for FI with a concentration value of 1278.35 µg ascorbic acid/mg. That is followed by FIII (1148.49 µg/mg) and FII (1036.70 µg/mg). The lowest capacity was detected for crude extract with a concentration value corresponding to 678.81 µg ascorbic acid/mg sample. It was not possible to report any results for FVI due to the reason that there was not enough yield. When the HPLC chromatograms are investigated, it is observed that the most abundant phenolic in FI, FII and FIII fractions is oleuropein. Peak area of oleuropein in FI, FII and FIII is 90 %, 78 % and 54 % respectively. It should be taken into account that FI fraction has the highest peak area value and also the highest water soluble antioxidant capacity value. As the FIII fraction has higher water soluble antioxidant capacity value than the fraction FII, it is not possible to say that the AOC value is just directly proportional with the oleuropein. When the chromatograms in Appendix C are investigated it is observed that fraction FII contains oleuropein, rutin and verbascoside. Although the main peak is oleuropein in FIII fraction, the existence of rutin, verbascoside and luteolin-7-glucoside should attract the attention. Moreover, rutin and oleuropein are the phenolics of fraction FIV and the phenolics of FV are rutin and vebascoside.

When antioxidant capacity of FII fraction is investigated, it is observed that the presence of rutin and verbascoside results into a decrease in water soluble antioxidant

capacity value. On the other hand, the combination of oleuropein, rutin, verbascoside and luteolin-7-glucoside in FIII, increases the water soluble antioxidant capacity value. This may be the positive effect of luteolin-7-glucoside on the radical scavenging capacity. The combination of rutin and vebascoside is recognized in fraction FV and this fraction has a lower water soluble antioxidant capacity value (807.03 μg/mg) indicating that the coupling of these phenolics results into a decrease in water soluble antioxidant capacity value. This can be also an explanation of why FII fraction has lower water soluble antioxidant capacity value than the FI fraction. The presence of rutin and verbascoside beside oleuropein in FII yields with a lower water soluble antioxidant capacity value, whereas the situation completely changes with the existence of luteolin-7-glucoside in FIII. In accordance with this knowledge, it can be thought that olive leaf phenolics show a synergic behaviour in radical scavenging capacity.

Garcia, et al. (2000) studied the $ABTS^{\bullet+}$ scavenging capacity of olive leaf phenolics and they found that the most effective flavonoid for scavenging the $ABTS^{\bullet+}$ radical was rutin with a TEAC value 2.75 mM. Water soluble antioxidant capacity analysis of this study showed that although rutin was found to be a promising flavonoid in scavenging $ABTS^{\bullet+}$ radical, it showed a synergic behaviour when mixed with oleuropein and verbascoside.

Antioxidant Capacity of Lipid Soluble Compounds:

ACL protocol was applied for the detection of the antioxidant capacity of lipid soluble compounds. Curves for the blank and the samples were constructed according to the protocol and they are given in Figure 5.12.

Antioxidant capacity, in terms of Trolox concentration, was calculated from the same equation as in the case of ACW, only differentiating in the molar mass value. Trolox was used in the ACL calculations and Trolox equivalent concentration values were calculated. A sample calculation is given in Appendix D. ACL results are shown in Figure 5.13.
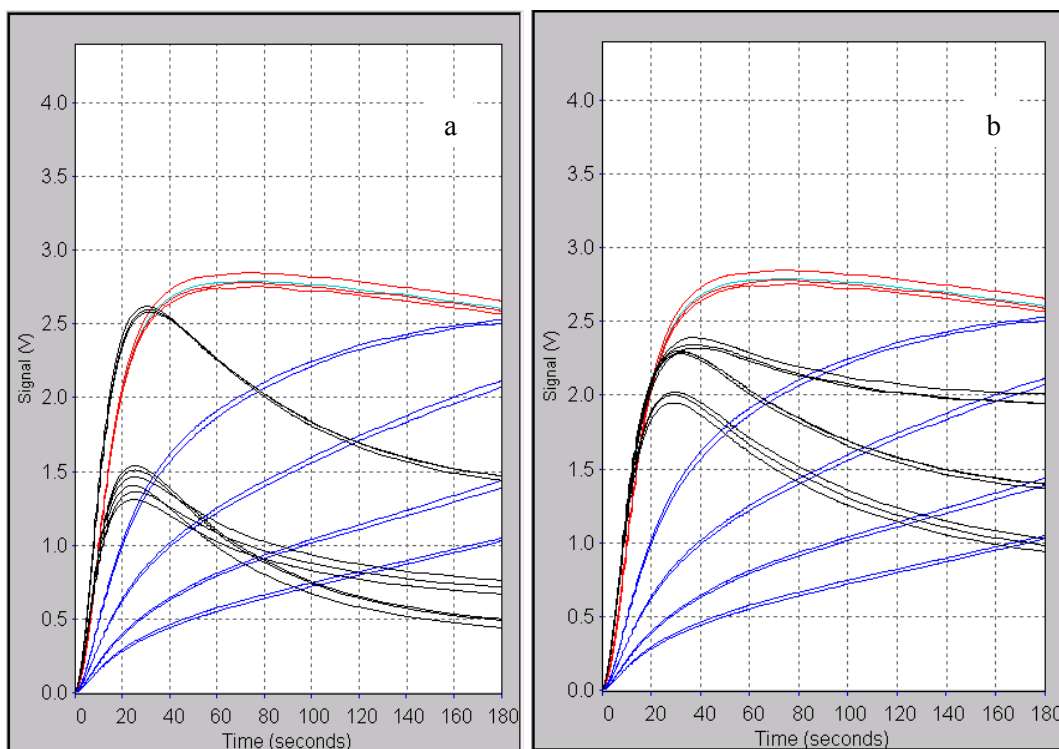
Figure 5.12. ACL curves for the olive leaf crude extract and the fractions (Figure 5.12.a. for the fractions FI, FII and FV, Figure 5.12.b. for samples CE, FIII and FIV)
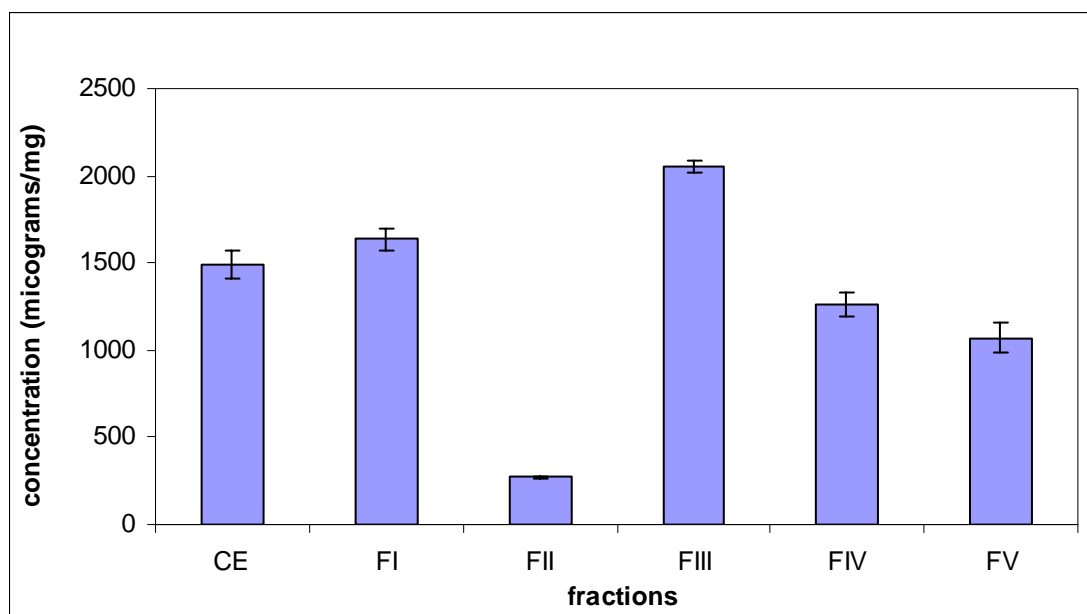

Figure 5.13. Trolox equivalent concentration values (µg/mg) for AOC of lipid soluble compounds in olive leaf extract and its fractions

According to the results, the highest lipid soluble antioxidant content was detected for FIII with a concentration value of 2051.46 μg/mg. This is followed by FI (1634.63 μg/mg), CE (1495.30 μg/mg), FIV (1260.51 μg/mg) and FV (1070.42 μg/mg). The lowest lipid soluble antioxidant content was observed for FII corresponding to 270.49 μg/mg concentration value. When the results are investigated, a direct comparison between the ACL and ACW results cannot be observed. ACL results just depend on the lipid soluble compounds. Although the higher water soluble antioxidant capacity values of FI and FIII fractions, they also have higher lipid soluble antioxidant capacity values. Crude extract has higher lipid soluble antioxidant capacity value than FIV, FV and FII fractions which is a demonstration of synergic behaviour of olive leaf phenolics.

5.5. Total Phenol Analysis

Total phenol contents of the olive leaf extract and its fractions were analyzed with folin-ciocalteu reagent and the results were expressed as milligrams of gallic acid equivalents per gram weight.

For the total phenol analysis, calibration curve of gallic acid standard was used and it is given in Figure 5.14.
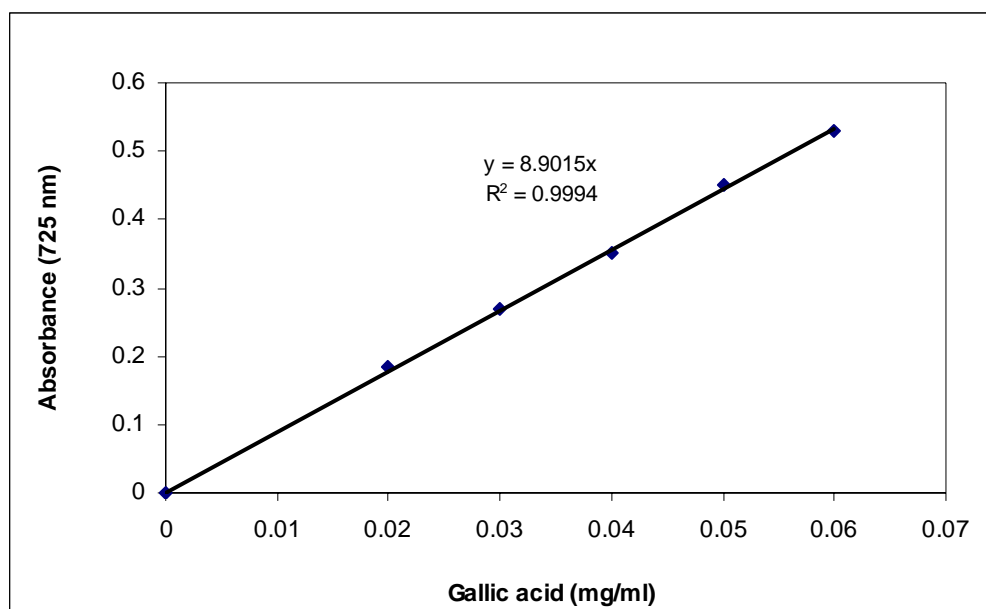

Figure 5.14. Calibration curve of gallic acid

Total phenol content of the crude extract was calculated as 195.09 mg GAEq/g sample. Total phenol content of the water soluble fractions (FI, FII, FIII and FIV) were found as 309.57, 376.63, 309.86 and 249.94 mg GAEq/g sample respectively. Total phenol content of the least polar fraction (FV) was calculated as 334.78 mg GAEq/g sample. The results are schematically represented in Figure 5.15. The highest total phenolic content was detected for FII that is followed for FV. As it is seen from the graph, non-phenolic compounds are eliminated by fractionation that results an increase in the total phenol content.
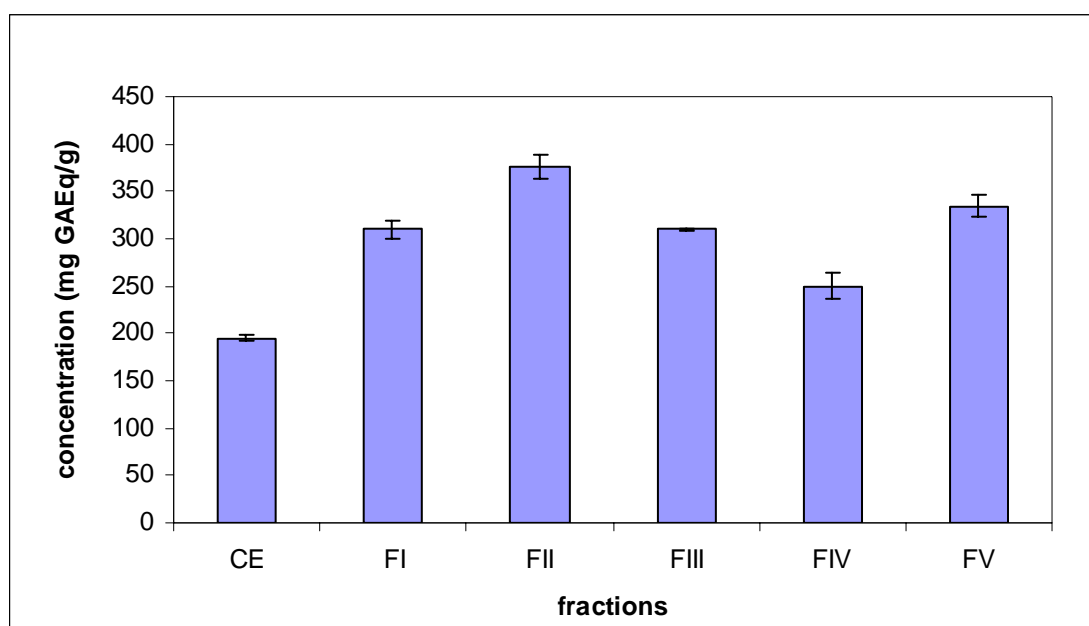
Figure 5.15. Total phenol content of crude extract and olive leaf extract fractions

5.6. Biosensor Construction

Carbon paste biosensors were constructed by mixing graphite powder and mineral oil as top components and the bottom part of the biosensor was prepared by immobilization of laccase from *Trametes versicolor* by addition of cross-linking agent into the carbon paste. Laccase enzyme concentration and crosslinking agent amount were the key components in this study. Laccase enzyme concentration (1, 5, 10 mg/ml) and glutaraldehyde amount (6.40, 12.03 and 17.01 % vol. of the biosensor bottom part) were changed in three levels in order to observe their effects on biosensor performance. So, nine different carbon paste biosensors and a chemical sensor as a reference sensor were prepared in this study.

5.7. Cyclic Voltammetry Experiments for Oleuropein

Before running the cyclic voltammetry experiments for oleuropein, Gamry Physical Electrochemistry Software was checked with the working electrode. $FeCl_3$ solution was used for this test. Cyclic Voltammogram of 1.25 M $FeCl_3$ solution at 25ºC and 10mV/s scan rate is given in Figure 5.16. It was concluded from this figure that the voltammogram had well characteristics and the experiments for oleuropein could be followed.
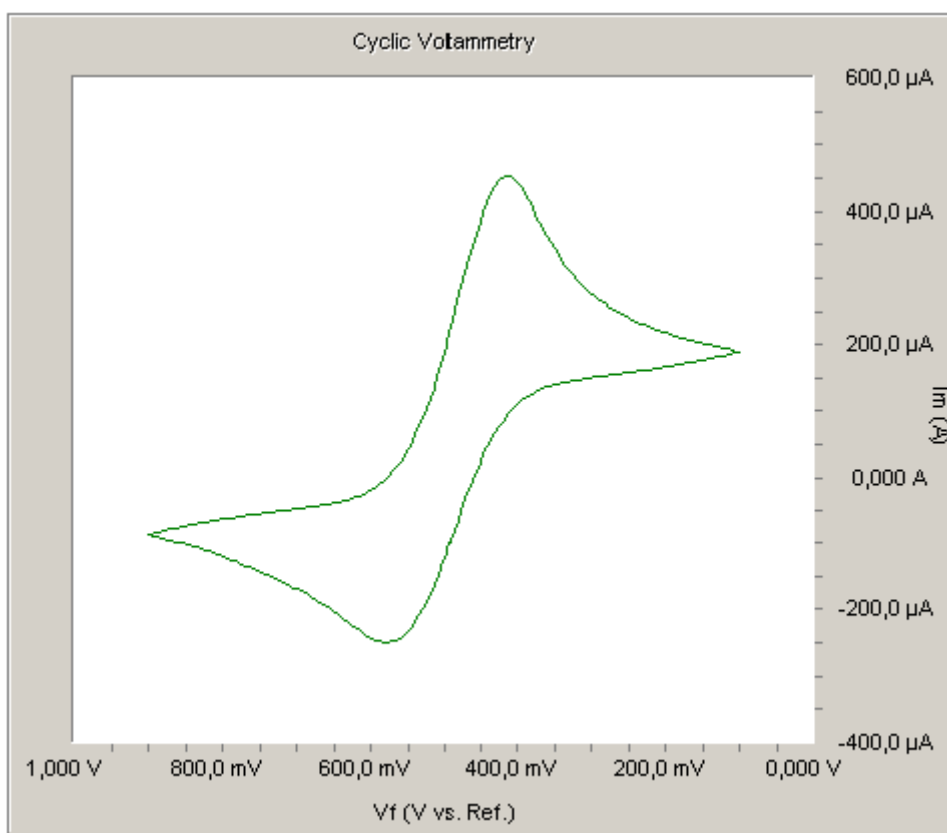

Figure 5.16. Voltammogram of 1.25 M $FeCl_3$ solution at 25ºC and 10mV/s scan rate

Presence of one or more hydroxylated benzene rings is the basic feature of all phenols. Phenolic compounds also contain hydroxyl group(s) attached to the ring structures. Antioxidant activity of polyphenols is known as radical scavenging property. In a typical scavenging reaction, H atom from the O-H group of a polyphenolic compound is donated to the radical and this is associated with the oxidizing capacity of the polyphenol (Filipiak 2001). In respect of Filipiak's publication, measuring an oxidation potential in cyclic voltammetry can be used as an analytical tool in the

determination of oxidizability of a compound. So, electrochemical behaviour of a phenolic compound, oleuropein, was studied in this work by cyclic voltammetry experiments. Figure 5.17 represents a typical cyclic voltammogram of oleuropein.
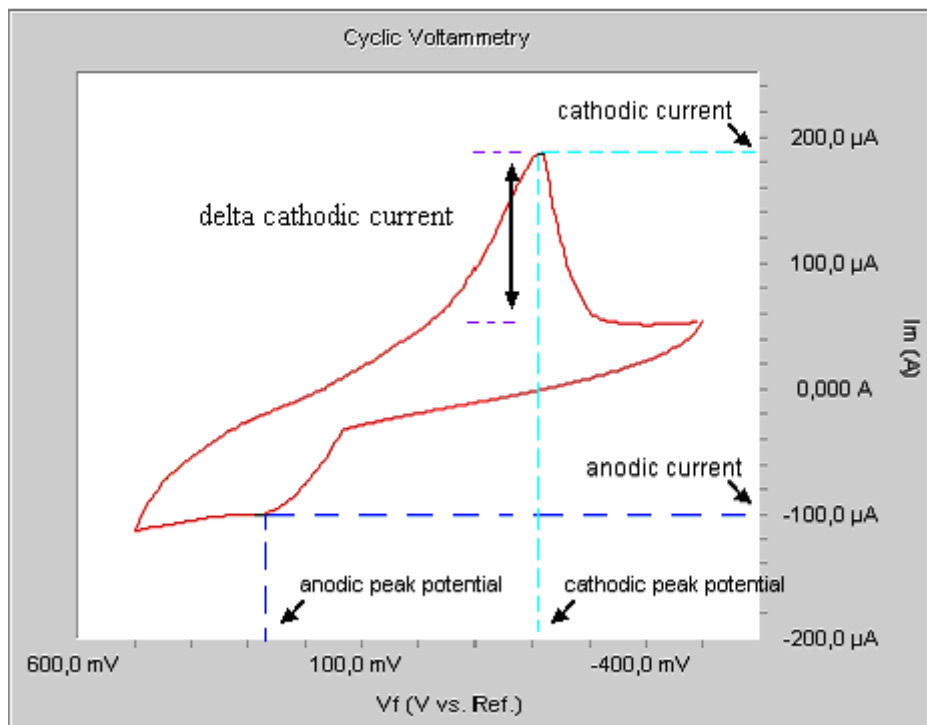

Figure 5.17. Cyclic Voltammogram of 2 mg/ml of oleuropein at 25ºC and 10mV/s scan rate

As a first step, different experimental conditions were studied in cyclic voltammetry experiments in order to check reversibility and to find out the suitable working potential values. As it is seen from the above figure, working between -0.5 V and 0.5 V potential values, yields a typical, reversible cyclic voltammogram for oleuropein.

Filipiak performed cyclic voltammetry experiments for some flavonoids and some phenolic acids. All investigated phenolics, both flavonoids and phenolic acids exhibit one or two oxidation peaks (anodic wave) at about 200-300 mV and 500-700 mV. It was reported that oxidation peaks are associated with the oxidation centers present in polyphenolic molecules. Oxidation peak existed at about 300 mV correspond to 3', 4'-dihyroxyl moiety at B ring (catechol moiety) and the second oxidation peak at about 600 mV comes from the OH group at position 3 at C ring or some additional OH groups at A and C (Filipiak 2001).

Some polyphenols have both an oxidation and a reduction peaks on their voltammograms showing reversibility on oxidation process (Filipiak 2001). When the

cyclic voltammogram for oleuropein is investigated, it is realized that the top scan of the cyclic voltammogram represents the reduction of oleuropein and this is achieved by generating a cathodic current. Anodic current occurs by peaking at a particular electrode potential. On the reverse scan, reduced form of oleuropein is oxidized back to its original form and in this situation, a positive peak potential is produced indicating to the anodic current value. The lower the potential of oxidation, the more powerful the compound as a reducing agent (Kilmartin and Hsu 2003).

Therefore, a huge number of information on the mechanism of polyphenols oxidation can be obtained by investigating their cyclic voltammograms. Base on this information, the typical cyclic voltammogram of oleuropein (Figure 5.17) is investigated to learn more about the electrochemical behaviour of oleuropein.

Oleuropein shows one oxidation peak at 285.4 mV (anodic peak potential, E_{pa}) and this can be associated with the catechol moiety at the B ring. Besides, oleuropein does not show a peak at 600 mV in cyclic voltammetry experiments. This is due to the fact that, oleuropein does not contain hydroxyl group attached to the 3 position of C ring. Oleuropein also shows a reduction peak at 212.3 mV (cathodic peak potential, E_{pc}). The reduction potential (E^o) was calculated as 248.85 mV which is the average of the oxidation and reduction potentials (Gosser 1993). The anodic peak current (I_{pa}) of oleuropein is 100.7 μA and the cathodic peak current (I_{pc}) is 187.8 μA.

As Filipiak reported, phenolics having a low oxidation potential value (below +300 mV), shows a high radical scavenging activity. In the case of oleuropein, the E_{pa} value was found as 285.4 mV. This value is lower than 300 mV that exhibits a high radical scavenging activity of oleuropein.

Several criteria can be utilized to confirm a single reversible electron transfer to characterize the phenolics as reducing agents. First, the difference in anodic (E_{pa}) and cathodic (E_{pc}) peak potentials is around 57-60 mV depending on the switching potential. Mathematically;

$$\Delta E_p = \text{abs } [E_{pc} - E_{pa}] \approx 58 \text{ mV}$$

But, in actual experiments the expected 58 mV value is rarely observed due to the small distortions and electronic or mathematical smoothing of the data. The value of ΔE_p for a reversible electron transfer is often accepted as 60-70 mV (Gosser 1993).

ΔE_p value is calculated as 73.1 mV from the cyclic voltammogram of oleuropein and this value is quite closer to the 70 mV. So, electrochemical behavior of oleuropein can be defined to have a reversible electron transfer mechanism.

Other criterion for a reversible electron transfer mechanism is the ratio of the cathodic peak current (I_{pc}) and shifted ratio of the anodic peak current (I^{*}_{pa}). If this ratio is unity, it is possible to deal with a fully reversible process. The shifted ratio of the anodic peak current is calculated from the baseline that is moved to a value that can be predicted from the decaying portion of the cathodic peak (Kilmartin and Hsu 2003, Gosser 1993).

The shifted ratio of the anodic peak is calculated as 151.7 μA from Figure 5.13 and the shifted ratio of the cathodic to anodic currents is calculated as 1.24. This value is also quite closer to unity and this criterion also proves the reversibility of oleuropein in cyclic voltammetry experiments.

It is clear that a number of parameters can be extracted from the cyclic voltammetry curves to characterise the phenolics as reducing agents. Up to this point it is intended to show how to use cyclic voltammetry to understand the redox properties of polyphenols.

5.8. Effect of Laccase Enzyme on Biosensor Response

Oleuropein is known to attach to the binding site of laccase by hydrophobic interaction and it is believed that it shows good substrate behavior for laccase enzyme (Quan, et al. 2004). To prove this belief, both the laccase biosensors and the electrochemical sensor were used to determine the redox properties of oleuropein in the same experimental conditions. Figure 5.18 is graphical representation of the cyclic voltammetry experiments with oleuropein. As it is seen from Figure 5.18, there is a sharp distinction between the behavior of laccase biosensor and the electrochemical sensor in oleuropein oxidation. Laccase biosensor has some distinctive features on their voltammograms such as, oxidation potential, reduction potential, anodic peak current and cathodic peak current, whereas, chemical sensor has recessive characteristics of these properties. The investigation of these features led us to have some useful information about the redox properties of oleuropein. So, cyclic voltammetry curves of laccase biosensor can be successively used to characterize oleuropein as a reducing agent. After this part of the work, it is proved that oleuropein showed a good substrate behavior for laccase enzyme.
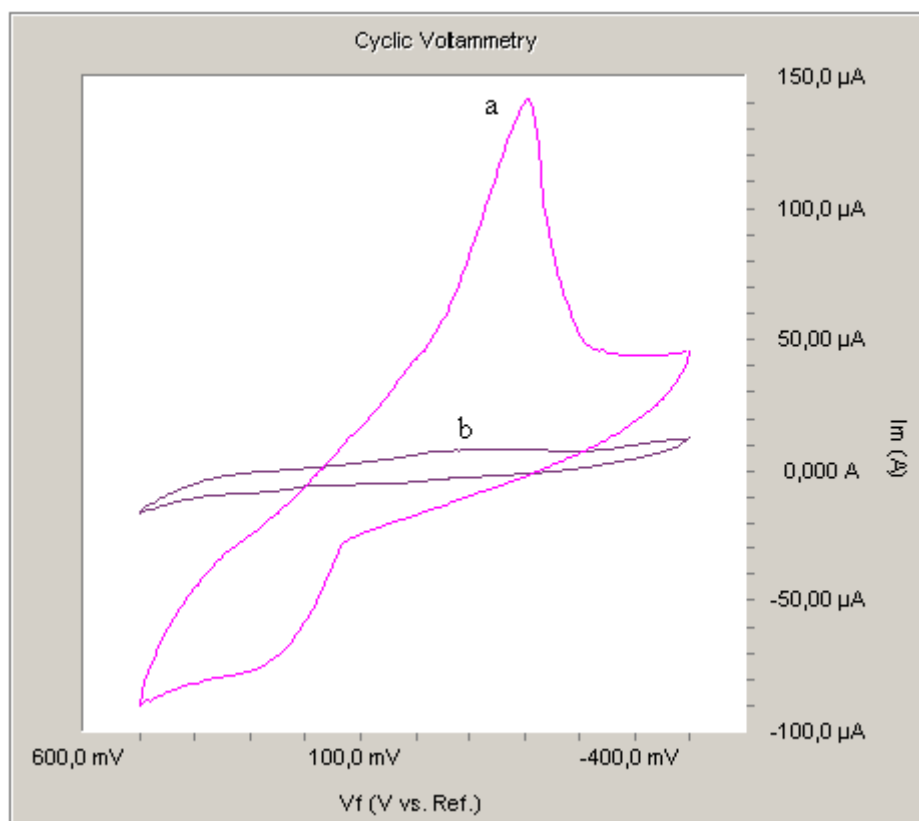
Figure 5.18. Cyclic voltammograms of (a) laccase biosensor and (b) electrochemical sensor in 2 mg/ml of oleuropein concentration at 25 ºC with ± 0.5 V and 10 mV/s scan rate

The effect of laccase concentration on biosensor performance was also investigated in this study. In order to achieve this, biosensors with laccase concentration of 1, 5 and 10 mg/ml were used and the cyclic voltammetry experiments were conducted with these biosensors. The voltammograms showing the effect of laccase concentration when glutaraldehyde amount was 6.40 % vol. of the biosensor bottom part are given in Figure 5.19.
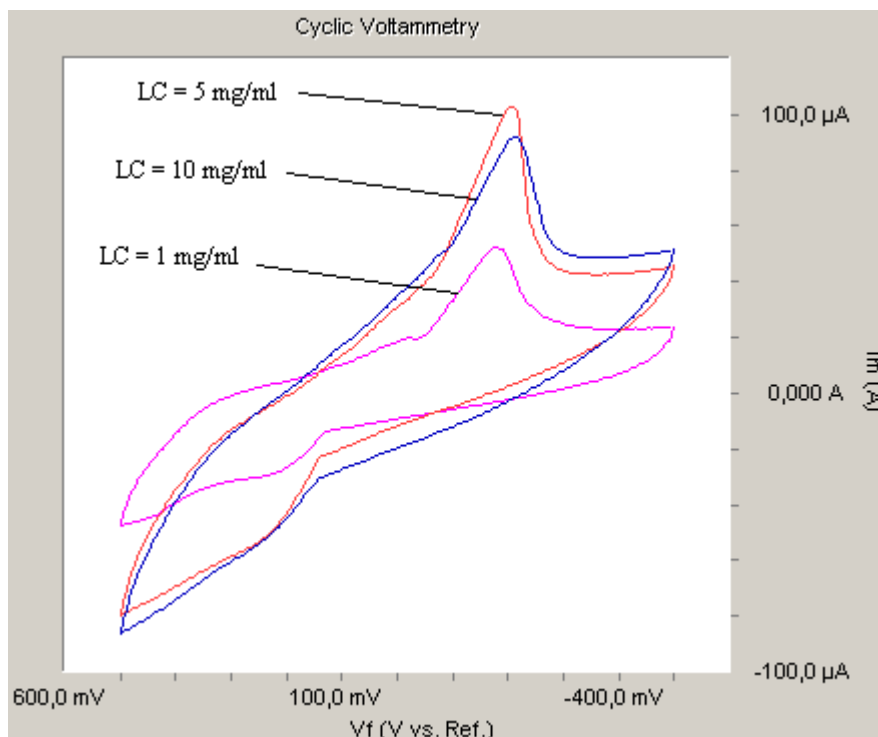
Figure 5.19. Cyclic voltammograms of biosensors with laccase concentration of 1, 5 and 10 mg/ml when GA = 6.40 % vol. of the biosensor bottom part (oleuropein concentration of 2 mg/ml, at 25 ºC with ± 0.5 V and 10 mV/s scan rate)

This figure proves that the best biosensor performance was obtained with the biosensor having laccase concentration of 5 mg/ml when the crosslinking agent amount was fixed at 6.40 % vol. of the biosensor bottom part. The situation was nearly same when the glutaraldehyde amount was changed to 12.03 and 17.01 % vol. of the biosensor bottom part. The voltammograms showing the effect of laccase concentration when glutaraldehyde amount was 12.03 and 17.01 % vol. of the biosensor bottom part are also given in Figure 5.20 and Figure 5.21 respectively.
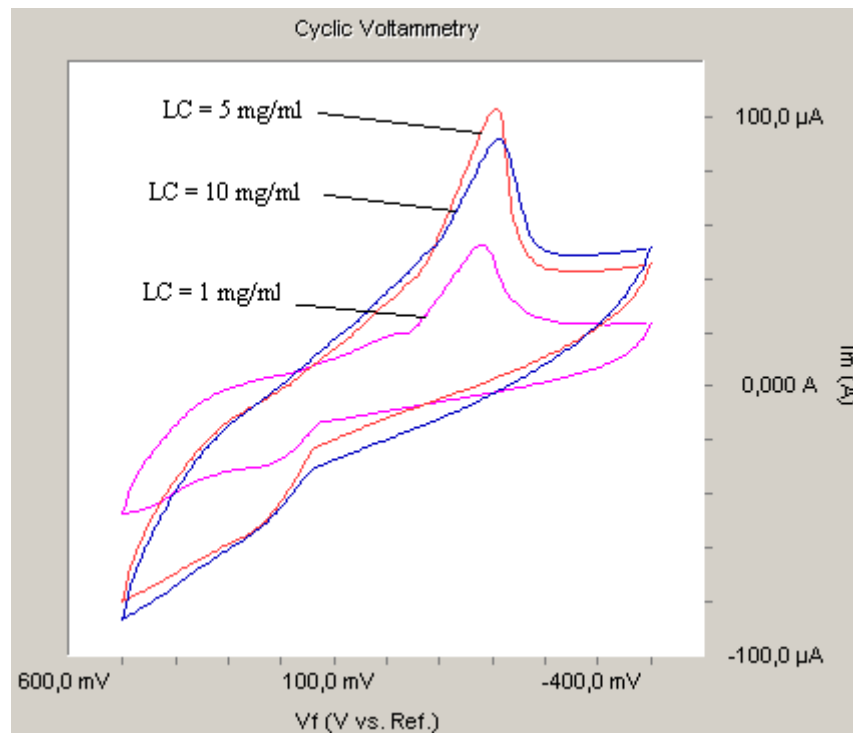
Figure 5.20. Cyclic voltammograms of biosensors with laccase concentration of 1, 5 and 10 mg/ml when GA = 12.03 % vol. of the biosensor bottom part (oleuropein concentration of 2 mg/ml at 25 ºC with ± 0.5 V and 10 mV/s scan rate)
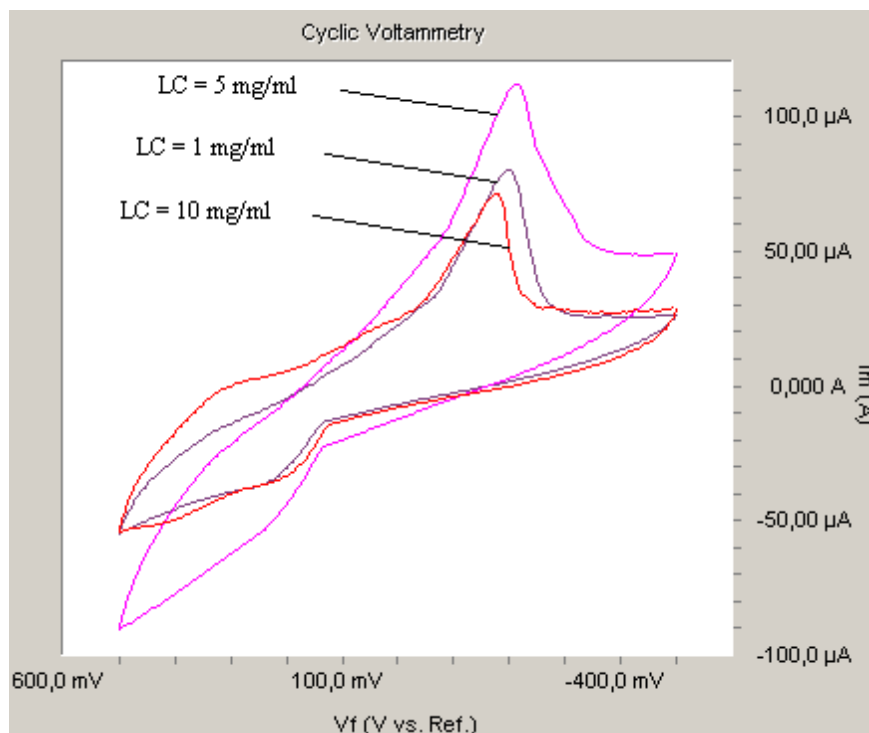
Figure 5.21. Cyclic voltammograms of biosensors with laccase concentration of 1, 5 and 10 mg/ml when GA = 17.01 % vol. of the biosensor bottom part (oleuropein concentration of 2 mg/ml, at 25 ºC with ± 0.5 V and 10 mV/s scan rate)

As, it is seen from the above figures, 5 mg/ml is the optimum laccase concentration compared to 1 and 10 mg/ml. So, it is clear that the enzyme amount is not directly proportional with the biosensor response. This is due to the phenomenon that is explained below.

The magnitude of the biosensor response can be related to the amount of enzyme. This relation can be summarized as, as the rate of enzymatic oxidation increases as the amount of enzyme increases. On the other hand, as the amount of enzyme increases, enzyme layer thickness also increases which leads to the increase of the diffusional limitations. Due to the diffusional limitations, a resistance occurs for the enzymatically oxidized substrate to arrive to the electrode surface and this contributes to the decrease in the current response (Quan, et al. 2004). So, it can be concluded that 10 mg/ml laccase concentration was resulted in the occurrence of the diffusional resistance to provide the oxidized form of oleuropein to arrive to the biosensor surface for re-reduction.

5.9. Effect of Crosslinking Agent on Biosensor Response

Quan and his friends reported that the stability of the sensors based on polymer entrapped laccase or carbon paste mixed laccase was generally about 1-2 weeks (Quan, et al. 2004). During this study, we encountered with a similar problem that was the disintegration of the carbon paste mixed laccase as soon as the biosensor surface was interacted with the analyte. Therefore, a crosslinking agent, glutaraldehyde, was added to the carbon paste mixture in order to enhance the stability of the immobilized enzyme. After this work, it is understood that using glutaraldehyde, dramatically enhanced the integrity of the biosensor and this is schematically shown in Figure 5.22.

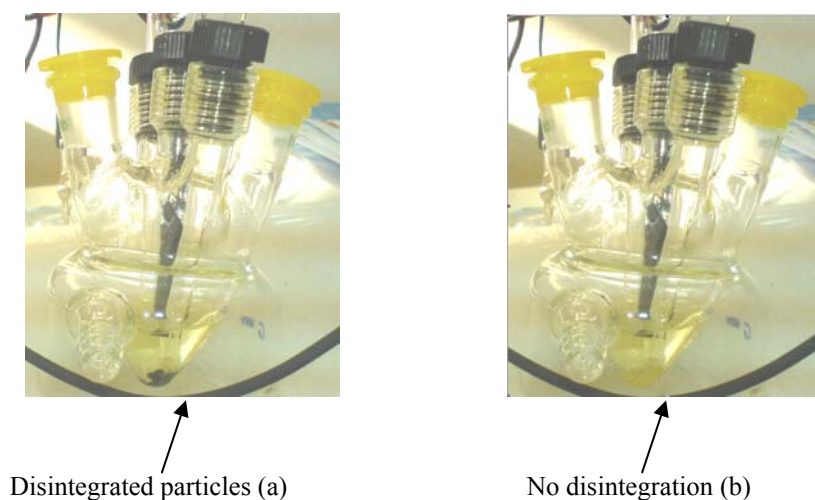Disintegrated particles (a)   No disintegration (b)

Figure 5.22. Biosensor system in contact with the analyte (a) without GA (b) with GA

Other concern in this part is the investigation of the effect of glutaraldehyde amount on biosensor performance. For this purpose, cyclic voltammograms obtained by using biosensors changing in glutaraldehyde amounts were analyzed and they are given in Figure 5.23, 5.24 and 5.25.

Figure 5.23. Cyclic voltammograms of biosensors with glutaraldehyde amount of 6.40, 12.03 and 17.01 % vol. of the biosensor bottom part when LC = 1 mg/ml (oleuropein concentration of 2 mg/ml, at 25 ºC with ± 0.5 V and 10 mV/s scan rate)

Figure 5.24. Cyclic voltammograms of biosensors with glutaraldehyde amount of 6.40, 12.03 and 17.01 % vol. of the biosensor bottom part when LC = 5 mg/ml (oleuropein concentration of 2 mg/ml, at 25 ºC with ± 0.5 V and 10 mV/s scan rate)
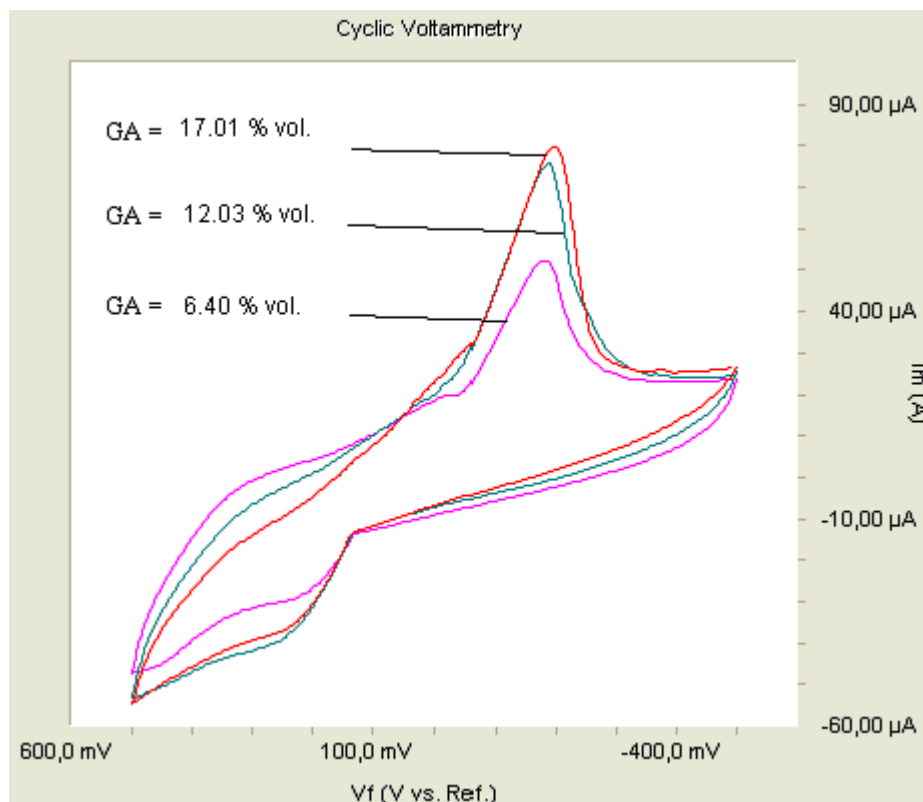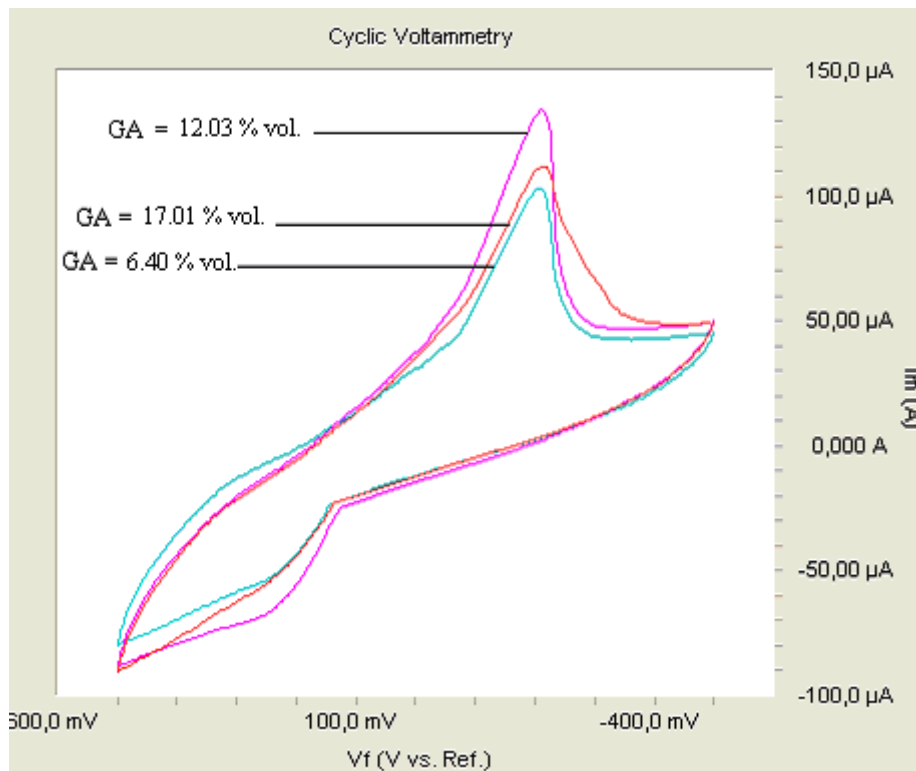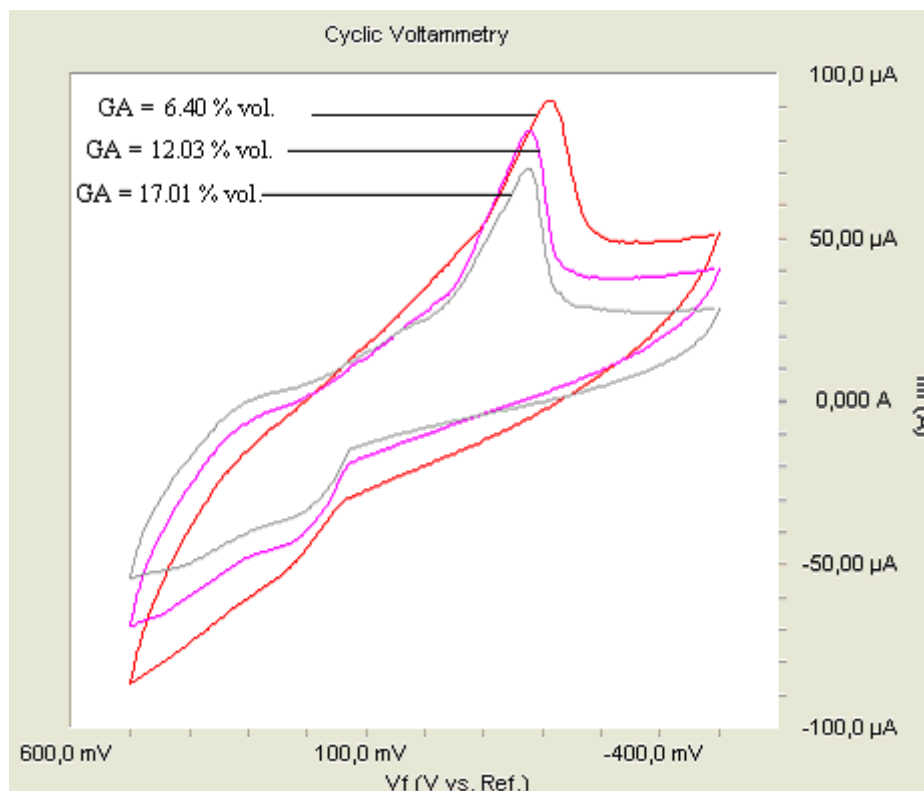
Figure 5.25. Cyclic voltammograms of biosensors with glutaraldehyde amount of 6.40, 12.03 and 17.01 % vol. of the biosensor bottom part when LC = 10 mg/ml (oleuropein concentration of 2 mg/ml, at 25 ºC with ± 0.5 V and 10 mV/s scan rate)

When these figures are investigated, it is understood that it is not easy to find out a direct relationship between the glutaraldehyde amount and catholic peak current.

5.10. Cyclic Voltammetry Experiments with Different Biosensors

After learning how to use the cyclic voltammograms, the experiment was run for 2 mg/ml oleuropein solution with nine biosensors and the chemical sensor. The response in terms of delta cathodic peak current for nine biosensors was investigated and the biosensor giving the best performance was reported in this part of the work. While the biosensor response in terms of cathodic peak current was determined, the response of chemical sensor was substracted from the response of each biosensor. The cyclic voltammetry experiments for oleuropein solution were repeated for two times with each biosensor. The cyclic voltammetry results are given in Table 5.4. Moreover, the graphical representation of the average cathodic peak current data is given in Figure 5.26.

Table 5.4. Cyclic voltammetry results

Biosensor no	L.C.* (mg/ml)	G.A.* amount (% vol.)	Delta current (μA)	Delta current (μA)	Average current (μA)
Biosensor 01	1	6.40	28.6	20.9	24.8
Biosensor 02	1	12.03	51.4	50	50.7
Biosensor 03	1	17.01	53.8	52.4	53.1
Biosensor 04	5	6.40	59.8	64.1	62
Biosensor 05	**5**	**12.03**	**87.2**	**73.8**	**80.5**
Biosensor 06	5	17.01	62.6	63.4	63
Biosensor 07	10	6.40	42.5	33.2	37.9
Biosensor 08	10	12.03	44.4	43	43.7
Biosensor 09	10	17.01	43.5	44.9	44.2

L.C.*: Laccase concentration
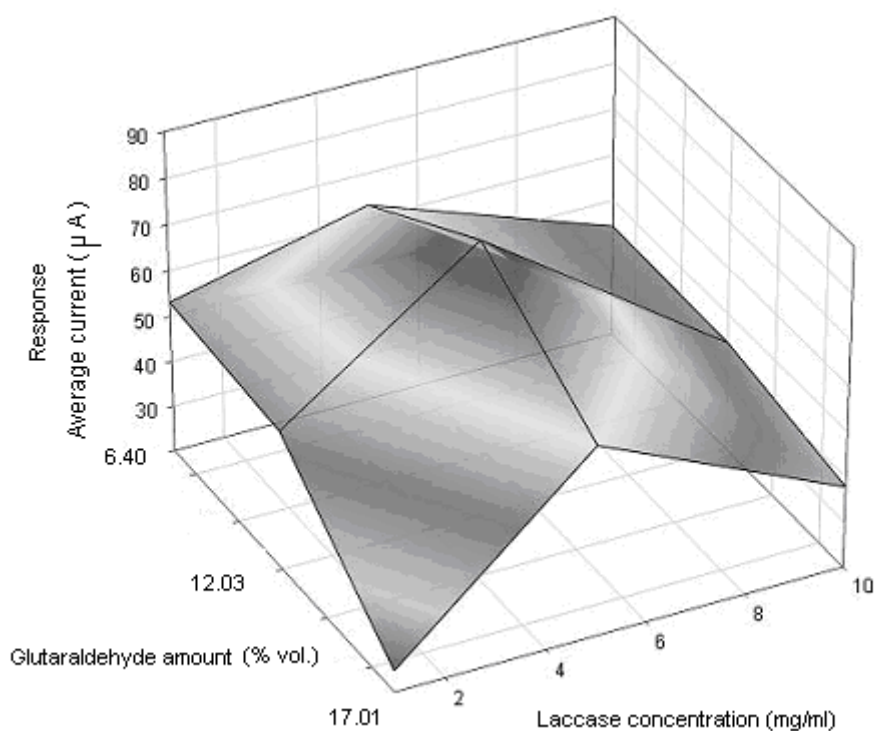G.A.*: Glutaraldehyde

Figure 5.26. Graphical representation of the average cathodic peak current with respect to laccase concentration and glutaraldehyde amount

When the results are investigated, it is easy to notice that Biosensor 05, having the highest average cathodic peak current value, showed the best performance in cyclic

voltammetry experiments. It is also possible to say that laccase concentration of 5 mg/ml and glutaraldehyde amount of 12.03 % vol. of the biosensor bottom part were the most suitable levels for this experiment.

The best biosensor performance was determined and after this point, the following experiments were followed with Biosensor 05. But before that, the effects of the factors were investigated. Due to this reason the experimental data were analyzed with Design Expert Software (30 days trial version). Full factorial design was followed and according to the analysis it was found that the model terms of laccase concentration and glutaraldehyde amount and their interaction are significant. It was also found that the most significant model term is the laccase concentration having a p value smaller than 0.0001. P value calculated for glutaraldehyde amount and the interaction of the model terms are 0.0003 and 0.0063 respectively. P values for these terms are smaller than 0.05 which indicates the model terms and their interaction are significant.

5.11. Effect of Scan Rate on Biosensor Response

Scan rate defines the speed of the potential sweep during data acquisition. Scan rate is one of the most important parameters which effects the redox properties of the substrates in cyclic voltammetry experiments. The influence of scan rate on the anodic and cathodic peak currents was investigated by performing cyclic voltammetry experiments in the range of 1-500 mV/s scan rate. The voltammograms are given in Figure 5.27.
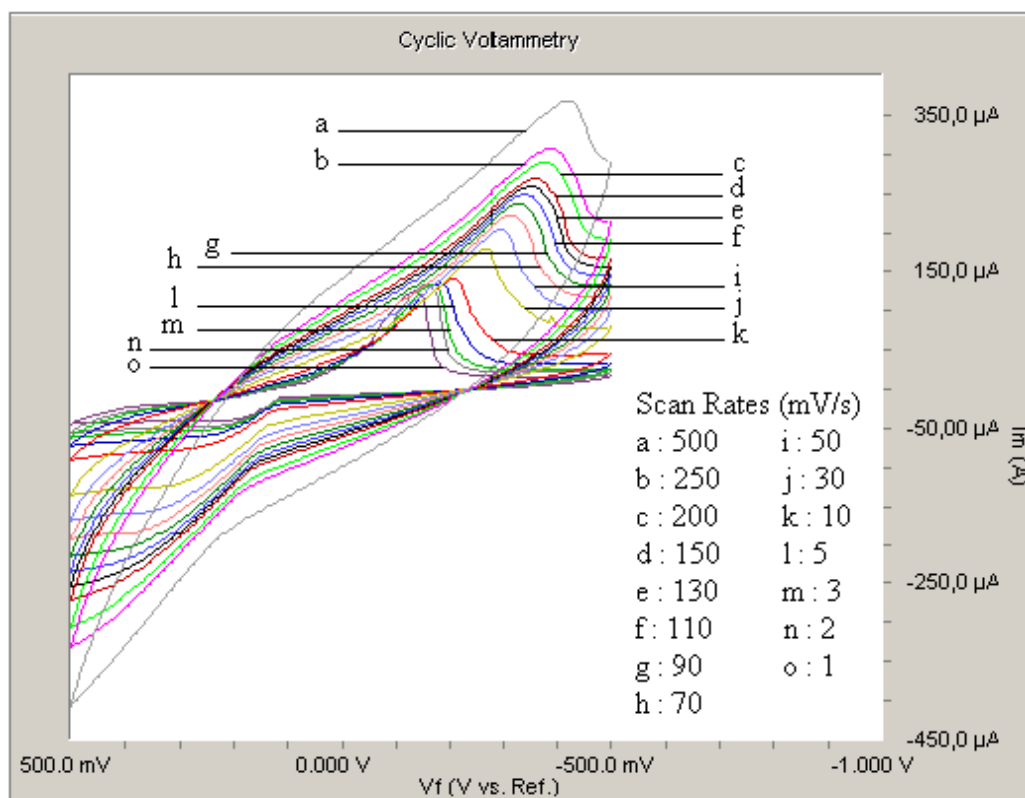
Figure 5.27. Cyclic voltammograms of laccase biosensor for 2 mg/ml oleuropein solution, at 25ºC, in ± 0.5 V potential interval, with various scan rates

It was observed that, delta value of the cathodic peak current decreases as the scan rate increases. In other words, the response of biosensor, in terms of cathodic peak current is inversely proportional with the scan rate. Moreover, anodic peak current is a distinctive feature of the voltammograms in the low scan rate values, whereas, oxidation peak cannot be observed as the scan rate increases hence, it is not possible to pronounce a term called anodic peak current in the high scan rate values. This situation occurs because, increasing scan rate results in the increase of the rate of diffusion of the reduced substrate from the electrode surface (Gosser 1993). In addition, higher scan rates may yield inaccurate data due to inability of the software to acquire data points fast enough. So, lower scan rates are suitable for the reduction of oleuropein. However, a considerable change on biosensor response cannot be observed below 10 mV/s scan rate values. Besides, working below 10 mV/s scan rate values requires extended experimental time. Then, 10 mV/s is selected as the suitable scan rate value for the detection of oleuropein with amperometric laccase biosensor.

Square root of scan rate can be also used as a key factor in the confirmation of the reversibility. Plots of I_{pc} and I_{pa} versus square root of the scan rate show the reaction of

oxidation of oleuropein to be reversible. Then, I_{pc} versus square root of scan rate is plotted and it is given in Figure 5.28. Figure 5.28 shows that there exists a proportional relationship between the cathodic peak current and square root of scan rate, verifying reversibility of oleuropein oxidation.
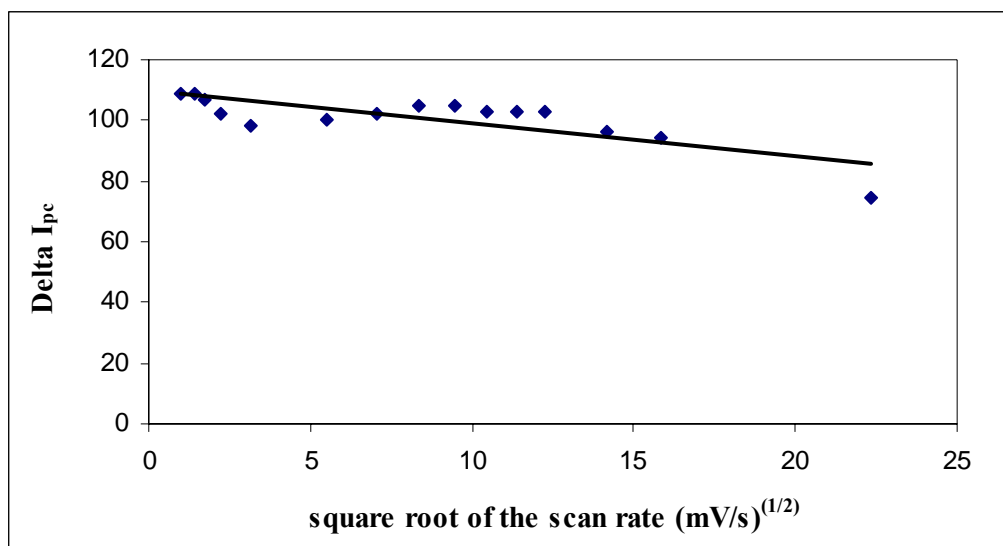

Figure 5.28. Cathodic peak current versus square root of scan rate (2 mg/ml oleuropein, at 25ºC, in ± 0.5 V potential interval, with 10 mV/s scan rate)

5.12. Effect of Temperature on Biosensor Response

Temperature dependency of the enzyme catalyzed reactions has already been known. Laccase enzyme was used in this study to construct the enzyme biosensors and oxidation and reduction reactions took place in the surface of the enzyme biosensors. Redox properties of oleuropein can be understood from the oxidation and reduction reactions. As these reactions were enzyme catalyzed reactions, the oxidation and reduction of oleuropein was also dependent on temperature. To sum up, it is understood that the biosensor response is highly affected from the temperature and the best working temperature should be found for the detection of oleuropein. In this respect, cyclic voltammetry experiments were done in the temperature range of 2 ^{0}C – 60 ^{0}C. The voltammograms are shown in Figure 5.29. It can be clearly seen from this figure that the response of the biosensor (cathodic peak current) increases with increasing temperature. But it was reported that the phenolic compounds which act as antioxidants loss their radical scavenging activity above 40^{0}C (Altıok 2003). So, the working temperature

should be kept below this value. Optimum working temperature for oleuropein oxidation with laccase biosensor was chosen as 25 ^{0}C in order to avoid possible activity loss.
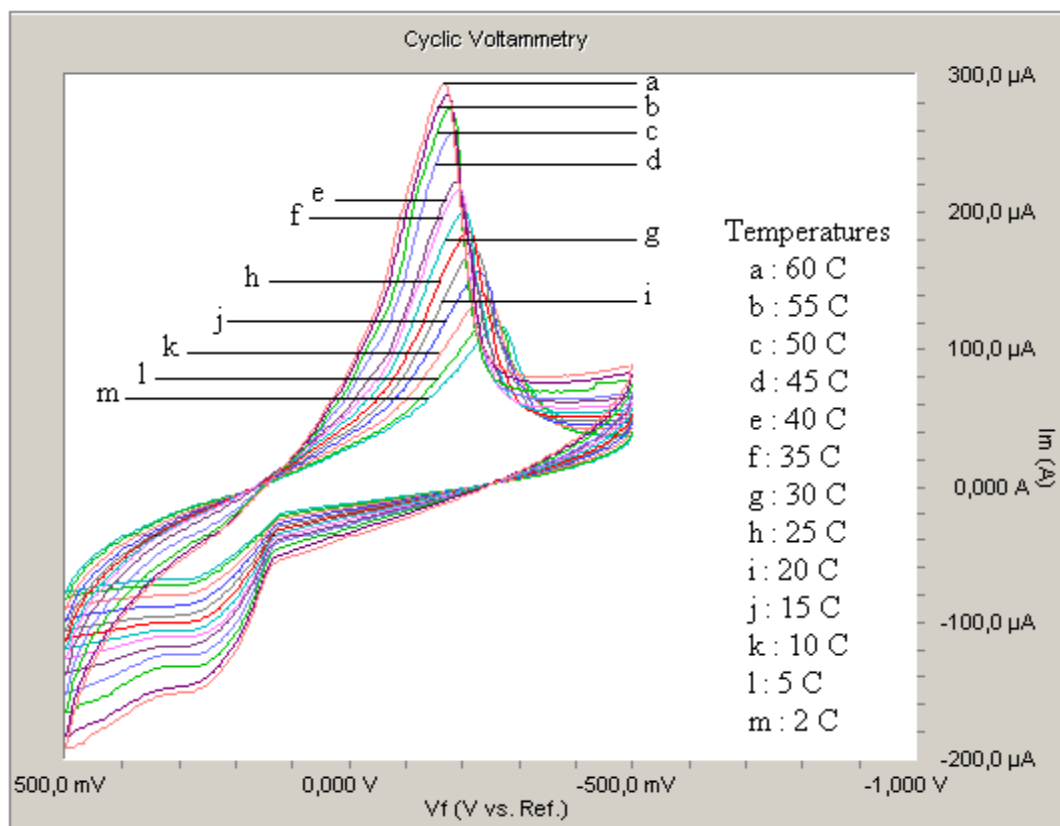

Figure 5.29. Cyclic voltammograms of laccase biosensor for 2 mg/ml oleuropein solution, in ± 0.5 V potential interval, with 10 mV/s scan rate, at various temperatures

5.13. Calibration Curve of Oleuropein

Amperometric measurements depend on the current resulting from the electrochemical oxidation and reduction of electroactive species and it is known that the resulting current response can be easily correlated to the bulk concentration of the electroactive species or its production or consumption rate within the adjacent biocatalytic layer. Biocatalytic reaction rates are often chosen to be first order dependent on the bulk analyte concentration thus, steady state current response is usually accepted to be proportional to the bulk analyte concentration (Thevenot, et al. 2001). This correlation can be obtained from the calibration curve and only by this

curve it is possible to find out the relation between the concentration of the analyte and the current response.

After investigating the effect of various parameters on biosensor response, cyclic voltammetry experiments were conducted with "Biosensor 05" in different analyte (oleuropein) concentrations. Cyclic voltammograms of oleuropein solutions, differentiating in their concentrations, are given in Figure 5.30. The voltammograms in the figure were obtained with the oleuropein concentrations of 1.05, 1.25, 2, 3.2, 5.18, 7.33 and 9.18 mg/ml.
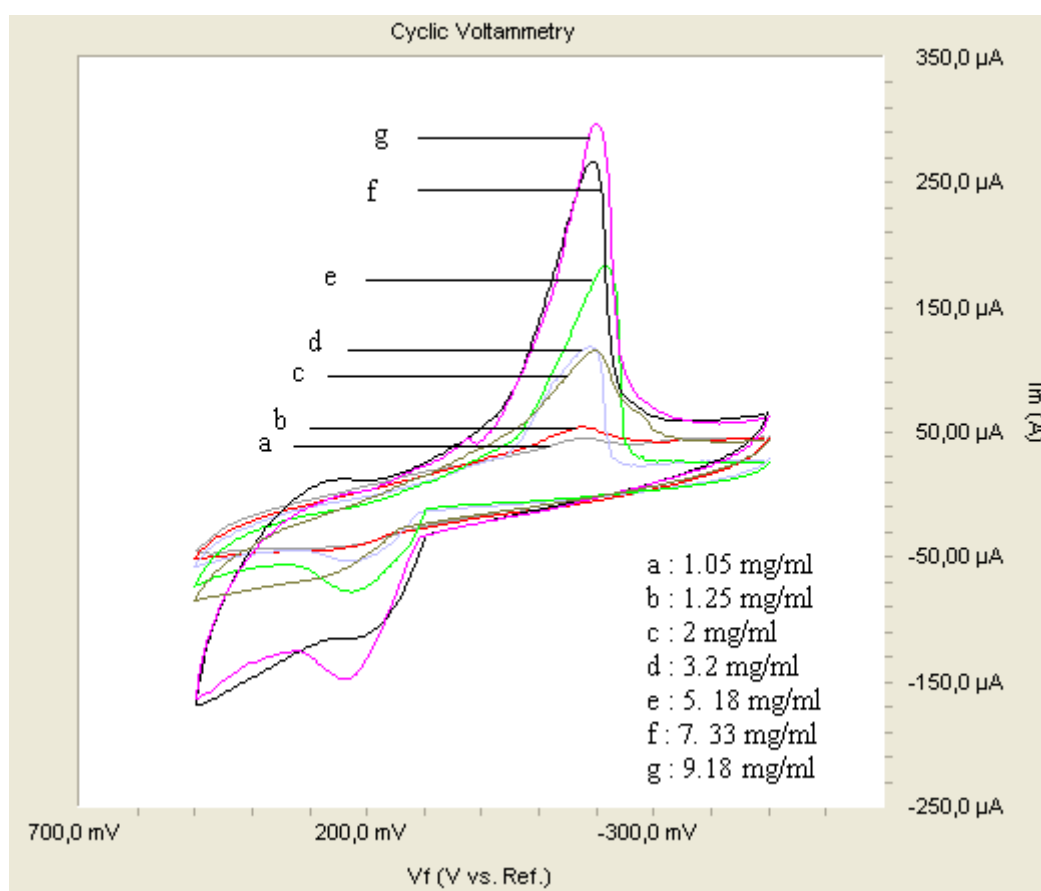
Figure 5.30. Cyclic voltammograms of oleuropein solutions in a temperature of 25 ^{0}C, scan rate of 10 mV/s and the potential range of -0.5 to 0.5 V

It is seen from the above figure that the cathodic current response for oleuropein solution increases as the concentration of oleuropein increases. This was an expected result yielding to a correlation which can be extracted from the calibration curve.

Steady state delta cathodic current response values were recorded for each analyte concentration and these current values were plotted with respect to the

corresponding oleuropein concentration. The resulting linear curve was called the "calibration curve". The calibration curve for oleuropein is given in Figure 5.31.
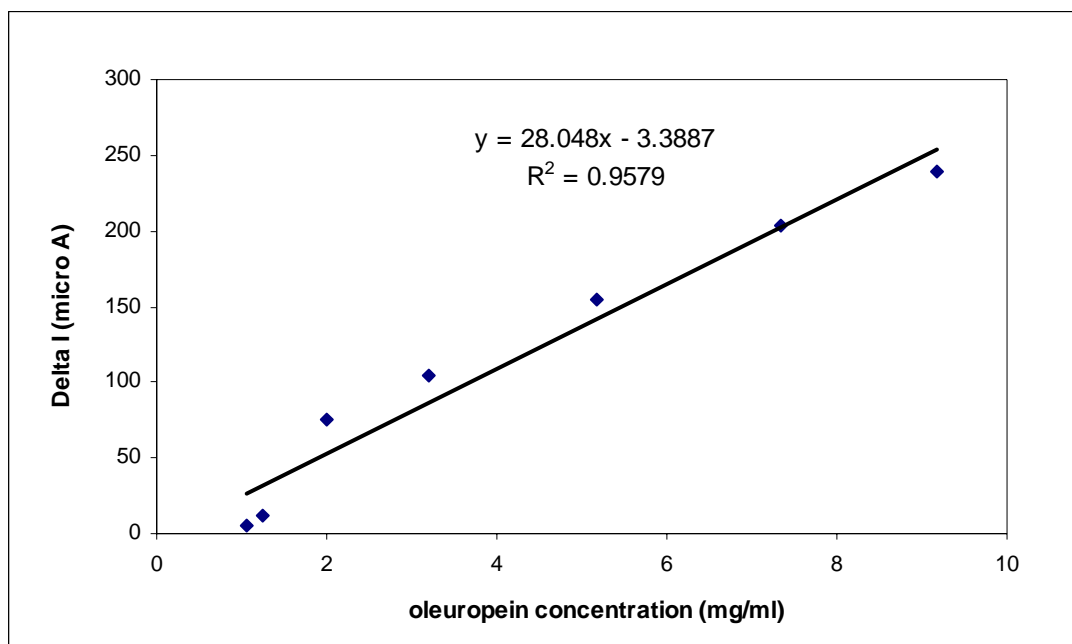

Figure 5.31. Calibration curve for oleuropein

As it is seen from the above figure, the calibration curve is a linear line with an R-sguare of 0.9579 and the calibration equation is given on the figure. The linear calibration curve was very significant in this study due to the fact that it would be used in the following experiments for the determination of oleuropein amount in an analyte solution with an unknown oleuropein concentration.

5.14. Response of Laccase Biosensor to OLE and Its Fractions

Laccase immobilized carbon paste biosensors showed a considerable response for oleuropein solution in cyclic voltammetry experiments. So it was clear that, this type of biosensor could detect oleuropein. After this finding, the question coming to mind was that; could the laccase immobilized carbon paste biosensor detect oleuropein amount in olive leaf extract or not. So, in the further experiments of this work it was decided to investigate the detection capability of laccase biosensor in olive leaf extract and its fractions. To achieve this goal, first 3 mg/ml of the analytes which were olive leaf extract, FI, FII, FIII, FIV and FV were prepared by solving the extracts in the working solution. Then, cyclic voltammetry experiments were run with laccase

biosensor in the potential range of -0.5 to 0.5 V, temperature of 25^0 C and scan rate of 10 mV/s.

Cyclic voltammograms for the olive leaf extract, FI, FII, FIII, FIV and FV are given in Figure 5.32, 5.33, 5.34, 5.35, 5.36 and 5.37 respectively. Voltammograms of laccase biosensor in FVI could not be obtained because of the fact that there was not enough yield of this fraction.
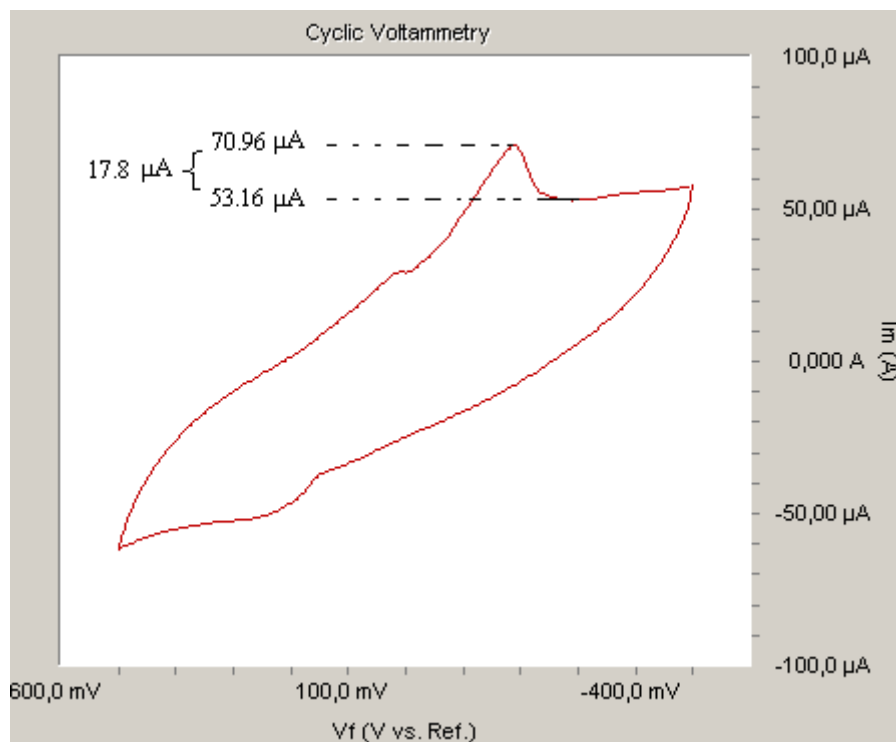

Figure 5.32. Cyclic voltammogram of laccase biosensor in 3 mg/ml of OLE

Cyclic voltammogram of laccase biosensor in 3 mg/ml of olive leaf extract can be seen in Figure 5.32. When this figure is investigated, it is found that the delta value of the cathodic current was 17.8 µA with a vertex value of 70.96 µA and the baseline of 53.16 µA.
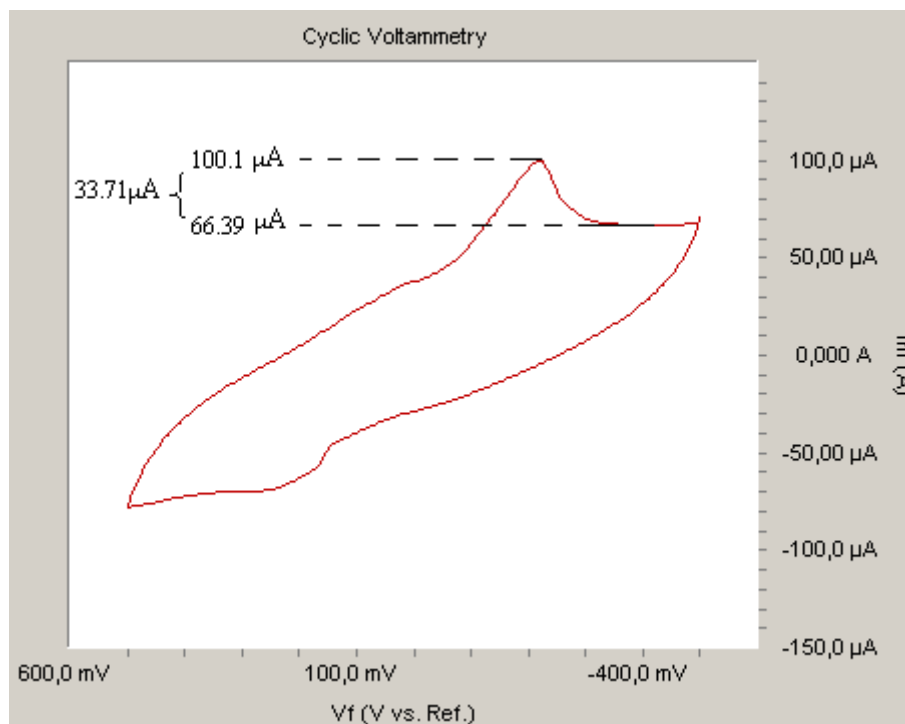
Figure 5.33. Cyclic voltammogram of laccase biosensor in 3 mg/ml of FI

According to the above voltammogram, the delta value of the cathodic peak current of the laccase biosensor in FI was observed as 33.71 µA. This value was calculated by subtracting the baseline of the peak (66.39 µA) from its vertex value (100.1 µA).

Figure 5.34 shows the cyclic voltammogram of laccase biosensor in FII. The highest cathodic peak current was observed in this fraction. As it is seen from the figure below, delta value of the cathodic peak current for this fraction was calculated as 66.13 µA.

The voltammogram of laccase biosensor in FIII can be seen in Figure 5.31. It is observed that the cathodic peak current of this voltammogram had a vertex value of 55.69 µA and a baseline of 30.50 µA. So, delta value of the cathodic peak current of this voltammogram was found as 25.19 µA.

Figure 5.34. Cyclic voltammogram of laccase biosensor in 3 mg/ml of FII

Figure 5.35. Cyclic voltammogram of laccase biosensor in 3 mg/ml of FIII

Figure 5.36. Cyclic voltammogram of laccase biosensor in 3 mg/ml of FIV

Figure 5.37. Cyclic voltammogram of laccase biosensor in 3 mg/ml of FV
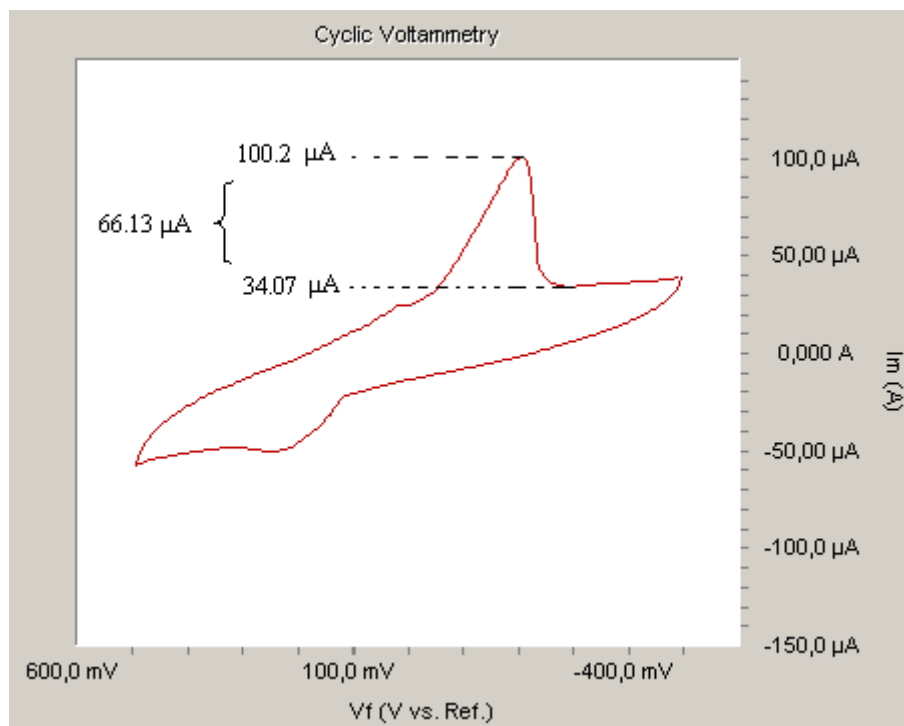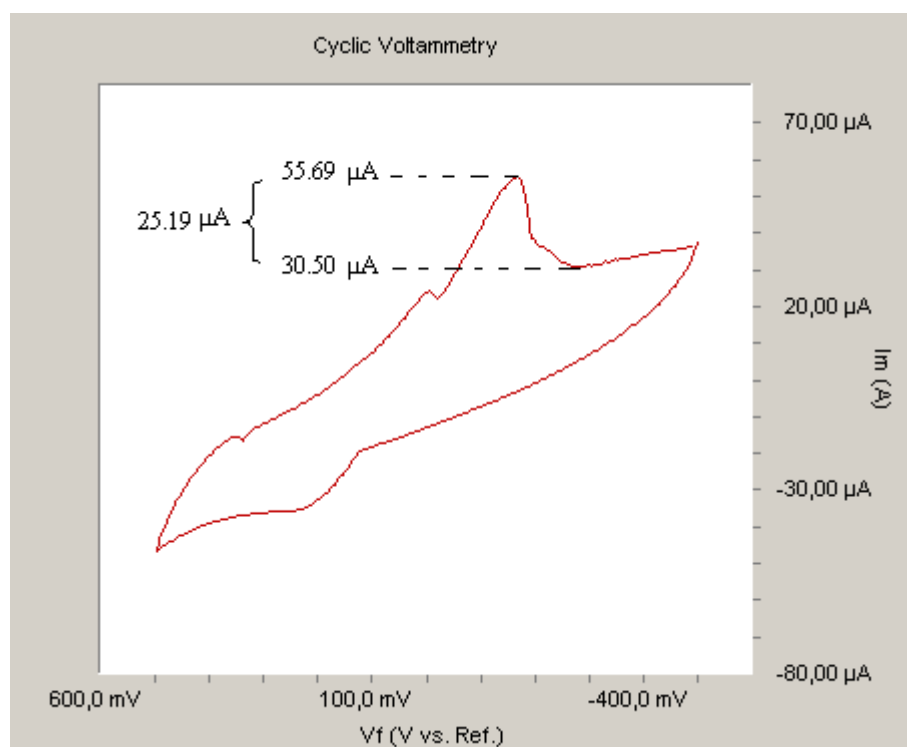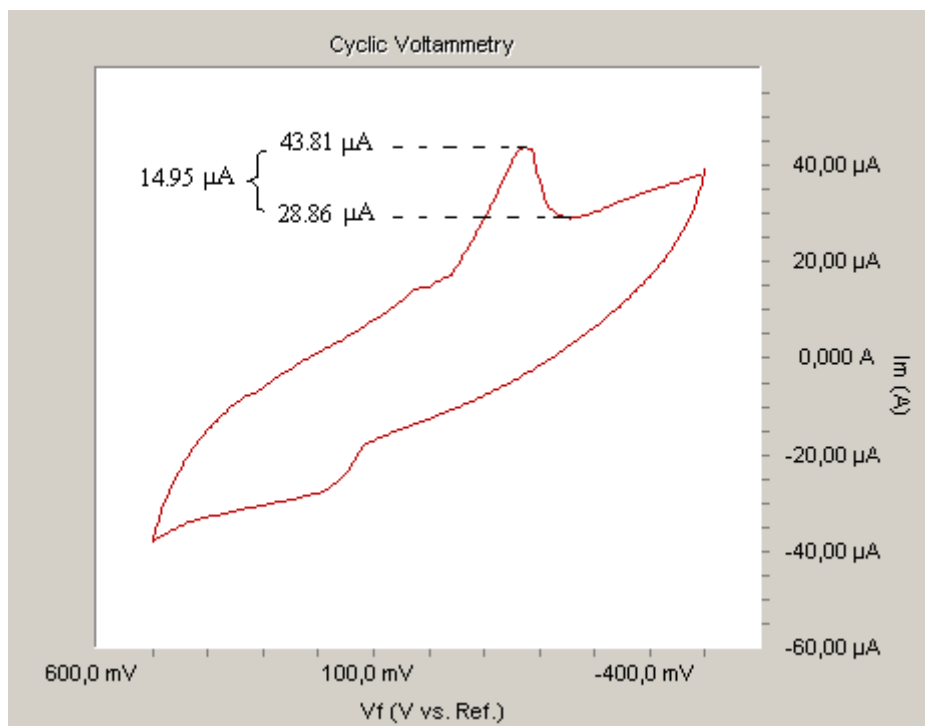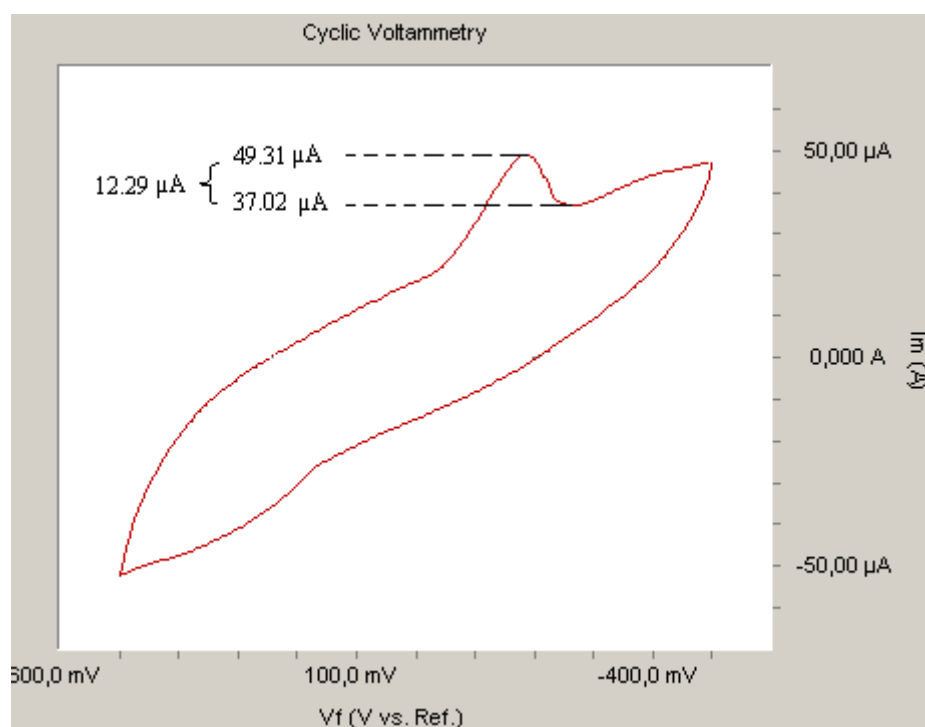
Cyclic voltammogram of laccase biosensor in fraction IV is given in Figure 5.36. Delta value of the cathodic peak current of this fraction is calculated as 14.95 μA which was found by subtracting the baseline of the peak (28.86 μA) from its vertex value (43.81 μA).

Cyclic voltammogram of laccase biosensor in fraction FV can be seen in Figure 5.37. Delta value of the cathodic peak current was found by subtracting the baseline of the peak (49.31 μA) from its vertex value (37.02 μA). It was found as 12.29 μA for this fraction.

5.15. Response of Laccase Biosensor to Catechin, Rutin and Caffeic Acid

Oleuropein is the major polyphenolic component of olive leaf that contributes to the total antioxidant capacity. However rutin, catechin and caffeic acid have known with a considerable antioxidant effect. In this respect the response of laccase biosensor to rutin, catechin and caffeic acid was also studied.

However, in the first trials, no noticeable anodic or cathodic peaks could be observed with these standards. Then, the experiment was run for various cycles and an acceptable anodic and cathodic peaks began to appear with catechin and caffeic acid in the potential range of -500 to 500 mV. On the other hand, no appreciable response was achieved for rutin no matter how many cycles the experiment was conducted. The number of the cycles was fixed and the voltammograms obtained from the last cycle for caffeic acid, catechin and rutin were respectively given in Figures 5.38, 5.39 and 5.40.

Figure 5.38. Cyclic voltammogram of laccase biosensor in 2 mg/ml of caffeic acid
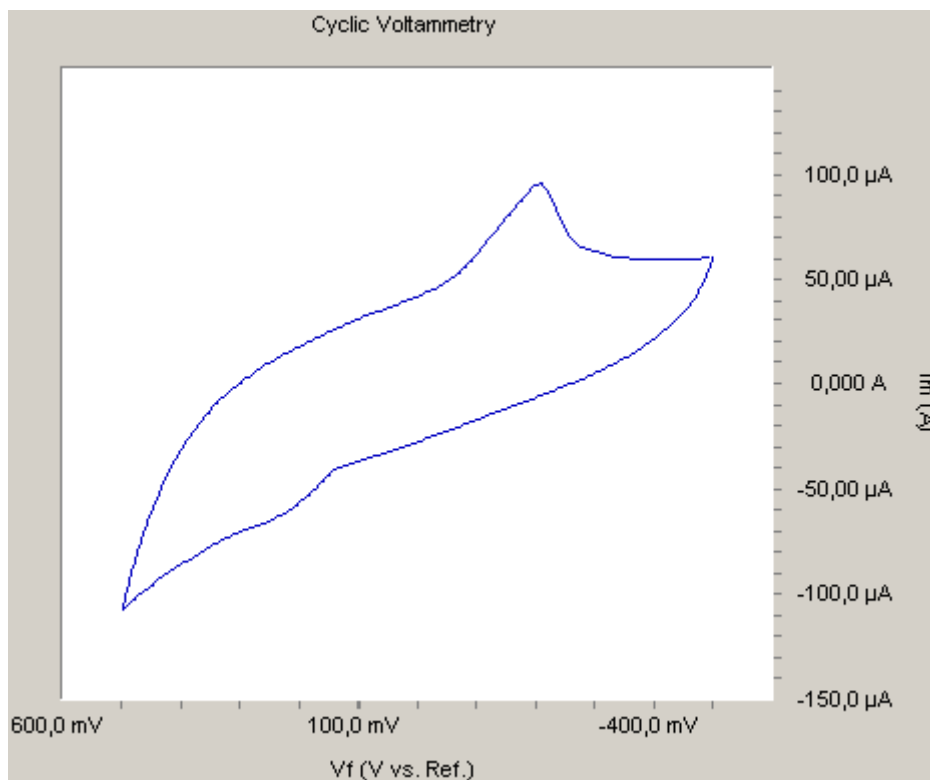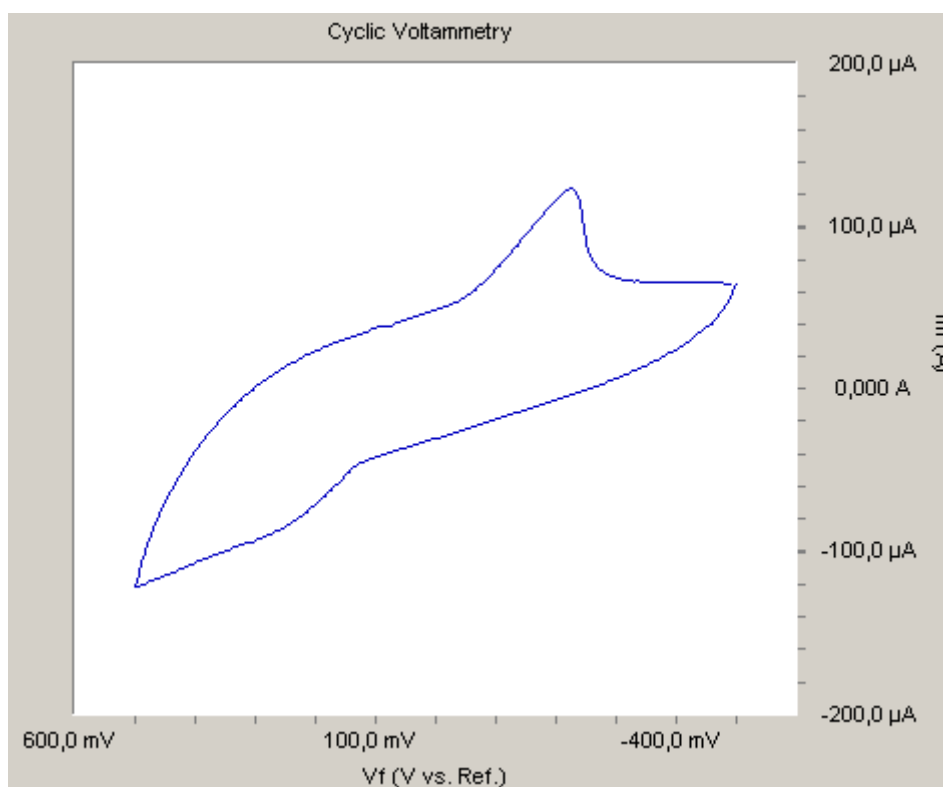

Figure 5.39. Cyclic voltammogram of laccase biosensor in 2 mg/ml of catechin
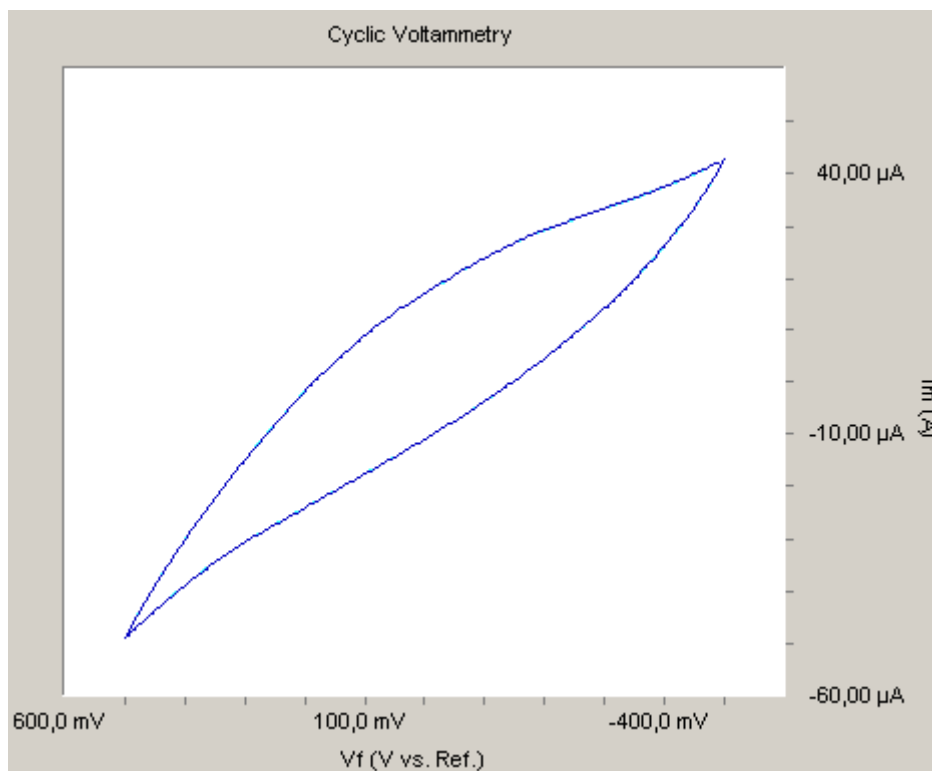
Figure 5.40. Cyclic voltammogram of laccase biosensor in 2 mg/ml of rutin

Structures of these polyhenols are given in Figure 5.41.
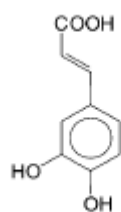
caffeic acid
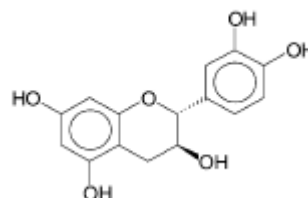
catechin
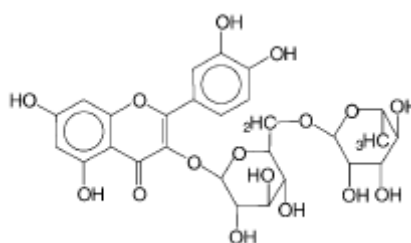
rutin

Figure 5.41. Structure of polyphenols: caffeic acid, catechin, rutin

(Source: Gomes, et al. 2004)

When the cyclic voltammograms of polyphenols are investigated together with their structures, it can be concluded that the electron transfer mechanism in caffeic acid and catechin oxidation is not a completely reversible process. Conducting the experiment for various cycles resulted into the accumulation of the oxidation products into the system and cathodic and anodic peaks were observed for the oxidation products of caffeic acid and catechin. However, the situation was different in the case of rutin oxidation. It was considered that no considerable oxidation and reduction occurred in the rutin solution since no anodic and cathodic peaks were observed. Laccase may not be a convenient enzyme for the oxidation of rutin.

5.16. Comparison of Biosensor Response with HPLC Response, Antioxidant Capacity and Total Phenol Analysis

Up to this point, HPLC analysis, antioxidant capacity analysis (ACW, antioxidant capacity of water soluble antioxidants; ACL, antioxidant capacity of lipid soluble antioxidants), total phenol analysis and cyclic voltammetry experiments were performed and the results were obtained for olive leaf crude extract and its fractions. These results were tabulated and they are given in Table 5.5.

Table 5.5. The results of antioxidant capacity analysis, total phenol, HPLC analysis and cyclic voltammetry experiments

	Antioxidant Capacity Analysis		Total Phenol Analysis	Biosensor Results	HPLC Results
	ACW Analysis (μg ascorbic acid /mg)	ACL analysis (μg trolox /mg)	(mg gallic acid/g sample)	oleuropein (% wt)	oleuropein (% wt)
CE	678.81	1495.3	195.09	25.18	14.16
FI	1278.35	1634.63	309.57	44.09	56.6
FII	1036.7	270.49	376.63	82.62	60.5
FIII	1148.49	2051.46	309.86	33.96	25.3
FIV	807.03	1260.51	249.94	21.79	10.4
FV	807.02	1070.42	334.78	18.63	No oleuropein peak

As it is seen from Table 5.5, ACW results were given as μg ascorbic acid standard/mg sample, while ACL results were given as μg trolox standard/mg sample. Total phenol results were reported as mg gallic acid standard/g sample. On the other hand, biosensor and HPLC results were given in the form of weight percentage of oleuropein in olive leaf extract.

ACW, ACL and total phenol results were not representative results for only oleuropein whereas, HPLC and biosensor results were representative for oleuropein. When the biosensor and HPLC results were investigated, it was seen that although the weight percentages were not completely same, they were quite closer with each other.

As it was noticed from the table, the highest oleuropein amount was found in FII fraction by both HPLC and biosensor analysis. FII fraction having the highest oleuropein amount also has the highest ACW result in terms of ascorbic acid equivalent antioxidant capacity. As, water soluble component, oleuropein is known with its antioxidative property, such a result is not suprising.

It was noticed that, FI and FIII contained additional water soluble antioxidants other than oleuropein. This prediction could also be proved by the HPLC chromatograms of the fractions which are given in Appendic C.

In addition, it was clear that non-phenolic components were removed from fractionation and the lowest total phenol amount was observed for CE.

To sum up, when antioxidant capacity, total phenol analysis, HPLC and biosensor results were investigated, a general relationship could not be obtained for all of them but some similarities were observed. This was not a surprising result because only HPLC and biosensor results were representative for oleuropein. On the other hand, ACW calculations were performed for water soluble antioxidants, ACL calculations were performed for lipid soluble antioxidants and total phenol analysis were predominantly for both water and lipid soluble antioxidants.

CHAPTER 6

CONCLUSION

The objective of this study was the development of an amperometric laccase biosensor for the determination of oleuropein concentration in olive leaf extract. The biosensor was prepared by immobilization of laccase enzyme from *Trametes versicolor* by addition of crosslinking agent, glutaraldehyde, into the carbon paste.

The behaviour of electrochemical sensor and laccase biosensor to oleuropein was investigated. As a result of the comparison of the responses of electrochemical sensor and laccase biosensor, it was concluded that the laccase enzyme has a capability to oxidize oleuropein. In other words, oleuropein shows a good substrate behaviour for laccase enzyme. After this confirmation, the effect of laccase concentration and glutaraldehyde amount on biosensor response was investigated. For this purpose biosensors having glutaraldehyde amount of 6.40, 12.03 and 17.01 % vol. of the bottom part and enzyme concentration of 1, 5 and 10 mg/ml were prepared. After the cyclic voltammetry experiments were performed with these biosensors, it was found that the best performance was observed with the biosensor having glutaraldehyde amount of 12.03 % vol. of the bottom part and 5 mg/ml of laccase enzyme.

The effect of scan rate and temperature on the response of the biosensor was also studied. To find the suitable values for the best biosensor performance, the cyclic voltammery experiments were performed at various scan rates and temperatures. The scan rate of 10 mV/s was decided to be the optimum for the amperometric detection of oleuropein considering the fastest response and maximum reduction current. 25^0C was chosen as an optimum temperature value due to the maximum laccase activity and capability of oleuropein acting as an antioxidant.

The cyclic voltammetry experiments were followed to obtain a calibration curve between the current response and the concentration of oleuropein. To construct a calibration curve, cyclic voltammetry experiments were performed with oleuropein solutions differentiating in their concentration in a temperature of 25^0C, scan rate of 10 mV/s and the potential range of -0.5 to 0.5 V. After the cyclic voltammograms were

obtained, the calibration curve was constructed and a good linearity was observed with the biosensor response with an R^2 value of 0.9579.

Extraction of olive leaf was also performed in this study. Isolation of polyphenols from olive leaf extract is a very important task and because of that fractionation was followed for further purification. By fractionation, the extract was divided into six fractions (FI, FII, FIII, FIV, FV and FVI) varying in their oleuropein amounts such as polar fractions and relatively less polar fractions. Therefore, biosensor performance was investigated for fractions containing different type of phenolics. Oleuropein amount in crude extract and its fractions was calculated by the biosensor method. Crude extract and the fractions (FI, FII, FIII, FIV, FV) were found to contain 25.18, 44.09, 82.62, 33.96, 21.79 and 18.63 % wt. oleuropein respectively. Oleuropein amount of fraction FVI could not be detected because of the inadequate yield value.

HPLC analyses of the fractions were also performed in this study. According to the HPLC results, it can be observed that CE, FI, FII, FIII and FIV contained 14.16, 56.6, 60.5, 25.3 and 10.4 % wt. oleuropein. Oleuropein peak could not be observed in FV and FVI fractions.

Moreover, total phenol content and antioxidant capacity of the fractions were determined by conventional methods. Total phenol content of CE and its fractions were found by Folin- ciocalteu method as 195.09, 309.57, 376.63, 309.86, 334.78 and 249.94 mg gallic acid/g sample. ACW and ACL analyses were performed by PLC assay. Water soluble antioxidant capacity values for CE and its fractions were found as 678.81, 1278.35, 1036.7, 1148.49, 807.03 and 807.02 μg ascorbic acid /mg and lipid soluble antioxidant capacity values were calculated as 1495.3, 1634.63, 270.49, 2051.46, 1260.51 and 1070.42 μg trolox /mg respectively.

Rutin, catechin and caffeic acid are known with their antioxidant capacities. So, the performance of biosensor to these polyphenols was also studied. It was found that constructed biosensors could specifically detect oleuropein, in contrast they gave no appreciable anodic and catodic peak current responses to rutin at studied concentration, scan rate and potentials.

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APPENDIX A

OLEUROPEIN CALIBRATION IN HPLC

Calibration studies should be applied as a necessary task to calculate the amount of oleuropein in olive leaf extract. External and internal calibration methods were applied in this study.

In external calibration, a stock solution of oleuropein was prepared by dissolving in 50 % acetonitrile-water solution at 25^0C. 1, 2, 3, 4, 5, 6, 7 and 8 mg/ml of oleuropein solutions were prepared by diluting the stock solution. Each of these concentrations was injected to HPLC and HPLC response, in terms of area, was recorded in each case. The injection was repeated twice for all the concentration values. The recorded area values for oleuropein are given in Table A1.

To find the minumum oleuropein concentration that could be detected by HPLC, low oleuropein concentrations were prepared by diluting the 2 mg/ml of stock oleuropein solution. First set was composed of 0.5 mg/ml, 0.3 mg/ml, 0.1 mg/ml of dilute oleuropein solutions and these three dilute concentrations yield a chromotogram in HPLC analysis. So it was understood that, the experiment should be conducted with more dilute oleuropein concentrations. For this reason, 0.05 mg/ml, 0.08 mg/ml, 0.09 mg/ml, 0.098 mg/ml and 0.099 mg/ml of oleuropein concentrations were injected to HPLC and no observable peaks could be seen in their chromatograms. As a result, 0.1 mg/ml of oleuropein concentration was accepted as the minimum detection limit in the HPLC anlysis and in order to check this result, detection of oleuropein amount with this concentration was repeated again.

Table A1. Data for HPLC response at each injection

Concentration (mg/ml)	Area of Oleuropein (mAU*s)	Area of Oleuropein (mAU*s)	Average Area of Oleuropein (mAU*s)
0.1	569.2	578.8	574
1	5465.8	5576.3	5521
2	11131.8	11161.4	11146.6
3	16826.9	16724.3	16775.6
4	21847.1	21758.5	21802.8
5	27077.2	26656.1	26866.7
6	31672.8	30569.8	31121.3
7	35904.8	34627.8	35266.3
8	43417.8	44502.5	43960.2

The concentration versus area was plotted for oleuropein and it is given in Figure A1. This curve is the oleuropein external calibration curve. Oleuropein external calibration curve is linear fit as it is expected with an R-square value of 0.9955.
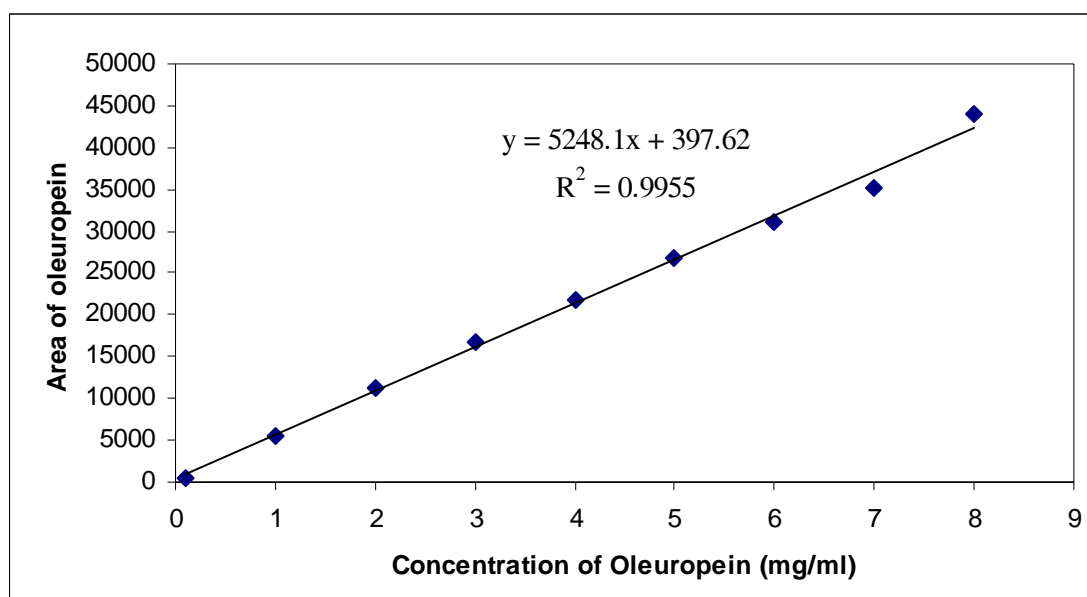

Figure A1. Oleuropein external calibration curve

In internal calibration, coumarin was used as a standard. The reasons of using coumarin as internal standard are as follows;

o Coumarin does not exist in olive leaf,

o It does not react with any phenolic in olive leaf,

o Its retention time in HPLC is very close to the retention time of oleuropein and

o It has a similar structure with the polyphenols in olive leaf.

In internal calibration method, stock solutions of oleuropein and coumarin were prepared. While preparing the stock solutions, both of the standards either oleuropein or coumarin was dissolved in 50 % acetonitrile-water solution at 25^{0}C. Different concentrations of oleuropein and coumarin were prepared by diluting the stock solution. For the oleuropein calibration, solutions containing 60% oleuropein and 40% coumarin were injected to the HPLC. The injection was repeated twice for all the concentration values. Then the responses of HPLC at different concentrations were recorded. The recorded area values for oleuropein and coumarin are given in Table A2.

Table A2. HPLC response for oleuropein and coumarin

Concentration of Oleuropein (mg/ml)	Area of Oleuropein (mAU*s)	Area of Coumarin (mAU*s)
0	0	0
1	3266.4	47088.9
2	6792.8	6379
3	10263	71777.5
4	13550.4	76306.4
5	17119.25	80666.4
6	19490.5	83323.9
7	22678.9	85278.2

The ratio of the area of oleuropein to area of coumarin versus concentration of oleuropein was plotted. The plot is the oleuropein internal calibration curve and it is given in Figure A2. Oleuropein internal calibration curve is linear fit with an R-square value of 0.9531.
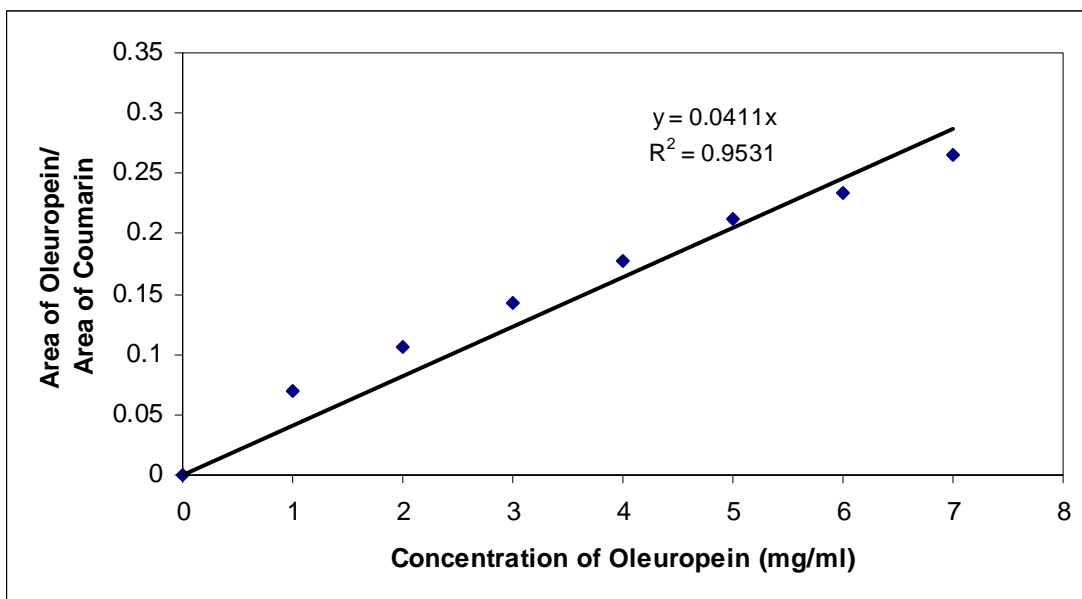
Figure A2. Oleuropein internal calibration curve

APPENDIX B

CALCULATION OF THE OLEUROPEIN AMOUNT IN OLE AND ADSORBED AMOUNT OF OLEUROPEIN ON SILK FIBROIN

HPLC response, in terms of area, was used to find out the oleuropein amount in olive leaf extract. The area value was put into the calibration equation and oleuropein concentration was calculated. For instance, in the case of olive leaf crude extract, 37,558 was recorded as the area value. The external calibration equation for oleuropein is;

$y = 5248.1x + 397.62$

From the calibration equation x is calculated and found as,

$x = 7.08$ mg/ml

7.08 mg/ml * 20 = 141.6mg oleuropein

As solid/liquid ratio of the extract was 1/20, 1 gram of extract contains 141.6mg oleuropein.

In order to find the adsorbed amount of oleuropein; first, calibration curves were used and the responses of HPLC in terms of area were converted to concentration values. Then the following equation was used.

$$q = \left(C_i - C\right)\frac{V}{m}$$

where,

q = Solute phase concentration in equilibrium (mg oleuropein/g silk fibroin)

C_i = Initial concentration of liquid phase (mg oleuropein/ml extract)

C = Equilibrium solute concentration in the aqueous phase (mg oleuropein/ml extract)

V = Volume of liquid phase (ml olive leaf extract)

m = Mass of the adsorbent (g silk fibroin)

$C_i = 7.08$ mg/ml and

$C = 6.17$ mg/ml

$$q = (7.08 - 6.17)\frac{1}{0.05}$$

q = 18.2 mg oleuropein/g silk

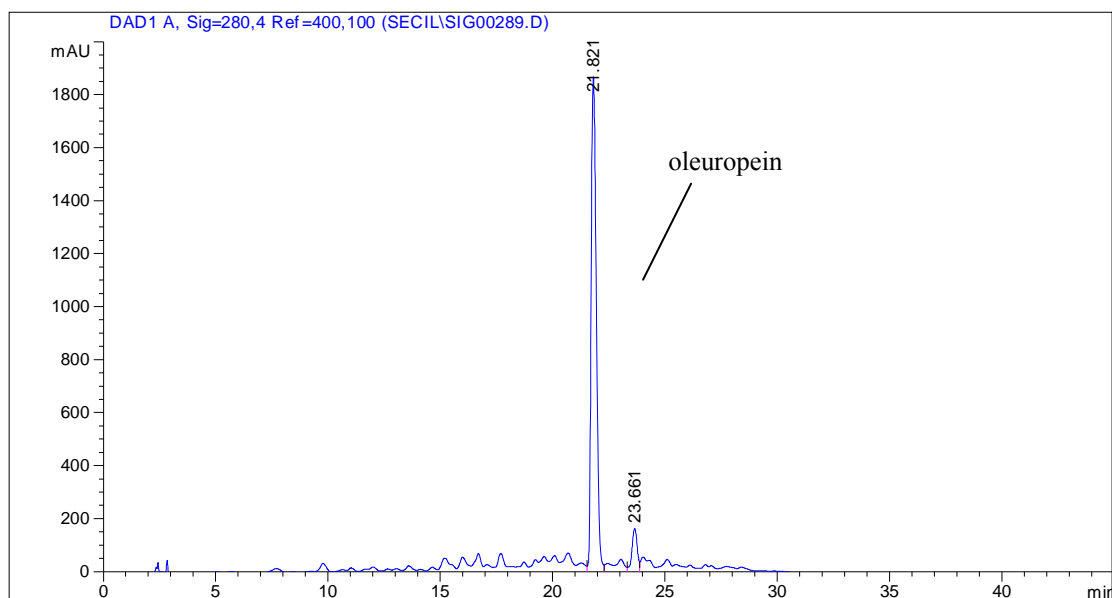# APPENDIX C

HPLC CHROMATOGRAMS OF OLIVE LEAF FRACTIONS

Figure C1. Chromatogram of FI (W1+W2)

```
Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|

   1  21.821 VV   0.2574 3.01225e4  1875.30090  90.9553
   2  23.661 VV   0.2707 2995.42041  170.68356   9.0447
```
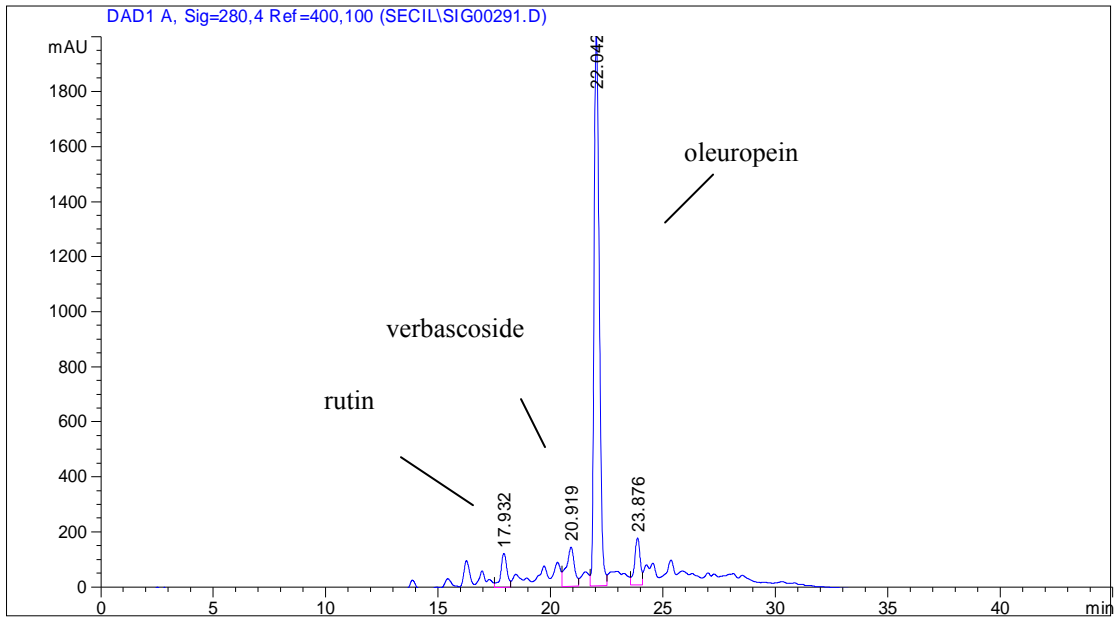
Figure C2. Chromatogram of FII (W3+W4)

```
Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|

  3  22.042  VV    0.2579  3.21681e4  1997.15039  78.1535
```
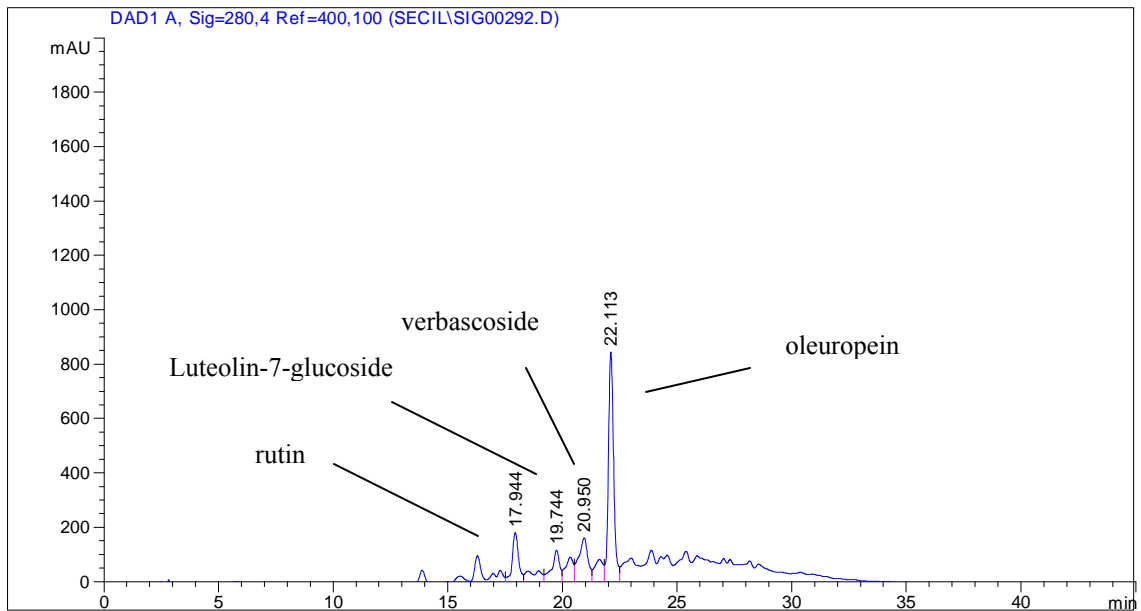

Figure C3. Chromatogram of FIII (W5+W6)

```
Peak RetTime Type  Width     Area       Height     Area
  #   [min]         [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|

  1  17.944 VV    0.2967 3619.87476  184.65550  14.4789
  2  19.744 VV    0.3472 2982.26782  120.32397  11.9286
  3  20.950 VV    0.4016 4722.24316  165.15726  18.8882
  4  22.113 VV    0.2521 1.36767e4   848.44305  54.7044
```
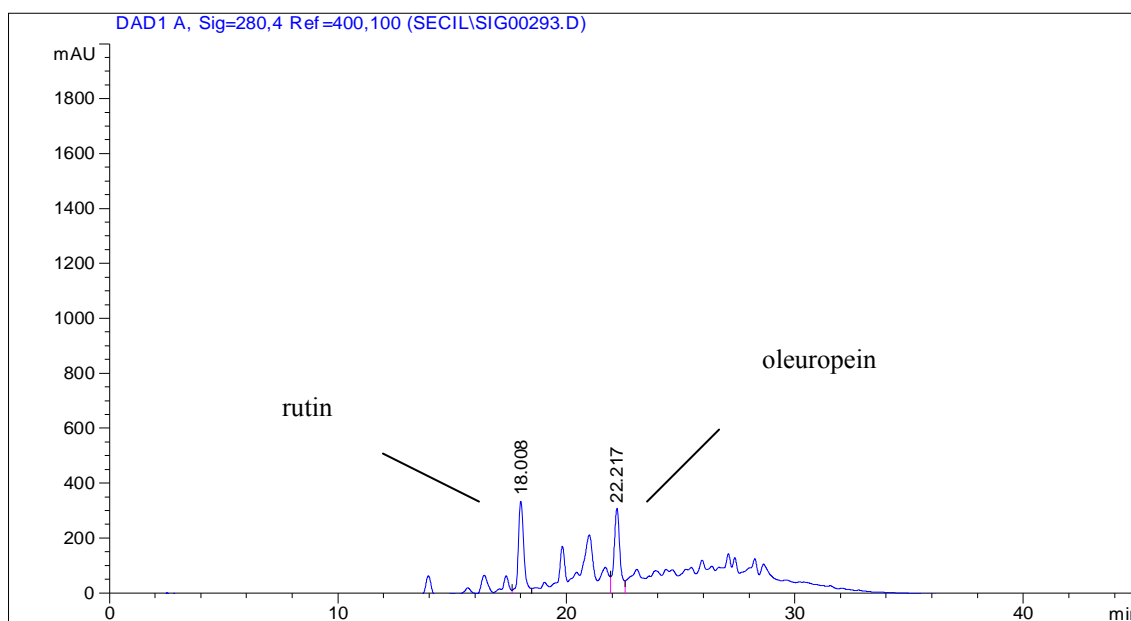

Figure C4. Chromatogram of FIV (W7+W8+W9)

```
Peak RetTime Type  Width     Area       Height     Area
  #   [min]         [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|

  1  18.008 VV    0.2680 5995.58643  342.83884  50.5684
  2  22.217 VV    0.2754 5860.80615  317.40268  49.4316
```
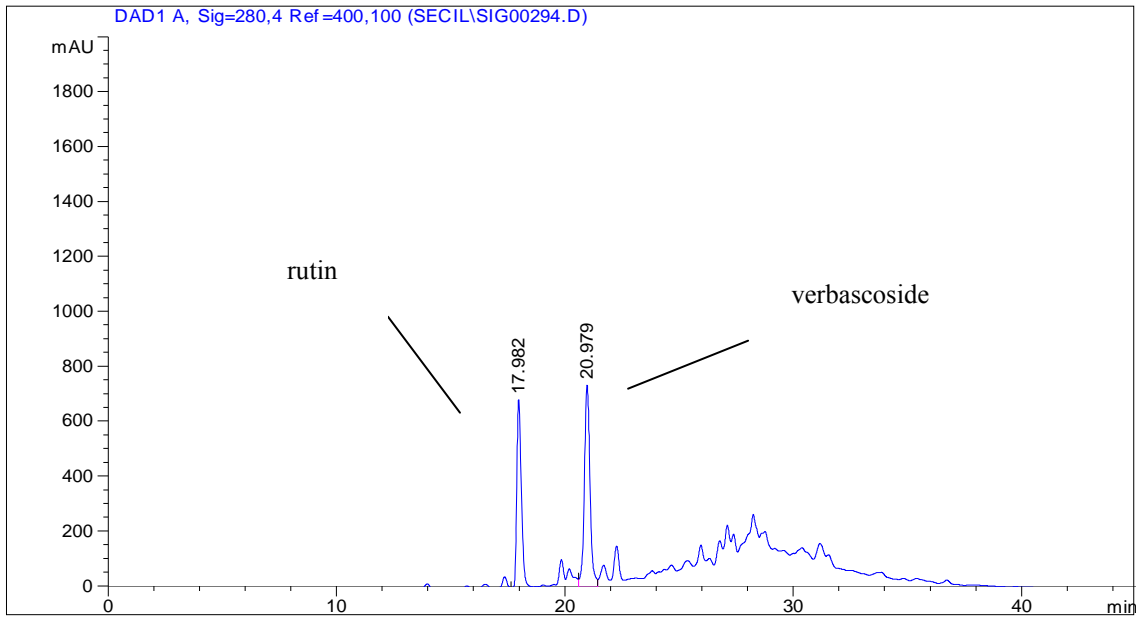
Figure C5. Chromatogram of FV (E1+E2)

```
Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]      [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  17.982 VB   0.2140 9490.01855  683.42554  42.8763
   2  20.979 VV   0.2646 1.26435e4   735.14966  57.1237
```
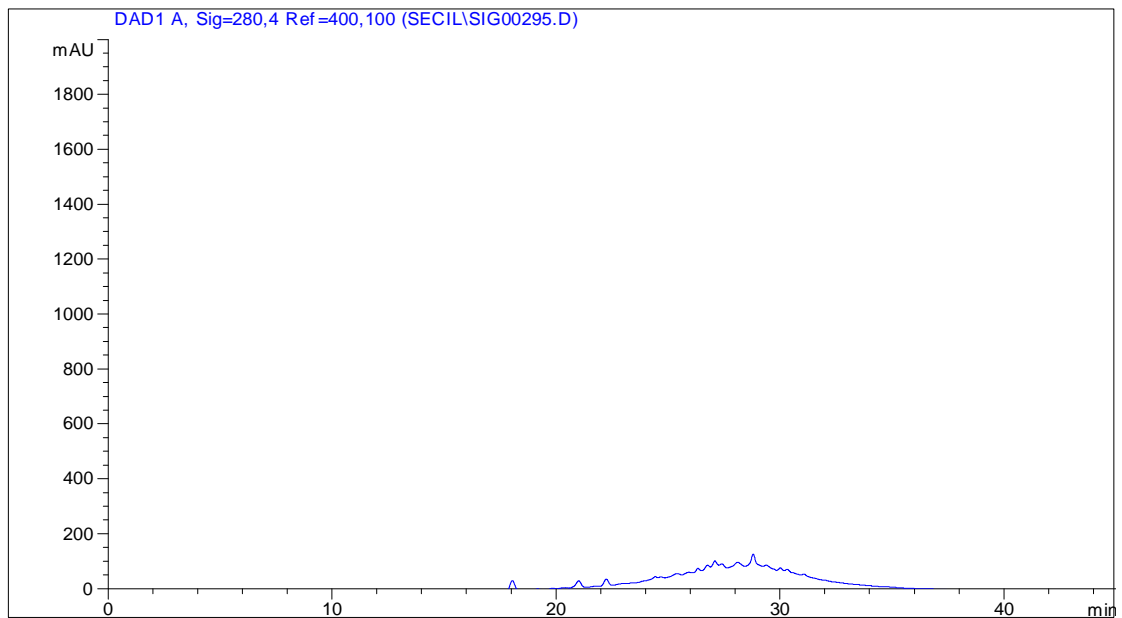

Figure C6. Chromatogram of FVI (E3+E4)

```
Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]      [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  28.807 VV   0.4575 4556.82129  127.96131 100.0000
```

APPENDIX D

ANTIOXIDANT CAPACITY OF WATER AND LIPID SOLUBLE COMPOUNDS

Antioxidant capacity of water soluble compounds, in terms of ascorbic acid concentration, was calculated from the following equation;

$$Concentration(\mu g / mg) = \frac{(Quantity)(Dilution)(M)(Volume)}{(Pipetted volume)(Weighted sample)}$$

where,

Quantity: nmol (Ascorbic acid equivalents),

M: molar mass of ascorbic acid (176.13 ng/nmol),

Pipetted volume: used volume in the vial in μl,

Weighted sample: initial weighted sample in mg,

Volume: extraction volume in ml,

Dilution: (at 1:10 dilution factor, dilution =10)

Quantity values were calculated from the ACW curves and substituting other values into the equation yields ascorbic acid equivalent concentration values. For example, quantity value was found as 1.29 nmol for one of the samples then, ascorbic acid equivalent antioxidant capacity of this sample is found as in the following,

$$Concentration(\mu g / mg) = \frac{(1.29)*(1500)*(176.13)*(20)}{(10)*(1000)}$$

$$Concentration(\mu g / mg) = 681.62 \ \mu g / mg$$

Antioxidant capacity of lipid soluble compounds, in terms of trolox concentration, was calculated from same equation;

$$Concentration(\mu g / mg) = \frac{(Quantity)(Dilution)(M)(Volume)}{(Pipetted volume)(Weighted sample)}$$

where,

Quantity: nmol (trolox equivalents),

M: molar mass of trolox (250.3 ng/nmol).

Pipetted volume: used volume in the vial in µl,

Weighted sample: initial weighted sample in mg,

Volume: extraction volume in ml,

Dilution: (at 1:10 dilution factor, dilution =10)

In this case quantity values were found from the ACL curves as nmoles of trolox equivalents and simultaneously molar mass of trolox was used in the equation. Antioxidant capacity of one of the samples yielding a quantity value of 1.996 nmol trolox equivalents is found as in the following,

$$Concentration(\mu g/mg) = \frac{(1.996)*(1500)*(250.3)*(20)}{(10)*(1000)}$$

$$Concentration(\mu g/mg) = 1498.8 \ \mu g/mg$$