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Development of Biosensors for Determination of the Total Antioxidant Capacity of Olive Leaf Extract

Oguz Bayraktar, Seçil Çoban

***Abstract*— In the present work, a laccase biosensor was investigated 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 Trametesversicolor by addition of cross-linking agent, glutaraldehyde, into the carbon paste electrode. The best biosensor performance was determined for biosensor having glutaraldehyde amount of 12.03 % and 5 mg/ml of laccase enzyme. The scan rate of 10 mV/s was decided to be the optimum for the amperometric detection of oleuropein based on the fastest response and maximum reduction current. Biosensor performance was also investigated for olive leave extract fractions varying in their oleuropein amounts. Therefore, biosensor performance was investigated for fractions containing different types of phenolic compounds. High Performance Liquid Chromatography (HPLC), total phenol content and antioxidant capacity analyses of the fractions were also carried out for comparison with biosensor results.**

***Index Terms*— Biosensor, antioxidant, oleuropein, olive leaf.**

I. 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 [1]. Olive tree is one of the potential natural antioxidant sources because of its phenolic content. Various methods are 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 [2, 3].

Biosensors are attractive alternative techniques offering several advantages. The unique characteristics of biosensors are selectivity, low cost, miniaturization, easy automation, time saving and simplicity of operation and manufacturing [3, 4]. Thus, the development of biosensors for the polyphenolic fraction of olive leaf is a great on-going challenge. Biosensors can be defined as an analytical tool or system consisting of an immobilized biological material in intimate contact with a suitable transducer [5]. 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 [6]. The transducer element in a biosensor system converts the biological signal into an electrical one. Biosensors can be classified into several types according to their transducer type such as amperometric, potentiometric, conductimetric, optical, acoustic, piezoelectric and thermal [6, 7].

Enzymes are extremely specific and selective for the substrates which they interact with. Problems like selectivity and slow response characteristics of biosensors can be overcome 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 oxido-reductase 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 [8].

An amperometric biosensor may be more attractive among the other biosensor systems due to its promising properties such as high sensitivity and wide linear range [9]. Therefore, in literature phenolic biosensor based on a zirconium oxide/polyethylene glycol/tyrosinase composite film for the detection of phenolic compounds has successfully been explored [10]. 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 [7].

In this study [11], an amperometric laccase biosensor was developed for determination of the oleuropein concentration which contributes dominantly to the total antioxidant capacity in olive leaf extract. 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 (OLE), in an easy and economical way. Also, this study will give an idea about how to determine the antioxidant capacities of different products in food industry.

II. MATERIAL AND METHODS

A Reagents, Standards and Samples

Graphite powder (synthetic <20 microns) was purchased from Sigma-Aldrich (Steinheim, Germany).

***Oguz Bayraktar**, Department of Chemical Engineering, Ege University, İzmir, Turkey
Seçil Çoban, Department of Chemical Engineering, Izmir Institute of Technology, İzmir, Turkey.

Mineral oil (0.85gml-1), glutaraldehyde (50% solution) and Trametesversicolorlaccase (EC 1.10.3.2) (0.72 Umg-1) were obtained from Fluka (Steinheim, Germany). Oleuropein and coumarin were purchased from Extrasynthese (Genay, France), whereas rutin was purchased from Merck (Darmstadt, Germany). Olive leaves were collected from the olive trees grown in the campus of İzmir Institute of Technology (İzmir, Turkey). Hydrophobic silk fibroin was used as an adsorbent for the adsorption of olive leaf phenols and it was obtained from Silk Biochemical Co.,Ltd. (Hangzhou, China). All solvents were of high-performance liquid chromatography (HPLC) grade. Acetonitrile from Sigma-Aldrich and acetic acid from Merck were used as mobile phases in HPLC analysis. Ethanol was used in the extraction of olive leaves and it was obtained from Riedel-de Haën (Seelze, Germany).

B Electrochemical Assays

Gamry Electrochemical Measurement Apparatus was used for the amperometric detection. This system consists of three electrodes, a 7.5 cm platinum electrode as an auxiliary electrode, a saturated Ag/AgCl electrode as a reference electrode and a working electrode which is enzyme immobilized carbon paste biosensor.

C Pretreatment and Extraction of Olive Leaves

Collected olive leaves were washed with deionized water and then dried at 37 ºC for 3 consecutive days. The dried leaves were grinded until the leaves become fine powder to about 90-150 µm particle size. The leaves were extracted in 70% aqueous ethanol solution since the highest oleuropein and rutin yields were obtained with this ratio in the literature [12]. After 2 hours of extraction at 25 ºC, the solvent was removed by rotary evaporator at 38 ºC and 120 rpm. Then, solvent-free olive leaf extract was put in a freeze-dryer at -52 ºC and 0.2 mbar. After all these steps, olive leaf crude extract was obtained and it was stored in light-protected glasses till further usage.

D HPLC Analysis and Identification of Phenolic Compounds

HPLC analysis given in the literature was used for the quantification of oleuropein [1]. The HPLC equipment used was a Hewlett-Packard series HP 1100 equipped with a diode array detector. The stationary phase was a C18 LiChrospher 100 analytical column (250mm x 4mm i.d.) with a particle size of 5 µm thermostated at 30 °C. The flow rate was 1 ml/min and the absorbance changes were monitored at 280 nm. The mobile phases for chromatographic analysis were (A) acetic acid/water (2.5:97.5) and (B) acetonitrile. A linear gradient was run from 95% (A) and 5% (B) to 75% (A) and 25% (B) during 20 min; it changed to 50% (A) and (B) in 20 min (40 min, total time); in 10 min it changed to 20% (A) and 80% (B) (50 min, total time), after reequilibration in 10 min (60 min, total time) to initial composition. Oleuropein and rutin in olive leaf extract were identified by comparison of their retention times with the corresponding standards. Coumarin was used as internal standard for the quantification of oleuropein and rutin. Because the retention time of coumarin is between the retention times of oleuropein and rutin and because it does not react with the polyphenols in olive leaf extract, coumarin was preferred as an internal standard in this study.

E Fractionation of Olive Leaf Antioxidants

Silk fibroin was understood to be a promising adsorbent for the purification of oleuropein in olive leaf extract from the studies given in the literature, thus silk fibroin was used as an adsorbent in this study for the fractionation of olive leaf phenols [1, 13]. Crude olive leaf extract was dissolved in deionized water in 1/20 solid/liquid ratio at room temperature with magnetic stirring. Then the solution was centrifuged at 5000 rpm for 15 min. A column of 20 cm in length and 2 cm internal diameter with Teflon fittings was used after filling it with 7.8 g of powdered silk fibroin. The column was preconditioned by washing with 50 ml of deionized water, ethanol and deionized water respectively. Then, the clear solution of olive leaf crude extract was loaded to the preconditioned column with constant flow rate 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 saturation of the column with oleuropein and rutin, desorption was followed. Deionized water and 70% aqueous ethanol solution were respectively used as eluting solvents in the desorption stage. At the end of the desorption process, 9 batches of polar and 4 batches of relatively less polar fractions were analyzed in HPLC in order to determine the desorption efficiency. Depending on the HPLC results, fractions were collected in 6 groups as FI, FII, FIII, FIV, FV and FVI. These fractions were in the form of solution and they were processed in rotary evaporator and then freeze dried. So, fractions were collected in the form of powder and they were stored at +4 °C to be used in biosensor experiments.

F Antioxidant Capacity Analysis

Photochemiluminescence (PCL) assay is known to be a time and cost effective system for the determination of the antioxidative capacity. This assay was described by Popov and Lewin and was commercialized by Analytic Jena AG (Jena, Germany) under the name PHOTOCHEM [13, 14]. PHOTOCHEM antioxidant analyzer was used to determine the antioxidant capacity of OLE and its fractions. Antioxidant capacity analysis by this system is first based on the generation of the radicals and then quantification of the generated radicals. PCL method can be conducted by two

different protocols, ACW and ACL. ACW permits the measurement of the antioxidant capacity of water soluble compounds and ACL permits the measurement of the antioxidative capacity of lipid soluble compounds. In ACW protocol, OLE and its fractions were firstly dissolved in DMSO in a presolution ratio of 1 g extract in 20 ml DMSO. Then the samples were diluted with deionized water. Next, ACW kit procedure was followed. The same presolution ratio was applied for the ACL protocol but in this method dilutions were made with methanol. Next, ACL kit procedure was performed.

G Total Phenol Analysis

Total phenol analysis of OLE and its fractions were performed by Folin-ciocalteu method using Gallic acid as standard. In this method, diluted samples were mixed with folin-ciocalteu reagent. Then Na2CO3 was added to the mixture and after incubating for 1 hour, absorbance values were measured at 725 nm.

H Biosensor Preparation and Amperometric Detection

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 Trametesversicolor by addition of cross-linking agent into the carbon paste. Nine different biosensors were prepared in this study by changing the amount of crosslinking agent and the concentration of the enzyme solution. While constructing biosensors, first the top mixture and then the bottom mixture were filled into a pipette tip. Finally, preparation of biosensors was completed by immersing silver wire into the pipette tip. Carbon paste biosensors were polished by using a polishing paper just before using and storage. The biosensors were stored at about +4 °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.

After the construction of biosensors, cyclic voltammetry (CV) experiments were followed for oleuropein standard with these biosensors. Effect of enzyme concentration and glutaraldehyde amount were investigated by CV experiments. After determination of the best biosensor performance, effect of scan rate and temperature were investigated with this biosensor. Then a linear calibration curve was constructed for oleuropein. Finally, CV experiments were conducted for OLE and its fractions.

III. RESULTS AND DISCUSSION

A HPLC Analysis of OLE 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.

Oleuropein, the major phenolic compound of olive leaf extract, was first qualitatively determined by comparing its retention time with its standard. From the chromatogram, retention time of oleuropein is observed as 21.58 min. Identification of oleuropein was followed by the quantitative determination of oleuropein. By using the calibration curves, it was found that 1 g of OLE contains 141.6 mg of oleuropein. It was reported that the oleuropein amount in olive leaf extract as 9.25 % [11]. In addition to this study, the oleuropein amount of 14 cultivated olive trees was determined and the results showed that oleuropein content changed from 9.04 % to 14.32 % depending on the variety of olive tree [15]. The oleuropein amount of the olive trees that are found in our region was calculated as 14.16 % in this study and this result is in accordance with the findings reported in the literature.

Besides OLE, HPLC analyses of OLE fractions were also performed. For the analysis, fractions were dissolved in 50% acetonitrile-water solution and then they were injected to HPLC. HPLC results, oleuropein amount as weight percentages, are given in Table1.
According to the HPLC analysis Fraction I (FI) and Fraction II (FII) were found to be the fractions that are richer with their oleuropein contents. Fraction V (FV) and Fraction VI (FVI) showed no oleuropein peak in their chromatograms meaning that there was no oleuropein in these fractions.

B Antioxidant Capacity Analysis

ACW protocol was applied for the detection of the antioxidant capacity of water soluble compounds in OLE and its fractions. Curves for the blank and the samples were constructed according to the ACW protocol and antioxidant capacities (AOC) in terms of ascorbic acid concentration were calculated. The results were given in Table 1. As it is seen in Table 1, the highest water-soluble antioxidant capacity was observed for FI with a concentration value of 1278.35 µg ascorbic acid/mg sample. That was followed by FIII (1148.49 µg/mg) and FII (1036.70 µg/mg). The lowest capacity was detected for crude extract (CE) with a concentration value corresponding to 678.81 µg ascorbic acid/mg. It was not possible to report any results for FVI due to the reason that there were not enough yields.

Table 1. The results of HPLC analysis, antioxidant capacity analysis (ACW and ACL), total phenol analysis and cyclic voltammetry experiments

Samples	Antioxidant Capacity Analysis	Total Phenol Analysis (mg gallic	Biosensor Results Oleuropein	HPLC Results Oleuropein

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

[a]Crude Olive leaf Extract :CE; [b]Fractions of olive leaf: FI, FII, FIII, FIV, FV, FVI

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 ACL protocol and antioxidant capacities, in terms of Trolox concentration, were calculated. ACL results are shown in Table 1. According to the results, the highest lipid soluble antioxidant content was detected for FIII with a concentration value of 2051.46 µg Trolox/mg. This is followed by FI (1634.63 µg Trolox/mg), CE (1495.30 µg Trolox/mg), FIV (1260.51 µg Trolox/mg) and FV (1070.42 µg Trolox/mg). The lowest lipid soluble antioxidant content was observed for FII corresponding to 270.49 µg Torlox/mg concentration value.

When the results are investigated, a direct comparison cannot be observed between the ACL and ACW results. ACW results just depend on the water soluble compounds and ACL results 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.

C Total Phenol Analysis

Total phenol contents of OLE and its fractions were analyzed with folin-ciocalteu reagent and the results are shown in Table 1 as gallic acid equivalents. The lowest total phenol content value was calculated for crude extract as 195.09 mg GAEq/g sample. The highest total phenolic content was detected for FII that was followed for FV. As seen from the results, non-phenolic compounds are eliminated by fractionation that results an increase in the total phenol content.

D . Biosensor Analysis

Antioxidant activity of polyphenols is known as radical scavenging property. In a typical scavenging reaction, H atom from the OH group of a polyphenolic compound is donated to the radical and this is associated with the oxidizing capacity of the polyphenol [16]. Hence, measuring the oxidation or reduction potential in cyclic voltammetry can be used as an analytical tool in the determination of oxidizability of a compound. So, electrochemical behaviour of oleuropein was studied by cyclic voltammetry experiments. A typical cyclic voltammogram of oleuropein is given in Figure 1.

As it is seen from the chromatogram, working between -0.5 V and 0.5 V potential values, yields a typical, reversible cyclic voltammogram for oleuropein. Oleuropein shows one oxidation peak at 285.4 mV (anodic peak potential, Epa) and this can be associated with the catechol moiety at the B ring. Oleuropein also shows a reduction peak at 212.3 mV (cathodic peak potential, Epc). The reduction potential (Eo) was calculated as 248.85 mV which is the average of the oxidation and reduction potentials [17]. The anodic peak current (Ipa) of oleuropein is 100.7 µA and the cathodic peak current (Ipc) is 187.8 µA.

As earlier reported, phenolics having a low oxidation potential value (below +300 mV), shows a high radical scavenging activity [16]. In the case of oleuropein, the Epa value was found as 285.4 mV that exhibits a high radical scavenging activity of oleuropein.

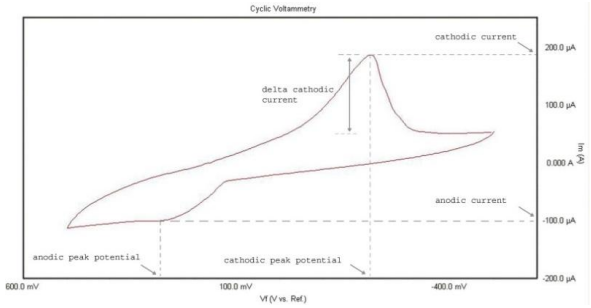


Figure 1. Cyclic Voltammogram of 2 mg/ml of oleuropein at 25ºC and 10mV/s scan rate.

E 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 [8]. To prove this, both laccase biosensors and the electrochemical sensor were used to determine the redox properties of oleuropein in the same experimental conditions. Figure 2 is graphical representation of the cyclic voltammetry experiments with oleuropein.

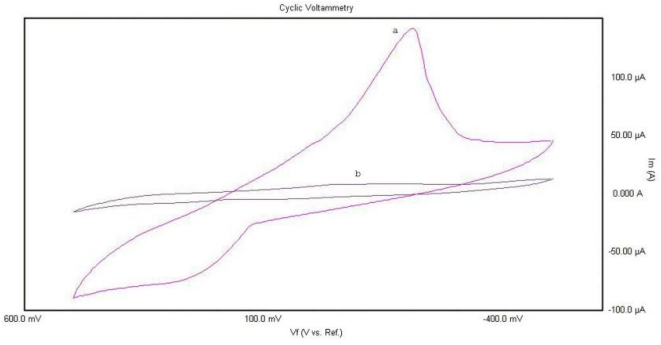


Figure 2. Cyclic voltammograms of (a) laccase biosensor and (b) electrochemical sensor in 2 mg/ml of oleuropein concentration at 25 ºC with ± 0.5 V and 10 mV/s scan rate.

As it is seen from Figure 2, 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. In other words, it is proved that oleuropein shows good substrate behavior for laccase enzyme.

F Effect of Laccase Concentration and Glutaraldehyde Amount on Biosensor Response

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. The cyclic voltammetry experiments were run for 2 mg/ml oleuropein solution with nine biosensors and the chemical sensor. To determine the biosensor response in terms of delta cathodic peak current, the response of chemical sensor was subtracted from the response of each biosensor. The cyclic voltammetry experiments for oleuropein solution were repeated for two times with each biosensor and the response in terms of average of the delta cathodic peak current was investigated for 9 biosensors. Cyclic voltammetry results are given in Table 2.

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 was 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.

Table 2. Biosensors and their cyclic voltammetry results

Biosensor No	Laccase concentration (mg/ml)	Glutaraldehyde amount (% vol.)	Average delta current (µA)
Biosensor 01	1	6.40	24.8
Biosensor 02	1	12.03	50.7
Biosensor 03	1	17.01	53.1
Biosensor 04	5	6.40	62.0
Biosensor 05	5	12.03	80.5
Biosensor 06	5	17.01	63.0
Biosensor 07	10	6.40	37.9
Biosensor 08	10	12.03	43.7
Biosensor 09	10	17.01	44.2

The laccase concentration of 5 mg/ml was the optimum value 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.

There is a relation between the magnitude of the biosensor response and the amount of the enzyme. This relation can be summarized as, 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 [8]. 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.

During this study, we encountered with a problem that was the disintegration of the carbon paste mixed laccase as soon as the biosensor surface was interacted with the analyte. Therefore, this problem was eliminated by the addition of crosslinking agent, glutaraldehyde, to the carbon paste mixture. So, it is understood that using glutaraldehyde, dramatically enhanced the integrity of the biosensor.

G Effect of Scan Rate

Scan rate is one of the most important parameters which affect the redox properties of the substrates in cyclic voltammetry experiments. The influence of scan rate on the biosensor response was investigated by performing cyclic voltammetry experiments in the range of 1-500 mV/s scan rates. The voltammograms are given in Figure 3.

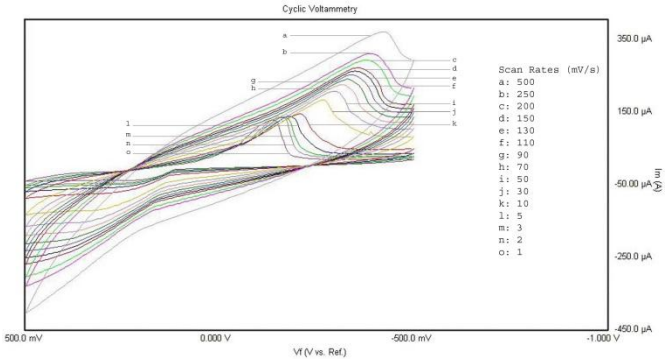


Figure 3. Cyclic voltammograms of laccase biosensor for 2 mg/ml oleuropein solution, at 25ºC, in ± 0.5 V potential interval, with various scan rates.

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 [17]. 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 was selected as the suitable scan rate value for the detection of oleuropein with amperometric laccase biosensor.

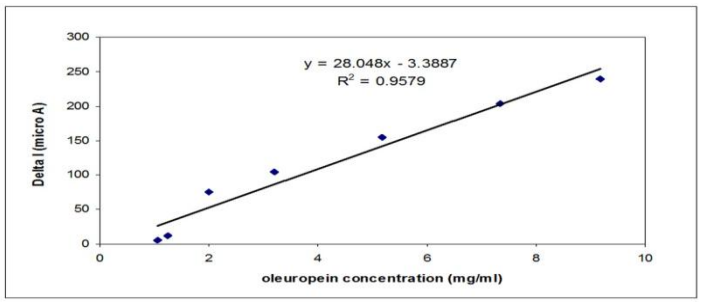
H . Effect of Temperature

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 performed in the temperature range of 2-60 ºC. 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 ºC [12]. So, the working temperature should be kept below this value. Optimum working temperature for oleuropein oxidation with laccase biosensor was chosen as 25 ºC in order to avoid possible activity loss.

I 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 [7]. 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.

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. To construct the calibration curve, cyclic voltammetry experiments were conducted with "Biosensor 05" in different analyte (oleuropein) concentrations. 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 4.



As it is seen from Figure 4, the calibration curve is a linear line with an R^2 of 0.9579 and the calibration equation is given on the figure.

Figure 4. Calibration curve for oleuropein.

J Response of Laccase Biosensor to Rutin

Oleuropein and rutin are most abundant polyphenols in olive leaf. So, the performance of laccase biosensor to rutin was also studied. It was found that constructed biosensors could specifically detect oleuropein, in contrast they gave no appreciable anodic and cathodic current responses to rutin at studied concentration, scan rate, temperature and potential values.

K Response of Laccase Biosensor to OLE and Its Fractions

In order to investigate the detection capability of laccase biosensor in olive leaf extract and its fractions, 3 mg/ml of the analytes were prepared by dissolving the extracts in the working solution. Then, cyclic voltammetry experiments were performed with laccase biosensor in the potential range of -0.5 to 0.5 V, temperature of 25 ºC and scan rate of 10 mV/s. The biosensor results are given in Table 1. The voltammogram of FVI could not be obtained because of the fact that there was not enough yield of this fraction.

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

In this study, HPLC analysis, antioxidant capacity analysis (ACW and ACL), total phenol analysis and cyclic voltammetry experiments were performed for OLE and its fractions and the results were obtained. These results were tabulated in Table 1. As it is seen from Table 1, ACW results were obtained as µg ascorbic acid standard/mg sample, while ACL results were obtained 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 is seen that although the weight percentages are not completely same, they were quite closer to each other.

From the table it was noticed that 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. Moreover, it is observed that, FI and FIII fractions contain additional water soluble antioxidants other than oleuropein. In addition, it is clear that non-phenolic

components were removed from fractionation and the lowest total phenol amount was observed for crude extract of olive leaf (CE).

To sum up, when total 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.

IV. Conclusion

It can be concluded that the laccase enzyme has a capability to oxidize oleuropein. In other words, oleuropein shows a good substrate behavior for laccase enzyme. It was found that the best performance was observed with the biosensor having glutaraldehyde amount of 12.03 % and 5 mg/ml of laccase enzyme.

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 ºC was chosen as an optimum temperature value due to the maximum laccase activity and capability of oleuropein acting as an antioxidant.

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. 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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Prof. Dr. Oguz Bayraktar received his MSc. and Ph.D. degrees in Chemical Engineering from West Virginia University, USA. He worked as a faculty member in İzmir Institute of Technology between 2001-2014. Now, he has been working as a professor at the Department of Chemical Engineering in Ege University. In 2006, he has established a company (Natural Products R & D Ltd. Co.) at İzmir Technology Development Zone. He published more than 30 publications, and 40 conference proceedings. He has 200 citations and his h-index is 7. His research interests include: biopolymers, bioactivities of natural compounds derived from plants, drug delivery systems and nanotechnology.

Seçil Çoban received her BS degree in Chemical Engineering from Izmir Institute of Technology. She studied in the area of biosensors under the supervision of Prof. Oguz Bayraktar and completed her MSc in 2008 at Izmir Institute of Technology.