

Scanning of Some Herbal Tissues to be Used with Biosensors as Polyphenol Oxidase Enzyme Source

Ayten Sağıroglu, Hakkı Mevlüt Özcan*, Özhan Hasancebi

Trakya University, Faculty of Science and Arts, Department of Chemistry, Edirne, Turkey

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Abstract

The purpose of this study is to examine levels of polyphenol oxidase (PPO) activities in the crude extracts of seventeen different herbal tissues (Banana peel, Jerusalem artichoke, black grape, buckthorn, fresh bean, pea, cactus apple, aloe vera, cabbage turnip grain, cabbage turnip vegetable, celeriac, quince, artichoke, aubergine, Trabzon palm, dry carob) with spectrophotometric method. The three herbal tissues (Anamur banana peel, Jerusalem artichoke and fresh broad bean) were found to have higher PPO activities than other tissues, 638, 3360, 3677 U/min.mg protein, respectively. Biologic oxygen biosensors were prepared by cross-linked thin film immobilization method, in the presence of gelatin and glutaraldehyde, with three herbal tissues used as biomaterials and these biosensors were used for detection of phenolic compounds via determining the concentration of consumed oxygen throughout the reaction medium. The typical calibration curves for the Anamur banana peel, Jerusalem artichoke and fresh broad bean based sensors revealed a linear range of 5-40 μM , 2-10 μM , 10-100 μM , respectively. In reproducibility studies, variation coefficients (CV) and standard deviations (SD) were calculated as 1.4%-0.3294 μM , 2%-0.8392 μM and 1%-0.0746 μM , respectively.

INTRODUCTION

Generally, pure enzyme based electrodes are designed for selective determination of phenolic compounds (PCs) in environmental matrices. Enzymes are mostly used in biosensor preparation due to their high specific activities and analytic sensitivities. Their application in biosensor preparation may be limited because of their time consuming nature, expensive enzyme purification,

and the need of cofactor/coenzyme. Herbals provide ideal alternatives to these handicaps. Many enzymes and cofactors that co-exist in the herbal cells provide these cells the ability to consume and hence detect large number of chemicals; however, this can compromise the selectivity. They can easily and directly be provided, are economic because there is no need of extreme isolation and purification, and are renewable. All of these make herbal tissues excellent biosensing elements. Herbal tissues, which contain enzyme systems, can be used in biomaterial of biosensor as an enzyme source [1-3].

In the world, industrial alteration occurred and with this alteration, precedence is given to production,

* Correspondence to: Hakkı Mevlüt Özcan

Trakya University, Faculty of Science and Arts, Department of Chemistry, Balkan Localization, 22030, Edirne, Turkey

Tel: +90284 235 9592 Fax: +90284 235 1198
E-mail: ozcanhakki@gmail.com

but the effects of these wastes on environment and living were not considered. Because of increasing industrial wastes which are released to environment, the damages of these wastes started to be noticed. One of these important waste materials is the PCs. Some industries, such as mining, paint, plastic or pharmaceutical, produce PCs that can be found in their waste waters. Some PCs are considered as most abundant pollutants in waste water due to their toxicity, even at low concentration levels; for humans, wildlife and aquatic organisms [4,5]. The result of this interaction with the PCs found in waste water in environment is the induction of serious pathologies such as abnormalities and carcinogenesis. In addition, the PCs also have some beneficial properties; these properties could be: antioxidant effect against radical reactions in body, protection of health against virutic infections, aromatic or coloring effects in fruit juices and gaseous or alcoholic beverages [6,7].

In this study, we used different herbal forms as PPO enzyme source. Relative PPO activities of crude extract of the tissues were detected by spectrophotometric method at 420 nm in order to use as a biomaterial of biosensors. And biosensors were prepared using selected three tissues with higher PPO activities for determination of PCs. The parameters of herbal tissues immobilization on electrode, detection limits of standard substrate and measurement conditions for determination PCs were studied with using the prepared biosensors.

MATERIALS AND METHODS

Materials

Turkish name, available site in Turkey, Latin name, family name and used form of the scanned herbal tissues are given in Table 1. The herbal tissues contain different enzyme systems as phenol-polyphenol oxidases that catalyze the oxidation reaction of several PCs, such as phenol and

polyphenols.

PCs, used as substrates, (catechol, phenol, resorcin, orsinol, p-cresol, pyrogallol, L-Dopa, Gallic acid mono hydrate) and glutaraldehyde (25% v/v), used as a cross-linking agent and spacer for tissue immobilization and buffer chemicals and other chemicals were supplied from Merck (Germany). Gelatin used as a gel matrix for immobilization and Folin-Ciocalteu reagent used for protein determination of extracts were purchased from Sigma-Aldrich Chemical Co. (USA).

Apparatus

Rotina 38R Type Centrifuge with refrigerator was used for collecting tissue extracts. All the electrochemical measurements were carried out at constant temperature with Nuve BM 302 type water bath with external circulation. For preparing phoshate buffers, 213 microprocessor type pH meters is used. A Hewlett-Packard type 8452A UV-visible spectrophotometer (Boise, ID, USA) with quartz cell was used for protein determination and PPO activity determination of herbal extracts. With using the prepared biosensors, Orion 3 star DO Bench top type dissolved Oxygen meter and Orion 3 star 080113 series dissolved oxygen (DO) probes have been used for PC determination, in relation to the level of dissolved oxygen in medium.

Preparation of herbal extracts

Fresh herbal tissues were purchased from local producers or markets from different sites within Turkey. Crude enzyme extracts of used forms of herbal tissues were extracted with phosphate buffer (20 mM, pH 7.0) cooled to 4°C. The procedure was performed two times for each of the herbal tissue and then the two extracts were joined and centrifuged at the 6000 rpm for 1/2 hour. Crude enzyme supernatants were stored in dark bottles at 4°C into deep-freeze.

Table 1. The scanning herbal tissues for PPO activity and same properties.

No	Turkish name	Provide site in Turkey	Latin name	Family	Used form
1	Muz	Anamur-Mersin	<i>Musa cavendishii</i>	<i>Musaceae</i>	Fruit peels
2	Taze bakla	Edirne	<i>Vicia faba</i>	<i>Fabaceae</i>	Vegetable
3	Yer elması	Edirne	<i>Helianthus tuberosus</i>	<i>Asteraceae</i>	Vegetable
4	Ayva	Eşme-Uşak	<i>Cydonia vulgaris</i>	<i>Rosaceae</i>	Fruit
5	Patlıcan	Edirne	<i>Solanum melongena</i>	<i>Solanoceae</i>	Vegetable
6	Kereviz	Edirne	<i>Apium graveolens</i>	<i>Apiaceae</i>	Vegetable
7	Alabaş tohum	Edirne	<i>Brassica oleracea var</i>	<i>Myrtaceae</i>	Seeds
8	Alabaş yumru	Edirne	<i>Brassica oleracea var</i>	<i>Myrtaceae</i>	Vegetable
9	Bezelye	Edirne	<i>Pisum sativum</i>	<i>Fabaceae</i>	Seeds
10	Taze fasulye	Edirne	<i>Phaseolus vulgaris</i>	<i>Fabaceae</i>	Vegetable
11	Hint inciri	Didim-Aydın	<i>Opuntia ficus-indica</i>	<i>Cartaceae</i>	Fruit
12	Keçiboynuzu	Didim-Aydın	<i>Ceratonia Siliqua</i>	<i>Fabaceae</i>	Fruit
13	Sarısabır otu	Didim-Aydın	<i>Aloe vera</i>	<i>Liliaceae</i>	Leaves
14	Enginar	İzmir	<i>Cynara scolymus</i>	<i>Asteraceae</i>	Vegetable
15	Hurma	Trabzon	<i>Diospyros kaki</i>	<i>Ebenaceae</i>	Fruit
16	Kara üzüm	Trabzon	<i>Vitis labrusca</i>	<i>Vitaceae</i>	Fruit
17	Güvem	Edirne	<i>Prunus spinosa</i>	<i>Rosaceae</i>	Fruit

PPO activity determination

PPO activity in herbal supernatants was determined by measuring the increase of absorbance relevant to the increase of color at 420 nm by spectrophotometer. PPO enzymes in extracts catalyze oxidation reaction of catechol (colorless) as a substrate to quinone (brown) at the presence of air oxygen. The reaction mixture in the cell contained crude enzyme solution (0.01-1 mL), Catechol substrate (1 mL, 20 mM), and phosphate buffer (20 mM, pH 7.0) at 30°C. Blank cell contained same amount of catechol and buffer solution without crude enzyme supernatant. Total cell volume of blank and sample cell was 5 mL. PPO enzyme activities were calculated from linear portion of the standard curve. One unit of PPO activity is defined as: amount that caused an absorbance change of 0.001 1/min [8]. Protein concentration was determined by the method of Lowry with bovine serum albumin as the standard [9].

Biosensor construction

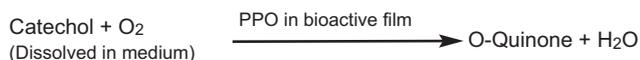
For biosensor construction, three PPO active herbal tissues (150 mg homogenate tissue in 0.75 mL

buffer) were directly used and were added to an eppendorf tube with gelatin (20 mg). This mixture was incubated at 37.5°C for 15 min to dissolve gelatin. 200 mL of gelatin-tissue-buffer mixture was dispersed over the dissolved oxygen probe and allowed to dry at + 4°C for 30 min and then the probe carrying bioactive layer was immersed into 0.625 % (v/v) glutaraldehyde solution in phosphate buffer (20 ml, 20 mM, pH: 7.0) and was allowed to rest for 5 min in this solution for cross-linking. Then the biosensor was washed with distilled water and it was ready to use.

Measurement procedure

For determination of the PCs, the biosensor based on herbal tissue was to dip into the thermostatic reaction cell containing 30 ml of working buffer (pH 7.0; 20 mM phosphate buffer) and was fixed at constant speed at 37.5°C. A few minutes later, dissolved oxygen concentration was equilibrated because of the diffusion of dissolved oxygen between working buffer and dissolved oxygen probe. At this moment, dissolved oxygen concentration was recorded. Then, catechol

substrate or samples were injected into the thermostatic reaction cell. Herbal tissue in the bioactive layer of the biosensor, which has polyphenol oxidase enzyme, is affected by catechol and so the dissolved oxygen concentration in the



reaction cell started to decrease accordingly as per the following reaction.

The time from the injection of the sample into the reaction cell until enzymatic reaction of polyphenol oxidase reaches the equilibration was determined as 10 min at our assay. At this moment, the dissolved oxygen concentration was recorded. Measurements were carried out by standard curves which were obtained by the determination of dissolved oxygen level (ΔDO) during reaction time (min). Same measurement procedures were used for assays conditions and biosensor characterizations.

RESULTS AND DISCUSSION

Selection of PPO active herbal tissue types

For selection of PPO active herbal tissue types, different volumes of crude enzyme supernatants of seventeen herbal tissue types as shown in Table 1 were scanned by spectrophotometric method. Derived data is used for preparation of curves, where values are; change of absorbance (420 nm) against reaction time (min) according to different crude enzyme volume (mL). Results were curved in Figure 1. Generally, the PPO activities displayed linear increase against the increase of enzyme volume and reaction times for all of the herbal tissues depending on the absorbance results in the curves.

During the measurements, to protect and

perpetuate herbal metabolic activity was the most important point and the protection of herbal metabolic activities during these assays could be obscured. When we compared the results in Figure 1 curves, three of herbal tissues (Banana fruit peel, Fresh broad bean and Jerusalem artichoke tissues) showed a higher PPO activity than other herbal tissues. Thus, banana peel, fresh broad bean, and artichoke tissues were chosen to be used as a polyphenol oxidase enzyme sources for the biosensors.

The amounts of protein in the crude enzyme supernatants of chosen three herbal tissues were detected by Lowry method as a standard bovine serum albumin. The relative PPO activities of the supernatants of herbal tissues were determined classically by measuring different absorbance against changing concentration of catechol substrate in the phosphate buffer (20 mM, pH 7.0), at 35°C). Relative PPO activities of three herbal tissues were given in Table 2. The results of PPO activity in table 2, displayed that, banana peel has a highest PPO activity than among of three herbal tissues, but as seen from table, the PPO activity of fresh broad bean and Jerusalem artichoke were not lower. Thus, we have chosen banana peel, Jerusalem artichoke and fresh broad bean tissues as PPO enzyme sources to use as biomaterials on biosensors. There is also a biosensor research that used Jerusalem artichoke as a PPO enzyme source for determination of phenol [10].

Table 2. Relative specific PPO activities of three active herbal tissues

Active herbal tissues	Relative PPO activity (U/ min. mg-protein)
Fresh broad bean	638
Jerusalem artichoke (vegetable)	3360
Banana fruit peel	3677

Optimizations of biosensor working conditions

The herbal based biosensors were prepared with chosen the three tissues. To optimize the

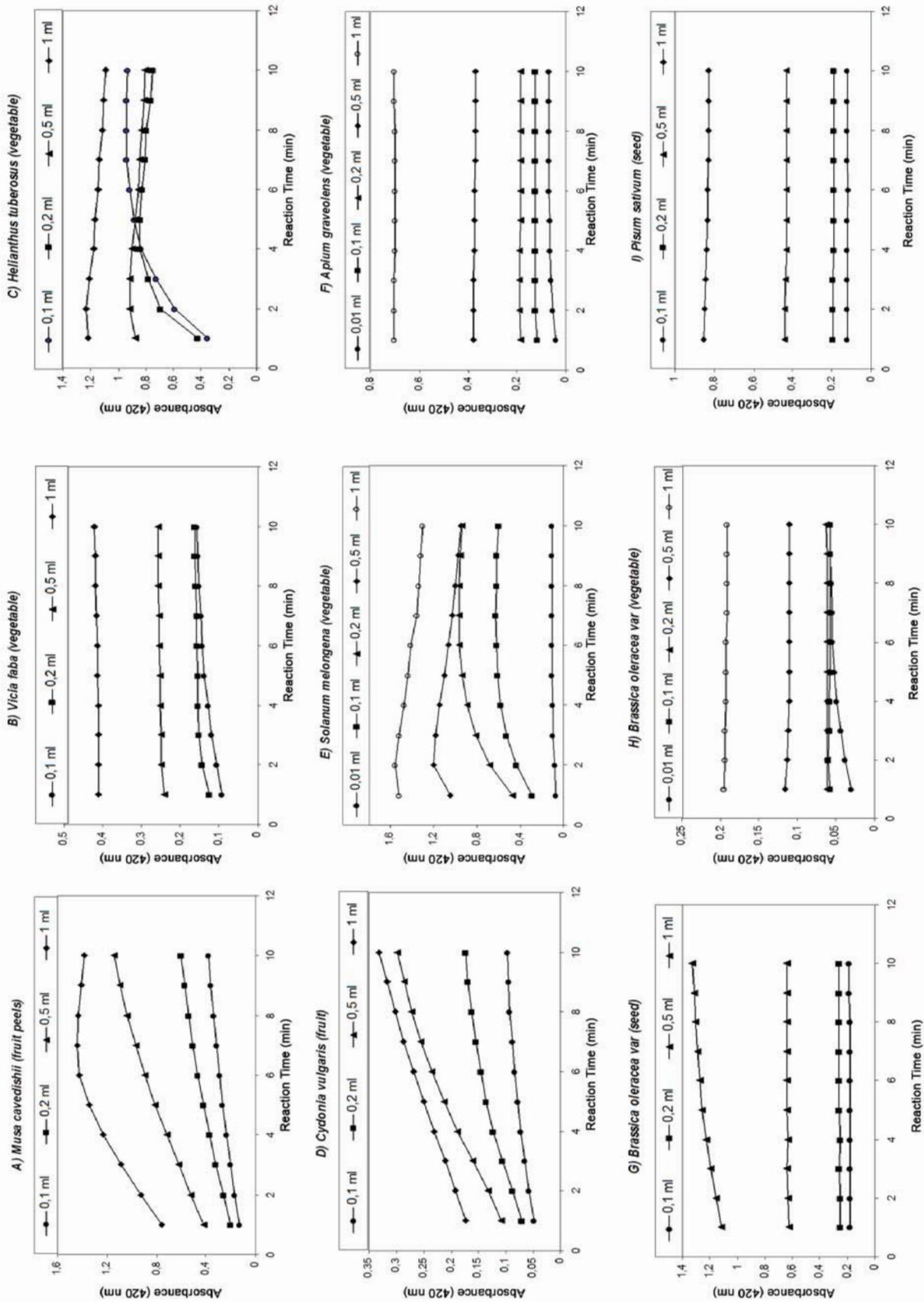


Figure 1a. Values changed absorbance against reaction time according to different crude herbals enzyme volume. A: *Musa cavendishii*, B: *Vicia faba*, C: *Helianthus tuberosus*, D: *Cydonia vulgaris*, E: *Solanum Melongena*, F: *Apium graveolens*, G: *Brassica Oleracea* var, H: *Brassica Oleracea* var, I: *Pisum sativum*.

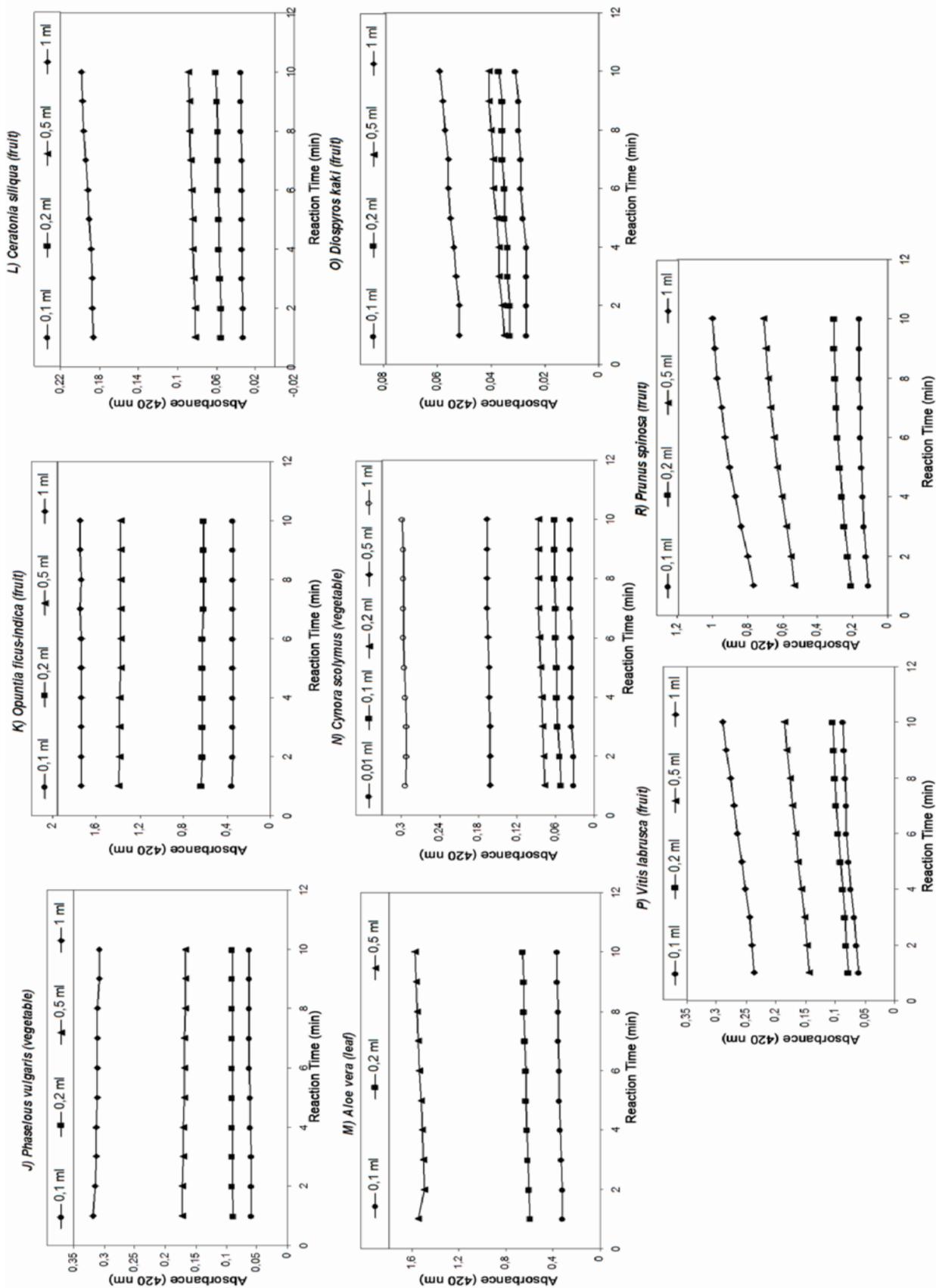


Figure 1b. Values changed absorbance against reaction time according to different crude herbals enzyme volume. J: *Phaseolus Vulgaris*, K: *Opuntia ficus-indica*, L: *Ceratonia Siliqua*, M: *Aloe vera*; N: *Cynara scolymus*, O: *Diospyros kaki*, P: *Vitis labrusca*, R: *Prunus spinosa*.

biosensors, various experimental parameters were investigated. In the early studies, as realized by our group, the best immobilization component compositions were found 32.6 mg of homogenized herbal tissue, 163 μ L phosphate buffer, 4.13 mg gelatin in 200 μ L of bioactive layer mixtures and 2.5 % of cross linking glutaraldehyde solution for three herbal tissue biosensors approximately.

For determined of substrate selectivity's of three tissues biosensors prepared as above were assayed catechol, phenol, resorcin, orsinol, p-cresol, pyrogallol, L-Dopa and Gallic acid mono hydrate such as substrates. The three tissues biosensors displayed specificity to catechol as substrate and addition, the fresh broad bean biosensor also displayed specificity to L-Dopa. The catechol as a standard substrate was used for further biosensor assays

The effects of the pH of the phosphate buffer on biosensor response were investigated between 4.0 and 9.0 in 20 mM buffer solutions (citric acid buffer used was pH 4, phosphatebuffer used was in the range of 5-8 and glycine buffer used was pH 9) in the presence of 50 mM catechol in reaction medium for the three tissue biosensors. The biosensor response as the consumed oxygen concentration, increased from 4.0 to 7.0, and achieved a maximum value between 6.5 and 7.5, before decreasing from 8.0 to 9.0. Optimum pH was found 7.5 for banana peel, 7.0 for fresh broad bean and 7.5 for Jerusalem artichoke tissues. Therefore pH 7.5 was used in further studies of all tissues. The effect of the buffer concentration (pH 7.5) on the response was also investigated between 10-100 mM with the presence of same catechol in the reaction medium. When we used 50 mM of buffer concentration for three tissue biosensors, the best responses were found.

The effect of varying the temperature from 20 to 60°C on the consumed oxygen concentration or on

the biosensor response was studied in the presence of optimized phosphate buffer and of the same amount of catechol. The highest responses were obtained at 37.5°C for the three tissue biosensors. Thus, these optimal conditions were used for the subsequent studies. Therefore, the optimization results of biosensor working conditions were given in Table 3.

Table 3. The optimization values of three active herbal tissues.

Tissues biosensors	Specific substrate	Opt.pH-Buffer Conc.(mM)	Opt. Temp. (°C)
Banana fruit peel	Catechol	7.50–50	37.5
Fresh broad bean	Catechol, L-Dopa	7.00–50	37.5
Jerusalem artichoke	Catechol	7.50–50	37.5

Biosensor characteristics

Linear response ranges

For the determination of linear measurement range of herbal tissues biosensors, as biosensor response was recorded amount of consumed oxygen in medium against changing catechol concentration by prepared biosensors under the optimized conditions. The curves between biosensor responses and catechol concentrations were obtained for biosensors. Linear response ranges of three herbal biosensors were determined using prepared curves.

The proposed biosensors showed a linear response range from 0.5 to 40 μ M of catechol for banana peel, from 10 to 100 μ M of catechol for fresh broad bean and from 2 to 10 μ M of catechol for Jerusalem artichoke biosensors. The linear response range of fresh broad bean biosensor was found as most wide (70 μ M of Catechol) other linear response ranges of two tissues biosensors (35 and 8 μ M of Catechol). The linear ranges curves can be seen in Figures 2-4. Thus, values in these linear ranges were selected for further experiments.

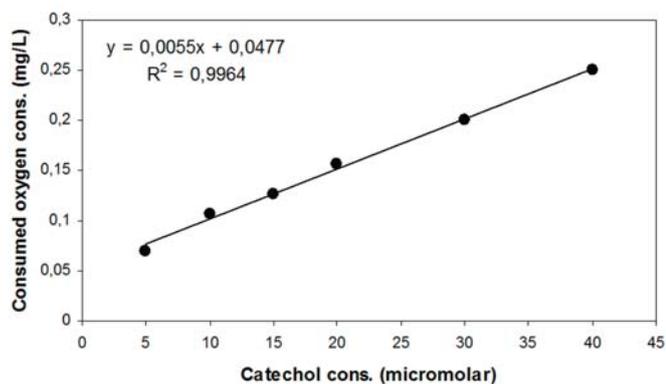


Figure 2. Linear range of biosensor based *Musa cavendishii* fruit pell against catechol.

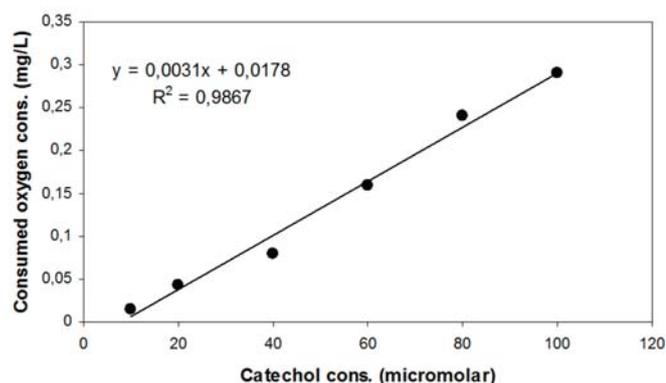


Figure 3. Linear range of biosensor based *Vicia faba* vegetable against catechol.

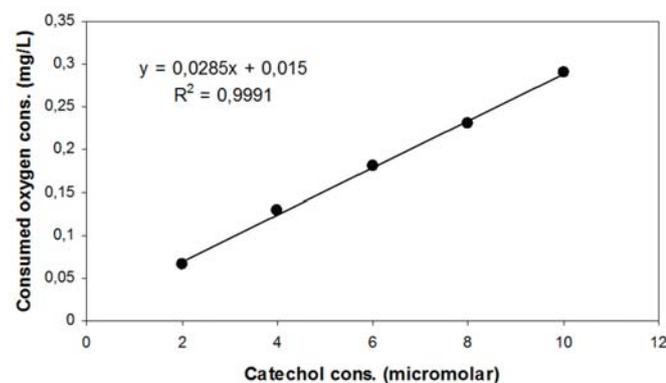


Figure 4. Linear range of biosensor based *Helianthus tuberosus* vegetable against catechol.

Repeatability's and operational stabilities

The repeatability of current responses for proposed three herbal biosensors were investigated. The relative standard deviations were less than 1.0% for 7 successive assays with all biosensors. The good repeatability may be due to the high sensitivity and efficiency of the immobilization of the herbal tissues on gelatin and cross linking of glutaraldehyde.

Standard deviation (S.D.) and variation coefficient (C.V.) were calculated for $n=7$. The used catechol concentrations, linear ranges, S.D. and C.V. are given Table 4. It was seen that when S.D. and C: V. determination, three herbal biosensor may be used repeat for $n=7$ assays.

Table 4. The linear ranges, used catechol concentration, standard deviation (S.D.) and variation coefficient (C.V.) values of prepared tissues biosensors.

Tissues biosensors	Linear range (μM)	Catechol Conc. (μM)	S.D. (μM)	C.V. (%)
Banana fruit peel	5-40	25	± 0.3294	1.4
Fresh broad bean (vegetable)	10-100	50	± 0.0746	1.0
Jerusalem artichoke (vegetable)	2-10	8	± 0.8392	2.0

Operational stability is considered to be one of the key factors in biosensor performance. The operational stabilities of the three herbal biosensors were tested with repeated measurements without surface renewal over a same day period. Initial biosensor responses of the three tissue biosensors were set as 100%. When biosensors were stored at 4°C and measured in the same day, no obvious changes were found in the responses for determined catechol concentrations. The results of operational stabilities for the three tissue biosensors were given in Figures 5-7. The good operational stabilities could be attributed to the fact that there were strong interactions among the polyphenol oxidases-tissues, which could be firmly immobilized on the cross linked gelatin films and provided biosensors better stability and performance.

CONCLUSION

This study showed that the developed oxygen biosensors based on three chosen herbal tissues could be a good alternative as being usable as a biomaterial without requiring pretreatments of tissues. Biosensors that are able to measure

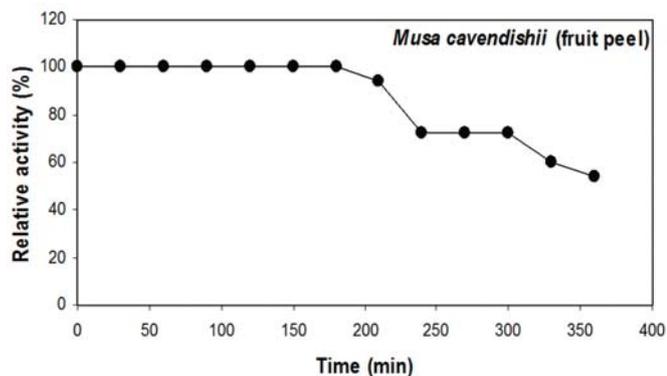


Figure 5. Operation stability of biosensor based *Musa cavendishii* fruit peel against time.

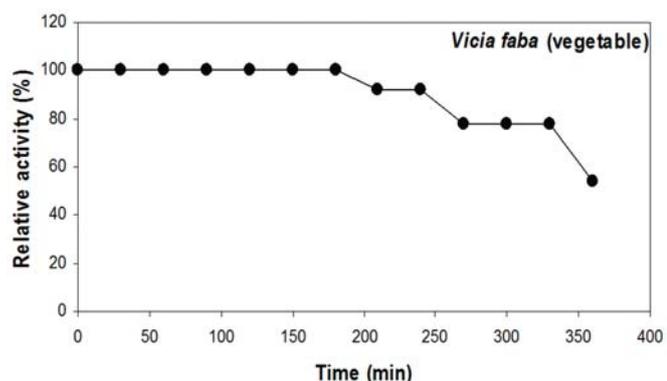


Figure 6. Operation stability of biosensor based *Vicia faba* vegetable against time.

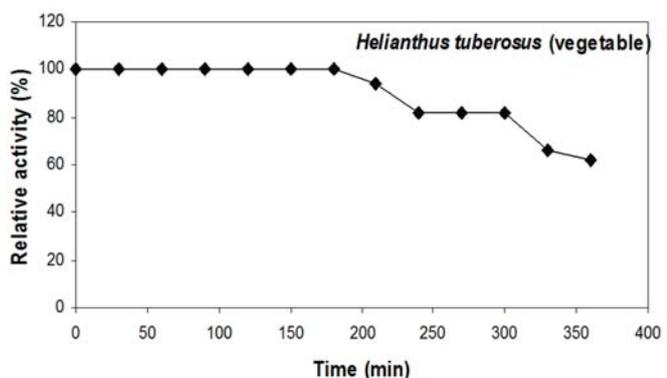


Figure 7. Operation stability of biosensor based *Helianthus tuberosus* vegetable against time.

phenolic compounds, catechol, and L-dopa will be useful to studies search ing to understand the total level of PC's. All the measurements showed that the obtained biosensor could be used simply and rapidly, and also has an advantage of inexpensive equipment. The total analysis time of biosensors takes 10-12 min and is sufficiently stable. This biosensor research also demonstrates a successful application of three herbal tissues, based on the measurement of oxygen consumption in the

medium during the oxidation reaction of phenolic compounds. The measurement of oxygen consumption based biosensor via using banana fresh peel, fresh broad bean vegetable and Jerusalem artichoke vegetable tissues for catechol is not constructed and is not characterized in literature as yet.

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