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Investigation of Kinetics of Polyphenol Oxidase-Gallic Acid Reaction in Selected Fruits

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Abstract: *Enzymatic browning that causes losses in fruits occurs when the enzyme polyphenol oxidase (PPO) (monophenol oxidase E.C.1.14.18.1 or catechol oxidase E.C.1.10.3.1) reacts with oxygen. In this study, the interaction of PPO in red apple, banana and jerusalem artichoke with an antioxidant namely gallic acid was investigated. Gallic acid is presented in well-known database (BRENDA) as a substrate and/or inhibitor of the PPO. In order to investigate the reactions in between different concentrations of gallic acid solution (0-800 µl) with the PPO prepared, no additional substrate was added in its natural environment, and the media were analyzed by two methods. The findings of the study in a holistic way showed that the spectrophotometric and colorimetric method results of experiments of all fruits and all gallic acid concentrations studied were compatible with each other. It was observed that 50 µl stock gallic acid solution could stop the browning reaction, but when this concentration was exceeded, a different reaction which forms a green product was observed. In the study, the possible reaction mechanism between gallic acid-PPO was also determined. It was concluded that specific concentration of gallic acid can be used to inhibit PPO in production of goods, instead of using protective additives.*

Key Word:*Polyphenol oxidase, Kinetics, Gallic acid, Substrate, Inhibitor*

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I. Introduction

As a result of the mechanical disruption of fruits, phenolic compounds in foods of herbal origin causes the brown color formation because of the reaction of polyphenol oxidase enzyme (PPO) (monophenol oxidase E.C.1.14.18.1 or catechol oxidase E.C.1.10.3.1) [1]. Browning of grated apple [2] and peeled/sliced banana [3] are the result of oxidation of those cellular materials with oxygen in the air. Substrates that cause enzymatic browning in red apples are chlorogenic acid, catechin and epicatechin [4]. Altough, most of the polyphenolics acts as substrates in enzymatic browning reactions, the others behave neither a substrate or an inhibitor [2].

As the number of hydroxy groups in the phenol ring increases, the antioxidant effect increases in phenolics. Antioxidants at lower concentrations than oxidizable substrates severely prevent/delay the oxidation of the substrate that had been strated via oxidation of pro-oxidants. The chemical structures, solubility and natural sources of antioxidants are the most important factors that determine their role in human health [5]. Gallic acid (3, 4, 5-trihydroxybenzoic acid; $C_7H_6O_5$), caffeic acid and gentisic acid are phenolics having the most antioxidant property. Gallic acid is used in foods, medicines and cosmetics to prevent lipid peroxidation/degradation caused by decay [6].

When the tissue is damaged, PPO-polyphenol interaction occurs as a result of breaking of the vacuole, where phenolic compounds are stored, and plastids containing PPO [7]. This reaction is followed by the non-enzymatic polymerization of quinones that cause the formation of melanins, and then formation of high molecular weight pigments and dark color (browning)[8]- [9].

PPO should be inactivated to reduce the nutritional loss of fruits/vegetables from browning during transportation and storage. Anti-browning agents used in the methods for this purpose are heat, ray, chemical agent, supercritical carbon dioxide, pulsed electric field and high pressure applications [1]. Although these methods are effective in reducing/stopping the enzyme activity, they cause the nutritional value of the food to decrease and also increase the cost of the process [10]-[11].

On the BRENDA web-page, which is a database based on the researches in literature, gallic acid is stated as both the substrate and the inhibitor of the PPO enzyme, and some contradictions present in this regard BRENDA [12]. Although gallic acid is a phenolic component, studies explaining its relationship with PPO have not been carried out, as in in the case of some other phenolics.

Therefore, in this study, it itn was aimed to determine the possible mechanism by examining the interaction of PPO of red apple, banana and Jerusalem artichoke with gallic acid.

II. Material And Methods

In this study, gallic acid and sodium dihydrogen phosphate monohydrate were bought from Sigma-Aldrich and ISOLAB firms, respectively. Red apple, banana and jerusalem artichoke were obtained from a greengrocer in Isparta region of Turkey.

Each of the fruits (10g) were pressed in the juice extractor (ARCELIK) and immediately after added into pre-prepared 50 ml of sodium dihydrogen phosphate monohydrate buffer solution (pH 5). 13 ml of this mixture were transferred into the tubes and centrifuged at 4000 rpm for 30 minutes in the machine (NUVE NF 400). The supernatant containing PPO and polyphenolics obtained were used in the experiments directly without adding any additional substrate. In industrial fruit juice production, since there is no addition of extra polyphenols to the environment, experiments were designed to carry out with its natural substrates.

In the study, all experiments were carried out at room temperature. Spectrophotometric and colorimetric methods were used to analyze the products of browning reaction. Additionally, images of the samples at the end of the specified period were photographed.

In monitoring the reaction with the spectrophotometric method, 2.5 µl of enzyme solution was taken into the spectrophotometer cuvette and determined volume (0 -50-100-250-500 µl) of gallic acid stock solutions (4.7×10^{-4}M) were added. The color of the medium was analyzed at every 15 or 30 seconds in a UV spectrophotometer (CARRY 60- UV-VIS) at a wavelength of 420 nm. In the red apple and banana, the procedure was repeated with 800 µl and 70 µl gallic acid solution, respectively, in addition to the above-mentioned volumes. Experiments in which red apples and bananas used were studied for 53 minutes, while those in which jerusalem artichoke used were studied for 35 minutes. The change of the absorbance values versus time were graphed.

In colorimetric analysis of enzyme-substrate/inhibitor media prepared by following the same procedure and amounts, L*, a*, b* values were read in every 15 or 30 seconds via colorimeter (MINOLTA). Enzyme mechanism was predicted by comparing all the graphics and photographs obtained by drawing the graphs of colorimetric analysis results over the periods specified in the spectrophotometric method.

III. Results and Discussion

In this study, by using its natural substrates in composition of red apple, banana and jerusalem artichoke, determination of PPO enzyme kinetics and the effects of gallic acid addition in the medium on the reaction mechanism were investigated. For this purpose, by adding different concentrations (50-800µl) of gallic acid solutions, the changes in absorbance values as a result of the reaction carried out at room temperature were determined for 53 minutes for red apple and banana juice, and for 35 minutes for jerusalem artichoke (Figure 1-3).

In the experiments, media without gallic acid were considered as reference. It was observed that at the very beginning of reaction in where enzyme and substrate first met, the red apple had a hyperbolic curve (Figure 1), while banana (Figure 2) and jerusalem artichoke juice had a linear relationship (Figure 3). This situation, which is also observed in chymotrypsin enzyme reactions in the literature, is called steady-state kinetics, and in hyperbolic curves, the point where the line drawn from the linearization of the curve intersects the absorbance axis indicates the amount of free enzyme in the environment [13]-[14]-[15].

When Figure 1 is examined in the light of this information, it is observed that the amount of free enzyme in the environment decreased by 23% with the addition of gallic acid that has a concentration in between 50-250µl.

In red apple experiments, addition of 800µl gallic acid to the medium caused a different result from other concentrations (Figure 1). At the end of the period determined in the study, the media image also confirmed this finding (Figure 4f). When the amount of gallic acid in the medium was 500µl, no significant difference was observed with the reference at the early stages of the reaction, the absorbance values decreased in the following period. As a result of PPO activity at the end of the period determined in the reference medium, a dark brown product was formed (Figure 4a). The absorbance values in the medium with 800µl gallic acid were lower than the reference (Figure 1) and the image was dark green (Figure 4f). When this findings compared to the media in the presence of 500 µl gallic acid, a brown-light green mixture of color formation (Figure 4e) and a decrease in absorbance (Figure 1) indicated that similar reactions occured. Increasing the gallic acid solution from 500µl to 800µl caused the green color to darken. At lower concentrations, gallic acid was observed to retain the yellow color of apple juice (Figure 4a-d). According to the spectrophotometric results, it was observed that the addition of 250 µl gallic acid caused lower absorbance values and the addition of 50 and 100 µl produced similar results (Figure 1).

In the medium obtained from banana, the addition of gallic acid at concentrations of 250 µl and above was observed to cause brown-light green color compared to reference (Figure 5e). It was determined that the absorbance values read from the spectrophotometer at these concentrations were also separated significantly from the others (Figure 2). Although the addition of 50 and 100µl gallic acid resulted a decrease in the

absorbance values compared to the reference, an extra experiment was carried out with addition of 70 µl gallic acid solution due to both supply yellow color formation and an acceptable long-term decrease in absorbance.
It was observed that the addition of gallic acid at this concentration played a more effective role in inhibiting the browning reaction than higher concentrations (Figure 2, 5). Industrially, instead of the transparent image of the reference, the light yellow color of the medium would be more applicable to get customer acceptance.

Similar results obtained in banana-PPO medium were observed in the jerusalem artichoke juice (Figure 3, 6). In the presence of 50 and 100 µl gallic acid, a light yellow colored product (Figure 6b-c) was observed, and as the concentration increased, the color turned to light green (Figure 6d-e).

When the results of spectrophotometric analysis were evaluated as a whole, it was concluded that the absorbance values decreased because of the presence of a certain gallic acid concentration in all selected fruits, and that at higher concentrations the presence of gallic acid caused green color formation. Depending on the fruit's own PPO enzyme amount, it was found that a concentration of 250 µl generally played a critical role in this color change. Considering both the formation of the lowest absorbance value and for customer satisfaction, it was determined that the most suitable value for red apple and jerusalem artichoke juice were 50 µl and for banana juice was 70 µl.

The color values in the enzyme media prepared in the study were analyzed also by a colorimetric device. Comparisons were made by plotting the graphs containing the readL*, a*, b* values. Here, as it was mentioned above, instead of presenting all the findings the results of the experiments realized by the addition of 50 µl gallic acid solution for the red apple and jerusalem artichoke juices, and 70 µl for banana juice were given (Figure 7-12).

Addition of gallic acid to the medium did not cause a significant change in the L* value of the red apple juice, wheras it caused an increase in the jerusalem artichoke juice which means that the whitening of the color (Figure 7, 10). These findings fits well with the images of the mixtures. Comparing Figures 8 and 9, it was seen that the presence of gallic acid did not change the a* values but increased the b* values. As is known, positive a* values represent red color, negative a* values represent green; and positive b* values represent yellow, negative b* values represent blue colors. Therefore, the fact that 50 µl gallic acid solution, determined to provide maximum PPO enzyme inactivation by spectrophotometric analysis, has a more yellow color than reference could be explained by an increase in b* value observed in colorimetric analysis. The effect of gallic acid in jerusalem artichoke was that both a* and b* values decreased (Figure 11, 12). In a simple proportionality in between those values, the reduction in red color was 60%, whereas the reduction in yellow color was about 43%. In other words, the red-to-yellow color ratio in the medium without gallic acid was decreased from 0.28 to 0.2 by gallic acid presence. Therefore, the color observed in the jerusalem artichoke juice was different from that in red apple juice. The findings of not only L*, but also a* and b* analysis confirmed the appearance of the apple of jerusalem artichoke juice given in Figure 6. It is thought that the different effects of gallic acid in the two juices are due to the change in PPO and polyphenol components/amounts in the fruits' own composition.

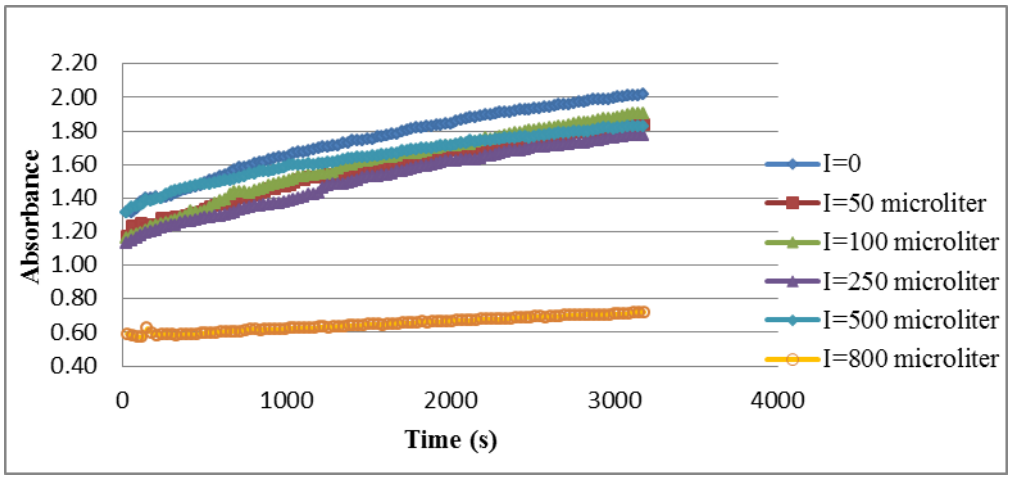

Figure 1: The activity of PPO in red apple juice at 25°C.

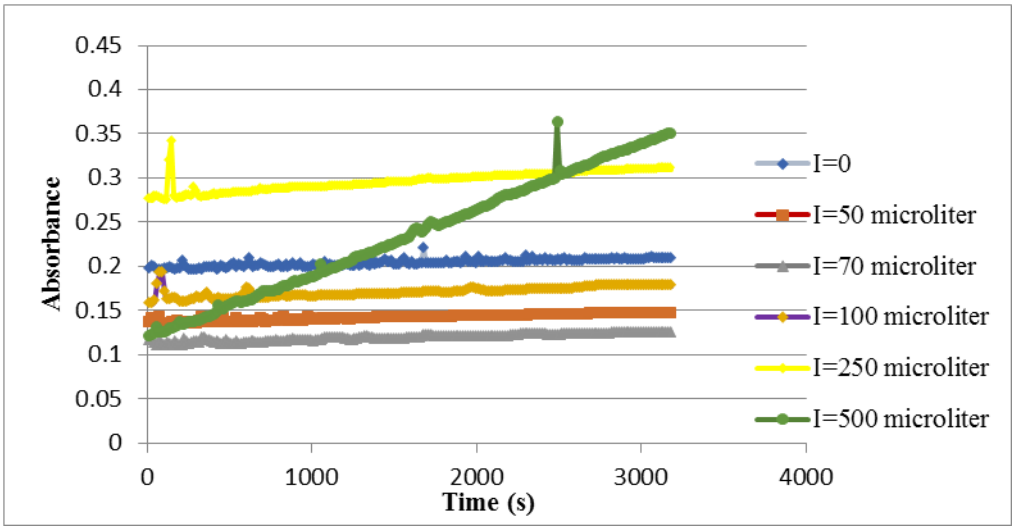

Figure 2: The activity of PPO in banana juice at 25°C.

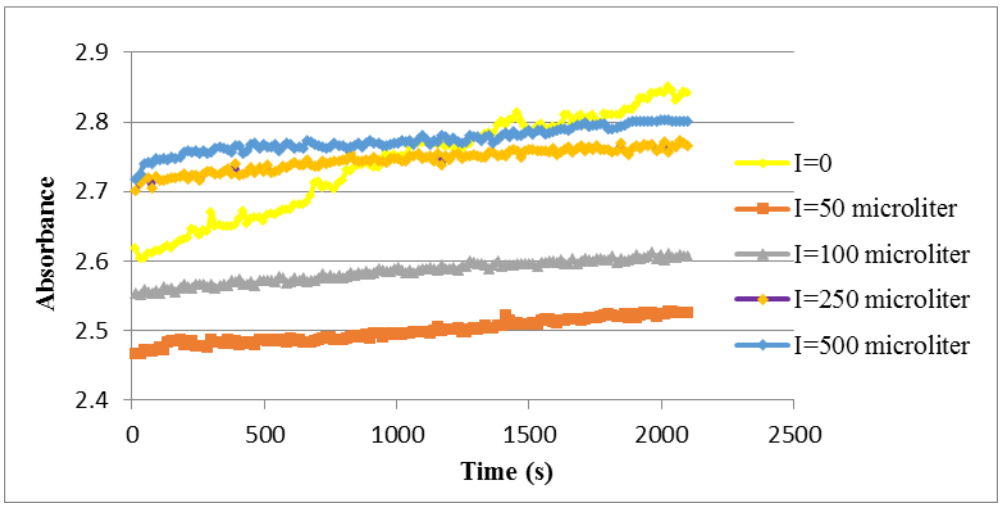

Figure 3: The activity of PPO in jerusalem artichoke juice at 25°C.

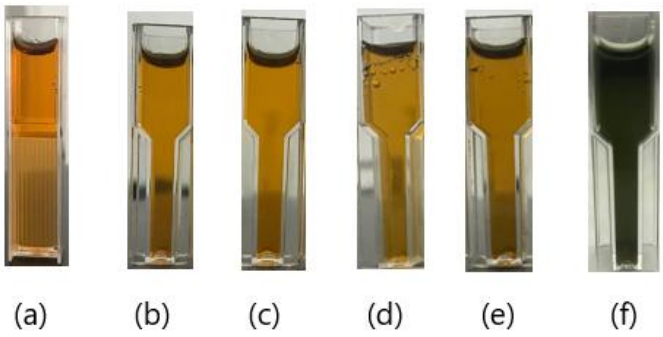

Figure 4: The appearance of red apple juice in the presence of (a) 0 µl, (b) 50 µl, (c) 100 µl, (d) 250 µl, (e) 500 µl, and (f) 800 µl gallic acid in the medium at the end of 53 min.

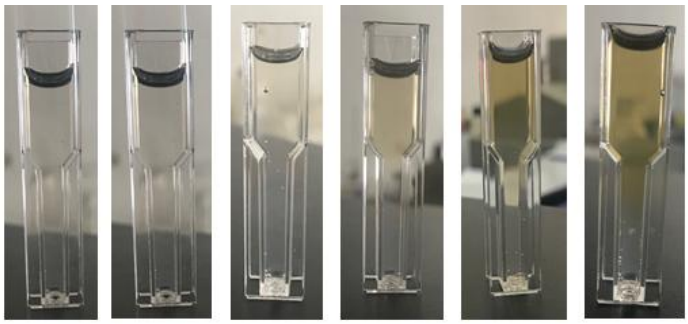

Figure 5: The appearance of banana juice in the presence of (a) 0 µl, (b) 50µl, (c) 70 µl, (d) 100 µl, (e) 2500 µl, and (f) 500 µl gallic acid in the medium at the end of 53 min.

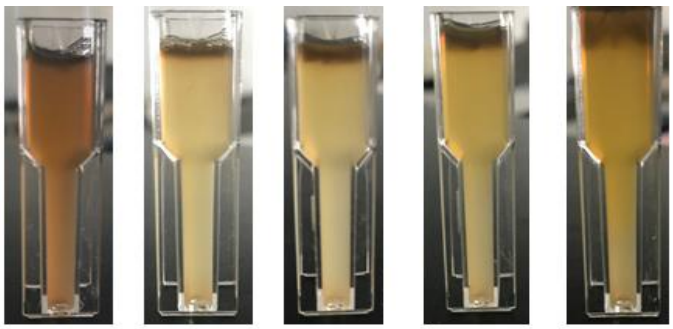

Figure 6: The appearance of jerusalem artichoke juice in the presence of (a) 0 µl, (b) 50 µl, (c) 100 µl, (d) 250 µl, and (e) 500 µl gallic acid in the medium at the end of 35 min.

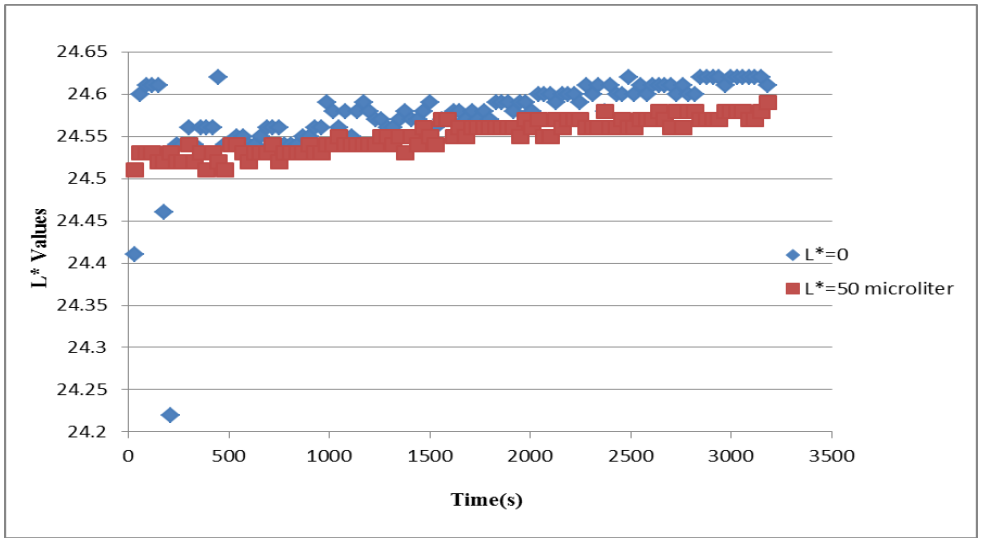

Figure 7: L* values of red apple juice.

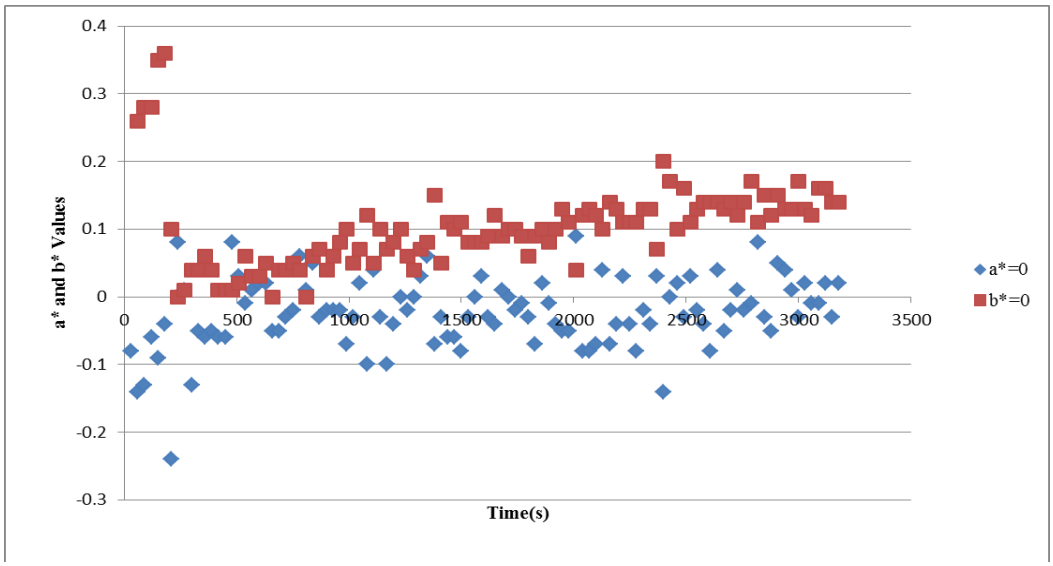

Figure 8: a* and b*values of red apple juice in the absence of gallic acid.

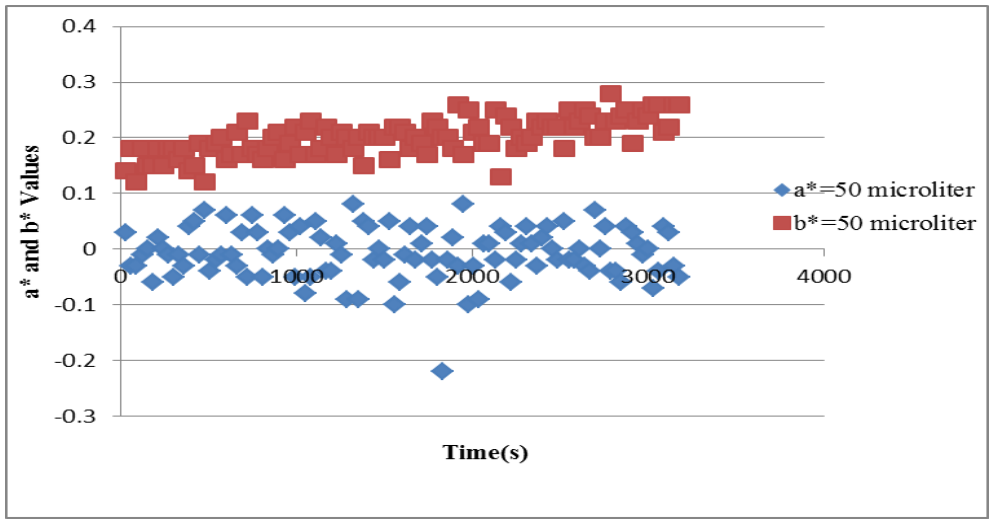

Figure 9: a* and b* values of red apple juicein the presence of 50µl of gallic acid in the medium

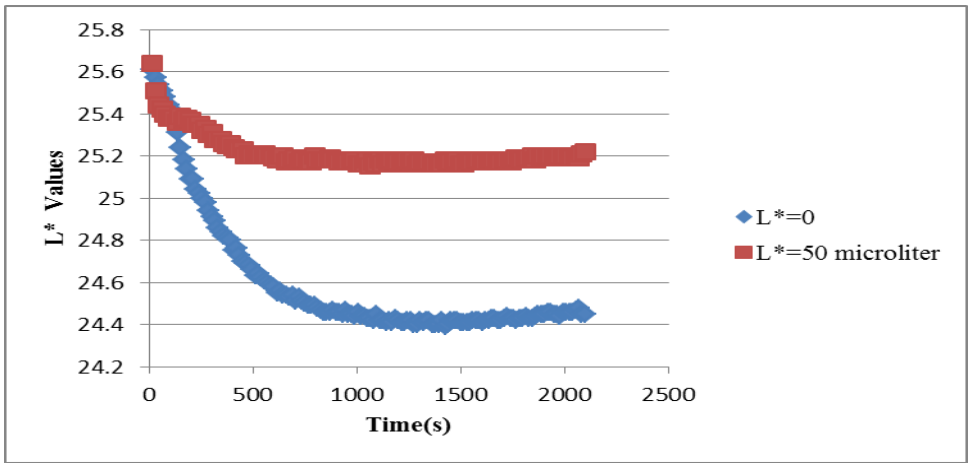

Figure 10: L* values of jerusalem artichoke juice.

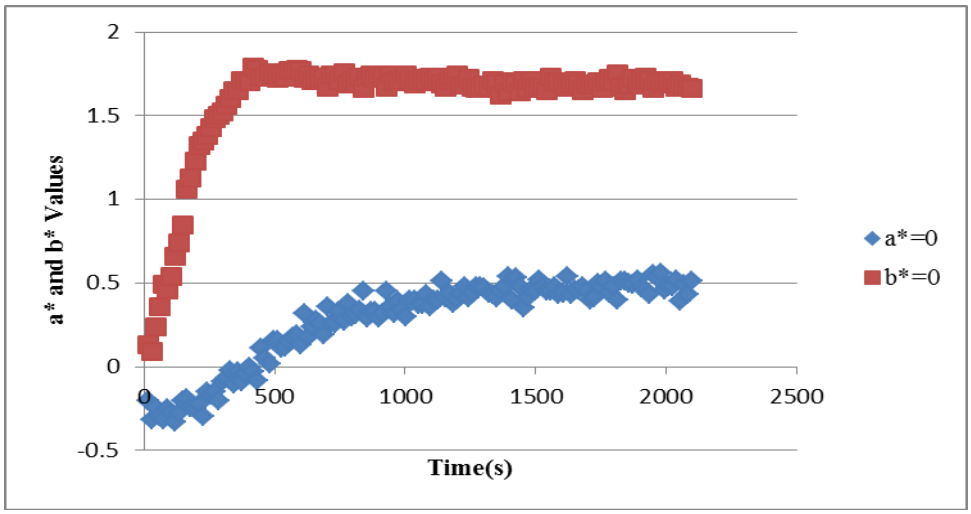

Figure 11: a* and b*values of jerusalem artichoke juice in the absence of gallic acid.

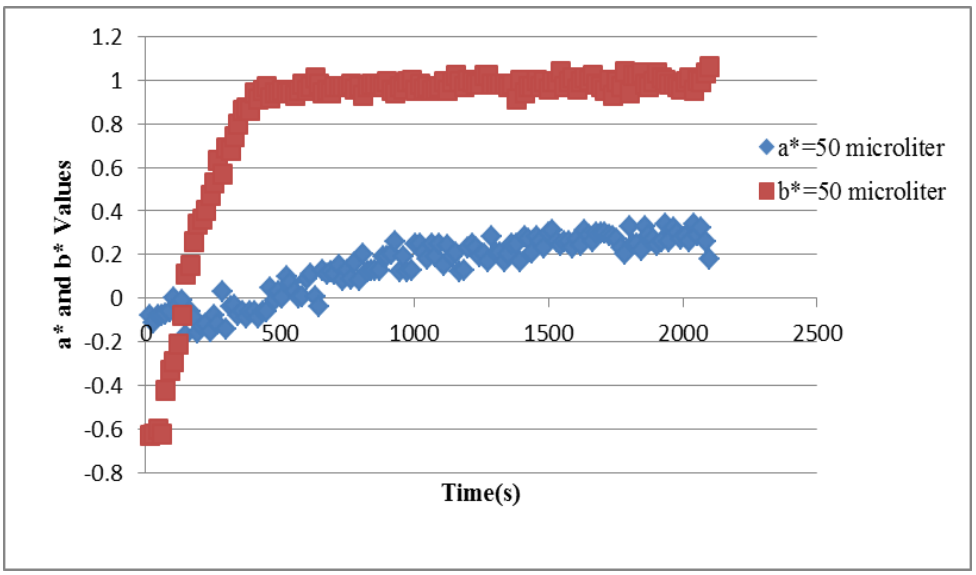

Figure 12: a* and b* values of jerusalem artichoke juicein the presence of 50µl gallic acid in the medium.

IV. Conclusion

In this study, in the presence of their own PPO enzyme and substrates of red apple, banana and jerusalem artichoke, PPO-gallic acid interaction was investigated using spectrophotometric, colorimetric methods and photographing technique. It was determined that the addition of gallic acid at different concentrations caused different effects on PPO. While the addition of gallic acid up to 250 µl provided inhibition of PPO enzyme in all three juices, it was determined at high concentrations that a different reaction was activated, which caused the color of the final product to turn green. Therefore, it was estimated that instead of the substrate of the first reaction, gallic acid dominated as a substrate at these concentrations. As a result, the possible enzyme mechanism to occur in juice media containing PPO-polyphenol-gallic acid was supposed to be the one given in Figure 13. According to this mechanism, the PPO enzyme can catalyze two different reactions; i.e. when it reacts with polyphenols it forms a brown color product as a result of the browning reaction and a green product was produced if it reacts with gallic acid. Therefore, gallic acid was predicted to cause competitive inhibition in the environment due to its ability to suppress the browning reaction at low concentrations. It seemed that which substrate will bound to the enzyme and which reaction will occur first or predominantly depends on the concentration of gallic acid in the environment. Because of this, color values was observed as scattered in colorimetric analysis. The decrease observed in browning reactions due to binding of gallic acid to the enzyme can be explained by either of the followings; i) the presence of the PPO-gallic acid molecule (PPOI) having a single active region where polyphenols cannot be attached to it, or ii) the change in structure of the enzyme having at least two active sites due to gallic acid binding. In the ping-pong mechanism stated in the literature studies supports the predicted mechanism. The mechanism results from the binding of

molecules A and B (polyphenol and gallic acid) to the active site of the enzyme (PPO) randomly or in a certain order (Figure 14). Some oxidoreductases have been reported among those enzymes with ping-pong mechanism [16].The findings obtained in this research also clarifies the reason why gallic acid, is included as both substrate and inhibitor of the PPO enzyme on the BRENDA page which is a database based on the literature studies. Thus, it was concluded that in order to prevent brown color formation and produce yellow-colored fruit juice that can provide customer satisfaction, it was concluded that a natural herbal material namely gallic acid at a concentration of 50 or 70 µl/13 ml of fruit-juice can be an alternative to the chemicals used today in red apple, banana and jerusalem artichoke juice production industrially.

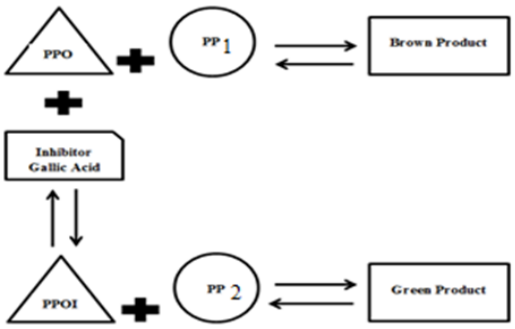

Figure 13: Predicted reaction mechanism in selected fruit juices

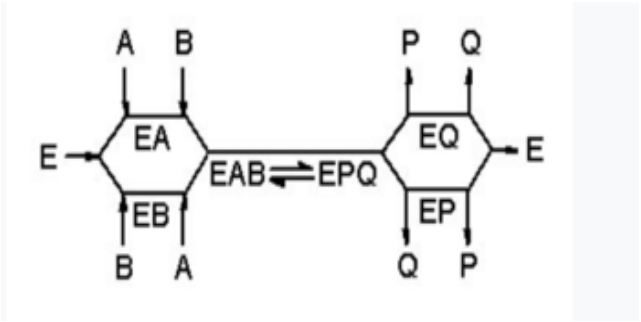

Figure 14: Ping-pong mechanism

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