Removal of phenolic compounds in pomegranate juices using ultrafiltration and laccase-ultrafiltration combinations

Neslihan Alper[1] and Jale Acar[2]

Phenolic compounds of fruit juices are responsible for haze and sediment formation as well as for color, bitterness and astringency. The influence of ultrafiltration (UF) and laccase-UF combination was investigated on phenolic contents of pomegranate juices and on filtration output. Laccase-treated and then ultrafiltered pomegranate juices have shown a rapid increase in their color, when compared to only ultrafiltered (control) samples. Kinetic parameters of laccase were also determined. During the oxidation period, the changes occurring in pomegranate juices were estimated from phenolic contents, color and anthocyanin measurements. Results have shown that laccase oxidation produced a significant decrease in phenolic content of pomegranate juices while juice color the increased. However, in recent literatures, the possibility to remove polyphenols in apple juices was reported. We decided in this study that laccase treatment can not be applied due to the loss of natural red color and unwanted dark brownish color formation in pomegranate juice.

1 Introduction

Pomegranate juices are important commercial products responsible for bitterness and astringency and are used for color and flavor in a wide range of juice products, beverages and other food products. Phenolic compounds contribute to the characteristic flavor and also play a large role in the acquisition of sensory properties (color, bitterness, astringency, *etc.*) of pomegranate juices like other fruit products [1–4]. Polyphenols have been also found to be responsible for haze and sediment formation [2]. Ultrafiltration (UF) for the production of clear juices becomes more and more common in fruit juice industry and replaces conventional fining and filtration methods. This technique provides an efficient and reliable method for clarification of non-cloud type fruit and vegetable juices. For example, for clarification of apple juice, UF offers increased juice yield, better juice clarity, reduced time of filtration and reduced material lost [5, 6].

A new technique using polyphenol oxidase (PPO) for enzymatic oxidation of polyphenols and UF in combination has been also examined for juice stability [7–9]. The oxidation of the reactable phenols in conjunction with effective separation of polymerization complexes in the subsequent UF has been reported to produce a stable juice low in color [10]. Related researches have shown that it is possible to remove polyphenols by UF process after polymerization by laccase in the presence of excess molecular oxygen [7, 8, 10, 11]. The reactive phenolic compounds that cause the haze are oxidized by laccase and react spontaneously further to polymers with a large molecular weight. In doing so the juice must be ventilated, in order to have available the oxygen that is needed for the oxidation. These polyphenols (in practice dark browning products) are retained by UF [11].

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Abbreviations: PPO, polyphenoloxidase; **UF**, ultrafiltration

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Laccase oxidation produces a significant decrease in the phenolic content of juices, associated with a remarkable color increase [8]. This technique is also still under investigation for the stabilisation of fruit juices by removing polyphenols. Related researches have shown that in apple juices the removal of polyphenols by UF after polymerisation by laccase in the presence of excess molecular oxygen is possible [7, 8, 10, 11]. No reports have been found in literature on the effect of this method on dark colored fruit juices. This method is quite simple and does not require any additional cost in the existing juice processing line. The purpose of the presented study was to evaluate the effect of the UF method and the laccase-UF combination for the removal of phenolic compounds of pomegranate juice.

2 Materials and methods

2.1 Reagents and enzyme source

Catechol and Folin reagent were from Sigma (St. Louis, MO, USA). Other chemicals used in research were Merck (Darmstadt, Germany) quality. Laccase from *Trametes versicolor* was obtained from the Consortium für elektrochemische Industrie GmbH (Munich, Germany) and stored at 4 °C prior to the experiments.

2.2 Evaluation of the kinetic and thermodynamic activation and standard parameters

Laccase activity was measured by increase of absorbance at 420 nm for catechol with a Schimadzu UV-VIS Scanning Spectrophotometer (UV-2101 PC). 300 µL of catechol as substrate (0.5 M in McIlvaine buffer) was added to a sample cuvette containing 2.6 mL of buffer solution (pH 4.0 McIlvaine buffer). The reaction was started with the addition of 100 µL of enzyme solution (1 mg/mL). The increase in absorbance at 420 nm was recorded at 25 °C automatically with 0.3 s intervals for 10 min. The rate of reaction was calculated from the initial slope of the progress curve. The rate of laccase catalysed oxidative coupling reaction of polyphenols following a pattern typical of a Michaelis-Menten model. For the determination of Michaelis-Menten kinetic parameters, different initial catechol concentrations (0.25, 0.50, 0.75, 1, 2, 4, 6, 14, 20 and 50 mM, respectively) were used. The reactions were carried out at constant enzyme concentrations at 50 °C and pH 4.5 which were found as optimum for laccase enzyme. The kinetic (V_m and

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K_m) and activation parameters were calculated from the Lineweaver-Burk plot. The activation energy (E_a) of laccase was calculated from the slope of the Arrhenius plot.

2.3 Pomegranate juice preparation

Pomegranate juice was obtained from fresh pomegranates grown in Turkey (İzmir, selection 1999) by squeezing with a laboratory-type press. The juices were heated to 50 °C in a water bath and divided into two equal parts. One part of pomegranate juice was oxidised with laccase (500 U/L) and aerated with an air pump during oxidation. The other part of pomegranate juice (control) remained untreated. The medium temperature was 50 °C and the reaction time was set to 2 h. Samples were collected at 10 min intervals.

2.4 Ultrafiltration

UF treatments were carried out with a cross-flow filtration laboratory device (Amicon 8200, stirred cell). The cell capacity of the UF apparatus was 200 mL, the filtration operating pressure was 2 bar and concentration ratio 5 : 1. Pomegranate juice filtration studies have been performed with UF membranes. The commercial UF membranes were made of cellulose acetate and of the type YM10 with 10 000 Da MWCO (Amicon). The total surface area of the UF membrane used for all experiments was 30.19 cm^2 and effective membrane area was 28.7 cm^2. Flux rate was 20 L/m^2 h for water at 1 bar.

2.5 Chemical analysis

Color (as absorbence at 420 nm, on samples filtered through a 0.45 µm membrane), hue (as the ratio between absorbence at 520 nm and absorbence at 420 nm) and clarity (as absorbence at 650 nm) values were measured by using a Shimadzu UV-VIS Scanning Spectrophotometer (UV-2101-PC). Total phenolics were determined with Folin-Ciocalteu (FC) reagent and referred to as mg/L of gallic acid [12]. Anthocyanine analyses was done spectrophotometrically [13].

2.6 Statistical analysis

Statistical examinations of data were performed by using SPSS software (9.05 for windows) package. The effect of variables is evaluated with least significant difference (LSD) test 95% confidential.

3 Results and discussion

3.1 Enzyme kinetics

The pH optimum of laccase enzyme was found to be 4.5. A rapid loss of activity is observed above this optimum. Experiments conducted to study laccase activity as a function of temperature showed a maximum at 50 °C, then the enzyme activity fell gradually, with 20% activity still left at 10 °C and nearly 7% at 80 °C. An increase in the assay temperature from 25 °C to 50 °C effected a 2.6-fold increase in activity. The optimum temperature obtained in this study is within the range values reported for PPOs from other sources between 20 °C and 55 °C [10, 14, 15].

The Lineweaver-Burk plot of laccase is shown in Fig. 1. The Michaelis-Menten constant (K_m) and maximum reaction rate (V_{max}) were calculated from this graph. The x intercept is equal to $1/V_{max}$ and the slope is equal to K_m/V_m. These parameters for

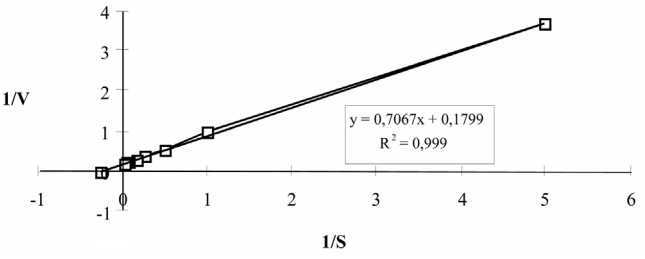

Figure 1. Lineweaver – Burk plot of laccase enzyme.

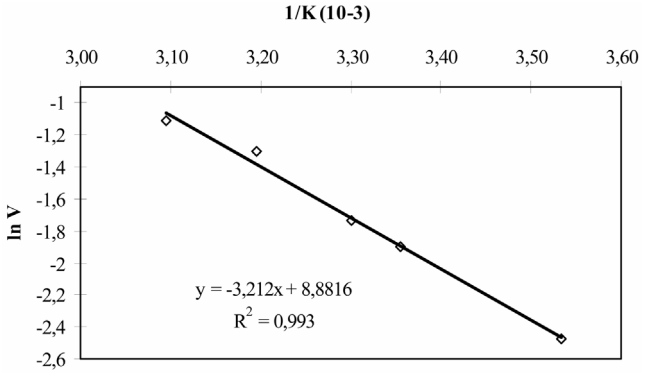

Figure 2. Arrhenius plot activation energy (E_a) of laccase. The correlation coefficient of the model is 0.993.

laccase was found as V_{max} = 5.559 and K_m = 3.928 mM with the correlation coefficient of R^2 = 0.999 at 50 °C. The effect of temperature on enzyme activity is presented as Arrhenius plot (Fig. 2). The temperature dependence of the reaction rate obeyed the Arrhenius law. The Arrhenius activation energy of the biochemical reaction catalysed by laccase was calculated to be 6.38 kcal · mol^{-1}.

3.2 Laccase treatment

The pomegranate juices were oxidated with laccase enzyme by means of molecular oxygen. The changes in color of pomegranate juice at 420 nm and clarity at 650 nm with time during laccase application is shown in Figs. 3 and 4, respectively. As it is seen in Fig. 3, the color value increased higher with time in laccase-treated samples. The effect of laccase treatment on color, hue value and clarity of pomegranate juice is also given in Table 1. Laccase-treated pomegranate juices showed signifi-

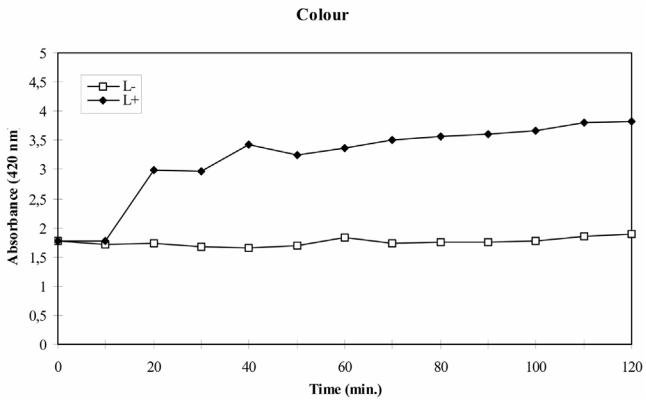

Figure 3. Change of colour of pomegranate juice with and without laccase.

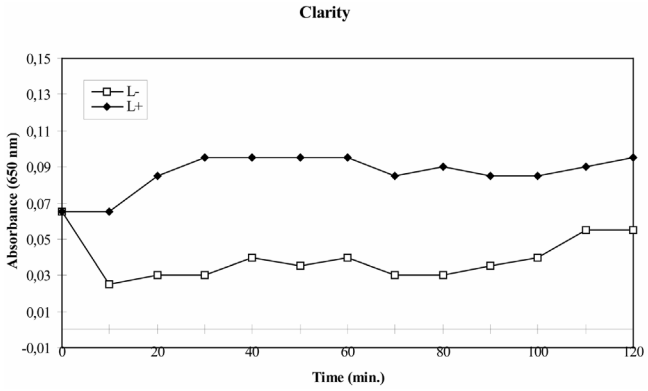

Figure 4. Change of clarity of pomegranate juice with and without laccase.

Table 1. Effect of UF-laccase combination treatment on color and clarity of pomegranate juice

	Color (*A*420 nm)		Hue (*A*520 nm/*A*420 nm)		Clarity (*A*650 nm)	
	without laccase	with laccase	without laccase	with laccase	without laccase	with laccase
Initial	1.775	1.775	0.960	0.960	0.065	0.065
After oxidation	1.885	3.820	1.000	0.460	0.060	0.090
After UF	1.580	3.265	0.950	0.450	0.060	0.090

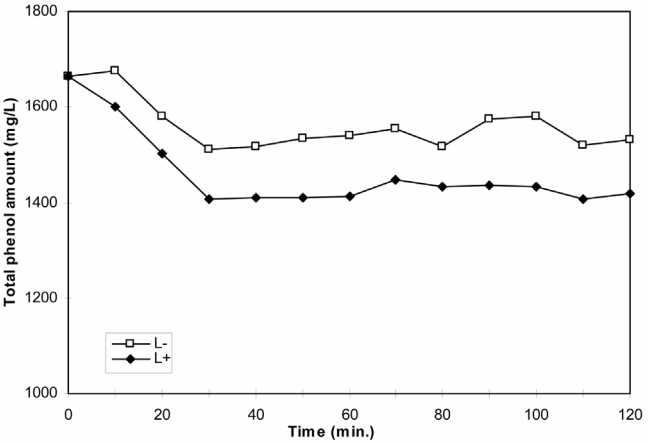

Figure 5. Change of total phenol amount of pomegranate juice with and without laccase.

cant changes ($p < 0.05$) in color, hue and clarity value. Also, the clarity of the laccase-treated samples was found to be higher than those of the corresponding untreated juice and these values increased with time (Fig. 4). The effect of laccase treatment on clarity was found to be statistically significant ($p < 0.05$). Pomegranate juices treated with laccase proved to be clearer.

The hue value gives information about the juice color. This value decreased after oxidation of pomegranate juice with laccase, *i.e.*, the dark colored matters increased during the oxidation process. The change of the total phenol amount of pomegranate juice with time during laccase treatment is demonstrated in Fig. 5. Laccase oxidation of phenolic compounds of pomegranate juice resulted in a decrease of these components during application. In pomegranate juice treated with laccase, total phenolics were oxidised and dark colored matters could be formed (Table 1).

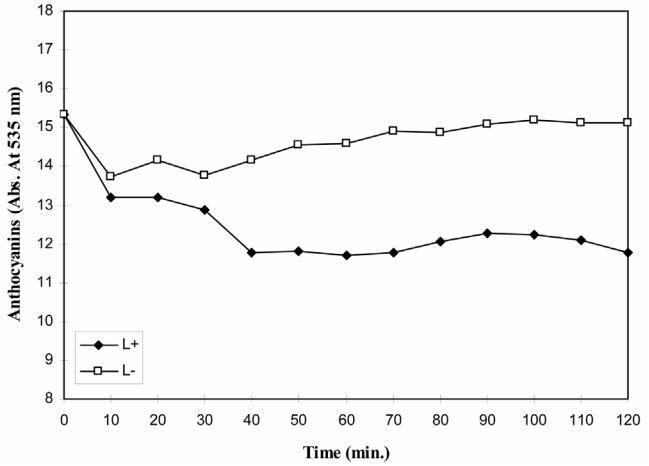

Figure 6. Change of anthocyanin amount of pomegranate juice with and without laccase.

Maier *et al.* [7] reported that in apple juices more than 60% of the phenolic compounds could be removed and a stable apple juice with attractive color could be produced. In pomegranate juices, only 15.5% of the phenolic compounds could be removed while the red color of the juice took on brown color by oxidation with laccase and simultaneous aeration. The effect of laccase treatment on anthocyanin amount of pomegranate juice was found to be statistically significant ($p < 0.05$). In laccase-treated samples, anthocyanin amounts decreased with time (Fig. 6). Gonzales *et al.* [16] reported that PPO activity may also be responsible for loss of red color of some fruits by degrading anthocyanin pigments. Anthocyanins are fairly poor substrates for PPOs. However, they were degraded by laccase application with time. This degradation mechanism could be due to the presence of other phenols, which are good substrates for PPOs. The coupled oxidation mechanism can affect the degree of oxidation of anthocyanins [1].

3.3 Ultrafiltration

The initial fluxes of the cellulose acetate membrane with a 10 kDa cutoff were found as 20 and 12.7 L/m^2 h·bar for water and pomegranate juices, respectively. The natural colloid matters present in the pomegranate juice caused a decrease in flux values. During the UF of pomegranate juice, the flux rate of the membrane reached a stable value after 20 mL of permeate volume. The high amount of phenolic compounds in pomegranate juice was also the major factor on flux decline and membrane fouling. So, the retention of these compounds on the surface of the membrane resulted in forming a dark-red colored deposit layer. The fouling of membranes with polyphenols and color components of pomegranate juice could be easily removed by regenerating with 0.1 N NaOH solutions for 30 min.

Table 1 shows the characteristics of the juices obtained by UF with and without laccase treatment. The initial color value of the samples between 11.0–14.5% has been reduced by UF. Laccase-treated and further ultrafiltered pomegranate juices have shown a rapid increase in their color, when compared with only ultrafiltered samples. This was due to hyperoxidation applied to all juice samples just prior UF. The color of the samples increased by ~115.2% after oxidation of pomegranate juice with laccase. The hue value increased while the clarity value decreased during laccase treatment. A 9.05% removal of

total phenols is achieved by using 10 kDa UF membranes without pretreatment of pomegranate juice with laccase. Cloudy pomegranate juices became clear with UF. A 97.58% removal of cloudy particles could be achieved. Laccase treatment increased the percentage removal of polyphenols from pomegranate juices. The ultrafiltrates of aerated and laccase-treated juices showed lower phenolic contents than ultrafiltered samples without laccase oxidation. Retention of polyphenols was clearly enhanced by laccase treatment for membrane. 8.82% of polyphenols present in the feed solution initially have been removed by the application of the laccase-UF combination when compared to only ultrafiltered samples (Fig. 4).

4 Concluding remarks

Laccase, which catalyses the initial phase of browning, remains active throughout processing and causes discoloration, resulting in poor acceptance of the final product, which cannot comply with technical specifications and/or customer's requirements. However, in recent researches, it has been shown that laccase treatment could be applied successfully to remove phenolic compounds for the production of stable juices, especially apple juices. Laccase application resulted in the decrease of phenolic compounds in pomegranate juices but it is not applicable due to the turning of natural red color of pomegranate juice to the unwanted dark brownish color. UF application after laccase treatment can not be sufficient for the improvement of color, while only UF was found to be applicable for pomegranate juices due to the attractive color and appearance. Moreover, sufficient reduction of bitterness and astringency can be reached by UF. UF was found to be suitable for one for our purposes of having a proper amount of phenolic compounds that do not cause astringency and bitterness. UF alone seems to be less effective than laccase-UF treatments for reduction of phenolic compounds.

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