

Investigating the Relationship Between Acid Concentration in Solution and the Onset of Enzymatic Browning in Granny Smith Apples

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Abstract

Finding an anti-browning agent for apples is crucial for preserving the look of a freshly cut apple, and is known to be correlated with acidity. This study investigated the effect of a solution's citric acid concentration on the browning of apples. There were five sample groups for the sliced apples, two controls and three treatments. The apples were soaked in the solutions with different levels of lemon juice and then observed for browning over a 24 hour period and the area of browning was measured with a 1x1cm² grid. We hypothesized that increasing lemon juice will decrease the area of browning because the juice pH is more acidic than the optimal pH needed for polyphenol oxidase (PPO) to oxidize the apple flesh. This experiment concluded that there was a significant difference in the area of browning between the five sample groups ($p < 0.05$) with the highest lemon juice concentration sample group having the least area of browning and the lowest lemon juice concentration sample group having the most. The results of this study showed the effectiveness of lemon juice as an anti-browning agent and provide insight for future studies on preventing the browning of different food substances.

Introduction

Browning is the darkening of the flesh that occurs shortly after fruits such as apples and pears are cut, exposing the flesh to air, making fruits unsightly and unappetizing to eat. However, browning is not toxic to humans since the pigment is composed of melanin (Deutch, 2018). The main cause of browning is polyphenol oxidase (PPO), an enzyme that catalyzes the first two steps of converting tyrosine into melanin in the presence of oxygen (Deutch, 2018). Physical and mechanical stresses from slicing fruit exposes PPO to oxygen, starting the browning process (Tinello & Lante, 2018). Past studies have investigated the effects of different consumable solutions on browning, and citric acid, found naturally in fruit juices, was identified to

be a moderate to high inhibitor of browning (Son et al., 2001; Tortoe et al., 2007). Citric acid can delay the onset of browning by lowering the pH of PPO's environment so that the pH is outside the optimal pH range of 6-7 required for PPO to oxidize the flesh (El-Shimi, 1993). Although other agents such as chelators and antioxidants can also

inhibit PPO through reduction and inhibition, citric acid is more commonly found in foods, especially fruit juices that, when applied to apples, are more likely to preserve the apples' taste (Moon et al., 2020).

Despite citric acid being effective for delaying browning, the concentrations at which it is effective are unknown. To find these concentrations, bottled lemon juice from concentrate was used since it is known to be composed of approximately 6% citric acid, and lemon juice is one of the highest natural sources of this acid (Yapo, 2009). Lemon juice also contains other acids, like ascorbic acid and malic acid, but their concentrations are negligible since citric acid comprises about 95% of the acid content of lemon juice (Yapo, 2009). Granny Smith apples (*Malus domestica*) were used in this experiment as they are a common fruit consumed in households and are known to brown quickly after being sliced. Since the pH level of Granny Smith apples is approximately 4, and the optimal pH for PPO activity is between pH 6 and 7, lowering the flesh's pH delays the onset of enzymatic browning, (Abasi et al., 2019; El-Shimi, 1993). It was hypothesized that lemon juice will delay the onset of browning due to the citric acid making the juice's pH too acidic for polyphenol oxidase to initiate the conversion of tyrosine into melanin. If lemon juice delays browning, it was predicted that the surface area of browning that appears on apple slices over a set period would decrease as the concentration of lemon juice the apple is exposed to increases.

Methods

The first experiment done was with vinegar solutions. Ambient temperature was noted at the beginning of each experiment. Each apple was sliced into ten slices. The outer skin was kept on and seeds were removed. Area of bruising was noted before the start of the experiment. A Ziploc bag was filled with 300 ml of the desired solution for each of the five treatments: 0% vinegar and 100% water, 25% vinegar and 75% water, 50% vinegar and 50% water, 75% vinegar and 25% water, 100% vinegar and 0% water. The bags were placed in an area with no direct sunlight. The apple slices were

submerged in their designated open bag filled with the liquid. The apples were left inside the solution for 24 hours and then the area of browning of each apple slice on both sides excluding the top and the peel for each treatment was measured. A new method was devised to obtain more conclusive results.

The revised experiment had four identical experiments which were performed separately. Ambient temperature was noted at the beginning of each experiment, ranging from 19°C to 23.5°C between the four experiments. Five sample groups per experiment were prepared before apple slicing. Control 1 was a non-liquid treatment in which the apples were not soaked in anything, representing a situation in which no preventative measures are taken to delay the onset of browning. Control 2 consisted of 1 cup of water and was included as a liquid control with no acid component since our treatment conditions involved using lemon juice dilutions. Treatment 1 consisted of 1 cup of water and 3 teaspoons of lemon juice, making a 1:16 ratio of lemon juice to water. Treatment 2 consisted of $\frac{1}{2}$ cup of water and $\frac{1}{2}$ cup of lemon juice, making a 1:1 ratio of lemon juice to water.

Treatment 3 consisted of 1 cup of lemon juice. Fifteen Granny Smith apples were used in this experiment. Each apple was sliced into ten pieces and equally distributed into the sample groups, so each treatment had twenty slices in total (5 in each treatment per replicate). Any seeds were removed. Using clear 1x1 cm clear grids, the area of both sides of each apple slice was measured. The area of bruising present at the beginning of the experiment in applicable samples was noted and excluded from the percent coverage of browning. After measuring each slice, they were placed in their designated solutions to soak for five minutes. Ten-minute intervals were allotted between each sample group testing to ensure minimal time between initial area measuring and time 0. Time 0 commenced once the apples finished soaking. The apples were then placed in dishware uncovered, with the peel contacting the dishware surface. The samples sat for 24 hours, with check-ins at 12 hours to ensure that there were no inconsistencies in browning between sample groups. After 24 hours, the area of browning on each sample was measured using the same 1x1 cm grids. Any area that was discoloured from the initial colour of the apple flesh upon slicing was counted as browned area; comparisons were made using reference pictures from the start of the experiment. For consistency, areas with apple peel were not included in the measurements.

A.



B. C.



Figure 1. Setup and measuring process before and after the experiment. **(A)** Experimental setup for all 5 sample groups. **(B)** Measuring surface area (cm²) for each slice - both sides. Clear grid sheet consists of 1x1 cm squares. **(C)** Measuring browning coverage after experiment. This panel shows an example with a slice from the failed vinegar trial.

Equation (1) shows how the percent coverage of browned surface area was calculated for each apple slice. Data were compiled into Microsoft Excel to calculate and visualize the mean percent cover in each sample group in a box and whiskers plot. A one-way ANOVA was performed with five independent treatments to determine if there is any significant difference in browning percent coverage between the treatments. A Tukey test was done to determine where the significant differences exist by comparing the means of each sample group.

$$\% \text{ Coverage of Browned Surface} = \frac{\text{Browned area (cm}^2\text{)}}{\text{Total surface area (cm}^2\text{)}} \times 100\% \quad (1)$$

The pH of each treatment solution was calculated using the steps in Figure 2.

We calculated the pH values of the treatment groups to further confirm our prediction.

Citric acid = $C_6H_8O_7$ $K_a(C_6H_8O_7) = 7.4 \times 10^{-4}$ 6% $C_6H_8O_7$ in lemon juice

A.
total grams lemon juice in solution (sol'n) $\times 0.06 = x$ grams of $C_6H_8O_7$ in sol

↓

B. x grams $C_6H_8O_7 \times \frac{\text{mol } C_6H_8O_7}{192.124 \text{ g } C_6H_8O_7} = x \text{ mol } C_6H_8O_7$

$$\frac{x \text{ mol } C_6H_8O_7}{\text{total volume of liquid (L)}} = x \text{ mol/L } C_6H_8O_7 = x \text{ M } C_6H_8O_7$$

	$C_6H_8O_7$	+	H_2O	\rightleftharpoons	$C_6H_7O_7^-$		H_3O^+
I	$x \text{ M}$				0		0
C	$-x$				$+x$		$+x$
E	$x \text{ M} - x$				x		x

$$K_a = \frac{[C_6H_7O_7^-][H_3O^+]}{[C_6H_8O_7]} = \frac{x^2}{x - x} = 7.4 \times 10^{-4}$$

Quadratic formula:

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \quad (\text{Find } x \text{ for } [H_3O^+])$$

↓

C.

$$pH = -\log [H_3O^+] \quad (\text{pH of solution})$$

$$pH = -\log [x]$$

Figure 2. Example calculations for finding pH of treatment solutions

- (A) Calculating for the amount of citric acid (grams) in the solution. (B) Finding the concentration of H_3O^+ ($[H_3O^+]$) in solution using an ICE table and the quadratic formula. (C) Finding the pH of the solution using the calculated value of $[H_3O^+]$.

Results

Five apple slices each in a water control treatment and four vinegar dilutions were meant to be observed after 24 hours. However, after 5 hours, it was observed that several apple slices had browned significantly in the vinegar treatment groups. Results were found to be inconsistent as a similar amount of browning was found amongst the vinegar treatment groups.

Twenty samples in each control and lemon juice treatment were observed over

24 hours, with a halfway check-in. After 12 hours, significant browning was observed in both controls, minimal browning was observed in treatment 1, and no browning was observed in treatment 2 and treatment

3. Observations from the 12-hour check-in showed that the samples were exhibiting consistent results, allowing the experiments to move on to the next 12 hours.

After 24 hours, each slice maintained its overall surface area and size but was not as rigid to the touch. The slices in controls 1 and 2 appeared to have the same amount of browning as they did at 12 hours. Treatment 1 showed significantly more browning than it did at 12 hours. Treatments 2 and 3 showed little to no browning. Results were analyzed as percent coverage of browning from each slice.

Figure 2 shows the mean percent coverage of browning: control 1 (93.2%), control 2 (91.7%), treatment 1 (87.7%), treatment 2 (9.8%), and treatment 3 (<1%). Figure 2 shows a negative correlation in percent coverage of browning with the increasing presence of liquid from control 1 to 2 and increasing concentrations of lemon juice. An α of 0.05 was used and the ANOVA produces a p-value of 1.11×10^{-16} , indicating that the differences between the treatment groups were statistically significant. The Tukey HSD analysis showed that significant differences were present between treatment 2 and treatment 3 with every sample group.

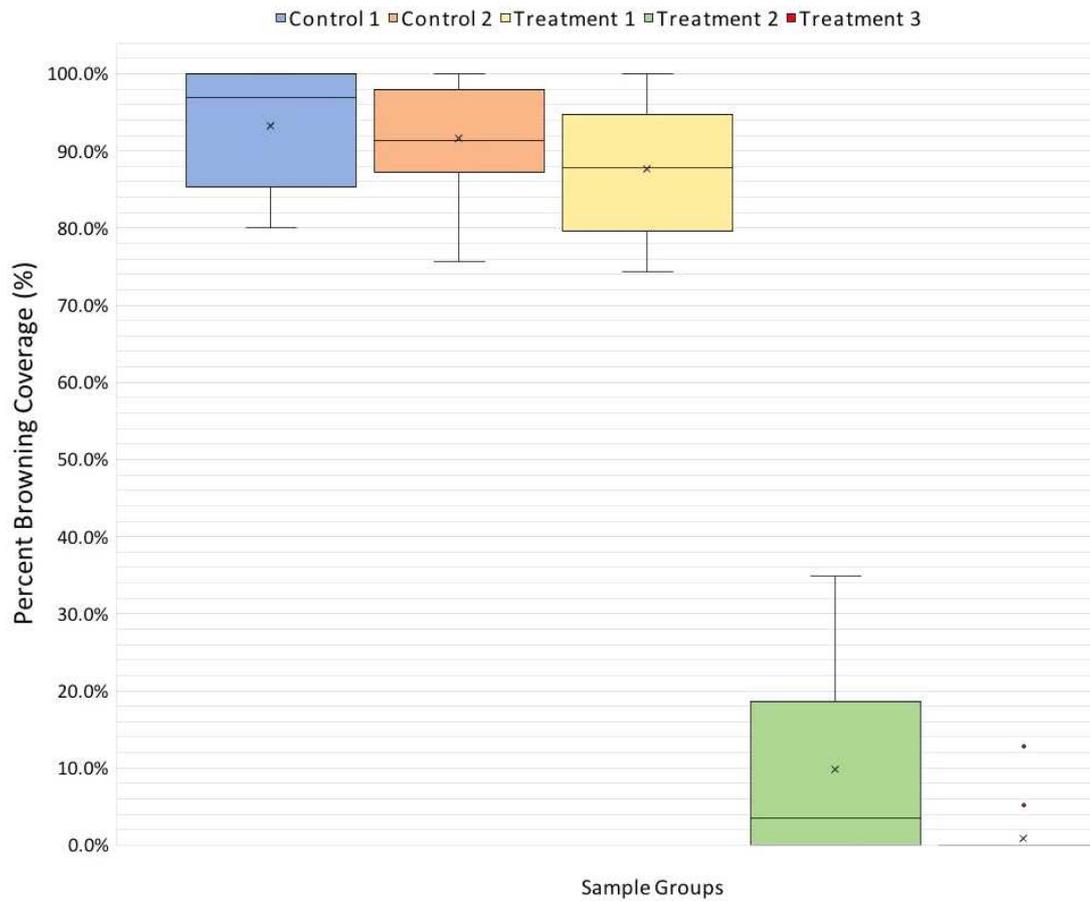


Figure 3. Mean percent coverage of browning for all 100 apple slices.

Each box (n = 20) shows the percentage of browning after 24 hours for each control and treatment. The 'X' in each box represents the average percent coverage of browning. Box plots represent the range of percent coverage, excluding the 25% and 75% percentiles (horizontal line, median). Data from Treatment 3 has two outliers, indicated by the red dots. The upper and lower whiskers show the minimum and maximum percent coverage of browning in each group except Treatment 3 ($P < 0.05$).

Discussion

This study aimed to determine whether there were any statistical differences in the area of browning of apple slices that had been soaked in solutions with different concentrations of lemon juice. Based on the p-value produced by the ANOVA test, which was less than the significance level of 0.05, we reject the null hypothesis that there is no significant difference in the area of browning

among the five treatment groups. This indicated that the difference in the area of browning among the five treatment groups was significant and that the citric acid in lemon juice effectively inhibited PPO enzymatic activity. Moreover, increased lemon juice concentration in the solution delayed browning.

The data was in agreement with the findings of Azevedo et al. (2018), where increasing lemon juice concentration in the solution reduces the enzymatic browning of apples. The function of PPOs is pH-dependent, where the optimal pH range is 6 to 7 and the activity of PPOs is significantly reduced when the pH is below 3 (El-Shimi, 1993; Castañer et al., 1996). Since the pH of Granny Smith apples is approximately 4, citric acid slows down enzymatic browning by compromising the performance of PPO by acidifying the solution to a pH that is less than the optimal range of the enzyme (Abasi et al., 2019; El-Shimi, 1993; Castañer et al., 1996). The calculated final pH of the solution in treatments 1, 2 and 3 were 2.483, 1.989, and 1.847, respectively. Since all pH values of the treatment groups were less than 3, the PPO activity was significantly inhibited, consistent with the observation of reduced browning in lower pH treatments compared to the controls.

White vinegar was initially selected as the anti-browning agent; however, severe browning was observed in the treatment groups and the degree of browning was found to be positively correlated with the amount of vinegar in the solution. This result contradicted the previous studies by Son et al. (2001) and Castañer et al. (1996), where acetic acid demonstrated a certain degree of enzymatic browning inhibition, though not as effective as citric acid. Moreover, in these studies, acetic acid lowers the pH of the solution to less than 3, which is sufficient to reduce PPO activity since PPO is inactivated below pH 3 (Castañer et al., 1996; Son et al., 2001; Moon et al., 2010). The reasons behind the inconsistency are yet to be determined; one possible explanation

was that the experiment was performed in an environment where the temperature was close to 20°C, which is the optimal temperature for PPO that results in maximum enzyme activity and thus browning (Mahmood, 2009).

Several factors may have impacted the reliability of the results. The same experiment was repeated at four different locations. Although we tried to minimize variations by experimenting in areas free from direct sunlight, other factors such as ambient temperature, humidity, and airflow were challenging to manipulate to be constant among the four locations. In addition, the grid sheet used to measure the area of browning was only precise to the nearest 1x1 cm²; using a more precise scale likely would have resulted in more accurate data.

Although we were able to reject the null hypothesis, more in-depth studies could be done to further analyze factors that may affect the enzymatic browning by PPO. Based on the Tukey test, it is worth noting that the difference between the water to lemon juice ratios for Treatment 1 (1:16) and Treatment 2 (1:1) were fairly large, thus further experiments can be conducted to test concentrations of lemon juice in between those of Treatments 1 and 2. Testing a ratio between 1:16 and 1:1 may find a lower threshold concentration of lemon juice that significantly delays browning. It would be advisable to repeat the experiment at a higher or lower temperature, where the PPO activity is not maximized, to further study the effect temperature has on enzymatic browning. Furthermore, there is still a lack of research on the relationship between humidity and PPO activity; exploring the impact of humidity on PPO activity and thus the enzymatic browning effect could be helpful for industries that manage fruit and vegetable storage.

Conclusion

In conclusion, sample groups with a higher lemon juice concentration had significantly less areas of browning, which matched with our prediction. This result supported the hypothesis that increasing lemon juice concentration has a significant difference in the delay of apple browning due to the low pH of the juice compared to the pH at which apple flesh browns. These results provide insight for future studies to find more effective anti-browning agents and further investigate environmental temperatures to avoid browning.

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Appendix

6% citric acid in lemon juice

1 tbs = 3 tsp = 0.18 tsp citric acid

1 tsp lemon juice = 4.8 g lemon juice → conversion used from Cake Lab

1 tbs = 3 tsp lemon juice = 14.4 g lemon juice

14.4 g lemon juice × 0.06 = **0.864 g citric acid in treatment 1**

236 g water in treatment 1 (googled 1 cup water to grams conversion)

1 lem. juice : 16 water ratio → 6.25% lem. juice, ∴ 0.375% citric acid in soln

1/2 cup lemon juice = 24 tsp lem. juice

24 tsp lem. juice = (4.8 g lem)(24)
= 115.2 g lem. juice

1:1 ratio of lem. juice to water
50% lemon juice, ∴ 3% citric acid in soln

115.2 g lem. juice × 0.06 citric acid = **6.912 g citric acid in treatment 2**

118 g water in treatment 2

1 cup lem. juice = 48 tsp lem. juice

48 tsp lem. juice = (4.8 g lem)(48)
= 230.4 g lem. juice

100% lem. juice, ∴ 6% citric acid in soln

230.4 g lem. juice × 0.06 citric acid = **13.824 g citric acid in treatment 3**

58 mg ascorbic acid / 100g lemon juice
= 0.058 g ascorbic acid / 100g lem juice
↳ ~0.06% ascorbic acid in lemon juice
negligible

Figure 4. Calculating the amount of citric acid present in each treatment solution with ~6% of citric acid present in bottled lemon juice concentrate.