

Application of Linear Mixed Model in Longitudinal Study to Optimize Effect of Ultrasound on Color of Minimally Processed Granny Smith Apple

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This study aims to identify and optimize ultrasonic bath process parameters to improve the quality of minimally processed apples during storage. Specifically, it investigates how exposure time and power affect the browning index over time using a longitudinal (time-dependent) approach. To capture the complexity of food quality changes during storage, a linear mixed model (LMM) was employed, accounting for both fixed effects and random variation among samples. The adopted experimental design was a 2^2 with 3 replications, treating storage time (ST) and quadratic storage time (QST) as covariates. The LMM fitting is improved when random intercept and slope are included in the model. The estimated variance of the random effect associated with the intercept and storage time was 3,79 and 0,13, respectively. The results also revealed significant interactions between linear and quadratic time and process factors, as well as a notable interaction between process factors ($p=0,032$), where power exhibited a buffering effect on exposure time. The optimal process parameters are 400 W and 3 minutes. Given the correlation between repeated measurements and the biological variability of the samples, identifying the optimal combination of process parameters was crucial for improving the quality attributes of perishable, short-shelf-life food products. Compared to traditional models, LMM provides more precise and accurate results.

1. Introduction

In highly perishable food products, statistical analysis is often used to assess the impact of various technologies on shelf life. The ready-to-eat apple market has experienced significant economic growth in recent years (FactorMR 2024) driven by consumer demand for convenient, nutrient-rich alternatives to fast-food (Szakály et al., 2012). The shelf life of sliced apples is primarily determined by their color (Kramer, 1965; Harker et al., 2003), as enzymatic activity causes the exposed fruit surface to brown rapidly, limiting consumer appeal (Toivonen and Brummell, 2008). Power ultrasound is an emerging technology, often used to enhance extraction processes (Di Caprio et al., 2021; Lima et al., 2021), and useful to enhance dipping treatments (e.g., ascorbic acid and calcium chloride), further improving color stability (Jang and Moon, 2011; Yildiz et al., 2020). However, the effect of power ultrasound depends on process parameters (e.g., amplitude, temperature, exposure time, and frequency), making it challenging to determine the optimal conditions (Zang et al., 2017). Factorial ANOVA and general linear models are commonly used to analyze factor combinations and their effects (Granato et al., 2014). However, sliced apples exhibit significant variability, and color measurements are taken repeatedly on the same samples over time. This leads to a longitudinal study involving measurements of the same individuals taken repeatedly over time, thus allowing the direct study of change (Fitmaurice, 2011). Because these repeated measurements introduce correlation and non-independence in the data, traditional repeated-measures ANOVA is limited, as it does treat time as a continuous factor (Muhammad, 2023). To address these limitations, a linear mixed model (LMM) is more suitable for analyzing longitudinal data (Sheek and Ma, 2011). Using a LMM

improves the precision of the estimated treatment effects and enhances the power to detect significant differences. Additionally, it allows the inclusion of random effects, such as slopes, to account for individual variations in response (Schober and Vetter, 2018). In this study, the effects of ultrasound process parameters (exposure time and power) on the dependent variable, browning index, of sliced Granny Smith apples over time (pre-treatment, post-treatment, and after 7 and 14 days of storage) were tested. The experimental design, combined with the LMM-based data analysis, provides deeper insights into the longer-term effectiveness of ultrasound treatments in slowing apple color deterioration.

2. Materials and methods

Apples (cv: Granny Ramsey Smith) were purchased from a local supermarket in Valencia, Spain. Ascorbic acid and calcium chloride were obtained from Scharlab (Spain).

2.1 Sample preparation

The apples were first washed, dried, and then sliced using a stainless-steel knife into discs of approximately 1,5 cm thick. The central core of each disc was removed using a stainless-steel corer. For each apple, three discs were prepared and subsequently divided into 4-5 triangular-shaped pieces. The apple samples were subjected to the following treatments: they were immersed in an ultrasonic (US) bath (ATM 12 L, ATU, Paterna, Valencia) containing deionized water with 2 % ascorbic acid and 1 % calcium chloride in solution. The ultrasound treatment was conducted at a frequency of 24 kHz and temperature 25°C, with two exposure times (1 or 3 minutes) and two power levels (200 W or 400 W). To maintain a constant temperature during the ultrasonic treatment, a temperature measurement probe and an automatic water recirculation system were used, ensuring temperature oscillation did not exceed $\pm 1^\circ \text{C}$ based on the experimental parameters. Each treatment group was replicated three times. After treatment, the apple samples were dried with a paper towel, packed in polyethylene bags, labeled, and stored under cold conditions ($4^\circ \text{C} \pm 0,5$) for 14 days. All analyses were conducted at three preset times: day 0 (both pre-treatment and post-treatment), day 7, and day 14 to monitor changes over time.

2.2 Experimental design

A complete 2-factor, 2-level (2^2) multifactorial experimental design was performed, resulting in a total of four treatments. Each apple was randomly assigned to one of the treatments. All samples underwent ultrasound treatment under consistent conditions (25°C, 24 kHz frequency, and the presence of ascorbic acid and calcium chloride). The two categorical factors were the exposure time (Time): 1 minute (-1 level) and 3 minutes (+1 level), and the power (P): 200 W (-1 level) and 400 W (+1 level). The storage time (ST) and quadratic storage time (QST) were treated as continuous factors. Browning index (BI) was the continuous dependent variable.

2.3 Color

Color was measured using CIELAB coordinates with a colorimeter (CM – 2500d, Konica Minolta, Tokyo, Japan). The specular component excluded (SCE) mode was used, with a D65 illuminant reference and 10° observation angle. The colorimeter was calibrated with a white standard plate. Color variables, including L^* (lightness/darkness), a^* (redness/greenness), and b^* (yellowness/blueness) in the Hunter Lab color scale (Hunter Lab, 1996), were measured on each freshly cut apple sample subjected to treatments. The results were expressed as the mean value of three replicates, each consisting of three measurements taken at two positions on the sample. The browning index (BI) was calculated using the following equations (Pathare et al., 2013):

$$BI = \frac{[100(x-0.31)]}{0.17} \quad (1)$$

$$x = \frac{(a^*+1.75L^*)}{(5.645L^*+a^*-3.012b^*)} \quad (2)$$

2.4 Statistical method for data analysis

Exploratory visual data analysis was conducted to assess the mean evolution of the data and select appropriate models for variable (West et al., 2007). The dataset consisted of 36 subjects (ID_sample), with nine subjects per experiment (four combination of factors), and four measurements per subject (pre-treatment, post treatment, after 7 days, and after 14 days of storage). The dataset was complete and balanced, as the number of measurements at each time point is equal. The pre-treatment measurement served as the baseline control. Linear mixed models (LMMs) were used to analyze the correlated continuous data. According to Searle et al. (1992), the linear mixed model for the longitudinal apple browning index (y) dataset is given as:

$$y = X\beta + Zu + \varepsilon \quad (3)$$

where y is the $n \times 1$ vector of responses, X is the $n \times p$ design/covariate matrix for the fixed effects β , and Z is the $n \times q$ design/covariate matrix for the random effects u . The $n \times 1$ vector of errors is assumed to be multivariate normal with mean 0 and variance matrix $\sigma^2 \epsilon R$. The Maximum Likelihood Estimation (MLE) method was used to estimate fixed effects (P, Time) and their interactions, while the Restricted Maximum Likelihood Estimation (REML) (Thompson 1962) was used to estimate random effects (ID_sample and time points (ST), nested within the ID_sample) and correlation functions. A quadratic storage time (QST=ST*ST) was included in the model to test for significant curvature effects. These estimation methods (REML and ML) were used to fit a linear mixed model to measured response browning index (BI), consisting of random terms for the design used and fixed terms. To fit the model, a combination of the Likelihood Ratio Test (LRT) and the Akaike (AIC) and Bayesian Information Criteria (BIC) was used. The LRT (Casella and Berger, 2001) was employed to test hypotheses about covariance parameters and fixed effect. In general, the LRT requires that both the nested model (null hypothesis) and the baseline model be fitted to the same subset of data. The LRT statistic was calculated by subtracting -2 times the log likelihood for the baseline model from that of the nested model. The significance of the LRT statistic was determined by referring to a χ^2 distribution with the appropriate degrees of freedom (dF). The Akaike information criterion (AIC) (Akaike, 1973) was calculated based on the log-likelihood (ML or REML), yielding different values depending on whether the ML or REML estimate was used. AIC serves as an estimator of prediction error. The BIC, in contrast, applies a larger penalty for models with more parameters, as it multiplies the number of estimated parameters by the natural logarithm of n , where n is the total number of observations. For both AIC and BIC, "smaller is better", meaning that lower values indicate a better model fit.

The LMM approach used to model fixed effects, random effects, and correlations in the residual errors. Following Weiss (2005), the selection of random effects was performed by considering only the intercept and storage as fixed effects. Starting with a simple linear regression model, all random effects associated with the intercepts and slopes for ID_sample were tested using a selection strategy based on REML estimation and AIC/ BIC values. Correlation functions were used to model covariance structures between random effects. Once the optimal random effect's structure was identified using REML and AIC/BIC values, the next step was to determine the fixed effects structure. The ML estimation method was used, and fixed effects were gradually added based on their significance, starting with the main effects (P, Time) and then sequentially incorporating 2-way, 3-way and 4-way interactions. Finally, the model was refined by removing non-significant terms ($p > 0,05$) from higher-order interaction terms. All analyses were conducted using SPSS 21 (SPSS Inc., Chicago, IL, USA, software).

3. Results

3.1 Selection model

The individual profile plots show variability both within and between apple samples. The variability between samples is evident (Figure 1a), justifying the inclusion of a random intercept in a linear mixed model. Additionally, BI values per sample follow different trends over time, possibly nonlinear (quadratic), suggesting that an appropriate should also include a random slope. Consequently, random effects associated with the intercept and slope, relative to both linear storage (ST) and quadratic storage time (QST), were both tested.

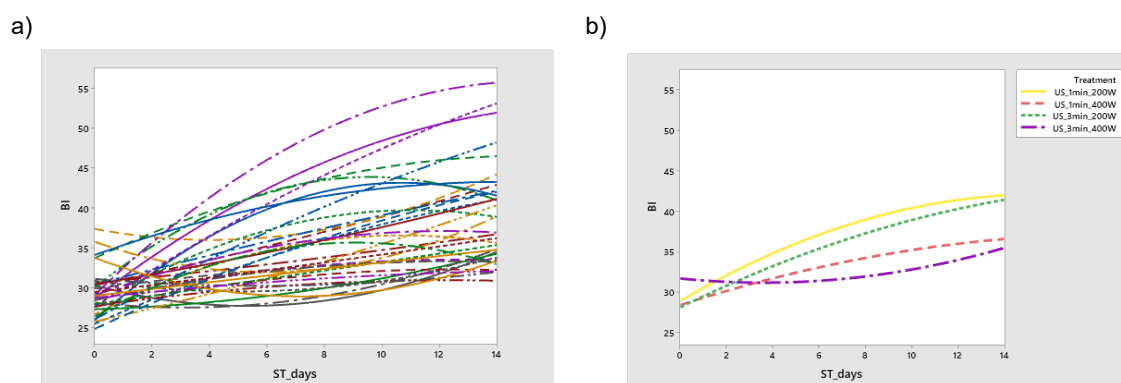


Figure 1 a-b: 36 individual profile plots of browning index by ID_sample (a). The mean profile plot of browning index by treatment (b)

The best-fitting model, determined by the lowest AIC and BIC values, included both the intercept and slope (ST) (AIC 760,89; BIC 769,69, $p < 0,0001$). The optimal covariance structure between the intercept and random slope was identified as a variance component (VC) model, as attempts to fit other covariance structure (unstructured, compound symmetry, autoregressive type 1) resulted in higher AIC and BIC values. The mean treatment effect by storage time, shown in Figure 1b, indicates an interaction effect between the categorical factors and continuous time, highlighting that the mean evolution may follow a quadratic trajectory over time. A full factorial model was tested. The model was subsequently re-fitted removing the non-significant interaction term (Time*P*QST). This adjustment led to a decrease in AIC (from 738,48 to 738,45) and BIC (783,03 to 780,03), indicating an improved model fit. Following the selection of fixed and random effects structures, residual analysis was conducted. The residuals were found to be normally distributed (Kolmogorov-Smirnov test, $p > 0,200$). Therefore, it was unnecessary to test correlation functions between residual errors, and a scaled identity function was adopted.

3.2 Results of the final fitted linear mixed model

The output of the final fitted linear mixed model is summarized in Table 1. The Type III Tests of Fixed Effects provide the overall statistical significance for each predictor in the model, along with their corresponding 95% confidence intervals and p-values for both main and interaction terms.

Table 1: Type III test fixed effects

	Numerator dF	Denominator dF	F	Sig.
Intercept	1	43,768	5276,87	0,000
P	1	43,768	3,598	0,064
Time	1	43,768	2,397	0,129
ST	1	107,262	57,061	0,000
Time*P	1	37,662	4,947	0,032
P*ST	1	107,262	24,842	0,000
Time*ST	1	107,262	9,225	0,003
Time*P*ST	1	37,662	1,629	0,210
QST	1	75,290	5,529	0,021
P* QST	1	75,290	11,523	0,001
Time * QST	1	75,290	8,144	0,006

The analysis indicates that Time ($p=0,129$) and P ($p=0,064$) are not significant, while linear time (ST) and quadratic time (QST) are significant. The estimated effect of linear time was negative (-0,14), whereas that of quadratic time was positive (0,29) (Table 2). This suggests that the average BI initially decreases before gradually increasing at a linear rate, though the magnitude of this change varies. Significant interactions were observed between the process factors P*Time ($p=0,032$) and between individual factors and time (ST and QST) in both two-way and three-way interactions. Figure 3 shows that the highest power level (W) has a buffering effect on time, lowering BI values. Specifically, the simple effect estimate for Time_1min*P 200 W indicates an increase of 3,45 in the mean BI score compared compared to other levels.

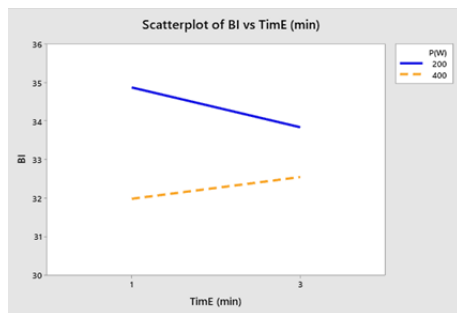


Figure 3: Interaction plot power*time exposure

The interaction between storage time (ST) and P (power) (Figure 4a) shows that P 200*ST had a significantly higher slope (1,40) on average than P 400*ST. Additionally, the interaction with QST indicates that P 200*QST had a significantly lower slope (-0,51) on average than P 400*QST. This suggests that the P 200 W treatment results in a faster linear increase in the BI response over time.

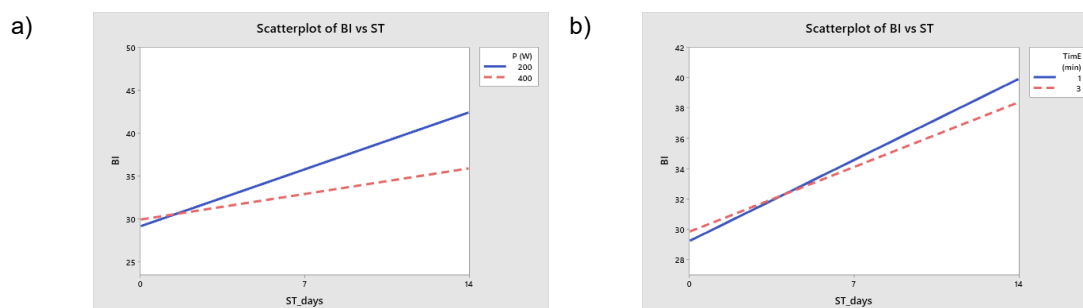


Figure 4 a-b: Interaction plot power*time storage (a) and time exposure*storage time (b)

Furthermore, the interactions Time*ST and Time*QST are significant. The simple effects estimates indicate a significant difference between levels, with increase in slope ($p=0,001$) of 0,92 for the Time_1*ST level compared to the Time_3*ST level, and a decrease of -0,43 for Time_1*QST level compared to Time_3*QST. This suggests that samples treated with 1 minute of ultrasound exposure and dipping exhibit faster growth in BI response. The P*Time*ST and P*Time*QST interactions were not significant, indicating that the combined effect is simply the sum of the effects observed in lower-level interactions. The best combination to slow down the browning phenomenon over time was Time_3*P 400 W, as it had an immediate effect after treatment, allowing a linear increase in the response with a lower slope over time. The random effects, which account for initial variability between samples and their different evolution over time, showed a variance of 3,79 for the intercept and 0,13 for ST nested in ID_sample. The residual variance from repeated measures of residual errors was 3,27.

Table 2: Estimates of fixed effects

Parameter	Estimate	St. Err	dF	t	Sig.	95 % confidence interval	
						Lower Bound	Upper Bound
Intercept	31,517934	0,798079	42,231	39,492	0,000	29,907606	33,128263
[Time=1]	-2,969889	1,117581	40,699	-2,657	0,011	-5,227397	-0,712380
[Time=3]	0 ^b	0
[P=200]	-3,250947	1,117581	40,699	-2,909	0,006	-5,508456	-0,993439
[P=400]	0 ^b	0
ST	-0,141564	0,223459	107,805	-0,634	0,528	-0,584507	0,301379
[Time=1]*[P=200]	3,444622	1,548705	37,662	2,224	0,032	0,308508	6,580736
[Time=1]*[P=400]	0 ^b	0
[Time=3]*[P=200]	0 ^b	0
[Time=3]*[P=400]	0 ^b	0
[Time=1]*ST	0,916835	0,279480	102,210	3,280	0,001	0,362501	1,471169
[Time=3]*ST	0 ^b	0
[P=200]*ST	1,396953	0,279480	102,210	4,998	0,000	0,842619	1,951287
[P=400]*ST	0 ^b	0
QST	0,029284	0,013016	75,290	2,250	0,027	0,003357	0,055211
[Time=1]*QST	-0,042889	0,015029	75,290	-2,854	0,006	-0,072828	-0,012951
[Time=3]*QST	0 ^b	0
[P=200]*QST	-0,051017	0,015029	75,290	-3,395	0,001	-0,080955	-0,021079
[P=400]*QST	0 ^b	0

4. Discussion

Setting the process parameters at 3 minutes and 400 W records a positive effect of ultrasound on the browning index. The index shows a slowdown in growth of the value over time. This effect could be due to the influence of ultrasound on the polyphenol oxidase enzyme as already reported in other studies. Ultrasound, especially when combined with immersion treatments, effectively reduced PPO activity (Putnik et al., 2017; Jang et al., 2009). Furthermore, the effectiveness of ultrasound on the secondary and tertiary structure of the enzyme at a power higher than 200 W has already been found in melon (Liu et al. 2017).

5. Conclusions

Linear mixed model focusing on the kinetics of variation of the groups of treated samples, establishes the best treatment in line with the final objective: an apple that browns more slowly. Apple treated at 400 W for 3 min has a slope with a starting mean, at the baseline, of 32,00 and a final mean of 35,42. Differently apple treated 1 min or 3 min at 200 W returns samples with the highest kinetics of evolution over time with baseline mean of 29,56 and 28,59 a final mean of 41,90 and 41,37. A General Linear Model (GLM) or a repeated measures ANOVA would not have been suitable to statistically determine which process parameters significantly slow browning over time, because the measurements are not independent. The use of linear mixed models should be expanded in food science and technology, especially when working with biological materials where initial variability and

time-dependent effects play a crucial role. However, the linear mixed model has potential limitations in its use also due to the complexity of the model and assumptions.

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