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Natural dye ultrasound extraction from beetroot: role of extraction solvent pH on color and enzyme inactivation

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Abstract: The presence of enzymes in agricultural commodities causes quality changes, including color, flavor, and nutritional losses. The use of non-thermal and innovative technologies for the production of quality extracts derived from plant products is a recent trend in food processing. The aim of this study was to evaluate the effect of ultrasound applying different pH in the extraction solvent for obtaining a natural dye from beetroot on the color and enzyme inactivation. The natural beetroot dye was obtained using the extraction solvent (Mc Ilvaine buffer) at pH of 3.5, 5.0 and 6.5, ultrasound amplitude of 40, 60 and 80% and time of processing of 2, 6 and 10 minutes. Physicochemical parameters, color and enzymes were determined on the dye extracted, additionally, the microstructure of

beetroot mash was analyzed. It was found that the physicochemical parameters were minimally modified when the pH of the solvent extraction was changed during ultrasound application. Concerning to the color characteristics, hue angle showed intense red color for ultrasound treatments at 3.5 pH. In the same way, this pH leads to obtaining the complete inactivation of peroxidase (100%) and 98.35% of inactivation for polyphenol oxidase for treatments at 10 min and 40% amplitude, which coincides with the treatment selected as optimal. From the microstructural analysis, it was possible to observe the formation of microchannels on the tissue surface beetroot as a result of ultrasonic waves. Therefore, ultrasound extraction at acidic pH could be employed for extracting a natural dye from beetroot in an effective way.

Keywords: Peroxidase; polyphenol oxidase; colorant; ultrasonic waves; microstructure.

1. INTRODUCTION

Recently there has been increasing interest in how synthetic colorants can contribute to the incidence of diseases or disorders not only in adults but also in children[1]. As a result of which, research has focused on evaluating the potential of natural dyes to be used in foodstuffs that are safe and harmless for human consumption. Red dyes in the food industry have some interest, these are pigments E120 (carmine red) obtained from the dry shell of the fertilized females of the insect cactus, E163 (anthocyanins), ranging from the red to bluish violet obtained from the extraction of blackberries, strawberries, currants, grapes, raspberries and black maize, as well as E162 (dark red) dye obtained by pressing and extracting beetroot or beet. The use of these dyes in the food industry extend to dairy products, confectionery, beverages and desserts mainly[2]. Particularly beetroot (*Beta vulgaris*) is a potential source of valuable water-soluble nitrogenous pigments, the so-called betalains, which are composed of two main groups, the red betacyanins, and the yellow betaxanthins. Thus betanin, which is the mayor pigment of beetroot, has been permitted for use as a natural food colorant under the color additive amendment to the food, drugs and cosmetic act in the form of beet juice concentrate or dehydrated beet powder[3]. When betalains are used as food colorants, color stability is a major concern. However, there are several environmental factors that have been recognized to affect the stability of these pigments: temperature, light, water activity, oxygen, pH and presence of some enzymes[4,5]. Pedreño and Escribano[3] studied the effect of temperature, light, and pH on the antiradical activity of betanin, finding that the acidic pH as well as the lower temperatures favored the antiradical activity of betanin and that light has no significant effect. In the same way, a color study was carried out from cactus juices, where it was demonstrated that betalains keep their appearance over a broad pH range[6] from 3 to 7. During the commercial development of natural beet pigments must be considered the possible enzymatic decolorizing problems of betalains, mainly caused by peroxidase (PO) and polyphenol oxidase (PPO) enzymes. With regards the PPO, it oxides the phenolic components to quinones that finally polymerize to colored melanin[7] while PO is associated with the development of unpleasant flavor and colored pigments[8]. The high-pressure carbon dioxide treatment was studied to inactivate enzymes in beetroot juice, led to obtain high-quality non-acidified beetroot juice, however, the use of thermal pasteurization as a traditional method allowed maximum inactivation of the enzymes[9]. In this context more studies should be carried out that contribute to obtaining maximum enzymatic inactivation while preserving the quality characteristics of the product. Ultrasound is a promising technology in preservation of food[8] in it, the ultrasonic waves emitted by a generator are characterized by using low frequencies and high power (≤1MHz, 10-1000W/cm^2) which are going to travel through the material or on the surface at the characteristic velocity of the wave and the

material through propagation. The effect of ultrasound on liquid systems is mainly related to the cavitation phenomena and the formation of free radicals. When the power is high enough, the rarefaction cycle can be higher than the attractive forces in the liquid molecules, forming cavitation bubbles formed by the nucleic gas that exists inside the fluid. These bubbles grow up to a critical size due to the alternant cycles, becoming unstable and violently collapse[10], when this phenomenon happens, there is a rupture in the wall cell in the treated material, size reduction and improvement of mass transfer throughout the cell membrane[11]. Due to the fact that the effectiveness of ultrasound to control enzymatic activity is greatly influenced by extrinsic and intrinsic factors such as enzyme concentration, temperature, pH and the medium composition, the aim of this investigation was to obtain a natural dye from beetroot using different ultrasound conditions with solvent at different pH and to evaluate their effect on the color and enzyme inactivation.

2. MATERIALS AND METHODS

2.1 Raw material:

The beetroot obtained from a local supermarket was washed with tap water, peeled and size reduction by means of a food processor (Model FPSTFP4255, Oster, and México). Then, the material was passed through a mesh to obtain a particle size of 2.33mm.

2.2 Ultrasound extraction:

The beetroot mash was immersed in the extraction solvent in relation 1:10 w/v using a glass beaker, which was placed in an ice bath. The treatment under ultrasound (US) was carried out using three pH in the Mc Ilvaine solvent 3.5, 5.0 y 6.5, amplitudes of 40, 60 y 80% and times of 2, 6 and 10 minutes. At the same time the preparation of the control sample (C, without ultrasound extraction) was carried out using distilled water as a solvent. Then the ultrasound and control samples were centrifuged at 3000 rpm during 10 min (Corning® LSE™ Compact Centrifuge, model 6755, USA). The supernatant was recovered and placed in black tubes to avoid the light exposure. The ultrasound was applied using an interchangeable tip of 40 mm diameter and frequency of 26 kHz (Model UP-200Ht, Hielscher Ultrasound Technology, Teltow, Germany).

2.3 Analysis

2.3.1. Physicochemical parameters: Physicochemical parameters were evaluated by the AOAC[12]: soluble solids (932.12), total solids (920.151) and pH (981.12).

2.3.2. Color analysis: Color measurement was carried out by the Hunter Lab Color Flex EZ 45/0 (Hunter Associates Laboratory Inc., Virginia, E.U.A). The color was expressed according to the CIELCh (L*C*h°) scale: L* (luminosity/darkness), C* (croma), h° (hue). Using the values of L*, a* and b*. The croma was calculated by the equation (1), and the hue angle according to the equation (2).

$$C^* = \sqrt{a^2 + b^2} \qquad \text{Equation (1)}$$

$$h^\circ = \arctan\left(b^* / a^*\right) \qquad \text{Equation (2)}$$

2.3.3. Determination of PO residual activity: The activity of the peroxidase enzyme was measured according to the procedure described by Kwak *et al.*[13]. The extract was centrifuged at 10,000 g for 10 min at

4 °C. The reaction mixture contained, in a total volume of 3 mL: 0.1 mL of centrifuged sample (or 0.1 mL of phosphate buffer for blank sample), 2.1 mL of distilled water, 0.32 mL phosphate buffer (100 mM, pH 6.0), 0.32 mL of pyrogallol (5% w/v) and 0.16 mL of H_2O_2 (O.15 M). The reaction was initiated by the addition of hydrogen peroxide and the increase in A_{420nm} recorded in 20 secs during 3 min for both, the sample and the blank. One unit of PO activity is defined as that forming 1 mg of purpurogallin from pyrogallol in 20 secs at pH 6.0 at 20°C. The calculation was performed according to Equation (3).

$$U = [(\Delta Abs_{420}/20secsample - \Delta Abs_{420}/20secblank) \cdot V_T \cdot DF] / (\varepsilon \cdot V_s) \qquad \text{Equation (3)}$$

The results were expressed as percentage of residual activity (%RA) according to equation (4).

$$\%RA = 100 \cdot [U_{sample}/U_{control}] \qquad \text{Equation (4)}$$

2.3.4. Determination of PPO residual activity: The enzymatic activity of the polyphenol oxidase was determined according to the method described by Abid *et al.*[14]. The extract sample was centrifugated at 3000 rpm for 10 min at 4°C. The reaction mixture was prepared with 1.5 mL of the centrifuged sample, 0.5 mL de catechol (0.05M) and 3 mL of potassium phosphate buffer (0.2M, pH 6.8) resulting in a total volume of 5 mL. The increment in the absorbance was measured at 410 nm every 30 secs during 5 min. The results were expressed as percentage of residual activity (%RA) according to equation (5).

$$\%RA = 100 \cdot [Abs_{sample}/Abs_{control}] \qquad \text{Equation (5)}$$

2.3.5. Response optimization: The election of the optimal treatment for obtaining a natural dye using ultrasound extraction was based on the residual activity of PO and PPO enzymes. The methodology used to carry out this election is denominated response optimization and it was useful to evaluate the impact of variables of: time, amplitude and pH in only one response. The individual desirability (d) for each response is achieved by stating the goals, i.e., minimize, maximize or target the response, in this case we tried to minimize the residual activity of the enzymes tested. The global desirability (D) evaluates the way in which the configuration optimizes a set of responses in general desirability has a range from zero to one. One represents the ideal situation, while zero indicates that one or more responses are outside acceptable limits.

2.3.6. Microstructure analysis: The mash morphology was analyzed by scanning electron microscopy JSM-6510 LV (JEOL) from secondary electrons, with acceleration voltage of 20kV at high vacuum. Because the samples are not electricity conductors, they were coated with a thin layer of gold in an equipment Electron Microscopy Sciences model EMS 550, at pressure vacuum of $7x10^{-2}$ mB and 40 mA, in argon atmosphere.

2.3.7. Statistical analysis: The statistical analysis was performed by the Software Minitab version 17. All the parameters were analyzed by triplicate and the results are reported as the media ± standard deviation. The data were analyzed by ANOVA ($P<0.05$) and the difference between homogeneous groups was performed by the Tukey test. The optimization response was realized taking into account the results of enzymatic activity.

3. RESULTS AND DISCUSSION

3.1 Physicochemical parameters:

Table 1 shows the mean value for each physicochemical parameter obtained under all the ultrasound conditions. The soluble solids and total solids increased as the pH in the extraction solvent was increased, with an increment in the mean value of 0.33 °Bx and 0.17%, respectively between each pH value. The set of values obtained under the different ultrasound conditions (time and amplitude) for each pH of the extraction solvent showed not significant difference (data not shown). In the same way, the pH of the obtained extract increased slightly, but there was not a significant difference between corresponding samples at the same pH, but it was with the control. In researches carried out with fruit juice, it was reported that the ultrasound treatment did not have a significant influence on the pH, soluble solids and total solids in guava, orange and grape juice[15-17]. Başlar and Ertugay[18], observed that the ultrasound did not affect the pH values in apple juice and reported that under the condition of 10 min at 60 °C increased the soluble solids in 0.5 °Bx probably due to the evaporation.

Table 1: Effect of pH extraction solvent on physicochemical characteristics of natural beetroot dye

CONTROL	SOLUBLE SOLIDS [°Bx]	TOTAL SOLIDS [%]	pH
	0.80 ± 0.00	0.80 ± 0.02	6.45 ± 0.01
Mc Ilvaine Buffer 3.5 pH	2.88 ± 0.13	2.81 ± 0.04	3.64 ± 0.03
Mc Ilvaine Buffer 5.0 pH	3.23 ± 0.03	3.00 ± 0.03	5.08 ± 0.02
Mc Ilvaine Buffer 6.5 pH	3.54 ± 0.07	3.15 ± 0.02	6.50 ± 0.01

3.2 Color characteristics

The color evaluation as a quality parameter is a really important determination in the development of food products, as some processes lead to its degradation. **Figure 1** shows the effect of pH on CIELCh parameters of natural dye, finding the higher values at pH of 3.5 where exists a tendency to increase the L* value as the application time of ultrasound increases. An increase in the luminosity values is a desirable characteristic because it indicates that some enzymatic reactions are being inhibited. Stintzing *et al.*[19] carried out the evaluation of color in prickly pear Opuntia juice and reported an increment in the luminosity value when the pH of the media was diminished. In the same way, Cejudo-Bastante *et al.*[20] observed the highest values of luminosity in acidic ulluco (*Ulluco* tuberosus) extracts, pH 4.0, which was also observed in this study. The croma parameter is a measure of intensity or saturation of color, all the processed samples presented higher values in respect to the control, reflecting an increasing in color purity of extracts and representing more color vividness, specifically the processed samples at pH of 3.5 with superior values of 70, which represent 23% more than the control. Additionally, the trend is to increase the croma value as the time of ultrasound application is increased, reaching the maximum values at 10 min and amplitudes of 60 and 80% without significant difference. The main reason of this increment could be due to the disintegration of compounds of enzymatic browning, which was caused by the acoustic cavitation. The color of the extracts, define the hue parameter, corresponding to the angle (h°), the desirable value is the one that maintains the value of the

control or that found under the control because an increment indicates the shift to orange. At pH of 3.5, the sample treated for 10 minutes increased its value with the increment in the amplitude, reaching a maximum value of 37.4 at the amplitude of 80%, being the sample with the higher difference in respect to control for all the treatments analyzed. However, that difference did not reach the value of 5 grades, indicating that the color characteristics of the juice (red) are maintained in all the treatments. Fonteles *et al.*[21] reported a similar behavior, where all the treatments applied to melon juice showed a variation of less than 5°, maintaining their color. In study carried out to evaluate the color of fruit extracts, Hurtado *et al.*[22] reported that an acidic pH improvement the red color stability of tamarillo fruit (*Solanum betaceum* Cav.).

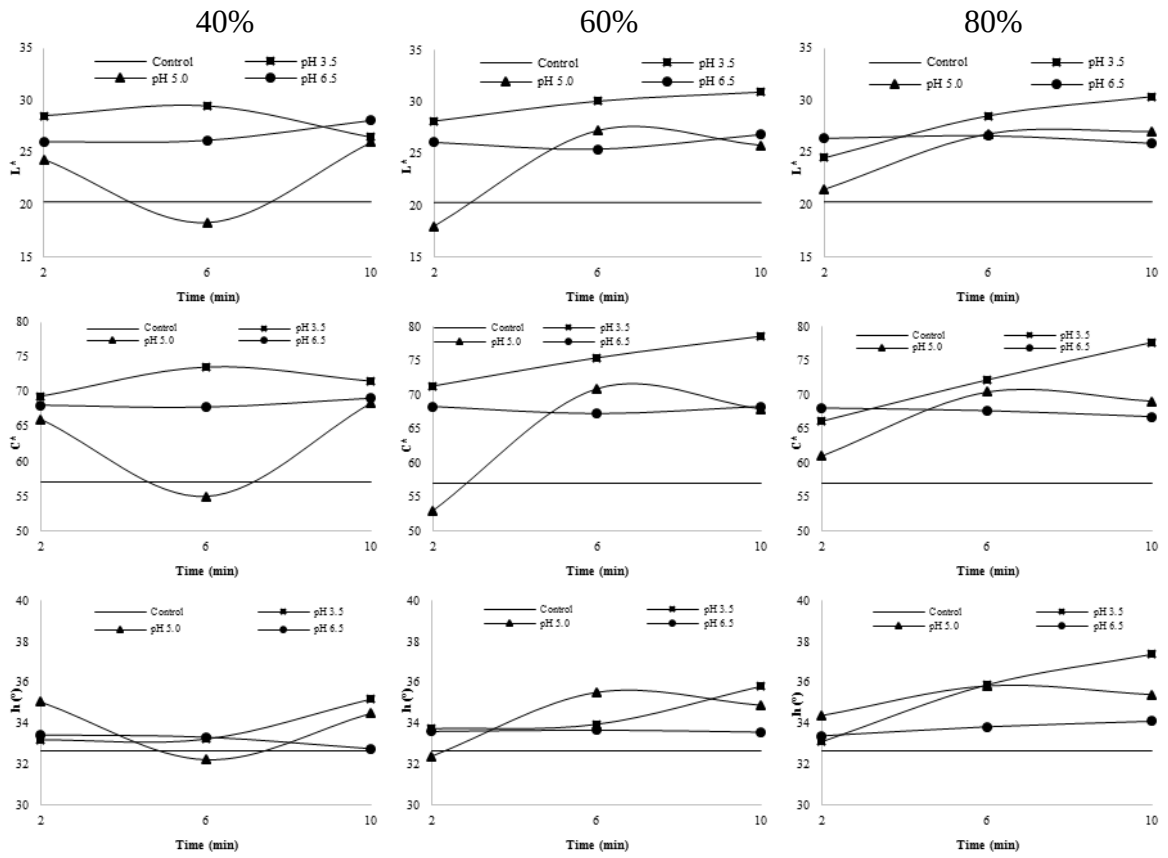

Figure 1: Effect of pH on CIELCh scale of natural beetroot dye at different ultrasound conditions of amplitude and time

3.3 Enzymatic behavior

The effect of ultrasound processing on the enzymatic activity depends of factors such as pH, temperature, food matrix, ultrasonic intensity and time of processing. The effect of ultrasound on the residual activity of peroxidase enzyme under different experimental conditions is presented in **Figure 2**, where, well defined changes were observed between the treatment groups of different pH. The enzymes, being proteins have properties that are highly sensible to the pH. Most of the proteins are active in a pH range (typically from 5 to 9), this is the result of the effect of pH on a combination of factors such as the link between substrate and enzyme, the catalytic activity, the ionization of the substrate, and the variation of protein structure by the

influence of the amino acids with charge[23]. From **Figure 2,** it can be observed that the natural dye extraction with acid pH (3.5) was the one that favored the inactivation of the enzyme, in this case, the complete inactivation was reached with 10 min and 40 % of amplitude, while with 10 min and 60 %, as well as with 10 min and 80 % presented an activation of 2.08 and 2.54 % respectively. O´Donnell *et al*.[24] have mentioned that under acidic pH the enzyme inactivation takes place when the velocity of denaturation of the enzyme is higher that the velocity of release, which is the main responsible for the inactivation, either for the formation of free radicals in the water sonolysis or due to the shear forces that resulted from the formation or collapse of bubbles of cavitation. A different behavior presented the samples treated at pH 5, where the increment in time from 2 to 6 minutes, increased the residual activity of the enzyme, then, when the time was increased to 10 min the activity of the enzyme was diminished. This can be explained due to the release of the enzyme, product of the rupture of the cell wall, where the time of 6 min favored the release and once released it was exposed to the shear forces, and finally resulting in the denaturation. The same behavior was observed by Costa *et al*.[25] in pineapple juice processed with ultrasound.

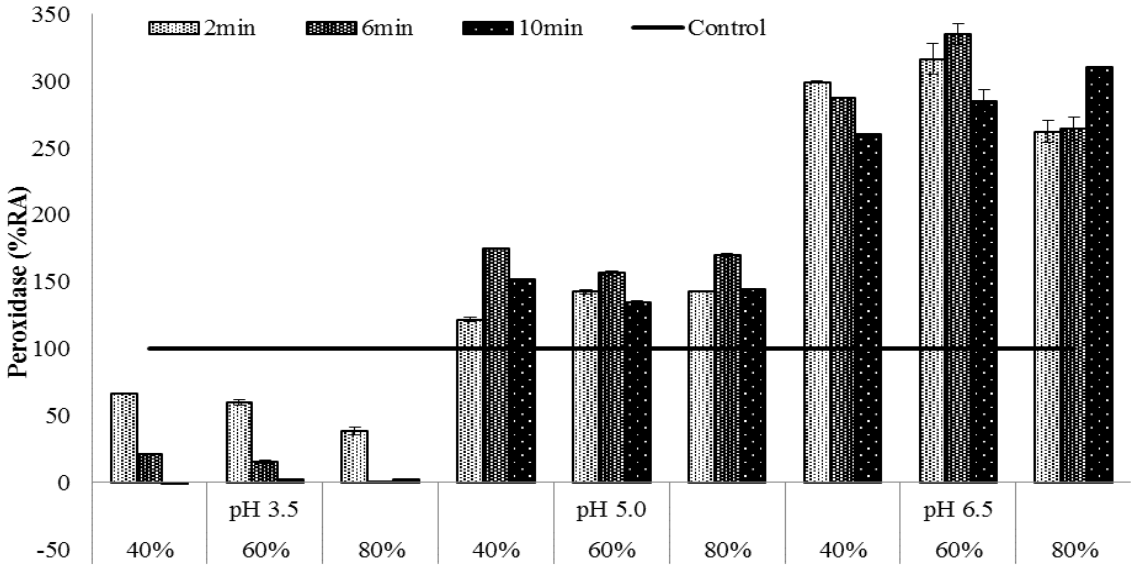

Figure 2: Effect of ultrasound treatment on the residual activity of PO enzyme at different pH values

Figure 3 shows the behavior of the polyphenol oxidase enzyme, the maximum reduction of the residual activity (RA) between different pH, was reached at the pH of 3.5 with values from 1.24 to 6.75 %, compared with the pH of 5.0 (65.24 to 105.37 % RA) and 6.5 (34.34 to 130.20 % RA). The treatment conditions that favored the maximum reduction of PPO activity were the time of 10 minutes and 60 % of amplitude (1.24 ± 0.00 %), although without significance difference with the treatment at 10 minutes and 40 % of amplitude (1.65 ± 0.41 %). This behavior could be attributed to the effect of the acoustic cavitation caused by the increment in pressure (1000 MPa) and temperature (5000 K) in the focalized points of collapse of bubbles[18]. In the same way, the formation of cavitation bubbles results in a shear force that can change the proteins conformation; the enzymes can be denatured by free radicals generated during sonolysis of water molecules[21].

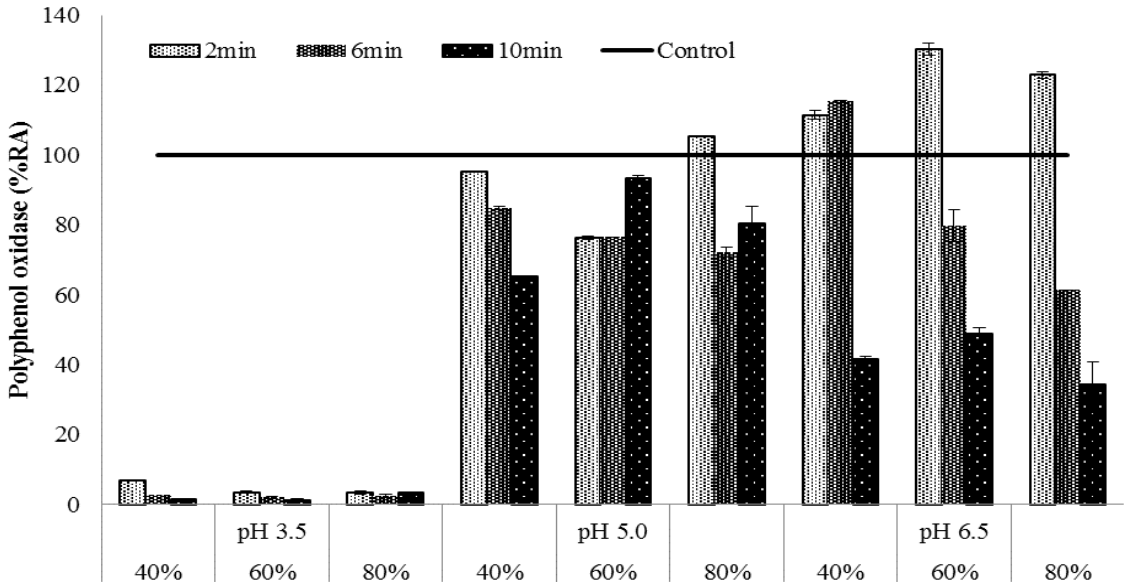

Figure 3: Effect of ultrasound treatment on the residual activity of PPO enzyme at different pH values

3.4 Response optimization

The individual desirability (d) values obtained from the response optimization (**Figure 4**) was 0.983 and 0.886 for PO and PPO respectively, combining these desirability values, the global desirability (D) resulted in 0.9340 value. From the results, it was found that the optimum treatment according to the evaluated variables was at 10 minutes, 40% of amplitude and pH of 3.5.

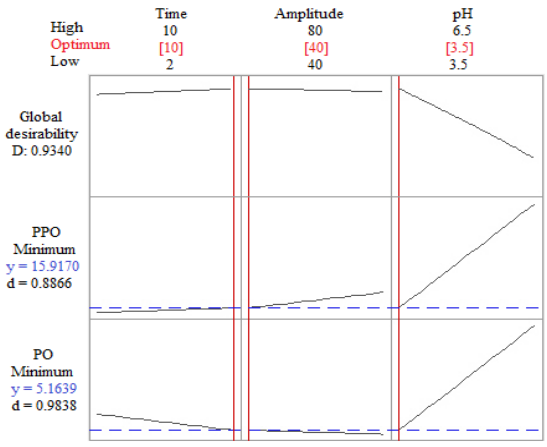

Figure 4. Response optimization

3.5 Microstructure

In base to the response optimization, the microstructure analysis was carried out on samples processed at pH of 3.5. The micrographs of beetroot tissue without treatment and treated with ultrasound under different level

of amplitude, are presented in **Figure 5**. It was possible to observe microchannels at any amplitude tested. Under 40 and 60% amplitudes, the microchannels presented spherical forms and they are heterogeneous, with diameter from 64 to 86 µm. On the contrary, at amplitude of 80% the microchannels are more homogeneous and present symmetry (32-40µm). An important observation on the microstructural analysis is the surface of the tissue, which was also altered when the ultrasound was applied (**Figure 6**).

The control sample presents a smooth surface and with the application of ultrasound it starts to be modified, becoming rugose, increasing as the amplitude was also increased, being clear that the modifications in the tissue were caused by strong mechanical forces, the pressure fluctuations into the solvent, and the impact of the microjets on the solid interphase, which was produced by the quickly collision of bubbles of acoustic cavitation. The rupture of the physical structure of the beetroot mash, could lead to a direct migration of the components towards the solvent of extraction. Several studies were based on the effect that has the ultrasound on the microstructure of the treated material, finding that the ultrasound disturb the cell wall, facilitating the extraction of the content[26], In the same manner microfractures were observed in soybean flakes, turning the surface more porous[27], in the same way it present dents and holes on the surface of starch granules[28].

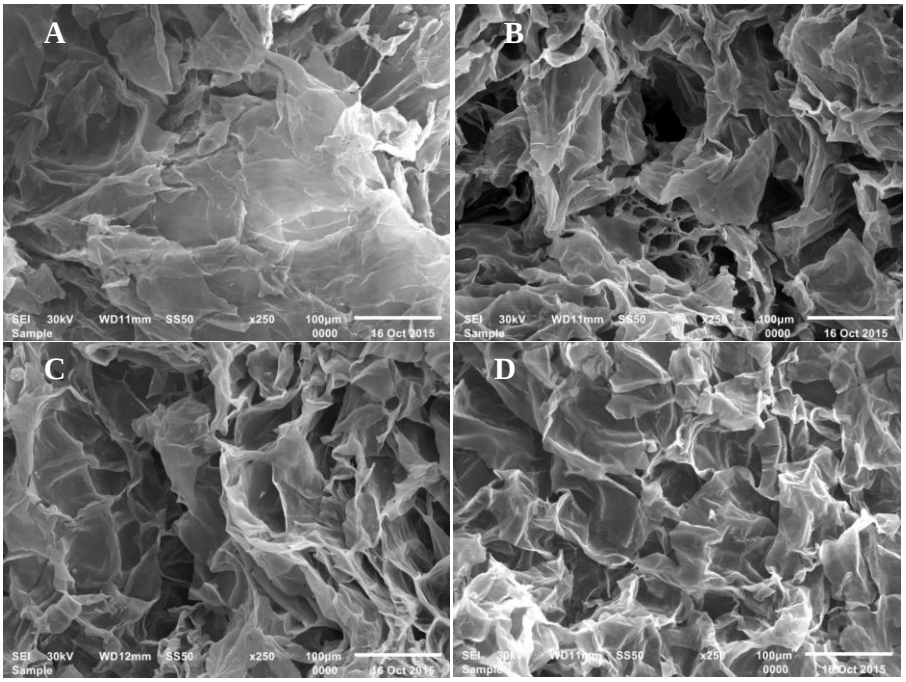

Figure 5: SEM micrograph of beetroot mash treated at amplitude 0% (A), 40 %(B), 60% (C) and 80 %(D) at 250x magnification

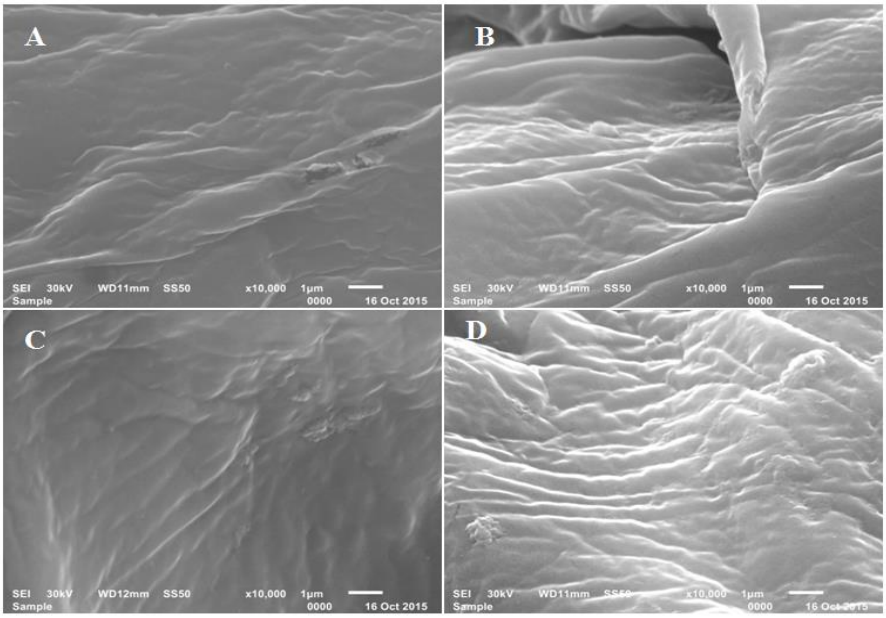

Figure 6: SEM micrograph of beetroot mash treated at amplitude 0% (A), 40 % (B), 60% (C) and 80 % (D) at 10000x magnification

4. CONCLUSIONS

The pH of the medium on which the ultrasonic waves were applied to obtain a natural beetroot dye, had great influence on color, enzymatic activity, and microstructure. Our study showed that the pH of 3.5 was the most effective to enhance the highest inactivation of both enzymes under the ultrasound conditions of 10 minutes and 40% of amplitude. Samples treated with ultrasound (pH 3.5) presented a more luminous and saturated color, therefore it can be inferred that this extract can be used as a natural colorant. The ultrasonic waves were able to modify the structure of the beetroot tissue. Based on our study, it can be concluded on the convenience to acidify the extraction solvent in an ultrasound system in order to enhance the enzyme inactivation and the maintenance of color in a natural beetroot dye to be used as an ingredient in a food product. Future work should involve studies about the stability of the beetroot extract and its preservation.

ABBREVIATIONS

PO	Peroxidase	**b***	Yellow/blue
PPO	Polyphenol oxidase	**RA**	Residual activity
CIE	Commission Internationale de L'Eclairage	**SEM**	Scanning Electronic Microscopy
L*	Luminosity/darkness	**C**	Control sample
C*	Croma	**US**	Ultrasound samples
h°	Hue angle	**d**	Individual desirability
a*	Red/green	**D**	Global desirability

NOMENCLATURE

DF	Dilution factor	**U**	Enzyme units/mL
E	Extinction coefficient of 1 mg / mL of purpurogallin at 420nm	**Abs**	Absorbance
V_T	Total volume of assay (mL)	**mB**	millibar
V_S	Sample volume (mL)	**mA**	milliampere
%RA	Residual activity percentage	**mM**	millimolar

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