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PECTINASE ENZYME (PEC-600) AS A CLARIFIER IN ORANGE WINE MAKING (*CITRUS SINENSIS*) (FERMENTED BEVERAGE)

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Abstract–The main problem in the process of obtaining fermented alcoholic beverages from orange, (*Citrus sinensis*) is the turbidity of the product, so this work proposes the application of pectinase enzyme (PEC-600) as a clarifier. For this purpose, an experimental model suitable for evaluating two types of oranges was considered on the basis of their availability in the zone (National and Tangelo). Likewise, two organisms were evaluated: bread yeast *Saccharomyces cerevisiae (levapan)* and lyophilized yeast or active dry yeast of wine. Once fermentation ended, two concentrations of pectinase enzyme (0.2 and 0.4%) as clarifier were applied. Wort conditioning rectifying was carried out at 23° Bx to this *Saccharomyces spp.* was applied, and the fermentation process took approximately fifteen days, then pectolytic enzyme was added at 45 ^{0}C. Once cleared, the beverage was allowed to stand for 60 days in storage conditions at 25 ^{0}C for finally bottling as an ended product. A statistical analysis have been done with completely randomized factorial block A*B*C, eight treatments with three repetitions were evaluated by using a statistical tool ANOVA. The product obtained from national orange was obtained as the best product with the addition of 0.2% pectinase and dry yeast, the resulting product presented: >5% of suspended solids (after clarification pH 3.45, acidity 0.39 % and soluble solids 6.67 % with a content of 13.2^0 of alcohol.

INTRODUCTION

Fermented fruit beverages are those obtained from the transformation of contained sugars in the wort, which becomes alcohol from the yeast. According to Paéz, (2010), in the biological sense, fermentation is a process of obtaining energy in anaerobic conditions. From a biochemical point of view, it is a metabolic process of obtaining energy from organic compounds without the participation of an exogenous oxidizing agent (Bourdichon *et al.*, 2012). The fermented orange beverage is made by handcraft manner presents the problem of turbidity as a result of incorrect clarification, which affect negatively in the appearance of the final product.

Clarification is an important stage in the processing of fermented fruit beverages, and most of the times it is made either by microfiltration, enzymatic treatment or by the common use of clarifying agents such as gelatin, bentonite, silica, or the combination of these compounds (Chatterjee *et al.*, 2004).

Since so many decades different population were dedicated for the production of alcoholic beverages. Due to its great importance, the scientific and technological investigation related to alcoholic beverages, has concentrated great efforts since the last century. This beverage industry, among biotechnological industries is the most economically important in the world- together with recently generated advances in knowledge, have has been extrapolated to many applications of biotechnology and food technology (García *et al.*, 2004).

Therefore, this paper seeks to determine (the adequate orange variety for the application of this enzyme; and also, the concentration of pectinase to be applied in wine). Once the fermentation process has ended, as well the types of yeast, which show

the best responses to the clarification process of the fermented beverage.

METHODS AND MATERIALS

Experimental Design

In this investigation, 24 experimental units were used in each treatment: 23 oranges weighting 4.949 g, 3 g. of yeast (*Saccharomyces cerevisiae*), 1.050 g refined sugar and 6 g of pectinase enzyme (PEC-600), and 2.699 ml of orange wine were obtained. The treatments were designed intended to evaluate two concentrations of pectinase enzyme, 0.2% and 0.4%, two varieties of oranges -National and Tangelo- and two types of yeast (dry or wine).

Fermentation Process

The wort fermentative process was made from selected oranges in this manner disinfection and washing were carried out with a 2% concentration of sodium hypochlorite. Then oranges were blanched at 70ºC for a period of two to five minutes, and the fruit was cut longitudinally for extraction of juice. At that point, Brix degrees' content and juice pH were measured and a heat treatment was made to avoid microorganism proliferation, the wort was conditioned with sugar added to reach amounts of 23 ºBrix and pH of 3.06 and 1 gram of dry or wine yeast was applied to each liter.

Fermentation was made in two autoclave bioreactors provided with a bio controller system ADI 1030 (Applikon, Schiedam, Netherlands) they were used for simultaneous saccharification and fermentation (SSF) of pretreated with MCPW. Both have the control of temperature, pH. and agitation intensity; and a small aperture with GyroVent PTT filter of 0,2 lm, allowing CO_2 release from fermentation. The wort was put in the bioreactors with a capacity of 5 liters for a period of 10 to 15 days in which fermentation was achieved, and after this, clarification was attained by putting the wort in amber colored bottles with 5 liters capacity, with the pectinase enzyme, an air trap was used to avoid contamination.

Application of pectinase enzyme (PEC-600)

Once the fermented beverage was obtained, pectinase enzyme was added at a concentration of 2% and 4%, according of what had been established by the statistical model, the application was made at 45 ^{0}C, it was allowed to stand during 2 months for its maturation, the intention was to accentuate flavor, odor and color characteristics and to obtain a clearer product (free of suspended solids). Once this process finished, packaging, sealing and storaging took place

Physicochemical analysis

The carbohydrate content in the samples clarified by centrifugation (4,000g every 2 min.) before and after fermentation, was measured with a digital portable refractometer (Atago, EUA) and expressed in Brix degrees (percentages W/V of free sugars). To determine pectinase influence in the process of wine obtaining, the pH. Chemical analysis was made: 50 ml of the fermented beverage were put in a beaker, the electrode was introduced, avoiding the contact with container walls; to what 10 ml of fermented beverage was required, 10ml of distilled water were added in the preparation of the sample, and then it was titered with 0.1N sodium hydroxide and phenolphthalein. Soluble solids (0Brix), was measured through refractometry; alcoholic content was measured through INEN 340 rule, To achieve this, the previously prepared sample was put in a clean and dry test tube, where the thermometer and alcoholmeter were introduced, and maintained for 10 minutes, after stirring the sample slightly to mix well and read the temperature, the sample was allowed to stand until the disappearance of air bubbles, and finally the alcoholmeter reading was performed considering the real liquid level and not the meniscus elevation. A magnifying glass was used for this. A sedimentation analysis by centrifugation was also performed, for this, 10ml of the sample were put into the test tube, and then they were put in the centrifuge, and inserted into each recipient in the opposite way.

Statistical Analysis

In this study a completely randomized factorial block (A*B*C) was applied A (orange varieties), B (pectinasa concentrations) and C (yeast types). An analysis of Variance and Tukey's test for separating the levels of treatments ($p<0.05$) were performed. The tabulation of the results was performed through statistical pack Statpectinasaics centurion of University of Massachusetts (Table 1)

RESULTS AND DISCUSSION

Analysis of variance considering study factors valued

The results obtained in fermented orange product

(Citrus sinensis) and subsequent application of pectinase enzyme (PEC-600) as clarifier of two studied varieties (grapefruit and Orange). In figure no. 1 pH, 3.50 (a_1) and 3.67 in a_0 were distinguished, and these amounts were found to be similar to be the de ones reported by Selli *et al*., (2008) who obtained a pH. of 3.50 in a similar fermented beverage. With regard to acidity, amounts of 0.33 % were found in grapefruit (a1) and 0.39% in orange (a_0) and these were below the 0.59 ± 0.97, amounts obtained by Olivero *et al.,* (2011). In Brix degrees, amounts of 10.25 were observed in orange (a_0) and 11.50 in grapefruit (a_1) resulting in a sweeter fermented beverage than the 8.28 ± 0.28 reported by Leal *et al.,* (2011) in his investigation. In alcoholic degrees 13.03% was obtained in grapefruit wine (a_1) and 12,00% in orange wine (a_0), which, in turn are higher than the amounts exposed by Hoyos, *et al*, (2010) in their investigation with a maximum value of: 8.25 ± 6.27.

Also, considering the signification test of Tukey ($p>0.05$), a substantial difference in pH, acidity, and alcoholic content was found, reporting less acid amounts of the National Orange (pH 3.67), and acidity 0.39 and in alcoholic degrees 13.0, statistically higher results than the wine obtained from Tangelo, while Brix degrees showed a similar behavior, in both national orange wine and Tangelo, because no significant differences were found between a_0 and a_1, this is why, with addition of sugar, wort Brix degrees value was rectified at the beginning of the process (Figure 1). If we consider variables pH, acidity and alcoholic degrees, we can specify that National Orange showed better conditions than Tangelo in the obtaining of clarified wine with pectinase.

With regard to the difference between pectinase concentrations applied in the process of orange wine obtaining (Figure 2). The analysis of two different concentrations: 2% (b0) and 4% (b1), showed a pH of 3.50 in b_0 and 3.67 in b1 amounts similar to those obtained by Cano *et al*., (2013) namely 5.44 ± 0.05. In Brix degrees 10, 0% in b_0, and 11.75% in b1 were obtained, these were higher than the amounts reported by Cayetano *et al*., (2001) in his investigation Rheological behavior of "Orange wine": Influence of temperature and composition which were of 10.5%± 8.50. Concerning alcoholic degrees, amounts of 12.03% in b1 and 13.00% in b0 were found, close to the 12.6% of alcohol obtained by Helebek *et al.,* (2009).

The analysis of the amounts reported by Tukey's rank signification test ($p>0.05$) considering the application of two different percentages of pectinase (2% and 4%). Figure 2, showed a significant difference in ph. Brix and alcoholic degrees, thus finding more basic amounts in treatments with addition of 4% of pectinase (pH 3.67) of the same way, this level presented higher Brix degrees than the addition of 2% of pectinase and while alcoholic degrees were lower (12.03), we observed that Brix degrees' content higher than 2% percent concentration coincides with the lower alcoholic conversion in the 4% pectinase addition. In on the

Table 1. Statistical design of the investigation (DBCA, A x B x C) for the pectinase enzyme application as clarifier of orange wine. The chart shows the study factors and its respective levels, and treatments to evaluate.

Study factors		Levels to evaluate		Symbology	Treatments combination
A:	**Orange varieties**	$\mathbf{a_0}$ $\mathbf{a_1}$	Tangelo orange	$a_0b_0c_0$	National orange + pectinasa concentration of 0.2 % + dry yeast.
				$a_0b_0c_1$	National orange + pectinasa concentration of 0.2 % + wine yeast.
				$a_0b_1c_0$	National orange + pectinasa concentration of 0.4 % + dry yeast.
B:	**Concentration of (PEC-600)**	$\mathbf{b_0}$ $\mathbf{b_1}$	0.2 % 0.4 %	$a_0b_1c_1$	National orange + pectinasa concentration of 0.4% + wine yeast.
				$a_1b_0c_0$	Tangelo orange + pectinasa concentration of 0.2 % + dry yeast.
				$a_1b_0c_1$	Tangelo orange + pectinasa concentration of 0.2 % + wine yeast.
C:	**Yeast types**	$\mathbf{c_0}$ $\mathbf{c_1}$	Dry yeast Wine yeast	$a_1b_1c_0$	Tangelo orange + pectinasa concentration of 0.4 % + dry yeast.
				$a_1b_1c_1$	Tangelo orange + pectinasa concentration of 0.4% + wine yeast.

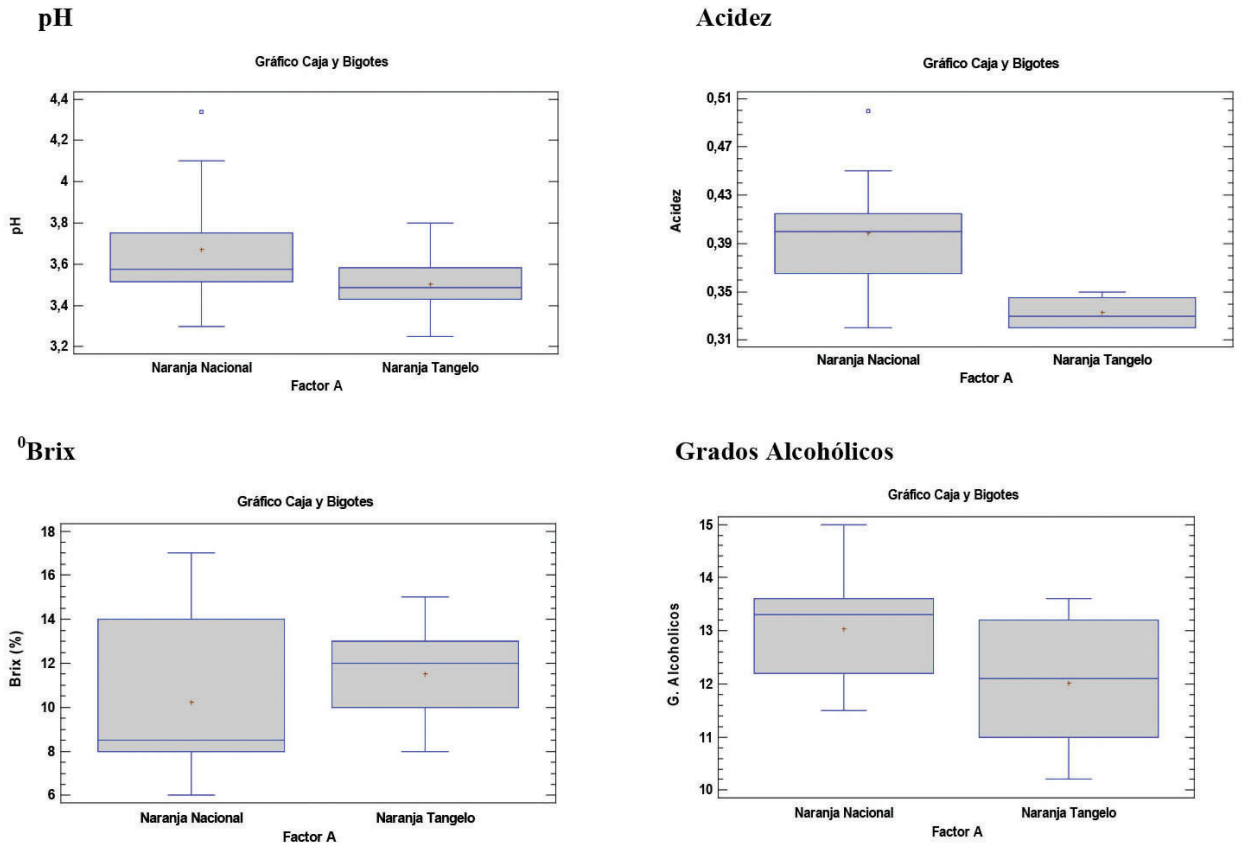

Fig. 1. Mean difference considering Physic-chemical analysis results in the study of two orange varieties a0: orange amount and a: tangelo. The chart shows ph, acidity, Brix degrees and alcoholic degrees mean difference. Tukey's rank test (p>0.05.

other hand, we did not see a considerable difference regarding to the citric acid as citric acid reference.

Regarding to the two types of yeast: dry yeast (c_0) and wine yeast (c_1) in orange wine obtaining (*Citrus sinensis*) through the application of pectinase enzyme (PEC-600) as clarifier (Figure 3), pH values of 3.05 in C0 and 3.67 in C1, were found, lower amounts than the ones registered by (Ferreyra, 2006), averaging 4.16 ± 3.55. In acidity, amounts of 0.35% in c_0 and 0.37% in c_1 were obtained, which resulted lower than the 0.59 ± 0.97 represented by Olivero *et al.*, (2010). Reported amounts of Brix degrees of 9.08 for C_1 and 12.66 in C_0 were higher than those obtained by Corazza *et al.*, (2001) that were in the order of 8% of solids. Also, alcoholic degrees were of 12.3% in C_1 and 12.74% in C_0. These amounts coincide with the ones obtained by Selli *et al.*, (2003) namely 12.2% of alcohols.

The Graphic N°3, shows a significant difference between: dry yeast and wine yeast, regarding the ph. and Brix amounts obtained in the Tukey's signification test (p>0.05) placing the highest ph. value in wine obtained from wine yeast, same happened with Brix degrees' content, this could involve better wine yeast conditions because a less acid product was obtained. Although sugar conversion was lower than the one in the yeast utilization, this might be because of the pectinase influence. On the other hand, No significant differences could be seen regarding to acidity and alcoholic degrees, although dry yeast showed higher numeric amounts (alcoholic degrees), which could be proportional to the Brix degrees' amounts.

In the figure 2 it can be observed in what concerns pH., the interaction in which intervened (National Orange +0,4% of pectinase and wine yeast), presents, higher amounts this results, less acidification than the remaining interactions. These amounts do not coincide with acidity considering this interaction presents higher amounts with respect to Brix degrees, interaction (National Orange+0.2% of pectinase + dry yeast), presented

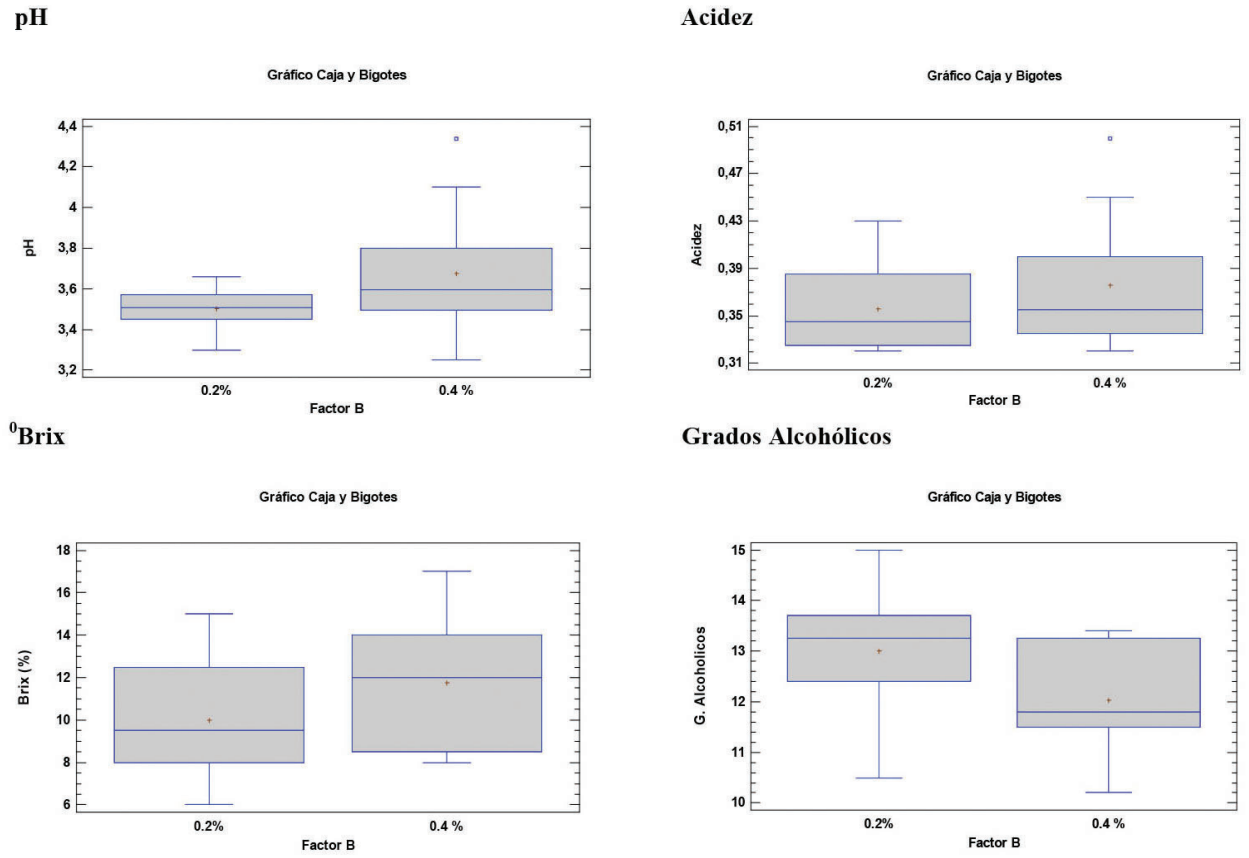

Fig. 2. Results of physic-chemical analysis of orange wine considering two pectinasa concentrations b_0: 2% y b_1: 4%, the Chart indicates pH, acidity and Brix degrees results. Tukey's ranks test (p>0.05).

the lowest value, which involves a better conversion of sugars in alcohol, this concurs with alcoholic degrees obtaining because this interaction presented the highest alcoholic degree. (13.9).

The figure 3 shows the percentage of wine purity under pectinase enzyme action. The amounts were taken depending on the number of suspended solids present in the fermented beverage, considering the percentage of purity after centrifugation, for which the solid that precipitated giving way to pure wine was measured. This allows to see that the best treatment contain 10% solids, that is to say with the help of this enzyme we can achieve a 90% of purity by employing: National Orange + 0.2% of pectinase + dry yeast. This implies that the application of this enzyme should take into account other factors such as the variety, it is important to consider that national orange (Ecuador), has its own characteristics namely a thicker peel with its different components that could be part of the wort and this leads to other considerations that could be discussed.

CONCLUSION

The combination of levels of National Orange *pectinase concentrations of 0.2%*dry yeast allows to obtain a final product with a 90% clarity in sediments with excellent organoleptic characteristics.

The application of the PEC-600 enzyme in the orange fermented beverage obtaining (*Citrus sinensis*), improves its chemical characteristics and phenolic composed extractability through sediment degradation in the mature period. Scalding stage was performed in order to avoid oxidation and take advantage of the highest content in the juice Orange extraction.

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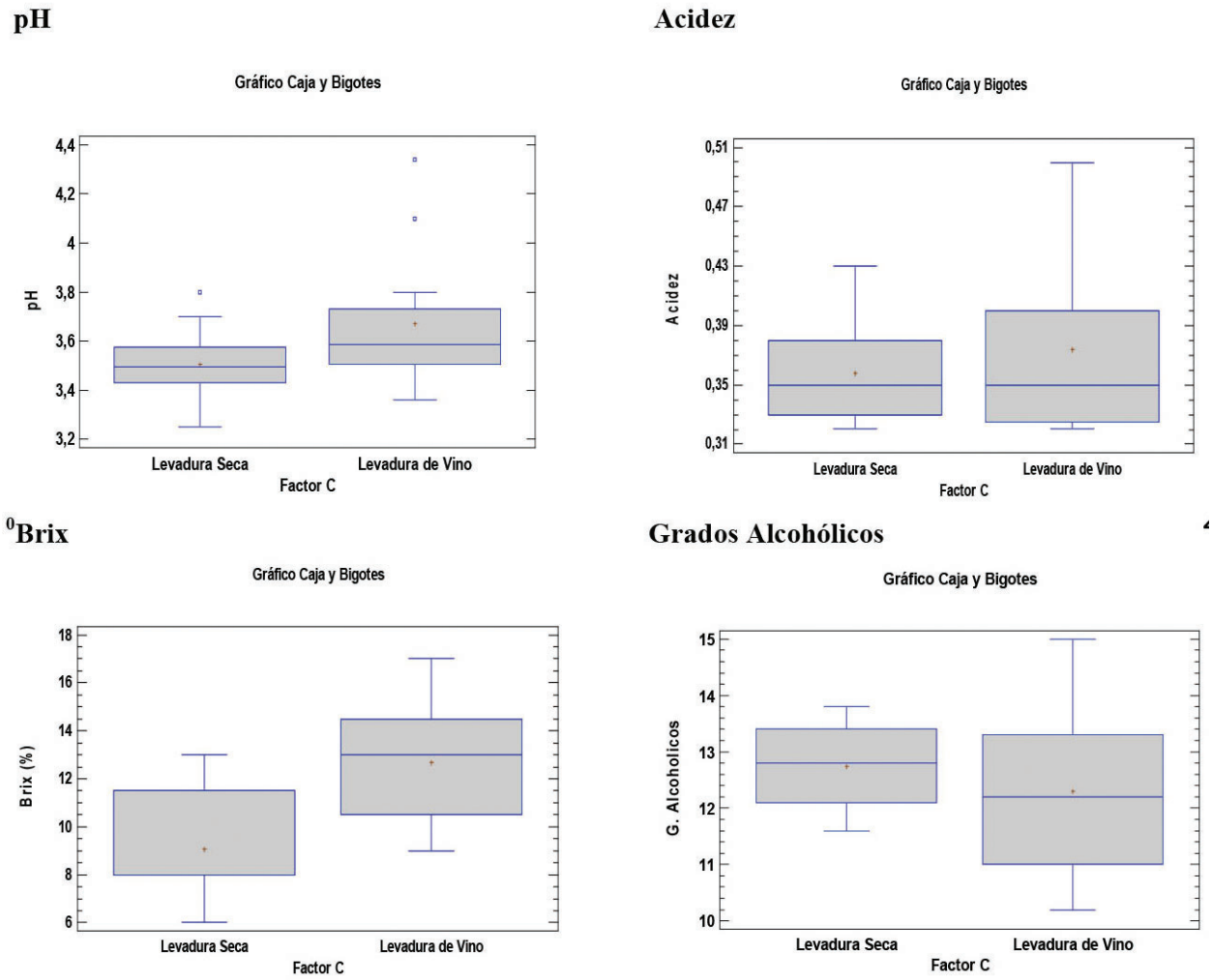

Fig. 3. Tukey's test results (p>0,05) in Physic-chemical analysis considering two types of yeast: dry yeast (c_0) and wine yeast (c_1), the chart shows pH, acidity, Brix degrees and alcoholic degrees data.

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