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Effect of fermentation conditions (yeast content and fermentation time) on the ethanol and bioactive compounds of Ca na (*Elaeocarpus hygrophilus* Kurz) wine by using response surface methodology

Ca na (*Elaeocarpus hydrophilus*) fruits were used as a raw material for wine processing, which will help increase the value, diversify products from Ca na fruit, and increase income for growers. Optimizing fermentation by response surface methodology (RSM) was conducted with 2 factors including yeast content [0.1 – 0.3 % w/w (compared to raw materials)] and fermentation time (8 – 12 days). The central composite design used for optimizing the effect of yeast content and fermentation time were in agreement with the quadratic regression models well with $R^2 > 0.80$. Based on the response surface plots, the optimal parameters of fermentation were the initial yeast addition of 0.24 % (w/w, compared to raw materials) and 11.5 days of fermentation time. Contents of ethanol and bioactive compounds (in 100 g of dry matter) were 9.32 % v/v, 9.18 g tannic acid equivalent (TAE), 3.66 g quercetin equivalent (QE) and 3.242 g TAE, respectively.

Descriptors: *Elaeocarpus hygrophilus*, ethanol, fermentation time, optimize, *Saccharomyces cerevisiae*

1 Introduction

Ca na (*Elaeocarpus hydrophilus*) fruit is a wild, ovate, about 3 cm long tree with a pointed tip and a pale yellow color when ripe (Fig. 1). The fruits contain 12 % carbohydrates, 0.024 % calcium, iron, vitamin C, phenolics, and triterpenoids [1–3]. Ca na fruits are mainly used to process products such as lactic fermentation, jam making, pickling with rice wine and at household scale, so the efficiency is not high. Using this source of raw materials for the production of a variety of beverage products will help increase the value and diversify products from Ca na fruits and raise income for growers. With the orientation of applying Ca na fruits to wine processing, it is also an effective way to use raw materials. However, controlling the fermentation process with *Saccharomyces cerevisiae* is a good technical measure to control the quality of the product. In addition, the response surface methodology (RSM) is based on the complete collection of experimental data, at the same time, determining the influence of the factors and reflected by the multivariate equation [4]. In this study, yeast content and fermentation time by using RSM were performed to determine the optimal conditions for Ca na wine fermentation.

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2 Materials and methods

2.1 Materials

Ca na (*Elaeocarpus hygrophilus*) fruits were harvested at Thoai Son district, An Giang province, Vietnam with the same maturity, weight and without damage and then transported to The Experimental-practical Area – An Giang University (Vietnam) within 1 hour. Ca na fruits were washed and collected the flesh.

Saccharomyces cerevisiae (3×10^{10} cfu.g^{-1}) was provided by Angel Yeast Co. Ltd (China). Before entering to the main fermentation *S. cerevisiae* passed the activation twice. In the first activation, 2 g of yeast was added into 10 mL of sterilized Hansen liquid medium in the test tube, incubated in the room temperature for 24 hours.

Fig. 1 Ca na (*Elaeocarpus hydrophilus*) fruit

After that, 10 mL of yeast medium that had been activated in the first time was put into the 250 mL erlen, which contained 45 mL Hansen medium and 45 mL of Ca na juice. Saccharose was added to the mixture for the fermentation to get 18 °Brix and the pH was modified to get 4.5 by using Na_2CO_3. The mixture was ready for the incubation and it was carried out at room temperature (28 – 30 °C) on the shaking machine in 24 hours to perform the 2nd activation of *S. cerevisiae*. In this state, the density of yeast cell was 10^8 cfu.mL^{-1} [5,6].

2.2 Experimental Design

The optimization of fermentation parameters was designed according to Response Surface Methodology (RSM) using Central Composite Design (CCD) 2^3+star (the STAGRAPHICS centurion software, version 16.1 was used), including 5 replications of central points and 12 numbers of treatments.

The experiment was arranged with two factors: yeast content (0.1 – 0.3 %) (compared to raw materials, w/w) and fermentation time (8 – 12 days). Each factor was surveyed with 5 levels, coded from – α to + α (with α = ± 1.4142). The level of encrypted variables and experimental layout was shown in table 1 and table 2.

Table 1 **Variable coding and survey levels of yeast content and fermentation time**

Variables	Codes	Levels				
		– α	– 1	0	+ 1	+ α
Yeast content (%)	A	0.06	0.1	0.2	0.3	0.34
Fermentation time (days)	B	7	8	10	12	13

Table 2 **Experimental layout according to central composite design**

Runs	Yeast content (%)	Fermentation time (days)
1	0.2 (0)	13 (+ α)
2	0.1 (– 1)	8 (– 1)
3	0.2 (0)	10 (0)
4	0.1 (– 1)	12 (+ 1)
5	0.34 (+ α)	10 (0)
6	0.06 (– α)	10 (0)
7	0.2 (0)	10 (0)
8	0.2 (0)	10 (0)
9	0.2 (0)	10 (0)
10	0.2 (0)	7 (– α)
11	0.3 (+ 1)	12 (+ 1)
12	0.3 (+ 1)	8 (– 1)

2.3 Experimental Methods

Each 500 g of flesh was crushed with water at ratio of 1:2 (w.v^{-1}) for 5 minutes and adjusted to 23 °Brix and pH 4.2 (with saccharose and Na_2CO_3, respectively); heated at 85 °C for 15 minutes; cooled to 40 °C; added Saccharomyces ceresiviae after 2nd activation and fermented at room temperature (28 – 30 °C) as designed in table 2.

2.4 Analytical Methods

2.4.1 Determination of °Brix and pH

°Brix and pH of samples was measured by using Atago hand-held refractometer (Japan) with 0 – 53 °Brix of detection level range and pH meter (Hanna, USA).

2.4.2 Determination of total acids content

The total acids content was measured by titration [7]. Acid-base titration is a quantitative analytical method to determine the concentration of an acid by precisely neutralizing it with a standard solution of a known base concentration (0.1 N NaOH). The concentration of total acids is calculated using equation 1:

$$A_x = \frac{n\ x\ V_1}{V_2\ x\ V_3} \qquad \text{(Eq. 1)}$$

Where, n is volume of 0.1 N NaOH to titrate (mL); V_1 is volumetric flask capacity (mL); V_2 is volume of raw sample (mL) and V_3 is volume of diluted sample to titrate (mL).

2.4.3 Determination of total phenolic content

Total phenolic content (mg TAE.kg^{-1} of dry matter) was determined using Folin-Ciocalteu reagent [8]. 150 µL of sample was mixed with 1200 µL of distilled water and 450 µL of 5 % (w.v^{-1}) Na_2CO_3 in the test tube. The mixture was added with 0.1 mL of Folin-Ciocalteu reagent and let at room temperature for 90 minutes. Phenolic in extract react with Folin-Ciocalteu to form a blue complex in alkaline medium that is phosphomolybdenum complex. The concentration of total phenolics was calculated as equal to standard tannic acid graph (TAE), y = 0.0021x + 0.0064 (R^2 = 0.9999), where y is the absorbance and x is the concentration of solution in the tube.

2.4.4 Determination of total flavonoid content

This assay was performed using aluminum chloride colorimetric method described by [9] with some modifications. The principle related to $AlCl_3$ creating a stable acid complex with the C-4 keto groups and the hydroxyl C-3 or C-5 group of the flavon and flavonol. 100 µL of sample was added with 1200 µL of distilled water and 30 µL of 5 % (w.v^{-1}) $NaNO_2$. The mixture was mixed with 10 % (w.v^{-1}) $AlCl_3.H_20$ (60 µL); 200 µL of 1 M NaOH and 110 µL of water. Solution was measured at 510 nm. The concentration of total flavonoid was calculated as equal to standard quercetin graph (QE), y = 8.2634x + 0.0182 (R^2 = 0.9999), where y is the absorbance and x is the concentration of solution in the tube.

2.4.5 Determination of tannin content

Tannin content was determined by Folin-Denis method [10]. 0.5 mL of sample was added 0.5 mL of distilled water, 0.5 mL of Folin-Denis and 2 mL of 20 % Na_2CO_3. The mixture was shaken well, warmed on boiling water for 1 minute and cooled to room temperature. Solution was measured at 700 nm. The concentration of tannin was calculated as equal to tannic acid graph (TAE), $y = 0.0098x + 0.0478$ ($R^2 = 0.9999$), where y is the absorbance and x is the concentration of solution in the tube.

2.4.6 Determination of ethanol content

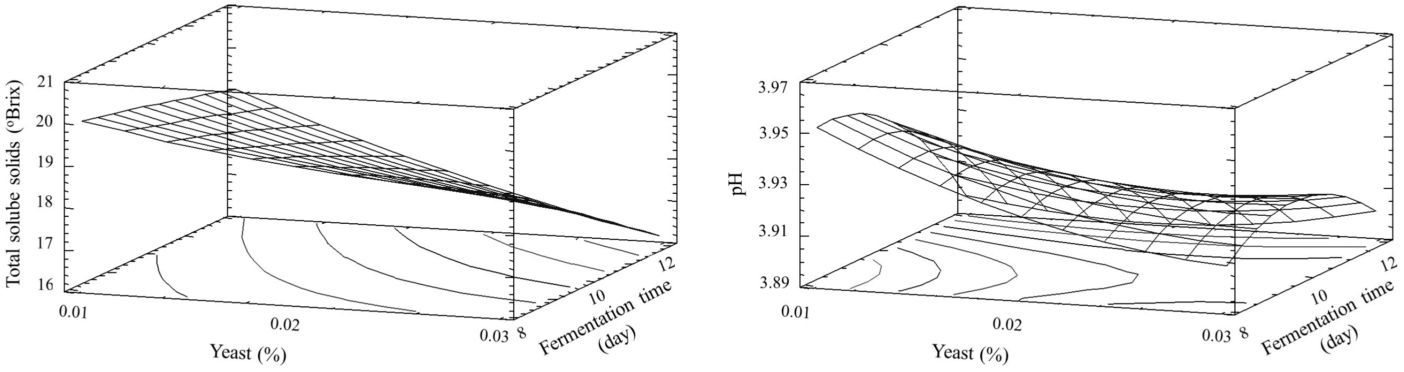
Ethanol content (% v.v^{-1}) was measured by distillation method [11]. A volume of 200 mL of sample was put into 500 mL distillation flask containing 20 – 30 mL of saturated NaCl; distilled; collected the distillate into a 200 mL volumetric flask and cooled to 20 °C. % ethanol was determined by ethanolmeter.

2.4.7 Determination of saccharose content

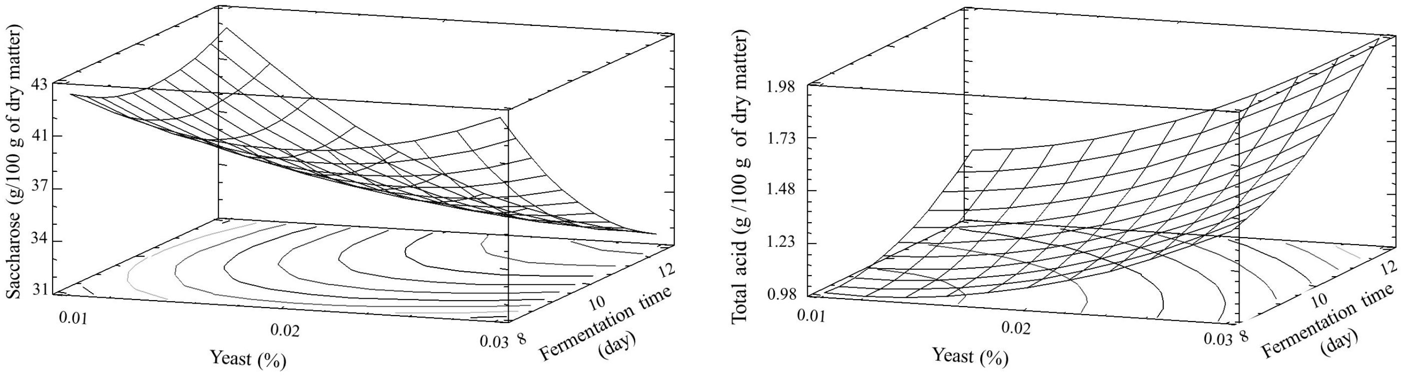
Saccharose content (g.100 g^{-1} of dry matter) was measured by the DNS method [12] with some modifications. A volume of 1 mL of sample was put in the test tube and then 2 mL of reagent DNS was added. This method is based on the oxidation of C = O group by 3,5-Dinitrosalicylic acid from a yellow colour to orangered in an alkaline medium. The tubes of the blank, solution of standard glucose and samples were placed in boiling water for 10 minutes. After that, 7 mL of distilled water was added to the tubes. The solution was analysed with an absorption of 575 nm. The concentration of saccharose was based on a standard curve of glucose, $y = 23885x + 0.126$ ($R^2 = 0.9999$), where y is the absorbance and x is the concentration of the solution in the tube.

2.5 Data analysis methods

Data were collected and processed by STAGRAPHICS Centurion 16.1 software for analysis variance (ANOVA), LSD test to conclude the difference between the average of experiments at 5 % confidence (P = 0.05) and Microsoft Excel software for calculating and graphing.



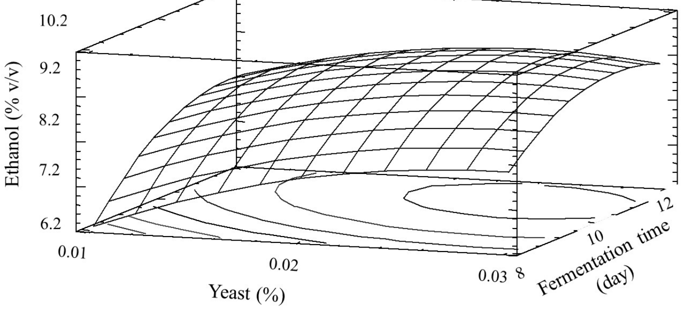
a. °Brix **b. pH**



c. Saccharose **d. Total acid**

$$Y = -16.191 + 37.134X_1 + 3.793X_2 + 46.432X_1^2 - 1.250X_1X_2 - 0.158X_2^2$$

$R^2 = 0.835$; $P_{\text{value (Lack-of-fit)}} = 0.154$



e. Ethanol

Fig. 2 The response surface and plots (a) °Brix, (b) pH, (c) saccharose, (d) Total acid and (e) ethanol influenced by yeast addition and fermentation time

The appropriateness of the predicted model was assessed through the correlation coefficient R². Equation optimize the response surface of general form experiments according to equation 2.

$$Y = \beta_0 + \sum_{j=1}^{k} \beta i X i + \sum_{j=1}^{k} \beta_{ii} X_i^2 + \sum_{j=1}^{k} \sum_{j=1}^{K} \beta_{ij} X_i X_j \quad \text{(Eq. 2)}$$

Where, Y is object function, β_o is constant, β_i is linear coefficient, β_{ii} is square coefficient, β_{ij} is interaction coefficient and X_i, X_j are survey variables.

3 Results and discussion

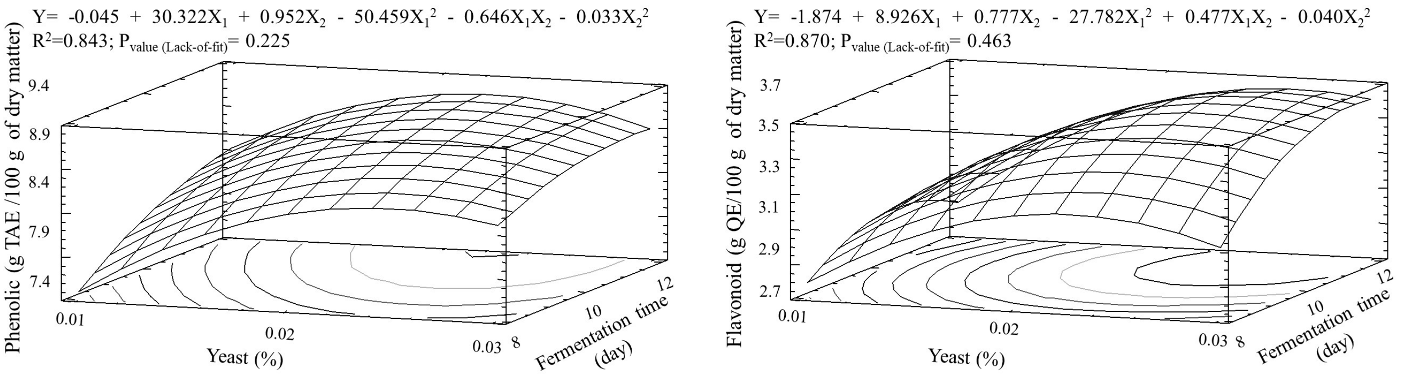
3.1 Effect of added yeast content and fermentation time on chemical composition and ethanol content of wine

Yeast growth involves the transport and assimilation of nutrients, followed by their integration into many cellular components for increased biomass and ultimately division. The main purpose of yeast cells is to reproduce instead of making alcohol. However, in wine fermentation, alcohol production and yeast growth are closely related. Ethanol is formed when yeast cells try to maintain redox balance and produce enough ATP to continue growing. In fact, ethanol cannot be produced efficiently without significant yeast cell growth. Therefore, in the process of wine fermentation, in addition to providing adequate nutrients for yeast to perform fermentation, the additional yeast content and fermentation time to avoid yeast overgrowth will lose ethanol [13]. The response surface model showing the effect of yeast addition and fermentation time on wine quality was constructed (Fig. 2).

The results showed that when increasing the added yeast content and wine fermentation time, the Brix and pH of the post-fermentation solution decreased, in contrast to the total acid content. In addition, the sucrose content decreased with fermentation time and decreased with increasing yeast content to an optimal value then increased with continued yeast content. In addition, the ethanol content produced increased to an optimal value, then gradually decreased with increasing yeast content and fermentation time. Results obtained with the optimal ethanol content when adding 0.25 % (w/w, compared to the weight of the material) and fermenting for 11 days.

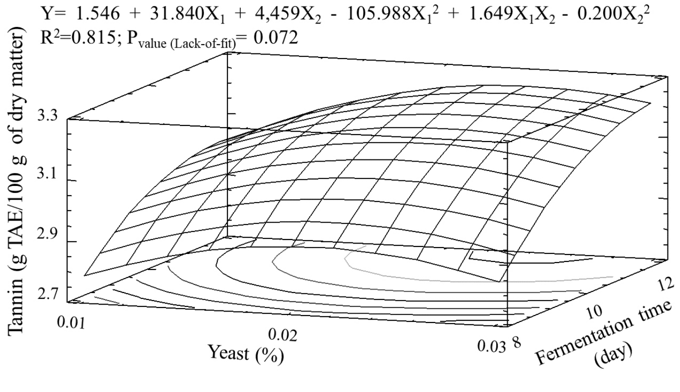
Although ethanol is the main product of fermentation, at a certain concentration it will again inhibit the growth and fermentation capacity of yeast [14]. During alcoholic fermentation, increasing ethanol will inhibit not only the vital activities of yeast but also the activity of many enzymes that convert sugar into alcohol [13]. When the alcohol concentration in the medium is about 2 %, the reproduction and growth of yeast will slow down and stop when the alcohol concentration is higher than 5%. In addition, the generation and release of CO_2 will make the fermentation environment agitated, prolonging the suspension state of the yeast.

S. cerevisiae reproduces vegetatively by budding, in which shoots emerge from different locations on the surface of the parent cell. If low yeast content is added, the possibility of further budding



a. Phenolic

b. Flavonoid



c. Tannin

Fig. 3 The response surface and plots (a) phenolic, (b) flavonoid, (c) tannin influenced by yeast addition and fermentation time

Table 3 **Effect of yeast addition and fermentation time on sensory evaluation and preferred level of Ca na wine**

Yeast (%)	Fermentation time (day)	Color	Odor	Taste	Preferred level	General comment
0.2	13	4.00^{a*}	4.00^{a}	4.0^{bc}	7.00^{a}	The aroma of Ca na fruit
0.1	8	3.00^{d}	3.25^{c}	3.50^{de}	5.25^{c}	Little aroma of Ca na fruit and sweet taste
0.2	10	3.63^{b}	3.63^{b}	3.75^{cd}	7.00^{a}	The aroma of Ca na fruit; harmonious sweet and sour taste
0.1	12	3.25^{cd}	3.50^{bc}	4.25^{ab}	6.00^{b}	The aroma of Ca na fruit and sweet taste
0.34	10	3.25^{cd}	3.25^{c}	4.50^{a}	6.00^{b}	The strong aroma of Ca na fruit and less sweet taste
0.06	10	3.00^{d}	3.25^{c}	4.25^{ab}	6.00^{b}	Little aroma of Ca na fruit and sweet taste
0.2	7	3.00^{d}	3.25^{c}	3.25^{e}	5.25^{c}	The aroma of Ca na fruit and sweet taste
0.3	12	4.00^{a}	3.50^{bc}	3.50^{de}	7.00^{a}	The strong aroma of Ca na fruit; harmonious sweet and sour taste
0.3	8	3.50^{bc}	4.0^{a}	4.25^{ab}	6.00^{b}	The strong aroma of Ca na fruit and slightly sweet taste

*Values are expressed as means of triplicate testing. Values with a different superscript in each column are statistically significantly different at 95 % statistically significant.

and fermentation time will be prolonged. These batches of fermentation often give poor efficiency and poor quality of finished products [13]. The higher the yeast content, the faster the alcohol production rate because the conversion of sugar into alcohol is carried out quickly, but it can hinder further fermentation [15]. In addition, with the initial yeast content being too high, the carbon source will be lost because about 10 % of glucose is used for yeast to increase biomass [16], resulting in ethanol content. not only did not increase, but also decreased [17]. Moreover, the prolongation of fermentation time will enhance the time and cost of the processing, and also reduce the efficiency of fermentation due to the competition for nutrients by yeasts. Besides, the longer the fermentation time, the lower the ester content will affect the quality of the product [17,18]. *Phong* et al. [19] also chose the fermentation time of soursop wine is 11 days to save time and cost when applied in practical production.

3.2 Effect of yeast content and fermentation time on the composition of bioactive compounds of Ca na wine

The response surface model showing the effect of yeast addition and fermentation time on the bioactive content of Ca na wine was constructed (Fig. 3). The results showed that with increasing yeast content and fermentation time, the content of phenolics, flavonoids and tannins of wine increased to an optimal value, and then gradually decreased. The results obtained optimal phenolic, flavonoid and tannin content (calculated per 100 g of dry matter) were 9.22 g TAE, 3.67 g QE and 3.25 g TAE, respectively, when adding 0.22 ÷ 0.26 % of yeast (w/w, compared to raw materials) and fermented from 11.30 ÷ 12.42 days. Previous studies also showed that the wine fermentation time is from 9 – 12 days [19, 20].

The main role of the yeast *S. cerevisiae* in the winemaking process is fermentation, however they are also responsible for the biochemical, enzymatic and physical reactions in this process and are therefore important to the composition of the wine. bioactive components of wine [21]. The effects of different yeast strains on phenolic compounds in wine have been studied by scientists [22]. Enzymes produced by yeast, such as pectinase and beta-glycosidase, influence the extraction of phenolics from raw materials and hydrolysis of glycosidic bonds of phenolic compounds [20]. In addition, yeast cell walls and autolysis can also interact with phenolic compounds in wine such as binding of mannoproteins – a component of the cell wall – with phenols [23]. In addition, the formation of esters during wine fermentation contributes to the fruit flavor and organoleptic characteristics of the wine. however, the difference in ester content was not statistically significant when increasing the fermentation time from 12 to 16 days [18]. Furthermore, with prolongation of fermentation time, esterification with methanol and ethanol reduces phenolic compounds due to yeast metabolism [25].

The results of sensory evaluation of wine presented in the table 3 showed that the product had a characteristic aroma of Ca na fruit, a harmonious sweet and sour taste, and the highest level of liking in the sample with the added yeast content of 0.2 ÷ 0.3 % (w/w, compared to raw materials) and fermented for 10 – 12 days.

Furthermore, the results of simultaneous optimization of many response surfaces have found the optimal parameters for Ca na wine to have a high content of ethanol and bioactive compounds,

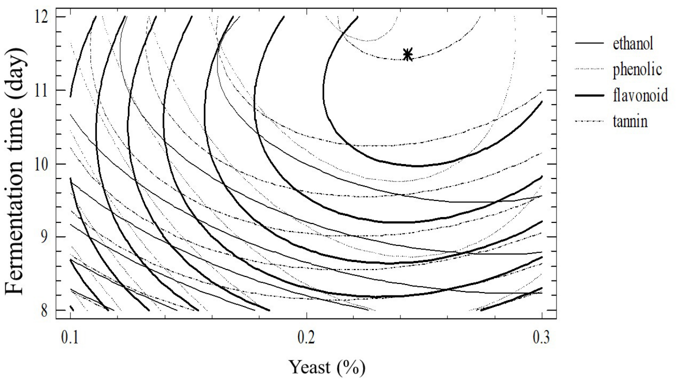
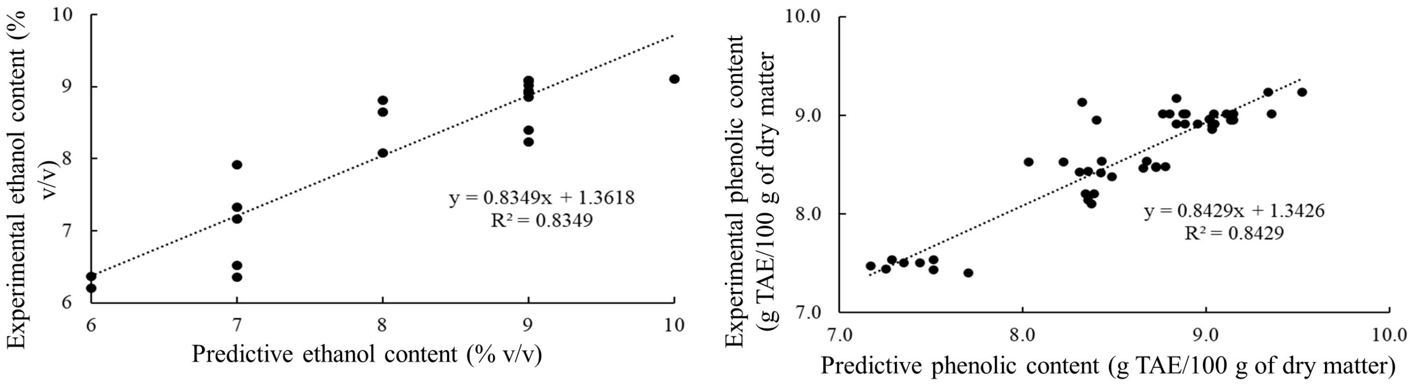
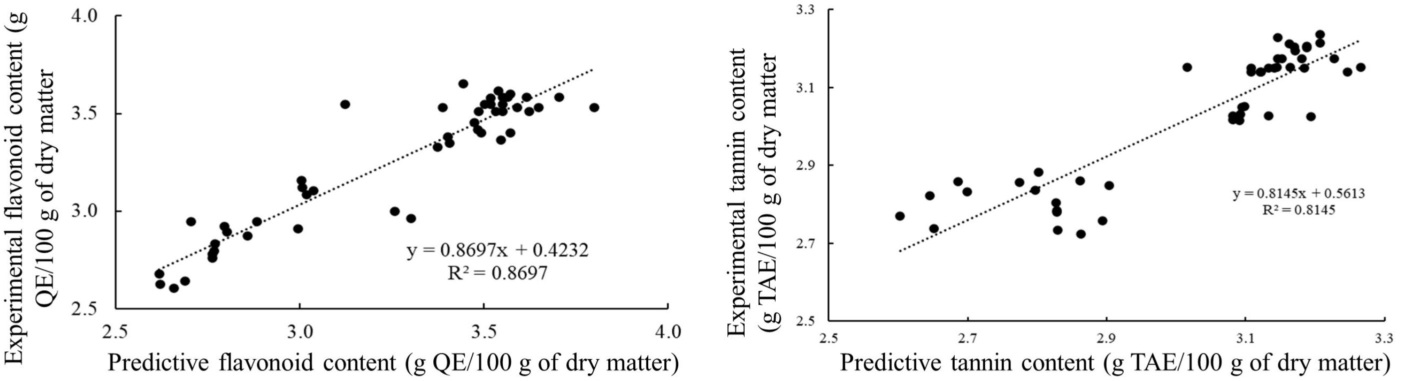


Fig. 4 **The contour plots of optimization of multiple response surfaces (ethanol, flavonoid, phenolic and tannin) to yeast addition and fermentation day**



a. Ethanol

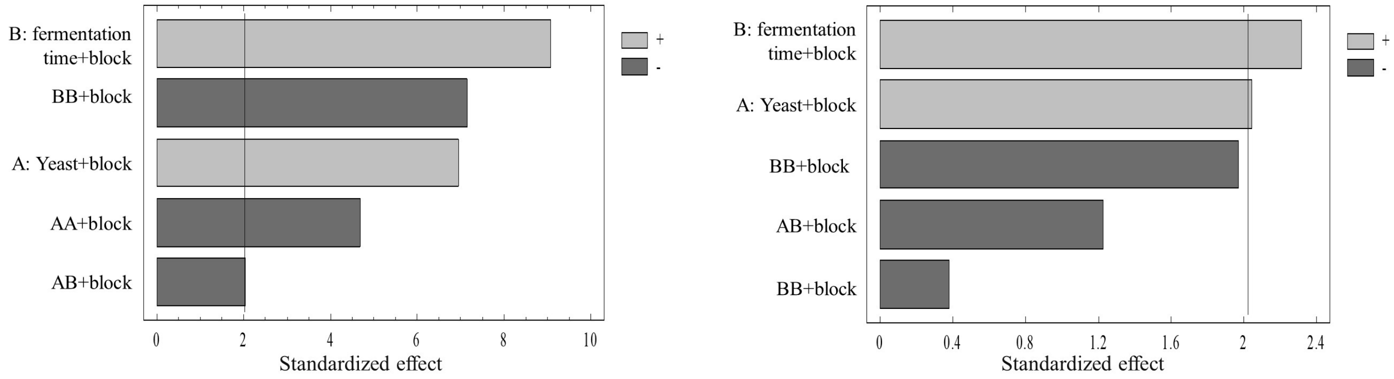
b. Phenolic



c. Flavonoid

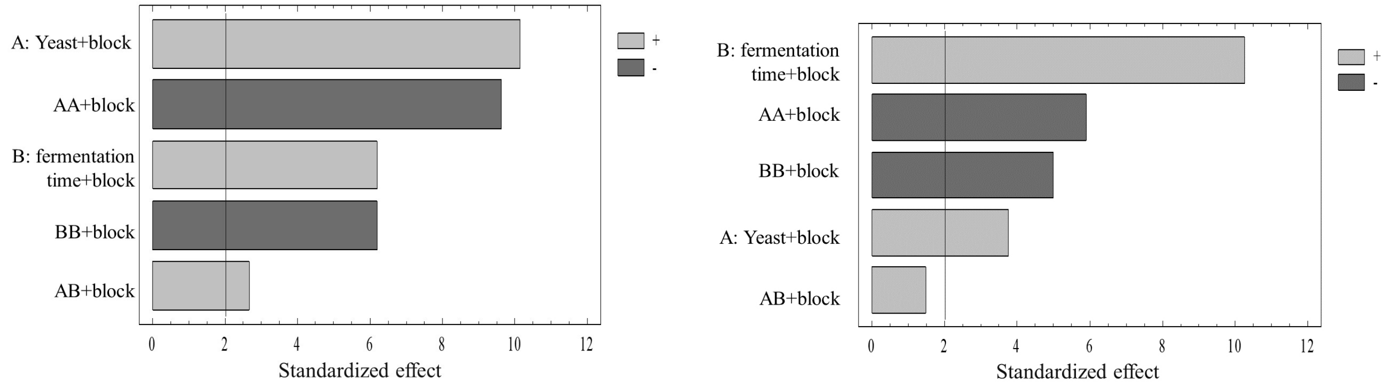
d. Tannin

Fig. 5 **The compatibility between experimental and theoretical data by regression equation for (a) ethanol, (b) phenolic, (c) total flavonoid and (d) tannin**



a. Ethanol

b. Phenolic



c. Flavonoid

d. Tannin

Fig. 6 **The level of influence of yeast addition and fermentation time and their interactions on the (a) ethanol, (b) phenolic, (c) total flavonoid and (d) tannin**

which was the addition of yeast with a content of 0.24 % and fermented for 11.5 days (Fig. 4). With these optimal parameters, the phenolic, flavonoid and tannin contents (in 100 g of dry matter) were 9.18 g TAE, 3.66 g QE and 3.24 g TAE, respectively; ethanol content was 9.32 % v/v.

In addition, the regression equations showing the relationship between the added yeast content and fermentation time to the ethanol content and bioactive compounds of the wine had $R^2 > 0.80$; Lack-of-fit values had no statistical significance ($p > 0.05$) (Fig. 2e and Fig. 3) and a high degree of compatibility between experimental data and predicted data ($R^2 > 0.80$) (Fig. 5) for the ability of the predictive model to fit the objective function is very high [26].

Furthermore the results from the Pareto plot (Fig. 6) also showed that the ethanol, phenolic and tannin contents were most affected by the fermentation time, while the flavonoid content was most affected by the added yeast content. Thus, the added yeast content of 2.4 % and the fermentation time of 11.5 days were selected as parameters for further studies.

4 Conclusion

The study had built regression equations showing the relationship between the added yeast content and fermentation time to the ethanol content and bioactive compounds of the wine. The optimal values for carrying out the fermentation processing were 0.24 % (w/w) of initial yeast content and 11.5 days of fermentation time. Ca na wine had a harmonious sweet and sour taste and the aroma of Ca na fruit.

Conflict of interest

The authors declare no conflict of interest.

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