Cider Production from King Mandarin (*Citrus nobilis* Lour.) and Its Antioxidant Activity

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Abstract. With the necessity of diversifying alcoholic beverages, cider has become a kind of drink that can fulfill this demand. This is because the cider will be diversified depending on the kinds of fruit that are chosen to be used for the cider fermentation. Therefore, this study aims to investigate the effects of dilution ratio, Brix, pH, and yeast concentration on the production of cider from king mandarin (*Citrus nobilis Lour.*), and to evaluate the analytical characteristics and antioxidant activity of the product. After the investigation, it can be claimed that the dilution of the juice causes the ethanol content to decrease, whereas the increase of Brix, pH, and yeast concentration makes the ethanol content increase. However, the proportional increase in the ethanol content with Brix, pH, and yeast concentration has its limitations. Specifically, when the Brix and the yeast concentrations were, respectively, higher than 16°Brix and 0.04%, the ethanol content tended to maintain the same. This is also the same when the pH was lower than 4.5. In addition, by using the DPPH and $ABTS^{\bullet+}$ methods, the antioxidant activity of cider is estimated to be lower than the one of the juice before fermentation, which is smaller than 3.78 times for the DPPH method and 3.76 times for the $ABTS^{\bullet+}$ method.

Keyword. Antioxidant activity, Cider, *Citrus nobilis*, King mandarin, *Saccharomyces cerevisiae*

1 Introduction

With the development of modern society, alcoholic beverages have become popular worldwide, but the issue of overusing these beverages has become a major concern in causing many unwanted incidents [1]. However, it still cannot be denied that these beverages have offered many benefits to human life. Many studies have pointed out that consuming these kinds of drinks at low levels of ethanol concentration will enhance the digestive system as well as mental health [2, 3]. Therefore, consuming low-ethanol beverages is an alternative way to obtain the benefit of alcoholic products instead of consuming beverages with higher ethanol concentrations. Cider is a low ethanol concentration beverage that is first fermented from apple juice. In addition, apple is not the only material that is used for cider fermentation, cider can also be fermented from different kinds of fruit depending on the differences in geographical locations and fruit sources.

Vietnam is a tropical country, which is suitable for growing fruit trees. Therefore, in Vietnam, some research has been carried out to investigate cider production from a variety of different kinds of fruit, such as *Docynia indica* fruit [4], strawberry [5], acerola [6], pitaya [7], etc. As many mentioned kinds of fruit, king mandarin, or known as *Citrus nobilis Lour.*, can also be used as the initial material for cider fermentation. This fruit is a cultivar of citrus fruit, which has a balanced flavor between sweetness and sourness, and is also rich in nutrients and health values, which can be included as anti-cancer, anti-inflammatory, antioxidant, cardiovascular protective and neuroprotective [8].

Despite having potential health benefits, studies targeting cider production from king mandarin are insufficient. Therefore, the objectives of this research were to investigate the effects of dilution ratio, initial Brix, pH, and yeast concentrations on the production of cider fermented from king mandarin, and to evaluate the analytical characteristics of the product and its antioxidant activity. Furthermore, this research will indirectly solve the problem of food waste from the excessive product, which is king mandarin. Therefore, this research will play a role in suggesting any beverage developer or company an idea of cider production from king mandarin. From that, the excessive of produced king mandarin will reduce, which also reduces food waste, and partly save the environment.

2 Materials and Methods

2.1 Materials and Chemicals

Materials: King mandarin was collected from Vinh Long province, Vietnam. Yeast *Saccharomyces cerevisiae* BV818 was purchased from Angel Group (distribution by ICFOOD Vietnam Co., Ltd.).

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Chemicals: Na_2CO_3, vitamin C, pectinase, $NaHSO_3$, tri-n-butyl phosphate (TBP), dinitrosalicylic acid (DNS), $K_2Cr_2O_8$, $KNaC_4H_4O_6.4H_2O$, NaOH 0.1N, H_2SO_4, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) are analytical grade chemicals from Xilong Scientific (China) and Merck KGaA (Germany).

2.2 Methods

2.2.1 Investigation of the Effect of Dilution Ratio and Initial Brix on the Cider Fermentation

King mandarin juice after being filtered was investigated with two dilution ratios, which were using pure juice and a ratio of 1:1 between juice and water. In addition, the filtered juice was also added an amount of sucrose to be adjusted to the desired Brix. There were a total of 6 levels of Brix to be investigated, and they were 10, 12, 14, 16, 18, and 20°Brix. After finishing the initial setup, the pH of the juice was also set to 4.5, and the juice will be pasteurized in 2 h by using 140 mg/L of $NaHSO_3$. Then, yeast powder was added to the juice of which the concentration is 0.04% (w/v), and the mixture was left to be fermented in 48 h at 30°C. After the fermentation, the product will be taken to measure ethanol content and reducing sugar content by respectively using TBP and DNS methods.

In the TBP method, a standard ethanol solution will be used to form the standard curve. The ethanol content of the sample will be measured by mixing 1 mL of TBP and 1 mL of the sample. In the mixture, 2 layers will be formed and 500 µL of the upper layer will be continuously mixed with 500 µL $K_2Cr_2O_7$. The mixture continuously forms layers, and the below one having cyan color will be used for the measurement. The measurement will be done with a wavelength of 595 nm (using a UV-Vis spectrophotometer; Genesys 10-S, Thermo Corporation, USA). The result will be compared with the standard curve to calculate the sample ethanol content [9].

In the DNS method, glucose will be used to form the standard curve. The reducing sugar content of the sample will be measured by mixing 2 mL of the sample and 2 mL of DNS solutions. The mixture will be heated in the water bath, and then let cool. Finally, the measurement will be done with a wavelength of 540 nm using a UV-Vis spectrophotometer (Genesys 10-S, Thermo Corporation). The result will be compared with the standard curve to calculate the sample ethanol content [10].

2.2.2 Investigation of the Effect of pH and Yeast Concentration on the Cider Fermentation

After successfully selecting the dilution ratio and initial Brix, the effects of pH and yeast concentration on the juice were spontaneously investigated. The process is similar to the previously described with four pH levels (4.0, 4.5, 5.0, and 5.5) and four yeast inoculations (0.02, 0.03, 0.04, and 0.05% w/v). Finally, the fermentation time was 48 h. After the fermentation, ethanol content and reducing sugar content of the product will be measured by TBP and DNS methods, respectively.

2.2.3 Evaluation of the Analytical Characteristics of the Product

The fermented product was evaluated with the following criteria: Physicochemical characteristics, heavy metals, and microbiological characteristics. All these criteria will be conducted based on the requirements of the regulation in Vietnam, which is regulated in QCVN 6-3:2010/BYT.

2.2.4 Evaluation of the Antioxidant Activity of the Product by Using DPPH Method

The antioxidant activity of the product was evaluated by the IC_{50} value of the product after using the DPPH method. Vitamin C was used as the control sample, and the juice before the fermentation and the product were used as the experimental samples. 0.2 mL of each sample was mixed with 1 mL of 39.4 µg/mL of DPPH and was left to react in the dark for 15 min. Then, the absorbance at 715 nm wavelength was measured by UV-Vis spectrophotometer (Genesys 10-S, Thermo Corporation). In addition, for each sample, a set of concentrations was used for the investigation, which was 8, 10, 12, 14, and 16 µg/mL for vitamin C; 40, 80, 120, 160, and 200 µL/mL for the juice before the fermentation; and 200, 250, 300, 350, 400 µL/mL) for the final product [11].

2.2.5 Evaluation of the Antioxidant Activity of the Product by Using ABTS$^{\bullet+}$ Method

Besides using the DPPH method, the ABTS$^{\bullet+}$ method can also be used to evaluate the antioxidant activity of the product. The overall process of this method is similar to the DPPH method with the same set of concentrations for the juice before fermentation and the product, but instead of using vitamin C, trolox solution is used as the control sample of which the used concentrations is 12.5, 25, 50, 100, 200, and 400 µM. The process started with the preparation of ABTS$^{\bullet+}$ by mixing 2 mL of 7mM of ABTS•+ with 2 mL of 2.45 mM of $K_2S_2O_8$ and letting it react in the dark for 16 h. The wavelength used for the investigation was 734 nm using a UV-Vis spectrophotometer (Genesys 10-S, Thermo Corporation). After that, the mixture of 10 µL of each sample at different concentrations and 990 µL of ABTS$^{\bullet+}$ was left to react in the dark for 6 min [12].

3 Results and Discussions

3.1 The Effect of Dilution Ratio and Initial Brix on the Cider Fermentation

With the differences in the dilution ratio and Brix, the ethanol contents of each combination resulted differently (Table 1). Specifically, among the diluted treatments, the highest ethanol content was recorded to be 6.11% (v/v), which is the result of the combination of using the diluted juice and the adjustment of Brix to 20°Brix. Similarly, the combination of pure juice and 16°Brix yielded the highest ethanol content, which was 6.90% (v/v). In contrast, the smallest ethanol contents when using pure juice and diluted juice were 4.50% and 4.39% (v/v), respectively.

With the results, the range of ethanol contents when using different dilution ratios was estimated to be from 4.39% to 6.11% v/v for using diluted juice and from 4.50% to 6.90% for using pure juice. In order to ensure the product ethanol content will yield in the range of 4-6%, and to reduce the cost of production, the dilution ratio of 1:1 between the juice and the diluted water is more reasonable, which corresponds to the previous study of the investigation of low-alcohol fermented passion fruit juice [13].

With the increase in Brix from 10 to 14°Brix when using the diluted juice, the ethanol content increased from 4.39% to 5.91% (v/v), which was correlated with previous research [4] showing that Brix was proportional to the yields of ethanol contents. Despite an increase in the ethanol content from 4.39 to 5.91% (v/v) when increasing Brix from 14 to 20°Brix, these results were not different from each other in the mean of statistical significance with $p < 0.05$. In addition, the reduced sugar content was also reported to be increased proportionally with Brix, and in the range of Brix from 14 to 20°Brix, the reduced sugar contents were recorded to be 4.66, 6.69, 7.27, and 6.59 g/100 mL. However, only the reduced sugar content of the 14°Brix treatment showed a difference in the mean of statistical significance (p-value < 0.05) with the 3 other treatments. Based on the obtained results, the dilution ratio and the Brix level were chosen to be a ratio of 1:1 and 16°Brix, which was chosen to ensure that the product ethanol content would yield in the range of 4-6% v/v, to yield the highest reduced sugar content and to reduce the cost of production for the next experiments.

Table 1. Results of ethanol content after fermentation with different dilution ratios and Brix values.

Treatment	Factors		Ethanol content (% v/v)	Reduced sugar content (g/100 mL)
	Dilution ratio	Brix		
1	Pure	10	5.19^{d}	0.74^{f}
2	Pure	12	4.50^{e}	0.71^{f}
3	Pure	14	5.83^{bc}	0.80^{f}
4	Pure	16	6.90^{a}	3.14^{d}
5	Pure	18	6.35^{b}	5.83^{c}
6	Pure	20	6.12^{b}	8.02^{a}
7	1:1	10	4.39^{e}	0.76^{f}
8	1:1	12	5.32^{cd}	2.43^{e}
9	1:1	14	5.91^{b}	4.66^{cd}
10	1:1	16	5.89^{b}	6.69^{b}
11	1:1	18	5.95^{b}	7.27^{b}
12	1:1	20	6.11^{b}	6.59^{b}

***Note:** Each result is the average of 3 replicates. Different lowercase letters indicate significantly different values within each column at p-value < 0.05.

3.2 The Effect of pH and Yeast Concentration on the Cider Fermentation

In previous studies, the ethanol content tended to increase proportionally with the increase in pH and the yeast concentration [4,13], which was caused by the higher yeast inoculation accelerated rate of conversion from sucrose into ethanol. Therefore, when more yeast appeared in the juice, the ethanol content increased.

In Table 2, at the same pH, treatment with higher yeast concentrations yielded higher ethanol contents. Specifically, when pH was 4.0, the ethanol contents produced by the treatment with 0.02% to 0.05% of yeast increased from 5.30% to 6.60% (v/v). Hence, this trend was observed similarly in other pH treatments. Despite having an increase when there was an increase in yeast concentration, there was no difference in ethanol content yielded from 0.04% and 0.05% yeast inoculations (v/v). Overall, the data from Table 2 suggested that the ethanol content when adding 0.04% and 0.05% of yeast concentrations were no different in the mean of statistical significance (p-value < 0.05) at every pH level. Similarly, at the same yeast inoculation, the increase in pH led to an improvement in ethanol concentrations. Specifically, with 0.05% yeast concentration, the pH from 4.0, 4.5, 5.0, and 5.5 yielded 6.02%, 6.06%, 7.28%, and 7.63% ethanol contents of each treatment, respectively. From this result, the ethanol contents of the treatments at pH 4.0 and 4.5 were not different from each other in the mean of statistical significance ($p<0.05$). Meaning that the ethanol content only increased when the pH of the treatment was adjusted from 4.5 to 5.5.

Table 2. Results of ethanol content after fermentation with the investigation of pH and yeast concentration.

Treatment	Factors		Ethanol content (% v/v)	Reduced sugar content (g/100 mL)
	pH	Yeast concentration (% w/v)		
1	4.0	0.02	5.30^{k}	10.82^{a}
2	4.0	0.03	5.48^{j}	7.75^{de}
3	4.0	0.04	5.99^{h}	6.93^{f}
4	4.0	0.05	6.02^{h}	6.60^{gf}
5	4.5	0.02	5.39^{jk}	9.00^{c}
6	4.5	0.03	5.62^{i}	7.99^{d}
7	4.5	0.04	6.04^{h}	7.64^{de}
8	4.5	0.05	6.06^{h}	7.30^{e}
9	5.0	0.02	6.49^{g}	9.50^{b}
10	5.0	0.03	6.80^{f}	9.32^{b}
11	5.0	0.04	7.16^{d}	8.91^{c}
12	5.0	0.05	7.28^{c}	8.60^{cd}
13	5.5	0.02	6.99^{e}	9.04^{c}
14	5.5	0.03	7.46^{b}	8.94^{c}
15	5.5	0.04	7.61^{a}	8.07^{d}
16	5.5	0.05	7.63^{a}	8.11^{d}

Note: Each result is the average of 3 replicates. Different lowercase letters indicate significantly different values within each column at p-value < 0.05.

3.3 Evaluation of the Analytical Characteristics of the Product

The analytical characteristics of the product were evaluated based on the regulation in QCVN 6-

3:2010/BYT from the Ministry of Health of Vietnam. The result of the comparison between the product characteristics and regulations is presented in Table 3.
The results in Table 3 indicate that the product is compatible with the requirements of the regulation. Specifically, the product's physicochemical characteristics, which were the methanol, hydrocyanic acid, and sulfur dioxide content, were all smaller than the limitation of the regulation. For heavy metals, such as cadmium, arsenic, mercury, zinc, and copper were not detected in the product. Similarly, some pathogenic bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* were also not detected in the product. Overall, the product demonstrated that its characteristics were appropriate for the regulation of QCVN 6-3:2010/BYT.

Table 3. Results of analytical characteristics of the product.

Analytical characteristics	Product	QCVN 6-3:2010/BYT
Methanol (mg/L)	657	< 10,000
Sulfur dioxide (mg/L)	36.4	< 150
Hydrocyanic acid (mg/L)	nd	< 70
Volatile acidity (mEq/L)	nd	-
Lead (mg/L)	nd	< 0.2
Cadmium (mg/L)	nd	-
Arsenic (mg/L)	nd	-
Mercury (mg/L)	nd	-
Zinc (mg/L)	nd	-
Copper (mg/L)	nd	-
Total aerobic microorganisms (CFU/mL)	39	$< 10^3$
Fungal (CFU/mL)	< 1	$< 10^2$
E. coli (CFU/mL)	< 1	0
Coliforms (CFU/mL)	< 1	0
Clostridium perfringens (CFU/mL)	< 1	0
Streptococcus feacalis (CFU/mL)	< 1	0
Staphylococcus aureus (CFU/mL)	< 1	-
Pseudomonas aeruginosa (CFU/mL)	< 1	-

***Note**: nd – not determined

3.4 The Antioxidant Activity of the Product by using DPPH Method

Figures 1 and 2 illustrate that the inhibition percentage of all three samples increased when the concentration of each sample increased. Specifically, when the concentration of vitamin C is increased from 8 µg/mL to 16 µg/mL, the percentages are recorded from 31.96% to 75.03%, respectively. Despite using different ranges of concentrations, both the juice and the cider are investigated at the concentration of 100 µL/mL and at this concentration there was a different result between the two samples. The percentage of inhibition of the juice at a concentration of 200 µL/mL is reported to be 87.03%, whereas the cider was only 26%. This means that both samples were investigated at the same concentration, but there was a change in the inhibition percentage before and after fermentation.

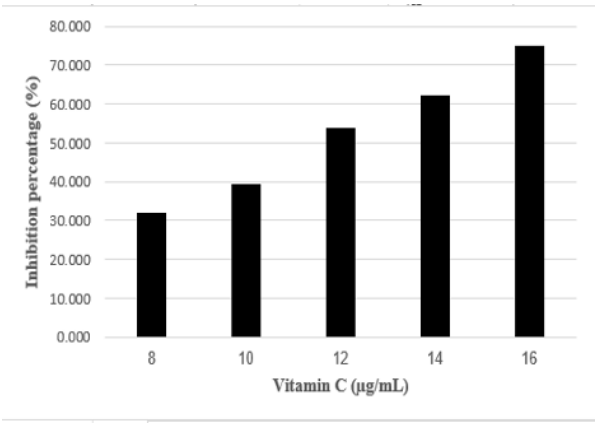

Fig. 1. DPPH free radical inhibition percentage of vitamin C.

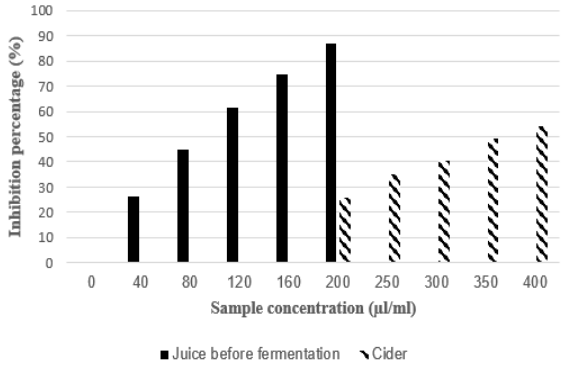

Fig. 2. DPPH free radical inhibition percentage of juice and cider.

As the results shown in Figures 1 and 2, the standard curve as well as the linear equation of each sample is established, and with the formed equation, the IC_{50} value of each sample is calculated. The IC_{50} value of a sample is the required concentration of a sample to inhibit 50% of free radicals in which the smaller the IC_{50} value is, the greater the antioxidant activity of a sample is [14]. The IC_{50} values of all samples are illustrated in Table 4.

Table 4. IC_{50} value of vitamin C, unfermented juice and cider when using DPPH method.

Samples	IC_{50} value
Vitamin C	11.27 µg/mL
Unfermented juice	96.37 µL/mL
Cider	363.94 µL/mL

*** Note:** Each result is the average of 3 replicates.

It can be seen that both the juice and the cider show significant antioxidant activity (Table 4). However, as mentioned, the smaller the IC_{50} value was, the greater the antioxidant activity of the sample. Therefore, the results from Table 4 show that the IC_{50} value of the juice before

fermentation was smaller by about 3.78 times than the IC_{50} value of the cider. In addition, with the calculated IC_{50} value of each sample, the needed concentrations of the juice and cider were respectively 96.37 µL/mL and 363.94 µL/mL, which was also equal to 11,27 µg/mL of vitamin C.

3.5 The Antioxidant Activity of the Product by using $ABTS^{\bullet+}$ Method

Figures 3 and 4 illustrate that the inhibition percentage of all three samples increases when the concentration of each sample increases. Specifically, when the concentration of vitamin C was increased from 12.5 µM to 400 µM, the percentages were recorded from 3.71% to 83.98%, respectively.

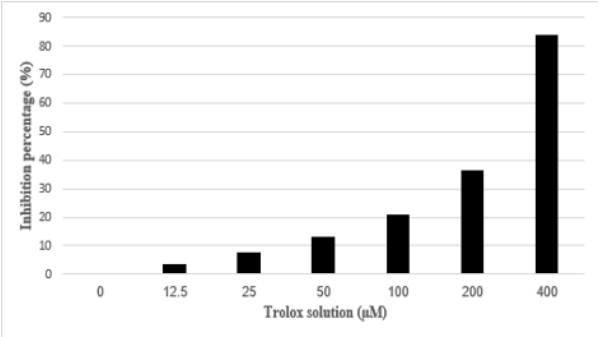

Fig. 3. $ABTS^{\bullet+}$ free radical inhibition percentage of trolox solution.

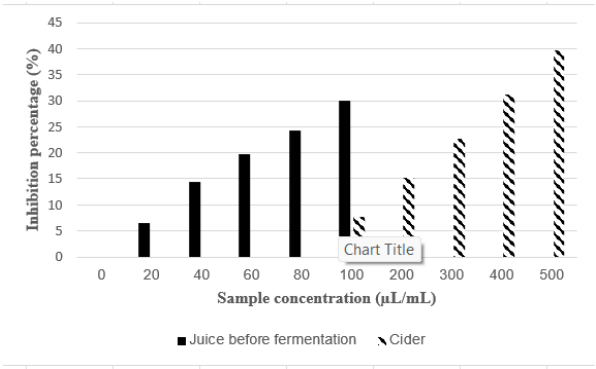

Fig. 4. $ABTS^{\bullet+}$ free radical inhibition percentage of juice and cider.

Despite using different ranges of concentrations, both the juice and the cider were investigated at the concentration of 100 µL/mL and at this concentration, there was a different result between the two samples which was similar to the DPPH method. The percentage of inhibition of the juice at the concentration of 100 µL/mL was reported to be 29.98%, whereas the cider was only 7.75%. This means that both samples were investigated at the same concentration but there was a change in the inhibition percentage before and after fermentation. Similar to the DPPH method, the IC_{50} value of each sample when using $ABTS^{\bullet+}$ was calculated and illustrated in Table 5.

Table 5. IC_{50} value of vitamin C, unfermented juice and cider when using $ABTS^{\bullet+}$ method.

Samples	IC_{50} value
Trolox	242.66 µM
Unfermented juice	168.63 µL/mL
Cider	633.86 µL/mL

***Note:** Each result is the average of 3 replicates.

In Table 5, it can be seen that both the juice and the cider show significant antioxidant activity. However, as mentioned, the smaller the IC_{50} value was, the greater the antioxidant activity of the sample. Therefore, the results from Table 5 show that the IC_{50} value of the juice before fermentation was smaller by about 3.76 times than the IC_{50} value of the cider. Meaning that the antioxidant activity of the juice was greater by about 3.76 times than that of cider. In addition, with the calculated IC_{50} value of each sample, it was believed that the needed concentrations of the juice and cider were respectively 168.63 µL/mL and 633.86 µL/mL, which was also equal to 242.66 µM of trolox solution.

It can be claimed that the product shows antioxidant activity after using DPPH and $ABTS^{\bullet+}$ methods. However, the results show that the product performed the antioxidant activity less greatly than the juice before fermentation, in which the ratio was 3.78 times for the DPPH method and 3.76 times for the $ABTS^{\bullet+}$ method. This result corresponded to a previous study on the determination of the antioxidant activity of *Syzygium cumini* fermented juice [15]. Even though there is a decrease in antioxidant activity of the product after fermentation, the product is still having its own benefits compared to only pure juice. Cider is a kind of beverage that can be preserved much longer than fresh juice. Normally, it is recommended to use fresh juice within 24 hours. In contrast, cider can be preserved within several weeks without changing the original flavor when the product is stored at a temperature of 0 – 2 °C [16]. Furthermore, with the need of beverages diversity, cider, specifically fermented from king mandarin, can be considered as a fresh candidate, which will accomplish the demand of brand-new products consumption.

4 Conclusions

The ethanol content of the product increased proportionally with the increase in Brix, pH, and yeast concentration. However, the ethanol content resulted in no difference in the mean of statistical significance ($p < 0.05$) when the Brix and the yeast concentration were, respectively, higher than 16°Brix and 0.04%. In contrast, when pH was lower than 4.5, the ethanol content resulted in no difference in the mean of statistical significance ($p < 0.05$). In addition, it can be claimed that the product is appropriate for the requirements of the regulation. Lastly, cider showed significant antioxidant activity but there was a decrease compared with the juice before fermentation.

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