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Effects of Supplementation with Needles of *Pinus densiflora* on the Fermentation Characteristics of Honey Wine

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솔잎 첨가 벌꿀주의 발효 특성

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Abstract

The goal of this study was to evaluate the effects of supplementation with the needles of *Pinus densiflora* on the fermentation characteristics of honey wine (*Pinus densiflora*-honey wine). Honey without supplementation with needles of *Pinus densiflora* (honey wine) was included as a control. Physiochemical changes were investigated during 30 days of fermentation at 20℃, and following aging. At the beginning of fermentation, pH and viable cell counts of *Pinus densiflora*-honey wine changed rapidly, while °Bx decreased gradually. Viable yeast counts reached maximum levels at 5 to 10 days of fermentation. At day 0, the pH of *Pinus densiflora*-honey wine was 3.8, while the non-supplemented honey wine had a pH of 3.4. Decease in °Bx was faster in *Pinus densiflora*-honey wine than in non-supplemented honey wine. Supplementation of honey with needles of *Pinus densiflora* prior to fermentation shifted the initial pH to a more neutral pH, and the presence of *Pinus densiflora* needles increased the fermentation speed. The final °Bx, pH, ethanol content, and total titratable acidity of *Pinus densiflora*-honey wine were 13.7°Bx, pH 3.05, 13.5%, and 0.37%, respectively. A sensory evaluation demonstrated that addition of 4% (w/v) fructose to honey wine supplemented with neddles of *Pinus densiflora* raised the level of product acceptability.

Key words : Fermentation, honey, mead, neddle of *Pinus densiflora*, wine.

INTRODUCTION

Honey has been used as a natural seasoning and a healthy food material for thousands of years, and it has been recently received much attention from the cosmetics and pharmaceutical companies (Lee *et al* 1997). Honey contains sugars, vitamins, minerals, and fatty acids, which provide some physiological functions, such as the release of fatigues and alcohol detoxication (Lee *et al* 1997, Jung *et al* 1999). The sugar composition of honey was detected to have the monohydrate fructose and glucose as the major carbohydrates, and some sucrose and oligosaccharides. The ratio of fructose/glucose was ranged between 2 and 28, and the excellent sweet tastes of honey might be due to high fructose contents (Kim & Rhee 1995).

In honey wine, the carbohydrates (monosaccharides and disaccharides) are converted to ethanol by the single-fermentation of yeasts (Jung *et al* 1999). So far, the honey wine has not been popular, because materials of honey wine were expensive, and the taste is too sweet, and easily contaminated by some spoilage microorganisms for a long fermentation period. However, the short fermentation process by adding substrates for yeasts and the filtration system diminished the problems of the honey-wine fermentation (Kim *et al* 1999).

Korean red pine (*Pinus densiflora* Siebold et Zuccarini) with needle-like leaves is distributed over the Korean Peninsula, and grows to 35 m. Pine leaves have been used as traditional medicines for neurodiseases, cardiovascular and liver diseases (Kang *et al* 1995). And the extract of pine leaves was investigated to have chlorophylls, vitamin A, C, proteins,

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lipids, enzymes, and essential oils (plant volatile terpenes) (Jung *et al* 1999). The physiological functions of pine leaves were performed for lipid metabolisms in serum (Kim *et al* 1991, Kim *et al* 2005) and for enzymes in serum and liver of high fat diet induced mouse (Kang *et al* 1996a, Kang *et al* 1996b). In addition, the pine leaves have the anti-aging activity of free radical scavenging (Choi *et al* 2001) and antibacterial activity (Choi *et al* 1997). Recently the life style seeking well-being increases the interest of wines containing medicinal plant extracts, which are expected to have several physiological functions and the preventive activity for illness (Kim & Park 2007, Lee *et al* 2008).

Several researchers have been tried to use the pine leaves and honey as a novel food material for the health improvement. However, little is known of the original copy for the fermentation of pine leaves-honey wine. In this study, we will report the fermentation characteristics of honey wine containing pine leaves extracts.

METHODOLOGY

1. Materials

Honey was collected from *Robinia pseudo-acaci* trees at Wonju (Kangwon, Korea) in 2006. The pine leaves were obtained from *Pinus densiflora* Siebold et Zuccarini at Samchuck (Kangwon, Korea) in May 2006, washed with tap water, and dried. Yeast (*Saccharomyces bayanus*, EC-1118) for wine-fermentation was purchased from Lallemand Co. (Mountreal, ON, Canada). Sucrose and potassium metabisulfite ($K_2S_2O_5$) were obtained from Cheiljedang Co. (Gangwon-do, Korea) and Sigma Co. (St. Louis, MO., USA), respectively.

2. Preparation of Pine Leaves-Honey Extracts

Pine leaves (2 kg) were mixed with honey (4 L), and were stored for 30 days at room temperature. For the adjustment of sugar contents to 24.0 °Bx, the honey (2,600 mL) filtrated with a sterile cotton-cloth was mixed with water (6,940 mL).

3. Fermentation of Honey Wine

Pine leaves-honey extracts (9,540 mL), diammonium hydrogen phosphate (10 g), which is a stimulator for yeast growth, and potassium metabisulfite (1 g) were mixed in 20 L of fermentor with air-lock for 16 hr (Table 1). For Honey wine fermentation, pine leaves-honey extracts was replaced by diluted honey (9,540 mL). Dry yeast (2.5 g) were transferred onto fermentor and then allowed to grow at 20～21℃ for 30 days. Samples (50 mL) were taken during fermentation period, and stored at −20℃ for the analysis.

Table 1. Proportion of *Pinus densiflora*-honey wine and honey wine

Component	*Pinus densiflora*-honey wine	Honey wine
Pinus densiflora extract (mL)[1)]	9,540	−
Diluted honey (mL)[2)]	−	9,540
$(NH_4)_2HPO_4$ (g)	10	10
$K_2S_2O_5$ (g)	1	1
Dry yeast (g)	2.5	2.5

1) Honey extracts of *Pinus densiflora* were prepared by mixing of honey(4,000 mL) and needle of *Pinus densiflora* (2 kg) at room temperature for 30 days.
2) Honey was diluted with distilled water to give diluted honey (24.0°Bx).

4. Analysis of Carbohydrates

The sugar contents of fermented mixture were determined by the hand-held refractometer (model N-1α, ATAGO, Japan). Quantitative determination for glucose, fructose, sucrose, and oligosaccharides was conducted by HPLC with a refractive index detector and gel filtration column (300×8 mm, Shodex Ionpack KS-802, Japan) using deionized water as a mobile phase (0.4 mL/min).

5. Determination of pH and Acidity

The change of pH in fermented mixture was measured by pH meter (model 725p, Istek Co., Seoul, Korea). For determination of total titratable acidity (TTA), the fermented mixture (10 mL) with 1～2 drops of phenolphthalein was titrated with 0.1N NaOH. TTA was calculated by the following equation using consumed amounts (mL) of 0.1N NaOH at the end-point (pink color).

Total titratable acidity % (TTA, %) =
[(mL of 0.1 N NaOH) × (N NaOH) × 0.075
(DL-tartaric acid coefficient) 100] / mL sample

6. Determination of Alcohols and Fusel Oils Contents

The alcohols and fusel oils of fermented mixture was measured by a gas chromatograph (6890, Agilent Technologies

Inc., Santa Clara, CA, USA) equipped with HP-INNOWax column (0.25 μm, 30 m × 0.25 mm, Agilent Technologies Inc.). The column temperature was programmed to hold constant at 35℃ for 5 min, to increase to 150℃ with 5℃/min, and to 250℃ with 20℃/min, and to hold at 250℃ for 2 min. Injection volume was 10 μL, and the temperature of injection port was 225℃. The temperature of the detector port with FID detector (split ratio was 10:1) was 260℃.

7. Assessment of Viable Yeast Number

The diluted fermented-mixture was spread on YPD agar plate (DIFCO, St. Louis, MO, USA) and incubated at 30℃ for 48 hr under aerobic conditions, and the viable yeasts were counted. Result was expressed as the average of triplicate.

8. Color Measurement

The color of honey-wine was determined by spectrophotometer (UV-visible spectrophotometer UV-1650 PC, Shimadzu, Japan) using 10 mm quartz cuvette, and the distilled water was used as the blank. Total phenol content, the brightness, the anthocyanin content, colority and shade were expressed as A_{280}, A_{420}, A_{520}, A_{420}+A_{520}, and A_{420}/A_{520}, respectively.

9. Statistical Analysis

Results were analyzed using 1-way analysis of variance (ANOVA) at 95% level of significance (Albright *et al* 1999). Experiments were carried out in triplicates. The results were presented as mean standard deviations (SD). Thestatistical analysis of sensory evaluation was performed using SPSS 12.0 software. The preferences for color, flavors, and the taste of honey-wines were evaluated by Friedman test, which is the nonparametric method (the number of samples <30). Statistical significance was considered at p<0.05.

RESULTS AND DISCUSSIONS

1. Characteristics of Honey Wines

The pine leaves were extracted by honey with a high osmolarity and subsequently used for wine fermentation after supplemented with nitrogen source. Potassium metabisulfite ($K_2S_2O_5$, 105 ppm) was added to fermented mixture for the prevention of oxidation, the color stability and the spoilage prevention. Honey without supplementation of neddle of *Pinus densiflora* (Honey wine) was also included as control. The supplement of sugars is necessary for the fermentation of fruit wines, such as grape wine and Korean Black Raspberry wine (*Bokbunjaju*, *Rubus coreanus* Miquel). However, the honey wine had the high sugar contents so that a dilution process was necessary for the success fermentation. The carbohydrate contents of *Pinus densiflora*-honey wine were fructose > glucose > sucrose, with 1.4 of the fructose/glucose-ratio (Table 2). Similar results were found in honey wine. These results were consistent to other reports regarding sugar composition of honey (Lee *et al* 1997, Kim & Rhee 1995). The ratio of fructose / glucose was further increased, as the fermentation proceeded. The fructose/glucose of 0 day, 14 day and 30 day fermentation of *Pinus densiflora*-honey wine were 1.4, 2.8 and 2.9, respectively. Based on this, it is expected that the sweetness of honey wine is due to the presence of fructose in the honey wine (Lee *et al* 1997, Kim & Rhee 1995). Several researchers recommend that initial sugar content should be between 22 to 24°Bx because it affects ethanol concentration and fermentation progress (Kim SK 1996, Kim *et al* 2008). Kim *et al* (2008) found that the mulberry wine fermented at 24°Bx showed excellent characteristics in terms of ethanol production. In the present study, the initial sugar content of both *Pinus densiflora*-honey wine and honey wine was equally 24°Bx. It is interesting to note that decrease in °Bx was faster in *Pinus densiflora*-honey wine than Honey wine (Fig. 1A).

Not only carbon sources, but also nitrogen sources are im-

Table 2. Changes in the relative sugar compositions (%) in *Pinus densiflora*- honey wine and honey wine during alcohol fermentation at 20℃ for 30 days

	Fermentation period		
	Days 0	Days 14	Days 30
Pinus densiflora-honey wine			
Sucrose	11.0	17.7	28.6
Glucose	36.5	22.0	18.3
Fructose	52.5	60.5	53.2
Fructose/glucose	1.4	2.8	2.9
Honey wine			
Sucrose	7.8	13.9	18.7
Glucose	33.9	23.1	17.7
Fructose	58.2	62.9	63.6
Fructose/glucose	1.7	2.7	3.6

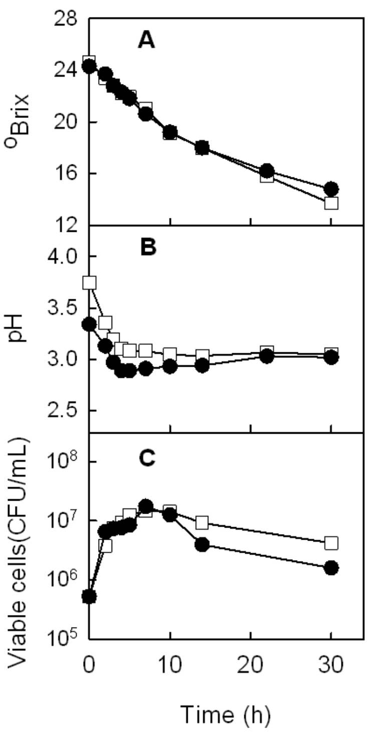

Fig. 1. Changes of sugar concentration (°Bx) (A), pH (B), and viable yeast cells(colony forming unit/mL) (C) of *Pinus densiflora*-honey wine and honey wine from alcohol ferentation at 20℃ for 30 days. Open symbol; *Pinus densiflora*-honey wine, closed symbol; Honey wine.

portant for honey wine fermentation (Jung *et al* 1999, Hwang *et al* 2004). In the optimization of the water melon wine fermentation using 17 *Saccharomyces* sp., Hwang *et al* (2004) reported that 0.2% (w/v) of $(NH_4)_2HPO_4$ among several nitrogen sources provided the highest ethanol contents (12.4%). Otherwise, Jung *et al* (1999) reported that ethanol level was 13.7% (v/v) of the fermented mixture containing 0.1% $(NH_4)_2SO_4$, 0.05% K_3PO_4, 0.005% $NaHSO_4$, 0.002% peptone, 0.144% tartaric acid, 0.233% malic acid, 0.0005% thiamine, 0.00025% Ca-pantothenate, 0.0002% inositol, 0.000025% pyridoxine, and 0.000002% biotin. In the present study, $(NH_4)_2HPO_4$ (0.1%) was used as sole nitrogen source for fermentation.

2. Change of pH and Viable Yeast Number

At day 0, the initial pH of *Pinus densiflora*-honey wine and honey wine were 3.8 and 3.4, respectively. In both honey wines, decrease in pHs were only seen within 5 days in fermentation. For instance, the pH of fermented mixture in *Pinus densiflora*-honey wine was decreased from pH 3.8 (0 day) to pH 3.1 (4 day) and pH 3.0 was maintained throughout extended fermentation period (Fig. 1B). For the fermentation of *Citrus*-honey wine, the change of pH was from pH 4.4 (0 days) to pH 2.9 (13 days), and for *Maesil* (*Prunus mume*)-honey wine was from pH 3.1 (0 days) to pH 3.0 (13 days). *Citrus*-honey wine and *Maesil*-honey wine fermented successfully, but low concentration of hydrogen ion (pH 2.8) made the honey wine fermentation difficult. Based on these results, authors suggested that adjustment of initial pH in fermentation medium is needed for the successful ethanol fermentation (Jung *et al* 1999). Taken together with the data from our work and others, it can be concluded that supplementation of needle of *Pinus densiflora* to honey prior to fermentation shifted the initial pH to more neutral pH, so that the presence of needle of *Pinus densiflora* in honey wine increased the fermentation speed.

Fermentation rate in honey wine is dependant on the concentration of minerals. Potassium contents (< 300 ppm) provide the decrease of pH to below 2.7, so that the honey wine fermentation was terminated (Schramm K 2005). Leaves of Korean domestic *Pinus densiflora* contain 514 mg% of potassium, 294 mg% of calcium, 84 mg% of magnesium, 18 mg% of manganese, 16 mg% of sodium, 5 mg% of iron, and 19 mg% of phosphorous and, among minerals, the potassium content showed the highest value (Chung *et al* 1996). Honey-extraction of minerals from pine leaves for a long time may supplement the potassium for successful honey wine fermentation.

Among *S. bayanus*, *S. cerevisiae*, *S. uvarum*, *S. formosensis* and *S. sake, S. bayanus* is reported to be the best yeast strain for honey wine fermentation, showing ethanol production (13.8%) in low temperature and low pH for 17 days fermentation (Jung *et al* 1999). The number of viable yeast in *Pinus densiflora*-honey wine was 6.4×10^5 colony forming unit (CFU)/mL at day 0, and reached to 1.5×10^7 CFU/mL at day 7 of fermentation, then decreased to 4.2×10^6 CFU/mL (Fig. 1C, Table 3). In case of honey wine, viable yeast cells were 5.3×10^5 CFU/mL at day 0, 1.7×10^7 CFU/mL at day 7, and 1.6×10^6 CFU/mL at day 30, respectively.

3. Fermentation Characteristics of Honey Wines

Fermentation characteristics of *Pinus densiflora*-honey wine and honey wine were summarized in Table 3. Kahoun *et al* (2008) reported that 19 phenolic compounds were detected from 46 kinds of honey wines by HPLC, and its composition was dependant on the kind of honey, fermentation process, heating process and storage conditions. The phenolic compounds in wines were determined by absorbance at specific wave-

Table 3. Ethanol concentration, pH, titratable acidity (TA), Bx, viable yeast cell, and color properties of *Pinus densiflora*-honey wine and honey wine from alcohol fermentation at 20℃ for 30 days

	Pinus densiflora-honey wine	Honey wine
Ethanol(%, v/v)	13.5	13.4
pH	3.05	3.00
Total titratable acidity(%)	0.37	0.45
Bx	13.7	14.8
Yeast(CFU/mL)	4.2×10^6	1.6×10^6
A_{280}	2.45	2.29
A_{420}	0.33	0.70
A_{520}	0.22	0.62
A_{320}	0.79	1.01
Color intensity(A_{420}+A_{520})	0.55	1.32
Shade(A_{420}/A_{520})	1.50	1.13

length using UV-spectrophotometry (Sarneckis *et al* 2006). Total phenolic compounds (Sarneckis *et al* 2006) were evaluated by A_{280}, and the colority of wines by a combined value of A_{520} and A_{420} (Kim & Kim 1997). *Pinus densiflora*-honey wine had 2.45 (A_{280}) of total phenolic compounds and 0.55 of colority (A_{420}+A_{520}). Honey wine had 2.29 (A_{280}) and 1.32 of colority (A_{420}+A_{520}) (Table 3). Five kinds of rice wines (*Yakju*) showed 11.2~25.5 of total phenolic contents at 280 nm of wavelength (Lee *et al* 2007).

According to the patent (Kwon *et al* 2005), the fermentation and maturity of red ginseng-honey wine with supplementation of nutrients provided 12.0% of ethanol content and 233 mg/L of fusel oil. Otherwise without supplementation of nutrients, ethanol and fusel oil contents was investigated to be 6.2% and 162 mg/L, respectively. It is reasonable to suppose that the supplementation of nutrients helps success the fermentation of wines. In this report, the contents of fusel oils, such as 1-propanol, *iso*-butyl alcohol, *iso*-amyl alcohol and 1-butanol, was 376 mg/L, and might provide rich pine flavor, providing the high preference for pine leaves-honey wine (Table 4).

4. Sensory Evaluation for Pine Leaves-Honey Wines

By the panel of 19 members (9 males and 10 females), the sensory evaluation for *Pinus densiflora*-honey wine was shown in Table 5. *Pinus densiflora*-honey wine containing fructose

Table 4. Volatile compounds of *Pinus densiflora*-honey wine from alcohol fermentation at 20℃ for 30 days

	Pinus densiflora-honey wine
Ethyl acetate(mg/L)	104.7±25.8
Methanol(mg/L)	324.3±169.5
1-propanol(mg/L)	26.7±12.3
iso-Butyl alcohol(mg/L)	68.7±34.3
iso-Amyl alcohol(mg/L)	274.7±149.7
1-butanol(mg/L)	6.3±6.3
Phenethyl alcohol(mg/L)	202.0±140.0

Values are means±S.D. of 3 observations.

gained the highest score, 5.9, when 3% (w/v) of sugars and sugar alcohol were added, however it did not seem in significantly differences ($p<0.05$). Preference for *Pinus densiflora*-honey wine containing 1~5% of fructose was further evaluated by sensory analysis. Color and favor for each honey wine with different fructose content did not show the difference of preference with significant difference. However, the taste's score for 4% fructose group was 6.7, and its preference was much better than the control group (without supplementation of fructose) with significant difference.

In addition, the preference of *Pinus densiflora*-honey wine supplemented with and without 4% fructose and two kinds of commercial rice wine (*Yakju)* were evaluated by sensory analysis. *Yakju*-A and *Yakju*-B had 16.5% and 13.0% of ethanol content, pH 3.9 and pH 3.7, and 10.0°Bx and 11.0°Bx of sugar contents, respectively. For color preference, *Pinus densiflora*-honey wine supplemented with 4% fructose received the highest appearance values, but the significant difference was not shown.

Pinus densiflora-honey wine supplemented with 4% fructose showed better appearance scores for flavor than *Yakju*-B, but pore appearance than *Yakju*-A. According to the report of sensory evaluation(Lee *et al* 2003), the major factor for preference of Korean red wines was the taste and then color and flavor. The further study will be focused on maintaining pine leaves-flavor in *Pinus densiflora*-honey wine supplemented with 4% fructose for extended storage.

In this report, we investigated the fermentation characteristics and the sensory preference of *Pinus densiflora*-honey wine. The change of viable yeast number and decrease of pH showed

Table 5. Sensory quality *Pinus densiflora*-honey wine supplemented with various sugars and sugar alcohol

		Sensory attribute[1)]		
		Color	Flavor	Taste
Test 1	Mead[2)]	6.6±1.4[3)]	5.4±2.3	5.0±2.1
	Mead supplemented with 3% glucose	6.7±1.4	4.8±1.9	5.7±2.7
	Mead supplemented with 3% fructose	6.8±1.5	5.5±2.4	5.9±2.9
	Mead supplemented with 3% sucrose	6.7±1.5	5.6±2.0	5.5±2.7
	Mead supplemented with 3% maltose	6.3±1.8	5.4±2.5	5.4±2.4
	Mead supplemented with 3% sorbitol	6.1±1.7	5.8±2.2	5.0±2.1
Test 2	Mead	7.1±0.8	5.3±2.3	4.8±2.1[a4)]
	Mead supplemented with 1% fructose	7.0±1.0	5.6±2.0	5.2±2.2[a]
	Mead supplemented with 2% fructose	6.8±1.6	5.8±2.1	5.4±2.1[a]
	Mead supplemented with 3% fructose	6.7±1.5	5.9±1.8	6.0±2.1[ab]
	Mead supplemented with 4% fructose	5.9±1.5	5.6±1.8	6.7±2.1[b]
	Mead supplemented with 5% fructose	6.5±1.9	5.7±1.8	5.7±2.6[ab]
Test 3	Mead	6.7±1.5	5.4±2.2[ab]	5.2±1.7
	Mead supplemented with 4% fructose	7.0±1.1	5.8±2.0[ab]	6.5±1.8
	Commercial rice wine A	6.2±1.4	6.3±2.3[b]	5.3±2.2
	Commercial rice wine B	5.8±2.3	4.8±2.3[a]	4.7±2.7

[1)] 1 : dislike very much, 9 : like very much.
[2)] Abbreviation: mead, *Pinus densiflora*-honey wine obtained from alcohol fermentation at 20℃ for 30 days.
[3)] Values are mean±S.D. (standard deviation).
[4)] Values with different superscript letters are statistically significant among different type drinks by Friedman test.

the successful honey wine fermentation for 7 days fermentation. However the sugar content was decreased slightly for 30 days of whole fermentation period and fermentation was terminated with pH 3.05, 0.37% of acidity, and 13.5% of ethanol content. Additionally, the level of fusel oil and phenethyl alcohol in honey wine were 376 and 202 ppm, respectively. The sensory analysis showed the better preference for tastes of *Pinus densiflora*-honey wine supplemented with 4% fructose. The further studies for maintaining the pine leaves-flavor and shortening fermentation period by supplementation of nutrient might be necessary for the commercial products.

국문초록

이 연구의 목적은 솔잎을 꿀로 추출한 추출물을 이용한 발효주를 개발하는 것이다. 솔잎이 첨가되지 않은 꿀을 사용한 그룹을 대조군으로 비교 평가하였다. 솔잎-벌꿀주와 벌꿀주 등 2개의 그룹을 20℃에서 30일간 발효한 후 숙성하였다. 솔잎-벌꿀주에서는 발효 초기에 pH와 효모 생균수 변화는 빠른 속도로 변화하였으나, 당도(°Bx)는 점진적으로 감소하였다. 효모는 발효 시작 후 5~10일에서 가장 높은 생균수를 보였다. 발효 전에는 솔잎-벌꿀주에서 발효액의 pH는 3.8으로 벌꿀주의 pH 3.4보다 높았다. 벌꿀주와 비교시 솔잎-벌꿀주에서 발효중 당도 감소 속도가 높게 나타났으며, pH는 초기의 산성 pH에서 중성쪽으로 변화하였고, 발효 속도는 증가하였다. 발효와 숙성을 거친 솔잎-벌꿀주는 13.7°Bx, pH 3.05, 에탄올 함량 13.5%, 산가 0.37%를 보였다. 솔잎-벌꿀주의 관능적인 선호도를 높이기 위한 시도에서는 과당을 4% (w/v) 수준으로 첨가시 가장 결과가 좋았다.

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