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Article

Comparison of Volatile Composition between Alcoholic Bilberry Beverages Fermented with Non-*Saccharomyces* Yeasts and Dynamic Changes in Volatile Compounds during Fermentation

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ABSTRACT: The profile of volatile compounds was investigated using headspace solid-phase microextraction coupled with gas chromatography−mass spectrometry (HS-SPME−GC−MS) during bilberry juice fermentation with nine non-*Saccharomyces* yeasts, including *Pachysolen tannophilus*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Torulaspora delbrueckii*, *Zygosaccharomyces bailii*, *Schizosaccharomyces pombe*, *Lachancea thermotolerans*, *Issatchenkia orientalis*, and *Saccharomycodes ludwigii*. Dynamic changes in volatile compounds were determined simultaneously with the development of ethanol concentration during fermentation. *H. uvarum* or *I. orientalis* produced more ethyl acetate than other yeast strains throughout fermentation, while fermentation with *M. pulcherrima* resulted in high accumulation of higher alcohols. *S. pombe* was associated with high productions of pentane-2,3-dione, 3-hydroxybutan-2-one, 2-methylbutanal, and 3-methylbutanal. Among the 59 volatile compounds detected, generally, higher alcohols and monoterpenes accumulated constantly and reached the maximum concentration at the middle or later fermentation stage, whereas aldehydes, ketones, and acetals accumulated first followed by a significant drop. The production and accumulation dynamics of metabolites were highly dependent on the yeast species and the developing ethanol content.

KEYWORDS: *non-Saccharomyces yeasts, volatile composition, dynamic changes, alcoholic bilberry beverages, HS-SPME−GC−MS*

INTRODUCTION

Over the past few years, there has been a growing interest among consumers in novel and unique fermented alcoholic fruit beverages made from local crops particularly in some European countries. This has promoted the development of alcoholic beverages fermented from diversified nongrape fruits, such as plums, blackberry, pineapple, strawberry, pomegranate, and cherry.[1] According to the report from European Cider and Fruit Wine Association (AICV), in recent years, fermented alcoholic fruit drinks are among the fastest growing ones of all alcoholic beverages. Bilberry (*Vaccinium myrtillus* L.) is one of the most economically valuable wild berries in Northern Europe and is gaining increasing attention primarily due to its pleasant aroma and richness in nutritional and bioactive compounds.[2] However, only 5−8% of the total bilberry yield in Nordic countries (>500 million kg/year) is exploited annually,[2,3] which may be partly due to the lack of innovative products from bilberries other than jam, juice, and concentrate.[4] Hence, the development of novel products from bilberry, such as alcoholic bilberry beverages (ABBs), is necessary to meet the new trends of the market for products of premium quality.

Aroma is one of the most crucial sensory features determining the quality of fermented fruit drinks, which is highly affected by the qualitative and quantitative composition of volatile compounds. During yeast fermentation, the generation and degradation process of aromatic compounds is dynamic and complicated. The monitoring of these compounds during fermentation is a matter of active research to understand their evolution patterns over time and the potential impact on both specific aroma attributes and the overall flavor. In recent years, there has been increasing interest in winemaking to investigate, for example, the dynamic changes in the main secondary metabolites of higher alcohols and esters from yeast metabolism,[5] the formation of volatile sulfur compounds arising from sulfurous precursors,[6] or the constant conversion of glycosidically bound monoterpenoids to their corresponding free monoterpenoids with the participation of hydrolytic enzymes (especially *β*-glucosidases).[7] However, the metabolic pathways and the biochemical processes involving volatile compounds during fermentation of ABBs still remain poorly understood.

Non-*Saccharomyces* yeasts were originally considered as problematic microorganisms for use in alcoholic beverage production due to their poor fermentation ability and low tolerance to ethanol and SO_2.[8] Nowadays, it is widely accepted that non-*Saccharomyces* yeasts may play an important role in determining the sensory quality of final beverages through the production of more diversified profiles of volatile compounds compared with conventional *Saccharomyces cerevisiae*. A number of studies have inoculated non-*Saccharomyces* yeasts to modify the aroma profiles of alcoholic beverages. For example, inoculation of *Metschnikowia pulcherrima* was used to increase the production of 2-phenylethanol.[9] *Torulaspora delbrueckii* has

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been documented to produce less off-flavor compounds, such as acetaldehyde, 3-hydroxybutan-2-one (acetoin), and acetic acid compared with other non-*Saccharomyces* strains.[10,11] In comparison with inoculation with *S. cerevisiae*, fermentations with *Hanseniaspora* species and *Lachancea thermotolerans* strain reduced the production of higher alcohols[8,12] and those with *Schizosaccharomyces* species generated a higher amount of acetaldehyde,[13] whereas fermentation with *Issatchenkia orientalis* resulted in lower production of acetaldehyde, propan-1-ol, butan-2-ol, and 3-methylbutan-1-ol.[14] However, such results were mainly obtained from the analysis of completely fermented wines or beers and no detailed studies have been reported on the dynamic changes of these metabolites during non-*Saccharomyces* yeast fermentation.

Headspace solid-phase microextraction (HS-SPME) technique has been widely used in the analysis of volatile compounds in alcoholic beverages due to its properties of solvent free, fast extraction, and higher sensitivity and reproducibility in comparison with the classical analytical approaches of liquid−liquid extraction (LLE) and solid-phase extraction (SPE).[15,16] HS-SPME coupled with gas chromatography−mass spectrometry (GC−MS) can detect volatiles at concentrations even below the level of ng/L.[16] During the HS-SPME process, an equilibrium is established between three phases: liquid sample matrix, gaseous headspace, and the stationary phase of fiber coating. Any changes in the sample matrix, such as ionic strength, ethanol concentration, and pH, may affect the partition coefficient between these phases and thus influence their adsorption rate and the concentration on the fiber.[15,16] Ethanol is continuously produced and accumulated during yeast fermentation, and the change in ethanol concentration has been demonstrated to affect the equilibrium of other volatile compounds through the alteration of solubility of the analytes in the liquid phase and the increase of competitive occupation of active sites in the stationary phase.[15,17] Hence, to carry out a reliable quantitation of volatiles during fermentation, it is important to take into account the changes in ethanol content, thus making the whole process more laborious. This fact may partly explain the scarcity of studies on the evolution of volatile compounds during fermentation of alcoholic fruit beverages.

The aims of this study were to (1) quantitate volatile compounds in alcoholic bilberry beverages using HS-SPME−GC−MS by minimizing the effects of ethanol during the extraction of analytes, (2) characterize and compare the volatile profiles of ABBs fermented with different species of non-*Saccharomyces* yeasts, including *Pachysolen tannophilus*, *M. pulcherrima*, *Hanseniaspora uvarum*, *T. delbrueckii*, *Zygosaccharomyces bailii*, *Schizosaccharomyces pombe*, *L. thermotolerans* (previously classified as *Kluyveromyces thermotolerans*), *I. orientalis*, and *Saccharomycodes ludwigii*, and (3) monitor and compare the dynamic changes of volatile compounds during fermentation with the nine non-*Saccharomyces* yeasts. Fermentation with *S. cerevisiae* was included in this study as a reference for comparison. This work provides useful information about the potentials of diverse non-*Saccharomyces* yeasts in the production of fruit wines or beverages. Moreover, the monitoring of volatile compounds during fermentation may help fermentation practitioners to optimize and control fermentation to improve the quality of final products.

MATERIALS AND METHODS

Chemicals. Volatile standards of propan-1-ol, 2-methylpropan-1-ol, butan-1-ol, 3-methylbutan-1-ol, hexan-1-ol, heptan-2-ol, 3-ethoxypropan-1-ol, (*Z*)-hex-3-en-1-ol, (*E*)-hex-2-en-1-ol, heptan-1-ol, 2-ethylhexan-1-ol, octan-1-ol, 3-(methylthio)-1-propanol, 2-phenylethanol, 4-methyl-2-pentanol, ethyl acetate, ethyl 2-methylpropanoate, 2-methylpropyl ethanoate, ethyl butanoate, ethyl 3-methylbutanoate, methyl hexanoate, ethyl hexanoate, ethyl 2-hydroxypropanoate, ethyl octanoate, ethyl decanoate, 2-phenylethyl acetate, ethyl dodecanoate, butane-2,3-dione, 3-hydroxybutan-2-one (acetoin), 6-methyl-5-hepten-2-one, acetaldehyde, hexanal, benzaldehyde, 1,1-diethoxyethane, 2-methylpropanoic acid, heptanoic acid, 3,7-dimethylocta-1,6-dien-3-ol (linalool), 2-(4-methylcyclohex-3-en-1-yl)propan-2-ol (α-terpineol), and 1,2-xylene with purity >98% and an alkane mixture (C5−C20) were purchased from Sigma-Aldrich (St. Louis, MO). Ethanol (>99.5%) was obtained from ALTIA Oyj (Rajamäki, Finland), sodium hydroxide (>98%) from Mallinckrodt Baker (Deventer, The Netherlands) and citric acid (>99%) from Alfa Aesar GmbH Co. (Karlsruhe, Germany). Food grade sucrose was purchased from Kesko Oyj (Kirkkonummi, Finland).

Bilberry Juice Preparation. Wild bilberries (*V. myrtillus* L.) were harvested in 2017 in Finland and frozen at −20 °C before processing. Bilberry juice preparation was performed according to our previous protocol with minor modifications.[18] First, a series of processes were carried out to obtain diluted juices, including thawing in a microwave for 5 min, pressing with a juice presser, and dilution with ultrapure water at the ratio of 1:1 (v/v). Subsequently, the juices were pooled into a sealed plastic bucket and stored at +6 °C in darkness for 24 h to separate supernatant from the solids. The supernatants were transferred to a new bucket. The separation procedure was carried out twice successively. Afterward, the juices were pasteurized in a water bath at 95 °C for 5 min and cooled to room temperature. The pH and degree Brix values of the resultant juice were adjusted to 3.5 and 14.0 using sodium hydroxide and sucrose, respectively.

Yeast Strains and Laboratory-Scale Fermentation. Yeasts of *S. cerevisiae* Lalvin V1116 (SC1116) and *T. delbrueckii* 291 (TD291) were obtained from Lallemand Inc. (Montreal, Canada). Strains of *S. pombe* 70572 (SP70572), *S. ludwigii* 3447 (SL3447), *M. pulcherrima* 70321 (MP70321), *L. thermotolerans* 3434 (LT3434), *I. orientalis* 3433 (IO3433), *H. uvarum* 26650 (HU26650), *P. tannophilus* 70352 (PT70352), and *Z. bailii* 70492 (ZB70492) were purchased from DSMZ Institute (Braunschweig, Germany). Before inoculation, the 10 cultures were propagated in YEPD medium (1% yeast extract, 2% peptone, and 2% dextrose) at 25 °C for 48 h according to our published method.[18]

Fermentations were carried out in sterile and sealed Duran bottles (volume = 100 mL) with aliquots of 50 mL of sterilized juice at 25 °C in darkness. The cell count for each inoculation was approximately 10^7 CFU/mL. During fermentation, the caps of bottles were unscrewed every day under an aseptic condition to release CO_2 produced from yeast growth. Fermentations were monitored by measuring Brix values and the weight loss of the bottles every 3 days till the completion of fermentation when bottle weights and Brix values remained constant during two consecutive monitoring time points. It is worth noting that to eliminate the possible impact of volume reduction caused by repeated sampling during fermentation on the chemical profiles of fermented bilberry samples, a series of bottles of juices (total 12 bottles) was inoculated for each yeast strain. The fermented samples were successively taken every 3 days and then immediately centrifuged at 4500*g* for 10 min to remove yeast pellets and precipitates. The supernatants were collected and stored at −80 °C. Figure S1 shows the fermentation procedure.

Determination of Ethanol Content. Ethanol concentration in fermented bilberry juice was determined in triplicate by Shimadzu GC-2010 Plus gas chromatography with a flame ionization detector (GC-FID, Shimadzu, Japan) equipped with an HP-INNOWax column (30 m × 0.25 mm i.d., 0.25 μm, Hewlett-Packard, Avondale, PA). The GC analysis was performed using a previously reported external standard method.[18] Briefly, the column temperature went from a steady 40 °C for 8 min to 240 °C with a gradient of 10 °C/min and was kept at 240 °C for 2 min. The injector and detector temperatures were 220 and 280 °C, respectively. Helium was used as the carrier gas at 1.5 mL/min flow with a split ratio of 1:25. A calibration curve

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(R^2 = 0.996) was constructed using standard solutions of ethanol at concentrations of 0, 2, 4, 6, 8, and 10%.

Determination of Volatile Compounds. *Preparation of Standard Mixture Solutions.* Standard solutions were prepared with varying concentrations of ethanol to take into account the impact of ethanol concentration on the volatile profile in the headspace. Stock solutions of authentic volatile standards were prepared in 5 mL ethanol. The initial concentrations of the standards are listed in Table S1. Six synthetic ABB matrices were also prepared with ethanol at varying concentrations of 0, 2, 4, 6, 8, and 10% (v/v), respectively. All matrices contained 7 g/L citric acid, and the pH values were adjusted to 3.5 with 1 M NaOH. Afterward, all stock solutions were mixed and diluted using the first synthetic matrix (0% ethanol) to establish five standard mixture solutions with increasing ethanol content of 2, 4, 6, 8, and 10% (v/v). Nine dilutions of the standard mixture solutions were prepared using the synthetic matrices with the same ethanol percentage. The diluted solutions (concentration ranges shown in Table S2) were kept at −20 °C in darkness until analysis.

HS-SPME−GC−MS Analysis. HS-SPME was used for the extraction of volatile compounds of fermented bilberry juices and standard mixture solutions as described previously.[19] 4-Methyl-2-pentanol (802 μg/mL in methanol) was used as the internal standard. Two milliliters of each sample, 0.2 g of sodium chloride and 10 μL internal standard were placed in a 20 mL glass vial. A 2-cm SPME fiber coating with divinylbenzene/carboxen/poly(dimethylsiloxane) (DVB/CAR/PDMS, 50/30 μm, Supelco, Bellefonte, PA) was used to extract volatile compounds. The fiber was conditioned at 250 °C for 60 min prior to extraction. The extraction process was carried out at 45 °C for 30 min with agitation. The extracted volatile compounds were analyzed in a Trace 1310 gas chromatography coupled with a Triplus RSH autosampler (Thermo Scientific, Reinach, Switzerland) and a TSQ 8000 EVO mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The column was DB-WAX capillary column (60 m × 0.25 mm i.d. × 0.25 μm film thickness; J&W Scientific, Folsom, CA). Helium was used as the carrier gas at a flow rate of 1.6 mL/min. The injector temperature was 240 °C. The oven temperature program was as follows: 50 °C held for 3 min, increased at a rate of 5 °C/min to 220 °C, and held for 8 min. Mass spectra were detected in electron impact (EI) mode at 70 eV with a scan range m/z 33−300. The temperatures of the MS transfer line and the ionization source were 200 and 220 °C, respectively. The HS-SPME−GC−MS analysis of each sample was carried out in triplicate.

Identification and Quantitation of Volatile Compounds. The volatile compounds in fermented bilberry samples were identified by comparing their retention indices (RIs) and mass spectra with those of authentic standards. The RIs were obtained from the injection of the C5−C20 alkane mixture under the same chromatographic conditions. When the corresponding authentic standards were not available, tentative identifications were conducted by matching mass spectra in the standard NIST 17 library and comparing the RIs with those reported in the literature and NIST database.[20]

The quantitation of the detected volatile compounds was performed using calibration curves built with authentic standards of interest from nine different concentrations in synthetic ABB matrices and internal standard for correcting any possible variations in SPME fiber performance along the sample sequence. In the calibration equations ($y = ax + b$), x and y represented the peak area ratio and concentration ratio of volatile standard to the internal standard, respectively. Five standard calibration curves were obtained for an individual volatile compound with ethanol concentration at 2, 4, 6, 8, and 10% (v/v), respectively (Table S2). On the basis of the ethanol concentration in fermented bilberry samples, an appropriate calibration curve was selected for the quantitation of volatile compounds following the principle of proximity of ethanol content. When the corresponding authentic standards were unavailable, the compounds were quantitated on the basis of the calibration curves obtained from the standards of the same chemical group with similar chemical structures. The method linearity was evaluated by the determination coefficient (R^2) for each standard addition curve. Limits of detection (LOD) and quantitation (LOQ) for volatile standards were estimated as the concentration of the analytes that provided a signal-to-noise ratio (S/N) of 3 and 10, respectively by injecting a series of diluted standard solutions. The values of LOD and LOQ for analytes were different in matrices with different ethanol concentrations (Table S2).

Statistical Analysis. All results were expressed as mean ± standard deviation from three replicates. One-way analysis of variance (ANOVA) followed by the Duncan test was employed to determine the difference between means using R software (version 3.6.1) with "agricolae" package. Significance was set at $p < 0.05$. Multivariate models, including principal component (PC) analysis (PCA) and partial least squares discriminant analysis (PLS-DA), were created with Unscrambler X software (version 11.0, Camo Inc., Norway). PCA was applied to investigate the sample groupings and correlations among volatile profiles (X-data) of all 10 bilberry beverages. PLS-DA was used to further study the difference of volatile composition of the samples that clustered into the same group in PCA. Heatmaps analysis using data normalized as mean-centered and divided by the standard deviation of each variable illustrating the dynamic evolution of volatile compounds during ABB fermentation was performed using online software MetaboAnalyst 4.0 (McGill University, Canada).

RESULTS AND DISCUSSION

Evolution of Ethanol during Fermentation. Figure 1 shows the progress of ethanol content during the 10 different

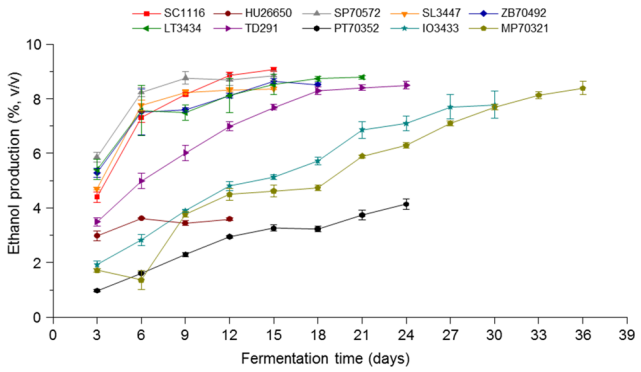

Figure 1. Evolution of ethanol concentration during the production of alcoholic bilberry beverages fermented with 10 different yeasts. SC1116, HU26650, SP70572, SL3447, ZB70492, LT3434, TD291, PT70352, IO3434, and MP70321 are *S. cerevisiae* Lalvin V1116, *H. uvarum* 26650, *S. pombe* 70572, *S. ludwigii* 3447, *Z. bailii* 70492, *L. thermotolerans* 3434, *T. delbrueckii* 291, *P. tannophilus* 70352, *I. orientalis* 3433, and *M. pulcherrima* 70321, respectively.

fermentations. The strains SC1116, SP70572, SL3447, ZB70492, and LT3434 showed strong fermentation capacities as indicated by higher production of ethanol or shorter fermentation duration compared to the other strains. Remarkably, a rapid release of ethanol was observed already at the early stage of these fermentations as more than 80% of the final ethanol content was generated during the first 6 days. These results are consistent with previous studies.[21−24] Although the ethanol production rates of the inoculations with TD291, IO3433, and MP70321 were lower than those of the aforementioned yeasts, their fermentation kinetics showed a linear trend with time, with ethanol concentration peaking at 8%, approximately. The results indicated that *T. delbrueckii*, *I. orientalis*, and *M. pulcherrima* all possessed stable fermentation activity required for the production of ABBs. The ethanol level in ABB fermented with PT70352 was 4.14% after 24 days of fermentation, being only 45.6% of the ethanol content found in the beverage fermented with SC1116. This result is in agreement with a previous finding reporting a sugar consumption of 47.7% by *P. tannophilus* during

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fermentation of a synthetic grape juice (glucose 75 g/L, fructose 75 g/L, tartaric acid 3 g/L, pH 3.5).[23] The final concentration of ethanol in the fermentation product with HU26650 was 3.6%, significantly lower than the levels after fermentation with other yeast cultures ($p < 0.05$). The poor fermentation ability and low ethanol tolerance of *H. uvarum* were verified in Montepulciano d'Abruzzo wine, with a viable cell count of *H. uvarum* increasing to a maximum at 5 days after inoculation followed by a rapid decrease.[25]

Overall, the fermentation with the non-*Saccharomyces* yeasts produced less ethanol compared to *S. cerevisiae* due to their poorer sugar conversion capacities. The combination of non-*Saccharomyces* and *Saccharomyces* yeasts, for example sequential and simultaneous inoculations, has been considered as a promising approach for lowering ethanol content of fermented beverages.[26,27]

Effect of Ethanol on the Extraction of Volatile Compounds. A constant decrease in the peak areas was observed with increasing ethanol content for most volatile compounds in the synthetic ABB solutions (Figure 2). The results are in agreement with previous studies in wine and synthetic wine solutions.[15,17,28] However, the biggest decline in the peak area generally occurred when ethanol concentration increased from 2 to 4%. This might have been due to the solubility of the studied volatile compounds in model solutions progressively increased with the increase of ethanol content and reached a critical state of saturation thereafter. Following this saturation point, the solubility of the compound in the liquid phase kept reducing with the increasing ethanol content from 4 to 10%, leading to a higher proportion of the analytes adsorbed on the HS-SPME fiber. These findings confirmed the necessity for taking into account the ethanol concentration in quantitation of volatiles in products submitted to a fermentation process.

Comparison of the Volatile Profiles of Final Alcoholic Bilberry Beverages. As discussed above, ethanol concentration was found to be a factor that influences the matrix properties and affects the quantitation of other volatiles. Moreover, ethanol concentration varied during the fermentation process and differed substantially among the 10 different finished ABBs (Figure 1). For quantitation of the volatile compounds, we constructed a series of calibration curves with different ethanol levels covering the entire range of ethanol percentage found in our samples (Table S2).

Totally, 59 volatile compounds, including 20 higher alcohols, 20 esters, 4 ketones, 4 aldehyde, 4 fatty acids, 3 acetals, 2 monoterpenes, and 2 benzenes, were identified and quantitated in bilberry products. Their concentrations in completely fermented bilberry beverages are listed in Table 1. To better understand the effect of microorganisms on the aroma differentiation of ABBs at the end of fermentation, an unsupervised classification using the PCA model was carried out to reduce the dimensionality of the data (Figure 3A). The first three principal components (PCs) explaining 62% of the total variance were used to separate the finished ABBs on the basis of the concentration differences of the 59 compounds detected. A clear separation of the 10 different samples into two groups was observed along the PC-1 (accounting for 30% of the total variation). The first group locating on the right part of PC-1, namely, the ABBs, produced with SC1116, SP70572, SL3447, and MP70321, was characterized by high abundance of most volatile compounds analyzed, particularly higher alcohols, esters, monoterpenes, aldehydes, and acetals, in comparison with the second group with the fermentations with HU26650, IO3433,

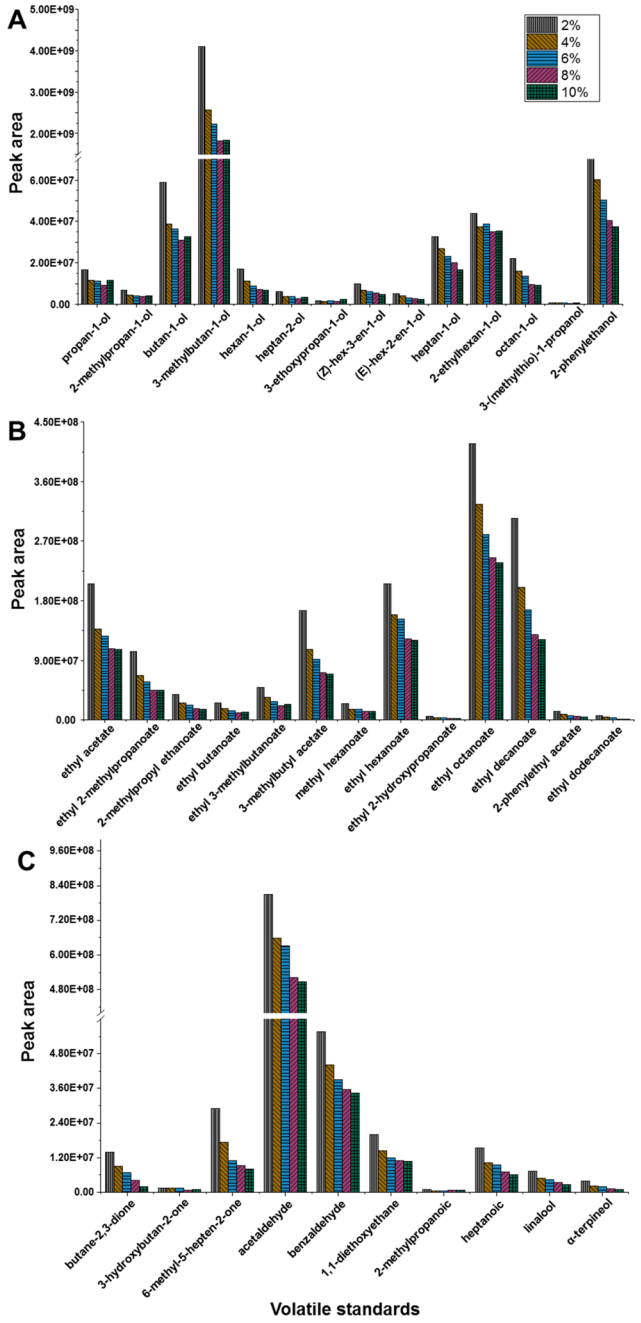

Figure 2. Effect of ethanol concentration on the HS-SPME extraction (expressed as extracted peak area) of a constant content of authentic standards. (A) Higher alcohols; (B) esters; (C) ketones, aldehydes, acetals, acids, monoterpenes, and benzenes.

PT70352, LT3434, ZB70492, and TD291 (Figure 3A). The concentrations and compositions of volatile compounds determine the overall aroma profile and odor properties of fermented beverages;[29] therefore, the overall aroma profile of the ABB samples in the first group may possess higher complexity than those of the second group. Bilberry beverages produced with HU26650 and IO3433 were clearly separated from the second group on the PC-2 (with 21% of the variation) and PC-3 (11%), respectively (Figure 3A). The sample fermented with HU26650 was characterized by the high concentrations of methyl acetate (variable E21 in Table 1), ethyl acetate (E22), and ketone compounds, including butane-2,3-dione (K41), pentane-2,3-dione (K42), and 3-hydroxybutan-2-one (K43).

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Table 1. Concentrations (μg/L) of Volatile Compounds Detected in Finished Bilberry Beverages Fermented by 10 Different Yeasts[a]

		fermentation[b]									
code	compounds	SC1116	HU26650	SP70572	SL3447	ZB70492	LT3434	TD291	PT70352	IO3433	MP70321
HA1	propan-1-ol	6525.54 ± 157.73 fg	5139.44 ± 537.20 g	8821.46 ± 879.08 e	7151.22 ± 611.62 f	19 155.33 ± 1819.45 a	13 183.72 ± 376.00 c	13 257.55 ± 316.42 c	17 756.40 ± 775.88 a	14 870.91 ± 1541.39 b	10 396.92 ± 378.49 d
HA2	2-methylpropan-1-ol	21 740.43 ± 145.15 e	11 987.29 ± 1408.89 f	27 847.34 ± 966.28 c	24 918.28 ± 1759.77 e	72 950.88 ± 6824.84 a	42 827.78 ± 855.46 b	31 429.20 ± 432.83 c	31 097.21 ± 585.73 cd	26 446.70 ±2916.76de	44 326.55 ±1424.57b
HA3	pentan-2-ol	1889.58 ± 9.01 b	1233.04 ± 147.44 e	1587.56 ± 168.05 bcd	1480.43 ± 116.16 cde	531.70 ± 55.00 f	1264.55 ± 10.3 de	328.46 ± 16.55 f	1681.60 ± 24.16 bc	3784.77 ± 523.86 a	1765.54 ± 38.20 bc
HA4	butan-1-ol	328.33 ± 4.31 d	82.61 ± 6.76 h	259.53 ± 19.26 e	206.75 ± 10.41 f	144.03 ± 11.63 g	675.47 ± 25.16 a	580.77 ± 15.66 b	368.46 ± 14.43 c	587.74 ± 58.10 b	677.13 ± 10.71 a
HA5	hexan-2-ol	191.71 ± 2.48 b	70.23 ± 19.12 ef	218.99 ± 15.45 a	123.72 ± 7.33 d	44.25 ± 4.79 g	50.62 ± 5.73 fg	135.13 ± 12.29 cd	74.16 ± 7.43 ef	76.24 ± 10.14 e	148.99 ± 27.93 c
HA6	2-methylbutan-1-ol	59 757.39 ± 5356.97 a	4917.25 ± 629.26 g	40 347.29 ± 2271.86 d	33 562.31 ± 3853.74 e	46 502.85 ± 4605.58 c	30 336.36 ± 844.22 e	54 508.32 ± 901.86 b	23 596.21 ± 1190.86 f	20 409.12 ± 2102.27 f	62 092.03 ± 1716.70 a
HA7	3-methylbutan-1-ol	13 9572.96 ± 527.16 b	22 024.55 ± 2622.50 h	97 115.39 ± 2361.86 d	93 845.86 ± 8587.26 d	80 214.56 ± 6878.70 e	82 965.34 ± 4149.72 e	12 3821.51 ± 7323.50 c	38 671.08 ± 1274.26 g	51 352.31 ± 4409.79 f	169 345.51 ± 3486.88 a
HA8	4-methylpentan-1-ol	201.28 ± 0.89 c	69.56 ± 6.32 f	349.39 ± 38.95 a	158.76 ± 15.51 d	121.14 ± 20.16 e	68.70 ± 11.74 f	89.82 ± 5.05 f	59.49 ± 5.60 f	78.39 ± 13.78 f	296.98 ± 18.90 b
HA9	heptan-2-ol	3.50 ± 0.07 b	3.28 ± 0.22 bc	3.49 ± 0.34 b	3.04 ± 0.15 bcd	12.66 ± 0.79 a	2.56 ± 0.17 d	2.62 ± 0.05 d	2.76 ± 0.06 cd	2.76 ± 0.16 cd	3.42 ± 0.24 b
HA10	3-methylbutan-2-ol	89.86 ± 7.74 e	837.85 ± 184.10 a	119.72 ± 8.84 e	228.63 ± 22.02 d	353.05 ± 24.90 c	571.69 ± 20.57 b	103.71 ± 3.79 e	317.15 ± 14.08 cd	302.18 ± 45.01 cd	257.34 ± 37.81 cd
HA11	3-methylpentan-1-ol	818.56 ± 59.39 c	100.35 ± 16.06 e	1629.90 ± 174.41 b	928.73 ± 97.10 c	142.81 ± 17.75 de	127.63 ± 1.69 de	133.99 ± 8.65 de	255.31 ± 15.86 d	205.60 ± 26.54 de	2004.27 ± 57.81 a
HA12	hexan-1-ol	1855.3 ± 109.34 b	1372.56 ± 189.59 d	2274.83 ± 231.79 a	1506.71 ± 145.11 cd	1323.15 ± 98.78 d	825.14 ± 29.93 e	1448.96 ± 48.40 cd	1347.85 ± 50.01 d	731.87 ± 70.54 e	1594.36 ± 20.70 c
HA13	3-ethoxypropan-1-ol	144.41 ± 15.05	181.63 ± 35.08	47.88 ± 14.38	22.60 ± 3.02	149.65 ± 9.16	1405.93 ± 15.53	3973.68 ± 156.92	210.94 ± 50.05	nq	60.91 ± 28.80
HA14	(*Z*)-hex-3-en-1-ol	41.38 ± 0.91 abc	46.17 ± 8.45 a	44.75 ± 3.29 a	37.98 ± 1.75 bc	38.58 ± 1.90 bc	37.16 ± 0.35 c	38.56 ± 0.62 bc	43.46 ± 1.64 ab	36.08 ± 1.44 c	41.77 ± 0.54 abc
HA15	(*E*)-hex-2-en-1-ol	20.47 ± 2.21 g	112.51 ± 30.84 d	108.84 ± 20.26 ef	174.18 ± 27.60 b	151.63 ± 12.69 bc	127.53 ± 11.32 cd	80.80 ± 13.77 f	98.89 ± 7.69 ef	10.41 ± 3.68 g	211.52 ± 15.56 a
HA16	heptan-1-ol	7.77 ± 1.11 b	8.62 ± 0.60 a	6.86 ± 0.15 c	6.72 ± 0.48 cd	6.49 ± 0.23 cd	6.49 ± 0.09 cd	5.27 ± 0.02 e	7.68 ± 0.16 b	6.35 ± 0.14 cd	6.00 ± 0.65 d
HA17	2-ethylhexan-1-ol	1.55 ± 0.28 de	5.32 ± 1.99 ab	2.63 ± 0.17 cd	1.67 ± 0.37 cde	3.03 ± 0.50 c	2.58 ± 0.23 cd	0.74 ± 0.12 e	4.35 ± 0.54 b	4.87 ± 0.78 ab	5.75 ± 0.41 a
HA18	octan-1-ol	6.58 ± 0.46 a	5.55 ± 0.27 cd	6.60 ± 0.26 a	5.66 ± 0.08 c	5.54 ± 0.03 cd	5.05 ± 0.07 f	5.27 ± 0.07 ef	5.59 ± 0.09 cd	5.42 ± 0.08 cd	6.19 ± 0.13 b
HA19	3-(methylthio)-1-propanol	1536.69 ± 21.63 c	160.97 ± 27.57 f	1834.67 ± 101.34 b	857.04 ± 84.81 d	1786.42 ± 186.87 b	305.45 ± 6.63 e	809.88 ± 37.60 d	63.43 ± 1.80 f	411.29 ± 9.31 e	2492.81 ± 22.72 a
HA20	2-phenylethanol	14 578.49 ± 214.98 bc	4650.29 ± 1158.67 g	12 647.79 ± 1070.01 de	13 249.81 ± 1396.80 cd	15 414.86 ± 1235.34 b	7128.82 ± 100.19 f	20 212.09 ± 845.14 a	5249.63 ± 554.50 g	11 259.72 ± 643.09 e	20 766.21 ± 618.18 a
	total higher alcohols	249 311.76 ± 5371.03 b	53 009.05 ± 3983.13 f	200 213.62 ± 7826.74 c	178 470.09 ± 16 291.62 d	239 052.63 ±21 707.20-b	181 918.54 ±6067.49 d	250 966.35 ±7508.32 b	120 911.67 ± 3939.82 e	130 562.9 ±11 695.97-e	316 500.21 ± 7053.58 a
E21	methyl acetate	1047.05 ± 62.69 cd	12 134.39 ± 3355.49 a	1010.81 ± 138.12 cd	1156.97 ± 159.60 cd	1506.53 ± 161.58 cd	467.64 ± 2.83 d	590.62 ± 35.46 d	2996.09 ± 25.10 c	5992.48 ± 581.65 b	1438.33 ± 91.10 cd
E22	ethyl acetate	31 995.61 ±1198.68 cd	345 560.72 ± 87 956.59 a	25 688.70 ± 2720.71 d	85 326.43 ± 7703.17 c	76 892.99 ±6063.69 cd	33 143.81 ±2328.79c-d	33 221.49 ±1407.40c-d	27 481.79 ± 304.36 d	200 761.56 ±19 873.93-b	59 600.85 ±1972.85c-d
E23	ethyl propanoate	51.32 ± 2.53 ef	139.88 ± 24.16 d	46.71 ± 5.31 f	183.28 ± 20.47 c	227.48 ± 21.14 b	56.54 ± 3.64 ef	209.22 ± 5.2 bc	44.09 ± 0.72 f	404.58 ± 44.00 a	83.16 ± 3.24 e

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Table 1. continued

code	compounds	fermentation[b] SC1116	HU26650	SP70572	SL3447	ZB70492	LT3434	TD291	PT70352	IO3433	MP70321
E24	ethyl 2-methylpropanoate	38.08 ± 1.33 de	26.50 ± 1.42 f	22.86 ± 2.75 f	43.16 ± 3.87 d	121.62 ± 11.02 a	108.29 ± 8.52 b	95.30 ± 4.69 c	27.69 ± 0.55 ef	114.24 ± 13.18 ab	25.49 ± 0.76 f
E25	2-methylpropyl ethanoate	18.81 ± 0.39 fg	33.50 ± 7.20 c	14.28 ± 0.91 g	17.64 ± 3.59 fg	25.15 ± 2.59 de	62.34 ± 1.85 a	14.61 ± 1.2 g	46.79 ± 0.53 b	29.05 ± 2.31 cd	21.67 ± 1.20 ef
E26	ethyl butanoate	100.71 ± 0.42 b	32.35 ± 3.27 f	66.11 ± 8.38 cd	74.40 ± 2.88 c	50.43 ± 2.81 e	17.62 ± 1.07 g	60.77 ± 3.42 de	23.10 ± 0.49 fg	163.64 ± 16.43 a	73.64 ± 2.08 c
E27	ethyl 3-methylbutanoate	11.50 ± 0.87 b	11.40 ± 0.25 b	9.09 ± 1.38 cd	6.47 ± 0.54 ef	5.24 ± 0.29 fg	4.59 ± 1.02 g	8.65 ± 1.65 cd	7.76 ± 0.16 de	9.98 ± 1.26 bc	23.61 ± 0.76 a
E28	3-methylbutyl acetate	515.70 ± 5.03 b	237.38 ± 20.75 f	430.82 ± 45.17 c	321.95 ± 21.27 d	92.32 ± 9.38 g	247.81 ± 13.32 ef	72.98 ± 3.50 g	315.38 ± 3.92 de	968.25 ± 112.24 a	403.68 ± 13.18 c
E29	methyl hexanoate	6.15 ± 0.23 a	5.65 ± 0.01 b	4.41 ± 0.07 c	3.51 ± 0.11 e	3.26 ± 0.02 f	3.32 ± 0.07 f	3.83 ± 0.10 d	5.67 ± 0.03 b	3.32 ± 0.06 f	3.56 ± 0.16 e
E30	ethyl hexanoate	109.80 ± 0.49 a	58.13 ± 0.54 ef	84.69 ± 5.07 c	88.46 ± 1.63 b	40.47 ± 0.39 g	40.60 ± 0.21 g	60.98 ± 1.31 e	57.01 ± 0.08 f	40.53 ± 0.25 g	74.1 ± 1.77 d
E31	hexyl acetate	111.10 ± 7.52 a	82.36 ± 6.39 de	97.29 ± 11.92 bc	84.50 ± 7.54 cde	77.67 ± 3.29 de	89.53 ± 5.42 cd	108.65 ± 14.25 ab	78.31 ± 1.25 de	73.39 ± 5.28 e	87.89 ± 6.38 cde
E32	ethyl (*Z*)-hex-3-enoate	6.17 ± 0.02 a	5.76 ± 0.08 b	4.74 ± 0.18 c	4.38 ± 0.17 d	3.59 ± 0.07 f	3.72 ± 0.10 f	4.47 ± 0.15 d	5.73 ± 0.08 b	4.11 ± 0.08 e	4.93 ± 0.26 c
E33	ethyl (E)-hex-3-enoate	6.23 ± 0.06 a	5.76 ± 0.09 b	4.84 ± 0.17 c	4.42 ± 0.14 d	3.60 ± 0.06 f	3.72 ± 0.10 f	4.48 ± 0.15 d	5.75 ± 0.05 b	4.13 ± 0.11 e	4.93 ± 0.26 c
E34	ethyl 2-hydroxypropanoate	660.70 ± 53.21 cd	502.32 ± 72.96 d	803.39 ± 32.16 c	444.03 ± 54.87 ef	1152.29 ± 154.20 b	6288.03 ± 328.12 a	1325.29 ± 44.64 b	276.50 ± 18.44 f	540.23 ± 52.35 d	764.63 ± 7.16 c
E35	methyl 2-hydroxy-3-methylbutanoate	697.28 ± 40.45 a	695.63 ± 170.5 a	664.23 ± 98.68 ab	10.16 ± 0.81 bc	222.63 ± 16.17 d	471.69 ± 25.43 c	281.02 ± 25.06 d	312.55 ± 13.29 d	448.82 ± 18.75 c	612.56 ± 23.45 ab
E36	ethyl octanoate	62.71 ± 0.10 b	14.39 ± 0.28 f	58.70 ± 3.95 bc	121.44 ± 13.00 a	26.63 ± 0.19 de	26.09 ± 0.17 e	32.42 ± 0.62 d	14.20 ± 0.09 f	27.45 ± 0.26 de	53.96 ± 1.84 c
E37	ethyl decanoate	61.63 ± 0.50 b	10.35 ± 3.01 c	43.49 ± 5.3 bc	351.40 ± 66.69 a	23.02 ± 0.41 bc	22.87 ± 0.79 bc	21.77 ± 0.39 bc	6.81 ± 0.28 c	22.44 ± 0.28 bc	61.12 ± 1.18 b
E38	ethyl 9-decenoate	14.13 ± 0.36 e	6.56 ± 0.11 f	21.71 ± 0.38 c	24.75 ± 0.55 a	20.96 ± 0.02 d	20.98 ± 0.07 d	20.90 ± 0.09 d	6.56 ± 0.09 f	20.94 ± 0.02 d	22.55 ± 0.15 b
E39	2-phenylethyl acetate	14.18 ± 0.19 c	14.44 ± 3.12 c	15.09 ± 0.62 c	10.16 ± 0.81 cd	104.22 ± 8.85 a	4.38 ± 0.03 d	9.06 ± 0.63 cd	52.03 ± 7.15 b	15.32 ± 0.80 c	8.76 ± 0.16 cd
E40	ethyl dodecanoate	45.81 ± 6.01 b	32.12 ± 1.80 cd	41.43 ± 0.69 b	85.36 ± 9.41 a	41.27 ± 3.13 b	40.77 ± 0.71 b	39.9 ± 0.90 bc	31.22 ± 1.39 d	40.95 ± 1.39 b	43.27 ± 1.67 b
	total esters	35 574.66 ± 976.14 d	359 609.62 ± 91 526.85 a	29 133.39 ± 3065.49 d	88 914.53 ± 7941.12 c	80 641.38 ± 6438.75 c	41 124.35 ± 2240.19c-d	36 186.42 ± 1451.88d	31 795.03 ± 312.98 d	209 685.40 ± 20 716.06-b	63 412.69 ± 2082.86c-d
K41	butane-2,3-dione	1458.75 ± 14.58 bcd	4547.69 ± 553.50 a	1362.47 ± 125.58 cd	1193.19 ± 40.54 d	1304.87 ± 67.65 d	1699.44 ± 19.28 bc	1428.46 ± 33.36 bcd	585.98 ± 9.89 e	1389.90 ± 88.04 cd	1747.78 ± 51.53 b
K42	pentane-2,3-dione	97.15 ± 2.22	540.44 ± 35.70	274.68 ± 38.29	100.03 ± 8.15	120.95 ± 4.01	214.70 ± 20.00	34.59 ± 5.39	24.39 ± 2.33	nq	nq
K43	3-hydroxybutan-2-one	1052.11 ± 107.19 b	38 780.30 ± 9646.81 a	1774.40 ± 139.70 b	1485.43 ± 113.12 b	1683.79 ± 82.95 b	4593.18 ± 155.27 b	1439.90 ± 61.05 b	1270.44 ± 19.87 b	846.34 ± 42.62 b	976.38 ± 95.72 b
K44	6-methyl-5-hepten-2-one	2.21 ± 0.23 a	1.50 ± 0.07 b	1.62 ± 0.22 b	1.46 ± 0.07 bc	0.90 ± 0.02 e	0.99 ± 0.05 de	1.09 ± 0.03 d	1.29 ± 0.03 c	1.05 ± 0.05 de	1.47 ± 0.07 bc
	total ketones	2610.23 ± 64.08 b	43 869.93 ± 9311.03 a	3413.17 ± 298.22 b	2780.10 ± 157.49 b	3110.50 ± 146.20 b	6508.30 ± 116.57 b	2904.03 ± 83.41 b	1882.11 ± 28.93 b	2237.24 ± 96.63 b	2722.61 ± 110.50 b
AL45	acetaldehyde	19 534.48 ± 1057.28 b	5065.22 ± 1609.09 e	26 176.64 ± 585.52 a	28 111.68 ± 1452.28 a	8935.28 ± 213.86 d	6380.61 ± 1225.63 e	15 990.54 ± 434.29 c	8750.48 ± 412.95 d	26 383.19 ± 2209.75 a	15 686.16 ± 423.97 c

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Table 1. continued

code	compounds	fermentation[b]									
		SC1116	HU26650	SP70572	SL3447	ZB70492	LT3434	TD291	PT70352	IO3433	MP70321
AL46	2-methylbutanal	4.06 ± 0.04	4.57 ± 0.64	19.09 ± 4.40	14.31 ± 3.33	nq	nq	nq	4.17 ± 0.21	nq	0.99 ± 0.26
AL47	3-methylbutanal	13.10 ± 1.20	6.95 ± 2.00	55.30 ± 8.29	37.96 ± 3.04	nq	nq	nq	5.33 ± 0.24	9.86 ± 1.85	14.4 ± 1.26
AL48	benzaldehyde	nq	14.21 ± 0.49	27.18 ± 0.26	27.55 ± 0.21	27.27 ± 0.20	26.13 ± 0.06	25.95 ± 0.06	13.15 ± 0.06	25.94 ± 0.03	27.09 ± 0.21
	total aldehydes	19 075.06 ± 669.48 b	5090.95 ± 1607.86 e	26 278.21 ± 588.20 a	28 191.49 ± 1446.30 a	8958.37 ± 213.18 d	6396.55 ± 1226.11 e	16 009.93 ± 435.94 c	8773.14 ± 413.19 d	26 417.71 ± 2211.33 a	15 728.63 ± 424.28 c
AC49	1-ethoxy-1-methoxyethane	2809.94 ± 71.68 e	7856.81 ± 3302.63 d	10 422.28 ± 138.93 c	17 052.87 ± 1584.74 a	7665.10 ± 146.99 d	5887.39 ± 443.44 d	10 031.52 ± 88.50 c	13 583.38 ± 539.40 b	560.19 ± 99.46 f	10 491.72 ± 137.13 c
AC50	1,1-diethoxyethane	46 474.62 ± 2367.92 b	14 402.77 ± 5119.68 g	37 560.37 ± 3731.13 cd	56 567.63 ± 4722.88 a	26 349.52 ± 2825.82 e	17 949.24 ±1029.71fg	34 637.50 ± 214.40 d	23 099.34 ± 772.44 ef	42 635.52 ±5390.93bc	39 583.48 ±818.94 cd
AC51	1-(1-ethoxyethoxy) pentane	1102.77 ± 173.33 c	479.52 ± 29.23 g	927.00 ± 51.27 d	1315.37 ± 45.58 b	623.99 ± 35.90 ef	524.50 ± 43.05 fg	899.53 ± 59.46 d	704.30 ± 26.53 e	693.59 ± 104.31 e	1491.32 ± 93.24 a
	total acetals	50 387.33 ±1746.25 bc	22 739.10 ± 8408.23 e	48 909.65 ± 3678.64 bc	74 935.87 ± 3239.49 a	34 638.61 ± 2950.72 d	24 361.14 ± 1044.05 e	45 568.55 ±175.45 bc	37 387.02 ± 1324.14 d	43 889.29 ± 5590.46 c	51 566.53 ± 863.15 b
FA52	2-methylpropanoic acid	2852.26 ± 387.34 cd	1988.50 ± 437.66 cd	2261.46 ± 224.78 cd	2252.57 ± 293.37 cd	8783.81 ± 2244.57 a	7539.62 ± 210.60 a	5125.00 ± 152.68 b	2243.46 ± 291.32 cd	3344.44 ± 157.01 c	1578.94 ± 113.64 d
FA53	pentanoic acid	8207.11 ± 204.76 c	2033.72 ± 523.40 f	9461.07 ± 1230.91 b	6332.52 ± 697.80 d	8702.58 ± 623.80 bc	3688.12 ± 102.94 e	4628.54 ± 534.60 e	1973.00 ± 178.31 f	15 629.12 ± 420.59 a	9107.30 ±1045.24b-c
FA54	heptanoic acid	776.73 ± 22.65 a	305.70 ± 28.32 e	481.85 ± 11.58 d	592.91 ± 38.2 c	299.17 ± 11.38 e	282.35 ± 3.67 ef	289.33 ± 9.17 ef	260.27 ± 2.86 f	303.35 ± 7.05 e	709.93 ± 12.06 b
FA55	octanoic acid	7025.54 ± 863.13 c	1616.63 ± 467.66 e	7736.84 ± 960.78 c	14 716.02 ± 1499.50 b	3374.48 ± 212.88 d	1105.82 ± 215.87 e	1145.46 ± 179.76 e	559.34 ± 107.44 e	1748.70 ± 170.31 e	17 102.96 ± 2060.62 a
	total fatty acids	18 861.64 ± 458.56 c	5944.53 ± 1398.99 e	19 941.21 ± 1561.25 c	23 894.02 ± 2381.96 b	21 160.04 ± 2838.92 c	12 615.90 ± 290.38 d	11 188.34 ± 595.20 d	5036.08 ± 393.78 e	21 025.61 ± 651.06 c	28 499.13 ± 1070.44 a
M56	3,7-dimethylocta-1,6-dien-3-ol	9.30 ± 0.43 a	8.52 ± 2.39 abc	9.18 ± 0.22 a	7.56 ± 0.59 bcd	6.47 ± 0.34 d	6.21 ± 0.24 d	7.27 ± 0.78 cd	6.49 ± 0.50 d	6.52 ± 0.42 d	8.97 ± 0.31 ab
M57	2-(4-methylcyclohex-3-en-1-yl)propan-2-ol	11.53 ± 0.71 bcd	10.2 ± 2.06 d	11.82 ± 0.07 bc	11.96 ± 0.17 b	10.43 ± 0.71 cd	10.11 ± 0.25 d	10.66 ± 0.83 bcd	8.65 ± 0.63 e	12.11 ± 0.41 ab	13.45 ± 0.18 a
	total monoterpenes	20.83 ± 1.01 ab	18.71 ± 4.45 bcd	21.01 ± 0.21 ab	19.52 ± 0.65 abc	16.90 ± 1.03 cde	16.31 ± 0.26 de	17.93 ±1.50 bcde	15.15 ± 1.11 e	18.63 ± 0.59 bcd	22.42 ± 0.13 a
B58	1,3,5-trimethylbenzene	5.64 ± 0.01 e	7.78 ± 1.91 d	12.65 ± 1.53 ab	12.09 ± 0.93 bc	12.42 ± 0.31 ab	11.47 ± 0.41 bc	10.75 ± 0.38 c	7.33 ± 0.31 d	4.82 ± 0.01 e	13.85 ± 0.72 a
B59	1,3-di-*tert*-butylbenzene	8.15 ± 0.30 a	6.41 ± 0.41 b	8.29 ± 1.70 a	8.41 ± 0.42 a	8.39 ± 0.59 a	7.13 ± 0.24 ab	7.92 ± 0.64 a	8.58 ± 1.21 a	7.46 ± 0.28 ab	8.53 ± 0.14 a
	total benzenes	13.79 ± 0.31 cd	14.19 ± 2.31 cd	20.94 ± 2.61 ab	20.49 ± 1.25 ab	20.81 ± 0.74 ab	18.60 ± 0.58 b	18.68 ± 0.32 b	15.91 ± 0.93 c	12.28 ± 0.28 d	22.38 ± 0.77 a

[a]Results represent the mean ± SD (n = 3). Values in the same raw with different letters (a–h) are significantly different according to Duncan test ($p < 0.05$). nq, not quantitated (<LOQ). [b]SC1116, HU26650, SP70572, SL3447, ZB70492, LT3434, TD291, PT70352, IO3434, and MP70321 represent *S. cerevisiae* Lalvin V1116, *H. uvarum* 26650, *S. pombe* 70572, *S. ludwigii* 3447, *Z. bailii* 70492, *L. thermotolerans* 3434, *T. delbrueckii* 291, *P. tannophilus* 70352, *I. orientalis* 3433, and *M. pulcherrima* 70321, respectively.

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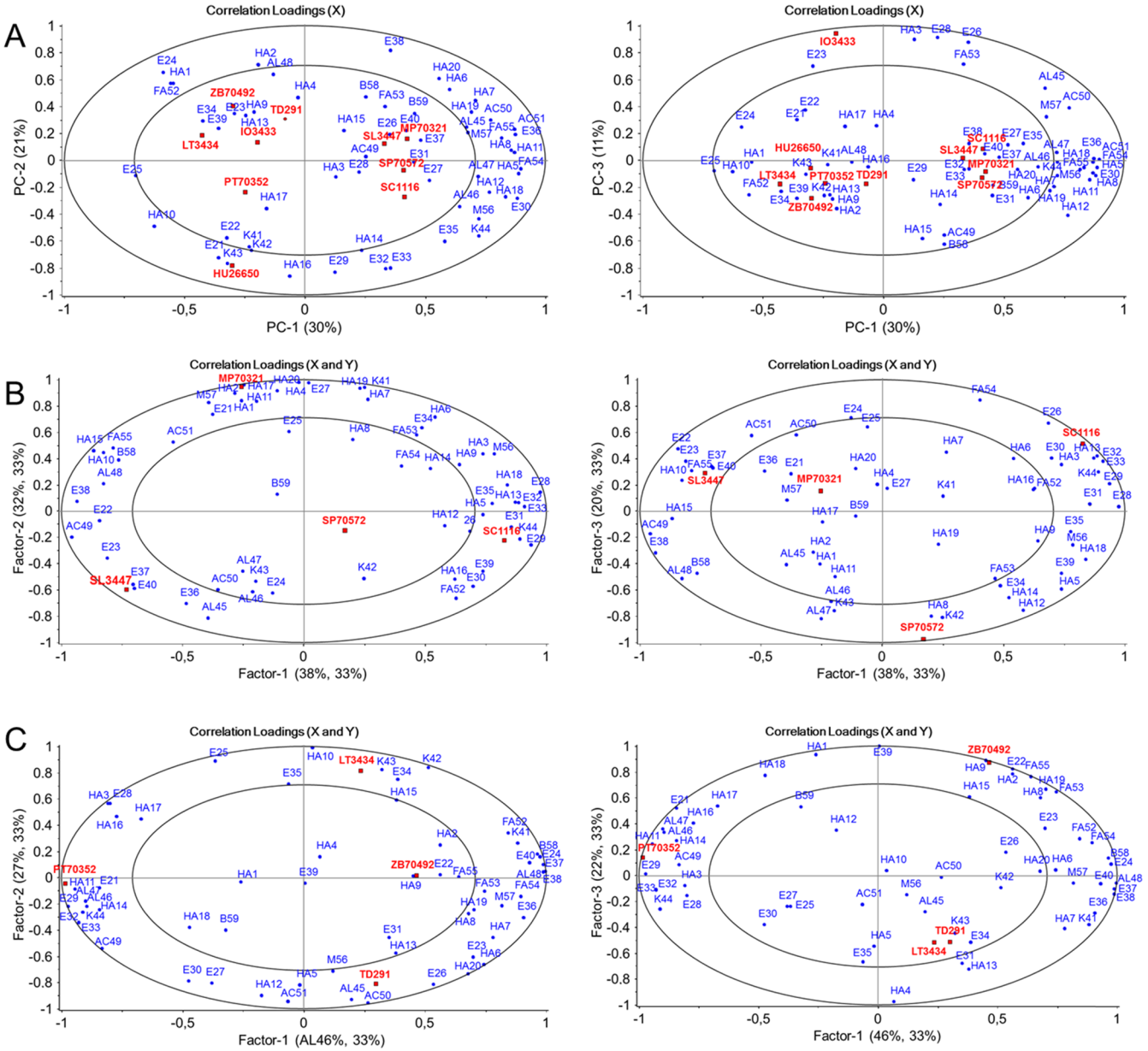

Figure 3. PCA and PLS-DA models using contents of volatile compounds (n = 59) to explain the differences between alcoholic bilberry beverages fermented with different yeasts (n = 10 in PCA; n = 4 in PLS-DA). The variable codes and abbreviations of yeasts refer to those in Table 1.

It is worth noting that ethyl acetate was reported to contribute to a pineapple fragrance at a low concentration, while it is likely to produce a chemical odor of varnish at a concentration above 150 mg/L.[30,31] Moreover, butane-2,3-dione, pentane-2,3-dione, and 3-hydroxybutan-2-one are reported as the main fatty odor contributors,[31,32] especially 3-hydroxybutan-2-one accounted for approximately 40−90% of the total amount of ketones in the finished bilberry beverages (Table 1). The high content of this compound in the fermentation product with *H. uvarum* strain is consistent with a previous finding reported by Romano and Suzzi suggesting that with low ethanol tolerance of *Hanseniaspora* preferentially utilize pyruvate for the biosynthesis pathway of 3-hydroxybutan-2-one rather than ethanol formation.[33] Therefore, the high generation of these undesirable compounds from the strain *H. uvarum* 26650 might confer a negative impact on the overall aroma of its corresponding bilberry beverage. PC-3 separates bilberry beverage produced with IO3433 from other samples primarily due to the high production of volatile compounds with generally pleasant odors, such as ethyl propanoate (E23), ethyl butanoate (E26) and 3-methylbutyl acetate (E28). These compounds were described as important contributors to fruity odors from banana, apple, and pineapple to strawberry.[19,31] However, a high concentration of pentan-2-ol (HA3), pentanoic acid (FA53), and ethyl acetate (>150 mg/L, Table 1) resulted from the inoculation with *I. orientalis* strain may have endowed ABB with bitter almond and cheese aromas.[34,35]

Despite the similarities in the volatile profiles within each of the two sample groups (SC1116, SP70572, SL3447, and MP70321 or ZB70492, TD291, LT3434, and PT70352), some variables contributing to the differences may have the ability to distinguish them from each other using more targeted multivariate analysis. Therefore, two additional PLS-DA models were established (Figure 3B,C).

Samples SC1116, SP70572, SL3447, and MP70321 were classified in the first PLS-DA model with four validated factors (R^2 = 0.989; validated R^2 = 0.972) (Figure 3B), indicating that

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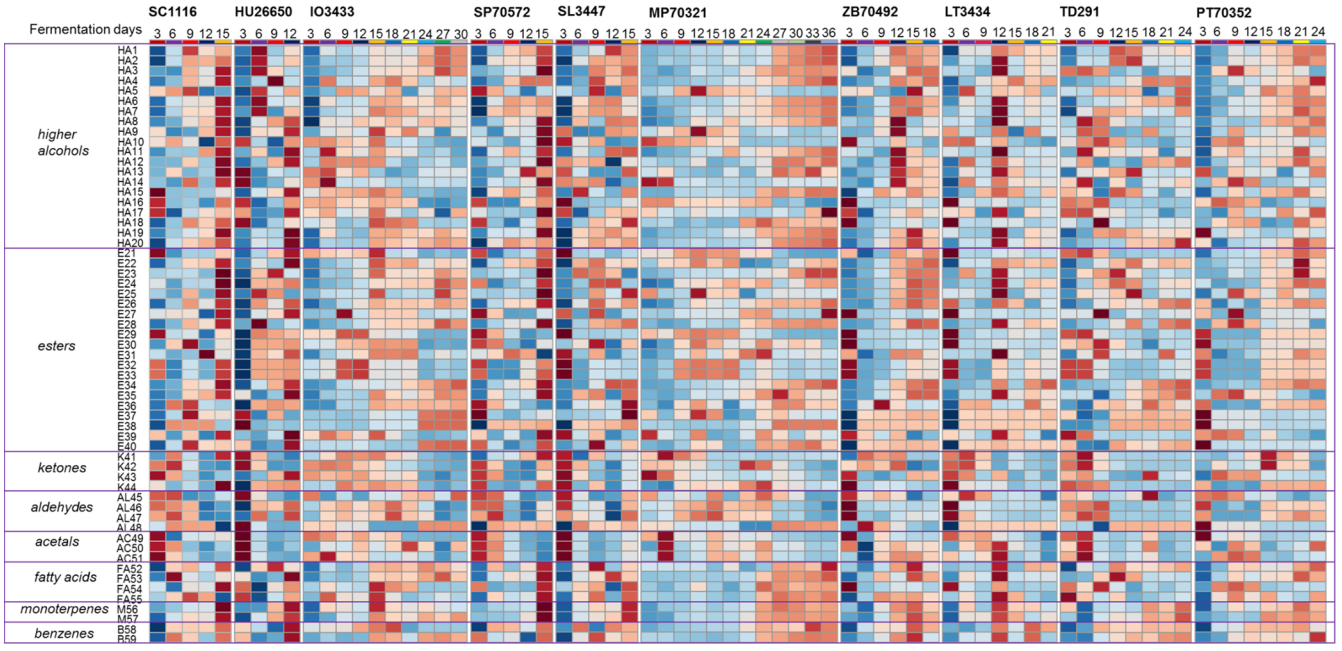

Figure 4. Heatmap visualization of the dynamic change in the concentration (based on normalized concentration) of the detected 59 volatile compounds during alcoholic bilberry beverage fermentations with 10 different yeasts. Each row on the heatmap represents the normalized concentration of an individual volatile compound (three replicates). Each column represents one fermentation with a particular strain after a particular period. The color scheme from blue to red represents the normalized value from low to high.

each of the four samples possessed a unique volatile profile. Fermentation with SL3447 was distinctly separated to the other three samples by its high production of ester compounds, like ethyl acetate (E22), ethyl propanoate (E23), ethyl octanoate (E36), ethyl decanoate (E37), and ethyl dodecanoate (E40), resulting in a 2.5 times higher amount of total esters than that with the control SC1116 (Table 1). Fermentation with MP70321 was characterized by a high content of higher alcohols as propan-1-ol (HA1), 2-methylpropan-1-ol (HA2), butan-1-ol (HA4), 3-methylbutan-1-ol (HA7), 3-methylpentan-1-ol (HA11), 2-ethylhexan-1-ol (HA17), 3-(methylthio)-1-propanol (HA19), and 2-phenylethanol (HA20). MP70321 was the only among the nine non-*Saccharomyces* samples that showed a significantly higher content of higher alcohols than the control SC1116 (316 vs 249 mg/L) (Table 1), which may result from the high decarboxylase activity in the conversion of keto acids in this species. The high yield of higher alcohols was also detected in the productions of sparkling wine and red wine inoculating with *M. pulcherrima*.[36,37] High production of unpleasant compounds, such as pentane-2,3-dione (K42), 3-hydroxybutan-2-one (K43), 2-methylbutanal (AL46), and 3-methylbutanal (AL47), differed in sample SP70572 from the other samples on factor-3 (Figure 3B).

Similarly, a clear separation of fermentations with PT70352, ZB70492, LT3434, and TD291 was observed in the second PLS-DA model with four validated factors (R^2 = 0.992; validated R^2 = 0.985) (Figure 3C). Fermentation with PT70352 was on the negative side of factor-1 due to the high concentration of 3-methylpentan-1-ol (HA11), (*Z*)-hex-3-en-1-ol (HA14), methyl acetate (E21), methyl hexanoate (E29), ethyl (*Z*)-hex-3-enoate (E32), ethyl (*E*)-hex-3-enoate (E33), 6-methyl-5-hepten-2-one (K44), 2-methylbutanal (AL46), 3-methylbutanal (AL47), and 1-ethoxy-1-methoxyethane (AC49). Because of the significant difference in the concentration of aldehydes, ketones, and acetals, ABBs produced with LT3434 and TD291 were located on the opposite side of factor-2. The final product fermented with ZB70492 differed from the samples produced with the other three yeasts partly due to the concentration of fatty acids.

Evolution of Volatile Compounds during Fermentation. Figure 4 shows the evolution of 59 volatile compounds during the fermentation process. The compounds were grouped into eight groups on the basis of their chemical classes. The monitoring analysis allowed us to assess the behavioral difference of each volatile compound during the 10 different fermentations. Generally, the concentrations of higher alcohols increased constantly and reached their maximum at the later stage of the fermentations with SC1116, SP70572, SL3447, ZB70492, PT70352, and MP70352. Nevertheless, the concentrations of higher alcohols increased sharply during the early to middle stages of fermentation followed by significant declines were also observed in the fermentations with HU26650, TD291, and LT3434. The decrease in these alcohols might partly be ascribed to the esterification reaction to yield their corresponding esters.[38] Among the 20 higher alcohols detected, 3-methylbutan-1-ol (HA7) and 2-methylbutan-1-ol (HA6) dominated throughout all 10 fermentations with different yeast strains (Table S3). 2-Phenylethanol (HA20), a floral odor contributor and metabolite formed in the Ehrlich pathway through the catabolism of phenylalanine,[39] followed a constant trend of increase all of the way through the fermentation with SC1116, HU26650, SP70572, SL3447, ZB70492, TD291, PT70352, and MP70321. 3-(Methylthio)-1-propanol (HA19), a compound derived from the catabolism of methionine during fermentation,[39] is the only sulfur compound detected in the ABBs. It is worth noting that this compound, often contributing to off-flavor in alcoholic beverages, reached its highest concentration after 12 days of inoculations with all yeast strains except PT70352. The concentration of 3-(methylthio)-1-propanol peaked after only 3 days of fermentation with PT70352,

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indicating a higher conversion efficiency of methionine of this yeast strain compared with the other ones.

Esters are another main group of secondary products produced by yeast metabolism during the fermentation of ABBs. The effect of the yeast strain on the change of the ester profile during ABB productions is complex. However, in general, the highest amounts of ethyl 3-methylbutanoate (E27), methyl hexanoate (E29), and ethyl hexanoate (E30) were obtained within the first 3 days of fermentations with SP70572, ZB70492, LT3434, and PT70352, whereas the concentrations of ethyl propanoate (E23), ethyl 2-methylpropanoate (E24), ethyl butanoate (E26), 3-methylbutyl acetate (E28), ethyl 2-hydroxypropanoate (E34), methyl 2-hydroxy-3-methylbutanoate (E35), and 2-phenylethyl acetate (E39) in the fermentation with SC1116 and SP70572 peaked at the end of the process. Interestingly, the evolution trend of esters of rising first followed by a significant concentration drop till the end of fermentation was more common in the samples fermented for a relatively longer time, such as fermentation with LT3434, TD291, PT70352, IO3433, and MP70321. The increasing release of cellular esterases along with fermentation might result in the decline of esters.[40] Ethyl acetate was the major compound accounting for more than 75% of the total ester content in ABBs (Table S3). Ethyl acetate is also known as being responsible for aroma deterioration.[41] The evolution of ethyl acetate in the fermentation could be distinguished into three different patterns: continuous accumulation throughout fermentation as in SC1116, HU26650, SP70572, and ZB70492 and PT70352; an initial sharp increase followed by a slow but constant decline as in SL3447, LT3434, TD291, and IO3433; finally, an increase at the early stage followed by fluctuation in the later stage as in MP70321.

Fatty acids play an extremely important role not only in the determination of the flavor feature of fermented beverages but also in the biosynthesis of fatty acid ethyl esters.[42] Generally, the concentrations of fatty acids increased for a certain time followed by a significant decrease, except those in the fermentations with SP70572 and MP70321, which showed a gradual increase. The reduction of fatty acids may be related to the occurrence of enzyme-mediated esterification between fatty acids and ethanol and to their absorption within yeast cell walls.[1,42]

Carbonyl compounds, including four aldehydes and four ketones, are intermediates in the formation of ethanol and higher alcohols from sugars and amino acids and generally are the early metabolic by-products of fermentation.[43] The transformation of carbonyl compounds, such as the reactions converting acetaldehyde (AL45) to ethanol and butane-2,3-dione (K41) to 3-hydroxybutan-2-one (K43), constantly occurred during yeast fermentation. Therefore, these carbonyl compounds generally showed a similar pattern of accumulating at the early stage followed by a significant decrease in the fermentation with all yeast strains. Acetals, including 1-ethoxy-1-methoxyethane (AC49), 1,1-diethoxyethane (AC50), and 1-(1-ethoxyethoxy)-pentane (AC51) were detected in this study. They are metabolites from fermentation through the reactions between acetaldehyde and alcohol.[38] Consequently, the concentration of acetals showed a similar changing pattern to that of the aforementioned aldehydes during yeast fermentation.

Monoterpenes and benzenes are the two minor groups in the varietal volatile compounds as their concentrations in all samples were lower than 20 μg/L throughout fermentation. Monoterpenes are reported to be responsible for floral odor in alcoholic beverages.[44] However, in this study, 3,7-dimethylocta-1,6-dien-3-ol (M56, linalool) and 2-(4-methylcyclohex-3-en-1-yl)propan-2-ol (M57, α-terpineol) may not contribute to the overall bouquet of ABBs due to their lower concentrations than their reported odor thresholds.[45] In all of the fermentation with each of the 10 yeast strains, the concentrations of monoterpenes showed a gradual increase and peaked at the end of the fermentation. The results are in line with the previous findings where the contents of α-terpineol, β-citronellol, borneol, and β-phellandrene increased to the levels above their odor thresholds at the later stage of fermentation of blueberry wine.[46] The concentration of benzenes (B58 and B59) reached the highest at the middle or middle-end stage and remained at the high level until the completion of fermentation.

In conclusion, to the best of our knowledge, this is the first report on the dynamic evolution of volatile compounds during the production of alcoholic bilberry beverages using non-conventional yeasts. Fermentations with non-*Saccharomyces* yeasts, especially with *H. uvarum* and *P. tannophilus*, produced less ethanol than that with *S. cerevisiae*. Ethanol content affected the extraction efficiency of other volatile compounds to HS-SPME fiber, highlighting the necessity of taking ethanol concentration into account when quantitating the volatile compounds in fermented bilberry samples. This conclusion could be extended to other fermentation procedures in which dynamic changes occur in the alcohol content. The evolution of volatile compounds during fermentation is yeast dependent. The new information on the diverse profiles of volatile compounds in the fermented bilberry beverages and on the dynamic changes in these compounds during fermentation will facilitate a better understanding of the biochemistry of the non-*Saccharomyces* yeasts in nongrape matrices. Moreover, since non-*Saccharomyces* yeasts are currently exploited in sequential or simultaneous inoculation with *S. cerevisiae* to provide diversity to the aroma profiles of alcoholic beverages, this study provides novel findings, which can be used for reducing or eliminating the accumulation of volatile compounds having potentially negative impact on aroma of alcoholic beverages during fermentation by non-*Saccharomyces* yeasts.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c01050.

Diagram of laboratory-scale fermentation (Figure S1) (PDF)

Initial concentrations of the authentic volatile standards (Table S1); identification and quantitation indexes of volatile compounds (Table S2) (PDF)

Dynamic change of volatile compounds during alcoholic bilberry beverage fermentation (Table S3) (XLSX)

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