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ORIGINAL PAPER

Effects of spontaneous fermentation on Karalahna and Cabernet Sauvignon young red wines: volatile compounds, sensory profiles and identification of autochthonous yeasts

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Abstract

The purposes of the present study were to evaluate the volatile compounds and sensory characteristics of young red wines produced by spontaneous and inoculated fermentations of Karalahna (KL) and Cabernet Sauvignon (CS) grapes and to identify the yeasts responsible for spontaneous fermentation by molecular methods. A total of 28 volatile compounds in KL wines and 35 compounds in CS wines were identified and quantified by GC–MS. The concentration of higher alcohols and esters differed significantly among spontaneously fermented and inoculated wines. Spontaneous fermentation resulted in greater amount of higher alcohols in KL wines, while inoculated wines had greater amount of higher alcohols in CS wines. Spontaneously fermented KL and CS wines showed greater amounts of esters than inoculated wines. KL wines obtained by spontaneous fermentation had significantly higher scores than inoculated wines based on fruity and green aromas, body and overall impression. Spontaneously fermented CS wines were found significantly higher in fruity and floral aromas than inoculated wines.

Keywords Red wine · Spontaneous fermentation · Volatile compounds · Sensory profile · Yeasts

Introduction

Aroma is one of the most important aspects of wine quality. Wine aroma is a complex mixture of hundreds of volatile compounds [1]. These aromatic compounds are closely related to the sensory quality of wine which is extremely important for consumers. In general, wine aroma is divided into three categories based on origins as primary, secondary and tertiary aromas. Primary aromas are grape-derived volatile compounds responsible for varietal character, while secondary aromas are produced by yeasts through the winemaking process and also known as "yeast bouquet". Tertiary aromas arise in finished wines during maturation and aging process [2, 3].

Yeasts play a key role in wine fermentation and contribute to the sensory characteristics of wine. Microflora in grape must vary depending on indigenous grape microflora, winery flora, harvest method (handpicked or mechanical), temperature, transportation from vineyard to cellar and production techniques [4]. Wine fermentation can occur naturally by indigenous yeasts (spontaneous fermentation) or by the inoculation of selected strains (inoculated fermentation). Inoculated fermentation provides a dominant strain in the fermentation and ensures a proper fermentation process in order to obtain reproducible products. However, the use of pure yeast cultures can reduce the formation of some desirable compounds which are important for the wine quality [5].

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Spontaneous fermentation, also referred as natural fermentation, is a complex microbial process performed by different strains of *Saccharomyces* and non-*Saccharomyces* yeasts. Spontaneous fermentation is generally associated with the slower fermentation rate or stuck fermentation and the formation of undesirable flavor compounds. However, it brings improved characteristics to wine-like aroma complexity and mouthfeel (body and finish) [6].

In recent years, high-quality wine production with indigenous wine yeasts became very important and highly appreciated in wine-producing countries like France, Italy and Spain. Numerous studies have showed that unique and typical regional wines can be produced using autochthonous wine yeasts [7, 8]. Among wine yeasts, non-*Saccharomyces* yeasts have great attention due to their desired enological effects for wine, such as high levels of aroma production [7]. In fact, non-*Saccharomyces* yeasts may contribute to the wine flavor by synthesizing secondary metabolites such as higher alcohols, esters, acids, volatile thiols and extracellular enzymes like β-glucosidases [9]. Among non-*Saccharomyces* yeasts, especially *Hanseniaspora* and *Candida* genera, are known as prevalent yeasts found on grapes and at the early stages of the fermentation process [4, 6]. They can affect the quality attributes of wine either positively or negatively [6, 10]. In recent years single or mixed inocula of non-*Saccharomyces* yeasts have been studied extensively to understand how they affect wine characteristics [11]. Therefore, there is a growing interest in isolation and characterization of non-*Saccharomyces* yeasts which can be used in wine fermentations [12].

Çanakkale is one of the most important wine-producing regions in Turkey, located in the northwest of the country. Karalahna is one of the well-known local grape varieties of Bozcaada (*Tenedos*: the island in the Eagean Sea) in Çanakkale. Since Karalahna wine has a dark color, it has been used in wine blending to enhance wine color mostly. In recent years, its single varietal production both by spontaneous and inoculated fermentations became very popular among the regional winemakers. However, to the best of our knowledge there is no study on the volatile composition and sensory profile of Karalahna wine. Also, the volatile composition and sensory profile of Cabernet Sauvignon wines originating from this region (Çanakkale, Turkey) have not been documented. Thus, the aims of this study were to characterize the wines produced from Karalahna and Cabernet Sauvignon grapes by spontaneous and inoculated fermentations with regard to volatile compounds and sensory properties and to identify the indigenous yeasts responsible for spontaneous fermentation by molecular methods.

Materials and methods

Chemicals and reagents

All standard compounds were purchased from Sigma–Aldrich (St. Louis, MO, USA). Purity of all standards was of gas chromatographic grade. 4-Methyl 2-Pentanol (4M2P) and methyl nonanoate (MN) were used as the internal standards. All other chemicals used in the study were of analytical grade.

Winemaking

Wine productions were performed in 2014 vintage. Grapes of Karalahna grown in Bozcaada (Tenedos), Çanakkale and Cabernet Sauvignon grown in Eceabat, Çanakkale were harvested at maturity considering pH, brix, titratable acidity and taste. All grapes were transported to the winery (Vinero, Canakkale, Turkey) for winemaking. Grapes were destemmed and crushed after elimination of impurities. General properties of the must samples are shown in Table 1. Musts were filled in 25 L glass vessels, SO_2 (15 mg/L) was added to the musts and then they were allowed to cold soak for 4 days at 7–8 °C. After cold maceration, the temperature of the musts was increased to 13 °C to inoculate wine yeasts. Wine productions were performed in 3 replicates for each variety as spontaneous and inoculated fermentations. Inoculations were carried out using a commercial *S. cerevisiae* strain (Zymaflore FX10 Laffort) at 20 g/hL. During alcoholic fermentation the must cap was punched down two times in a day and mixed well for uniform must. Fermentations were followed by daily measurement of density and temperature. Higher fermentation rates were observed in inoculated fermentations for both grape varieties (data were not shown). Spontaneous fermentations took 4 and 7 days longer in Karalahna and Cabernet Sauvignon wines compared to inoculated fermentations. The fermentation process completed after 20 days. After fermentations, SO_2 was added to ensure a final concentration of 25 mg/L free SO_2

Table 1 General properties of the must

	pH	°Brix	Total acidity[a] (g/L)	Reducing sugar (g/L)
Karalahna (KL)	3.15 ± 0.01	25.2 ± 0.1	6.64 ± 0.34	242.60 ± 3.72
Cabernet Sauvignon (CS)	3.95 ± 0.01	21.5 ± 0.1	4.35 ± 0.23	219.8 ± 0.6

The results were expressed as mean values ± standard error

[a]Expressed as tartaric acid

and wines were bottled. All analyses were performed after 6 months of maturation in bottle.

Basic wine composition

Determinations of pH (Sartorius PB-11, Goettingen, Germany), titratable acidity, dry matter and ash content, reduced sugar by Luff-Schoorl method, density by pycnometer, alcohol content by ebulliometer (Dujardin-Salleron, Noizay, France), volatile acidity, total and free SO_2 were carried out [13]. All analyses were performed in duplicate. Physical and chemical characteristics of the young wines are presented in Table 2.

Isolation and identification of yeasts

For the isolation of the yeast strains that play a role in spontaneous fermentation, 100 mL of wine samples was taken from each grape variety at the beginning (alcohol content 1%), middle (alcohol content 4%) and the end (alcohol content > 9%) of the fermentation in aseptic conditions. Wallerstein Laboratory (WL) nutrient agar containing 10 mg/L streptomycin and lysine agar medium containing 10 mg/L ampicillin were used for isolation yeast species of *Saccharomyces* and non-*Saccharomyces*, respectively. The incubation conditions of petri dishes were at 28 °C for 2–6 days. Ten of yeast colonies from each sample were selected and purified on GYP (glucose–yeast extract-peptone) agar medium [2% (*w*/*v*) glucose, 1% (*w*/*v*) peptone, 0.5% (*w*/*v*) yeast extract, 2% (*w*/*v*) agar] [14, 15].

The molecular identification of the yeast strains isolated from wine samples was carried out by using Restriction Fragment Length Polymorphism (RFLP) method. In this method, the genomic DNA of the yeast isolates was extracted using an isolation kit (Thermo GeneJET). PCR amplification of the internal transcribed spacers between the 18S and 26S rDNA genes (ITS1-5.8S-ITS2) and subsequent restriction analysis, in the yeast strains were performed as reported by Esteve-Zarzoso et al. [16]. PCR products were digested without further purification with restriction enzymes *Cfo*I, *Hae*III and *Hinf*I (Thermo Scientific FastDigest). Restricted fragments were separated by electrophoresis in 2% agarose gels and 1.0X TBE buffer. The obtained profiles were visualized and photographed under UV light [16–18].

Determination of volatile compounds

Volatile compounds of wine samples were isolated by Headspace-Solid Phase Microextraction (HS-SPME). 5 mL of wine, 10 µL internal standard mixture (4-methyl 2-pentanol and methyl nonanoate) and 1 g NaCl were placed in 40 mL amber colored vials (Supelco, Bellefonte, PA, USA) capped with a PTFE/silicon septum [3]. Different concentrations

Table 2 Physicochemical properties of Karalahna and Cabernet Sauvignon wines

	Density (g/mL)	Alcohol (v/v)	pH	Total acidity[A] (g/L)	Volatile acidity[B] (g/L)	Ash (g/L)	Reducing sugar (g/L)	Total SO_2 (mg/L)	Free SO_2 (mg/L)
Karalahna (KL)									
Spontaneous	0.999 ± 0.01^{a}	14.28 ± 0.07^{a}	3.12 ± 0.01^{a}	10.16 ± 0.09^{a}	0.61 ± 0.01^{a}	2.84 ± 0.14^{a}	4.10 ± 0.07^{a}	31.2 ± 1.83^{a}	16.1 ± 0.76^{a}
Commercial yeast inoculated	0.999 ± 0.01^{a}	14.08 ± 0.08^{a}	3.13 ± 0.01^{a}	10.21 ± 0.28^{a}	0.62 ± 0.01^{a}	2.97 ± 0.05^{a}	3.83 ± 0.31^{a}	29.8 ± 0.48^{a}	18.3 ± 0.67^{a}
Cabernet Sauvignon (CS)									
Spontaneous	0.996 ± 0.001^{x}	11.7 ± 0.1^{x}	3.84 ± 0.01^{x}	6.26 ± 0.11^{x}	0.85 ± 0.06^{x}	3.54 ± 0.04^{x}	2.34 ± 0.09^{x}	32.3 ± 9.9^{x}	14.7 ± 0.9^{x}
Commercial yeast inoculated	0.996 ± 0.001^{x}	11.6 ± 0.1^{x}	3.93 ± 0.01^{y}	6.13 ± 0.17^{x}	0.63 ± 0.04^{y}	3.55 ± 0.07^{x}	1.74 ± 0.04^{x}	21.5 ± 0.8^{y}	9.5 ± 0.5^{y}

The results were expressed as mean values ± standard error ($n = 6$)

$^{a-b}$Different letters within a column mean significant differences for KL wines ($p < 0.05$)

$^{x-y}$Different letters within a column mean significant differences for CS wines ($p < 0.05$)

^{A}Expressed as tartaric acid

^{B}Expressed as acetic acid

of internal standard (IS) mixtures were used for the quantification of volatiles in Karalahna and Cabernet Sauvignon wines. Internal standards were prepared in ethanol. It includes 0.005 µL methyl nonanoate and 0.002 µL 4-methyl 2-pentanol in 1 mL ethanol for Karalahna wines while including 0.1 µL methyl nonanoate and 1 µL 4-methyl 2-pentanol in 1 mL ethanol for Cabernet Sauvignon wines.

A gas chromatography–mass spectrometry system (GC–MS) (GC 6890 N, MS 5975C, Agilent Technologies, Wilmington, DE, USA) equipped with a HP5 column (30 m × 0.25 mm i.d. × 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA) was used for the separation of volatile compounds. Helium gas was used at the flow rate of 1.2 mL/min. The temperature programme was from 40 °C (2 min) to 120 °C at 2 °C/min, from 120 °C (3 min) to 250 °C at 8 °C/min, and then 250 °C for 2 min. Data were obtained in the electron impact (EI) mode with an ionization voltage of 70 eV.

Mixtures of aliphatic hydrocarbons (C_6–C_{25}) (Aldrich, MO, USA) were injected at the same chromatographic conditions and the retention indices (Kovats indices) were calculated according to Van Den Dool and Kratz [19]. National Institute of Standards and Technology and Wiley Registry of Mass Spectral Data libraries were used for identification of volatile components. The concentration of alcohols was calculated based on 4-methyl 2-pentanol internal standard. Amounts of esters and other compounds (vitispirane, γ-butyrolactone and limonene) were determined based on methyl nonanoate.

Sensory evaluation

The wines were evaluated by six trained panelists whose ages ranged from 24 to 45. 20 h training session was performed for panelists by an enologist. Evaluations were conducted with the Spectrum™ descriptive method [20]. By the first tasting panel, panelists developed the best descriptors that define the wine samples. Twelve descriptive terms were developed for the evaluation of wine samples. Descriptive terms and reference standards used in sensory evaluation were given in Table 3. Wine samples were rated using a 0–10 scale (1: very low intensity and 10: very high intensity). 25 mL of wine samples were served at 13–14 °C in a tulip-shaped wine tasting glass. All wine samples aerated 30 min before evaluation. Salt-free crackers were served to the panelists in order to neutralize their mouths between the wine samples.

Statistical analysis

In comparison of inoculated and spontaneous fermented young wines, one-way analysis of variance (ANOVA) was performed using the SPSS 22 Statistical software program (SPSS Inc., Chicago, IL, USA). Kruskal–Wallis test was used to compare the means for non-parametric data. Young wines from each grape varieties were evaluated individually.

Table 3 Descriptive terms and reference standards

Descriptive terms	References
Red fruit	Grape, plum
Alcohol	Ethyl alcohol
Green	Grass
Floral	Rose
Sour	Citric acid solution (0.08%)
Sweet	Sucrose solution (2%)
Astringency	Alum solution (0.5%)
Sweet spice	Cinnamon, clove
Body	Weight of wine as mouthfeel
Color	Clarity, intensity
Finish	Final taste sensations of wine after swallowed
Overall impression	Balance and harmony of all taste sensations

Results and discussion

Isolation and identification of yeasts

Isolation and identification of yeasts during spontaneous fermentation of KL and CS grapes were evaluated at the beginning, middle and end of the fermentation. A total of 178 isolates were obtained during fermentation stages. 138 of these isolates were identified as yeast and further molecular analyses were applied in these isolates. The distribution of yeasts in different stages of the spontaneous fermentation was given in Table 4. RFLP profiles of yeast strains isolated from wine samples are shown in Supplementary Figure S.1, S.2 and S.3.

Candida albicans, Zygosaccharomyces bisporus, *Dekkera anomala* (formerly *Brettanomyces anomalus*) and *Pichia terricola* (formerly *Issatchenkia terricola*) were isolated at the beginning of the alcoholic fermentation of KL grapes (alcohol content 1%). Among these, *Candida albicans was determined as the* dominant flora at this stage. On the other hand, *Dekkera anomala* was found as dominant flora at the beginning of the fermentation of CS grapes. *Candida albicans* and *Candida apicola* were also isolated at this stage. *Hanseniaspora* (*Kloeckera*), *Candida* and *Metschnikowia* species are mostly found at the beginning of fermentation. Also, *Pichia*, *Issatchenkia* and *Kluyveromyces* species can grow in some cases [21]. *Dekkera anomala* is noted as a kind of yeast that can be found in every step of fermentation. Rodrigues et al. [22] reported that *Dekkera* species can be isolated from the cellar and grapes. *Zygosaccaharomyces bisporus* and *Pichia terricola* species can also be isolated from cellar environment.

Table 4 Distribution of yeasts during the spontaneous fermentation of KL and CS grapes

Grape variety	Beginning of the fermentation		Middle of the fermentation		End of the fermentation	
	Yeast species	Number of isolates	Yeast species	Number of isolates	Yeast species	Number of isolates
KL	*Candida albicans*	14	*Candida albicans*	19	*Candida albicans*	13
	Zygosaccharomyces bisporus	7	*Dekkera anomala*	6	*Dekkera anomala*	9
	Dekkera anomala	4				
	Pichia terricola	3				
Total		28		25		22
CS	*Dekkera anomala*	14	*Dekkera anomala*	7	*Candida apicola*	20
			Candida apicola	3	*Candida albicans*	11
			Candida albicans	5	*Dekkera anomala*	3
Total		14		15		34

At the middle (alcohol content 4%) and end of the fermentations (alcohol content > 9%), *Candida albicans* was found predominantly and *Dekkera anomala* species ranked second for KL wines. *Candida apicola* (as dominant flora), *Candida albicans* and *Dekkera anomala* were found at the end of the fermentation in CS wines. It is known that most non-*Saccharomyces* yeast strains cannot tolerate 5–7% ethanol concentration during fermentation process. However, in low temperature (15–20 °C) *Hanseniaspora* and *Candida* species can tolerate ethanol and grow as much as *S. cerevisiae* [21]. The isolation of *Candida apicola* and *Candida albicans* species at the end of fermentation of KL and CS wines may be explained by this finding.

It is stated that the yeast species in the wines are derived from grapes, vineyard, equipments used in cellars and cultures if used in the fermentation [6]. Although *Saccaharomyces cerevisiae* is known as the main species of wine fermentation, the other yeast species have also important impacts. Usually the yeast load of immature grapes is low (10–10^3 cfu/g) and it increases to 10^4–10^6 cfu/g population level when the grapes get mature to harvest. *Rhodotorula*, *Cryptococcus* and *Candida* species were detected predominantly on immature grapes [21]. Since *S. cerevisiae* is not prevalent (10-100 cfu/g) on wine grapes, difficulties in isolation of *S. cerevisiae* from healthy mature grapes were reported [21, 23]. In fact, we were not able to identify *Saccharomyces cerevisiae* in this study, even in the middle and at the end of the spontaneous fermentation of KL and CS grapes. Surface chemistry of grapes, tolerance characteristics of the yeast strains against some environmental conditions including temperature, sunlight, irradiation, chemical inhibitors, and interactions with other microorganisms may influence the dynamics of yeasts during the fermentation [21].

In recent years, several researches based on quantitative polymerase chain reaction (PCR) and denaturating gradient gel electrophoresis (DGGE) have shown that autochthonous non-*Saccharomyces* yeasts are not inhibited entirely during the early stages of alcoholic fermentation. They can subsist during the fermentation, even in the case of inoculation of active dried yeasts [24].

Volatile compounds of wines

A total of 28 compounds in KL wines and 35 compounds in CS wines were identified and quantified by GC–MS using HS-SPME technique. Volatiles were given in Tables 3 and 4 including retention time, retention index and odor descriptors of the compounds. Higher alcohols were the most abundant in all wines consisting about 99.6% and 99.1% of the total volatiles identified in KL and CS wines, respectively. On the other hand, esters accounted for only about 0.3% and 0.8% of the total volatiles in KL and CS wines, respectively.

Higher alcohols

Alcohols with more than two carbon atoms are known as higher alcohols. Many of these compounds are produced during fermentation and contribute to the wine complexity at low concentrations [25].

Composition and concentration of higher alcohols differed among KL and CS wines. For KL wine samples, 9 higher alcohols were identified and quantified (Table 5), while 17 higher alcohols were identified and quantified in CS wines (Table 6). Isoamyl alcohol had the highest concentration followed by phenyl ethyl alcohol and 2-methyl 1-butanol in all wine samples. Among higher alcohols of KL wines, 1-octen-3-ol, 6-methyl-5-hepten-2-ol and phenyl ethyl alcohol showed significantly higher concentrations in spontaneously fermented wines. However, no significant differences were found in other higher alcohols of KL wines. For CS wines, the concentrations of isoamyl

Table 5 Concentration of some volatile compounds in young Karalahna wines produced by spontaneous and inoculated fermentations

Compounds	Retention time (min)	Retention Index (RI)[A]	Odor descriptor	Spontaneous	Inoculated
Higher alcohols (mg/L)					
Isoamyl alcohol	3.1	734	Cheese[B]	36.67 ± 1.04[a]	35.38 ± 1.10[a]
2-Methyl 1-butanol	3.2	739	–	11.80 ± 0.44[a]	12.38 ± 0.39[a]
1-Hexanol	6.6	865	Green, Grass[C]	1.68 ± 0.04[a]	1.66 ± 0.05[a]
1-Heptanol	11.4	968	Sweet[B], lemon[C]	0.94 ± 0.04[a]	0.84 ± 0.04[a]
1-Octen-3-ol	11.8	975	Mushroom[C]	0.27 ± 0.01[a]	0.21 ± 0.01[b]
6-Methyl-5-hepten-2-ol	12.5	988	–	0.05 ± 0.01[a]	0.03 ± 0.01[b]
1-Octanol	17.5	1069	Citrus, rose[B]	0.29 ± 0.02[a]	0.24 ± 0.02[a]
Phenyl ethyl alcohol	19.7	1104	Rose, honey[C]	16.57 ± 0.51[a]	8.93 ± 0.54[b]
1-Nonanol	24.1	1170	Raspberry[C]	0.48 ± 0.03[a]	0.49 ± 0.18[a]
Σ Higher alcohols (mg/L)				68.75	60.16
Esters (µg/L)					
Ethyl acetate	1.8	616	Fruity, sweet[D]	26.81 ± 1.20[a]	30.32 ± 1.15[a]
Isobutyl acetate	3.8	772	Banana[C]	2.26 ± 0.18[a]	2.43 ± 0.20[a]
Ethyl butanoate	4.4	803	Apple[C]	5.42 ± 0.43[a]	4.65 ± 0.26[a]
Propanoic acid, 2-hydroxy ethyl ester	4.7	811	–	2.13 ± 0.21[a]	1.75 ± 0.23[a]
Butanoic acid, 2-methyl ethyl ester	6.0	848	–	0.68 ± 0.03[a]	0.73 ± 0.05[a]
Butanoic acid, 3-methyl ethyl ester	6.1	850	–	1.86 ± 0.10[a]	1.91 ± 0.12[a]
Isoamyl acetate	6.9	874	Banana[C]	16.12 ± 0.56[a]	18.97 ± 0.84[b]
2-Methylbutyl acetate	7	877	Pear[C]	4.64 ± 0.14[a]	5.92 ± 0.28[b]
Ethyl hexanoate	13.1	1000	Fruity, anise[B]	48.86 ± 1.72[a]	45.28 ± 2.96[a]
Ethyl-2-hexanoate	15.7	1040	–	0.56 ± 0.02[a]	0.54 ± 0.10[a]
Ethyl heptanoate	19.3	1097	Fruity[C]	5.97 ± 0.21[a]	4.48 ± 0.45[b]
Octanoic acid, 2-methyl–methyl ester	23.4	1159	–	1.18 ± 0.07[a]	1.10 ± 0.04[a]
Diethyl succinate	24.8	1180	Fruity[C], melon[E]	40.70 ± 2.34[a]	31.68 ± 2.92[b]
Ethyl octanoate	25.9	1197	Pineapple, pear, floral[B]	53.65 ± 3.72[a]	39.60 ± 10.01[b]
Benzene acetic acid, ethyl ester	28.6	1237	–	9.51 ± 0.33[a]	7.69 ± 0.65[b]
Phenylethyl acetate	29.4	1250	Floral, rose[C]	1.10 ± 0.06[a]	1.03 ± 0.09[a]
Ethyl nonanoate	32.4	1295	Floral, fruity[C]	0.58 ± 0.07[a]	0.44 ± 0.07[a]
Ethyl decanoate	38.6	1393	Fruity[C]	3.19 ± 0.30[a]	3.08 ± 0.65[a]
Σ Esters (µg/L)				225.22	201.60
Other (µg/L)					
Vitispirane	30.6	1268	Fruity[F]	2.72 ± 0.14[a]	2.07 ± 0.13[b]

The results were expressed as mean values ± standard error ($n = 6$)

Different letters in the same row means significant difference in concentrations according to One-way ANOVA ($p < 0.05$)

[A]Calculated RI for HP5 column

[B][27]

[C]Soares et al. [39]

[D]Tao and Li [40]

[E][26]

[F][25]

alcohol, 1-pentanol, (S)-(+)-3-Methyl-1-pentanol, 1-hexanol, 1-heptanol, (s)-3-ethyl-4-methylpentanol, benzyl alcohol, 1-octanol and phenyl ethyl alcohol were significantly higher in inoculated wines.

Isoamyl alcohol was the main higher alcohol accounting for about 55% of higher alcohols determined in all wine samples. This compound is released as a secondary product of yeast metabolism and generally associated with cheese-like odor. There is no significant difference between spontaneous (36.67 mg/L) and inoculated KL wines (35.38 mg/L) in terms of isoamyl alcohol content. Isoamyl alcohol concentrations of spontaneous and inoculated CS wines were found

Table 6 Concentration of some volatile compounds in young Cabernet Sauvignon wines produced by spontaneous and inoculated fermentations

Compounds	Retention time (min)	Retention Index (RI)[A]	Odor descriptor	Spontaneous	Inoculated
Higher alcohols (mg/L)					
Isoamyl alcohol	3.1	734	Cheese[B]	23.92 ± 0.85[a]	40.51 ± 1.74[b]
2-Methyl 1-butanol	3.2	739	–	6.23 ± 0.27[a]	6.78 ± 0.36[a]
1-Pentanol	3.6	761	Balsamic[C]	0.04 ± 0.01[a]	0.07 ± 0.01[b]
(S)-(+)-3-Methyl-1-pentanol	5.7	840	Soil, mushroom[D]	0.04 ± 0.01[a]	0.10 ± 0.01[b]
(Z) 3-Hexen-1-ol	6.2	854	Green, bitter[C]	0.04 ± 0.01[a]	0.04 ± 0.01[a]
1-Hexanol	6.6	865	Green, Grass[C]	3.93 ± 0.04[a]	5.09 ± 0.26[b]
1-Heptanol	11.4	968	Sweet[B], lemon[C]	0.20 ± 0.01[a]	0.35 ± 0.03[b]
1-Octen-3-ol	11.8	975	Mushroom[C]	0.18 ± 0.01[a]	0.25 ± 0.02[a]
3-Octanol	12.8	994	–	0.05 ± 0.01[a]	0.07 ± 0.01[a]
(s)-3-Ethyl-4-methylpentanol	14.3	1019	–	0.60 ± 0.08[a]	1.30 ± 0.11[b]
Benzyl alcohol	14.8	1027	Citrus[B], sweet[B]	0.49 ± 0.06[a]	0.76 ± 0.09[b]
1-Octanol	17.5	1069	Citrus, rose[B]	0.36 ± 0.01[a]	2.26 ± 0.20[b]
2-Nonanol	19.4	1099	–	0.26 ± 0.11[a]	0.10 ± 0.03[a]
Phenyl ethyl alcohol	19.7	1104	Rose, honey[C]	7.55 ± 0.88[a]	14.58 ± 1.60[b]
(6Z)-Nonen-1-ol	24.0	1168	–	0.09 ± 0.01[a]	0.10 ± 0.01[a]
1-Nonanol	24.1	1170	Raspberry[C]	0.37 ± 0.04[a]	0.47 ± 0.06[a]
1-Decanol	30.7	1270	Flower[B]	0.29 ± 0.03[a]	0.29 ± 0.06[a]
Σ Higher alcohols (mg/L)				44.64	73.12
Esters (µg/L)					
Ethyl acetate	1.8	616	Fruity, sweet[D]	297.50 ± 34.78[a]	106.02 ± 7.81[b]
Isobutyl acetate	3.8	772	Banana[C]	4.26 ± 0.29[a]	1.69 ± 0.15[b]
Ethyl butanoate	4.4	803	Apple[C]	1.97 ± 0.17[a]	3.73 ± 0.28[b]
Isoamyl acetate	6.9	874	Banana[C]	41.43 ± 2.07[a]	23.22 ± 1.12[b]
2-Methylbutyl acetate	7	877	Pear[C]	10.22 ± 0.57[a]	8.33 ± 0.60[a]
Ethyl hexanoate	13.1	1000	Fruity, anise[B]	46.77 ± 3.30[a]	65.28 ± 4.36[b]
Hexyl acetate	13.9	1013	Pear[C], apple[E]	4.14 ± 0.32[a]	2.96 ± 0.20[b]
Isoamyl lactate	17.2	1064	–	1.84 ± 0.16[a]	3.36 ± 0.17[b]
Ethyl heptanoate	19.3	1097	Fruity[C]	0.83 ± 0.07[a]	0.55 ± 0.02[b]
Octanoic acid. 2-methyl–methyl ester	23.4	1159	–	2.68 ± 0.08[a]	2.88 ± 0.04[a]
Diethyl succinate	24.8	1180	Fruity[C], melon[E]	5.93 ± 0.68[a]	68.73 ± 11.09[b]
Methyl salicylate	25	1183	–	11.84 ± 0.54[a]	27.73 ± 1.96[b]
Ethyl octanoate	25.9	1197	Pineapple, pear, floral[B]	47.47 ± 3.14[a]	100.77 ± 9.21[b]
Phenylethyl acetate	29.4	1250	Floral, rose[C]	5.07 ± 0.38[a]	3.26 ± 0.23[b]
Ethyl salicylate	30.1	1261	–	3.03 ± 0.28[a]	3.10 ± 0.19[a]
Ethyl decanoate	38.6	1393	Fruity[C]	9.81 ± 0.52[a]	18.90 ± 2.44[b]
Σ Esters (µg/L)				494.79	440.51
Others (µg/L)					
γ-Butyrolactone	8.6	915	Sweet, caramel[E]	0.19 ± 0.05[a]	1.30 ± 0.16[b]
Limonene	14.5	1022	Floral, green, citrus[B]	0.32 ± 0.01[a]	0.13 ± 0.06[b]

The results were expressed as mean values ± standard error ($n = 6$)

Different letters in the same row means significant difference in concentrations according to One-way ANOVA ($p < 0.05$)

[A]Calculated RI for HP5 column

[B][27]

[C]Soares et al. [39]

[D]Tao and Li [40]

[E] [26]

as 23.92 and 40.51 mg/L, respectively. It is seen that commercial yeast strain provided higher formation of isoamyl alcohol. Similarly, Romano et al. [5] who studied the aromatic profiles of wines obtained by the inoculation of different wine yeast species to the Aglianico grape must reported that wines produced by *S. cerevisiae* had higher isoamyl alcohol compared to the wines obtained by the inoculation of non-*Saccharomyces* species.

Phenyl ethyl alcohol, which is known to contribute to floral (rose-like) aromas of wine, was the second most abundant higher alcohol identified in all wines [25]. KL wines obtained by spontaneous fermentation had significantly greater amount of phenyl ethyl alcohol (Table 5). On the contrary to KL wines, spontaneously fermented CS wines had lower concentration of phenyl ethyl alcohol (Table 6). The amount of phenyl ethyl alcohol in wines can vary depending on grape variety and maturity and winemaking techniques [26, 27]. It was stated that phenyl ethyl alcohol content of CS wines produced by different *S. cerevisiae* strains ranged from 7.87 to 23.17 mg/L. In our study, phenyl ethyl alcohol concentrations of wines ranged from 7.55 to 16.57 mg/L for both grape varieties. The amount of this compound was found above the odor threshold value (14.00 mg/L) in the spontaneous KL and inoculated CS wines [26]. In a similar study conducted on Parellada musts, notable production of 2-phenyl ethanol was found in the spontaneously fermented samples [28].

2-Methyl 1-butanol concentrations of KL wines were 11.80 and 12.38 mg/L in spontaneous and inoculated wines, respectively. As seen, no significant differences were found between spontaneous and inoculated wines. 2-Methyl 1-butanol concentrations were found as 6.23 and 6.78 mg/L in spontaneous and inoculated CS wines, respectively. Similar to KL wines, amount of 2-methyl 1-butanol was not affected by the fermentation technique in CS wines. However, it was stated that spontaneous fermentation resulted in higher amount of 2-methyl 1-butanol content in Chardonnay wine [3].

Regarding 1-hexanol which is described as green aromas, inoculated CS wines (5.06 mg/L) had higher amount of this compound, whereas no significant differences were found in KL wines (1.66–1.068 mg/L). It was stated that 1-hexanol concentration of CS wines fermented by different *S. cerevisiae* strains ranged from 1.98 to 3.08 mg/L [29]. Similar to our results, Godello wines obtained by commercial yeast fermentation had higher 1-hexanol concentration than spontaneously fermented wines with the concentration of 1.57 mg/L [1]. However, in the mentioned study, spontaneously fermented Albarino wines had higher 1-hexanol concentration. The source of 1-hexanol compound is generally known as grape variety, but it may also be related to the yeast strains [1].

1-Heptanol which is characterized with sweet-lemon aroma showed no significant difference in spontaneous and inoculated KL wines with the concentration of 0.94 and 0.84 mg/L, respectively. On the other hand, CS wines had lower concentration of 1-heptanol, but its amount was statistically higher in inoculated CS wines. 1-Octen-3-ol gives mushroom odors to wine and its concentration was significantly higher in KL wines produced by spontaneous fermentation, whereas no significant difference was found in CS wines. This compound may come from the activity of spoilage yeast and bacteria and formed by the enzymatic breakdown of linoleic acid [30]. Benzyl alcohol is another important aroma compound of wine with citrus-sweet odor. It was quantified only in CS wines and inoculated CS wines had statistically higher amount of benzyl alcohol. Also, 1-octanol was significantly higher in inoculated CS wines (2.26 mg/L), but no significant difference was found in KL wines.

Considering the total concentrations of higher alcohols, spontaneous fermentation resulted in greater amount of higher alcohols in KL wines. On the contrary, inoculated wines had greater amount of higher alcohols in CS wines. Varela et al. [3] stated that Chardonnay wines produced by spontaneous (natural) fermentation had higher concentrations of higher alcohols than inoculated wines. In a recent study, it is suggested that inoculated Cabernet Sauvignon wines had a greater amount of higher alcohols [7]. Godello and Albariño wines produced using a commercial yeast strain presented a greater amount of higher alcohols and 2-phenyl ethanol [1]. In another study, no significant differences were found in the concentration of total higher alcohols between spontaneous and inoculated Parellada wines [28]. Thus, it is seen that the effect of yeast on higher alcohols shows different trends for different materials.

Esters

Esters are desirable compounds giving floral and fruity notes to wines. They have large impact on wine aroma even in very small quantities [31]. In wine, the ester compounds are presented in two different forms as esters of fatty acids and alcohols, and acetates of higher alcohols.

In KL wines, 18 ester compounds were identified and quantified (Table 5). Main ester compounds were ethyl octanoate, ethyl hexanoate, diethyl succinate, ethyl acetate and isoamyl acetate in KL wine samples. Among these compounds, ethyl octanoate and diethyl succinate were higher in the spontaneous KL wines. However, isoamyl acetate that is responsible from banana aroma was greater in inoculated KL wines.

16 esters were determined in CS wines (Table 6). The main ester compounds were ethyl acetate, ethyl octanoate, ethyl

hexanoate, isoamylacetate and diethyl succinate. Among them, ethyl acetate and isoamyl acetate were significantly higher in spontaneously fermented CS wines although ethyl octanoate and diethyl succinate were greater in inoculated CS wines.

Regarding the total concentrations of esters, spontaneously fermented KL and CS wines showed greater amounts of ester compounds than inoculated wines. Therefore, it is revealed that wild yeasts contributed to the ester formation in spontaneous fermentation due to the higher concentration of total ester compounds in wine samples. Similar to our results, it was stated that wines produced by spontaneous fermentation had higher concentrations of esters [1, 32, 33].

Esters give a desirable odor and contribute positively to the wine quality [25]. Acetate esters are synthesized by the reaction occurred between acetyl CoA and higher alcohols [34]. The acetic esters of higher alcohols have intense odors and enhance the aromatic complexity of wines. On the other hand, they can also mask some varietal aromas. The formation of these ester compounds is greater in the case of slower fermentation rate [25]. In fact, ethyl acetate which is one of the most important volatile compounds of wines was greater in spontaneously fermented CS wines. This was likely due to the fact that the rate of spontaneous fermentation was lower than that of inoculated fermentation and non-*Saccharomyces* synthesize more ethyl acetate than *Saccharomyces* strains [28]. Ethyl acetate was the main ester compound in CS wine samples with the highest concentration among all esters. Its concentrations were 26.81 and 30.32 µg/L in spontaneous and inoculated KL wines, respectively. Whereas no significant differences were detected in KL wines, ethyl acetate concentration of spontaneous CS wines (297.50 µg/L) was nearly 2.8-times of wines produced by commercial yeast inoculation (106.02 µg/L). Similarly, Romano et al. [5] investigated the metabolic profiles for different wine yeasts in early fermentation phases of Aglianico grape must and suggested that *Candida stellata* was characterized by higher production of ethyl acetate than *S. cerevisiae*. Higher ethyl acetate formation might be resulting from representatives of *Candida* yeasts (*C. albicans* and *C. apicola*) identified in the spontaneous fermentation of CS grapes. Lower concentrations (< 50 mg/L) of ethyl acetate may be desirable and contribute to the complexity of wine aroma, although it may cause off-flavor in wine above 150 mg/L [28].

Isoamyl acetate has a banana aroma that may contribute to aroma profiles of young wines. Its concentration was found under its perception threshold (30 µg/L) in all wine samples except for spontaneous CS wine. Isoamyl acetate concentrations of spontaneous and inoculated KL wines were 16.12 and 18.97 µg/L, respectively. On the other hand, the amounts of this compound in spontaneous and inoculated CS wines were 41.43 and 23.22 µg/L, respectively. Formation of isoamyl acetate in wines had variable trends in some studies. For example, Garde-Cerdan and Ancin-Azpilicueta [28] suggested that isoamyl acetate concentration was found lower in the spontaneous fermentation than in the inoculated fermentation. On the contrary, Blanco et al. [1] stated that isoamyl acetate concentration of Godello wines was greater in spontaneously fermented wine while its concentration was higher in commercial yeast inoculated Albarino wines. It is seen that the concentration of isoamyl acetate in wine can vary depending on yeast diversity and grape variety. Also, isobutyl acetate was found significantly greater in spontaneous CS wines. These results agreed with the results of Puertas et al. [33] who suggested that spontaneous fermentation led to higher contents of isobutyl acetate in Chardonnay and Verdejo wines.

Phenylethyl acetate has floral, fruity and honey aromas and can enhance the aroma profile of young wines [25]. The amount of phenylethyl acetate ranged from 1.03 to 5.07 µg/L in wine samples. Its concentration was significantly higher in spontaneous CS wines. Similarly, 2-phenylethyl acetate was produced in greater concentration during the spontaneous fermentation while it did not form in the inoculated fermentations of Parellada grapes [28]. No differences were detected in KL wines in terms of phenylethyl acetate concentration (Table 5).

Ethyl esters of fatty acids are formed by enzymatical reactions during yeast fermentation and by ethanolysis of acetylCoA. The concentration of these esters can vary depending on the yeast strains presented in the fermentation and may be affected by the temperature and aeration degree during the fermentation process [34]. These compounds have a positive impact on general wine quality with their typical fruity aromas [27]. Among ethyl esters of fatty acids, the most abundant compounds were ethyl octanoate, ethyl hexanoate and diethyl succinate for both grape varieties. Ethyl octanoate was statistically higher in spontaneous KL wines, whereas it was significantly higher in inoculated CS wines. Ethyl hexanoate concentrations were found to be between 45 and 65 µg/L in all wine samples that is above odor threshold value (5 µg/L). No significant differences were found in ethyl hexanoate concentrations of KL wine samples. However, inoculated CS wines had higher amount of this compound with the concentration of 65.28 µg/L. Inoculated CS wines had also higher concentration of diethyl succinate (68.73 µg/L) than that of spontaneous wines (5.93 µg/L). Diethyl succinate concentrations of KL wines were 40.70 and 31.68 µg/L for spontaneous and inoculated fermentations, respectively. Ethyl decanoate concentrations were 9.81 and 18.90 µg/L in spontaneous and inoculated CS wines, respectively. Inoculated CS wines had about 2 times higher amount of ethyl decanoate (18.90 µg/L) than spontaneous wines, but no significant difference was found in ethyl decanoate concentrations of KL wines. It is reported that ethyl decanoate did not form in spontaneous fermentation while it appears in appreciable level in the pure *S.*

cerevisiae inoculated fermentations [28]. Methyl salicylate was detected in CS wines and its concentration was higher in inoculated wines. When the concentrations of these esters (ethyl esters of fatty acids) are compared between the spontaneous and the inoculated wines, it can be seen that *S. cerevisiae* contributed to their formation in inoculated CS wines. However, the formation of these compounds was promoted by the indigenous yeasts in spontaneously fermented KL wines. Benzene acetic acid, ethyl ester was detected in KL wines not in CS wines and it was higher in KL wines obtained from spontaneous fermentation.

Our results demonstrated that spontaneous fermentation resulted in higher concentration of total ester compounds in KL and CS wines. Similarly, Cabernet Sauvignon wines produced by spontaneous fermentation were characterized by a greater amount of esters [7]. On the other hand, Varela et al. [3] stated that Chardonnay wines produced by spontaneous fermentation had lower concentrations of acetate esters than inoculated wines.

Other compounds

Beside esters and alcohols, other compounds such as vitispirane, γ-butyrolactone and limonene were also determined in wine samples (Table 5 and 6). Vitispirane has fruity or camphor-like odor and it is possibly formed during bottle aging [25]. Statistically significant differences, with a small variation, were observed in vitispirane concentrations of spontaneous and commercial yeast inoculated KL young wines (Table 5). Similar results were reported in Riesling icewines for two consecutive vintages [35]. γ-Butyrolactone is the best known lactone present in wine with its small quantities. This compound is formed by the lactonization of γ-hydroxybutyric acid which is an unstable molecule released by deamination and decarboxylation of glutamic acid. Lactones in wines mainly originate from grapes, but they can be also formed during fermentation and aging processes. The sensorial effects of lactones has not been clearly established [25]. γ-Butyrolactone was higher in inoculated CS wines with the concentration of 1.30 µg/L. It is possibly produced by the commercial yeast strains used for the inoculated fermentation. Limonene is a monoterpene compound with resin-like odor and mostly considered to be originated from grapes. This compound was identified only in CS wines and it was higher in spontaneously fermented wines. On the other hand, it is reported that different yeast species, particularly non-*Saccharomyces* yeasts can perform the biotransformation of free terpenes [36].

Sensory profiles of wines

Figures 1 and 2 show the sensory characteristics of KL and CS wines, obtained by spontaneous and inoculated fermentations, respectively. Fermentation techniques have led to important differences in sensory profiles of wines due to the presence of different yeasts. As known, yeasts used in winemaking play a key role in the fermentation and lead to significant differences in the sensory properties of final wine [1, 10, 37].

In spontaneously fermented KL wines, the certain sensorial characteristics including fruity and green aromas besides sour taste had the higher scores compared to the inoculated wines. On the other hand, no considerable differences were

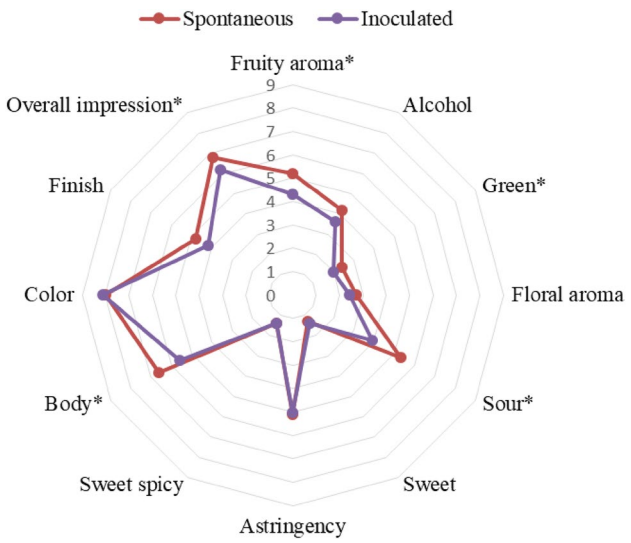

Fig. 1 Sensory profiles of young Karalahna wines produced by spontaneous and inoculated fermentations. Statistical significance is given by "*" $p < 0.05$

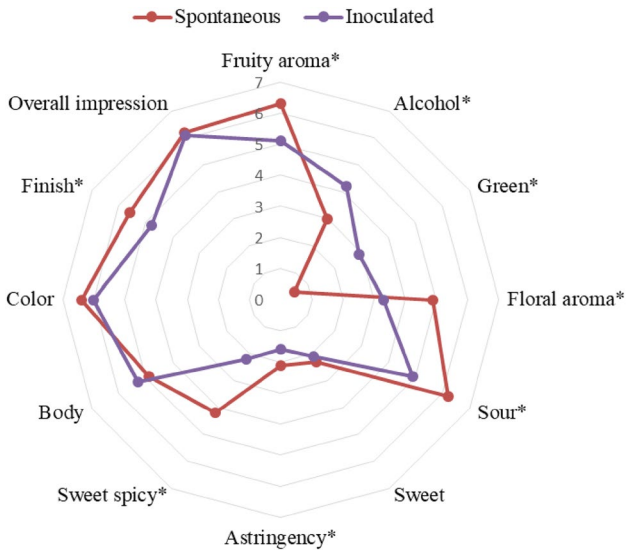

Fig. 2 Sensory profiles of young Cabernet Sauvignon wines produced by spontaneous and inoculated fermentations. Statistical significance is given by "*" $p < 0.05$

detected in alcohol-feel, floral aroma, sweetness, astringency, sweet spicy, color and finishes of the KL wines (Fig. 1). CS wines obtained by spontaneous fermentation had higher scores by judges based on fruity and floral characters. These wines were also evaluated as more astringent than those produced from commercial yeast inoculation. Sour taste and sweet spicy aroma were also greater in spontaneous CS wines. On the other hand, alcohol-feel and green aroma were higher in inoculated CS wines. Regarding the finishes of CS wines, spontaneously fermented wines had greater scores by the judges (Fig. 2).

Non-*Saccharomyces* yeasts may have contributed to the fruity aroma of spontaneously fermented wines by improving the ester synthesis [7]. Renault et al. [10] investigated the formation of esters and sensorial effects of non-*Saccharomyces* species (*Torulaspora delbrueckii*) when used in association with *S. cerevisiae* and indicated that mixed inoculation had an increasing effect on the complexity and fruity aromas of wines compared to pure culture of *S. cerevisiae*. Similarly, Patrignani et al. [9] stated that spontaneously fermented wines had significantly higher scores in taste and odor complexity compared to the inoculated wines. More recently, Puertas et al. [33] who evaluated the impact of grape variety and inoculation technique on wine volatiles and aromas stated that spontaneous wines (Chardonnay and Verdejo) showed the highest total ester amount and gained the highest scores in various fruit aromas, complexity, balance and persistence. On the other hand, there is no clear and singular way to explain how non-*Saccharomyces* yeasts impact the wine flavor and chemistry owing to different strains and their interactions [12, 38]. These results confirm the fact that spontaneous fermentation can contribute to the quality of local wines by enhancing the pleasant fruity aromas and improving the organoleptic complexity.

Conclusions

In conclusion, the results of this study demonstrated that the volatile compounds of wines produced by spontaneous and inoculated fermentations differed significantly. Based on volatiles of CS wines, spontaneous fermentation was characterized by higher amount of esters while inoculated fermentation was characterized by greater amount of higher alcohols. On the other hand, results showed that spontaneous fermentation promoted the production of alcohols and esters in KL wines. It is also suggested that indigenous wine yeasts provided fruit nuances to the wines obtained from KL and CS grapes. Spontaneous fermentation contributed to wine aroma (especially fruit nuances) and increased complexity of wines based on sensory profile. Local yeast species and strains found in spontaneous fermentation, especially non-*Saccharomyces* yeast species known as good producers of esters, seemed to be responsible from these results. However, more studies are needed to understand entirely of the enological properties of non-*Saccharomyces* yeasts and their effects on wine characteristics.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements The authors have no conflicts of interest and that sensory testing with human subjects was conducted with appropriate informed consent.

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