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STUDY INTO AN ALTERNATIVE TREATMENT METHOD TO SULPHUR DIOXIDE IN MULBERRY WINEMAKING

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ABSTRACT

The characteristics of alcohol fermentation of mulberry juice treated under four different conditions of ultra-high pressure (100 MPa/10 min, 200 MPa/10 min, 300 MPa/10 min, 400 MPa/10 min), sulphur dioxide (60 mg/L), and a combination of ultra-high pressure and sulphur dioxide (30 mg/L) were assessed in this work. The volatile aroma constituents of mulberry wine made from mulberry juice using different treatments were isolated by solid phase microextraction (SPME) and identified by gas chromatography-mass spectrometry (GC-MS). A total of twenty five volatile aroma compounds of mulberry juice were identified, while nineteen volatile aroma compounds (five alcohols, four acids, and ten esters) were detected in mulberry wine. Ethyl acetate, butanoic acid ethyl ester, octanoic acid ethyl ester, acetic acid 2-phenylethyl ester, decanoic acid ethyl ester and ethyl 9-decenoate were the major esters and the main components of the mulberry wine, followed by 2-methyl, 1-propanol. Aldehydes were the major volatile fraction in mulberry wine, followed by alcohols. Pressure processing maintained the original flavour distribution of the juice. There were no significant differences among different mulberry wines in the types and concentrations of the volatile aroma components. It could be concluded that ultra-high pressure (300 MPa/10 min, 400 MPa/10 min) treatment could replace sulphur dioxide in winemaking in order to improve safety while maintaining the flavour compounds of mulberry wine.

Keywords: Ultra-high pressure processing, sulphur dioxide, mulberry wine, alcohol fermentation; SPME, GC-MS, aroma compounds

INTRODUCTION

In recent years, increased interest in human health, nutrition and disease prevention have led to increased consumer demand for functional foods including fruits and their products such as wine. Epidemiological evidence has been provided showing that constituents in fruits are beneficial to human health and contribute to the prevention of degenerative processes caused by oxidative stress **(Kaur and Kapoor, 2001; Vinson *et al*., 2001)**. Fruits contain many different dietary phytonutrients including phenolics, flavonoids, phenolic acids, carotenoids, and vitamins **(Kaur and Kapoor, 2001; Tomas-Barberan *et al*., 2001; Vinson *et al*., 2001)**. Dietary intake of plant phenolics is inversely related to coronary heart disease **(Hertog *et al*., 1997).** Plant phenolics have anti-ulcer, antispasmodic, antisecretory, or anti-diarrhoeal effect on the gastrointestinal tract **(Hussain *et al.*, 2015)**. Mulberry fruit is rich in anthocyanin which is a good source of natural antioxidants **(Yang and Tsai, 1994)**. Mulberry trees are extensively grown in Southern Europe, India, and China for their leaves (as silkworm feed) and fruits as food. There are three kinds of mulberry: white mulberry (*Morus alba* L.), black mulberry (*Morus nigra* L.), and red mulberry (*Morus rubra* L.). White mulberry originated from Western Asia, red mulberry in North and South America, and black mulberry is from Southern Russia. **Wills *et al*. (1987)** studied the chemical composition of mulberry and reported the approximate composition as 89.3% water, 2.2% protein, 0.2% fat, 2% glucose, 2.3% fructose, 2.2% dietary fibre, 0.19% malic acid, 0.59% citric acid, 0.8% ash, 121 kJ energy, 10 mg vitamin C, 0.01 mg thiamine, 0.01 mg riboflavin, 0.7 mg niacin, 0.01 mg β-carotene, 310 mg potassium, 6 mg sodium, 20 mg calcium, 12 m magnesium, 0.3 mg iron and 0.2 mg zinc per 100 g edible portion. Mulberry (*M. alba* L.) has long been used in Chinese medicine to treat fever, protect the liver, improve eyesight, strengthen joints, facilitate discharge of urine and lower blood pressure **(Zhishen *et al*., 1999)**. Leaves of mulberry species are consumed in Korea and Japan as antihyperglycemic nutraceutical food by patients with diabetes mellitus because the leaves contain 1-deoxynojirimycin, known to be one of the most potent a-glycosidase inhibitors **(Kim *et al*., 2003)**. In Japan, consumption of mulberry-leaf tea has been increasing (**Katsube *et al.*, 2006**). Mulberry wine has been studied extensively for many years (**Jung *et al.*, 2005; Kim *et al*., 2006**). However, wine makers use sulphur dioxide to inhibit or kill microorganisms, as an antioxidant, and a clarificant (**Sacchi *et al.*, 2005)**. The dosage of sulphur dioxide is strongly being limited by many countries and World Health Organization (WHO) because of its toxicity to our body (**WHO, 2006**). The current trend is to reduce the dosage of sulphur dioxide, stopping usage entirely, or to find an alternative in the wine making process. The main purpose of this present study was to investigate the influence of different treatment methods on the flavour compounds of mulberry wine as a way of finding an alternative method for preventing microbial growth in wine.

MATERIAL AND METHODS

Reagents and standards

All reagents used were of analytical quality. Sodium chloride was purchased from SINOPHARM CHEMICAL REAGENT CO., LTD, CHINA. The series of straight- chain alkanes (C5-C19) were purchased from TIANJING CHEMICAL REAGENT WHOLESALE COMPANY, China. The Pure chemicals (≥95%) used as internal standards, 1-Propanol, 3-octanol and 2, 3-Pentanedione, were supplied by SHIJIAZHUANG DONGFENG CHEMICALS CO., LTD, CHINA. Pure water was obtained from a Milli-Q purification system (MILLIPORE, BEDFORD, MA).

Source of sample and preparation

Mulberry fruits were harvested in July, 2013 from SHIYEZHOU, ZHENJIANG, CHINA. All berries were picked at the commercially ripe stage. The berries were selected according to uniformity of shape and colour. The fruits were thoroughly washed using potable water and then stored ° Brix in polyethylene bags at -18 ºC until needed for the experiment.

Wine making

Fresh, healthy and ripe mulberry (5 kg) fruits were carefully crushed using a

blender. Pectinase (20 m g/kg of must) (PECTINEX BE XXL, NOVOZYMES, DENMARK) was added and the mixture placed in a water bath for 60 min at 55 ^{o}C to increase the yield of juice. The mixture was centrifuged at 5000 rpm for 20 min and the supernatant was filtered. Before alcohol fermentation, the total soluble solids (TSS) (12^{o} Brix) of the juice was adjusted to 20^{o} Brix by adding inspissate cider in order to obtain a sufficient alcohol level. The mixture was divided into seven uniform parts and respectively marked: AA (control), BB (ultra high pressure processing [100 MPa, 10 min]); CC (ultra high pressure processing [200 MPa, 10 min]); DD (ultra high pressure processing [300 MPa, 10 min]); EE (ultra high pressure processing [400 MPa, 10 min]); FF (sulphur dioxide [60 mg/L]); GG (ultra high pressure processing [300 MPa, 10 min] + sulphur dioxide [30 mg/L]). The Intelligent Ultra High Pressure Machine with 3 L capacity (MANUFACTURED BY JIANGSU UNIVERSITY AND BAOTOU SCIENTIFIC DEVELOPMENT COMPANY, CHINA) was used for the ultra high pressure processing. To each mixture was added 200 mg/L yeast culture (Angel high-temperature yeast) and fermented at 20 ^{o}C till the fermentation was completed (216 h).

Determination of total soluble solids, residual sugar, pH, titratable acidity, ethanol content, and volatile acidity

The total soluble solids (TSS) of the samples were determined using the Abbe Refractometer (WAY-2S, GERMANY), with temperature compensation, and the values were expressed in degree brix (°Brix). The pH of the samples was determined using a pH Meter (PHS-2C PRECISION PH/MV METER, CHINA), earlier calibrated using buffer solutions of pH 7 and 4. The dinitrosalicyclic (DNS) method as described by **Miller (1972)** was used to determine the residual sugar content of the samples. Titratable acidity (TA) was measured as described by **Sadler and Murphy (2010),** and the results were expressed in terms of g/L malic acid. The alcohol content was measured by the method described by **Caputi *et al*. (1968),** and the results expressed as %v/v. **AOAC (1960)** method was used to determine the volatile acid content and the result was expressed in g/L acetic acid.

SPME analysis of volatile compounds

The method as described by Kataoka *et al*. (2000), with some modifications, was used in analysing the volatile compounds in all the samples. The SPME fibre used was a Stable Flex Divinylbenzene/ Carboxen/ Polydime- -thylsiloxane (DVB/CAR/PDMS) (SUPELCO, BELLEFONTE, PA), which is designed for flavour analysis. For each SPME analysis, the sample was saturated with sodium chloride (1g) for HS-SPME and 5 ml of sample for each extraction were placed into a 15 ml glass vial. The sample was placed in a vial with a small stir magnet at a speed of 350 rpm. Mulberry wine was spiked with two internal standards comprising 50 µL of water solution of 1-Propanol (100 µg/L) and 3-octanol (800 µg/L) and 50µL of water solution of two internal standards 3-octanol (200 µg/L) and 2, 3-Pentanedione (200 µg/L) were added to the wine. The vial was then placed in a water-bath at 40 ^{o}C. The vial was sealed with a silicone septum. The SPME needle was used to pierce the septum and the fibre was extended through the needle to place the stationary phase in contact with the headspace of the sample. The fibre was withdrawn into the needle after 30 min. Finally, it was removed from the vial and inserted into the injection port of the gas chromatograph (GC) for 3 min. The extracted chemicals were desorbed thermally and transferred directly to the analytical column. The fibre was conditioned for 1 hour at 270 ^{o}C before use.

GC-MS Parameters and Analysis

SPME fibres were desorbed at 250 ^{o}C for 3 min in the injection port of an Agilent 6890/5973 GC-MS (AGILENT, USA) with a DB-1701 (cross linked [14%-Cyanopropyl -phenyl)-methylpolysiloxane, Agilent] column (30 m, 0.25 mm i.d., 25 µm film thickness) for 31 min runs. The injection port was operated in splitless mode, with ultrahigh-purity helium (99.9995%) as carrier gas at a flow rate of 1 mL/min. The initial oven temperature was 50 ^{o}C, held for 10 min, ramped at 6 ^{o}C min^{-1} to 150 ^{o}C and then at 8 ^{o}C min^{-1} to 200 ^{o}C, and held for 3 min. The Agilent 5973 quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV, a source temperature of 230 ^{o}C quadrupole at 150 ^{o}C, with a continuous scan from *m/z* 33 to 330. The HP ChemStation software (D.00.00) was used to collect data and searched against the NIST98 libraries. Compounds were preliminarily identified by library search, and the identities of most of them confirmed by GC retention time (RT), MS ion spectra, authentic compounds or a homologous series, and a retention index (RI). The RI's from a series of straight-chain alkanes (C5-C19) were used to calculate the RI's of all identified compounds.

Antioxidant capacity determination

The clearing (scavenging) effect of 1, 1-Diphenyl-2-picrylhydrazyl (DHHP) radical was used to evaluate the antioxidant capacity of mulberry wine using the method as suggested by **Schimada *et al*. (1992)**. To 5 ml of wine sample was added 5 ml of 0.008% DPPH in 50% ethanol. Decolourisation of DPPH was monitored by measuring absorbance at 528 nm.

Microbiological determination

Plate count agar was used for bacteria population determination. Serial dilution and the pour plate method were used for this work **(Benson, 1994)**. All determinations were done in triplicate and bacteria populations determined as colony forming units per millilitre (cfu/mL) of mulberry juice and wine.
The yeast population, during fermentation of mulberry wine samples, were monitored using the staining method as described by **Zoecklein *et al*., 1995**.

RESULTS AND DISCUSSION

General composition of mulberry wine

The general composition of the mulberry wine samples at the end of fermentation is presented in Table 1. In terms of total soluble solids (TSS), there were no significant differences among samples except sample EE, which had a significantly lower mean value, but statistically same as sample BB. The values obtained were slightly lower than those obtained for papaya, banana, orange and lime by **Gavimath *et al*. (2012)**. However, **Owusu *et al*. (2014)** recorded relatively lower TSS values for wine samples produced using tomato. For residual sugar, samples AA, BB and DD had values which were not significantly different ($p>0.05$) but were significantly higher ($p<0.05$) than the rest of the samples. All the values obtained in this work for residual sugar were slightly higher than those obtained by **Tchabo (2015)** for mulberry wine samples. The differences may be attributed to the different pre-treatment methods employed by the authors. High residual sugar content of wine might reduce its bitterness and astringency (**Mena *et al*., 2011; Sokolowsky *et al*., 2015**), but can also reduce the microbial stability of the wine (**Du Toit & Pretorius, 2000**). Also, according to **Towantakavanit et al. (2011)**, sweetness is an important parameter for wine acceptance. The alcohol content of the samples ranged between 9.98±0.11 and 11.58±0.08 % (v/v). This range compares favourably with that obtained by **Tchabo (2015)**, but **Owusu *et al*. (2014)** obtained values in the range 8.59±0.28 - 9.61±0.17 for tomato wine samples. Relatively lower alcohol values were obtained by **Yan *et al*. (2012)** for blueberry wine. For pH, there were no significant differences among all the wine samples, although sample EE had the least value. The values obtained are similar to those obtained by **Tchabo (2015**), **Owusu *et al*. (2014)** and **Yan *et al*. (2012)** in their work. The titratable acidity obtained for sample EE was the highest but was not significantly different from sample GG. The titratable acidity obtained in this work (8.36±0.11 – 9.26±0.06) was higher than that obtained by **Tchabo (2015)** (4.50±0.03 – 4.51±0.05). For volatile acidity, sample EE had a significantly higher value than the rest of the samples. There were no traces of volatile acidity in samples BB and DD. The different treatment regimes may account for the differences observed.

Table 1 General composition of mulberry wine after fermentation

Sample	Total soluble solids (^{o}Brix)	Residual sugar (g/L)	Alcohol content (%, V/V)	pH	Titratable acidity (g/L malic acid)	Volatile acidity (g/L acetic acid)
AA	9.00±0.28^{a}	11.70±0.28^{a}	10.53±0.17^{b,c}	4.01±0.13^{a}	8.36±0.11^{c}	0.49±0.03^{c}
BB	8.40±0.28^{a,b}	11.10±0.14^{a}	11.54±0.16^{a}	4.01±0.14^{a}	8.60±0.14^{b,c}	0±0.00^{d}
CC	9.00±0.35^{a}	9.30±0.14^{c,d}	10.02±0.18^{c}	3.87±0.13^{a}	8.75±0.14^{b,c}	0.36±0.03^{c}
DD	8.50±0.07^{a}	11.70±0.14^{a}	11.42±0.17^{a}	3.95±0.08^{a}	8.35±0.10^{c}	0±0.00^{d}
EE	7.50±0.14^{b}	10.00±0.28^{b,c}	9.98±0.11^{c}	3.83±0.10^{a}	9.26±0.06^{a}	1.08±0.13^{a}
FF	9.00±0.20^{a}	8.99±0.18^{d}	10.90±0.28^{a,b}	3.86±0.06^{a}	8.43±0.10^{c}	0.15±0.01^{d}
GG	9.00±0.14^{a}	10.30±0.14^{b}	11.58±0.08^{a}	4.13±0.06^{a}	8.95±0.20^{a,b}	0.73±0.03^{b}

Legend: AA: control; BB: ultra high pressure processing (100 MPa, 10 min); CC: ultra high pressure processing (200 MPa, 10 min); DD: ultra high pressure processing (300 MPa, 10 min); EE: ultra high pressure processing (400 MPa, 10 min); FF: add sulphur dioxide (60 mg/L); GG: ultra high pressure processing (300 MPa, 10 min) + sulphur dioxide (30 mg/L).

Total number of bacteria colonies in sample after different treatments

T

he total number of colonies was significantly reduced ($p<0.05$) from the control (AA) to sample EE (Figure 1). No colonies were detected in sample GG, and there was no significant difference between samples EE and GG. The result obtained for sample EE was similar to that obtained by **López-Malo *et al*. (1998)** under similar conditions. In their work, **García-Graells *et al*. (2003)** concluded that the initial number of bacteria affects the effectiveness of UHT treatment. The total number of microorganisms present, before inoculation, could affect the initiation of alcohol fermentation and quality of mulberry wine **(King and Beelman, 1986)**. It could be seen from Figure 1 that, ultra high pressure (UHP) processing at 300 MPa/10 min or 400 MPa/10 min achieved a better result than sulphur dioxide (60 mg/L) treatment. Sulphur dioxide could therefore be replaced by UHP processing if used at 300 or 400 MPa for 10 min to treat the mulberry juice. The dosage of sulphur dioxide decreased greatly when ultra high pressure processing and sulphur dioxide were used together (sample GG).

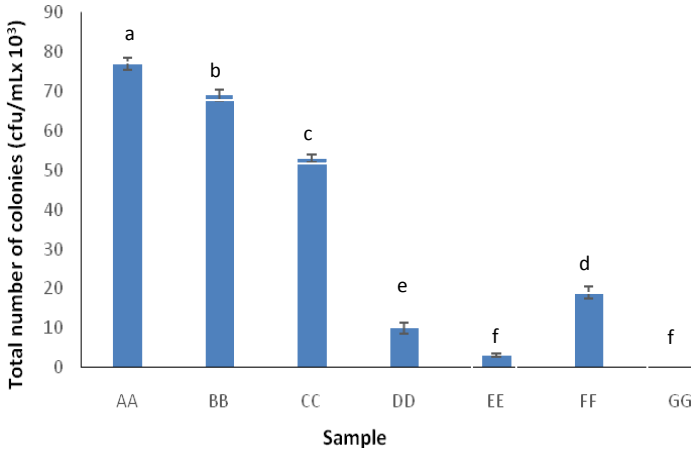

Figure 1 Total number of bacteria colonies in sample after different treatments
Legend: AA: control; BB: pressure treatment at 100 MPa/10 min; CC: pressure treatment at 200 MPa/10 min; DD: pressure treatment at 300 MPa/10 min; EE: pressure treatment at 400 MPa/10 min; FF: sulphur dioxide (60 mg/L); GG: pressure treatment (300 MPa, 10 min) + sulphur dioxide (30 mg/L).

Changes in total soluble solids, pH and total number of yeast during alcohol fermentation

Figures 2, 3, and 4 show the changes in TSS, pH and total number of yeast cells during alcohol fermentation, respectively. The TSS reduced gradually until it reached 8.0%–10.0% at the end of fermentation (216 h) (Figure 2). All of the mulberry juice samples, except AA (control), had same change tendency. The relatively higher number of initial microorganisms in AA may have slowed down the utilization of sugar in the juice by the yeast cells. The TSS usually reduces with the progression of fermentation and becomes stable at the end of the process (**Duarte *et al*., 2010; Pino and Queris, 2011**). Figure 3 shows that, the pH value of mulberry juice samples changed intensively during fermentation from 0 h to 72 h. It decreased from 0 h to 24 h but increased from 24 h to 72 h, and then it became almost stable. The pH value of mulberry wine reached 3.85-3.90 when alcohol fermentation was completed. The change in the pH of EE (400 MPa/10 min) was more gradual than the others after 72 h. This observation may be plausibly due to the relatively low number of bacteria colonies (Figure 3) present as well as the gradual death of yeast cells (Figure 4). At the end of the fermentation process, the pH for all the samples were slightly higher than the initial values, deviating from what had been reported by other workers (**Reddy *et al*., 2010; Towantakavanit *et al*., 2011**).

From Figure 4, yeast growth in mulberry juice (DD, EE, FF and GG) was in logarithmic growth phase from 0 h to 48 h. However, other logarithmic growth phases were delayed to 36 h (BB and CC) or 72 h (AA). The credible reason for this observation may be due to the presence of different numbers of colonies in the differently treated mulberry juice samples. The presence of large number of microorganisms would delay yeast growth. This may explain the reason why alcohol fermentation was variably affected according to the number of microorganisms present in the mulberry juice samples. The quality of mulberry wine was better if the yeast reached the logarithmic growth phase early **(Torija, 2003)**.

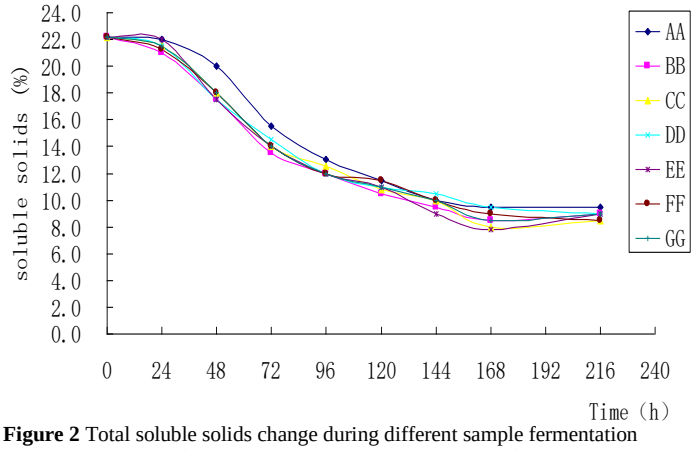

Figure 2 Total soluble solids change during different sample fermentation
Legend: AA: control; BB: pressure treatment at 100 MPa/10 min; CC: pressure treatment at 200 MPa/10 min; DD: pressure treatment at 300 MPa/10 min; EE: pressure treatment at 400 MPa/10 min; FF: sulphur dioxide (60 mg/L); GG: pressure treatment (300 MPa, 10 min) + sulphur dioxide (30 mg/L)

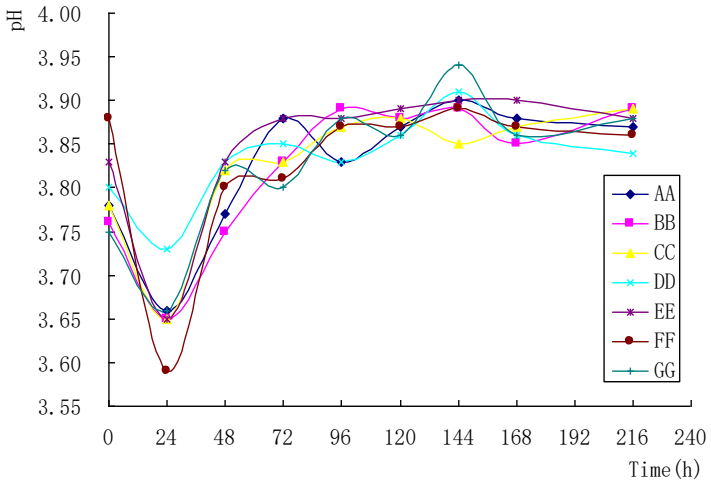

Figure 3 pH changes during different sample fermentation
Legend: AA: control; BB: pressure treatment at 100 MPa/10 min; CC: pressure treatment at 200 MPa/10 min; DD: pressure treatment at 300 MPa/10 min; EE: pressure treatment at 400 MPa/10 min; FF: sulphur dioxide (60 mg/L); GG: pressure treatment (300 MPa, 10 min) + sulphur dioxide (30 mg/L)

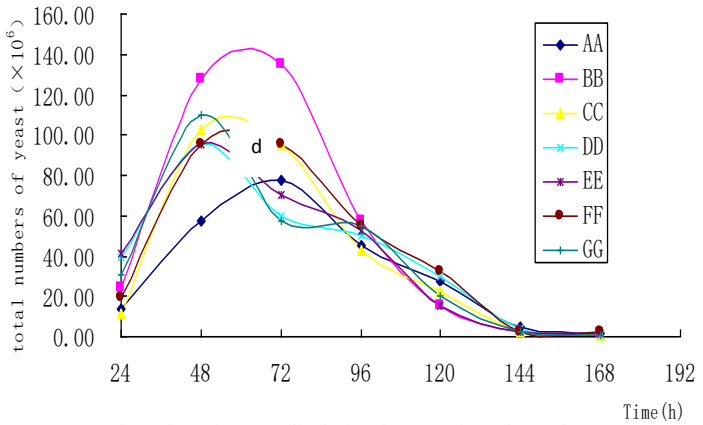

Figure 4 Total number of yeast cells during fermentation of samples
Legend: AA: control; BB: pressure treatment at 100 MPa/10 min; CC: pressure treatment at 200 MPa/10 min; DD: pressure treatment at 300 MPa/10 min; EE: pressure treatment at 400 MPa/10 min; FF: sulphur dioxide (60 mg/L); GG: pressure treatment (300 MPa, 10 min) + sulphur dioxide (30 mg/L)

Volatile compounds of mulberry juice

A total of 25 volatile aroma compounds of mulberry juice (AA) were identified by means of solid phase micro extraction (SPME) followed by gas chromatography (GC) and mass spectrometry (MS). Table 2 shows the compounds isolated from the headspace of mulberry juice (AA–GG). In Figure 5, a representative total ion chromatogram is presented. Major volatile compounds were identified by MS analysis, including 5 alcohols, 3 esters, 3 acids, 5

aldehydes, 4 ketones, 4 hydrocarbons and 1 miscellaneous compound (Table 2). Previous reports for mulberry essential oils have tabulated many more compounds **(Butkhup *et al.*, 2011)**. However, because of different extraction methods and possible oxidation or chemical alteration, less volatile compounds and/or possible defects are in those lists. The SPME method recovers mainly low- to mid molecular weight semi volatile and volatile compounds (**Butkhup *et al.*, 2011**), which could be used as the indicator for the volatile composition of the juice. This current work recorded fewer overall compounds recovered.

The majority of compounds recovered in mulberry juice with this SPME-GC-MS method were 1-hexanol, 2-ethyl-, furfural, benzene acetaldehyde, hexanal, γ-terpinene, hexanoic acid and ethyl acetate. These compounds may be indicative that mulberry juice has sweet, fruity, acid, musky and woody-fresh aroma characteristics **(Butkhup *et al.*, 2011)**.

Table 2 shows that the volatile compounds during high-pressure treatment were not changed when compared with the fresh fruit juice. It seems that high pressure treatment has no effect on the volatile components and compositions of the juice. **Laboissière *et al.* (2007)** compared the volatile flavour components of passion fruit juice during high-pressure processing and pasteurization and reported that, high-pressure processing did not change the volatile components. **Lambert (1999)** also observed that, strawberry coulis maintained the freshness and the original flavour during high-pressure processing. Again, **Yen (1999)** reported that guava juices treated with high pressure and heat processing, could maintain the original flavour distribution of the juice. Cyclopentanone, 2-cyclopentylidene- and oxime-, methoxy-phenyl- were not identified in the mulberry juice in which SO_2 was added (FF and GG) as the adsorption of the two volatile components by the headspace-SPME would be affected by SO_2.

Table 2 Type and concentration* of volatile compounds of mulberry juice samples

RI	Compound name	AA	BB	CC	DD	EE	FF	GG
<500	Sulphur dioxide						1.38	0.13
	Alcohols							
543	Ethanol	0.34	0.27	0.27	0.27	0.27	0.31	0.3
849	1-Butanol, 3-methyl-	0.38	0.4	0.42	0.48	0.51	0.3	0.25
1137	1-Hexanol, 2-ethyl-	8.84	8.57	9.04	8.97	9.1	9	8.9
1287	Phenylethyl Alcohol	0.45	0.46	0.48	0.44	0.45	0.47	0.45
1686	2,4-Di-tert-butylphenol	1.68	1.69	1.67	1.59	1.6	1.58	1.65
	Esters							
589	Acetic acid, methyl ester	0.53	0.55	0.63	0.49	0.6	0.59	0.57
671	Ethyl Acetate	0.69	0.61	0.55	0.54	0.53	0.55	0.6
862	Butanoic acid, ethyl ester	0.29	0.21	0.21	0.26	0.31	0.24	0.25
	Acids							
827	Acetic acid	3	2.58	2.57	2.62	2.7	2.8	2.49
1032	Butanoic acid, 2-methyl-	1.23	1.05	1.06	1.07	113	1.2	1.1
1161	Hexanoic acid	1.28	1.22	1.14	1.19	1.05	1.12	1.22
	Aldehydes							
884	Hexanal	1.27	1.09	1.07	1.16	1.15	1.2	1.01
966	2-Hexenal, (E)-	0.46	0.32	0.31	0.35	0.27	0.29	0.27
989	Heptanal	0.37	0.36	0.39	0.29	0.36	0.31	0.26
1188	Benzene acetaldehyde	2.41	2.38	2.45	2.44	2.33	2.3	2.13
979	Furfural	10.06	10.87	10.47	10.82	10.26	10.94	9.86
	Ketones							
1049	Cyclopentanone, 2-ethyl-	0.75	0.77	0.6	0.66	0.74	0.75	0.67
1207	Acetophenone	0.79	0.78	0.83	0.8	0.79	0.81	0.84
1514	2-Buten-1-one,1-(2,6,6-trimethyl-1, 3-cyclohexadien-1-yl)-	0.24	0.28	0.21	0.3	0.31	0.29	0.27
1544	Cyclopentanone, 2-cyclopentylidene-	0.24	0.22	0.23	0.24	0.25	0	0
	Miscellaneous Compound							
1127	Oxime-, methoxy-phenyl-	0.28	0.25	0.29	0.31	0.28	0	0
	Hydrocarbons							
1197	3-Carene	0.66	0.63	0.64	0.59	0.68	0.6	0.64
1275	γ-Terpinene	1.76	1.7	1.64	1.73	1.69	1.71	1.66
1332	p-cymenene	0.38	0.37	0.3	0.34	0.32	0.38	0.34
1530	Benzene, 2-(2-butenyl)-1,3,5-trimethyl-	0.22	0.2	0.23	0.21	0.24	0.25	0.26

*Results are given as mean concentration (μg/L) of duplicate determinations

Legend: AA: control; BB: pressure treatment at 100 MPa/10 min; CC: pressure treatment at 200 MPa/10 min; DD: pressure treatment at 300 MPa/10 min; EE: pressure treatment at 400 MPa/10 min; FF: sulphur dioxide (60 mg/L); GG: pressure treatment (300 MPa, 10 min) + sulphur dioxide (30 mg/L)

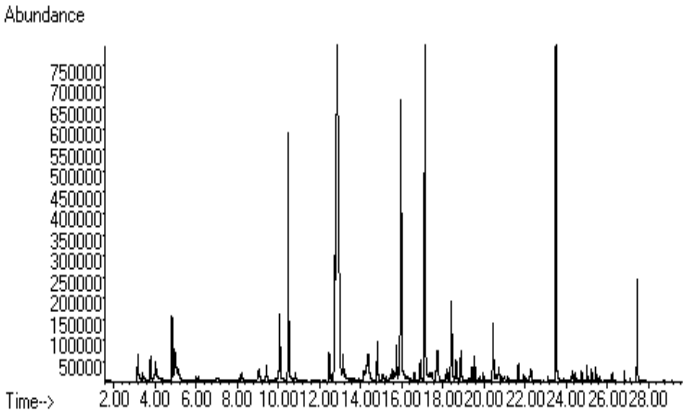

Figure 5 Typical total ion chromatogram of volatile constituents from mulberry juice obtained by SPME in the headspace sampling mode

Volatile compounds of mulberry wine

Table 3 shows the volatile flavour compounds of mulberry wine, with the mean of replicate figures expressed in (µg/L). A total of nineteen volatile flavour compounds were identified by GC-MS (Figure 6), including alcohols (five), acids (four) and esters (ten). As can be seen in Table 3, esters were found to be the most abundant aroma compounds identified by the HS-SPME techniques. These results agree with that obtained by Kalua and Boss (2009) who indicated that esters were the major volatiles characteristic for berries**. Tchabo *et al.* (2015)** also had similar results in their work. Among the esters, ethyl acetate, butanoic acid ethyl ester, octanoic acid ethyl ester, acetic acid 2-phenylethyl ester, decanoic acid ethyl ester and ethyl 9-decenoate were the major esters of the mulberry wine. The detected volatile esters of the mulberry wine can both originate from the raw material and the ones synthesized during alcoholic fermentation by yeast (**Tchabo *et al.*, 2015**). Ethyl acetate possesses a sweet, fruity and wine aroma, butanoic acid ethyl ester has a fruity aroma, octanoic acid ethyl ester has a fruity aroma, and acetic acid 2-phenylethyl ester has fruity, flowery aroma **(Forney *et al.*, 2000; Jordán *et al.*, 2003)**. Another important ester considered for its aroma contribution to wine is ethyl-9-decenoate, known for providing a very pleasant aroma **(Kafkas *et al.*, 2006)**. The most abundant alcohols detected in mulberry wine were 1-propanol, 2-methyl- and 1-butanol, 3-methyl-. 1-butanol, 3-methyl- is a major aliphatic alcohol and among the aliphatic alcohols which have a higher concentration in wines. It can both originate from the raw material or attributed to enzymatic action of yeast during fermentation. 1-propanol, 2-methyl-, an aromatic alcohol, had a sweet aroma above its perception threshold value. Also, Phenylethyl alcohol was detected in appreciable quantities in the mulberry wine. It is mainly formed during juice fermentation and is largely responsible for rose-like aroma in wines **(Juan *et al.*, 2012)**. Acetic acid, hexanoic acid, octanoic acid and n-decanoic acid were detected in the mulberry wine. Their contribution to the aroma of mulberry wine cannot be considered important because of the fact that their concentrations were much lower than their odour threshold (values not provided).

When the volatile flavour compounds of mulberry wines made from differently treated mulberry juice and untreated one are compared, the types and concentrations of the volatile components were found to be similar. It seems that high pressure treatment does not change the composition of mulberry juice and has no effect on the enzymatic action of yeast during fermentation.

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Table 3 Volatile compounds of mulberry wine and their concentration*

RI	Compound name	AA	BB	CC	DD	EE	FF	GG
	Alcohols							
738	1-Propanol, 2-methyl-	50.27	48.91	49	49.71	50.89	48.11	48.23
850	1-Butanol, 3-methyl-	20.48	21.62	21.27	20.65	21.29	20.06	20.15
980	2,3-Butanediol	0.46	0.48	0.49	0.46	0.5	0.5	0.51
989	1,3-Butanediol	0.12	0.12	0.11	0.1	0.12	0.13	0.11
1283	Phenylethyl Alcohol	3.17	2.99	2.86	2.83	2.84	2.88	2.97
	Acids							
822	Acetic acid	0.95	1.12	1.13	1.2	1.02	0.96	0.97
1157	Hexanoic acid	0.16	0.16	0.18	0.15	0.18	0.17	0.18
1353	Octanoic Acid	0.73	0.77	0.75	0.78	0.8	0.76	0.77
1562	n-Decanoic acid	0.16	0.17	0.15	0.16	0.15	0.17	0.17
	Esters							
678	Ethyl Acetate	44.49	47.56	46.12	49.52	43.56	45.77	45.73
862	Butanoic acid, ethyl ester	0.09	0.12	0.11	0.13	0.11	0.09	0.07
943	1-Butanol, 3-methyl-, acetate	1.55	1.57	1.78	1.74	1.58	1.86	1.54
945	1-Butanol, 2-methyl-, acetate	0.19	0.19	0.21	0.2	0.18	0.26	0.21
1063	Hexanoic acid, ethyl ester	0.88	0.82	0.87	0.88	0.9	0.91	0.88
1263	Octanoic acid, ethyl ester	5.46	5.18	5.38	5.53	5.86	5.32	5.72
1386	Acetic acid, 2-phenylethyl ester	1.05	0.95	0.96	0.99	1.01	0.94	1.02
1472	Decanoic acid, ethyl ester	3.12	3.01	3.04	2.89	2.97	3.23	3.06
1475	Ethyl 9-decenoate	1.27	1.3	1.28	1.27	1.28	1.28	1.3
1697	Dodecanoic acid, ethyl ester	0.57	0.58	0.6	0.61	0.58	0.59	0.58

*Results are given as mean concentration (µg/L) of replicates. There were no significant differences ($p>0.05$) among the samples in terms of the concentration of the volatile compounds detected in the samples.

Legend: AA: control; BB: pressure treatment at 100 MPa/10 min; CC: pressure treatment at 200 MPa/10 min; DD: pressure treatment at 300 MPa/10 min; EE: pressure treatment at 400 MPa/10 min; FF: sulphur dioxide (60 mg/L); GG: pressure treatment (300 MPa, 10 min) + sulphur dioxide (30 mg/L)

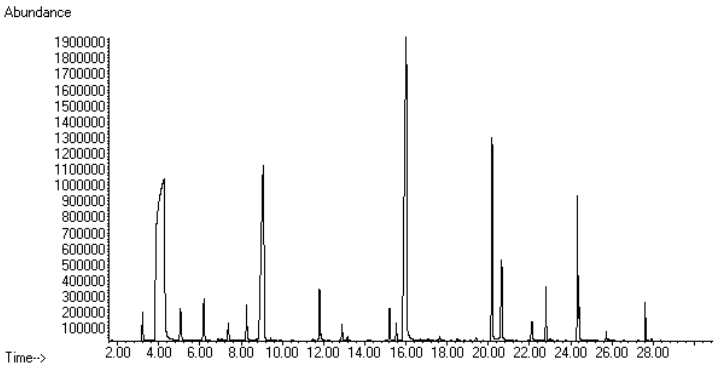

Figure 6 Typical total ion chromatogram of volatile constituents from mulberry wine obtained by SPME in the headspace sampling mode

Antioxidant capacity of mulberry wine

The ultra high pressure processed mulberry wines reduced the DHHP radical clearance rate to different extent. The antioxidant capacity of mulberry juice is attributed to the high amount of anthocyanins present **(Tsai *et al*., 2005; Sadiq Butt *et al*., 2008; Song *et al*., 2009)**. The free radical clearance rate of DHHP in mulberry wines treated at 100 MPa/10 min, 200 MPa/10 min, 300 MPa/10 min, 400 MPa/10 min, sulphur dioxide (60 mg/L), 300 MPa /10 min + 30 mg/L sulphur dioxide treatment were 9.97%, 11.43%, 13.13%, 12.33%, 14.60%, and13.17%, respectively (Figure 7). The control sample had 15.03% clearance rate. The DHHP clearance rate tended to increase with increasing pressure up to 300 MPa/10 min, after which it dropped a little when the pressure was increased to 400 MPa/10 min. This observation may be due to the chemical destabilization of the anthocyanins' structure by the high pressure, thereby affecting the biological activity of anthocyanins **(Stintzing *et al*., 2002; Liu, 2003; Matsumoto *et al*., 2003)**. Again, it is plausible that as pressure was increased, there was the inactivation of *β*- glycosidase, peroxidase and polyphenol oxidase enzymes, which are known to hydrolyse and reduce anthocyanins **content (Garcia-Palazon *et al*., 2004)**.

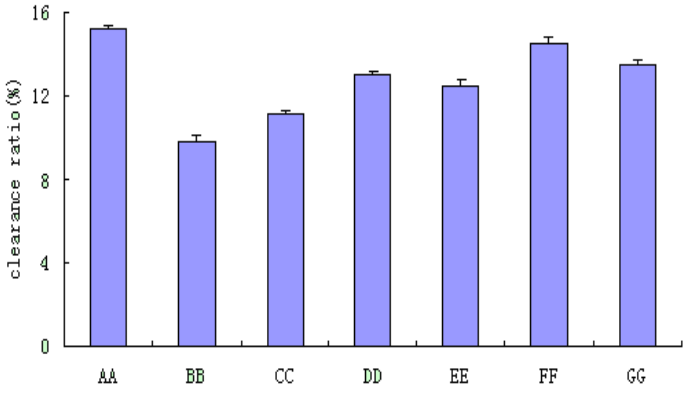

Figure 7 Antioxidant capacity of mulberry wine made by different treatment methods

Legend: AA: control; BB: pressure treatment at 100 MPa/10 min; CC: pressure treatment at 200 MPa/10 min; DD: pressure treatment at 300 MPa/10 min; EE: pressure treatment at 400 MPa/10 min; FF: sulphur dioxide (60 mg/L); GG: pressure treatment (300 MPa, 10 min) + sulphur dioxide (30 mg/L)

CONCLUSION

In this work, a total of nineteen volatile flavour compounds were identified in mulberry wine samples. These included alcohols (5), acids (4) and esters (10), with the esters being the main compounds. Largely, there were no significant differences among different mulberry wines in the types and concentrations of the volatile components. The major volatile fractions in mulberry juice were aldehydes and alcohols, imparting sweet, fruity, acid, musky and woody-fresh aroma characteristics to the juice. High pressure had no effect on the original flavour. Mulberry juice treated at 300 MPa/10 min, 400 MPa/10 min, sulphur dioxide (60 mg/L) and 300 MPa/10 min plus sulphur dioxide (30 mg/L) had similar characteristics of alcohol fermentation.

The number of microorganisms present in mulberry juice was different for differently treated conditions of ultra high pressure processing and sulphur dioxide, with that obtained for 300 MPa/10 min and 400 MPa/10 min being better than that treated with 60 mg/L sulphur dioxide.

It can therefore be concluded that, the traditional way of adding sulphur dioxide during winemaking could be replaced by using pressures of 300 MPa/10 min or 400 MPa/10 min.

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