JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 11209–11217 11209
DOI:10.1021/jf9025053

Influence of Prefermentative Treatments and Fermentation on the Antioxidant and Volatile Profiles of Apple Wines

Pawel Satora,* Tomasz Tarko, Aleksandra Duda-Chodak, Pawel Sroka, Tadeusz Tuszyński, and Maria Czepielik

Department of Fermentation Technology and Technical Microbiology, University of Agriculture, ul. Balicka 122, Krakow, Poland

The aim of this study was to determine the influence of different factors such as fruit processing, pectinolytic enzyme application, strain of yeast (Johannisberg-Riesling, Steinberg), cell immobilization on the alginate, and type of fermentation on the antioxidant profile and volatile composition of apple wines. Champion and Idared apples were used during experiments. The factors used influenced significantly ($p < 0.05$) antioxidant capacity, polyphenol profile, and volatile composition of apple wines. Pulp fermentation caused formation of higher amounts of ethanol and favorably influenced the antioxidant activity of wines. Procyanidins B2 and C1 as well as epicatechin and catechin prevailed among polyphenols in these samples. Cell immobilization positively affected the ethanol content, but decreased the antioxidant activity, of wines. Volatile composition of wines was mainly influenced by strain of yeast and type of fermentation. Apple wines fermented spontaneously were characterized by more esters and methanol and fewer higher alcohols compared to inoculated samples.

KEYWORDS: Apple wines; prefermentative treatment; antioxidant activity; polyphenols; volatile compounds

INTRODUCTION

The moderate climate of Poland is conducive to high apple production. The average annual harvest of these fruits has now been estimated at about 1.5 million tonnes, and the industry processes about 700,000 tonnes. Apples are valuable raw materials for juice, stewed fruit, jelly, dry fruit, concentrate, cremogen, puree, and wine production. For many years, apple wine production constituted a significant food-processing section. Most often, late cultivars with smaller fruits and larger rates of skin surface to flesh are used. The wines produced from this kind of fruit are spicy and more aromatic.

The quantitatively most important volatiles in apple wine are the higher alcohols, esters, and lower fatty acids (*1*). These compounds are formed during fermentation and mainly derived from raw material, as well as metabolic processes of yeast under anaerobic conditions. Both qualitative and quantitative characterizing aroma-producing compounds in apple wine and their formation during fermentation are desired to provide quality control of apple wine.

Phenolic compounds are the second most important group of apple wine constituents because they greatly contribute to their sensory properties and other attributes. In particular, polyphenolic compounds have antioxidant activity and free radical scavenging capacity and exhibit pro-healthy properties, reducing the risk of coronary heart disease and cancer. Furthermore, phenolics are associated with bitterness, astringency, and color stability, and some of them have been used for detecting adulterations in apple products and could be inhibitors for microbiological growth-avoiding processes (*2*). From the quantitative point of view, there are five major groups of polyphenols found in apple wines: flavan-3-ols, procyanidins, flavonols, dihydrochalcones, and hydroxycinnamic acids and derivatives (*3*, *4*).

The aim of this study was to determine the influence of different factors such as fruits processing, pectinolytic enzyme application, strain of yeast, cells immobilization, and type of fermentation on the antioxidant profile and volatile composition of apple wines. Champion and Idared apples, chosen for the research, are among the most popular varietes of dessert apples grown in Poland. However, in terms of chemical composition and sensory features they represent a valuable raw material for the food processing industry, including the wine industry (*5*). Selection of proper prefermentative treatments and fermentation technology could result in the production of a beverage with favorable antioxidant properties and sensory features.

MATERIALS AND METHODS

Chemicals. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), and Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from the Sigma-Aldrich Co. Potassium persulfate ($K_2S_2O_8$) was obtained from the POCh Co., Poland, and 96% ethanol from the ChemPur Co., Poland.

Yeast and Plant Material. Active wine yeast *Saccharomyces cerevisiae* cv. Johannisberg-Riesling and Steinberg, obtained from the

*Corresponding author (telephone ++48 12 662 47 97; fax ++48 12 662 47 98; e-mail psatora@ar.krakow.pl).

Published on Web 11/13/2009

Table 1. Chemical Composition of Apple Musts Used for Winemaking

apple musts			g/L						
			extract	total sugars	reducing sugars	sucrose	sugar-free extract	titratable acidity[a]	pH
Champion	WO[b]	juice extractor	111.3	103.47	96.40	6.71	8.19	1.39	4.62
		juice extractor, + pulp	125.1	109.23	99.50	9.24	16.36	1.52	4.60
		vertical basket press	110.0	95.47	92.00	3.29	14.71	1.35	4.63
	WE[b]	vertical basket press	111.0	103.5	99.10	4.08	7.72	1.39	4.60
		vertical basket press, enzymes	115.0	112.40	105.73	6.33	2.94	1.56	4.69
Idared	WO	juice extractor	102.0	94.13	86.93	6.84	8.23	2.82	4.20
		juice extractor, + pulp	108.5	97.20	88.20	8.55	11.75	2.96	4.15
		vertical basket press	100.0	89.60	82.80	6.46	10.74	2.56	4.13
	WE	vertical basket press	104.2	93.23	91.37	1.27	11.26	2.74	4.13
		vertical basket press enzymes	114.0	100.93	96.00	4.31	13.69	3.19	4.39

[a] Expressed as grams per liter of malic acid. [b] WO, fruits without endocarp; WE, fruits with endocarp.

Table 2. Chemical Composition of Young Apple Wines

Champion											
apple wines				g/L							
strain	method of must obtaining	fruit	other treatment	extract	sugar-free extract	total sugars	reducing sugars	sucrose	ethanol	titratable acidity[a]	
Johannisberg-Riesling	juice extractor	WO[b]	+ pulp	25.0a (±0.0)	19.7 (±0.2)	5.4ab (±0.2)	3.4a (±0.1)	1.9a (±0.0)	90.7a (±1.6)	3.99a (±0.08)	
		WO		17.5bc (±0.5)	14.3 (±0.6)	3.3a (±0.1)	2.2a (±0.1)	1.0b (±0.0)	89.5a (±0.7)	3.03b (±0.01)	
	vertical basket press	WO		15.5b (±0.5)	13.2 (±0.6)	2.3a (±0.1)	2.3a (±0.1)	0.0c	82.6b (±0.6)	2.04c (±0.04)	
		WE[b]		18.6c (±1.6)	12.2 (±7.0)	6.4ab (±5.4)	6.4ab (±5.4)	0.0c	81.1b (±1.4)	3.86ad (±0.04)	
Steinberg	vertical basket press	WE		18.7c (±1.0)	9.2 (±6.0)	9.5b (±5.6)	9.5b (±5.6)	0.0c	80.8b (±6.5)	3.92a (±0.09)	
		WE	+ pectinolytic enzymes	24.5a (±1.7)	15.0 (±2.9)	9.5b (±1.2)	9.2b (±1.7)	0.2c (±0.4)	89.8a (±2.1)	5.42e (±0.19)	
		WE	(cell immobilization)	17.2bc (±2.5)	14.8 (±2.4)	2.4a (±1.0)	2.2a (±0.8)	0.2c (±0.3)	80.3b (±2.0)	3.86ad (±0.02)	
significance[c]				***	ns	**	**	***	***	***	

Idared										
apple wines				g/L						
strain/type of fermentation	method of must obtaining	fruit	other treatment	extract	sugar-free extract	total sugars	reducing sugars	sucrose	ethanol	titratable acidity
Johannisberg-Riesling	juice extractor	WO	+ pulp	19.0a (±0.5)	18.3a (±0.6)	0.7a (±0.1)	0.7a (±0.1)	0.0a	76.7ab (±2.4)	3.62a (±0.04)
		WO		14.0b (±2.0)	13.1b (±2.1)	0.9ab (±0.1)	0.9a (±0.1)	0.0a	80.0a (±0.7)	4.10b (±0.02)
	vertical basket press	WO		24.5c (±0.5)	13.3b (±0.8)	11.2c (±0.3)	11.2b (±0.3)	0.0a	78.5ab (±1.2)	3.51a (±0.13)
		WE		15.2b (±0.3)	14.0b (±0.5)	1.4b (±0.4)	1.0a (±0.2)	0.2ab (±0.4)	87.0c (±2.4)	4.43b (±0.12)
Steinberg	vertical basket press	WE		18.0a (±0.0)	17.0a (±0.1)	1.0ab (±0.1)	1.0a (±0.1)	0.0a	83.5d (±1.0)	5.01c (±0.08)
		WE	+ pectinolytic enzymes	25.0c (±0.5)	18.2a (±0.4)	6.8d (±0.4)	6.8c (±0.4)	0.0a	86.0cd (±2.4)	5.15c (±0.45)
		WE	(cell immobilization)	18.5a (±0.5)	17.5a (±0.6)	1.0ab (±0.1)	1.0a (±0.1)	0.0a	91.6e (±2.2)	5.16c (±0.09)
spontaneous	vertical basket press	WE		45.0d (±2.0)	18.6a (±2.6)	26.4e (±0.6)	26.1d (±0.6)	0.3b (±0.0)	75.8b (±2.5)	3.35a (±0.04)
significance				***	***	***	***	*	***	***	

[a] Expressed as grams per liter of malic acid. [b] WO, without endocarp; WE, with endocarp. [c] Significance: *, **, and *** display significance at 5, 1, and 0.5% by least significant difference; ns, not significant. Values with different roman letters (a−e) in the same column are significantly different according to the Duncan test ($p < 0.05$).

Collection of Pure Industrial Microorganism Cultures of the Institute of Fermentation Technology and Microbiology, Technical University of Lodz (collection ŁOCK 105 and 39, respectively), were used for the fermentation. Apple musts were obtained from Champion and Idared apples, harvested in October 2005 from the experimental apple orchard in Garlica Murowana near Krakow.

Fermentation Assays. *Juice Extractor*. Apple fruits were washed, dried, and divided into fourths. Endocarp with seeds was removed. Then the fruits were disintegrated using a juice extractor. To part of the samples the pulp obtained after fruit disintegration was added. Then sucrose (up to 200 g/L) and citric acid (up to 4.5 g/L) were added, and the musts were pasteurized for 15 min at 104 °C. Alcoholic fermentation was conducted

Table 3. Antioxidant Activity of Apple Wines Produced from Different Musts

Champion						
apple wines				antioxidant activity (mg of TE/100 mL)		
strain/type of fermentation	method of must obtaining	fruit	other treatment	DPPH	ABTS	total polyphenol content (mg of GAE/L)
Johannisberg-Riesling	juice extractor	WO[a]	+ pulp	97.7a (±3.4)	1087.1a (±74.9)	742.5a (±28.8)
		WO		86.6a (±4.5)	761.2b (±29.1)	515.6b (±19.2)
	vertical basket press	WO		75.8a (±5.4)	489.0c (±12.3)	601.9c (±9.5)
		WE		99.6a (±17.2)	464.2cd (±5.0)	634.7d (±26.4)
Steinberg	vertical basket press	WE		99.4a (±11.1)	450.7cd (±17.4)	657.2d (±17.0)
		WE	+ pectinolytic enzymes	142.7b (±7.6)	399.7de (±5.8)	642.9d (±10.5)
		WE	(cell immobilization)	124.6b (±26.3)	337.4e (±68.4)	210.3e (±18.0)
	significance[b]			***	***	***
Idared						
apple wines				antioxidant activity (mg of TE/100 mL)		
strain/type of fermentation	method of must obtaining	fruit	other treatment	DPPH	ABTS	total polyphenol content (mg of GAE/L)
Johannisberg-Riesling	juice extractor	WO	+ pulp	13.4a (±1.0)	664.8a (±13.5)	261.6ab (±6.6)
		WO		12.1a (±1.5)	616.8b (±37.4)	240.5ac (±8.3)
	vertical basket press	WO		5.6b (±2.2)	179.6c (±5.6)	242.7ac (±10.9)
		WE		13.3a (±2.4)	132.7d (±3.8)	236.8c (±17.9)
Steinberg	vertical basket press	WE		13.1a (±3.8)	127.7d (±16.4)	272.0b (±9.9)
		WE	+ pectinolytic enzymes	13.3a (±1.1)	143.8d (±4.1)	251.6abc (±6.8)
		WE	(cell immobilization)	19.6c (±1.3)	111.8d (±20.1)	96.7d (±4.1)
spontaneous	vertical basket press	WE		11.5a (±1.4)	174.2c (±7.0)	252.3abc (±6.4)
	significance			***	***	***

[a] WO, fruits without endocarp; WE, fruits with endocarp. [b] Significance: *, **, and *** display significance at 5, 1, and 0.5% by least significant difference; ns, not significant. Values with different roman letters (a−e) in the same column are significantly different according to the Duncan test ($p < 0.05$).

for 28 days at 25 °C in 3 L glass flasks containing 1.5 L of apple must inoculated with free cells of yeast (0.5 g dry weight/L).

Vertical Basket Press. Apple fruits were washed, dried, and divided into fourths. Endocarp with seeds was removed from part of the fruits (WO samples). Then the fruits were disintegrated using an apple crusher. The obtained pulp was next pressed using a vertical basket press. Some of the pulp was treated with pectinolytic preparation Pektopol PT-400 (0.3 mL/kg; Pektowin, Jasło, Poland) for 3 h at 28 °C, before pressing (samples after pectinolytic enzymes treatment).

Then sucrose (up to 200 g/L) and citric acid (up to 4.5 g/L) were added, and the musts were pasteurized for 15 min at 104 °C. Alcoholic fermentation was conducted for 28 days at 25 °C in 3 L glass flasks containing 1.5 L of apple must inoculated with free or immobilized in alginate beads cells of yeast (0.5 g dry weight/L).

Spontaneous Fermentation. Unwashed Idared apple fruits with endocarps and seeds were disintegrated using an apple crusher. The obtained pulp was next pressed using a vertical basket press. Then sucrose (up to 200 g/L) and citric acid (up to 4.5 g/L) were added. Alcoholic fermentation was conducted for 28 days at 25 °C in 3 L glass flasks containing 1.5 L of apple must.

After the fermentation, the young wine was separated from the sediments by careful pouring into another vessel and kept for further clarification for 48 h at 4 °C. Clarified young wines were subjected to the analysis. All samples were done in triplicate.

Cell Immobilization in Calcium Alginate Beads. *S. cerevisiae* cells, grown in YPED and collected after 24 h, were washed with distilled water, mixed with a sterile sodium alginate solution (2.85%, w/v, final concentration) up to a final cell density of 50 mg/mL (*6*), and added dropwise to a 5% $CaCl_2$ cross-linking solution. The flasks with obtained beads were kept for 3 h at low temperature to harden them.

Enological Parameter Analysis. The ethanol content, pH, total acidity, total dry matter, and reducing sugars and sucrose concentrations were determined using official methods (*7*).

ABTS Radical Cation Decolorization Assay. The antioxidant activity of the wines was determined according to the method of Tarko et al. (*8*). ABTS radical was generated in the chemical reaction between the 7 mM diammonium salt of the ABTS and 2.45 mM potassium persulfate. To terminate the reaction and to stabilize the ABTS cation radical, the solution was kept overnight in the dark at ambient temperature. Prior to analysis, the radical solution was diluted with phosphate buffer saline (pH 7.4) in such a way that allowed a final absorbance of $A = 0.70 \pm 0.02$ ($ABTS_{0.7}$) measured at 734 nm to be obtained.

Samples of wines (0.1 mL) or Trolox solution (concentration = 1−10 mg per 100 mL) were added to 1 mL of $ABTS_{0.7}$, and the absorbance was measured 6 min after mixing. Antioxidant capacity was calculated with the use of a standard curve obtained by measuring the absorbance of synthetic vitamin E solutions (Trolox) and expressed in milligrams of Trolox per 100 mL of wine.

DPPH Radical Scavenging Assay. In the determination procedure with a DPPH method used, antioxidants present in the sample investigated reduce a stable DPPH radical and cause a drop in the absorbance value measured at a wavelength of 515 nm. The scavenging capacity of DPPH radical was assessed on the basis of modified methods (*9*). An amount of 200 μL of the infusion analyzed (properly diluted with a redistilled water) or Trolox solutions (their concentrations ranging from 0 to 2.5 mg/100 mL) was added to 800 μL of a 225 μL ethanol solution of DPPH and, then, the rate of absorbance disappearance was measured in the 10th minute upon mixing of reagents in a cuvette. The antioxidant capacity of wines was calculated using a standard curve developed for Trolox and expressed as milligrams of Trolox per 100 mL.

Total Polyphenol Index (TPI). The amount of total polyphenols in wines was determined according to the Folin−Ciocalteu colorimetric method (*10*). Wine samples were diluted with water (1:4). A 1 mL volume of the standard or sample solution was added to 5 mL of Folin−Ciocalteu reagent (1:10 dilution, Sigma-Aldrich), 50 mL of deionized water, and 20 mL of sodium carbonate (20% (w/v)). The reaction mixture was then made up to the mark in a 100 mL volumetric flask and left to stand for 30 min before measuring the absorbance at 765 nm (spectrophotometer Beckman DU-650). A calibration curve was obtained with gallic acid solutions (concentration range = 0.4−5 mg/L; Fluka), and the results are expressed as milligrams of gallic acid per liter of wine.

Polyphenol Analysis (HPLC). For this analysis the samples that were differentiated on the basis of ABTS, DPPH, and Folin−Ciocalteu methods were chosen.

A HPLC apparatus consisting of a Merck-Hitachi L-7455 diode array detector and a quatenary pump L-7100 equipped with a D-7000 HSM

Table 4. Polyphenol Profile of Selected AppleWines Produced from Different Musts

apple wines				mg/L						
method of obtaining must	strain/type of fermentation	fruit	other treatment	chlorogenic acid	derivative of caffeic acid	caffeic acid glucoside	*p*-coumaric acid	*p*-coumarylquinic acid	(+)-catechin	(−)-epicatechin
					Champion					
juice extractor	Johannisberg-Riesling	WO[a]	+ pulp	25.7a (±4.3)	0.0a	2.6a (±0.2)	1.1 (±0.1)	5.6a (±0.2)	8.4a (±1.4)	41.1a(±6.1)
		WO		21.2bc (±1.3)	12.6b (±1.2)	1.9bc (±0.5)	1.1 (±0.2)	5.6a (±0.1)	4.3b (±0.3)	30.7b (±1.3)
vertical basket press		WE		24.2ab (±1.8)	0.0a	2.3ab (±0.2)	1.0 (±0.4)	4.0b (±0.9)	3.5bc (±0.8)	4.1c(±0.3)
	Steinberg	WE		17.5c (±0.9)	0.0a	0.5d (±0.1)	1.3 (±0.2)	0.4c (±0.0)	1.2d (±0.3)	8.7c (±0.7)
		WE	(cell immobilization)	32.1d(±2.2)	0.0a	1.7c (±0.1)	1.1 (±0.0)	4.7d (±0.4)	8.4a (±1.0)	29.2b (±1.2)
					Idared					
vertical basket press	Johannisberg-Riesling	WE		18.1c (±0.8)	0.0a	2.8a (±0.3)	0.9 (±0.2)	3.5b (±0.4)	1.5d (±0.3)	4.1c (±0.5)
	spontaneous	WE		34.6d (±3.4)	0.0a	2.0bc (±0.3)	1.0 (±0.2)	5.2ad (±0.2)	2.2cd (±0.6)	15.0d(±2.0)
	significance[b]			***	***	***	ns	***	***	***

multisolvent delivery system (Merck-Hitachi, Tokyo, Japan) was employed. Separation was performed on a Synergi Fusion RP-80A 150 × 4.6 mm (4 μm) Phenomenex (Torrance, CA) column thermostated at 30 °C. The mobile phase was composed of solvent A (2.5% acetic acid) and solvent B (acetonitrile). The program began with a linear gradient from 0% B to 36 min 25% B, followed by washing and reconditioning of the column. The flow rate was 1 mL/min, and the runs were monitored at the following wavelengths: flavanols at 280 nm, phenolic acids at 320 nm, flavonols at 360 nm, and anthocyanidins at 520 nm. Retention times and spectra were compared to those of pure standards within 200−600 nm.

In addition, enzymatic hydrolysis of flavonol glycosides in citrate buffer solution (pH 5.0) was performed. Afterward, specific enzymes were added: β-glucosidase, β-xylosidase, β-galactosidase, and β-hesperidinase (Sigma, Steinheim, Germany). The disapearance of single peaks in the chromatogram and formation of the corresponding aglycon were observed using HPLC after 1 h of incubation at 38 °C with a specific enzyme.

Results (expressed as mg/100 mL of apple wine) were read from standard curves developed for the corresponding standards: chlorogenic acid, caffeic acid, *p*-coumaric acid, *p*-coumarylquinic acid, (+)-catechin, (−)-epicatechin, phloridzin, and quercetin glycoside manufactured by the Sigma-Aldrich Co. To determine the magnitude of error in the selected series, the assays were repeated. A standard error in the HPLC assays was below 10%.

Volatile Compounds Analysis (GC-SPME). Two milliliters of each wine sample was transferred to a 15 mL amber vial having a screw cap (Supelco) with a magnetic stirrer and 1 g of NaCl, which was then spiked with 2 μL of internal standard (4-methyl-2-pentanol; Fluka). The SPME device (Supelco Inc., Bellefonte, PA) coated with PDMS (100 μm) fiber was first conditioned by inserting it into the GC injector port at 250 °C during 1 h. For sampling, the fiber was inserted into the headspace under magnetic stirring (300 rpm) for 35 min at 40 °C. Subsequently, the SPME device was introduced into the injector port for chromatographic analysis and remained in the inlet for 2 min.

GC-SPME analysis was performed on a Hewlett-Packard 5890 series II chromatograph system. The tested components were separated on a capillary column HP-INNOWAX (cross-linked polyethylene glycol stationary phase; 30 m × 0.53 mm i.d. with 1.0 μm film thickness). The detector and injector temperatures were 250 °C, and the column was heated using the following temperature program: 35 °C for 5 min at an increment of 5 °C/min to 110 °C, then 40 °C/min to 220 °C, and maintaining a constant temperature for 3 min. The carrier gas was helium at a 20.0 mL/min flow. Hydrogen flow speed was 33.0 mL/min, and that of air was 400 mL/min.

The qualitative and quantitative identification of volatile substances (acetaldehyde, ethyl acetate, methanol, propanol, isobutanol, butanol, amyl alcohols, pentanol, hexanol, 2-phenylethanol, and acetic acid; Sigma-Aldrich) was based on the comparison of retention times and peak surface area read from sample and standard chromatograms.

All tests were carried out three times.

Statistical Analysis. SPSS 13.0 software was applied for statistical results analyses. Statistically significant differences between results ($p = 0.05$) were evaluated using one-way analysis of variance (ANOVA). Comparisons of mean values were made using the Duncan test ($p < 0.05$).

RESULTS AND DISCUSSION

Enological Characteristics of Apple Wines. The chemical composition of musts obtained from Idared and Champion apples is presented in **Table 1**. Due to the relatively low titratable acidity of these musts (1.35−3.20 g/L), to increase acidity citric acid was added. According to Polish legislation (2004), dry fruit wines should contain 9−11% volume of ethanol. To fulfill this requirement, musts before fermentation were sweetened with sucrose up to 200 g/L (20 °Blg).

All obtained young apple wines were characterized by diversified levels of extract and total sugars (**Table 2**). In most samples relatively high sugar consumption (>95%) was found. Only wines produced spontaneously using natural microbiota present on the fruit surface contained higher amounts of unfermented carbohydrates (total sugars, 26.4 g/L). This could be caused by the lower resistance of these microorganisms to the increased ethanol concentration (*11*), compared to *S. cerevisiae* strains, that could lead to fermentation inhibition when the ethanol concentration exceeded a specific value.

The higher sugar consumption in Idared wines than in Champion was also affirmed. Idared musts contained about 10% less sugars than those obtained from Champion fruits; therefore, the amount of sucrose added to them was higher. It should be supposed that this carbohydrate dissolved in apple must was used by yeast cells prior to others or relatively quickly hydrolyzed to simple sugars (*12*), which is indicated by lower concentrations of sucrose after fermentation in comparison with other sugars such as glucose and fructose. Very important also is the bioavailability of other nutritious components (such as nitrogen compounds and vitamins) and the presence of compounds that could inhibit yeast growth (*13*).

The level of sugar-free extract was rather even in all analyzed samples and several times higher than that found in apple musts. It is connected with higher amounts of glycerol by yeast cells that

Table 4. Continued

mg/L										
procyanidin B1	procyanidin B2	procyanidin C1	phloridzin	phloretin xyloglucoside	quercetin galacto-side	quercetin gluco-side	quercetin arabino-side	quercetin xyloside	quercetin rhamno-side	total poly-phenols
					Champion					
5.6a (±0.4)	55.9a (±3.1)	19.7a (±1.5)	2.3a (±0.2)	4.9a (±0.3)	3.2a (±0.3)	0.0	1.6a (±0.4)	5.9a (±0.6)	5.6a (±0.5)	189.2a(±8.2)
3.3bc(±0.3)	49.5b (±0.5)	8.5b (±0.6)	2.2a (±0.2)	4.7a (±0.2)	0.0b	0.0	0.0b	0.0b	0.0b	145.6b (±0.5)
4.1b (±0.6)	7.2c (±0.9)	4.5c (±0.3)	1.1b (±0.4)	0.5b (±0.1)	0.4b (±0.2)	0.0	0.0b	0.1b (±0.0)	0.4b (±0.1)	57.2c(±4.4)
1.6d (±0.4)	2.1d (±0.0)	0.4d (±0.0)	1.6b (±0.2)	2.9c (±0.3)	3.6c (±0.4)	0.0	0.9c (±0.1)	0.1b (±0.0)	2.7c (±0.3)	45.7d(±1.6)
2.5cd(±0.4)	3.4d (±0.4)	6.8e (±0.4)	4.7c (±0.4)	1.2d (±0.1)	0.0b	0.0	0.0b	0.0b	0.0b	95.8e (±1.2)
					Idared					
2.0d (±0.1)	3.2d (±0.2)	4.7c (±0.3)	1.1b (±0.2)	1.1de (±0.1)	0.3b(±0.2)	0.0	0.0b	0.1b (±0.0)	0.2b (±0.0)	43.5d (±1.3)
9.7e (±1.2) ***	10.7e (±0.3) ***	4.3c (±0.3) ***	3.6c (±0.5) ***	0.8be (±0.2) ***	0.1b (±0.0) ***	0.0	0.0b ***	0.0b ***	0.3b (±0.1) ***	89.6e (±1.6) ***

[a] WO, fruits without endocarp; WE, fruits with endocarp. [b] Significance: *, **, and *** display the significance at 5, 1, and 0.5% by least significant difference; ns, not significant. Values with different roman letters (a−e) in the same column are significantly different according to the Duncan test ($p < 0.05$).

is a byproduct of ethanol fermentation and is formed by microorganisms to protect their cells against unfavorable impact of environmental factors (*14*). Increased concentration of sugar-free extract was detected in wines obtained after fermentation of pulp and application of pectinolytic enzymes. This could be associated with progressive release of pulp components such as nitrogen compounds, cellulose, hemicelluloses, pectic acids, and others to the fermentation solution.

Analyzed wines were characterized by statistically significant differences in ethyl alcohol level. A relatively high amount of this compound was found in samples fermented using immobilized cells (Champion, 80.3; Idared, 91.6 g/L). Trapped inside alginate beads, yeast cells produced intensively reserve and structural carbohydrates, which protected them against higher ethanol amounts (*15*). The response of immobilized microorganisms on the increasing ethanol concentration could be also changes of membrane fatty acid composition (*16*). Application of a juice extractor for fruit processing caused formation of higher amounts of ethanol (about 90 g/L) in Champion wines. Probably more sugars and other nutritious compounds were released to must as a result of improved disintegration of fruit tissues and cells; these substances were also better available for yeast cells. A similar dependence was found in samples when a pectinolytic preparation was used.

Apple wines obtained during experiments showed relatively low titratable acidity, from 2.04 to 5.42 g/L, expressed as malic acid. The major factor influencing its level was the strain of yeast used for the fermentation. Samples fermented by Johannisberg-Riesling strain were characterized by decreased titratable acidity, which could be connected with malic acid consumption by yeast cells. This kind of phenomenon was found in our earlier studies (*5*); it is generally known that *S. cerevisiae* yeast has the ability to decompose from 0 to 3 g/L malic acid during alcoholic fermentation (*17*). The second yeast strain used, Steinberg, increased titratable acidity of wines. A relatively high reduction of titratable acidity was also observed in samples fermented spontaneously (3.35 g/L). In the case of mixed cultures, competition for carbon sources in fermenting medium can cause an increase in malic acid consumption. Samples obtained from fermentation of musts treated with pectinolytic preparation were also distinguished by relatively high titratable acidity, which could depend on the degree of methylation of pectins in fruits, which is higher in apples compared to other fruits (*18*). Formed as a result of demethylation of pectins carboxyl groups and free pectin acids may significantly increase the obtained value of acidity determined by titratable method. According to the chemical equation of the demethylation process, release of 500 mg/L methanol during enzymatic treatment could raise the titratable acidity level by 1.5 g/L.

Antioxidant Activity and Polyphenolic Composition of Apple Wines. Apple fruits contain relatively high amounts of various polyphenols with anioxidant activity. Most of these compounds pass from the fruits to juices, wines, or other products and remain active. However, changes of their profile as well as phenolic degradation during disintegration of raw material, fermentation, and wine aging have been reported (*19*).

The fermentation process favorably influences the antioxidant activity of apple wines. Idared and Champion musts were characterized by almost 2 times lower content of antioxidants than fermented beverages (*20*). The weaker antioxidant activity of musts depends on polyphenol composition and their amounts as well as bioavailability (*21*). Polyphenols of must are more polymerized and complexed, and their solubility in water is lower. However, they are better soluble in 10% alcohol and released to wine during fermentation.

Because Champion apples possessed higher free radical scavenging than Idared (*5*), wines produced from this variety of apples were characterized also by relatively high antioxidant activity. Besides variety of apples, the content of individual polyphenolic compounds in fruits depends as well on the degree of maturity and conditions of their storage (*22*).

The method of obtaining must (juice extractor, apple crusher + vertical basket press) influenced significantly the antioxidant capacity (**Table 3**) and polyphenol profile (**Table 4**) of apple wines. Total antioxidant activity assay (TAA) with ABTS radical showed a >2 times higher value in samples obtained using the juice extractor. Epicatechin and procyanidin B2 predominated among polyphenols, and their concentrations were, respectively, 10 and 7 times higher than in wines produced from must that was pressed by vertical basket press. A stronger homogenization process made by juice extractor could lead to easier release of polyphenolic compounds to the solution. In the case of pulp pressing on the vertical basket press, a significant part of the compounds remained in the pulp and was removed with pomace.

Table 5. Volatile Composition of Apple Wines Produced from Different Musts

Champion apple wines				mg/L				
strain/type of fermentation	method of must obtaining	fruit	other treatment	acetaldehyde	acetoine	carbonyl compounds	ethyl acetate	total esters
Johannisberg-Riesling	juice extractor	WO[a]	+ pulp	13.1ab (±3.1)	0.0a	96.9a (±17.6)	4.7a (±0.3)	70.4a (±35.2)
		WO		34.5c (±10.2)	0.6ab (±0.2)	88.1a (±0.0)	7.0a (±0.2)	52.8a (±17.6)
	vertical basket press	WO		5.8a (±0.8)	0.2a (±0.1)	88.1a (±8.8)	14.0ab (±0.3)	123.2b (±26.4)
		WE		22.2bc (±13.7)	0.8ab (±0.1)	38.2bc (±26.9)	20.3bc (±0.8)	146.7b (±26.9)
Steinberg	vertical basket press	WE		6.9a (±3.0)	0.6ab (±0.2)	52.9b (±0.0)	27.0c (±5.7)	246.4c (±35.2)
		WE	+ pectinolytic enzymes	22.5bc (±7.8)	1.9c (±0.6)	45.5c (±20.8)	49.0d (±15.3)	199.5c (±20.3)
		WE	(cell immobilization)	13.5ab (±4.6)	1.3bc (±0.1)	35.2bc (±8.8)	30.2c (±4.5)	140.8b (±30.5)
	significance[b]			***	***	***	***	***

Idared apple wines				mg/L				
strain/type of fermentation	method of must obtaining	fruit	other treatment	acetaldehyde	acetoine	carbonyl compounds	ethyl acetate	total esters
Johannisberg-Riesling	juice extractor	WO	+ pulp	8.6a (±1.0)	0.0	105.7a (±8.8)	3.2a (±0.6)	88.0a (±17.6)
		WO		19.2b (±8.7)	0.0	96.9ab (±8.8)	3.8a (±0.1)	70.4a (±17.6)
	vertical basket press	WO		8.5a (±3.0)	0.0 (±0.0)	70.5bc (±35.2)	13.0a (±0.2)	193.6bc (±35.2)
		WE		4.3a (±0.9)	0.0 (±0.0)	38.2d (±10.2)	13.0a (±1.0)	146.7bc (±26.9)
Steinberg	vertical basket press	WE		6.4a (±7.3)	0.0 (±0.0)	36.7d (±11.1)	30.1b (±8.0)	228.8c (±17.6)
		WE	+ pectinolytic enzymes	12.1ab (±2.2)	0.6 (±0.1)	16.2cd (±9.2)	31.2b (±12.1)	176.0b (±46.6)
		WE	(cell immobilization)	9.5a (±4.7)	0.5 (±0.1)	22.0d (±4.4)	33.9b (±1.4)	187.7bc (±36.6)
spontaneous	vertical basket press	WE		11.3ab (±3.7)	0.3 (±0.1)	79.3ab (±0.0)	24.3b (±0.9)	211.2c (±35.2)
	significance			*	ns	***	***	***

Additionally, pressing increased aeration of samples. Tuszyński and Sroka (*23*) proved that during fruit processing in the oxygen conditions the majority of polyphenolic compounds could undergo decomposition. For this reason aeration during fruit processing and pulp pressing as well as during must and wine storage should be strictly avoided.

Fermentation of must with pulp addition favorably affected the antioxidant activity of studied apple wines. These samples were characterized by relatively high free radical scavenging capacity (Champion, 1087.0 mg of TE/100 mL; Idared, 664.8 mg of TE/100 mL) that was directly connected with their polyphenolic compound concentration measured by HPLC method. Procyanidins B2 (55.9 mg/L) and C1 (19.7 mg/L) as well as epicatechin (41.1 mg/L) and catechin (8.4 mg/L) prevailed among these compounds. Relatively high amounts of glycosidic derivatives of quercetin were found, compounds that were nearly absent in the other samples. Pulp includes a comparatively greater amount of homogenized peel. It is well-known that apple peel contains up to several times higher concentration of glycosidic derivatives of quercetin, epicatechin, phloridzin, and phloretin xyloglucoside (*24*) than apple flesh. All of these compounds could be released to the wine during the fermentation process.

Application for the fermentation musts produced from fruits without endocarp and seeds did not influence significantly the total antioxidant activity determined using ABTS method. However, the results obtained using DPPH radical were about 10% higher in the case of samples having endocarps and seeds present during fruit processing. According to Lu and Foo (*25*) apple seeds contain large amounts of phloridzin, phloretin xyloglucoside, and quercetin galactoside, which during fruit processing gets into the must.

The strain of yeast used for the fermentation did not affect the antioxidant activity of studied wines, and obtained results remained on a similar level. Samples fermented spontaneously were characterized by about a 20% increase of free radical scavenging capacity, however, only when it was determined by ABTS method. Polyphenolic compound analysis using the HPLC method showed an almost 2 times higher amount of polyphenols in wines fermented spontaneously compared to other samples. Among analyzed components prevailed chlorogenic (34.6 mg/L) and *p*-coumarylquinic acids (5.2 mg/L) as well as catechin, epicatechin, and its oligomers. Differences were found also in polyphenol profile of apple wines produced using Johannisberg-Riesling and Steinberg strains. The samples obtained with the latter strain were distinguished by a considerably higher level of epicatechin, but a lower level of procyanidins, which could be caused by degradation of oligomers to monomers in the presence of microbial enzymes (*26*). Because epicatechin and procyanidins are characterized by similar antioxidant activities (*25*), hence summary results obtained by ABTS and DPPH methods were similar.

Pectinolytic treatment of pulp did not change significantly the antioxidant activity of apple wines, but a small increase in its value was detected, especially in the Idared wines. Enzymes (Pektopol PT) used during apple processing to improve pulp-pressing efficiency contributed to the release of pulp polyphenols. However, relatively great contact of must with air during treatment (3 h) could cause degradation of many of these compounds as well as deepened color of the must. Maceration under anaerobic conditions prevents oxidation of polyphenolic compounds but equally protects against microbial spoilage.

Yeast immobilization using alginate increased the antioxidant activity determined by the DPPH method and decreased the activity measured with ABTS radical. Cell immobilization caused also a rise of chlorogenic acid, catechin, and epicatechin concentrations. It is possibile that some polyphenols could interact with the surface of alginate beads by adsorption outside or inside it. Adsorption of polymerized polyphenols (such as procyanidins or quercetin derivatives) on the carrier surface increases contact of these compounds with yeast cells, and microbial enzymes can effectively decompose them to monomers (*26*).

Table 5. Continued

Champion mg/L									
methanol	propanol	isobutanol	butanol	amyl alcohols	pentanol	hexanol	phenylethanol	total fusels	acetic acid
343a (±106)	12.3a (±0.6)	81.9a (±0.6)	12.0ab (±0.0)	228.0abc (±0.4)	0.7 (±0.0)	4.6a (±0.2)	30.9a (±2.2)	370.4ab (±1.3)	67.2a (±4.8)
72b (±13)	6.0b (±0.4)	109.1b (±1.3)	10.3a (±0.1)	280.6ade (±1.6)	1.1 (±0.6)	3.6a (±0.2)	39.0ab (±1.1)	449.6ac (±2.0)	48.0b (±12.0)
65b (±1)	17.5c (±0.3)	91.2ac (±0.5)	14.7ab (±0.1)	336.7d (±3.1)	1.0 (±0.0)	3.5a (±0.1)	48.8b (±7.1)	513.5c (±10.6)	60.0ab (±12.0)
121b (±4)	20.6d (±0.9)	95.0c (±5.3)	20.2b (±0.9)	322.6de (±20.7)	1.3 (±0.3)	5.2a (±0.2)	38.4a (±3.4)	503.4c (±28.7)	76.0a (±6.9)
63b (±15)	9.7a (±0.4)	31.0d (±6.3)	21.6b (±2.5)	200.8bc (±32.3)	1.5 (±0.7)	4.4a (±2.8)	29.2a (±2.3)	298.1b (±40.9)	68.0a (±6.9)
567c (±183)	10.6a (±1.8)	43.7d (±15.7)	41.3c (±13.1)	260.6abe (±79.6)	1.0 (±0.3)	12.6b (±3.5)	60.8c (±11.7)	430.0ac (±27.1)	100.0c (±6.9)
74b (±14)	10.1a (±3.4)	39.0d (±5.7)	32.0d (±2.4)	190.0c (±23.2)	1.3 (±0.3)	8.3c (±0.6)	32.5a (±3.3)	313.7b (±30.4)	68.0a (±6.9)
***	***	***	***	***	ns	***	***	***	***

Idared mg/L									
methanol	propanol	isobutanol	butanol	amyl alcohols	pentanol	hexanol	phenylethanol	total fusels	acetic acid
17a (±10)	12.7a (±0.7)	111.3a (±0.1)	10.5a (±0.1)	219.5a (±0.8)	0.0a	4.4 (±0.1)	36.2a (±0.8)	394.6 (±1.3)	72.0a (±12.0)
trace a	13.0a (±0.5)	115.9a (±1.3)	11.0a (±0.1)	242.5ab (±1.4)	0.7ab (±0.1)	3.8 (±0.2)	40.0a (±1.7)	426.7 (±2.4)	72.0a (±12.0)
trace a	34.0bc (±0.2)	120.6a (±0.3)	16.8ab (±0.1)	365.5c (±0.7)	5.0c (±0.2)	3.5 (±0.1)	45.2a (±4.2)	393.8 (±34.1)	96.0ab (±24.0)
72a (±39)	38.8b (±21.2)	159.0b (±14.9)	20.9bc (±2.0)	321.5bcd (±33.2)	2.7d (±1.5)	4.9 (±3.8)	58.3b (±6.1)	606.1 (±78.4)	124.0b (±18.3)
20a (±11)	23.2abcd (±1.6)	110.0a (±16.6)	27.1cd (±4.1)	329.0cd (±39.5)	1.4b (±0.2)	6.2 (±0.8)	44.2a (±6.2)	541.1 (±67.1)	92.0a (±18.3)
383b (±77)	20.7acd (±5.4)	58.1c (±22.3)	26.1cd (±7.7)	271.8abd (±97.6)	1.1b (±0.0)	6.6 (±1.9)	34.5a (±2.3)	418.9 (±35.3)	88.0a (±6.9)
36a (±7)	31.1bcd (±5.8)	107.4a (±9.0)	29.3d (±2.5)	281.4abd (±25.6)	1.0ab (±0.2)	5.3 (±0.4)	33.6a (±1.2)	489.1 (±35.2)	100.0ab (±18.3)
769c (±83)	17.6ad (±0.2)	99.9a (±0.3)	22.6bc (±0.0)	82.7e (±0.3)	5.3c (±0.2)	4.2 (±0.2)	8.4c (±1.8)	240.7 (±14.1)	72.0a (±0.0)
***	**	***	***	***	***	ns	***	ns	***

[a] WO, fruits without endocarp; WE, fruits with endocarp. [b] Significance: *, **, and *** display the significance at 5, 1, and 0.5% by least significant difference; ns, not significant. Values with different roman letters (a−e) in the same column are significantly different according to the Duncan test ($p < 0.05$).

Volatile Composition of Apple Wines. The obtained apple wines were characterized by significantly differentiated volatile compositions (**Table 5**).

Carbonyl compounds are some of the most important aroma components of alcoholic beverages. The studied apple wines contained relatively low amounts of these compounds, generally below 100 mg/L. Samples produced from fruits without endocarps and seeds were distinguished by a >2 times higher level of carbonyl compounds compared to other samples. Using a juice extractor for fruit disintegration also increased the amount of carbonyl compounds in apple wines. It could be connected with a higher release of carbonyl compounds and their precursors mainly from peel to the must. The most abundant aldehydes in apples are *n*-hexanal and (*E*)-2-hexenal. These compounds are related to grass/tallow/leaf odor and originate from fatty acids by β-oxidative enzymes or lipoxygenases. These enzymatic reactions, caused by cell disruption, can occur during apple juice production (*27*).

Acetaldehyde and acetoin constituted <50% of these components, and their concentrations were similar in all samples (up to 35 and 2 mg/L, respectively). The flavor threshold of acetaldehyde in cider and apple wines has been established as approximately 30 mg/L. Its production during fermentation depends on the yeast species or strain used as well as temperature and oxygen concentration (*5*). In the presence of oxygen a higher amount of this compound is formed as a result of chemical and enzymatic oxidation of ethanol. When present in excess, acetaldehyde imparts an undesirable green, grassy, applelike aroma, which is usually masked by the addition of SO_2. Higher concentrations of acetaldehyde are also unfavorable because it can bind catechins and other phenolics (*28*).

Another analyzed carbonyl compound, acetoin, is synthesized during fermentation mainly by lactic acid bacteria and yeasts. Romano and Suzzi (*29*) reported that the amount of acetoin in wines ranged from 2 to 25 mg/L. In the studied apple wines, trace amounts (below 1.9 mg/L) of this compound were found, and most of the Idared wines did not contain it. Acetoin formation depends on the yeast strain used, and it is generally known that non-*Saccharomyces* yeasts produced more of it (*29*).

Methanol is formed as a result of pectin esterase (EC 3.1.1.11) action on methoxy groups of pectins in the crushed fruit. The formation of larger amounts of methanol depends mainly on the content and level of methylation of pectins (fruit variety), the activity of the native pectin methylesterase in the fruit, and, sometimes, processing, which causes tissue homogenization (addition of enzymatic preparations) or the yeast strain used for fermentation (*11*). We detected high concentrations of methanol in samples in which pectinolytic preparation (Pektopol) was used: 567 mg/L (Champion) and 383 mg/L (Idared). An increased level of methanol was also found in Champion wines obtained after fermentation of musts with pulp addition (343 mg/L). Champion apples generally include highly methylated pectins (*5*), which are present in higher amounts in pulp than in must. Additionally, pulp can contain more native pectin methylesterase that is released to the fermentation medium and carries out demethylation during fermentation process. Other apple wines were characterized by relatively low methanol contents, below 120 mg/L. The highest methanol concentration was detected in samples obtained after spontaneous fermentation (567 mg/L). Earlier studies (*30*) showed that wild strains of yeast present on the apple surface such as *Rhodotorula* and *Candida* spp. can be characterized by higher activity of pectin esterases than *S. cerevisiae*. Also, within one species the activity of pectinolytic enzymes can vary over a wide range (*31*).

Acetic acid is responsible for volatile acidity (VA) in wine, and it constituted >75% of all volatile acids. At normal levels in wine (<300 mg/L), acetic acid can be a desirable flavorant, adding to the complexity of taste and odor. However, if it is >300 mg/L, it progressively gives wine a sour taste and taints its fragrance (*28*). The studied apple wines were distinguished by relatively low concentrations of acetic acid, ranging from 48 to 124 mg/L. The

highest values were found in Idared wines as well as in samples for which pectinolytic preparation was used during must processing. The content of this compound in wines increases with sugar concentrations above about 20% (w/v), and its formation is also higher below pH 3.2 (*32*).

During ethanol fermentation many esters are formed, among which the most important for sensory characteristics of alcoholic beverages are acetate esters, mainly ethyl acetate (*33*). The total concentration of esters in analyzed apple wines ranged from 53 to 246 mg/L. Their higher level were detected in samples obtained using Steinberg yeasts. Increased amounts of esters were also found in wines fermented spontaneously (211 mg/L). All samples mentioned above were also characterized by relatively high conntents of ethyl acetate (24−49 mg/L). Esterase activity in different yeast strains is an important factor that influenced the concentration of individual esters in wines. It is also generally known that some non-*Saccharomyces* yeasts, such as *Pichia* or *Hanseniaspora*, are better producers of these compounds than *S. cerevisiae* (*34*). The highest levels of ethyl acetate were determined in apple wines obtained after cell immobilization (Champion, 30 mg/L; Idared, 34 mg/L) and the lowest from must pressed by juice extractor (Champion, 5−7 mg/L; Idared, 3−4 mg/L), respectively. The amount of ethyl acetate depends directly on acetic acid concentration, which can react nonenzymatically with ethanol, forming ethyl acetate (*28*).

Higher alcohols are quantitatively the largest group of flavor compounds in alcoholic beverages and are a secondary product of alcoholic fermentation. The content of fusel alcohols in analyzed apple wines depends mainly on apple variety. Ten percent more higher alcohols were found in Idared samples than in Champion wines. This tendency was already observed in previous studies (*5*) and was probably associated with the presence of individual amino acids in fruits.

The amounts of propanol, isobutanol, and amyl alcohols were strongly influenced by strain of yeast used. In the case of samples fermented using the Johannisberg-Riesling strain > 2 times more of these compounds was found than in wines obtained with the Steinberg yeast. Other factors such as application of pectinolytic preparations and cell immobilization had less of an impact on their concentration. According to Torrea et al. (*35*), a strain with a higher nitrogen demand produces a higher concentration of esters during fermentation and gives rise to a wine with a somewhat lesser content of higher alcohols. This tendency was strictly detected in the studied apple wine samples.

Other higher alcohol levels (butanol, pentanol, hexanol, 2-phenylethanol) were mainly dependent on prefermentative treatments. Increased amounts of these compounds were found in wines produced from must treated with pectinolytic preparations. Butanol, pentanol, and hexanol originated first of all from a raw material (*11*). Pectinolytic and celullolytic enzymes present in Pektopol preparations loosen the fruit pulp and cause the release of these compounds to the must, and throughout fermentation their concentration remains constant.

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Received for review July 21, 2009. Revised manuscript received October 23, 2009. Accepted October 27, 2009. The research was financed in part by the Polish Committee for Research in the years 2003−2006 as scientific project 094/P06/2003/28.