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Evaluation of Methanol Concentration in Hellenic Traditional Alcoholic Beverages after Grape Pomace Fermentation at Different Conditions

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Abstract: Methanol is a toxic and harmful substance to human health. The consumption of alcoholic beverages with high concentration of methanol can cause blindness, even death. The European level on methanol in alcoholic beverages from distilled grape pomace (solid residue consists seeds, stems and skins left after must extraction for wine production) is 1000 mg/100 mL of absolute alcohol or 400 mg/100 mL of 40% vol and for United States 280 mg/100 mL of 40% vol. The aim of this study was to investigate the methanol levels of distillates from different representative viticultural regions of Hellas via the fermentation conditions of the row material. The empirical analysis of this study 150 samples (75 red and 65 white samples from grape varieties (*Vitis vinifera* L.) was determined by gas chromatography Flame Ionization Detection (FID).The time of fermentation and the pectolytic enzyme treatments on the methanol concentration of the red and white grape pomaces also were investigated. Grape pomace samples were fermented for 10, 15, 20, 25, 30, 35 and 40 days at temperatures of 0, 20 and 30°C, respectively. The use of the enzyme treatments increased the methanol content of distillate from red grapes during in all the stages of fermentation.

Key words: Methanol concentration, alcoholic beverages, grape pomace, fermentation, FID

INTRODUCTION

Alcoholic beverages from fermented grape pomace are very popular in Mediterranean countries. Very popular are those produced from fermented grape pomaces, such as the Hellenic tsipouro and tsikoudia (Apostolopouloy *et al*., 2004; Gerogiannaki-Christopoulou *et al*., 2004, 2006, 2007; Kana *et al*., 1991; Soufleros *et al*., 2001, 2004), the Italian grappa (Da Porto, 1998, 2002; Odello, 1994; Versini, 1995), the Portuguese Bagaceiras (Silva and Malcata, 1998, 1999; Silva *et al*., 1996), Aguardiente (wine or marc spirit) (Rogerson and Freitas, 2001), the French grape marc or eau-de vie de marc (Soufleros and Bertrand, 1987), the Cypriote zivania (Kokkinofta *et al*., 2003), etc.

Grape pomace or grape marc is a solid residue consists seeds, stems and skins left after juice extraction for wine production. Traditional distilled alcoholic beverages from grape pomace made by natural fermentation or inoculation with commercial dry yeast (Da Porto, 1998, 2002; Da Porto and Freschet, 2003; Gerogiannaki-Christopouloy *et al*., 2004, 2006, 2007; Soufleros *et al*., 2001, 2004; Hang and Woodams, 2007) and distilled immediately or stored, depending on the availability equipment in a distillery (Gerogiannaki-Christopoulou *et al*., 2004, 2006, 2007; Da Porto, 1998, 2002; Da Porto and Freschet, 2003).

The regulation 1576/89 of EU., established the general production procedures for all these traditional alcoholic beverages and fixed common analytical composition limits. European Regulations can specify certain production procedures and in particular cases, more restrictive analytical parameters for particular geographical denominations (EEC, 1989; Versini, 1995). The European commercial grape pomace distillates have an alcohol limit on methanol 37.5% (v/v) and the legal limit on, ethanol is 1000 mg/100 mL of 100% alcohol (Regulation 1576/89 of EU) and 700 mg/100 mL of 100% (absolute alcohol)-(United States Code of Federal Regulations, 2003).

Methanol (CH_3OH, methyl alcohol) is a toxic chemical (The Merck Index, 2001) that has been shown to exert adverse effects on human health. Methanol is infinitely soluble in other alcohols, water and ethers. It is a highly toxic and inflammable material whose ingestion or inhalation can cause blindness or death (Gnekow and Ough, 1976) and is much more slowly oxidized in the body than ethanol. Even after two days; one-third of it still remains in the body. It damages the central nervous system, acting specifically upon the optic nerve. Bertrand (1974), found that as long methanol does not exceed 4% by volume there appears to no danger from ingesting it. In the human body methanol is accumulated in tissues with high water concentration, destroys the optical nerve and in high concentrations is lethal.

Methanol found in grape pomace alcoholic beverages, is formed from the demethoxylation of esterified methoxyl groups of the pectin polymer and is a product resulted from the hydrolysis of grape pectins by the enzyme, pectin methylesterase as follows:

$$\text{Pectin} + H_2O \xrightarrow{\text{Pectin methylesterase}} \text{Pectic} + \text{Methanol}$$

Pectin is composed of the methyl ester of alpha-1,4-linked, D-galacturonopyrasose units and is the general term for pectic substances which form the characteristic sugar-acid gels.

Methanol concentration have been reviewed by many researchers. Da Porto and Freschet (2003) in Italy reported that the methanol concentration of grape pomace distillates (grappa) ranged from 2685-1576 mg/3939 mg/ 100 mL absolute alcohol or 1074-1576 mg/100 mL of 40% alcohol, depending on the methods used in the pre-treatment of grape pomace prior to fermentation. Soufleros and Bertrand (1987) and Soufleros *et al*. (2003), demonstrated values of methanol in Hellenic grape pomace distillates ranging from 504-840 mg/100 mL of 100% alcohol. Silva *et al*. (1996) and Silva and Malcata (1998, 1999) for Portuguese grape pomace distillates presented mean value of methanol concentration equal to 755 mg/100 mL in 100% alcohol. Gerogiannaki-Christopoulou *et al.* (2006) presented methanol concentration in mono-varietal (from white and red grapes (*Vitis vinifera* L.) pomace distillates fractions from 165.7-955.8 mg/100 mL of 100% alcohol.

This study evaluate the effects of time and temperature of fermentation, grape variety and pectin enzyme on the methanol content of Hellenic grape pomace distillates. This knowledge should lead to a better standardization process and a more uniform quality of this alcoholic beverages.

MATERIALS AND METHODS

Grape pomace samples: Eleven grape varieties were collected from commercial wineries-seven from white grape varieties (*Vitis Vinifera* L.). Athiri and Asyrtico from a commercial winery in the island of Santorini-volcanic area in Central Cyclades of Aegean Archipelagos, Robolla, a grape variety from the island of Kefalonia-Ionian Sea, Savatiano from commercial winery of Mesogaia-Attica of continental Central Hellas, Soultanina from Korinthos in Peloponisso(the island of the ancient King Pelopa). Five from red grape varieties (*Vitis Vinifera* L.). Xinomavro from the area of Naoussa Macedonia in North Hellas, Kalambaki, an ancient grape variety from Limnos-North Aegean Archipelagos (the island of Argonauts and Amazons), Mantilaria from the Myconos-Central Cyclades-Aegean Archipelagos), Agiorgitico from the Nemea-Central Peloponisos and Fokiano from Central area of Attiki. All samples were collected under the recommendations of FAO/WHO (1986). When the grapes pomace (skins, stems and seeds) were received from the commercial wineries after the must extraction, each one of the varieties was divided into separate lots. One lot of each variety (without pectinase addition) was placed in a refrigerator, left to equilibrate to that temperature 0°C and two lots from each variety placed at 20°C (room temperature) and 30°C in climatizated store rooms.

Samples of each grape variety and for each selected temperature transferred in barrels where they remained for 10, 15, 20, 25, 30, 35 and 40 days with pectinase addition (1 g/20 kg of grape pomace).

After the predetermined time the samples were transferred in a distillation apparatus. Distillates collected and were stored at 5°C until gas chromatographic analysis.

Pectic enzymes: The grape pomace from selected grape varieties (*Vitis vinifera* L.) used for the pectinase studies: The enzyme preparations were with the addition of pectinase (1 g/20 kg of grape pomace). The pectinase utilized (ULTRAZYM 100 G, NOVO Nordisk, Bagavaerd, Denmark) treatments were made according to Silva and Malcata (1998).

Distillation: The distillation is realized with the traditional copper alembics of 130 L, which is a simplified type of the Charentais alambic. The fermented raw material transferred to the vessel up to the 3/4 of its capacity in order to be distilled. Before the beginning of heating, the traditional copper distillation apparatus (alambic) is hermetically closed in order to prevent any vapor leakage. When the temperature reaches 80-90°C, the liquid spirit starts to run from the funnel and collected in glass bottles.

The distillation of the grape pomace is being done without the use of any aromatic plants or seeds. In this case, the aroma of the spirit is deriving exclusively from the raw material, the fermentation process.

Reagents: Standard (chromatographic grade) of methanol were purchased from Sigma (St. Louis, USA) and pentanol-3 from Sigma (St. Louis, USA) was used as an internal standard.

Gas Chromatographic (GC) analysis: Analysis of methanol was by direct injection of distilled samples. A 5 mL aliquot of each sample was mixed with 50 mL of internal standard solution (50 g of pentanol-3 per liter of absolute ethanol). A gas chromatograph (Hewlett Packard 5890 series II) equipped with FID has been used for the ana1ysis of methanol. Samples of 0.2 µL were injected into the gas chromatograph. The injector of the gas chromatograph was maintained at 200°C and operated under split mode.

Elution was achieved in a 50×0.25 mm i.d.×0.2 µm capillary CBWAX 57 (Chrompack, Middelburg, The Netherlands) cross-linked polyethylene glycol column. The oven temperature program was as follows: 40°C for 5 min, a linear ramp from 40 to 200°C at 30°C/min and hold at 200°C for 20 min. The FID was kept at 200°C. Helium was used as the carrier gas at a split ratio of 1:60.

The flow rate of the carrier gas was 2 mL min^{-1}.

The fermentation experiments were conducted in triplicate 1 L jars with U-tube airlocks at room temperature (19-20°C).

The reproducibility was estimated by calculating the Coefficient of Variation (CV) of total peak areas of the four chromatograms.

Statistical analysis of our experiments were carried out in quadruplicate and the results are expressed as mean±standard deviation.

RESULTS AND DISCUSSION

In this study some novel results concerning the concentration of methanol, a major volatile with questions about their toxicity, in Hellenic traditional grape pomace alcoholic beverages, consumed by a lot of people in Hellas and other Mediterranean countries, have been presented. The farmers and the industry they leave the grape pomace for fermentation in plastic or wooden containers at different time and storage temperature and the knowledge about the methanol concentration in the produced grape pomace distillates is very few. White and red grape varieties (*Vitis vinifera* L.), studied after fermentation at different temperature and time of fermentation of the row material and without and with the addition of pectinase. The results are summarised in Table 1, 2 and Fig. 1, 2.

Table 1: Methanol concentration (mg/100 mL) in red grape varieties (*Vitis vinifera* L.), after storage of grape pomace at different time and temperature, with and without the use of pectinase

		Methanol					
Variety	Time (days)	(mg/100 mL) 0°C	(mg/100 mL) 20°C	(mg/100 mL) 30°C	(mg/100 mL) 0°C	(mg/100 mL) 20°C	(mg/100 mL) 30°C
Xinomavro	10	66.0	92.7	139.0	102.0	148.0	196.2
	15	92.8	107.0	172.0	132.0	177.0	215.8
	20	108.0	129.0	202.0	172.3	196.0	257.0
	25	127.0	164.0	236.0	198.0	226.0	280.0
	30	157.0	195.0	278.9	228.0	262.0	299.3
	35	177.0	217.0	306.9	250.0	296.0	335.0
	40	204.0	246.0	349.0	287.0	336.0	378.0
Kalambaki	10	93.1	109.8	149.6	129.6	196.5	288.9
	15	99.8	118.0	174.0	148.0	219.0	310.2
	20	108.5	129.5	198.9	159.8	235.8	321.9
	25	116.0	144.0	234.4	178.0	252.1	349.8
	30	127.5	149.8	258.9	193.2	281.2	378.4
	35	139.7	168.1	274.0	217.0	289.9	399.1
	40	146.6	182.3	299.3	236.3	310.1	415.7
Mantilaria	10	60.0	87.0	140.1	110.0	168.0	323.0
	15	78.0	118.0	170.5	135.0	197.0	354.7
	20	98.1	145.0	203.0	164.0	240.0	372.1
	25	116.0	166.0	225.2	194.0	267.0	395.4
	30	134.0	184.0	263.7	224.0	295.0	411.3
	35	158.0	199.0	289.9	257.0	330.0	439.6
	40	180.0	226.0	325.2	285.0	363.0	459.3
Agiorgitiko	10	65.0	96.9	141.0	113.0	210.8	329.6
	15	82.0	121.0	189.9	159.9	239.4	356.9
	20	105.0	145.0	226.0	184.0	265.6	385.7
	25	136.0	181.1	277.5	215.0	300.0	420.0
	30	167.0	211.4	321.2	252.0	328.0	453.0
	35	190.0	233.8	346.0	272.0	365.0	496.5
	40	226.0	266.0	385.0	300.0	400.0	543.0
Fokiano	10	70.0	122.0	185.0	96.4	143.2	238.3
	15	95.1	153.1	228.0	125.0	167.1	278.1
	20	127.0	182.1	272.0	163.0	212.0	318.8
	25	150.0	207.1	302.1	184.0	235.4	365.2
	30	175.0	238.1	327.0	217.0	270.0	389.1
	35	210.0	271.0	357.0	250.0	300.0	422.3
	40	222.0	318.0	399.0	297.0	344.1	459.8

*RSD from (0.9-7.6)

Table 2: Methanol concentration (mg LG^{1}) in white grape varieties (*Vitis vinifera* L.), after storage of grape pomace at different time and temperature, with and without the use of pectinase

Variety	Time (days)	Methanol (mg/100 mL) 0°C	(mg/100 mL) 20°C	(mg/100 mL) 30°C	(mg/100 mL) 0°C	(mg/100 mL) 20°C	(mg/100 mL) 30°C
Athiri	10	40.0	78.3	117.0	60.0	89.6	159.9
	15	62.0	99.6	163.0	85.0	125.8	189.1
	22	93.0	155.0	212.0	132.0	185.0	253.0
	26	110.0	183.0	265.0	157.0	223.0	288.0
	30	129.0	208.0	301.0	180.0	250.0	327.0
	35	150.0	250.0	349.0	208.0	289.2	373.0
	40	176.0	293.0	388.0	250.0	333.0	416.0
Savatiano	10	56.0	90.0	162.0	75.0	105.0	276.0
	15	68.0	109.4	197.0	89.5	143.0	305.0
	20	87.0	140.0	236.0	112.0	180.0	334.0
	25	104.0	166.0	263.0	133.0	218.0	357.0
	30	124.0	184.5	299.0	151.0	249.0	375.0
	36	137.0	218.9	320.0	182.0	278.0	402.0
	40	151.0	241.1	342.5	201.0	305.0	419.6
Soultanina	10	32.0	69.0	168.9	46.7	98.4	211.0
	15	51.0	94.0	189.3	74.0	1154.0	234.0
	20	68.0	121.0	211.0	94.0	143.2	260.0
	24	86.0	145.0	238.0	114.0	170.0	280.0
	30	108.1	160.0	263.4	138.0	198.0	300.0
	35	120.4	183.0	279.9	155.0	223.0	324.0
	40	132.0	204.0	301.2	172.0	243.0	340.0
Asyrtiko	10	39.0	68.0	110.0	83.5	95.6	158.7
	15	56.0	77.0	135.9	94.0	111.0	185.0
	20	65.0	93.3	158.4	105.0	132.0	215.0
	25	77.0	103.0	185.5	118.0	150.0	240.0
	30	93.1	115.8	208.0	127.0	176.0	261.0
	35	104.3	127.0	230.0	140.0	198.0	292.0
	40	120.0	145.0	255.0	158.0	216.0	327.0
Robolla	10	20.0	62.1	159.8	65.0	92.3	199.6
	15	44.0	75.0	180.0	86.0	120.3	221.0
	20	53.0	90.0	208.0	103.0	150.0	259.9
	25	70.0	112.3	236.9	130.0	178.5	287.1
	30	87.0	129.0	265.0	155.0	200.0	306.0
	35	107.9	144.0	298.5	177.0	218.0	330.0
	40	130.0	162.3	330.0	199.0	250.0	356.0

RSD (1.1-9.5)

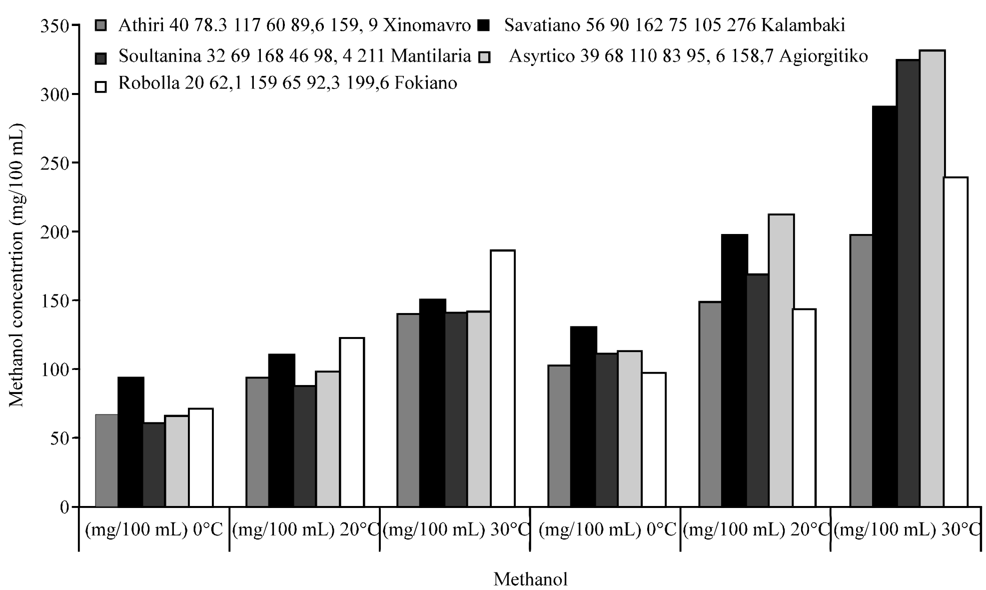

Fig. 1: Mean values of methanol concentration in white and red grape varieties (*Vitis vinifera* L.) At different temperature and time of fermentation, with and without pectinase

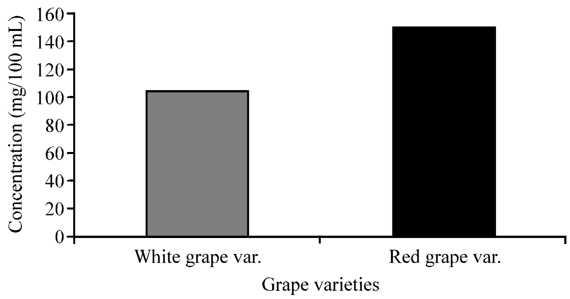

Fig. 2: Varietal methanol concentration in red and white grape varieties (*Vitis vinifera* L.)

As it can be seen from Table 1 and 2 in all samples measured, grape pomace fermented with the addition of pectinase had higher methanol content compared to samples fermented without any addition. These differences in all grape pomace distillates were from 20 to 419.6 mg/100 mL of 100% alcohol for white grape varieties and from 65 to 459.3 mg/100 mL of 100% alcohol for red grape varieties.

This means that the amount of methanol existing in the fermented grape marc distillates (*Vitis vinifera* L.) is not stable and it is affected by the time and temperature of grape marc fermentation and the amount of pectinase present in the fermented mixture(Kana *et al*., 1999). The pectinase treated samples at 0°C increase of mean values for methanol production compared to the non treated samples from 37.4-60 mg/100 mL in 100% alcohol (White grape varieties and from 70.8-110.2 mg/100 mL in 100% alcohol for red grape varieties. At 20°C the corresponding increase was from 73.5-96.2 mg/100 mL in 100% alcohol for white grape varieties and from 101.7-173.3 mg/100 mL (100% alcohol)for red grape varieties. At 30°C the increase was from 143.5-201.0 mg/100 mL (W) and 110.2-275.2 mg/100 mL(R) absolute alcohol.

Table 1 and 2 also shows the amounts of methanol produced from the grape varieties. From these data it can be seen that the use of pectinase increases the methanol content of grape pomace distillates regardless of variety. We can summarize that red grape pomace spirit have higher methanol than white grape pomace distillates and this agree with the research results of Kana *et al*. (1991) and Silva and Malcata (1998). It can be seen that for the temperature 20 and 30°C exists a gradual increase of methanol production. It has been found that several species of microbes and yeasts transfer pectinesterase in the fermenting mixture and therefore can substantially increase its methanol content. Accordingly, this increase in methanol concentration was very probably due to the increase of pectinesterase production from microbial population. During the fermentation at 30°C there was a strong initial production of methanol. This was very probably due a) to the proximity of fermentation temperature (30°C) to the optimum temperature of action for pectinase that induced a high initial production of methanol and b) due to the initial hydrolyses of methoxy group substrate enzyme their amount was considerably decreased and that lead to a continuously higher production of methanol during the subsequent time period.

Figure 1 shows the mean values of methanol formed by all grape pomace varieties following storage at the three selected temperatures. From these graphs there appears to be little difference between grape pomace stored at 0°C, at 20°C and big different in grape pomace stored at 30°C. In all grape pomace varieties stored at 30°C, however, there was high increase for all grape pomace varieties were kept at 30°C for 40 days. The difference between the methanol content of the red variety Agiorgitiko and the white variety Athiri was extremely interesting.

According to the European legislation (EEC 1576/89), the distillate must have a methanol concentration lower than 1000 mg/100 mL Absolute Alcohol. The levels for our samples were found to be much lower than the European limit (Table 1, 2).

Soufleros and Bertrand (1987) demonstrated values of methanol in Greek grape pomace distillate ranging from 50.4 to 840 mg/mL Absolute Alcohol. Silva and Malcata (1996, 1998, 1999) presented for bagaceiras a much more higher concentration of methanol with a mean value equal to 755 mg/100 mL Absolute Alcohol or higher than the European limit, ranging from 1021 to 1031 mg/100 mL Absolute Alcohol. These values are dependent mainly on the applied technique of the grape treatment and the distillation and secondly from the grape variety. In other reports, the concentrations of methanol in grape pomace distillates range between 5300 and 1590 mg/mL Absolute Alcohol (Cordonnier, 1971), from 39 to 2860 mg/100 mL Absolute Alcohol (Amerine *et al*., 1972), from 205 to 1157 mg/10 mL Absolute Alcohol (Bertrand, 1974).

CONCLUSION

This study shows that the manipulation of the raw material, the temperature of the fermentation and the use of pectolytic enzymes in red and white grape varieties are convenient to monitor the methanol levels during grape pomace storage and are useful for the distillers to produce high quality alcoholic beverages.

As a general conclusion in could be said that enzymatic methanol production from grapes is almost never quantitative and its production depends on several factors as: fermentation temperature, intentional addition of pectic enzymes during must production for improvement of must yield and also unintentional introduction of pectic enzymes by microbes and yeasts attacking the fermenting grape pomace.

If someone is interested in the production of a alcoholic beverage from fermented grape pomace these findings permit the application of counter measures in order to avoid the excessive production of unwanted toxic methanol.

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