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Determination of Pectinesterase Activity in Grape Varieties (*Vitis vinifera* L.) During Vinification

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Abstract: Twenty red and white grape varieties (*Vitis vinifera* L.) were studied for pectin methyesterase activity during alcoholic fermentation of grape must. Methanol production in final wines from those grape samples was quantified. For the concentration of pectin methyl esterase activity a titrimetric method was used. Methanol concentration in final monovarietal wines quantified by Gas Chromatography Flame Ionization Detector (GC-FID). Methanol levels ranged from 30.5-121.4 mg L^{-1} in white wines and 61-207 mg L^{-1} in red wines. The highest level was found in Agiorgitiko from Nemea. Methanol content of red wines increased with fermentation time because pectinesterase activity grows with the microorganism's activity of must. All the results show that methanol levels of monovarietal red and white wines are under the maximum acceptable limits of International Office of Vine and wine (O.I.V.) and do not represent a risk to consumer health.

Key words: Grapes, pectins, vinification, pectin methyl esterase, *Vitis vinifera* (L), methanol

INTRODUCTION

Pectin, a major biopolymer of grapes, is composed primarily of essentially linear polymers of D-galactopyranosyl uronic acid units joined in α-D- (1→4) glycosidic linkages. This regular structure is interrupted with L-rhamnopyranosyl units and with side chains containing other neutral sugars. The intervening regions of the chain, which are largely composed of galacturonic acid, are etserified with methanol up to 80% (Mangos and Haas, 1997). The degree of methoxylation varies with age and location within the plant tissue (biosynthetic conditions) and with the method of extraction. A good knowledge of the chemical structure of pectins is of major importance from the technological point of view and also aids the understanding of the role of pectic substances in the cell walls (Rexova and Markovie, 1976).

Pectinesterase catalyze the hydrolysis of the galactopyrasonyl uronic acid methyl esters of pectin, converting methoxy pectins and methanol.

Plant pectinesterase participate in the conversion of protopectin to soluble pectin and pectete and are involved in plant maturation processes aw well as in mechanisms that protect the plant from infection (Rexova and Markovie, 1976) plant pectinesterase do not hydrolyze methyl esters in a more or less random fashion, as do microbial ones, but act processively on the galacturonan chain reating blocks of free carboxyl groups. These blocks are extremely prone to complex formation and participation with calcium ions (Pilnic and Voragen, 1991). Fractionation of pectins by ion exchange chromatography is mainly based on the degree of esterification of the pectin. The distributions of free and methoxylated carboxylated carboxyl groups influence the elution behavior of pectic molecules during high-performance ion-exchange chromatography (Richard and Noat, 1986).

The activity of pectinesterase on a pectin fraction in relation to the degree of methoxylation after partial alkaline saponofication may give valuable information on the intermolecular distribution of free and esterified carboxyl groups in pectin.

Many methods for the determination of the enzymatic activity of pectin methyl esterase were studied by many researchers.

Method described by titrimetry (Rhodes, 1980; Pressey and Woods, 1992; Tijskens *et al*., 1999) by spectometry (Hagerman and Austin, 1986) and by manometry have been reported.

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The most frequently used method of determining the activity of pectinesterase is the real-time titration of the liberated carboxyl groups with dilute alkali (Versteeg *et al*., 1980) while the pH is kept constant (pH-stat). Demethoxylation of pectin by plant pectinesterase exhibits optimum activity at pH 7.5.Using a titrimetric assay at the optimum pH of pectinesterase corrections must be made for partial dissociation of the liberated carboxyl groups, whose pK values vary with the concentration of the pectin, the degree of esterification and the mode of desterification.

This project designed for the determination of pectin methyesterase in 10 Greek grape varieties in three different time of alcoholic fermentation of must. Methanol is a product resulted from the hydrolysis of fruit pectins (Brown and Ough, 1981; Gigliotti and Bucelli, 1993; Revilla and Gonzalez-Sanjosè, 1998) and its concentration in final wines was calculated.

MATERIALS AND METHODS

Samples: Ten different white grape varieties Savatiano, Muscat of Alexamdria, Robolla, Soultanina, Athiri, Malagouzia, Vilana, Batiki, Ìoschofilero and ten red grape varieties Negosca, Kalambaki, Agiorgitiko, Mantilaria, Xinomavro, Muscat of Hamburg, Mavrodafni, Kotsifali, Liatiko, were collected from private vineyards when a good degree of ripeness (15.2-17.8 °Brix and 3.2-4.9 g L^{-1} of tartaric acid for white grape varieties and 15.2-18.8 °Brix and 3.6-4.2 g L^{-1} of tartaric acid for red grape varieties). The grapes were harvested at the 1st h in the morning. The grapes were pressed without being destemmed. A traditional vertical hydraulic press was used, applying pressure lower than 1kg m^{-2} and quickly after must extraction transported in different wooden barrels for spontaneous fermentation. The concentration of pectin methyl esterase was studied at 4 different times of spontaneous fermentation of extracted must (at 36, 48 and 60 h). Also studied methanol concentration of the samples by GC-FID.

Preparation of pectin methyl esterase extraction from grapes must (*vitis vinifera* L.) : Pectin methyl esterase was extracted by homogenizing 50 g of grape must in the presence of 100 mL ice cold water containing 0.5% suspended Polyclar AT in an Kenwood Blender for 5 min and stirred for 1h at 4°C. After centrifugation at 10.000×g for 10 min, the sediment was re-suspended in 50 mL ice cold 1M NaCl, stirred for 1 h and centrifuged at 10,000×g for 10 min.The pH of the supernatant was adjusted to 7.0 with NaOH and used as such for enzyme analysis. Extraction were conducted in triplicate.

Pectin methylesteras activity assay: The titrimetric method was used for Pectin methyl esterase activity assay. Twenty five mililiter pectin solution (Citrus pectin, DE 75%. Sigma Chem., USA in 0.2 2M K_2HPO_4) was incubated in a 150 mL water-heated vessel at 30°C. The reaction started by adding 1 mL of enzyme solution and was allowed to proceed for 15min. The amount of 0.01 N NaOH delivered with the 665 Dosimat automatic titrator (Metrohm, Switzerland) was recorded (Tijskens *et al*., 1999). The temperature during the assay was kept at 30°C (Hagerman and Austin, 1986). The results were expressed as nkatal mL^{-1}: the carboxylic groups in nmoles formed per second per mL of grape must.

Chromatographic analysis: Methanol content was determined by Gas Chromatography (GC) direct injection method on distilled wine samples by adding an internal standard according to reference method of OIV (1990).

Distillation: One hundrad mililiter of each wine sample was distilled by a simple distillation equipment as explained by OIV; 100 mL of each distillate was collected in a volumetric flask at low temperature to prevent losses. Each sample was distilled in triplicate. A standard solution of methanol (Merck, Switzerland) was distilled in the same way to determine the recovery of methanol, which was 99.2%. Distilled sample of 100 mL was added with 10 mg of pentanol-3 (Merck) as internal standard (injected one microlitre pentanol-3, Merck (Darmstadt,Germany) as internal standard. It is well known that the enzymatic system with high pectinesterase activity degrades the pectic compounds with methanol release (Cabaroglu, 2004; Revilla and Gonzales, 1998).

RESULTS AND DISCUSSION

Table 1 and 2 shows the physicochemical parameters of grapes which used for this experiment. pH was from 3.6-4.9 and °Brix from 15.2-19.2 for white grape varieties and 14.8-18.8 and 3.6-4.6 for red grape varieties, respectively. The activity of the crude pectin methyl esterase in white and red grape (Fig. 1) shows that white grape contain lower levels of the enzyme from red grape varieties at different times of must fermentation. The concentration of pectin methyl esterase in white grape varieties (Fig. 2) was 15-64 units mL^{-1} in three different time of fermentation (after 36, 48 and 60 h. As shown in histograms 1 and 2 methanol concentration of produced wines were from 85-154 mg L^{-1} after 36 h of fermentation, from 108-172 mg L^{-1} after 48 h of fermentation and from 125-179 mg L^{-1} after 60 h of fermentation in must from white grape varieties.

In red grape varieties methyl pectinesterase concentration (Table1) was from 32-66 mg L^{-1} after 36 h of fermentation, from 44-78 mg L^{-1} after 48 h and from 57-89 mg L^{-1} after 60 h. As shown in histograms 3 and 4 methanol concentration of produced wines were from 85-154 mg L^{-1} after 36 h of fermentation, from 108-172 mg L^{-1} after 48 h of fermentation and from 125-179 mg L^{-1} after 60 h of fermentation in must from white grape varieties. The European legal limit on methanol in red wines is 300 mg L^{-1} (Fig. 3).

The results of the present investigation clearly indicate that the concentration of methyl pectinesterase increase during fermentation time of must match more in red grape varieties than in white grape varieties and also the methanol concentration is more in red than in white grape varieties. The methanol content of red wines from different countries were reported as follow (Nycanen and Suomalainen, 1983) : In French wines, from 100-200 mg L^{-1} with an saverage of 163 mg L^{-1}; In Italian wines, from

Table 1: Physicochemical parameters of White grape varieties

White grape varieties	Geographical area	°Brix	pH
Savatiano	(Mesogaia-Attiki	16.4	4.6
Muscat of Alexandri	LimnosI, Lesvos	19.2	4.9
Robolla	Kefalonia ionian island	17.4	3.8
Soultanina	Korinthos, Peloponissos	19.2	4.4
Athiri	Santorini,Cyclades	16.8	3.2
Malagouzia	Nafpactos, Aitoloakarnania	17.8	4.1
Vilana	Chania cretaa	15.2	3.6
Batiki	Timavos thessalia	16.2	3.9
Ìoschofilero	Mantinia, Peloponisos	17.4	4.7
Aidani	Santorini cyclades	16.2	4.8

Table 2: Physicochemical parameters of Red grape varieties

Red grape varieties	Geographical area	°Brix	pH
Negosca	Florina Macedonia	15.4	4.1
Kalambaki	Limnos Lesvos	15.2	3.6
Agiorgitiko	Nemea Peloponnisos	15.8	4.1
Mantilaria	Naxos Cyclades	16.2	3.9
Xinomavro	Xino Nero Macedonia	14.8	4.2
Muscat of hamburg	Trikala Thessalia	18.8	3.6
Mavrodafni	Patra Thessalia	15.8	3.7
Kotfifali	Hraklio Creta	15.2	4.4
Liatiko	Hraklio Creta	16.2	4.6
Mavro mesenikola	Karditsa Thessalia	16.9	4.1

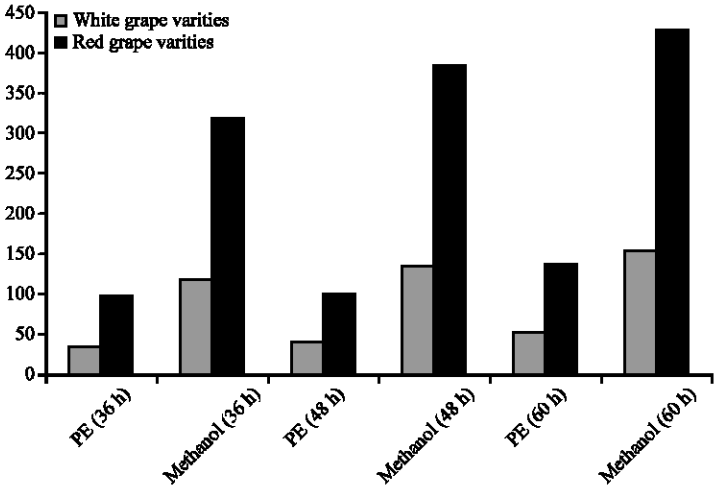

Fig. 1: Compared histograms for pectinesterase activity and methanol concentration in white and red grape varieties (*Vitis vinifera* L.)

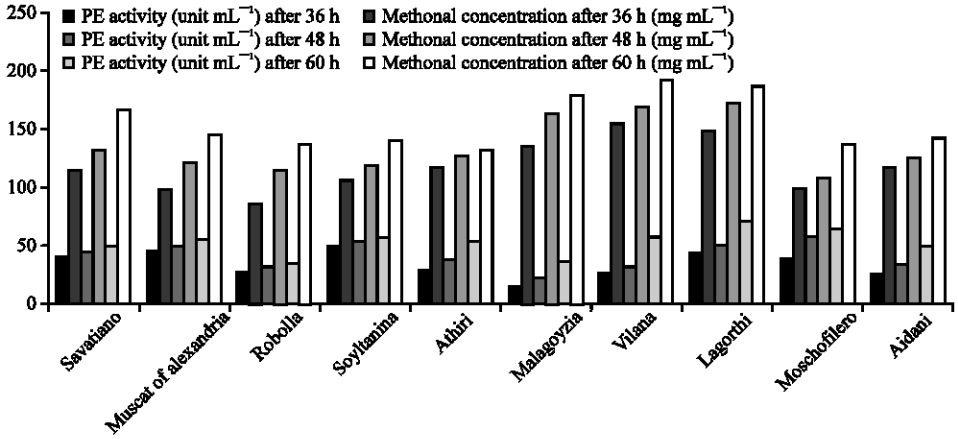

Fig. 2: Histogram for methanol concentration and pectinesterase activity in white grape varieties (*Vitis vinifera* L.)

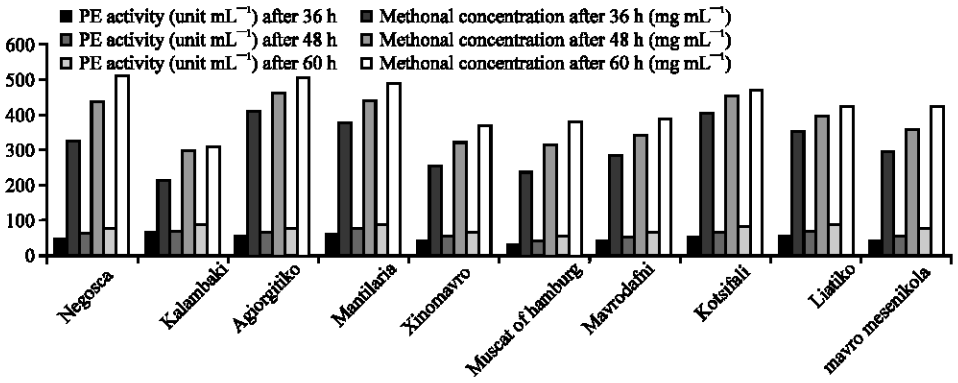

Fig. 3: Histogram for methanol concentration and pectinesterase activity in red grape varieties (*Vitis vinifera* L.)

0-635 mg L^{-1} with an average of 103 mg L^{-1}; in Spanish wines, from 39-624 mg L^{-1} with an average value of 145 mg L^{-1}; in Portuguese wines from 165-264 mg L^{-1} with an average of 195 mg L^{-1}. According to OIV, maximum acceptable level for methanol in red wines is 300 mg L^{-1}. Previous studies from other researchers showed that methanol levels of wines were generally under the maximum limits (150 mg L^{-1} for white and rosé, 300 mg L^{-1} for red. However, levels above the given limits were reported by Cordonnier, (1987) Gnekow and Ough (1976) and Nycanen and Suomlainen (1983).

The results of the present investigation clearly indicate that the activity of methyl pectinesterase during the alcoholic fermentation of grape must in white and red grape varieties is important for the methanol formation. The significance of this research is that the wine Industry will benefit economically from the conversion of grape must into a qualified healthy wine and also will control methanol release during alcoholic fermentation.

CONCLUSION

This study of varietals wines from white and red grapes shows that methanol concentration depends from the activity of pectin methylesterase during the alcoholic fermentation. This activity increase with the time (36, 48 and 69 h) of fermentation. The concentration of this enzyme shows that methanol increased more in red than white grape varieties. It is important for wineries, to control the use of pectolytic enzymes to avoid any risk of methanol formation because it is well known that the high pectinesterase activity degrades the pectic compounds with methanol release.

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