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Detection of pectinmethylesterase activity in presence of methanol during grape pomace storage

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Abstract

Methanol is an unwanted component in the production of spirit beverages. Its presence in grape pomace stored until distillation greatly affects the composition of the final product, which requires redistillation, modifying the aromatic characteristics of the distillate. Consequently, reduction of methanol, by controlling its formation during pomace storage, can increase the quality of grappa. The aim of this work was to monitor pectinmethylesterase activity during grape pomace storage, in order to identify its presence related to methanol release. The enzyme activity was detected during various storage times by spectrophotometric and electrophoretic methods. Results show that yeasts do not contribute to PME production. Moreover, by using paranitrophenyl acetate, a specific substrate for esterase, also as PME substrate, we demonstrate the presence of several enzymes hydrolysing ester bonds.

Keywords: Methanol; Grape pomace; Grappa; Pectinmethylesterase; Esterase; Electrophoresis

1. Introduction

Methanol is a colourless volatile compound with a mild alcohol odour. It is toxic to humans and is readily adsorbed by ingestion and inhalation and, more slowly, through the skin. In the body, methanol is metabolised in the liver, and converted first to formaldehyde and then to formate. High levels of formate after excessive methanol intake can cause severe toxicity and even death (Lamiable, Hoizey, Marty, & Vistelle, 2004). Low doses of methanol may be ingested in fruit, vegetables, fermented beverages, and foods sweetened with aspartame, which breaks down to methanol in the gastrointestinal tract (Newsome, 1993). For the above reasons, low levels are now fixed for methanol concentration in foods. EU regulations (ECC 1576/89) set minimum levels of methanol in distilled fruit spirits, where it is naturally present as a consequence of enzymatic degradation of pectins (Andraous, Claus, Lindemann, & Berglund, 2004). Pectins occur as structural polysaccharides in the middle lamella and primary cell walls of higher plants (Lee, Smith, & Nelson, 1979). Pectic substances are polymeric molecules with a variable quantity of rhamnogalacturonan backbone; this structure comprises homogalacturonan regions in which galacturonic acid is esterified by methyl groups (Pérez, Mazeau, & Hervé du Penhoat, 2000). Pectinmethylesterase (PME) de-esterifies pectins to low-methoxyl pectins, resulting in the formation of methanol (Micheli, 2001).

Grape pomace, or marc, is the solid residue obtained from must or wine at the end of alcoholic fermentation. It includes skins and seeds and, to a varying extent, stalks. This by-product is extensively used in producing spirit beverages, called ''bagaceiras'' in Portugal, ''marc'' in France and ''grappa'' in Italy (De Pina & Hogg, 1999).

The storage of pomace before distillation is a key step as regards the quality of Italian grappa, because the biochemical transformations occurring during storage greatly affect the composition of the final product (Da Porto, 2002). It is widely accepted that distillation techniques can never improve the characteristics of a spirit deriving from low-

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quality raw material. In this respect, methanol is an unwanted component, its contents in the raw material being a critical factor determining methanol concentration in the end-product. The possibility of controlling methanol formation during pomace storage can enhance the quality of grappa without the need for redistillation, which may modify the aromatic characteristics of the distillate. Moreover, a low level of methanol promotes marketing of grappa in countries with more stringent limits to alcohol contents.

In this paper, we studied the activity of PME on stored grape pomace in order to examine the contribution to PME activity due to both plant raw material and microorganisms. For better understanding of methanol release during grape pomace storage, we related PME activity with methanol concentration.

2. Materials and methods

2.1. Pomace sampling and storage

Pomace from grape berries (*Vitis vinifera* cv. Prosecco), vintage of 2002, were studied. No moulds were present. Grape berries were harvested and washed with distilled water to eliminate indigenous yeast (sample T_0). The washing water was filtered through a 0.22-μm membrane. The membrane was then placed on YPG agar in a Petri dish and incubated at 28 °C for 24 h, and the total number of colonies formed was recorded. Pilot-scale experiments were performed in 400-kg ensilage boxes in essentially anaerobic conditions, fermentation being effected at the prevailing ambient temperature between 20 and 40 °C. Fermented pomace from sample berries was drawn from the ensilage boxes 5 (T_1), 20 (T_2), and 50 (T_3) days after grape crushing. Progressive samples were frozen at −80 °C for subsequent extraction and chemical analysis.

2.2. Determination of methanol

Methanol concentration was determined by quantitative HS-SPME-GC, partially following Huisman, Oosterveld, and Schols (2004). Fiber: Supelco 65 μm Carbowax Divinylbenzene. A Thermo Finningan Trace GC Series gas chromatograph equipped with a flame ionization detector and an automatic injection system was used. Column: Chromopack capillary column PoraPLOT Q 27.5 m, 0.32 mm, 10 μm, was set at 60 °C for 3 min, and programmed to 140 °C at a rate of 8 °C/min. Helium was used as carrier gas at a flow rate of 0.9 ml/min. The injector was set at 200 °C and the detector at 300 °C. Samples (100 g of grape pomace) were distilled prior to analysis for methanol contents. Aliquots of the distillates were heated for 15 min at 50 °C in a water-bath, and extraction fiber time was 10 min. Methanol concentrations were determined from a standard curve and expressed as mg/100 g of grape pomace fresh weight.

2.3. Grape pomace saponification

The total amount of methanol present in grape pomace pectin was determined after saponification (5 N NaOH, 25 °C, overnight). Quantification was performed by HS-SPME-GC, as reported above.

2.4. Total protein

Pomace, without seeds, was reduced to powder with liquid nitrogen. Protein extraction was carried out according to Barnavon et al. (2001) as follows: 40 g of pomace were homogenised for 3 h in 80 ml of 0.5 M Tris–HCl pH 8.5 buffer, 1% (w/v) Triton X-100, 20% (v/v) glycerol, 5% (w/v) polyvinylpirrolydone, 2 M NaCl and 14 μl β-mercaptoethanol. The suspension was centrifuged at 1100*g* at 4 °C for 30 min and the first supernatant was removed. The same extraction process was re-applied and the second supernatant was combined with the first. The sample was concentrated by ultrafiltration through a 1-kDa membrane (Amicon). The protein contents of samples were determined according to Bradford (1976). Nitrogen ($N \times 6.5$) of grape pomace was measured by the micro-Kjeldahl method, according to AOAC and AACC procedures (1995).

2.5. PME activity

Protein extract PME activity was spectrophotometrically determined according to Hagerman and Austin (1986), with some modifications, as follows: the substrate was 2 ml of 0.5 (w/v) pectin (apple pectin, Sigma), 0.80 ml water and 0.15 ml of 0.04% (w/v) bromothymol blue in phosphate-buffered saline (0.01 M Na-phosphate, 0.15 M NaCl PBS), pH 7.5. To achieve a constant starting pH for the reaction, all solutions were adjusted to exactly pH 7.5 with NaOH. PME activity was monitored by adding 50 μl of protein extract solution and read at 620 nm in a Jasco 7800 UV/Vis spectrophotometer at room temperature for 40 min. Blanks were made without blue bromothymol.

Specific PME activity was defined as a decrease in absorbance A_{620} min/g grape pomace fresh weight, analysed in three replicates.

2.6. Esterase activity

Esterase activity was spectrophotometrically determined as described by Lomolino, Rizzi, Spettoli, Curioni, and Lante (2003). Fifty microliters of each sample was added to a substrate solution containing 1.9 ml phosphate saline buffer 10 mM (PBS), pH 7.5 and 50 μl of 20 mg *p*-nitrophenyl acetate/ml of 10% (w/v) Triton X-100 in PBS. Activity was spectrophotometrically monitored at 400 nm for 60 min. Specific esterase activity is defined as an increase of absorbance A_{400} min/g grape pomace fresh weight, analysed in three replicates.

2.7. Electrophoretic analyses

All electrophoretic procedures were carried out at room temperature in a mini-Protean 2 apparatus (Bio-Rad Laboratories, Milan, Italy). Electrophoresis of samples was performed according to Davis (1964) on a 10% polyacrylamide gel containing 0.2% (w/v) pectin. 50-μg aliquots of protein were loaded and N pectin-PAGE was carried out at 30 mA until the tracking dye bromophenol blue ran off the gel. Gels were stained by Coomassie Brilliant Blue (CBB) or used for detection of PME and esterase activity. Tricine SDS-PAGE was performed according to Shägger and Von Jagow (1987).

2.8. Detection of esterase and PME activity on gels

After electrophoresis, gels were washed exhaustively three times for 10 min in PBS, pH 7.5. Esterase activity was detected by incubating them in 100 ml PBS containing 20 mM β-naphthyl acetate or β-naphthyl butyrate and Fast Blue BB at 37 °C for 45 min, as described by Rizzi et al. (2003). PME activity was highlighted with ruthenium red, as described by Kawano, Dos Santos Cunha Chellegatti, Said, and Vieira Fonseca (1999).

2.9. Statistical analysis

All data are reported as means ± standard error of the mean of three replicates.

3. Results and discussion

Spirits are the result of combined biochemical activities of microbial and vegetal origin. PME activity is involved in the production of methanol, but this enzyme may be related to raw materials or released by microorganisms.

Sampling the grape pomace in order to follow PME activity, the control (T_0) was obtained by crushing sound grapes, previously washed in distilled water, and checking the absence of microbes, in order to avoid microbial enzyme activities interfering with the detection of grape enzymes. Instead, the other marc samples, T_1, T_2, and T_3, were fermented by indigenous microorganisms and stored for differing periods of time.

Preliminary experiments to find the best way of extracting protein from T_0 samples showed that the extraction method plays an important role because, as reported by Knee, Paull, Ben, and Hawker (1991), soluble proteins comprise only a fraction of the total. Therefore, the solubilisation capacity of increasing concentrations of NaCl (from 0.1 to 1 M without buffer) was tested (Hagerman & Austin, 1986). The poor results in protein extraction led us to choose other procedures. The best yields, i.e., 1322 mg protein/g grape pomace fresh weight (Fig. 1) were obtained by applying the procedure of Barnavon et al. (2001) which: (i) reduces S–S bonds and protects proteins against oxidation, (ii) destabilises hydrophobic interactions among proteins, and (iii) absorbs the phenols in the sample. Knee et al. (1991) reported that the amount of phenols in fruit is higher than that of protein, and that phenol interactions with proteins are due to hydrogen bonds between their hydroxyl groups with the carbonyl function of peptide bonds. Our results show that extracted protein decreases with storage time, even when the amount of nitrogen in samples (0.23 ± 0.05), determined by the Kjeldahl method, remained unvaried. This behaviour may depend both on protease activity by fermenting microrganisms and the change in protein solubility due to the presence of oxidised phenols, which are more reactive towards proteins (Hsu & Heatherbell, 1987). Instead, PME activity, determined in the soluble fraction, increased during ensilage (Fig. 2). This result may be due to the differing enzyme solubility of our samples, probably because of microbial hydrolases, which may play an important role in cell wall degradation, relaxing the "backbone" which is involved in enzyme release. Also in grape, PME is considered to be a cell wall enzyme (Micheli, 2001). As a consequence of this release, PME activity appears to increase in the soluble fraction. At the same time, sugar concentrations also affect PME activity, as reported by Deng, Wu, and Li (2005), and enzyme inhibition may lead to as much as a 40% loss of activity at 15% sucrose. In our case, the sugar concentration (8.24%) was monitored only in sample T_0, in which enzyme activity was the lowest. After that phase, alcoholic fermentation converted grape sugars into ethanol, carbon dioxide, and other by-products.

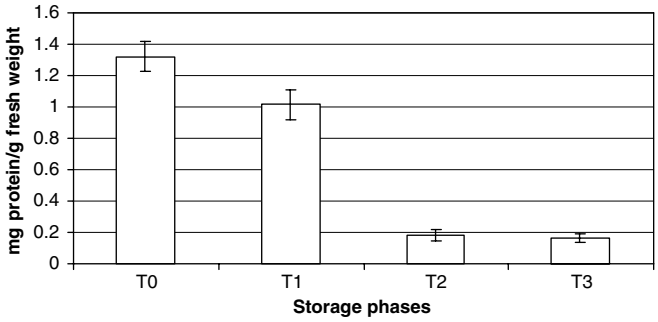

Fig. 1. Protein yield (mg/g grape pomace fresh weight), calculated on vegetal extracts drawn at four storage phases (T_0, T_1, T_2, and T_3).

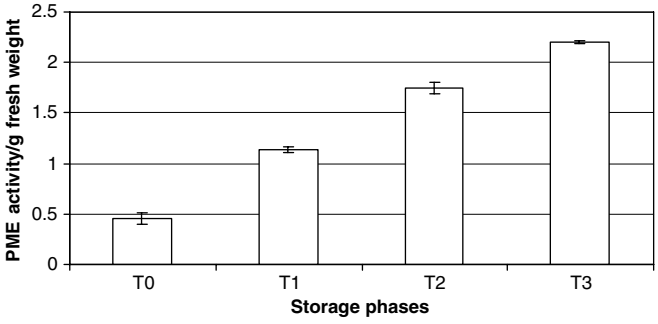

Fig. 2. PME activity expressed as Δ absorbance/min/g grape pomace fresh weight, calculated on vegetal extracts drawn at four storage phases.

Methanol contents determined on the same samples reached their highest values at phase T_2, but thereafter the accumulated methanol remained unvaried (Fig. 3), without any link with enzyme activity. For better understanding of methanol release during grape pomace storage, we saponified the grape pomace to achieve total pectin hydrolysis. The results (data not reported) showed that the pectin demethylation carried out by the enzyme was incomplete. The reduced rate of methanol production (Fig. 3) indicated a possible change in the PME enzyme, which is known to display end-product inhibition (Pilnik & Voragen, 1991) or a change in its environment. This change converts the enzyme into a poorly active form which does not reflect what occurs in vitro, employing an extracted form of PME (Anthon & Barrett, 2006).

In order to detect enzymes in the various samples, visualising PME activity on electrophoretic gels was considered. Grape extracts were analysed in native conditions to visualise PME activity after gel staining with ruthenium red, as reported by Kawano et al. (1999). The zymogram in pectin gel shows two activity bands (Fig. 4), with the same electrophoretic mobility in all samples, demonstrating that yeast (samples T_1, T_2, and T_3) does not contribute to PME production. These results are in agreement with those of Rombouts and Pilnik (1980), who studied the occurrence of pectic enzymes in fungi, yeast and bacteria. They reported high levels of PME activity only in *Aspergillus niger*. The Coomassie stained protein patterns of our four samples do not reveal qualitative differences (see later, Fig. 8). The zymographic technique reveals the incomplete disappearance of the PME band with higher mobility, probably involved in the change of the PME enzyme, as hypothesised above. The presence of two PME isoforms fits the results of Rombouts, Wissenburg, and Pilnik (1979) on citrus PME and of Alonso, Rodriguez, and Canet (1995) on the PME of ripe cherry (*Prunus avium* L.).

Since several authors (e.g., Hagerman & Austin, 1986) have proposed paranitrophenyl acetate, a specific substrate for esterase, also as PME substrate, we tested this substrate with our samples. The results (Fig. 5) suggest the presence of esterases in the extracts. However, the trend of this activity was different from that obtained with pectin as substrate

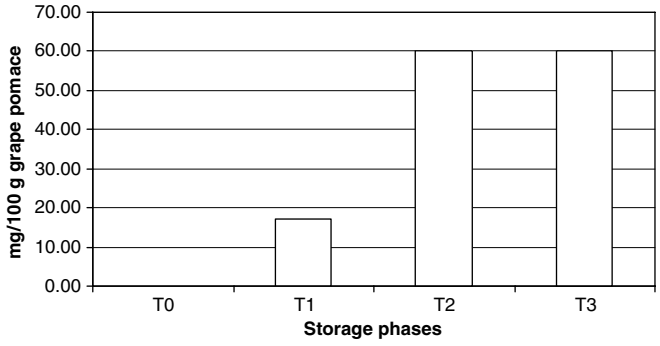

Fig. 3. Evolution of methanol release during grape pomace storage (mg/100 g fresh weight).

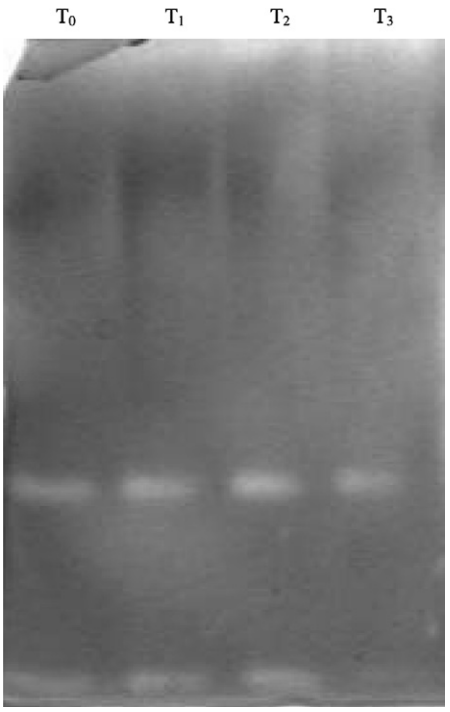

Fig. 4. PME activity zymogram of vegetal extracts, drawn at four storage phases. Zymographic profiles obtained after electrophoresing with N pectin PAGE, T 10%, and staining with ruthenium red.

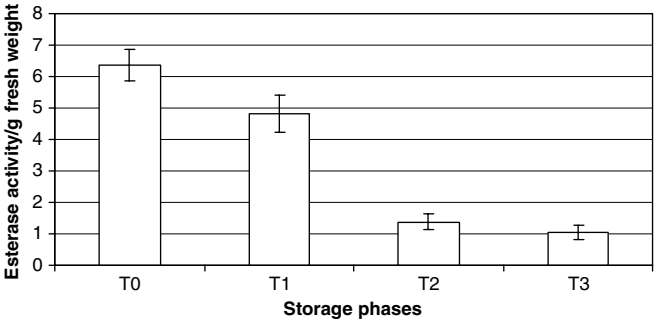

Fig. 5. Esterase activity expressed as Δ absorbance/min/g grape pomace fresh weight, calculated on vegetal extracts drawn at four storage phases.

(Fig. 2), and may be related to another enzyme. The question regarding the different behaviour due to the two substrates was solved by electrophoretic analysis. To avoid problems with electrophoretic mobility and thus comparison of patterns, pectin was added when making the gels. Cut in two, gels were stained with ruthenium red and β-naphtylacetate Fast BB (Fig. 6) or β-naphthyl butyrate (Fig. 7) substrates, respectively. In Fig. 6, the β-napthyl acetate shows two esterase isoforms, different from those revealed with ruthenium red. This finding proves: (i) the aspecificity of β-napthyl acetate as a PME substrate and (ii) the presence of different enzymes hydrolysing the ester bond when β-naphtyl butyrate is used as substrate (Fig. 7). An esterase activity band, completely different from the others, was detected and, as demonstrated by Lomolino, Lante, Rizzi, Spettoli, and Curioni (2005), the

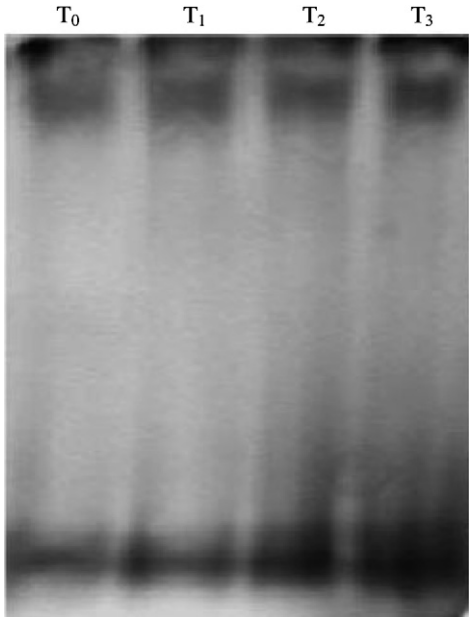

Fig. 6. Esterase activity zymogram of vegetal extracts, drawn at four storage phases. Zymographic profiles obtained after electrophoresing samples in N pectin PAGE, T 10% and β-naphthyl acetate, and staining with Fast BB.

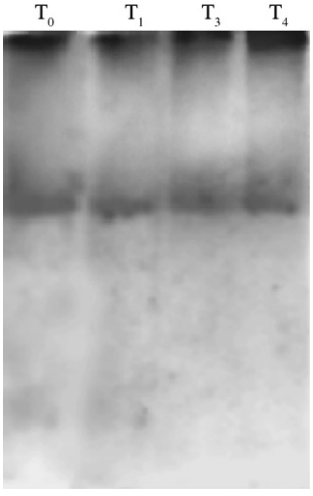

Fig. 7. Esterase activity zymogram of vegetal extracts, drawn at four storage phases. Zymographic profiles obtained by electrophoreting samples in N pectin PAGE, T 10%, and β-naphthyl butyrate, and staining with Fast BB.

zymographic results depend to a great extent on the substrate used. The Coomassie stained protein patterns of our four samples do not show qualitative differences (Fig. 8), as in most vegetal extracts the number of bands is low, and the complexes between proteins and polyphenols could be visible at the sides of each lane. The Tris–Tricine–PAGE (Fig. 9), a technique which visualises electrophoretic bands of low molecular weight, confirms the absence of peptides and shows that grape skin has low protein content. The gels clearly show that the number of protein bands is the same in the four samples, but their

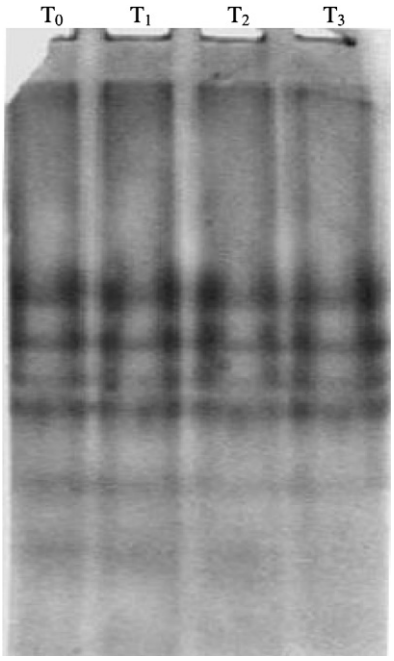

Fig. 8. N pectin PAGE of vegetal extracts, drawn at four storage phases, Coomassie staining.

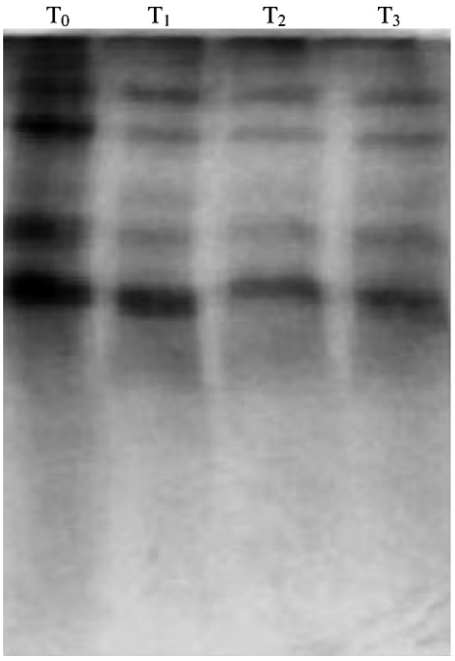

Fig. 9. SDS–Tris–Tricine–PAGE of vegetal extracts, drawn at four storage phases, Coomassie staining.

dye has different intensities. We quantified these differences by densitometric analysis (Fig. 10) which compares the relative quantity of protein in each band with the total protein profile. The results revealed both constant and variable bands, indicating a ''storage effect'' on the protein contents of the samples. Further study of this aspect may be of interest.

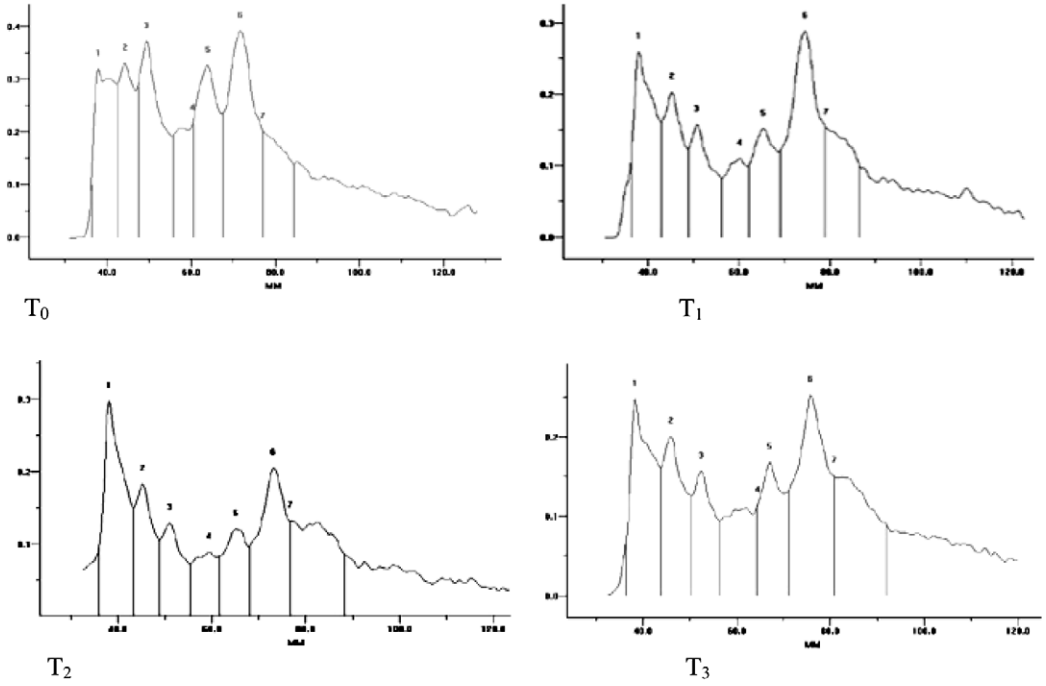

Fig. 10. Densitograms of protein profiles of Fig. 9.

4. Conclusions

Our results show that PME activity may be detected in grape pomace and that enzymatic activity evolves during storage. Evidence is presented that yeasts do not contribute to PME production or the modification of pectins involved in methanol release.

Last but not least, our zymograms show that PME and esterase are different enzymes with peculiar technological effects. The possibility of visualising various vegetal enzymes with esterase activity may be a promising step in the study of flavour evolution during grape pomace storage. These preliminary results are thus a starting-point in clarifying the biochemical mechanisms which underlie the transformation of plant material as grape pomace, into a alcoholic beverage such as Italian grappa.

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