

Enzymes as Potential Markers of Wine Aging

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ABSTRACT

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There is no analytical method currently available in Spain to determine elapsed time in wine aging. The only control used is based on the levels of esters and other secondary metabolites as important indicators related to wine quality. The main goal of this work was to establish a useful method to differentiate among young, “*crianza*”, “*reserva*” and “*gran reserva*” wines based on the study of the enzymatic decay of different hydrolases present in the wines and also to determine which enzyme(s) may be applicable in fraud detection as regards time and storage conditions of the wine. We elaborated red wine based on “Tempranillo” grapes from the Rioja Alta Alavesa and fermented with *Saccharomyces cerevisiae* USC 013, followed by malolactic fermentation by *Oenococcus oeni* CECT 630 in order to evaluate the different enzymes. One of the enzymes present, i.e., invertase, which is resistant to inactivation, could be a useful marker of wine aging.

Key words: Glucanase, glucosidase, invertase, polygalacturonase, *Saccharomyces cerevisiae*, wine.

INTRODUCTION

Wine is one of the oldest beverages obtained by fermentation in the world and currently plays a very important economic role in many countries⁴.

Spanish regulations classify wines, depending on the spent time in the cask and in the bottle, as “*crianza*”, “*reserva*” and “*gran reserva*”¹ (Table I). The distinction, however, as to which of these categories a given wine belongs, relies to a certain extent on certain physicochemical

Table I. Wine classification according to the time elapsed in the barrel and in the bottle.

		Minimum time (months)		
		Barrel	Bottle	Total
Crianza	White	6	18	24
	Red	6	18	24
Reserva	White	6	18	24
	Red	12	24	36
Gran Reserva	White	6	42	48
	Red	24	60	84

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parameters of the wine and, to a greater extent, on what is certified by the producer.

Preliminary studies carried out at this laboratory revealed that some of the enzymes present in grape must or released mostly by *Saccharomyces cerevisiae* (i.e. β -glucanases, polygalacturonases, β -glucosidases and invertases) vary in wine according to the time elapsed from fermentation¹². The first type of enzymes may be 1,3- β -D-glucanases (EC 3.2.1.6) or 1,6- β -D-glucanases (EC 3.2.1.58) and for many years these have been known to be present in the supernatant broths of ascomycetous yeasts as the result of cell leakage during active growth⁹. Both aryl and alkyl β -D-glucosidases (EC 3.2.1.21) may be also secreted by *S. cerevisiae*².

Polygalacturonases (EC 3.2.1.15) belong to the pectinase group and have also been described in many yeasts since the early description by Luh and Phaff⁶. Finally, invertase (EC 3.2.1.26) is formed in high amounts by different yeasts and is involved in the hydrolysis of sucrose into fructose and glucose³.

MATERIALS AND METHODS

Strain and media

Alcoholic fermentation was carried out by *S. cerevisiae* USC 013, isolated in this laboratory from spontaneous fermentations⁵. Malolactic fermentation was performed with *Oenococcus oeni* CECT 630, also isolated in this laboratory¹⁰. The yeast was grown and maintained in YPD medium (1% yeast extract, 2% peptone, 2% glucose) and the bacterium was kept in MRS broth (prepared and sterilized as suggested by the manufacturer, Cultimed[®] (Panreac Química, Spain). Media were solidified with 2% Bacto agar when necessary.

Wine-making

Red wine (300 L) was elaborated with sulphited must (35 gL⁻¹ of potassium metabisulphite) from *Vitis vinifera* of the Tempranillo variety (Rioja Alta Alavesa, North-Eastern, Spain). Alcoholic fermentation was carried out for 45 days at 20°C and then, after pressing, malolactic fermentation lasted 15 additional days at 25°C. During this time, 100 mL samples were periodically withdrawn and the wine parameters and activity of several enzymes were analysed.

When the secondary fermentation had halted, part of the wine was bottled (wine A); another portion was subjected to the “*crianza*” process (wine B); a third portion was subjected to the “*reserva*” process (wine C) and a

Table II. Basic properties of young wine A.

Alcoholic degree gL ⁻¹ (% vol)	Density (gcm ⁻³)	Glycerol (gL ⁻¹)	Acetic acid (gL ⁻¹)	Malic acid (gL ⁻¹)	Lactic acid (gL ⁻¹)	pH
110.40 (14°)	994	5.94	0.43	0.51	1.33	4.03

Table III. Analysis of alcohols and esters in the different wines.

	Wine A	Wine B	Wine C	Wine D	Wine E
Higher alcohols (gL ⁻¹)					
1-Butanol	0	0	0	0	0
Isoamyl alcohol	86.06	74.25	81.76	53.98	63.05
1-Hexanol	0.8624	0.8294	0.8736	0.0198	0.0165
trans-Hexanol	0	0	0	0	0
cis-Hexanol	0	0	0	0	0
2-Octanol	1	1	1	1	1
1-Heptanol	0.1768	0.0723	0.073	0.0086	0.0525
2-Ethyl-1-hexanol	0.0175	0.0089	0.0108	0.0132	0.0143
1-Octanol	0.0402	0.0248	0.0319	0.0205	0.0232
Benzyl alcohol	0.0289	0.0483	0.046	0.0395	0.0243
2-Phenylethanol	73.771	63.57	69.76	81.13	63.9
Total alcohols	160.96	138.81	152.56	135.22	127.08
Esters (gL ⁻¹)					
Propyl propanate	0	0	0	0	0
Isoamyl acetate	0.0106	0.0069	0.0062	0.0053	0.006
Ethyl hexanoate	0.2203	0.1994	0.234	0.0873	0.195
Hexyl acetate	0.004	0.0037	0.0082	0.0039	0.0036
Ethyl caprylate	0.1569	0.174	0.1799	0.0795	0.1821
Ethyl hydroxybutyrate	0.0287	0.0281	0.0382	0.0156	0.042
2-Phenylethanol acetate	1.4915	0.7198	0.557	0.148	0.4733
Ethyl cinnamate	0	0	0	0.0075	0.0047
Total esters	1.912	1.132	1.029	0.347	0.907

further fourth and fifth wine aliquots were subjected to accelerated aging by storage in the presence of oak shavings at 60°C for 6 h (wine D) and at 25°C for 4 days (wine E).

Analysis of must and wines

Evaluation of glucose, ethanol, malic acid, glycerol, lactic acid and acetic acid was carried out using commercial kits (Boehringer-Mannheim). The reducing power was determined by Somogyi's method¹¹ as modified by Nelson⁷. All measurements were carried out in triplicate.

Higher alcohols and esters were determined by gas chromatography, using a Hewlett Packard 5890 series II chromatograph equipped with an ionising flame detector and a Carbowax 20M (25 m × 0.2 mm) capillary column. These samples were analyzed at the Department of Biotechnology (Instituto de Agroquímicas y Tecnología de los Alimentos, Valencia, Spain).

Enzymatic assays

The above-described enzymes were evaluated in both commercial and experimental wines previously decolourised as recommended by Regulation 1990-CEE 2676/90. Before enzyme analysis, the samples were filtered through 0.22 µm membranes and dialysed for 24 h against 0.05 M sodium acetate buffer, pH 5.5. A typical reaction mixture contained 0.5 mL sample and 0.5 mL of the appropriate substrate (1%) in 0.05 M sodium acetate buffer pH 5.5. Thus, 1,3-β-D-glucanase was assayed on laminarin (Sigma), 1,6-β-D-glucanases on pustulan (Calbiochem),

polygalacturonase on polygalacturonic acid (Sigma), invertase on sucrose (Merck), and β-glucosidase on salicin (Senn Chemicals). Enzymatic reactions were carried at 30°C for different times and enzyme activity was evaluated by quantifying the reducing power using Somogyi's method¹¹ modified by Nelson⁷. The data were obtained, three in triplicate, and were analyzed by ANOVA statistical method (SPSS 11.5). One enzyme unit (U) was defined as the amount of enzyme that released one nanomole of product (or the equivalent in reducing power) per hour at 30°C.

RESULTS AND DISCUSSION

Evolution of enzymes in experimental wines

The red wines A, B, C, D and E were elaborated in the experimental cellar of this University, starting from grape must with 22.3°Brix, a density of 1095, and pH 3.7. After the primary (*S. cerevisiae* USC 013) and secondary (*O. oeni* CECT 630) fermentations, a young wine (A) was obtained that served as the starting point for elaborating B, C, D and E.

As seen, the basic values of wine A (Table II) lay well within those reported for red wines⁸. The total amount of alcohols did not vary significantly, regardless of their being subjected to the "crianza" or "reserva" manipulations. This was not the case of esters, since these were dramatically reduced when the samples were subjected to manipulations using oak casks and high temperatures (60°) (Table III). Therefore, the evaluation of esters in wines

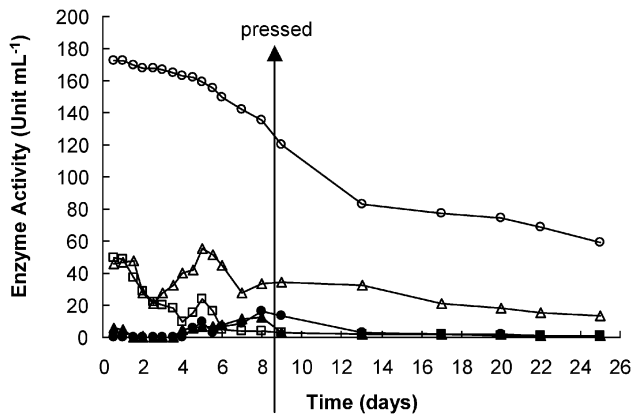


Fig. 1. Enzyme activity during the alcoholic fermentation. Invertase (○), polygalacturonase (△), β -1,3-glucanase (□), β -1,6-glucanase (●) and aryl- β -D-glucosidase (▲). Arrow marks the time of pressing.

classified as “crianza”, “reserva” or “gran reserva” may be employed in order to detect malpractice in wine making.

Regarding the evaluation of the different enzymes, these were tested from the very beginning of the alcoholic fermentation (Fig. 1). As shown, invertase exhibited the highest activity. The number of units of all the enzymes tested varied from sample to sample before pressing; after

pressing, the total amount of units diminished, although measurements were more reliable in terms of lack of fluctuations. Fluctuations in enzyme levels before pressing should be taken as an indication that a number of biochemical reactions are taking place, probably related to the presence of the grape skins and of uncontrolled biological elements. After pressing, an important decrease in enzyme activities together with value stabilization was detected. This may be related to the fact that the ecosystem was now more homogeneous and also to the possible presence of grape proteases released by pressing.

It is worth noting that all enzyme activities, except that of 1,6- β -D-glucanase, were already present at the beginning of fermentation. This suggests that their origin would lie in both *Vitis vinifera* and *Saccharomyces cerevisiae*. As seen in Fig. 2a, overall invertase activity followed a downward trend from the very start of fermentation and throughout the primary and secondary fermentations (ca. day 80 after the beginning). Only when glucose had been depleted was there a new increase in invertase activity in the untreated wines (A, B and C) until the end of the experiment. When the same wine was maintained in the presence of oaks (D and E), the profile of invertase was rather different from that of bottled wine. Thus, when it was maintained in the presence of oak casks at 25°C (E) the enzyme activity showed a maximum followed by a fairly rapid decrease; when the temperature was 60°C (D),

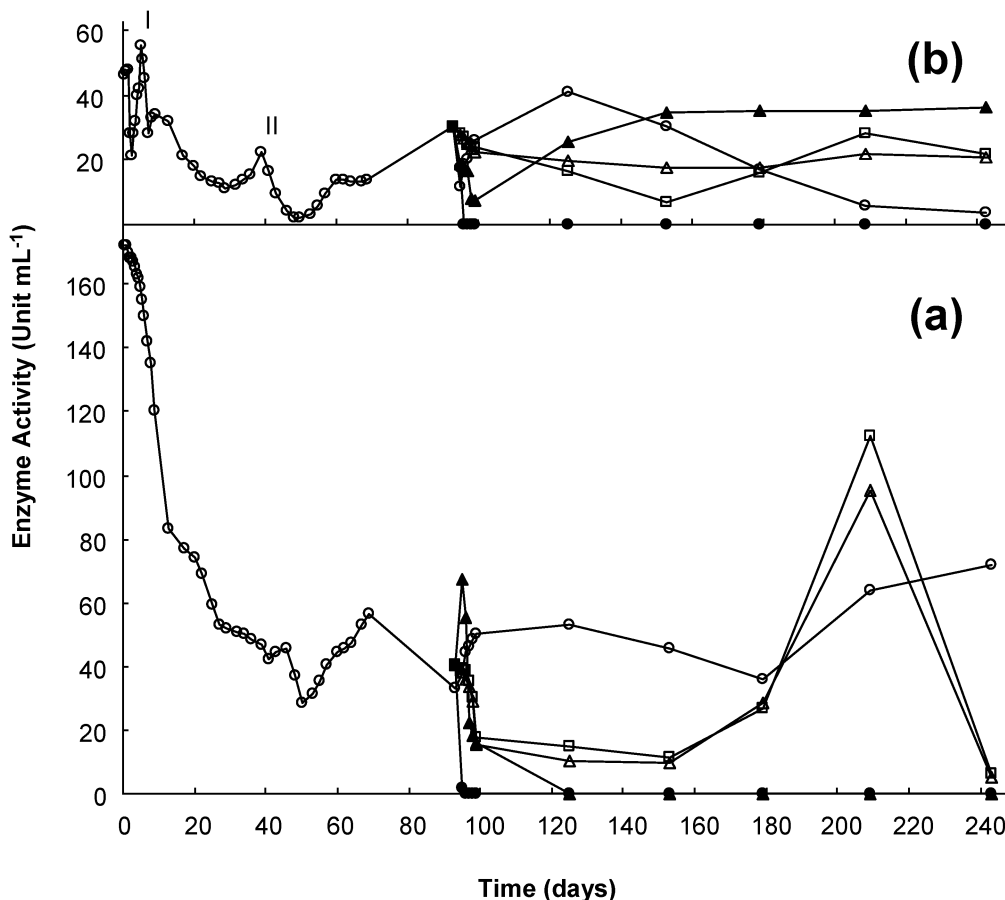


Fig. 2. Invertase (a) and polygalacturonase (b) activities in elaborated wines. Wine A (○), wine B (△), wine C (□), wine D (●) and wine E (▲).

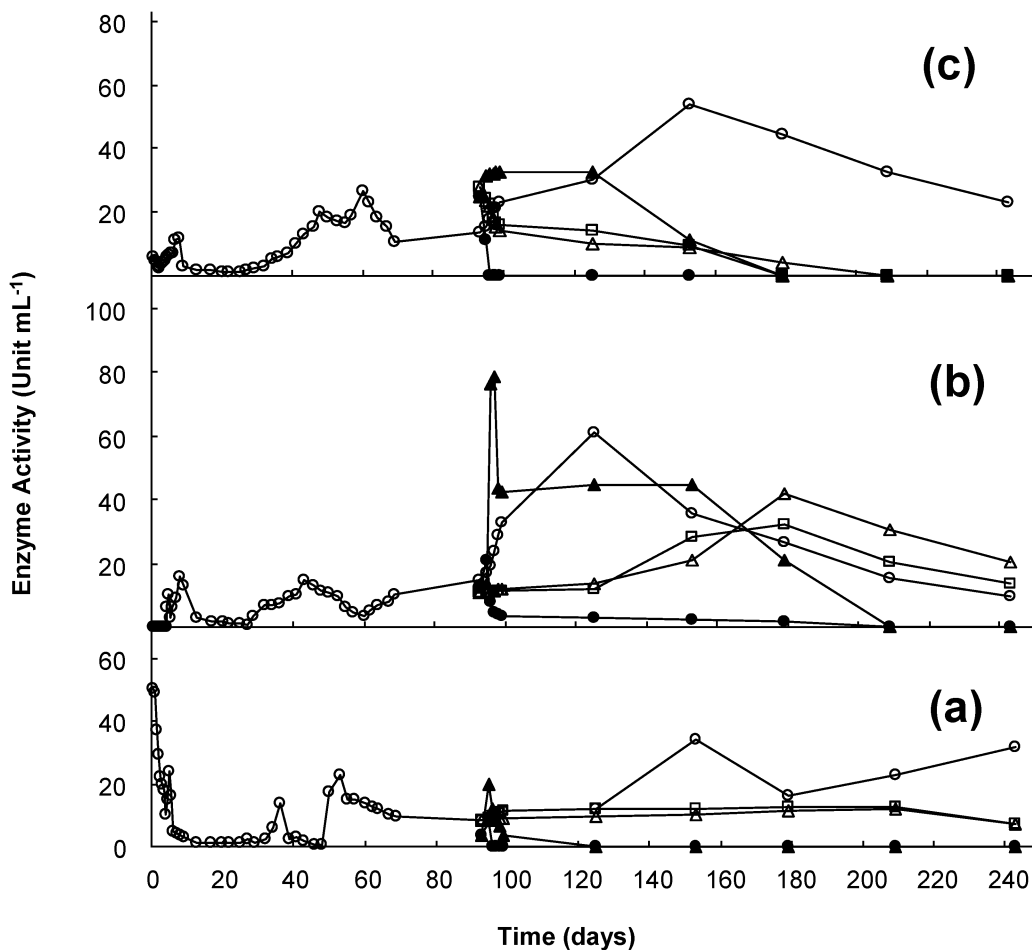


Fig. 3. β -1,3-Glucanase (a), β -1,6-glucanase (b) and aryl- β -D-glucosidase (c) activities in elaborated wines. Wine A (○), wine B (△), wine C (□), wine D (●) and wine E (▲).

the enzyme was rapidly inactivated, as expected. When the wine (E) was subjected to the oak-cask treatment, after showing an initial decrease, the invertase exhibited a relevant and unexpected maximum. Accordingly, all these data suggest that some components present in oak wood are somehow able to interact with the invertase molecule, thereby regulating its activity, exactly the same as occurs in barrelled wines (B and C), although later.

As regards polygalacturonases present in the must, after an initial decline in the number of units, they were complemented by those secreted by *S. cerevisiae* during active primary fermentation, the profile thus showing a clear increase (peak I in Fig. 2b). Thereafter, being sensitive to changing environmental conditions, these enzymes underwent a slow inactivation and, after a second increase (peak II in Fig. 2b), became almost undetectable. (*ca.* day 50). However, when the wine had been in contact with oak wood the activity of the enzyme did not decline and persisted for months (B and C), as though some component of the wood somehow had the ability to protect the enzyme from inactivation. In the case of young wine (A), which was bottled immediately after fermentation, a small increase in activity was detected and then a slow decline. As expected, the wine treated with oak casks at 60°C (D) showed dramatic decrease in activity. However the wine maintained at 25°C in the presence of oak casks (E) first

showed a decrease in the enzyme activity and then an increase up to the end of the experiment.

1,3- β -D-Glucanase activity (Fig. 3a) was also present in fresh must and this must be due to the callase activity of grape must. The activity of this enzyme declined as from the beginning of fermentation. The transient peaks observed were due to the enzyme secreted by *S. cerevisiae* during active fermentation. After pressing (day 80), activity remained more or less constant for several months, even in the samples maintained in oak casks. However, the enzyme was rapidly lost, after an increase, when the wine was treated with oak casks at 25°C. Only the data obtained for young wine (A) revealed fluctuations.

Finally, the patterns for 1,6- β -D-glucanase and aryl- β -D-glucosidase were very similar (Fig. 3b and 3c, respectively). The grape juice was entirely devoid of the first enzyme and very low amounts of the second were found. The 1,6- β -D-glucanase showed two peaks (Fig. 3b) before pressing, and it exhibited a third clear peak in bottled wine and a slow decline as the months passed. Maximum activity was observed when the wine was treated with oak casks at 25°C, followed by a typical decrease. The main source of this enzyme in wine must be *S. cerevisiae*.

Aryl- β -D-glucosidase activity was very low during the first 30 days of fermentation, after which it showed a clear increase that was even stronger in bottled wine. Again, as

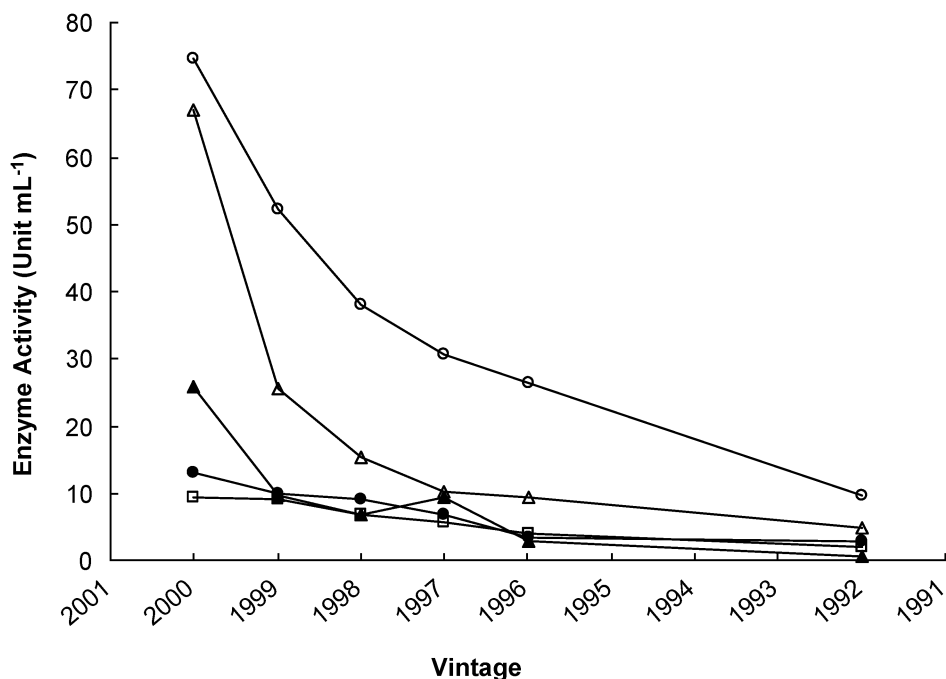


Fig. 4. Enzyme activity in commercial wines. Invertase (○), polygalacturonase (△), β -1,3-glucanase (□), β -1,6-glucanase (●) and aryl- β -D-glucosidase (▲).

shown in the present paper, the presence of oak in the wine-making process tended to diminish or even abolish these kinds of hydrolases.

Enzyme analysis in commercial wines

Enzyme analysis was performed in commercial wines from the *Rioja* region elaborated (on average) with 80–88% tempranillo grapes and 20–12% garnacha grapes, and catalogued by the manufacturers as “*crianza*”, “*re-*

serva” and “*gran reserva*”. The wines were treated as specified in Material and Methods and the five enzymes were evaluated (Fig. 4). As may be seen, invertase was the most resistant enzyme and it was still detectable ten years after the wine had been elaborated. Polygalacturonase was less resistant since it was difficult to detect at six years. The 1,6- β -D-glucanase and 1,3- β -D-glucanase were almost undetectable after five years and the same was the case of aryl- β -D-glucosidase.

Table IV. Mean and standard deviations for the enzymes in commercial wines.

Year		U (nmol/h) invertase	U (nmol/h) polygalacturonase	U (nmol/h) β -1,3-glucanase	U (nmol/h) β -1,6-glucanase	U (nmol/h) aryl β -D-glucosidase
1992	Mean	9.6000	4.8100	1.9500	2.9000	.5900
	N	3	3	3	3	3
	Stand. dev.	1.44010	1.64763	.41328	1.20000	.50408
1996	Mean	26.3000	9.3000	3.9000	3.4000	2.8000
	N	3	3	3	3	3
	Stand. dev.	3.10000	1.41067	.81854	1.70587	.79373
1997	Mean	30.7000	10.2000	5.7000	6.9000	9.3000
	N	3	3	3	3	3
	Stand. dev.	1.70880	1.91572	1.42380	1.60935	1.22882
1998	Mean	37.9000	15.3000	6.8000	9.1000	6.9000
	N	3	3	3	3	3
	Stand. dev.	1.85203	1.66433	1.22882	2.61534	1.47986
1999	Mean	52.2000	25.4000	9.0000	9.9000	9.6000
	N	3	3	3	3	3
	Stand. dev.	2.93087	1.94679	1.07503	2.51595	1.13578
2000	Mean	74.5000	67.0500	9.3500	13.0000	25.7000
	N	3	3	3	3	3
	Stand. dev.	2.68514	2.58200	1.62028	3.30454	.90000
Total	Mean	38.5333	22.0100	6.1167	7.5333	9.1483
	N	18	18	18	18	18
	Stand. dev.	21.21940	21.81356	2.88371	4.14970	8.37267

Table V. Mean and standard deviations for the enzymes in elaborated wines.

Wine		U (nmol/h) invertase	U (nmol/h) polygalacturonase	U (nmol/h) β -1,3-glucanase	U (nmol/h) β -1,6-glucanase	U (nmol/h) aryl β -D-glucosidase
A	Mean	48.1582	20.8148	15.8764	25.1015	26.9455
	N	33	33	33	33	33
	Stand. dev.	11.80323	11.03234	9.22611	13.92465	12.12354
B	Mean	31.0000	22.9345	9.1909	17.5670	13.0973
	N	33	33	33	33	33
	Stand. dev.	23.99371	4.41609	1.60361	9.89022	9.23803
C	Mean	33.8918	22.5700	10.5000	16.0315	13.4027
	N	33	33	33	33	33
	Stand. dev.	27.78622	7.04852	1.88116	9.35075	9.74077
D	Mean	3.8682	4.3491	1.0173	7.3058	3.3036
	N	33	33	33	33	33
	Stand. dev.	11.86456	9.81013	2.37607	9.33338	7.71110
E	Mean	19.8818	24.0182	4.8600	32.0485	20.8491
	N	33	33	33	33	33
	Stand. dev.	23.56917	10.75791	6.16991	27.13111	14.33556
Total	Mean	27.3600	18.9373	8.2889	19.6108	15.5196
	N	165	165	165	165	165
	Stand. dev.	25.42857	11.54146	7.21581	17.48389	13.40302

Statistical analysis of the data from both elaborated and commercial wines

Tables IV and V show the statistical analysis of all the data obtained for invertase, polygalacturonase, β -1,3-glucanase, β -1,6-glucanase and aryl β -D-glucosidase based on analysis of variance through means comparison (ANOVA). The statistical analysis revealed that there were significant differences for invertase among commercial young wines (Table IV) and “crianza”, “reserva” and “gran reserva” wines.

In elaborated wines (Table V), it was found that invertase did differentiate young wine (A) from those treated with oak casks (D and E); it also differentiates wines maintained in barrels (B and C) and those treated with oak casks (D and E). Therefore, invertase may be used as an enzyme to mark the aging of wines and, in particular, to detect whether accelerated-aging practices have been employed.

Moreover, the statistical analysis for polygalacturonase showed the existence of significant differences between commercial young wines and those labelled as “crianza”, “reserva” and “gran reserva”, and revealed that in the case of elaborated wines it was able to differentiate wines kept in barrels from those subjected to accelerated maturation by means of oak casks. In conclusion, as in the case of invertase, study of this enzyme could be used for fraud detection. The same applies for β -1,6-glucanase aryl β -D-glucosidase and β -1,3-glucanase (Tables IV and V).

It should be kept on mind that the enzymes evaluated could indeed be different isoforms. An electrophoretic analysis as well as an analysis of the DNA using PCR and RFLP could help to identify the origin of the enzymes studied here, the type of yeast utilized as starter, wild yeast, bacteria or other biological debris.

It must also be borne in mind that the level of the enzymes may vary considerably, depending on the year, the geographic region, or the *Vitis vinifera* variety employed. We are currently trying to evaluate all these enzymatic parameters using grapes of other varieties and other geo-

graphic regions, which should allow us to obtain more precise results.

In conclusion, in the present paper we propose that the sensory description of a wine by itself is not enough to determine its aging and that the enzymes reported here could be used as potential markers of wine aging for the detection of fraud.

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