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Enzymatic activities in the varieties of hazelnuts (*Corylus avellana* L.) grown in Tarragona, Spain

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Enzymatic activities of lipase, esterase, lipoxygenase (LOX), peroxidase (POD) and polyphenoloxidase (PPO) of the main local, Italian and Turkish varieties of hazelnuts grown in Tarragona, with and without irrigation, were determined. Enzymatic extract protein was estimated by calculating Δ Abs/min.mg of protein with spectrophotometry. Oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acid percentages were determined using high resolution gas chromatography (HRGC). Data were statistically analysed using General Linear Models and Duncan's multiple test. It is observed that variety is a determining factor for all enzymatic activities, especially lipase and peroxidase activity. Negret (Negret and Pauetet varieties), the most cultivated variety in Tarragona, has a high $C_{18:2}$ content and high enzymatic activities. Italian varieties have lower lipase, POD and PPO activities. All varieties show minimum activity of esterase and lipoxygenase. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

Hazelnut oil becomes rancid as a result of hydrolytic oxidation and chemical auto-oxidation (Rothe *et al.*, 1967; Heimann & Schreier, 1971; Schreier & Heimann, 1971). In the former, the activity of lipase depends on moisture and water activity (A_w), producing fatty acids. Also, high content of oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids enhances auto-oxidation. Once primary oxidation takes place, peroxidase favours secondary and terminal oxidation. Thus, enzymatic activity and $C_{18:2}$ content are determining factors in hazelnut preservation. Most large cocoa-derivate producing firms are also the largest hazelnut consumers and demand homogeneous quality. Now that new Italian varieties, that grow easily in Turkey and are supposedly easier to preserve, are reaching the market there is increasing competition for Tarragona's hazelnut production (Rivella, 1984; Farràn Codina & Serra Bonvehí, 1992; Serra Bonvehí & Ventura Coll, 1992). This situation has triggered a research programme that aims to typify the morphology, composition and oxidation stability of the main local, Italian and Turkish varieties cultivated in Tarragona (Serra Bonvehí & Ventura Coll, 1993*a,b,c*, 1996; Serra Bonvehí, 1995; Serra Bonvehí *et al.*, 1996). Of all the defined parameters, enzymatic activity may be the criterion best-defining preservation capacity. Therefore, hazelnut enzymatic activities have been determined [lipase, esterase, lipoxygenase (LOX), peroxidase (POD) and polyphenoloxidase(PPO)].

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MATERIALS AND METHODS

Materials

Forty samples of the most commercially available local, Italian and Turkish varieties cultivated in Tarragona in the 1990 crop were studied (Table 1). Samples were directly collected from the main producers and belonged to two areas best representing cultivation methods: a non-irrigated mountain area (Falset) and an irrigated area (Reus). Hazelnuts were collected from the soil and dried in a kiln at 40°C in their shells, the water content of the meat of the nut was below 5 g/100 g, and kept in darkness and at room temperature. Analyses were all done in triplicate.

Methods

Enzymatic extract

Twenty grammes of hazelnuts were triturated in a coffee grinder until a homogeneous paste was obtained. For each enzyme, 5 g of paste were comminuted in 40 ml of extract buffer using a mortar and polytron. The extract buffer contained 1 g of insoluble polyvinylpyrrolidone

Table 1. Hazelnut sampling: site of origin

Sample No.	Variety No.	Variety	Geographical origin	Farming method	Province
1	1	Castanyera	Falset	Dry-farmed	Tarragona
2	2	Culplà	Falset	Irrigated	Tarragona
3	2	Culplà	Falset	Dry-farmed	Tarragona
4	2	Culplà	Reus	Irrigated	Tarragona
5	3	Gironell	Falset	Irrigated	Tarragona
6	3	Gironell	Reus	Irrigated	Tarragona
7	4	GLM	Reus	Irrigated	Tarragona
8	5	Negret	Reus	Irrigated	Tarragona
9	5	Negret	Falset	Irrigated	Tarragona
10	5	Negret	Falset	Dry-farmed	Tarragona
11	6	Pauetet	Falset	Irrigated	Tarragona
12	6	Pauetet	Reus	Irrigated	Tarragona
13	7	San Giovanni	Reus	Irrigated	Italy
14	8	Tonda Giffoni	Falset	Irrigated	Italy
15	8	Tonda Giffoni	Reus	Irrigated	Italy
16	9	T. G. de Langhe	Falset	Irrigated	Italy
17	10	Tonda Italiana	Reus	Irrigated	Italy
18	11	Tonda Romana	Falset	Irrigated	Italy
19	11	Tonda Romana	Reus	Irrigated	Italy
20	12	Tomboul	Reus	Irrigated	Turkey

and 40 ml of 0.01 M K-phosphate buffer solution, pH 5.8. The extract was stirred for 1 h in an ice bath and then filtered through a 500 μm nylon cheesecloth and centrifuged (18 000*g* for 30 min at 0–5°C). The extract was further clarified with six drops of Carrez I solution [(15 g $K_4[Fe(CN)_6].3\ H_2O$)/100 ml H_2O] and Carrez II solution [(30 g $Zn(AcO)_2.2H_2O$)/100 ml H_2O] using a Pasteur pipette. The new solution was softly homogenized and filtered once more through Whatman paper No. 541. Enzymatic extract was obtained after discarding the first few millilitres. The clarified extract was kept cold until analysed.

Protein content of the supernatant was determined by the method of Bradford (1976).

Lipase activity was measured according to the modified method of Kazi & Cahill (1971). The reaction mixtures containing 3 ml of enzymatic extract were combined with 50 μl of Al_2O_3 column-purified olive oil (1% isopropanol), 0.2 ml of 5% $CaCl_2$ and 0.6 ml of 0.5% bromothymol blue aqueous solution; pH was adjusted to 6–7 using 0.05 M NaOH. This solution was incubated 30 min at 44±2°C. The time curve was recorded directly on the spectrophotometer at 620 nm against the reference cuvette without enzymic extract. A linear regression equation of the curve was used to calculate ΔAbs/min.mg protein.

Peroxidase (POD) activity was measured according to the modified method of Ridge & Osborne (1970). The reaction mixture contained 1 ml of enzymatic extract, 1 ml of 35% formaldehyde, 1 ml of 20 mmol/litre of 2,2′-azino-bis-(3-ethylbenzotiazol-6-sulphonic) (ABTS) solution and 1 ml of 1% H_2O_2. The time curve was recorded directly on the spectrophotometer at 430 nm for 10 min against the reference cuvette without enzymic extract. A linear regression equation of the curve was used to calculate ΔAbs/min.mg of protein (Fig. 1).

Polyphenoloxidase (PPO) activity was measured according to modified method of Udayasekhara Rao & Deosthale (1987). The reaction mixture contained 1 ml of enzymatic extract and 1 ml of 1% pyrocatechol in 0.005 N oxalic acid. The time curve was recorded directly on the spectrophotometer at 540 nm for 10 min against the reference cuvette without enzymic extract. Linear regression was used to calculate ΔAbs/min.mg of protein (Fig. 2).

Esterase activity was measured according to the modified method of Purr (1962). The reaction mixture

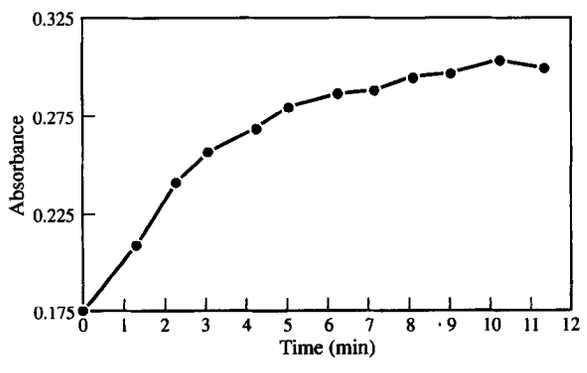

Fig. 1. Peroxide activity. The rate of reaction increases.

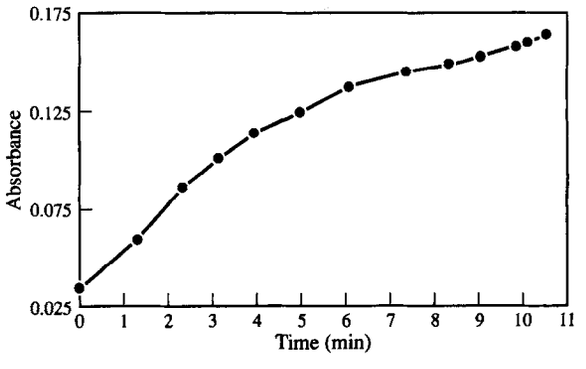

Fig. 2. Polyphenoloxidase activity. The rate of reaction increases.

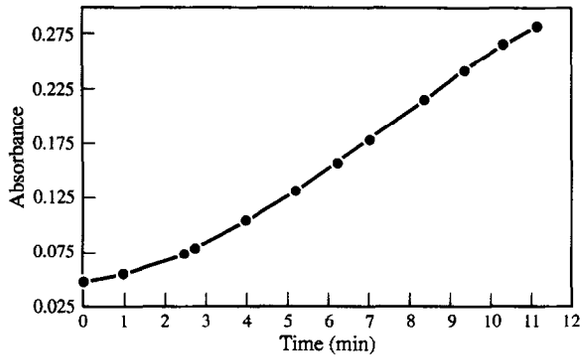

Fig. 3. Esterase activity. The rate of reaction increases.

contained 2 ml of enzymatic extract and 0.2 ml of 0.4% indoxylacetate in ketone. The time curve was recorded directly on the spectrophotometer at 660 nm for 10 min against the reference cuvette without enzymic extract. Linear regression was used to calculate Δ Abs/min.mg of protein (Fig. 3).

Lipoxygenase (LOX) activity was measured according to method of Galliard (1986) with an O_2-electrode. Oil was extracted from 5 g of hazelnut paste using 50 ml of ethyl ether. Extraction was repeated twice more. After the residual solvent was discarded, using a vacuum pump and nitrogen current, 3 g of the sample were combined with 10 ml of 0.1 M imidazole–HCl buffer (pH 6.9). The solution was homogenized using a vortex mixer, agitated in an ice bath for 1 h and centrifuged at 18 000*g* for 30 min at 0–5°C. The O_2-electrode cell was kept at 25°C. Two millilitres of 0.1 M imidazole–HCl buffer solution and 0.5 ml of linoleic acid solution were added [1 g of $C_{18:2}$/35 ml of ethyl ether containing butylhydroxytoluene (BHT) (5 mg/100 ml)]. The cell was closed lowering the occluding cylinder to expel the air located in the headspace. The solution was magnetically stirred. Once the electrode was stabilized, 20–200 μl of enzymatic extract was injected and oxygen consumption was registered. The activity of the sample was estimated (μmol O_2 consumed/min) in relation to protein content or sample weights, bearing in mind that a saturated oxygen solution yields 240 μmol O_2/ml.

Oil extraction

Shelled hazelnuts (30 g) were homogenized in a coffee grinder. Triplicate 10 g samples were extracted with petroleum ether (40–60°C) for 6 h using a Soxhlet apparatus. Oil was determined as the difference in weight of dried samples before and after extraction.

Fatty acid content was determined using a capillary gas chromatography column following Serra Bonvehí & Ventura Coll (1993*b*).

Free acidity

One gramme was extracted under pressure and hazelnut oil was dissolved in ethanol and ethyl ether (1:1). The solution was titrated with a 0.01 M NaOH solution, using phenolphthalein as an indicator. The result was expressed as oleic acid percentage per 100 g of hazelnut oil.

Peroxide value (PV)

One gramme was extracted under pressure, and hazelnut oil was dissolved in 25 ml of acetic acid–chloroform (3:2) solution. A nitrogen current was passed through the solvent solution and 1 ml potassium iodide IK solution (20 IK/26 ml H_2O) was added. The solution

Table 2. Moisture, enzymatic activity, acidity, peroxide value, linoleic and oleic acid content*

Sample No.	Moisture		POD (Abs/min.mg)	PPO (Abs/min.mg)	Esterase (Abs/min.mg)	Lipase (Abs/min.mg)	Acidity (g oleic acid/100 g oil)	Peroxide value (PV) (meq O_2/kg)	$C_{18:2}$ (%)	$C_{18:1}$ (%)
01	7.85*	4.62**	700	451	156	482	0.60	0	10.0	81.8
02	8.26	4.37	565	206	330	413	0.90	0	6.65	83.8
03	9.38	5.51	995	424	145	468	0.80	0	7.10	83.3
04	8.97	4.54	515	339	158	1030	0.60	0	7.80	81.1
05	7.39	5.79	683	586	106	619	0.58	0	10.8	81.9
06	9.89	3.73	376	410	196	1176	0.64	2.8	12.6	78.8
07	10.52	6.18	415	183	113	949	0.68	0	13.6	77.6
08	7.69	5.04	563	777	108	403	0.55	0	16.7	73.8
09	8.92	4.91	352	444	198	480	0.61	1.5	13.1	78.7
10	9.49	5.86	566	538	104	696	0.86	0	21.1	71.5
11	6.77	5.62	413	515	165	801	0.31	2.9	10.1	82.2
12	9.97	5.11	512	764	99.0	419	0.68	2.9	9.75	81.2
13	10.54	5.83	691	207	123	482	0.77	0.9	13.2	77.1
14	9.32	5.55	834	642	82.2	626	0.56	0	10.5	82.5
15	10.22	6.47	520	374	98.2	426	0.68	3.6	12.3	80.2
16	8.62	5.39	656	670	205	575	0.80	0	10.2	82.3
17	11.10	4.81	418	348	194	1046	0.63	0	13.6	78.6
18	7.67	5.80	456	775	78.2	379	0.68	3.5	8.64	82.8
19	10.11	4.14	517	401	143	364	0.67	0	9.10	81.9
20	10.69	5.58	467	451	91.1	705	0.79	1.6	21.4	71.2

*With shell (g/100 g); **without shell (g/100 g).

Table 3. Means for lipase, esterase, peroxidase and polyphenoloxidase (Abs/min. mg protein)

Level	Count	Lipase		Esterase		Peroxidase		Polyphenoloxidase	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	2	482	29.9	156	8.40	700	1.50	451	2.97
2	6	646	123	251	37.8	692	102	323	40.9
3	4	648	236	151	26.0	529	90.0	498	52.2
4	2	949	55.4	113	8.23	415	6.26	183	1.27
5	6	526	60.1	137	19.5	493	46.3	586	62.8
6	4	610	118	132	19.6	462	30.2	640	72.6
7	2	482	68.4	123	7.26	691	8.52	207	1.77
8	4	526	68.0	90.2	5.70	677	91.4	508	77.6
9	2	575	11.3	205	1.93	656	0.29	670	15.8
10	2	1047	20.7	194	4.17	418	7.36	348	10.1
11	4	371	21	111	19.2	486	28.7	588	108
12	2	705	26.9	91.2	0.27	467	6.41	451	0.98
Total	40	603	36.4	145	8.02	561	23.1	475	20.7

Table 4. Analysis of variance ($P < 0.05$) between the parameters of the study

Enzyme	Significant difference			
	Variety	Farming	Geographical origin	Province
Lipase	X	—	—	—
Esterase	X	—	—	X
POD	X	X	X	—
PPO	X	—	X	—

was agitated for 1 min and left to rest in darkness for 5 min. Immediately, 75 ml of distilled water were added and iodine was titrated with 0.01 N $Na_2S_2O_3$. The result was expressed as meq O_2/kg.

Moisture was determined using 5 g of hazelnut paste in a conventional kiln at 103–105°C for 3–4 h, until a constant weight was reached (Keme & Messerli, 1976).

Statistical analyses

Data were introduced into a Lotus 123 spreadsheet and averages and standards deviations were calculated using Statgraphics. Sas/Stat was used for the General Linear Model (GLM) and for an average multiple comparison with Duncan's test (Steel & Torrie, 1985).

RESULTS AND DISCUSSION

Table 2 shows the enzymatic activities of the various varieties as well as water content, acidity, peroxide content and percentages of $C_{18:1}$ and $C_{18:2}$. Table 3 shows lipase, esterase, peroxidase and polyphenoloxidase activities for each analysed sample. Table 4 presents statistical analyses. Free acidity and peroxide value are determined to compare lipase and peroxidase activities. Not all varieties share the same number of parameters, thus the General Linear Model (GLM) is applied, as the ANOVA test is not applicable (Affifi & Azen, 1977). Discrete parameters (variety, farming method, geographical origin and province) and continuous parameters (enzymatic activity of lipase, esterase, peroxidase and polyphenoloxidase) are defined. Lipoxygenase shows no activity, as already mentioned by Serra Bonvehí & Ventura Coll (1993c). Most varieties exceed the maximum water content limit at the moment of collection (5 g/100 g) (Table 2) (Serra Bonvehí & Ventura Coll, 1992).

Lipase

All varieties exceed maximum acidity (0.3 g of oleic acid/100 g of oil) in freshly collected hazelnuts (Keme *et al.*, 1983*a*,*b*) (Table 2). Higher acidity may account for

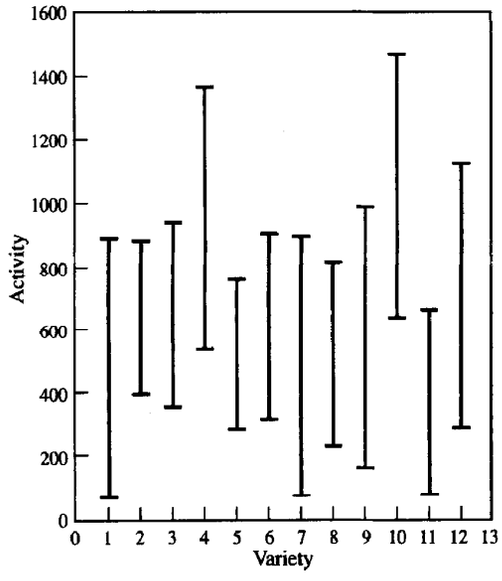

Fig. 4. Lipase activity (ΔAbs/min.mg protein).

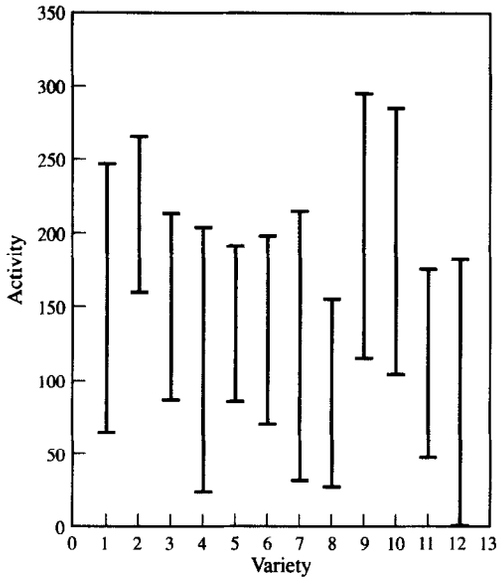

Fig. 5. Esterase activity (ΔAbs/min.mg protein).

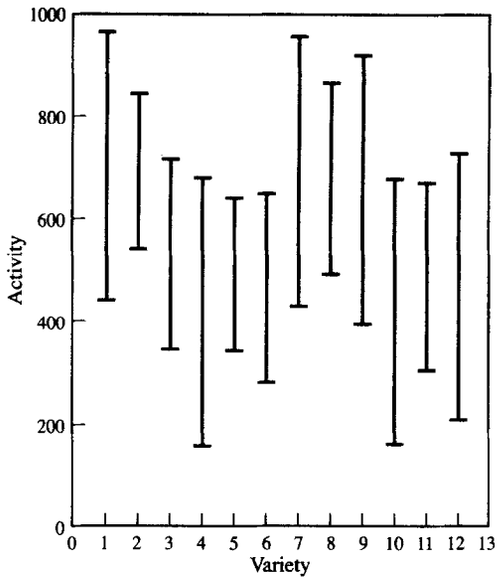

Fig. 6. Peroxidase activity (ΔAbs/min.mg protein).

the activities found in 1990, lower than those registered in 1989 for the same varieties (Serra Bonvehí & Ventura Coll, 1993*c*). This difference may be caused by the excessive water content of present in hazelnuts if they are not collected and dried in minimum time (Table 2). Highest lipase activity was detected in the varieties Tonda Italiana, GLM and Tomboul (Fig. 4). Low activities were found in Tonda Romana, Castanyera and San Giovanni.

Significant differences ($P < 0.05$) were found between varieties, but not between farming method, geographical origin and province (Table 4).

Esterase

Despite having found significant differences ($P < 0.05$) between varieties and province, this parameter offers little information as esterase activity is very low, as stated by Hadorn *et al.* (1978) and Serra Bonvehí & Ventura Coll (1993*c*). The varieties Culplà, Tonda Gentile de Langhe and T. Italiana show the highest activities. Low activities were found in T. Giffoni and Tomboul (Fig. 5).

Peroxidase (POD)

Some samples showed peroxidase indices above 1.40 meq O_2/kg, the maximum amount in freshly collected hazelnuts (Zürcher & Hadorn, 1975; Serra Bonvehí & Ventura Coll, 1993*c*). High peroxide levels are only found in irrigated varieties, so higher POD activities were expected in these samples. Varieties S. Giovanni, T. Giffoni, T. G. de Langhe and Tarragona (Castanyera, Culplà and Gironell) showed the highest peroxidase activities. Lower activities were found in T. Italiana and GLM varieties, indicating that higher lipase activity means lower peroxidase activity as stated by Serra Bonvehí & Ventura Coll (1993*c*). Italian varieties show higher peroxidase activity that those from Tarragona (Fig. 6). Statistical analysis confirms significant differences ($P < 0.05$) among varieties, farming methods and geographical origins, but not among provinces (Table 4). Dry-farmed hazelnuts show higher peroxidase activity.

Polyphenoloxidase (PPO)

The obtained results are very similar to those presented by Serra Bonvehí & Ventura Coll (1993*c*). Except for the variety S. Giovanni, the most peroxidase-active varieties are also the most polyphenoloxidase-active. As PPO shows maximum activity during fruit formation, polyphenolic compounds may also be oxidized. These, with microlepidopter perforation, originate necrotic areas in the hazelnut (Uyemoto *et al.*, 1986). The Falset

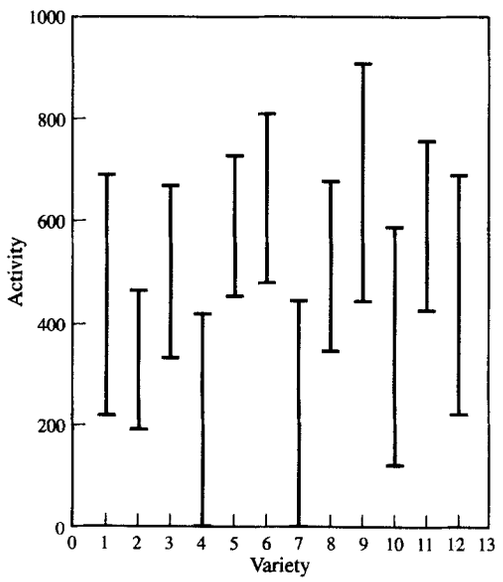

Fig. 7. Polyphenoloxidase (ΔAbs/min.mg protein).

area shows the highest polyphenoloxidase activity. Italian varieties show a different pattern. In general, polyphenoloxidase activities are lower compared to Negret and Pauetet (commercial type 'Negret'). Results appear very disperse (Fig. 7). Significant differences ($P < 0.05$) are detected between varieties and geographical origin but not between farming methods and province (Table 4).

Enzymatic comparisons among the varieties, show that all can give hydrolytic and/or auto-oxidation rancidity. Castanyera is the only variety showing minimum lipase, esterase and polyphenoloxidase activity, but not peroxidase. The Negret commercial variety shows high lipase and peroxidase activity, as stated by Serra Bonvehí & Ventura Coll (1993c). High $C_{18:2}$ percentages are found in Tomboul and Negret, and lower percentages in GLM, San Giovanni and T. Italiana. Minimum $C_{18:2}$ quantities are detected in Culplà, Castanyera and T. G. de Langhe varieties.

CONCLUSION

Variety is a key factor in enzymatic activity. Of the five studied enzymes, lipase and peroxidase clearly negatively affect preservation. Polyphenoloxidase enhances polyphenolic compounds, oxidation originating necrotic areas in the hazelnut. PPO and POD show significant differences ($P < 0.05$) between varieties and geographical origin. Only PPO shows significant differences between farming methods. In general, enzymatic activity is lower in Italian varieties.

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