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A disposable Laccase–Tyrosinase based biosensor for amperometric detection of phenolic compounds in must and wine

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ABSTRACT

An amperometric biosensor for the detection of polyphenols in wine has been developed immobilizing the two enzymes Tyrosinase and Laccase on graphite screen printed electrodes modified with ferrocene. Different immobilization procedures have been carried out, the sensor operational parameters have been optimized, determining the best conditions and the analytical method for the analysis of samples. The biosensor has been then tested with real samples, using wines and musts supplied by Astra, experimental winery, in Imola (Italy). The biosensor gave good results when employed for wine analysis, showing a good agreement with the spectrophotometric data obtained with the Folin-Ciocalteu test, the official method for polyphenols' analysis in wine. On the other hand, the measurements on musts and wines recently bottled, were seriously affected by the presence of an high level of free sulphur dioxide. SO_2 is the likely responsible for enzyme activity inhibition on the sensor. Further studies are currently proceeding to find out the most suitable conditions to obtain results not influenced by the presence of sulphur dioxide.

1. Introduction

Polyphenols are secondary metabolites of plants and they are mainly present in berry skins and seeds, but almost absent in grapes' juice. They are responsible for the sensory characteristics of wine such as colour, flavour, astringency and hardness.

Both polyphenolic content and composition of wines depend on geographical and atmospherical factors as well as on different production systems. In red wines the content of polyphenolic compounds varies from 2 to 5 g/L on average, while in white wines it is about 100 mg/L [1–3]. These compounds are essential nutritional elements and great relevance is given to their antioxidant properties, which have a definitely positive effect on human health [4]; as a consequence, it is very useful to characterize a wine by its polyphenolic content. The most common techniques used for the determination of phenolic compounds in wines are HPLC and GC [5,6], but these methods need complex and time-consuming sample pre-treatment procedures, and are unsuitable for on site analyses. Biosensors provide an alternative solution to the determination of polyphenols for their low costs and because they can be easily used to carry out analyses on field during the fermentation process [7–10].

In a previous article [11] the development of an amperometric biosensor for the determination of phenols in wines, based on screen printed graphite electrodes (SPEs), modified by mixing into the graphite's ink a redox mediator, namely ferrocene, was described. Tyrosinase enzyme was immobilized on the working electrode using different immobilization techniques [12,13].

In the present work, Laccase (p-diphenol oxidase containing copper ions) [13,14] has been co-immobilized with Tyrosinase in a sol–gel matrix of diglycerysilane (DGS) [15] with the aim of widening the range of phenolic compounds detected, due to the different catalytic activity of such enzymes.

After a preliminary study of the Laccase–Tyrosinase biosensor parameters and optimization of the suitable analytical conditions for measurements in Flow Injection Analysis (FIA), wines and samples of must, have been analyzed during the fermentation process. Spectrophotometric analyses of the samples, the Folin-Ciocalteu method [16] and the measure of the absorbance of wines at 280 nm [17], have been carried out as well, in order to compare the results obtained with the biosensor with those obtained with reference methods.

The interfering effect of sulphur dioxide, usually added during wine-making [1], in the determination of phenols by this biosensor method, is also briefly discussed.

2. Experimental

2.1. Chemicals and materials

Tyrosinase from *mushroom* (monophenol monooxigenase EC 1.14.18.1) with a specific activity of 2060 U/mg of solid, Laccase

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(EC 1.10.3.2, *p*-benzenediol:oxigen oxidoreductase) from *Trametes versicolor* 30.6 U/mg, tetraethyl orthosilicate (TEOS) 99.99%, glycerol anhydrous 99.5%, 3-amino propyl-triethoxysilane (APTES), ferrocene powder, gallic acid, phenol, caffeic acid, catechin, potassium metabisulphate $K_2S_2O_5$, monobasic and dibasic sodium phosphate, potassium chloride, Folin-Ciocalteu reagent were purchased from Sigma–Aldrich. All solutions were prepared using deionized water obtained from Millipore Synergy Milli-Q system. Screen printed electrodes (SPEs) modified with ferrocene were prepared as described in previously published works [11,18,19].

2.2. Immobilization procedure

The electrode was left under constant stirring for 1 h in a solution of APTES 10% in 0.1 M PB and 0.1 M KCl, then washed with a phosphate buffer solution to eliminate the excess of the reagent used.

DGS, prepared according to the synthesis reported by Brook et al. [15], (20 mg), was dissolved in H_2O (30 μL) and sonicated at room temperature for at least 5 min. An enzyme solution was freshly prepared by dissolving 2 mg of Tyrosinase and 1 mg of Laccase in 10 μL of buffer solution. 5 μL of the DGS solution were rapidly mixed with 5 μL of the enzyme solution. Then, 2.5 μL of this final solution were dropped on the working electrode surface of the ferrocene modified SPEs. Polymerization at room temperature usually occurred within 15 min. When Tyrosinase was immobilized individually, the enzymatic solution was prepared adding 1 mg of the enzyme in 20 μL of buffer phosphate; for the Laccase biosensor, 1 mg of the enzyme was dissolved in 10 μL of buffer solution. Then, for both the single enzyme immobilizations, the procedure described above was exactly followed. Enzyme concentrations used showed maximum sensor stability and sensitivities (data not shown).

Enzymatic activity for both free and immobilized Laccase and Tyrosinase was experimentally assayed by using an electrochemical method. Tyrosinase and Laccase immobilized in DGS showed respectively an enzymatic activity yield value of 26% and 16%. Such values were expected, due to the effect of enzyme entrapment in gel.

2.3. Apparatus for amperometric measurements

All measurements were carried out in a FIA system including a peristaltic pump Gilson™ Model Minipuls 3, an injector valve from Omnifit with a 100 μL fixed volume sample injector loop and a Perspex homemade flow cell [20]. Amperometric measurements were performed using a portable, lab-developed, chronoamperometer (Domotek Instr, Italy) and an applied potential of 0.050 V versus Ag/AgCl reference electrode (RE) was used throughout all the experiments.

2.4. Spectrophotometric methods

For comparison purposes, wines were also analyzed by spectrophotometry. The Folin-Cioacalteu test was carried out according to the following procedure: 1 ml of sample (wines were diluted 1:100 or 1:50), 7 mL of deionized water, 0.5 mL of Folin-Ciocalteu reagent and 1 mL of a 20% sodium carbonate solution were added in this order to a 10 mL beaker and diluted to volume with deionized water. The resulting solution was stirred and was allowed to react for half and an hour at room temperature in darkness. The absorbance was then read at 760 nm by a CaryWin 200 spectrophotometer. The total phenolic content, expressed in molar equivalents of gallic acid, was evaluated from the absorbance value by interpolation into the calibration plot obtained with gallic acid standard solutions, multiplying the resulting value by 10 and by the proper dilution rate.

For the determination of the total content of polyphenolic compounds of wines the polyphenol index I_{280} was also considered: wine was diluted with water (1:100 or 1:50) and the absorbance was measured directly at 280 nm. The value of I_{280} for each sample was given as the absorbance multiplied by the proper dilution rate. In both methods the blank solution was an hydro alcoholic solution (12%, v/v ethanol) of tartaric acid 3 g/L.

3. Results and discussion

3.1. Optimization of the operational parameters of the bi-enzyme biosensor

The main operational parameters of the Laccase–Tyrosinase biosensor have been studied in FIA, determining the best conditions for the analysis of the samples. The variation of the response of the bi-enzyme biosensor was investigated as a function of the applied potential at the graphite electrode in the range between −50 and +450 mV versus Ag/AgCl obtaining the maximum current intensity at +50 mV (Fig. 1). The optimal pH for phosphate buffer which gave the best value of the amperometric response was 6.0 (Fig. 2) and the chosen flow rate of the carrier was 0.45 mL/min as the best compromise between the intensity of the signals and the time of analysis (Fig. 3).

Laccase/Tyrosinase (w/w) ratio, used in the immobilization reaction, was investigated in the range from 0.5:1 to 1:2, for both the enzymes, versus the sensitivity values of the corresponding calibration curves for phenol. Sensors with an enzymatic 1:2 ratio were chosen for the following measurements as it corresponds to the highest sensitivity value of the calibration curve of phenol, moreover, they gave stable electrical signals and were least affected by enzymatic leaching effects.

Operational stability of the bi-enzyme biosensor, for repetitive injections of a 0.1 mM phenol standard solution over a period of 5 days, was also studied (Fig. 4); the data show error bars related to the measurements on a single day (about 25 injections/day).

As regards the repeatability of the Laccase–Tyrosinase biosensor, it was obtained respectively a value of relative standard

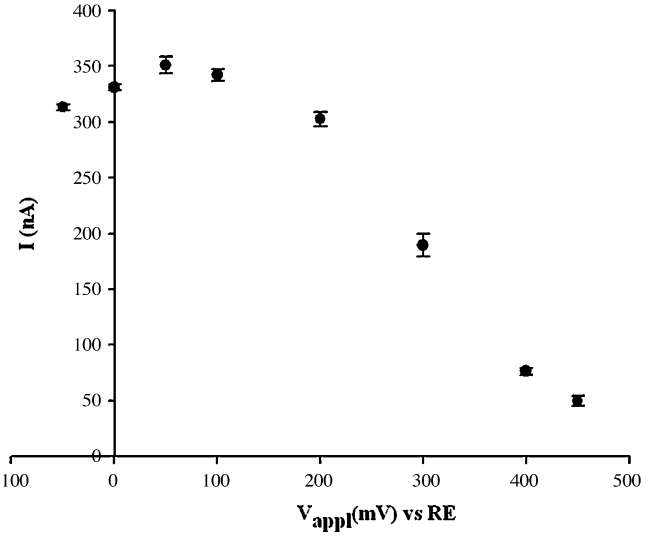

Fig. 1. Amperometric response of the Laccase–Tyrosinase biosensor after injection of 0.05 mM phenol solution as a function of applied potential. Experimental conditions—carrier: phosphate buffer 0.1 M containing KCl 0.1 M (pH 6.0) with a flow rate of 0.45 mL/min; injection valve with a loop volume of 110 μL.

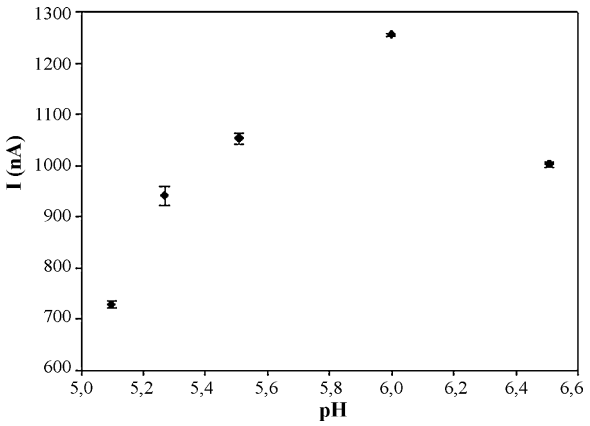

Fig. 2. Amperometric response of a Laccase–Tyrosinase biosensor after injection of 0.1 mM phenol solution as a function of the pH of phosphate buffer solutions. Experimental conditions—V_{appl} = +50 mV vs. Ag/AgCl, carrier with a flow rate of 0.45 mL/min; loop volume 100 μL.

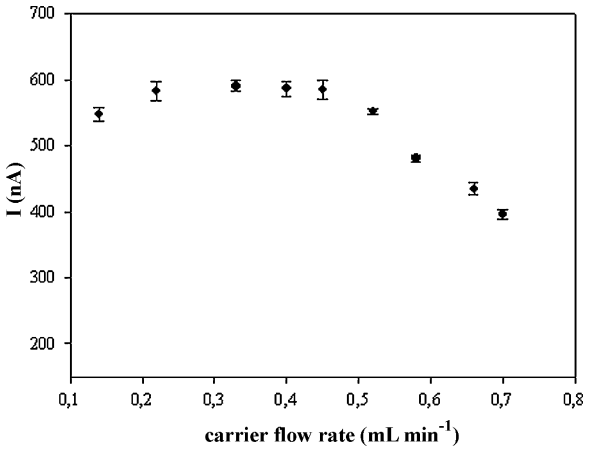

Fig. 3. Effect of the carrier flow rate on the amperometric response of the Laccase–Tyrosinase screen printed ferrocene modified electrode; 100 μL of 0.05 mM phenol were injected into the carrier stream. The carrier was 0.1 M phosphate buffer (pH 6.0) and the applied potential was +50 mV vs. Ag/AgCl.

deviation (RSD) of 6.9% (n = 12, with n number of consecutive measurements) for a 0.1 mM phenol standard solution and a RSD of 9.8% (n = 12) for a 0.1 mM gallic acid solution. These values indicate a good repeatability of the biosensor, which can be used for different substances retaining its analytical performances. In Fig. 5 it is shown an example of FIA responses of a Laccase–Tyrosinase biosensor for repetitive injections of a 0.05 mM phenol standard solution.

Reproducibility is also quite good with a RSD value of 4.9% obtained for gallic acid with five different biosensors, produced

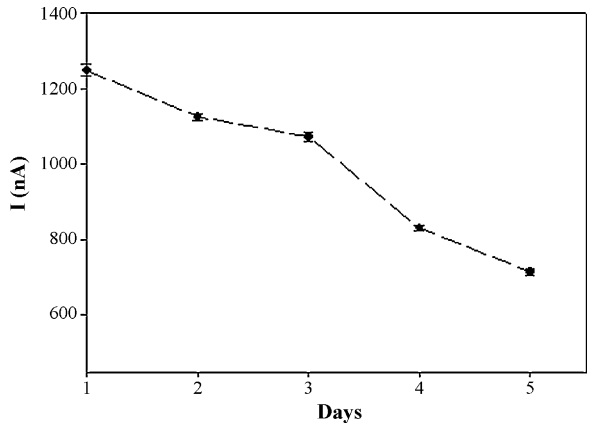

Fig. 4. Operational stability of a Laccase–Tyrosinase biosensor obtained after injections of a 0.1 mM phenol solution in FIA. Experimental conditions—V_{appl} = +50 mV vs. Ag/AgCl RE, carrier: phosphate buffer 0.1 M containing KCl 0.1 M (pH 6.0) with a flow rate of 0.45 mL/min, injection valve with a loop volume of 110 μL.

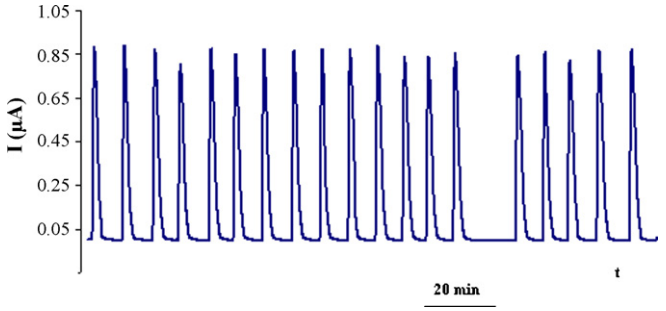

Fig. 5. FIA responses of a Laccase–Tyrosinase biosensor for repetitive injections of a 0.05 mM phenol standard solution. Experimental conditions as reported in Fig. 4.

with the same technique following the same enzyme immobilization procedure.

3.2. Comparison of the analytical performances of a Laccase–Tyrosinase biosensor with a Tyrosinase biosensor and Laccase one

We compared the calibration parameters measured for a Laccase–Tyrosinase based biosensor with those detected for a Tyrosinase and a Laccase biosensor in the same FIA conditions (data shown in Table 1). As expected, the co-presence of the two enzymes on the sensing area allows to measure a wider range of phenolic compounds. It can be noticed that the LOD of the single enzyme sensors for different and complementary phenolic compounds is sensibly higher. As we are interested in an estimate of total phenolic compounds, the slight loss in LOD, showed by the bi-enzyme biosensor for some phenolic substrates, is fully compensated for by

Table 1
Comparison of sensitivity, correlation coefficient (R^2) and limit of detection (LOD) values for different phenolic compounds obtained with a bi-enzyme biosensor, a Tyrosinase biosensor and a Laccase biosensor in FIA.

	Laccase biosensor			Tyrosinase biosensor			Laccase–Tyrosinase biosensor		
	Sensitivity ± σ_n (μA/mM)	R^2	LOD (μM)	Sensitivity ± σ_n (μA/mM)	R^2	LOD (μM)	Sensitivity ± σ_n (μA/mM)	R^2	LOD (μM)
Phenol	0.072 ± 0.004	0.9927	620	4.814 ± 0.028	0.9984	10	11.067 ± 0.134	0.9994	2
Gallic ac.	0.103 ± 0.008	0.9889	380	0.173 ± 0.030	0.9871	58	0.339 ± 0.008	0.9977	50
Caffeic ac.	6.255 ± 0.494	0.9816	6	0.853 ± 0.040	0.9580	78	1.218 ± 0.024	0.9992	24
Catechin	1.028 ± 0.047	0.9939	20	0.207 ± 0.029	0.9630	140	0.435 ± 0.021	0.9930	40
Catechol	3.021 ± 0.216	0.9849	10	–	–	–	–	–	–

Experimental conditions: V_{appl} = +50 mV vs. Ag/AgCl, carrier: phosphate buffer 0.1 M containing KCl 0.1 M at pH 6.0 (for the bi-enzyme biosensor and the Laccase sensor) and at pH 6.5 (for the Tyrosinase biosensor) with a flow rate of 0.45 mL/min, injection valve with a loop volume of 110 μL.

Table 2
Analytical characteristics of some amperometric biosensors for the determination of phenol.

Biosensor	E_{appl} (mV)	Technique	Sample matrix	Linear range (10^{-4} M)	Sensitivity (μA/mM)	R^2	LOD (10^{-6} M)	Stability	Ref.
Tyr-nAu-GCE	−100	Batch	Wine	0.01–0.4	82	0.993	0.21	18 days	[22]
Tyr-Os-complex-EDP-Pt wire	−150	On line sequential analyzer	Phenolic compounds	0.2–1.0	0.15	–	0.1	45 h[a]	[23]
PCS(Tyr)-HRP-SPCEs	−100	Batch	Waters	0.25×10^{-3} to 0.45	62.43	–	2.5×10^{-3}	92% of the initial activity retained after 60 days	[24]
Lac-GCE	−200	FIA	Wine	2.3×10^{-3} to 0.12[b]	3.15[b]	0.999 [b]	0.2[b]	4 days[b]	[9]
Lac-Tyr/Sonogel-Carbon electrode	−150	Batch	Beer	10^{-3} to 0.15[b]	14.10[b]	–	19×10^{-2}[b]	80% of its stable response for 3 weeks[b]	[25]
PPO-GCPE	−100	–	Tea, wine	Up to 0.7	9.0	–	1.7	90% of the value of sensitivity for 4 months	[26]

nAu-GCE: glassy carbon electrode modified with electrodeposited Gold nanoparticles;
Os-complex-EDP-Pt wire: osmium complex modified electrodeposition polymer on Pt wire;
PCS-: poly(carbamoylsulfonate) hydrogel;
Tyr: tyrosinase;
HRP: horseradish peroxidase;
SPCEs: screen printed carbon electrodes;
Lac: laccase;
PPO: polyphenoloxidase;
GCPE: glassy carbon paste electrode.

[a] For catechol.

[b] For gallic acid.

the larger number of phenols that can be revealed. The LOD values were graphically calculated as proposed by Meyer and Zund [21] and are summarized in Table 1.

The analytical characteristics of some amperometric biosensors for polyphenols detection, described in more recent publications, are summarized in Table 2. It can be seen for each analytical parameter a high variability of values due to the different nature of the considered biosensor. A direct comparison of the methods is not so straightforward, because different electrodes are used and reactions with completely different mechanisms (detection of the oxygen consumption, re-oxidation of the enzymatic reaction products, mixing of redox mediators) are involved.

3.3. Results on wine samples

Samples of must and wines, supplied by ASTRA, an experimental winery in Tebano-Faenza, Italy, were analyzed using the two spectrophotometric methods and the amperometric bi-enzyme biosensor under FIA. The results of the measurements are quite different in values as they are obtained with analytical methods based on different principles. In fact, the Folin-Ciocalteu method consists in a redox reaction of phenols with the phosphomolybdic reagent followed by the colorimetric detection of the resulting products, and the other spectrophotometric method is a direct detection of the absorbance at 280 nm, while our biosensor is based on an enzyme oxidation.

However, reporting in a graph the data of both spectrophotometric determinations and of the Laccase–Tyrosinase biosensor as a function of time, the resulting curves (Fig. 6) have similar shapes. This demonstrates that the biosensor method is consistent with both reference spectrophotometric methods, therefore the presented analytical device can be practically used as an index of total polyphenolic content in wine during fermentation. This is an important result for wine-industry, because the detection of polyphenolic compounds can be carried out during all the stages of the wine-making from the arrival of the grapes at the winery to the final product on the bench.

3.4. Sulphite in wine samples as interfering substance

In some moments during fermentation, SO_2 and sulphite are added to must and wine; their use in wine-making is still necessary to control the state of a must, preventing oxidation and microbial spoilage. These substances, unfortunately, act as inhibitors of catalytic activity of both Laccase and Tyrosinase, and consequently, they seriously compromise the biosensor responses. All the same, the measurements of polyphenols can still be carried out adding a simple pre-treatment step along the procedure. The sample of

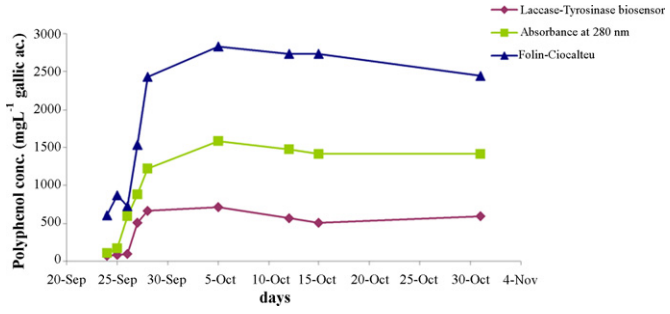

Fig. 6. Polyphenolic content, expressed in mg/L of gallic acid, of must and wine samples collected during fermentation obtained with the reference spectrophotometric methods (squares and triangles) and the Laccase–Tyrosinase biosensor (diamonds). The analysis of the biosensor was performed in FIA using the experimental conditions of Fig. 3.

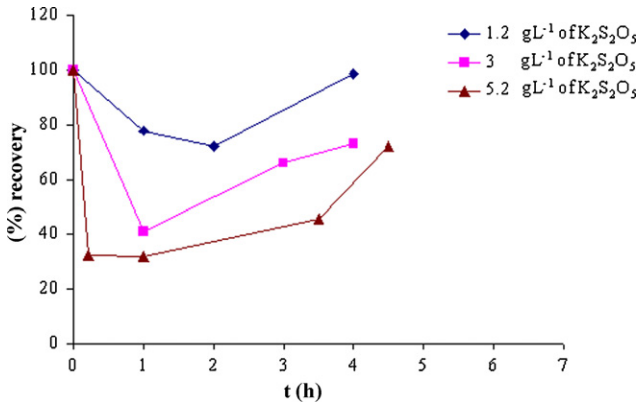

Fig. 7. Comparison between the recovery percentages of differently spiked sample of wines with potassium metabisulphite over a period of time. Measurements performed in FIA in the same conditions of Fig. 3.

wine freshly added with SO_2 or sulphite was acidified with HCl to pH <2.0, stirred for about 5 h and then diluted with phosphate buffer to the optimum pH for the enzymes. The results of the measurements, shown in Fig. 7, were obtained with samples of wine spiked with, largely overestimated quantities of $K_2S_2O_5$; it can be noticed that after this sample treatment the percentage of recovery is between 75% and 100%. Further experiments are currently going on to reduce the time required to minimize this interference.

4. Conclusions

An amperometric biosensor was realized co-immobilizing Laccase and Tyrosinase on the working electrode of a ferrocene modified screen printed electrodes. The immobilization procedure of the enzymes in DGS is very simple and quick and it can be carried out just before starting the measurements. The bi-enzyme biosensor shows good analytical performances comparable with data appearing in literature, allowing to perform measurements at a low applied potential value so to greatly reduce the effect of interfering substances. Moreover, it can be used as an index of phenolic compounds in samples of wine providing results consistent with those obtained with the reference spectrophotometric methods. During fermentation, immediately after the addition of sulphite, this method requires, at the moment, a pre-treatment to eliminate sulphur dioxide responsible for the enzyme inhibition. Further experiments are being addressed in order to reduce the time needed for the pre-treatment. Such cheap and disposable device can usefully support and substitute traditional chromatographic techniques for in field measurements through all the steps of wine-making, allowing for real time monitoring of phenols during fermentation and in the final product.

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