APPLICATION OF A REVERSED-PHASE HPLC METHOD FOR QUANTITATIVE *P*-COUMARIC ACID ANALYSIS IN WINE

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Abstract

Aims: This paper presents a rapid chromatographic method to monitor the concentration of *p*-coumaric acid in wine and in bioconversion studies.

Methods and results: RP-HPLC method was validated in synthetic wine medium and in natural red wine. Mobile phase composition was water 77%, acetonitrile 23%. Formic acid was added to control pH at 3.5. The flow was 0.7 mL/min and the temperature 30 °C. The detection was done using UV at 305 nm. The linearity range was validated between 0.5 and 15 mg/L. The resolution was respectively 5.35 and 2.99. The detection and quantification limits were 0.01 mg/L and 0.04 mg/L. This method was used to study p-coumaric acid bioconversion into 4-ethylphenol and 4-vinylphenol, and to study this acid adsorption in enological conditions.

Conclusions: This paper presented a simple HPLC method to monitor the concentration of *p*-coumaric acid in synthetic media and natural wine. It was used to study the *p*-coumaric acid bioconversion rates and mechanism.

Significance and impact of the study: This method is useful to monitor p-coumaric acid concentration, which helps to predict amounts of 4-ethylphenol or 4-vinylphenol that can be produced in wine. This method can be helpful to control undesirable phenolic flavors potential in wine.

Key words: *p*-coumaric acid, 4-ethylphenol, *Brettanomyces* sp., *Saccharomyces* sp., HPLC

Résumé

Objectifs : Cette étude présente une méthode chromatographique rapide pour le dosage de l'acide *p*-coumarique dans les vins et lors des études de sa bioconversion.

Méthodes et résultats : Une méthode RP-HPLC a été validée dans un milieu synthétique et dans un vin rouge naturel. La phase mobile a été : eau 77%, acétonitrile 23%. Le pH a été ajusté à 3.5 par l'acide formique. Le débit a été 0.7 mL/min et la température 30 °C. La détection a été effectuée par UV à 305 nm. La zone de linéarité a été validée entre 0.5 et 15 mg/L. La résolution a été respectivement 5.35 et 2.99. Les limites de détection et de quantification ont été 0.01 mg/L et 0.04 mg/L. La méthode a été utilisée pour étudier la bioconversion de l'acide *p*-coumarique en 4-éthylphénol et en 4-vinylphénol. Elle a été utilisée aussi pour étudier l'adsorption de cet acide en conditions œnologiques.

Conclusion : Une méthode HPLC pour le dosage de l'acide *p*-coumarique en milieu synthétique ou dans un vin naturel a été validée et utilisée pour étudier le rendement de la bioconversion de l'acide *p*-coumarique.

Signification et impact de l'étude : Cette étude est utile pour le dosage de l'acide *p*-coumarique, ce qui aide à prédire les quantités de 4-éthylphénol ou 4-vinylphénol qui peuvent être potentiellement produites. Cette méthode peut aider ainsi à contrôler l'apparition des odeurs phénoliques indésirables dans les vins.

Mots clés : acide *p*-coumarique, 4-éthylphénol, *Brettanomyces* sp., *Saccharomyces* sp., HPLC

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INTRODUCTION

Polyphenols have received considerable attention over the past years because of their diverse activities in plants and food (Garcia Sanchez *et al*., 1988). Some of these phenolic compounds are the cause of adverse tastes and color changes in food products (Krygier *et al*., 1982). Hydroxycinnamic acids are included in the large polyphenol family (BudicLeto *et al*., 2003). The present study focuses on one specific hydroxycinnamic acid, the *p*-coumaric acid.

In grapes, *p*-coumaric acid is a polyphenol precursor, especially for flavonoids, flavones and flavonols (Hrazdina *et al*., 1984). It is a primary substrate for enzymes to generate resveratrol. In winemaking, *p*-coumaric acid is released into the grape juice during the maceration process and its concentration can reach 60 mg/L. After fermentation and clarification, the *p*-coumaric acid concentration will not exceed 15 mg/L in red wine (Chatonnet *et al*., 1997, Goldberg *et al*., 1998).

The monitoring of *p*-coumaric acid in wine has become crucial since it has been demonstrated that the sequential action of two enzymes of *Brettanomyces* yeast on this compound is the principal cause of 4-ethylphenol production (Baumes and Cordonnier, 1986, Chatonnet and Boidron, 1988, Chaudray *et al*., 1968, Dubois *et al.*, 1971, Etievant, 1981, Schimidzu and Watanabe, 1982). The cinnamate decarboxylase first converts *p*-coumaric acid into 4-vinylphenol, which is subsequently converted into 4-ethylphenol by the vinylphenol reductase. The first enzyme is common to many microorganisms in wine, especially *Saccharomyces cerevisiæ*. Volatile phenol synthesis by *Saccharomyces sp*. yeast depends on the nature of the strain and the presence of certain polyphenolic inhibitors, and it is strictly limited to alcoholic fermentation (Chatonnet *el al*., 1993). The second enzyme is specific to a few microorganisms; the most important one is known to be *Bretanomyces sp*. (Chatonnet *et al*., 1992, Dias *et al*., 2003, Edlin *et al*., 1995). The 4-ethylphenol molecule is associated with a non desirable organoleptic characteristic: the horse sweat odor. Therefore, its presence in wine causes great economic losses (Chatonnet *et al*., 1997).

We have recently demonstrated that *p*-coumaric acid is involved in many physical, chemical and biochemical interactions in wine (Salameh *et al*., 2008). The analysis and the monitoring of *p*-coumaric acid during fermentation, in wine or synthetic media, can provide valuable data which help us to determine precisely its available concentration and predict its biosynthetic fate. This is useful to study its specific bioconversion kinetics into 4-vinylphenol or 4-ethylphenol, and to evaluate the 4-ethylphenol production (Dias *et al*., 2003, Edlin *et al*., 1995, Salameh *et al*., 2008).

Knowing that yeasts and wine enological components can adsorb *p*-coumaric acid (Morata *et al*., 2003, Morata *et al*., 2005), a way to avoid its bioconversion into 4-ethylphenol would be to eliminate its presence in wine (Salameh *et al*., 2008). Therefore, it is very important to have a precise, easy and fast method to study *p*-coumaric acid adsorption in wine conditions.

There are several published methods for phenolic compounds analysis in wine. These methods include fluorimetric techniques (Garcia Sanchez *et al*., 1988), polarographic techniques (Shleev *et al*., 2004), but the most common are chromatographic techniques (Ho *et al*., 1999, O'Neill *et al*., 1996, Rizzo *et al*., 2006, Tuzen and Ozdemir, 2003, Vanbeneden *et al*., 2006). These methods are mainly different in their analysis time, the eluent phase, the detection used or the sample preparation. All these techniques are well adapted to detect *p*-coumaric acid and many other polyphenols in wine. The problem is that these methods can be time consuming for enological use, especially when *p*-coumaric acid is to be specifically detected in fundamental studies.

Since *p*-coumaric acid is the crucial substrate for 4-ethylphenol occurrence in wines, it is important to study precisely its disappearance from the media as 4-ethylphenol is produced. To our knowledge, there is no validated HPLC method to monitor specifically *p*-coumaric acid in wine, in the purpose of studying its bioconversion or its physical availability in laboratory or enological conditions. For all these reasons, it is important to optimize an analytical technique adapted to monitor specifically this hydroxycinnamic acid in the media. Hence, the aim of this work is not to add another classical method among others of the same type, but to present a rapid and simple application of an HPLC-UV method to determine *p*-coumaric acid concentration in wine.

MATERIALS AND METHODS

1. Reagents, solvents and materials

Tartaric acid, fructose, glucose, KH_2PO_4 and $MgSO_4$ powder were purchased from Merck Darmstadt. Mobile phase components as acetonitrile, formic acid and pure ethanol were all analytical grade and purchased from Merck Darmstadt. Polyvinylpolypyrrolidone povidone (PVPP), *p*-coumaric acid, citric acid, malic acid and glycerol were purchased from Sigma. The yeast extract powder was purchased from Oxoid, the yeast cell wall powder from Oenofrance, and the *Saccharomyces cerevisiæ* strain from Lallemand Inc.

2. Synthetic media

The synthetic wine medium composition was as follows : glucose 10 g/L , fructose 10 g/L, yeast extract 0.5 g/L, $(NH_4)_2SO_4$ 0.5 g/L, citric acid 0.3 g/L, malic acid 3 g/L, tartaric acid 2 g/L, $MgSO_4$ 0.4 g/L, KH_2PO_4 5 g/L, and glycerol 6 g/L. The pH was adjusted to 3.5 before autoclaving. Ethanol was added to 10% v/v after autoclaving.

The synthetic grape juice medium composition was as follows : glucose 100 g/L, fructose 100 g/L, yeast extract 1 g/L, $(NH_4)_2SO_4$ 2 g/L, citric acid 0.3 g/L, malic acid 5 g/L, tartaric acid 5 g/L, $MgSO_4$ 0.4 g/L, and KH_2PO_4 5 g/L. The pH was adjusted to 3.5 before autoclaving.

3. Quantification of *p*-coumaric acid in wine

A Merlot wine was used to validate the RP-HPLC method under natural wine conditions. The *p*-coumaric acid concentration in the wine was 2 mg/L. To check whether the peak detected at 12.3 min corresponded to *p*-coumaric acid, *p*-coumaric acid was added to the wine to a final concentration of 5 mg/L. Samples were injected after filtration on a 0.4 µm membrane. Considering that the maximum concentration of *p*-coumaric acid in wines is in the range of 10 mg/L, no dilution was done.

4. High performance liquid chromatography system

All the samples were filtered through a 0.4 µm membrane and analyzed by direct injection on a Thermo separation High Performance Liquid Chromatography system, equipped with a Spectra Series UV 150 detector and an AS 100 autosampler. PC 1000 ChromStation - Thermoseparation products™ - was used for data acquisition and analysis. Phenolic compounds were separated on an ODS-2 5 µ™ (Waters®) column (4.6 x 250 mm) following a Spherimarge ODS-2™ pre-column.

The automatic injector was set to full loop of 20 µL. The mobile phase composition was as follows: water 77% and acetonitrile 23%. Formic acid was added to a concentration of 0.12 g/L, which corresponded to 120 µL of formic acid per liter of mobile phase solution, to adjust the final pH to 3.5. The flow rate was fixed at 0.7 mL/min and the temperature was fixed at 30 °C. The detector used was a UV spectrophotometer set at 305 nm. Quantification was based on peak areas as determined by the software.

5. Method validation

The method was validated with respect to specificity, robustness, stability, linearity, recovery and repeatability, in accordance with the European Commission Decision 2002/657/EC (ICH, 1996).

The specificity was tested for matrix interference. A significant number of representative blank samples of each matrix (five of each matrix of synthetic media) were analyzed and tested for any interference in the region of the chromatogram, where the targeted analytes are expected to elute (10-15 min).

To estimate the robustness of the method, the effect of minor changes in experimental conditions was tested. Besides room temperature, samples of *p*-coumaric acid were analyzed at three different temperatures (25, 30 and 35 °C). The effect of pH (mobile phase and matrix) was studied by preparing 3.2, 3.5 and 3.7 pH solutions. Finally, three different flow rates (0.6, 0.7 and 0.8 mL/min) were tested.

The linearity of the calibration curve was validated with five concentration points, including the limit of quantification (LOQ), assayed in triplicate in the range of 0.04 -15 mg/L. The LOQ and the limit of detection (LOD) were calculated on the basis of the standard deviation of the response and the slope obtained from the linearity plot of *p*-coumaric acid, as described in the ICH guideline (ICH, 1996). LOD and LOQ were calculated as 3.3 α/S and 10 α/S, respectively, where α is the standard deviation of the y-intercept and S is the slope of the regression line.

Since no certified reference materials were available for *p*-coumaric acid solutions, the precision, recovery, and repeatability were investigated. Recovery was determined by experiments using samples at three different concentration levels including the LOQ. The quantification potential of the method was tested in a merlot wine.

For the repeatability tests, fresh samples were prepared and tested at six different concentration levels including the LOQ. The analysis of each concentration level was performed in six replicates in 1 day (intraday precision). To determine the reproducibility of the method, the same concentrations of the 10 mg/L fresh solution of the calibration curve were analyzed for six consecutive days after being stored in darkness at -20 °C.

Stability was checked for the calibration curve solutions and also for samples taken from synthetic wine fermentation. For calibration curve solutions, each concentration sample was analyzed directly after preparation and after being kept in darkness at 4 °C for 1 day, 1 week and 1 month. Similarly, the stability of the samples resulting from fermentation was tested. The supernatants of these samples were analyzed directly after being collected and after being stored in darkness at -20 °C for 1 day, 1 week, and 1 month.

The efficiency value (N), which gives an indication of the effectiveness of the total system, was calculated by applying:

$$N = 16 \left(\frac{T_r}{W} \right) 2$$

where W is the peak width at base line, and T_r is the retention time of the *p*-coumaric acid. We calculated as well the peak asymmetry, defined by

$$As = \frac{B}{A}$$

where B and A are respectively the right and left part of the segment crossing the peak at 10% peak height. The capacity factor K' of the *p*-coumaric acid is a measure of the degree to which that component is retained by the column relatively to Tm, an unretained solvent. It is calculated using the following formula:

$$K' = \frac{(T_r - T_m)}{T_m}$$

where T_r is the retention time of *p*-coumaric acid, T_m is the retention time of the solvent. The separation factor α of the separation between the *p*-coumaric acid peak and the mean peak of the synthetic wine medium is defined by:

$$\alpha = \frac{K'_b}{K'_a}$$

where K'_b is the capacity factor of the second peak, and K'_a is the capacity factor of the first peak. Another indication revealing the good separation and quantification of the *p*-coumaric acid in our HPLC conditions is the resolution. In fact, Rs is defined as the amount of separation between two adjacent peaks. It is given by:

$$Rs = \frac{1}{4}\sqrt{N_b}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{K'_b}{1 + K'_b}\right)$$

6. Method application

Two fermentations were carried out to study the bioconversion of *p*-coumaric acid. The first fermentation was carried out in a synthetic wine medium, using a *Brettanomyces bruxellensis* yeast strain isolated from a winery situated in the South West of France. The second fermentation was carried out in a synthetic grape juice medium, using a commercial *Saccharomyces cerevisiæ* yeast strain. Fermentation conditions are as described by Salameh *et al*., 2008. Briefly, both culture media were inoculated with yeast at a cell density of 3.10^6 cells/mL. Culture was carried out in 500 mL Erlenmeyer flask in 300 mL of liquid medium, with shaking at 250 rpm. After inoculation, cultures were incubated at 30 °C. To reproduce its natural concentration range in wine and grape juice, *p*-coumaric (dissolved in 1mL of pure ethanol) was added at 10mg/L final concentration in the *Bretanomyces sp*. fermentation culture and 100 mg/L in the *Saccharomyces sp*. fermentation culture, when populations reached stationary growth phase. Two minutes after adding *p*-coumaric acid, the first sample was collected, centrifuged, and the supernatant was analyzed by HPLC as described above to determine the *p*-coumaric acid concentration. A dilution of *p*-coumaric acid (1/10) was done in the *Saccharomyces* fermentation samples. The 4-vinylphenol and 4-ethylphenol concentration in samples was detected by GC/MS analysis at the CRAO Laboratory (Toulouse-France). Samples were collected at different time points for 100 hours to monitor the *p*-coumaric acid consumption in time.

Different levels of *p*-coumaric acid adsorption were checked by using PVPP and yeast cell walls in a synthetic wine medium, where the adsorbent concentration was 3 g/L and the contact time was 2 hours. The temperature was fixed at 25 °C. In all the cases, a medium free of yeasts or adsorbent, but containing the same *p*-coumaric acid concentration, was monitored in parallel.

7. Gas chromatography system for 4-vinylphenol and 4-ethylphenol analysis

The concentrations of 4-vinylphenol and 4-ethylphenol were measured using gas chromatography, after pre-concentration on SPME fibers, 0.85 µm polyacrylate film (Supelco inc.) and using head space method. Detection was performed by mass spectrometry ion trap system. Detection was acquired at ion mass [106,5-107,5 and 121,5-122,5] for 4-ethylphenol and [90,5-91,5 and 119,5-120,5] for 4-vinylphenol, using single ion monitoring (SIM) mode. Dimethyl Phenol (DMP) was used as internal standard. After adsorption on SPME fiber, phenolic compounds desorption was done directly in the injection room at 240 °C for 3 min. Splitless mode was used. The mobile gas was helium with a 1 mL/min flow.

RESULTS AND DISCUSSION

1. Method validation

Statistical analyses were performed at different stages on the values obtained from different peaks at different concentrations. Three different blank matrix samples (hydroalcoholic solution - 10% v/v ethanol, synthetic wine medium, and synthetic grape juice) were analyzed to check the specificity of the method. None of them showed major interference at the retention times of the eluted *p*-coumaric acid.

The effect of variations in the analytical conditions was studied to evaluate the robustness of the method. Solutions of *p*-coumaric acid at a concentration of

10 mg/L were monitored under varying parameters (temperature, mobile phase and sample pH, and flow rate). Analyses were carried out at 25° C, 30 °C and 35 °C and the method was validated at 30 °C. No differences in peak shape and resolution were recorded. Non significant changes in retention times were recorded.

Three different pHs were tested for the mobile phase and the samples. The retention time generally decreased when the pH decreased, still this variation could not be considered significant because of the small variation for the retention. Moreover, the specificity remained elevated and there is low pH variation in wine (3.1 - 3.8). Similarly, the retention time decreased as the pump flow increased. Higher flow rate reduced the analysis time by approximately 6 minutes. The resolution factors did not remain constant with flow or pH variation. All together, these tests lead us to set the pump flow at 0.7 mL/min, mobile phase pH at 3.5 and temperature analysis at 30 °C.

The calibration curves were obtained by least-squares linear regression analysis of the peak area versus concentration of analyte in hydroalcoholic solutions 10%v/v ethanol - water. The test showed a good linearity in the tested range (0.5 - 15 mg/L). The area response obeyed the equation y = mx + C, where the intercept C was zero within 95% confidence limits and the square correlation coefficient (R^2) was always greater than 0.999.

The linearity of the peak area versus phenolic acid concentration was validated in the range of the concentrations tested. After validating the calibration curve, the analysis on synthetic wine medium was acquired. The detection limit of *p*-coumaric acid was found to be 0.01 mg/L and the quantification limit of *p*-coumaric acid in this method was found to be 0.04 mg/L.

The recovery studies were performed in the calibration curve solutions as described above. The mean recovery of *p*-coumaric acid was found to be 99.76% ± 2.3 (Table 1).

The quantification potential of the method was tested in natural wine. The peak found had an area of 529750 ± 435 which corresponds to 1.95 ± 0.02 mg/L. After adding *p*-coumaric acid at a concentration of 3 mg/L, the peak area increased to 1318453 ± 523. The difference in the areas was 788703 ± 632 which corresponds to 2.95 ± 0.03 mg/L (figure 2). This emphasizes the good quantification of the *p*-coumaric acid by the method. Even though the *p*-coumaric acid is known to be unstable in analysis (Salameh *et al*., 2008, Herrera *et al*., 1998, Dugelay *et al*., 1995), the chromatographic conditions described above let us analyze this acid in natural and synthetic wine if new calibration curve solutions are used, where *p*-coumaric acid is dissolved in hydroalcoholic solution (10% ethanol-water).

The percentage coefficient of variation (% CV) for each well-recovered injection in the repeatability tests was under the acceptance range of 5% (Table 2), thus confirming that the method is sufficiently precise.

The stability of samples was checked. The results showed that the calibration curve solutions were unstable over time, even if stored at 4 °C. It is thus important to prepare fresh hydroalcoholic solutions of *p*-coumaric acid. On the contrary, sample supernatants were stable when stored at -20 °C, over the time period tested (1 month).

2. Method suitability

The uniformity and normality of the peaks were first studied in a hydroalcoholic solution 10%, then in a synthetic wine medium, and finally in natural wine. The retention time for the *p*-coumaric acid in the hydroalcoholic solution 10% was 12.54 ± 0.23 min. Tests performed on *p*-coumaric acid, in synthetic wine medium and in natural wine, revealed that the *p*-coumaric acid

Table 1. Recovery test of p-coumaric acid

	Detected concentration (mg/L)			Mean (mg/L) ± SD	Recovery (%)	Mean Recovery (%) ± SD
Theoretical added concentration (mg/L)	inj.1	inj.2	inj.3			
0.04	0.04	0.04	0.03	0.04 ± 0.01	101.6	99.7 ± 2.4
3	2.9	3	2.9	2.9 ± 0.1	97.1	
8	8	7.9	8.1	8 ± 0.1	100.5	

Table 2. Repeatability tests of *p*-coumaric acid

Repeatability (intra day) Theoretical concentration (mg/L)	Found concentration (mg/L) inj.1	inj.2	inj.3	inj.4	inj.5	inj.6	Mean (mg/L) ± SD	%CV
0.04	0.04	0.03	0.04	0.04	0.03	0.04	0.04 ± 0.1	4.9
1	1	1	1.1	0.9	0.9	1	1 ± 0.1	5.0
3	2.9	2.8	2.9	3	3.1	2.9	2.9 ± 0.2	3.9
6	6	6.2	5.9	5.8	6.2	6.1	6.1 ± 0.1	2.9
8	8.1	8	7.9	8.2	7.9	7.9	8.1 ± 0.1	1.7
10	9.9	9.9	10.2	9.9	10	10.1	10.1 ± 0.1	0.9

Repeatability (inter day) Theoretical concentration (mg/L)	Found concentration (mg/L) inj.1	inj.2	inj.3	inj.4	inj.5	inj.6	Mean (mg/L) ± SD	%CV
10	9.9	9.8	10.1	9.9	10.2	10.1	10 ± 0.1	0.8

peak is well separated from the other medium peaks (figure 1 and 2). The retention time of the *p*-coumaric acid under the conditions used is acceptable, and even better when compared to the retention times of other more sophisticated methods. In our conditions, the efficiency value (N) was found to be 3964 for the hydroalcoholic medium, 3183 for the synthetic wine medium, and 3783 for the natural merlot wine. The asymmetry factor had a value of 1.05, which is under the accepted threshold of 1.2. The value of K' was found to be 1.66 for the hydroalcoholic medium, 3.37 for the synthetic wine medium, and 4.11 for the natural merlot wine. We consider in our case that the first peak has the retention time of the closest peak in the peaks group situated on the left side of the 12.5 min *p*-coumaric peak. The calculated α value was 1.97 for the synthetic wine medium, and 1.32 for the merlot wine. It is an acceptable value, knowing that the acceptable threshold for the separation factor is higher than 1. That test helped us validate the application of the method in our synthetic wine medium. The calculated resolution value (Rs) was 5.35 for the synthetic wine medium, and 2.99 for the merlot wine, knowing that the Rs value is accepted if it is higher than 2.5.

3. Method application

The method described and validated above was used for many purposes. First, it was used to monitor the bioconversion of *p*-coumaric acid into 4-vinylphenol then into 4-ethylphenol in a synthetic wine medium containing *Brettanomyces bruxellensis* yeast. In a previous paper, we showed that *p*-coumaric acid can be adsorbed on *Brettanomyces* yeast in wine conditions (Salameh *et al.*, 2008). The consumption of the available quantity of *p*-coumaric acid was monitored by the method we described above (figure 3a). The acid consumption was total and its equivalent quantity was transformed into 4-ethylphenol.

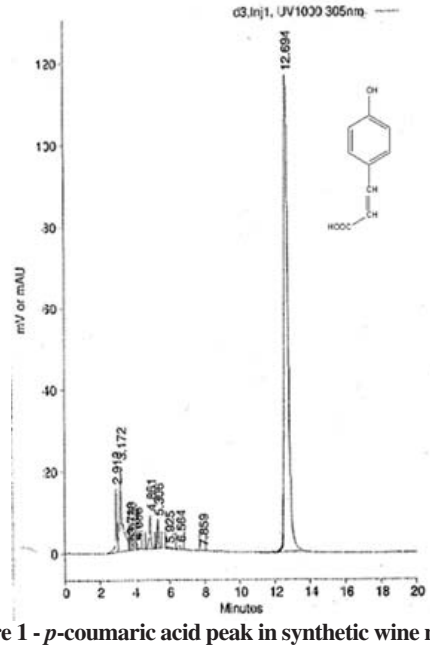

Figure 1 - *p*-coumaric acid peak in synthetic wine media - 10% ethanol

Table 3. Adsorption tests of *p*-coumaric acid

Adsorbant (3 g/l)	PVPP		Yest cell wall	
Initial *p*-coumaric concentration (mg/L)	20	2,5	20	2,55
Adsorbed mean ± SD (%)	6.1 ± 1.1	99.4 ± 1	44.7 ± 12	89,9 ± 13

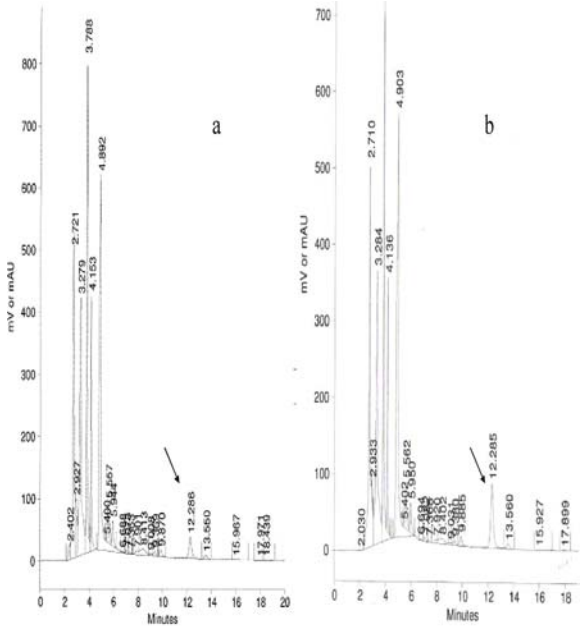

Figure 2 - p-coumaric acid peak in wine
(a) - p-coumaric acid peak in wine after adding p-coumaric acid (b)

The same method was used to monitor the *p*-coumaric acid consumption and bioconversion into 4-vinylphenol in synthetic grape juice, where the fermentation agent was *Saccharomyces cerevisiæ*. The results showed that the available quantity of *p*-coumaric acid disappeared totally from the medium. The acid consumption was total, and its equivalent quantity was transformed into 4-vinylphenol (figure 3b). The aim of this paper is not to discuss the bioconversion kinetics nor the appearance of 4-vinylpheol or 4-ethylphenol in different conditions. Still, the examples we showed above validated the field application of the chromatographic method we described in this study.

Because of the adsorption of *p*-coumaric acid on wine enological adsorbents and yeast, another application of this method was the study of *p*-coumaric acid adsorption on industrial material like the PVPP and yeast cell walls. Table 3 shows the different concentration of *p*-coumaric acid used and the quantity adsorbed at equilibrium, when the adsorbent concentration was 3g/L. The recovery in the adsorbent-free medium for each concentration was 99.86% ± 1.2. Again, these results showed the good quantification of the method. They also showed that PVPP is a better *p*-coumaric acid adsorbent in wine conditions than yeast cell walls.

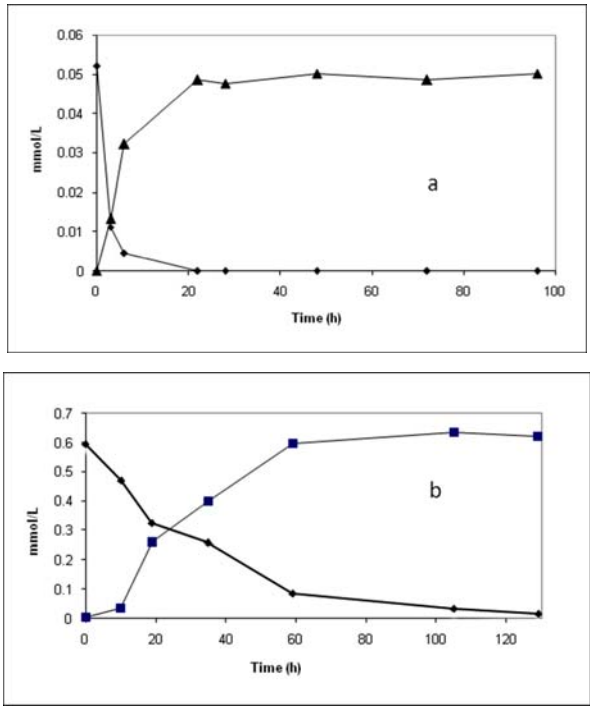

Figure 3. *p*-coumaric acid consumption (◆) and 4-ethylphenol appearance (▲) by *Brettanomyces bruxellensis* (◆), and *p*-coumaric acid consumption (◆) and 4-vinylphenol appearance (n) *Saccharomyces cerevisiæ* (b).

CONCLUSION

Even though there are several chromatographic methods in the field of polyphenol analysis, the novelty of our method is its simplicity and application. It is adapted to study the *p*-coumaric acid reactivity in the conditions described above; hence it was validated in a natural wine medium. Knowing that this phenolic acid adsorbs well on yeast cell walls and on synthetic adsorbents, this method is precise and fast to study these phenomena in wine media, or in enological adsorption treatment processes when compared to other similar chromatographic methods. As an example of application, we showed that *p*-coumaric acid adsorbs better onto PVPP than yeast cell walls in wine conditions.

The study of *p*-coumaric acid bioconversion into 4-ethylphenol was based until now on the detection of 4-ethylphenol, which is the final product of that reaction. In this work, the monitoring of the substrate (*p*-coumaric acid) consumption by yeast in wine or grape juice conditions provided a precise tool to study the

bioconversion kinetics. This will help us to study the reaction rates, and to predict the maximum quantity of 4-ethylphenol that can be produced under *Brettanomyces* yeast contamination in wine. This method can be useful as well to predict the maximum amount of 4-vinylphenol that can be eventually produced by *Saccharomyces* yeast during fermentation.

We presented in this paper a rapid, simple and sensitive chromatographic method to detect and monitor *p*-coumaric acid in several conditions. In comparison to similar methods, the specificity of *p*-coumaric acid monitoring in wine analysis and enological context can be considered improved. The application fields of this method are large because of the good separation, specificity and quantification indications of the *p*-coumaric acid peak, as well as the low detection limit (0.01 mg/L) and the good quantification limit (0.04 mg/L).

More studies and condition optimizations are being carried out, allowing us to separate different chemical aspects of *p*-coumaric acid, such as coumaroyltartric and *p*-coumaric acid esters.

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