

Conversion of hydroxycinnamic acids into volatile phenols (in a synthetic medium and in red wine) by *Dekkera bruxellensis*

Transformação de ácidos hidroxicinâmicos em fenóis voláteis (em meio sintético e em vinho tinto) por Dekkera bruxellensis

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Abstract

The conversion of *p*-coumaric acid, ferulic acid, and caffeic acid into 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol was studied in *Dekkera bruxellensis* ISA 1791 under defined conditions in a synthetic medium and in a red wine. Liquid chromatography (HPLC-DAD) was used to quantify the phenolic acids, and gas chromatography (GC) coupled to a FID detector was used to quantify volatile phenols using a novel analytical methodology that does not require sample derivatization. Identification was achieved by gas chromatography-mass detection (GC-MS). The results show that phenolic acids concentration decreases while volatile phenols concentration increases. The proportion of caffeic acid taken up by *Dekkera bruxellensis* is lower than that for *p*-coumaric or ferulic acid; therefore less 4-ethylcatechol is formed. More important, 4-ethylcatechol synthesis by *Dekkera bruxellensis* in wine has never been demonstrated so far. These results contribute decisively to a better understanding of the origin of the volatile phenols in wines. The accumulation of these compounds in wine is nowadays regarded as one of the key factors of quality control.

Keywords: phenolic acids; volatile phenols; 4-ethylcatechol; red wine.

Resumo

A transformação do ácido *p*-cumárico, ácido ferúlico e ácido cafeico em 4-etilfenol, 4-etilguaiacol e 4-etilcatecol foi estudada na presença de *Dekkera bruxellensis* ISA 1791, sob condições controladas, em meio sintético e em vinho tinto. Os compostos fenólicos foram doseados por cromatografia em fase líquida (HPLC-DAD) e os fenóis voláteis foram doseados por cromatografia em fase gasosa (GC) com detector de ionização de chama (FID), recorrendo a uma metodologia que não requer prévia derivatização da amostra. A identificação dos compostos foi comprovada por espectrometria de massa (GC-MS). Os resultados demonstram que os teores em ácidos fenólicos diminuem enquanto se observa um aumento dos teores em fenóis voláteis. O ácido cafeico é dos três ácidos em estudo, o menos utilizado pela levedura daí os teores em 4-etilcatecol serem os mais baixos observados. Esta síntese de 4-etilcatecol por *Dekkeras bruxellensis* em vinhos nunca tinha sido demonstrada. Os resultados obtidos contribuem, decisivamente, para um melhor conhecimento sobre a origem precisa dos fenóis voláteis nos vinhos. A acumulação destes compostos no vinho é, hoje em dia, considerado como um dos factores chave do controle de qualidade.

Palavras-chave: ácidos fenólicos; fenóis voláteis; 4-etilcatecol; vinho tinto.

1 Introduction

The accumulation of volatile phenols in wine has been a cause of great concern in modern enology being now a key point in the control of wine quality. Vinylphenols (4-vinylphenol and 4-vinylguaiacol) and ethylphenols (4-ethylphenol and 4-ethylguaiacol) may be produced in wine, in a sequential pathway, due to microbial activity, imparting undesirable odours and flavours. The quality of wine is considered to be mainly affected by the accumulation of 4-ethylphenol and 4-ethylguaiacol, whose presence is commonly described as responsible for sensorial notes related to “leather”, “horse sweat”, “animal”, and “medicinal”.

The precise origin(s) of volatile phenols has been under discussion for some time, but most likely they come from hydroxycinnamic acids or their corresponding tartaric esters.

The production of these compounds has been recognised as an important characteristic of the yeast *Brettanomyces/Dekkera* (LOUREIRO; MALFEITO-FERREIRA, 2006).

A number of microbial species have been reported as able to decarboxylate hydroxycinnamic acids, such as ferulic, *p*-coumaric, and caffeic acids. The products of these decarboxylations are ethylphenols that give rise to strong smoky and aromatic odours and flavours, and are regarded as the source of phenolic “off-flavours” in several beers and wines (EDLIN et al., 1998). These “off-flavours”, described as the “phenolic character”, can appear in all red wines at different stages during production and ageing processes.

The metabolism of hydroxycinnamic acids by *Brettanomyces/Dekkera* yeasts is due to a sequential action of two enzymes: a

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cinnamate decarboxylase, which cleaves the phenolic acid (namely ferulic, caffeic, and *p*-coumaric acids) directly into the corresponding vinylphenol and a vinylphenol reductase, which converts the vinyl into the corresponding ethylphenol (CHATONNET; DUBOURDIEU; BOIDRON, 1995; EDLIN et al., 1995). Benito et al. (2009) studied possible ways of controlling these organisms in wineries by studying several factors affecting the enzymatic activity of *Dekkera/Brettanomyces. D. bruxellensis* shows hydroxycinnamate decarboxylase and vinyl reductase activity under oenological conditions to such an extent that the species is considered an undesirable yeast capable of producing high concentrations of 4-ethylphenol (SUÁREZ et al., 2007). Recently, Godoy et al. (2008) have shown the presence of a *p*-coumarate decarboxylase (CD) and a Vinylphenol Reductase (VR) in *B. bruxellensis*. The authors demonstrated that both enzymatic activities were stable at pH 3.4, but in the presence of ethanol the CD activity decreased drastically while the VR activity was more stable.

D. bruxellensis is nowadays considered to be the main cause of wine spoilage, especially of fashionable premium red wines matured in oak casks, and can be responsible for serious economic losses (SUÁREZ et al., 2007; LOUREIRO; MALFEITO-FERREIRA, 2003).

Several studies report the finding of 4-ethylphenol and 4-ethylguaiacol in wines (PIZARRO; PÉREZ-DEL-NOTARIO; GONZÁLEZ-SÁIZ, 2007; VALENTÃO et al., 2007). The presence of 4-ethylcatechol, on the other hand, has been reported only once, by Hesford et al. (2004), until Carrillo and Tena (2007) reported the presence of 4-ethylcatechol in some wines affected by *Brettanomyces*. More recently Larcher et al. (2008) developed a HPLC-CEAD method for quantification of 4-ethylcatechol and applied it to Italian wines.

The aim of this work was to evaluate the disappearance of cinnamic acids and consequent appearance of volatile phenols by a strain of *D. bruxellensis* with emphasis on 4-ethylcatechol using a very simple sample preparation method. The present study was carried on in a model solution as well as in a red wine.

2 Material and methods

2.1 Yeast strain and inocula preparation

D. bruxellensis ISA 1791 strain was isolated from a phenolic tainted red wine (RODRIGUES et al., 2001) and kept in GYPC medium (20 g.L⁻¹ glucose (Merck, Darmstadt, Germany), 3 g.L⁻¹ yeast extract (Difco Laboratories, Detroit, USA), 5 g.L⁻¹ peptone (Difco), and 20 g.L⁻¹ agar, pH 6.0, 5 g.L⁻¹ of calcium carbonate (Merck), at 4 °C.

A synthetic medium (6.7 g.L⁻¹ YNB (Difco); 2% (w/v) glucose, 10% (v/v) ethanol, pH 3.5), filtered by 0.22 µm sterilized membranes filters was used to grow the yeast strain. Stirring was applied at 120 rpm, and temperature was kept at 25 °C. Biomass concentration was followed everyday by optical density measurements at 640 nm. When biomass reached OD_{640nm} = 1, the liquid medium was divided into 12 (500 mL) flasks (samples B1 to B12). Each flask received 10⁴ cell.mL⁻¹.

2.2 Samples

Case 1. Synthetic medium

10 mg.L⁻¹ of caffeic acid were added to samples B1, B2, and B3; 10 mg.L⁻¹ of *p*-coumaric acid were added to samples B4, B5, and B6; and 10 mg.L⁻¹ of ferulic acid were added to samples B7, B8, and B9. Samples B10, B11, and B12 were added 10 mg.L⁻¹ of each acid (30 mg.L⁻¹ total amount). All samples were kept at 25 °C under anaerobic conditions.

50 mL of each sample were collected at days 0, 2, 4, 6, and 8 for HPLC and GC analysis.

Case 2. Wine inoculated with same yeast strain

A young red wine from 2007 vintage, collected after alcoholic fermentation and prior to malolactic fermentation, was divided in two parts: one was the control (C) and the other was inoculated with *D. bruxellensis* ISA 1791 strain (Y). To half of wines C and Y, 10 mg.L⁻¹ of each phenolic acid (C+A and Y+A) were added. The wines were kept at 25 °C under anaerobic condition and samples were collected after one and two months. The experiment was performed in triplicate.

Case 3. Naturally contaminated wine

A red wine from Trincadeira variety fully described in a previous study (CABRITA et al., 2008) aged in a 20 L glass container and developed a strong “horse” flavour, indicating a possible contamination with *D. bruxellensis*. Samples were collected and phenolic compounds and volatile phenols were analysed.

2.3 Reagents and standards

Water was previously purified in a Mili-Q system (Millipore, Bedford, MA, USA). HPLC grade methanol, n-pentane, ethanol, and anhydrous sodium sulphate were from Merck (Darmstadt, Germany). Analytical grade diethyl ether was from Lab-Scan Analytical Sciences (Dublin, Ireland).

Ferulic acid was supplied by Extrasynthese (Genay, France). Caffeic acid and *p*-coumaric acid from Sigma-Aldrich (St Louis, MO). 4-ethylphenol, 4-ethylcatechol, 4-ethylguaiacol and 3,4-dimethylphenol were supplied respectively from Merck, ABCR (GmbH&Co), TCI (Tokyo Casei), and Sigma-Aldrich (St Louis, MO).

2.4 HPLC analysis

Sample preparation

The samples were extracted by liquid-liquid extraction using diethyl ether: 5 mL of wine with pH adjusted to 2 were extracted twice with 5 mL of diethyl ether for ten minutes. The organic phase was dried over Na₂SO₄; the solvent was evaporated under nitrogen; the dry residue obtained was dissolved in methanol–water (1:1, v/v) and filtered through a 0.45 µm Nylaflo membrane filter (Gelman) before being injected into

the chromatograph. 20 µL of each extract were used for HPLC analysis.

Chromatographic conditions

The column used was a Superpher® 100, C18 (5 µm packing, 250 × 4.6 mm i.d.) (Merck, Germany) installed into a Hewlett Packard 1050 equipment, and a Hewlett Packard Diode Array 1100 detector (Agilent Technologies, Germany), was used. Flow rate was 1 mL/minute. The mobile phase was solvent A: water:acetic acid (98:2, v/v) and solvent B: water:methanol:acetic acid (68:30:2, v/v). A gradient program was adopted as follows for solvent A: from 95 to 70% (12 minutes); from 70 to 45% (15 minutes); from 45 to 23% (6 minutes); isocratic for 9 minutes; from 23 to 5% (5 minutes); from 5 to 0% (3 minutes); isocratic for 5 minutes, and a post-run of 5 minutes for re-equilibration of the initial gradient. The detection wavelength used was 320 nm. The sample preparation and chromatographic conditions were fully described in a previous study (CABRITA et al., 2008).

Calibration curves

A stock solution containing caffeic, ferulic, and *p*-coumaric acids was prepared in ethanol 12% (v/v). Six working solutions with concentration between 0.39-50.0 mg.L⁻¹ of caffeic and *p*-coumaric acids and between 0.78-50.0 mg.L⁻¹ of ferulic acid were extracted as described above. Each standard was injected three times. Calibration parameters are presented in Table 1.

2.5 GC analysis

Sample preparation

To 10 mL of each sample, 200 µL of internal standard (3,4-dimethylphenol 800 mg.L⁻¹) were added. The samples were extracted twice using 5 mL of diethyl ether/pentane (1:1) for 10 minutes. The organic phases were collected, dried over Na₂SO₄, and the solvent evaporated under a gentle stream of nitrogen until approximately 0.5 mL, prior to injection.

Chromatographic conditions

A GC-FID HP 6890 (Agilent Technologies, Germany) series with a RTX-Wax column from Restek

(30 m × 0.25 mm × 0.25 µm) was used. The injector, in splitless mode, at 230 °C, the injection volume was 1 µL, and the detector temperature was 250 °C. Helium was used as carrier gas at a flow of 1.4 mL/minutes. The oven was initially set at 80 °C, raised to 140 °C at 10 °C/minutes, to 155 °C at 5 °C/minutes, to 200 °C at 10 °C (held for 2 minutes), and finally up to 220 °C at a rate of 20 °C/minutes.

Calibration curves

The internal standard was prepared in ethanol 75% (v/v). A stock solution containing 80 mg of 4-ethylcatechol, 80 mg of 4-ethylguaiaicol, and 50 mg of 4-ethylphenol was prepared in ethanol 75% (v/v). The working solutions were prepared by diluting the stock solution with a model solution (4 g.L⁻¹ tartaric acid; 3 g.L⁻¹ malic acid; 0.1 g.L⁻¹ acetic acid; 0.025 g.L⁻¹ magnesium sulphate; 0.1 g.L⁻¹ potassium sulphate and 90 g.L⁻¹ ethanol; pH = 3.30). Final concentrations of 0.24, 0.50, 1, 2, 4, 8, and 16 mg.L⁻¹ were obtained. Each standard was injected three times. The LOD and LOQ limits were established using calibration data and regression statistics considering the y-intercept and standard deviation of the regression according to Miller and Miller (1993). Calibration parameters are presented in Table 2.

2.6 GCMS analysis

A GC HP 5890 series II coupled to a HP 5972 series mass selective detector (Agilent Technologies, Germany), equipped with a column HP-5HS from Agilent Technologies Inc. (30 m × 0.25 mm × 0.25 µm), was used for 4-ethylcatechol identification.

The injector in splitless mode was at 250 °C, injection volume of 1 µL, delay was 3 minutes, and helium flow rate was at 1 mL per minute. The oven was programmed from 80 to 200 °C at 8 °C/minutes. Transfer line was 280 °C. Electron Impact (EI) ionization mode at 70 eV was used; Spectra were recorded in Selective Ion Monitoring mode (SIM). Mass spectral data and retention times were determined for standard compounds. The ions monitored were as follows: 4-ethylcatechol, m/z 123 + 138, 4-ethylguaiaicol, m/z 137 + 152, 4-ethylphenol, and 3,4-dimethylphenol, m/z 107 + 122.

Table 1. Parameters for the calibration of phenolic acids.

Compounds	RT ± SD (minutes)	Equation	r ²	LOD (mg.L ⁻¹)	LOQ (mg.L ⁻¹)
Caffeic acid	27.20 ± 0.98	y = 391.03x - 167.74	0.9993	1.34	4.47
<i>p</i> -Coumaric acid	37.68 ± 1.05	y = 521.08x - 107.10	0.9999	0.58	1.94
Ferulic acid	43.94 ± 1.40	y = 421.86x - 276.27	0.9986	2.01	6.70

RT ± S.D. retention time ± estimated standard deviation (n = 3); r² determination coefficient. LOD limit of detection, LOQ limit of quantification.

Table 2. Parameters for the calibration of volatile phenols.

Compounds	RT ± SD (minutes)	Equation	r ²	LOD (mg.L ⁻¹)	LOQ (mg.L ⁻¹)
4-ethylguaiaicol	6.68 ± 0.01	y = 0.0504x + 0.0054	0.9993	0.031	0.091
4-ethylphenol	8.01 ± 0.01	y = 0.0611x - 0.0083	0.9999	0.015	0.029
4-ethylcatechol	14.67 ± 0.05	y = 0.0376x - 0.0004	0.9993	0.019	0.065

RT ± S.D. retention time ± estimated standard deviation (n = 3); r² determination coefficient. LOD limit of detection, LOQ limit of quantification.

2.7 Statistical analysis

The evaluation of statistical significance was determined by ANOVA, followed by Fisher-LSD test using SPSS 7.0 software. The level of significance was set at $p < 0.05$.

3 Results and discussion

Case 1. Synthetic medium

Table 3 presents the results obtained for the quantification of phenolic acids and volatile phenols in the studied samples. It is clear that phenolic acids concentration decrease with time, while volatile phenols concentration increases. 4-ethylcatechol is the last one to appear, and it is the least significant.

Both 4-ethylphenol and 4-ethylguaiaicol were detected at day 2 after yeast inoculation and reached concentration of 7.69 and 7.25 mg.L⁻¹ at day 8, while the content of their precursor (*p*-coumaric and ferulic acids) decreased up to 0.28 and 0.72 mg.L⁻¹ at the same day.

Due to vicinal OH groups, 4-ethylcatechol is thermally unstable at the required analysis temperature; moreover it can also react with most of the commercial available stationary phases used for GC columns. Detection and quantification is usually observed only after chemical derivatization (LARCHER et al., 2008).

4-ethylcatechol was detected four days after yeast inoculation, and the analysis was performed without chemical derivatization. The final amount detected reached 1.24 mg.L⁻¹ (at day 8), while caffeic acid (the precursor) concentration decreased up to 5.51 mg.L⁻¹.

Conversion rate of hydroxycinnamic acids into volatile phenols in a synthetic medium (Table 4) was obtained through the ratio of the maximum concentration obtained for volatile phenols and the theoretical concentration obtained from 10 mg.L⁻¹ of hydroxycinnamic acids.

Conversion rates greater than 90% were obtained for the conversion of *p*-coumaric acid into 4-ethylphenol and from ferulic acid into 4-ethylguaiaicol, but a rate smaller than 20% was found for the conversion of caffeic acid into 4-ethylcatechol. These results may justify why only recently the presence of 4-ethylcatechol in wines was reported (HESFORD et al., 2004). Although caffeic acid is present in wines in considerable amounts and has a structure similar to the other phenolic acids, these results seem to indicate that *p*-coumaric and ferulic acids are easily used by yeast metabolism.

A difference between the initial concentration, 10 mg.L⁻¹ of caffeic, ferulic and *p*-coumaric acids, and the concentration detected by HPLC, immediately after yeast inoculation, was observed (samples B1 to B9). Similar results were described by other authors (MEDAWAR, 2003; SALAMEH et al., 2008). The loss of the hydroxycinnamic acids in a synthetic medium might be explained either by their instability at high temperature, by esterification reaction with ethanol, or by the ability of yeast cell walls to adsorb these compounds. Salameh et al. (2008) showed that although *p*-coumaric acid was stable at oenological temperatures, it can, like polyphenols, react with ethanol, and it can also be adsorbed into *Brettanomyces* yeast.

The results regarding the samples in which the three acids were added simultaneously (B10 to B12) are presented in Table 5. When compared with the results obtained in previous samples (B1 to B9), no clear differences could be observed. Yeast strain seems to have similar behaviour either in the presence of only one acid or in the presence of the three acids together. A preferential use of a single acid was not observed either.

Case 2. Wine inoculated with same yeast strain and added 10 mg.L⁻¹ of each phenolic acid

The results from this assay are shown in Table 6. In wine samples without yeast inoculation, the content of hydroxycinnamic acids increased with time; probably due to the hydrolysis of hydroxycinnamic esters. The presence of volatile phenols was slightly detected, more likely due to lactic bacteria activity, which also has the ability to convert vinyl phenols into ethyl phenols.

In wine samples inoculated with *D. bruxellensis*, a clear decrease in the *p*-coumaric and ferulic acids content as well as an increase in the corresponding volatile phenols could be observed. This result was clearer in samples to which acids were added. One month after inoculation, the content of hydroxycinnamic acids were near the added amounts (10 mg.L⁻¹).

Table 4. Conversion rate of hydroxycinnamic acids into volatile phenols in synthetic medium.

Compound	Obtained concentration (mg.L ⁻¹)	Theoretical concentration (mg.L ⁻¹)	Conversion rate (%)
4-ethylguaiaicol	7.25 ± 0.53	7.84	92.47
4-ethylphenol	7.69 ± 0.36	7.44	103.36
4-ethylcatechol	1.24 ± 0.34	7.66	16.19

Table 3. Phenolic acids and volatile phenols (mg.L⁻¹) in samples B1 to B9.

Day	Caffeic acid	<i>p</i> -coumaric acid	Ferulic acid	4-ethylguaiaicol	4-ethylphenol	4-ethylcatechol
0	7.13 ± 0.49 ^a	3.80 ± 0.66 ^a	2.56 ± 0.35 ^a	nd	nd	nd
2	8.61 ± 0.58 ^a	2.10 ± 0.59 ^b	2.90 ± 0.24 ^a	4.56 ± 0.42 ^a	3.67 ± 0.83 ^a	nd
4	7.67 ± 0.60 ^a	0.97 ± 0.22 ^c	1.40 ± 0.11 ^b	6.50 ± 0.75 ^b	6.09 ± 0.54 ^b	0.13 ± 0.03 ^a
6	5.80 ± 1.25 ^b	0.36 ± 0.06 ^d	0.89 ± 0.07 ^c	7.35 ± 0.79 ^b	7.28 ± 0.42 ^b	0.52 ± 0.11 ^b
8	5.51 ± 0.64 ^b	0.28 ± 0.01 ^d	0.72 ± 0.07 ^c	7.25 ± 0.53 ^b	7.69 ± 0.36 ^b	1.24 ± 0.34 ^c

Different letters in a row denote a significant difference with 95% confidence level in the Fisher's LSD multiple comparison test. nd – not detected.

Table 5. Phenolic acids and volatile phenols (mg.L⁻¹) in samples B10 to B12.

Day	Caffeic acid	<i>p</i> -coumaric acid	Ferulic acid	4-ethylguaiacol	4-ethylphenol	4-ethylcatechol
0	7.24 ± 0.36 ^a	6.78 ± 0.50 ^a	4.87 ± 0.38 ^a	Nd	Nd	Nd
2	9.26 ± 1.66 ^b	3.32 ± 0.98 ^b	3.24 ± 0.60	5.18 ± 0.21 ^a	3.38 ± 0.13 ^a	Nd
4	6.77 ± 0.84 ^a	1.67 ± 0.18 ^c	1.35 ± 0.26 ^c	7.36 ± 0.42 ^b	5.94 ± 0.14 ^b	0.46 ± 0.05 ^a
6	6.22 ± 0.39 ^a	0.62 ± 0.03 ^d	0.58 ± 0.08 ^d	8.41 ± 0.16 ^b	7.00 ± 0.13 ^c	0.99 ± 0.36 ^b
8	4.71 ± 0.31 ^c	0.58 ± 0.09 ^d	0.53 ± 0.02 ^d	8.37 ± 0.47 ^b	7.17 ± 0.21 ^c	1.89 ± 0.18 ^c

Different letters in a row denote a significant difference with 95% confidence level in the Fisher's LSD multiple comparison test. nd – not detected.

Table 6. Phenolic acids and volatile phenols (mg.L⁻¹) in wine contaminated with yeast inoculation.

	Sample	Caffeic acid	<i>p</i> -coumaric acid	Ferulic acid	4-ethyl guaiacol	4-ethyl phenol	4-ethyl Catechol
1 month	C	4.15 ± 0.07	2.44 ± 0.11	0.87 ± 0.05	1.28 ± 0.04	nd	Nd
	C+A	15.39 ± 0.28	13.23 ± 0.27	12.79 ± 1.18	1.10 ± 0.06	nd	Nd
	Y	4.98 ± 0.28	2.95 ± 0.07	0.99 ± 0.01	0.90 ± 0.34	nd	Nd
	Y+A	18.21 ± 0.36	15.31 ± 0.24	16.86 ± 0.39	4.16 ± 0.00	nd	Nd
2 month	C	5.42 ± 0.55	3.08 ± 0.27	1.01 ± 0.08	0.84 ± 0.47	nd	0.07 ± 0.00
	C+A	17.41 ± 1.46	14.59 ± 1.47	15.30 ± 0.22	0.80 ± 0.14	0.18 ± 0.00	0.04 ± 0.01
	Y	4.72 ± 0.22	1.44 ± 0.08	0.87 ± 0.02	1.26 ± 0.14	0.47 ± 0.00	0.24 ± 0.00
	Y+A	20.17 ± 1.36	8.00 ± 1.03	6.90 ± 0.98	8.34 ± 0.31	3.97 ± 0.17	0.33 ± 0.00

C – control, Y – yeast; A – acids; nd – not detected.

Table 7. Phenolic acids and volatile phenols (mg.L⁻¹) in wine naturally contaminated.

Caffeic acid	<i>p</i> -coumaric acid	Ferulic acid	4-ethylguaiacol	4-ethylphenol	4-ethylcatechol
13.12 ± 0.39	3.00 ± 0.30	1.01 ± 0.12	1.44 ± 0.20	2.24 ± 0.12	0.96 ± 0.36

By that time, 4-ethylguaiacol was the only volatile phenol whose presence was detected. Although degradation occurred slower than in synthetic medium, after two months the *p*-coumaric and ferulic acids contents were half the initial concentration and all three volatile phenols were detected. 4-ethylguaiacol presented the highest concentration (8.34 mg.L⁻¹).

The observed decrease of the ferulic acid content is not in accordance with previous reported results. Silva et al. (2005) argue that although ferulic acid is also a precursor of volatile phenols, no decrease of its content was observed in inoculated samples of Dao red wine with various strains of *D. bruxellensis*.

Case 3. Naturally contaminated wine

After two years in 20 L glass carboy, a red wine previously described (CABRITA et al., 2008) developed a strong “horse” smell. Table 7 shows the phenolic acids and the amounts of volatile phenols for this wine.

The presence of 4-ethylcatechol in wine samples, without prior derivatization was confirmed by GC-MS analysis.

A higher value was found for 4-ethylphenol (2.24 mg.L⁻¹), followed by 4-ethylguaiacol (1.44 mg.L⁻¹). 4-ethylcatechol (0.96 mg.L⁻¹) presented the smallest amount. Values obtained for 4-ethylphenol and 4-ethylguaiacol are higher than the reported sensory perception thresholds for 4-ethylphenol and

4-ethylguaiacol alone in a red wine (605 and 100 µg.L⁻¹, respectively) (CHATONNET et al., 1992), and thus in accordance with the strong horse smell detected. The values obtained in this study are within the limits detected and reported by several authors for red wines produced and stored under different conditions (CHATONNET et al., 1992; POLLNITZ; PARDON; SEFTON, 2000a,b).

During aging, wines phenolic acids content increases from an average value of 10.24 to 17.13 mg.L⁻¹ due to caffeic acid (data not show) transformation/degradation. It has been stated that storage conditions may affect wine phenolic content since they can induce hydrolysis, oxidation, and complexation reactions (ZAFRILLA et al., 2003). Hydrolysis, enzymatic or not, seems to be the major cause of free phenolic acids increase. Degradation by light or temperature (CHEYNIER; FULCRAND, 2003) has also been reported.

4 Conclusions

Bioconversion of hydroxycinnamic acids into volatile phenols by *Brettanomyces/Dekkera* yeasts is a major problem in modern enology. The results obtained show that the *D. bruxellensis* strain used produces 4-ethylphenol from *p*-coumaric acid, 4-ethylguaiacol from ferulic acid and 4-ethylcatechol from caffeic acid, either in synthetic medium or in a regular wine. All phenolic acids presented clear decreases in accordance with the increase in the corresponding volatile phenols. 4-ethylcatechol was analysed without derivatization.

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