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Influence of *Dekkera bruxellensis* on the contents of anthocyanins, organic acids and volatile phenols of Dão red wine

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Abstract

The anthocyanin, organic acid and volatile phenol compositions of red wine obtained from Touriga Nacional grapes growing in the Dão region (Portugal) were determined by HPLC/DAD, HPLC/UV and GC/FID, respectively. By these means, nine anthocyanic compounds (malvidin-3,5-*O*-diglucoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, delphinidin, cyanidin, pelargonidin and malvidin), six organic acids (ketoglutaric, tartaric, malic, quinic, lactic and shikimic acids) and two volatile phenols (4-ethylguaiacol and 4-ethylphenol) were identified and quantified. Malvidin-3-*O*-glucoside, the pair lactic plus shikimic acids and 4-ethylguaiacol were the main anthocyanin, organic acids and volatile phenol, respectively. The effects of nine different *Dekkera bruxellensis* strains on these chemical parameters were also evaluated. The results obtained indicate that some strains of *D. bruxellensis* yeast are able to cause deterioration of red wine from the Dão region during its maturation by the production of volatile phenols, namely 4-ethylphenol.

Keywords: Dão red wine; *Dekkera bruxellensis*; Anthocyanins; Organic acids; Volatile phenols

1. Introduction

The importance of phenolics in viticulture and enology is well known. Phenolic compounds play a major role in wine quality as they contribute to sensory properties, in particular colour, flavour, bitterness and astringency (Atanasova, Fulcrand, Cheynier, & Moutounet, 2002; Macheix, Fleuriet, & Billot, 1990). Wines may have very complex phenolic compositions that change during their shelf-life, being influenced by several factors, such as the grape composition (Andrade, Mendes, Falco, Valentão, & Seabra, 2001; Andrade, Oliveira, et al., 2001; Atanasova et al., 2002; Ramos et al., 1999; Soleas, Dam, Carey, & Goldberg, 1997), the enological practices (Atanasova et al., 2002; Ramos et al., 1999; Zafrilla et al., 2003) and evolution of phenolics during storage and ageing of wine (Atanasova et al., 2002; Zafrilla et al., 2003). Among phenolic compounds, anthocyanins have been identified as chemical markers for differentiating red grape cultivars and red wines made with different cultivars, and also for classifying wines according to geographical origin (de Villiers, Vanhoenacker, Majek, & Sandra, 2004; Gambelli & Santaroni, 2004; Revilla, García-Beneytez, Cabello, Martín-Ortega, & Ryan, 2001).

Several simple phenols have been described as normal components of wine aroma. Depending on the concentration levels and the aromatic properties, some of them contribute positively to wine aroma, but others are responsible

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for wine off-flavours. Among these off-flavours, ethylphenols are the most significant in red wines and affect their quality by giving them a horsy character when present at high concentrations (Martorell, Martí, Mestres, Busto, & Guasch, 2002; Mejías, Marín, Moreno, & Barroso, 2003). The unpleasant odours may appear in the wine at different stages. However, the alteration usually occurs during ageing, prior to bottling, especially when wines are kept in barrels, particularly old barrels, which are seldom or never racked. (Chatonnet, Dubourdieu, & Boidron, 1995; Chatonnet, Viala, & Dubourdieu, 1997).

On the other hand, wines can be modified by microorganisms during the ageing process. Contaminating yeasts of the genus *Dekkera*/*Brettanomyces*, especially *D. bruxellensis* species, are able to produce very important amounts of ethylphenols, mainly 4-ethylguaiacol and 4-ethylphenol, when developed in wine, leading to its deterioration and to great economical losses on a world-wide level (Chatonnet et al., 1995; Chatonnet et al., 1997). These spoilage yeasts are not easily detected in the microbiological routine control, as they are slow growing. They have fastidious nutritional requisites that include complex exogenous nutrient sources, vitamin supplements or the presence of auxotrophic microorganisms that can donate vitamins. In addition, their populations are usually minor, due to the presence of more numerous or faster growing species in wine (Fugelsang, Osborn, & Muller, 1993). Once malolactic and alcoholic fermentation is completed, these yeasts grow easily on traces of residual sugars. Careful hygiene and adequate sulfuring of wines and containers can prevent the development of these undesirable yeasts (Chatonnet et al., 1995; Chatonnet et al., 1997).

In addition, the determination of organic acids, mainly tartaric, malic and lactic acids, is important for monitoring the fermentation process. The organic acid contents in wine have a substantial effect on the balance of the flavour, taste and colour, but also influence the chemical stability and pH, and thus the wine quality (Esteves, Lima, Lima, & Duarte, 2004; Kerem, Bravdo, Shoseyov, & Tugendhaft, 2004).

Dão is a unique region with important viticulture traditions, located in north central Portugal, from which the excellent edapho-climatic conditions are turned to advantage for vineyard culture, which corresponds to 20,000 ha. The Dão region presents a temperate climate, although cold and rainy in winter and frequently very hot in summer. Dão wines with *Denomination d'Origine Controllée* (DOC) arise from vineyards established in granite land, between 400 and 500 m altitude. Touriga Nacional is the most important grape cultivar for the elaboration of Dão red wines, being responsible for organoleptic characteristics and for the prestige that Dão wines have gained in the course of time. The wine presents an intense and wild aroma, with delicate flavour, ruby colour, high alcoholic degree and an elevated acidity, which allows harmonisation of its aroma and balance of body. As far as we know, only the non-coloured phenolic composition of the wine produced from this cultivar growing in Dão and the effect of *Dekkera bruxellensis* strains contamination on its content have been reported (by our group) (Silva et al., 2005).

Due to the above-mentioned important role of anthocyanins, organic acids and volatile compounds in enology, owing to their contribution to wine sensory properties, in the sequence of our previous study (Silva et al., 2005), the anthocyanin, organic acid and voltatile phenol compositions of red wine from Touriga Nacional cultivar from Dão were determined. The alterations induced by nine different *Dekkera bruxellensis* strains on their levels were also evaluated.

2. Materials and methods

2.1. Standards and reagents

The standards were purchased from Sigma (St. Louis, MO, USA) and from Extrasynthése (Genay, France). Methanol, formic and hydrochloric acids, ethyl ether, *n*-hexane and ethanol were obtained from Merck (Darmstadt, Germany) and sulfuric acid from Pronalab (Lisboa, Portugal). Polyvinylpolypyrrolidone (PVPP) was from Sigma (St. Louis, MO, USA). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade.

2.2. Microorganisms

The various strains of *D. bruxellensis* used in this study were isolated from red wine from Dão in Sabouraud medium with chloramphenicol (Pronadisa, Spain). Yeasts were identified by a series of biochemical characteristics, according to the PROLEWINE system (Velázquez, Cruz-Sánchez, Mateos, Monte, & Chordi, 1993), as previously reported (Silva et al., 2005).

2.3. Wine samples

The red wine from Touriga Nacional grapes growing in the Dão region was treated according to a described procedure (Silva et al., 2005). Briefly, the wine (5 l) was removed from a stainless steel tank, sterilised by filtration and divided by 12 glass flasks. Samples W1 and W2 did not received any microorganism and were used as witness samples. Samples D1 to D9 were inoculated with nine different strains of *D. bruxellensis*, and sample S was inoculated with *Saccharomyces cereviseae* (control sample). Inocula were prepared by suspending several colonies of each yeast strain in sterile water up to a concentration of 8×10^6 cells/ml and aliquots of 2 ml were inoculated into each glass flask. All samples were kept in a stove at 18 °C for three weeks, with the exception of sample W1, which was stored in a refrigerator (3 °C) and used to evaluate the effect of the temperature. The growth in the inoculated samples was measured by count of viable cells in the med-

ium at the end of the incubation (about 1.5×10^6 CFU/ml). The samples were then frozen prior to the preparation of the extracts.

2.4. Anthocyanins analysis

2.4.1. Sample preparation

Sample preparation was simple, consisting only in the acidification of 5 ml of wine with 20 μl HCl. 20 μl of the acidified samples were analysed by HPLC/DAD.

2.4.2. HPLC/DAD analysis

The extracts were analysed on an analytical HPLC unit (Gilson), using a Spherisorb ODS2 (25.0 × 0.46 cm; 5μm, particle size) column (Silva et al., 2000). The solvent system used was a gradient of water–formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15%B at 3 min, 25%B at 13 min, 30%B at 25 min, 35%B at 35 min, 45%B at 39 min, 45%B at 42 min, 50%B at 44 min, 55%B at 47 min, 70%B at 50 min, 75%B at 56 min, and 100%B at 60 min, at a solvent flow rate of 0.9 ml/min. Detection was achieved with a Gilson diode array (DAD) detector. The compounds in each sample were identified by comparing their retention times and UV–Vis spectra in the 200–600 nm range with the library of spectra previously compiled by the authors. Peak purity was checked by means of the Gilson 160 SpectraViewer Software Contrast Facilities. Anthocyanin quantification was achieved by the absorbance recorded in the chromatograms relative to external standards (malvidin-3,5-*O*-diglucoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, delphinidin, cyanidin, pelargonidin and malvidin).

2.5. Organic acids analysis

2.5.1. Sample preparation

For the determination of organic acids, phenolic compounds were removed according to a described procedure (Kerem et al., 2004) and 20 μl of the clear samples were analysed by HPLC/UV.

2.5.2. HPLC/UV analysis

The separation was carried out as previously reported (Silva, Andrade, Mendes, Seabra, & Ferreira, 2002) with some modifications. An analytical HPLC unit (Gilson), and an ion exclusion column Nucleogel® Ion 300 OA (300 × 7.7 mm) column, in conjunction with a column heating device at 30 °C, were used. Elution was carried out at a solvent flow rate of 0.2 ml/min, isocratically, with 0.01 N sulfuric acid as the mobile phase. Detection was performed with a UV detector set at 214 nm.

Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Lactic and shikimic acids were quantified together as lactic acid.

2.6. Volatile phenols analysis

2.6.1. Sample preparation

50 ml of a mixture containing the wine sample and 50 μl of 4-decanol (1 g/l in 60% ethanol) (internal standard) were extracted with 4 ml of ethyl ether:*n*-hexane (1:1) for 5 min, followed by extraction with 2 ml of ethyl ether *n*-hexane (1:1) (twice). The volatile fractions were gathered and 2 μl were analysed by GC/FID.

2.6.2. GC/FID analysis

The GC analysis was carried out in a Chrompack 9000 chromatograph (Elnor, USA), equipped with a flame ioni-

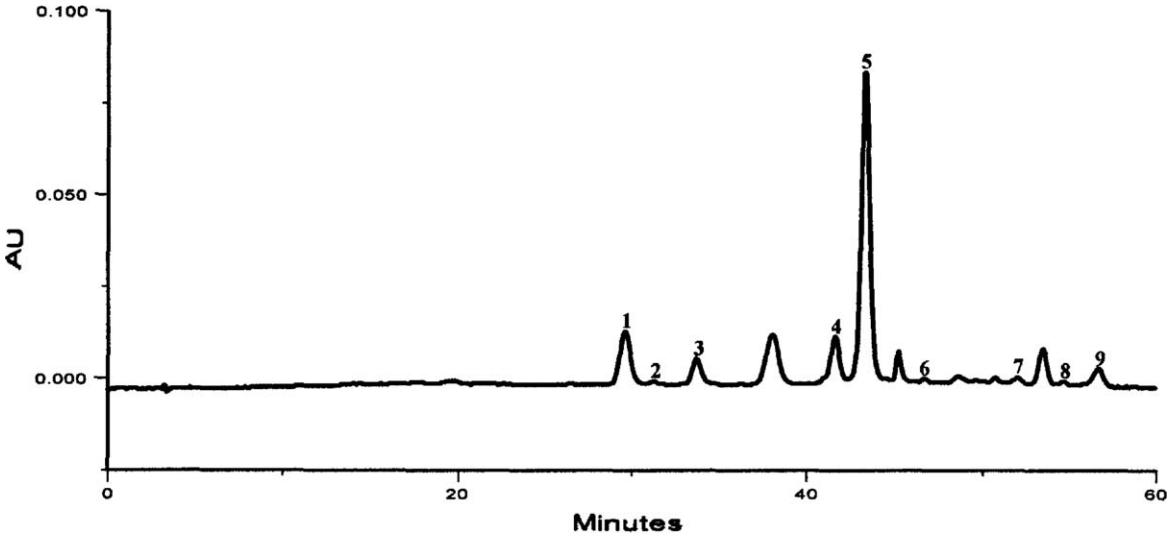

Fig. 1. HPLC anthocyanins profile of wine samples. Detection at 500 nm: (1) malvidin-3,5-*O*-diglucoside; (2) cyanidin-3-*O*-galactoside; (3) cyanidin-3-*O*-glucoside; (4) peonidin-3-*O*-glucoside; (5) malvidin-3-*O*-glucoside; (6) delphinidin; (7) cyanidin; (8) pelargonidin; (9) malvidin.

sation detector (FID) and a CP-WAX 58-(FFAP)CB (50 m × 0.25 mm) column. The injection port was a split–splitless one, working at 200 °C, in splitless mode for 0.5 min and split ratio 30:1. The carrier gas was hydrogen, with a flow rate of 2.8 ml/min. The oven temperature was as follows: 40 °C (5 min), 3 °C/min to 200 °C (20 min), and the detector temperature was set at 250 °C.

The detector signals were recorded and processed by Chrom-Card for Windows software (Fisions, USA). The compounds were identified by comparing their retention times with those from standards.

Volatile phenols quantification was achieved by the external standard method.

3. Results and discussion

Wines include a number of polyphenolic constituents (Andrade, Seabra, Ferreira, Ferreres, & García-Viguera, 1998; Andrade et al., 2001; Ramos et al., 1999) that have shown interesting biological properties, related to their antioxidant capacities (Frankel, Waterhouse, & Teissedre, 1995; Zafrilla et al., 2003). Among them, there are anthocyanins, which are extracted from the skins of black grapes during winemaking, becoming responsible for the wine colour. However, during conservation and ageing of red wines the levels of anthocyanins decrease, as they react with other wine constituents, resulting in the change of the colour and loss of astringency (Atanasova et al., 2002; de Villiers et al., 2004).

In the present work, the analysis of wine samples by reversed-phase HPLC/DAD allowed the identification of nine anthocyanic compounds: malvidin-3,5-*O*-diglucoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, delphinidin, cyanidin, pelargonidin and malvidin (Fig. 1). The presence of cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside is not surprising, as they could be expected on the basis that these compounds were described in Touriga Nacional grapes, though collected in another distinct Portugal region (Douro Valley) (Mateus, Machado, & de Freitas, 2002).

All the analysed samples exhibited a profile composed by the nine identified anthocyanic compounds, ranging from 235 to 290 mg/l (Table 1). Despite the differences observed in each anthocyanin content, there are common characteristics in the profile obtained. All of the samples exhibited malvidin-3-*O*-glucoside as the major compound, representing ca. 65% of total compounds. This is in accordance with previous works that reported malvidin-3-*O*-glucoside as the main compound in Touriga Nacional grapes from the Douro valley (Mateus et al., 2002) and in several red wines made with other grape varieties (de Villiers et al., 2004; Revilla et al., 2001). Cyanidin derivatives and the four anthocyanidins were always minor compounds (Table 1). Thus, the anthocyanin composition was not affected, either by the refrigeration or the inoculation with *D. bruxellensis*.

Table 1
Anthocyanins content in wine samples (mg/l)[a]

Sample	Anthocyanin									Total
	Malvidin-3,5-*O*-diglucoside (Rt 29.6 min)	Cyanidin-3-*O*-galactoside (Rt 30.6 min)	Cyanidin-3-*O*-glucoside (Rt 33.7 min)	Peonidin-3-*O*-glucoside (Rt 41.5 min)	Malvidin-3-*O*-glucoside (Rt 43.2 min)	Delphinidin (Rt 46.7 min)	Cyanidin (Rt 51.9 min)	Pelargonidin (Rt 54.6 min)	Malvidin (Rt 56.7 min)	
W1	70.5 (12.0)	nq	nq	14.7 (2.9)	179.1 (33.6)	2.0 (0.2)	3.1 (0.2)	2.3 (0.5)	10.0 (2.8)	281.5
W2	73.0 (0.6)	nq	nq	15.0 (0.0)	189.5 (1.4)	nq	4.7 (0.2)	nq	8.0 (4.9)	290.3
D1	63.5 (1.3)	nq	nq	14.8 (0.2)	168.4 (3.2)	1.6 (0.3)	3.7 (0.0)	nq	11.1 (5.2)	263.2
D2	67.1 (0.0)	nq	nq	14.0 (0.7)	168.9 (0.5)	1.3 (0.1)	4.0 (1.2)	nq	8.4 (0.2)	263.8
D3	54.9 (2.3)	nq	nq	11.7 (0.5)	155.6 (7.1)	1.2 (0.5)	3.5 (0.0)	1.5 (0.1)	6.5 (0.6)	234.8
D4	57.9 (1.1)	nq	nq	13.3 (0.3)	150.6 (1.5)	2.0 (0.5)	3.3 (0.0)	3.2 (0.8)	6.7 (0.9)	237.1
D5	69.6 (0.4)	nq	2.7 (0.1)	14.3 (0.3)	165.6 (3.0)	1.6 (0.1)	3.7 (0.2)	2.6 (1.2)	8.4 (0.1)	268.5
D6	55.5 (0.2)	nq	1.4 (0.5)	13.5 (0.1)	159.4 (0.7)	2.3 (0.4)	2.9 (0.2)	nq	7.9 (0.5)	242.8
D7	65.4 (1.9)	nq	2.1 (0.6)	14.0 (0.5)	178.3 (5.6)	1.9 (0.0)	4.6 (0.4)	2.1 (0.6)	8.6 (0.6)	277.0
D8	66.4 (2.3)	nq	nq	12.9 (0.1)	163.2 (1.2)	2.1 (0.3)	3.4 (0.1)	1.6 (0.2)	8.8 (0.1)	258.4
D9	59.0 (1.9)	nq	nq	12.0 (0.7)	158.4 (5.6)	2.1 (0.0)	2.8 (0.2)	1.4 (0.4)	9.0 (0.0)	244.8
S	62.8 (4.7)	nq	nq	13.2 (0.5)	158.8 (7.4)	1.5 (0.2)	3.1 (0.1)	1.6 (0.4)	5.2 (1.2)	246.2

[a] Values are expressed as means (standard deviation) of three determinations; nq, not quantified.

The nature and concentration of organic acids are important factors influencing the organoleptic characteristics of fruit and vegetables, i.e. their flavour (Vaughan & Geissler, 1997). Acids are known to have a lower susceptibility to change during processing and storage than other compounds, such as pigments and flavour components (Cámara, Díez, Torija, & Cano, 1994). Besides, organic acids may have a protective role against various diseases due to their antioxidant activity (Silva et al., 2004). Wine includes the acids from grape juice and those produced during fermentation (Esteves et al., 2004). The HPLC/UV analysis allowed the identification of six organic acids: ketoglutaric, tartaric, malic, quinic, lactic and shikimic acids (Fig. 2).

The sum of quantified organic acids in all of the samples ranged from ca. 6 to 9 g/l (Table 2). Lactic plus shikimic acids were the main acids, corresponding to ca. 80% of total compounds, followed by tartaric acid (ca. 13% of total acids). Ketoglutaric acid was the compound present in the lowest amount, with the exception of sample W2, in which quinic acid was the minor acid. Malic acid, which was not present in all of the samples inoculated with *D. bruxellensis* (Dl–D9), was noticed in the witness sample (W2), although in very small amounts (Table 2). This could suggest an influence of the yeast in malolactic fermentation, but an increase of lactic acid content was not observed. Additionally, for quinic acid, the results obtained with sample W1 were quite different from those of sample W2. When compared with sample W2, data from sample W1 seem to indicate that refrigeration leads to the doubling of quinic acid content (from ca. 3% to 6% of total identified compounds).

Depending on their concentration levels, volatile phenols can be considered as normal constituents or as the cause of deterioration. Red wine quality is affected by levels of ethylphenols (4-ethylguaiacol + 4-ethylphenol) above 425 μg/l (Chatonnet et al., 1995; Chatonnet et al., 1997). These compounds arise mainly from the metabolism of hydroxycinnamic acids by *Brettanomyces*/*Dekkera* yeasts, which involves the sequential action of two enzymes. First, a cinnamate decarboxylase cleaves the phenolic acids (namely ferulic, caffeic and *p*-coumaric acids) directly into the corresponding vinylphenol. Then, a vinylphenol reductase converts the vinylphenol into the corresponding ethylphenol (Chatonnet et al., 1995; Edlin, Narbad, Dickinson, & Lloyd, 1995).

Both 4-ethylguaiacol and 4-ethylphenol were detected in all analysed samples (Table 3). In contrast to what happened with the witness (W2), all of the inoculated samples (D1–D9) exhibited levels of 4-ethylphenol higher than those of 4-ethylguaiacol, especially sample D7: inoculation led to an increase in the production of 4-ethylphenol, ranging from 19% to 54%, when compared with the witness sample. Besides, in samples D1, D2, D3, D4, D5 and D7 an increase of volatile phenol contents was noticed, but only the yeast strain used to inoculate sample D7 led to a production of these compounds to values above which the wine is considered deteriorated (Table 3). The decrease of *t*-CAFTA, *t*-COUTA caffeic and *p*-coumaric acids in inoculated samples, already reported (Silva et al., 2005),

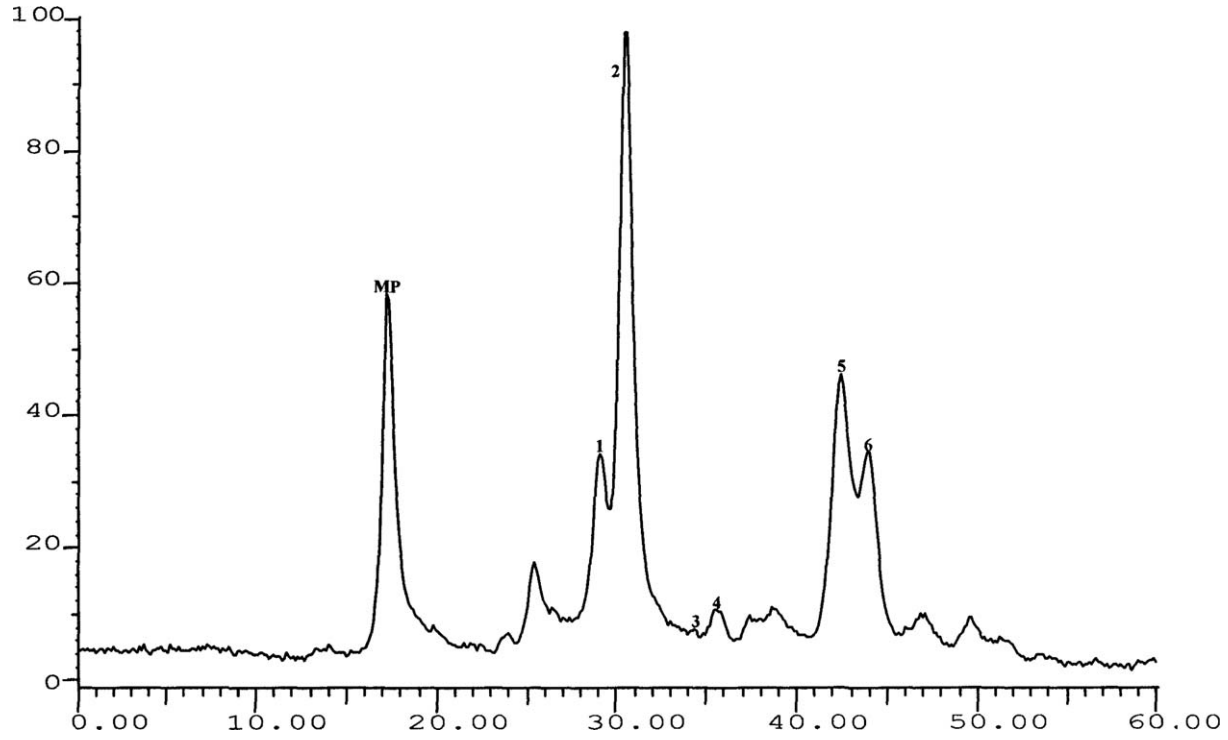

Fig. 2. HPLC organic acids profile of wine samples. Detection at 214 nm (MP) mobile phase; (1) ketoglutaric acid; (2) tartaric acid; (3) malic acid; (4) quinic acid; (5) lactic acid; (6) shikimic acid.

Table 2
Organic acids content in wine samples (mg/l)[a]

Sample	Organic acid					Total
	Ketoglutaric acid (Rt 28.8 min)	Tartaric acid (Rt 59.5 min)	Malic acid (Rt 34.3 min)	Quinic acid (Rt 35.5 min)	Lactic acid + shikimic acid (Rt 42.3, 44.0 min)	
W1	207 (6.4)	1235 (6.4)	40 (15.0)	489 (20.4)	6641 (40.7)	8611
W2	227 (18.8)	1113 (2.8)	nq	194 (3.2)	6004 (222.7)	7538
D1	169 (1.5)	1014 (1.4)	–	536 (29.6)	5584 (154.6)	7303
D2	101 (1.6)	625 (6.8)	–	195 (65.7)	5843 (157.7)	6765
D3	190 (13.8)	926 (73.9)	–	372 (5.3)	6185 (76.2)	7673
D4	274 (19.8)	1235 (0.6)	–	584 (30.4)	7052 (44.2)	9145
D5	83 (5.0)	786 (19.3)	–	152 (14.3)	4825 (222.6)	5848
D6	217 (10.8)	671 (4. 7)	–	438 (19.0)	5738 (246.8)	7065
D7	146 (5.2)	996 (5.8)	–	203 (14.9)	5914 (44.8)	7259
D8	107 (5.2)	911 (1.6)	–	379 (63.0)	5415 (52.4)	6812
D9	131 (2.0)	760 (8.4)	–	342 (36.0)	5284 (119.4)	6519
S	72 (3.7)	692 (3.9)	–	150 (17.0)	5154 (4.6)	6068

[a] Values are expressed as means (standard deviation) of three determinations; nq, not quantified.

Table 3
Volatile phenols content in wine samples (μg/l)[a]

Sample	Volatile phenol		Total
	4-Ethylguaiacol (Rt 48.0 min)	4-Ethylphenol (Rt 52.4 min)	
W1	47.7 (0.0)	46.8 (0.0)	94.5
W2	52.2 (0.0)	29.4 (0.0)	81.6
D1	38.0 (0.0)	46.8 (0.0)	84.8
D2	52.0 (0.1)	91.2 (0.0)	143.2
D3	32.9 (0.0)	62.9 (0.0)	95.8
D4	46.0 (0.1)	50.5 (0.0)	96.5
D5	38.4 (0.0)	59.7 (0.0)	98.1
D6	10.8 (0.0)	53.9 (0.0)	64.7
D7	49.3 (0.0)	448.7 (0.0)	498.0
D8	nq	nq	nq
D9	18.0 (0.0)	29.0 (0.0)	47.0
S	53.7 (0.0)	42.4 (0.0)	96.1

[a] Values are expressed as means (standard deviation) of three determinations; nq, not quantified.

partly explains the rise of 4-ethylphenol, as the free acids are substrates for volatile phenols production (Edlin et al., 1995). However, other precursors should also be involved, which could justify the remarkable increase of this compound in sample D7. The results obtained with sample W1 seem to indicate that refrigeration also contributes to higher 4-ethylphenol amounts (Table 3).

In general, the effect of *D. bruxellensis* seems to be identical to that of *S. cereviseae*. However, in the sample inoculated with *S. cereviseae*, 4-ethylguaiacol was the main volatile phenol (Table 3).

In conclusion, the results obtained in the work herein indicate that some strains of *D. bruxellensis* yeast are able to cause deterioration of red wine from the Dão region during its maturation by the production of volatile phenols, namely 4-ethylphenol, conferring unpleasant organoleptic characterisitcs. As far as we know, this is the first report on the anthocyanin organic acid and volatile phenol compositions of red wine from the Dão region and about the effect of different *D. bruxellensis* strains on these constituents.

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