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Analysis of non-coloured phenolics in red wine: Effect of *Dekkera bruxellensis* yeast

Luís R. Silva [a,b], Paula B. Andrade [c,*], Patrícia Valentão [c], Rosa M. Seabra [c], Martha E. Trujillo [b], Encarna Velázquez [b]

[a] *Escola Superior Agrária de Viseu, Departamento das Indústrias Agro-Alimentares, Estrada de Nelas-Quinta da Alagoa, 3500-606 Viseu, Portugal*
[b] *Departamento de Microbiologia y Genética, Universidad de Salamanca, Salamanca, Spain*
[c] *REQUIMTE, Serviço de Farmacognosia, Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha, 4050-047 Porto, Portugal*

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Abstract

The non-coloured phenolic composition of red wine obtained from Touriga Nacional grapes growing in the Dão region (Portugal) was determined by HPLC/DAD and 16 compounds were identified and quantified. The effect of nine different *Dekkera bruxellensis* strains on the levels of phenolic acids and flavonoids of the wine were also evaluated. In all samples (inoculated and non inoculated), phenolic acids predominated over flavonoids. In inoculated samples a considerable rise in the amounts of gallic acid was observed (about 33%) while the amounts of *t*-CAFTA, *t*-COUTA, caffeic and *p*-coumaric acids suffered a statistically significant decrease.

Keywords: Dão red wine; *Dekkera bruxellensis*; Non-coloured phenolics; HPLC/DAD

1. Introduction

The quality of a wine depends on its numerous constituents, with the presence/absence and amount of a given chemical playing a considerable role. Phenolic compounds are always present and they contribute markedly to the colour, flavour, bitterness and astringency of the final product (Macheix, Fleuriet, & Billot, 1990). In addition to their direct role, phenolic compounds may also contribute to the sensory and chemical qualities of wine because of their interaction with other compounds, namely proteins, polysaccharides or other polyphenols. Moreover, interest in phenolic compounds in wine has increased in recent years because of their potential beneficial effects on human health (Frankel, Waterhouse, & Teissedre, 1995; Zafrilla et al., 2003).

The phenolic composition is deeply influenced by three sets of factors: the nature of the raw material (grape variety, its degree of maturation, the nature of the soil and the climate) (Andrade, Oliveira, Seabra, Ferreira, Ferreres, & García-Viguera, 2001; Ramos, Andrade, Seabra, Pereira, Ferreira, & Faia, 1999; Soleas, Dam, Carey, & Goldberg, 1997), the vinification techniques (Ramos et al., 1999; Zafrilla et al., 2003) and evolution of phenolic compounds during the ageing of wine (Zafrilla et al., 2003). In comparison with the original grapes, qualitative modifications occur, mainly due to hydrolysis, oxidations and complexations. Hydrolysis (enzymatic or not) is mainly responsible for the appearance or increase of simpler compounds, such as tyrosol and free phenolic acids. Oxidations and complexations are also described; for instance, in the case of hydroxycinnamic derivatives, one-third are found unchanged in wine, one-third are oxidized and linked to sulfhydryl compounds and the rest are found as complexes with nucleic acids (Macheix et al., 1990). Any of these transformations can be caused, or not, by enzymes that can be native to the grapes or have their origin in the microorganisms (Macheix et al., 1990).

A number of microbial species have been reported to decarboxylate hydroxycinnamic acids, such as ferulic,

* Corresponding author. Tel.: +351-222-078934; fax: +351-222-003-977.
E-mail address: pandrade@ff.up.pt (P.B. Andrade).

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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 many beers and wines (Edlin, Narbad, Gasson, Dickinson, & Lloyd, 1998). This off-flavour, described as the phenolic character, can appear in all red wines at different stages in the production and ageing processes. Contaminating yeasts of the genus *Dekkera*/*Brettanomyces*, especially *D. bruxellensis* species, are able to form very significant amounts of ethylphenols when developed in wine. However, the growth of this type of yeast during fermentation is rather uncommon if elementary hygiene precautions are taken. On the other hand, this problem usually occurs during aging, prior to bottling, especially when wines are kept in barrels, particularly old barrels, if rarely or never racked. Once malolactic and alcoholic fermentations are completed, these yeasts grow easily on traces of residual sugars (Chatonnet, Dubourdieu, & Boidron, 1995; Chatonnet, Viala, & Dubourdieu, 1997).

Touriga Nacional is an important red grape cultivar, highly esteemed in Dão (north central Portugal) for its ability to produce high quality wines, being responsible for the prestige that Dão wines have acquired. As far as we know, no previous report exists concerning the phenolic composition of the wine produced from this cultivar growing in Dão nor about the effect of yeast contamination on its phenolic content. In the present work, phenolic acids and flavonoid composition of wine from Touriga Nacional cultivar from Dão and the alterations induced by different *D. bruxellensis* strain contaminations were investigated.

2. Materials and methods

2.1. Standards and reagents

Standards were from Extrasynthese (Genay, France). HPLC grade methanol and formic acid were obtained from Merck (Darmstadt, Germany). *trans*-Caffeoyltartaric acid (*t*-CAFTA) and *trans*-*p*-coumaroyltartaric acid (*t*-COUTA) were kindly supplied by Dr. C. Garcia-Viguera (CEBAS-CSIC, Murcia, Spain). 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) (Table 1).

2.3. Wine samples

The red wine used in the present study is a commercial one and was produced in a winery (Sogrape) from Touriga Nacional grapes growing in the Dão region, collected in September, 2000. Five litres of wine were removed from the stainless steel tank, sterilised by filtration through a 0.45 μm membrane filter (Millipore, USA) and divided in to 250 ml glass flasks. Samples W1 and W2 did not receive any microorganism and served as witness samples. Samples D1–D9 were inoculated with different strains of *D. bruxellensis*, while sample S was inoculated with *Saccharomyces cerevisiae* (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 in 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 was used to evaluate the effect of the temperature (Fig. 1). The growth in the inoculated samples was measured by count of viable cells in the medium at

Table 1
Biochemical characterization of inoculated *Dekkera* sp. yeasts[a]

Tests	Sample[b]								
	D1	D2	D3	D4	D5	D6	D7	D8	D9
Urease production	−	−	−	−	−	−	−	−	−
Nitrate growth	+	+	+	+	+	+	+	+	+
Starch	−	−	−	−	−	−	−	−	−
Raffinose	+	+	−	−	w	+	+	w	−
Lactose	−	−	−	−	−	−	−	−	−
Galactose	w	−	−	w	−	−	w	w	−
Melezitose	+	+	−	+	+	−	+	+	+
Erythritol growth	−	−	−	−	−	−	−	−	−
D-glucose fermentation	+	+	+	+	+	+	+	+	+
Growth 0,1% cycloheximide	+	+	+	+	+	+	+	+	+

[a] +: Positive, −: negative, w: weak.
[b] Identity of samples as in Fig. 1.

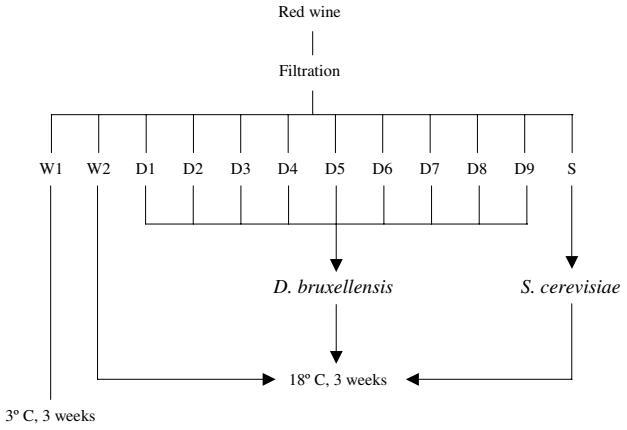

Fig. 1. Schematic representation of wine samples. W1 and W2: witness samples; D1–D9: samples inoculated with different strains of *D. bruxellensis*; S: sample inoculated with *S. cereviseae*.

the end of the incubation. From each glass flask, 1 ml was recovered under sterile conditions and was serially diluted in sterile peptone water, plated in Saboureaud cloramfenicol plates and incubated for 1–2 weeks at 25 °C to obtain the number of CFU (colony forming units) per mililitre. At the end of the incubation, the media contained about 1.5×10^6 CFU/ml. The samples were then frozen until the preparation of the extracts.

2.4. Preparation of extracts

Wine samples (20 ml) were extracted three times with 30 ml of diethyl ether for 5 min, with agitation. The ether fraction was then separated and concentrated to dryness using a rotary evaporator. The residue obtained was redissolved in 1 ml of methanol for HPLC analysis.

2.5. HPLC/DAD analysis of phenolic compounds

Twenty microlitres of each extract were analysed using a Gilson HPLC unit and an ODS-Hypersil reversed-phase column (20×0.21 cm, 5 μm particle size). Elution solvents were water/formic acid (19:1) (A) and methanol (B), with a flow rate of 0.3 ml/min, using a linear gradient starting at 2% B, increasing to 62% B after 60 min (Ramos et al., 1999). Detection was achieved with a Gilson DAD. Spectral data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 280, 320 and 350 nm. The data were processed on Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. As the available amounts of *t*-CAFTA and *t*-COUTA standards were not enough, these compounds were quantified as caffeic and *p*-coumaric acids, respectively. Epicatechin was quantified as catechin and the other compounds were quantified as themselves.

2.6. Statistical analysis

The evaluation of statistical significance was determined by ANOVA, followed by Newman-Keuls test. The level of significance was set at $p < 0.05$.

3. Results and discussion

The Dão region is located in north central Portugal, with a 376,000 ha geographical surface, from which 20,000 ha correspond to vineyards. The unique characteristics of this region result from the presence of three mountainous groups, Serra do Caramulo and Serra do Buçaco in the west, Serra da Nave in the north and Serra da Estrela in the east, which constitute an important barrier against humid masses from the coast and continental winds. As a result, Dão has a temperate climate, although cold and rainy in winter and frequently very hot in summer. The vineyards from Dão that give origin to wines with *Denomination d'Origine Controllée* (DOC) are implanted in granite land, of little fertility, between 400 and 500 m altitude. The wine presents a deep ruby colour, a delicate scent and velvety taste, with a distinctive herbaceous bouquet, followed by an aroma of forest fruits.

In the present work, the analysis of wine samples by reversed-phase HPLC/DAD allowed the identification of sixteen non-coloured phenolic compounds: gallic, 3,4-di-hydroxybenzoic, *t*-CAFTA, *t*-COUTA, caffeic, syringic, *p*-coumaric, ferulic, sinapic and ellagic acids, epicatechin, myricetin, quercetin, kaempferol, isorhamnetin and tyrosol (Fig. 2). These results were the expected ones for a wine prepared with Touriga Nacional grapes, once we had found the aglycones of the flavonoidic heterosides (myricetin, quercetin, kaempferol and isorhamnetin glycosides) present in these grapes (Andrade, Mendes, Falco, Valentão, & Seabra, 2001), which are hydrolysed by the enzymes of the plant material during the vinification process (Macheix et al., 1990). On the other hand, as mentioned above, the vinification techniques have marked effects on the phenolic composition of a wine. So, it was not surprising that wine from Dão exhibited a phenolic profile distinct from that obtained of Port wine prepared with the same grape variety (Andrade, Seabra, Ferreira, Ferreres, & García-Viguera, 1998).

The metabolism of hydroxycinnamic acids by *Bretanomyces/Dekkera* yeasts involves the sequential action of two enzymes. The first is a cinnamate decarboxylase, which cleaves the phenolic acid (namely ferulic, caffeic and *p*-coumaric acids) directly into the corresponding vinylphenol. The second enzyme is a vinylphenol

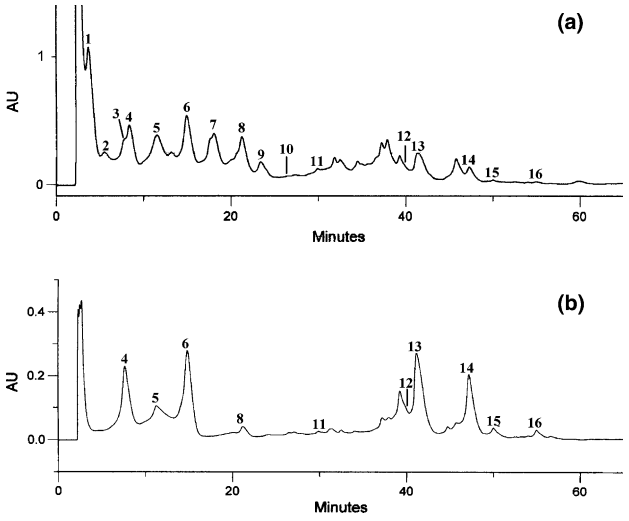

Fig. 2. HPLC profile of wine samples. Detection at (a) 280 and (b) 350 nm: (1) gallic acid; (2) 3,4-di-hydroxybenzoic acid; (3) tyrosol; (4) *trans*-caffeoyltartaric acid; (5) *trans p*-coumaroyltartaric acid; (6) caffeic acid; (7) syringic acid; (8) *p*-coumaric acid; (9) epicatechin; (10) ferulic acid; (11) sinapic acid; (12) ellagic acid; (13) myricetin; (14) quercetin; (15) kaempferol; (16) isorhamnetin.

reductase, which converts the vinyl into the corresponding ethylphenol (Chatonnet et al., 1995; Edlin, Narbad, Dickinson, & Lloyd, 1995). In order to verify if there were other phenolic substrates for this yeast, we have evaluated the non-coloured phenolic composition of samples inoculated with several *D. bruxellensis* strains.

Although all the analysed samples exhibited a common phenolic profile, there were differences in the amounts of each identified compound (Table 2). The results obtained with sample W1 were quite different from those of sample W2. The results from sample W1 seem to indicate that refrigeration leads to higher contents of 3,4-di-hydroxybenzoic and syringic acids and epicatechin (9%, 7% and 35% of total identified compounds, respectively) when compared with sample W2, in which these compounds were present in trace amounts. Storage at low temperatures also leads to decreases in gallic acid (from 25% to 13%), *t*-CAFTA acid (from 15% to 9%), *t*-COUTA acid (from 11% to 6%), caffeic acid (from 26% to 12%) and *p*-coumaric acid (from 8% to 4%) contents. In all inoculated samples, gallic acid was the most abundant phenolic, representing 33% of total identified compounds and, when present, epicatechin, was also a major compound (29%). In samples D1–D9, the epicatechin contents were significantly higher than that of sample W2. This significant increase, observed in inoculated samples, may possibly be due to the instability of the tannins at wine pH (Ibern-Goméz, Andrés-Lacueva, Lamuela-Raventós, Lao-Luque, Bruxaderas, & de la Torre-Boronat, 2001)

Table 2
Phenolic compounds content in wine samples (mg/l)[a]

Sample[b]	Phenolic compound[b]															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
W1	12.0 (0.93)	8.0 (3.17)	nq	8.2 (0.07)	5.5 (0.11)	11.4 (0.09)	6.2 (0.05)	3.4 (0.03)	32.0 (0.13)	nq	0.7 (0.00)	nq	2.5 (0.01)	2.4 (0.29)	nq	nq
W2	10.1 (0.81)	nq	nq	6.1 (0.23)	4.6 (0.10)	10.5 (0.39)	0.2 (0.02)	3.4 (0.22)	nq	nq	0.6 (0.01)	nq	2.2 (0.09)	2.1 (0.10)	0.2 (0.05)	0.5 (0.02)
D1	31.7 (0.78)	9.9 (2.26)	nq	7.5 (0.30)	5.4 (0.18)	10.8 (0.31)	6.5 (0.49)	3.6 (0.19)	31.7 (1.05)	nq	0.4 (0.02)	nq	1.3 (0.00)	1.7 (0.04)	nq	nq
D2	27.6 (1.51)	5.8 (0.52)	nq	6.3 (0.14)	4.6 (0.12)	10.2 (0.19)	6.0 (0.07)	3.6 (0.05)	30.9 (0.57)	nq	0.6 (0.00)	nq	2.0 (0.39)	2.3 (0.47)	nq	nq
D3	34.5 (2.22)	nq	nq	6.6 (0.03)	4.9 (0.03)	10.9 (0.18)	5.5 (0.25)	3.3 (0.08)	30.6 (2.90)	nq	0.8 (0.01)	nq	3.9 (0.02)	3.2 (0.13)	0.5 (0.00)	nq
D4	29.9 (0.98)	nq	nq	5.5 (0.00)	4.2 (0.05)	8.8 (0.10)	5.1 (0.10)	2.8 (0.02)	23.9 (0.65)	nq	0.7 (0.00)	nq	2.4 (0.01)	1.9 (0.08)	nq	nq
D5	19.1 (0.08)	nq	nq	3.5 (0.15)	2.5 (0.20)	6.1 (0.08)	4.7 (0.08)	2.2 (0.02)	nq	nq	nq	nq	1.5 (0.06)	1.4 (0.08)	nq	nq
D6	37.9 (0.07)	294.1 (5.44)	nq	2.3 (0.16)	5.7 (0.05)	8.7 (0.16)	3.9 (0.15)	3.2 (0.10)	nq	nq	0.6 (0.00)	nq	1.7 (0.03)	1.8 (0.04)	nq	nq
D7	27.0 (1.21)	nq	nq	4.5 (0.06)	3.9 (0.14)	9.3 (0.30)	0.8 (0.00)	3.0 (0.05)	nq	nq	0.4 (0.03)	nq	2.7 (0.38)	1.8 (0.28)	nq	nq
D8	34.0 (0.02)	9.7 (3.74)	nq	4.9 (0.10)	4.2 (0.16)	10.8 (0.62)	5.7 (0.10)	3.6 (0.17)	29.5 (2.16)	nq	0.5 (0.00)	nq	2.3 (0.07)	1.6 (0.07)	nq	nq
D9	33.4 (0.50)	9.7 (2.00)	nq	6.9 (0.32)	4.8 (0.19)	0.1 (0.00)	6.5 (0.45)	3.7 (0.70)	28.6 (3.34)	nq	0.5 (0.12)	nq	2.0 (0.03)	1.4 (0.01)	nq	nq
S	30.0 (3.55)	nq	nq	3.6 (0.31)	3.7 (0.13)	9.6 (0.12)	5.4 (0.07)	3.2 (0.08)	26.9 (0.08)	nq	0.3 (0.03)	nq	1.9 (0.15)	1.7 (0.08)	nq	nq

[a] Values are expressed as mean (standard deviation) of three determinations; nq: not quantified.
[b] Identity of samples as in Fig. 1.
[c] Identity of compounds as in Fig. 2.

and to the presence of enzymes, in the yeast, that use tannins as substrate.

Significantly lower contents of *t*-CAFTA, *t*-COUTA, caffeic and *p*-coumaric acids were obtained with inoculated samples (Table 2). Generally, the decrease of *t*-CAFTA and *t*-COUTA acids in the course of the fermentation process is not accompanied by a proportional accumulation of free acids, because hydrolysis products are rapidly further metabolised (Karagiannis, Economou, & Lanaridis, 2000). These results may be explained by the *D. bruxellensis* conversion of caffeic and *p*-coumaric acids into volatile phenols (Edlin et al., 1995; Ibern-Goméz et al., 2001). The yeast strain used to inoculate sample D6 seems to affect the amount of 3,4-di-hydroxybenzoic acid, since a high level of this compound was found in this sample (Table 2). A significant reduction was found for quercetin content in samples that received *D. bruxellensis* (Table 2).

Although ferulic acid is also a precursor of volatile phenols (Edlin et al., 1995; Ibern-Goméz et al., 2001), no decrease of its content was observed in inoculated samples, since it was a minor compound in both witness samples (Table 2). Tyrosol, ellagic acid, kaempferol and isorhamnetin were always minor compounds.

The effect of *D. bruxellensis* on the phenolic constituents seems to be identical to that of *S. cereviseae*. In conclusion, the presence of different strains of *D. bruxellensis* leads to the same alterations in the phenolic composition of red wine, being caffeic and *p*-coumaric acids being the main substrates for volatile phenol production. As far as we know, this is the first report about the phenolic composition of red wine from the Dão region and about the effect of different *D. bruxellensis* strains on its phenolic profile. In the course of this work, the ethyl and vinyl-phenolic composition should be studied, in order to check for a possible correlation with the percursors of the volatile phenols.

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