# Volatile compounds contribution of *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* during red wine vinifications

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A B S T R A C T

This work aims to investigate the contribution of a selected non-*Saccharomyces* yeast species, *Hanseniaspora guilliermondii*, to higher alcohols, esters, fatty acids and heavy sulphur compounds composition of red wine. Red grape must vinifications of 100 l were performed and an inoculated fermentation with *H. guilliermondii* was compared to a spontaneous fermentation. The presence of apiculate yeasts was observed in both fermentations; however, *Hanseniaspora uvarum* was the only apiculate yeast isolated from the spontaneous fermentation. Apiculate yeasts dominated the fermentation until an ethanol concentration of 6% (v/v) was attained and remained in considerable high levels for an ethanol concentration of 12.5% (v/v). The grape must inoculated with *H. guilliermondii* led to the production of wine with higher concentrations of 1-propanol, 2-phenylethyl acetate and 3-(methylthio)propionic acid, and lower amounts of ethyl hexanoate, pentanoic acid, free fatty acids, 2-methyltetrahydrothiophen-3-one and acetic acid-3-(methylthio)propyl ester, than wine resulting from the spontaneous fermentation. The present study shows that the use of specific apiculate yeasts in grape must fermentations may lead to the production of wines with different chemical profiles, emphasising the importance of *Hanseniaspora* yeasts as mixed starter cultures with *Saccharomyces cerevisiae*, in winemaking.

## 1. Introduction

Traditionally wine fermentation has been carried out in a spontaneous way, by indigenous yeasts present on grapes when harvested, or passively introduced from contact surfaces during the vinification process (Constantí, Poblet, Arola, Mas, & Guillamón, 1997). Many qualitative studies have shown the sequential development of yeast species during spontaneous wine fermentations. It is well known that the early stages are characterised by growth of non-*Saccharomyces* yeasts, mostly apiculate yeasts (Beltran et al., 2002; Fleet, 2003; Fleet & Heard, 1993; Fugelsang & Edwards, 2007, pp. 84–86; Granchi, Ganucci, Messini, Rosellini & Vincenzini, 1998). *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* can grow up to $10^6$–$10^8$ cells/ml during the first 4–6 days of fermentation, after which they die off largely as a result of the increasing ethanol concentration produced by *Saccharomyces cerevisiae*, which has higher fermentation activity and ethanol tolerance (Fleet, 2003; Fugelsang & Edwards, 2007, pp. 84–86; Granchi et al., 1998; Jolly, Augustyn, & Pretorius, 2006; Zott, Miot-Sertier, Claisse, Lanvaud-Funel, & Masneuf-Pomarede, 2008). Generally, the species of *Hanseniaspora*, *Candida*, *Pichia*, *Metschnikowia* found in grape juice are not tolerant to ethanol concentrations exceeding 4–7%, and this explains their decline and death as the fermentation progresses beyond the mid-stage (Fleet, 2003; Fleet & Heard, 1993; Fugelsang & Edwards, 2007, pp. 84–86; Moreira, Mendes, Guedes de Pinho, Hogg, & Vasconcelos, 2008). However, the diversity of yeast population in grape musts, the interaction between different yeast species and strains, the *S. cerevisiae* inoculum concentrations, the nutrient composition of grape musts and the processing conditions (sulphur dioxide addition, oxygen content and fermentation temperature) also affect growth of non-*Saccharomyces* yeasts during alcoholic fermentation (Erten, Tanguler, Cabaroglu, & Canbas, 2006; Fleet, 2003; Hansen, Nissen, Sommer, Nielsen, & Arneborg, 2001; Jolly et al., 2006; Nissen & Arneborg, 2003; Nissen, Nielsen, & Arneborg, 2003; Pérez-Nevado, Albergaria, Hogg, & Gírio, 2006; Zott et al., 2008).

Apiculate yeasts have become an object of increasing interest, as their proliferation in competition with *S. cerevisiae* can impact on the sensory quality of wine, because of higher esters production (Moreira et al., 2008; Plata, Millán, Mauricio, & Ortega, 2003; Rojas, Gil, Piñaga, & Manzanares, 2003; Viana, Gil, Genovés, Vallés, & Manzanares, 2008). Nevertheless, few reports are available in

literature concerning the contribution of non-*Saccharomyces* yeasts to fatty acids and heavy sulphur compounds composition of wines. These compounds, formed mainly during alcoholic fermentation, can contribute negatively to wine aroma; due to their impact on wines organoleptic profile, it is important to evaluate how growth of non-*Saccharomyces* yeasts influences the composition of wine.

Fatty acids are produced by yeasts as intermediates in the biosynthesis of long-chain fatty acids, important constituents of yeast membrane (Lambrechts & Pretorius, 2000). When present above certain concentrations, short- to medium-chain fatty acids can also act as inhibitors of growth and survival of *S. cerevisiae*, causing stuck fermentations (Fleet & Heard, 1993). The production of these acids varies significantly with yeast species and strains and may influence the sequential growth of yeasts during fermentation (Fleet, 2003).

Sulphur compounds in wines come mainly from the metabolism of yeast, being associated to the reduction of sulphate and, consequently, the production of sulphur amino acids (methionine and cysteine). Heavy sulphur compounds are volatile compounds present in wines in low amounts and are characterised by their low odour thresholds (Anocibar Beloqui & Bertrand, 1995; Mestres, Busto, & Guasch, 2000). They may play an attractive or repulsive role, according to their nature and quantity present in wine (Landaud, Helinck, & Bonnarme, 2008).

The main heavy sulphur compound reported in wine is 3-(methylthio)-1-propanol(methionol), usually found at concentrations above its threshold value, which attributes a cauliflower, cabbage, and cooked-potatoes aroma (Anocibar Beloqui & Bertrand, 1995; Landaud et al., 2008). Most of the many other heavy sulphur compounds identified in wines, with low detection limits, are usually found at levels below their threshold value; these include 2-mercaptoethanol (poultry-like aroma), 2-methyltetrahydrothiophen-3-one (metallic, natural gas odour), 2-(methylthio)ethanol (French bean aroma), acetic acid-3-(methylthio) propyl ester (mushroom and garlic odour), 3-mercapto-1-propanol (roasted, potato, broth), 4-(methylthio)-1-butanol (chive-garlic aroma) and 3-(methylthio)propionic acid (chocolate, roasted flavour) (Anocibar Beloqui & Bertrand, 1995; Landaud et al., 2008; Lavigne, 1996; Mestres et al., 2000; Rauhut, 1993; Swiegers & Pretorius, 2007). A family of volatile thiols was identified as a positive contributor to complexity and varietal character of wines (Sarrazin et al., 2007; Swiegers & Pretorius, 2007; Tominaga & Dubourdieu, 2006).

In order to understand the growth behaviour of non-*Saccharomyces* yeasts, in particular apiculate yeasts, during grape must fermentation, and its influence on higher alcohols, esters, fatty acids and heavy sulphur compounds composition of wines, a *H. guilliermondii* NCYC 2380 inoculated fermentation was compared to a spontaneous fermentation of the same must.

## 2. Materials and methods

### 2.1. Yeast strain

The yeast strain used in this work was *H. guilliermondii* NCYC 2380 (National Collection of Yeast Cultures, Norwich, UK). This apiculate yeast was initially isolated from grape must used for Port wine production in the Douro region of Portugal. The strain was maintained on Yeast Malt agar slants (YM agar, Difco Laboratories Detroit, IN, USA) at 5 °C prior to use.

### 2.2. Fermentation conditions and media

Red grapes of *Tinta Roriz* variety from the Dão region of central Portugal were used. Grapes were obtained before entrance into the winery to exclude contamination from winery surfaces. The must, obtained by the traditional foot treading technique, had a pH of 3.7 and a sugar content of approximately 205 g/l. Sulphur dioxide was added to the must at a concentration of 6 mg/l.

Two fermentations (100 l each from the same batch of grapes), a spontaneous fermentation and a *H. guilliermondii* NCYC 2380 inoculated fermentation (1% v/v inoculum) were carried out using identical open cylindrical vessels (depth of must about 0.5 × diameter of vessel) with an environmental temperature of around 21 °C. The inoculum of *H. guilliermondii* NCYC 2380 was grown at 25 °C for 24 h in YM medium (Difco, Detroit, IN, USA) under aerobic conditions (active oxygenation, achieved by orbital shaking at 150 rpm). Under these growth cultivation conditions, cells of *H. guilliermondii* NCYC 2380 showed a high viability when grown in presence of high ethanol contents (Pina, Santos, Couto, & Hogg, 2004). The inoculation was carried out immediately after grape crushing in order to obtain an initial cell concentration of approximately $10^6$ cfu/ml.

After 5 days of maceration, the skins and seed were removed by dejuicing and pressing. Alcoholic fermentation continued to completion (less than 2 g/l of residual sugar). After 14 days of fermentation, wines were bottled and stored at room temperature.

Fermentation tests were carried out daily for yeast counting and sugar monitoring. The number of yeast cells, expressed as cfu/ml, was determined using the pour plate method, after incubation of plates at specific temperatures for 48 h on selective and non-selective media as described by Moreira, Mendes, Hogg, and Vasconcelos (2005). The various macroscopic colonies formed were counted, and representative colony forms were isolated and maintained on YM agar slopes at 4 °C prior to identification. The identification of typical colonies was performed using the morphological, physiological and biochemical tests according to the scheme of Barnett, Payne, and Yarrow (1990). Sugar assimilation tests were carried out using API/ID 32C® galleries (bioMérieux, Mercy l'Etoile, France) with Yeast Nitrogen Base (Difco, Detroit, IN, USA) as suspending diluent.

### 2.3. Analytical determinations

Concentration of ethanol, glucose and fructose were determined by High Performance Liquid Chromatography (HPLC) using a Beckman (System Gold) chromatograph. Separation was performed on an Aminex® HPX-87H column (300 × 7.8 mm, Bio-Rad) and detection was assessed by refractive index. The mobile phase was a 0.5 mM sulphuric acid solution, with a flow rate of 0.5 ml/min, at 30 °C.

The free α-amino acids content of musts was analysed by HPLC using a Beckman (System Gold) chromatograph, according to the method described by Pripis-Nicolau, Revel, Marchand, Anocibar Beloqui, and Bertrand (2001).

Aliphatic higher alcohols (1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol) and ethyl acetate were analysed using a Hewlett–Packard 5890 gas chromatograph equipped with a flame ionisation detector and connected to a Hewlett–Packard 3396 Integrator. 50 μl of 4-methyl-2-pentanol at 10 g/l were added to 5 ml of wine as internal standard. The wine (1 μl) was injected (split, 1:60) into a CP-WAX 57 CB column (Chrompack) of 50 m × 0.25 mm and 0.2 μm phase thickness. Temperature program was 40 °C (5 min) to 180 °C (0 min) at 3 °C/min. Injector and detector temperatures were set at 250 °C. The carrier gas was $H_2$ at 1–2 ml/min.

The determination of 2-phenylethanol, acetates of higher alcohols (isoamyl acetate, 2-phenylethyl acetate and hexyl acetate), ethyl esters of fatty acids (ethyl butyrate, ethyl hexanoate and ethyl octanoate), ethyl lactate, volatile fatty acids (pentanoic, isobutyric and butyric acid) and free fatty acids (hexanoic, octanoic and decanoic acids) was performed in a Perkin–Elmer Autosystem gas

chromatograph, equipped with a flame ionisation detector. 50 ml of wine, with 4-decanol at 1.5 mg/l as internal standard, were extracted successively with 4, 2 and 2 ml of ether-hexane (1:1 v/v) for 5 min. The organic phase (1 μl) was injected (splitless, 0.3 min) into a CP-WAX 58 (FFAP)-CB column (Chrompack) of 50 m × 0.32 mm and 0.3 μm phase thickness. Temperature program was 40 °C (5 min) to 220 °C (20 min) at 2 °C/min. Injector and detector temperatures were set at 250 °C. The carrier gas was $H_2$ at 1–2 ml/min.

Heavy sulphur compounds were determined according to the method described by Moreira, Guedes de Pinho, and Vasconcelos (2004). The sulphur compounds analysed were 2-(methylthio) ethanol, 2-methyltetrahydrothiophen-3-one, acetic acid-3-(methylthio)propyl ester, 3-(methylthio)propionic acid, methionol (3-(methylthio)-1-propanol), 3-(ethylthio)-1-propanol, 3-mercapto-1-propanol, 4-(methylthio)-1-butanol, *trans*- and *cis*-2-methyltetrahydrothiophen-3-ol. Concentrations of commercially available sulphur compounds were expressed as μg/l. For those compounds whose reference standard was not available, the amounts were expressed as the ratio of peak area/peak area of internal standard.

### 2.4. Statistical analysis

An analysis of variance (ANOVA) was applied to the experimental data; results were considered significant if the associated *P* value was below 0.05. The significant differences were determined by Tukey tests. All statistical analyses were performed using the software SPSS for Windows, version 10.0.

## 3. Results and discussion

### 3.1. Yeast succession

The succession of yeasts during the spontaneous fermentation and the *H. guilliermondii* NCYC 2380 inoculated fermentation is shown in Fig. 1. Fresh grape must (prior to inoculation) contained a mixture of non-*Saccharomyces* yeasts composed by the genera *Hanseniaspora*, *Rhodotorula*, *Cryptococcus* and *Debaryomyces* (identification probability ≥ 96%), which represented a total initial cell concentration of $10^6$–$10^7$ cfu/ml. The *Hanseniaspora* yeasts where further identified to species level as *H. uvarum*. The level of non-*Saccharomyces* yeasts found in fresh must was high. Granchi et al. (1998) stated that, depending on the vintage, density of non-*Saccharomyces* yeasts in freshly extracted grape musts may range from about $10^3$–$10^6$ cfu/ml. In the experiences performed, *S. cerevisiae* was never isolated from fresh musts by usual plating media, so its initial presence should have been always in much lower numbers than the non-*Saccharomyces* species, which is also in accordance to other quantitative studies (Beltran et al., 2002; Combina et al., 2005; Fugelsang & Edwards, 2007, pp. 84–86; Granchi et al., 1998). The disappearance of non-*Saccharomyces* non-apiculate yeasts at 5–6 days of fermentation was observed in both fermentations. *S. cerevisiae* was only detected after 4 days of fermentation, with a concentration of $10^7$ cfu/ml, becoming the dominant species after 5–6 days until the end of fermentation.

Growth and succession of yeasts did not differ significantly in both fermentations, except for the presence of *H. guilliermondii* NCYC 2380 in the inoculated fermentation (Fig. 1B). A dominance of apiculate yeasts was observed in the first 4–5 days of fermentation, reaching total counts of $10^8$ cfu/ml. *H. uvarum* was the only apiculate yeast isolated from the spontaneous fermentation and died off after 4–5 days, but remained in countable numbers until the end of fermentation (Fig. 1A). The decrease of *H. guilliermondii* NCYC 2380 was slower, becoming undetectable after 11 days.

The growth behaviour of the yeast species that dominated the early stages of both fermentations is in good accordance with most

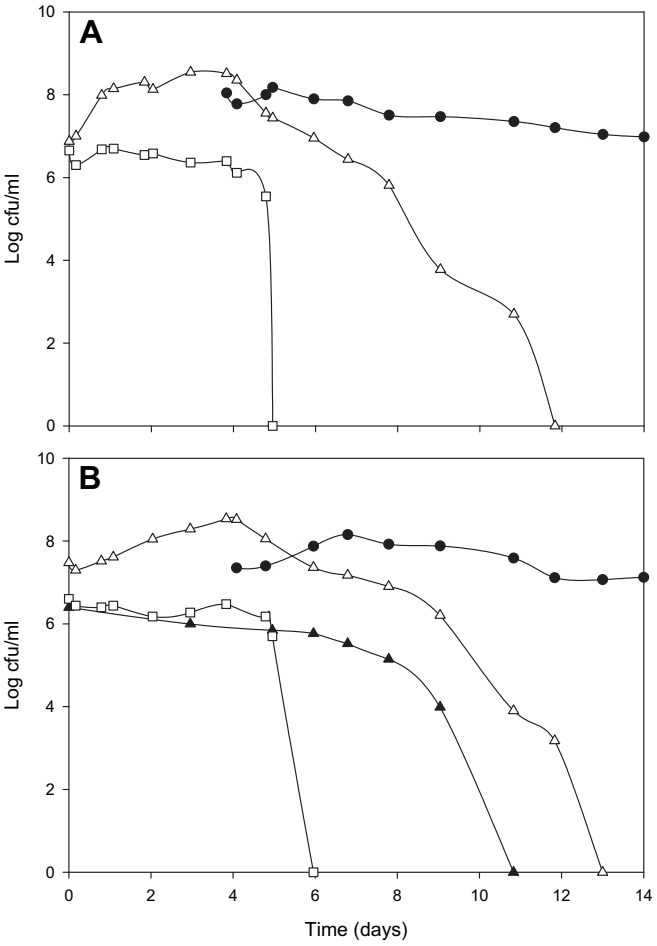


**Fig. 1.** Evolution of yeasts population (log cfu/ml) during red grape must vinification in (A) spontaneous fermentation and (B) *Hanseniaspora guilliermondii* NCYC 2380 inoculated fermentation. (●) *Saccharomyces cerevisiae*, (△) total apiculate yeasts, (▲) *H. guilliermondii* NCYC 2380 and (□) other non-*Saccharomyces* non-apiculate yeasts. Values for colony forming units are the average values of results obtained from four determinations.

observations reported in literature, which refer the dominance of apiculate yeasts within the first 3–4 days of fermentation, corresponding to an ethanol concentration of about 4–7% (v/v) (Fleet, 2003; Fleet & Heard, 1993; Fugelsang & Edwards, 2007, pp. 84–86; Moreira et al., 2008). The inoculated *H. guilliermondii* NCYC 2380 strain and the indigenous *H. uvarum* remained viable, at considerable high concentration levels, until the end of the fermentation. Furthermore, the decline phase of these populations was very slow, cells being detected at levels of about $10^6$–$10^7$ cfu/ml by the 6th fermentation day, with an ethanol level of 12.5% (v/v) present in the medium. Pina et al. (2004) showed that a pre-culture regime of *H. guilliermondii* NCYC 2380 with active oxygenation is a good method to enhance ethanol tolerance.

### 3.2. Sugar, amino acids and ethanol contents during fermentation

The grape must from Tinta Roriz variety presented an initial sugar content of 205 g/l and an initial total amino acid level of 451 mg N/l. Similar results for sugar and amino acids consumption and ethanol production were obtained in both fermentations. Sugar was totally consumed in 6–8 fermentation days, reaching a final ethanol concentration of 12.5% (v/v). The depletion of amino acids from the media was parallel to the consumption of sugar, presenting low values at the end of fermentation.

### 3.3. Volatile composition of wines

#### 3.3.1. Higher alcohols

Similar contents of higher alcohols were found in both wines, except for the production of 1-propanol which was higher in the wine resulting from the *H. guilliermondii* NCYC 2380 inoculated fermentation (Table 1). The contents of total aliphatic higher alcohols (mean values of 363 and 373 mg/l) and 2-phenylethanol (21.2 and 22.3 mg/l) were within the usual range described for wines, 150–550 mg/l for aliphatic higher alcohols and 10–100 mg/l for 2-phenylethanol (Ribéreau-Gayon, Glories, Manjean, & Dubourdieu, 2000). Excessive concentration of aliphatic higher alcohols can result in wines with a strong, pungent smell and taste, whereas balanced contents contribute to aromatic complexity. 2-Phenylethanol contributes with floral, rose aroma to wines and presents an aroma threshold of 10 mg/l in hydroalcoholic solution (Ribéreau-Gayon et al., 2000; Swiegers, Bartowsky, Henschke, & Pretorius, 2005). Previous studies on grape musts showed that apiculate yeasts, when used as mixed starter cultures with *S. cerevisiae*, decrease the total aliphatic higher alcohols level and may not enhance considerately the production of 2-phenylethanol (Moreira et al., 2008).

#### 3.3.2. Esters

Similar amounts of esters were found in both wines, except for 2-phenylethyl acetate and ethyl hexanoate levels (Table 1). The inoculated fermentation led to a higher content of 2-phenylethyl acetate, 0.733 mg/l, whereas 0.356 mg/l were obtained in the spontaneous fermentation. The significant difference observed is in agreement with previous studies showing that *H. guilliermondii* produced significant levels of 2-phenylethyl acetate (Moreira et al., 2008; Rojas et al., 2003; Viana et al., 2008). This compound contributes positively to wine aroma, bringing rose, honey, fruity and flowery odours, and presents an aroma threshold of 0.25 mg/l in hydroalcoholic solution (Lambrechts & Pretorius, 2000). A significant difference was found for the concentration of ethyl hexanoate, which was higher in the wine produced from the spontaneous fermentation (0.263 mg/l vs 0.163 mg/l). The ethyl esters of fatty acids also present pleasant floral and fruity odours in wines (Lambrechts & Pretorius, 2000).

Herraiz, Reglero, Herraiz, Martín-Alvarez, and Cabezudo (1990) found that the production of ethyl acetates of fatty acids and isoamyl acetate was significantly higher in wines produced by a pure culture of *S. cerevisiae* than in wines obtained from the sequential inoculation of apiculate yeasts. However, Rojas et al. (2003) showed that, in general, *S. cerevisiae* produces wines with higher contents in isoamyl acetate, ethyl hexanoate and octanoate, whereas *H. guilliermondii* produces higher levels of 2-phenylethyl acetate and hexyl acetate. The ability of apiculate yeasts to produce acetates was also reported by Plata et al. (2003).

The content of ethyl acetate in wines was similar (858 vs 791 mg/l). The high production of ethyl acetate and acetic acid by apiculate yeasts had been widely reported (Ciani & Maccarelli, 1998; Ciani & Picciotti, 1995; Herraiz et al., 1990; Rojas et al., 2003; Romano, Fiore, Paraggio, Caruso, & Capece, 2003). Ethyl acetate is produced both in presence of acetyl-CoA, by acetiltransferases, and in presence of ethanol and acetic acid, by a reverse reaction of esterases; its level in wines increases considerably with aeration and glucose concentration of the media (Plata et al., 2003). The high production of acetic acid by apiculate yeasts, as well as the aeration level during fermentation, could be the main reason why high levels of ethyl acetate were present in

**Table 1**
Concentration of volatile compounds in wines produced by spontaneous fermentation and by *H. guilliermondii* NCYC 2380 inoculated fermentation.

| Concentration (mg/l) | Spontaneous fermentation | *H. guilliermondii* NCYC 2380 inoculated fermentation | Sig. |
|---|---|---|---|
| *Higher alcohols* | | | |
| 1-Propanol | 62.5 (4.2) | 73.0 (0.8) | † |
| 2-Methyl-1-propanol | 81.7 (4.7) | 86.5 (2.3) | ns |
| 2-Methyl-1-butanol | 63.2 (4.3) | 59.8 (3.6) | ns |
| 3-Methyl-1-butanol | 156 (3) | 154 (4) | ns |
| Total aliphatic higher alcohols | 363 (16) | 373 (11) | ns |
| 2-Phenylethanol | 21.2 (0.2) | 22.3 (0.5) | ns |
| *Esters* | | | |
| Isoamyl acetate | 4.44 (0.70) | 3.34 (0.50) | ns |
| Hexyl acetate | 0.088 (0.009) | 0.081 (0.013) | ns |
| 2-Phenylethyl acetate | 0.356 (0.033) | 0.733 (0.089) | † |
| Total acetates of higher alcohols | 4.88 (0.74) | 4.15 (0.60) | ns |
| Ethyl butyrate | 0.730 (0.047) | 0.738 (0.027) | ns |
| Ethyl hexanoate | 0.263 (0.008) | 0.163 (0.005) | † |
| Ethyl octanoate | 0.377 (0.027) | 0.207 (0.004) | ns |
| Total ethyl esters of fatty acids | 1.37 (0.08) | 1.11 (0.04) | ns |
| Ethyl acetate | 858 (60) | 791 (72) | ns |
| Ethyl lactate | 3.81 (0.75) | 3.58 (0.91) | ns |
| *Fatty acids* | | | |
| Butyric acid | 1.64 (0.28) | 1.18 (0.11) | ns |
| Isobutyric acid | 2.51 (0.39) | 2.80 (0.17) | ns |
| Pentanoic acid | 1.07 (0.07) | 0.850 (0.074) | †† |
| Total volatile fatty acids | 5.22 (0.74) | 4.83 (0.35) | ns |
| Hexanoic acid | 0.582 (0.038) | 0.344 (0.053) | †† |
| Octanoic acid | 2.15 (0.18) | 1.40 (0.14) | †† |
| Decanoic acid | 0.537 (0.155) | 0.218 (0.037) | ns |
| Total free fatty acids | 3.27 (0.37) | 1.96 (0.23) | † |

Values in parenthesis are standard deviations from four determinations.
Sig.: significance, † and †† displays the significance at 1 and 5%, ns – not significant.

**Table 2**
Concentration of heavy sulphur compounds in wines produced by spontaneous fermentation and by *H. guilliermondii* NCYC 2380 inoculated fermentation.

| Concentration (μg/l) | Spontaneous fermentation | *H. guilliermondii* NCYC 2380 inoculated fermentation | Sig. |
|---|---|---|---|
| Methionol | 1036 (111) | 959 (213) | ns |
| 2-(Methylthio)ethanol | 33.9 (3.0) | 30.5 (1.9) | ns |
| 4-(Methylthio)-1-butanol | 30.8 (5.0) | 28.9 (3.2) | ns |
| Acetic acid-3-(methylthio)propyl ester[a] | 1.21 (0.12) | 0.901 (0.146) | †† |
| 3-(Methylthio)propionic acid[a] | 0.380 (0.131) | 0.534 (0.136) | † |
| 2-Mercaptoethanol | 56.7 (3.5) | 58.9 (3.6) | ns |
| 3-Mercapto-1-propanol | 34.0 (1.4) | 33.6 (0.8) | ns |
| 2-Methyltetrahydrothiophen-3-one | 127 (4) | 104 (3) | † |
| *cis*-2-Methyltetrahydrothiophen-3-ol[a] | 0.073 (0.001) | 0.074 (0.002) | ns |
| *trans*-2-Methyltetrahydrothiophen-3-ol[a] | 0.028 (0.003) | 0.024 (0.005) | ns |

Values in parenthesis are standard deviations from four determinations.
Sig.: Significance, † and †† displays the significance at 1 and 5%, ns – not significant.
[a] Amounts are expressed as the ratio of peak area/peak area of internal standard.

the produced wines. Ethyl acetate is the main ester occurring in wine and possesses an unpleasant odour (acetic odour). This ester contributes significantly to defect aroma at contents of 150–200 mg/l (Lambrechts & Pretorius, 2000). The negative effect of ethyl acetate might be reduced during bottle aging (Rojas et al., 2003). However, to prevent a high level of ethyl acetate, wine must be produced under low oxygen concentrations, with a rapid fermentation beginning, maintenance of an adequate level of $SO_2$ and control of bacterial growth.

### 3.3.3. Fatty acids

No significant differences were found for the amounts of butyric, isobutyric and decanoic acids in both wines (Table 1); the total volatile fatty acids was also similar in both wines (4.83 vs 5.22 mg/l). However, the *H. guilliermondii* NCYC 2380 inoculated fermentation led to the production of wine with lower contents in pentanoic, hexanoic and octanoic acids than the wine produced from spontaneous fermentation. The total content of free fatty acids was also significantly lower in the inoculated fermentation (1.96 vs 3.27 mg/l). In literature, the total volatile and free fatty acids in red wines were found at levels up to 5.2 and 7.8 mg/l respectively (Escudero, Campo, Farina, Cacho, & Ferreira, 2007; Maicas, Gil, Pardo, & Ferrer, 1999; Ortega, López, Cacho, & Ferreira, 2001; Rocha, Rodrigues, Coutinho, Delgadilho, & Coimbra, 2004; Selli et al., 2004). Although fatty acids are present in wines in low amounts, they can contribute negatively to final aroma. Odour descriptors of fatty-rancid, fruity, cheesy and sweaty characterise the impact of isobutyric and butyric acids in wines, with an odour threshold lower than 0.4 mg/l; free fatty acids are associated with fatty-rancid, grass, cheesy, dairy, dry and woody like-odours, and present odour thresholds lower than 6.70 mg/l (Rocha et al., 2004).

### 3.3.4. Heavy sulphur compounds

No significant differences were found for the amounts of methionol (959–1036 μg/l), 2-(methylthio)ethanol (30.5–33.9 μg/l), 4-(methylthio)-1-butanol (28.9–30.8 μg/l), 2-mercaptoethanol (56.7–58.9 μg/l), 3-mercapto-1-propanol (33.6–34.0 μg/l), *trans*- and *cis*-2-methyltetrahydrothiophen-3-ol in both wines (Table 2).

The methionol levels obtained for both wines are close to the odour threshold value (1000 μg/l; Escudero et al., 2007); similar concentrations were obtained in red wines by Fedrizzi, Magno, Badocco, Nicolini, and Versini (2007), who also reported values from literature up to 4.5 mg/l for methionol, 70 μg/l for 2-(methylthio)ethanol and 180 μg/l for 4-(methylthio)-1-butanol and 2-mercaptoethanol.

The wine obtained from the *H. guilliermondii* NCYC 2380 inoculated fermentation presented higher amounts of 3-(methylthio) propionic acid and lower levels of acetic acid-3-(methylthio)propyl ester and 2-methyltetrahydrothiophen-3-one than the wine resulting from spontaneous fermentation. Acetic acid-3-(methylthio)propyl ester is present at trace amounts in wines, normally below its perception threshold, having little influence on wine quality (Anocibar Beloqui & Bertrand, 1995; Mestres et al., 2000). 3-Methylthiopropionic acid can be found in wines at higher contents, up to 1800 μg/l (Landaud et al., 2008). In general, 2-methyltetrahydrothiophen-3-one is present in wines at values lower than 100 μg/l (Mestres et al., 2000).

A previous study showed that, when *H. guilliermondii* NCYC 2380 and *H. uvarum* were grown in mixed cultures with *S. cerevisiae*, wines presented amounts of heavy sulphur compounds similar to those produced by a pure culture of *S. cerevisiae* (Moreira et al., 2008). These results emphasise the fact that apiculate yeasts may not enhance the production of undesirable sulphur compounds.

## 4. Conclusions

The experiments performed underlie the fact that apiculate yeasts can survive throughout the alcoholic fermentation for longer periods than previously thought. It was observed that these yeasts survived in the presence of ethanol concentrations considerably high (12.5% v/v). Significant higher levels of 2-phenylethyl acetate, 1-propanol and 3-(methylthio)propionic acid were observed in wine resulting from the *H. guilliermondii* NCYC 2380 inoculated fermentation, whereas a higher content of total free fatty acids, pentanoic acid, ethyl hexanoate, 2-methyltetrahydrothiophen-3-one and acetic acid-3-(methylthio)propyl ester were found in wine produced by the spontaneous fermentation. These results highlight the fact that certain apiculate yeasts have the capacity to influence, in a positive way, the aromatic profile of wines, and can be used in mixed starter cultures with *S. cerevisiae*. Furthermore, considering that the only apiculate yeast isolated from grape juice, and found at the end of spontaneous fermentation, was *H. uvarum*, research on this strain should be carried out. Moreover, the contribution of apiculate yeasts to the final volatile composition of wines, as well as the contribution of aromas from the grape variety, should be studied further, aiming to obtain different and good quality wines.

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## References


Anocibar Beloqui, A., & Bertrand, A. (1995). Study of sulfur compounds in wine: preliminary results. *Italian Journal of Food Science, 3*, 279–288.

Barnett, J. A., Payne, R. W., & Yarrow, D. (1990). *Yeasts, characteristics and identification*. Canbridge: Cambridge University Press.

Beltran, G., Torija, M., Novo, M., Ferrer, N., Poblet, M., Guillamón, J., et al. (2002). Analysis of yeast population during alcoholic fermentation: a six year follow-up study. *Systematic and Applied Microbiology, 25*, 287–293.

Ciani, M., & Maccarelli, F. (1998). Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology, 14*, 199–203.

Ciani, M., & Picciotti, G. (1995). The growth kinetics and fermentation behaviour of some non-*Saccharomyces* yeasts associated with wine-making. *Biotechnology Letters, 17*, 1247–1250.

Combina, M., Elías, A., Mercado, L., Catania, C., Ganga, A., & Martinez, C. (2005). Dynamics of indigenous yeast populations during spontaneous fermentation of wine from Mendoza, Argentina. *International Journal of Food Microbiology, 99*, 237–243.

Constantí, M., Poblet, M., Arola, L., Mas, A., & Guillamón, J. (1997). Analysis of yeast populations during alcoholic fermentation in a newly established winery. *American Journal of Enology and Viticulture, 48*, 339–344.

Erten, H., Tanguler, H., Cabaroglu, T., & Canbas, A. (2006). The influence of inoculum level on fermentation and flavour compounds of white wines made from cv. Emir. *Journal of the Institute of Brewing, 112*, 232–236.

Escudero, A., Campo, E., Farina, L., Cacho, J., & Ferreira, V. (2007). Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. *Journal of Agricultural and Food Chemistry, 55*, 4501–4510.

Fedrizzi, B., Magno, F., Badocco, D., Nicolini, G., & Versini, G. (2007). Aging effects and grape variety dependence on the content of sulphur volatiles in wine. *Journal of Agricultural and Food Chemistry, 55*, 10880–10887.

Fleet, G. H. (2003). Yeast interactions and wine flavour. *International Journal of Food Microbiology, 86*, 11–22.

Fleet, G. H., & Heard, G. M. (1993). Yeasts-growth during fermentation. In G. H. Fleet (Ed.), *Wine microbiology and biotechnology* (pp. 27–55). Chur, Switzerland: Harwood Academic Publishers.

Fugelsang, K., & Edwards, C. (2007)*Wine microbiology, practical applications and procedures, Vol. 2*. New York: Springer Science+Business Media/LLC.

Granchi, L., Ganucci, D., Messini, A., Rosellini, D., & Vincenzini, M. (1998). Dynamics of yeast populations during the early stages of natural fermentations for the production of *Brunello di Montalcino* wines. *Food Technology and Biotechnology, 36*, 313–318.

Hansen, H., Nissen, P., Sommer, P., Nielsen, J. C., & Arneborg, N. (2001). The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed cultures fermentations of grape juice with Saccharomyces cerevisiae. *Journal of Applied Microbiology, 91*, 541–547.

Herraiz, T., Reglero, G., Herraiz, M., Martín-Alvarez, P., & Cabezudo, M. D. (1990). The influence of the yeast and type of culture on the volatile composition of wines fermented without sulfur dioxide. *American Journal of Enology and Viticulture, 41*, 313–318.

Jolly, N., Augustyn, O., & Pretorius, I. (2006). The role and use of non-*Saccharomyces* yeasts in wine production. *South African Journal of Enology and Viticulture, 27*, 15–39.

Lambrechts, M. G., & Pretorius, I. S. (2000). Yeast and its importance to wine aroma. A review. *South African Journal of Enology and Viticulture, 21*, 97–129.

Landaud, S., Helinck, S., & Bonnarme, P. (2008). Formation of volatile sulfur compounds and metabolism of methionine and other sulfur compounds in fermented food. *Applied Microbiology and Biotechnology, 77*, 1191–1205.

Lavigne, V. (1996). Recherches sur les composés soufrés volatils formés par la levure au cours de la vinification et l'élevage des vins blancs sec, Ph.D. thesis, University of Bordeaux II, Bordeaux, France.

Maicas, S., Gil, J., Pardo, I., & Ferrer, S. (1999). Improvement of volatile composition of wines by controlled addition of malolactic bacteria. *Food Research International, 32*, 491–496.

Mestres, M., Busto, O., & Guasch, J. (2000). Analysis of organic sulfur compounds in wine aroma. *Journal of Chromatography A, 881*, 569–581.

Moreira, N., Guedes de Pinho, P., & Vasconcelos, I. (2004). Method for analysis of heavy sulphur compounds using gas chromatography with flame photometric detection. *Analytica Chimica Acta, 513*, 183–189.

Moreira, N., Mendes, F., Guedes de Pinho, P., Hogg, T., & Vasconcelos, I. (2008). Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *International Journal of Food Microbiology, 124*, 231–238.

Moreira, N., Mendes, F., Hogg, T., & Vasconcelos, I. (2005). Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. *International Journal of Food Microbiology, 103*, 285–294.

Nissen, P., & Arneborg, N. (2003). Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. *Archives of Microbiology, 5*, 65–71.

Nissen, P., Nielsen, D., & Arneborg, N. (2003). Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by cell–cell contact-mediated mechanism. *Yeast, 20*, 331–341.

Ortega, C., López, R., Cacho, J., & Ferreira, V. (2001). Fast analysis of important wine volatile compounds. Development and validation of a new method based on gas chromatographic-flame ionisation detection analysis of dichloromethane microextracts. *Journal of Chromatography A, 923*, 205–214.

Pérez-Nevado, F., Albergaria, H., Hogg, T., & Gírio, F. (2006). Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology, 108*, 336–345.

Pina, C., Santos, C., Couto, J. A., & Hogg, T. (2004). Ethanol tolerance of five non-*Saccharomyces* wine yeasts in comparison with a strain of *Saccharomyces cerevisiae*-influence of different culture conditions. *Food Microbiology, 21*, 439–447.

Plata, C., Millán, C., Mauricio, J. C., & Ortega, J. M. (2003). Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. *Food Microbiology, 20*, 217–224.

Pripis-Nicolau, L., Revel, G., Marchand, S., Anocibar Beloqui, A., & Bertrand, A. (2001). Automated HPLC method for the measurement of free amino acids including cysteine in musts and wine; first applications. *Journal of the Science of Food and Agriculture, 81*, 731–738.

Rauhut, D. (1993). Production of sulfur compounds. In G. H. Fleet (Ed.), *Wine microbiology and biotechnology* (pp. 183–242). Chur, Switzerland: Harwood Academic Publishers.

Ribéreau-Gayon, P., Glories, Y., Manjean, A., & Dubourdieu, D. (2000). *Handbook of enology*. In *The chemistry of wine stabilization and treatments, Vol. 2*. Chichester: John Wiley & Sons Ltd.

Rocha, S., Rodrigues, F., Coutinho, P., Delgadilho, I., & Coimbra, M. (2004). Volatile composition of Baga red wine. Assessment of the identification of the would be impact odourants. *Analytica Chimica Acta, 513*, 257–262.

Rojas, V., Gil, J., Piñaga, F., & Manzanares, P. (2003). Acetate ester formation in wine by mixed cultures in laboratory fermentations. *International Journal of Food Microbiology, 86*, 181–188.

Romano, P., Fiore, C., Paraggio, M., Caruso, M., & Capece, A. (2003). Function of yeasts species and strains in wine flavour. *International Journal of Food Microbiology, 86*, 169–180.

Sarrazin, E., Shinkaruk, S., Tominaga, T., Bennetau, B., Frérot, E., & Dubourdieu, D. (2007). Odorous impact of volatile thiols on the aroma of young Botrytized sweet wines: identification and quantification of new sulfanyl alcohols. *Journal of Agricultural and Food Chemistry, 55*, 1437–1444.

Selli, S., Cabaroglu, T., Canbas, A., Erten, H., Nurgel, C., Lepoutre, J. P., et al. (2004). Volatile composition of red wine from cv. Kalecik Karast grown in central Australia. *Food Chemistry, 85*, 207–213.

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., & Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *The Australian Journal of Grape and Wine Research, 11*, 139–173.

Swiegers, J. H., & Pretorius, I. (2007). Modulation of volatile sulphur compounds by wine yeast. *Applied Microbiology and Biotechnology, 74*, 954–960.

Tominaga, T., & Dubourdieu, D. (2006). A novel method for quantification of 2-methyl-3-furanthiol and 2-furanmethanethiol in wines made from *Vitis vinifera* grape varieties. *Journal of Agricultural and Food Chemistry, 54*, 29–33.

Viana, F., Gil, J., Genovés, S., Vallés, S., & Manzanares, P. (2008). Rational selection of non-*Saccharomyces* yeasts for mixed starters based on ester formation and enological traits. *Food Microbiology, 25*, 778–785.

Zott, K., Miot-Sertier, C., Claisse, O., Lanvaud-Funel, A., & Masneuf-Pomarede, I. (2008). Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. *International Journal of Food Microbiology, 125*, 197–203.