Hinc patriam sustinet

Instituto Superior de Agronomia
Universidade Técnica de Lisboa

Studies on the incidence of *Dekkera bruxellensis* in Turkish wines and effect of low temperatures on 4-ethylphenol production

Mehmet Yigit KESKIN

Dissertação para obtenção do Grau de Mestre em

Vinifera EuroMaster – European Master of Sciences of Viticulture and Oenology

Orientador: Professor Manuel Malfeito Ferreira

Júri:
Presidente: Olga Laureano, Investigador Coordenador, UTL/ISA
Vogais: - Doris Rauhut, Professor, Hochschule RheinMain, Geisenheim
- Manuel Malfeito Ferreira, Professor, UTL/ISA
- Luísa Brito, Professor, UTL/ISA

Lisboa, 2012

Şükran'cığıma ve Kazım'cığıma...

Index

TABLES

Page

FIGURES

Page

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to:

Malfeito Manuel Ferreira, Ph.D. of the Instituto Superior de Agronomia in Lisbon, Portugal for acting as my supervisor, his guidance and advice and above all the fact that his door was always open,

Turgut CABAROGLU, Ph.D. of the Cukurova University in Adana, Turkey for being my co-supervisor and for his support.

Ana Carla Silva for tireless guidance and endless help regarding my laboratory work.

My colleagues in Microbiology Laboratory especially Ines Oro, Ana Ramos, Rita Abreu, Ana Teles, Sandro Martins and Viviana Monteiro for endless support and help.

Mahesh Chandra, Ph.D. and Paula Cunha for their guidance

All Family of microbiology laboratory for helping me to feel part of them and to pushed me to speak Portuguese.

D. Helena and D. Manuela for help me with laboratory material and to improve my Portuguese knowledge.

My Casa da Vinifera friends for always keep me in company for their brothership.

My all family for the support throughout my studies period.

ABSTRACT

Brettanomyces/Dekkera bruxellensis has the ability to produce significant amounts of 4-ethylphenol (4-EP) and cause important organoleptic defects in affected wines. In this work we investigated the incidence of *B. bruxellensis* and of 4-ethylphenol in Turkish red wines randomly collected at winery facilities. Among 40 samples, this species was only recovered from one sample aged in oak barrels. In 4 samples, culturable population was not found but the 4-EP concentration indicated previous *Dekkera bruxellensis* activity. Thus, following potential Brettanomyces-sourced aroma impacts in wine using 4-Ethylphenol and 4-Ethylguaiacol concentrations as proxies should only be considered reliable at analyte levels >100 µg/l (Rayne and Eggers, 2007). Furthermore, we studied the influence of storage temperature on the production of 4-EP by wild of *D. bruxellensis* in 3 different Portuguese red wines. Temperatures of 10 ºC and 15ºC were not effective in preventive the increase of 4-EP over 600 µg/l during 90 days. At 3 ºC one sample showed similar 4-EP increase while in two other samples its concentration was kept unchanged. Production of 4-EP at 3ºC and 10ºC was confirmed when the strain TR 26 was incubated in red wine for 49 days. However, the total amount of 4-EP produced was below 150 µg/l while at 15ºC and 20ºC the values reached 1600 µg/l. Therefore, the effect of low temperatures acts by delaying microbial growth which results in lower concentrations of 4-EP but once temperature increases 4-EP production is readily stimulated.

Key words: *Brettanomyces/Dekkera*, storage conditions, temperature, horse-sweat taint, 4-ethylphenol, Turkish wine.

RESUMO

A *Brettanomyces/Dekkera* tem a capacidade para produzir quantidades significativas de 4-etilfenol (4-EF) e causar importantes defeitos organolépticos em vinhos contaminados. Neste trabalho, investigou-se a incidência de *Brettanomyces bruxellensis* e a produção de 4-etilfenol em 40 vinhos tintos de origem Turca, provenientes de diversas adegas. De entre as 40 amostras estudadas apenas uma se encontrou contaminada, sendo esta proveniente de um vinho envelhecido em barricas de carvalho. Em quatro amostras de vinho tinto, a população de 4-EF indica que foi encontrada actividade da *Dekkera.* Assim, o impacto dos seguientes potenciais de arome, usaudo o 4-Etilfenol e 4-Etilguaiacol so deveram sei conseideradas em concentrações proximas niveis analiticos, neste caso maio que 100 µg/l (Rayne and Eggers, 2007). Além disso, estudou-se a influência da temperatura de armazenamento na produção de 4-etilfenol pela *Dekkera bruxellensis* em três vinhos tintos diferentes. As Temperaturas de 10^{o}C e de 15^{o}C, não foram eficientes na prevenção do aumento de 4-EF acima dos 600 µg/l durante 90 dias. A 3 ^{o}C, uma amostra apresentou um crescimento semelhante de 4-EF enquanto que em duas outras amostras a concentração permaneceu inalterada. A 3 ^{o}C e 10^{o}C, a produção de 4-EF foi contirmada pela estırpe TR 26, quando inoculada em vinho tinto durante 49 dias. No entanto, a quantidade total de 4-EF produzida foi inferior 150 µg/l enquanto que a 15 ^{o}C e 20 ^{o}C, a quantidade produzida atingiu as 1600 µg/l. Portanto, baixas temperaturas, atrasam o crescimento microbiano, resultando em baixas concentrações de 4-EF. Com o aumento das temperaturas, a produção deste composto é estimulada, aumentando a concentração de 4-EF.

Palavras-chave: *Brettanomyces/Dekkera*, condições de armazenamento, temperatura, suor de cavalo, 4-etil fenol, vinho Turco.

1. Introduction

Wine is appreciated by consumers due to its variety of colors, aromas and flavours. This variability in organoleptic qualities is due to many organic and non-organic chemical compounds that it contains and there are various ways that they interact with each other. It is not possible to define perfectly which compounds have negative or positive effects to quality of wine due to complex physicochemical reactions occurring during fermentation, aging, and finally the tasting of wine, like beauty, is in the eye of the beholder.

Some compounds have a negative effect, if at some concentration beyond the perception threshold they may be perceived negatively and/or mask desired aromas; in this case the wine is considered to have a fault. Wine faults have various causes which can occur during production and storage. Besides, poor hygiene practice at the winery, incorrect quantities of oenological products used in wine production, preservatives and insufficient filtering, may cause to the development of wild yeast and/or bacteria populations which could produce compounds in unwanted concentrations, respectively.

In wine production, yeasts may have either beneficial or damaging activities. *Saccharomyces cerevisiae* is mainly responsible for transforming grape juice into wine but this species and several other yeasts may also show undesirable effects in wines. Among all effects, winemakers are particularly concerned with the production of off-flavours that may occur during all stages of winemaking. Mainly, the spoilage occurs due to production of volatile phenols by *Dekkera bruxellensis* during storage or after bottling (Malfeito-Ferreira, 2010). *Dekkera* is quite different from the yeast responsible for fermentation of grape must (Barata, 2002).

Summarily; the classical wine taints (e.g. volatile acidity, hydrogen sulphide and oxidation) have been under great concern during the last decade. There are also other taints which are related to the phenolic compounds development. Wine character known as “Brett” is comprised one of the most arguable issues of recent times. Sensory of these compounds are described like “stable” and “horse sweat” (Fugelsang, 1997). For the production of volatile phenols is responsible yeast of genus *Dekkera*, or its anamorph *Brettanomyces* (Chatonnet *et al.*, 1995, 1997; Loureiro and Malfeito- Ferreira, 2006). These yeast genera are able to convert efficiently grape cinnamic acids (*p*-coumaric and ferulic acids) into the correspondent volatile phenols such as 4-ethylphenol or 4-ethylguaiacol.

However, good manufacturing practices must be strengthened to deal with volatile phenol production problems in red wines (Malfeito-Ferreira, 2010).

1.1. *Brettanomyces / Dekkera*

1.1.1. History of *Brettanomyces*

Claussen first described the yeast of genus *Brettanomyces* in 1904 while investigating the spoilage causes in English stock beers. The flavours produced by this yeast became characteristic of the British beers of that time and so the name '*Brettanomyces*' was derived from 'British brewing fungus'. Afterwards some of *Brettanomyces* strains were mentioned using different names (Licker *et al.*, 1997). It was not until the 1940s, when M.T.J. Custers performed the first systematic study on *Brettanomyces* yeast, that *Brettanomyces* was associated with wine (Custers, 1940). Although this study included 17 strains, of which most were isolated from beer, one strain originated from a French wine (Krumbholz and Tauschanoff, 1933).

1.1.2. Different species in wine

The taxonomy of the genus *Brettanomyces* has seen numerous reclassifications over the years (table 1.1.). Originally, these species included *Brettanomyces bruxellensis*, *Brettanomyces lambicus, Brettanomyces clausenii, Brettanomyces anomalus* and *Brettanomyces intermedius,* which reproduced asexually by means of budding (Custers, 1940; Van der Walt and Van Kerken, 1958). The genus Dekkera was introduced to the taxonomy in 1964 after the production of ascospores (sporulating-form) was observed (Van der Walt, 1984). Currently, the five species jointly belonging to the genera *Brettanomyces* and *Dekkera* are: *Brettanomyces custersianus, Brettanomyces naardenensis, Brettanomyces nanus, Brettanomyces anomalus* and *Brettanomyces bruxellensis* (Kurtzman and Fell, 2000; Cocolin *et al.*, 2004). Teleomorphs (perfect state) are known for the last two species, *Dekkera anomala* and *Dekkera bruxellensis,* respectively (Kurtzman and Fell, 2000). The details regarding the morphological, biochemical and physiological characteristics of these species are well described in recent classification manuals (Barnett *et al.*, 2000; Boekhout *et al.*, 2002; Kurtzman and Fell, 2000). In brief, budding cells are spheroidal, subglobose to ellipsoidal, frequently ogival or cylindroidal to elongate. Pseudomycelium and branched, one celled, non- septate, mycelium are sometimes formed (Kurtzman and Fell, 2000) (Figure 1.1 and Figure 1.2).

The physiology of *B. bruxellensis* has been well studied several times due to its unusual aromatic bi-products and because it is an example of the Custer effect, which is the

inhibition of alcoholic fermentation under anaerobic conditions due to high production of acetic acid and redox imbalance (Wedral *et al.*; 2010).

Table1.1. Chronology of *Dekkera/Brettanomyces* Identification (adapt. Madeira, 2011).

1904	**- First References of *Brettanomyces***
	- First References of *Brettanomyces* in wines
	- Classification of four species of *Brettanomyces*
	B. bruxellensis *B. anomalus* *B. lambicus* *B. clausenii*
	- Reference of turbidity and increasing of Volatile acidity
	- Discovery of ascospores - *Dekkera*
	- New species description of *Brettanomyces*
	B. abstinenes *B. anomalus* *B. intermedius* *B. bruxellensis* *B. custersii* *B. claussenii* *B. custersianus* *B. lambicus* *B. naardenensis*
	- Two species description of *Dekkera*
	D. bruxellensis *D. intermedia*
	- Four species description of *Brettanomyces*
	B. bruxellensis *B. anomalus* *B. custersianus* *B. naardenensis*
	- New species description of *Dekkera*
	D. anomala *D. bruxellensis* **D. custeriana* **D. naardensis*
	- New species description of *Brettanomyces*
	B. anomalus *B. bruxellensis* *B. naardensis* *B.custerianus* *B. nanus*
	- Two species description of *Dekkera*
2011	*D. bruxellensis* *D. anomala*

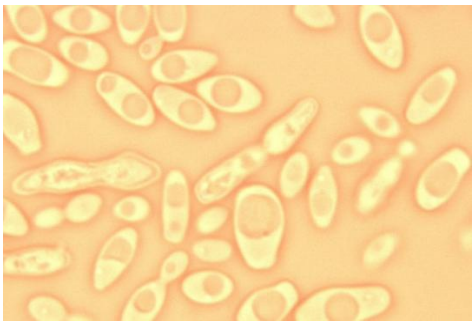


Figure 1.1. Observation with an optical microscope of *Brettanomyces sp.* cells (100x with immersion oil; Photogaphed by author)

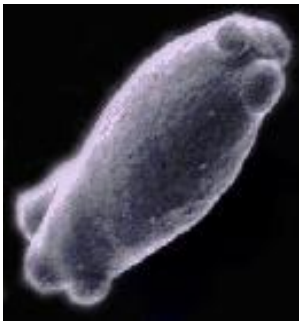


Figure 1.2. Observation with an electronic microscope of *Brettanomyces sp.* cells (Licker *et al.*,1997)

Among the five species currently known, the species primarily associated with winemaking is *B. bruxellensis* (*D. bruxellensis*) (Egli and Henick-Kling, 2001; Stender *et al.*, 2001; Rodrigues *et al.*, 2001 Cocolin *et al.*, 2004; Loureiro and Malfeito-Ferreira, 2006), although *B. anomalus* (*D. anomala*) and *B. custersianus* isolations from must fermentations have been reported in two instances (Querol *et al.*, 1990; Esteve-Zarzoso *et al.*, 2001). With advances in DNA based methods, recent wine-related investigations often include *D. anomala* along with the predominant species *D. bruxellensis* as conventional methods had showed difficulty in differentiating between these two species (Loureiro and Malfeito-Ferreira, 2006). Although current taxonomical classifications suggest that *Dekkera* should be used in reference with the species *D. bruxellensis* and *D. anomala* (Boekhout *et al.*, 1994), many discrepancies exist and some authors frequently prefer using the technically incorrect naming of *B. bruxellensis* and *B. anomalus* when referring to these yeasts in a winemaking context. This is largely attributed to the fact that the sexual or sporulating form of *Dekkera*, is yet to be found in wine (Oelofse *et al.*, 2008).

Dekkera is an auxotrophic yeast and it can grow very slowly in absence of vitamins (Gill, 1996). Biotin and thiamine are very essential for its growth (Silva, 1998). Some authors suggested that thiamine is the most important, and by stimulating the consumption of sugars allows the higher population growth (Dias *et al.*, 2003).

Additionally, these yeasts show the Custer effect where under anaerobic conditions and lack of any compounds that may act as proton acceptors glucose fermentation to ethanol and acetic acid is strongly inhibited (Barrio *et al.*, 2006). *Dekkera* can use various carbon sources and have some resistance against sulfites (Conterno *et al.*, 2006).

Some authors have made the point that the separation of *Brettanomyces* and *Dekkera* in the context of wine is meaningless because current molecular DNA techniques reveal no distinction between the anamorph and teleomorph forms (Loureiro and Malfeito-Ferreira, 2006). This might explain why it is not uncommon to see the use of '*Brettanomyces/Dekkera* spp.' in wine research (Oelofse *et al.*, 2008). In this study, the same context will be used as the original authors were using the naming in their publications. This can either be *B. bruxellensis* or *D. bruxellensis.*

1.1.3. Occurrence and dissemination during winemaking

Brettanomyces/Dekkera spp. are ubiquitously distributed in nature. Their occurrence and spoilage activities have been well summarized by Loureiro and Malfeito-Ferreira (2006). The majority of reports associate *Brettanomyces/Dekkera* spp. with fermented food products ranging from cheeses and fermented milk to various alcoholic beverages including wine, beer, cider, kombucha (fungus-tea) and tequila (Davenport, 1976; Kumara and Verachtert, 1991; Lachance, 1995; Kosse *et al.*, 1997; Licker *et al.*, 1998; Gadaga *et al.*, 2002; Teoh *et al.*, 2004; Loureiro and Malfeito-Ferreira, 2006). Less frequent reports of their isolations from other sources (bees, fruit-flies, olives and carbonated drinks) are also available (Van der Walt and Van Kerken, 1958; Phaff *et al.*, 1978; Jong, Lee, and Bengston 1985; Deak and Beuchat, 1995; Kotzekidou *et al.*, 1997).

In the 1950s and 1960s the yeast was identified as *Brettanomyces* ssp. and was isolated from wine in France, Italy and South Africa, but it was not until the 1980s and 1990s that the yeast was characterized for its ability to impart characteristic aroma to wine (Henschke *et al.*, 2007). *Brettanomyces/Dekkera* spp. has been and still are isolated from wines and wineries all around the world, predominantly from red wines. These yeasts are less frequently isolated from white wines (Licker *et al.*, 1998; Dias *et al.*, 2003) although their loss of viability and the consequent non-existence of ethylphenol levels in white wines is largely ascribed to the efficiency of sulfur dioxide (SO_2) at lower pH conditions (Loureiro and Malfeito-Ferreira, 2006). Hence, the focus of the research on these yeasts has primarily fallen on their occurrence in red wine (Oelofse *et al*., 2008).

The winemaking process hosts multiple sources where *Brettanomyces/Dekkera* spp. can survive and numerous debates about the initial source and dispersion of these yeasts have occurred (Licker *et al.*, 1998). The vineyard provides many sources, including the soil, rootlets, bark, leaves and grapes. Davenport (1976) investigated all of these but could not isolate any *Brettanomyces* spp. In 1987, Guerzoni and Marchetti reported their isolation from grapes damaged by sour rot. This agrees with recent knowledge suggesting a connection between *Brettanomyces/Dekkera* and damaged grapes (*Botrytis*-affected) (Taillandier, 2007). Unexpectedly, several scientific reports are published about their isolations present from grapes, particularly from sour rot damaged grapes (Lonvaud Funel *et al.,* 2006, 2012; Malfeito-Ferreira *et al.*, 2012). Despite the fact that *Dekkera* was isolated many times from fermenting musts during earlier researches (Wright and Parle, 1973; Licker *et al.*, 1998; Pretorius, 2000; Jolly *et al.*, 2003; Prakitchaiwattana *et al.*, 2004; Van de Water, 2004). The poor detection of *Brettanomyces/Dekkera* spp. on grapes has been speculated to be the

result of their low cell numbers surrounded by a diverse microbial ecosystem where other wild yeast and bacterial species dominate. However, this problem has been overcame by developing an enrichment medium that enabled them to detect *B. bruxellensis* on grape berries. (Lonvaud-Funel *et al.*, 2006) The same authors were subsequently able to detect this yeast from several vineyards and at different stages of grape berry development.

Following the initial stages of winemaking, *Brettanomyces/Dekkera* spp. have been more consistently associated with wine and cellar equipment (Fugelsang and Edwards, 1998). As their populations are usually minor in the presence of numerous other rapidly fermenting yeasts, their increase in numbers only occurs during more nutritionally favorable conditions that suit their slow growing characteristics (Fugelsang *et al.*, 1993). These conditions are created once alcoholic fermentation is completed and traces of residual sugars allow them to proliferate more easily. Malolactic fermentation (MLF) and ageing in used barrels recognized as the most critical stages of wine production for *Brettanomyces/Dekkera* contamination (Chatonnet *et al.*, 1992, 1995; Fugelsang *et al.*, 1993; Licker *et al.*, 1998; Renouf *et al.*, 2006b; Suarez *et al.*, 2007). During MLF, *Brettanomyces/Dekkera* spp. is presented with conditions of low free sulfur dioxide, low residual sugar concentrations and yeast autolysis with the release of nutrients occurring along with modest microbial competition (Oelofse *et al.*, 2008). The main characteristics of oak barrels (new and old) that are beneficial to *Brettanomyces/Dekkera* growth are the porous microstructure, which allows the influx of small amounts of oxygen (Swaffield and Scott, 1995; Loureiro and Malfeito-Ferreira, 2006) due to not properly clean and sterilized after usage of winemaking (Suarez *et al.*, 2007). Moreover, the presence of cellobiose can serve as sugar resource (Boulton *et al.,* 1996). In addition, difficulty of old barrels sanitation is favorable to established *Brettanomyces/Dekkera* populations and promotes contamination of wine (Pollnitz *et al.*, 2000; Yap *et al.*, 2007). When MLF is performed in barrels these characteristics can aid the growth of *Brettanomyces/Dekkera*. These yeasts have also been recovered from wines in concrete or stainless steel tanks is more likely due to other reasons of survival than those pertaining in barrels (Chatonnet *et al.*, 1992; Rodrigues *et al.*, 2001). Furthermore, numerous finished and bottled wines have also been known to host *Brettanomyces/Dekkera* populations. These wines linked to prior conditions of long periods of barrel ageing, lower SO_2 concentrations and less filtration prior to bottling (Herezstyn, 1986a; Arvik *et al.*, 2002). Wineries and equipment that have been investigated revealed the presence of *Brettanomyces/Dekkera* yeasts in winery air samples and on cellar walls, drains, pumps, transfer lines and other pieces of equipment that are difficult to sterilize (Van der Walt, 1984; Alguacil *et al.*, 1998; Fugelsang, 1998; Connel *et al.*, 2002). It is therefore not surprising that wineries are often considered as the primary source of

Brettanomyces/Dekkera contamination, as opposed to grapes. However, as its occurrence is often inconsistent, each winery can present a unique situation that requires the determination of the specific origin and route of contamination.

1.2. Volatile phenols

Volatile phenols greatly influence the aroma of wine. The most important molecules are 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol in this class (Chatonnet *et al.*, 1992). Elevated concentrations of 4-ethylphenol in red wine are associated with disagreeable aromas often described as ‘‘phenolic’’, ‘‘leather’’, ‘‘horse sweat’’, ‘‘stable’’ or ‘‘varnish’’, etc. (Chatonnet *et al.*, 1992, 1993; Rodrigues *et al.*, 2001).

The origin of volatile phenols involves the sequential action of two enzymes (Figure 1.3.) on a hydroxycinnamic acid (ferulic, *p*-coumaric or caffeic acid) substrate. Hydroxycinnamate decarboxylase first turns these hydroxycinnamic acids into hydroxystyrenes (vinylphenols) (Edlin *et al.,* 1998), which are then reduced to ethyl derivatives by vinylphenol reductase (Dias *et al.*, 2003). The enzyme that facilitates decarboxylation is present in large number of bacteria, fungi, and yeasts, but the reduction step is only performed by the species *Dekkera bruxellensis* and *Dekkera anomala* (Chatonnet *et al*, 1995, 1997; Dias *et al.*, 2003; Edlin *et al.,*1995).

Initially, the presence of ethylphenols in wine was attributed to lactic acid bacteria. Indeed, these are capable of producing significant quantities of vinyl phenols, but under oenological conditions they only produce small amounts (Suarez *et al.*, 2007). Other yeasts present in wines, such as *Saccharomyces cerevisiae*, *Pichia* spp., *Torulaspora* spp. and *Zygosaccharomyces* spp. can produce 4-vinylphenol but do not reduce it to 4-ethylphenol (Dias *et al.*, 2003).

The relationship between high concentrations of 4-ethylphenol in wines and the activity of *Brettanomyces*/*Dekkera* was thoroughly studied during the 1990s (Chatonnet *et al.*, 1995, 1997; Cullere, Escudero, Cacho, and Ferreira, 2004; Fugelsang and Zoecklein, 2003; Kelly, 2003; Parish *et al.*, 2003; Suarez-Lepe, 2001). *D. bruxellensis* shows hydroxycinnamate decarboxylase and vinyl reductase activity under oenological conditions to the extent that the species are considered an undesirable yeast capable of producing high concentrations of 4-ethylphenol. Perceptible threshold was explained for 4-vinylphenol is 770 *µ*g/l; 4-viniguaiacol 440 *µ*g/l, 4-ethylphenol 620 *µ*g/l and for 4-ethylguaiacol 140 *µ*g/l (Chatonnet *et al.*1997). Over the threshold gives "animal" phenolic odors, "barnyard" and "stable" sensory to wine (Etievant *et al.,*1989; Chatonnet *et al.,*1990, 1997; Ribéreau-Gayon *et al.*, 2006).

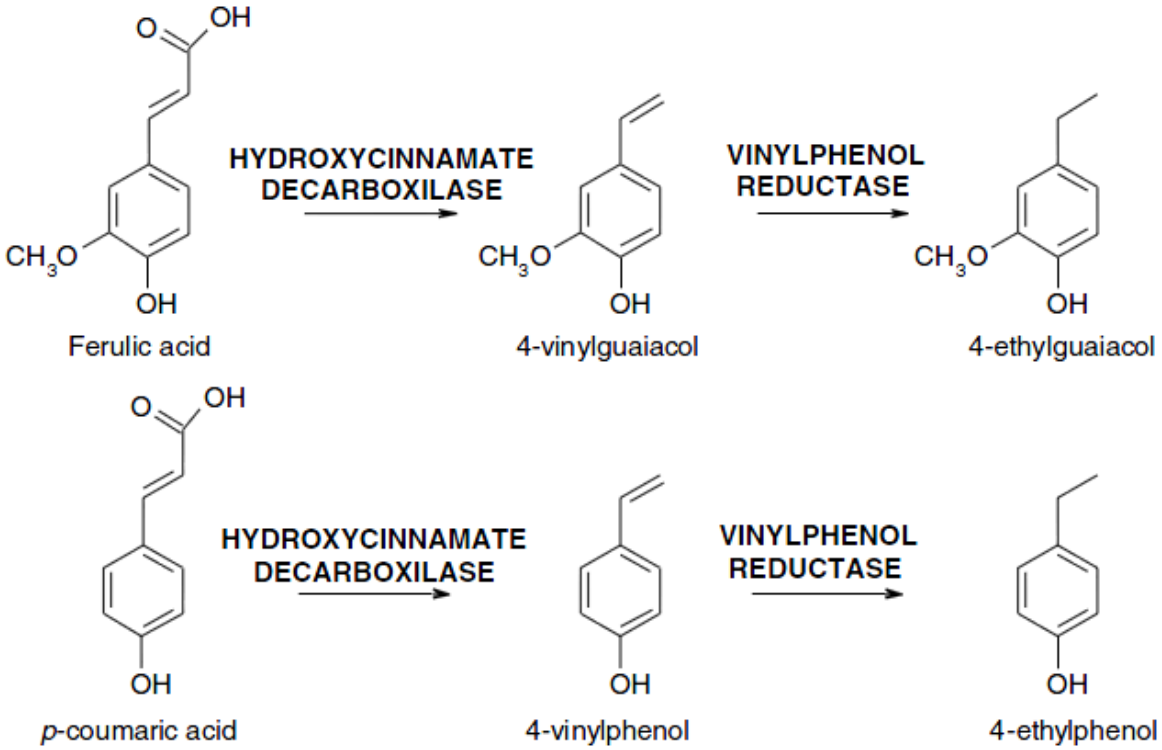


Figure 1.3. Formation of ethylphenols from their hydroxycinnamic precursors (Suarez *et al.,*2007).

Different strains of *Brettanomyces* can show great differences in their production of volatile phenols (Joseph and Bisson, 2004). The variety of grape used also affects the sensorial perception of ethylphenols. Physter and Mills (2004) indicate detection thresholds to be high in mono varietal Cabernet Sauvignon wines, and lower in Tempranillo wines (Suarez *et al.*, 2007).

1.2.1. Thresholds

Thresholds were explained by Chatonnet *et al.,*1992.

Table 1.2. Thresholds of 4-Ethylphenol and 4-Ethylguaiacol

Molecule	Perception Threshold (PT) µg/l	Recovery Threshold (RT) µg/l		Preference Threshold (PrT) µg/l	
		White wine	Red Wine	White wine	Red Wine
4-Ethylphenol	400	600	605	-	620
4-Ethylphenol + 4-Ethylguaiacol (10:1)	-	-	369	-	426

PT: Detection in alcoholic solution

RT: Detection in wine

PrT: 50% of panellists said 'wine spoiled'.

Moreover, researches showed that more than 100 µg/l of 4-Ethylphenol indicatives of *Brettanomyces* activity (Rayne and Eggers, 2007).

In international Wine Challenge contest, more than 10.000 wines tasted in order to make research in taint incidence. This research has done for three years. Results shown that panellists were not approved taints in wines (Goode and Harrop, 2008). Especially, Horse sweat taints were increased year by year.

Table 1.3. Evaluation of Taints in three years

Taint	2006	2007	2008
Cork	27,83	29,68	31,11
Horse sweat	10,59	12,82	15,79
Oxidation	24,29	22,88	19,11
Reduction	29,18	26,53	28,89
Tainted wines	7,1	-	5,88

1.2.2. Volatile acidity and tetrahydropyridines

Brettanomyces/Dekkera are acetic acid producers (Freer *et al.*, 2000; 2003; Suarez *et al.*, 2004). As well as stimulating the growth of *Brettanomyces*, oxygen also appears to stimulate its production of acetic acid. However under anaerobic conditions acetic acid production was suggested very low (Aguilar-Uscanga, 1998) or even null (Blondin *et al.*,

1982; Larue *et al.*, 1991). Derivatives of amino acids such as 2-acetyl-1,4,5,6-tetrahydropyridine are recorded the cause of the ‘‘mousy’’ flavour (Heresztyn, 1986) of some wines. The prevention of this aroma requires the control of undesirable yeast populations by avoiding unnecessary aerobiosis.

1.2.3. Anthocyanin degradation

As well as producing volatile compounds with disagreeable aromas, under favourable situations *Brettanomyces* can hydrolyze anthocyanins, releasing glucose and destabilizing the aglycone (Mansfield *et al.*, 2002). This may be the reason why wines contaminated by *Brettanomyces* have an undesirable colour.

1.3. Factors affecting *Dekkera* growth and volatile phenol production

The factors affecting the production of 4-ethylphenol, especially as regards the production of wine, are not well understood (Dias, 2003). Mainly; they are affected by (i)O_2 (ii)Temperature (iii)pH (iv)sugar content (v)precursors and (vi) preservatives.

Brettanomyces/Dekkera yeasts exhibit a particular metabolism. The inhibition of alcoholic fermentation under anaerobic conditions (Custer effect), in fact, has been considered as biochemical characteristic of *Brettanomyces/Dekkera* (Scheffers 1966). This particular behaviour may affect the growth in the fermentation products. Carrascosa *et al.* (1981) suggested that the Custer effect was a consequence of a redox unbalance caused by the reduction of NAD^+ during the oxidation of acetaldehyde to acetic acid. That is why Ciani and Ferraro (1997) suggested that oxygen concentration exerted a strong influence on both growth and acetic acid production by *Brettanomyces* yeasts in winemaking. According to their study; full aerobiosis lead to large production of acetic acid causing a block of metabolic activity. Semi-aerobiosis resulted in the best condition for alcoholic fermentation (Custer effect) combined with acetic acid production. In anaerobic condition *Brettanomyces* yeasts did not result in high acetic acid production and a pure alcoholic fermentation, even if slow, occurred. The absence of an increase in acetic acid in wines, does not exclude the active presence of *Brettanomyces* yeast since the characteristic high acetic producer in *Brettanomyces* yeast is linked to the presence of oxygen (Ciani and Ferraro, 1997). Malfeito-Ferrera *et al.* (2001) suggested that oxygen stimulated yeast growth and production of 4-ethyl phenol and moreover if oxygen levels get higher than 7mg/l (82% saturation concentration) by stimulating of growth was responsible for faster production. The same authors suggested that total amount of 4-ethylphenol production was decreased 80% with no

measurable levels of oxygen (< 2% saturation) in red wines. They also suggested that presence of oxygen stimulated the production of acetic acid increasing wine volatile acidity.

Several studies have been done with intent to establish the values of temperature (Charoenchai *et al.*, 1998; Barata *et al.*, 2008a). Besides, Correia (2004) suggests that the temperature does not increase the concentration of 4-ethylphenol itself, so that it does promote to increase the reaction kinetics. As known, accelerates the conversion of cinnamic acid into 4-vinylphenol and subsequently the 4-ethylphenol.

Dias *et al.* (2003) made a study and inoculated *D. bruxellensis* in samples subjected to various temperatures and observed that the optical density (OD) of samples at 30°C was greater than 16°C. They obtained growth rate was 0.07 h^{-1} at the 30°C temperature and growth rate was 0.02 h^{-1} at the 16°C temperature. It was found that the production of 4-ethylphenol reaches the maximum value of 620 g/l during the first hours at 30°C. However, growth of these strains was unfavorable at increased temperature. Barata *et al.* (2008a) found that at temperatures 30°C to at 35°C, the cells lose viability in less than 12 hours and at temperatures 36°C to at 40°C cell death occurs in the first 24 hours. Same authors also observed that at 25°C there was no cell death and increased production of ethylphenol was about 400 g/l. Blomqvist *et al.* (2010) explained that the lowest temperature such as at 25°C which the solubility of oxygen point is higher and this affect increase of 4-ethylphenol production and accordingly affects increase of 4-ethylphenol production.

Jensen *et al.* (2009) observed that at 10ºC the yeast *D. bruxellensis* does not grow, in no matter what the alcohol content is. Couto *et al.* (2005) defined thermal inactivation temperature degrees for *Brettanomyces / Dekkera*. Their studies suggested that when heating was performed in wine, a slight effect on cell and survival was observed until at 32,5^{o}C, significant inactivation has begun at 35^{o}C but measurable thermal inactivation has begun at 50^{o}C. Moreover, temperature is not the only affect factor that stimulates growth of organisms but also it is influenced by other factors such as pH (Charoenchai *et al.*, 1998). According to same authors the maximum cell biomass of yeasts was highest at pH 3.5. Dias *et al.* (2003) also suggested adjustment of pH as 3.5 ±0.01.

In winemaking process, the main reaction is transformation of the grape sugars into ethanol (Licker *et al.*, 1998). According to Dias *et al.* (2003) the alcohol content directly

influences the production of 4-ethylphenol. There are several studies the problem of tolerance to ethanol by yeasts *Dekkera* / *Brettanomyces* (Froudiere and Larue, 1989, Delia *et al.* 1997). Barata *et al.* (2008b) found that in 14% (v/v) ethanol conditions there was no cell growth, even at pH 3.5. However, under the same conditions of pH and 12% (v/v) ethanol was observed the recovery of viability after an initial phase of death. Content of more than 13% (v/v) ethanol results in a very low growth rate; production of 4-ethylphenol consequently is limited (Dias *et al.,* 2003). Barata *et al.* (2008b) suggested that cell growth only when the ethanol content was 8% (v/v) and wine at pH 3.0.

Yeasts of the genus *Dekkera* can also develop in the presence of sugar (Barata, 2002) hence growth rate will be increased by glucose concentration (Barata *et al.*, 2008a). However, the technical limit of the residual sugar content of wine is 2 g/l; it does not mean that this quantity is limited for the production of volatile phenols in significant concentrations. In fact, Barata *et al.*, (2008a) found that residual sugar concentration is sufficient to permit the production of considerable amounts of volatile phenols when combined with other factors which it is suitable to growth of *Brettanomyces/Dekkera*. Similarly, Chatonnet *et al.* (1995) reported that in the case of *Dekkera*, consumption of 300 mg/l of fermentable sugars (glucose, fructose and galactose) was sufficient to produce significant changes in aromatic wines. However, Massini *et al.* (2010) concluded that if wine contains residual sugar this is not the most important factor that interferes with cell growth and even 6 g/l of residual sugar contained wine at the temperature of 6°C, cell growth and production of volatile phenols were not observed due to the temperature. In other words, temperature is the factor which it is most prevalent rather than sugar content. Barata *et al.* (2008a) described the growth and production of 4-ethylphenol by *D. bruxellensis* as affected by sugar concentration and temperature under conditions mimicking wine production.

The precursors of the reaction production of volatile phenols are (i)cinnamic acid (ii)*p*-coumaric acid (iii)ferulic and (iv)less quantity of caffeic acid. These acids are present on grape cell walls (Lynd *et al.*, 2002) and all mechanisms are potential sources of winemaking process (Esti and Tamborra, 2006). Crushing, prolonged macerations and other treatments intensively contribute to the increase of cinnamic acids in wine (Baumes *et al.*, 1986; *cit in* Correia 2004). Increasing the temperature of maceration verified that at the end of fermentation extraction of phenolic compounds can also increase, especially cinnamic acids. It was suggested that cinnamic acids exist in grapes at different concentrations. This existence depends on variety and winemaking process (Reguant *et al.*, 2000). Dias *et al.* (2003) also reported that 4-vinylphenol can be considered as precursor and formation of this

phenol can be in the absence of volatile and cinnamic acid. Same authors also studied the population of growth in media with addition of 4-vinylphenol. They found that the production of 4-ethylphenol increases during the first hours as reaching sugar concentration of 50 mg /l.

Among the possible preservative is (i) sulfur dioxide (SO_2) to help inhibit growth of the organism. Sulfur dioxide considered as an anti-bacterial, anti-oxidants, anti-oxidase and improving the clarity of aroma due to help in the process of maceration. Therefore; sulfur dioxide considered the most common and effective inhibitors for growth of *Dekkera/Brettanomyces*. However, sulphur dioxide cannot be added continuously because it is subject to maximum legal limits. In the EU, these limits are a function of wine type (Loureiro and Malfeito-Ferreira 2003), while in the US the maximum level of total sulphite is 350 mg/l (Fugelsang and Edwards 2007). After addition, a fraction of the sulphite is combined and loses its antimicrobial activity the use of high doses of sulphur dioxide before fermentation can increase the production of acetaldehyde by fermenting yeasts. Therefore additions should be controlled by sulphite measurement after treatment (Malfeito- Ferreira, 2010). To prevent microbial growth, common advised usage levels are 0.5–0.8 mg/l molecular sulphur dioxide (Fugelsang and Edwards 2007) but *D. bruxellensis* has resistance up to 75 mg/l total SO_2 (Malfeito- Ferreira, 2010). In addition, growing populations are more resistant, 1 mg/l molecular sulphite being required to prevent the proliferation of *D. bruxellensis* (Barata *et al.* 2008a).

Massini *et al.* (2010) studied the influence of the concentration of SO_2 in interaction with temperature and the cell growth of *Dekkera* yeasts and they observed that under low temperatures SO_2 keep its presence in order to act against to *Dekkera*. Du Toit *et al.* (2005) kept wine with low pH values with this purposes they aimed enhancing the properties of SO_2, due to under these conditions they conserved existence of free form of SO_2.

Potassium metabisulphite ($K_2S_2O_5$) can also use as white crystals, typically using a pinch of it or it may apply with sulfur solutions (5% to 6%) in water or in gaseous form with pure sulfur dioxide. This preservative is also known as very effective application against to *Dekkera*. This compound is extremely toxic that is why it should be use very carefully. Although in recent times there is legislation limiting the amount of application of this compound. For wine with a sugar content of less than 5 g /l total SO_2 it is allowed to 160 mg /l for red wines and 210 mg/l to white wines (Ribéreau-Gayon *et al.*, 2006

Other preservatives used in the wine in order to decrease of *Dekkera* populations. Such as sorbic acid is weak acid, the free form of which is present in higher proportions at lower pH values (Malfeito-Ferreira, 2010). The maximum legal limits are 200 mg/l in the EU and 300 mg/l in the US (Fugelsang and Edwards 2007). Due to its higher solubility, potassium sorbate is used as the vehicle of sorbic acid. Its usage is advised, together with sulphur dioxide, at bottling of sweet wines to inhibit fermenting yeasts. It is metabolized by lactic acid bacteria, originating the “geranium taint”. More than 950 mg/l doses it is not effective against *D. bruxellensis* (Malfeito-Ferreira, 2010). This agent has a selective effect on microorganisms of the wine to against the yeast growth without interfering with bacterial gowth (Ribereau-Gayon, *et al.*, 2006).

Finally, dimethyl dicarbonate (DMDC) is another preservative very effective at low concentrations against to wide variety of some fungi and yeast (Delfini *et al.*, 2002; Costa *et al.*, 2008). DMDC has been recently approved in the EU for use at the maximum amount of 200 mg/l at bottling of wines with more than 5 g/l residual sugar. In the US it may be used during the storage of wine in regular amounts up to the maximum level of 200 mg/l (Fugelsang and Edwards 2007). Its efficiency depends on the initial microbial contamination, with a maximum of 500 viable cells/ml wine advised (Malfeito-Ferreira, 2010). Bacteria are more resistant than yeasts and this preservative should not be regarded as a sterilant when used alone (Costa *et al*. 2008). Therefore, in wineries, if legally authorized, DMDC should be used routinely together with sulphite during wine storage or at bottling (Malfeito-Ferreira, 2010). Its activity depends on adequate homogenization, which is achieved by a costly dosing apparatus. Another factor requiring precautions is its human toxicity (Fugelsang and Edwards 2007). Renouf *et al.*, (2008) suggested that when DMDC applied at the maximum dose, may cause an increase in the concentration of methanol in wine which it extremely contains high concentrations for human health.

1.4. Controlling *Brettanomyces/Dekkera sp.* in wines

Various techniques have been proposed to avoid or reduce the wine faults associated with volatile phenols (Table 1.2.); these can be broken down in two groups.

Table 1.2.. Various methods for controlling ethylphenols in wines.

Treatment	Result	Drawback
Protein clarification	Reduces *Brettanomyces*/*Dekkera* populations by flocculation.	Loss of colour and aroma.
Filtration	Reduces *Brettanomyces*/*Dekkera* populations by physical separation.	Loss of colour and aroma.
Physicochemical variables	Establishes physicochemical conditions that reduce the viability of *Brettanomyces*/*Dekkera*.	These variables can be difficult to modify and may be incompatible with aging.
Reduction of precursor concentration	Prevents the solubilization of hydroxycinamic acids (the precursors of volatile phenols).	May cause a loss of colour and aroma.
Additives	Inhibit the growth of *Brettanomyces*/*Dekkera* and prevent the conditions that favour the formation of ethylphenols.	Some of the products are not authorised for use in the wine sector or are still experimental.
High pressure processing	Destroys microorganisms in wine without seriously affecting its organoleptic properties.	High equipment costs.
Biological techniques	Inhibit the growth of *Brettanomyces*/*Dekkera*.	Use of these techiniques in wine is usually experimental.
Genetic engineering	Genetically engineered yeasts that prevent the growth of *Brettanomyces*/*Dekkera*.	Not currently allowed in winemaking.
Reverse osmosis [a]	Reduces ethylphenol contents in wine.	High equipment costs, this kind of use is not currently allowed.
Fining agents [b]	Reduces ethylphenol contents in wine.	Loss of colour and aroma.
β-Glucanases[c]	Reduces wine spoilage yeasts	Possible negative effect on wine quality
Cold Pasteurisation [d]	Reduces ethylphenol contents in wine.	Loss total anthocyanins content

Table adapted from Suárez R. *et al.*, 2007, with additions from: **a** Ugarte P*. et al.*, 2005, **b** Lisant M. T. *et al.*, 2008 and **c:** Enrique M., 2009 **d:** Morata A. *et al.*, 2012

1.5. Aim of research

Taking in consideration the absence of data from Turkish wines and the requirement for validating previous results suggesting the influence of storage temperature on the prevention of phenolic taint in wines, we established the following objective for the present work:

To investigate the incidence of *Dekkera bruxellensis* and the levels of 4-ethylphenol in randomly selected Turkish red wines.

To investigate influence of storage temperature on the production of 4-ethylphenol by wild of *D. bruxellensis* in 3 different Portuguese red wines.

To investigate the effect of different storage temperatures on *Dekkera* growth and volatile phenol production.

2. MATERIAL AND METHODS

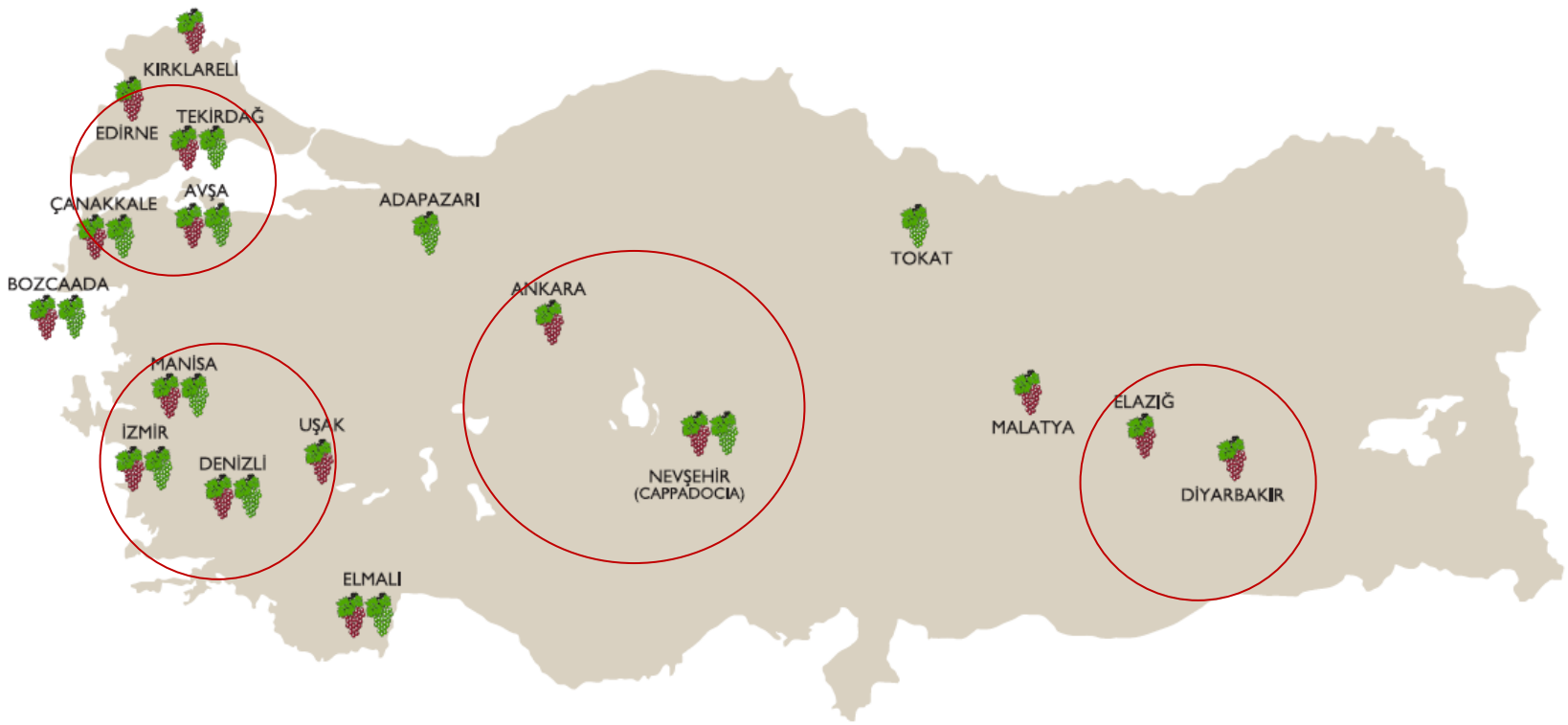
2.1.1 Wine samples

In these work wine samples were obtained from Turkish and Portuguese wineries. Samples were randomly chosen and supplied by Turkish companies. Turkish red wines were collected from different regions of Turkey (figure 2.1).For this aim, wines are collected from all wine regions and to try to make the regions more homogenous in order to provide objective results. The Portuguese red wine samples are listed in table 2.1. Wine region and wine characterization for Turkish red wines were given in table 2.2.

Table 2.1 Portuguese red wine samples

Sample	Lab. Code	Type of vessel	Vintage
Tank 5	1	5000 lt Inox	2010
Tank 6	2	5000 lt Inox	2010
Tank 9	4	2500 lt Inox	2010

TURKEY'S WINE GRAPE PRODUCTION



ADAPAZARI: Sauvignon Blanc.
ANKARA: Boğazkere, Kalecik Karası, Öküzgözü.
AVŞA: Adakarası, Cabernet Sauvignon, Chardonnay, Merlot, Shiraz.
BOZCAADA: Cabernet Sauvignon, Çavuş, Karalahna, Kuntra, Merlot, Vasilaki.
ÇANAKKALE: Cabernet Sauvignon, Chardonnay, Grenache, Merlot, Sauvignon Blanc, Shiraz, Cabernet Franc.
DENİZLİ: Boğazkere, Cabernet Franc, Cabernet Sauvignon, Chardonnay, Çalkarası, Dimrit, Kalecik Karası, Merlot, Narince, Öküzgözü, Petit Verdot, Pinot Noir, Sangiovese, Sauvignon Blanc, Shiraz, Sultaniye.
DİYARBAKIR: Boğazkere.
EDİRNE: Cabernet Sauvignon, Merlot, Shiraz.
ELAZIĞ: Boğazkere, Öküzgözü.

ELMALI: Boğazkere, Cabernet Sauvignon, Chardonnay, Kalecik Karası, Malbec, Merlot, Öküzgözü, Pinot Noir, Sauvignon Blanc, Shiraz.
İZMİR: Alicante Bouchet, Bornova Misketi, Cabernet Sauvignon, Carignan, Chardonnay, Grenache, Merlot, Petit Verdot, Sangiovese, Sauvignon Blanc, Shiraz, Viognier.
KIRKLARELİ: Merlot, Papazkarası.
MALATYA: Öküzgözü.
MANİSA: Alicante Bouchet, Bornova Misketi, Carignan, Chardonnay, Grenache, Kalecik Karası, Merlot, Öküzgözü, Sangiovese, Sauvignon Blanc, Shiraz, Malbec, Tempranillo.
NEVŞEHİR: Chardonnay, Dimrit, Emir, Kalecik Karası, Narince, Öküzgözü, Sauvignon Blanc, Malbec, Tempranillo.
TEKİRDAĞ: Cabernet Franc, Cabernet Sauvignon, Cinsault, Gamay, Kalecik Karası, Merlot, Papazkarası, Riesling, Sauvignon Blanc, Semillon, Shiraz, Viognier.
TOKAT: Narince.
UŞAK: Boğazkere, Kalecik Karası, Öküzgözü, Shiraz.

Figure 2.1. Turkey's wine grape production. (www.winesofturkey.org)

Table 2.2. Wine region and wine characterization for Turkish red wine samples

Wine Samples	Origin	Year	Characterization			
			Barrel Usage	Organic	Bulk	Bottled
TR 1	CAPPADOCIA	2010	+		+	
TR 2	CAPPADOCIA	2006	+			+
TR 3	KAYSERİ	2010	+	+		+
TR 4,5,6	DENİZLİ-CAL	2008			+	+
TR 7,8,9	CAPPADOCIA	2011	+		+	
TR10,11,13,14,15	DENİZLİ	2011	+		+	
TR 12	DİYARBAKIR	2011	+		+	
TR 16,32	ELAZIĞ	2011	+		+	
TR 17,22	TEKİRDAĞ	2011	+		+	
TR 18	DENİZLİ	2011	+		+	
TR 19,20	ANKARA	2011	+		+	
TR 21,23,24	DENİZLİ	2011	+		+	
TR 25	MARMARA	2011	+		+	
TR 26	DENİZLİ	2010	+		+	
TR 27,29	DENİZLİ	2011			+	
TR 28	ELAZIĞ	2010			+	
TR 30	DİYARBAKIR	2011			+	
TR 31	ELAZIĞ	2011			+	
TR 33,34,35	EGE	2010			+	
TR 36	ANKARA	2011			+	
TR 37,38	ANKARA	2009			+	
TR 39	CAPPADOCIA	2011			+	+
TR 40	CAPPADOCIA	2008			+	+

2.2. Microbiological Analyses

2.2.1. DBDM Differential Media

DBDM (*Dekkera / Brettanomyces* Differential Medium) have been used as a differential selective nutrient media for detection of *Dekkara* yeast strains which it was developed by Rodrigues *et al.* (2001). Analyses have taken in place in microbiology laboratory of Instituto Superior de Agonomia. DBDM has two different differential media phase which are solid and liquid.

Composition of selective nutrient media was:

YNB (Yeast Nitrogen Base)	6.7 g/L
Ethanol (96%)	60 ml
p – Coumaric Acid	0.1 g/L
Cycloheximide (0.25g/25 ml)	1 ml
Clorofenicol (2.5g/25 ml)	1 ml
Bromocresol Green	0.022 g/L
Agar – Agar	20 g
Distillated Water	940 ml

Solution 1: 0.022 g of Bromocresol Green (Sigma) has been dissolved in 800 ml of distillated water with the aid of Ultrasonic bath (15 minutes) and pH: 5.4 (±0.1) was verified with NaOH 1M and DBDM base media was put in autoclave(120^{o}C, 20 minutes) after addition of 20 g of Agar-Agar. Agar-Agar was added only usage of DBDM in solid phase media.

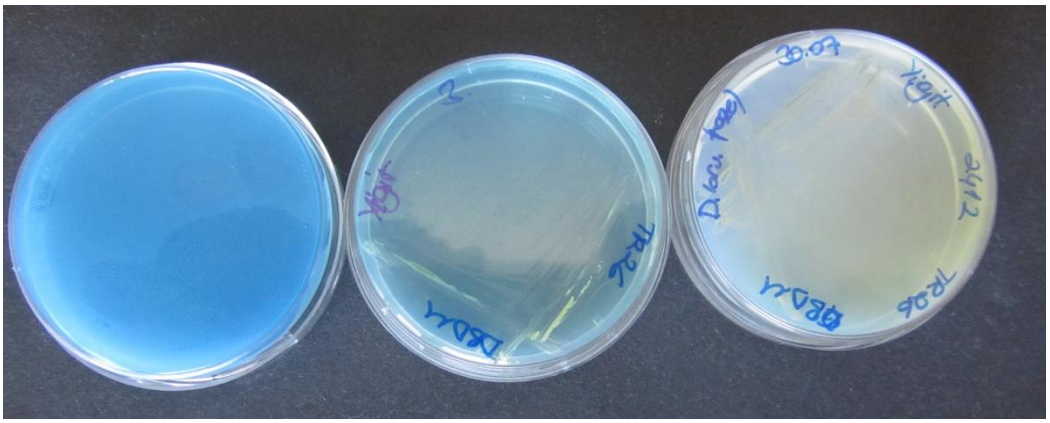
Solution 2: A) 6. 7 g of YNB (Difco) dissolved in 140 ml distillated water with the aid of ultrasonic bath. **B)** 0.1 g of *p*– Coumaric Acid was dissolved in 60 ml of ethanol with the aid of ultrasonic bath. Those two solution (A and B) were jointed and pH was adjusted on 5.4 (±0.1). Afterwards 1 ml of Cycloheximide (0.25g/25 ml) and 1 ml of Clorofenicol (2.5g/25 ml) were added to prepared solution latterly solution was filtered via membrane filtration 0.22 μm, 47mm diameter (Millipore).

Prepared two solution were joined after cooling solution 1 in a water bath at about 55^{o}C. Afterwards solution distributed to Petri dishes.

Liquid phase of DBDM media was used in order to obtain effect of sensory analyses of *Dekkera* sp. as mentioned by (Rodrigues *et al.*,2001). In order to prepare DBDM liquid phase all preparation pathway fallows as explained above but for this occasion Agar-Agar were not added for preparation of liquid phase.

Incubation time of DBDM was *Dekkera* sp. was 12 days at 25 ^{o}C (Rodrigues *et al.* 2001). DBDM has along with yeast nitrogen base (YNB) comprise two antimicrobial agents (ethanol and cycloheximide), a pH indicator (Bromocresol green) to indicate media actification and a substrate (*p*– coumaric acid). In case of present of *Dekkera* sp. in the nutrient media was transformed blue colour to light yellow colour (figure 2.2.) due to addition of acetic acid and it showed typical phenolic smell which they are barnyard, horse sweat, medicinal and mousy due to in the case of addition of *p* – coumaric acid to nutrient media. This typical smell was already explained by many authors (Chatonnet e*t al.*, 1995; 1997; Fugelsang, 1997; Rodrigues *et al.*, 2001; Malfeito-Ferreira, 2003; Loureiro and Malfeito-Ferreira, 2006). Besides, it also showed that other characteristic structure of *Dekkera* sp. was colourable changes in colonies. According to researchers, the inclusion of *p*-coumaric acid substrates for the production of 4-ethylphenol, enabled the differentiation by smell of *Dekkera/Brettanomyces* sp. (Rodrigues *et al.,* 2001).

Figure 2.2 Colour change of DBDM



First day | 6 days incubated | 12 days incubated

Figure 2.2. Colour change of DBDM

2.2.2. Ringer Solution (Biokar)

Ringer's Solution 1/4 strength is a diluent used to prepare diluted solutions for the microbiological control of wine samples. Accordingly; usage description of Ringer's solution, two instant Ringer's tablets has been dispersed in 1 liter distillated water with the aid of ultrasonic water bath and used for to dilute the wines which were in different temperature. For preparation of wine dilutions; 9 ml of ringer solution was mixed with 1 ml of wine in experimental test tubes and this process repeated 3 times. 1 ml of diluted wine samples has been inoculated to Petri dishes and incubated as referred by Rodrigues *et al.*(2001).

2.2.3. YNB Media (Yeast Nitrogen Base) (Difco)

Concentrated suspension was prepared from the cell culture media into fresh YNB (Difco) at a concentration of 0.67% (w / v) with 10% ethanol (v / v), and 2% glucose, pH 3.5 and filtered with membrane (0.20 μM, 47 mm diameter, Millipore). Afterwards ethanol content of suspension was measured 11%. The suspension was placed in Erlenmeyer flasks homogenized and kept on orbital agitation of 120 rpm at 25 ° C. The optical density was determined with the inoculum absorbance reading at 640 nm (S20 Boeco Spectrophotometer) at the initial moment. This process followed accompanying increase of cell until reaches values between 0.3 and 0.5. Counting observation of this increase took in a place under the microscopic conditions and it was made by hemocytometer counts of viable cells in order to calculate the exact volume of suspension to be inoculated.

2.2.4. GYP (Glucose Yeast Peptone) Media (Wickerham)

GYP medium was used (5g / L of yeast extract (Oxoid), 5 g / L peptone (Oxoid), 20 g/L glucose (Merck) and 20 g / L agar, pH 6.0) in order to evaluate of present of *D. bruxellensis* from red wines samples.

Besides, obtained Turkish strain TR 26 has been inoculated to GYP in order to evaluate presence of *Dekkera bruxellensis* in different storage temperature condition.

2.2.5. Sterilized wine

Parameter correction and sterilized wine was done in order to stabilize all laboratory results. That is the reason why, alcohol content has adjusted 11% (v/v) through dilution. For this purpose, 5g / L tartaric acid (Merck) solution was used in order to change the total acidity of wine. Therefore; wine supplied from in microbiology laboratory of Institute Superior de Agonomia, reducing alcohol content 4.8% with distilled water and a water solution of tartaric acid concentration 5 g / L. The pH was adjusted to 3.50 (± 0.2), using a pH meter (Radiometer). For this purposes, NaOH (Merck) and HCl (Merck) of several dilutions of solutions were used. Free sulfur was removed by addition of acetaldehyde (Ribéreau-Gayon et al., 2006) at concentrations necessary to remove the whole of this compound.

2.3. Evaluation of *Dekkera bruxellensis* growth

2.3.1. Microbiological Analyses

2.3.1.1. Preparation of Inoculation

All inoculation process has been done under Biohazard safety cabinet conditions. Required equipments for preparation were:

DBDM solid and liquid phase (3 Petri or experiment tube for each wine samples)

Millipore membrane filters (0.22 μm pore sized, 47mm diameter)

Funnel (Millipore)

Büchner Flask (Vacuum Filter Flask)

Compressed Air Pump (Millipore)

Glass Straws

Provided wine samples have been filtered with the aid of funnel and compressed air pump. Liquid and solid phases of DBDM had been prepared for analysis. Obtained filtered membranes inoculated solid DBDM media or liquid DBDM media.

Accordingly, 10 ml of liquid phase of DBDM has been put in experiment tubes in order to provide growing conditions in the DBDM for *Dekkera* sp. for evaluate odor of the sample. For this purposes, filtered membranes were inoculated with liquid DBDM. Only 1 ml of wine sample was poured directly to liquid media.

Additionally, solid DBDM was used in order to obtain *Dekkera bruxellensis* colonies. For this occasion, three different amount of wine have been filtered via funnel and membrane filtration such as 1ml, 10ml and 50ml and 0.22 *µ*m, (47mm diameter, Millipore) pore sized membrane filter was used. Obtained membranes were put on Petri dishes and incubated in 12 days as refereed by Rodrigues *et al.*, 2001.

2.3.1.2. Usage of GYP

After growing of Turkish strain TR 26 in YNB: It inoculated to sterilized and parameter corrected wine to see evolution of growing factor of *D. bruxellensis* in strain TR 26, 25*µ*l of samples has been inoculated to GYP Petri dishes in the three different concentrations. For this purposes, dilution was done by Ringer solution. Firstly 900 *µ*l of ringer solution was put in three different eppendorf tubes for each wine sample, they were diluted with 100 *µ*l wine sample which it is contaminated by TR 26. Dilution was done from 10^{-1} until 10^{-3}. After calculations take in a place with formula;

$$Number\ of \frac{cells}{mL} = Number of\ colonies\ x \frac{\mathbf{1}}{0,\ 025} x \frac{\mathbf{1}}{dilution\ factor\ (\mathbf{10^{-1}}, \mathbf{10^{-2}}, \mathbf{10^{-3}}..etc.)}$$

2.3.1.3. Incubation period of *Dekkera bruxellensis*

Filtered membranes were kept in incubator 12 days at 25 ^{o}C as refereed by (Rodrigues *et al.,* 2001). At the end of incubation period, colonies and described odor observed. Secondly, already affected wild *Dekkera bruxellensis* three different Portuguese wine were kept in three different storage conditions (3 ^{o}C, 10 ^{o}C, 15 ^{o}C) in order to see evaluation of different temperature low storage conditions. This stage of experiment took in a place for 90 days. Afterwards, Turkish strain (TR 26) have been kept in different storage conditions (3 ^{o}C, 10 ^{o}C, 15 ^{o}C and 20 ^{o}C), without agitation to see evolution of *Dekkera bruxellensis.* This part of work has taken a place for 3-4 days in incubator because in this point GYP media was used. End of the 3-4 days was observed, counted growing colonies.

2.3.1.5. Colony Investigations and Microscopy

Colony types were obtained on DBDM nutrient agar and GYP media were investigated under microscopy conditions (Leitz, Dialux 20, Germany, 40X). Microscopic sample preparation comprised taking a minor portion of individual colony from the agar plate and re-suspending it in distilled water directly on a microscopic slide prior to viewing.

After 12 days incubation period with DBDM nutrient media, Petri dishes were taken off from incubator and were counted colonies which are on the membranes that used with the help of colony counter (Figure 2.3.).

Figure 2.3. *Dekkera bruxellensis* colonies on DBDM

2.3.2. Chemical Analyses

Chemical analyzes of samples were the alcoholic strength by volume (EtOH %), total acidity (g/L), fixed acidity (g/L), volatile acidity (g/L), pH, sugar (g/L) and verification of malolactic fermentation. The analyses were all made according to standards OIV (Anonymous, 2005). Those analyses had taken place in Laboratory of Enology, Institute Superior de Agronomia, Lisbon, Portugal.

2.4. Extraction of 4- Ethylphenol by Gas Chromatography

The extraction of 4-ethylphenol is the separation of the organic phase. Analysis by gas chromatography was performed according to the protocol described by Roberts *et al.* (2001). For this reason; wine samples in different temperatures had been extracted. This procedure is performed in a 50 ml sample of wine adjusted pH 8 with NaOH (Merck) and was added 0.5 mL of a solution of 3.4-dimethyl-phenol (internal standard, 100ppm diluted in 75%

of ethanol) and 0.05 ml of 4-ethylphenol solution in 50 ml of volumetric flask immediately. This 50 ml of sample has been transferred to 100 ml of volumetric flasks. Those volumetric flasks had 4 ml of mixture of ether-hexane (1:1). The extraction of three replications were done. First time 100 ml volumetric was treated 4 ml of ether and hexane once and after 2 ml of ether and hexane twice. After each addition the samples were stirred by magnetic stirrer for five minutes. Separating funnels had been used in order to collect the organic phases of samples, after each shaking flasks. Small flasks where the organic phases stay were identified accurately. Organic phase of sample was carefully transformed with the aid of Pasteur pipettes. The extract was analyzed immediately whenever possible. However, in case of late analyze, the tubes were reserved in the freezer (-4 ° C) before being analyzed by gas chromatography least maximum 1 day. For the analysis in a gas chromatograph was used (Varian CP-3800 Gas Chromatograph) comprises a Restek Stabilwax ® column (30 m, 0.25 mID, 0.25 umdf and column flow was 2.0mL/min J & W Scientific) and an injector split that injects a volume of 2 µL. Oven final temperature was 250 ° C. The carrier gas was hydrogen at 25 kPa and the separation of the compounds is achieved by an FID detector. Is coupled to the chromatograph equipped with a computer software called Galaxie GC Worldstation WS for Windows (Varian) that allows data collection.

Alcohol degree corrected wine which it is contaminated with TR 26 by has been analyzed once per week in order to see evolution of 4-ethylphenols in Gas Chromatograph.

2.5. HPLC (High-performance liquid chromatography)

Analysis by HPLC (High performance liquid chromatography) was performed on 40 different Turkish red wines. HPLC analyzes the compounds considered important to evaluate the evolution of the samples over time during the cell growth process. The constituents were analyzed, gluconic acid, galactronic acid, succinic acid, glycerol, acetic acid, tartaric acid, fructose lactic acid and ethanol. For this purposes, wine samples were filtered with syringes and nylon filters of 0.2 ,13 mm (Frilabo) and 1 ml (±0.1) sample of wine has been transferred to HPLC vials accurately identified. The filtrate was injected into a column (Schodex 1011) maintained at 65 °C. The mobile phase used consisted of 5.2 mM sulfuric acid at a flow rate of 0.6 ml / min. The separation of the constituents was achieved by detecting a refractive index (486 Waters Corporation, Milford, MA) and results were processed using the software HPLC (Empower). The HPLC was calibrated with standard solutions of known concentrations and the samples were injected in duplicate (Appendix 1).

3. Results and Discussion

3.1. Chemical and HPLC analyses of Turkish wines

Conventional chemical analyses of Turkish red wines have taken place in laboratory of enology of Instituto Superior do Agonomia. Those Turkish wine samples were also analyzed by HPLC. Results of Chemical analyses and HPLC analyses were given in Appendix 2. The aim of those analyses was to obtain correlation between chemical analyses and HPLC analyses and compare with *Dekkera* countings. Unfortunately, we could not obtain big amount of *Dekkera* colonies from Turkish wine samples so that is the reason why, we cannot achieve to make correlation. However according to results of HPLC and of chemical analysis, it was possible to obtain correlations between volatile acidity and acetic acid and between ethanol content obtained by both methods. The results of the correlations are shown in Appendix 3. Correlation between volatile acidity and acetic acid showed a R^2 value of 0.88, while a poor correlation was obtained for ethanol. In this way results from ethanol were only obtained from conventional analysis.

3.2. Microbiological and 4-Ethylphenol Analyses

3.2.1. Turkish red wine samples

The results of microbiological analyses of Turkish red wines are given in table 3.1. Only one strain (TR 26) was isolated out of randomly chosen 40 Turkish red wine samples. This strain was purified and used for further studies of this thesis. 4 ethylphenol results and *Dekkera bruxellensis* cell countings were shown in table 3.1 for Turkish red wine samples. Other samples showed levels of volatile phenols higher than the detection threshold but did not show contamination by viable cells of *Brettanomyces*.

Table 3.1. Results of 4-Ethyl phenol contains and microbiological analyses of Turkish Red Wines

Wine Samples	4-EP GC* ppb	Dekkera Countings (CFU/ml)
TR 1, 18	4	0
TR 2	223	0
TR 3	94	0
TR 4	631	0
TR 5	556	0
TR 6	547	0
TR 7, 12, 28	6	0
TR 8,11, 13, 14, 15, 16, 17,22 27, 29, 33, 34, 35, 36, 37, 38, 39, 40	0	0
TR 9	10	0
TR 10, 32	3	0
TR 19, 25, 30	5	0
TR 20	1	0
TR 21, 23, 31	2	0
TR 24	7	0
TR 26	0	6

* Gas Chromatography

3.2.2 Portuguese Red Wine Samples

In this work the evolution of *Brettanomyces* contamination and volatile phenol concentrations were monitored in real winery samples taken from a Portuguese winery. In this way it was possible to check the effect of winery operations on the levels of both determinations. The results are shown in table 3.2.

According to these results it was shown that, although wines were contaminated by wild *Dekkera*, 4-ethylphenol levels were maintained below the detection threshold. The main operations done in winery were addition of sulphur dioxide up to levels of 30-35 mg/l free sulphite and a filtration by K100 filter sheets. This filtration reduced the levels of contamination but was not fully efficient. On the contrary, the adjustment of sulphite levels was essential to prevent the increase in volatile phenol concentration.

210^{th} day of samples selected as a base of Day zero (D-0) and separated for three different storage temperatures in order to link with further studies of this work.

Table 3.2. 4-Ethylphenol and microbiological analyses from Portuguese red wines in winery conditions.

Samples	**Dates**	22.08.2011[3]	29.09.2011[2]	20.10.2011	28.10.2011	15.11.2011	20.12.2011[3]	19.03.2012	30.05.2012	06.07.2012
	Days	0	38	59	67	85	120	210	282	319
Tank 5	4-EP (ppb)	138	165				204	339	123	54
	Dekkera counts	>100 UFC/ 100ml	<1UFC / ml				>300 UFC / ml	<1 UFC / 100 ml	<1 UFC / 100 ml	<1 UFC / 100 ml nd[1]
Tank 6	4-EP (ppb)	130	242	246	146	178	245	305	118	50
	Dekkera counts	>100 UFC/ 100ml	140 UFC / ml		36 UFC / ml	70 UFC / ml	>300 UFC / ml	19 UFC / 100 ml	<1 UFC / 100 ml	<1 UFC / 100 ml nd[1]
Tank 9	4-EP (ppb)	121	151	250	150	251	140	295	112	59
	Dekkera counts	Nd	50 UFC /ml		281 UFC /ml	>100 UFC/ 100ml	>300 UFC / ml	<1 UFC / 100 ml	<1 UFC / 100 ml	<1 UFC / 100 ml nd[1]

[1] : non - detected

[2] : Filtration

[3] : SO_2 Addition

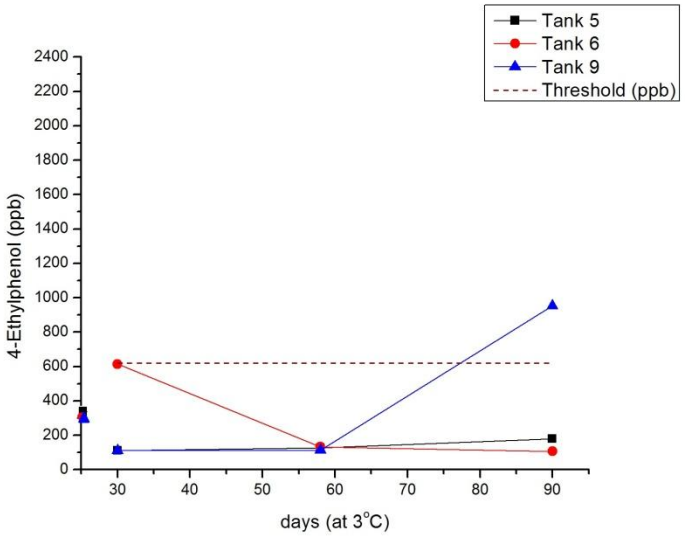
3.3. Volatile phenol production and growth of *Dekkera bruxellensis* under different storage temperatures

3.3.1. Evolution of volatile phenol naturally contaminated by *Dekkera bruxellensis*

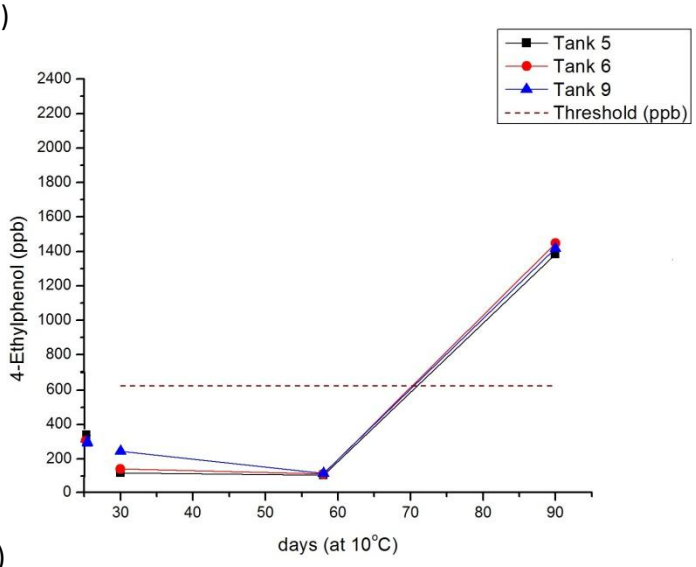
Several samples obtained from real wineries contaminated by *Dekkera* were stored at different temperatures to evaluate its effect on volatile phenol production. For this reason, 210th day of results assumed day zero (D-0) from table 3.2.4. Results are showed (figure 3.1) that, broadly, only at 3ºC it was possible to prevent an excessive increase in 4-ethlphenol. However in one sample (tank 9) 4-ethylphenol increased perhaps because of sample manipulation.

On the contrary of previous study refereed by Jensen *et al.* (2009), results showed that wild *Dekkera bruxellensis* strain was not able to produce 4-ethylphenol at 10^{o}C with 11% of ethanol content. Although Jensen *et al.* (2009) referred 40 days incubation, in our study wines took in place 90 days incubation under different storage temperature conditions. In figure 3.1.b showed that 10^{o}C with 11% of ethanol content is a favorable conditions for *Dekkera bruxellensis* growth in long term. At 15^{o}C (figure 3.1. c) 4-ethylphenol was increased after 58th days as foreseen several authors (Charoenchai *et al.,* 1998; Malfeito-Ferreira *et al.,* 2001; Dias *et al.,* 2003; Barata *et al.*,2008; Jensen *et al.*, 2009).

a)



b)



c)

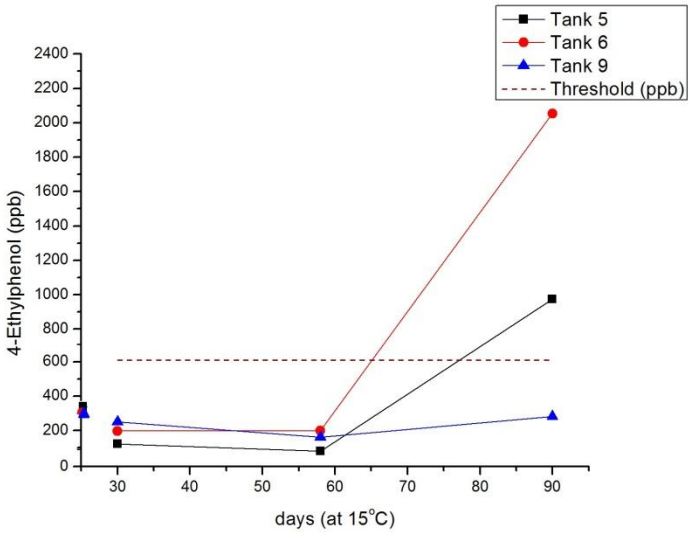


Figure 3.1. Effect of different storage conditions in growth and 4-EP production of wild *Dekkera bruxellensis* population in laboratory conditions

3.3.2. Growth and volatile phenol production by strain TR 26

The previous results showed that decreasing storage temperature may be an efficient measure to control volatile phenol production without using sulphur dioxide. However, the observation high 4-EP levels in one sample maintained at 3 ºC led us to study more deeply the influence of temperature on 4-EP production. In this case we used sterile filtered wine inoculated with a pure culture of the strain TR 26 previously isolated from a Turkish wine sample.

In figure 3.2 viability of cells (log CFU/ml) (A) and 4-ethylphenol contents (B) are shown for temperatures of 3 ^{o}C, 10^{o}C, 15 ^{o}C and 20 ^{o}C, respectively. At 3^{o}C all results were shown either viable cell countings or 4-EP production were also decreased as foreseen by Jensen *et al.* (2009) and Malfeito-Ferreira (2010). At 10 ^{o}C the viable yeast cell countings increased as well as production of 4-EP. After 37th day of experiment production of 4-EP were decreased although viable cells increased.

The effect of temperature on the growth and 4-ethylphenol production of *Dekkera bruxellensis* is similar when glucose is used as single energy and carbon source (Dias *et al.*, 2003). Under warm conditions yeasts can consume more glucose until dryness is reached (Fugelsang, 1997). Barata *et al.* (2008) explained that at 15^{o}C and 20^{o}C are very favorable temperatures for both viable yeast cell growth and production of 4-EP. Our experiment took a place at 15^{o}C and 20^{o}C (A), (B). At the result either viable cell countings or production of 4-EP increased as foreseen. At 20^{o}C, formation of 4-EP occurred quicker due to consumption of glucose was quicker.

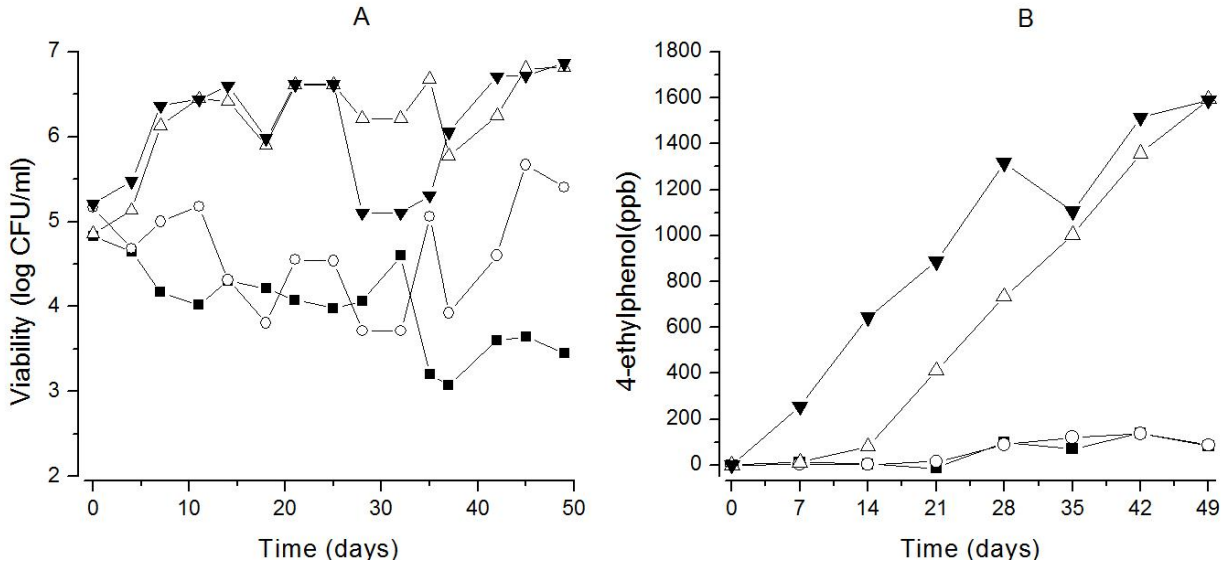


Figure 3.2. Viability of cell gowth (A) and volatile production of TR 26 (B) gown in red wine at 3^{o}C, 10^{o}C, 15 ^{o}C and 20 ^{o}C.

Symbols: ■ 3^{o}C; ○ 10^{o}C; △ 15 ^{o}C, ▼ 20 ^{o}C.

4. Conclusions

Over the last two decades many researchers attempted to improve wine quality, strengthen risk management and decrease the processing cost of winemaking. In addition, the more knowledgeable consumers and the increasing consumer expectation for higher quality products have initiated stricter regulations concerning modern winemaking. This approach brought about trends such as producing lower alcoholic wines and using less sulfur dioxide (SO_2) during the winemaking process. In latter case, the consequent risk lies in the lower levels of free SO_2 that are available to act as antimicrobial agent, especially under high pH conditions. These conditions generate a favorable environment for opportunistic spoilage microbes. The main spoilage microbes that cause great economical losses in the wine industry belong to the non-*Saccharomyces* yeasts and lactic acid bacteria. This dissertation focused on one particular spoilage yeast species that is classified in the non-*Saccharomyces* or wild yeast group. Yeast of the genus of *Brettanomyces* or its telemorph form *Dekkera* have been identified as one of the most controversial spoilage microorganisms in the wine, largely due to their volatile phenol production.

In this study, we studied the incidence of these species in Turkish wines and the effect of temperature on their growth and volatile phenol production.

The results showed a low incidence of *Dekkera bruxellensis* and, therefore, an expected low percentage of wines with high levels of 4-EP. The explanation may be related with the type of wineries that sent samples to analysis because most of them did not use oak barrels during wine aging. In fact, the single sample contaminated had been obtained from a oak barrel. We believe that a wider screening should be done to determine the real incidence of these problems in Turkish wines.

Regarding the influence of storage temperature on yeast growth and 4-EP production we observed that the delay of cell growth in wine induced by low temperature may be used as a tool to prevent the horse sweat taint. In fact, lower 4-EP production rates give more time for the winemaker to use other preventing measures like filtration, pasteurization or sulphur dioxide addition. However, it should be kept in mind that our results showed that relatively low temperatures, like 10ºC, may not be enough to efficiently delay the increase in 4-EP production. In future research, other preventive measures should be studied together with temperature to establish which effective technological alternatives may be advised to winemakers.

5. Bibliography

Aguilar-Uscanga, G. (1998). Caracterisation cinetique et metabolisme d'une souche de *Brettanomyces*. These I. N.P. Toulouse, France

Aguilar-Uscanga, M.G., Delia, M.L. & Strehaiano, P., (2003). *Brettanomyces bruxellensis*: Effect of oxygen on growth and acetic acid production. Appl. Microbiol. Biotechnol. **61**, 157‑162.

Albagnac, G. (1975). La decarboxylation des acides cinnamiques substitues par les levures. Ann. Technol. Agic. **12**:381-387.

Alguacil M., Fidalgo M., Jimenez J., Lozano J.I., Neva M.A. and Perdigones F., (1998) Deteccion de *Brettanomyces/Dekkera* en instalaciones de vendimia mediante PCR. Alimentacion, Equipos y Tecnologia K **10**: 81-85

Arvik, T.J., Conterno, L. & Henick-Kling, T., 2002. *Brettanomyces bruxellensis* in New York State wines: a global issue. In: Henick-Klink, T. (ed). Proc. 31st Annual New York Wine Industry Workshop, April 3-5, Cornell University, New York State, pp. 124-125.

Barata A., Pagliara D., Piccininno T., Tarantino F., Ciardulli W., Malfeito-Ferreira M., Loureiro V., (2008a) The effect of sugar concentration and temperature on growth and volatile phenol production by *Dekkera bruxellensis* in wine. FEMS Yeast Res **8**, 1097–1102

Barata, A., (2002). Detecção, identificação e comportamento de *Dekkera bruxellensis* em vinhos. Relatório de Estágio. Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologia.

Barata, A., Cladeira, J., Botelheiro, R., Pagliara, D., Malfeito-Ferreira, M., Loureiro, V., (2008b). Survival patterns of *Dekkera bruxellensis* in wines and inhibitory effect of sulphur dioxide. International Journal of Food Microbiology, **121**: 201 - 207.

Barbin, P., Cheval J.L., Gilis J.F., Strehaiano P. and Taillandier P. (2008) Yeast *Brettanomyces bruxellensis* is a contaminant found worldwide and is responsible for red wine spoilage due to the development of animal and phenolic off-odours. J. Inst. Brew. **114(1),** 69–75.

Barnett, J. A., Payne, R. W. e Yarrow, D., (2000). Yeasts: characteristics and identification. Cambridge University Press, Cambridge. 3rd ed.

Barrio E., Gonzales S., Arias A., Belloch C. and Querol A. (2006) Molecular Mechanisms Involved in the Adaptive Evolution of Industrial Yeasts. **In** Querol A., Fleet G. (eds) *Yeasts in food and beverages* (pp. 153-174). Berlin: Springer-Verlag.

Beech F.W., Davenport R.R., Mossel D.A.A., Kijkmann K.E., Koopmans M., de Jong J., Put H.M.C., Tilbury R.H. (1980) In: Media and methods for growing yeasts: proceedings of a discussion meeting. Academic Press; biology and Activites of Yeasts. 260-277

Bellon J., Robinson E., Cowey P., Gaves P., Field J., Godden P. and De Barros- Lopes M. (2003) The degree of genetic variation between strains of *Dekkera/Bretanomyces* yeast isolated from different Australian wineries and regions. Bell S.M., de Garis K.A., Dundon C.G., Hamilton R.P., Partridge S.J., Wall G.S., (eds.) Grape growing at the edge; Managing the wine business; Impacts on wine flavour: proceedings of a seminar. 10-11 Jul 2003 Barrosa Convention Center, Tanunda, S.A. Adelaide S.A., Austr. Soci. Of Vitic.&Enol., 51-55.

Beokhout T., Kurtzman C.P., O'donnell K., Smith M.T. (1994) Phylogeny of the yeast genera *Hansienaspora* (anamorph *Kloeckera*), *Dekkera (*anamorph *Brettanomyces),* and *Eeniella* as inferred from 26S ribosomal DNA nucleotide sequences. Int. J. Syst. Bacteriol. **44**:781-786

Bertrand A. (2002) uso del legno nella produsione e nella con servazione dei vini rossi, in: Atti del convego" La gestione rezionale dell'elevage dei vini". Ospedaletto di Pescantina, Verona, April 2002

Blomqvist, J., Eberhard, T., Schnurer, J. e Passoth, V., 2010. Fermentation characyeristics of *Dekkera bruxellensis* strains. Applied Microbial and Cell Physiology, **87**: 1487 - 1497.

Blondin, B., Ratomahenina, R., Arnaud, A., & Galzy, P. (1982). A study of cellobiose fermentation by a *Dekkera* strain. Biotechnology and Bioengineering, 24, 2031–2037.

Boidron, J. N., P. Chatonnet, and M. Pons.(1988) Influence du bois sur certains substances odorantes des vins. Connaiss. Vigne Vin 22:275-294.

Boulton, R.B., Singleton, V., Bisson, L.F. & Kunkee, R., 1996. Principles and Practices of Winemaking. Chapman & Hall Publishers New York, NY.

Brumstead, D. D., A. F. Lauterbach, and D. B. West. (1965) Proceedings of the American Society of Brewing Chemists Annual Meeting St. Louis 142.

Buron N., Coton M., Desmarais C., Ledauphin J., Guichard H., Barillier D., Coton E (2011) Screening of representative cider yeasts and bacteria for volatile phenol-production ability, Food Microbiology **28** : 1243-1251

Calderón F., Morata A., Uthurry C. A., Suárez Lepe, J.A. (2004) A method for estimating *Dekkera/Brettanomyces* populations in wines.Tecnología del vino: tratamientos y equipos para viticultura y enología ,**6:** 49-54

Charoenchai C., Gando M.S., Zini C., (1998) Effects of the temperature, pH and sugar concentration on the growth rates and cells biomass of the wine yeast. Am.J.Eno. Viti. **49**: 283-288

Chatonnet, P., Boidron, J. N., & Dubourdieu, D. (1993). Influence des conditions d'e´levage et de sulfitage des vins rouges en barriques sur leur teneur en acide ace´tique et en ethyl-phenols. Journal International des Sciences de la Vigne et du Vin, **27**, 277–298

Chatonnet, P., Boidron, J., & Pons, M. (1990). Elevage des vins rouges en futs de chêne: évolution de certains composes volatils et de leur impact aromatique. Science des Aliments, 10, 587–656.

Chatonnet, P., D. Dubourdieu, J. N. Boidron, and M. Pons. (1992) The origin of ethylhenols in wines. J. Sci. Food Agic. 60**:65:**178.

Chatonnet, P., Dubourdieu, D. and Boidron, J. N., (1995) The influence of Brettanomyces/Dekkera sp. yeast and lactic acid bacteria on the ethylphenol content of red wines. Am. J. Enol. Viti., , **46**, 463-468.

Chatonnet, P., Dubourdieu, D. and Boidron, J. N., (1997)The influence of Polyphenolic Components of Red Wines on the Microbial Systems of Volitale Phenols. . Am. J. Enol. Viti., , **48**, 443-448.

Chatonnet, P., Dubourdieu, D., & Boidron, J. (1995). The influence of Dekkera/Brettanomyces sp. yeast and lactic acid bacteria on the ethylphenol content of red wines. American Journal of Enology and Viticulture, **46**, 463–468.

Chatonnet, P., Dubourdieu, D., Boidron, J., & Pons, M. (1992). The origin of ethylphenols in wines. Journal of the Science of Food and Agiculture, **60**, 165–178.

Chatonnet, P., J. N. Boidron, and M. Pons. (1990) Elevage des vins rouges en futs de chene: evolution de certains composes volatils et de leur impact aromatique. Sci. Aliment. **10**:565-587

Chattonet P., Boidron J.N., Bons M., (1990) elevade des vin rouges en futs de chene: evolution de certains composes volatils et de leur impact aromatique. Sci. Des Aliments **10** : 565-587

Ciani M. and Ferraro L. (1997) Role of oxygen on acetic acid production by *Brettanomyces/Dekkera* in winemaking. J. Sci. Food Agi. **75**: 489-495

Claussen N.H., (1904) On a method for the application of Hansen's pure yeast system in the manufacturing of a well-conditioned English Stock beer. Jour.Inst. of Brewing **10**:308-331

Clemente- Jimenez J.M., Mingorance-Cozorla L., Martinez-Rodriguez S., Las Heras-Vazquez F.J., and Rodriguez-Vico F. (2005) Influence of sequential yeast mixtures on wine fermentation. Int. J.Food Microbi. **98**: 301-308

Cocolin L., Rantsiou, K., Iacumin L., Zironi R and Comi G. (2004) Molecular detection and identification of *Brettanomyces* / *Dekkera bruxellensis* and *Brettanomyces* / *Dekkera anomalus* in spoiled wines. App. Envirom. Microbio. **70**: 1347-1355

Connel, L., Stender, H., Edwards, C.G., 2002. Rapid detection and identification of Brettanomyces from winery air samples based on peptide nucleic acid analysis. Am. J. Enol. Vitic. **53**, 322– 324.

Conterno L., Joseph C.M. L., Arvik T. J., Henick-Kling T., and Bisson L. F. (2006) *Genetic and Physiological Characterization of Brettanomyces bruxellensis Strains Isolated from Wines.* American Journal of Enology and Viticulture 57(2):139-147.

Correia, P., 2004. Crescimento, viabilidade e produção de 4-etilfenol em mostos de uva e em vinhos por populações de *Pichia guilliermondii*. Relatório do Trabalho de Fim de Curso de Engenharia Agonómica. Universidade Técnica de Lisboa.

Costa A., Barata A., Malfeito-Ferreira M., Loureiro V. (2008) Evaluation of the inhibitory effect of dimethyl dicarbonate (DMDC) against wine microorganisms-Short communication- Food Microbiology **25** 422–427

Coulon, J., Perello, M. C., Lonvaud-Funel, A., Revel, G. E Renouf, V., (2009) Brettanomyces bruxellensis evolution and volatil phenols production in red wines during storage in bottles. Journal of Applied Microbiology, **108**: 1450 -1458.

Couto J.A. , Neves F. , Campos F., Hogg T., (2005) Thermal inactivation of the wine spoilage yeasts Dekkera/Brettanomyces **104 :** 337– 344

Cullere, L., Escudero, A., Cacho, J., & Ferreira, V. (2004). Gas chromatography–olfactometry and chemical quantitative study of the aroma of six premium quality Spanish aged red wines. Journal of Agricultural and Food Chemistry, 52, 1653–1660.

Custers M.T.J. (1940). Onderzeokingen over her Gistseglascht *Brettanomyces*. Tesi, Delft University.

Daudt C.E., Ough C.S. (1980) Action of dimethyldicarbonate on various yeasts. Am.J.Eno. Viti **31**: 21-23

Davenport, R.R., 1974. Microecology of yeasts and yeast like organisms associated with an English vineyard. Vitis **13**, 123– 130.

Deak T., Beuchat L.R., (1996) Handbook of Food Spoilage Yeast. CRC pres, Boca Raton, F.L.

Delfini C., Gaia P., Schellino R., Strano M., Pagliara A. And Ambro S. (2002) Fermentability of grape must after inhibition with dimethyl dicarbonate (DMDC). J.Agic. Food Chem. **50**: 5605-5611

Delia M.L., Phowchinda O. Alfenore S., Strehaiano P.(1997) *Brettanomyces* as a polluting yeast in alcoholic fermentations: study of its relations to *Saccharomyce.* International Specialized Symposi. On Yeast. 24-29,Bled, Slovenia

Dias L., Pereira-Da-Silva S., Tavares M., Malfeito-Ferreira M., Loureiro V., (2003a), Factors affecting the production of 4-ethylphenol by the yeast *Dekkera bruxellensis* in enological conditions. Food Microbi. **20**: 377-384

Dias, L., Dias, S., Sancho, T., Stender, H., Querol, A., Malfeito-Ferreira, M., et al. (2003). Identification of yeasts isolated from wine-related environments and capable of producing 4 ethylphenol. Food Microbiology, **20**, 567–574.

Du Toit, W. J., Pretorius, I. S. e Lonvaud-Funel, A., 2005. The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces brixellensis* isolated from wine. Journal of Applied Microbiology, **98**: 862- 871.

Dubois, P., G. Brule, and M. Illic. (1971) Etude des phenols volatils de deux vins rouges. Ann. Technoc. Agic. 20:131-139

Edlin, D. A. N., Narbad, A., Gasson, M. J., Dickinson, J. R., & Lloyd, D.(1998). Purification and characterization of hydroxycinnamate decarboxylase from Brettanomyces anomalus. Enzyme Microbial Technology, 22, 232–239.

Egli C.M., Henick-Kling T., (2001) Identification of *Brettanomyces/Dekkera* species based on polymorphism in the rRNA internal transcribed spacer. Am. J. Eno. Viti. **52**: 241-247

Esteve-Zarzoso B., Peris-Toran M.J., Garcia-Maiquez E., Uruburu F., Querol A. (2001) Yeast population Dynamics during the fermentation and biological ageing of sherry wines. Appl. Environ. Microbi.**67**: 2056-2061

Esti M. and Tamborra P. (2006) Influence of winemaking techniques on aroma precursors, Analytica Chimica Acta **563**: 173–179

Etievant, P. X. (1981) Volatile phenols determination in wine. J. Agic. Food Chem. 29:65-67.

Etievant, P. X., S. Issanchou, S. Marie, V. Ducruet, and C. Flanzy. (1989) Sensory impact of volatile phenols on red wine aroma: influence of carbonic maceration and time of storage. Sci. Aliment. **9**:19-33.

Ferrarini R., Zironi R., Celotti E., D'andrea E., (2001) Ruolo dell'ossigeno nei processi di vinificazione ed affinamento dei vini. L'enologo, Anno XXXVII, **11** :65-72

Freer S.N. (2002) Acetic acid production by Dekkera/Brettanomyces yeasts. World Journal of Microbiology & Biotechnology 18: 271–275, 2002.

Freer, S. N., Dien, B. S., Matsuda, S. & Rothast, R. J. (2000). Acetic acid production by *Brettanomyces* yeast. Abstracts of the General Meeting of the American Society for Microbiology (vol. 100, p. 503).

Freer, S. N., Dien, B., & Matsuda, S. (2003). Production of acetic acid by *Dekkera/Brettanomyces* yeasts under conditions of constant pH. World Journal of Microbiology and Biotechnology, 19, 101–105.

Froudiere, I., Larue, F., 1988. Condition de survie de Brettanomyces (Dekkera) dans le moût de raisin et le vin. Connaisance de la Vigne et du Vin, **2**: 296 - 303.

Fugelsang, K. C. (1997), Yeast and molds. In: Wine microbiology. Chapman & Hall: New York, pp.9-11,87-88,164-166. ISBN-13: 978-0-387-33341-0

Fugelsang, K. C., & Zoecklein, B. W. (2003). Population dynamics and effects of Brettanomyces bruxellensis strains on Pinot Noir (Vitis vinifera L.) wines. American Journal of Enology and Viticulture, 54, 294–300.

Fugelsang, K. C., Osborn, M. M. e Muller, C. J., 1993. *Brettanomyces* and *Dekkera*. Implications in winemaking in beer and wine production. B. H. Gump, **536**:110 - 129.
Gadaga, T.H., Mutukumira, A.N. & Narvhus, J.A., 2002. Identification of yeasts isolated from Zimbabwean traditional fermented milk. Int. Diary J. **10**, 459-466.

Gaia P. (1987) Caratterizzazioone tassonomica ed enologica di lieviti anomali fonte di inqunamento dei vini. Estratto da Rivista di viticoltura ed enologia di Conegliano. Annali dell istituto sperimentale per l'enologia di Asti. Vol XVIII, Publi.no 711.

Gaunt D.M., Degn H., Lloyd D. (1988) The influence of oxygen and organic hydrogen acceptors on glycolitic carbon dioxide production in (1988) The influence of oxygen and organic hydrogen acceptors on glycolitic carbon dioxide production in *Brettanomyces anomalus,* Yeast **4**:249-255

Geros H., Azevedo M., Cassio F., (2000) Biochemical studies on the production of acetic acid by the yeast *Dekkera anamola*. Food. Tech. Biotechnol. **38**: 59-62

Gilis J.F., Seiller I., Delia M.L. (1999) Effect de certains parameters physico-chemiques (pH e O_2 sur la cinetique de croissance de *Brettanomyces*) 6eme Symp. Int. Oenol. Bordeaux Tec.&Doc. Pp 244-267

Gilliland R.B., (1961) The *Brettanomyces* I : Occurrence, characteristics and effect on beer flavour. . Jour.Inst. of Brewing **67**:257-261

Guerzoni, E., Marchetti, R., 1987. Analysis of yeast flora associated with grape sour rot and of the chemical disease markers. Appl. Environ. Microbiol. 53, 571– 576.

Harris V. (2008) Physiological, Biochemical and Molecular characterisation of hydroxycinnamic acid catabolism by *Dekkera* and *Brettanomyces* yeast. PhD thesis, University of Adelaide.

Henschke, P., Curtin, C. e Girbin, P., 2007. Molecular characterization of the wine spoilage yeast – *Dekkera (Brettanomyces) bruxellensis*. Microbiology Australia, **28**: 76,78.

Heresztyn T. (1986) Formation of substituted tetrahdropyridines by species of *Brettanomyces* and *Lactobacillus* isolated from mousy wines. Am. J. Eno. Viti. **3**: 127-132

Heresztyn, T., 1986. Metabolism of phenolic compounds from hydroxycinnamic acids by *Brettanomyces* yeasts. Arch. Microbiol. 146, 96-98.

Jensen, S. L., Umiker, N. L., Arneborg, N., Edwards, C. G., 2009. Identification and characterization of *Dekkera bruxellensis*, *Candida pararugosa*, and *Pichia guilliermondii* isolated from commercial red wines. Food Microbiology, **26**: 915 - 921.

Jolly N.P., Augustyn O.P.H. and Pretorius I.S. (2003) The occurance of non-*Saccharomyces cerevisiae* yeast species over theree vintages in four vineyards and grape musts from production regions of the western cape, South Africa. S. Afr.J.Enol. Viti. **24**: 35-42

Jong, S. C., Lee, F.-L., & Bengston, L. (1985). Direct evidence of relationship between Dekkera and Brettanomyces. Mycotaxon, **23,** 271–273 cit in Springer Compendium Of The Microbiological Spoilage Of Foods And Beverages Oct. (2009) page 253

Joseph, C.M.L. & Bisson, L., 2004. Physiological diversity of *Brettanomyces/Dekkera* isolated from wine. In: Technical Abstracts, 55th Annual Meeting, San Diego, California, Am. Soc. Enol. Vitic., Davis, CA, p. 28.

Kelly, J. (2003). What is Brettanomyces and why are so many winemakers concerned about it? Australian and New Zealand Grape grower and Winemaker, 474, 54–57.

Kolfschten G.A. and Yarrow D. (1970) *Brettanomyces naardensis* a new yeast from soft drinks. Antonie van Leewenhoek **36**: 458-460

Kosse, D., Seiler, H., Amann, R., Ludwig, W. & Scherer, S., 1997. Identification of yogurt-spoiling yeasts with 18S rRNA-targeted oligonucleotide probes. Sys. Appl. Microbiol. **20**, 468-480.

Kotzekidou, P., 1997. Identification of yeasts from black olives in rapid systems microtitre plates. Food Microbiol. 14, 609-616.

Krumbholz, G. & Tauschanoff, W., 1933. Mycotorula intermedia n. sp., ein Beitragzur Kenntnis der Gärungserreger im Wein. Zentralblatt für Bakteriologie. **88,**366-373.

Kumara, H. & Verachtert, H., 1991. Identification of lambic superattenuating microorganisms by the use of selective antibiotics. J. Inst. Brew. **97**, 181-185.

Kurtzman C.P., Fell J.W. (1998). The yeast: a taxonomic study. Amsterdam, Fourth Edition. Pg. 114,118,174-177,450-453.

Kurtzman, C.P. & Fell, J.W., 2000. (4th ed. revised). The yeasts. A taxonomic study. Elsevier Science Publisher BV, Amsterdam, The Netherlands

.

Lachance M.A. (1995) Yeast communities in a natural tequila fermentation. Antone van Leeuwenhoek **68**: 151-160

Larue F., Rozes N., Froudiere I., Couty C. and Perreira G.P. (1991) Incidence du developpment de *Dekkera/Brettanomyces* dans les mouts et les vins. J. Int. Sci. Vigne Vin. **25**: 149-165

Larue, F., Rozes, N., Froudiere, I., Couty, C., & Perreira, G. P. (1991). Influence of development of *Dekkera/Brettanomyces* in wine musts. Connaisance de la Vigne et du Vin, 25, 149–165.

Licker J.l., Acree T.E., Henick-Kling T.E. (1997). What is "Brett" Brettanomyces flavour? A preliminary investigation. Am. Chem. Soc. Symp., 213th National Meeting San Francisco, C.A.,Published: Chemistry of wine flavour. A.L. Wathouse and S.E. Ebeler, eds. ACS Symposium Series, 1998, **714**,96-115

Lisant M. T., Piombino P., Gambuti A., Genovese A., Siani V.L. and Moio L. (2008*).* Analytical evaluation of remedial treatments for red and white wines contaminated by volatile phenols. Bulletin de l'OIV 81(923-925):45-55

Lonvaud-Funel A., Martins G., Miot-Sertier C., Lauga B., Claisse O, Soulas G, Masneuf-Pomarède I. (2012) Grape berry bacterial microbiota: Impact of the ripening process and the farming system International Journal of Food Microbiology **158**: 93–100

Loureiro V. and Malfeito-Ferreira M. (2006) Dekkera/Brettanomyces spp.. **In** Blackburn C. (ed.) Food spoilage microorganisms pg:353-398. Cambridge: Woodhead Publishing Limited. Woodhead ISBN-13-978-1-85573-966-6

Lynd, L. R., Weimer, P. J., Van Zyl, W. H. e Pretorius I. S., 2002. Microbial cellulose utilization: fundamentals and biotechnology. American Society for Microbiology, **66**: 506 - 577.

Malfeito-Ferreira M, (2010) Yeasts and wine off-flavours: a technological perspective, Ann Microbiol **61**:1:95-102

Malfeito-Ferreira M., Barata A., Loureiro V. (2012) The microbial ecology of wine grape berries International Journal of Food Microbiology **153** :243–259

Malfeito-Ferreira, M., Rodrigues, N., Loureiro, V., (2001). The influence of oxygen on the ''horse sweat taint'' in red wines. Ital. Food Bev. Technol. **24**, 34–38.

Mansfield, A. K., Zoecklein, B. W., & Whiton, R. S. (2002). Quantification of glycosidase activity in selected strains of *Brettanomyces bruxellensis* and *Oenococcus oeni*. American Journal of Enology and Viticulture, 53, 303–307.

Martorell P., Barata A., Malfeito-Ferreira M., Fernandez-Espinar M.T., Loureiro V.,and Querol A. (2006) Molecular typing of the yeast species *Dekkera bruxellensis* and *Pichia guillermondii* recovered from wine related sources. Int. Food. Microb. **106**: 79-84

Massini, L., Pereira-Lopes, F., Barnavon, L., Riou, C., Vuchot, P., (s/d). Etude physiologique de souches de Brettanomyces dans les vins de la vallée du Rhône. Retirado em 15 Junho, 2010, de www.institut-rhodanien.com/download/785.

Miniac, M., 1989. Contamination des fermentation alcooliques industrielles par les levures du genre *Brettanomyces*. Industries Alimentaires et Agicoles, **106**: 559 - 563

Morata A.; Benito S., Gonzalez M.C., Palomero F., Tesfaye W., Suarez-Lepe J.A. (2012) Cold Pasteurisation of red wines with high hydrostatic pressure to control *Dekkera/Brettanomyces* : effect on both aromatic and chormatic quality of wine. Eur.Food.Res. Technol. Vol.235, Number 1 (2012), 147-154.

Morrissey W.F., Davenport B. Querol A. and Dobson A.D. (2004) Role of indigenous yeasts in traditional Irish cider fermentations. J.Appl. Microbio. **97**: 647-655

Oelofse A, Pretorius I.S. and du Toit M., Significance of *Brettanomyces* and *Dekkera* during Winemaking: A Synoptic Review (2008) **S. Afr. J. Enol. Vitic., 29-2,** 128-144

Parish, M., Kelly, M., & Baldwin, G. (2003). Managing Brett. Australian and New Zealand Wine Industry Journal, 18, 12–15.

Phaff, H.J., Miller, M.W. & Mrak, E.M. 1978 (2nd ed). The Life of Yeasts. Harvard University Press, Cambridge, MA, p. 341.

Phister T.G., Mills D.A., (2003) Real-time PCR assay for detection and enumeration of *Dekkera bruxellensis* in wine. Appl.Envir. Microbio. **69**: 7430-7434

Physter, T. G., & Mills, D. A. (2004). Novel methods to detect *Brettanomyces* (*Dekkera*) in wine. In Technical abstracts, 55th annual meeting, San Diego, CA (p. 30). Davis, CA: American Society for Enology and Viticulture.

Pollnitz, A.P., Pardon, K.H. & Sefton, M.A., 2000. 4-Ethylphenol, 4-ethylguaiacol and oak lactones in Australian red wines. Aus. Grapegrow. Winemaker 438, 45-52.

Prakitchaiwattana C., Fleet G.H. and Heard G.M. (2004) Application and evaluation of denaturing gradient gel elecrophoresis to analyse the yeast ecology of wine grapes. Fems Yeast Res. **4**: 865-877

Pretorius, I.S., Van der Westhuizen, T., Augustyn, O., Khan, W. , 2000. Geographical distribution of indigenous *Saccharomyces cerevisiae* strains from vineyard in the coastal regional of the Western Cape in South Africa. S. Afr. J. Enol. Vitic. 21, 3-9.

Querol, A., Jiménez, M. & Huerta, T., (1990). Microbiological and enological parameters during fermentation of musts from poor and normal grape harvests in the region of Alicante, Spain. J. Food Sci. **55**, 1603-1606.

Ravelomanano R., Guiraud J., Vİncent J. and Glazy P. (1985). The yeast flora of cocoa bean fermentation in Ivory Cost. World Journal of Micbio.&Biotech. **1:** 319-326

Rayne, S. And Eggers, N.J. (2007) 4-Ethylphenol and 4-Ethylphenol in wines: Estimating non-microbial sourced contributions and toxicological considerations. Journal of Enviromental Science and Health- Part B, Food Contaminants and Agricultural Wastes, **42** (8), pp.887-897.

Reguant, C., Bordons, A., Arola, L., Rozes, N. (2000) Influence of phenolic compounds on the physiology of Oenococcus oeni from wine. Journal Applied Microbiology, **88**: 1065 - 1071.

Renouf V, Lonvaud-Funel A & Coulon J (2007) The origin of *Brettanomyces bruxellensis* in wines: a review. J Int Sci Vigne Vin **41**: 161–173.

Renouf, V., Strehaiano P., Lonvaud-Funel A., (2008). Effectiveness of dimethlydicarbonate to prevent Brettanomyces bruxellensis growth in wine. Food Control, **19**: 208 - 216.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche. B. e Lonvaud, A. 2006. Handbook of enology *The Microbiology of Wine and Vinifications*. John Wiley & Sons, Ltd. Vol 1.

Rodrigues, N., 1998. Leveduras do género *Dekkera*: despiste rápido em vinhos e capacidade de produção de 4-etilfenol. Relatório do Trabalho de Fim de Curso de Engenharia Ago-Industrial. Universidade Técnica de Lisboa.

Rodrigues, N., Gonçalves, G., Pereira-da-Silva, S., Malfeito-Ferreira, M.,& Loureiro, V. (2001 a). oxygen requirements of the food spoilage yeast oxygen requirements of the food spoilage yeast *Zygosaccharomyces baili*. In synthetic and comples media. Appl. Environ Microbio. **67** :2123-2128

Rodrigues, N., Gonçalves, G., Pereira-da-Silva, S., Malfeito-Ferreira, M.,& Loureiro, V. (2001 b). Development and use of a new medium to detect yeast of the genera Dekkera/ Brettanomyces sp. Journal of Applied Microbiology, 90, 588–599.

Rossini, R. (2003) Contaminazione da *Brettanomyces*: lo stato dell'arte,11,17-19,27, 39-49.

Scheffers, W. A., & Wiken, T. O. (1969). The Custer effect (negative Pasteur effect) as a diagnostic criterion for the genus *Brettanomyces.* Antonie van Leeuwenhoek. Supplement: Yeast Symposium, 35, 31–32.

Silva P., Cardoso H., Geros H. (2004) Studies on the wine spoilage capacity of *Brettanomyces/Dekkera* spp. Am.J.Eno.Vit. **55**: 1, 65-72

Silva, S., 1998. Leveduras do género Dekkera: incidência em vinhos portugueses e produção de etil- enol. Relatório do Trabalho de Fim de Curso de Engenharia Ago-Industrial. Universidade Técnica de Lisboa.

Smith M.T. and van Ginsven A.M. (1984) *Dekkera anomala* sp-nov. the telemorph of *Brettanomyces anomala* recoverd from spoiled soft drinks. Antonie van Leewenhoek **50:** 143-148

Smith, M. T., Yamazahi, M. e Poot, G. A., 1990. *Dekkera, Brettanomyces* and *Eeniella*: electrophoretic comparison of enzymes and DNA homology. Yeasts, **6**, 299-310.

Sponholz, W. R., 1992. Wine spoilage by microrganisms. In: wine microbiology and Biotechnology. Ed. G. H. Fleet, Harwood Academic Publishers. pp. 395 - 420.

Steels H., James S.A., Roberts I.N., Stratford M., (2000) sorbic acid resistance : the inoculum effect. Yeast **16** :1173-1183

Steinke R.D., Paulson M.C. (1964) The production of steam volatile phenols during the cooking and alcoholic fermentation of gain. Ag.Food Chem. **12**: 381-387

Stender H., Kurtzman C., Hyldig-Nielsen J.J., sorensen D., Broomer A., Oliveira K., Perry-O'Keefe H., Sage A., Young B., Coull J., (2001) Identification of *Dekkera bruxellensis* (Brettanomyces) from wine by fluoresence *in situ* hybridization using peptide nucletic acid probes. App.Envirm. Microb. **67**:938-941

Suárez R., Suárez-Lepe J. A., Morata A., and Calderón F. (2007) *The production of ethylphenols in wine by yeasts of the genera Brettanomyces and Dekkera: A review.* Food Chemistry 102(**1**):10-21.

Suarez, J. A., & Inigo, B. (2004). El genero Brettanomyces. In Microbiologia Enologica. Fundamentos de Vinificacio´ n (p. 422–424).Madrid: Ed. Mundiprensa.

Suarez-Lepe, J. A. (2001). Microbial spoilage of wine. From Pasteur to the 21st century. Semana Vitivinicola, 56, 6–16.

Swaffield C.H., Scoot J.A., Jarvis B. (1997) Observations on microbial ecology of traditional alcoholic cider storage vats. Food Microbi. **14**: 353-361

Swaffield, C.H. & Scott, J.A., 1995. Existence and development of natural microbial populations in wooden storage vats used for alcoholic cider maturation. J. Am. Soc. Brew. Chem.**53**, 117-120.

Taillandier, P., 2007. Humidité et azote, des facteurs aggravants. La Vigne. No. 189 de Juillet-Aout, p. 50.

Teoh, A.L., Heard, G. & Cox, J., 2004. Yeast ecology of Kombucha fermentation.Int. J. Food Microbiol. **95**, 119-126

.

Thurston, P. A., and R. S. Tubb. (1981) Screening yeast strains fortheir ability to produce phenolic off flavours. J. Inst. brew. **87**:177-179.

Turkey wine grape production map, Wines of Turkey, 2010, www.winesofturkey.org

Ugarte P, Agosin E., Bordeu E. and Villalobos J. (2005). Reduction of 4-ethylphenol and 4 ethylguaiacol in red wines using reverse osmosis and adsorption. American Journal of Enology and Viticulture **56**:30-36.

Van der Walt J.P. (1984). *Dekkera* van der Walt. In: N.J.W. Kreger-van Rij (ed.) The yeast, A taxonomic study, 3rd edition. Elsevier, Amsterdam, pp.146-150.

Van der Walt J.P. , van kekren A.E. (1961). Antonie van Leeuwenhoek, **27**:81-90

Van der Walt, J.P. & Van Kerken, A.E., 1958. The wine yeast of the Cape. Part I.A taxonomical survey of the yeasts causing turbidity in South African table wines. Antonie van Leeuwenhoek. **24***,* 239-252.

Viljoen B.C., Knox A.M., De Jager P.H. and Lourens-Hattingh A. (2003) Development of yeast populations during processing and ripening of blue veined cheese. Food Tech. & Biotech. **41**: 291-297

Wedral, D., Shewfelt, R. Frank, J., (2010) The challenge of Brettanomyces in wine. LWT- Food Science and Technology. **43**: 1474 -1479.

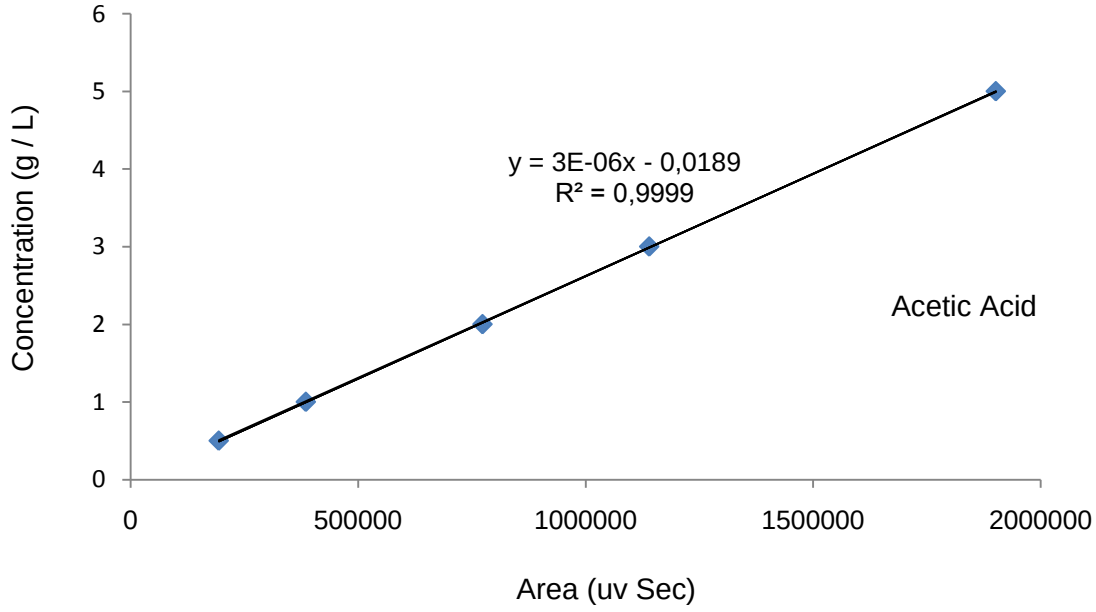
Welthagen J.J., Viljoen B.C. (1999) The isolation and identification of yeast obtained during the manufacture and ripening of Cheedar cheese. Food Microbio. **16**: 63-73

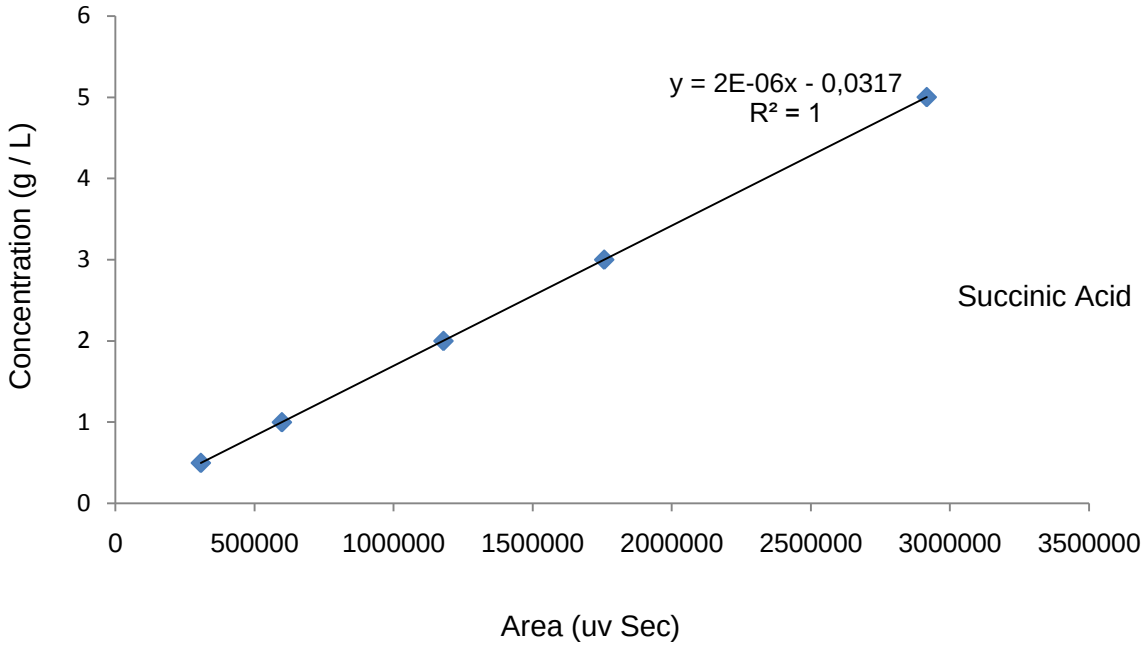
Wijsman M.R., van Dijken J.P., Bastiaan H.A. (1984) Inhibition of fermentation and growth in batch cultures of the yeast *Brettanomyces intermedius* upon a shift from aerobic to anaerobic conditions (Cluster effect) Antonie van Leeuwenhoek **50**: 183-192

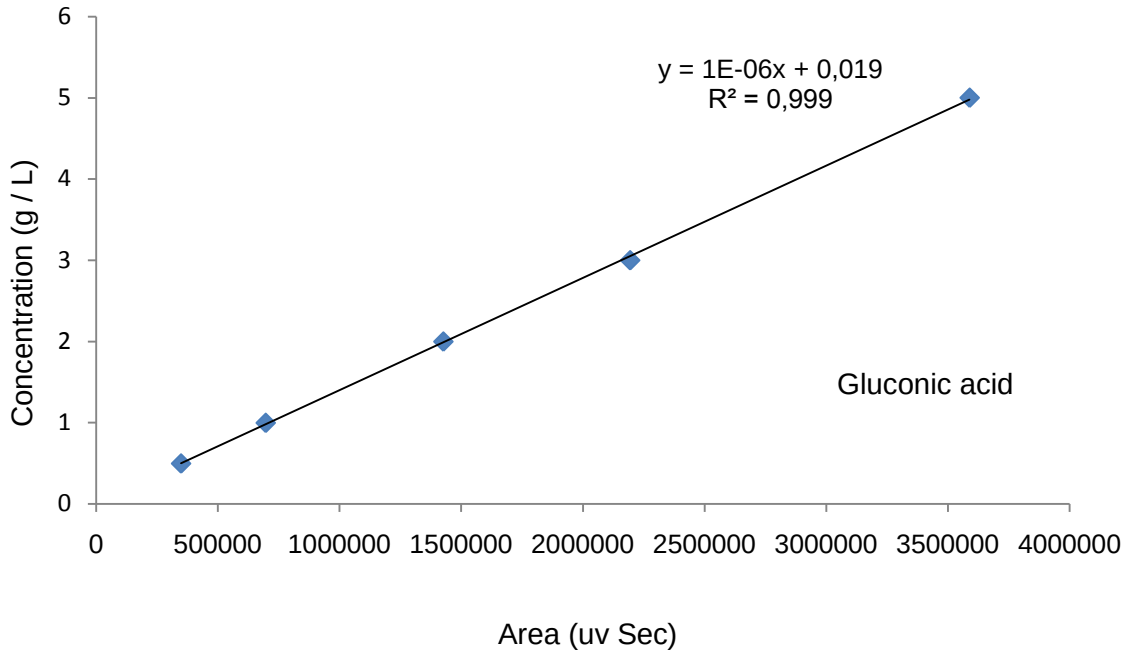
Wright J. and Parle I. (1974) *Brettanomyces* in New Zealand wine industry. New Zealand J.Agi. Res. **17**: 273-278

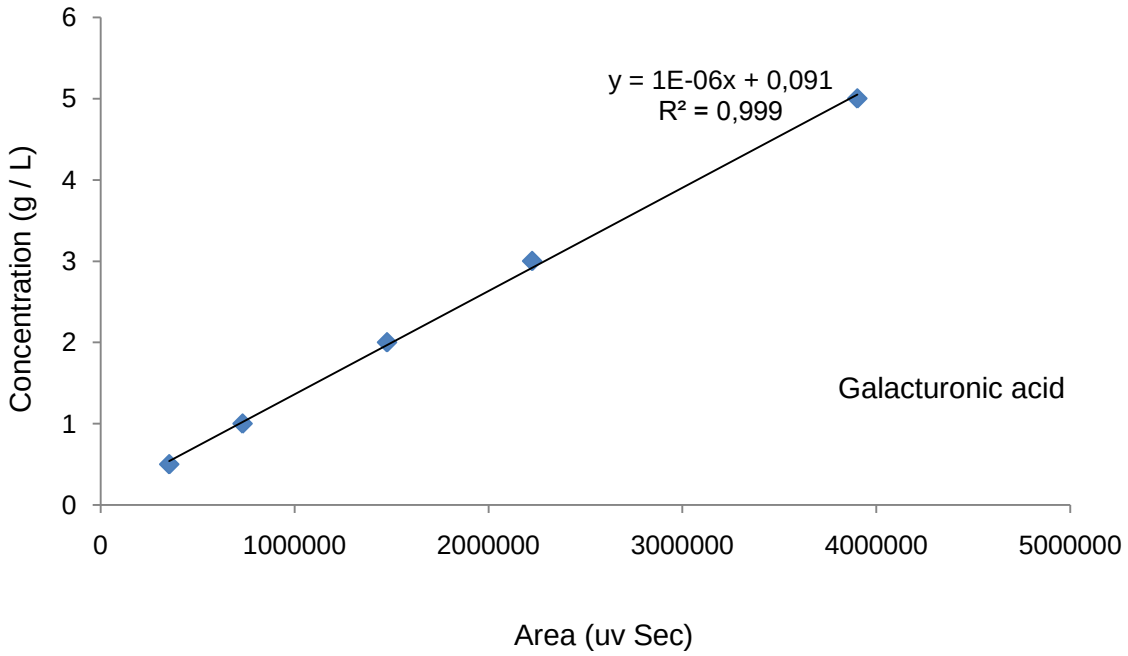
Yap, A., Jiranek, V., Grbin, P., Barnes, M. & Bates, D., 2007. Studies on the application of high-power ultrasonics for barrel and plank cleaning and disinfection. Aus. Wine Ind. J. 22, 96-104.

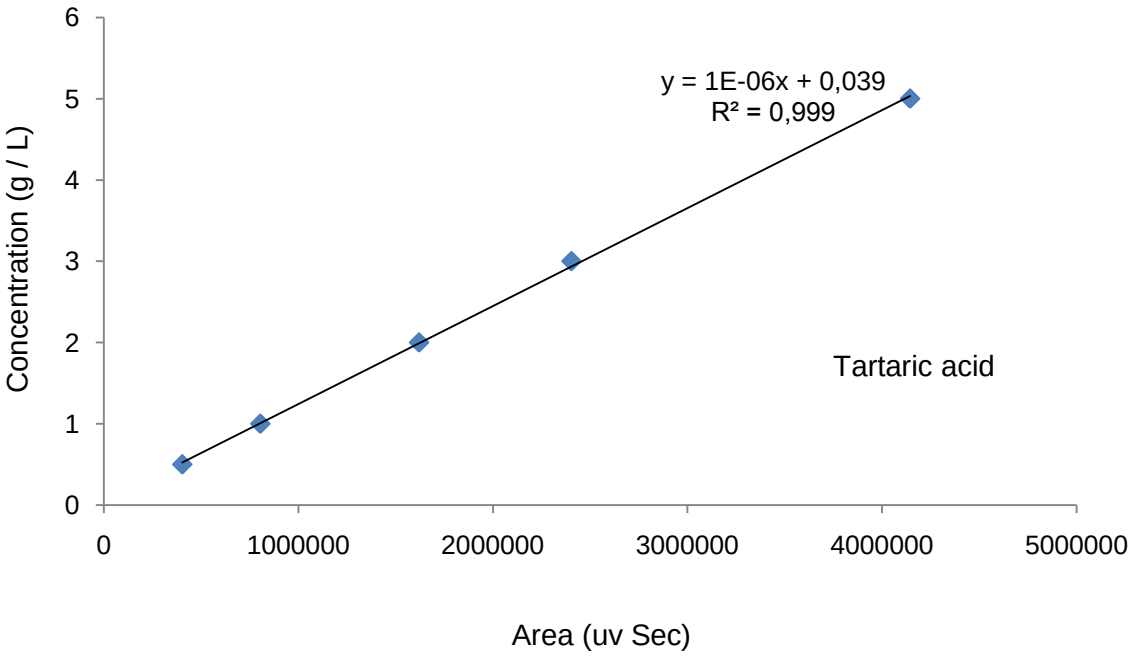
Appendix 1 . Calibration curves for the HPLC determination of concentrations of compounds, acetic acid, tartaric acid, gluconic acid, galacturonic acid, fructose, succinic acid, lactic acid, glycerol, and ethanol.

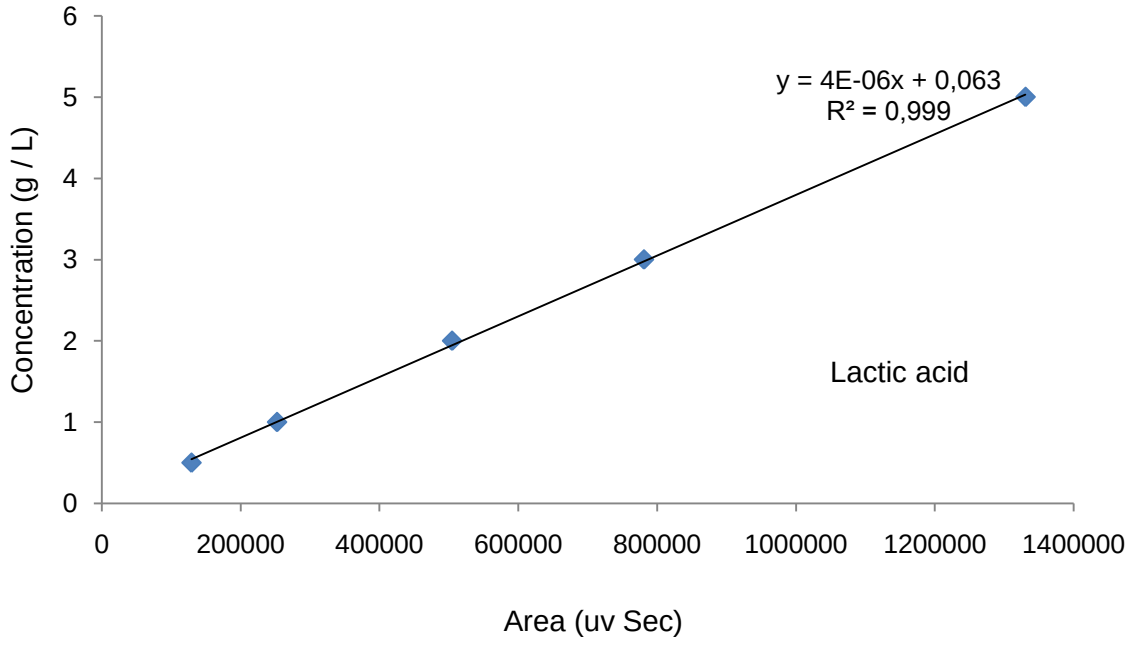


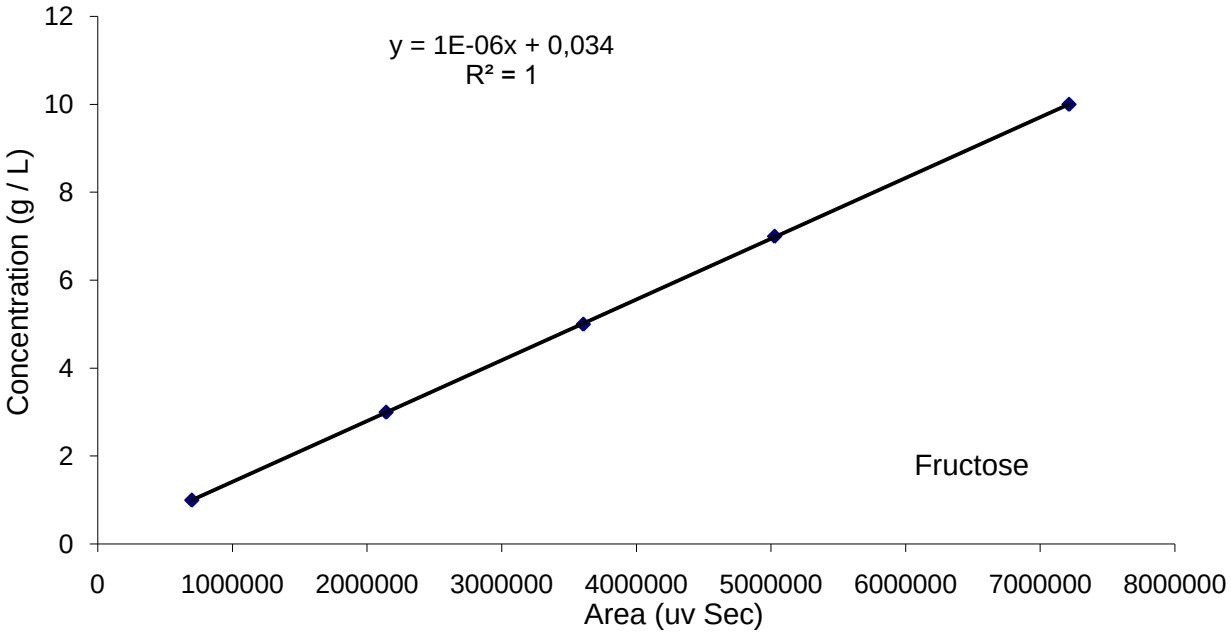


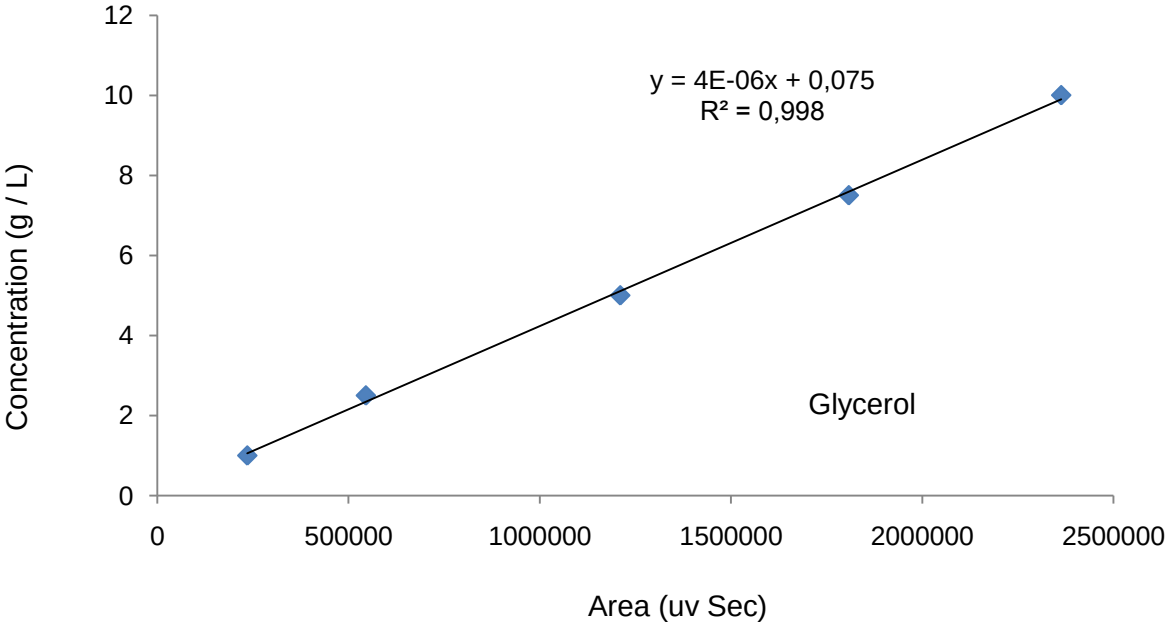

y = 1E-06x + 0,019
R² = 0,999
Gluconic acid
Concentration (g / L)
Area (uv Sec)
0
1
2
3
4
5
6
0
500000
1000000
1500000
2000000
2500000
3000000
3500000
4000000

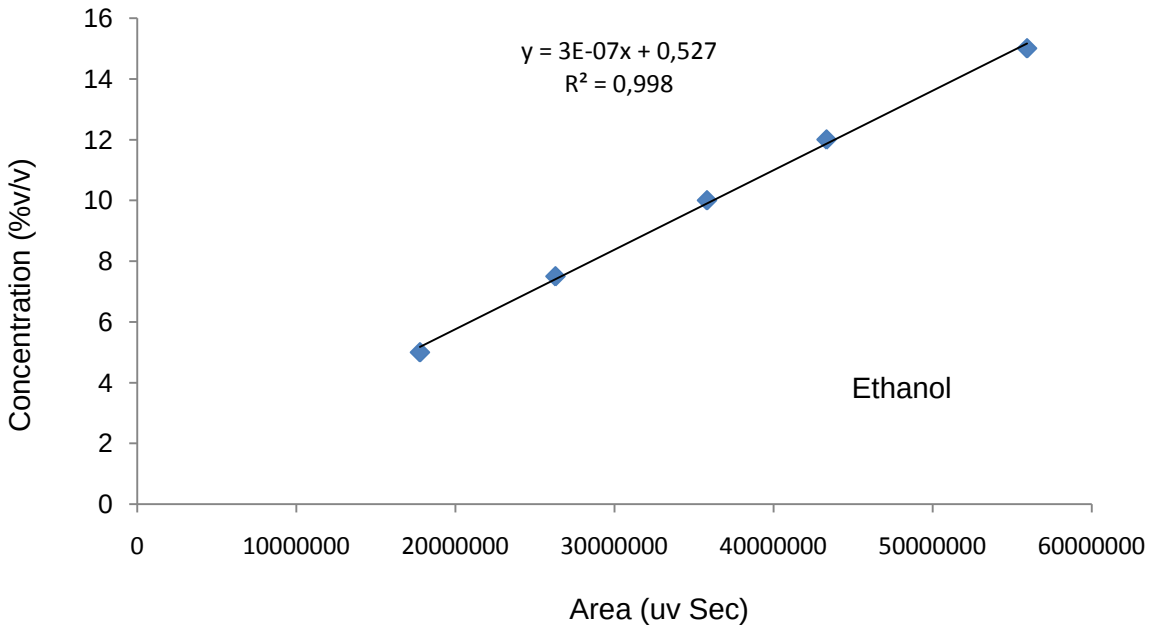

y = 1E-06x + 0,091
R² = 0,999
Galacturonic acid
Concentration (g / L)
Area (uv Sec)
0
1
2
3
4
5
6
0
1000000
2000000
3000000
4000000
5000000


y = 1E-06x + 0,039
R² = 0,999
Tartaric acid
Concentration (g / L)
Area (uv Sec)
0
1
2
3
4
5
6
0
1000000
2000000
3000000
4000000
5000000


y = 4E-06x + 0,063
R² = 0,999
Lactic acid
Concentration (g / L)
Area (uv Sec)
0
1
2
3
4
5
6
0
200000
400000
600000
800000
1000000
1200000
1400000


y = 1E-06x + 0,034
R² = 1
Fructose
Concentration (g / L)
Area (uv Sec)
0
2
4
6
8
10
12
0
1000000
2000000
3000000
4000000
5000000
6000000
7000000
8000000

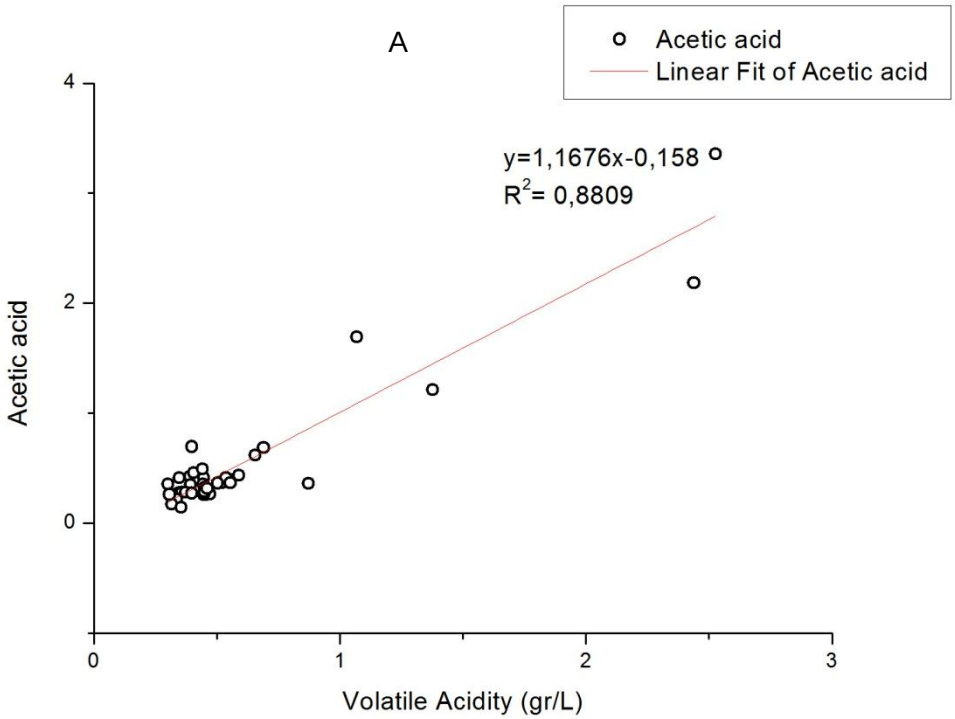

y = 4E-06x + 0,075
R² = 0,998
Glycerol
Concentration (g / L)
Area (uv Sec)
0
2
4
6
8
10
12
0
500000
1000000
1500000
2000000
2500000


y = 3E-07x + 0,527
R² = 0,998
Ethanol
Concentration (%v/v)
Area (uv Sec)
16
14
12
10
8
6
4
2
0
0
10000000
20000000
30000000
40000000
50000000
60000000

Wine Samples	Conventional Analysis						HPLC							
	EtOH (%)	Sugar (gr/L)	pH Values	Volatile Acidity (gr/L)	Total Acidity (gr/L)	Fixed Acidity (gr/L)	Gluconic Acid	Galacturonic Acid	Succinic Acid	Glycerol	Acetic Acid	Tartaric Acid	Fructose	Lactic Acid
TR 1	--	--	3,63	0,69	--	--	3,3	1,13	1,67	27,20	0,69	–	--	–
TR 2	--	--	3,35	1,07	--	--	2,3	0,61	1,16	22,20	1,69	0,07	--	–
TR 3	13,70%	2,2	3,75	0,87	5,03	3,94	2,8	0,90	2,25	30,11	0,36	0,38	--	–
TR 4	9,40%	0,8	3,44	2,53	6,99	3,83	2,1	0,83	1,42	22,28	3,36	–	0,83	–
TR 5	11,00%	1,0	3,4	2,44	6,90	3,85	2,2	0,63	0,96	21,15	2,19	–	0,45	–
TR 6	11,00%	1,2	3,35	1,38	5,78	4,06	2,3	0,61	0,99	21,46	1,21	–	0,41	–
TR 7	13,75%	1,3	3,59	0,45	4,91	4,35	2,8	0,97	1,45	26,82	0,26	–	0,90	–
TR 8	13,74%	2,9	3,66	0,39	5,38	4,89	3,8	1,10	1,89	30,04	0,43	–	--	–
TR 9	2,30%	1,9	3,65	0,55	4,64	3,95	2,6	0,54	0,99	22,44	0,40	0,15	--	–
TR 10	13,45%	0,9	3,64	0,43	3,89	3,35	2,0	1,07	1,35	22,24	0,39	–	--	–
TR 11	15,50%	0,7	3,67	0,35	3,94	3,51	1,4	0,37	0,97	19,59	0,27	–	--	–
TR 12	15,40%	0,5	3,6	0,41	4,69	4,19	2,4	1,40	1,74	25,12	0,46	0,25	--	–
TR 13	15,90%	0,6	3,48	0,45	4,39	3,83	1,8	1,71	1,37	20,61	0,41		--	–
TR 14	14,10%	1,3	3,17	0,42	5,95	5,42	2,7	0,80	1,19	20,39	0,32	0,13	--	–
TR 15	8,50%	0,6	3,45	0,47	4,25	3,66	2,1	0,87	1,37	20,55	0,26	0,12	--	–
TR 16	13,50%	2,6	3,55	0,52	4,81	4,16	2,6	1,45	1,15	22,12	0,36		--	–
TR 17	15,50%	0,9	3,52	0,35	4,83	4,39	2,4	0,84	1,95	23,35	0,41	0,28	1,23	–
TR 18	12,80%	1,2	3,49	0,30	4,64	4,27	2,3	0,81	1,36	22,25	0,36	–	0,86	–
TR 19	13,50%	0,1	3,54	0,41	4,38	3,87	1,8	0,79	1,32	21,64	0,30	–	--	–
TR 20	8,90%	0,2	3,32	0,36	5,74	5,29	3,1	1,31	1,09	16,86	0,28	–	--	–
TR 21	12,60%	0,6	3,67	0,39	5,15	4,66	1,7	1,63	2,73	26,23	0,35	3,21	--	–
TR 22	7,30%	0,9	3,67	0,45	5,06	4,50	--	1,43	2,50	23,19	0,26	2,37	--	3,29
TR 23	7,20%	0,1	3,66	0,34	4,94	4,52	--	1,48	2,51	24,02	0,22	3,24	0,20	3,11
TR 24	8,90%	0,7	3,55	0,32	5,38	4,98	1,6	1,32	--	22,82	0,17	2,84	0,22	3,07
TR 25	12,50%	0,8	3,51	0,36	5,06	4,62	0,2	0,53	1,70	23,24	0,15	1,78	--	2,23
TR 26	12,70%	1,8	3,44	0,66	4,93	4,11	0,6	2,38	1,89	26,51	0,62	2,44	--	3,09
TR 27	6,60%	1,5	3,22	0,31	6,83	6,45	0,3	1,16	1,93	20,83	0,26	2,01	--	1,31
TR 28	5,90%	1,8	3,68	0,44	4,56	4,01	0,6	2,55	--	22,08	0,36	3,06	--	1,75
TR 29	11,10%	2,1	3,67	0,45	3,83	3,26	1,0	0,92	2,18	27,60	0,33	3,11	--	3,07
TR 30	10,40%	0,3	3,54	0,40	5,06	4,56	0,5	2,06	2,24	21,73	0,70	3,27	--	2,51
TR 31	12,10%	1,1	3,81	0,45	4,04	3,47	0,2	1,04	1,28	20,60	0,27	2,87	--	1,35
TR 32	11,70%	2,2	3,53	0,54	5,02	4,35	0,5	1,72	1,56	20,73	0,41	2,72	--	1,68
TR 33	5,00%	0,8	3,67	0,50	3,96	3,33	--	1,71	1,95	22,41	0,36	2,83	--	3,64
TR 34	11,90%	0,9	3,66	0,37	4,32	3,85	--	1,72	1,54	19,36	0,28	2,76	--	3,43
TR 35	11,80%	0,8	3,41	0,40	5,48	4,98	--	0,86	1,71	19,40	0,27	3,16	--	2,76
TR 36	12,90%	1,7	3,5	0,44	4,61	4,06	0,8	2,15	1,78	25,94	0,49	2,57	--	1,93
TR 37	13,90%	2,5	3,53	0,59	4,91	4,18	0,9	1,24	1,71	26,90	0,44	2,98	--	1,73
TR 38	13,90%	1,1	3,67	0,56	4,38	3,69	0,7	1,02	1,64	26,87	0,37	2,73	--	2,47
TR 39	13,20%	0,8	3,64	0,45	3,05	2,49	0,5	1,76	1,84	23,33	0,29	2,42	--	2,68
TR 40	12,20%	2,4	3,57	0,46	4,26	3,69	0,7	0,82	1,52	23,94	0,31	2,34	--	2,13

Appendix 2 Chemical characterisation, Isolation and 4-EP determination in Turkish Red Wines

Appendix 3. Fit Linear Comparing HPLC and Chemical Analysis of Turkish red wines



A) Correlation of acetic acid (HPLC results - Volatile Acidity (chemical analysis results).

B) Correlation of ethanol (HPLC results) and ethanol (chemical analysis results)