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THE METHODOLOGICAL ASPECTS OF USING REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR) IN *BRETTANOMYCES/DEKKERA* DETECTION

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Abstract. Wine spoilage could be caused by a lot of genera and species of yeasts. One of the most damaging is *Brettanomyces/Dekkera* which causes severe quality problems in the wine industry. Timely detection of these organisms in wine is of crucial importance for preserving wine quality. The conventional methods of diagnostics are often time-consuming and laborious. For this, molecular-based methods of rapid testing, particularly real-time PCR are gaining increasing importance. In this work, the potential of the real-time PCR method in microbiological monitoring of wines was evaluated, and analysis process according to the PIKA Weihenstephan™ SO Detection Kit H *Brettanomyces/Dekkera* protocol was optimized.

***Key words*:** *Red wine, spoilage yeast, DNA extraction, detection kit Brettanomyces/Dekkera, real-time PCR, volatile phenols, cycle quantification (Cq).*

Introduction

Wine spoilage could be caused by a lot of genera and species of yeasts. One of the most serious yeast genus in wine spoilage is *Brettanomyces/Dekkera* which causes severe quality problems in the wine industry. This genus exists in two forms: *Brettanomyces*, the asexual, non-sporulating form, and *Dekkera*, the sexual, sporulating form [1] and was first described by Claussen in 1903 in beer production [2]. There are five recognized species of *Brettanomyces/Dekkera* genus: *D. anomala*, *D. bruxellensis*, *B. custersianus*, *B. naardenensis* and *B. nanus* [1, 3]

Brettanomyces/Dekkera species are isolated from the grape, during fermentations, ageing and after bottling [4]. *Brettanomyces/Dekkera* yeasts are well adapted to high ethanol concentrations and low pH which allows its to develop in difficult environments, such as wine [5].

Different strains of *Brettanomyces/Dekkera* species have been found on grape surfaces (in the field), in the wine samples, specially various red wine varieties and in the contaminated equipment in the winery as well as old oak barrels that have been insufficiently cleaned and disinfected after use. Also, insects, specially fruit flies, are important vectors. These potential spoilage yeasts have been identified in almost every wine-producing area of the world [6].

The growth of *Brettanomyces/Dekkera* in wine has been associated with various forms of spoilage including cloudiness and pellicle formation, have harmful effects on flavour. Depending on the conditions and precursors available, these yeasts can produce acetic acid, contributing to wine's volatile acidity; tetrahydropyridines that are reminiscent of mouse-urine (mousiness); isovaleric acid, known for a rancid cheesy aroma and volatile phenols, like 4-ethylphenol and 4-ethylguaiacol.

Table 1

The chemical compounds produced by *Brettanomyces/Dekkera* yeasts [7]

Compound	Character
4-EP (ethyl phenol)	Band-aid, elastoplast
4-EG (ethyl guaiacol)	Smoky, spicy, cloves
4-EC (ethyl catechol)	Sweaty, horsey
Isovaleric acid	Rancid, cheesy, vomit
Combination of all the above	Horsey, barnyard, mouldy

The quality of wine is considered to be mainly affected by the accumulation of 4-ethylphenol and 4-ethylguaiacol. Actually there are six compounds responsible for the phenolic flavour: 4-ethylguaiacol, 4-ethylphenol, 4-ethylcatechol and their precursors 4-vinylguiacol, 4-vinylphenol and 4-vinylcatechol. This is clearly seen in “Figure 1”.

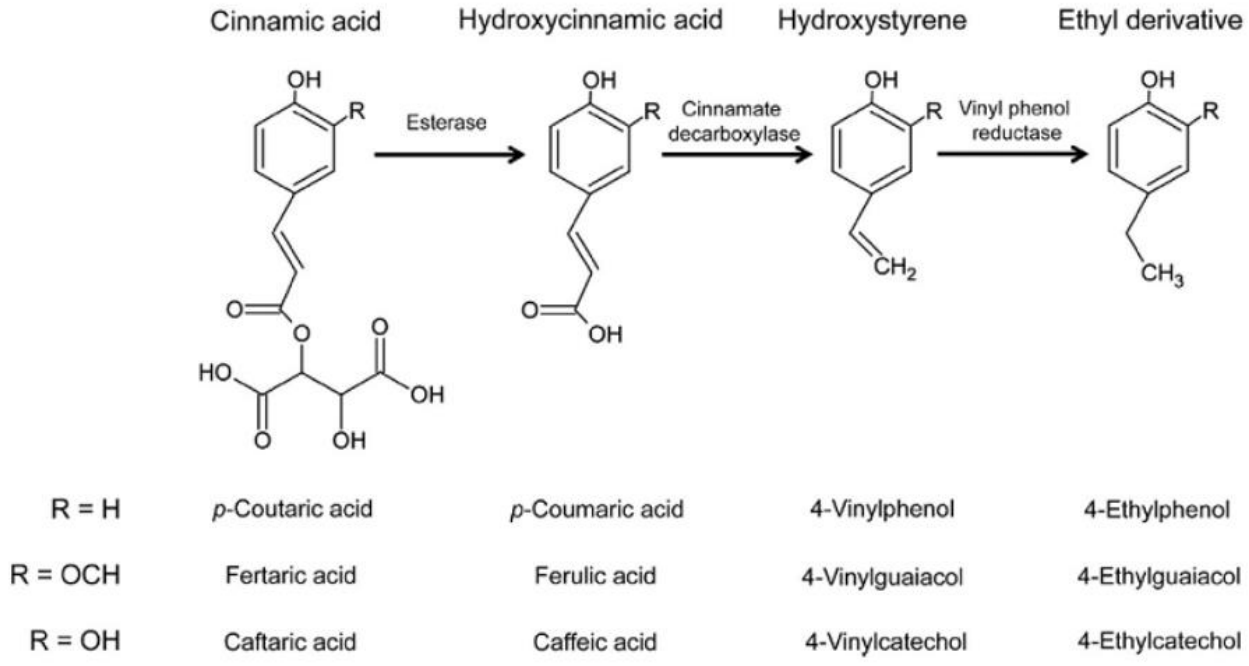

Figure 1. The formation of volatile phenols from their hydroxycinnamic acids precursors [8].

Volatile phenols found in wines are microbial derived product formed from hydroxynnamic acids naturally present in grapes [9]. Vinylphenols (4-vinylphenol and 4-vinylguaiacol) and ethylphenols (4-ethylphenol and 4-ehtylguaiacol) may be produced in wine, in a sequential pathway, due to microbial activity, imparting undesirable odours and flavours. Mainly, they are formed by metabolism of hydroxycinnamic acid (ferulic, *p*coumaric or caffeic acid) substrate by *Brettanomyces/Dekkera* yeast which involves the sequential action of two enzymes. Hydroxycinnamate decarboxylase first turns these hydroxycinnamic acids

into hydroxystyrenes (vinylphenols), which are then reduced to ethyl derivatives by vinylphenol reductase ("Figure 1").

Several strategies have been applied to prevent the development of *Brettanomyces/Dekkera* during wine production. Among them there are improved hygiene, monitoring of nutrients and residual sugars during the fermentation and at the end of it, temperature control, use of sulphur dioxide, avoidance of old oak barrels, etc.

The methods of detection and quantification of *Brettanomyces/Dekkera* contamination are essential to preventing wine spoilage. These methods conventionally can be divided into two groups: microbiological and molecular methods. Microbiological methods can take a longer time, 1 to 2 weeks and rely on growth on semiselective culture media or selective culture media, followed by final identification by biochemical and physiological analysis and morphology as determined by microscopic examination [6].

In this regard, the molecular biology methods for rapid detection and identification of *Brettanomyces/Dekkera* based on polymerase chain reaction (PCR) analysis of the isolated DNA have been developed.

Table 2

PCR methods used to identify *Brettanomyces/Dekkera* in wine [4]

Molecular tools of identification by PCR	DNA region targeted by PCR primers	References	Identifying information
Species specific PCR	RAD4 gene	[10]	Amplicon of certain size
PCR-RFLP	5,8 S rDNA gene and the two internal transcribed spacers (ITS1 and ITS2)	[11; 12]	Enzymatic restriction pattern (after amplicons digestion)
PCR-DGGE	D1/D2 domains of the rRNA 26S gene	[6;13]	Specific front of migration
Multiplex PCR	Internal transcribed spacer region (ITS) or 26S rDNA	[3]	Specific front of migration
LAMP-PCR	Internal transcribed spacer region (ITS)	[14]	Specific front of migration; real time melt curves analysis
Quantitative PCR	RAD4 gene	[15; 16]	Real time melt curves analysis

Traditional PCR methods require amplification of target DNA in a thermocycler and product separation by gel electrophoresis followed by visual detection [17]. This is a time-consuming, laborious and non-automated process. However, the products of the PCR can also be detected by using fluorescent probes in quantitative real-time PCR. The labelled probe anneals to the target DNA during the reaction and amid DNA extension emits a fluorescent signal that can be registered by a light cycler. The concentration of fluorescence released is proportional to the concentration of PCR product generated at each cycle. Real-time PCR assay is automated, sensitive and rapid, because it reduces or even eliminates lengthy enrichment and isolation processes [18]. It can also quantify PCR products with greater

reproducibility while eliminating the need for post-PCR processing, thus preventing carryover contamination.

Real-time PCR has already been successfully used as a tool to detect foodborne organisms in water [19] or in products such as wine [4; 15; 16; 20; 21; 22; 23; 24], fruit juice [25], milk [26], cheese [27].

The main goal of this research was to evaluate the potential of the real-time PCR method in microbiological monitoring of wines and to optimize the analysis process according to the PIKA Weihenstephan™ SO Detection Kit H *Brettanomyces/Dekkera* protocol [28]. In order to achieve the proposed goal we have studied:

1) the dependence of quantification results (Cycle quantification – Cq) of RT-PCR on analysed DNA concentration in solutions;

2) the suitability of Hard Shell PCR Plates Biorad and PCR tubes Rnase/Dnase/Endotoxin free for *Brettanomyces/Dekkera* detection by RT-PCR;

3) the suitability of two DNA amplification master mix, TaqMan®Universal Master Mix II and Platinum®PCR SuperMix for *Brettanomyces/Dekkera* detection by RT-PCR.

Materials and methods

The real-time polymerase chain reaction (real-time PCR) was first introduced in 1992 by Higuchi and coworkers [29] and allows precise quantification of specific nucleic acids in a complex mixture by fluorescent detection of labeled PCR products. Fluorophore-coupled nucleic acid probes interact with the PCR products in a sequence-specific manner and provide information about a specific PCR product as it accumulates. Detecting the PCR product in real-time involves the use of specific fluorescent probe (e.g., *Taq*man) or nonspecific dye (such as SYBR Green I) [30].

In our research molecular detection of *Brettanomyces/Dekkera* has been done using *Taq*man probe [28] at real-time PCR Detection Systems CFX96 Touch™ BIORAD as described in the protocol, supplied by the producer. The qPCR reaction was carried out according to manufacturer's protocol. Unless specified otherwise, TaqMan Universal Master Mix II, no UNG was used for the reaction.

For the serial dilutions experiment, we made 5, 25, 125 and 625 serial dilutions of the control DNA of Dekkera (supplied by the manufacturer as a positive control). For the negative control sample, the corresponding amount of water was used instead of DNA. The qPCR was carried out in the hard shell PCR plates "Biorad"

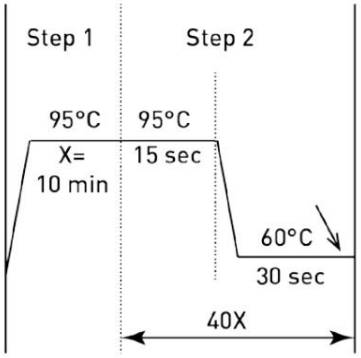

Figure 2. Thermal cycling program [PIKA].

For the tube suitability testing experiments, the tubes (ISOLAB Rnase/Dnase/Endotoxin free) were used instead of the plates. We used a negative control sample, a sample containing control DNA and a 5-fold dilution of the control DNA for this experiment.

For the reaction mixture suitability test, we used Platinum PCR Supermix (Invitrogen). A sample containing TaqMan Universal Master Mix II was used as a positive control.

The detection of *Brettanomyces/Dekkera* was done at **FAM™** (520 nm emission) channel, of Internal Positive Control at **VIC®** (550 nm emission) channel. Cycling conditions are shown in "Figure 2".

Results and discussion

At the first stage of our research the dependence of PCR quantification results (Cq) on analysed DNA concentration in solutions have been revealed. The DNA amplification and analysis have been done in hard shell PCR plates "Biorad". The analyzed DNA was supplied as a part of the PIKA Weihenstephan kit for *Brettanomyces/Dekkera* detection as a positive control.

As "Figure 3a" and "Figure 3b" show, the amplification was successful.

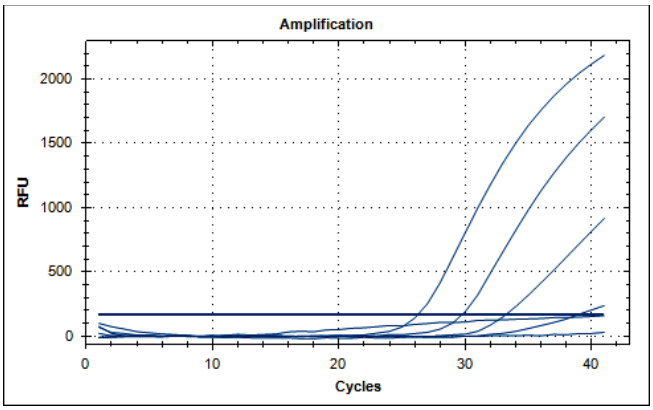

Figure 3a. Amplification plot obtained by fluorophor FAM for *Brettanomyces/Dekkera* detection**.**

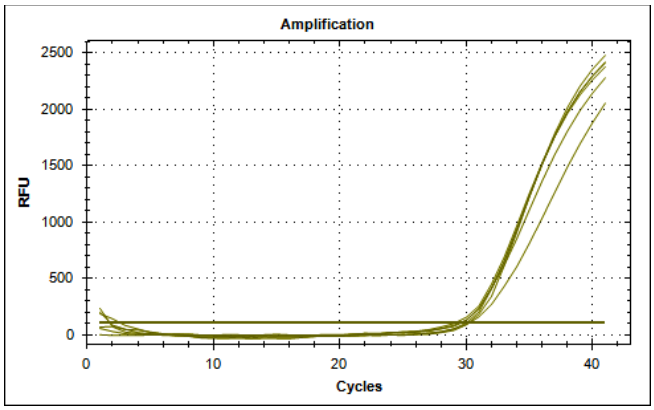

Figure 3b. Amplification plot obtained by fluorophor VIC for internal control.

Table 1

RT-PCR data analysis

Sample	DNA content	Cycle quantification, Cq	
		FAM*	VIC**
A 01	0 µL (NC)	N/A	29.98
B 01	5 µL	26.20	30.55
C 01	1/5 µL	29.68	29.92
D 01	1/25 µL	33.14	29.85
E 01	1/125 µL	38.78	30.09
F 01	1/625 µL	N/A	29.82

* – 520 nm: ** – 550 nm

In case of Internal Positive control ("Figure 3b", "Table 1" VIC), the Cq values are similar for all samples and lie in the range of 29.8 and 30.55, meaning no inhibition of qPCR. In case of *Brettanomyces/Dekkera* detection ("Figure 3a", "Table 1" FAM), the results range from no amplification in the negative control sample, with no DNA (FAM Cq value N/A) to Cq value 26.2 in case of undiluted DNA. Each subsequent dilution gave a higher Cq value (29.68 for a 5-fold dilution, 33.14 for 25-fold dilution and 38.78 for a 125-fold dilution. The 125-fold dilution was the last dilution where DNA could be detected, with the next dilution (625-fold) giving no Cq value, proobably because the amount of the DNA was below the detection limit.

"Figure 3c" shows the correlation of the cQ value with the DNA concentration with the correlation coefficient (R^2) 0,9838.

At the second stage of our research the suitability of PCR tubes (ISOLAB Rnase/Dnase/Endotoxin free) for *Brettanomyces/Dekkera* detection by the RT-PCR have been studied.

So, three samples (no DNA negative control, undiluted *Brettanomyces/Dekkera* DNA and a 5-fold dilution of this DNA) were tested in the ISOLAB tubes.

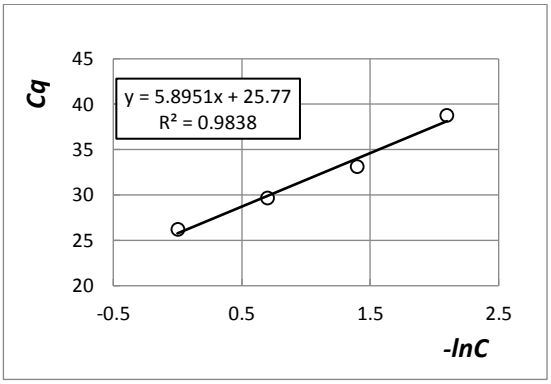

Figure 3c. Correlation curve of the Cq value with the DNA concentration.

As one can see from the "Figure 4", both amplification and fluorescence detection be done in these tubes using the instrument. "Figure 4b", "Table 2" VIC) show the results of the detection through VIC channel. Cq values are similar for all samples, ranging from 29.41 to 30.16, which mean that no inhibition of the reaction, problems with temperature regimen during amplification or fluorescence detection occurred.

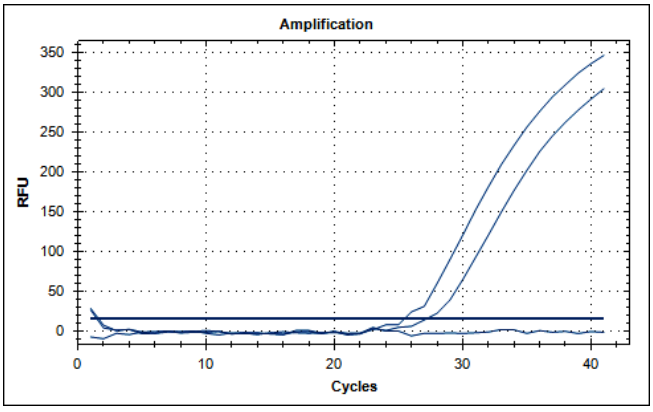

Figure 4a. Amplification plot obtained by fluorophor FAM for *Brettanomyces/Dekkera* detection.

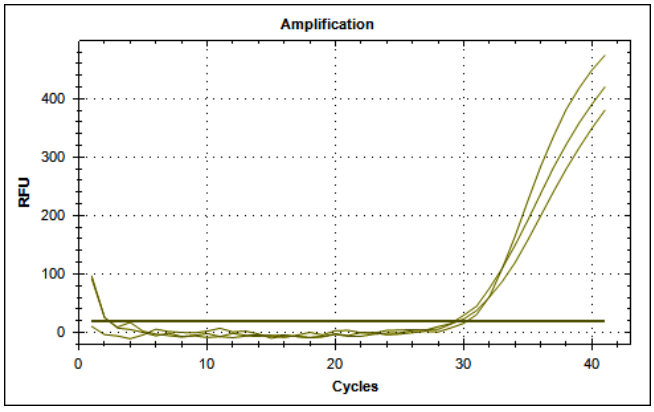

Figure 4b. Amplification plot obtained by fluorophor VIC for internal control.

The data obtained through FAM channel ("Figure 4a", "Table 2" FAM), corresponding to the *Brettanomyces/Dekkera* DNA also look as expected, with not detected Cq value for the negative control (no DNA sample), lowest Cq value 25.43 for the undiluted DNA, and higher Cq value 27.15 for the 5-fold diluted sample. Thus, the tested tubes were suitable for performing this kind of analysis.

Table 2

RT-PCR data analysis

Sample	DNA content	Cycle quantification, Cq	
		FAM*	VIC**
C04	0 µL (NC)	N/A	30.16
D04	1/5 µL	27.14	29.19
E 04	5 µL	25.43	29.41

* – 520 nm: ** – 550 nm

At the third stage of our research the suitability of two DNA amplification master mixes for *Brettanomyces/Dekkera* detection by the RT-PCR have been studied. One was TaqMan Universal Master Mix II, no UNG recommended by manufacturer, the other one was Platinum PCR supermix (Invitrogen). As it can be seen from "Figure 5", we used a no DNA negative control, a five-fold dilution of the *Brettanomyces/Dekkera* DNA and undiluted *Brettanomyces/Dekkera* DNA with Platinum PCR supermix, as well as undiluted *Brettanomyces/Dekkera* DNA with TaqMan Universal Master Mix II, no UNG as a positive control.

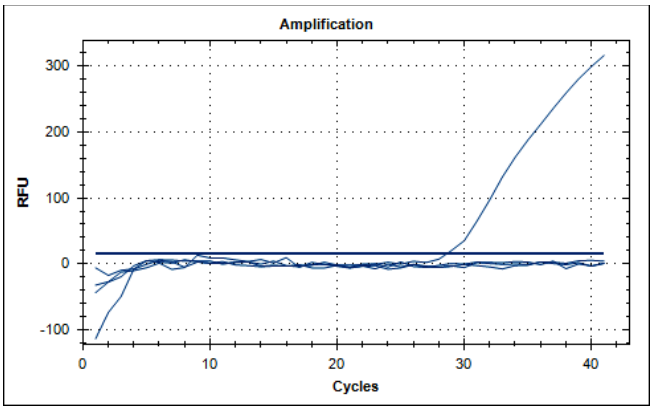

Figure 5a. Amplification plot obtained by fluorophor FAM for *Brettanomyces/Dekkera* detection.

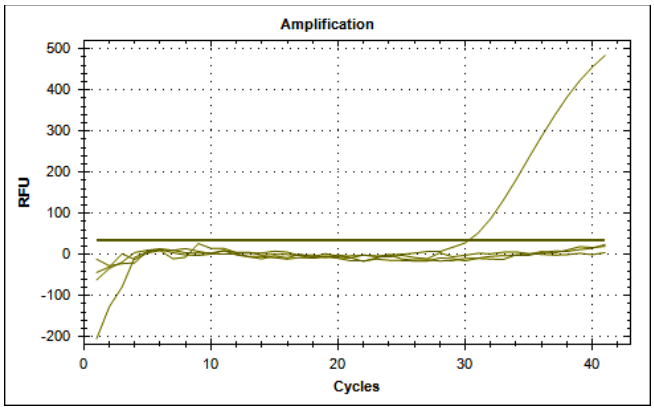

Figure 5b. Amplification plot obtained by fluorophor VIC for internal control.

As shown in "Figure 5a", "Figure 5b" and the "Table 3", the only sample in which fluorescent signal was detected was the sample containing TaqMan Universal Master Mix II, nTaqMan Universal Master Mix II, nTaqMan Universal Master Mix II, with Cq value 28.7 on FAM and 30.19 on VIC channel.

Table 3

RT-PCR data analysis

Sample	DNA content	Cycle quantification, Cq	
		FAM*	VIC**
B04	0 µL (NC)	N/A	N/A
C04	(1/5 µL)	N/A	N/A
D04	(5 µL)	N/A	N/A
E04	(5 µL)	28.70	30.19

* – 520 nm: ** – 550 nm

This means it was the only sample in which DNA amplification took place. No value on either FAM or VIC channel was detected for any of the samples containing Platinum PCR supermix, which means this PCR mix is not suitable for this kind of analysis.

Conclusions

The qPCR using PIKA Weihenstephan detection kit can be used for detection of *Brettanomyces/Dekkera* using TaqMan Universal Master Mix II. The Cq values have a good correlation with the amount of *Brettanomyces/Dekkera* DNA present in the analyzed sample.

The PCR tubes (ISOLAB Rnase/Dnase/Endotoxin free) are suitable for this kind of analysis with the real-time PCR Detection Systems CFX96 Touch™ BIORAD, and can be used as a substitution for hard-shell BIORAD 96-well plates.

Platinum PCR supermix (Invitrogen) is not suitable for qPCR amplification, and cannot be used as a substitution for TaqMan Universal Master Mix II.

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