

Application Proficiency-Testing Schemes (PTS) in Analytical Methods Evaluation for the Detection of Spoilage Yeast *Brettanomyces* in Wine

Caterina Mazzoni*, Anne Tirard, Romain Le Neve and Abdelkader Boubetra

Bureau Interprofessionnel d'Etudes Analytiques (BIPEA), France

*Corresponding Author: Caterina Mazzoni, Bureau Interprofessionnel d'Etudes Analytiques (BIPEA), Paris, France.

Received: September 24, 2019; Published: November 20, 2019

Abstract

The main goal of microbiological analysis of musts and wines is to ensure higher quality of wines, allowing the detection of all anomalies both during the different steps of production and in the final product. Among all possible microbial alterations of wines, volatile phenols production by the yeast *Brettanomyces* is one of the most feared by wine producers. The non-controlled accumulation of such molecules in wines leads to sensory defects which compromise their quality. The most used analytical methods for detection and quantification of *Brettanomyces* are culture microbial enumeration (Petri dish) and qPCR (quantitative Polymerase Chain Reaction), method based on the identification of the microorganisms by their DNA. These two analytical approaches are quite different: qPCR detects non-viable and viable microorganisms, whereas cultural methods enumerate only the viable and culturable ones. Proficiency-testing schemes (PTS) on wines spiked with *Brettanomyces bruxellensis* are being organized by BIPEA (Bureau interprofessionnel d'études analytiques) since February 2016. Data collected in these tests were subject to statistical studies to evaluate the comparability of results obtained by qPCR and culture microbial enumeration. Some differences in *Brettanomyces* quantification according to the performed method were highlighted: assigned values (robust means of the results) obtained using qPCR are always higher than the ones by culture enumeration. On the other hand, laboratories' results obtained using kits based on qPCR remain quite dispersed compared to those got by cultural methods.

Keywords: Proficiency-Testing Schemes; *Brettanomyces*; Microbiology; Wine Quality Control; Laboratory Performance; Culture Microbial Enumeration; qPCR

Introduction

Wine contamination with the spoil yeast *Brettanomyces* resulted in the production of phenolic compounds (4-ethylphenol and 4-ethylguaiacol) that effect wine fruity character [1-3]. The rapid detection of *Brettanomyces* at the early stage of wine production is of crucial importance for preserving wine quality. This is the reason why analyses' request of detection and quantification of these yeast has gradually increased in recent years with the consequent development of faster and more performing techniques.

Methods developed for detection and quantification of microorganisms in musts and wines can be grouped in two main categories: culture microbial enumeration (Petri dish) and qPCR (quantitative polymerase chain reaction), method based on the identification of the microorganisms by their DNA, used for *Brettanomyces* determination in particular [4-9].

The culture microbial enumeration on Petri dish is routinely used in the wine industry as simple and cost-effective test. On the other hand, qPCR allows a result in 24 - 48 hours versus 7 - 14 days for the culture microbial technique. Moreover, qPCR has the advantage of detecting non-viable and viable microorganisms, in contrast with the Agar plate method that enumerate only the viable and culturable ones. This aspect is very interesting because *Brettanomyces* can enter in a "viable but not cultivable" state after the addition of sulphur

Citation: Caterina Mazzoni, et al. "Application Proficiency-Testing Schemes (PTS) in Analytical Methods Evaluation for the Detection of Spoilage Yeast *Brettanomyces* in Wine". *EC Microbiology* 15.12 (2019): 01-06.

dioxide to wine [10] and these cells can be missed using Agar culture media. That is why several specific qPCR kits have been developed during the last years. However, commercial kits available for quantification of *Brettanomyces* have different extraction yields leading to different quantification results, which can cause an over or underestimation of these yeast in wines [11].

To respond to the need of laboratories to monitor the reliability of the results for both culture microbial enumeration and qPCR method, proficiency tests on *Brettanomyces* analyses in wine were organized.

Aim of the Study

The aim of this work is to compare laboratories results obtained in 3 different proficiency tests (PTs) for *Brettanomyces* quantification using commercially available kits based on real time qPCR and classical method (plate counting on selective medium).

Materials and Methods

Production and shipment of the samples

Samples were prepared by spiking a batch of homogenized red wine (100% Grenache, Côtes-du-Rhône, total SO₂ content: 1 mg/l) with a suspension of *Brettanomyces (Dekkera) bruxellensis* in well controlled proportions. Following the requirements of the ISO 13528 [12], homogeneity of the samples was verified by experimental studies on 10 samples *in duplo* taken randomly across the batch of manufactured samples. Stability of the product was proved by analyzing 3 samples *in duplo* during 7 days. For both studies, the analyses were performed according to the Compendium of the OIV (International Organization of Vine and Wine [13]). Samples were shipped at (5 ± 3)°C to laboratories participating in the test together with a water sample for monitoring the temperature of reception.

Collection of the results and statistical treatment of the data

Laboratories returned their analysis results *via* a specific reply form. In this form, participants were invited to enter some complementary information such as the date of the beginning of the analysis and, for the laboratories performing the cultural medium methods, growth medium used, incubation temperature and time and the type of plating. Given the nature of the product, the participants were invited to analyze the samples as soon as possible after the reception. Before any statistical treatment, the whole traceability of the procedure, from the sample production to the results of each participant, was checked. 29 laboratories on average returned their results. A statistical treatment of the data was performed according to ISO 13528 standard [11], which describes in its Annex B some robust statistics (algorithm A). Data of plating method and q-PCR were separated and evaluated distinctly. Assigned values (x_{pt}) were estimated using the robust means of all results transformed in log (except the incoherent ones) from application of robust algorithm A.

Results and Discussion

Results of three PTs are examined in details. Table 1 summarizes statistical data of these tests for qPCR and plate counting on selective medium methods. On average, 20 laboratories returned results by cultural method and 10 laboratories by qPCR. As expected, assigned values (x_{pt}) of qPCR method are always higher than the ones obtained by culture enumeration. However, it should be noted that coefficients of variation $CV(x_{pt})$, reflecting data dispersion according to assigned values, range from 6% to 9% for culture microbial enumeration analyses and from 8% to 16% for qPCR. As well, uncertainties, $u(x_{pt})$ and robust standard deviations, $s(x_{pt})$, of culture enumeration are lower than those obtained from the results obtained by qPCR. These results highlight that reference method based on plate counting provides reliable results.

For each proficiency test, means obtained by qPCR and by enumeration method were statistically compared taking into account the standard uncertainties $u(x_{pt})$ of the assigned values. This comparison is based on the estimations of the difference of two identified means ($d_{ptm1-m2}$) and the standard uncertainty of this difference ($u_{dptm1-m2}$), according to the following formula:

$$d_{ptm1-m2} = x_{ptm2} - x_{ptm1}$$

$$u_{dptm1-m2} = \sqrt{(u_{ptm1}^2 + u_{ptm2}^2)}$$

where x_{ptm1} and $u(x_{ptm1})$ are respectively the assigned value and the standard uncertainty obtained by qPCR and x_{ptm2} and $u(x_{ptm2})$ are the assigned value and the standard uncertainty obtained by culture microbial enumeration.

PTs	Analytical methods	x_{pt}^1 log (UG/ml)	$u(x_{pt})^2$ log (UG/ml)	$s(x_{pt})^3$ log (UG/ml)	$p(x_{pt})^4$	$CV(x_{pt})^5$ %
PT 1 November 2018	qPCR	4.953	0.167	0.401	9	8
	Culture microbial enumeration	4.640	0.078	0.278	20	6
PT 2 February 2019	qPCR	4.669	0.235	0.625	11	13
	Culture microbial enumeration	4.160	0.089	0.327	21	8
PT 3 May 2019	qPCR	4.640	0.297	0.753	10	16
	Culture microbial enumeration	4.631	0.119	0.426	20	9

Table 1: Main statistical parameters of PTs.

x_{pt} : Assigned value or conventionally true value, calculated by the robust algorithm A from ISO 13528 standard.

$u(x_{pt})$: Standard uncertainty of the assigned value; this value permits to quantify the confidence that can be given to the assigned value. It depends on the mathematical model applied (algorithm A) and is a function of the standard deviation and the number of results used for the estimation of the assigned value. It is calculated as indicated in § 5.6.2 of ISO 13528 standard.

$s(x_{pt})$: Robust standard deviation of the results, calculated by the robust algorithm A from ISO 13528 from all the results which participated to the estimation of the assigned value.

$p(x_{pt})$: Number of results taken into account for the estimation of the assigned value.

$CV(x_{pt})$: Coefficient of variation, this value permits to compare the dispersion of the results, whatever the mean is.

If $d_{ptm1-m2} > 2 * u_{dptm1-m2}$, then the two means are considered statistically different according to their standard uncertainties. A difference between assigned values higher than twice the standard uncertainty may be caused by a method bias, a difference from returned results or a default of the mean values' estimation.

Results of this study, summarized in table 2, highlight that, in two cases out of three (PT1 and PT3), twice the standard uncertainty of the difference is higher than the difference between the two assigned values. That leads to the conclusion that results obtained using these two different methods cannot be considered significantly different even if the assigned values of qPCR method are always higher than the ones obtained by culture enumeration. Quite the contrary, the statistical comparison of the assigned values of the second PT (PT2) underlines that two assigned values are statistically different and the results obtained by qPCR and culture enumeration are not comparable.

PTs	$d_{ptm1-m2}^1$ log (UG/ml)	$2 * u_{dptm1-m2}^2$ log (UG/ml)
PT 1 November 2018	0.313	0.369
PT 2 February 2019	0.509	0.504
PT 3 May 2019	0.009	0.641

Table 2: Statistical comparison of the assigned values obtained by qPCR and culture enumeration methods.

$d_{ptm1-m2}$: Difference of the assigned values obtained by qPCR and culture enumeration methods.

$2 * u_{dptm1-m2}$: Twice the standard uncertainty of the difference of the assigned values obtained by qPCR and culture enumeration methods.

On average, 7 laboratories out of all participants, sent back quantitative results obtained either by qPCR and by culture methods.

Figure 1 shows Youden plot confidence ellipse based on Jackson method comparing results obtained using the two different methods for each laboratory (represented as blue crosses). Results of culture microbial enumeration are reported on the ordinate axis and those obtained by qPCR on the abscissa one. A bisector is also plotted. Many information can be read in these graphs: the main one is that, even if assigned values of qPCR are higher than those of the plate counting on selective medium ones for all studied tests, some laboratories found lower results by qPCR. In detail, 3 laboratories out of 7 for the PT1, 3 out of 9 for the PT2 and only one out of 6 for the PT3 (crosses

on the top left of the bisector). It can be noted that, for each PT, all laboratories are inside the confidence ellipse at 5% and only one laboratory in PT1 and two in PT2 are out of the trueness area. The profile of these 3 curves varies widely and corresponds to the different cases observed on the 3 PT. For the two first tests (PT1 and PT2), the assigned values gap is almost equal to one standard deviation.

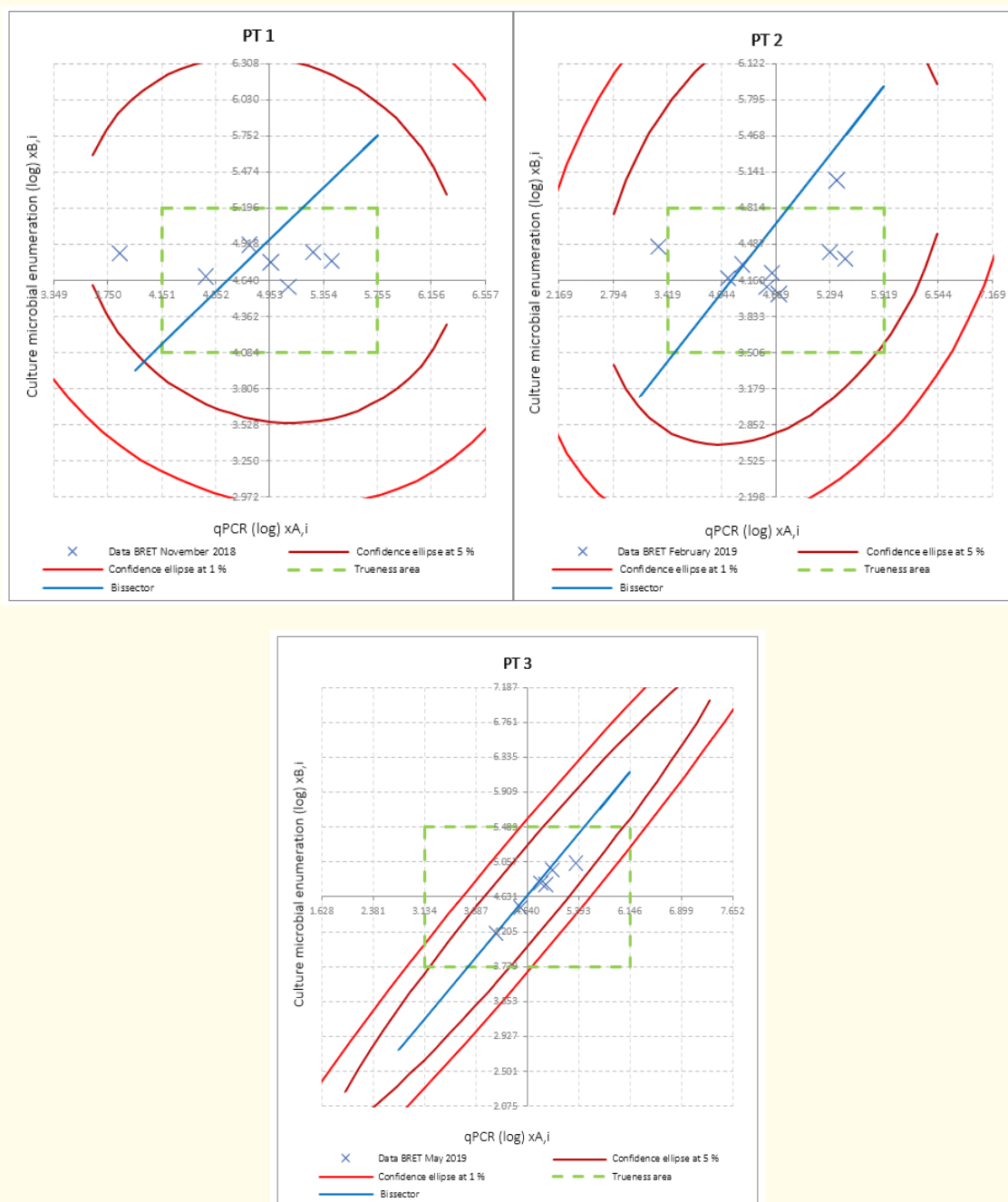


Figure 1: Comparison of laboratories' results obtained using qPCR or culture microbial enumeration method using Youden plot confidence ellipse based on that of Jackson.

These data confirm that, in most cases, qPCR overestimates *Brettanomyces* population in comparison with methods based on plate counts, as this technique do not discriminate live from dead or “viable but not cultivable” cells. However, some underestimations are also observed. This results heterogeneity can be due to the relatively recent introduction of qPCR method for the microbiological analyses of wine. Moreover, different kits used by laboratories can also play an important part in this dispersion of results. Commercial kits for the quantification of *Brettanomyces* have in fact different extraction yields leading to different quantification results.

Conclusion

Traditional methods to detect spoilage yeasts in wine based on media enumeration are still relevant, even if incubation time remains high, postponing any corrective action by the winemaker in case of contamination. This drawback conducts more and more laboratories to use qPCR method that allows winemakers to quickly determine *Brettanomyces* by directly measuring genetic material and eliminating the time-consuming incubation step.

Several studies, performed on other matrices and microorganisms, showed an equivalence of these two methods, though qPCR produces higher values under some circumstances [14-16]. This equivalence was observed in two tests out of the three described in this paper.

This work confirms that, in general, qPCR method overestimates the number of microorganisms in comparison with culture enumeration methods, that count only viable and cultivable cells. Anyway, an underestimation of *Brettanomyces* population by qPCR was observed by some laboratories, in particular in the first two studied PTs (PT1 and PT2). These results indicate that considerable work has to be done to increase performances of methods based on the identification of *Brettanomyces* DNA. Interpretation of obtained data by scientists must take into account the specific nature of the performed method and its pertinence according to analytical needs and expected results.

In conclusion, even if these methods are not always directly comparable, they are complementary and should be used in parallel when accuracy is required to avoid to negatively affect a winemaker’s decision and lead to wine spoilage due to over or underestimation.

Acknowledgements

Bipea acknowledges all the laboratories participating to these PTSs.

Bibliography

1. Moreno-Arribas MV and Polo C. “Wine Chemistry and Biochemistry”. Springer, New York, USA (2008).
2. Agnolucci M., et al. “*Brettanomyces bruxellensis* yeasts: impact on wine and winemaking” *World Journal of Microbiology and Biotechnology* 33 (2017): 180.
3. Lonvaud-Funel A., et al. “Microbiologie du vin, Bases fondamentales et applications”. Lavoisier, Paris, France (2010).
4. Suárez R., et al. “The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: A review”. *Food Chemistry* 102 (2007): 10-21.
5. Iland P., et al. “Microbiological Analysis of Grapes and Wine: Techniques and Concepts”. Patrick Iland Wine Promotions, Campbelltown, Australia (2007).
6. Bonder C. “Analyses et décisions en œnologie, Guide pratique du laboratoire et de la cave”. Lavoisier, Paris, France 46 (2014): 381-400.
7. Phister TG and Mills DA. “Real-time PCR assay for detection and enumeration of *Dekkera bruxellensis* in wine”. *Applied and Environmental Microbiology* 69 (2003): 7430-7434.
8. Cocolin L., et al. “Molecular detection and identification of *Brettanomyces/Dekkera bruxellensis* *Brettanomyces/Dekkera anomalus* in spoiled wines”. *Applied and Environmental Microbiology* 70 (2003): 1347-1355.

9. Tessonière H., *et al.* "Design and performance testing of a real-time PCR assay for sensitive and reliable direct quantification of *Brettanomyces* in wine". *International Journal of Food Microbiology* 129 (2009): 237-243.
10. Zuehlke JM and Edwards CG. "Impact of sulfur dioxide and temperature on cultivability and viability of *Brettanomyces bruxellensis* in Wine". *Journal of Food Protection* 76.12 (2013): 2024-2030.
11. Longin C., *et al.* "Evaluation of three *Brettanomyces* qPCR commercial kits: results from an interlaboratory study". *OENO One* 50 (2016): 223-230.
12. ISO. Statistical methods for use in proficiency testing by interlaboratory comparison (2015): 13528.
13. International Organisation for Vine and Wine - OIV Compendium of International Methods of Wine and Must Analysis 2.4 (2017).
14. Lavender JS and Kinzelman JL. "A cross comparison of qPCR to agar-based or defined substrate test methods for the determination of *Escherichia coli* and *Enterococci* in municipal water, quality monitoring programs". *Water Research* 43 (2009): 4967-4979.
15. Noble RT., *et al.* "Comparison of rapid quantitative PCR-based and conventional culture-based methods for enumeration of *Enterococcus* spp. and *Escherichia coli* in recreational waters". *Applied and Environmental Microbiology* 76 (2010):7437-7443.
16. Whitman RL., *et al.* "Relationship and variation of qPCR and culturable enterococci estimates in ambient surface waters are predictable". *Environmental Science and Technology* 44 (2010): 5049-5054.

Volume 15 Issue 12 December 2019

©All rights reserved by Caterina Mazzoni., *et al.*