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GENERAL PAPER

Microbiology proficiency testing schemes in wine

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Abstract
The number of laboratories performing microbiological analyses of wines has gradually increased in recent years over the world. However, the lack of regular proficiency testing schemes (PTS) in this field is an obstacle for the monitoring of the laboratory performance. The complexity of wine matrix in terms of bacterial ecology and biochemical aspects is a factor to be taken into account in developing a PTS, especially during the preparation of stable and homogeneous samples. Since February 2016, BIPEA set up tests on *Brettanomyces* analyses in wine, gathering more than 20 laboratories around the world. For each test, the statistical treatment of the data is performed according to ISO 13528. The assigned and tolerance values are calculated from the participants' data, and the performances of the laboratories are evaluated individually and collectively according to ISO/IEC 17043 and ISO/DIS 22117. The results obtained in these first tests are satisfactory with a progressive improvement of the dispersion of participants' results over the series. These data confirm that participating in a PTS is particularly important for the laboratories in order to assess and improve analytical performances and to obtain recognition of their analytical procedures by the accreditation bodies according to ISO/IEC 17025.

Keywords Proficiency testing schemes · *Brettanomyces* · Microbiology · Wine quality control · Laboratory performance

Introduction

Wine is a complex matrix from both physiochemical and microbiological points of view. Different technological or alteration microorganisms can coexist and constitute a complex microbial ecosystem, very difficult to understand [1–3]. Among all possible microbial alterations of wines, volatile phenols production by the *Brettanomyces bruxellensis* yeast is one of the most feared by winemakers and probably one of the most undesired by consumers [4]. The non-controlled accumulation of such molecules in wines leads to sensory defects which compromise the wine quality. Different descriptors such as *medicinal, smoked*, *animal* or *spiced* are used to qualify the odors conferred by these compounds [5].

The main goal of the 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 [6, 7].

The methods developed for detection and quantification of microorganisms in musts and wines can be grouped in three main categories: microscopy techniques (Malassez hemocytometer and epifluorescence), culture microbial enumeration (Petri dish) and PCR (polymerase chain reaction), a method based on the identification of the microorganisms by their DNA, used in particular for *Brettanomyces* determination [8–10].

Due to the importance of microbiological analyses of wines and the development of better performing methods, the number of laboratories interested in these analyses has gradually increased in recent years. However, the lack of regular proficiency testing schemes (PTS) in this field is an obstacle for the monitoring of the laboratory performance.

The complexity of wine matrix in terms of bacterial ecology is a factor to be taken into account in developing a PTS, especially during the preparation of stable and homogeneous samples.

This work describes the design, the implementation and the results of PTS for the analyses of wine samples spiked with yeasts. The goal of this PTS is to allow laboratories to demonstrate the reliability of their results and to compare

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with each other analytical data and protocols used for the enumeration of *Brettanomyces* in wine.

Experimental

One of the fundamental aspects for the implementation of a PT (proficiency test) is the preparation of homogenous and stable samples. The homogeneity and stability of the samples must be demonstrated to avoid misjudging laboratory performance owing to sample inadequacy.

For these PTs, samples are prepared by spiking a batch of homogenized red wine (100 % Grenache, Côtes-du-Rhône, total SO_2 concentration: 1 mg/L) with suspensions of *Dekkera bruxellensis* in well-controlled proportions. Different spiking concentrations, between 2000 CFU/mL and 12000 CFU/mL (colony-forming unit per mL), are proposed to allow the laboratories to test their analytical procedures with wine containing *Brettanomyces* with concentrations.

The homogeneity of the samples is verified by experimental studies on 10 samples in duplicate taken randomly across the batch of samples. According to BIPEA internal procedures for setting up new microbiology PTS, the samples are considered sufficiently homogeneous if the range between the minimum and the maximum values of this check is lesser than 0.5 in log10.

The stability of the product is proved by analyzing 3 samples in duplicate over a period of 7 days. Considering the nature of the tested microorganisms and the variability of the performed enumeration method, the stability of the samples is regarded as satisfactory if the maximum acceptable difference (in log10) between the averages of the results obtained at the first day of the study (D1) and the last one (D7) is situated near 1 in log10.

For both studies, the analyses are performed according to the Compendium of the OIV (International Organization of Vine and Wine) [11], on 1 mL of sample and successive decimal dilutions that are inoculated into empty Petri dishes. The agar (YGC) is added, and the samples are maintained at (25 ± 1) °C over a period of 8 days.

Once the homogeneity and the stability are demonstrated, the samples are shipped at (5 ± 3) °C to all of the participants who, given the nature of the product, are invited to analyze the samples as soon as possible after the reception. A sample of demineralized water is included in the package to allow the laboratories to monitor the temperature at reception. Laboratories' results are collected via a reply form available online over a period of 1 month. Results obtained using culture microbial enumeration and/or PCR methods must be entered in this form considering that only one single result per method is requested. Moreover, participants are invited to add in the reply form some complementary information such as the status of the sample on arrival, including temperature, the identity of the sample, the date of the beginning of the analysis and, for the laboratories performing the culture medium methods, growth medium used, incubation temperature, time and the type of plating.

The statistical treatments of the returned results are conducted according to ISO 13528 [12], and the performances of the laboratories are evaluated individually and collectively according to ISO/IEC 17043 and ISO/DIS 22117 [13, 15]. The assigned values (x_{pt}) are estimated using the robust means of the results from application of robust algorithm A. The performances of each laboratory are evaluated using a tolerance value (T_V) of twice the standard deviations. This value is used to identify an interval around the assigned value. Results out of this range are considered, at least, at the first sight, a warning signal for the laboratories. Moreover, the laboratories' results (x) are also evaluated through z-scores (z):

$$z = \frac{x - x_{pt}}{\frac{T_V}{2}}.$$

The results of the laboratories who have a z-score $\leq |2|$ are considered satisfactory, and those with a z-score $> |2|$ are classified as "questionable" or "unsatisfactory": if the laboratories' z-score is $|2| < z \leq |3|$ the result is considered questionable and $> |3|$ unsatisfactory. The results are published in a specific interlaboratory comparison report distributed to all the participants who can then classify their results and implement some corrective and/or preventive actions if necessary.

Results and discussion

Overview of the results of the first experimental test

The first PT on microbiological analyses of wine was set up in February 2016, gathering more than 20 laboratories around the world.

The results of the homogeneity check of this first experimental test are shown graphically in Fig. 1. These data show that the samples are sufficiently homogenous to meet the requirements of the PT, with a gap between the minimal and maximal values of 0.460 CFU/mL in log10.

The results of the stability checks showed a satisfactory recovery rate considering the expected concentration after storing the samples at (5 ± 3) °C over a period of 7 days (Table 1). The variability of the performed method can explain the difference between the means of the results collected from the first day (D0) to the last day of analysis (D7).

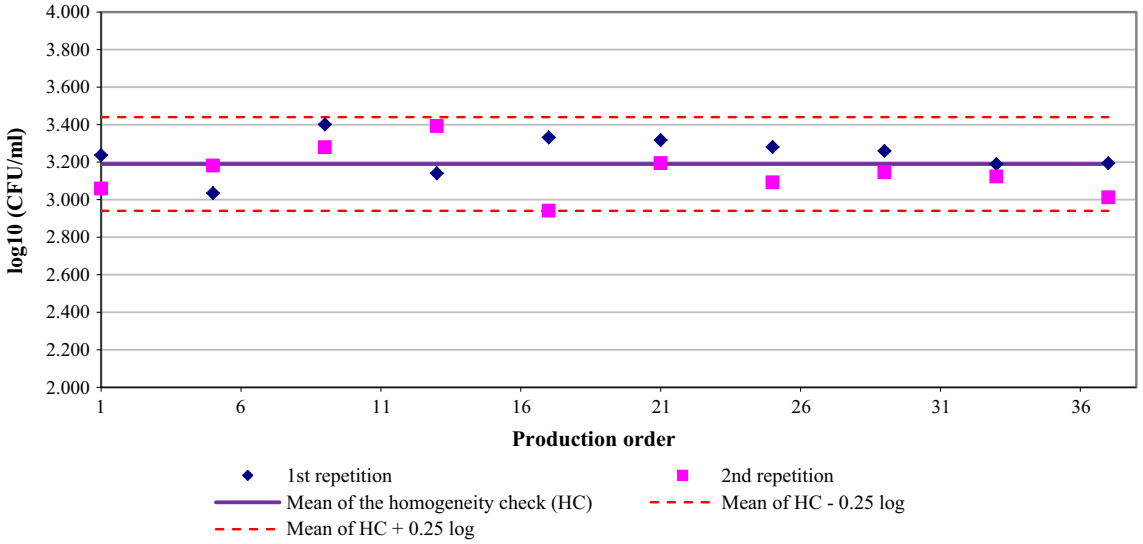

Fig. 1 Results of the homogeneity check of the samples of the first PT as a function of the production order

Table 1 Average results of the enumeration of *Brettanomyces* in wine over a period of 7 days at (5 ± 3) °C

Day of analysis	D0	D1 (D0 + 24 h)	D2 (D0 + 48 h)	D3 (D0 + 72 h)	D7 (D0 + 198 h)
Mean log10 (CFU/mL)	2.781	2.405	2.723	2.825	2.463

Sixteen laboratories out of twenty gave their results with useful information for the interpretation of the data. Among these results, fifteen were obtained using methods based on culture microbial growth and only one was collected performing the PCR method.

An assigned value (x_{pt}) of 2.462 log10 (CFU/mL) was calculated from the robust mean of all returned results except those of the three laboratories which indicated a date of analysis exceeding 7 days after the dispatch date of the samples. The result obtained using PCR was not taken into account too, as this method differs in principle from the analytical method used by the other laboratories which are based on culture microbial growth. The main statistical parameters of this PT, calculated according to ISO 13528 [12], are summarized in Table 2.

The laboratories' results are shown as histograms in Fig. 2. On this graph, assigned value and tolerance interval are indicated in the x-axis and the results of the laboratories are shown in different colors as a function of the performed method: OIV method (8 laboratories, gray), internal method (7 laboratories, black) and PCR method (1 laboratory, white). The means by method are also calculated and are shown in Table 3.

Participants of this first test use different growth media from many suppliers (see Fig. 3), and the major part of the laboratories perform the enumeration in surface. Incubation temperature and time vary from 25 to 30 °C and from 4 to 12 days, respectively. However, no tendency was highlighted as a function of the growth media or the performed incubation conditions.

Table 2 Summary of the statistical treatment of the data, log10 (CFU/mL)

Statistical parameter	Value
Assigned value for proficiency testing, x_{pt}	2.462
Standard uncertainty of the assigned value, $u(x_{pt})$	0.270
Robust standard deviation of the results, $s^*(x_{pt})$[a]	0.749
Number of results, p_x	12
Standard deviation for proficiency assessment, σ_{pt}[b]	0.749
Tolerance value, T_V[c]	1.498
Upper limit of the tolerance interval[d]	3.960
Lower limit of the tolerance interval[e]	0.964
Number of untrue results, p_D[f]	2

[a]Calculated from all the results which participated in the estimation of the assigned value

[b]Measure of dispersion used in the evaluation of results of proficiency testing

[c]Two times the standard deviation for proficiency assessment ($2 \times \sigma_{pt}$): it is a maximum tolerated deviation from the assigned value

[d]Assigned value + tolerance value: value of the parameter over which the result × is considered a warning signal

[e]Assigned value − tolerance value: value of the parameter below which the result × is considered a warning signal

[f]Laboratories' z-score > |2|

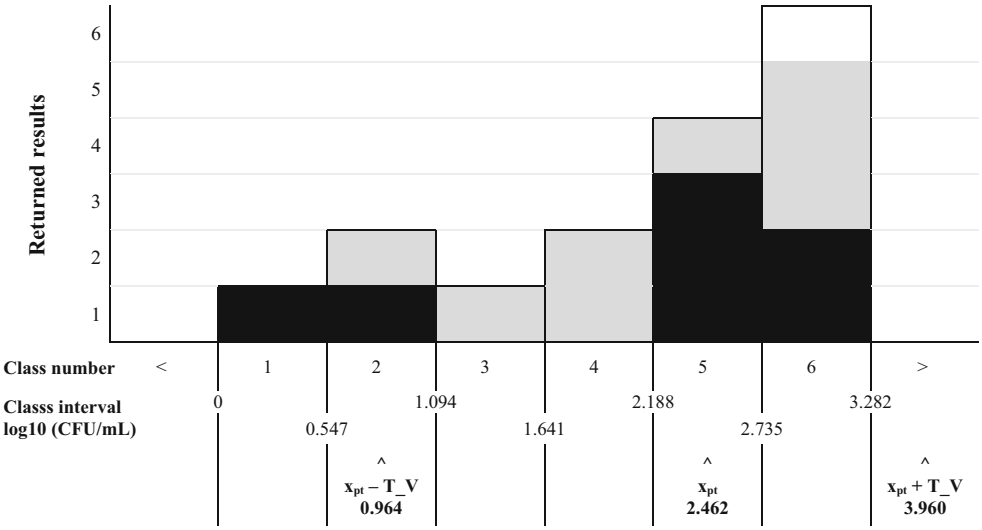

Fig. 2 PT results represented as a histogram

Table 3 Means by used method in PT, log10 (CFU/mL), calculated using the robust algorithm A according to ISO 13528 on each series of results by method

	OIV method	Internal method	PCR method
Mean, x^*_m	2.182	2.060	2.916
Standard uncertainty of the mean, u^*_{xm}	0.397	0.680	–
Standard deviation of the results, s^*_m	0.898	1.438	–
Number of the results, p_m	8	7	1

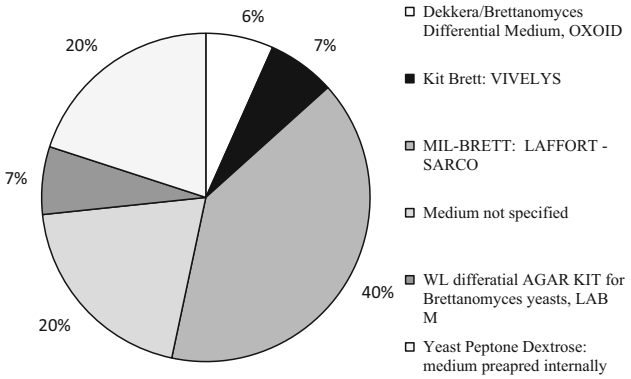

Fig. 3 Different media used by the laboratories

This first PT for *Brettanomyces* analysis in wine was successfully implemented, and the results were published in an interlaboratory comparison report distributed to the participants. This first PT has been transformed into a regular PTS, with 3 tests per year.

Overview of the results of regular proficiency testing

Three tests were scheduled during the 2016/2017 series, with 24 participating laboratories around the world. The samples of all PTS described below were prepared according to the procedure described in the Experimental section, as well as the homogeneity and the stability checks.

The active participation in these tests was satisfactory for the *Brettanomyces* by culture *medium* methods, with 16 returned results on average, contrary to the RT-PCR ones, where only 2 results on average where provided by the participants.

For each test, a statistical treatment could be performed on the results of the *Brettanomyces* by culture *medium* methods. As in the first test, the results obtained outside the deadline were excluded from the evaluation of the assigned values. The main statistical parameters of these PTS are summarized in Table 4.

Concerning methods performed for the analyses based on culture microbial growth, 7 laboratories followed the OIV procedures and 8 indicated another method (see Table 5 which summarizes the response rates and the means).

It can be noticed that the means of the results obtained by the OIV methods are, most of the time, higher than the means obtained from the other methods (mainly methods based on the OIV one, but modified internally by the laboratories). These data are in accordance with the results of the experimental test.

The treatment of information about growth *medium*, incubation temperature, time and type of plating shows that the participants use different growth *media* from many suppliers and the major part of the laboratories perform the

Table 4 Summary of the statistical treatment of the data of the 2016/2017 series, log10 (CFU/mL)

	Test 1	Test 2	Test 3
Assigned value for proficiency testing, x_{pt}	4.050	3.373	3.472
Standard uncertainty of the assigned value, $u(x_{pt})$	0.217	0.064	0.200
Robust standard deviation of the results, $s^*(x_{pt})$	0.672	0.177	0.619
Tolerance value, T_V	1.344	0.354	1.238
Number of returned results, p_{CA}[a]	15	16	17
Number of results taken into account for the estimation of the assigned value and the tolerance value, p_x[b]	15	12	15
Number of untrue results, p_D[c]	2	1	1
% of untrue results, p_D	13	6	6

[a]Including results given as quantification limit

[b]Data examination is performed according to the following criteria: traceability of the provided result (checking of the sample identification number), visual (expression of the result, data input error), technical (according to the Commission instructions), and/or statistic (tests, observed distributions)

[c]Laboratories' z-score $> |2|$

Table 5 Summary of the statistical treatment by method, log10 (CFU/mL)

	Test 1		Test 2		Test 3	
	OIV method	Other methods	OIV method	Other methods	OIV method	Other methods
Assigned value	4.050		3.373		3.472	
Mean, x^*_m	4.443	3.804	3.407	3.367	3.613	3.348
Standard uncertainty of the mean, u^*_{xm}	0.438	0.206	0.107	0.077	0.256	0.302
Standard deviation of the results, s^*_m	0.928	0.467	0.209	0.175	0.541	0.683
Number of the results, p_m	7	8	6	8	7	8

enumeration in surface for these two parameters. Incubation temperature and time vary from 25 to 37 °C and from 4 to 15 days. No tendency as a function of the growth *medium*, incubation temperature, time or type of plating was highlighted.

Conclusions

Proficiency tests enable the participating laboratories to draw up a general inventory of their analytical skills and improve their analytical performances in *Brettanomyces* detection and quantification analyses of wines.

A regular PTS for *Brettanomyces* analysis in wine is now available for the laboratories. This PT program has recently been approved and accredited by COFRAC (Comité Français d'Accréditation—French Accreditation Body). The PTS has since been further developed to include lactic and acetic bacteria in the samples to allow the laboratories to also demonstrate their performances for these microorganisms. Laboratories can now monitor punctually and/or continuously through time the reliability of their results and obtain recognition of their analytical procedures by the accreditation bodies according to ISO/IEC 17025 [14] for microbiological analyses of wines.

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