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Project acronym
BRETT MONITORING

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DEVELOPMENT OF DIAGNOSIS TOOLS
FOR BRETTANOMYCE MONITORING IN WINERIES.

Instrument Co-operative Research Project

FINAL ACTIVITY REPORT

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Publishable executive summary

Three innovating detection methods for Brettanomyces, which are reliable, fast and not very expensive.

The **Consortium**, has conceived, for the wine and drinks fields, an innovating system which can detect a Brettanomyces contamination; Thanks to its innovation, **the Consortium** allows to get an essential advance by marketing three revolutionary detection tests: immunological, genomic and micro biological.

This coming from a 30 months research in collaboration with the European Commission.

NKS Brettanomyces and agrar culture media:

Culture media on NKS dehydrated cardboard discs meet all the needs of controls imposed by health regulations.

The NKS culture media or agar Brettanomyces is based on a decoloration of its colour into yellow, due to the environment acidification. The kit include the specific culture media put in a sterile Petri dishes, with the adequate filtrating membranes.

Real time PCR

The polymerase chain reaction is a method of biology molecular. The PCR remains an in vitro targeted application technique. It makes it possible to obtain, from a complex and small sample, large quantities of specific and defined-length DNA from a DNA fragment.

This kit presents a lot of advantages : **Great sensitivity, Simplicity of implementation and less tire some preparation, Rapidity: results obtained in less than 4 hours, Possibility of automation**

ELISA method

ELISA method relies on the performance of an immunological test used for detection of Brettanomyces in wine.

This immunological test is intended to detect and/or dose a protein in an organic liquid. This kit presents a lot of advantages : **Time saving, Selectivity, Ease of use and preparation, Rapidity: results obtained in less than 4 hours, Reliability, Possibility of automation**

These 3 kits, advantageously make it possible to the producers to carry out analyses of Brettanomyces detection by themselves. In addition to the speed of analysis, guarantee safety, profitability, flexibility and savings.

Today the results of these kits are very promising but in the current state of the results, none of the 3 methods is usable and saleable. It remains a lot of R & D to finalise the product

Section 1 – Project objectives and major achievements during the reporting period

Overview of general project objective

The yeast genus *Dekkera* (anamorph: *Brettanomyces*) plays a significant role in flavour production in beer and wine. Low amount may be necessary for typical “flavours”, but oversynthesis of volatile phenols (4-ethyl phenol (4-EP) and 4-ethyl guaiacol (4EG)) or tetrahydropyridines, results in production with "animal" or “mousy” type odour. To avoid the development of *Dekkera*, winegrowers can only use preventive measures. Rigorous hygiene in wine cellar contributes to avoid the development of the micro-organisms.

Problems due to *Brettanomyces* appear mostly during storage and aging of wine. The problem is especially relevant in large wood barrels because they are difficult to clean and some species can use cellobiose as carbon source. Furthermore, the present trends in quality wine making, involving the production of very concentrated wines with high alcoholic degree, combined with the use of micro-oxygenation techniques, favour the development of *Brettanomyces* sp.

Because of the ubiquitous presence and high spoilage potential of *Dekkera* (*Brettanomyces*), there is a demand for a simple, fast and reproducible monitoring method. The earlier the detection in the wine, the better the chances for winegrowers to prevent further growth and spread to other wine batches.

It is today difficult to obtain a fast diagnosis of the presence and amount of *Dekkera* yeasts because methodologies of analysis are complex and poorly diffused among wine-makers. A few specialised laboratories are able to carry out analyses. The most widespread methods of analysis use customary media and techniques, where it can take a week or longer before growing colonies become visible, a far longer incubation time compared to other yeast, which may overgrow *Dekkera* colonies and prohibit proper detection. Strictly selective media are not available commercially. Other detection methods based on molecular techniques have been developed in the recent years (e.g. RAPD-PCR, in situ hybridisation with fluorescence detection, fatty acid profiling). These techniques are powerful but can only be implemented by specialised laboratories.

The project aims at improving methodologies for *Brettanomyces* monitoring. The project has four main objectives.

1. Gain new knowledge on Brett and the off flavour production mechanism

Research will determine if the off flavour production depends on the species, possibly on the strains of *Dekkera/Brettanomyces*. Other objectives will be to determine at which stage of the *Brettanomyces* growth, off flavour is produced, as well as the parameters influencing its production. This information is needed for an efficient monitoring in wineries.

2. Development of new specific media for optimal growth of *Brettanomyces*.

Existing media does allow a quantification of *Brettanomyces* in less than 5 days. The objective is to develop strain selective media that will allow colony detection after 24 – 36 h as with

other yeast. The specificity will be higher than with existing media inducing lower false negative and positive result.

3. Improvement of molecular techniques based on PCR for detection of Dekkera / *Brettanomyces* in laboratories

The final objective is to develop a PCR kit for Brett detection. Specific objectives are:

- the development of a quantitative detection system
- the development of a detection method able to obviate the necessity for DNA isolation, cultivation or preenrichment;
- the generation of a database for DNA sequences of strains belonging to the genus *Brettanomyces*, and DNA fingerprints of them.

4. Development of a new diagnostic test kit for easy detection of Dekkera / *Brettanomyces* in wineries

The final objective is to develop of new *Brett* diagnostic kits based on immuno detection methods (ELISA). The kit will be commercialised to wineries. Scientific objective will be to obtain a panel of monoclonal antibodies for *Brett* strain.

Summary of the work objectives performed and main achievements for the reporting period

BRETT MONITORING is organised in 7 work-packages: Research on Brett (WP1), Selective Media (WP2), PCR (WP3), Immunoassay probes (WP4), Validation test (WP5), Diffusion (WP6) and Co-ordination (WP7). The project has a duration of 30 months. The present report covers the period from January 2006 to November 2006.

All work packages have been addressed during the project life time and all contractors were involved in the activities except for the WP5 Validation Tests. An overview of the objectives, achievement and deviations is given hereunder.

Table 1: Summary of objectives & achievements of the project

| Tasks | Deadline | Objective of realisation reached | | | | Chance to reach objective | | | |
|---|------------|----------------------------------|---|-----|---|---------------------------|---|---|---|
| | | 0% | < | 50% | < | 100% | ☺ | ☹ | ☹ |
| WP 1 Research on Brett | | | | | | | | | |
| T1.1 Bibliography | 31/07/2004 | | | | | X | X | | |
| T 1.2 Elaboration of a protocol for measurement | 31/07/2004 | | | | | X | X | | |
| T 1.3 Experimentation | 31/05/2005 | | | | | X | X | | |
| T 1.4 Analyze of the results | 31/08/2005 | | | | | X | X | | |
| WP 2 Selective Media | | | | | | | | | |
| T2.1 Research on strain selective media | 12/2005 | | | | | X | X | | |
| T2.2 Development of the media | 07/2006 | | | | | X | | X | |
| T2.3 Test | 07/2006 | | | | | X | X | | |
| WP 3 PCR | | | | | | | | | |
| T3.1 Research on the primer | 05/2005 | | | | | X | X | | |
| T3.2 Design of quantitative primer | 12/2005 | | | | | X | X | | |
| T3.3 Research on DNA extraction | 09/2006 | | | | | X | X | | |
| T3.4 Optimisation of PCR protocol | 09/2006 | | | | | X | X | | |
| T3.5 Test | 09/2006 | | | | | X | X | | |
| WP 4 Immunoassay probes | | | | | | | | | |
| T4.1 Development of monoclonal antibody | 12/2005 | | | | X | | | X | |
| T4.2 Development of the analysis device | 07/2006 | | | | X | | | X | |
| WP5 Validation test | | | | | | | | | |
| T5.1 Validation tests of the three detection techniques | 10/2006 | | | | | X | X | | |
| T5.2 Protocol design and monitoring methodology | 11/2006 | | | | | X | X | | |
| WP6: Diffusion | | | | | | | | | |
| T6.1 Preparation to commercialisation | 11/2006 | | | | | X | X | | |
| T6.2 Diffusion of the results | 11/2006 | | | | | X | X | | |
| T6.3 Normalisation | 11/2006 | | | | | X | X | | |

All the objectives of the different workpackage of the project have been successfully reached.

A summary of the deliverables planed for the first year is given hereunder:

Table 2 : Summary of the deliverables planed for the first year

| N° | Title | WP no | Lead Participant | Nature | Delivery date | |
|------------|---|-------|------------------|--------|---------------|---------|
| | | | | | Planned | Real |
| D1 | Report on the bibliographical study on BRETT | 1 | FOEB | R | 07/2004 | 06/2005 |
| D2 | Protocol for sample collection and measurement campaign | 1 | FOEB | Co | 07/2004 | 07/2004 |
| D3 | Report on the identification of strains responsible for the development of the off flavour | 1 | FOEB | Co | 09/2004 | 05/2005 |
| D4 | Report on the growth of Brett and on the parameters influencing the production of off flavour | 1 | FOEB | R | 08/2005 | 01/2006 |
| D5 | Report formulating recommendation for detection strategy | 1 | FOEB | R | 07/2006 | 11/2006 |
| D6 | Selective media for strains identified in T1.3 | 2 | Moeller | D | 02/2005 | 12/2005 |
| D7 | Primer for Brettanomyces producing off flavour | 3 | CSIC | Co | 04/2005 | 05/2005 |
| D8 | Measurement protocol | 3 | UM1 | Co | 08/2005 | 02/2006 |
| D9 | Monoclonal antibodies for strains of Brettanomyces producing off flavour | 4 | CSIC | O | 12/2005 | 12/2005 |
| D10 | Kit of Brettanomyces detection | 4 | Bionostra | P | 09/2006 | 09/2006 |
| D11 | Result of the validation test | 5 | Predel | R | 11/2006 | 11/2006 |
| D12 | Validated protocol for Brett detection using plating on selective media | 5 | Moeller | R | 11/2006 | 11/2006 |
| D13 | Validated protocol for Brett detection using PCR | 5 | Bionostra | R | 11/2006 | 11/2006 |

| | | | | | | |
|------------|---|---|-----------|---|---------|---------|
| D14 | Validated protocol for Brett detection using immunoassay probes | 5 | Bionostra | R | 11/2006 | 11/2006 |
| D15 | Guide of good practise on Brett monitoring in wineries | 5 | Predel | R | 11/2006 | 11/2006 |
| D16 | Marketing plan | 6 | Predel | R | 11/2006 | 11/2006 |
| D17 | Report on diffusion activities (publications etc.) | 6 | Predel | R | 11/2006 | 11/2006 |
| D18 | Plan for using and disseminating knowledge | 6 | Predel | R | 11/2006 | 11/2006 |

The table shows that at the end of the project, all the deliverables have been delivered.

Section 2 – Work package progress of the period

Deviations of the Project compared to the Technical Annex

At the kick off meeting on PCR a major change has been decided in the work package 3: the strain-specific PCR has been abandoned and replaced by quantitative PCR.

At the 18-month meeting in Valencia, Spain, the project achievements were on average 4 months behind the initial schedule because of delays in the elaboration of the detection methods.

The Consortium agreed to ask for a 6-month extension of the project. The European Commission accepted. The BRETT MONITORING project ended on November 30, 2006. Despite this extension, the validation tests were limited to 3 months for the PCR, 2 months for the selective media and one month for the Elisa kit. The validation tests only concerned the validation of the kits and not the validation of the methods.

WP 1 Research on Brett

Objectives

The main objective is to determine which strain of *Brettanomyces* produce the off flavour. Other objectives are to determine at which stage of the *Brettanomyces* growth, off flavour is produced as well as the parameters influencing its production.

Progress towards objectives

Task 1.1 Bibliographical research

The bibliography has been received from most partners, either abstracts or reference titles. All the articles have been found and most of them have been analysed. The bibliography is classified in several chapters. The report comprise for each chapter some keywords and references to the paper. In reality this work was much more time consuming than previously thought since many articles have already been published. Several partners have sent the same references.

The final document has been completed. It represents the deliverable D1 “Report on the bibliographical study on Brett” (attached to this report).

Task 1.2 Elaboration of a protocol

During the first meeting in Bordeaux, a discussion between all the partners led to an agreement on the way to build the Collection of different strains of Dekkera/Brettanomyces present in wineries.

A protocol of sampling was written and sent to the partners. It represents the deliverable D2 “Protocol for sample collection and measurement campaign” (attached to this report). It acts of collecting wine samples of the partners to obtain various samples of wine from different countries to optimize the chance to gather a broad lots of Brett.

After 3-5 months delay, even if the protocol was not exactly followed, FOEB finally could analyse 93 samples of all isolates and wines from the different regions (42 wines, 29 plates and 22 tube cultures).

Task 1.3 Implementation of the experimentation

- Working collection

The partners sent a great number of wine sample and also *Brettanomyces* yeast strains isolated in contaminated wines.

| Number of wine sample | Number of strains isolated |
|-----------------------|----------------------------|
| 93 | 68 |

From wine samples, colonies were isolated. From plates and tubes, cultures were purified. All isolates were identified to the species level using 3 different primers sets already described in the literature. Of all isolates, 3 were identified as *Brettanomyces. Anomala*.

A deeper investigation using the PCR-DGGE method, targeting different regions of the genome, separated the collection into two main groups.

CSIC IFI, Bionostra, UM1 and DSMZ have received the whole collection (68 strains) with the 2 representative strains chosen by FOEB (CB63 + CB28). These 2 strains have been selected based on their origin (Germany, Spain, France) and their molecular profile PCR-DGGE. This represents the Deliverable 3 “Identification of strains responsible for off flavour production” (attached to this report).

DSMZ has realised biochemical measurements on the strains of the collection in using 40 parameters. According to DSMZ, *Brettanomyces. bruxellensis* - the specie most frequently associated with off flavour in the literature- is reported to have a highly active esterase enzyme, which could be used as selective marker using chromogenic substrates.

Two isolates were chosen by FOEB according to the PCR-DGGE pattern and were sent to Madrid for antibodies production.

FOEB also performed an attempt of strain typing by PCR. In spite of some strain differences the method did not prove to be solely reliable. Restriction analysis followed by PFGE analysis was evaluated.

For further experiments, FOEB has done a selection of 8 strains using several criteria : origin, cell morphology, colony aspect, PCR-DGGE group. Each one exhibited a unique REA-PFGE pattern.

-Experimentation:

The experimentation aims to evaluate the ability of Brett strains to produce volatile phenols and to study the influence of several factors. A growth medium supplemented with cinnamic acids was elaborated. Carbon sources and ethanol concentrations were adjusted at different levels. Growth was followed by OD 600 nm measurement and volatile phenols by GC-MS.

In this study FOEB include an additional task: comparison of HPLC and GC-MS for 4-vinyl and 4-ethyl phenol determination, also for 4-vinyl and 4-ethyl guaiacol (vinyl and ethyl derivatives).

Task 1.4 Analysis of the experimentation

According to the results, the existing Brett detection kit and the opinion pool from wineries and specialist of wine, a new direction and change of the detection strategy in particularly PCR method is proposed.

At the Madrid meeting it was approved to identify all 68 strains collected for the project by conventional methods too. A set of 37 carbon sources, 2 nitrogen sources and studies for growth at 37 and 40°C was used to set up individual strain profiles. The details of this study are summarised in a table submitted by DMSZ to PREDEL and may be interpreted as follows.

63 strains could be identified as *B. bruxellensis* in accordance with literature data

- The assimilation pattern of these strains is quite uniform, variable results have been obtained for ribose, N-acetylglucosamin and lysine as a nitrogen source as well as growth at 40°C.
- Some strains showed differences in growth velocity on certain substrates.
- Very weak growth was observed on some carbon sources not increasing with time. It is interpreted as growth on impurities or breakdown products from the original substrate and not as utilisation of these substrates.
- The media used for morphological studies (cornmeal or rice agar) failed to support growth. DSMZ are currently searching for a working replacement to fill in the missing data on pseudomycelium formation.
- 5 strains (CB 52, 55, 56 and CB 60, 61) do not belong to *Brettanomyces/Dekkera*. They represent 2 different species whose identity is checked currently using more data (e.g. ascospores)
- None of the carbohydrates presented itself as a candidate for a truly selective medium supporting growth of *Brettanomyces* only, thus confirming the results from literature research.

For FOEB, the main objective was to compare strains for 4 EP production. This could not reasonably be undertaken before being sure that the cultures were really individual strains. Therefore on the side of the project a REA-PFGE protocol was evaluated. This method is currently under development in the FOEB laboratory, as it proved to be suitable for strain typing. Therefore the 8 strains of *B. Bruxellensis* used for the experiments were certified to be different. Two more strains of *B. Anomala* were included in the assay.

After preliminary tests with 3 strains, a medium containing glucose 20 g/l, or glucose 2 g/l and ethanol 8 % was chosen for experiments. After several attempts, the medium with ethanol as only carbon source was discarded due to the very poor growth of cultures. As an additional precaution it was verified that autoclaving did not damage the cinnamic acids, but it was preferred to add them after autoclaving the media alone.

In the glucose medium growth reached a stationary phase after 6-7 days. Of the 8 strains, 3 grew faster and reached a higher total population than the others. The two *B. Anomala* strains grew the slowest. The 4 EP concentrations accumulated during the 7 days assay were very high, from 40 mg/l to 71 mg/l according to the culture. The level was not directly correlated to the final population : e.g strains CB 68 produced 71 mg/l and reached OD₆₀₀ ≈ 3.6, while strain CB 28 produced 50 mg/l for a final O.D₆₀₀ of ≈ 6.5.

Growth in Glucose-Ethanol medium was only possible after "conditioning" the strains to ethanol by successive transfers incrementing the ethanol concentration of the culture media.

Finally growth was performed with 8% ethanol. As expected, the final O.D was much lower than without ethanol. The differences between the *B. Bruxellensis* strains was lower too. *B. Anomala* did not grow at all. In these conditions even with a final O.D between 0.7 and 1.0, the 4 EP produced was high and very similar for all strains ranging from 45 to 52 mg/l (determined by HPLC) and 40 to 47 mg/l (determined by GCMS). In these experiments the 4 VP concentrations were significant, suggesting that reduction to 4 EP is much slower in this medium. This might be related to a slower metabolism in difficult growing conditions.

Deviation from the project WP

The project was not deviated. However the bibliography took time to be achieved without having a big input on the progress of the program.

The large wine sampling and the great number of Brett strains present in wineries increasing the number of analysis. Additional tasks have been added such as typing by REA PCR-DGGE and comparison between GC-MS and HPLC.

At the 6 month meeting it has been decided that DSMZ will realize biochemical measurements on the strains of the collection. Around 40-50 parameters should be provided for each isolate.

Deliverables

D1: Report on the bibliographical study on Brett.

D2: Protocol for sample collection and measurement campaign.

D3: Report on the identification of strains responsible for the development of the off flavour.

D4 Report on the growth of Brett and on the parameters influencing the production of off flavour.

D5 Report formulating recommendation for detection strategy.

Milestones

Milestone 1: Identification of strain responsible for the off flavour production (partial result of T1.3)

WP2 Selective Media

Objectives

The objective is to develop a selective media. The media will be used on paper board support.

Progress towards objectives

T2.1 Research on strain selective media, on the support and on solvent

On the basis of results from WP1 the following medium composition was divided:

- PD broth (pH 6.0) as basis
- 0.02% alpha-naphtylacetat and 0.05% FastRed TR salt (Serva 21317.01) as chromogenic enzyme substrate
- Actidione (25 mg/L) to exclude *S. cerevisiae*

This medium on agar plates and cardboard nutrient plates is going to be tested with 8 strains from the CB collections and several wild yeasts under incubation at 35°C for its discriminating power.

DSMZ and Moeller prepared this medium on the 20th of June 2005. The results were not satisfactory because there was no colour reaction. After 24 hours of growth some colonies were already visible in the different media.

No true selective medium could be devised as no criterium separating *Brettanomyces* from other yeasts could be found in the literature. The pronounced ability to secrete acid into the growth medium was selected as the main criterion for the distinction of *Brettanomyces* from other wine associated yeasts; the shift in pH was made visible by a buffer/indicator system which showed a prominent yellow colour in the medium and in the colonies of *Brettanomyces*. Comparison to 12 yeast species relevant for wine showed that an initial pH of 8.0 showed the best discrimination; all these species will grow on the medium devised but the colour reaction is much weaker or missing at all.

T2.2 Development of the Media

The medium was initially developed as an agar based system; the formula proved able to be used for the production of nutrient pads (NKS) by Möller & Schmelz.

Results from the primary testing phase necessitated changes in the medium composition: the basic buffer system was changed to HEPES (20 mM) because of problems to recover colonies from single cells or low cell densities. Other components remain unchanged; the production of nutrient pads was not affected.

The addition of actidione (cycloheximide) to suppress growth of the wine yeast (*S. cerevisiae*) was not done during the developmental phase, but will be included in the final test kit to facilitate the detection of *Brettanomyces* cells despite the presence of larger numbers of wine yeasts. This is an established protocol for the fermentation industry and not subject for this project.

T2.4 Test

Recovery rates were determined by DSMZ for 3 *Brettanomyces* strains (CB 30, CB 47, CB 68) and several other yeast species selected earlier using agar plates and nutrient pads of the Brett-Medium in comparison to Potato-Dextrose-Agar as a standard medium for yeasts; incubation was done for 6 days at 30°C. Serial dilutions of a yeast suspension were plated directly or passed through membrane filters (0.65 µm) which were placed on the test plates.

Colony counts were found to be erratic and irreproducible on the phosphate-buffered medium with all of the *Brettanomyces* strains.

A modified buffer (see T2.2) gave highly improved results showing comparable colony counts within the same dilution step or filter. Nutrient pads with the newly formulated medium have been distributed to interested participants for testing and validation.

Deviation

Brettanomyces yeasts do not differ from other yeast species found in wine in their physiological or nutritive characteristics. Therefore it was found impossible to build a truly selective growth medium which would allow only the growth of one, defined yeast species. This corresponds to results and publications of other research groups in this field. The aim of this study was thus modified to result in a medium with improved optical recognition of *Brettanomyces* in comparison to other yeasts.

Actually the media is not selective. So the contamination and the development of the other micro-organisms in the media do not allow the end users to have relevant results. It is also necessary to improve the filtration membrane according to the step of wine sampling.

Deliverables

D6: Selective media for strains identified in T1.3

Milestones

M2 : Selective media for strains of *Brettanomyces*

WP3 PCR**Objectives**

The objective is to improve the measurement using PCR probes. Specific objectives are:

- to develop specific primers for strains responsible for the off flavour production,
- to improve the DNA extraction in order to lower the detection threshold.

Progress towards objectivesT3.1 Research on the primer

For the design of primers useful for the detection and *Brettanomyces bruxellensis* in wine samples by Real Time Quantitative PCR it was decided that CSIC and Bionostra would focus in RAD4 gene sequences, because UM1 would focus on procedures based on ribosomal DNA sequences. It was also decided to use RAD4 sequences not previously published, in order to improve the potentiality of the results.

Sequencing and alignment of these 11 new sequences, as well as alignment of the resulting consensus sequence with that of the homologous genes from other yeasts and filamentous fungi, has led to the identification of the more suitable regions (conserved among *B. bruxellensis* isolates but different from other fungal species) for the design of Real Time Quantitative PCR primers for sensible and specific detection of *B. bruxellensis*.

Then an alignment of all the sequences was made. The alignment permitted Bionostra and CSIC to design primers (DB5 & DB6) to amplify in the different strains from *D. bruxellensis* regions upstream and downstream of the region between DB1-DB2 a known sequence.

The culture of the different strains was made for isolating the DNA of each one.



The primers designed was used to amplify this DNA, and then the fragment obtained was sequenced to know exactly the correct sequence of *D. bruxellensis* in order to design new specific primers from *D. bruxellensis*. The newly obtained sequences served for the design of a new non-degenerated oligonucleotide (DB7) that was used in turn for the amplification of a 700 bp fragment, containing the 500 bp new sequence from 11 Brettanomyces isolates.

T3.2 Design of quantitative primer

In base on the obtained sequences alignment of the fragment amplified was designed primers and probe with the aim to develop a system of detection in real-time PCR (RT-PCR).

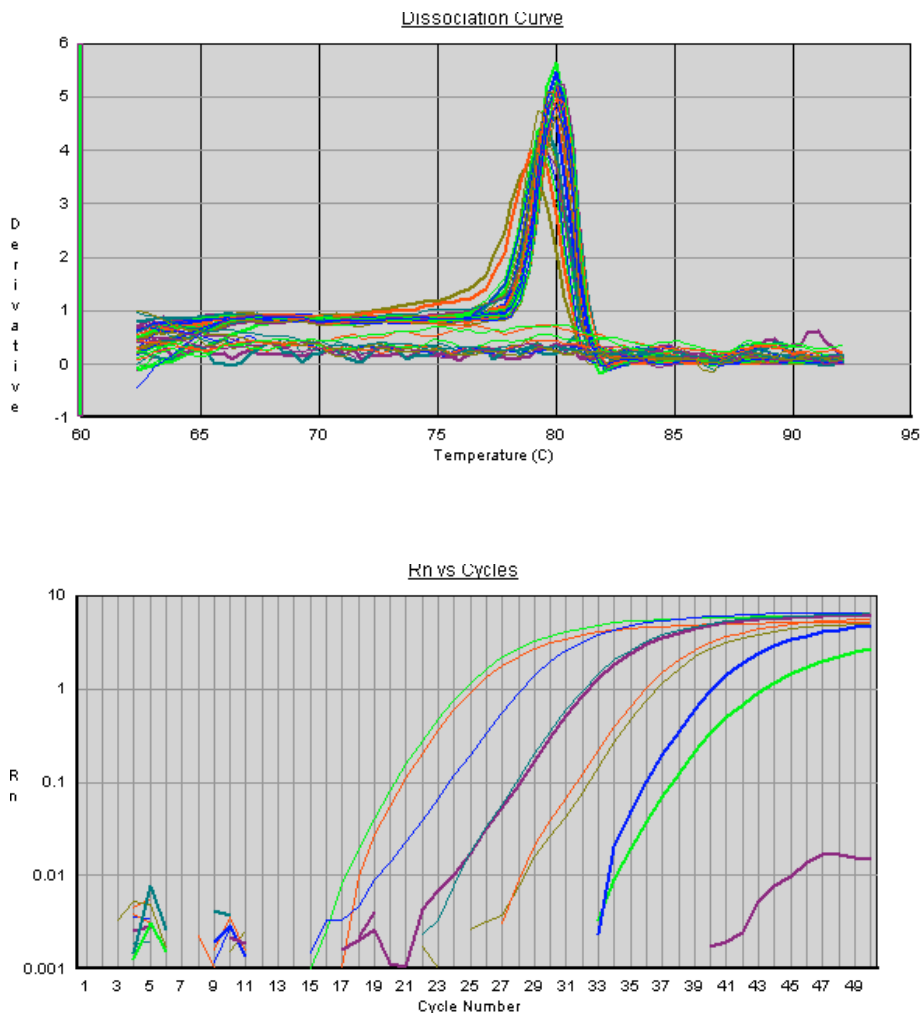
The primers designed by CSIC was tested in RT- PCR with Sybrgreen technology previously, to use the TaqMan technology. This experiments can give a first impression of the quality of its.

The samples used in this experiments are DNA extracted for different culture of the positive control of *D. bruxellensis* belonging to the collection of the project (9, 20, 28, 42, 44, 52, 60, 61 y 63). It is interesting to note that 52, 60 and 61 were genotyping like *D. anomala* and not *D. bruxellensis*. It was extracted DNA for culture of the other wine yeast different to *D. bruxellensis*, given by Bionostra and CSIC (*Hanseniospora quillermondii*, *H. uvarum*, *Sacharomyces cerevisiae*, *S. pastorianus*, *S. veronae*, *Rhodotorula mucilaginosa*, *Kluyveromyces lactis*, *K. marxianus*, *K. Zigosacharomyces ceveronae*, *Candida stellata*, *D. anomala*).

The PCR protocol used was: 50°C 2 min 1 cycle
 95°C 12 min 1 cycle
 50 cycles: 20 seg 95° C
 1 min 64°C

The results obtained are promising, because it not appears amplification curves in the negative control except to in *D. anomala* that the amplification curve are at the same level of the *D. bruxellensis* and the dissociation curves shown the same peak. The amplification of *D. anomala* is not a problem because these strains have been isolated from wines with the off flavour characteristic. Moreover, carrying out the amplification of the DNA of these strains with the primers DB1-DB2 followed by a nested PCR with DB3-DB4, it is possible to obtain a fragment in the nested PCR. This fragment was sequenced and the result showed that the sequence is the same that the corresponding to *D. bruxellensis*, indicating that both strains have a high similarity in the sequences.

In the following figure it is possible to see the amplification and dissociation curves.



These results represent the deliverable D7 “Primer for *Brettanomyces* producing off flavour” (attached to this report).

T3.3 Research on DNA extraction

UM1 had previously identified polyphenolic compounds as the main inhibitors of the PCR reaction. UM1 worked on the development of a method that would allow a quick separation of the yeast cells from the polyphenols. UM1 found that yeast cells could be separated efficiently from wine polyphenols by centrifugation through a layer of 60% glycerol. Most of the polyphenols are kept in a supernatant fraction while cells are found in the pellet. Using this separation system after collecting the cells by classical filtration UM1 have been able to detect *Brettanomyces* cells from wines with amounts as low as 100 cells/ml.

UM1 have examined different procedures of yeast cells recovery and treatment to increase the efficiency of the direct PCR detection.

The use of filtration was shown to be an effective manner to collect yeast cells. However, it seems that with wine containing high polyphenol amounts the filtration per se could favor association of polyphenols with yeast cells.

The use of centrifugation as a first step in yeast recovery was examined. UM1 observed that cells could be recovered with a good efficiency by a single centrifugation of wine if a limited

amount, 25 mL, was used. Since this procedure was easier to perform UM1 carried on the other experiments with this recovery method. A rapid step of filtration on a filter paper was however considered to remove big debris when dealing with “dirty” wines

UM1 treated in all the cases the cells suspension by centrifugation on a glycerol layer as proposed previously. Different procedures of cells washing after the glycerol separation step were examined on the PCR efficiency from a wine containing various amount of cells from 10^4 to 10 cells/mL.

UM1 examined the effect of the washing solution polarity on the detection threshold. The polarity of the medium is expected to influence the interaction between polyphenolic compounds and yeasts cells. In order to increase the polarity UM1 washed the cells with increasing amount of ethanol solution. UM1 found that washing the cells with a 30 % ethanol solution slightly decreased the detection threshold in our system (from $8 \cdot 10^2$ to $5 \cdot 10^2$ in the tested wine).

UM1 examined the effect of the pH of the medium used for yeast cells washing. UM1 did not observed any effect of repeated washing at low pH (2.5) on the detection threshold. When the pH was raised to the basic zone UM1 did observe an improvement of the detection suggesting that high pH are favourable to inhibitors removal. The pH used was 8.0, fixed by a 50 mM Tris-Cl buffer.

The effect of temperature of cells washing was examined. An incubation of the cells at 50°C during 10 min appear to slightly increase the PCR detection sensitivity indicating that inhibitors could be also eliminated by this step.

The different washing steps were combined and included in a new protocol. Using this protocol UM1 have been able to detect *Brettanomyces* cells from 10^2 to $7 \cdot 10^2$ cells/mL depending on the wine.

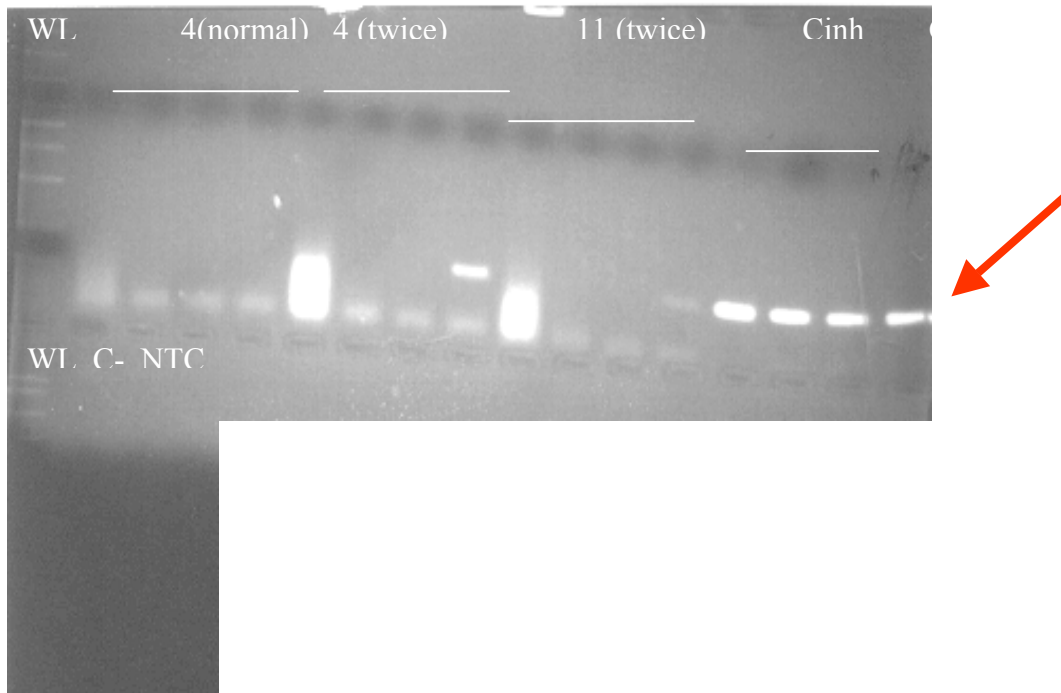
T3.5 Tests

Using the protocol sent by UM1 to clean cells, BIONOSTRA used wine samples sent by Agrovin - which were previously tested by culture medium (table 1) - to validate the extraction method.

| Number | Ufc/100 ml |
|--------|-----------------------------|
| 1 | 1 ufc/100 ml |
| 2 | 1 ufc/100 ml |
| 3 | $2,2 \cdot 10^3$ ufc/100 ml |
| 4 | $4 \cdot 10^2$ ufc/100 ml |
| 8 | 60 ufc/100 ml |
| 9 | $1,3 \cdot 10^3$ ufc/100 ml |
| 10 | $2,1 \cdot 10^4$ ufc/100 ml |
| 11 | $4 \cdot 10^4$ ufc/100 ml |
| 12 | $8 \cdot 10^4$ ufc/100 ml |
| 13 | $9,8 \cdot 10^3$ ufc/100 ml |
| 14 | $4,7 \cdot 10^3$ ufc/100 ml |
| 16 | $2,7 \cdot 10^4$ ufc/100 ml |

Table 1: Level of contamination of the wine tested

After the treatment of the samples BIONOSTRA realised the PCR protocol using different dilutions and all of them duplicate. In determinates cases, when the wine is very dirty or with a lot of sediments, it is necessary to pass twice by the glycerol column (fig 1) to the analysis work well.



Picture: 1. **4(once):** wine sample with 4×10^2 ufc/100ml, dilution from 1/10 until 1/1000. **4(twice):** wine sample with 4×10^2 ufc/100ml dilution from 1/10 until 1/1000, passed twice by glycerol column. **11(twice):** wine sample with 4×10^4 ufc/100ml, passed twice by glycerol column, dilution from 1/10 until 1/1000. This sample when is passed only one through the column it was not possible to had an amplification band (not sown in the picture. **C inh:** Inhibition control; **C+:** positive control. **WM:** weigh leader. **C-:** negative control. **NTC:** non template control.

To analyse the reproducibility and repetitiveness of the protocol, BIONOSTRA performed twice the same protocol with the same wine. We can observed that the protocol is not repetitive, because we do not have the same bands in the same replicas (figure 2).

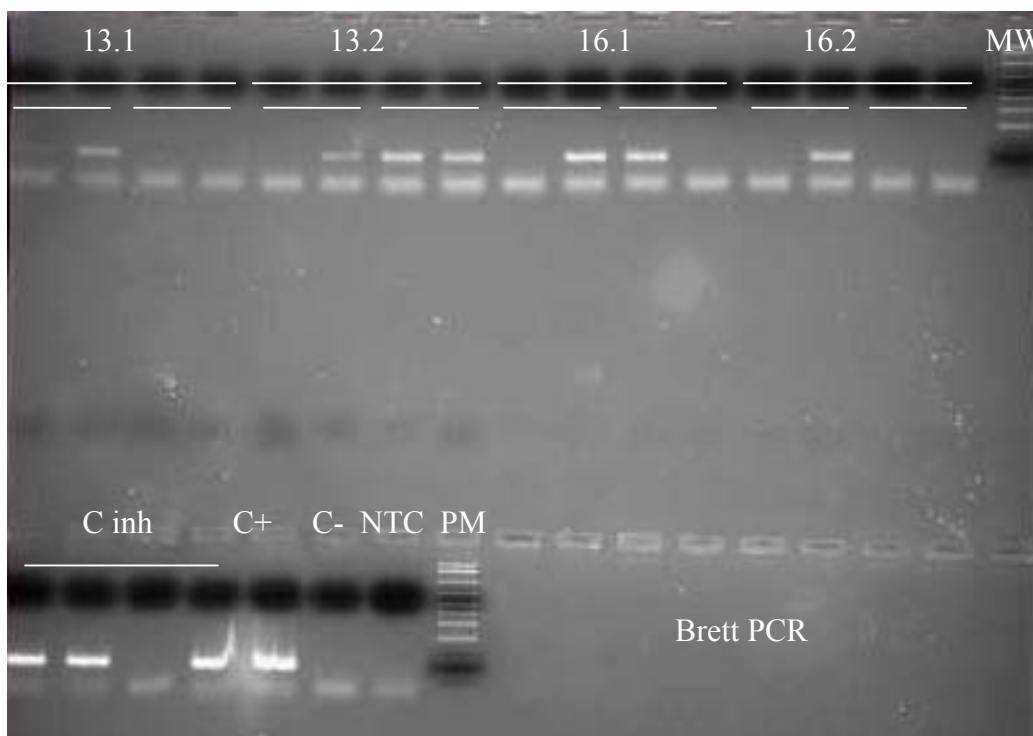


Figure 2: 13.1 and 13.2: samples from the same wine treated like different samples. The wine had $9,8 \times 10^3$ ufc/100ml. There was put twice dilution 1/100 and 1/1000. 16.1 and 16.2: samples from the same wine treated like different samples. The wine had $2,7 \times 10^4$ ufc/100ml. **C inh:** Inhibition control; **C+:** positive control. **WM:** weigh marker. **C-:** negative control. **NTC:** non template control.

It has been tested 12 different wines with different level of contamination of Brett, measured by culture medium (table 1), in which we detect until 60 ufc/100ml. This indicates that the sensibility of the detection system is 60 ufc/100ml.

BIONOSTRA designed a kit, what means a box whit bottles that contain the different reactive to perform the analysis (purification of the cells and PCR) and an instruction manual that explains how to proceed. BIONOSTRA prepared some kits to send to the end users and PREDEL.

To validate the kit BIONOSTRA received more wines from Agrovin (table 2) and made the analysis twice for each wine doing a purification of the cells following the protocol from UM1. Moreover, BIONOSTRA analyse twice again the same wine but introducing a modification in the protocol, consisting in a additional step of prefiltration to try to clean more the dirty wine.

| Muestra | Culture medium (ufc/100 ml) |
|----------|-----------------------------|
| (4031/2) | $6,3 \cdot 10^3$ |
| (4031/4) | $2 \cdot 10^4$ |
| (4032) | 4 |
| (4104-2) | $2,4 \cdot 10^2$ |
| (4104-3) | 4 |

Table 2: Level of contamination of wines tested

As you can see in the figure 3 this additional step does not improve the results. All the PCR were made with different dilutions and twice.

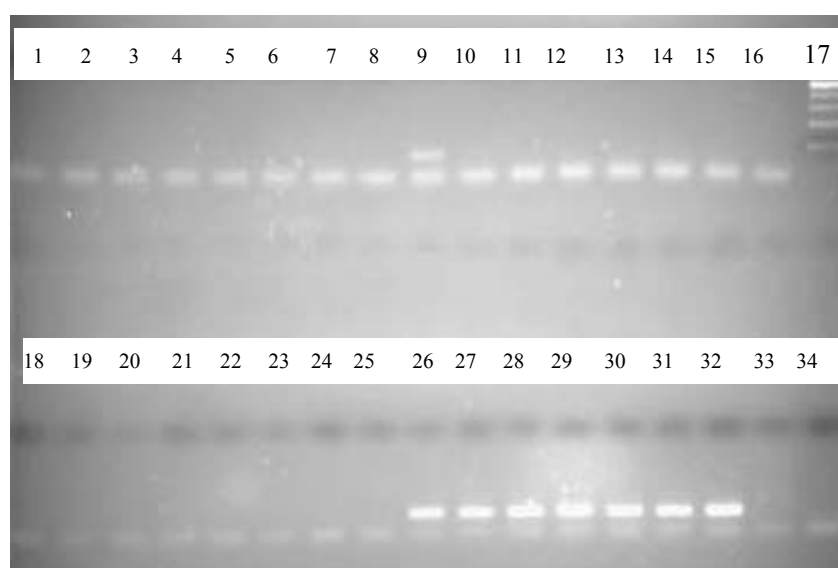


Figure 3: agarose gel that shows the results using the PCR detection kit

| | |
|-------------------------------------|--|
| 4031/2 dilution -2 | 4032 dilution -2 |
| 4031/2 dilution -3 | 4032 dilution -3 |
| 4031/2 dilution -4 | 4032 dilution -4 |
| 4031/2 dilution -5 | 4032 dilution -5 |
| 4031/2 with prefiltered dilution -2 | 4032 dilution prefiltered dilution -2 |
| 4031/2 with prefiltered dilution -3 | 4032 dilution prefiltered dilution -3 |
| 4031/2 with prefiltered dilution -4 | 4032 dilution prefiltered dilution -4 |
| 4031/2 with prefiltered dilution -5 | 4032 dilution prefiltered dilution -5 |
| 4031/4 dilution -2 | inhibition control sample 4031/2 |
| 4031/4 dilution -3 | inhibition control sample 4031/2 prefiltered |
| 4031/4 dilution -4 | inhibition control sample 4032/4 |
| 4031/4 dilution -5 | inhibition control sample 4031/4 prefiltered |
| 4031/4 with prefiltered dilution -2 | inhibition control sample 4032 |
| 4031/4 with prefiltered dilution -3 | inhibition control sample 4032 prefiltered |
| 4031/4 with prefiltered dilution -4 | positive control |
| 4031/4 with prefiltered dilution -5 | negative control |
| Molecular weigh | NTC: non template control |

The final conclusion are the kit works well with wines which do not contain a lot of debris, and always performing the PCR reaction with different dilutions and we tested all twice, data not shown, and BIONOSTRA advice to perform always an inhibition control.

Conclusions: It is necessary to improve the method to clean the wine and purify the cells to have good results in the posterior analysis.

Deliverables

D7: Primer for *Brettanomyces* producing off flavour.

D8: Measurement protocol.

Milestones

M3: Primer for *Brett* available.

WP4 Immunoassay

Objectives

The first objective will be to obtain a panel of monoclonal antibodies for each *Brett* strain identified in T1.3. The final objective will be the development of new *Brett* diagnostic kits based on immunodetection methods (ELISA).

Progress towards objectives

T4.1 Development of the monoclonal antibodies

Objectives

On this period CSIC focused its work in the production, purification and labelling of a panel of previously selected monoclonal antibodies to *Brettanomyces*. These monoclonals were evaluated in the ELISA sandwich format with the aim of developing a diagnostic test kit for easy detection of Brett in wineries.

Results

Antibody production and labelling.

From the panel of 19 monoclonals initially identify as able to recognise Brett, 10 hybridomas were finally selected for production at milligram quantities and evaluation in the sandwich format.

The cell lines were cultured in Petri dishes at 37 °C in an atmosphere containing 5% CO₂. The medium used was DMEM supplemented with fetal bovine serum (15%), hypoxanthine (100 µM), thymidine (16 µM), and non-essential amino acids (1 mM). Medium was collected in late stationary phase, centrifuged to remove cells and debris, and the supernatant precipitated with a saturated solution of ammonium sulphate. Antibodies were collected by centrifugation, purified by affinity chromatography on protein G-Sepharose, and quantified by UV spectroscopy. The table below shows the amount of monoclonal antibody obtained from each hybridoma line.

| Immunogen: Brett#28M strain | | Immunogen: Brett#63M strain | |
|-----------------------------|-------------|-----------------------------|-------------|
| Antibody | Amount (mg) | Antibody | Amount (mg) |
| 28M#1.1 | 0.72 | 63M#1.1 | 0.90 |
| 28M#1.2 | 2.98 | 63M#3.1 | 1.93 |
| 28M#2.1 | 1.78 | 63M#3.3 | 3.73 |
| 28M#3.3 | 3.36 | 63M#4.1 | 1.85 |
| 28M#3.4 | 2.87 | 63M#4.2 | 2.12 |

Table 1. List of monoclonal antibodies produced.

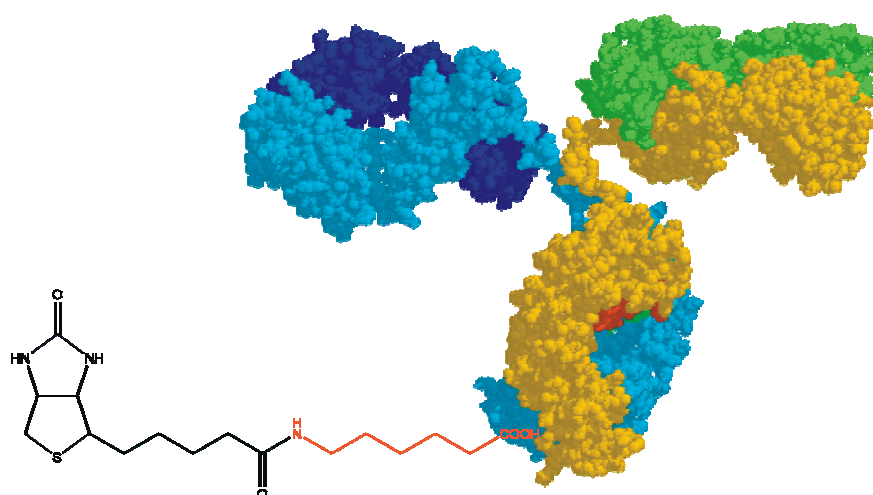


Figure 1. Schematic representation of an antibody molecule labelled with biotin.

An aliquot of each antibody was further labelled with biotin using the kit developed by Pierce. According to the manufacture instructions, each antibody should be labelled with 2-3 biotin molecules per antibody molecule. A schematic representation of one of these conjugates is shown in figure 1.

The functionality of labelled antibodies was assessed by indirect ELISA, where plates were coated with a number of yeast species. Both labelled and non-labelled antibodies were added to the wells, and their respective recognition pattern was compared (Figure 2).

Results clearly showed that biotin labelling did not affect at all antibody functionality; each labelled antibody recognised the same yeast species and at the same extent that its non-labelled counterpart did it.

At this point, we were ready to begin the development of the ELISA assay for Brett detection.

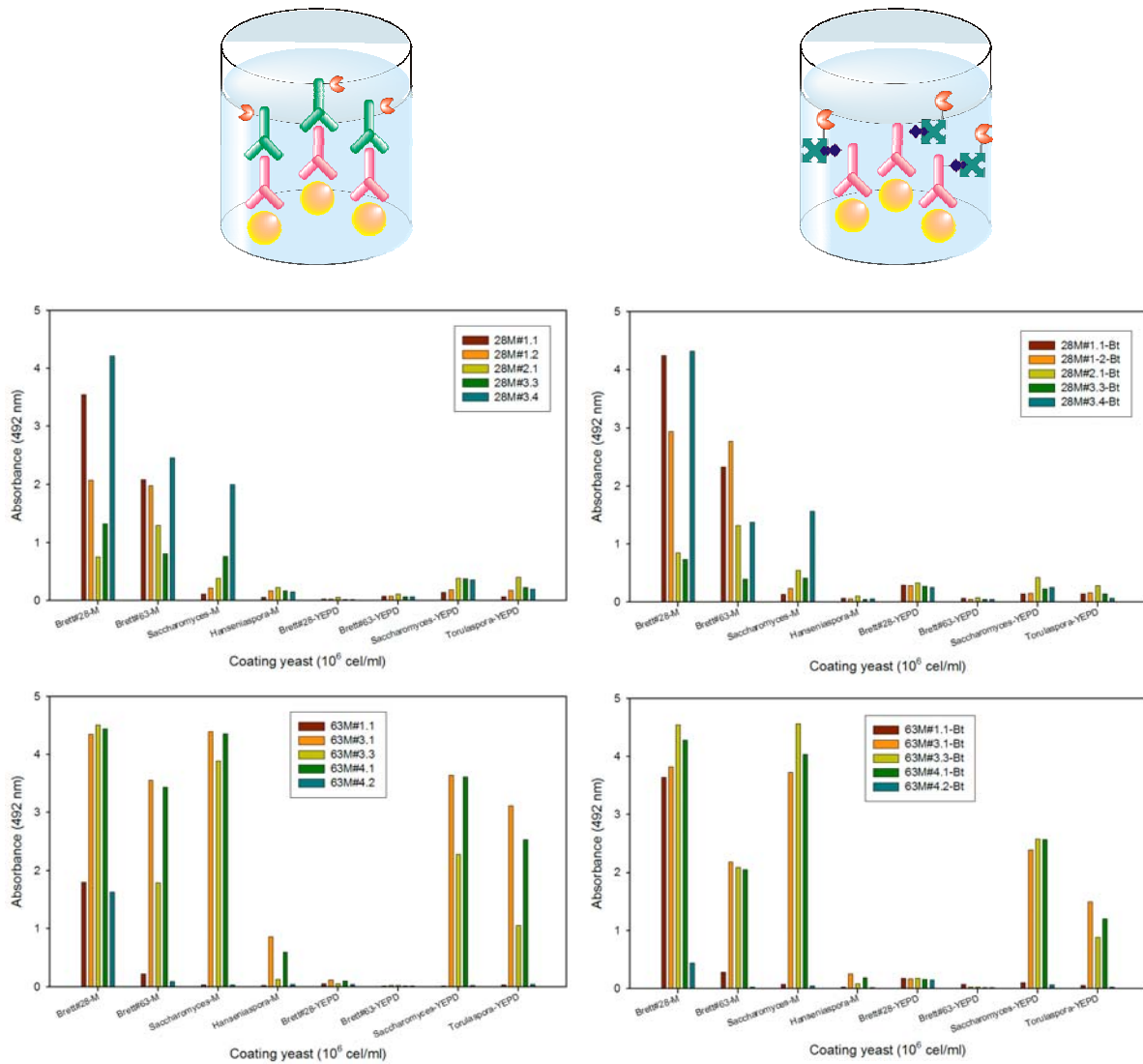


Figure 2. Recognition pattern of labelled and non-labelled antibodies by indirect ELISA against different yeast species.

T4.2 Development of the analysis device

CSIC tested the IATA antibodies against 28 and against 63 in order to try to clarify what happened with the antibody in the ELISA and to see if the antibodies recognise the same or different proteins. First of all, using together the monoclonal antibodies that proceed of the same antigens (culture of brett 63 or 28) and after one by one.



Figure 4: This shown three western blot using almost the same samples and all the antibodies for group 28. **RB1.1, RB1.2, M9.1, M9.2, M12.1, M12.2** are different cultures extracts of the same isolated of Brett (1, 2), + is positive control, they are different in the same western blot.

As the figure 4 shows the results are not reproducible, which corresponding with the ELISA assays. The same occurs with 63 antibodies.

CSIC also did the western with the five types of 28 group antibodies one by one, in which CSIC could see different bands in 28#M1.1 one of 30 KDa; 28#M3.3 one of 25 KDa; 28#M3.4 one of 39 KDa this last as in 28#M1.2 (data not shown).

In 63 groups of antibodies, only 3 of 5 worked in western analysis. This could be because the western analysis is made in denaturalised conditions and it is possible that the epitope that recognised the monoclonal antibodies were denaturalised two.

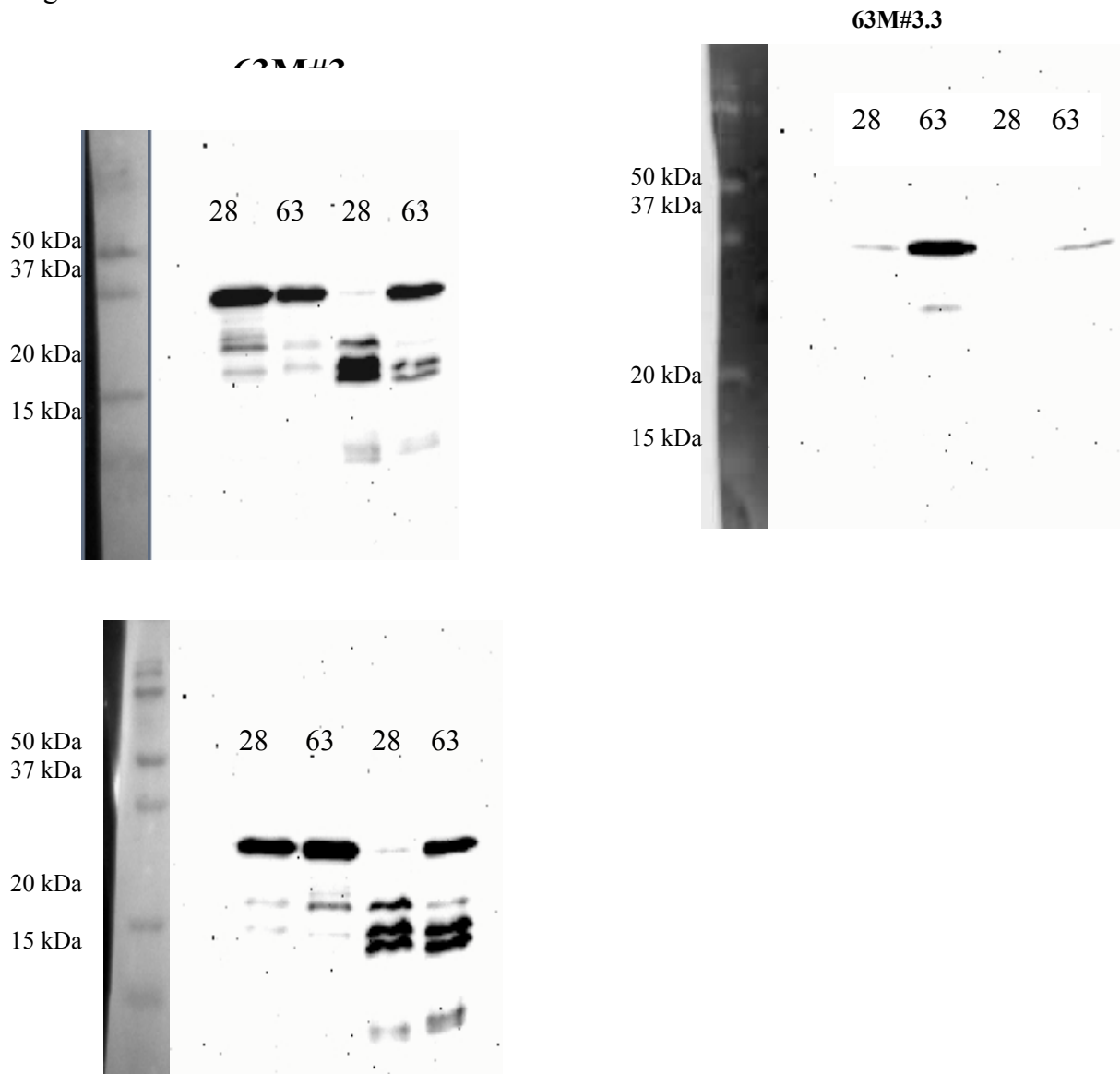


Figure 5: Western blots of 63 antibodies group. It is shown 3 western blots, each one with a different antibody **63M#4.1**; **63M#3.3**; **63M#3.1**; **28**: culture of this yeast. **63**: culture of this yeast. **28**: different culture of this yeast than the other. **63**: different culture of this yeast than the other

We saw that in western happened the same that in the ELISA assays, some time recognised sometime not.

CSIC also try to analyse wine samples in western analysis. CSIC prepared the samples in two different ways. One following, one centrifugation of the wine and the other one following the same protocol to clean the cell of the wine used in the PCR kit. The result are not reproducible.

The theory of CSIC and BIONOSTRA to explain this results (some times yes, some times not) is the situation of the culture (stress, oldness, freezer-tawed,...). When the culture is fresh and it have not suffer any alteration the result is negative and if it is old, it has suffered some freezer-tawed cycles, the result is positive. The experiments of ELISA performed by CSIC indicate this theory, and after they were confirmed by BIONOSTRA by western analysis. To demonstrate it, CSIC prepared new culture to analyse directly without any freezer step by ELISA in IATA and by western by BIONOSTRA. In theory, the result seems to be in the opposite of BIONOSTRA's idea, but this can have an explanation, that in this occasion the culture growth very bad and slowly, then the result confirms the theory (figure 6 and 7).

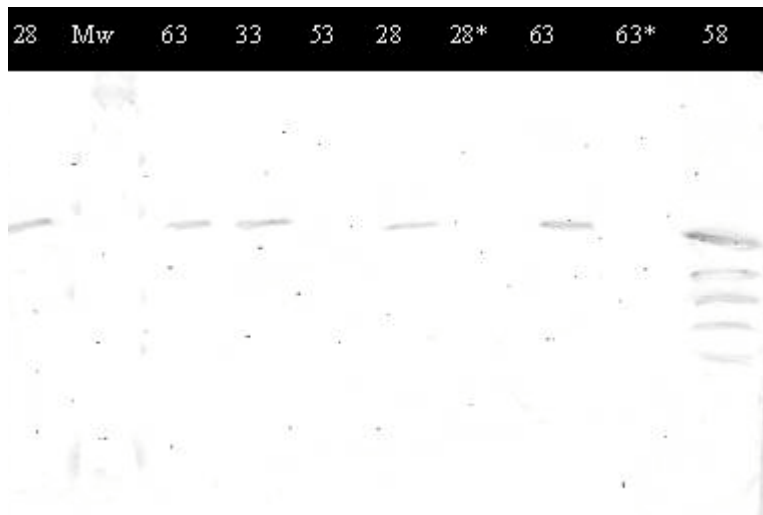


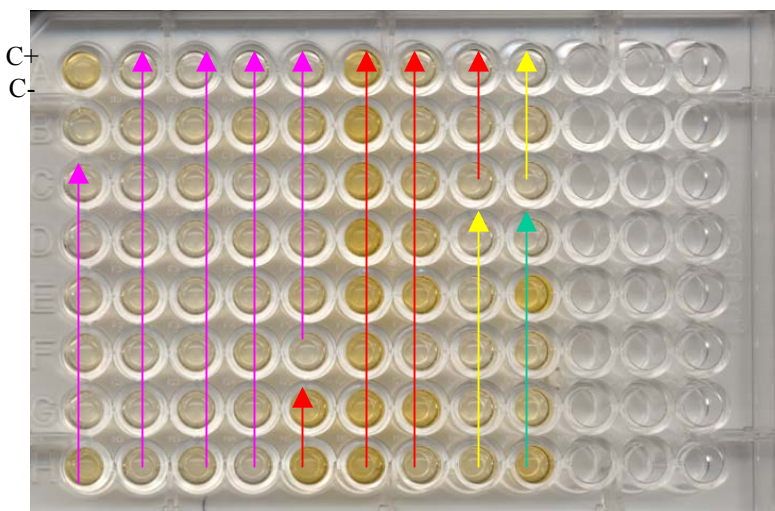
Figure 6: 28 pool western blot. All the numbers represent the name of the yeast culture. Has appeared two 28 and two 63, those are different cultures of the same yeast. Also appeared one 28 and 63 marker with *, it means that is the supernatant of the culture. **MW**: molecular weight



Figure 7: 63 pool western blot. All the numbers represent the name of the yeast culture. Has appeared two 28 and two 63, those are different cultures of the same yeast. Also appeared one 28 and 63 marker with *, it means that is the supernatant of the culture. **MW**: molecular weight

BIONOSTRA designed a kit to detect Brettanomyces by ELISA technology, this mean a box with the reactivs and plates coated by antibodies necessities to perform the analysis and the manual instruction. BIONOSTRA prepared some kits to send to the end users and PREDEL

To validate the kit firstly we tested the Elisa plates sent by CSIC. The wine samples were prepared in two different way, one centrifugation the wine and the other following the same protocol for the PCR kit (processed wines). In the first test, we put positives and negatives cultures and samples processed (figure 8). The positive control value was low, only the positive culture given a good signal. These plates were tested twice with identical results.



Wine samples centrifuged and processed
 Positive cultures
 Negatives cultures
 Wine samples processed

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|-------|-------|-------|--------|-------|-------|-------|-------|-------|
| A | 0,234 | 0,079 | 0,059 | 0,063 | 0,065 | 0,348 | 0,116 | 0,065 | 0,062 |
| B | 0,101 | 0,098 | 0,116 | 0,0157 | 0,153 | 0,386 | 0,126 | 0,085 | 0,155 |
| C | 0,078 | 0,063 | 0,103 | 0,066 | 0,063 | 0,313 | 0,153 | 0,106 | 0,096 |
| D | 0,063 | 0,081 | 0,083 | 0,111 | 0,076 | 0,451 | 0,219 | 0,65 | 0,83 |
| E | 0,085 | 0,092 | 0,068 | 0,084 | 0,118 | 0,325 | 0,271 | 0,06 | 0,489 |
| F | 0,102 | 0,058 | 0,079 | 0,118 | 0,057 | 0,277 | 0,084 | 0,73 | 0,162 |
| G | 0,089 | 0,066 | 0,085 | 0,098 | 0,269 | 0,324 | 0,297 | 0,102 | 0,16 |
| H | 0,211 | 0,098 | 0,128 | 0,063 | 0,316 | 0,316 | 0,126 | 0,138 | 0,307 |

Figure 8: Elisa using IATA plates. There are wines samples centrifuged and processed positive and negative cultures. In the table it is shown the Elisa results

BIONOSTRA coated new plates with the antibodies in the lab and the signal was higher, this indicated that perhaps the plate coated is not stable during the transport and it is for it that the plate included in the kits was sent with PBS buffer.

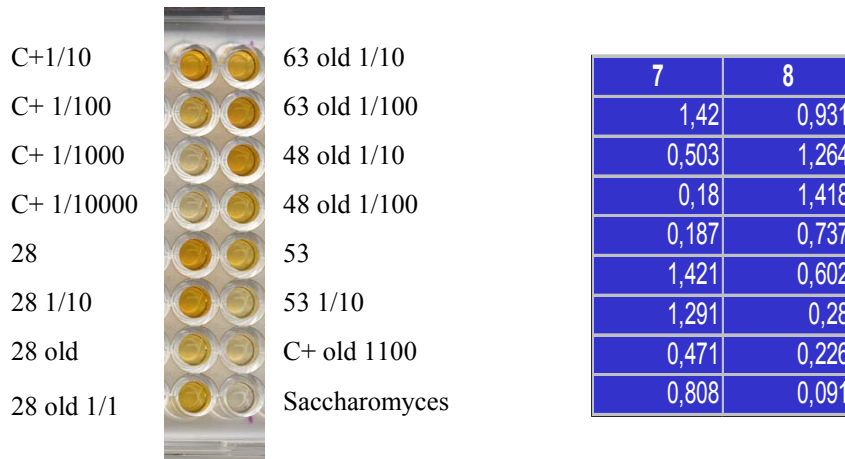
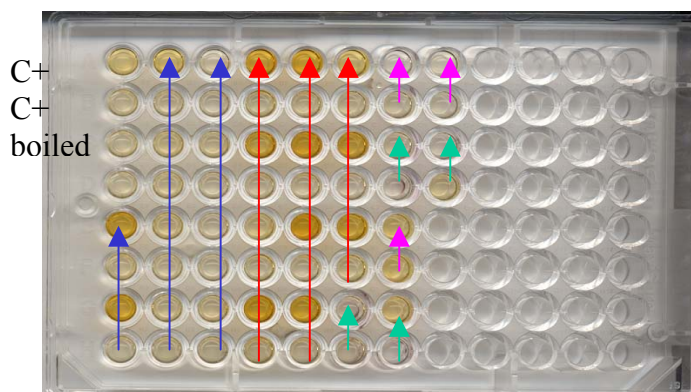


Figure 9: Elisa using antibodies coated by Bionostra. There are cultures of positive and negative controls.

In the following test was added wine samples processed and centrifuged, positives cultures. All the samples was added by duplicated, one normal and the next boiled. The results shows that the Elisa only recognises non boiled samples, which explain why did not had positive results in two of the five 63 group antibodies tested (figure 10).



Positive cultures dilution

Positive cultures boiled an not boiled.
Wine samples processed

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|-------|-------|-------|-------|-------|-------|-------|-------|
| A | 0,318 | 0,33 | 0,086 | 0,794 | 0,834 | 0,352 | 0,071 | 0,08 |
| B | 0,144 | 0,156 | 0,069 | 0,085 | 0,121 | 0,122 | 0,089 | 0,081 |
| C | 0,127 | 0,202 | 0,117 | 0,379 | 0,861 | 0,719 | 0,075 | 0,068 |
| D | 0,114 | 0,144 | 0,079 | 0,098 | 0,099 | 0,104 | 0,044 | 0,165 |
| E | 0,773 | 0,16 | 0,078 | 0,183 | 1,284 | 0,632 | 0,316 | 0 |
| F | 0,153 | 0,12 | 0,073 | 0,087 | 0,079 | 0,134 | 0,321 | 0 |
| G | 0,831 | 0,134 | 0,14 | 0,87 | 0,76 | 0,056 | 0,275 | 0 |
| H | 0,102 | 0,125 | 0,108 | 0,164 | 0,105 | 0,086 | 0,057 | 0 |

Figure 10: Elisa using BIONOSTRA plates. There are wines samples centrifuged and processed positive and negative cultures. All the samples put in the Elisa was added by duplicated one boiled and the other not. In the table it is shown the Elisa results

We can observe signal in some of the wine samples with a high amount of *Brettanomyces* and not very dirties. And it is not necessary in this case to purify the cells; it is enough with a previous centrifugation step of the wine.

Conclusions: It is necessary to improve the method to clean the wine and purify the cells to have good results in the posterior analysis.

Deviation from the project WP

Elisa kit has actually very high sensitivity so can not be applicable to allow a better monitoring of *Brettanomyces* contamination. To be useful, Elisa kit must be optimise to go down in sensitivity to analyse in the early step of *Brettanomyces* contamination during wine processing and in particular at the moment of bottling.

Deliverables

D9: Monoclonal antibodies for strains of *Brettanomyces* producing off flavour

D10 : kit of *Brettanomyces* detection

Milestones

M4: Monoclonal antibody for strains identified in T1.3

WP5 Validation and monitoring technology

Objectives

The objective are to validate the three detection techniques as well as elaborating a guide of good practises for efficient monitoring of *Brettanomyces* growth in wineries.

Progress towards objectives

T5.1 Validation tests

Agrovin Validation Tests

1) Culture media NKS.

Objective: Validation of NKS culture media in comparison with other media use to detect *Brettanomyces*.

Previous experiences: Agrovin's laboratory use to detect *Brettanomyces* in wine by means of DBDM agar media (Rodrigues *et al.*, 2001).

Samples: Potentially contaminated wines from different cellars of several Spanish wine regions. All of them are matured in oak from different harvest (from 2000 to 2005). Number of samples 20.

Comparative essay between DBDM agar media and NSK media. It is made at the same time with two different media. Indications given by each provider are followed:

| | NSK | DBDM |
|-------------------------------|------------------------------|--|
| Petri dish preparation | Add 3,5mL steril water. | Ready to use (agar prepared) |
| method | Filtración / membrane 0.60µm | Filtración /membrane 0.45µm |
| volumen | 100 mL. | 50-100 mL. |
| Incubation time | 3-5 days | 10-15 days |
| Colony identification | Yellow-orange colonies. | Yellow colonies that became darken with time Agar media turn from blue to yellow. Characteristic phenolic aroma. |

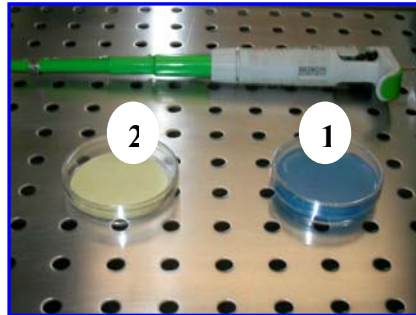


Fig. 1.- Detection of *Brettanomyces* with culture media: Before dish preparation
DBDM
2. MKS

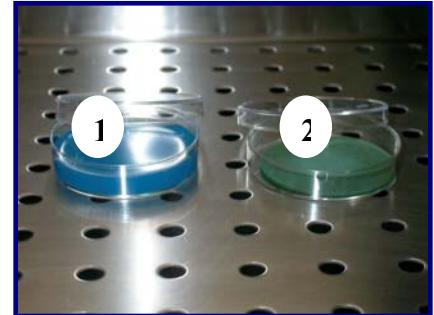


Fig. 2.- Detection of *Brettanomyces* with culture media: After dish preparation
1.- DBDM ready to use
2.- MKS after humidification

Colony identification: Microscopical identification of all the colonies to verify if they are *Brettanomyces* ssp. Use of optical microscopy (600A), looking for characteristic morphology of *Brettanomyces* ssp.

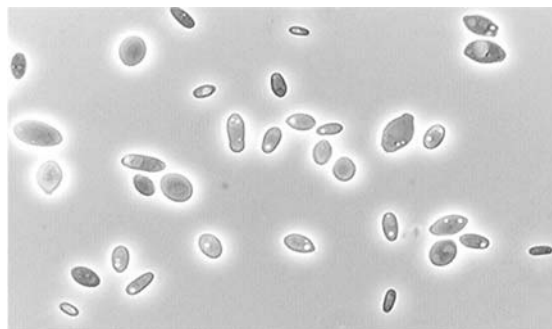


Fig. 3.- Characteristic morphology of *Brettanomyces* ssp. in culture media. (600A)

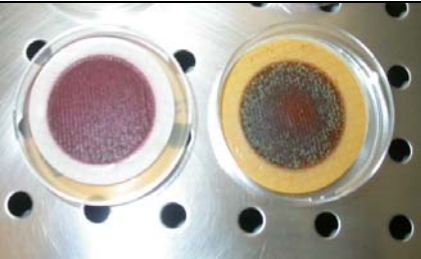

Results: Results are given in Table1

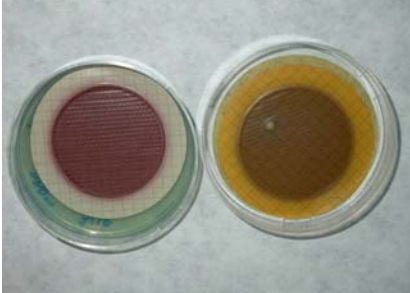

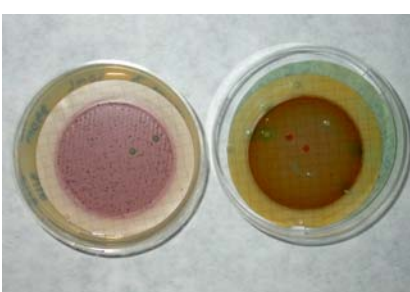
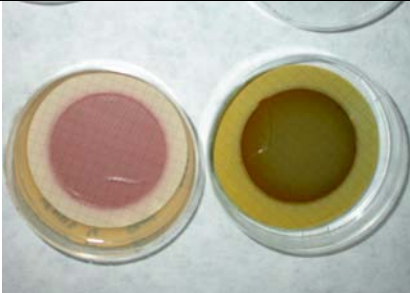

| Sample | DBDM (ufc/100 ml) | NKS (ufc/100 ml) |
|---------|----------------------|---------------------|
| A | < 1 | < 1 |
| B | < 1 | < 1 |
| C | < 1 | < 1 |
| D | < 1 | < 1 |
| 63 | $6,3 \cdot 10^3$ | $> 10^3$ |
| 90 | $2 \cdot 10^4$ | $> 10^3$ |
| L2B | <1 | < 1 |
| L7 | <1 | < 1 |
| Luz7 | 4 | < 1 |
| T.B. | <1 | < 1 |
| DEM | <1 | < 1 |
| VCZ 3L | <1 | < 1 |
| LOT 2 | <1 | < 1 |
| VCZ ROS | <1 | 5 |
| V1 | <1 | <1 |
| V2 | $2,4 \cdot 10^2$ | $1 \cdot 10^2$ |
| V3 | 4 | <1 |
| V4 | <1 | <1 |
| CM1 | <1 | <1 |
| CM2 | 38 | <1 |

In Yellow: Equivalent results in both media

In Pink: Not similar result in both media

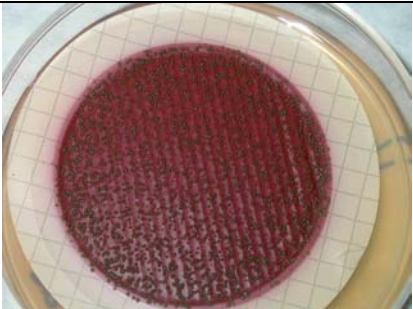
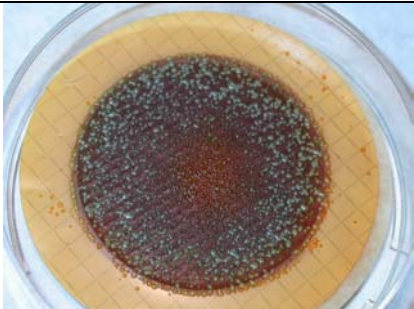
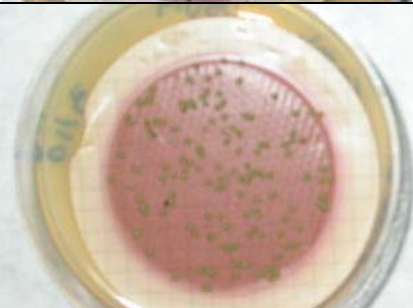
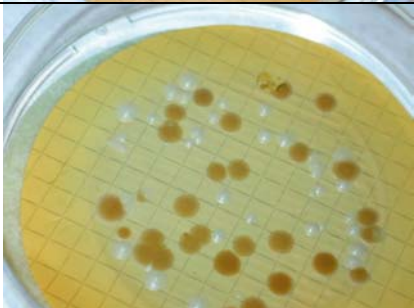
Equivalent results:

| Samples | MEDIO DBDM | MEDIO M&S NKS | Coments |
|---------|------------------|--|-----------------|
| | (ufc/100 ml) | Results (ufc/100 ml) | |
| 63 | $6,3 \cdot 10^3$ |  $> 10^3$ | Similar results |
| 90 | $2 \cdot 10^4$ |  $> 10^3$ | Similar results |

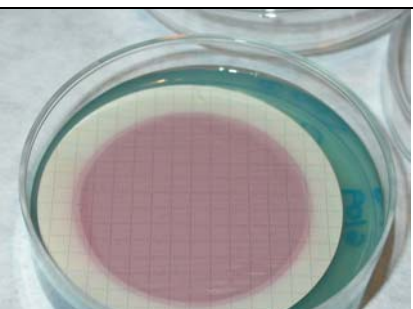
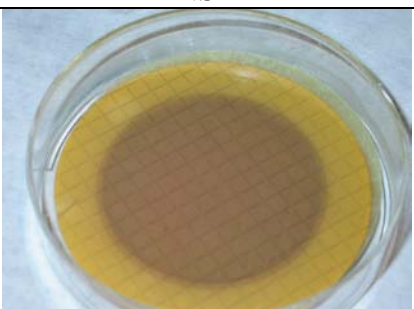


| | | | | |
|-------------------|---------------------------|--|-------------------------|---|
| <p>V1</p> | <p><1</p> |  | <p><1</p> | <p>No colonies in DBDM. One colony appears in NSK that is not Brett (microscopy 600 A).</p> |
| <p>V2</p> | <p>2,4·10²</p> |  | <p>1·10²</p> | <p>Homogeneous type of colonies in DBDM (Brett positive). Two different types of colonies in NKS</p> |
| <p>V3</p> | <p>4</p> |  | <p><1</p> | <p>Growth of two different types of colonies in DBDM. One of them is identified as Brett. In NSK media we distinguish three different types of colonies, none of them is identified as Brett.</p> |
| <p>V4</p> | <p><1</p> |  | <p><1</p> | <p>No colonies.</p> |
| <p>CM1</p> | <p><1</p> |  | <p><1</p> | <p>Colonies in DBDM that are not Brett. Colonies in NKS that are not Brett.</p> |

| | | | |
|-------------------|-----------|--|---|
| <p>CM2</p> | <p>38</p> |  | <p><1</p> <p>Two different colonies in DBDM. One of them is identified as Brett. In NSK media growth of colonies that are not identified as Brett.</p> |
|-------------------|-----------|--|---|

Positive results (Brett presence):

| BDBM | NSK |
|--|---|
|  |  |
|  |  |

Negative results (Brett absence):

| BDBM | NSK |
|---|--|
|  |  |
|  |  |

Conclusions:

Culture media DBDM and NKS are both **easy to use** in wine microbiology laboratory.

NKS medium in dehydrated pads, offers an advantage as **expiry date is longer and is easier to maintain.**

DBDM can detect *Brettanomyces* ssp at lower concentration: seems to be more accurate in few contaminated samples. **NKS is less accurate.**

DBDM is more selective, colonies grow in NKS medium need to be checked under the microscope. **NKS is not completely selective culture medium,**

Brettanomyces detection in DBDM is easier as can be noticed by phenolic odour in positive samples, change of medium colour and different colour of colonies. **NKS medium needs to have some experience in Brett identification** under microscope.

Agrovin considers that filtration procedure recommended by the producer(0,65um) is not enough to retain all Brett cells. **Filtration procedure could be improved**

2) PCR and Elisa.

Objective: Validation of PCR and Elisa procedure to detect Brettanomyces

Samples: Potentially contaminated wines from different cellars of several Spanish wine regions. All of them are matured in oak from different harvest (from 2000 to 2005).. All of these samples were analysed using culture media.

Samples were sent to BIONOSTRA to be analysed by PCR and ELISA techniques.

Results

| Samples | DBDM (ufc/100 ml) | NKS (ufc/100 ml) | PCR ADN r (presence/ausence) detection limit 60 ufc/ml | ELISA Results Purified cells.” | ELISA Results centrifuged wine |
|---------|-------------------|------------------|--|--------------------------------|--------------------------------|
| 63 | $6,3 \cdot 10^3$ | $> 10^3$ | Ausence | Ausence | ausence |
| 90 | $2 \cdot 10^4$ | $> 10^3$ | Presence | Presence | - |
| Luz7 | 4 | < 1 | Ausence | Ausence | Ausence |
| V2 | $2,4 \cdot 10^2$ | $1 \cdot 10^2$ | Presence | Presence | ausence |
| V3 | 4 | < 1 | Ausence | -- | ausence |

In Yellow: equivalent results with all the techniques

In Pink: Not similar results

Conclusions:

1.- Results with PCR , Elisa and culture media are quite similar. All the sample except one. The three technics can be use for detecting Brett cells.

2.- Not correspondance between culture media and PCR/Elisa **is in relationship with**

dirtyness of wine samples. Turbidity and solid particles can mask positive results.

3.- PCR and Elisa are faster than culture media.

4.-Elisa procedure with purified cells is more accurate than directly from centrifuged wine, but procedure is longer.

CA33 Validation Tests

In order to validate the three detection techniques, CA33 have first collected different samples from different wineries in Gironde. The wines were detected by its laboratories and they have collected the samples themselves and explain to the responsible the aim of the study done and the interest of the new methods studied included in a good practice methodology.

The samples were studied with the tree methods:

For the selective media:

A first evaluation has been done in the beginning of the year with the first type of media NPS compare to a non-saccharomyces media used as a control. Three pure species were evaluated in order to see the selectivity and eighteen samples from wineries.

A second evaluation has been done during the harvest and wine-making. Two selective media were studied (NKS and DSMZ) compared to non-saccharomyces media. Five pure species of yeast and bacteria were studied to evaluate specificity and twenty nine samples from wineries.

For each result obtained, three different dilution were used.

For the PCR kit:

Six samples from wineries were used for the evaluation. CA33 have first verify the contamination with Brettanomyces by classic PCR, growth on selective media non-saccharomyces and microscopic observation. For the evaluation of the Bionostra Kit, two different extractions and two repetitions have been done for each sample.

For the Elisa kit:

Ten samples from wineries were studied for the evaluation. The Elisa kit results were compared to growth on selective media non-saccharomyces and microscopic observation.

ITV France Validation Tests

ITV France tested all the detection kits.

- ITV France receive a first PCR kit in June 2006. When we performed this first kit, some problem were observed about reactive media.
- ITV France receive a second PCR kit after that. Because a period of intensive wine-making activity at ITV France, the kit were performed in October. The activity report about PCR was send to the consortium in November.
- ITV France receive the NSK media in September instead of July as previously attended. ITV France performed the NSK media and send the activity report about this media in September to the consortium.
- ITV France receive and performed the ELISA kit in November. The activity report was send to the partners as soon as possible before the last meeting.

PREDEL Validation Tests

PREDEL also tested the media, the ELISA and the PCR kits with wine samples including first version of the kits and modified kits:

- Analyse the results from the three validation centre and redaction of the deliverable.
- Identification of the critical points of brettanomyces contamination during the wine process in collaboration with the validation centre and redaction of the guide of good practices.

FOEB Comparison of culture media for Brett enumeration

FOEB made an additional work by making a comparison of culture media for BRETT enumeration.

Four media were tested :

Brett NKS: in Petri dishes of 55mm, dehydrated on a cellulose support. For analyses it is rehydrated using 3 ml water with 0.02 and 0.03g/L of actidione. The membranes provided are white and the porosity 0.6µm. The incubation time recommended is 3 to 5 days at 30°C. FOEB received 25 dishes for testing.

M&S media : provided by Möller & Schmetz, is an agar medium, green, in glass tubes closed by a screw cap. The medium is heated then poured in Petri dishes of 97mm. FOEB received 25 tubes.

Brett Millipore : It is provided in plastic bulbs of 2mL. It is poured on a cellulose membrane 0.6µm in Petri dishes of 55mm. The incubation time recommended is 5 days at 25°C. FOEB also received 25 dishes for the tests.

Non-*Saccharomyces* medium : It is the usual medium that we prepare and use in the laboratory. It contains actidione in order to prevent the growth of *Saccharomyces*. The incubation time is 7 days at 30°C. The samples are filtered on a membrane of 0.45µM porosity.

Tests for pure cultures :

In order to assess the selectivity of the media, pure cultures were used as samples. They are yeasts and bacteria from the Collection of the laboratory. Pure cultures are grown and a dilution is sprayed on the surface of the media.

Table 1 shows the results :

The sign + indicates that colonies developed after the incubation of 5days at 30°C. the sign – signifies that no growth occurred .

| | Brett NKS* | M&S | Brett Millipore | Non-<i>Saccharomyces</i> |
|-------------------------------|-------------------|----------------|------------------------|---------------------------------|
| <i>S.cerevisiae</i> | + | + | - | - |
| <i>H. uvarum</i> | + | + | + | + |
| <i>B. bruxellensis</i> | + | + | + | + |

| | | | | |
|----------------|---|---|---|---|
| <i>O. oeni</i> | + | + | - | - |
| <i>A aceti</i> | + | + | - | - |

These preliminary results show that only Brett Millipore and Non-*Saccharomyces* media support the growth of *Brettanomyces bruxellensis* and *H.uvarum*. On the other *Saccharomyces cerevisiae* and bacteria could grow.

Evaluation of the media with wine samples :

Samples are wines produced in 2006 in the Bordeaux area. All are at the moment in barrels for ageing. Samples are diluted 10 and 1000 times. Three filtrations are done on the membranes which are put on the nutrient media: 10mL of wine, 1mL of wine diluted 10times, 1mL of wine diluted 1000times.

Each wine sample and each culture media were done in triplicate. Tables 3 and 4 show the results obtained after respectively 3 and 7 days of incubation at 30°C .

| wine | Brett NKS | | M&S | Brett Millipore | Non- <i>Saccharomyces</i> |
|-----------|-----------|---------|-------------------------|-----------------|---------------------------|
| | 0.02g/L | 0.03g/L | | | |
| actidione | 0.02g/L | 0.03g/L | | | |
| 6373 | nd | nd | 2.1.10 ² /mL | nd | nd |
| 6593 | | | 6.5.10 ⁵ /mL | nd | nd |
| 84 | nd | nd | 1.2.10 ² /mL | nd | nd |
| 6719 | | | 1.9.10 ⁴ /mL | nd | nd |
| 6819 | | | 1.4.10 ³ /mL | nd | nd |
| 64 | nd | nd | 1.6.10 ³ /mL | nd | nd |

| wine | Brett NKS | | M&S | Brett Millipore | Non- <i>Saccharomyces</i> |
|-----------|-------------------------|-------------------------|-------------------------|-------------------------|---------------------------|
| | 0.02g/L | 0.03g/L | | | |
| actidione | 0.02g/L | 0.03g/L | | | |
| 6373 | 5.4.10 ² /mL | 4.1.10 ² /mL | 4.5.10 ³ /mL | 2.2.10 ² /mL | 7.5.10 ³ /mL |
| 6593 | | | 7.5.10 ⁵ /mL | < 1/10 mL | <1/10 mL |
| 84 | 1.3.10 ³ /mL | 1.2.10 ³ /mL | 4.5.10 ⁴ /mL | 7.1.10 ² /mL | 3.1.10 ⁴ /mL |

| | | | | | |
|------|----------------------|----------------------|----------------------|----------------------|----------------------|
| 6719 | | | $1.3 \cdot 10^6$ /mL | $3.2 \cdot 10^3$ /mL | $1.1 \cdot 10^4$ /mL |
| 6819 | | | $1.1 \cdot 10^6$ /mL | $2.4 \cdot 10^4$ /mL | $9.3 \cdot 10^3$ /mL |
| 64 | $6.3 \cdot 10^3$ /mL | $5.1 \cdot 10^2$ /mL | $2 \cdot 10^3$ /mL | $2.3 \cdot 10^2$ /mL | $4.2 \cdot 10^2$ /mL |

On the medium M&S , several times moulds developed on plates making difficult the colony counting. Some colonies were coloured. The yellow to orange colour appeared distinctly after 7 days. Other colonies were white , very flat and large on the surface of the medium. Cells observed under the microscope were round and large. They did not resemble *Brettanomyces bruxellensis*. At least two different species grew on this medium. Only on this medium colonies were visible after 3 days. For wines 6593 and 64, there was no more growth between day3 and 7. Especially for wines 6593, 6719 and 6819 the final counts at day 7 were much higher than in "Brett Millipore" and "non-Saccharomyces". Microorganisms other than *Brettanomyces bruxellensis* probably can grow in those conditions.

For all the other media, 7 days of incubation were required.

The "Brett Millipore" and the "Brett NKS" gave results rather close. For 3 wines out of 6 the population counted was lower than with "Non-Saccharomyces".

It is note worthy that on NKS some colonies are very small and clear, so that counting was difficult. For wine 64 only colonies were orange after 7days. Excepted for this wine, there was no difference between the two concentration of actidione.

In conclusion :

All these media were convenient for *Brettanomyces bruxellensis* growth. However other yeast and even bacteria could row on NKS and M&S media after 5 and 7 days of incubation. On M&S undesirable growth of moulds also occurred. On NKS, the colonies were small and difficult to count on the white membrane. The orange colour of some colonies, not all, shows that different yeast species could grow in the same conditions.

The M&S medium should not be recommended, since the results are very different from those obtained on all the other media.

NKS and Brett Millipore gave close results, but only 3 wines were compared.

The Non-*Saccharomyces* medium gave the highest counts.

Briefly, these results show the difficulty to determine the best culture medium for enumeration of *Brettanomyces bruxellensis* in the complex mixture of wine micro organisms. This kind of study should be repeated with much more wine samples in order to decide which one is the most adapted whatever the kind of wine and the level of population.

FOEB Comparison of the kit BIONOSTRA to another Brett detection kit newly proposed

FOEB also made a comparison of the kit BIONOSTRA to another Brett detection kit newly proposed even if it was not foreseen initially.

Firstly FOEB proposed to evaluate the reproducibility of the Bionostra kit and try the construction of standard curves in order to quantify the Brett population. The idea was to relate the response of the quantitative PCR, expressed as Ct, to the cultivable population on plate counts. However it rapidly appeared some problems. Therefore, FOEB decided to compare the Bionostra Kit to another one, newly proposed by Applied Biosystem.

The two kits differ by the DNA extraction protocol and by the PCR reaction itself. The objective of this study was to compare the two protocols as they are exactly described by the providers.

1. DNA extraction

1.1 Bionostra protocol

From the wine sample the extraction is duplicated. Wine is filtered in order to collect microorganisms and the membrane washed, then put in a test tube with 10 mL of buffer A. Then cells are harvested from the filter by ultrasons and after removing the membrane, they are collected by centrifugation for 5 min at 3000 g. The cell pellet is suspended in 2 ml of buffer A. Then poured in a test tube containing 4 ml of buffer I. After centrifugation for 5 min at 3000 g, the cells should be ready for PCR. However, at this step the pellet should not be too coloured. If it is the case the precedent washing procedure must be removed. However, the Bionostra kit should provide enough buffer A. When the pellet is suitable for continuation of the procedure, it is washed twice with 1 ml of water and finally suspended in 100 µl of water, or more if the biomass collected is abundant. The samples are finally diluted 1000 and 10 000 times.

1.2 Applied Biosystem protocol

Cells are collected by centrifugation and lysed by a chemical treatment and heating. Filtration on a Microcon 100 column removes cell debris and macromolecules larger than DNA. The protocol is as follows. One ml of wine is centrifugated at 13 000 g, the pellet suspended in 1 ml water, vortexed 15s, then again centrifugated for 5 min at 13 000 g. 200 µl of the Prepman Ultra are added to the pellet which is resuspended then heated at 100°C for 10 min. Before the filtration we added a centrifugation for 5 min at 13 000 g. A Microcon 100 column is added with 300 µl of water and 200 µl of cell lysate. It is centrifugated at 500 g and we extended the time at 40 min, instead of 15 min, in order that all the liquid passed through the column. 500 µl water are added on the column, which is again centrifugated for 15 min at 500 g and the new permeate is again discarded. 30 µl of water are added to the column which is vortexed for 10 s, then reversed and put in a new tube. After centrifugation for 5 min at 500 g or preferably for 2 min at 1000 g the liquid is collected and diluted 20 times.

2 PCR amplification

The PCR apparatus is the Thermocycler 7300 Applied Biosystem with the suitable software for realtime PCR.

2.1 Bionostra

For a final volume of 10 µl the mixture comprises : 5 µl Taqman Universal Master mix, 3.4 µl water, 0.3 µl Primer Q-Brett F, 0.3 µl Primer Q-Brett R, 1 µl Probe Q-BrettP, template 1 µl.

The templates are :

- DNA extracts after dilution 10^3 and 10^4 times. For each dilution two PCR reaction are done. So that finally for one wine sample, 8 PCR reactions are done.
- Positive control : *B. bruxellensis* DNA diluted 10 and 100 times.
- Specificity control : DNA from non *B. bruxellensis*
- Negative control : without DNA

2.2 Applied Biosystem

For a final volume of 30 μ l : 15 μ l of Environmental master mix 2X, 3 μ l Brettanomyces specific mix, 12 μ l template.

The templates are :

- DNA extracts diluted 20 times. For each extract, two PCR reactions are done.
- Since it was no provided in the kit we added a positive control of *B. bruxellensis* DNA from the laboratory.
- Negative control : without DNA

3. Results

3.1 Wine samples

For the comparison six wines were used. A preliminary analysis aimed to verify that they were contaminated by *B. bruxellensis*.

The analysis comprised a microscopic observation, a conventional PCR detection (qualitative non quantitative) and plate count enumeration (Table 1).

Table 1 : *B. bruxellensis* determinations in six wines by “conventional” methods

| Wine | PCR Signal | Plate counts UFC/mL |
|------|------------|---------------------|
| 1 | + | 8×10^4 |
| 2 | + | 8.2×10^2 |
| 3 | + | 1 |
| 4 | + | 2 |
| 5 | + | 3.6×10^3 |
| 6 | + | 1.6×10^3 |

The microscopic observation showed the usual *B. bruxellensis* morphology for all the yeast population. The PCR signal was positive for all samples. All wines contained *B. bruxellensis* but it very different levels.

Wines 3 and 4 were very lightly contaminated even if the PCR signal was intense. The presence of VNC or even recently dead cells was hypothesized.

3.2 Comparison of the quantitative PCR kits

The six wines were extracted according to the recommendations of Bionostra and Applied Biosystem as described above.

Then all extracts were PCR amplified by the two PCR reaction mixtures. Table 2 shows the results when the wines were treated according to the Bionostra protocol delivered in the kit. Table 3 corresponds to extraction as recommended by Applied Biosystem.

Table 2 : Results for DNA extraction with the Bionostra Kit

| Bionostra extraction | PCR Bionostra (Ct) | | PCR AB (Ct) | | IPC | |
|----------------------------------|--------------------|-------|--------------|-------|--------------|-------|
| | | | | | | |
| Wine 1 a 10-3 | | | 29,66 | 26,71 | 23,79 | 23,8 |
| Wine 1 b 10-3 | | | 29,13 | 29,28 | 23,79 | 23,86 |
| Wine 1 a 10-4 | | | 29,64 | 29,73 | 23,89 | 23,93 |
| Wine 1 10-4 | | | 29,27 | 29,41 | 23,65 | 23,81 |
| Wine 2 a 10-3 | nd | nd | 29,55 | 28,59 | 23,85 | 24,02 |
| Wine 2 b 10-3 | nd | nd | 29,45 | 29,5 | 23,88 | 23,92 |
| Wine 2 a 10-4 | nd | nd | 29,54 | 29,22 | 23,78 | 23,93 |
| Wine 2 b 10-4 | nd | nd | 29,31 | 29,43 | 23,8 | 23,8 |
| Wine 3 a 10-3 | | | 29,61 | 29,29 | 23,84 | 23,76 |
| Wine 3 b 10-3 | | | 29,39 | 30,27 | 23,88 | 24 |
| Wine 3 a 10-4 | | | 29,44 | 29,12 | 23,77 | 23,77 |
| Wine 3 10-4 | | | 29,15 | 31,72 | 23,82 | 23,84 |
| Wine 4 a 10-3 | nd | 37,08 | 29,59 | 32,38 | 23,91 | 24,2 |
| Wine 4 b 10-3 | nd | nd | 29,12 | 29,34 | 23,85 | 23,81 |
| Wine 4 a 10-4 | nd | nd | 29,18 | 28,71 | 23,9 | 23,81 |
| Wine 4 b 10-4 | nd | nd | 28,63 | 28,71 | 23,72 | 23,81 |
| Wine 5 a 10-3 | | | 27,77 | 28,09 | 23,84 | 23,78 |
| Wine 5 b 10-3 | | | 29,12 | 28,78 | 23,81 | 23,84 |
| Wine 5 a 10-4 | | | 28,77 | 28,69 | 23,83 | 23,83 |
| Wine 5 b 10-4 | | | 28,43 | 28,55 | 23,79 | 23,73 |
| Wine 6 a 10-3 | nd | nd | 27,81 | 28,09 | 23,63 | 23,93 |
| Wine 6 b 10-3 | nd | nd | 26,91 | 28,68 | 23,98 | 23,93 |
| Wine 6 a 10-4 | nd | nd | 29,27 | 28,52 | 23,87 | 23,8 |
| Wine 6 b 10-4 | nd | nd | 28,39 | 28,67 | 23,82 | 23,8 |
| T+ Bionostra 10-1 | | | | | | |
| T+ Bionostra 10-2 | | | | | | |
| T- Bionostra | | | | | | |
| T- PCR | | | 29,67 | | 23,38 | |
| ADN <i>B.bruxellensis</i> | | | 15,82 | | 25,14 | |

Table 3 : Results after DNA extraction with the Applied Biosystem Kit

| Applied extraction | Biosystem | PCR Bionostra (Ct) | | PCR AB (Ct) | | IPC | |
|--------------------|-----------|--------------------|-------|-------------|-------|-------|-------|
| | | | | | | | |
| Wine 1 a 1/20 | | 33,4 | 26,5 | nd | nd | nd | nd |
| Wine 1 b 1/20 | | 33,48 | 33,26 | nd | nd | nd | nd |
| Wine 2 a 1/20 | | 28,4 | 28,15 | 22,69 | 22,73 | 23,71 | 23,58 |
| Wine 2 b 1/20 | | 29,15 | 29,41 | 22,14 | 22,84 | 23,56 | 23,72 |
| Wine 3 a 1/20 | | 30,19 | 30,28 | 22,32 | 22,42 | 23,67 | 23,71 |
| Wine 3 1/20 | | 27,55 | 27,63 | 22,19 | 22,14 | 23,74 | 23,77 |
| Wine 4 a 1/20 | | 29,8 | 28,57 | 23,21 | 22,38 | 23,45 | 23,78 |
| Wine 4 b 1/20 | | 29,76 | 30 | 22,48 | 22,39 | 23,69 | 23,7 |
| Wine 5 a 1/20 | | 30,1 | 30,37 | 22,05 | 22,07 | 23,62 | 23,72 |

| | | | | | | |
|----------------------------------|-------|-------|-------|-------|-------|-------|
| Wine 5b 1/20 | 29,7 | 30,17 | 21,93 | 22,14 | 23,65 | 23,73 |
| Wine 6 a 1/20 | 32,57 | 33,4 | 24,63 | 24,93 | 23,41 | 23,82 |
| Wine 6 b 1/20 | 31,95 | 31,99 | 24,5 | 24,32 | 23,84 | 23,64 |
| Wine 7 a 1/20 | 29,59 | 29,12 | 25,46 | 25,02 | | |
| Wine 7 B 1/20 | 29,19 | 29,35 | 26,14 | 25,72 | | |
| T+ Bionostra 10-1 | 23,1 | 23,01 | | | | |
| T+ Bionostra 10-2 | 27,06 | 26,6 | | | | |
| T- Bionostra | nd | | | | | |
| T- PCR | nd | | 29,67 | 28,78 | 23,38 | 23,42 |
| DNA <i>B.bruxellensis</i> | | | 15,82 | | 25,14 | |

In each table, the three double columns give the results of PCR duplicates. The numbers are the Ct for each PCR reaction. They are obtained using the PCR Bionostra reaction mix and the Applied Biosystem reaction mix. For the later there are two columns names IPG. The last one corresponds to an internal control, that allows to verify that the reaction correctly occurs.

In the positive controls, "T + Bionostra 10⁻¹" and "T + Bionostra 10⁻²", "*B. bruxellensis* DNA from the laboratory, the DNA is detected both in the PCR Bionostra (Table 2) and PCR AB (Table 3).

In the negative controls "T-Bionostra" and "T-PCR" the results are consistent for the determination with the Bionostra kit, not for Applied Biosystem. This is probably due to a contamination of a reagent or of equipment. In addition (table 2 or table 3), the Ct for the sample "DNA *B. bruxellensis*" as positive control is lower than the one for the negative control "T-PCR". In spite of the contamination, this shows that there is amplification with the PCR Applied Biosystem mix.

For the DNA wine extracts, when using Bionostra extraction and Bionostra PCR no amplification occurred (Table 2). Only two positive results were obtained, but they were not confirmed in the duplicate for wines 4 and 5. When the same DNA wine extracts are analysed with the Applied Biosystem reagents, there is a response. Unfortunately, the Ct measured were all very close to the negative control (29.67). However, these results (table 2) confirm that the DNA extraction using the protocol of Bionostra was not conclusive. On the contrary, columns "PCR Bionostra" in Table 3 show that DNA was amplified in all the samples treated by Applied Biosystem for extraction and Bionostra for PCR. This confirms that the Bionostra kit is efficient for the PCR not for the extraction.

As for PCR Applied Biosystem, *B. bruxellensis* was detected as expected in all samples excepted wine n°1 where also the internal control was negative. The value of Ct lower for all the samples than for the negative control "T-PCR" (that should not be amplified) is consistent with a detection of Brett DNA in the wine extracts.

3.3 Linearity

The final interest of quantitative PCR is to enumerate the yeast cells present in wine using the CT value from the PCR reaction. That means that the Ct should be correlated to CFU. To this aim it would be interesting to establish standard curves giving the population expressed as CFU/mL as a function of the Ct obtained by quantitative PCR reaction. That was our previous objective but we could not achieve it due to lack of Bionostra reagents, problems in extraction and to lack of time in the frame of this contract.

However, we started to verify the linearity of the PCR amplification with the Bionostra kit and compared it to Applied Biosystem, using increasing amounts of *B. bruxellensis* DNA (figure 1 and table 3). The Ct is a linear function of the logarithm of DNA amount expressed in pg, for both PCR reagent kits. However the Ct values are lower with the Applied Biosystem kit. Its sensitivity seems to be better than Bionostra.

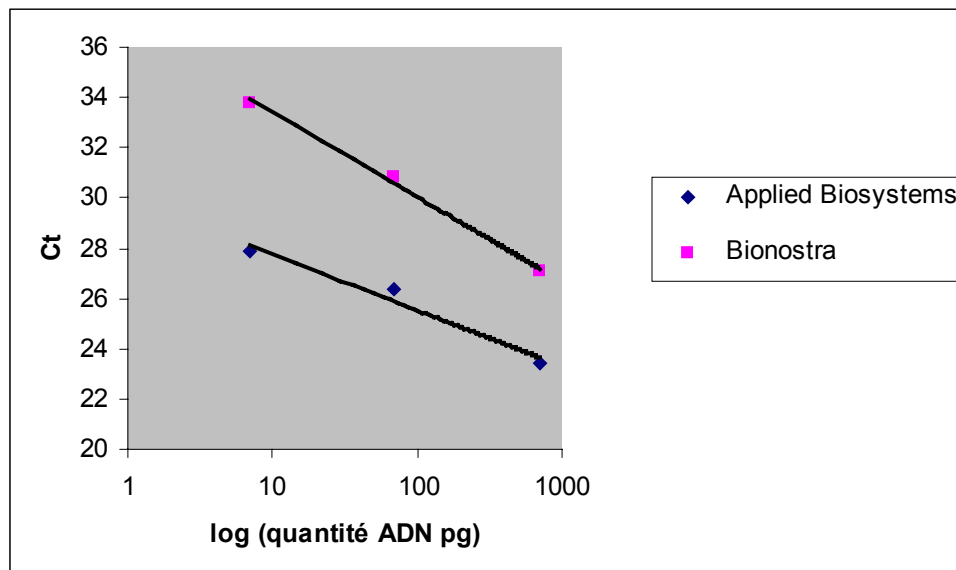


Figure 1 : Linearity of Ct values versus the DNA amount of *Brettanomyces bruxellensis* used as template.

Table 3

| Applied Biosystem extraction | PCR Bionostra (Ct) | PCR AB (Ct) | IPC |
|----------------------------------|--------------------|-------------|-------|
| ADN <i>B.bruxellensis</i> 7 pg | 33,81 | 27,86 | 23,5 |
| ADN <i>B.bruxellensis</i> 70 pg | 30,82 | 26,33 | 23,81 |
| ADN <i>B.bruxellensis</i> 700 pg | 27,07 | 23,43 | 23,62 |

Conclusion :

As it is presently proposed in the kit, the Bionostra extraction protocol is not conclusive. The critical point for this kit is extraction, not the PCR amplification itself. The attempts for establishing standard curves did not succeed for the reasons given above. However, to our opinion after the extraction of DNA will have been optimised it will be the necessary work to be done. The ideal would be to calibrate the Ct response to CFU/mL for the Brett populations present in several types of wine, mainly varying by their composition of polyphenols.

T5.2 Protocol design and monitoring methodology

Following these first results, PREDEL continue to search of different ways to improve these kits. PREDEL has develop collaborations with RTD performers to modify different parameters of the first draft protocol for the three methodologies.

Deviation from the project WP

There were big delays in the realisation of the validation tests as the research on the detection methods took more time. However the validation tests were all carried out in time. Some additional tests were even provided by FOEB and PREDEL.

Deliverables

D11: Result of the validation test

D12: Validated protocol for Brett detection using plating on selective media

D13: Validated protocol for Brett detection using PCR

D14: Validated protocol for Brett detection using immunoassay probes

D15: Guide of good practice for Brett monitoring in wineries

Milestones and expected results

Results

- A validated protocol for the measurement using the three different detection techniques
- Recommendation for Brett growth monitoring in wineries

WP6 Diffusion**Objectives**

The objective is to disseminate the result of the project and to prepare the commercialisation.

Progress towards objectivesT6.1 Preparation to commercialisation

PREDEL elaborated a business plan:

- Commercial organisation including the creation of an export service,
- Identification and contact of potential distributors, suppliers to produce the kit and partners for the equipment.
- Redaction of the business plan for the 3 SMEs

T6.2 Diffusion of the results

PREDEL elaborated a marketing plan:

- Creation of a marketing service which identified the relevant newspaper and technical magazine to publish article or advertise the kits,
- List of professional exhibitions to promote the products including the Vinitech event.
- Elaboration of an advertising booklet in French and English language and a website page about Brettanomyces problematic.

T6.3 Normalisation

PREDEL elaborated a report on normalisation activities:

Deliverables

D16: Marketing plan

D17: Report on diffusion activities (publications etc.)

D18: Report on normalisation activities

Milestones and expected results

Results

- Large diffusion of the project results in Europe.

Table 1: Deliverables List

List all deliverables, giving date of submission and any proposed revision to plans.

| Deliverable No ¹ | Deliverable title | WP no. | Lead participant | Estimated person-month | Nature ² | Dissemination level ³ | Delivery date ⁴ |
|-----------------------------|---|--------|------------------|------------------------|---------------------|----------------------------------|----------------------------|
| D1 | Report on the bibliographical study on Brett | 1 | FOEB | 3 | R | PP | September 2004 |
| D2 | Protocol for sample collection and measurement campaign | 1 | FOEB | 1 | O | CO | July 2004 |
| D3 | Report on the identification of strains responsible for the development of the off flavour | 1 | FOEB | 5 | R | CO | September 2004 |
| D4 | Report on the growth of Brett and on the parameters influencing the production of off flavour | 1 | FOEB | 6 | R | PP | August 2005 |
| D5 | Report formulating recommendation for detection strategy | 1 | FOEB | 4.5 | R | PP | July 2006 |
| D6 | Selective media for strains identified in T1.3 | 2 | Moeller | 9.5 | D | Co | February 2005 |
| D7 | Primer for Brettanomyces producing off flavour | 3 | CSIC | 8 | D | Co | April 2005 |
| D8 | Measurement protocol | 3 | UM1 | 10.5 | O | CO | August 2005 |

¹ Deliverable numbers in order of delivery dates: D1 – Dn

² Please indicate the nature of the deliverable using one of the following codes:

R = Report
P = Prototype
D = Demonstrator
O = Other

³ Please indicate the dissemination level using one of the following codes:

PU = Public
PP = Restricted to other programme participants (including the Commission Services).
RE = Restricted to a group specified by the consortium (including the Commission Services).
CO = Confidential, only for members of the consortium (including the Commission Services).

⁴ Month in which the deliverables will be available. Month 0 marking the start of the project, and all delivery dates being relative to this start date.

| Deliverable No ⁵ | Deliverable title | WP no. | Lead participant | Estimated person-month | Nature ⁶ | Dissemination level ⁷ | Delivery date ⁸ |
|-----------------------------|---|--------|------------------|------------------------|---------------------|----------------------------------|----------------------------|
| D9 | Monoclonal antibodies for strains of <i>Brettanomyces</i> producing off flavour | 4 | CSIC | 10 | P | Co | December 2005 |
| D10 | kit of <i>Brettanomyces</i> detection | 4 | Bionostra | 6.5 | D | PU | September 2006 |
| D11 | Result of the validation test | 5 | Predel | 6.7 | R | Co | November 2006 |
| D12 | Validated protocol for Brett detection using plating on selective media | 5 | Moeller | 2 | R | PU | November 2006 |
| D13 | Validated protocol for Brett detection using PCR | 5 | Bionostra | 2 | R | PU | November 2006 |
| D14 | Validated protocol for Brett detection using immunoassay probes | 5 | Bionostra | 2 | R | PU | November 2006 |
| D15 | Guide of good practice on Brett monitoring in wineries | 5 | Predel | 3 | R | PU | November 2006 |
| D16 | Marketing plan | 6 | Predel | 2 | R | CO | November 2006 |
| D17 | Report on diffusion activities (publications etc.) | 6 | Predel | 4 | R | Re | November 2006 |
| D18 | Plan for using and disseminating knowledge | 6 | Predel | 3.2 | R | Re | November 2006 |
| D19 | Management report | 7 | Predel | 3.2 | R | Re | May 2005 December 2006 |

⁵ Deliverable numbers in order of delivery dates: D1 – Dn

⁶ Please indicate the nature of the deliverable using one of the following codes:

- R** = Report
- P** = Prototype
- D** = Demonstrator
- O** = Other

⁷ Please indicate the dissemination level using one of the following codes:

- PU** = Public
- PP** = Restricted to other programme participants (including the Commission Services).
- RE** = Restricted to a group specified by the consortium (including the Commission Services).
- CO** = Confidential, only for members of the consortium (including the Commission Services).

⁸ Month in which the deliverables will be available. Month 0 marking the start of the project, and all delivery dates being relative to this start date.

Section 3 – Consortium management

Consortium management and co-ordination activities

The consortium management tasks are divided into “project management” and “administrative management”.

Project management

The activities related to the project management concerned the day-to-day follow up of the partners’ activities. This task is mainly realised by Euroquality. Advancement files have been used to follow the activities of the partners: every two month, a short report summarising the activities performed were sent to Euroquality. This ensures dissemination, co-ordination and follow up of the different activities. These advancement files can be consulted on the private web site.

Emails were sent from time to time to remind the partners the main deadlines and to share information about the project advancement. Deadlines and objectives for each partner are determined at each consortium meeting. Six meetings have been organised:

- Kick off meeting in Saint Loubès on the 3rd and 4th June 2004.
- 6-month meeting in Madrid on the 14th January 2005.
- 12-month meeting in Göttingen on the 23rd and 24th June 2005.
- 18-month meeting in Valencia, Spain on the 9th and 10th of January 2006.
- 24-month meeting in Montpellier, France on the 15th and 16th of June 2006.
- 30-month meeting in Bordeaux, France on the 27th of November 2006.

Details on each meeting (presentation, reports) are given in the project management web site (<http://www.euroqualityfrance.com/brett>).

As co-ordinator PREDEL has also helped to define and harmonise methodologies used to gather data and materials for producing relevant information that can be used by all and to optimise the preparation of each WP. PREDEL has conducted studies and reports on the state of the research and trends observed compared to the needs of the market.

Administrative management

The administrative management concerns the financial reporting. Euroquality elaborated templates for the financial reporting (time sheets, cost budget follow up table, tool to calculate the personnel cost) and helped the partners to complete the financial information.

Reports on the expenditures have been collected in January 2005 for the period June 2004 to December 2004. The information has been up-dated in May 2005 to cover the whole period of 12 months. Financial statements are given in the Management report.

Reports on the expenditures have been collected in June 2006 for the period from January 2006 to May 2006. The information has been up-dated in December 2006 to cover the whole last period. Financial statements are given in the Management report.

Main conclusions

The involvement of each partner is satisfactory. There is no particular problem to be reported. Requested deliverables have been generally delivered in time. The quality of the reports are considered adequate.

Project timetable and status

| Work program | Months | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|--------|----|----|----|----|----|------|----|----|----|----|----|----|----|----|----|----|----|------|----|----|----|----|----|----|----|----|-----|-----------------|
| | 2004 | | | | | | 2005 | | | | | | | | | | | | 2006 | | | | | | | | | | |
| | 06 | 07 | 08 | 09 | 10 | 11 | 12 | 01 | 02 | 03 | 04 | 05 | 06 | 07 | 08 | 09 | 10 | 11 | 12 | 01 | 02 | 03 | 04 | 05 | 06 | 07 | 08 | 09 | 10 |
| WP1: Research on Brett | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| T1.1 Bibliography | x | | | | | | | | | | | | | | D1 | | | | | | | | | | | | | | |
| T1.2 Elaboration of a protocol for measurement | x | | | D2 | | | | | | | | | | | | | | | | | | | | | | | | | |
| T1.3 Experimentation | | | | x | | | | | D3 | | | | | | | | | | | | | | | | | | | | |
| T1.4 Analysis of the results | | | | | | | | | | | | | | | D4 | | | | | | | | | | | | D5 | | |
| WP2: Selective Media | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| T2.1 Research on strain selective media | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| T2.2 Development of the media | | | | | | | | | x | | | D6 | | | | | | | | | | | | | | | | | |
| T2.3 Test | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| WP3: PCR | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| T3.1 Research on the primer | | | | | | | | x | | | D7 | | | | | | | | | | | | | | | | | | |
| T3.2 Design of quantitative primer | | | | | | | | | | | x | | | | D8 | | | | | | | | | | | | | | |
| T3.3 Research on DNA extraction | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| T3.4 Optimization of PCR protocol | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| T3.5 Test | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| WP4: Immunoassay probes | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| T4.1 Development of monoclonal antibody | | | | | | | | | | | | | | | | x | | | | D9 | | | | | | | | | |
| T4.2 Development of the analysis device | | | | | | | | | | | | | | | | | | | | | | | | | | | | D10 | |
| WP5 Validation test | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| T5.1 Validation tests of the three detection techniques | | | | | | | | | | | | | | | | | | | | | | | | | | | | | D11 |
| T5.2 Protocol design and monitoring methodology | | | | | | | | | | | | | | | | | | | | | | | | | | | | | D12 13 14 |
| WP6: Diffusion | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| T6.1 Preparation to commercialisation | | | | | | | | | | | | | | | | | | | | | | | | | | | | | D15 16 |
| T6.2 Diffusion of the results | | | | | | | | | | | | | | | | | | | | | | | | | | | | | D17 |
| T6.3 Normalisation | | | | | | | | | | | | | | | | | | | | | | | | | | | | | D18 |
| WP7: Co-ordination | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| T7.1 Co-ordination | | | | | | | | | | | | | | | | | | | | | | | | | | | | | D19 |

In : Actual work progress x : Planed deliverable or Milestone D/M : Deliverable or Milestone given