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Summary

1. Executive summary

Botrytis cinerea is a fungus that affects wine grapes and is responsible for the grey rot infection, which can strongly alter the quality of grapes and derived wine, causing: browning, destruction of flavour compounds, decrease of foaming properties, fermentation stopping. Grey rot is the largest infection of grapes in vineyards all over the world and is a major problem both for wine growers and wine producers, being very rapid and resistant to fungicides.

The SAFEGRAPE objective was to developed a simple and cheap instrument for the wine industry, able to provide a quantitative and reliable evaluation in a very short time of the presence of Laccase Activity, a by-product produced by the grey rot infection in grapes. The instrument is based on a biosensor system and on an innovative analysis method, that allows a precise and fast analysis procedure, i.e. in about 3 minutes, which is compatible with the quality control procedures applied to grapes before wine-making (measure of total acidity, sugar content).

The main result produced by the project is the development of the biosensor system integrated in two instruments:

1. First portable instrument for the measure of Laccase Activity, based on palm device, which can also be used in vineyard by winegrowers before or after grape-harvest. (Weight 0.6 kg)
2. An automatic instrument for the quality control in wine productions, to be integrated to the quality controls commonly applied to grapes when entering the cellars (sugar and total acidity content), using the same measurement principle but an automated measuring procedure.

The results produced by SAFEGRAPE consist in:

1. Biosensor based system and procedure for the measure of Laccase Activity.
2. Design of the electronic system (probe) allowing the amperometric measure from the biosensor in about 3 minutes, with a limit of detection of 0.2 ULA, limit of quantification of 0.7 ULA and reproducibility < 10%.
3. Design of electronic device for the handling of the measure in the portable device (palm) and of the mechanical system for the automatic instrument (sampling, charging, filtering, washing and strip sensor substitution)
4. Development of the data regression system for the analysis and calibration of the measure to provide the estimation of the Unit of Laccase Activity
5. Testing in real samples of the portable instrument and assessment with respect to the 2 commercial instruments available for use in win production facilities.

2. Project context and the main objectives

Botrytis cinerea is a grey **fungus that affects wine grapes**. In its benevolent form, botrytis is called **noble rot** and under certain climatic conditions (dry conditions), affects grapes which, if picked at a certain point during infestation, are used to produce distinctive sweet dessert wines.

However, the same fungus, in particular in wet or humid climatic conditions (very frequently in many EU producing countries), is responsible of the infection called **grey rot,** and of the presence of the oxidative enzymes and protease into grape juice.

Some of these enzymes are very resistant **to sulphites** and **can not be removed through clarification** and persist through to the wine. The botrytis enzymes may produce:

1. The browning of wine (through oxidation of the phenolics)
2. The destruction of grape derived flavour compounds.
3. The production of new undesirable flavour compounds, which apart from being unpleasant in themselves, can mask desirable grape derived and potential flavour compounds produced during fermentation and wine ageing.
4. The decrease of foaming properties of sparkling wines such as Champagne.

Apart from spoiling wine quality, *Botrytis* also complicates wine making by making fermentation more complex. In fact *Botrytis* produces an anti-fungal that kills yeast and often results in fermentation stopping.

Grey rot is presently the **largest infection of grapes in vineyards all over the world** and is a **major problem both for wine growers and wine producers**. The infection of Botrytis cinerea happens very rapidly during grape maturation and is very resistant to fungicides; moreover the countermeasures are very expensive.

The damage caused by grey rot has a **disastrous economic impact on numerous crops**, in particular vineyards, but also soft fruits such as strawberries and bulb crops. **Losses due to grey rot account for 20% of harvests of the affected crops in the world**, and their cost is estimated at **10-100 billion euros per year.** In the wine sector Botrytis cinerea c**auses losses for vineyards equal to 15-40% of harvests** depending on climatic conditions. Such losses can be estimated to overcome **15 billions of Euro per year**[1], over a total world wine market value at production level of 60 billions, i.e. **25% of the potential turnover is lost due to this problem**.

Other losses are estimated at 20-25% of the strawberry crops in Spain, and 20% of cut flowers in Holland. In 2006 a very violent attack was experienced in citrus productions in Sardinia, causing a

[1] Genoscope - Centre National de Séquençage

consistent loss for the sector. Moreover, fungicidal treatments against ***B. cinerea*** cost about **540 million euros year**, which represents 10% of the world fungicide market[2].

Grapes infected by grey rot must be eliminated before going to wine production. Quality control is usually done visually but the presence is sometime not easily visible. Presently available testing procedures to measure the presence of the botrytis are based on the use of antibodies, or on products produced by the metabolism of the fungus, or on enzymatic activity; hence they are basically lab based analysis which need several minutes and are highly complex and expensive.

The wine producers are strongly looking for simple instrumentations able to evaluate the presence of Botrytis:

- in the grape selection phase of wine production, to evaluate the quality of grapes before wine-making process by an on line instrumentation. This is particularly important to avoid the presence of Botrytis enzymes in must and therefore in wine and alter the quality of final product, or to select grapes for the production of different quality of wines.
- in the vineyard, in order to act in time (before it is visible and hard to eliminate) with proper countermeasure and avoid the spoilage of large amount of grapes.

These instrumentations will allow to **increase the wine quality** and **reduce the loss of harvest of about 10%**, by a fast intervention in the field. Hence they could grant to the European wine industry, which account for about 60% of total world production:

- an **increase of production of about 2.5%** from increased harvest, counting for about **1.5 billion Euro** for the whole European market.
- a **reduction of the use of fungicides** in vineyards and of **sulphites** in wines

The solution could also be used by producers of sweet wines produced through noble rot, to detect and avoid the turning into grey rot.

The SAFEGRAPE project aimed to develop for the first time **a simple and cheap instrument for the wine industry, able to provide a quantitative and reliable evaluation of the presence of grey rot infection in grapes in a very short time,** through the detection of Laccase Activity, a by-product produced by the grey rot infection in grapes. The instrument proposed is based on a biosensor system and on an innovative analysis method for the measure of the presence of botrytis in grapes, that allows a precise and very fast analysis procedure, i.e. in **less than one minute**, which is compatible with the quality control procedures applied to grapes before wine-making (measure of total acidity, sugar content).

The proposed new method for the detection of Botrytis in grapes is based on a **fast kinetic process** involving active components of the fungus and on **very sensitive transduction device** which permits to sense the reaction products with a **very low detection limit**. This system requires very short reaction times, (of the order of tens of seconds) and is characterized by a linear response and a

[2] Annual Report, UIPP, 2002

high sensitivity which permit to operate with a high dilution factor of the grape juice minimizing the possible interferences and therefore making simple the sample preparation.

The targets for the measurement method of SAFEGRAPE were:

- **Short response time** (1minute for the automatic instrument and 3 minutes for the portable). This allows **on-line measures in wineries** together with the common measure carried out (sugar content and total acidity), compatible with the selection procedure carried out on grapes entering the production site.
- **Compactness and portability** of the instrumentation for field application. The target is to produce a portable field instrument of **max 0.5 kilograms and on-line instrument of the order of few kg**, while present solutions are of the order of 50 kg and 50 cm side cube.
- **Very small amount of juice needed: less than 1 ml** instead of litres needed by present solutions
- **Linearity of the measure**, for a quantitative analysis of the presence of botrytis degradative enzymes in grapes.
- **High sensitivity and reproducibility** of the measure.

The main result expected from the project was the development of a **biosensor system for the quantitative analysis of the content of grey rot**, to be integrated into 2 types of instruments:

1. A **portable instrument for the measure in vineyard**, to be used by winegrowers to quantify the level of fungi infection of grapes in the field or after grape-harvest.
2. An **on-line instrument for quality control** of grapes in the loads entering the production site in cellars, to be integrated in the quality controls commonly applied (sugar and total acidity content) having the same response time.

These instrumentations improve the quality control in the wine production sector, allowing the control of the infection of grey rot in vineyards, and supporting quality control in wineries, including an on-line grey rot quantitative evaluation in the grape selection and quality control step before wine-making.

The **S&T objectives** of the SAFEGRAPE consisted in:

1. Development of the measurement system with particular regards to analysis time (kinetics, sample processing, etc.), to the transduction system and to the repeatability.
2. Engineering of the biosensor system with particular regards to micro fluidic aspects, the automatic charging system, the portability for field instrumentation
3. Development of the, data regression system, electronic (hardware and software) and user interface
4. Testing in wineries and in field and comparison with the state of the art solutions (gluconic acid, immuno-assay, etc.).

3. Main S & T results/foregrounds

3.1. Biosensor based measurement system

Preliminary study was carried out to evaluate the possibility to correlate the presence of Botrytis cinerea with the Laccase Activity. After a carefully regression study, the conclusion is that the calibration curve between the % of Botrytis contamination versus the laccase activity is not possible, given the dependence to several other unmeasurable factors (kind of strain, stage of development of the infection). Several studies support the conclusion stated. Research has indicated that levels of Botrytis infection do not always correspond to laccase activities in juice (Macheix et al. 1991, Perino et al. 1994). Conversely, Dewey et al.2008 stated that although high levels of laccase in grape juice are generally associated with significant levels of Botrytis no evidence was found for a direct relationship. For this reason it was decided to avoid this regression and provide the measure in terms of Unit of Laccase Activity (ULA).

A biosensor based measurement system based on an electrochemical method (and G-sensor strip from Ecobioservices), measuring the rate of oxidation of a specific substrate present in the test solution, which is in a linear relationship with the laccase concentration in the sample, expressed as Unit of Laccase Activity per ml of sample (ULA/ml).

The biosensor used to perform the measure is the G-Sensor, which is illustrated in figure 3. It's produced by EcoBioServices (Florence, Italy) and as already mentioned it constitutes a three electrode electrochemical cell.

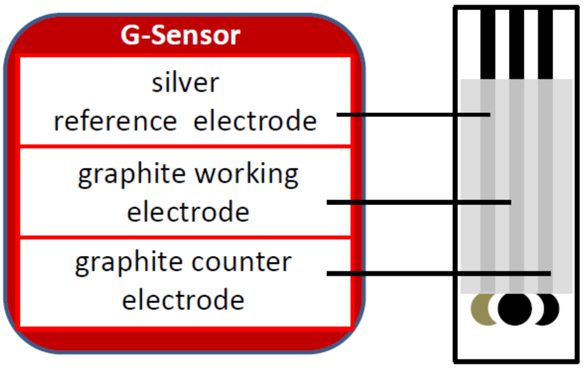


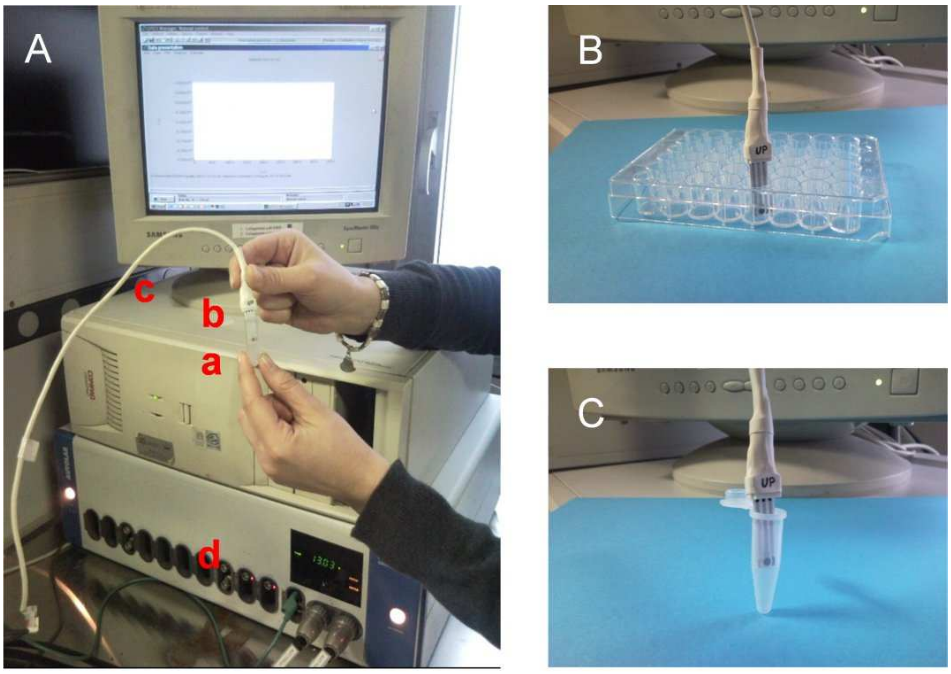
Figure 1: the biosensor "G-Sensor"

The strips are printed by screen-printing technology and the grey zone between the heads and the end of the electrodes is an insulating one to protect them. All the electrodes end with a graphite connection, so that the sensor can be inserted in a standard connector 2.54mm pitch. The working area of the working electrode is a circle of 7.06mm^{2}s.

The reduced dimensions (8x45mm), its planar shape, make the sensor suitable for a wide range of applications, mostly for devices "drop-on" types. It's low cost make it disposable and easy replaceable.

A laboratory scale alpha prototype was developed to evaluate the characteristics of the measurement system, in Figure 2 and Figure 3 the picture and in the scheme of the lab scale alpha prototype is reported, using a lab equipment (Autolab). The procedure to run this lab prototype is also reported.

Figure 2 Lab scale alpha prototype of portable biosensor and the relative scheme



A –a) test tube; b) screen –printed electrode; c) Connector for screen –printed electrode; d) Potentiostat (AUTOLAB PGSTAT12- GPES a fully computer controlled electrochemical system).
B – EBSR strip (G-sensor) and multiwell plate.
C – EBSR strip (G-sensor) and test tube.

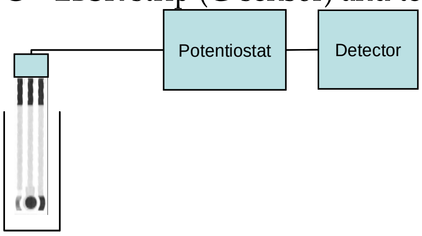
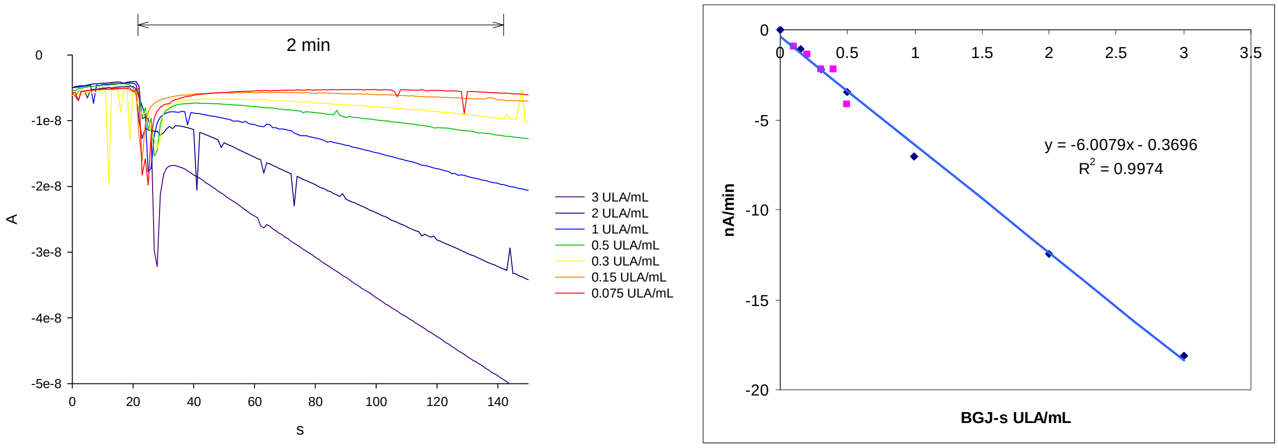


Figure 3: scheme of alpha prototype

EBSR strip (G-sensor): Working electrode: graphite; Reference electrode: silver; Counter electrode: gold.

The preliminary calibration of portable alpha prototype was carried out using both *Trametes versicolor* laccase and botrysed grape juice (BGJ-s) obtained from GRC. The linearity of response, the sensitivity, the precision, the response time and the matrix effect were assessed.
In Figure 4 an example of calibration of the portable alpha prototype is reported.

Figure 4. *Calibration of the portable alpha prototype with a botrysed grape juice*



Electrochemical response (left) and calibration curve (right) of an artificially infected juice. The electrochemical response was plotted versus the activity measured by the syringaldazine test.
A botrysed grape juice (BGJ-s from GRC) was used.

During the second year a beta prototype was implemented, without use of lab testing devices, and preliminary tests were carried out by INBB to assess the performances of the measuring method. In the figure a picture of the SAFEGRAPE Beta Test (SBT) prototype unit are shown.

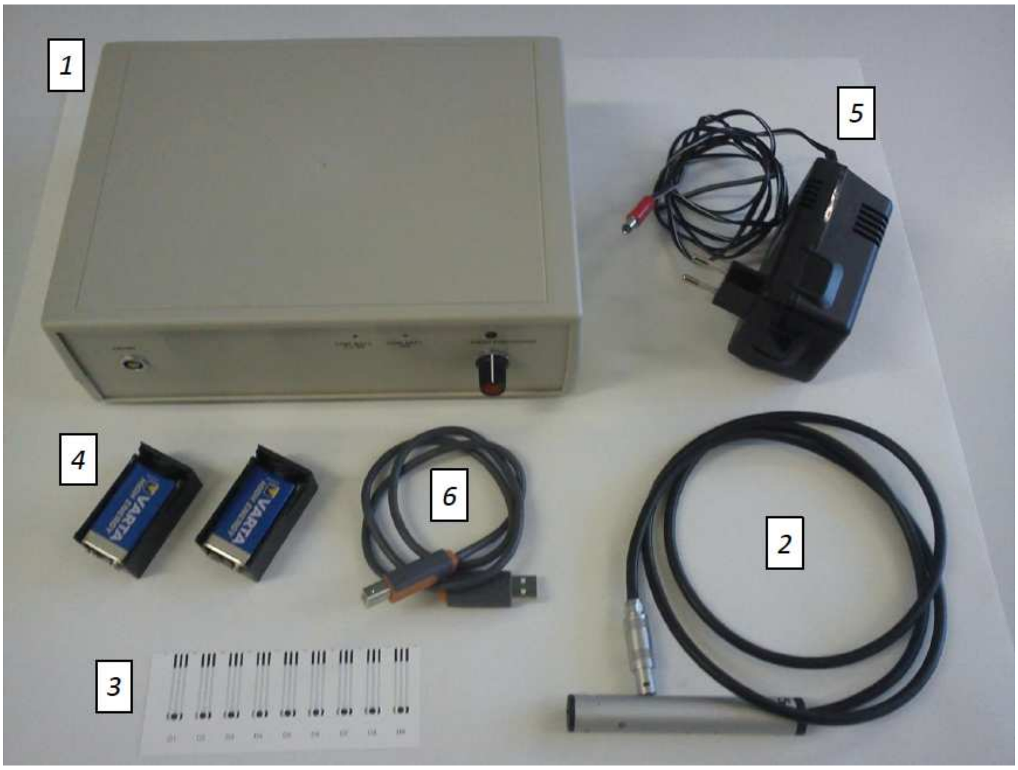


Figure 5: Safegrape Beta Test Prototype components

- 1) the main case, in ABS, having dimension of 250x180x80mm (LxWxH) - weight 1500g;
- 2) the analogical probe, having dimension of 16x105mm (ØxH);

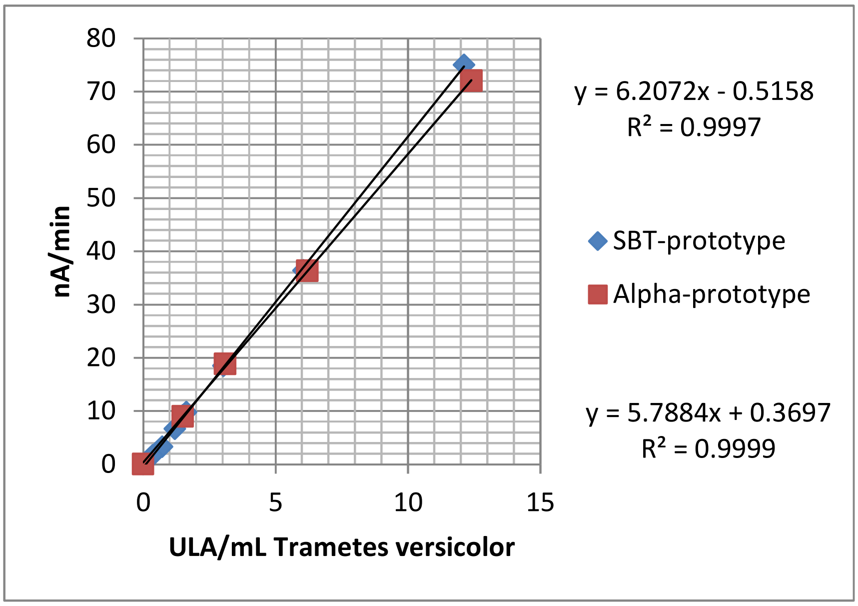
- 3) the three electrode cell, the G-Sensor strips;
- 4) two +9V batteries to power the probe;
- 5) the charge battery, to recharge the +12V battery pack that powers the case;
- 6) a 2.0USB Type A-B cable to connect the device to a personal computer.

Beta tests were carried out by INBB on the SBT prototype, as compared with the alpha-prototype by performing parallel measurements. This allowed to verify the functionality, suggest some corrections to the software of the SBT prototype and to define the optimal operative conditions for field testing.

Comparison of alpha and beta prototypes

To test the prototype responses the activity of *Trametes versicolor* laccase was measured by the two instruments. Measurements of baseline current, noise, and the calibration curves were performed in the test solution at room temperature. The results of the calibration tests are shown in Figure 6, where the two calibration curves were laid one upon the other. From this figure it clearly appears similar slopes and similar intercept values, and in both cases regression values (R^2) higher than 0.999 were calculated. As regards the evaluation of the electrical signal from Figure 7 and Table 1, it appears that the noise is higher in the case of the SBT prototype, but still acceptable. However the presence of strong spikes in the signal trace of the SBT prototype, probably due to the electrical noises, must be taken into account and must be reduced or removed, see Figure 7.

Figure 6: ***Comparison of the alpha and SBT prototypes***



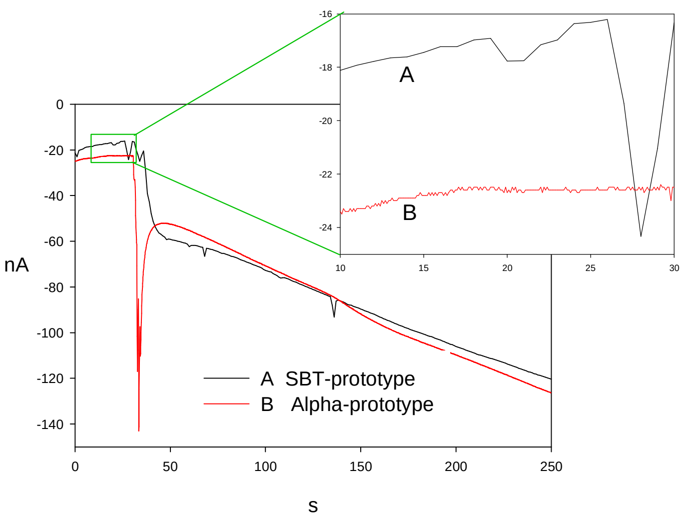
Comparison between calibration curves of Trametes versicolor laccase performed with SBTprototype and Alpha-prototype. Electrochemical response was plotted versus the activity measured by the syringaldazine test.

Table 1: ***Comparison between SBT prototype and alpha prototype***

		SBT-prototype	Alpha-prototype.
Baseline current	nA	14.1±3.2	18.8±2.7
Noise	nA	0.4 (with spikes >10 nA)	< 0.2 (with rare spikes)

Current was recorded in the same solution with two different G-sensor connected one to the alpha prototype and the other to the SBT prototype (n=7).

Figure 7: ***Comparison of the signal between alpha and SBT prototypes***



Further characterisation of the SBT prototype

INBB carried out some preliminary tests on the SBT prototype using musts obtained from grapes infected by *Botrytis cinerea* (infected juice, IJ), from healthy grapes (uninfected juice, UJ) or using artificially infected grape juice (AIJ) to assess the correct functioning and some characteristics of the system.

Detection limit

The detection limit of measurable laccase activity in the electrochemical cell resulted to be 0.15 ULA/mL which corresponds to about 1 ULA/mL in the analysed sample (dilution1/6).

Precision

The precision was tested with AIJ (BGJ-s sample) and resulted to be

2.78±0.08 ULA/mL (st.dev. ±2.9%)	n=5	Alpha prototype
2.62±0.15 ULA/mL (st.dev ±5.8%)	n=8	SBT prototype
3.33±0.23 ULA/mL (st.dev ±7%)	n=6	SBT prototype

<u>Dependence of the reaction rate on the reagents concentration</u>

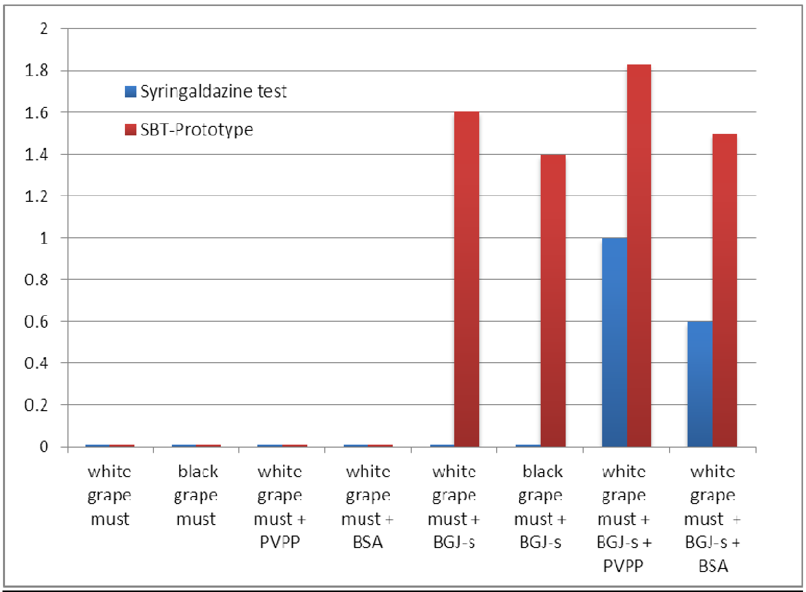
Also in the presence of high laccase concentrations a small amount of the reagents (substrate and oxygen) are consumed. These amounts should not modify significantly the reaction rate during the time course of the laccase activity test (3 min at maximum).

<u>Matrix effect</u>

INBB investigated the matrix effect on the SBT prototype. Matrix effect could be due to the competition for the active site of laccase between polyphenols present in must with the substrate used in the electrochemical test, or to a red-ox reaction of oxidized polyphenols with the substrate, or to the direct reduction at the electrode of oxidized polyphenols or other oxidized compounds present in must.

Figure 8, relative to musts obtained from healthy grapes (white and black) and to the same musts in which an amount of artificially infected grape juice was added, shows that in the case of the musts obtained from healthy grapes no laccase activity was measured both with the syringaldazine test and the SBT prototype. However when to these musts a botrysed grape juice (BGJ-s) was added, only the SBT prototype showed the presence of laccase. To obtain an indication of the presence of laccase with the syringaldazine test in these musts (must with added botrysed grape juice) they have to be incubated with PVPP or BSA. These compounds eliminate the presence of polyphenols so avoiding their competition with syringaldazine for the laccase. The incubation with PVPP or with bovine serum albumin (BSA) extends noticeably the time necessary to carry out the syringaldazine test (at least 10-15 minutes). Finally it must be pointed out that also in the case of the measurements carried out with the SBT prototype there is partial reduction of the laccase activity (about 30-40 %) when must is added to the botrysed grape juice. Moreover this issue should be re-examined using fresh prepared musts obtained from various type of botrysed and healthy grapes.

Figure 8: ***<u>Matrix effect in the testing laccase activity by the syringaldazine test and SBT prototype</u>***



Must from healthy grapes (black and white) and grape juice artificially infected by *Botrytis cinerea* (AIJ) were used.

<u>Examples of calibration curves</u>

Calibration curves of laccase activity of grape juices artificially infected by *Botrytis cinerea* (AIJ) and of must of botrysed grapes (IJ) were obtained with SBT prototype. The electrochemical responses were plotted versus the activity of the laccase present into the electrochemical cell measured according to the syringaldazine test. The curve slopes (sensitivity) were 5.4 and 5.3 (nA/min)/(ULA/mL) for AIL and IJ respectively, see **Table 2**. These slope values appear comparable with the slope value of calibration curve obtained using *Trametes versicolor* laccase, that is 6.2 (nA/min)/(ULA/mL).

Table 2: ***<u>Laccase activity calibration curves of artificially infected juice and of juice from infected grapes by SBT prototype</u>***

	T	**Curve slope**	**Curve Intercept** (0 ULA/mL)		**Concentration range**
	°C	-(nA/min)/(ULA/mL)	nA	R^2	ULA/mL
TvLc	26	6.2	-0.51	0.9997	0.2-12*
BGJ-s	26	5.4	-0.81	0.9957	0.2-5*
IJ	26	5.3	-0.18	0.9628	0-3.5*

(*) *Laccase activity in the electrochemical cell.*

3.2. Portable instrument

The SAFEGRAPE Portable (SP) instrument: is a completely portable device usable for the evaluation of the presence of Botrytis cinerea in grapes in the field (through the measure of Unit of Laccase Activity). This is a fully portable device, due to the reduced dimensions (150x100x50 mm), weight (less than 300g) and not requiring any connection to personal computer, through an accessible OLED display by means of a small integrated functions keys.

The main body is a palmtop of ABS, having dimensions of 150x100x50mm (LxWxH). The operator can handle it with a single hand and manipulate the parameters of the measure with a simple pressure of his thumb over the function keys available on the front panel of the device following the status of the instruments on the integrated OLED display, see figure.

Figure 9: Safegrape Portable prototype

Figure 10: Safegrape Portable prototype front view and rear view

The weight was further reduced to 350g, including the weight of the probe and the 4x+1.5V batteries. In fact, also the powering was torn to a total of+6V from the +30V necessary to power the Beta Test prototype, strongly reducing the power consumption of the device.

The probe of the SAFEGRAPE Portable prototype is based on a digital receiver, while the beta test prototype was based on an analogical one. The case is always made of aluminum but with dimensions further reduced to 16x75mm (ØxH). The use of a small PCB and the absence of leds had permitted this remarkable decrement in size.

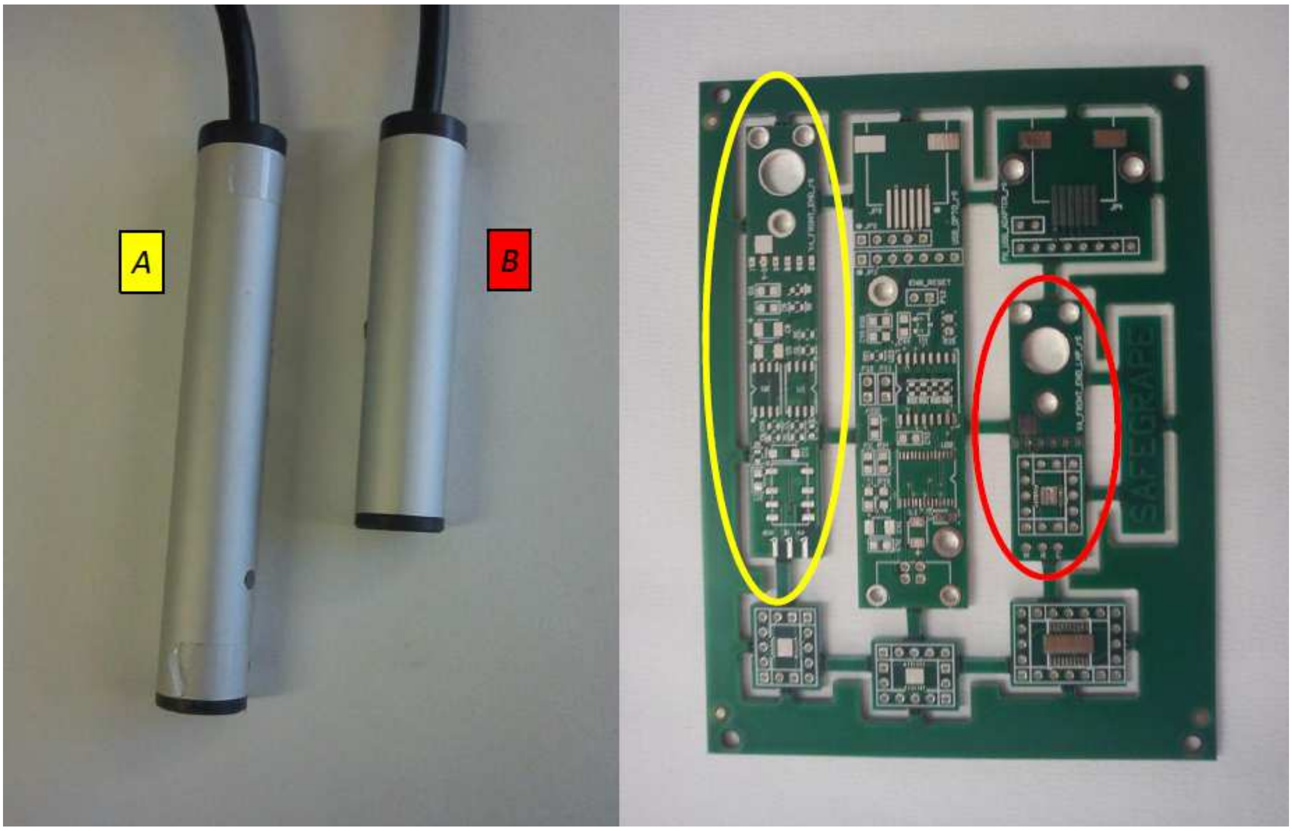


Figure 11: Safegrape Portable Probe (yellow) Vs Safegrape Beta Test Probe (red): comparison between body size (left) and PCBs (red)

The SAFEGRAPE Portable prototype is meant to work standalone through an integrated software which can be controlled with the keys on the front panel.

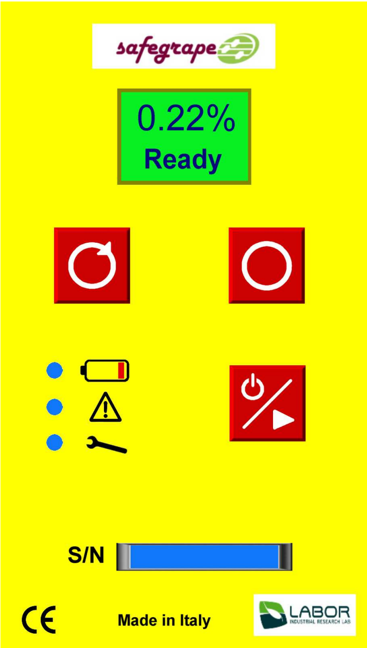


Figure 12: Safegrape Portable User Interface

There are three buttons, which different pressure sequences permit the setting of the parameters of the measure, perform an analysis, set the parameters and store the results of the measure.

The flow chart follow this possible associations of "key button" → "symbol":

-  is represented by the symbol 
-  is represented by the symbol 
-  is represented by the symbol , also used to switch "ON/OFF" the device;

The portable device has a USB connection with PC to download the measures done.

3.3. Automatic instrument

The SAFEGRAPE automatic instrument for the measure of the Laccase Activity in a grape juice or must adopts the same measuring system described in 3.1 and applied in the portable device.
The device is designed to automate every single action that in the portable system are realised by the operator. Essentially, they are:

- Sample preparation;
- Measuring;
- Cleaning

The list of the actions performed by the automation of the instrument is here presented, including a short description of the role that the automation will play to make the same action in a fully automated system.

Sample Preparation

Action 1: Gathering the sample must

- *Operator*: he gathers some must of the grape juice that needs to be analyzed through a disposable syringe;
  - *In-Line prototype*: the gathering of the must is an automated action realized through commercial samplers; the MCU switches on a peristaltic pump which fills a dedicated pipe line, a must line;

Action 2: Filtering the sample must (1)

- *Operator*: usually the collected must has a percentage of particulate which can interfere with the measure, so it has to be filtered. The operator connects a 0.45 micron PP (polypropylene) syringe filter to the syringe head;
  - *In-Line prototype*: the system automatically grabs the same syringe filter from a vending filter element and places it at the end of the must line;

Action 3: Filtering the sample must (2)

- *Operator*: he pushes the syringe plunger until a small amount of must (~2÷3 ml) is filtered and collected in a empty vial;
  - *In-Line prototype*: the MCU switches on an high precision pump to filter a fixed amount of must, 0,2ml, directly into the measuring cell of the system;

Action 4: Collecting the fixed amount of sample must (0.2 ml)

- *Operator*: to perform a measure, 0.2 ml of must are required. The operator collects this amount of sample through the use of a calibrated laboratory micropipette (M1) from the vial where it was previously filtered;
  - *In-Line prototype*: this action in already performed during Action 3;

Measuring

Action 5: Gathering of the reagent

- *Operator*: he gathers the fixed amount of reagent (1ml) necessary to a single measure through the use of another calibrated laboratory micropipette (M2);
  - *In-Line prototype*: the MCU switches on an high precision pump which fills a dedicated reagent pipe;

Action 6: Adding of the reagent

- *Operator*: he empties M1 in the measuring cell;
  - *In-Line prototype*: there is no need to filter the reagent, so the needed amount is already loaded in the measuring cell during Action 5;

Action 7: Insertion of the G-Sensor

- *Operator*: he deeps the G-Sensor into the measuring cell making attention that the level of the reagent his higher so that the heads of the electrodes are fully covered in liquid;
  - *In-Line prototype*: the deeping of the G-Sensor into the measuring cell is an automated motion;

Action 8: Acquiring of the baseline signal, i=f(t)

- *Operator*: it's a software task;
  - *In-Line prototype*: it's a software task;

Action 9: Adding of the sample must

- *Operator*: he empties M2 in the measuring cell;
  - *In-Line prototype*: this action in already performed during Action 3;

Action 10: Mixing of the solution

- *Operator*: he empties, then reloads and empties again the M2 micropipette in the measuring cell at least three time;
  - *In-Line prototype*: this action is performed by a vibrating element of the system;

Action 11: Gaining the slope of the signal, i=f(t)

- *Operator*: it's a software task which leads to the response of the measure;
  - *In-Line prototype*: it's a software task which leads to the response of the measure;

Cleaning

The measure is gained. The following steps are to be realised before starting a new measure.

Action 12: Emptying and cleaning of the measuring cell;

Action 13: Cleaning of the must pipe line;

Action 14: Replacing of the syringe filter;

Action 15: Replacing of the G-Sensor (if needed);

Figure 13 shows a global CAD design of the prototype which will be further described and itemized in its single elements, according to the "Actions" previously listed.

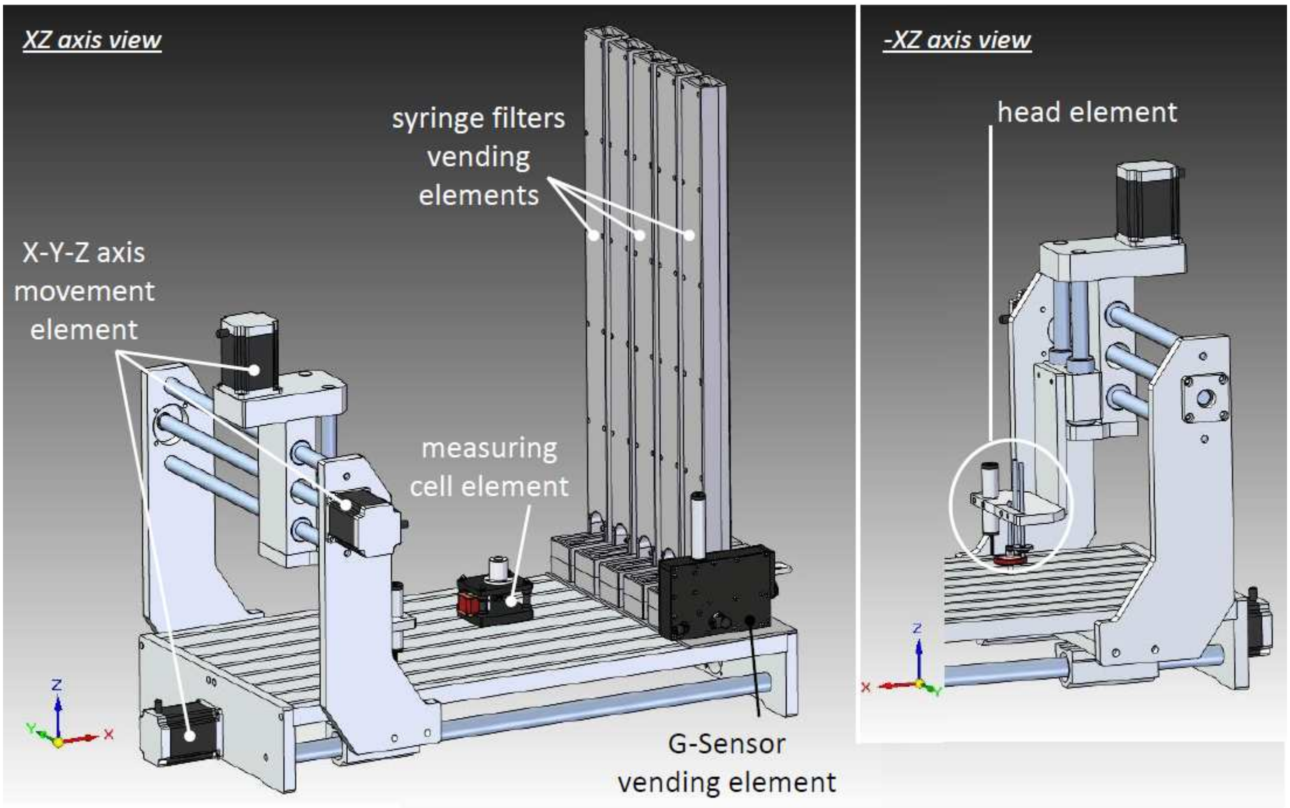


Figure 13: Safegrape In-Line prototype CAD design, XZ axis and –XZ view

Five main elements can be recognized in figure 11, they are:

- *The X-Y-Z axis movement element;*
- *The measuring cell element;*
- *The G-Sensors vending element;*
- *The syringe filters vending elements;*
- *The head element;*

The measuring cell is the location where the measure takes place, described in the following figure.

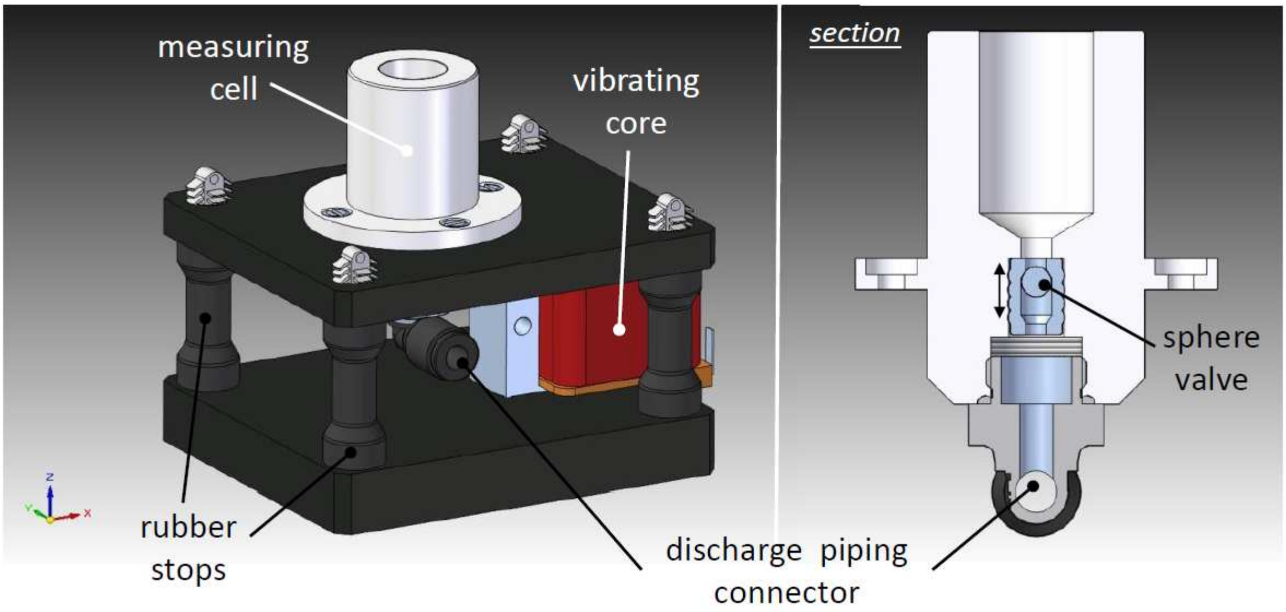


Figure 14: Safegrape In-Line prototype measuring cell element

The G-sensor and filter vending elements are described in the following figures.

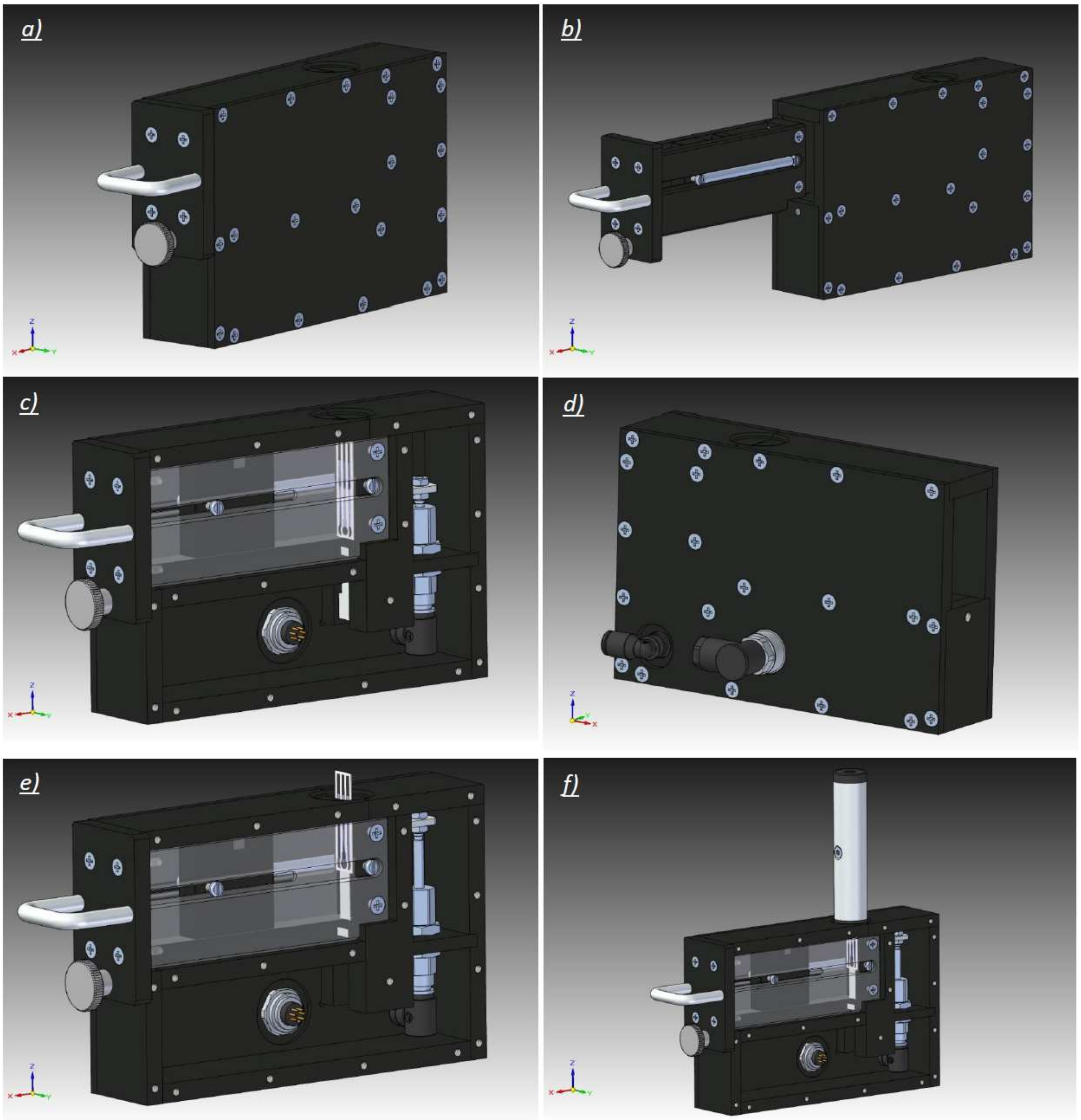


Figure 15: Safegrape In-Line prototype G-Sensor vending element

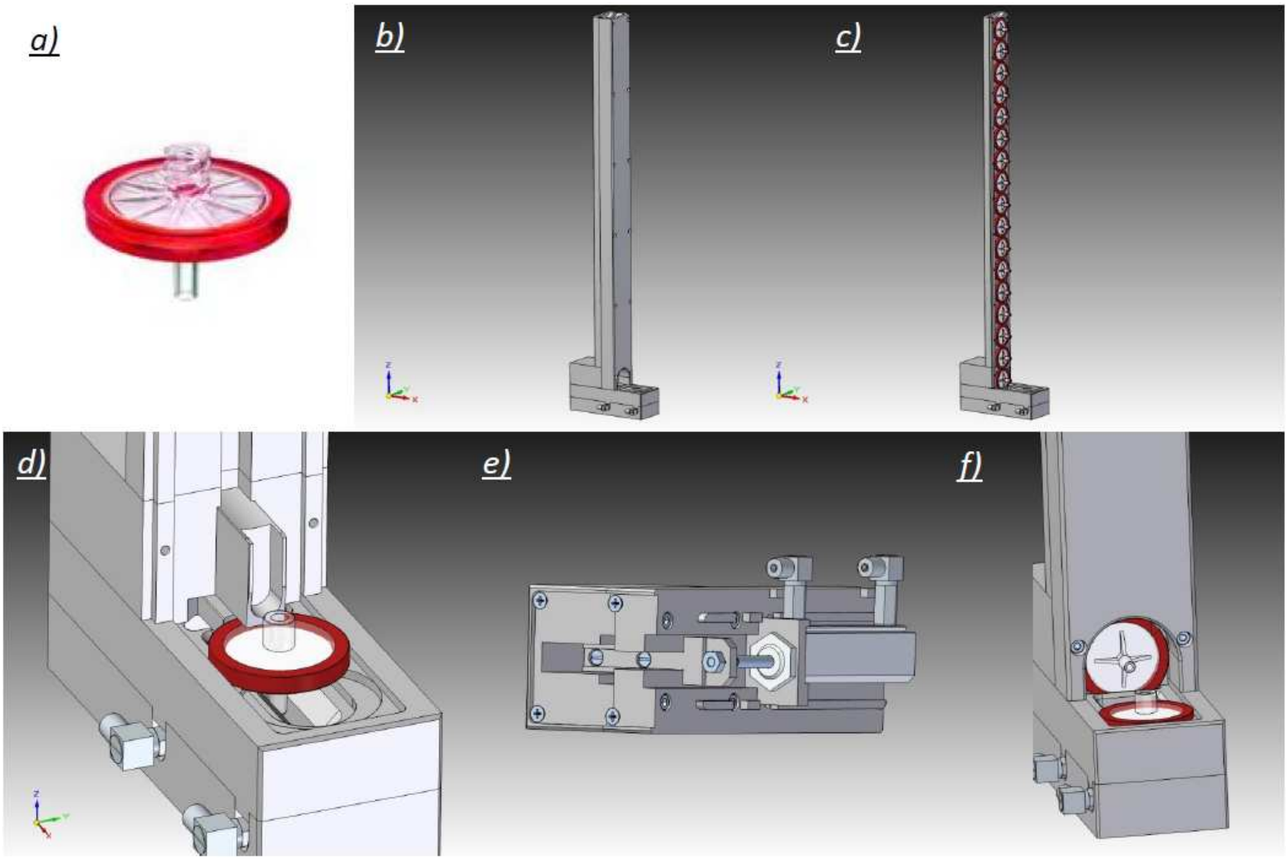


Figure 16 : Safegrape In-Line prototype syringe filters vending element

3.4. Field testing and results assessment

At UNIRIOJA, tests were carried out on 24 samples of white must type Viura, collected in La Rioja Region (Spain) with support of VITISTOP. Laccase activity measured with reference method ranged from 0 to 25.40 ± 1.26 ULAs.

On the other hand, tests were carried out by GRC during the same period, collecting and analyzing other kind of white must samples. Results were sent to UNIRIOJA in order to process them as an attempt to compare the behavior of the 2 prototypes, and also to test the application of the calibration models have developed for a type of must for predicting the laccase activity in other white musts (Chardonnay, Müller Thurgau).

The measures carried out by UNIRIOJA with the SAFEGRAPE prototype on the Spanish samples and the analysis of the results allowed:

1. To establish the most appropriate operational conditions and total working time for each test (250 secs)
2. To propose a signal smoothing by Kernel linear local regression as a diagnostic tool to select reliable measurements

3. To propose a R2 coefficient as a diagnostic tool in order to select/discard reliable/anomalous slope values for each time interval and replicate. Thus, a hard cut-off R2 of 0.999 was used in order to exclude any signal part significantly affected by perturbations
4. to evaluate the reproducibility of the electrochemical measurement
5. To evaluate the effect in the electrochemical measurement of the polyvinylpolypyrrolidone (PVPP) addition and to confirm this process not to be needed
6. To perform a calibration study between the syringaldazine method and the electrochemical measurement, through a linear regression analysis for different time intervals
7. To regression models for 3 different operative temperature, to be applied for prediction purposes, i.e. the regression model predicting the value of the ULAs reference value (obtained by the Syringaldazine test) [Y] versus the current gradient in the must computed from the electrochemical measurement [X].
8. To assess the main performances of the SAFEGRAPE prototype against the specifications. In short, the promising results obtained in the field tests performed could be seen as additional evidence that encourages the feasibility of using the SAFEGRAPE prototype as a preventive diagnosis tool in real vineyard samples

A picture of the testing interface of the portable instrument is presented. The first part of the measure is related to the acquisition of the baseling, the peak is due to the injection of must, while in the final part the slope is linearly related to the ULA/ml present in the sample.

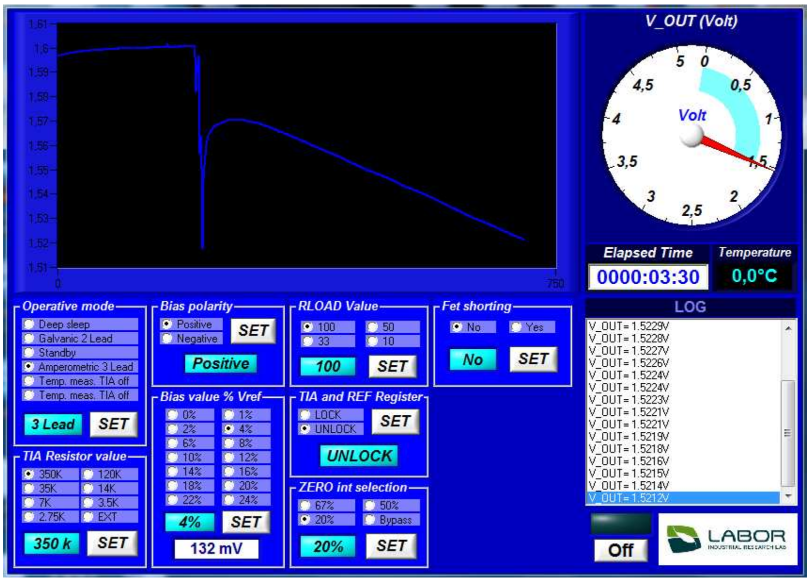


Figure 17: Testing interface of the portable device

In short, the promising results obtained in the field tests performed could be seen as additional evidence that encourages the feasibility of using the SAFEGRAPE prototype as a preventive diagnosis tool in real vineyard samples.

In the following table and overview of the performances of the SAFEGRAPE device as compared to commercial.

TABLE I: Safegrape Portable Prototype Assessment Specification			
Parameter	**RAISYTIS**	**BOTRYMAT**	**SAFEGRAPE portable**
Measuring method	**polargraphique - consumed oxygen**	**Colorimetric - syringaldazine**	**Electrochemical amperometric measure**
Measuring time	**3 m 40 s**	**2 min**	**3 minutes + 1 minute stabilization**
Repeatability (intra sensor)	**5.5 %**	**4.8%**	**3,7÷10,9%**
Reproducibility (inter sensor)	**13.8 %**	**low**	**4,6÷10,2%**
Limit of detection LOD	**1 ULA**	**0.1 ULA**	**0,2 ULA**
Limit of quantification LOQ	**1 ULA**	**Hard to detect low values**	**0,7 ULA**
Volume of sample must	**2 l**	**0.75 ml**	**0,2ml**
Dimensions	**H=550 mm L=600 mm P=400 mm**	**H= 1600 mm L=700 mm P=600 mm.**	**H=150 mm L=100 mm P=50 mm**
Weight	**50 kg**	**?**	**0.6 kg**
Automated	**YES**	**YES**	**NO**
Autonomy	**Grid connected**	**Grid connected**	**40 hours stand-by 4 hours measure**
Utilities	**Electricity Water compressed air specific reactive**	**Electricity Water compressed air specific reactive**	**Specific reactive**
Interface	**RS 232**	**?**	**USB**

Table 3: comparison of SAFEGRAPE with commercial benchmark (data for repeatability and reproducibility of benchmark are taken from the study "COMPARAISON DE DIFFERENTES METHODES D'ESTIMATION DE L'ETAT SANITAIRE DE LA VENDANGE" Jean- Christophe CRACHEREAU. Chambre d'Agriculture de la Gironde – Service VIN; April 2000).

4. Potential impact, dissemination activities and the exploitation of results

4.1. Socio economic impact

European vine cultivation and wine production has a **world dimension**. The European Union (EU) wine sector leads in terms of:

a) Production: European vineyards account for approximately 45% of the areas under vines in the world and produces, on average, 60% of world wine production;

b) Consumption: the EU accounts for almost 60% of world consumption;

c) Trade: the EU is both the leading world exporter and importer.

The **EU has more than 2.4 million holdings producing wine**, covering 3.6 million hectares, or 2 percent of EU agricultural area. **Wine production in 2006 represented 5% of the value of EU agricultural output**, with peaks of 10-14% for main producing countries and 20-30% in some specific regions. Competitors from New Countries (USA, Chile, Australia, Argentina and South Africa) are attacking the European wine industry position. If we consider the UK and Germany, which are the leading imports countries, **the new countries wine industries are gaining market shares while the old ones seem to loose their positions.**[3] Since the end of the 80s EU's (15) share of world wine exports declined from 81 per cent to 64 per cent in 2003, while the share of other countries, above all in North and South America and Australia, grew from 3 to 23 per cent. **Table 4** shows the changes in the export shares fro Macro-Regions between the 80s and the 2000s.

Table 4: *Evolution of the export composition Shares (%) on total export* **(Source: Pomarici 2005)**

Geographic Area	81/85	86/90	91/95	96/00	2003	
Main EU exporters*	76%	81%	75%	71%	64%	↘
USA and South Hemisphere Countries**	2%	3%	8%	15%	23%	↗
East Europe and Africa	14%	10%	5%	5%	4%	↘
Other countries	9%	8%	12%	10%	9%	=

* France, Italy, Spain and Portugal

** Chile, South Africa, Australia, Argentina

European production and marketing system based upon table wines and wines with Protected Designation of Origin is confronting to a new kind of producing and selling systems, adopted by the New Countries producers, in which variety of wines are offered under a brand and produced "industrially" in wineries. This strategy is based on mass production systems and it is not generally obliged to respect the numerous normative regulations existing in the EU countries.

[3] Source FAO GTI

It is evident that the wine market has experienced the creation of a new competitive scenario due to the affirmation on the international arena of producers from the *new world*, against the producers from the European Union and that the challenge can only be won through constant innovation and increase of product's quality.

This, together with the increase in market demand for quality wine at the expense of low quality table wine experienced in the last years, has pushed many European producers to specialise the production to organic productions, with no use of fungicides and reduced use of sulphites. Although organic wine makes up only 1% of the total wine market, the industry has been experiencing a steady growth of production, especially in USA which is about 20% per year; worldwide organic turnover in 2005 grew to about 20 billion Euro[4]. In Germany alone, last year's organic turnover rose by as much as 15% to 4 billion EUR[5].

Regions with very strong Botrytis problems due to the wet climatic conditions are Germany, north of Spain, north of Italy, north of France. These 4 countries (Germany, Italy, Spain, France) alone account for **more than 50% of world wine production**, as indicated in **Table 5.**

Country	Production (hl x 1.000)	% over world production
France	45.518	18%
Italy	42.050	16%
Spain	38.380	15%
Germany	8.000	3%
TOTAL	**133.948**	**52%**
WORLD	256300	-

Table 5: Wine production for SAFEGRAPE's target markets

The SAFEGRAPE devices will have relevant market opportunity, allowing for wine producers and growers:

- **the increase of wine quality**
- **the reduction of use of fungicides and sulphites**
- **the increase of harvest and production**.

Moreover, the further adaptation of the system to the needs of quality control in other fruit productions would open a much wider market.

[4] Organic Monitor
[5] ZMP, Zentrale Markt- und Preisberichtsstelle für Erzeugnisse der Land-, Forst- und Ernährungswirtschaft

4.2. Dissemination of results

Planned /actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible /involved
Available	*Web site*	*General public*	*ALL*		*BEGEROW*
18-10-2009	*Press Release*	*General public*	*SPAIN*		*UNIRIOJA*
21–04-2010	*Press Release- DWV*	*Wine producer*	*Germany*		*DWV, BEGEROW*
28-04-2011	*Workshop/Presentation*	*Wine producers*	*Germany*	*24*	*BEGEROW*
18-05-2011	*Press Release- Annual report of German wine grower association*	*Wine producer*	*Germany*		*DWV, BEGEROW*
23-05-2011	*Questionnaires*	*Wine producer*	*Germany*		*DWV, BEGEROW*
28-07-2011	*Presentation/wine tasting*	*Consortium of Rioja Wine producers*	*SPAIN*	*20*	*UNIRIOJA*
28-07-2011	*Press Release*	*General public*	*SPAIN*		*UNIRIOJA*
Feb. 2012	*Publication- Best practise*	*Consortium of EU projects*	*EU*		*BEGEROW*
30-03-2012	*Workshop/Presentation*	*Wine producers*	*GERMANY*		*BEGEROW, DWV, GRC*

1. Seminars

BEGEROW has organizes a specific seminar in Germany during April 2011, with support of DWV, who invites its associates, in order to show the results achieved. The seminar is supported by GRC, who provides scientific evidence of the research done and of the results achieved.
Another seminar was planned to be organized during the last part of the project with support of DWV. This event was shifted due to the delay in the finalization of the tests results and is planned to be organized by DWV and BEGEROW, with support of GRC during March 2012.

2. Presentation through association

Contacts with National and Regional wine producers associations in Italy, Spain and France have already been taken. They have shown interest to the SAFEGRAPE results and have declared to be keen to disseminate the results of the SAFEGRAPE to their associates as soon as available. A communicate indicating the results achieved by the SAFEGRAPE is sent to the associations, asking them to include it in the usual newsletters disseminated to the associates.

3. Press Releases

Dissemination through press has been carried out twice during the project:

- During the kick off meeting of the project (21/12/2009), Uni Rioja organized press release to present the aims of the project. The press release included:
    1. articles in 5 Spanish newspapers
    2. presentation during 3 radio and 3 TV news
- After the 6 month meeting, the project was published in a German magazine by DWV/Begerow:
    1. Article in the magazine Deutscher Weinbau (21/04/2010)
- An article in the annual report of the German wine grower association DWV was published (18/05/2011)
- During the meeting held at BODMED (28/07/2011), UNIRIOJA organized a presentation to the press, which included
    1. Articles in 4 Spanish newspapers
    2. Presentation during 8 radio and 2 TV news
    3. UNIRIOJA in the same occasion has also organized a presentation of SAFEGRAPE results and prototypes to press and to management of the PDO Rioja wine producers Consortium on 28/07/2011, together with wine tasting event, in the cellar of BODMED.

This was a great occasion to use the project results to improve the brand image and the quality pursue in the final phase of the project.

4. Web Site

A dedicated SAFEGRAPE web-site was produced at month 3 to increase the visibility of the project, with useful information, images and technical specifications, links to partners web pages. It is updated every 3 months. After the end of the project, it will be maintained to give specific information about the SAFEGRAPE project's scientific results and products technical specifications, in order to provide a proven scientific background to potential buyers of the devices.

5. Workshops

Dissementation of the results through presentations/Workshop.

- During a workshop of DWV the opinion leader and wine cellarmaster of the biggest cooperative in Germany Begerow / GRC presented the SAFEGRAPE project
    1. presentation 28/04/2011
    2. planned presentation 30/03/2012 – follow up

5.1. Exploitation of results

Exploitable Knowledge (description)	Exploitable product(s) or measure(s)	Sector(s) of application	Time for Commercial use	Patents or other IPR protection	Owner & Other Partners involved
Analysis Method for the measure of Laccase Activity through electrochemical device	Innovative Biosensor system for the measure of Laccase Activity	Wine production	2013		EBSR
	Portable instrument for the measure of Laccase Activity in grapes	Vine growing and wine production	2013		BEGEROW VITISTOP
	On-line instrument for the measure of Laccase Activity in grapes	Wine production	2013		BEGEROW VITISTOP
	Prototypes of the 2 different instruments and results of field tests	Wine production	2012		BEGEROW BODMED

1. Biosensor and Analysis Method

Description of result

Innovative biosensor system and analysis method for the measure of Laccase activity in grape juice and must, related to the presence of Botrytis (grey rot). A measuring system based on iron complexes compounds, able to react in presence of Laccase, and an electrochemical transduction system has been defined. In the project the biosensor system and analysis method was validated through field tests of real must.

The biosensor system is applicable both in the on-line and portable instruments developed in the project.

Methods applied so far are:

1. polargraphique method, which measure the consumed oxygen in grape juice.
2. colorimetrique methods, which is based on syringaldazine addition and measure of the colorisation in the presence of laccase.

Partners involved

EBSR specialised in the development of biosensors is the partner owning this result and involved in the exploitation.

Possible exploitation

EBSR intends to patent the "biosensor and method for the measure of Botrytis cinerea"
and to manufacture the SAFEGRAPE biosensor. EBSR will be free to use the biosensor
for the detection of Botrytis in other kinds of application than detection in grapes and must.

2. Online Instrument for quality control in wine production

Description of result

On-line instrument for quality control of grapes for the measure of the Laccase activity due to the presence of Botrytis (grey rot) in the loads entering the production site in cellars.

The device is to be integrated in the quality controls commonly applied (sugar and total acidity content) having the same response time.

Partners involved

BEGEROW and VITISTOP are the owners of this result.

Possible exploitation

Target application is quality control of grapes entering the production site in cellars.

The device is to be integrated in the quality controls commonly applied (sugar and total acidity content) having the same response time.

3. Portable instrument for field application in vineyards

Description of result

Portable instrumentation for the measure of grey rot content in grapes in vineyards. In the first period a lab set up of the portable device was implemented and tested, achieving a good reproducibility and sensibility. Possible layout of the portable device was defined.

Partners involved

BEGEROW and VITISTOP are owners of the result.

Possible exploitation

The target application is the vinegrowing, to detect the presence of Botrytis in advance and avoid the need of a-priori chemical treatments.

4. Prototypes and results of tests

Description of result

Prototype of the portable system is available and an executive design with feasibility analysis of the inline instrument has been produced.

Field tests have been realised with the prototype on real must showing the feasibility of the measure.

Partners involved

BEGEROW and BODMED are the owners of the prototypes.

Possible exploitation

BEGEROW will keep the prototypes for further tests and evaluations for production and commercialisation, agreeing with other partners for the temporary availability of the prototypes for testing.

5. Public website address and relevant contact details.

Project website: www.safegrape.eu

Coordinator contacts:

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SME participants:

- E. BEGEROW GmbH & Co. (Germany) www.begerow.com
- BODEGAS DEL MEDIEVO SL (Spain) www.bodegasdelmedievo.com
- ECOBIOSERVICES AND RESEARCHES SRL (Italy) www.ebsr.it
- VITIS TOP ASESORIA EN VITICULTURA SL (Spain) www.vitistop.com

Other participants

- Deutscher Weinbauverband e.V. - German Wine Growers Association e.V. www.dwv-online.de

RTD performers:

- ISTITUTO NAZIONALE BIOSTRUTTURE E BIOSISTEMI (Italy) www.inbb.it
- Forschungsanstalt Geisenheim (Germany) www.forschungsanstalt-geisenheim.de
- UNIVERSIDAD DE LA RIOJA (Spain) www.unirioja.es

Figure 18: SAFEGRAPE team with the prototype at the final meeting in Geisenheim