Introduction

Wine fermentation process still has some difficulties to be managed and its kinetic is often not easy to predict.

The use of wine yeast, and a complete set of correctly balanced nutrients, mainly nitrogen, vitamins etc., together with a correct temperature management, are responsible of the fermentation aptitude of musts, but the complete and rapid monitoring of vinification requires the management of much analytical information, leading to interpretation problems. The oenologist has two strategies to fulfil this task:

- The use of complex (and expensive) analytical equipments allowing rapid measurements of multiple analytes at the same time, like FT-IR spectrometer or similar. Biosensors are a possible alternative: they are small devices containing immobilized enzymes, antibodies or other biological molecules that can perform quick analyses on a small surface and with a reduced amount of sample, without requiring a biological or chemical background or skill.
- Apply information technologies that can manage a great number of complex data and, coupled to mathematical models expressly built up, help the operator to interpret phenomena and to solve complex problems.

The aim of the study was the monitoring of alcoholic and malolactic fermentations in a continuous way in a winery scale. This was done with a pressure sensor installed on the bottom of 300-hl fermentation tanks for monitoring the alcoholic fermentation, and for malolactic fermentation in a discontinuous way by using biosensors containing immobilized enzymes. It is the result of an international cooperation of partners having oenological, microbiological, electro-chemical and informatics knowledge.

Fermentation data were acquired by newly developed software that makes use of a mathematical model that describes (alcoholic) fermentation kinetics. Furthermore, the software manages data coming from sensors and from external sources, supporting in this way the oenologist on taking decisions about fermentation and the overall winemaking process.

Variables affecting fermentation kinetics

Wine fermentations differ in kinetics, depending on temperature, yeast strain and on grape must composition. In order to build up a mathematical model able to explain the whole fermentation curve, and to define the variables that can affect its parameters, we compared the kinetics of several commercial yeasts on the same musts, and then those of three yeasts on
musts to which nutrients have been added in different amounts and at different times. The inhibition of fermentation activity was studied after copper addition to the must, again comparing its effect on several commercial yeasts.

The carbon dioxide produced was measured as weight loss weighing the small bioreactors, closed with Muller valves, three-four times per day with a Sartorius CP 2202S balance connected to a computer by mean of SartoConnect software. Fermentation was considered to be over when the weight was unchanged for 48 hours.

The enological parameters that are critical in the definition of a fermentation curve are the lag phase, the maximum CO$_2$ production rate, and the fermentation duration, or Total Fermentation Time (TFT). Equations are known that allow the calculation of such parameters, the most common are the Gompertz and Verhulst-Pearl equations. Gompertz equation has been successfully modified by Zwietering et al. (1991), in order to calculate lag, $\mu_{max}$ and the Asymptote (A) of the microbial growth curve. The same equation can be used for derived parameters, like sugar consumption rate, ethanol or carbon dioxide production rate. In these cases the asymptote is less important, as it depends only on the amount of sugar to be fermented, but it is helpful to calculate TFT: we calculated TFT as the time required to reach the 99% of A value [Cavazza et al., 2004].

The stimulation and the inhibition of fermentation activity were measured on the two parameters lag phase and maximum CO$_2$ production rate. Experimental data fitting gave satisfactory results with the modified Gompertz equation (Zwietering et al., 1990), but only for sigmoid curves, i.e. those in which the fermentation conditions did not change during the process. However, poor fitting was found if nutrients were added during the fermentation, thus modifying the fermentation rate in a point inside the curve.

The following differential equation, describing growth on a sigmoid shape was then used:

$$\frac{dx}{dt} = \mu(t,x(t))x(t)$$

Where t is the time, and x(t) is the amount of produced carbon dioxide during alcoholic fermentation or L-malic/L-lactic acid concentration during malolactic fermentation.

The specific growth rate of the fermentation process $\mu(t,x(t))$, indicative of the process rate, is assumed to be dependent on exogenous factors, as temperature, pH, initial sugar concentration, nutrients… Depending on CO$_2$ production rate, different kinetics model can be used, as Verhulst-Pearl, Gompertz and extended Gompertz. The first two are well known, while the third is proposed now in order to have better results.

According to the form assumed by the specific growth rate, it is possible to characterize different mathematical models, such as:

1. Verhulst – Pearl (logistic): $\mu(t,x(t)) = r(1 - \frac{x(t)}{b})$

2. Gompertz: $\mu(t,x(t)) = k e^{-a t}$

3. Gompertz-extended: $\mu(t,x(t)) = k e^{-a t}(1 - e^{-b t})$

It can be verified that, with the above specific growth rates, the corresponding models describe a population growth that starting from a maximum value (model 1 and 2), gradually decrease to zero, as long as the substrate is consumed. Model 3 has been introduced, because
it seems to be more coherent with the dynamics of fermentation processes, since it starts from a zero value, and, after reaching a maximum value, decreases to zero.

The parameters characterizing each growth model (r and b in model 1, k and α in model 2, k, α and β in model 3) can be determined by using a specific parameter identification method. Identification consists of determining the pertinent parameters by minimizing a suitable cost function that takes into account the discrepancies between a set of measured and predicted concentration values. Some preliminary identification tests conducted on experimental alcoholic fermentation data confirm that a good validation can be achieved by using the three above presented models. An example of the good agreement between experimental and predicted data for Logistic, Gompertz and Gompertz-extended models is shown in Fig.1, 2 and 3. Such preliminary results related to experiments of alcoholic fermentation confirm that a good validation can be obtained with all the above presented models, even if the Gompertz-extended model seems to achieve the best performance in terms of predictive capability.

Fig.1 : Fitting of alcoholic fermentation by logistic model (time in days)
It can be concluded that the Gompertz-extended model is capable to predict the specific growth rate of CO2, particularly at the early fermentation stage.
**Fermentation monitoring in winery scale.**

The fermentation monitoring was transferred in a winery scale, by using pressure transducers to measure the weight decrease due to the production of CO\(_2\) and the decrease of density from must to wine. Three transducers have been installed in three 300-hl tanks in Armani winery (Dolcè-Verona). They were ST SA food-grade 0-1 bar pressure transmitters produced by Nova Fima (Invorio -Novara - Italy), with 4 – 20 mA output range. Pressure transducers have been connected with the DSP Compact Field Point of National Instruments, which acquired data every eight seconds and stored them on a connected PC. One DSP can acquire data from up to 32 tanks, and they can be then downloaded to a PC afterwards. By measuring the pressure variations fermentation curves were acquired, as shown in fig. 4, and the mathematical model was used to calculate fermentation parameters.

![Fig 4: Variation of pressure during fermentation in a 300-hl tank](image)

The system allowed also the control of tank filling and emptying, together with all the variations in the must level in the tanks. In this way it can be useful for wine traceability.

**BIOSENSORS AND MALOLACTIC FERMENTATION CONTROL**

The carbon dioxide amount produced by lactic acid bacteria during malolactic fermentation process is about 100 fold less than that detected during alcoholic fermentation. Since this quantity was not clearly detectable by the pressure transducers, and there were any feasible possibility to monitor this important transformation step continuously in a cheap way, it has been decided to control this process off-line developing a new concept electrochemical biosensors applied for L-malic acid and L-lactic acid detection in wine, that carry out fast and easy measurement in a selective way.

The development of electrochemical biosensors based on dehydrogenases using the NAD\(^+/\)NADH cofactor, is an attractive goal for many researchers mainly due to the large field of substrates to analyse. In theory the direct oxidation of NADH at the electrode surface can be used as an indicative reaction, in practice this is very often not appropriate due to the large anodic potential required that induces interferences as well as electrode poisoning. The use of electron transfer mediators is an attractive alternative to avoid such undesirable phenomena and to increase the biosensor selectivity. In spite of the advantages brought by the use of mediators, the problem of interferences is still, in many cases, to be solved in particularly
when the sample to analyse, such the wine, has a very complex matrix and contains many oxidizable products. We developed a new concept of redox-flexible bioassays or biosensors that gives a response to such challenge [Charpentier L. et al., 1995a; Charpentier L. et al., 1995b; Serban S. et al., 2006]. This concept can be applied to very large number of enzymes, virtually all oxidases and NAD+/NADH dependent dehydrogenases.

For a given substrate, when the corresponding oxidase enzyme (Sox) is available, the concept is easier to implement because it needs only the redox mediator (e.g. FcCH2OH) and two enzymes: the oxidase itself (Sox) and the Horse Radish Peroxidase (HRP). The reactions involved for the detection and quantification of the substrate are shown in Figure 5:

\[
\begin{align*}
    \text{FcCH}_2\text{OH} & \quad \rightarrow \quad \text{FcCH}_2\text{OH}^+ + e^- \quad \text{[anodic current]} \\
    \text{Substrate} + 2 \text{FcCH}_2\text{OH}^+ & \quad \rightarrow \quad \text{Product} + 2 \text{FcCH}_2\text{OH} + H^+ \quad \text{(2)} \\
    \text{Substrate} + O_2 & \quad \rightarrow \quad \text{Product} + H_2O_2 \quad \text{(3)} \\
    \text{H}_2\text{O}_2 + 2H^+ + 2\text{FcCH}_2\text{OH} & \quad \rightarrow \quad 2\text{H}_2O + 2\text{FcCH}_2\text{OH}^+ \quad \text{(4)} \\
    \text{FcCH}_2\text{OH}^+ + e^- & \quad \rightarrow \quad \text{FcCH}_2\text{OH} \quad \text{[cathodic current]} \\
\end{align*}
\]

Fig 5: Reaction scheme

The system is redox flexible because it can be applied either in oxidation or in reduction. In the first case reactions (1 and 2) take place and the anodic current is related to the substrate concentration. In the second case reactions (3, 4 and 5) are involved and the cathodic current is then proportional to the substrate concentration. The oxido-reduction potential of FcCH2OH/ FcCH2OH+ constitutes the border which separates the two applications. The first takes place at more positive potentials and the second at more negative potentials. It is obvious that when the matrix of the sample is complex and contains various products, the choice of the working potential can be set in a way to have less interference. The food samples normally contain many oxidizable products and the reduction mode makes it possible not to have interferences which distort the measured current.

When the enzyme oxidase is not available, the concept can be used with the corresponding dehydrogenases. The concept is then based on the use of the NADH oxidase (NAox) in the presence of a redox mediator (FcCH2OH) and horseradish peroxidase (HRP) associated with a preliminary reaction using the dehydrogenase and producing NADH. In this case the redox mediator can be involved in two different pathways depending on his oxidation state and creates at the electrode surface either an anodic or a cathodic current offering a large working potential range. Such flexible measurement conditions permit to avoid most of the interferences and to reach high sensitivities.

The redox flexible dehydrogenase bioassays and biosensors work according to the reactions scheme shown in Figure 6:
To follow the concentrations of lactic and malic acids, as substrates, during wine fermentation, the lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) are respectively used in reaction (6) while the other reactions remain the same.

For the realization of reagentless biosensor operating in reduction mode, the three enzymes dehydrogenase, NAox and HRP as well as the redox mediator (FcCH₂OH) will be fixed on the surface of the screen printed electrochemical cell. The sample, adequately diluted, is deposited on this surface and the current is measured by chronoamperometry. In this case reactions (6, 8, 9 and 5) take place and the measured current is proportional to the concentration in substrate in accordance, of course, with the kinetic effects. When a reagentless biosensor is used reaction (7) has to be considered. If the kinetic of reaction (7) is fast enough this will provoke the appearance of interference phenomena at the surface of the working electrode and the measured current will not account for the exact value of the substrate concentration. If the kinetic of reaction (7) is very slow, this effect will be negligible and measurements will be exact.

The use of bioassay mode for the measurement of a substrate makes it possible to isolate one or more reactions shown in the previous scheme. This has the advantage of carrying out the other reactions without interferences and also of carrying out them up to the end point. Thus the exhaustion of the substrate and the NADH generated by the reaction (6) allows a simple and an accurate analysis via reactions (8, 9 and 5).

In preceding studies carried out on various white and red wine samples we showed that the analysis by oxidation using biosensors was complicated because of the existence of a large number of oxidizable products in the samples [S. Serban et al., 2004]. For the first studies carried out according to this new concept, we used the method of bioassay with an electrochemical cathodic detection according to the following procedure: a wine sample is adequately diluted in a solution of phosphate buffer, 50µL of the obtained solution are placed for 2 minutes in a tube containing the NAox and either LDH or MDH. This solution is then placed on the top of the peroxidase biosensor and left for 1 minute then measurement, by chronoamperometry, is carried out for 20 seconds. The measured current is then referred to a calibration curve which makes it possible to measure the concentration in lactic or malic acid.

The L-lactic acid has the advantage of the availability of the two enzymes, the oxidase and the dehydrogenase whereas for the L-malic acid only the dehydrogenase is available. The results for the L-lactic acid obtained with the two types of enzymes will be shown. The calibration curve of the dehydrogenase presents a fairly large zone of linearity between 0.005 mM (0.45 mg/L) and 0.1 mM (9 mg/L). The limit of detection is 0.001 mM (0.09 mg/L). With the oxidase, the calibration curve presents a wider linear range (0.005 – 0.35mM), higher
sensitivity and the same detection limit. These characteristics show for both enzymes that the method is very sensitive and is capable to detect very weak concentrations in acid and dilution of the sample beforehand is needed.

The bio-electrochemical method was validated by measuring the concentrations of lactic acid in one wine in the course of fermentation (N°1) and four commercial wines (N° 2 to 5). The results of electrochemical bioassays were compared with those obtained by the spectrophotometric standard method. The comparative results are shown in the following table 1. These results, although preliminaries, show a good correlation between the method which we describe and that used with enzymatic kits utilising spectrophotometers, the method often used by winemakers.

Table 1: comparative results between the standard method and the bioassay method obtained on wine samples

<table>
<thead>
<tr>
<th>Wine</th>
<th>N° 1</th>
<th>N° 2</th>
<th>N° 3</th>
<th>N° 4</th>
<th>N° 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard method (g/L)</td>
<td>1.35</td>
<td>1.86</td>
<td>0.00</td>
<td>0.00</td>
<td>2.02</td>
</tr>
<tr>
<td>Bioassay method (g/L)</td>
<td>1.36</td>
<td>1.77</td>
<td>0.00</td>
<td>0.00</td>
<td>2.04</td>
</tr>
</tbody>
</table>

The originality of the concept we describe lies in the possibility that the amperometric bioassay or biosensor is able to function either in oxidation or in reduction and can cover large ranges of substrate concentrations and gives the chances to escape from interference problems. Moreover the great number of available dehydrogenases makes this generic concept applicable for many analyses and compels biosensors to become much more attractive to produce and to market in various fields of wine industry.

Wine Monitor 2006

Wine Monitor 2006, is a software for monitoring wine fermentation, developed at the Department of Information and Systems- University of Pavia. The task of this software is to control the process of wine fermentation by using real time measurement and knowledge of the previous processes. Wine Monitor 2006 has been developed using Visual C# and recent programming techniques, such as ADO.NET and NI Measurement Studio 7. Therefore it is very fast, reliable and relatively compact. In Figure 7 is shown the basic structure of Wine Monitor 2006:
This software has the following group of objects: Measurement Objects, User interface Objects, Simulation Objects, Analysis Objects, DSS Objects.

A first group of objects, “Measurement Objects”, are responsible for communication with measurement hardware by using some of the standard protocols.

From the second group of objects “User Interface Objects”, a graphical user interface (GUI) is built. On the Wine Monitor 2006 main form are located several diagrams, displays, knob, led diode and wine tank icon. All these elements represent so-called “virtual instruments”. On the menu there are located items for working with databases, simulation, data acquisition and for exporting data on several different ways and formats. In Figure 9 there is presented the main form of Wine Monitor 2006.

“Simulation” objects are responsible for performing “real time” and “off-time” simulation. Off – time simulation is based on the previous measurements (“knowledge” database). Real – time simulation is based on the previous measurements and on the real – time measurements (“knowledge” database and database “measurements”). With use of the real – time simulation it is possible, in every moment, to predict final results of fermentation and to suggest next actions with the intention to obtain the best results of the fermentation.

“Analysis” objects help us to better understand measured data. There are several different actions like zoom entire diagram or some part, searching for specific value on the curve, exporting values, etc.

“DSS objects” are responsible for making decision. These objects use data from Database “Knowledge” and Database “Measurements”. The algorithm is based on a Decision Support System (DSS), based on validated statistical tests and methods. Communication between DSS objects and databases is based on the ADO.NET technology, which is the latest implementation of Microsoft’s Universal Data Access strategy. Databases are very important elements in Wine Monitor 2006. This program communicates with MS Access 2003 database. For physical storing of data there are two options at our disposal, SQL Server and Access.
After a careful study it has been decided that MS Access 2003 is good enough to respond on the all demands of our project and that is no need for using SQL Server.

Wine Monitor software is expected to carry on the integration of real-time fermentation measurements, MSDF, identification of mathematical model of fermentation process, database management and decision support system.

References


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