Biosensor-based intelligent measurement system for wine fermentation monitoring

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Abstract

The paper presents an innovative distributed measurement system for wine fermentation monitoring. The system intelligence is distributed among some Local Measurement Units (LMU) and a Manager Unit (MU). LMU’s, made by a biosensor with its conditioning circuit plus a signal acquisition and processing module, can be integrated in complex multi-sensor schemes for process monitoring under control of a MU. The innovative approach proposed in this work allows to a fully automatized measurement process. A prototype of the system characterized for wine fermentation monitoring has been set-up as a case study and first experimental results are presented.

1. Introduction

During last years, biosensors had a large diffusion in many applicative fields, due to their performance in terms of measurement time, sensitivity, and cost.

For example, novel applications of biosensors appeared in health-care (e.g. glucose monitoring in diabetic patients), environmental monitoring (e.g. the detection of toxic substances in water), food industry (e.g. both in the determination of drug residues in food and for food quality) [1]-[7].

Among various interesting features, the major advantage of biosensors can be found in their selectivity. Thanks to the biological part, they are able to measure the concentration of a single component (usually named analyte), even in a very complex mixture [1]-[4].

Biosensors can be made using various transduction principles. There exist photometric, piezoelectric, thermometric and magnetic biosensors, although the electrochemical ones, and in particular amperometric biosensors, are widespread at the most.

Amperometric biosensors are normally based on the enzymatic catalysis of a reaction that produces or consumes electrons: the related electrical current is proportional to the analyte concentration [4]-[7].

In most of the applications, a batch-type measurement is made: at first the mixture to be measured is posed in contact with the enzyme, and then the generated current is evaluated.

The electrical signal from the transducer often shows a low level value and is superimposed on a relatively high and noisy baseline. Generally, the baseline is evaluated by means of a signal produced by the same transducer without any biocatalytic membrane. The measurement signal is obtained by difference between the “biosensor” signal and the baseline, successively filtered and amplified.

The significant uncertainty often characterizing the baseline reduces the reliability of the measurement [8]. Recently, flow injection analysis (FIA) come into widespread use, because of the high reliability this technique shows in measurements [9]-[12]. FIA is also characterized by a shorter response time so to allow the realization of on-line measurement procedures.

Nowadays, electronic measurement devices designed for conditioning biosensors and processing their output are more and more of common use in the scientific community, but one strong limit to their pervasive diffusion is the need to be locally controlled and set up by an expert user [13]. In fact, these instruments provide rough values of electrical currents that can be visualized on a computer, in order to allow the expert to select the current whose amplitude has to be evaluated. Once the correct value is selected, the analyte concentration can be esteemed on the basis of the biosensor characteristics. At the best of our knowledge, no existing measurement system implements the knowledge and the skill necessary for automatic management of a whole measurement procedure based on FIA.

In this paper, an innovative measurement system for automatic FIA is proposed. It is made of local measurement units and of a central control unit. The main innovations are concentrated in the digital signal processing: peak amplitude measurement is fully automatized because the proposed units are able to detect the correct peak. The biosensor characteristics can be memorized in order to provide
the analyte concentration as result. Furthermore, the LMU's can be interfaced via RS485 port to a controller in order to be used in a multisensor measurement system for FIA process automatic management. A novel unit playing the role of controller (Manager Unit) has been designed to control several LMU's and a set of actuators, e.g. pumps and valves. Its main task is making decisions based on both the continuous evaluation of measurement data reliability and the knowledge of static and dynamic characteristics of sensors and actuators. In the opinion of the authors, the proposed system can be considered a good candidate to contribute to a larger and pervasive utilization of biosensors.

In the following of this work, a detailed description of the LMU is presented so to focus the originality of the computing solutions. A case study of the application of the automatic measurement system to the monitoring of wine fermentation is then detailed and, finally, first experimental results are showed and briefly discussed.

2. Local measurement units

Each Local Measurement Unit is composed by three main parts: sensible element, conditioning circuit, processing unit. Its schematization is reported in Figure 1. The sensible element (the biosensor) is constituted by a sensitive biological element (immobilized enzyme) within an electrochemical cell. Using the electro-chemical properties quoted in the Introduction, the biosensor converts the concentration of the analyte under test into a current. The conditioning circuit transforms the current signal in a voltage signal, which is measurable by the processing unit. Finally, the acquired voltage is processed to evaluate the current parameters that, through the use of a biosensor calibration curve, lead to the analyte concentration calculation. The LMU can be connected with either a supervisor PC, for a single measurement [14], or with a Manager Unit able to automatize the FIA process.

2.1. Biosensors

The biosensors are realized covering a sensor substrate with a specific substance carrying the enzyme. The enzyme layer defines the biosensor selectivity: the sensor capability changes when substituting the enzyme. Depending on the nature of the specific enzyme, the reaction between the target analyte and the enzyme produces or consumes electrons. The sensor substrate contains three electrodes: the working electrode acting as cathode W; a reference electrode R, which is the anode, and a counter electrode C [15]-[16]. In Figure 2, a schematization of the electrochemical cell (Figure 2a) with its photo (Figure 2b) is shown.

The target analyte is involved in the reaction determining a flow of electrons. The magnitude of
this flow and the voltage drop between the working electrode W and the reference R depend on the analyte concentration. As a consequence, the current has to be measured at a fixed potential [18]-[19]. To this aim, the current is forced to flow through the counter electrode C instead of the reference one.

2.2. The conditioning circuit

The conditioning circuit is designed to both control the electrochemical cell and convert the biosensor current in a measurable voltage. As for the former task, it works as a potenziostat circuit [7]: a known voltage between working and reference electrode is generated by the D/A converter of the processing unit, while a zero current is imposed in the reference electrode by means of a J-FET operational amplifier.

Moreover, the current flowing from the counter into the working electrode (proportional to the analyte concentration) is first converted to a voltage signal and then amplified by the “transducer” (see Figure 3).

2.3. Processing unit

The signal acquisition, conversion and processing is carried out by a PIC18F4620 microcontroller (by Microchip™) that is featured with both analog to digital (ADC) and digital to analog (DAC) converters. The specifications of the PIC18F4620 are: 8 bit fixed point arithmetic; 10 MIPS; 13 channels analog multiplexer; 10 bit ADC, 100 kHz maximum sampling frequency; 3 timers for the event synchronization; an USART port for the PC interface to be used in the developing phase. The potentiostat working voltage value, generated by the DAC output, depends on the single biosensor (for example, the best operating voltage for a glucose biosensor is 700 mV while for the phenols biosensors is -50 mV [12], [17]). Both the conditioning circuit and the processing unit are integrated in a unique SMD technology board, see Figure 4, and closed in an aluminum case acting as a shield versus electromagnetic induced disturbance.

The communication between LMU and MU can be realized through a serial link: RS 485 or RS 232.

2.4. Digital signal processing

As mentioned in the Introduction, Flow-Injection Analysis (FIA) has the goal to monitor the analyte concentration. Once a suitable circuit has been realized to allow a continuous flow of current through a biosensor cell, FIA starts by injecting a reproducible volume of the substance to be analyzed (sample) into the cell.

When the sample passes through the biosensor cell, a transient peak of current is produced whose characteristics depend on the analyte concentration in the sample [11]. So, the electro-chemical current is constant (regime current) apart from the time when the sample reaches the biosensor. Under this circumstance, it increases and when the sample...
completely flew away from the biosensor it decreases back to the regime value.

The consequent current peak value (see Figure 5) depends on both the analyte concentration and the biosensor static and dynamic characteristics [5]-[6].

First novel features implemented in the measurement software of the LMU are the automatic detection of the current peaks generated by sample injections and their magnitude measurement in respect to the regime value.

After the low pass filtering obtained through a moving average of the signal samples, the measurement software performs the following four steps procedure:

1. regime condition identification;
2. regime current measurement;
3. detection of a current peak due to a new injection;
4. peak magnitude measurement and transmission to the supervisor PC.

In the following, each step is described in more details.

1. Every M samples the condition of regime is tested: is the difference between measured maximum and minimum values in the time window less than a threshold?
2. If yes, the regime condition is satisfied and a new regime current value is memorized.
3. In order to detect the injection, and in particular the beginning and the end of current peaks, a definite integration of the preprocessing signal is executed. At each new filtered sample, the absolute value of the difference between the present and the previous one is evaluated and the moving sum of the last N differences is upgraded. When this last quantity overcomes a suitable threshold, a new injection is detected. When this value comes back under the threshold, the end of a peak is identified.
4. Once a peak has been detected, the maximum current value is searched; the peak amplitude is then calculated as the difference between the measured maximum value and the regime one.

In order to avoid the missing of any injection, steps 1 and 2 are continuously run until an edge is detected. Then, steps 3 and 4 follow and after their execution the whole procedure starts again.

In Figure 6, the acquired current versus time in presence of two successive injections is reported, together with a zoom for the peak measurements. The reported acquisition includes a transitory phase with low dynamic corresponding to the biosensor first connection to the carrier flow).
2.5. Local measurement unit performance evaluation

The performance of the proposed digital signal processing software was estimated by means of several experimental tests.

Before describing such tests, it is important to underline that a specific software module have to be included with the aim of compensating the systematic errors revealed through the metrological characterization of the LMU's [13].

As far as concerning standard uncertainty of the measurements of current made by LMU's, tests were carried out by making successive injections firstly with a given concentration of a reference analyte (in our case, hydrogen peroxide) and then with different values of the quoted concentration. In these tests, a standard uncertainty of 2.0 nA was estimated in case of single injection. This value downs to about 1.0 nA, when the results of three successive injections are averaged for each measurement.

Processing time is evaluated to determine the maximum sampling frequency allowing the use of the system for real-time scenarios. Since the computational load changes during the measurement phase, only the maximum value of the processing time is considered. A value of 7.5 $\mu$s was measured in the final version of the software module and then a 100 kHz sampling frequency was adopted.

Figure 7 reports the quantities measured by the digital signal processing together with the measured current (blu line) versus time. In more detail, the Figure shows: the peak maximum values (dotted line), the regime current values (red), and the detected time when peak begins (green) and ends (lilac).

By analyzing such results, it is possible to verify that the software promptly detects the peak and properly defines the peak end.

The performance of the LMU processing software was evaluated for different types of biosensors: hydrogen peroxide, glucose, lactic acid, ethanol and phenols biosensors.

As for the peak detection capability, for hydrogen peroxide sensors, a correct detection equal to 100% was observed for current peaks greater than 5.0 nA.

<table>
<thead>
<tr>
<th>Biosensor</th>
<th>Threshold</th>
<th>Correct detection on 5 nA peak</th>
<th>Correct detection on 1 nA peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>5.0 nA</td>
<td>100 %</td>
<td>40 %</td>
</tr>
<tr>
<td>Glucose</td>
<td>7.0 nA</td>
<td>85 %</td>
<td>20 %</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>7.5 nA</td>
<td>80 %</td>
<td>20 %</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10 nA</td>
<td>70 %</td>
<td>10 %</td>
</tr>
<tr>
<td>Phenols</td>
<td>8.0 nA</td>
<td>75 %</td>
<td>15 %</td>
</tr>
</tbody>
</table>
For lower magnitude peaks, this percentage decreases reaching about 40% for 1.0 nA peaks due to the worse signal to noise ratio.

In the tests realized with glucose, lactic acid, ethanol and phenols biosensors, the performance decreases because of the greater noise level, see Tab. I. The reported threshold indicates the minimum peak value with a correct detection percentage equal to 100%.

3 Multi-sensors system for wine fermentation monitoring

Fermentation is the natural process followed by “must” (e.g., grape juice) to become wine. Since the evolution time of this process changes in accordance with a great number of environmental and process parameters, it must be monitored by winers and/or enologists through the continuous measurement of some analyte that can be considered status parameters. Off-line measurement of these parameters requires that a specialized laboratory is owned by each wine producer and that expert technicians support enologists.

The described LMU featured with proper biosensors (glucose, ethanol, etc...) proved to be suitable for realizing a multi-sensor on-line measurement system adequate to help wine producers to make decisions during fermentation. A Manager Unit was designed to control LMU's and a set of actuators like pumps and valves, so automating both measurements and sample injections otherwise requiring an expert human operator. The multi-sensor measurement system was configured for the wine fermentation monitoring but can be utilized in other field of application requiring on-line measurements for biological process monitoring.

In Fig. 8, a block diagram of the multi-sensors system for wine fermentation monitoring is shown. It is made of a Manager Unit, one LMU for each quantity to be measured (glucose, lactic acid, ethanol, phenols), a FIA circuit for the automation of the injection phase and a supervisor PC.

The Manager Unit is a microcontroller-based module controlling the FIA circuit via suitable actuators. The MU communicates with LMU's in order to obtain the required measures and it is interfaced with the final user by means of the supervisor PC. LMU's exchange data with the MU using a serial daisy chain connection (RS 485), while the communication with the supervisor PC is realized through a serial port (RS 232). Given such communication schema, the MU receives the final user queries and commands through the Supervisor PC and it returns the measurement results obtained from the LMU's to the same PC.

3.1 Communication protocols

The communication protocol between the Manager Unit and the Local Measurement Units is very simple. It is the well known master-slave approach with MU acting as master. In such a schema only the master can start the communication.

The data format is the same in both directions, each data record composed by four 8 bits fields:
- address – this field contains a number identifying the measurement unit;
- command – this field contains a character indicating the type of measurement or status required/provided;
- size – this field codifies the number of data records that will be transmitted;
- data – the measured value, the status, the condition and so on.

The master puts on the bus the command and then waits for the addressed slave answer; the slave responds to the master putting on the bus the required information.

Figure 8. Block diagram of multi-sensor system for wine fermentation monitoring.
A suitable communication protocol between the supervisor PC and the Manager Unit was also set-up. The command and data format are constituted by a message of 28 bytes, including one start and one stop characters, and messages have the same structure for both the communication directions. The message is composed by different fields, including a field defining the type of data (communication, testing, measurement, calibration, alarm); a field specifying the involved LMU; a field for the measurement results; an alarm field codifying possible errors, e.g. communication errors or biosensor errors (not working electrodes, non-linear behavior and so on).

As soon as a new message coming from the supervisor PC reaches the Manager Unit, it is de-codified and a corresponding routine is started.

3.2 Measurement procedures

Some procedures have been set-up, which implement the single steps of the FIA procedure.

Injection and current measurement - For each new measure required by the Supervisor PC, the Manager Unit waits until the regime status of the biosensor current is detected and signaled by the chosen LMU. Then, it sends to the LMU the command to start the sample injection entering a waiting state until a peak amplitude value is measured and transmitted by the LMU itself. In order to improve the measurement reliability, at least three injections are executed for each measurement point giving as the measurement result the mean of the measured peaks with the exclusion of those values that appear to be statistically unreliable.

Analyte measurement - The mean current value is converted in analyte concentration by means of the calibration curve, and the obtained value is given to the Supervisor PC.

Calibration The calibration is made in order to evaluate the current/analyte conversion factor. It is made giving known analyte concentration samples to the biosensor and measuring the corresponding current peaks. To this aim, following the previously described “Injection and current measurement” procedure, three known samples of the analyte are injected and the corresponding current peaks are measured. The known analyte values and the corresponding current peaks are used to trace the linear calibration curve by means of least squares method. Once the interpolated calibration curve is available, the calibration phase ends. Two error conditions are possible: i) a low determination coefficient is observed, then a message about a non-linear behavior of the sensor is sent to the Supervisor PC; ii) a low sensitivity coefficient is measured, then a corresponding alarm is generated to the Supervisor PC.

3.3 FIA circuit

As discussed above, during Flow Injection Analysis a fluid sample is mixed with a suitable carrier solution and then is forced to flow on the biosensor. The dosage of the mixture defines the exact conversion factor. As a consequence, it has to be strictly controlled by the Manager Unit by means of suitable valves and pumps. In Figure 9, a schematization of the realized FIA circuit is reported.

The measurement is realized by two phases: Load – a controlled sample of the fluid under test (wine, in this case) is mixed with a controlled sample of the carrier solution so to obtain a desired dosage. During this phase the carrier is continuously flowing through the biosensor; Inject – the loaded mixture is sent to the biosensor.

All the operations of the aforementioned phases are controlled by the Manager Unit turning on/off valves and running pumps.

The FIA circuit has been designed to allow four different measurements. Two types of carrier are adopted: one carrier (carrier 1, in Figure 9) can be used for glucose, ethanol and lactic acid, while the phenol measurement requires a different carrier (carrier 2, in Figure 9). Depending on the measurement, the Manager Unit enables, through a selector and some valves, the carrier to reach only the selected biosensor. When the biosensor is detected to be in regime condition, the Manager Unit drives the pumps to inject the specified volume of diluted wine that gets to the biosensor. At the end of the measurement the tank containing the mixture is automatically emptied. To increase the input ranges, it is possible to inject in the FIA system different concentrations of the wine sample through addition of a carrier of known volume inside wine sample. The mixture is realized with two pumps (see Figure 9) that pick up known volumes of carrier and sample wine respectively. The two fluids are stored in a tank standing on a magnetic stirrer in order to obtain a uniform mixture.
3.4 The Supervisor PC software

The Supervisor PC interacts with both the user and Manager Unit. Two different operating modes are implemented: debugging mode and monitoring mode.

The debugging mode allows checking and setting up both the biosensor and the conditioning circuit. All the acquired current samples are plotted versus time, together with the measured regime and peak values, so to put into evidence possible failures. The user interface in debugging mode is reported in Figure 10. As shown in the Figure, the user can also activate manually measurement procedures so to verify the correctness of provided results.

In the monitoring mode, the desired control strategy of the fermentation process is implemented in the Supervisor PC. In particular, the analyte to be measured, as well as the measurement frequency for each analyte, can be set-up by the user thanks to a simple interface. The Manager Unit executes the tasks required by the PC, and gives back the measured values which are displayed and stored. In case of errors and/or failures, detected by the Manager Unit the PC generates suitable alarms.

Figure 9. Multi-sensor system placed on the wine line. S: selection valves, V isolation valves, P pump, Diff diffuser.

Figure 10. The user interface of the supervisor PC in debugging mode.
3.5 Experimental results

The performance of the whole system was evaluated in the real case scenario of analysis and control of wine fermentation process. As well assessed in the scientific community, the performance of a measurement system like the proposed one can be defined on the basis of the following indices:

i. repeatability;
ii. measurement time;
iii. sensitivity;
iv. user-friendlyness.

i. In our case, to evaluate repeatability is equal to evaluate the uncertainty of the measurement. Analyzing the reported results, it is evident that, even if the uncertainty of current measurement by LMU’s is constant (see paragraph 2.5), the uncertainty of the system changes in dependence of the analyte under test. This is due to the fact that measurement of the concentration of analyte depends on the biosensor sensitivity and linearity range which are determined by specific factors related to the physical realization of the biosensor itself. This kind of parameters could not be taken into account during the realization of our prototypes but can be easily faced during industrial production of the system (see Tabs.2).

ii. All the reported results concern the averaged values evaluated on three injections, corresponding to a measurement time of about 40 minutes. This time is independent from the single analyte and is mainly due to the pump characteristics.

iii. Experimental results confirmed sensitivity values reported in paragraph 2.5. The peak detection capability ranges between 5 nA (Hydrogen peroxide) and 10 nA (Ethanol). However, the system is able to keep constant the sensitivity (see Tabs.2) by suitably diluting the samples.

iv. The user interface looks like being simple and intuitive. No particular skill is required to interface the system and it’s easy to start up a monitoring process. Some alarms are automatically put to the attention of the user in case of problems with biosensors. Results can be visualized and deeply analyzed.

All summarizing, the realized measurement system can be considered flexible, i.e. able to exhibit a constant performance even if significant variations of some parameters are experimented. The MU software implements reconfiguration rules intended to grant process automation keeping the same accuracy and even improving the reliability which can be obtained.

### Tables 2. Metrological characteristics of the analyte measurements for wine fermentation monitoring.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Analyte</th>
<th>Glucose</th>
<th>Lactic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biosensor sensitivity</td>
<td></td>
<td>110 nA/mM</td>
<td>70 nA/mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.6 nA/(mg/l)</td>
<td></td>
</tr>
<tr>
<td>Linearity range of the biosensor</td>
<td></td>
<td>0.025÷1 mM</td>
<td>0.05÷10 mM</td>
</tr>
<tr>
<td>(with no dilution)</td>
<td></td>
<td>4.5÷180 mg/l</td>
<td>5 mg/l÷9 g/l</td>
</tr>
<tr>
<td>Linear regression determination</td>
<td></td>
<td>R²&gt;0.999</td>
<td>R²&gt;0.9997</td>
</tr>
<tr>
<td>coefficient (R²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurement range with the possible</td>
<td></td>
<td>0.05÷200 g/l</td>
<td>0.05 – 100 mM</td>
</tr>
<tr>
<td>dilution</td>
<td></td>
<td>5mg/l÷9g/l</td>
<td>35 mg/l÷35 g/l</td>
</tr>
<tr>
<td>Resolution with dilution 1:10</td>
<td></td>
<td>0.05 mM-0.009 g/l</td>
<td>0.08 mM -11 mg/l</td>
</tr>
<tr>
<td>Uncertainty with dilution 1:10</td>
<td></td>
<td>0.2 mM-0.04 g/l</td>
<td>0.3 mM - 35 mg/l</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Analyte</th>
<th>Ethanol</th>
<th>Phenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biosensor sensitivity</td>
<td></td>
<td>36 nA/mM</td>
<td>70 nA/mM</td>
</tr>
<tr>
<td>Linearity range of the biosensor</td>
<td></td>
<td>0.1÷1 mM</td>
<td>0.05÷0.2 mM</td>
</tr>
<tr>
<td>(with no dilution)</td>
<td></td>
<td>6·10⁻⁴÷6·10⁻¹ (%o)</td>
<td>9÷38 mg/l</td>
</tr>
<tr>
<td>Linear regression determination</td>
<td></td>
<td>R²&gt;0.99999</td>
<td>R²&gt;0.99999</td>
</tr>
<tr>
<td>coefficient (R²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurement range with the possible</td>
<td></td>
<td>3·10⁻³ %o ÷ 30 %o</td>
<td>45 mg/l÷3.8 g/l</td>
</tr>
<tr>
<td>dilution</td>
<td></td>
<td>38 µM-0.3 10⁻³ %o</td>
<td>35 µM - 7 mg/l</td>
</tr>
<tr>
<td>Resolution with dilution 1:5</td>
<td></td>
<td>0.15 mM-0.5 10⁻³ %o</td>
<td>0.1 mM- 20 mg/l</td>
</tr>
<tr>
<td>Uncertainty with dilution 1:5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In any case, we have verified that the obtained results are very similar to those obtainable by human skilled experts using the same biosensors.
by manual systems. On the basis of this final consideration, it is possible to say in our opinion that the proposed system will help biosensors to be more and more largely used.

4. Conclusions

This paper describes an intelligent measurement system based on biosensors that can be used for automatic measurements in case of Flow Injection Analysis. A first prototype of distributed system was customized and configured for monitoring the wine fermentation process through the measurement of glucose, ethanol, acid lactic and phenols. During the experimental phase, the proposed system showed to be suitable and robust in conditioning biosensors and measuring current peaks of relative scarce amplitude (tenth of nA), that are typical of FIA. Furthermore, the reported metrological performance is fully comparable with that of traditional manual systems in terms of accuracy and sensitivity. This result can be considered very relevant in the direction of a pervasive diffusion of measurement systems based on biosensors.

Future work will be focused on allowing a simple customization of the proposed system for a wide range of different application fields and, as far as concerning the wine fermentation realm, even if the obtained measurement time is already compatible with the needs of enologists and winers, on a further reduction of the quoted measurement time so to allow a more accurate control on the fermentation process.

5. References