Supplementary Materials: Automated Bioanalyzer Based on Amperometric Enzymatic Biosensors for the Determination of Ethanol in Low-Alcohol Beers

Eva Vargas, Felipe Conzuelo, M. Asunción Ruiz, Susana Campuzano, Víctor Ruiz-Valdepeñas Montiel, Guillermo González de Rivera, Fernando López-Colino, Á. Julio Reviejo and José M. Pingarrón

CONTENTS.....PAGE

Parameters Optimization

Sampling Unit Design

The optimized variables were membrane pore diameter, number of membranes equipped in the device, and contact surface between acceptor and donor solutions. As expected, the larger number of membranes, the smaller pore size, and the smaller contact surface between donor and acceptor solutions meant that a lower amount of ethanol reached the detector. Accordingly, a lower sensitivity and a wider linear range were obtained (Table S1). Thus, a contact surface diameter of 10 mm and one PTFE membrane of 0.45 μm pore diameter were chosen for further work since they provided an adequate sensitivity for the analysis of non-alcoholic beers and beers with an ethanol concentration below 1.0% (*v*/*v*).

Table S1. Slopes values and upper ethanol concentration measured within the linear calibration plots constructed using the semiautomatic bioanalyzer.

Optimized Variable	Value	Slope, nA %$^{-1}$	Upper Ethanol Concentration in the Linear Range, % (*v*/*v*)
Number of membranes	1	(1.38 ± 0.04) × 10^3	1.5
	2	(9.7 ± 0.2) × 10^2	2.0
	3	(4.41 ± 0.04) × 10^2	4.5
	4	(3.98 ± 0.06) × 10^2	5.0
Pore diameter	0.45 μm	(1.38 ± 0.04) × 10^3	1.5
	0.20 μm	(7.0 ± 0.1) × 10^2	2.5
Contact diameter	10 mm	(1.38 ± 0.04) × 10^3	1.5
	20 mm	(2.46 ± 0.03) × 10	10

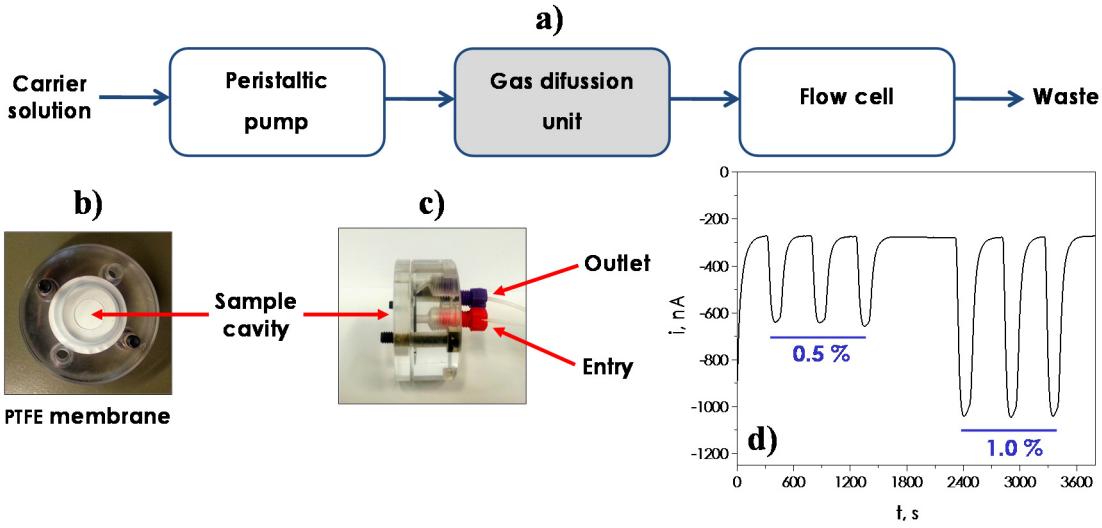

Figure S1. (**a**) Scheme of the continuous flow system constructed to evaluate the PTFE membrane-based sampling unit. (**b**) Front and (**c**) side views of the sampling unit. (**d**) Successive amperometric responses obtained for three successive additions of 0.5 and 1.0% (*v*/*v*) ethanol standards to the sampling unit. 10 mm simple sampling unit with one PTFE membrane (0.45 μm pore diameter). Carrier solution: 0.05 M phosphate buffer, pH 7.4. Q = 0.5 mL·min^{-1}. E_{app} = 0.0 V vs. Ag/AgCl.

Table S2. Determination of the ethanol content in standards of various concentrations at low temperatures using the continuous flow system.

Sample	Sample Temperature	[EtOH], % (*v*/*v*)	t_{exp}	t_{tab}
EtOH standard 0.040%	4 °C	0.041 ± 0.002 (RSD $_{n=4}$ = 3.0%)	1.063	3.182
EtOH standard 0.080%		0.08 ± 0.01 (RSD $_{n=3}$ = 4.8%)	1.645	4.303

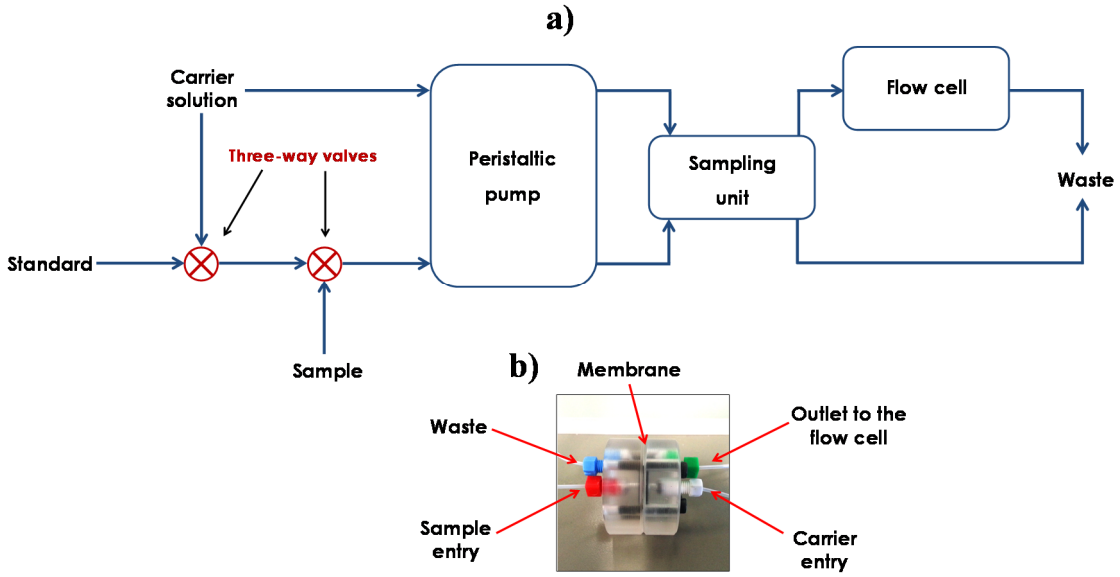

Figure S2. (**a**) Scheme of the continuous flow system implemented for the monitoring of ethanol concentration in beer at the production line. (**b**) Side view of the sandwich format sampling unit.

De-bubbler Unit Design

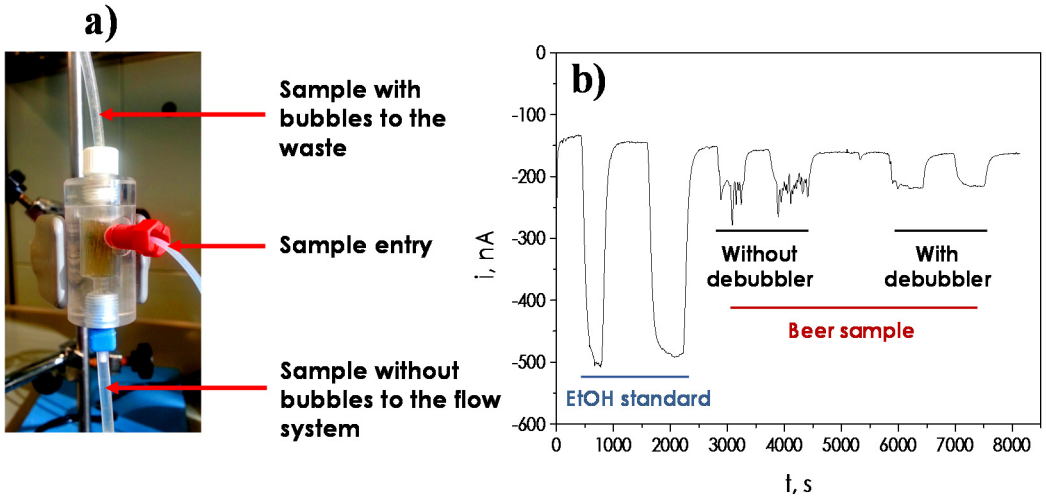

Figure S3. (**a**) Picture of the de-bubbler unit. (**b**) Amperometric responses obtained for 0.04% (*v*/*v*) ethanol standard and non-alcoholic beer sample before and after integrating the de-bubbler unit in the continuous flow system. Carrier solution: 0.05 M phosphate buffer, pH 7.4. Q = 0.5 mL·min^{-1}. E_{app} = 0.0 V vs. Ag/AgCl.

Automated Bioanalyzer for Ethanol Determination

Electrical Design

In the bioanalyzer, two parts should be controlled automatically: the amperometric detector, which applies a constant potential during the measurement and acquires the intensity values of current generated in the biosensor response, and an active interface or manager of the instrument, which allows the control of electronically active components of the flow system of the bioanalyzer (valves and pumps) to be carried out. There is also a computer-amperometric detector connection that enables the different experimental variables applied by the amperometric detector to be controlled and the intensity current data generated to be recorded; one active computer-interface

connection, which enables the manager to act on the various electronic components of the bioanalyzer; and a computer for acquiring and processing the data transmitted by the bioanalyzer and for controlling the amperometric detector and the active interface. Moreover, as well as being configured as a feedback system controller, needed in an automated instrument for decision making depending on the amperometric signals obtained, the computer is the part of the instrument that allows operator interaction with the bioanalyzer and the real-time display of the information collected during the monitoring process of the parameter under study.

Therefore, considering the elements that should constitute the control system of the bioanalyzer, the installation of the equipment components required an automatic operation of the prototype, which was carried out. As in Figure S4a, the amperometric detector and the printed circuit manager plate that controls the solenoid valves and peristaltic pumps were placed inside the instrument. The connections and the switch to turn on the instrument are placed outside the device (Figure S4b). Connection with the computer is made with a USB connector type. Finally, the equipment is powered with a power supply of 24 V.

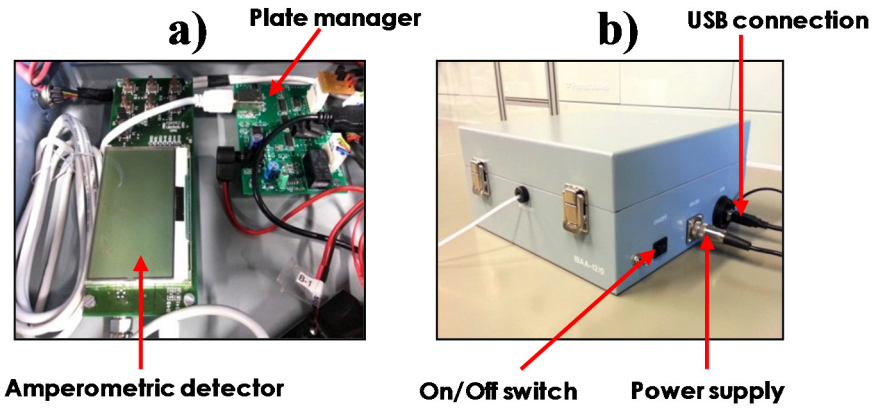

Figure S4. (**a**) Electrical installation of the bioanalyzer and (**b**) a picture of the developed bioanalyzer.

Software Applications

For the operation of the automated bioanalyzer, software applications were developed, which allowed the instrument to be controlled, the analytical information to be visualized, and a real feedback system to be obtained with respect to the registered information. With the objective of using the same prototype in both on-line and off-line analysis, the development of two softwares was realized.

Considering firstly the control software for on-line analysis, the user interface will allow the operator to display the recording parameters such as the working potential, the time interval for recording data of amperometric current, and the time for the analysis. A process indicator, which comprises a simplified diagram of the flow system and allows the analysis to be tracked; the faceplate, in which the current-time record is displayed in real time; the calculated ethanol concentration in the beer sample; and the concentration of the standard solution used as a reference are shown (Figure S5).

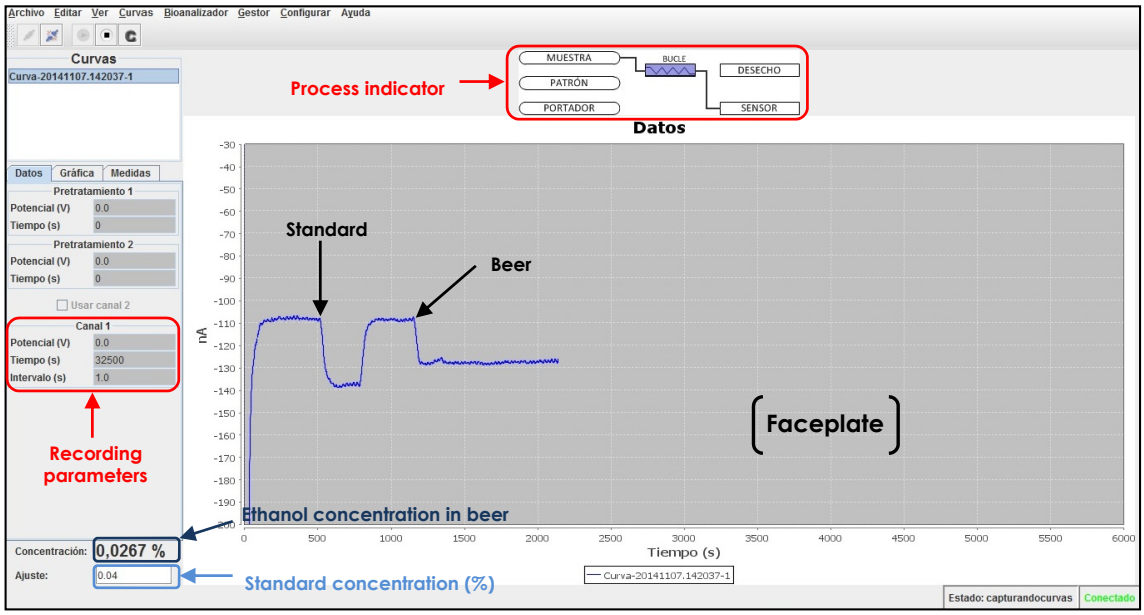

Figure S5. User interface picture for the control software for the on-line analysis of ethanol in beer.

To control the developed application, a set of configuration parameters, from which it is possible to program a method of analysis and operation for the bioanalyzer, was established. Thus, the analysis and decision making by the system are performed in predefined conditions. Depending on the recorded results and considering the parameters established from the configuration file of the application, the computer will send specific orders to the active interface so that the required operations at any time were performed.

The configuration parameters depended in a first stage on the working mode (CFA or FIA) and, thereafter, on the analysis phase at which the action is being performed (baseline stabilization or measurement). Thus, the values that have been introduced in the file of configuration parameters, characteristic of this bioanalyzer, consider the dimensions of the flow system, the working flow rate, and the behavior of biosensors, so that the current stabilization and separation of the signals are output in an efficient manner to obtain analytical measurements with adequate accuracy and reproducibility.

Once the measurement stage is finished, appropriate changes in the valve module are performed by the system, so that the stabilization stage restarts to carry out the next measurement once the baseline is reached again. Depending on the analysis method and configuration parameters established, it will correspond to the measurement of the standard solution or the sample.

With respect to the acquisition and data processing for the on-line application, such information is stored in measurement files (Figure S6). These files provide different information depending on the monitoring mode of the analytical signals. An example of a measurement file of the CFA mode, in which sample measurements have been recorded every second, is shown in Figure S6a. In Figure S6b a measurement file of the FIA mode is displayed, showing, for each measurement, date, time, ethanol concentration, amperometric peak current, and type of measurement (standard solution or sample and, in the second case, which replica it is).

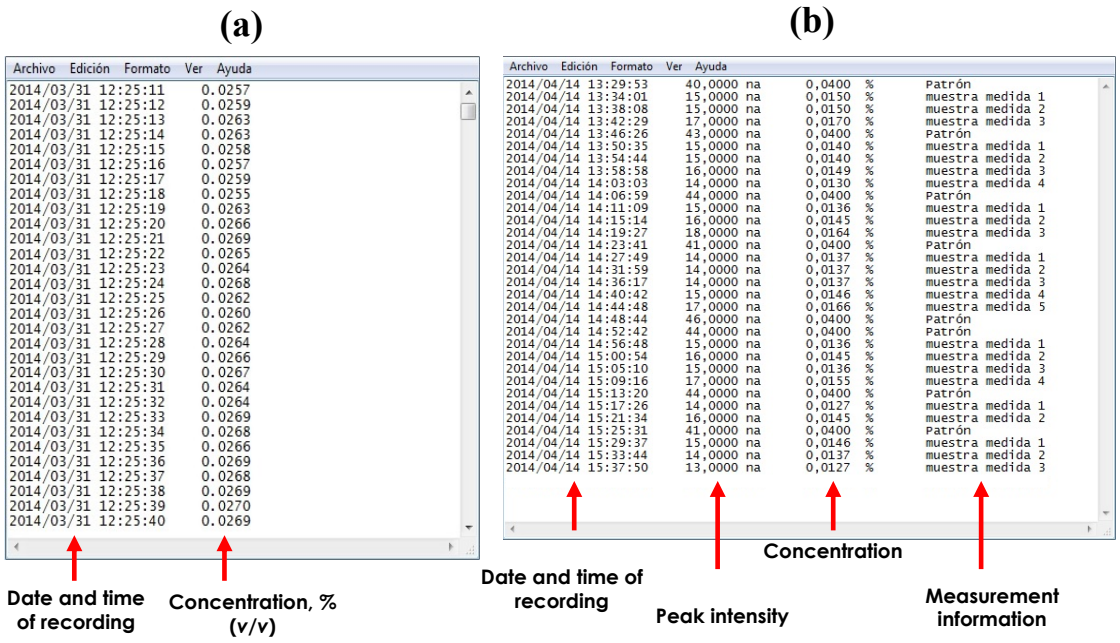

Figure S6. Measurement files supplied by the control software for the on-line application in the (**a**) CFA and (**b**) FIA modes.

The control software for the off-line application has a user interface, which will allow the operator to display, in addition the information commented above for the on-line application, a control panel from which it is possible to configure with which solution (standard or beer) each entry of the valve module in the sample selector is occupied, the number of replicas to be measured of each sample, and the number of sample measurements to be carried out before the system orders the bioanalyzer to be recalibrated (Figure S7). As shown in the figure, the application was developed for an eight-entries sample selector.

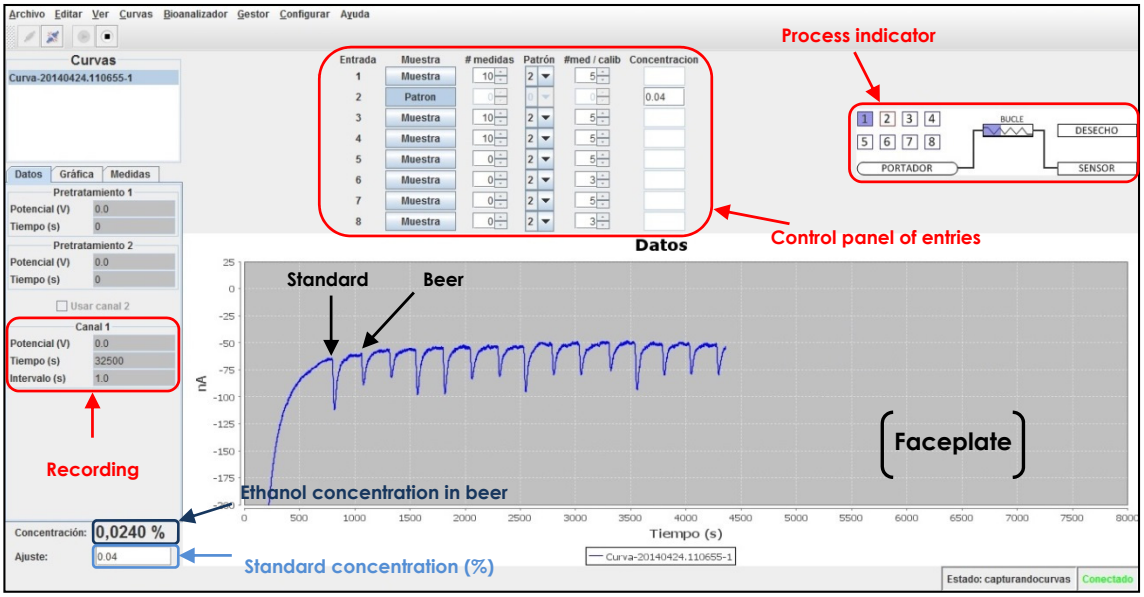

Figure S7. User interface display of the control software for the off-line analysis of ethanol in beer.

To control this application, a specific configuration file for this type of analysis was included, from which the analysis method to be performed is established so that the data introduced in such a file will appear in the control panel of entries of the user interface. Regarding the acquisition and data processing obtained by the system for the off-line application, in addition to the analytical information indicated above for the FIA mode of the on-line application, statistical analysis of each sample was included in the measurement file. Thus, the mean value, standard deviation, and relative standard deviation of the results were included (Figure S8).

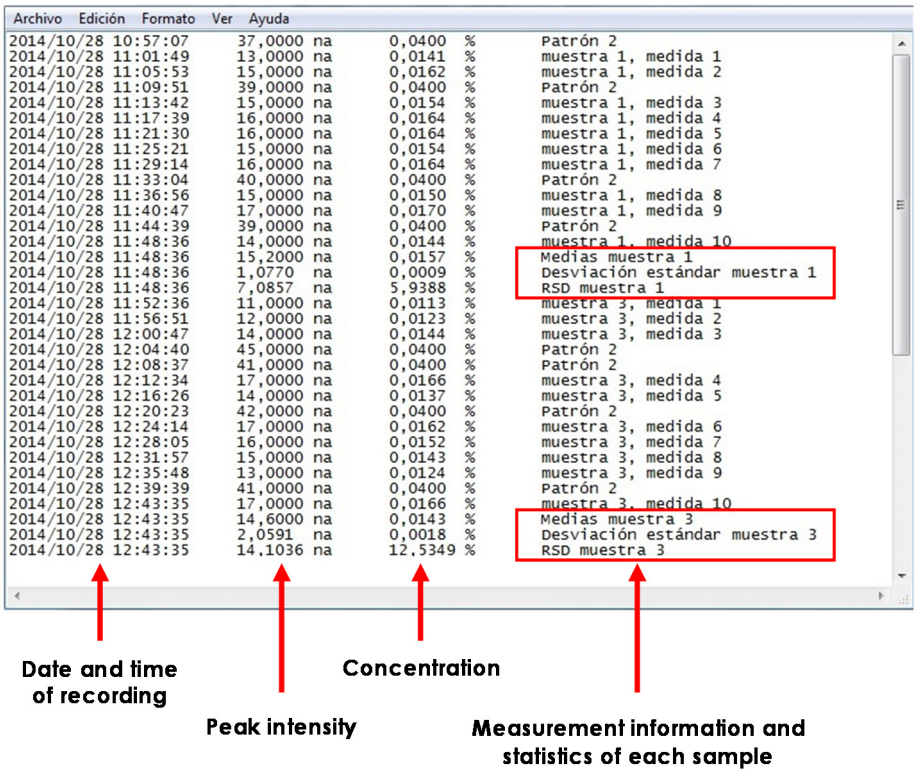

Figure S8. Measurement file supplied by the control software for the off-line application.

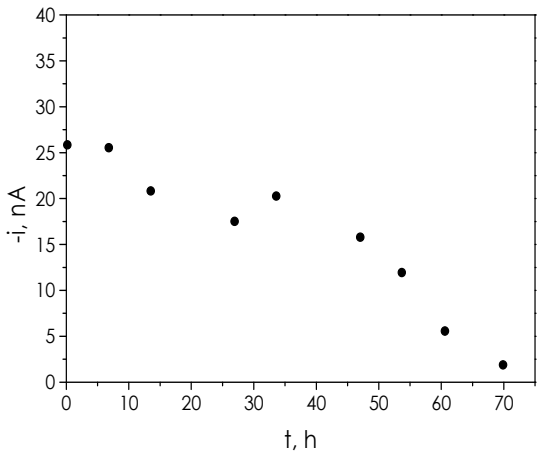

Figure S9. Variation of the peak current measured as a function of time for a graphite-Teflon composite ethanol biosensor when used in the automated bioanalyzer working as a CFA system. The plotted data correspond to the amperometric signals obtained for freshly prepared 0.04% (*v*/*v*) ethanol standard solutions. 20 mm sampling unit equipped with one PTFE membrane of 0.45 μm pore diameter. Carrier solution: 0.05 M phosphate buffer, pH 7.4. Q = 4.0 mL·min^{-1}. E_{app} = 0.0 V vs. Ag/AgCl.

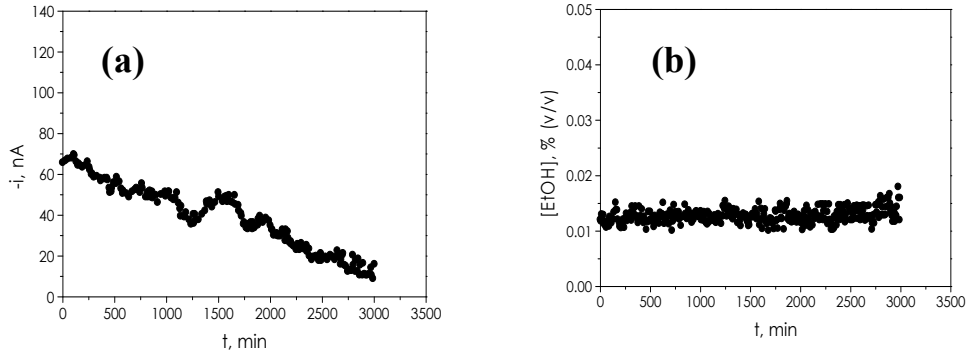

Figure S10. Variation of the peak current measured as function of time using the commercial ethanol biosensor as a detector in the automatic bioanalyzer working in the FIA on-line mode. (**a**) Amperometric signals obtained for freshly prepared 0.04% (*v*/*v*) ethanol standard solutions. (**b**) Ethanol concentration determined for a non-alcoholic beer sample when recalibration of the system was performed every five sample measurements. 20 mm sampling unit equipped with one PTFE membrane of 0.45 μm pore diameter. Carrier solution: 0.05 M phosphate buffer, pH 7.4. V_i = 500 μL. Q = 4.0 mL·min^{-1}. E_{app} = 0.0 V vs. Ag/AgCl.