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Monitoring β-Fructofuranosidase Activity through *Kluyveromyces marxianus* in Bioreactor Using a Lab-Made Sequential Analysis System

Edwin J. Barbosa-Hernández [1], Jorge E. Pliego-Sandoval [2], Anne Gschaedler-Mathis [1], Javier Arrizon-Gaviño [1], Alejandro Arana-Sánchez [3], Ricardo Femat [4] and Enrique J. Herrera-López [1,*,†]

[1] Biotecnología Industrial, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C., Camino Arenero 1227, Col. El Bajío del Arenal., Zapopan C.P. 45019, Mexico; edbarbosa_al@ciatej.edu.mx (E.J.B.-H.); agschaedler@ciatej.mx (A.G.-M.); jparrizon@ciatej.mx (J.A.-G.)
[2] Centro Universitario del Sur, Departamento de Ciencias Computacionales e Innovación Tecnológica, Universidad de Guadalajara, Av. Enrique Arreola Silva No. 883, Cólon., Cd. Guzman C.P. 49000, Mexico; jorge.pliego@cusur.udg.mx
[3] Departamento de Procesos Tecnológicos e Industriales, Instituto Tecnológico y de Estudios Superiores de Occidente, Anillo Perif. Sur Manuel Gómez Morín 8585., San Pedro Tlaquepaque C.P. 45604, Mexico; aarana@iteso.mx
[4] División de Control y Sistemas Dinámicos, Instituto Potosino de Investigación Científica y Tecnológica, Camino a la Presa San José 2055, Lomas 4a Sección., San Luis Potosi C.P. 78216, Mexico; rfemat@ipicyt.edu.mx
* Correspondence: eherrera@ciatej.mx
† Laboratorio para la Innovación en Bioelectrónica e Inteligencia Artificial, LINBIA®, Biotecnología Industrial, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C., Camino Arenero 1227, Col. El Bajío del Arenal, Zapopan C.P. 45019, Jalisco, Mexico

Abstract: The yeast *Kluyveromyces marxianus* has shown the potential to produce β-fructofuranosidases, which are enzymes capable of hydrolyzing β-fructofuranosides links of fructans to obtain fructooligosaccharides. The thriving market for fructose syrup and the quality standards imposed by food and pharmaceutical industries have generated an increased search for improved, monitored, and controlled production processes. Monitoring β-fructofuranosidase activity in a bioprocess requires the use of adequate sensors and the processing of information using efficient software algorithms; nevertheless, currently, such a sensor does not exist for this purpose. In this contribution, a sequential injection analysis system (SIA) developed in our laboratory was adapted to monitor at-line β-fructofuranosidase activity produced by the yeast *K. marxianus*. Samples were taken out automatically from the bioreactor and analyzed using 3,5-dinitrosalicylic (DNS). An algorithm was designed to operate the overall components of the lab-made SIA system. The enzymatic activity error obtained with the automatic SIA compared to the off-line laboratory determinations varied from 0.07% at high enzyme concentrations to 20.39% at low β-fructofuranosidase activity. Further development is required to improve the performance of the lab-made SIA system; nevertheless, such a device must be considered as a potential method for monitoring β-fructofuranosidase activity in real time.

Keywords: *Kluyveromyces marxianus*; β-fructofuranosidase; monitoring; at-line activity; sequential injection analysis; lab-made system

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1. Introduction

Kluyveromyces marxianus, a non-*Saccharomyces* thermotolerant yeast, has the potential to grow in a wide variety of substrates, at high specific growth rates, and displays a significant metabolite secretory capacity [1,2]. This yeast has been widely used for the production of aromatic compounds in ethanol fermentation [3], in simultaneous saccharification and fermentation processes [4], as a biocatalyst of renewable chemicals and for lignocellulosic ethanol production [5,6], and other applications in the food and biotechnology industry [7–10]. *K. marxianus* can assimilate inulin [11,12], and *Agave tequilana* fructans

(ATF) as substrate [4,13], and has the secretory capacity to synthetize β-fructofuranosidases. The fructooligosaccharides (FOS) used in producing fructose syrup are obtained from the hydrolysis of fructans. Interest in inulin has increased worldwide due to the growing demand for dietary supplements enriched with prebiotics and the synthesis of fructose as a sweetener [12,14]. Agave fructans are relevant for the production of tequila, which today is one of the most popular alcoholic beverages in the world [15]. Therefore, from an economic point of view, FOS production is relevant since the estimated market will be worth USD 3.52 billion by 2024 [16].

The most common method to determine β-fructofuranosidase activity at the laboratory is through using 3,5-dinitrosalicylic reducing sugars (DNS) [17]. Nevertheless, the standard DNS assay is carried out in a manual way, which is time-consuming, monotonous, and generates considerable waste. In the context of this issue, automatic analyzers such as flow injection analysis systems (FIAs) have been proposed, which support routine determinations that demand frequent sampling [18]; another solution is the use of sequential injection analysis systems (SIAs), which allow discontinuous or intermittent modes of operation reducing reagents and sample consumption [19]. DNS methodologies have been applied in diverse flow systems to detect glucose content in soft drinks and wines [20] to detect L-lactate during lactic acid fermentation [21], for monitoring and controlling a lactose production process [22], and for cell culture applications [23]. SIA systems have been applied in bioprocesses to evaluate enzyme activity such as proteases [24], phospholipases [25], horseradish peroxidase [26,27], glucose oxidases [28], and lipases and esterase [29], among other applications [30,31].

Regarding β-fructofuranosidase activity, this enzyme has been detected by an SIA system coupled to Fast Fourier infrared spectroscopy [32,33]. The SIA system was used to prepare a standard mixture of products where it was possible to determine the enzyme activities of two enzymes acting on two different substrates using the FTIR, although the authors stated that care had to be taken since the reactions were monitored off-line. An electrochemical sensor based on the oxidation of the carbonyl group with ferricyanide ions in a rotating disk electrode was used to detect agave tequila fructans. The method was able to detect concentrations of oligosaccharides as low as 0.71 mM [34]; however, this sensor detected FOS rather than β-fructofuranosidase activity.

FIA and SIA systems can be acquired from different commercial suppliers; however, these devices can be quite expensive. As an alternative, researchers can design and build their own SIA systems. This approach requires one to design the system, purchase and integrate the components, and write the control algorithm. Diverse algorithms to control flow injection systems have been reported in the scientific literature. An FIA system to determine lactose was designed in [22]. The system was controlled with commercial software called CAFCA™ (Computer Assisted Flow Control and Analysis). This software is available as a professional version (ANASYS, Hannover, Germany), and was designed in Borland Pascal 7.0; the system runs under the MS-DOS environment [35]. A methodology to measure glucose and lactate for immobilized cells in FIA systems by means of C++ was designed in [21]; the device used a manufactured electrode with an amperometric detector. An algorithm in Python was developed to control an injection valve Cavro XP3000 and a pH meter [36]; however, these types of systems are not suitable for hardware reconfiguration. The software LabVIEW™ (Austin, TX, USA) also has been used to control the SIA systems, where its operation was validated determining morphine [37], hydroquinone in plant tissue culture media [38,39], thiamine, and 1,10-phenanthroline [40].

Monitoring enzymes in a bioprocess requires the use of specialized instrumentation to measure the desired bioproduct accurately and simultaneously accomplish the strict high-quality standards imposed by the food and pharmaceutical industries. The search for specialized sensors to monitor enzyme activity in a bioprocess is currently an open field of research. To our best knowledge, SIA systems have not been used to detect β-fructofuranosidase from at-line measurements taken out from the bioreactor. DNS could be considered as a potential method to determine β-fructofuranosidase activity using a SIA

system. However, this technique is not easy to implement in such devices since it requires a complex process that involves sampling, combining substrate with reactants, homogenizing, heating, cooling, reading the mixture at a specific wavelength, and correlating the spectrum with the enzyme activity [28,41]. In addition, at high enzyme concentrations, the sample must be diluted. All these activities must be performed in an accurate and sequential reproducible way, that needs a computer algorithm to strictly control the routines, execution times, flow management, signal acquisition, and data processing [30].

In this contribution, a Lab-made SIA system previously designed in our laboratory [29] was modified and adapted to determine at-line β-fructofuranosidase activity. The SIA system now has the capability to cool, stir, and perform dilutions to samples automatically taken from the bioreactor; this way, it was possible to determine at-line enzymatic activity by means of the reducing sugar (DNS) method. In addition, we emphasize the LabVIEW™ algorithm designed to operate the components of the lab-made SIA system and describe the graphical user interface. The performance of the lab-made SIA system was evaluated by monitoring at-line β-fructofuranosidase activity produced by the yeast *K. marxianus* in an ongoing bioprocess. The β-fructofuranosidase activity determined during at-line measurements was validated through comparing the samples to regular laboratory measurements.

2. Materials and Methods

2.1. Microorganism

The yeast *Kluyveromyces marxianus* SLP1 belongs to the microorganism collection of the Centro de Investigation y Asistencia en Tecnología y Diseño del Estado de Jalisco; A.C was used as a case study for β-fructofuranosidase production. The strain was stored at 4 °C and subcultured every month in Petri dishes with YPDA (g/L): 20 yeast extract, 10 glucose, 20 bactopeptone, and 20 agar for maintenance.

2.2. Culture Media

The strain was grown in mineral medium (DMM) containing (g/L): 20.0 glucose, 3.0 KH_2PO_4, 3.0 $(NH_2)SO_4$, 1.5 $Na_2HPO_4{\cdot}2H_2O$, 1.0 glutamate, 0.4122 $MgCl_2{\cdot}6H_2O$, 0.0192 $ZnCl_2$, 0.0174 $CaCl_2$, 0.0117 $FeCl_2{\cdot}4H_2O$, 0.0044 $MnCl_2{\cdot}4H_2O$, 0.0006 $CuCl_2{\cdot}2H_2O$, 0.0005 $CoCl_2{\cdot}6H_2O$, 0.0004 $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.003 H_3BO_3. Vitamin solution (g/L): 0.000012 biotin, 0.005 thiamine HCl, 0.005 pyridoxine, 0.005 pantothenic acid, 0.005 nicotinic acid, 0.125 myoinositol, and 0.001 4-aminobenzoic acid.

2.3. Inoculums Conditions

Two sterile 500 mL flasks with 100 mL of YPD (YPDA without agar) medium were inoculated with two CFUs of the *K. marxianus* SLP1 obtained from properly conserved Petri dishes. The flasks were placed in a New Brunswick® Innova 44 rotary orbital for 12 h, with a controlled temperature of 30 °C and an agitation speed of 250 rpm. Afterward, two 500 mL flasks with 100 mL of DMM were inoculated with 1 mL of the previous culture for 10 h at the same operational conditions. After incubation, the flasks had approximately 4×10^8 cells/mL.

2.4. Bioreactor Conditions

Yeast propagation was carried out in a 3 L Applikon bioreactor, prepared with 1 L of DMM. The bioreactor was sterilized at 121 °C for 15 min. The following physical variables were controlled: pH 4.5, temperature 34 °C, and agitation were varied automatically depending on the dissolved oxygen saturation (from 500 to 800 rpm). The aeration was kept constant during the kinetics at 1.6 L/min (the air was supplied by a fish tank compressor and filtered by a 0.22 mm membrane for sterilization). The initial substrate concentration was 10 g/L. The fed-batch culture began when the substrate was depleted; afterwards, the bioreactor was fed at a constant flow of 70 mL/h with a carbon source concentration of 100 g/L of glucose.

2.5. Biomass Determination

Biomass was determined by dry weight. From each sampling, 5 mL was centrifuged for 15 min at 5554× *g*. Pellets were washed twice with 5 mL of distilled water and centrifuged under the above conditions. Subsequently, pellets were dried at 50 °C for 24 h and then placed in a desiccator until constant weight was attained.

2.6. Reducing Sugars Determination

The reducing sugar concentration in the medium was determined using 3,5-dinitrosalicylic acid reagent [17].

2.7. Off β-Fructofuranosidase Activity Determination at the Laboratory

β-fructofuranosidase enzymatic activity was determined in our laboratory using the reducing sugar method described in [42]. A sample taken out manually from a bioreactor was centrifuged to separate enzyme extract from the cells. A measure of 50 µL of supernatant was put in a microtube with 50 µL of substrate (100 mM sodium acetate + 100 mM acetic acid + 1 g/L sucrose, pH 5.0), and the sample was homogenized. The mixture was incubated at 50 °C for 15 min in a Thermo-Shaker® MS-incubator 100, the reaction was stopped by placing the samples in ice for 2 min. A measure of 100 µL of the DNS reagent was added to the samples and homogenized, in addition, 50 µL of enzyme extract was added to the blank. The mixture was placed in a water bath at 95 °C for 5 min, then put on ice for 5 min to stop the reaction. A measure of 800 µL of distilled water was added to the microtubes and then were homogenized. A measure of 200 µL of the described mixture was placed in the well of a microplate to be read at 540 nm in an ×Mark® microplate spectrophotometer from Bio-Rad® (Hercules, CA, USA) with endpoint determination. The sample was analyzed in triplicate and compared against a calibration curve of reducing sugars. In each experiment, a new calibration curve was constructed with an adjustment > 0.98. Equation (1) was used to calculate the enzymatic activity units:

$$Activity = \frac{U}{mL} = \frac{\mu_{mol}}{min \cdot mL} = \frac{F \cdot V_{rx}}{M_w \cdot t \cdot V_e} \quad (1)$$

where F = glucose concentration; V_{rx} = reaction volume; M_w = glucose molecular weight; t = incubation time; V_e = enzymatic extracted volume.

2.8. General Description of the Lab-Made SIA System

The hardware components integrating the lab-made SIA system [29] are a mini peristaltic pump S2 Laboratory, Watson-Marlow, (Wilmington, MA, USA), a holding coil, two interconnected multiposition valves of ten ports C25-3180EMH-FL, Valco (Houston, TX, USA). A reaction coil FT-COIL90 with a heating system. A spectrophotometric detector VIS-NIR USB4000 of Ocean Optics (Dunedin, FL, USA), with source light Tungsten-Halogen HL-2000-LL, and a flow cell (FIA-Z-SMA-SS) with 10 mm path length of stainless steel. An algorithm was designed in LabVIEW™ 11.0 to control the SIA system, installed in an assembled personal computer (W7 home premium, 2009 processor AMD Athlom™ X4 640 processor 3.00 GHz, 8 Gb Ram), as can be seen in Figure 1. The software is described in detail in the results section.

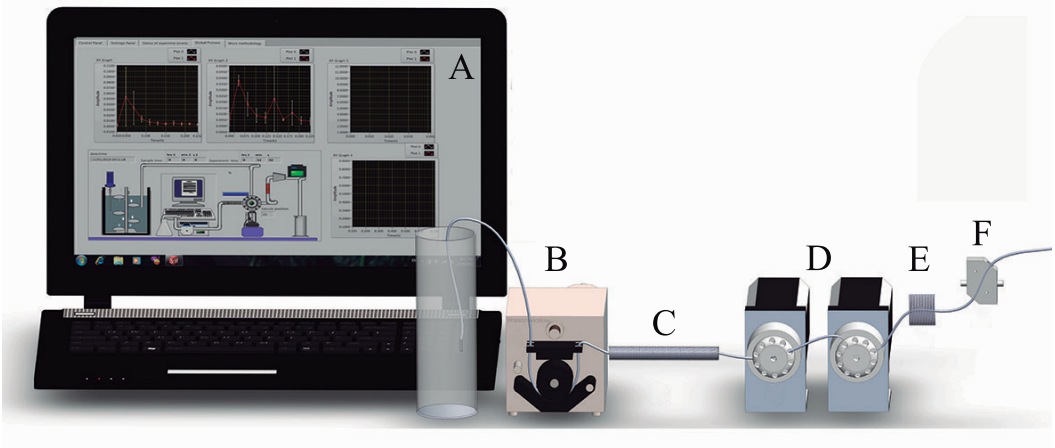

Figure 1. Main components of the SIA System with UV/visible spectrophotometer; (**A**) graphical user interface, (**B**) peristaltic pump, (**C**) holding coil, (**D**) multiposition valves, (**E**) reaction coil, and (**F**) flow cell.

2.9. β-Fructofuranosidase Activity Set Up in the Lab-Made SIA System

Figure 2 shows the configuration and description of the components of the SIA system that were used during the implementation of the methodology to monitor on-line *β*-fructofuranosidase activity.

- Free cell samples supernatant (m) was taken out automatically from the bioreactor at selected times and pumped into the holding chamber of the SIA system.
- An automatic dilution with the carrier water (a) was performed depending on the time of the culture and it was homogenized in the mixing chamber.
- The diluted sample (ds) was mixed in equal parts with the substrate (s) by suction cycles ds/s/ds/s.
- The mixture was injected into the incubation chamber, where it was maintained at 50 ± 2 °C for 15 min.
- The incubated sample (mi) was sent to the retention chamber and mixed with the reagent DNS (r) in suction cycles mi/r/mi/r. Due to the corrosive nature of phenol, this compound was not used in the preparation of the DNS reagent.
- The mixture was injected into the heating chamber, where it was maintained at 93 ± 2 °C for 5 min.
- Afterwards, the mixture was injected into the cooling chamber submerged on ice where it was kept for 1 min.
- The mixture was automatically diluted in a 1:4 ratio with a carrier and homogenized in the mixing chamber.
- Finally, the sample was injected into the detector where it was read at 540 nm. To eliminate any trace of the previous sample, a cleaning cycle was carried out after each analysis. All these hardware sequences were controlled with an algorithm designed with the software LabVIEW™. One second of operation of injection/suction is equivalent to a flow rate of 20 µL/s, with a standard deviation of 6%.

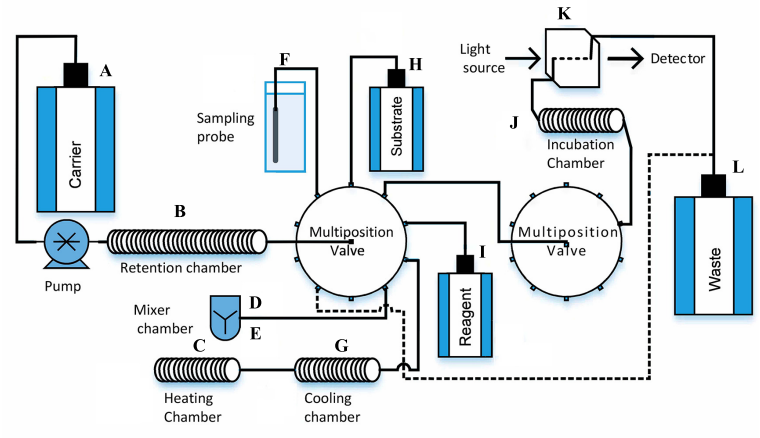

Figure 2. Specific components of the lab-made model used to determine $\beta-$fructofuranosidase activity. (**A**) Carrier, (**B**) retention chamber, (**C**) heating chamber, (**D**,**E**) mixer chambers, (**F**) bioreactor and sampling probe, (**G**) cooling chamber, (**H**) substrate, (**I**) reagents, (**J**) incubation chamber, (**K**) spectrophotometric detector, and (**L**) waste container.

2.10. Calculating β-Fructofuranosidase Activity

Following the steps described in Figure 2, a spectrum is obtained. The resulting area under the curve is directly proportional to the β-fructofuranosidase activity. The spectrum data were exported to the software Matlab™ 2015, where it was analyzed with the command "trapz", which returns the approximate integral of a function through the trapezoidal method. The calculated area was compared with a calibration curve performed in the SIA system. The units of β-fructofuranosidase activity were calculated with Equation (1).

2.11. Validation and Statistical Comparison

At-line β-fructofuranosidase measurements were compared with off-line microplate determinations, and their mean values were analyzed to find statistical differences.

3. Results

3.1. Lab-Made SIA Hardware Modifications to Determine β-Fructofuranosidase

To set up the DNS β-fructofuranosidase activity methodology, the lab-made SIA system reported in [29] was modified to include a cooler chamber to stop the chemical reaction produced by the DNS. To homogenize the samples, a mixer chamber controlled with an electronic Arduino UNO card was also integrated into the SIA system. On the other hand, the flow restrictor FR-902 was eliminated from the device to allow the return of the sample. The software was modified to include automatic dilution options.

3.2. Virtual Code to Control the Lab-SIA System

The software LabVIEW™ is known as a virtual instrument (VI), in which code is created using the graphical programming language G [43]. In LabVIEW, the programmer does not need to develop typing code; instead, the VI is used to create virtual code from interconnecting diverse functional icons. Due to its versatility and functionality, it was decided to use this software to control the SIA system. The algorithm designed in LabVIEW™ is composed of a front panel and the block diagram window. The front panel comprises all the information needed by the user to configure, enable, and operate the SIA system to determine enzyme activity. It also shows the status of the variables, tables, graphs, and indicators. The block diagram window contains the code to control the hardware components of the lab-made SIA system, such as the peristaltic pump, the multiposition valves, and the USB4000 spectrophotometer, which are briefly described below.

3.3. Virtual Code to Control the Peristaltic Pump

The peristaltic pump carries sample/reagents along the different components of the SIA system. The peristaltic pump was controlled enabling/disabling the pins 4/7 of the RS-232 port. The RTS/DTR submenu software controls the request to send (RTS) and data terminal ready (DTR) signals for the RS-232 serial communications interface for hardware handshaking. The STR/DTR signals allowed the interface to operate in a continuous way, as well as to automatically execute suction/injection of the sample/reagents and carrier, all functions were synchronized time-based. The functional block diagram to control the peristaltic pump is shown in Figure 3A.

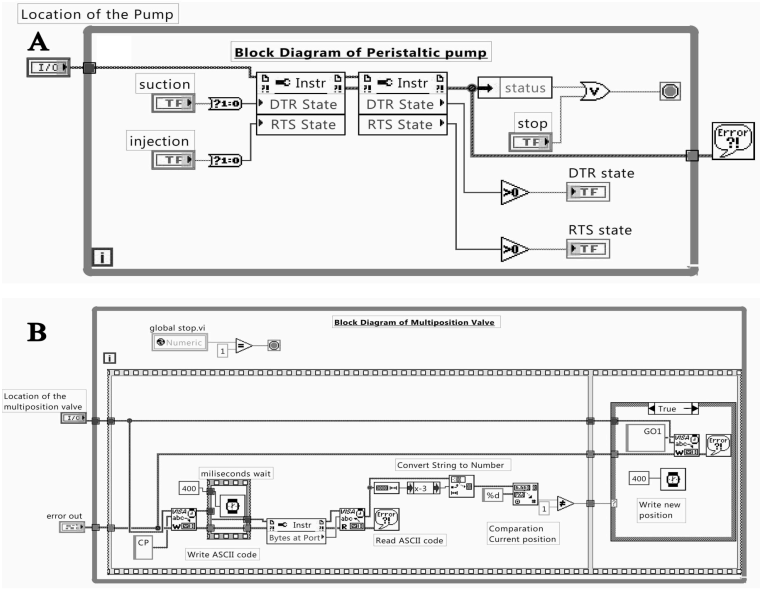

Figure 3. Virtual code diagram to control (**A**) the peristaltic pump and (**B**) the multiposition valves.

3.4. Virtual Code to Control the Multiposition Valves

The multiposition valve allows the user to select a specific port that may be connected via tubing to reagents, buffers, or other substances to be transported by the peristaltic pump to a specific component of the SIA system, i.e., holding coil or to the detector. The multiposition valves were controlled by means of an ASCII communication protocol used through pins 2–3 of the RS-232 port. The serial port requests the current position of the valve using ASCII code. The system transforms data from a string type to a numeric value to make a comparison between the current position and the location requested by the user. The block diagram to control the multiposition valves is shown in Figure 3B.

3.5. Spectrophotometer Control

To evaluate the analyte concentration in the SIA system the USB4000 spectrophotometer read the processed sample within the detector flow cell. This module was redesigned from the existing virtual instrument based in [44]. A Savitzky–Golay filter was implemented along the sampling periods, with wavelength filter selectors to smooth the signal obtained from the spectrophotometer. The filters were configured to be used at two different wavelengths. The designed algorithm allows the activation and deactivation at precise times of the spectrophotometer. Sampling intervals will be calculated according to the time interval required in each methodology. After the completion of each experiment, the information collected through the software was sent to an Excel™ spreadsheet (Microsoft 2016, Redmond, Washington, DC, USA).

3.6. General Virtual Code to Control the SIA System

The virtual code to control the overall components of the SIA system is integrated into a general block diagram window that is divided into six main parts, Figure 4. The "flat sequence" included in Figure 4A resets all the entries to zero, including graphs, synchronization times, tables, graphical and on/off indicators, and run, stop, and pause buttons. A "global stop" button synchronized with the SIA controller was included in all the sections of the control panels of the SIA system, Figure 4B. The block diagram of Figure 4C contains the addresses of the ports USB, and RS-232. In addition, it accounts for the initial settings of the system such as operating times, sequences for turning on and off the device, selection of wavelengths, enable/disable of automatic dilutions, and all the information is stored in proper arrays. Figure 4D, is related to the general software to manipulate the peristaltic pump, the multiposition valves, and the spectrophotometer USB4000 virtual instrument. This section was based on two main sub-virtual instruments: "VI of cut" and "VI of order". The first one considers the inclusion of a general matrix table displaying the sequences or steps to operate the peripherals. In addition, this VI sets the required time that a peristaltic pump must be enabled. The versatility of the software allows to change the order of the sequence within the action line. In Figure 4E, 21 sequences are contained as a case structure. This section allows the user to select a specific port and its configuration, i.e., the peristaltic pump selection actions such as suction, injection, stop, or execute a specific action with the multiposition valve or obtain a read from the spectrophotometer USB4000. To control the movement of a sample through the SIA components the VI located in Figure 4F is used, which is independently configured. Finally, Figure 4G closes the ports and stores all the information in a text document. All the virtual algorithms described previously need to be operated in a perfect synchronization sequence to carry out robust determination of the analyte.

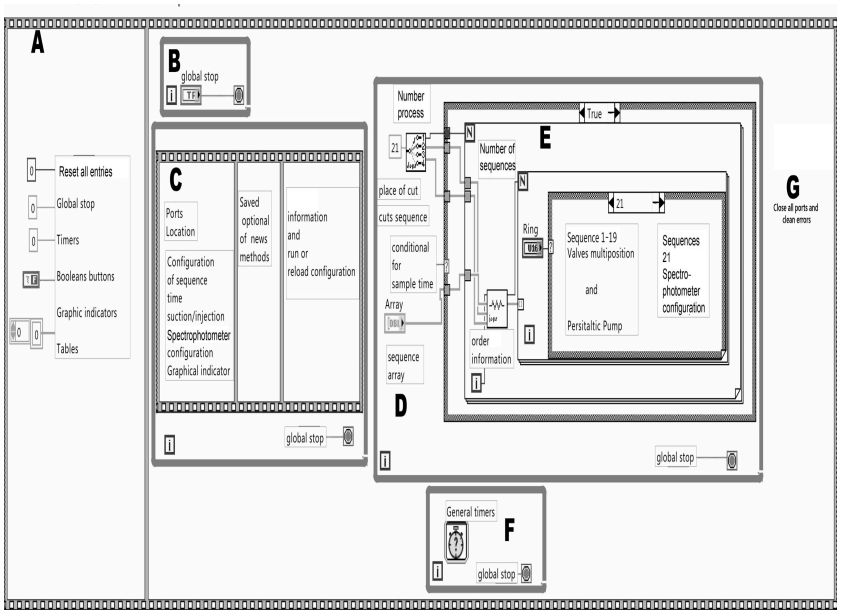

Figure 4. Virtual code to control the overall components of the SIA system. (**A**) initialization, (**B**) global stop, (**C**) USB port configuration, (**D**) control of the actuators, (**E**) sequences to activate components, (**F**) control of the sample, and (**G**) saving data.

3.7. Description of the User Interface That Controls the Lab-Made SIA System

To configure the operation of the hardware components of the SIA system a friendly user interface was designed in the front panel, which contained four different screens denoted as: control panel, setting panel, experiment status panel, and overall monitoring panel (see Figure 5). In the control panel Figure 5A, the user configures the components of the SIA system to be used in the determination of the enzyme. It is important that the

selected methodology is set up by the technician who commonly determines the enzymatic activity off-line at the laboratory, and consequently understands the technique. This window allows the user to set the appropriate sequence for the sample and reactants to be transported from a specific location to a selected position. For this purpose, the diverse inputs and outputs of the multiposition valve can be selected, enabled, and associated with the operation of a peristaltic pump. The spectrophotometer wavelengths can also be set in the control window, as well as the conversion factor between absorbance and the variable of interest that is displayed in this panel. The duration of the experiment and the sampling time can be set in this window. There is a section in the control panel in which a suitable dilution could be planned for the experiments.

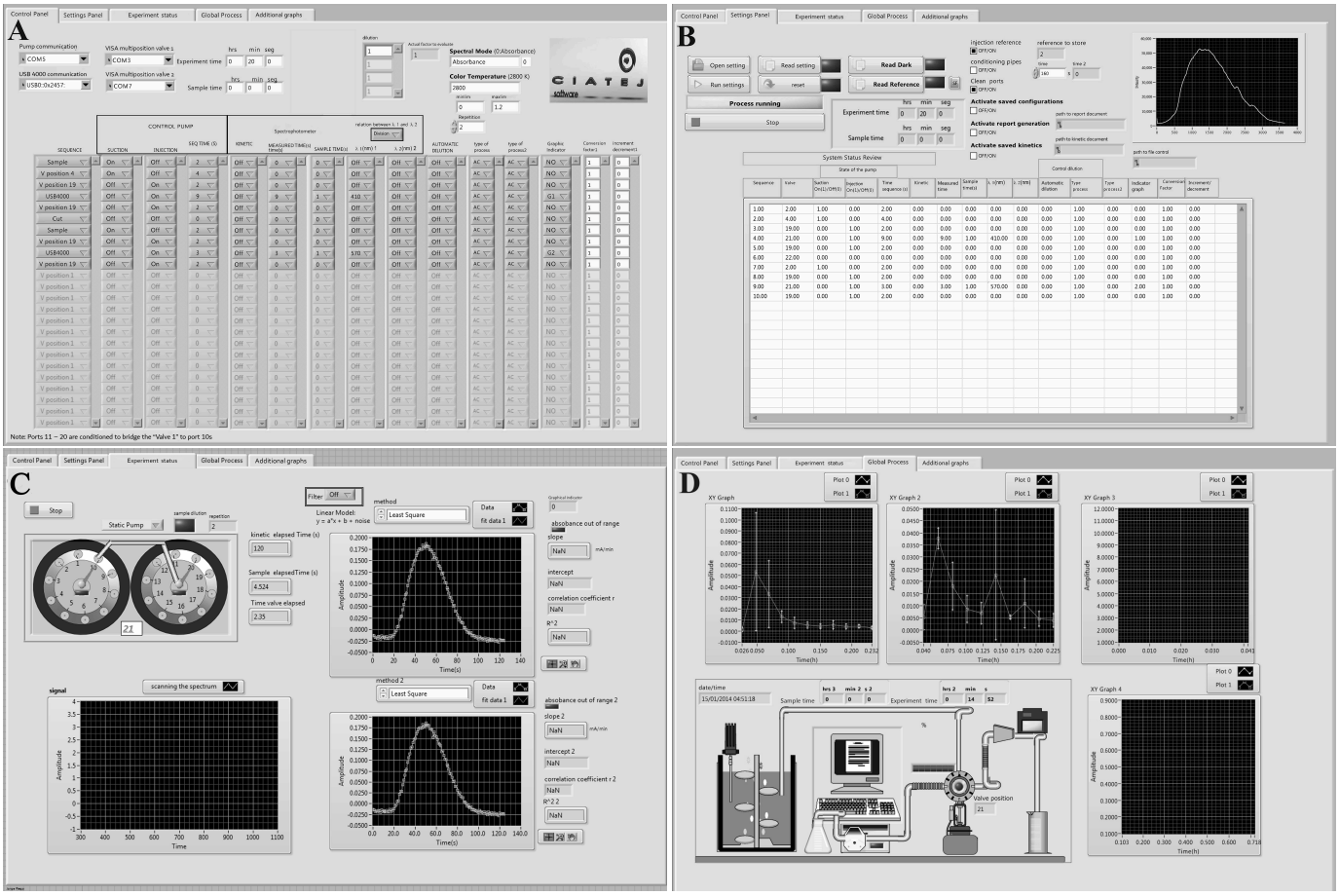

Figure 5. Front panel of the graphical user interface: (**A**) control panel, (**B**) settings panel, (**C**) experiment status, and (**D**) global process.

The setting panel shown in Figure 5B it is intended to load and save the diverse methodologies created in the SIA system. In this window, it is possible to produce the baseline for the spectrophotometer. It can also generate a report in Microsoft Excel. The sequence of operating components configured in the control panel is shown in the setting panel as it is executed by the SIA system. In the experiment status window Figure 5C the user can observe which input of the multiposition valve is enabled. The metabolite being measured can be seen as an end-point or as a kinetic, and the results are depicted in a graph in the same panel, in which it is possible to set a filter to eliminate the Schlieren effect [45], with the assignment of the wavelength of interest and the filter wavelength. Finally, Figure 5D shows one of the two windows developed in the software to monitor the overall measurements performed by the SIA system. The data are displayed in duplicate considering their respective standard deviation bars.

The SIA system is flexible in the sense that the user must configure the components of the device system according to the methodology to be implemented or alternatively, an already saved predefined technique can be loaded by the user. The operator must set the baseline and define the wavelengths of the measurements. In the second stage, the port to operate the inputs of the multiposition valves is selected, then the corresponding action of suction or injection performed by the peristaltic pump is specified, along with other possible actions. The algorithm repeats the sequence as many times as set by the technician corresponding to the sampling events. The results are displayed in diverse graphs with the possibility of generating a report.

3.8. β-Fructofuranosidase Activity Determination Using the Lab-Made SIA System

As already described, diverse hardware modifications were performed in the SIA system that allowed us to set up the DNS methodology in this device. Determination of the *β*-fructofuranosidase activity with the SIA system requires to perform the following steps.

3.8.1. Calibration Curve

Firstly, it was necessary to set up in the SIA system the DNS methodology to determine *β*-fructofuranosidase activity described in the methodology. Calibration curves were generated, relating the response of the system to the concentration of reducing sugars in a sample. The curve considered five known concentrations of glucose: 0.0, 0.5, 1.0, 1.5, and 2.0 g/L. Flow rate was set to 20 µL/s. The DNS methodology adapted to the SIA system was repeated for each point of the curve considering three repetitions for every determination. Figure 6 shows that each group of curves for a given concentration was close to each other denoting the repeatability of the method. The coefficient of determination was 0.997. Due to the limits imposed by the calibration curve, the methodology developed for the determination of *β*-fructofuranosidase was able to measure enzymatic activity in the linear range of 0.01–1.4 U/ml. Enzymatic activities outside that range are required to dilute or concentrate the sample.

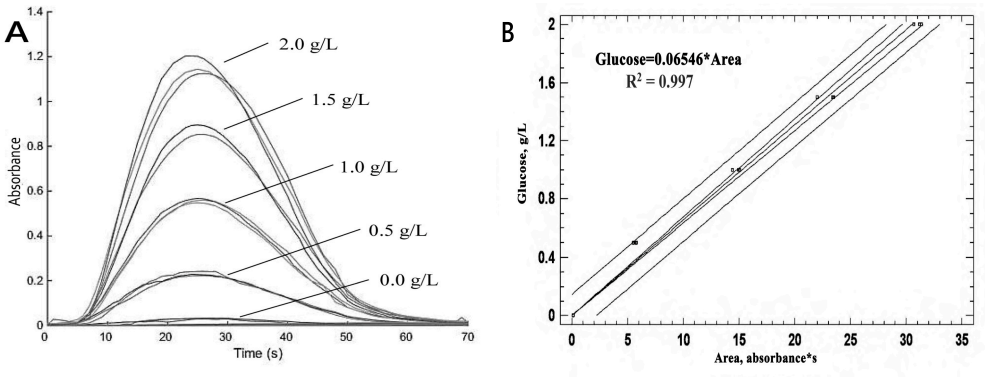

Figure 6. Response from SIA: (**A**) response of the SIA system in front of different substrate concentrations; (**B**) calibration curves.

3.8.2. Sequence Followed in the SIA System to Determine *β*-Fructofuranosidase Activity

To identify the most appropriate sequence to detect enzymatic activity in the SIA system, diverse configurations of injection and suction times were carried out. Table 1 details the parameters programmed in the flow system for the determination of *β*-fructofuranosidase activity from a sample taken manually from the bioreactor and processed with the SIA system.

The holding coil of the SIA system allowed us to obtain dilutions up to 10 times; however, for sample dilutions greater than that, it was necessary to include a dilution module to achieve proportions of 1:100 or even 1:1000, where it was required to implement and repeat the sequence shown in Table 2. The dilution module included storage of the sample, the dilution process, and the washing of the system to avoid contamination with undiluted samples.

Table 1. Protocol for the determination of $\beta-$fructofuranosidase activity in the SIA system.

Described Action	Sequence	Valve Port Position	Pump Operation	Time (s)
Sample	0	2	Suction	4
Dilution 1:10	1	7	Injection	40
Air	2	8	Suction	2
To mixer chamber	3	7	Injection	4
Agitation	4	22	Stop	30
		Dilution stage if this is required 1:10		
To retention chamber	5	7	Suction	5
Supernatant	6	1	Suction	1
To retention chamber	7	7	Suction	1
Supernatant	8	1	Suction	1
Air	9	8	Suction	2
To incubation chamber	10	10	Injection	35
To retention chamber	11	7	Suction	40
Waste	12	5	Injection	55
Cleaning mixer system *	13–16		Cleaning process	280
Washing chamber **	17	8	Injection	120
To retention chamber **	18	8	Suction	65
Waste **	19	5	Injection	70
To retention chamber **	20	8	Suction	20
Waste **	21	5	Injection	30
Incubation 50 C	22	10	Stop	200
To retention chamber	23	10	Suction	30
Reagent DNS	24	9	Suction	1
To retention chamber	25	10	Suction	1
Reagent DNS	26	9	Suction	1
To retention chamber	27	10	Suction	1
Reagent DNS	28	9	Suction	2
To retention chamber	29	10	Suction	3

*—mix chamber washing process; **—washing process for the heating and cooling chamber.

Table 2. Protocol description for the dilution module 1:10.

Described Action	Sequence	Valve Port Position	Pump Operation	Time (s)
To retention chamber	5	7	Suction	8
Air	6	8	Suction	2
To storage	7	10	Injection	8
To retention chamber	8	7	Suction	40
Waste	9	5	Injection	55
Cleaning mixer system	10	7	Injection	180
To retention chamber	11	7	Suction	65
Waste	12	5	Injection	75
To retention chamber	13	10	Suction	8
Air	14	7	Injection	44
Dilution 1:10	15	8	Suction	2
To mixing chamber	16	7	Injection	4
Agitation	17	22	-	30
Waste	18	10	Injection	60

3.8.3. Automatic β-Fructofuranosidase Activity Determination

Diverse β-fructofuranosidase activity determinations were performed with the lab-made SIA system during its configuration process. Several samples were taken manually from shake flasks and from running bioreactors. The samples were analyzed by the SIA system and the results were compared to laboratory determinations concluding that the device had a satisfactory performance.

To demonstrate the potential of the lab-made SIA system to determine β-fructofuranosidase activity at-line, selected samples were taken automatically from a fed-batch bioreactor. Four

culture samples taken at times 1 h (A1), 2 h (A2), 7 h (B1), and 8 h (B2), were extracted automatically from the bioreactor with a specialized probe that included a ceramic filter that retained cells just providing supernatant, Figure 7. The automatic sampling was performed with the sequence shown in Table 3. The samples were processed with the SIA system and the resulting enzymatic measurements were compared to microplate determinations.

Figure 7. At-line β-fructofuranosidase activity determination in a real bioprocess.

Table 3. Protocol for the suction of supernatant from the bioreactor using the sampling probe.

Described Action	Sequence	Valve Port Position	Pump Operation	Time (s)
Air	0	3	Suction	1
From bioreactor	1	2	Suction	60
Waste	2	5	Injection	80
Air	3	3	Suction	1
From bioreactor	4	2	Suction	60
Waste	5	5	Injection	80
Air	6	3	Suction	1
From bioreactor	7	2	Suction	60
Waste	8	5	Injection	90

4. Discussion

The yeast *K. marxianus* has the potential to produce β-fructofuranosidases, which are enzymes capable of hydrolyzing β-fructofuranosides links of fructans to obtain fructooligosaccharides. It is important to understand the dynamics associated with the production of the enzyme under favorable and inhibited conditions in the bioreactor [46,47]. The DNS methodology has been applied in diverse flow systems [20–22], i.e., to detect sugar content in beverages or to validate self-made amperometric sensors. Fructose is a reducing sugar that reacts with free amino groups giving the Maillard reaction [48]. The DNS method applied for enzymatic activity is based on the detection of fructose produced by β-fructofuranosidase which reduces 3,5-Dinitrosalicylic acid (DNS) to 3-amino-5-nitrosalicylic acid, which has a maximum absorption at 540 nm and can be read spectrophotometrically. The increasing absorption is proportional to the production of 3-amino-5-nitrosalicylic acid and consequently to fructose production [49].

To study the accuracy of the lab-made SIA system, the β-fructofuranosidase activity, performed in microplates, was measured against the reaction in the sequential injection system in a kinetic of 8 h. The values obtained by both methods showed a correlation

of 0.9999%. The precision was determined with the standard deviation of the samples with a (n = 2). The standard deviation shown in this study was 6.5% for samples with low activity (A1–A2) and 3.56% for samples with high activity (B1–B2), with a mean of 5.03%. Compared to the standard deviation of 8.75%, the maximum error in this investigation was approximately 20.39% in samples with low activity (A1–A2), which is attributable to the enzymatic activity. The SIA system loses sensitivity since Miller's method has low sensitivity at low concentrations [50,51]. The precision and accuracy of the method depend on the analyte reagent molar ratio, the changes and reaction times, and temperatures [17,52,53]. In another study, the accuracy of the use of a DNS in a spectrophotometric method and the accuracy of enzymatic assays are compared, showing variations in glucose dependence with an error of 5–35% [54]. The error could be decreased if the SIA system is coupled to Raman or FTIR spectroscopies.

Comparing the average activity between both techniques, the lower the activity, the greater the degree of separation, with up to 21% difference, as in sample A2. It turns out that as the activity in the sample increases, the difference between the averages of the two techniques decreases, Table 4. The difference in standard deviation is associated with the sensitivity of the designed equipment, although it is worth mentioning that samples B1 and B2 showed larger enzymatic activity. The results obtained demonstrated that the DNS methodology implemented in the SIA systems has the potential to be used as an at-line process to determine β-fructofuranosidase activity in samples taken out automatically from a bioreactor Figure 8. Nevertheless, the apparition of bubbles in the tubing during the processing of the sample provoked perturbations in the measurements, a problem that must be considered in future determinations to improve repeatability. Syringe injection results in lower standard deviation among the sample due to the decrease in pulses caused by the peristaltic pump [39]; this could in part explain the repeatability obtained achieving standard deviations fluctuating from 0 to 21%. Nevertheless, the use of multiposition valves and peristaltic pumps makes the SIA system versatile allowing the use of multiple reactants to determine diverse analytes in a bioprocess.

Table 4. Validation of microplate vs. SIA technique.

Sample	Microplate Enzymatic Activity (U/mL)	Std Desv.	SIA Enzymatic Activity (U/mL)	Std Desv.	% Error SIA vs. Microplate
A1	8.45	1.20	7.20	3.56	14.79
A2	10.47	0.80	8.34	2.18	20.39
B1	74.45	1.21	74.50	5.33	0.07
B2	83.70	1.95	83.70	6.50	0.00

The automation of the SIA system with the software designed in this manuscript presented substantial improvements to what was reported in [36,37]. The algorithms have the advantage of being easily reconfigurable, with the option to save new methodologies of different sequences, sample auto-dilution, and the opportunity to perform kinetics under a stop–flow and endpoint with a continuous flow or stopped flow. In addition, it is possible to select and manage up to two wavelengths, scanning, and monitoring at wavelengths in the range of 345–1000 nm, and real-time graphs of the variables measured; all this is presented in a friendly, versatile user interface which is easy to manipulate. This is relevant since the new trends in bioprocess are related to the use of micro bioreactors for enzymatic determination [55,56], which will require samplings with less volume from the culture medium. On the other hand, it will be required to monitor more complex bioprocesses with multiple metabolites to draw information about the physiological state of the cells [57,58], with the SIA system being a strategic tool to develop better control algorithms.

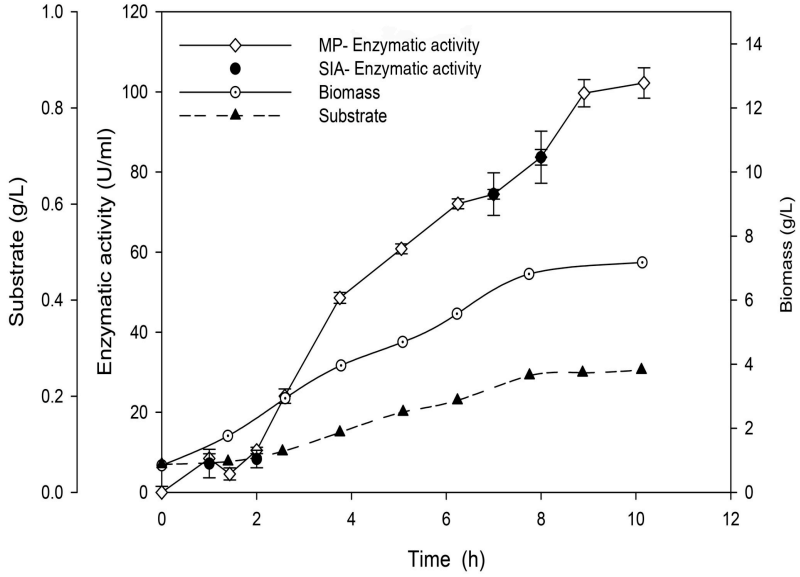

Figure 8. Comparison of β-fructofuranosidase activity determination at selected point with low and high activity in a fed-batch culture.

As a difference between this research and the SIA system reported in [29], it can be stated that in the former manuscript, a flow restrictor was used to pressurize the sequential injection system line at 0.2 MPa. This accessory allows the reduction of bubbles in the SIA line; however, the use of the flow restrictor under the current configuration of the equipment (at the end of the flow cell) does not allow the return of the sample. The modification made in this system was to remove the flow restrictor and include a mixing chamber and a cooling chamber whose purpose is to stop the reaction with the DNS ("quenching reaction") reagent. With respect to the at-line methodology, the determination of the area under the siagram curve is made, which allows the measurement of the generated chromophore, thus reducing the loss of concentration due to the concentration gradient (dilution factor) [29]. On the other hand, in this new system in each determination of reducing sugars during at-line measurement, a calibration curve is created since the DNS continuously degrades. Regarding SIA systems diverse LabVIEW™ algorithms have been reported [37–40]. As a difference to our study, Wagner et al. (2002) [40] reported up to 40 software instructions to control the flow system compared to the 22 commands used in our algorithm. However, in our approach, we can measure samples with the UV-VIS detector reading up to two wavelengths (filter entry), and at-line analysis of the sample, with the possibility to perform analysis in continuous or stopped flow (enzymatic kinetics). Another difference is that in this research we used multiposition valves while in other studies Cavro XP3000 syringe pumps are considered [38–40].

Designing a lab-made SIA system can be quite a challenge, from the point of view of integrating the components, designing the algorithm, and the configuration and validation stages. The built system can be used to monitor different types of metabolites in a bioreactor at the laboratory level in universities, research centers, or even in industry. Designing an SIA system is a challenging research project for Ph.D. students, in which it will be necessary to develop interdisciplinary strategies such as electronics, software development, mechanics, and of course biotechnology resources.

5. Conclusions

A lab-made SIA system was designed to detect at-line β-fructofuranosidase activity. The SIA was controlled with a LabVIEW™ algorithm allowing the user to configure and set up new methodologies in the SIA system from already developed off-line determinations of metabolites in a bioprocess. The system is a potentially useful tool for real-time monitoring

of metabolites since it can automatically sample from the bioreactor, increasing efficiency and lowering the need for manual intervention. The evaluation of the SIA system and the software allowed us to conclude that the device was able to determine the enzymatic activity of β-fructofuranosidase, showing a standard deviation below 7% at activities lower than 11 U/mL with standard deviations below 4% and errors below 21% in comparison with the measurement performed off-line. These experiments demonstrated that the SIA system can perform enzymatic kinetics and end point, under a clever scheme of auto configuration according to the conditions that the culture media showed; however, the performance of the flow device must be improved to decrease the determination times in the presence of dilution and to enhance repeatability.

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Abbreviations

The following abbreviations are used in this manuscript:

DNS	3,5-dinitrosalicylic acid
FIA	flow injection analysis
FOS	fructooligosaccharides
Mp	microplate technique
SIA	sequential injection analysis

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