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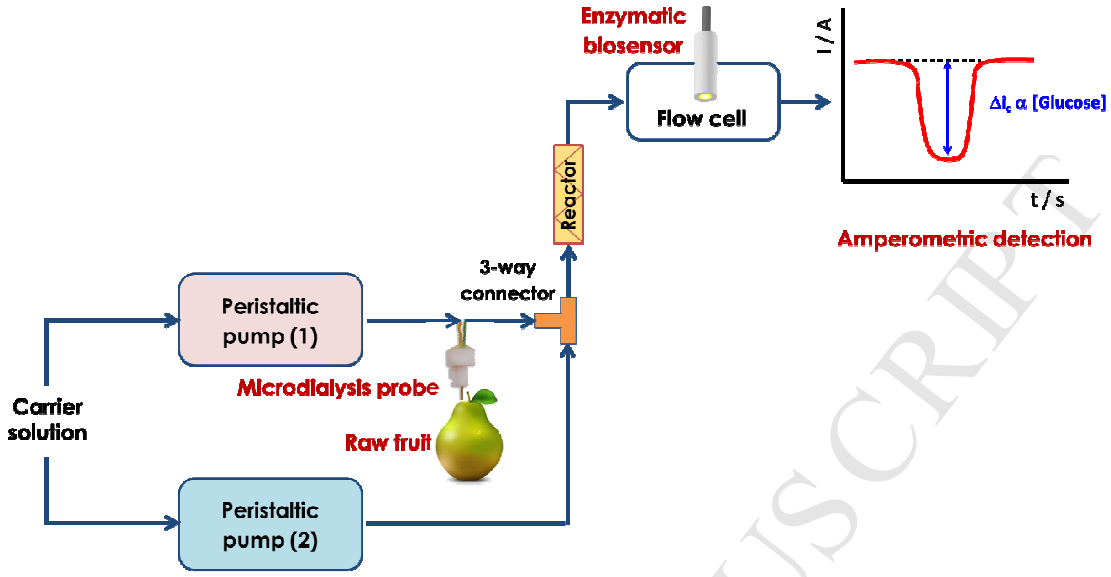
Graphical Abstract



Non-invasive determination of glucose directly in raw fruits using a continuous flow system based on microdialysis sampling and amperometric detection at an integrated enzymatic biosensor

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Abstract

A non-destructive, rapid and simple to use sensing method for direct determination of glucose in non-processed fruits is described. The strategy involved on-line microdialysis sampling coupled with a continuous flow system with amperometric detection at an enzymatic biosensor. Apart from direct determination of glucose in fruit juices and blended fruits, this work describes for the first time the successful application of an enzymatic biosensor-based electrochemical approach to the non-invasively determination of glucose in raw fruits. The methodology correlates, through previous calibration set-up, the amperometric signal generated from glucose in non-processed fruits with its content in % (w/w). The comparison of the obtained results using the proposed approach in different fruits with those provided by other method involving the same commercial biosensor as amperometric detector in stirred solutions pointed out that there were no significant differences. Moreover, in comparison with other available methodologies, this microdialysis-coupled continuous flow system amperometric biosensor-based procedure features straightforward sample preparation, low cost, reduced assay time (sampling rate of 7 h^{-1}) and ease of automation.

Keywords: microdialysis sampling; raw fruits; continuous flow system; amperometric detection; glucose enzymatic biosensor.

1. Introduction

The sugar (fructose, sucrose and glucose) content in ripened fruits is correlated with their glycaemic index. Diabetic patients often question and worry whether it is safe for them to eat fruit because it can contain large quantities of sugar. Glucose is a major monosaccharide found in almost all fruits and is easily absorbed through gastrointestinal tract to increase blood glucose levels. Moreover fruit-ripening process is closely associated with increasing glucose levels. Thus, the glucose content attracts great attention as an indicator of the glycaemic index for fruits and control of their ripening process. Therefore, the development of simple, direct, reliable and economical analytical methodologies for measuring glucose in fruits is highly desirable. Application of such methodologies in the fruit industry can have an economic impact as well as health benefits to end users who are diabetic patients [1].

Although different methods based on infrared [2], titrimetric [3], colorimetric [4] and chromatographic [5] techniques have been described in the literature for glucose determination in fruits, electrochemical glucose biosensors have become an intense topic because fast and easy detection is important in lots of fields such as clinical diagnostics, food industry, and biotechnology [6]. Howewer, recently efforts are being focused on the development of biosensors for nondestructive assortment in real-time mode [7].

Microdialysis is well known to allow *in-situ* sampling and sampling clean up [8]. Two liquids of different composition are separated by a semipermeable membrane, which allows only the transport of small molecules up to a certain molecular weight. During this passage, small molecules and ions diffuse through the membrane and enrich the perfusion liquid to form a continuous sample stream, called the dialysate. This process allows a clean sample stream to be obtained without potentially sensor-fouling substances such as cells or proteins and as the dialysis process has a minimal effect on the surrounding fluid; it is viewed as a tool

for continuous monitoring [9]. The dialysate or analyte-enriched carrier can be transported through a flow analysis system directly to a detector, or be collected in a vial for further analysis [10]. Optimal recovery of the analytes in the dialysate is obtained by using long (up to 10 nm) dialysis probes in conjunction with slow perfusion rates (e.g. 0.3 μL min^{-1}). Slow perfusion rates, however, require even longer sample periods if conventional, off-line detection techniques are used. These drawbacks can be overcome by using slow microdialysis coupled to different continuous systems and fast and sensitive biosensor-based detection techniques. This coupling exhibits potential ability for automatic sampling and applicability for monitoring analytes in all types of matrices and on-line (near) real-time measurements [10,11].

Although microdialysis has been used extensively in the clinical field, currently microdialysis probes have started also to be used as sampling systems in automatic systems related to other sample matrices (foods, seawages and industrial effluents) [12]. In this context, the combination of this attractive sampling technique to a biosensor detection system is particularly convenient since it avoids the direct contact of the biosensor with non-desirable components of the matrix sample, therefore preventing its fouling and extending its stability. Moreover, it also allows carrying out the monitoring of an analyte in a noninvasive way for the sample to enable the extraction function [13]. Taking into account that, depending on experimental conditions (perfusion flow rate, temperature, characteristics of the membrane and physicochemical properties of the sample and analyte) recoveries are usually below 15%, when working with dialysis probes [14]), the microdialysis probes can find applications also to perform dilutions "in-line" and analyse samples with a high concentration of the analyte [15,16]. Although microdialysis probes have been coupled to different continuous systems using amperometric detection at glucose biosensors to perform glucose determination in different samples (fermentation broth, rat brain, blood, liquor, wine, wood biomass and

beverages) [11,17–22], to the best of our knowledge no application to the direct determination of glucose in non-processed fruits has been reported yet.

This paper reports for the first time a non-invasive methodology suitable for the rapid, accurate and in real-time determination of glucose directly in non-processed fruits combining microdialysis sampling on line with continuous flow system and amperometric detection at an enzymatic biosensor without any sample treatment.

2. Experimental

2.1. Apparatus and electrodes

Amperometric measurements were carried with a single channel amperometric detector purchased from InBea Biosensores S.L. (Madrid, Spain). A P-Selecta ultrasonic bath, a Velp Scientifica Microstirrer, a Crison Basic 20+ pH-meter, a Philips Blender and microdialysis probes TP-100-10, DZ-6-02 and AZ-6-02 from EICOM were also used. Flow experiments were carried out using Spetec Perimax-12 and Gilson Minipuls-2 peristaltic pumps.

A glucose commercial biosensor developed previously in our research group [23] and commercialized by InBea Biosensores S.L. was employed to carry out the amperometric detection. This biosensor is based on the glucose oxidase (GOD)/peroxidase (HRP)/ferrocene (Fc) system. A BAS MF-2052 Ag/AgCl/KCl (3 M) reference electrode and a Pt wire counter electrode were also employed. A 10–mL glass electrochemical cell (Pobel®) and a homemade methacrylate wall-jet cell (10 mL) were employed for amperometric measurements in stirred solutions and in the continuous mode, respectively.

2.2. Reagents and solutions

Stock 100 g L^{-1} D(+)-glucose (Panreac) solutions were prepared in 0.05 mol L^{-1} phosphate buffer of pH 7.4. All chemicals used were of analytical-reagent grade and water was obtained from a Millipore Milli-Q purification system.

Different fruits (grapes, pears, kiwis and plums) were purchased in a local supermarket.

2.3. Procedures

2.3.1. Amperometric detection in stirred solutions

Amperometry in stirred solutions was performed by introducing the glucose biosensor, the reference electrode and the auxiliary electrode into the 10-mL glass electrochemical cell containing 10 mL of 0.05 M phosphate buffer (pH 7.4) and applying a potential value of 0.00 V (vs Ag/AgCl). After stabilisation of the baseline, aliquots of the appropriate standard glucose solution were added and the current variation monitored until the steady-state current was reached (~ 7 min).

2.3.2. Amperometric detection with the continuous flow system

The analytical device used for the continuous measurement relied on the combination of a microdialysis sampling system and a continuous flow system using an enzymatic biosensor (see Fig. 1b). The flow cell was connected to peristaltic pumps and three-way connector valves were placed at the beginning of each of the two flow channels to control the passage of carrier or sample solution. The carrier stream used were 0.05 mol L^{-1} phosphate buffer of pH 7.4 at flows of 24 µL min^{-1} (Pump 1) and 3.61 mL min^{-1} (Pump 2). Equilibration of the system was allowed until a stable base line was reached (0.00 V vs. Ag/AgCl). Thereafter, the microdialysis probe was manually immersed in the standard solution/liquid sample under study or inserted in the piece of the raw fruit to be analysed and the current was

continuously monitored. Subsequent measurements can be made as soon as the baseline was reached again.

2.3.3. Analysis in real samples

Microdialysis was used for sampling, sample clean up and dilution before on-line injection of the samples into the continuous flow system [8]. Microdialysis probe without cannula was directly introduced in the fruit juices or in the homogenates resulting after cutting the fruit into small pieces and blending. Conversely, microdialysis probe inside the cannula was introduced to perform the direct determination in the non-processed fruits.

For comparison purposes the same samples were analyzed with the same commercial amperometric biosensor but under batch conditions following the protocol described in Section 2.3.1. The glucose concentration in % (w/w) was calculated using the InBea protocol with a standard calibration plot between 4.5×10^{-3} and 9×10^{-3} g L^{-1}. Fruit samples were prepared by dissolving 0.7 g (pear, kiwi and plum) or 0.2 g (grape), weighed accurately, in 10 mL of 0.05 M phosphate buffer (pH 7.4).

The determination of glucose in raw fruits was accomplished using two-point calibrations in order to reduce the assay time. These calibrations include the (0, 0) point and therefore only the amperometric measurement of one standard solution of similar concentration to that of the analyzed fruit (7, 16, 25 and 59 g L^{-1} for pear, plum, kiwi and grape, respectively, which was previously measured in the fruit juice) was required.

3. Results and Discussion

A diagram of the experimental set-up employed for the continuous monitoring of glucose using rapid sampling microdialysis and a continuous flow system with enzymatic biosensor-based amperometric detection is shown in Fig. 1b). The dialysates were introduced

on-line into the flow system. Since the linear range available with the commercial glucose biosensor (3.8–250.0)×10^{-4} g L^{-1} is far below the usual glucose concentration in fruits (0.5–9.5 % (w/w)), a careful optimization of the key variables involved in the developed methodology such as the type of microdialysis probe, the bioanalyzer design and the flow rate was required to allow the direct determination of glucose in raw fruits.

3.1. Optimization of working variables

The performance of the microdialysis probe depends on several factors such as flow rate, probe length and type of analyte. Therefore, assays to find optimal extraction conditions for glucose were carried out.

3.1.1. Selection of the microdialysis probe

Three different types of commercial microdialysis probes (see Fig. 2) were evaluated. The DZ-X-0Y probe showed practical problems to be efficiently coupled in the continuous system giving rise to the introduction of bubbles or the rupture of the dialysis membrane by overpressure. On the other hand, the AZ-X-0Y probe was fabricated with a rigid material which resulted in an easy rupture of the membrane when it was inserted into a solid sample such as non-processed fruit. Furthermore, the above mentioned probes required longer flow system tubes connected to the inlet and outlet of the probe to enable its introduction and correct orientation into the sample. Accordingly, the TP-X-Y probe, with a 10 mm length, was selected for further work taking into account that its design and dimensions facilitated the integration into the continuous flow system thus resulting in increased durability due to its flexibility.

3.1.2. Optimization of the bioanalyzer design

Two different bioanalyzer designs were implemented and compared. The one schematized in Fig. 1a comprised of a peristaltic pump, the microdialysis probe and the wall jet electrochemical cell. This simple system was characterized using glucose standards at concentrations between 0.4 and 2.5 g L^{-1} and a flow rate of 24 μL min^{-1}. The upper limit of the resulting linear range was 1.44 g L^{-1} (see left inset in Fig. 1a) which is still far below the glucose concentration found in fruits. This indicated that, under the experimental conditions used, the dilution capacity of the dialysis probe was not enough to allow the direct determination of glucose in fruits. A possible solution would be to increase the flow rate so that the contact time would be shorter and less glucose would be extracted, thereby decreasing the analyte concentration in the dialysate. However, as it was previously mentioned, high flow rates affected adversely the stability of the microdialysis probe. Therefore, we proceeded to change the bioanalyzer design in order to dilute the sample in the system itself. This was made by using two peristaltic pumps and a second flow channel by placing a three-way connector after the microdialysis probe (Fig. 1b). A reactor of appropriate length was also coupled to this three way connector in order to achieve a reproducible mixture of the diluting solution and the dialysate. Accordingly, the flow of this second channel needed to be optimized also in order to achieve a sample dilution able to allow the direct measurement of glucose concentration in fruits.

3.1.3. Optimization of the hydrodynamic variables

Both the perfusion (peristaltic pump 1) and dilution (pump 2) flow rates should be considered. However, the perfusion flow rate was not optimized but fixed at 24 μL min^{-1} which was the lowest value that the employed pump could provide with the used tubes. This flow rate was high in comparison with those normally used in microdialysis (0.1–5.0 μL min^{-1}) [24]. However, the use of lower flow rates would be meaningless in this case because it

would entail a larger extraction of the analyte and consequently an undesired lower analyte dilution in the dialysate [14]. Moreover, at the selected perfusion flow rate, the stability of the microdialysis probe was shown to be adequate as it will be described below.

In order to optimize the flow provided by pump 2, used for dilution and transporting the dialysate to the flow cell, calibration plots for glucose were constructed at flow rates between 0.37 and 3.61 mL min^{-1}. The steady state currents were measured after immersing the probe in standard solutions of increasing glucose concentration. Fig. 3 shows as the biosensor response was highly dependent on the dialysate flow rate. The sensitivity was remarkably larger with the lower flow rates due to the longer enzyme-catalyzed reaction time. On the contrary, the range of linearity was narrower and the sample throughput decreased with respect to the use of higher flow rates [25]. With the aim of making possible to measure glucose in a wide concentration range, a flow rate of 3.61 mL min^{-1} was selected for the peristaltic pump 2. It is important to note that this value allowed the levels of glucose concentrations in fruits were included within the range of linearity provided by the bioanalyzer.

3.2 Stability studies

The developed bioanalyzer comprised two disposable components the microdialysis probe and the enzymatic biosensor. Therefore, the lifetime of both components was checked.

3.2.1 Glucose biosensor stability

Different aspects concerning the stability of the integrated glucose biosensor were evaluated.

The repeatability of the continuous flow amperometric measurements was tested (Fig. 4). Twenty repetitive measurements of a 40 g L^{-1} glucose standard solution yielded a RSD

value of 7.0% thus pointing out the good stability of the bioelectrode under the used hydrodynamic conditions.

Moreover, the lifetime of one single glucose biosensor was evaluated by performing daily amperometric measurements for a 40 g L^{-1} glucose solution. After using, the biosensor was stored in phosphate buffer 0.05 M of pH 7.4 at 4 °C. The results obtained (not shown) demonstrated that the commercial biosensor used as amperometric detector in the bioanalyzer was operative for about 10 days. During this period of time an average of 90 samples could be analyzed with the same biosensor. It is interesting to note that other enzyme biosensors based on similar electrode configurations and immobilization strategies exhibited different storage stabilities (from 10 days for a lactate oxidase (LOD)/HRP/Fc L-lactic acid biosensor to 20 days for an ethanol biosensor using alcohol oxidase (AOD)/HRP/Fc). Therefore, it can be concluded that the stability of the enzymatic system seems to play a determinant role in the storage stability of the developed biosensor in comparison with the small Fc leakage (although the mediator was immobilized in excess and exhibits low solubility in water [26]) occurring from the electrode surface."

3.2.2 Microdialysis probe stability

Regarding the stability of the microdialysis probe, the results obtained with 3 different probes demonstrated that, under the selected optimal conditions, each microdialysis probe could be used an average of 20 days without observing membrane rupture.

All these results led us to conclude that the two disposable components of the bioanalyzer could be continuously used with minimal risk of failure for one week.

3.3. Analytical characteristics of the continuous flow methodology

Under the optimized working conditions, the analytical characteristics shown in Table 1 were obtained for glucose standards. The limits of detection (LOD) and determination (LOQ) were calculated according to the $3\times s_b/m$ and $10\times s_b/m$ criteria, respectively, where m is the slope of the linear calibration plot and s_b is the standard deviation of the blank current which was calculated as the current value measured 300 s before the glucose measurement.

Given the well-known interference of ascorbate, able to reoxidize HRP [27], and its high concentration in fruits, the potential interference of the ascorbic acid with the developed biosensor was evaluated prior to the analysis of real samples. Since glucose has been reported to be between 30 and 6,300 times more concentrated than ascorbic acid in kiwi, pear and grape juices [28,29] the amperometric signals provided by the developed biosensor for a glucose concentration of 6.1×10^{-3} g L^{-1} and that measured for a mixture solution containing the same glucose concentration plus a 29-times lower concentration of ascorbic acid (6.1×10^{-3} g L^{-1} glucose + 2.1×10^{-4} g L^{-1} ascorbic acid) were compared. The results obtained (not shown) demonstrated that this latter was only 3.2% larger than the amperometric measurement obtained with no ascorbic acid. These results confirmed the great selectivity and the low detection potential required by the developed biosensor ensure the absence of any significant interference from the presence of this common naturally occurring acid in fruits.

3.4. Determination of glucose in real samples

The analytical usefulness of the developed bioanalyzer was evaluated by determining glucose in three different types of real samples: fruit juices, blended fruits and raw fruits. No statistically significant differences were observed between the slope values of the calibration plots constructed with glucose standards and the calibration graphs obtained for blended red plum spiked with increasing concentrations of glucose, and for different blended fruits (pear, red plum, green kiwi and white grape) containing different known endogenous glucose

content. Accordingly, we could conclude that no significant matrix effects existed indicating that the microdialysis sampling was also effective for sample clean-up [30]. Therefore, glucose quantification could be accomplished by simple interpolation of the measured current from the samples into the calibration plot constructed with glucose standard solutions.

3.4.1 Determination of glucose in blended fruits

Glucose was determined in a homemade green Kiwi smoothie. The continuous flow amperometric signals measured in the non-diluted smoothie were interpolated in the two-point calibration plot constructed by triplicate measuring of 40 and 80 g L^{-1} standard glucose solutions (Fig. 5).

The same sample was analyzed by applying a method validated by InBea S.L. that used the same biosensor as amperometric detector in stirred solutions. This method involved the addition of 50 μL of the 20 times diluted kiwi smoothie (in phosphate buffer 0.05 M pH 7.4) to an electrochemical cell containing 20 mL of phosphate buffer 0.05 M pH 7.4 and interpolation of the measured steady state current into a glucose calibration plot constructed over the 4.5×10^{-3}–9.0×10^{-3} g L^{-1} concentration range. Results from four replicates yielded mean glucose concentrations of (39.8 ± 0.9) g L^{-1} with the continuous flow method and (38 ± 2) g L^{-1} using InBea's method, the confidence intervals being calculated for a significance level of 0.05. A comparison of the mean values obtained with both methods, after checking that there were no significant differences between the standard deviations values, indicated that the experimental Student t value (2.461) was smaller than the tabulated one (2.776) for a significance level of 0.05, thus demonstrating the absence of significant differences between the results obtained by the two methodologies.

In addition, glucose was determined by using the flow continuous mode in a plum smoothie spiked with 5 and 10 g L^{-1} of glucose. Mean recoveries of (102 ± 19) and (97 ± 26) % were obtained, respectively, with the confidence intervals calculated for $\alpha = 0.05$ (n = 4).

These results demonstrated fairly well the usefulness of the developed bioanalyzer for the reliable determination of glucose in smoothies without any sample treatment.

Moreover, with the aim to verify that the microdialysis probe behaved similarly when glucose was measured in standard solutions and fruits, at any level of concentration, the relative recovery "RR" parameter was evaluated. RR is an important parameter in quantitative microdialysis expressing the relative performance in which the analyte has been extracted from the sample as the extraction fraction [10] or extraction efficiency [15]. RR, in %, was calculated using the following expression [31]:

$$RR, \% = \frac{C_d}{C_m} \times 100 \quad (1)$$

where C_d and C_m are the analyte concentration in the dialysate and the sample, respectively.

RR values were calculated in glucose standard solutions with concentration ranging between 10 and 90 g L^{-1} by introducing the microdialysis probe in the solutions and collecting the corresponding dialysate using phosphate buffer of pH 7.4 as perfusion fluid. Glucose concentration was calculated by amperometry in stirred solutions at the InBea's biosensor.

For the estimation of the RR values in the blended fruits, the glucose concentration in the samples (C_m) was determined using the continuous flow method by interpolation of the amperometric signals obtained from the non-diluted blended fruits into the calibration plot constructed with glucose standard solutions over the 10 to 80 g L^{-1} concentration range. Moreover, a similar protocol to that described for glucose standard solutions was employed to calculate the concentration in the dialysates (C_d) collected upon introducing the microdialysis probe in the blended fruit.

The results obtained in these studies are summarized in Table 2 with the confidence intervals calculated for 5 replicated and for a significance level of 0.05. As it can be deduced from this Table, the mean RR value obtained in blended fruits (2.4 ± 0.2) was not statistically different than that calculated with glucose standards (2.43 ± 0.05) ($t_{exp,}$ 0.132; $t_{tab,}$ 2.776) demonstrating that the glucose extraction efficiency of the microdialysis probe is similar in the standards solutions and blended fruits.

Furthermore, the glucose concentrations in blended fruits were also calculated by applying the mean RR value obtained in standard solutions (2.43 ± 0.05) over the dialysate concentration determined by amperometry in stirred solutions. The results are shown in Table 3, where the concentration values obtained by the continuous flow mode using the bioanalyzer are also included.

As it is clearly seen, the statistical comparison between mean values for each type of sample obtained either directly with the continuous flow method or indirectly using amperometry in stirred solutions with the glucose biosensor, allowed us to deduce that no significant differences (t values were calculated using paired data, with t_{exp}, 0.085, being lower than t_{tab}, 3.182). These results demonstrated once again that the microdialysis probe behaved similarly in terms of analyte extraction both in glucose standard solutions and blended fruits.

Fig. 6 shows the regression plot constructed with data from both methodologies. The resulting linear least-squares regression graph (r = 0.9992) yielded a slope value of (0.98 ± 0.03) and an intercept of (1 ± 1). As expected, the correlation found was highly satisfactory since the confidence intervals (at a significance level of 0.05) for the slope and intercept included the unit and the zero values, respectively. These results indicated that the continuous flow method involving the bioanalyzer did not exhibit systematic errors and can be successfully used for the reliable determination of glucose in blended fruits.

3.4.2 Direct determination in raw fruits

In order to perform the direct determination of glucose in non-processed fruit pieces, a correction factor *K*, related to the different extraction efficiency of the analyte with the microdialysis probe in solid and in blended fruit, as well as a conversion factor between the concentration units used with standard solution (g L^{-1}) and those commonly used to describe the glucose content in fruits (% (w/w)), were calculated.

3.4.2.1 Factor K determination

Calculation of the K factor correction was needed to obtain the glucose concentration simply by interpolation of the corresponding current measurement into the calibration plot constructed with glucose standard solutions. The factor was defined as the ratio between the measured current in the raw fruit (i_f) and the current measured in the blended fruit (i_b):

$$K = \frac{i_f}{i_b} \quad (2)$$

To determine the K factor, the piece of fruit was divided into two halves. One of them was blended and used to measure the corresponding amperometric signal (i_b) as described in the previous section. On the other hand, the microdialysis probe was inserted in the other fruit half by means of a cannula to prevent rupture of the microdialysis probe (Fig. 7a), and the measured current was used to obtain the i_f value. An example of the recorded amperograms for a white grape sample is displayed in Fig. 7b. As it can be observed, there was a noticeable difference between the heights of both amperometric signals which depended on the physical condition of the fruit. Table 4 summarizes the mean values of the K factor for each fruit which were calculated from data obtained by performing different determinations for each piece and using 2–3 different fruit pieces. It is important to remark that no significant differences in the measurements were observed when the microdialysis probe was inserted in

different parts of a same piece of fruit as well as for different orientation angles of the membrane and therefore for different depths.

Data given in Table 4 show an acceptable reproducibility in the factor *K* determination considering the simple and direct experimental approach used. Moreover, we could conclude that this correction factor was a parameter characteristic for each type of fruit according to the uniqueness of its physical properties.

3.4.2.2 Conversion factor g L^{-1} to % (w/w)

The glucose content in fruits is commonly expressed in % (w/w). However, when the continuous flow methodology using microdialysis probe sampling was employed to perform this determination, the glucose content in the piece of fruit could not be directly obtained in these units since the standard calibration plots were constructed in g L^{-1} and the sample weight is unknown. Therefore, the equivalence between glucose content in g L^{-1} and % (w/w) was calculated. In order to do that the glucose concentration in blended fruits was determined in g L^{-1} by the continuous flow method using the bioanalyzer while the amperometric method in stirred solutions using the InBea's biosensor was applied to determine it in % (w/w) since in this latter case it is also possible to know the fruit weight.

The correlation found between both set of results is shown in Figure 8a (r = 0.998) according to the equation:

$$[\text{glucose}],\ \%\ (w/w) = (0.162 \pm 0.006) \times [\text{glucose}],\ g\ L^{-1} - (0.2 \pm 0.2) \quad (3)$$

which can be used to exchange the glucose concentration units both in standard solutions and blended fruits.

3.4.2.3 Direct determination of glucose (in %, w/w) in raw fruits

The determination of % glucose in non-processed fruits was carried out by introducing the microdialysis probe (inside the cannula) into various points of each raw fruit and recording the current as a function of time. Glucose quantification in % (w/w) was made using the following expression (4):

$$[\mathrm{glucose}],\ \%\ (w/w) = \frac{i_f / K}{i_s} \times [\mathrm{standard}] \quad (4)$$

where i_f and i_s are the current values measured in the piece of fruit and in the glucose standard solution, respectively, and K is the mean correction factor value characteristic of each type of fruit (see Table 4). The obtained results using the continuous flow method with the bioanalyzer and applying equation 4 are summarized in Table 6. Moreover, they are compared with those given in Table 5 using the InBea methodology. It is important to mention that the values obtained are in agreement with the range of glucose content reported in fresh fruits (0.5–9.5 % (w/w)).

The results provided by both methodologies were statistically compared by constructing the corresponding regression plot (Fig. 8b). An r value of 0.999, with slope and intercept values of (1.02 ± 0.02) and (−0.1 ± 0.1), respectively, indicated that both methods led to statistically similar results.

It is worth to mention also that the damage made in the fruit by the introduction of microdialysis probe inside the cannula is so minimal that besides being invisible by the eye has not been shown to produce a fast development of molds and fruit rottenness. These results demonstrated the successful applicability of the developed methodology, based on coupling a continuous flow system with fast sampling microdialysis and selective biosensor-based amperometric detection, as non-destructive approach to provide on-line monitoring of glucose directly in raw fruits in a reliable manner thus showing potentiality for *on-site* assessment or inspection of quality parameters even in unpicked fruits.

4. Conclusions

This work described for the first time a microdialysis sampling, which also provided on-line sample clean-up, coupled with flow continuous set-up and amperometric detection at integrated enzymatic biosensor for the direct determination of glucose in non-processed fruits without any sample preparation. This new analytical system has demonstrated to possess adequate sensitivity to track directly the concentration of glucose in raw fruits in a non-destructive way with a lag time of less than 20 min (including standard calibration). The methodology exhibited great robustness and can be advantageously compared to other reported methods in terms of cost and assay time, elimination of stability problems, direct application to the sample without pretreatment and automated analysis for on-line monitoring. The simplicity of the developed methodology and easy adaptability make it a versatile tool for routine analysis in the food industry or to perform *on-site* determinations, a feature that cannot be considered for the existing methods based on electrochemical (bio)sensors which therefore would allow, apart from determination in fruit juices and beverages, the real-time glucose monitoring even in unpicked fruits using a non-destructive protocol, thus allowing to left the fruit maturing in the tree until the optimal time of collection arrive. Moreover, taking into account the excellent performance of the developed approach which demonstrates its great potential as non-invasive technique for glucose assessment, other interesting applications such as glucose determination at different depths in the meat, to ensure freshness and microbiological quality, or evaluation of human tissues healthiness through glucose content could be also envisioned.

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Table 1. Analytical characteristics obtained for glucose standards with the bioanalyzer with the bioanalyzer set-up shown in Fig. 1b.

Parameter	Value
Linear range, g L^{-1}	3.0–80
Slope, nA g^{-1} L	2.18 ± 0.02
Intercept, nA	1 ± 1
r	0,9996
LOD, g L^{-1}	0.9
LOQ, g L^{-1}	3.0

Table 2. Mean RR values calculated in blended fruits and in glucose standard solutions (C_m was determined by applying the continuous flow mode with the bioanalyzer while C_d was calculated by amperometry in stirred solutions).

		C_m, g L^{-1}	C_d, g L^{-1}	RR, % (n = 5)	Mean RR, %
Standards	1	10	0.25 ± 0.03	2.5 ± 0.3	(2.43 ± 0.05)
	2	18	0.44 ± 0.02	2.4 ± 0.1	
	3	20	0.49 ± 0.04	2.4 ± 0.2	
	4	25	0.64 ± 0.01	2.55 ± 0.04	
	5	45	1.01 ± 0.01	2.25 ± 0.03	
	6	60	1.48 ± 0.03	2.47 ± 0.04	
	7	90	2.1 ± 0.2	2.4 ± 0.2	
Blended fruits	Pear	5 ± 1	0.122 ± 0.005	2.4 ± 0.1	(2.4 ± 0.2)
	Green Kiwi	21.6 ± 0.8	0.49 ± 0.04	2.3 ± 0.2	
	Red plum	24 ± 1	0.60 ± 0.02	2.50 ± 0.08	
	White grape	63 ± 2	1.6 ± 0.1	2.5 ± 0.2	

Table 3. Glucose concentration determined in blended fruits (in g L^{-1}) calculated by applying the mean RR value obtained in standard solutions to the C_d values using amperometry in stirred solutions and the continuous flow mode with the developed bioanalyzer.

Blended fruit	Amperometry in stirred solutions	Bioanalyzer
Pear	5.0 ± 0.2 ($RSD_{n=3}$ = 1.7%)	5 ± 1 ($RSD_{n=3}$ = 7.8%)
Green Kiwi	20 ± 2 ($RSD_{n=5}$ = 6.3%)	21.6 ± 0.8 ($RSD_{n=5}$ = 2.9%)
Red plum	24.8 ± 0.8 ($RSD_{n=5}$ = 2.5%)	24 ± 1 ($RSD_{n=5}$ = 4.9%)
White grape	64 ± 4 ($RSD_{n=5}$ = 5.2%)	63 ± 2 ($RSD_{n=5}$ = 3.2%)

Table 4. Mean K factor values calculated for different types of fruit.

Fruit	Mean K value
Pear	0.53 ± 0.02 ($RSD_{n=42}$ =10.7%)
Green Kiwi	0.78 ± 0.03 ($RSD_{n=27}$ =11.3%)
Red plum	0.65 ± 0.02 ($RSD_{n=33}$ =10.6%)
White grape	0.59 ± 0.02 ($RSD_{n=45}$ =9.5%)

Table 5. Glucose concentration determined in blended fruits by the continuous flow method using the bioanalyzer (in g L^{-1}) and by amperometry in stirred solutions using InBea's methodology (in w/w %).

	[Glucose], g L^{-1}	[Glucose], % (w/w)
Pear	7.0 ± 0.3 ($RSD_{n=9}$ = 5.8%)	1.00 ± 0.05 ($RSD_{n=14}$ = 8.2%)
Green Kiwi	25 ± 1 ($RSD_{n=9}$ = 5.2%)	3.6 ± 0.1 ($RSD_{n=15}$ = 6.0%)
Red plum	16.0 ± 0.8 ($RSD_{n=11}$ = 7.5%)	2.03 ± 0.09 ($RSD_{n=15}$ = 8.2%)
White grape	59 ± 1 ($RSD_{n=19}$ = 4.0%)	9.5 ± 0.2 ($RSD_{n=20}$ = 4.6%)

Table 6. Glucose concentration in raw fruits in % (w/w) using the bioanalyzer with the continuous flow mode and the InBea methodology using amperomewtry in stirred solutions.

	Bioanalyzer	InBea
Pear	0.9 ± 0.1 ($RSD_{n=5}$ = 8.4%)	1.00 ± 0.05 ($RSD_{n=14}$ = 8.2%)
Green Kiwi	3.0 ± 0.5 ($RSD_{n=5}$ = 12.7%)	3.6 ± 0.1 ($RSD_{n=15}$ = 6.0%)
Red plum	2.0 ± 0.1 ($RSD_{n=10}$ = 8.4%)	2.03 ± 0.09 ($RSD_{n=15}$ = 8.2%)
White grape	10.0 ± 0.8 ($RSD_{n=3}$ = 3.1%)	9.5 ± 0.2 ($RSD_{n=20}$ = 4.6%)

Figure captions

Fig. 1. Different types of bioanalyzer set-ups evaluated for the direct determination of glucose in fruits.

Fig. 2. Types of commercial microdialysis probes assayed: DZ-X-0Y (a), AZ-X-0Y (b) and TP-X-Y (c).

Fig. 3. Dependence of the amperometric responses obtained using the bioanalyzer set-up shown in Figure 2b with the flow rate of Pump 2 for glucose standard solutions of increasing concentration. Carrier solution: phosphate buffer 0.05 mol L^{-1} pH 7.4; flow rate pump 1: 24 µL min^{-1}; E_{ap} = 0.00 V vs. Ag/AgCl. Error bars estimated as the triple of the standard deviation (n = 3).

Fig. 4. Successive amperometric responses obtained for 40 g L^{-1} glucose with the bioanalyzer set-up shown in Figure 2b. Carrier solution: phosphate buffer 0.05 mol L^{-1} pH 7.4; flow rate Pump 1: 24 µL min^{-1}; flow rate Pump 2: 3.61 mL min^{-1}; E_{ap} = 0.00 V vs. Ag/AgCl.

Fig. 5. Amperometric responses obtained in the continuous flow mode with the bioanalyzer set-up shown in Figure 2b for 40 and 80 g L^{-1} glucose standard solutions and the non-diluted kiwi smoothie. Carrier solution: phosphate buffer 0.05 mol L^{-1} de pH 7.4; flow rate Pump 1: 24 µL min^{-1}; flow rate Pump 2: 3.61 mL min^{-1}; E_{ap} = 0.00 V vs. Ag/AgCl.

Fig. 6. Correlation plot between the results obtained in the determination of glucose in blended fruits using the continuous flow method with the developed bioanalyzer and those

provided by amperometry in stirred solutions with the InBea's biosensor and applying the mean RR value calculated with standard solutions (data from **Table 3**).

Fig. 7. Microdialysis probe used to perform the direct continuous flow determination of glucose in raw fruits (a). Amperogram recorded after introducing the microdialysis probe in blended and different places of non-processed white grape pieces (b). Other conditions are similar to those given in **Fig. 5**.

Fig. 8. Correlation between the glucose concentration determined in blended fruits in g L^{-1} and % (w/w) (a), and between the glucose concentration calculated in % (w/w) in blended fruits by using the continuous flow mode with the developed bioanalyzer and the InBea methodology by amperometry in stirred solutions (b).

Fig. 1

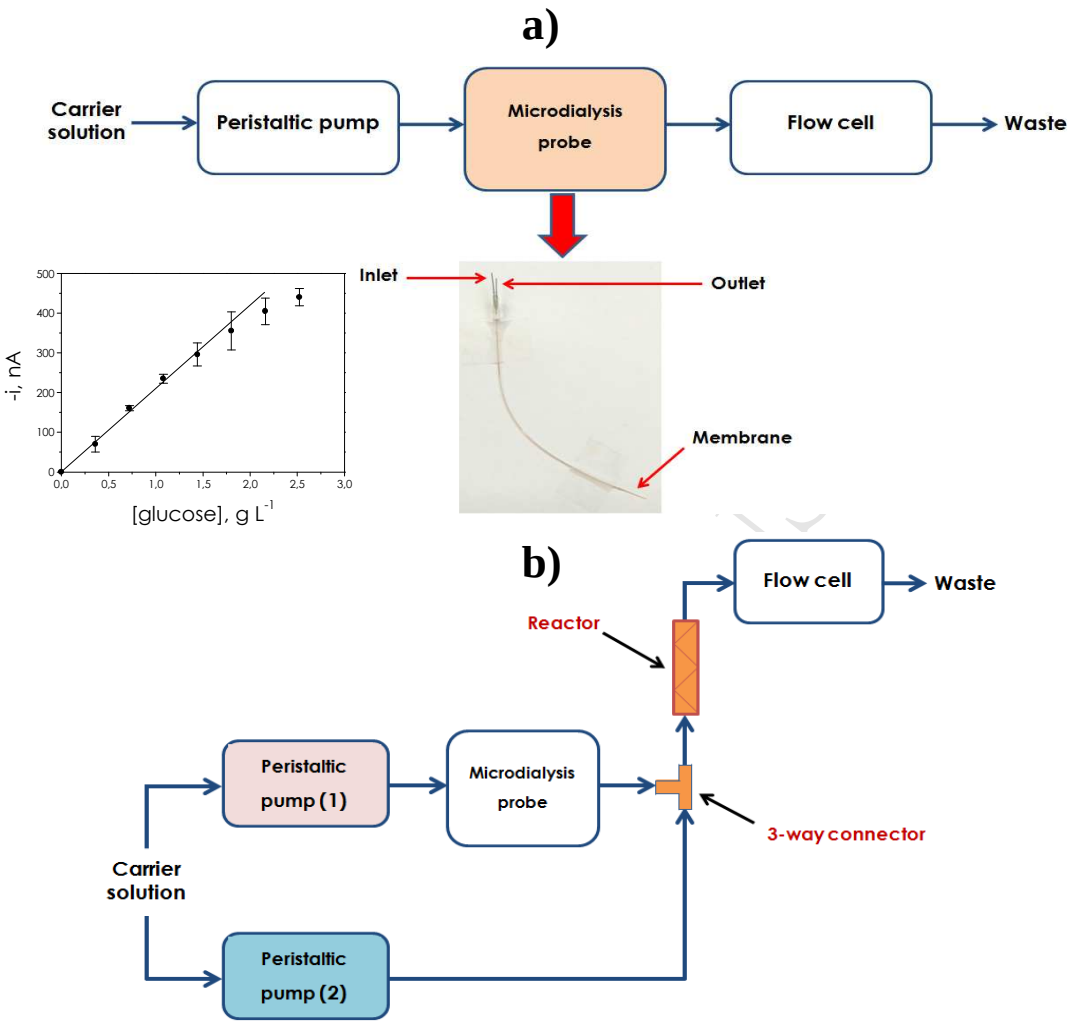

a)
Carrier solution
Peristaltic pump
Microdialysis probe
Flow cell
Waste
Inlet
Outlet
Membrane
-i, nA
[glucose], g L-1
b)
Flow cell
Waste
Reactor
Peristaltic pump (1)
Microdialysis probe
3-way connector
Carrier solution
Peristaltic pump (2)

Fig. 2

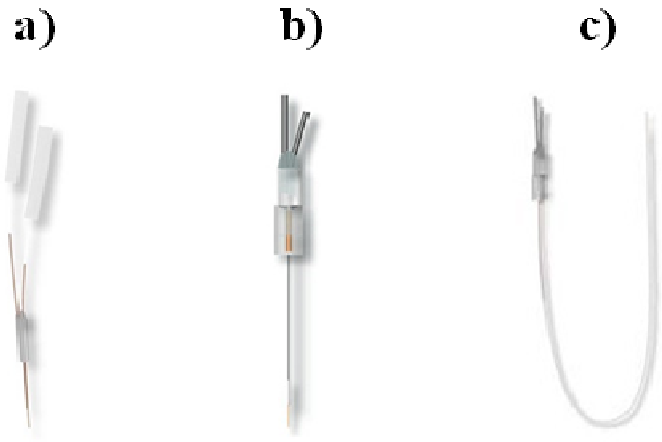


Fig. 3

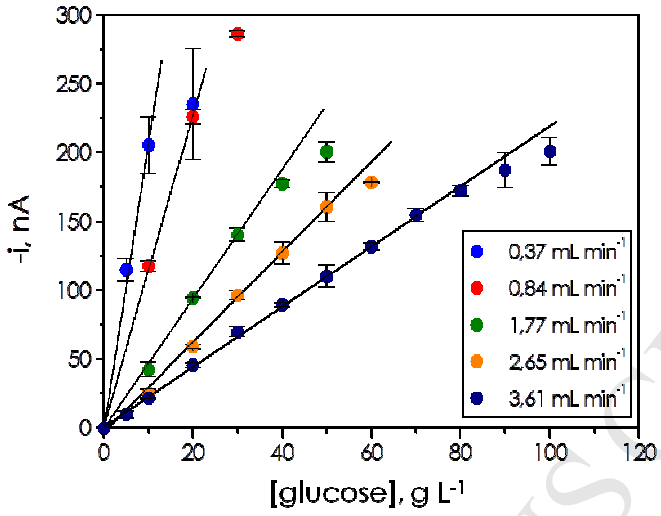


Fig. 4

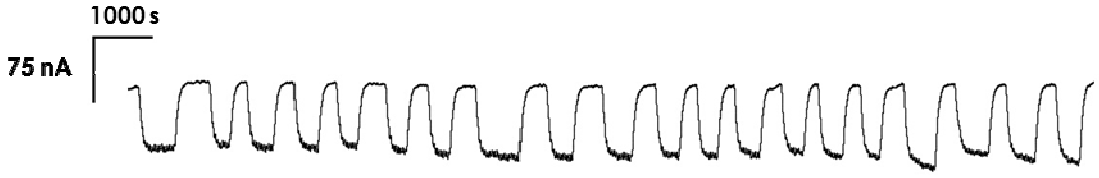


Fig. 5

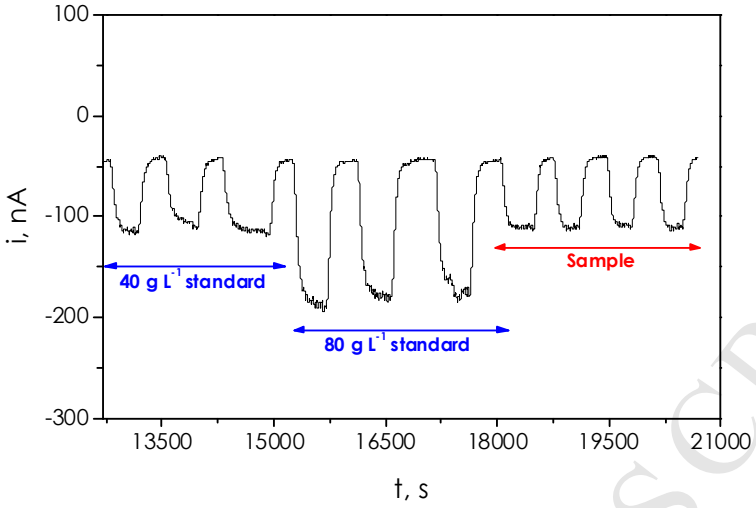


Fig. 6

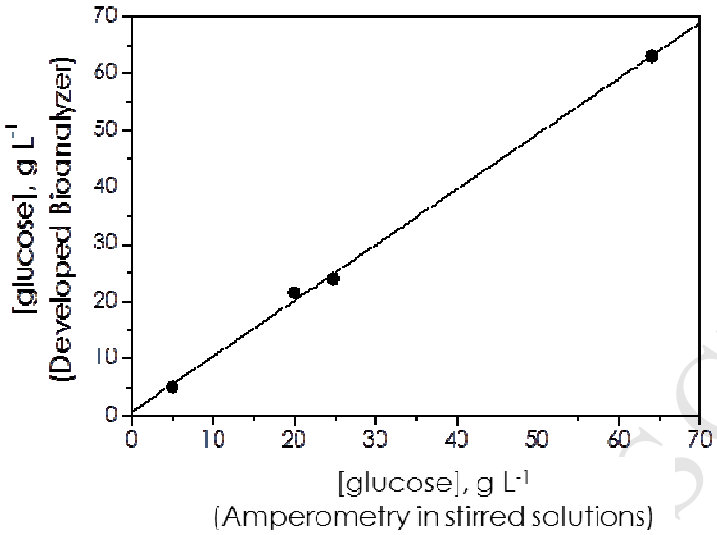


Fig. 7

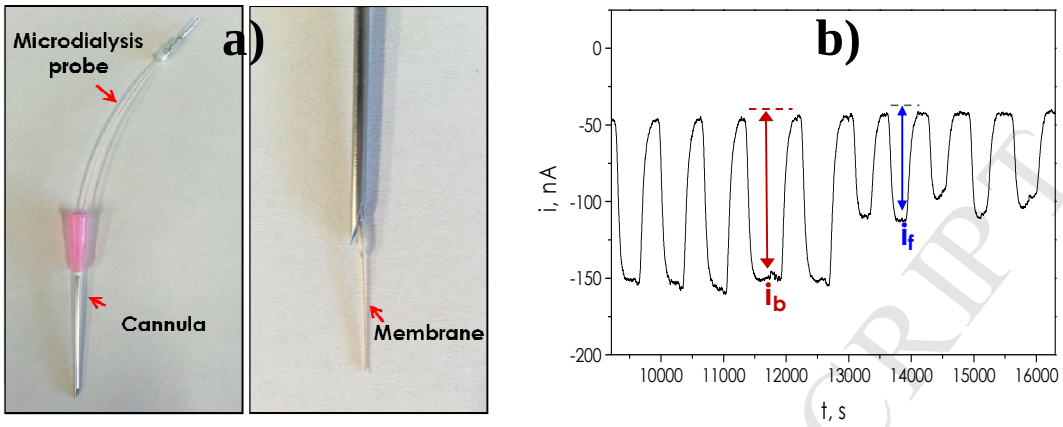
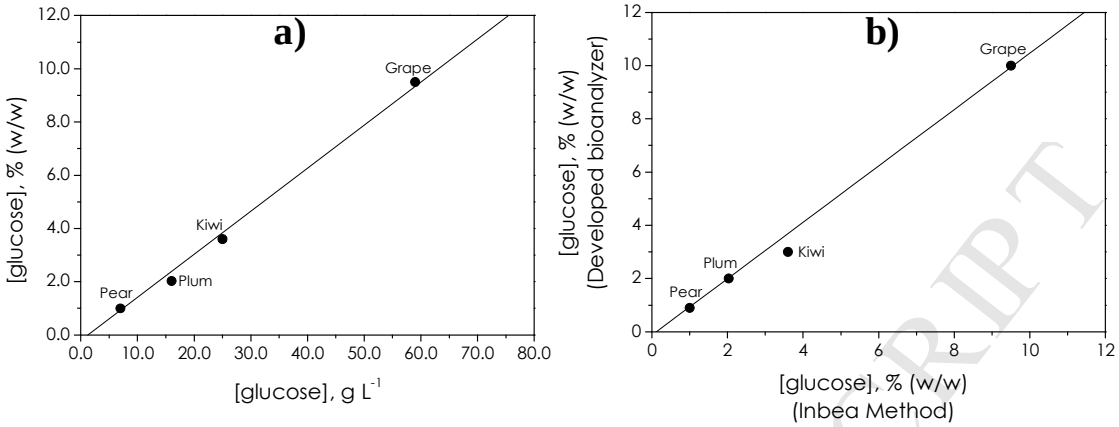


Fig. 8



Highlights:

- First electrochemical biosensor applied to non-invasively determination of glucose directly in raw fruits.
- On-line microdialysis sampling coupled to an amperometric enzymatic biosensor.
- Versatile tool for routine analysis in the food industry with a sampling rate of 7 h^{-1}.
- Feasible to perform *on-site* assessment or inspection of quality parameters.