

Multicommutated Flow System for Glycerol Determination in Alcoholic
Fermentation Juice Using Enzymatic Reaction and Spectrophotometry

Eloísa A. M. Kronka¹, Pedro R. Borges¹, Rodrigo Latanze¹, Ana Paula S. Paim²,
Boaventura F. dos Reis^{2*}

¹Universidade de Ribeirão Preto, Centro de Tecnologia e Desenvolvimento Agroindustrial

²Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, CP 96, CEP 13400-
970, Piracicaba, SP, Brazil

Abstract.

An automatic multicommutated flow procedure for glycerol determination in alcoholic fermentation of sugar-cane juice is proposed. The method was based on the reaction of the adenosine triphosphate (ATP) catalyzed by the glycerol-kinase to form quinoneimine detected at 540 nm. The flow system was controlled by microcomputer running a software written in Quick BASIC 4.5 that provide facilities to permit also data acquisition. The feasibility of the system was ascertained by processing a set of fermented sugar-cane juice samples. Comparing results with those obtained by the manual procedure no significant difference at 95% confidence level was observed. Other advantageous features such as a linear response ranging from 0.200 to 1.000 % (w/v) glycerol; a 1.3 % relative standard deviation (n= 9); a low reagent consumption, 30 µl enzymatic solution per determination; and an analytical throughput of 60 samples were also achieved.

Keywords: flow injection analysis; glycerol; enzymatic reaction; multicommutation

Introduction

In alcoholic fermentation of sugar-cane juice the glycerol (or glycerin) is a by-product, and its production is

dependent of parameters such as initial sugar concentration in the juice, kind of yeast employed, temperature, medium

*Corresponding author
E-mail: reis@cena.usp.br

acidity, etc. These effects caused by these parameters are more pronounced in the begin of the fermentation process and glycerol concentration is an indicative of the fermentation condition. Therefore, its determination is mandatory to assure the control of the alcohol production [1]. The A.O.A.C. method is based on glycerol oxidation with periodic acid to form formaldehyde prior to spectrophotometric detection [2]. Glycerol had been also determined employing enzymatic reaction [3]. These procedures are laborious and time consuming, thus, these difficulties have been minimized by using flow injection approach. In this sense, procedures for glycerol determination using enzymatic reaction and several detection techniques have been reported [4-13].

In the mill of alcohol which use sugar-cane as raw material, usually, analytical steps are performed in batch employing either oxidation with periodic acid or enzymatic reaction followed by spectrophotometric detection. To follow the fermentation process, a large amount of determination is required, thus, an automatic analytical procedure as those base on flow injection technique would be appreciated [14].

The multicommutation and binary sampling approach [15] is a branch of the flow injection process (14) which presents as main features abilities to handle two or more solutions employing a flow network with a single pumping channel [16].

In this work, we have developed a multicommutated flow procedure for determination of glycerol in samples of fermented sugar-cane juice using the reaction of glycerol with adenosine triphosphate (ATP) to form quinoneimine that was detected by spectrophotometry at 540 nm.

1. Experimental

1.1. Reagents solutions and samples

All solutions were prepared with analytical reagents. Distilled and deionized water were used throughout.

A 0.100 % (m/v) glycerol solution was prepared by weighing 0.100 g of glycerol (Merck) into a volumetric flask and completing the volume to 100 ml with water. Working solutions 0.200, 0.400, 0.600, 0.800, 1.000 % (w/v) were prepared by appropriate dilution with water. A 20 ml enzyme solution, 0.85 U ml⁻¹ Peroxidase plus 0.2 U ml⁻¹ Glycerokinase plus 0.13 U ml⁻¹ Glycerol

Phosphate Oxidase plus 0.43 U ml⁻¹ Lipase was prepared from an enzyme kit purchased from Merck. This solution was prepared by dissolving the enzymes in the buffer solution pH 7.5 (PIPES, Merck) that was maintained at 2 °C when not used.

Samples of fermented sugar-cane juice were centrifuged, the overflow liquid were collected in polyethylene bottles and maintained in refrigerator at 6 °C. Before use they were equilibrated to the laboratory temperature (25 °C).

Samples were analyzed by the reference method recommended by the International Commission for Uniform Methods of Sugar Analysis (ICUMSA)[3].

1.2. Apparatus

The flow set-up comprised an Ismatec mp4 peristaltic pump equipped with Tygon pumping tube; a 432 Femto spectrophotometer furnished with a flow cell 100 μL inner volume (optical path = 10 mm); three Nresearch 161T031 three-way solenoid valves; a microcomputer equipped with a PCL 711S Advantech Co and running a software written in QuickBasic 4.5; a home made electronic interface [17]; junction made of Perspex;

reaction coils and flow lines of polyethylene tubing (i.d. = 0.8 mm).

2.3. Procedure

The flow network was designed to implement the multicommutation and the binary sampling approaches [15], and its flow diagram are shown in Fig.1.

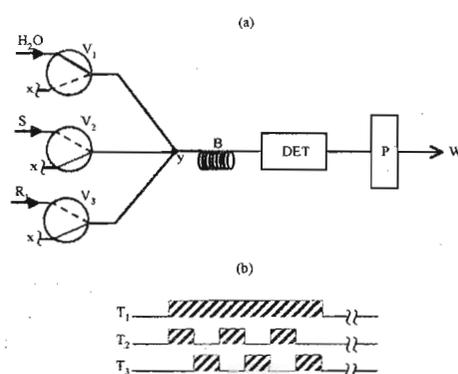


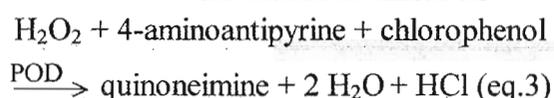
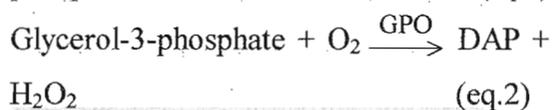
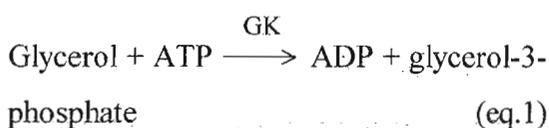
Fig.1a. Flow diagram of the system. V₁, V₂ and V₃ = three way solenoid valves; B = coils of PTFE, 0.8 mm i.d., 200 cm long; DET = spectrophotometer at 540 nm; P = peristaltic pump, flow rate at 30 μl s⁻¹; S = sample solution; R = enzymatic solution; x = valve inlet stopping; y = joint point; W = waste. Solid line inside of valve symbol indicates flow pathway when it is switched off, while dotted lines indicate flow pathway when it was switched on. Fig.1b, Valves timing course to switch on/off. Dashed line indicated valve on.

All valves are switched off and only the carrier solution is flowing through the valve V_1 and the reaction coil (B) towards to waste (W) by aspiration. Prior to begin the analytical run valves (V_1 and V_2) and (V_1 and V_3) were sequentially switched on during 5 s to fill the flow line connecting valves to the joint point y . After a delay time of 10 s to wash the reaction coil B with the carrier solution, the solenoid valves were switched on/off as indicated in the valves timing course (Fig.1b). As can be seen in this figure, valve V_1 was maintained on while valves V_2 and V_3 were alternately switched on/off three times. During this sampling step, the carrier solution stream was halted and three slugs of sample solution was inserted into the reaction coil B in tandem with three slugs of reagent solution. Afterwards, all valves were switched off and the carrier solution flowed again through the reaction coil. To carry out the analytical run this sampling pattern was repeated several times to load the reaction coil with a string comprising slugs of sample solution in tandem with slugs of reagent solution. When the loading step was completed, all valves were switched off, and carrier solution flowed again displacing the sample string

towards the detector (DET). The generated signal was read by the microcomputers as a time function, stored for further treatment and also displayed on the computer screen to allow its visualization at real time.

The flow rate was constant during loading step, thus the slugs volumes of the solutions were optimized by varying the time interval (Δt) to switch on valves V_2 and V_3 , which control the insertion of the sample and reagent solutions. To optimize the ration between slugs volumes of sample and reagent solutions, time interval to switch on valve V_3 was maintained at 0.2 s and time interval to switch on valve V_2 was varied from 0.1 to 0.8 s. To verify the effect of sample volume on the analytical signal, the number of sampling cycle (V_2 and V_3 on/off one times) was changed from 5 to 30 maintaining the time interval to switch on valves V_2 and V_3 at 0.2 s. Once the system variables have been established, a set of sample solution was processed in order to prove the feasibility of system. The laboratory was maintained at 25 °C, so that solutions could be handled without any degassing step, although inner pressure of the system was lower than that of atmosphere.

The method was based on the reaction of glycerol with adenosine triphosphate (ATP) catalyzed by the glycerol kinase (GK) to form adenosine-5-diphosphate (ADP) and glycerol-3-phosphate (eq.1) that was oxidized by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (eq.2). The peroxide reacted with 4-aminoantipyrine and chlorophenol to produce quinoneimine (eq.3) that was detected at 540 nm [3].



2. Results and discussion

The time interval to switch on valve to insert sample solution slug (V_2 , Fig.1) was varied from 0.1 up to 0.8 s (slug volume, 3-24 μl) maintaining the time interval to insert reagent solution at 0.2 s (slug volume, 6 μl).

The generated signals increased with the slug volume up to 15 μl , and for higher values signals decreased and its profile presented shoulders. These effects

could be interpreted as a poor distribution of the reagent solution into the sample bulk. Considering this results, the time interval to switch on valve V_2 must be less than 0.5 s.

The effect of the sample string volume was verified by varying the sampling cycle number and maintaining the ratio between slugs of sample and reagent solutions yielding results showed in Fig.2. The curve presents an asymptotic behavior as in the usual FIA system, tending to a constant value when sample volume was higher than 150 μl . This volume should be selected to achieve high sensitivity.

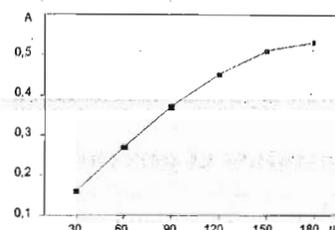


Fig. 2. Effect of the sample volume. A = absorbance. A 0.6 % (w/v) glycerol solution was used as reference. Operational conditions flow rate at 30 $\mu\text{l s}^{-1}$, slugs volumes of sample and reagent solution were maintained at 6.0 μl .

By settling as an operational condition, a sample string of 96 μl comprised of 8 sampling cycles (sample

solution slug, 6 μ l; enzyme solution slug, 6 μ l) a set of samples and standard solutions were processed yielding a linear response ranging from 0.200 to 1.000 % (w/v) glycerol. Afterwards, a set of samples (Fig. 3) comprising fermented sugar-cane juice was analyzed and results are shown in Table 1.

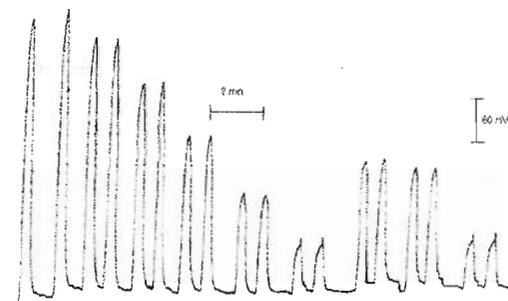


Fig. 3. Records of the results. From the left to right, reference solutions 1.0, 0.8, 0.6, 0.4, 0.2 and 0 % (w/v) glycerol followed of 3 samples, in duplicate.

Table 1. Results comparison. Glycerol concentration at percent (w/v)

| Samples | Proposed Method* | Batch Method* |
|---------|-------------------|-------------------|
| 1 | 0.251 \pm 0.005 | 0.262 \pm 0.008 |
| 2 | 0.273 \pm 0.004 | 0.258 \pm 0.006 |
| 3 | 0.281 \pm 0.006 | 0.291 \pm 0.005 |
| 4 | 0.492 \pm 0.009 | 0.478 \pm 0.013 |
| 5 | 0.513 \pm 0.008 | 0.511 \pm 0.008 |
| 6 | 0.463 \pm 0.007 | 0.451 \pm 0.012 |
| 7 | 0.487 \pm 0.008 | 0.495 \pm 0.011 |

*Results average of three consecutive measurements.

Accuracy was assessed by comparing results with those obtained using manual procedure as described elsewhere [3]. Applying the paired *t*-test no significant difference at the 95% confidence level was observed. Other profitable features such as, an analytical throughput of 60 determinations per hour; a relative standard deviation of 1.3 % (n = 9) for typical sample presenting 0.60 % (w/v) glycerol; low consumption of enzymatic solution, 48 μ l, were also obtained.

3. Conclusions

The system presented a good stability, working continuously during four hours no significant variation on baseline and signal magnitude were observed, being also very simple to run and robustness. Others advantages concerning to the consumption of enzymatic solution and effluent generation were estimated as 96 % and 97 %, respectively, lower than those of manual procedure [3].

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