SEQUENTIAL INJECTION ANALYSIS

USE OF YEAST CRUDE EXTRACT FOR SEQUENTIAL INJECTION DETERMINATION OF CARBOHYDRATES

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ABSTRACT

A sequential injection procedure for enzymatic-potentiometric determination of non-structural carbohydrates content (sucrose, fructose and glucose) in forrage plants was developed. In the proposed methodology, a yeast crude extract (Saccharomyces cerevisiae) solution was employed as source of invertase to promote the sucrose hydrolysis. Periodate reduction monitoring was used as quantitative parameter. The periodate remained from reduction reaction between IO₄⁻ ions and sugar reducing units was monitored using a periodate tubular selective electrode. Agreement results were obtained when the developed procedure was compared with the spectrophotometric method used for carbohydrate determination (anthrone). Detection limit of 0.13% (mv/C₀) sucrose, relative standard deviation <2.0% (n=10) and...

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analytical frequency of 24 samples h\(^{-1}\) could be observed when 280 \(\mu\)l of sample and yeast crude extract with activity around 5,000 I.U. were employed. The use of crude extract solution associated with sequential injection approach was easy to perform and presented reproductive results.

*Key Words:* Yeast crude extract; Invertase enzyme; Carbohydrates.

**INTRODUCTION**

Carbohydrates are the most abundant biomolecules in the earth and their oxidation is the main way of energy obtaining by non-photosynthetic cells. These compounds are usually classified as monosaccharides, simple sugars composed by a single poly-hydroxy-aldehyde or poly-hydroxy-ketone unit; oligosaccharides, composed by short chains of monosaccharide units linked by glucosidic bonds; and polysaccharides, composed by long chains of monosaccharide units (1). The carbohydrates, besides acting as an energetic source, have a fundamental role in storage and preservation of foods based on fermentative processes (2). In anaerobic conditions, suitable microorganisms transform the carbohydrates available to fermentation process (non-structural carbohydrates) into organic acids that are responsible for preservation of stocked material. The silage production is based on this principle and different materials can be preserved using this food preservation process. However some characteristics of stocked material have been taking into account for obtaining a storage process with good quality. The non-structural carbohydrate content is one of these characteristics. Sucrose, glucose and fructose are the major components of this carbohydrate fraction (2).

Normally the sucrose, glucose and fructose determination is based on the reducing propriety of their monomer unit. Non-specific reduction reactions have been used to develop flow methods with different detection systems (3–7). In these methods, the determination is indirectly performed, involving acid or enzymatic hydrolysis for sucrose quantification. Periodate oxidant action on some organic compounds is well known. Flow injection systems exploring this property were proposed for spectrophotometric and potentiometric determination of sucrose and reducing sugar (5,7). Other reduction reaction, employing hexaferrocyanate (II) alkaline solution or Fehling reagent, have also been used for carbohydrate determination (3,4,6).
In the present work, the use of a yeast crude extract (Saccharomyces cerevisiae) to promote sucrose hydrolysis and total non-structural carbohydrates (glucose, fructose and sucrose) determination was studied. A sequential injection manifold was developed to automate the determination and to perform in line the sucrose enzymatic-hydrolysis. A tubular periodate selective electrode (8) was used as detector for potentiometric monitoring of periodate remained from the reaction between the reducing sugar monomer units and IO$_4^-$ ions. The main experimental parameters involved in sequential injection determination were systematically evaluated. The proposed procedure was applied to carbohydrate determination in sugar cane (Saccharum officinarum L.) and maize (Zea mays) samples.

**EXPERIMENTAL**

**Instrumentation**

Yeast Crude Extract Preparation

A centrifuge (Beckman J2-21, USA) and a spectrophotometer (Femto 432, São Paulo, Brazil) were used to prepare the yeast crude extract solution and to assess the invertase activity, respectively. The spectrophotometer was also used in the carbohydrate spectrophotometric determination (anthrone) employed to check the accuracy of the proposed SI approach.

Sequential Injection System

The sequential injection system presented in Figure 1 was built using a single channel peristaltic pump (Gilson Miniplus 3, Vilier-le-Bell, France) and a 6-way electrically actuated solenoid valve (NResearch 225T091, Houston, USA). The direction and rotation speed of peristaltic pump and 6-way selector valve were controlled by a computer equipped with an interface card Advantech PCL 711-S (Advantech Co, Taiwan) and a home-made program developed in Quick Basic software package (Microsoft, USA). The manifold was built with 0.8 mm i.d. polyethylene tubing and connected to a peristaltic pump through a 0.5 mm i.d. Tygon pumping tube. Controlled-temperature bath (Fanem, São Paulo, Brazil) was used to ensure constant temperature for enzymatic reaction. A stripchart recorder (Kipp & Zonen BD111, Delft Holland) was used for data acquisition. The periodate tubular selective electrode was prepared as previously described by Montenegro et al. (8) and coupled to a potentiometer (Mettler Toledo 355, USA).
All solutions were prepared using analytical grade reagents and distilled and deionized water as solvent (Milli-Q® 18 MΩ cm).

In the flow diagram represented in the Figure 1, 0.50 mol L⁻¹ Na₂SO₄ solution with 1.0×10⁻² mol L⁻¹ NaIO₄ was used in the carrier stream. For promoting sucrose enzymatic-hydrolysis, yeast crude extract solution, prepared as described follow, was used.

A 0.15 mol L⁻¹ of NaHCO₃ solution was used for the yeast crude extract preparation. For invertase activity assessment, enzymatic kit (Boehringer, USA) containing 4-aminoantipyrine, p-hydroxybenzene sulphate, glucose oxidase (GOD) and peroxidase (POD), was employed.

The anthrone reagent solution was prepared as described by Silva (9). A volume of 760 ml concentrated H₂SO₄ was carefully added to 230 ml H₂O. After that, 1.00 g of tiourea and 1.00 g of anthrone were dissolved in the resulting acid solution and the volume filled up to 1,000 ml with H₂O.

The used analytical curve, 0.20, 0.40, 0.60 and 0.80% (m v⁻¹) sucrose, was prepared after suitable dilution of a 10% (m v⁻¹) sucrose stock solution with water.

Figure 1. Flow diagram proposed for enzymatic-potentiometric determination of nonstructural carbohydrates. S, sample (280 μl); EE, yeast crude extract (280 μl); C, carrier solution (NaIO₄ 1.0 × 10⁻² mol L⁻¹ + Na₂SO₄ 0.50 mol L⁻¹, 3.4 ml min⁻¹); HC₁ and HC₂, holding coils (3.0 ml and 250 cm, respectively); SV, selector valve; ISE, tubular periodate selective electrode; RE, reference electrode and W, waste.
Preparation of Yeast Crude Extract Solution
(Saccharomyces cerevisae)

For the enzymatic crude extract solution preparation, 60.0 g of a commercial biological yeast (Saccharomyces cerevisae) was homogenized in 250 ml of 0.15 mol·L⁻¹ NaHCO₃ solution. The resulting mixture was placed in a bath with controlled temperature at 45°C for 24 h and 30-min centrifuged (10,000 rpm and 4.0°C) after a 24-h period. The supernatant solution was used as enzymatic source of invertase.

Yeast Crude Extract Solution Activity Assessment

The glucose oxidase/peroxidase (GOD/POD) enzymes were used to determine the activity of prepared yeast crude extract solutions. An aliquot of 1.0 ml of the yeast crude extract solution was transferred and placed in a controlled-temperature bath (25°C). After the temperature stabilization, 1.0 ml of 10% (m/v) sucrose in 0.20 mol·L⁻¹ phosphate buffer solution (pH 4.6) was added. A reaction time of 5 min was necessary before the addition of 1.0 ml of 1.0 mol·L⁻¹ HCl solution to stop the enzymatic reaction. The pH of the solution was adjusted to 4.6 and the volume filled up to 100 ml with water. At this pH value, the concentrations of glucose and fructose are equivalent. Therefore, the invertase activity could be assessed from the amount of glucose produced by the enzymatic sucrose inversion. An aliquot of 1.0 ml of this solution was taken to develop the enzymatic reaction by the GOD/POD enzymes.

The glucose produced in the sucrose hydrolysis was indirectly determined through oxidative action of the GOD enzyme that produced H₂O₂, which is responsible for the condensation between 4-aminoantipyrine and p-hydroxybenzene sulphate in POD presence. The reaction product had a reddish color and its absorbance was determined at 505 nm. The yeast crude extract activity was expressed in international units (I.U.), where one I.U. is defined as the amount of enzyme that hydrolyze 1.0 µmol of sucrose to fructose and glucose, in 1 min at pH 4.6 and 25°C.

Sample Preparation

Amounts of 10.0 g of sugar cane (Saccharum officinarum L.) and maize (Zea mays), previously dried at 60°C for 72 h and milled with 20 mesh sieve, were homogenized in 100 ml of water for 60 min. After that, the
mixtures were filtered and the resulting extracts were used for the proposed determinations.

**Carbohydrate Spectrophotometric Determination – Anthrone**

The anthrone reagent was used as chromogenic reagent for total soluble carbohydrate determination, glucose, fructose and sucrose. An aliquot of 2.0 ml of the extract obtained in the sample preparation section was taken and placed in a test tube. After that, 10 ml of the anthrone reagent solution was added to the sample aliquot and the test tube transferred to a bath with temperature controlled at 90°C. The reaction time of 10 min was require. The hydroxymethyl-furfural formed from non-structural carbohydrates in the presence of concentrated H₂SO₄ reacts with the anthrone producing a greenish complex. The measurements were performed in triplicate at 625 nm (9).

**SI System**

In the flow-setup represented by Figure 1, a peristaltic pump (P) and a selector valve (SV), were used to insert sequentially in a holding coil (HC₁) sample (S) and yeast crude extract solution aliquots (EE). By using a reversal flow, the sample zone formed in HC₁ was then addressed to HC₂ that was immersed in a 35°C controlled-temperature bath. The carrier flow stream was then 30 s stopped and subsequently reestablished for pumping the sample zone through the tubular detector. The sample zone passage through the tubular periodate selective and reference electrodes produced a transient signal proportional to periodate consume in reduction reaction between the IO₄⁻ ions and sugar reducing units. The sequence of selector valve and peristaltic pump acting is described in Table 1.

**RESULTS AND DISCUSSION**

With the use of yeast crude extract, only the enzymatic solution was required to accomplish the sucrose hydrolysis, avoiding the use of a second reagent in the flow-setup that could be act inefficiently for the present proposal due to low dispersion inherent to SI systems. The yeast crude extract preparation in 0.15 mol1⁻¹ NaHCO₃ allowed the potentiometric detector use without accuracy damage. The neutral conditions established for the
sample zone when it was passing through the tubular periodate electrode avoided the electrode response to H\(^+\) and OH\(^-\) ions.

The prepared yeast crude extracts had invertase enzyme in solution, that was responsible for the sucrose hydrolysis by its \(\beta\)-fructofuranosidase action. The determination of activity of these extracts presented average value of 5,000 I.U. These activities were different among extractions in view of time of production and yeast storage conditions that affect the enzyme amount present in the yeast. Otherwise, during non-structural carbohydrates determination the activity is kept constant. Using the same crude extract for a 7-day period, no changes in the sensitivity were observed.

In order to establish the experimental conditions for proposed determination, the sample and crude extract volumes and the reaction time were evaluated. The time of stopped-flow, concerning the reaction time, was set taking into account the hydrolysis efficiency of a 0.20% (m v\(^-1\)) sucrose solution, and was selected as a compromise between sensitivity and analytical frequency. These experiments were performed with the use of a yeast crude extract with 4,800 I.U. activity. The results are present in the Figure 2.

The sample and yeast crude extract zones could not be extremely long in view of low dispersion inherent to SI systems which decrease the overlap between sample and reagent aliquots, reducing the hydrolysis reaction efficiency. The time chosen for stopped-flow step had to produce maximum efficiency for sucrose hydrolysis, even decreasing the analytical frequency.

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### Table 1. Sequence of Selector Valve and Peristaltic Pump Acting

<table>
<thead>
<tr>
<th>Analytical Sequence</th>
<th>Port</th>
<th>Time (s)</th>
<th>Flow-Rate (ml min(^{-1}))</th>
<th>Pump Direction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaning step</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample aspiration (280 µl)</td>
<td>2</td>
<td>10</td>
<td>1.7</td>
<td>a</td>
</tr>
<tr>
<td>Sample discharge</td>
<td>3</td>
<td>10</td>
<td>3.4</td>
<td>p</td>
</tr>
<tr>
<td>Sampling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample aspiration (280 µl)</td>
<td>2</td>
<td>10</td>
<td>1.7</td>
<td>a</td>
</tr>
<tr>
<td>Yeast crude extract aspiration (280 µl)</td>
<td>1</td>
<td>10</td>
<td>1.7</td>
<td>a</td>
</tr>
<tr>
<td>Enzymatic reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample zone pumping to HC(_2)</td>
<td>4</td>
<td>20</td>
<td>2.5</td>
<td>p</td>
</tr>
<tr>
<td>Stopped-flow</td>
<td>30</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reading step</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample zone pumping through detector</td>
<td>4</td>
<td>60</td>
<td>3.4</td>
<td>p</td>
</tr>
</tbody>
</table>

* a, aspiration; p, pumping.
Hence, the sample and yeast crude extract volumes and stopped-flow time were, respectively, 280 \( \mu l \) and 30 s. With these conditions, the hydrolysis percentage was evaluated using individual solutions of 0.20% (m v\(^{-1}\)) sucrose, fructose and sucrose prepared in a pH 4.6 buffer solution. The analytical signals obtained for glucose and fructose solutions without the enzymatic extract were summed and compared with the in line hydrolyzed sucrose signal. Inversion efficiency of 70% was founded for enzymatic reaction.

Figure 2. Evaluation of the experimental conditions. (a) Sample and yeast crude extract solutions volume. (b) Stopped-flow time.
Figure 3. Potentiometric recorder for non-structural carbohydrate determination in maize and sugar cane samples. From the left, analytical curve (blank, 0.20, 0.40, 0.60 and 0.80% m v⁻¹ of sucrose) in triplicate, two samples in quadruplicate, one sample measured 5 times and the analytical curve (0.80 – blank % m v⁻¹ of sucrose) in triplicate.

The signal corresponding to analytical curve blank in routine diagram (Figure 3) was due to dilution of periodate present in the carrier flow-stream by the analytical blank and yeast crude extract aliquots. When the sample zone passed through detector, the periodate concentration was smaller in the center than in the extremities of the sample zone. The periodate electrode detected the absence of IO₄⁻ ion in the center of the sample zone, producing a positive difference of potential.

After establishing the sequential injection parameters, the procedure was applied to samples of sugar cane and maize that would be used for silage production. The results were compared with spectrophotometric procedure (9) and agreement results were obtained by both methods, as can be observed in Table 2. The non-structural carbohydrates amounts were expressed as sucrose percentage in view of the impossibility of distinguishing
the sucrose from the reducing sugar content. The proposed procedure is not selective relating to these species.

A baseline noise, owing to different forms of IO$_4^-$ ions migration in the selective membrane surface during the stopped-flow and flow-stream re-establishing steps, can be observed in the potentiometric recording presented in Figure 3. This noise was constant and reproductive during the analytical cycles represented in Table 1, and no interfering in the analytical results. High-speed recorder for one cycle of sampling is presented in Figure 4.

The detection limit, determined as suggested by IUPAC (10), was 0.13% (m v$^{-1}$) sucrose. An analytical frequency of 24 samples h$^{-1}$ and a

Table 2. Non-structural Carbohydrate Contents in Sugar Cane and Maize Samples Determined by the Proposed Sequential Injection Approach (SI) and by the Used Spectrophotometric Method (Anthrone). Values Expressed Based on Dry Material

<table>
<thead>
<tr>
<th>Sample</th>
<th>SIA (sucrose g kg$^{-1}$)</th>
<th>Anthrone (sucrose g kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar cane 1</td>
<td>369 (± 40)*</td>
<td>385 (± 23)</td>
</tr>
<tr>
<td>Sugar cane 2</td>
<td>321 (± 19)</td>
<td>312 (± 28)</td>
</tr>
<tr>
<td>Maize 1</td>
<td>64.5 (± 1.0)</td>
<td>67.0 (± 1.0)</td>
</tr>
<tr>
<td>Maize 2</td>
<td>44.0 (± 0.3)</td>
<td>40.5 (± 0.2)</td>
</tr>
</tbody>
</table>

* rsd based on a triplicate sample.
relative standard deviation < 2.0 % (n = 10) were also observed with the use of the proposed procedure.

CONCLUSIONS

The use of yeast crude extract solution to promote the sucrose hydrolysis and following non-structural carbohydrates determination presented favorable characteristics such as the use of only one low cost reagent, making easier the analysis. The obtaining results confirm the potentiality of the use of biological materials for analytical purposes.

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