Introduction

Carbohydrates in plants can be classified into two main categories, recognized as structural and nonstructural compounds. Structural carbohydrates are found mainly in the cell wall, cellulose and hemicellulose, starch and pectic substances. The nonstructural carbohydrates comprise sucrose, glucose and fructose, which are sources of substrates, which can undergo fermentation process caused by anaerobic bacteria, thus permitting their use to produce ensilage material. Under anaerobic conditions, naturally existing bacteria in the raw material can convert these nonstructural carbohydrates to organic acids (lactic, acetic, propionic, butyric, etc.), which can be used as feed for ruminants. Considering that the useful life of these organic acids is higher than that of reducing sugar and nonstructural carbohydrate, ensilage is a common practice in agriculture related to ruminant nutrition. The raw material quality can affect the amount and nature of the ensilage material, therefore, the determination of reducing sugar and nonstructural carbohydrate before the ensiling step should be recommended in order to save costs. In this sense, the availability of a reliable analytical procedure should be appreciated.

Glucose and fructose comprise the nonstructural carbohydrate family. Its reducing feature has been exploited to develop spectrophotometric procedure for its determination. On another hand, sucrose can be hydrolyzed to glucose and fructose using chemical or enzymatic reactions, thus allowing its determination by spectrophotometric procedure similar to that employed for the determination of reducing sugars. Spectrophotometry is a widespread detection technique employed in analytical procedures for the determination of nonstructural carbohydrates in forage material. Among them, those based on a flow-analysis process are useful for high throughput and low reagent consumption. These features are appreciated when many samples must be analyzed.

Analytical procedures based on flow analysis for reducing sugar determination in several matrices have been proposed for detection techniques, such as spectrophotometry, fluorometry, amperometry, gravimetry and chemiluminescence. Nevertheless, there is a scarcity of procedures concerning to the determination of nonstructural carbohydrate in forage material. Sucrose determination by spectrophotometry usually requires a hydrolyzing step to convert it to glucose and fructose, which has been carried out using either an enzymatic reaction or a warmed acid medium. Among the spectrophotometric procedures, those based on an oxidizing reaction using copper(II), or hexacyanoferrate(III) or periodate as an oxidizing reagent have been widely used. Nevertheless, when an acid hydrolysis step was employed, an alka lization step was required, thus increasing the complexity of the system manifold. The manifolds of the cited studies comprised several flow lines, thus requiring an equal number of pumping channels, which is a feature due to earlier flow-injection process. On another hand, a flow system based on a multicommutation approach can handle several solutions using a single pumping channel. A decrease in reagent consumption is another advantage that is easily achieved by employing a multicommutation approach.

In this work, we intend to develop an automatic flow procedure for the simultaneous spectrophotometric determination of nonstructural carbohydrates and reducing sugar in forage material by employing a flow system based on a multicommutation approach. A method based on the reaction of copper(II) with reducing sugars followed by the reaction of resulting copper(I) with neocuproine was selected for both analytes. In this sense, the nonstructural carbohydrates must undergo acidic hydrolysis prior to a reaction with copper(II).
this sense, the flow system will be designed with active hardware controlled by a microcomputer. To do this, software will be developed to control the flow system, in order to carry out the simultaneous determination of nonstructural carbohydrate and reduction sugar and to perform data acquisition.

Experimental

Reagents and solutions

All of the chemicals used were of analytical grade. Distilled and deionized water (conductivity ca. 0.1 μS cm⁻¹) was used throughout.

The chromogenic reagent solution was 0.06% (w/v) 2,9-dimethyl-1,10-phenanthroline (neocuproine) plus a 0.03% (w/v) copper(II) sulfate pentahydrate solution. It was prepared by dissolving 15.0 mg of neocuproine in 1 ml of ethanol. Afterwards, 7.5 mg of CuSO₄·5H₂O was added to them, and after dissolution the volume was made up to 25 ml with water. A 5.0% sucrose plus 5.0% fructose plus 5.0% glucose (w/v) stock standard solution was prepared by dissolving 5.000 g of each solid in 100 ml of water. Standard solutions (0.0, 0.2, 0.4, 0.6 and 0.8% (w/v)) were prepared by appropriate dilutions of the stock solution with water. These solutions were prepared every day.

An amount of forage material (ca. 50 g dried) was maintained at 60°C for 72 h and them grounded. A mass of 10.0 g was added to a beaker with 100 ml of water. This suspension was stirred for 60 min, and afterwards it was filtered and stored in an amber bottle. By maintaining this extract at 4°C, it could be used during one week. Before use, it was equilibrated to the laboratory temperature (25°C).

Apparatus

The equipment set-up consisted an IPC-4 Ismatec peristaltic pump furnished with Tygon pumping tubes; a 432 Femto spectrophotometric equipped with a 100 μL inner flow cell, 10 mm optical path; five 161T031 NRResearch three-way solenoid valves; a Pentium II microcomputer equipped with a PCL-711S Advantech interface card running a software written in QuickBASIC 4.5; mixing coils of PTFE tubing, 1.6 mm i.d.; joint devices machined in acrylic; a glass de-bubbling chamber, 150 mm optical path; five 161T031 NRResearch three-way solenoid valves; a Pentium II microcomputer equipped with a PCL-711S Advantech interface card running a software written in QuickBASIC 4.5; mixing coils of PTFE tubing, 1.6 mm i.d.; joint devices machined in acrylic; a glass de-bubbling chamber, 150 mm optical path; a temperature controlled water bath.

Procedure

The flow system, which was designed to implement the multicommutation approach, is depicted in Fig. 1. In this configuration, all valves are switched off and the carrier fluid (Cₛ) flows through the coils (B₁, B₂) towards the spectrophotometer (Det). When an analytical run is started, the microcomputer running the software sends a set of electric pulses to switch on/off the solenoid valves, following the sequence depicted in the valves-timing course, assigned as tᵣ.

To improve the precision of the inserted solutions aliquots, the sampling step is synchronized with pumping pulsation. In this sense, prior to beginning an analytical run, the microcomputer waits for an electric pulse coming from the peristaltic pump. To permit this synchronization, the roller counting output of the peristaltic pump was coupled to the analog input (Aᵢ) of the PCL-711S interface card.

As shown in the valves timing course, in the first step of the analytical run (tᵣ) valves V₁ and V₂ are switched on/off alternately 3 times, while valve V₃ is maintained switched on. Under this condition, the stream of the carrier fluid (Cₛ) is halted, and 3 slugs of sample solution are inserted into the hydrolyzing coil (B₁) in tandem with 3 slugs of hydrochloric acid solution (R₁). The volumes of the solution slugs are vᵢ = t₁φ, vᵢ = t₁φ (φ = flow rate, t₁ and t₂ = time intervals to switch on valves V₁ and V₂, respectively). Afterwards, all valves are switched off for time tₖ, and the carrier fluid (Cₛ) flows again to displace the sample zone through coil B₁, where the hydrolyzing reaction to form reducing sugar occurs. Afterwards, valves V₄, V₂, and V₁ are switched on/off following the pattern assigned as tᵣ (Fig. 1) to insert the reagent solutions R₂ and R₃ to the sample zone. After the tᵣ event, all valves are switched off and the carrier solution (Cₛ) flows again to displace the sample zone through coil B₁ where the hydrolyzing reaction to form reducing sugar occurs. Afterwards, valves V₄, V₂, and V₁ are switched on/off following the pattern assigned as tᵣ (Fig. 1) to insert the reagent solutions R₂ and R₃ to the sample zone. After the tᵣ event, all valves are switched off and the carrier solution (Cₛ) flows again to displace the sample zone through coil B₁, where the hydrolyzing reaction is carried out. In this way, the analytes are separated and the carrier fluid is replaced by de-bubbling device, draining flow rate (W₁) at 0.4 ml min⁻¹. Solid and dashed lines in the valves symbols indicate the flow pathways when valves are switched on or off, respectively; x = stopper; y = flow line joint device; T₁ = temperature water bath at 60°C; arrow indicates flow direction. T₁, T₂, ..., T₅ = valves timing course; shadow surfaces indicate that the corresponding valve was switched on; tᵣ, sample inserting stage; tₖ, sample zone displacing time settled to 10 s; tᵣ, reagents inserting stage; and t₁, t₂, t₃ and t₄, time intervals for switching on valves V₁, V₂, V₃ (only in tᵣ stage), and V₄, respectively, each one was settled at 0.5 s and tₖ that was maintained at 1.0 s.

Fig. 1 Flow diagram of the system. V₁, V₂, ..., V₅, three-way solenoid valves; B₁, hydrolyzing coil (25 cm long, 1.6 mm i.d.); B₂, reaction coil (60 cm long, 0.8 mm i.d.); Cₛ, carrier fluid, water, flow rate at 2.0 ml min⁻¹; S, sample solution; R₁, hydrochloric acid solution; R₂, sodium hydroxide solution; R₃, chromogenic reagent solution; P, peristaltic; Det, spectrophotometer (460 nm); W₁ and W₂, waste; D, de-bubbling device, draining flow rate (W₁) at 0.4 ml min⁻¹.

Under this condition, the stream of the carrier fluid (Cₛ) is halted, and 3 slugs of sample solution are inserted into the hydrolyzing coil (B₁) in tandem with 3 slugs of hydrochloric acid solution (R₁). The volumes of the solution slugs are vᵢ = t₁φ, vᵢ = t₁φ (φ = flow rate, t₁ and t₂ = time intervals to switch on valves V₁ and V₂, respectively). Afterwards, all valves are switched off for time tₖ, and the carrier fluid (Cₛ) flows again to displace the sample zone through coil B₁, where the hydrolyzing reaction is carried out. In this way, the analytes are separated and the carrier fluid is replaced by de-bubbling device, draining flow rate (W₁) at 0.4 ml min⁻¹. Solid and dashed lines in the valves symbols indicate the flow pathways when valves are switched on or off, respectively; x = stopper; y = flow line joint device; T₁ = temperature water bath at 60°C; arrow indicates flow direction. T₁, T₂, ..., T₅ = valves timing course; shadow surfaces indicate that the corresponding valve was switched on; tᵣ, sample inserting stage; tₖ, sample zone displacing time settled to 10 s; tᵣ, reagents inserting stage; and t₁, t₂, t₃ and t₄, time intervals for switching on valves V₁, V₂, V₃ (only in tᵣ stage), and V₄, respectively, each one was settled at 0.5 s and tₖ that was maintained at 1.0 s.

Analytical Sciences December 2003, Vol. 19
on/off alternately to insert into coil B₁ slugs of the sample solution in tandem with slugs of the carrier solution to cause a previous dilution in order to match the analyte concentration within the analytical range of the procedure. The steps to inserting the reagents solutions, R₂ and R₁ are carried out following the same sequence described in last paragraph.

In the valve timing courses assigned as tᵢ and tᵣ in Fig. 1, valves Vᵢ₁, Vᵢ₂, Vᵢ₄ and Vᵢ₅ are switched on/off 3 times. Therefore, the number of switching can be increased to insert the solution volumes required for the analytical procedure. The pumping flow rate is maintained at 2.0 ml min⁻¹ throughout.

Assays to verify the temperature effect were carried out by varying the water bath temperature from 50 to 70°C. It was performed using an 0.8% (w/v) nonstructural carbohydrates reference solution, a 0.75 mol l⁻¹ hydrochloric acid solution (R₁) and a 1.5 mol l⁻¹ sodium hydroxide. The on/off switching of the valves was repeated 9 times, and the time intervals assigned (as tᵢ, tᵣ, tᵢₛ and tᵣₛ) were each set at 0.5 s. Unless otherwise mentioned, the parameters settled in this paragraph were maintained to perform the further experiments settling the water bath temperature at 60°C.

The acid concentration effect on nonstructural carbohydrates hydrolysis to convert it to reducing sugar was studied using 0.5 and 0.75 mol l⁻¹ hydrochloric solutions. The alkalinity effect was studied varying from 0.75 to 1.5 mol l⁻¹ the concentration of sodium hydroxide solution and maintaining the concentration of the hydrochloric acid solution at 0.75 mol l⁻¹.

The hydrolysis of the nonstructural carbohydrates occurred in reaction coil B₁, and its dimensions were maintained at 25 cm length and 1.6 mm i.d. while the length of coil B₂ was changed from 40 to 80 cm maintaining the inner diameter at 0.8 mm. Using as R₂ a 1.0 mol l⁻¹ sodium hydroxide solution, additional experiments were carried out by varying sample volume from 100 to 250 µL and chromogenic reagent solution (Rᵢ) volume from 100 to 350 µL. These values were achieved by varying the time intervals to switch on valves Vᵢ₂ and Vᵢ₃ from 0.3 to 0.8 s and 0.3 to 1.2 s, respectively, and repeating the on/off cycle 9 times.

All steps of the analytical procedure were accomplished by microcomputer using the control parameters summarized in the caption of Fig. 1, which were saved on the hard disk, which were recovered when the software was run.

To demonstrate the usefulness of the proposed system, extracts of forage materials were analyzed. Samples were also analyzed employing reference methods¹¹ to permit results comparison.

### Results and Discussion

#### Effects of the temperature and acidity

The determination of nonstructural carbohydrate comprised two sequential steps: the hydrolysis to produce the reducing sugar, followed by reaction to form the compound to be detected. Considering that the temperature affected the reactions involved in both steps, it was the first parameter studied. The water bath temperature was varied from 40 to 70°C, and a linear relationship (signal = -0.088 + 0.01516T; r = 0.9979) was observed using a reference solution containing 0.8% (w/v) nonstructural carbohydrate. The reaction coils B₁ and B₂ (see Fig. 1) were immersed into water bath. Therefore, the increase in the signal could be attributed to the overall effect caused in both steps.

The nonstructural carbohydrate concentration in sludge samples comprised range up to 30% (w/w). Therefore, the sensibility of the analytical procedure was not a critical parameter. In this sense, to avoid excessive bubble gas delivering into sample bulk, which could impair the precision of the results, the temperature of the water bath was maintained at 60°C to carry out additional experiments.

The results commented above were obtained using a 1.0 mol l⁻¹ hydrochloric acid solution to acidify the sample zone to permit hydrolysis of the nonstructural carbohydrate. Similar results were obtained using a 0.75 mol l⁻¹ hydrochloric acid solution. This acid concentration was selected because using lower values caused decrease in signal.

Although the nonstructural carbohydrate hydrolyzing reaction required an acid medium, the reaction between reducing sugar and Cu(II) occurred in an alkaline medium. To find better condition, experiments varying the hydroxide concentration from 0.75 to 1.50 mol l⁻¹ were carried out. Because no significant augment in signal was observed using hydroxide concentration higher than 1.0 mol l⁻¹, this value was selected.

### Sample analysis

To demonstrate the feasibility of the proposed system, samples comprising different forage materials were processed, yielding the results shown in Table 2. The reducing chemicals species, such as citric acid, carminic acid, sulfate, thiourea and ascobic acid, could cause interference.²⁰ Nevertheless, according to McDonald et al.²¹ in forage material these interferences were not observed; thus, no assays to verify its effect were carried out. Comparing the results with those obtained employing independent procedures,²¹ no significant difference at the 90% confidence level was observed.

Other favorable features such as: relative standard deviation of < 2.0% (n = 10) for a typical sample presenting 0.6% (w/v) of both analytes; a linear response ranging from 0.2 to 0.8% (w/v) (r = 0.999); a throughput of 32 determinations per hour and a detection limit of 0.12 and 0.15% (w/v) for reducing sugars and nonstructural carbohydrate, respectively, estimated as suggest by IUPAC²²; reagent consumption of 0.9 mg copper(II) sulfate and 0.45 mg neocuproine per sample analysis, were also achieved.

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### Table 1 Comparison of results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reduction sugar (mg g⁻¹ in MS)</th>
<th>Nonstructural carbohydrates (mg g⁻¹ in MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reference procedure</td>
<td>Proposed procedure</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>8</td>
<td>55 ± 2</td>
<td>55 ± 1</td>
</tr>
</tbody>
</table>

Results average of three consecutive measurements.

Reduction sugar, t_{exp} = 0.043, t_{theoretical} = 1.895 for 90% confidence level for seven degrees of freedom.

Nonstructural carbohydrates, t_{exp} = 0.054, t_{theoretical} = 1.895 for 90% confidence level for seven degrees of freedom.
Conclusions

In the proposed flow system, five solutions were handled using a single pumping channel, which is a feature due to manifold based on multicommutation. It was easily accomplished using the control software.

The long-term stability of the system was ascertained by running a 0.6% (w/v) nonstructural carbohydrate during 4 h. No drift of the base line and no significant variation of signal were observed. Repeating this assay on different days similar behavior was observed, thus confirming the very good robustness of the system.

The system is very easy to operate. When the software was run, the control parameters summarized in the caption of the Fig. 1 were recovered from the hard disk and the analytical procedure was carried out without any operator assistance.

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