

Uncertainty in the Determination of Glucose and Sucrose in Solutions with Chitosan by Enzymatic Methods

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O objetivo deste trabalho foi determinar a aplicabilidade de dois métodos enzimáticos um para a quantificação da glucose e outro da sacarose em soluções aquosas contendo quitosano. Os métodos analíticos foram validados e os principais parâmetros determinados como limite de detecção, intervalo de linearidade, precisão e exatidão. A incerteza global do método enzimático da glucose foi inferior a 10% no intervalo de 700 a 2000 mg L⁻¹, para o método de análise da sacarose a incerteza global foi inferior a 3% para todo o intervalo de análise (100-1000 mg L⁻¹). Apesar da semelhança das performances com relação a outros métodos analíticos, o método enzimático provou ser mais adequado, em particular do que a cromatografia líquida de alta eficiência (HPLC), principalmente se um grande número de amostras deve ser analisado (permitindo um resultado rápido) e ainda devido às propriedades mucoadesivas do quitosano que dificultam as metodologias analíticas por HPLC, criando problemas de estabilidade na coluna cromatográfica.

The purpose of this work was to evaluate the applicability of two enzymatic methods to quantify glucose and sucrose in aqueous solutions with chitosan. The analytical methods were validated and the main parameters, as limit of detection, linearity range, precision and accuracy were determined. The global uncertainty for glucose enzymatic method was less than 10% for concentration levels between 700 and 2000 mg L⁻¹, and for the sucrose enzymatic method, the global uncertainty showed values less than 3% for all concentration levels analyzed (100-1000 mg L⁻¹). Despite the similarities of performances with respect to other analytical methods, the enzymatic method proved to be better than others, in particular high performance liquid-chromatography (HPLC), mainly if a great number of samples needs to be analyzed, allowing a quick result, and because the mucoadhesive properties of chitosan make difficult the HPLC analytical methodology, creating stability problems in the chromatographic column.

Keywords: uncertainty, enzymatic methods, glucose, sucrose, chitosan

Introduction

Chitosan is a polycationic polymer obtained commercially by alkaline deacetylation of chitin.^{1,2} The chitosan molecule is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine and differs in the degree of *N*-acetylation (40-98%) and molecular weight (50-2000 kDa).¹⁻³ Chitin is the second most abundant natural polymer in nature after cellulose and is found in the structure of a wide number of invertebrates (crustaceans, exoskeleton insects, cuticles) among others. Chitosan can be also considered as a biodegradable and a non-toxic product and is currently receiving a great deal of

interest for medical, pharmaceutical, industrial and food applications.¹⁻³

The main reasons for this increasing attention to this natural product are certainly its interesting intrinsic properties associated to the reactive amino functional groups that give the potential to be used in many different fields.^{1,4-6}

Other properties with special interest for food industry are related to the anticholesterolemic,^{7,8} antioxidant⁹ and antimicrobial properties⁹ of chitosan.

Chitosan is an attractive biomacromolecule, presenting, however, a disadvantage: it is a water-insoluble material. Chitosan is soluble only in acidic solutions because of its rigid crystalline structure and deacetylation, which limits its application. However it is possible to modify the chitosan

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structure to form water soluble chitosan which is easily soluble in neutral aqueous solutions.⁴⁻⁸

Chitosan has been widely used in pharmaceutical and food industry, for example as a carrier for drug delivery. The use of chitosan microparticles and chitosan films as controlled drug delivery systems for conventional drugs, protein drugs and bioactive compounds has attracted increasing attention since the beginning of 1990's.^{2,10-15} Chitosan can also be used as a support for enzyme immobilization.¹⁶ On the other hand, carbohydrates are known to play a variety of roles in the welfare of mankind. The analyses of carbohydrates are the most widespread chemical analyses that are performed within the industries of food, beverage, forage, biomass, pulp and paper, pharmaceuticals, among others.¹⁷ These industries intend the development of analytical methods based on low cost, easiness of operation and portable instrumentation.¹⁸⁻²⁰ An example of the importance of the control of carbohydrates is the sugar refining process (crystallization, centrifugation or carbonation).²¹

Several methods and protocols have been revised and new generations of instruments have emerged to give response to the increased importance of carbohydrate analysis in the last years. Although there is a large number of analytical methods for carbohydrates, few studies include validation parameters and the uncertainty determination. Some methods with validation parameters for carbohydrates analysis, such as biosensor-based, refractometry, Fourier transform infrared spectroscopy (FTIR), enzymatic with spectrophotometry analysis and high-performance liquid chromatography (HPLC) have been discussed by Estevinho *et al.*²²

In the literature, the most of analytical methods for carbohydrates analysis are enzymatic¹⁸ and chromatographic methods,^{17,23-25} but, to the best of our knowledge, the only methodology for the determination of glucose in aqueous solutions with chitosan was developed by Estevinho *et al.*²⁶ These authors studied the interference of chitosan in the analysis of glucose aqueous solutions by HPLC with evaporative light scattering detection (ELSD), considering the advantages of this detector relatively to others.²³⁻²⁵ The HPLC-ELSD methodology was considered adequate for analysis of glucose in aqueous solution in the presence of chitosan. However, the mucoadhesive properties of chitosan, which is known to interact with a large kind of molecules, make difficult the analytical methodology, particularly creating stability problems in the chromatographic column, affecting efficiency and resolution of glucose peak.²⁶ These authors minimized the effects of the chitosan interactions by using a gradient programme with acetonitrile:water as eluent and special procedures between runs, for a total time of analysis of 20 min.

The objective of this work was to evaluate the applicability of an enzymatic method to quantify glucose and sucrose in solution with chitosan, minimizing the effects of the chitosan interactions, the time of analysis and the problems associated to the use of HPLC methods with chitosan. The analytical methods were validated and the main parameters, as the limit of detection, linearity range, precision, accuracy and uncertainty, were obtained.

Experimental

Reagents

All reagents were of analytical grade purity. D-Glucose was supplied by Merck (Darmstadt, Germany) (Ref. 1.08337.1000), sucrose was from Merck (Darmstadt, Germany) (Ref. 1.07687.1000), chitosan (medium molecular weight with deacetylation degree between 75 and 85% and viscosity of 200 to 800 mPa s) was purchased from Aldrich (St. Louis, USA) (Ref. 448877-50G) and acetic acid (glacial) 100% anhydrous was from Merck (Darmstadt, Germany) (Ref. 1.00063.2511).

For the preparation of the enzymatic solution used in glucose determination, the following reagents were used: 3-(*N*-morpholino)propanesulfonic acid (MOPS) with a purity degree 99.5% from Sigma (Ref. M1254), phenol from Riedel-de Haën® (Ref. 33517), 4-aminophenazone from Riedel-de Haën® (Ref. 33528), glucose oxidase (from *Aspergillus niger*) from Sigma (Ref. G6766-10KU-023K3792), peroxidase type I (from horseradish) from Sigma (Ref. P8125-25KU-031K7465), potassium dihydrogen phosphate from Panreac (Ref. 131509.1211) and anhydrous sodium carbonate from Pronalab (Cod. 98 No. 1717).

For sucrose determination a commercial enzymatic kit from Sigma (Ref. SCA-20) was used.

Preparation of the standard and simulated samples

Glucose and sucrose standard solutions were prepared from stock solutions prepared with deionized water. Glucose calibration standards with concentrations of 50, 150, 300, 500, 700, 1000 and 2000 mg L⁻¹ of glucose were prepared from the stock solution of 10 g L⁻¹ of glucose in deionized water.

Sucrose standards with concentrations of 100, 250, 500, 750 and 1000 mg L⁻¹ of sucrose were prepared from the stock solution, with the concentration of 1 g L⁻¹ of sucrose, in deionized water.

Simulated samples were used in the experiments of optimization of the method by diluting glucose or sucrose in a chitosan (1% w/v) aqueous solution with 1% (v/v) acetic acid.

Preparation of enzymatic solutions

The glucose enzymatic solution was prepared considering the following final concentration for each compound: 5.16 g L⁻¹ MOPS, 1.04 g L⁻¹ phenol, 0.16 g L⁻¹ 4-aminophenazone, 0.71 g L⁻¹ glucose oxidase, 0.04 g L⁻¹ peroxidase, 5.25 g L⁻¹ potassium dihydrogen phosphate and 1.21 g L⁻¹ anhydrous sodium carbonate.

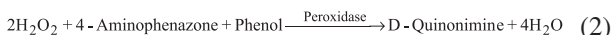
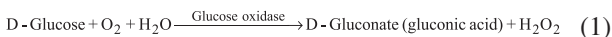
The sucrose enzymatic solution was prepared according to the sigma kit instructions. The sucrose assay reagent and glucose assay reagent solutions were reconstituted in water.

Equipment

The equipments used were a spectrophotometer UV-Visible V-530 (Jasco) and a thermostatic bath Stuart Scientific Co. Ltd (test tube, heater SHTD, 5006, UK).

Glucose enzymatic method

This method was adapted from Skoog and Leary,²⁷ and was based on the utilization of enzymes that catalyze the reaction of glucose degradation with the formation of a colored compound (quinonimine), considering the following equations:



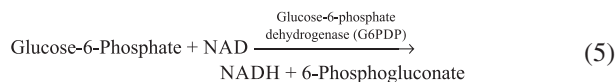
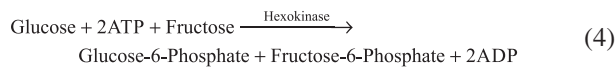
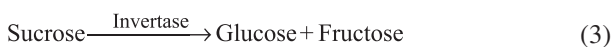
Samples and standards were prepared as described in Table 1. All samples and standards were incubated for 1 h at 37 °C. The absorbance at 500 nm is proportional to the concentration of glucose in the sample, according to the Beer law.

Table 1. Preparation of the samples and standards for the enzymatic reaction

| | Water / μL | Standard / μL | Sample / μL | Enzymatic solution / mL |
|----------|-----------------------|--------------------------|------------------------|-------------------------|
| Standard | – | 50 | – | 5 |
| Sample | – | – | 50 | 5 |
| Blank | 50 | – | – | 5 |

Sucrose enzymatic method

For the analysis of sucrose, a commercial kit was used (Sigma sucrose assay kit). In this process, the following equations are involved:



Samples were prepared for the enzymatic reaction as described in the instructions of the sucrose assay kit (Table 2).

Table 2. Preparation of samples and standards for the sucrose enzymatic reaction

| | Sucrose assay reagent / mL | Sample / mL | Water / mL |
|-----------------------------|----------------------------|-------------|------------|
| Sucrose assay reagent blank | 0.1 | – | 0.1 |
| Sample blank | – | 0.1 | 0.1 |
| Glucose assay reagent blank | – | – | 0.2 |
| Test | 0.1 | 0.1 | – |

After 10 min of ambient temperature incubation, 2 ml of glucose assay reagent were added to the samples and incubated for more 15 min. The consequent increase in absorbance at 340 nm was directly proportional to sucrose concentration (Beer law). The sucrose concentration (g L⁻¹) was determined according to the following equations:

$$C_{\text{sucrose}} = (A_{\text{samples}} - A_{\text{total blank}}) f_{\text{dilution factor from sample preparation}} \cdot 1.21 \quad (6)$$

$$A_{\text{total blank}} = (A_{\text{sample blank}} - A_{\text{glucose assay reagent blank}}) + A_{\text{sucrose assay reagent blank}} \quad (7)$$

where A represents absorbance, C the concentration of sucrose and f the dilution factor from sample preparation.

Results and Discussion

The analysis of glucose and sucrose are two of the most frequent chemical analyses in industry (food, beverages, pharmaceuticals, among others), either for quality control purposes or chemical composition evaluation, so requiring the necessity of implementing selective and sensitive analytical methods like the enzymatic methods.

An adequate and validated method for the analysis of any nutrient or compound is a fundamental step in achieving a good analytical result. Few enzymatic methods have a study of validation method, and from our knowledge, no one including the determination of uncertainty. For example, Garbelotti *et al.*²⁸ studied the determination and validation of dietary fibers (chitosan can have the function of a fiber in human digestion) in food by an enzymatic gravimetric method. These authors determined the precision (approximately 20%), the accuracy by the analysis of the recovery (70-120%) and the coefficient of correlation of the methods (0.9999). Liu *et al.*²⁹ developed and validated

a direct enzymatic HbA1c assay for human whole blood samples, and also in this case, the validation study was limited to the determination of the precision and accuracy. Accuracy studies were completed by comparing the direct enzymatic assay to the existing HPLC and immunoassay methods. Also, Woollard *et al.*³⁰ did the enzymatic determination of carnitine in milk and infant formula studying some validation parameters (precision and accuracy). Zhou and Prognon³¹ tested the implementation of International Conference on Harmonisation (ICH) guidelines for validation of analytical methods in the case of two enzymatic assays of determination of superoxide dismutase (SOD) activity. These authors determined precision and accuracy by the analysis of the recovery, specificity and coefficients of correlations of the methods.

The validation of the enzymatic methodologies (glucose enzymatic and sucrose enzymatic methods) were performed, not only to establish the main characteristics of the methods (linearity range, limit of detections, accuracy and precision), but also to assess the global uncertainty associated to the results.

Validation of the glucose enzymatic method

For the glucose enzymatic method, the calibration curve was obtained for 7 glucose standards with concentrations ranging from 50 to 2000 mg L⁻¹, whenever the samples were measured. Figure 1 presents one of these calibration curves. The coefficients of correlation were higher than 0.998 and the limit of detection was 39.5 mg L⁻¹.

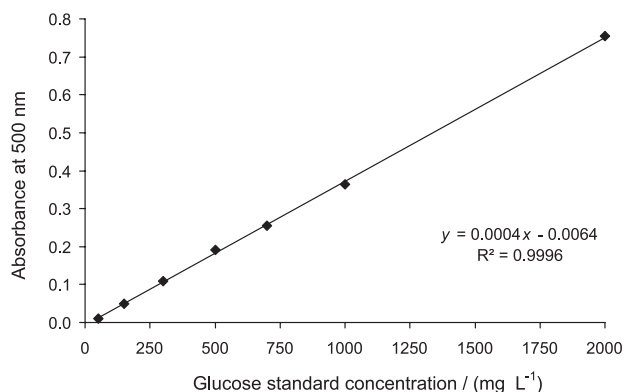


Figure 1. Example of one calibration curve obtained for the glucose enzymatic method for 7 glucose standards with concentrations ranging from 50 to 2000 mg L⁻¹.

The intermediate precision of this method was evaluated taking into account the relative standard deviation (RSD) of all the standards, in different days, at the optimized conditions. The variation coefficient (CV in %) was 26.9% on average, the bigger contribution being from the

standards with lower concentration. The smaller standard concentrations (50 and 150 mg L⁻¹) had a variation coefficient higher than 40%.

The recovery factor (Re) was defined as the ratio between the obtained concentration and the expected one. Accuracy, expressed by the percentage of recovery, was 102.6%, on average. The recovery factors ranged between 98.8 and 120%, related to the standards of 300 and 50 mg L⁻¹, respectively.

In the present work, the bottom-up approach was used to estimate the overall uncertainty by identifying, estimating and combining all the sources of uncertainty associated to the analytical results, as mentioned in Eurachem/Cytec.³²

It is considered an overestimation of the uncertainty, but it has the advantage of weighing the individual contributions, and therefore, allowing the detection of the most significant sources.

Global uncertainty (U) combines the contributions of all the sources of error linked to the analytical procedure and can be calculated from the following equation:

$$U = \sqrt{U_1^2 + U_2^2 + U_3^2 + U_4^2} \quad (8)$$

U_1 , U_2 , U_3 and U_4 are the uncertainties associated to standard preparation, calibration curve, precision and accuracy, respectively.

The uncertainty associated to the standard preparation (U_1) was calculated for each standard according to equation 9, considering the relative error associated to each mass or volume measurement and the law of propagation of uncertainty

$$U_1 = \sqrt{\left[\left(\frac{\Delta m_{\text{glucose}}}{m_{\text{glucose}}} \right)^2 + \left(\frac{\Delta V_1}{V_1} \right)^2 + \left(\frac{\Delta V_2}{V_2} \right)^2 + \left(\frac{\Delta V_3}{V_3} \right)^2 \right]} \quad (9)$$

where $\Delta m_{\text{glucose}}$ is the uncertainty associated to the mass measurement (m_{glucose}), ΔV_1 is the uncertainty associated to the preparation of the standard stock solution in a volumetric flask of 100 mL (V_1), ΔV_2 is the uncertainty associated to the preparation of the standards (25 mL, V_2), and ΔV_3 is the uncertainty associated to the pipetted volume (V_3) of the stock solution to prepare the different standards.

The uncertainty associated to the calibration curve (U_2) was calculated, for each standard by the calibration curve considering the following equations:

$$U_2 = \frac{\left(\frac{S_y}{x} \right)}{b} \sqrt{\left(\frac{1}{m} \right) + \left(\frac{1}{n} \right) + \left(\frac{(y_i - y_{av})^2}{b^2 \sum (x_i - x_{av})^2} \right)} \quad (10)$$

$$S_{\frac{y}{x}} = \sqrt{\frac{\sum (y_i - y_{ical})^2}{n-2}} \quad (11)$$

where $S_{\frac{y}{x}}$ represents the standard deviation of the linearization, b represents the slope of the regression curve, m the number of replicates performed for each x_i value, n the number of standards used to build the calibration curve ($n = 7$), y_i the experimental value of y (absorbance), y_{ical} the value of y calculated by the regression curve for the concentration x_i , y_{av} the average of y_i values, x_i the concentration of standards (x) used in the calibration and x_{av} the average of x_i values.

The uncertainty associated to the precision (U_3) was estimated considering the precision of the measurement for each standard. In the following formula, s represents the standard deviation of precision assays.

$$U_3 = \frac{s}{x\sqrt{n}} \quad (12)$$

The uncertainty associated to the accuracy (U_4) was calculated from equation 13, where η represents the recovery of the assays with spiked solutions at different levels:

$$U_4 = \frac{s(\eta)}{\eta_{average}} \quad (13)$$

In Figure 2, the relative weight of each individual source of uncertainty for glucose analysis is represented.

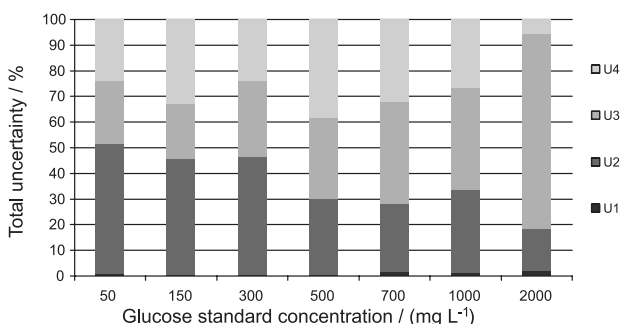


Figure 2. The relative contribution of the uncertainty sources on different calibration levels in the glucose enzymatic method.

The relative contribution of these four sources is decisively dependent of the calibration levels, being the uncertainty associated to the calibration curve (U_2), the main responsible for the correspondent variation for lower glucose concentration. For the upper glucose concentration levels, the influences of precision (U_3) and accuracy (U_4) achieved a combined contribution around 70%.

Figure 3 presents the variation of the global uncertainty with the glucose concentration levels. The global

uncertainty was less than 10% for concentration levels between 700 and 2000 mg L⁻¹. For lower glucose concentrations, the uncertainty increased in an exponential mode. For glucose concentration less than 150 mg L⁻¹, the values increased significantly to values higher than 25%.

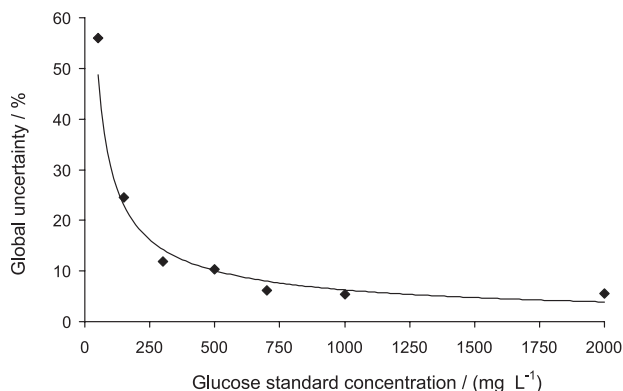


Figure 3. Global uncertainty for glucose enzymatic method.

This method was applied to analyze simulated samples prepared by mixing glucose with a chitosan (1% w/v) aqueous solution with 1% (v/v) acetic acid. Several simulated solutions were prepared with chitosan and glucose. The first tested solution did not have glucose and was used to evaluate if the chitosan gave any kind of response with this methodology. One can conclude that chitosan does not interfere with glucose analysis. The other solutions were tested in the range between 700 and 2000 mg L⁻¹ in which the global uncertainty was less than 10%. Four solutions with glucose concentrations close to 770 mg L⁻¹ and four other solutions with average concentrations of 1605 mg L⁻¹ were tested giving a recovery factor of 104.0 and 99.9%, on average, respectively.

Validation of the sucrose enzymatic method

For the sucrose enzymatic method, the validation was done in a range of concentrations between 100 and 1000 mg L⁻¹. The sucrose concentration was determined from equation 6 and the limit of detection was 134.0 mg L⁻¹. The intermediate precision of this method was 0.6% (CV) and the accuracy, evaluated in terms of recovery factor, was 93.0%.

The global uncertainty (U) was also determined in a bottom-up approach, considering the contributions of all the sources of error linked to the analytical procedure and can be calculated from equation 14. In this formula, the uncertainty associated to the error propagation of the concentration formula (equation 6) $\Delta C/C$ and the uncertainties associated to precision (U_3) and accuracy (U_4) were considered.

$$U = \sqrt{\left(\frac{\Delta C}{C}\right)^2 + U_3^2 + U_4^2} \quad (14)$$

Equations 15 and 16 were obtained considering the volume errors (ΔV) associated to the dilutions in the sample preparations and to errors associated to the final absorbance (ΔA_{final}). The final absorbance was limited by the errors associated to the sample blank (ΔA_{sample}) and to reagent blanks (ΔA_{sb} , ΔA_{garb} and ΔA_{sarb}).

$$\frac{\Delta C}{C} = \sqrt{\left(\frac{\Delta A_{final}}{A_{final}}\right)^2 + \left(\frac{\Delta V_1}{V_1}\right)^2 + \left(\frac{\Delta V_2}{V_2}\right)^2} \quad (15)$$

$$\Delta A_{final} = \sqrt{(\Delta A_{sample})^2 + (\Delta A_{sb})^2 + (\Delta A_{garb})^2 + (\Delta A_{sarb})^2} \quad (16)$$

Figure 4 presents the relative weight of each individual source of uncertainty in sucrose analysis. The uncertainty associated to the error propagation of the concentration formula ($\frac{\Delta C}{C} = U_1^*$) is crucial for the lowest concentration levels. The influence of U_1^* decreased with the increase of the sucrose standard concentration. The influences of precision (U_3), and, mainly, of the accuracy (U_4) increased for the higher concentration of sucrose.

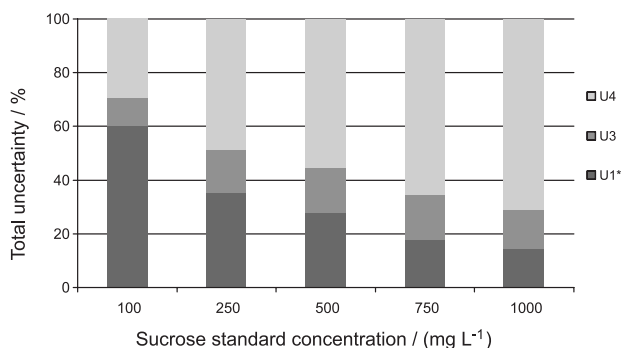


Figure 4. The relative contribution of the uncertainty sources on different calibration levels in the sucrose enzymatic method.

In Figure 5, the global uncertainty presents lower values (less than 3%) for all the analysis range (between 100 and 1000 mg L⁻¹).

Comparing the glucose and the sucrose enzymatic method, the glucose method has a larger range of analysis (50-2000 mg L⁻¹) and a smaller limit of detection (39.5 mg L⁻¹). However, the sucrose method has a smaller global uncertainty (less than 3%) for all the analytical range (100-1000 mg L⁻¹).

A similar study regarding the effect of chitosan was made for sucrose. One solution without sucrose was used

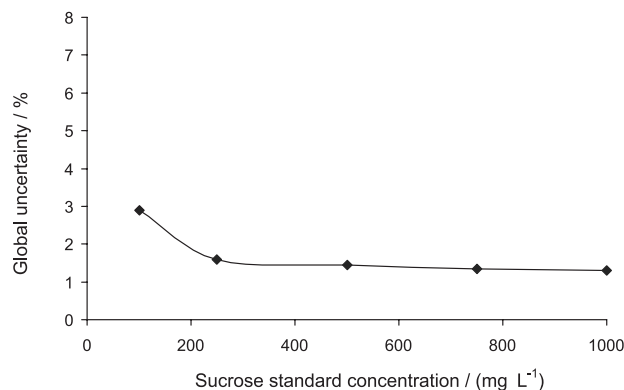


Figure 5. Global uncertainty for sucrose enzymatic method.

to evaluate if the chitosan gave any kind of response with this methodology. A similar conclusion to the glucose case was obtained. Several solutions with average sucrose concentrations of 645 and 1000 mg L⁻¹ were tested giving a recovery factor, of 99.7 and 98.1%, on average, respectively.

Glucose enzymatic method versus glucose HPLC-ELSD method

The HPLC-ELSD method was developed by Estevinho *et al.*²⁶ for samples with chitosan and glucose. These authors minimized the effect of the chitosan interactions by using a gradient programme with acetonitrile:water as eluent and special procedures between runs, for a total time of analysis of 20 min. The validation of the analytical methodology showed a linear response between 50 and 500 mg L⁻¹, with coefficient of correlation of 0.999 and limit of detection of 10.7 mg L⁻¹. Global uncertainty associated with the results was 4.1%, on average.

Some samples were analyzed by the two methods (enzymatic and HPLC-ELSD). Results of glucose in simulated samples obtained by the validated enzymatic method were compared with those obtained by HPLC-ELSD (Table 3).

Table 3. Glucose concentration in samples containing chitosan given by the two methods (enzymatic and HPLC-ELSD)

| Sample (chitosan and glucose) | HPLC method / (mg L ⁻¹) | Enzymatic method / (mg L ⁻¹) |
|-------------------------------|-------------------------------------|--|
| 1 | 334.1 | 347.4 |
| 2 | 341.9 | 341.6 |
| 3 | 0.0 | 0.0 |

The concentration selected to compare the two analytical methods was limited by the analytical range of the HPLC-ELSD method (50-500 mg L⁻¹) and by the

high values of the global uncertainty obtained for low concentrations of glucose for the enzymatic method (for glucose concentrations less than 150 mg L⁻¹, the uncertainty values increased significantly to values higher than 25%). By these reasons, the comparison was made for concentrations near 300 mg L⁻¹.

The results obtained by the two methods are very similar. The HPLC method was developed for a more restricted range (50-500 mg L⁻¹), presenting a smaller limit of detection (10.7 mg L⁻¹).

The enzymatic method for glucose presented higher values of global uncertainty than the HPLC method. However, the enzymatic method has the advantage of avoiding the problems found in the chromatographic column provoked by the mucoadhesive properties of chitosan. The use of the HPLC method has high costs involved with acquisition and maintenance of the equipment, and acquisition of solvents and columns. The enzymatic methods have smaller costs involved with the equipment. The most expensive part in the application of the enzymatic method is the acquisition of the enzymes/enzymatic solutions.

The enzymatic method is also more flexible and simple in the utilization, allows a large number of sample analyses in a small period of time and presented less operational problems.

The applicability of the enzymatic method on the analysis of glucose/sucrose in complex chitosan samples proved to be a good option.

Conclusions

The presented enzymatic methods intended to be used as a fast way of determining the glucose and sucrose contents in solutions with chitosan.

Glucose method was developed in the range of concentrations between 50 to 2000 mg L⁻¹, with a limit of detection of 39.5 mg L⁻¹. The intermediate precision was 26.9% (CV), on average, and the accuracy, expressed by the percentage of recovery, was 102.6%. The global uncertainty was less than 10% for concentration levels between 700 and 2000 mg L⁻¹.

Sucrose method was applied in the range of sucrose concentrations between 100 and 1000 mg L⁻¹, with a limit of detection of 134.0 mg L⁻¹. The intermediate precision of this method was 0.6% (CV), the accuracy, evaluated in terms of recovery factor, was 93.0% and the global uncertainty showed values less than 3% for all concentration levels analyzed.

The enzymatic method proved to be more suitable than HPLC, mainly if a high volume of samples needs to be

analyzed, because chitosan has mucoadhesive properties that create stability problems in the chromatographic columns.

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