

Quantification of Reducing Sugars Based on the Qualitative Technique of Benedict

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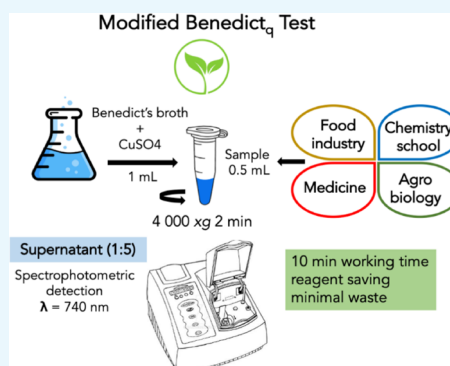
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ABSTRACT: Determination of reducing sugars is carried out routinely in the food industry, in biological research, or pharmaceutical and biomedical quality control to estimate metabolically assimilable sugars. Widespread detection methods are complex, expensive, or highly polluting. Here, we propose the use of spectrophotometric quantification for reducing sugars (Benedict_q) based on the qualitative method of Benedict. The protocol was validated, to verify its reproducibility and precision. With the proposed method (Benedict_q), the reducing sugar glucose can be determined in a range of 0.167–10 mg mL⁻¹, with an R^2 of 0.997 and accuracy (expressed as % of recovery) greater than 97%. Other reducing sugars, such as maltose, fructose, and lactose, showed similar values. The method robustness was verified for pH values greater than or equal to 4. In the case of protein presence, a correction is proposed in the range of 0–1.67 mg mL⁻¹. Modifications implemented in the protocol reduce cost, working time, and reaction volumes with respect to the original assay without detracting in accuracy and precision. In addition, waste reduction represents an important contribution of the method.



INTRODUCTION

Carbohydrate determination is a routine test in the industry or research laboratories to determine the metabolically assimilable sugars.¹ This methodology is important to study the dynamics of many sugars as an indicator of metabolic state² and the amount of carbohydrate in alternative sources for energy.^{3,4} Usually, in research laboratories and industries, the choice methodologies to estimate reducing sugars are 3,5-dinitrosalicylic acid (DNS)^{4,5} or phenol-sulfuric⁶ methods, while in clinics, the glucose oxidase method is the most used. However, these methods are very expensive, highly polluting, or both.

Benedict's method for reducing sugars was developed by Stanley R. Benedict for qualitative detection (Benedict_q) of glucose in urine.⁷ This method is still used in the qualitative determination of reducing sugars in the clinic, industry, and research.⁸ The reaction mechanism is based on the reducing capacity of free carbonyl groups in glucose, which are able to reduce a wide range of metal ions, including Cu²⁺. In an alkaline medium, copper is reduced to Cu⁺ and precipitates as Cu₂O. The main contribution of Benedict's reagent was the rapid detection of reducing sugars by color change, using stable alkaline agents that were not very corrosive.⁷ While initially the method only indicated the presence or absence of glucose in a test sample, later, Benedict himself proposed a modification to make it semi-quantitative (Benedict_{sq}) by indirectly estimating the resulting copper sulfate after a reduction reaction. The

Benedict_{sq} method involves the use of potassium thiocyanate and ferrocyanide to produce copper thiocyanate, which precipitates and could be titrated. With this method, Benedict established that a certain amount of glucose reduces a given amount of copper (9 mg mL⁻¹ of copper sulfate in the reagent is reduced by 1 mg mL⁻¹ of glucose). The procedure requires keeping the reaction components at the boiling point while dripping the problem sample to be titrated until the disappearance of the blue color.⁷ This makes it very impractical when handling a large number of samples.

On the other hand, the original Benedict_{nq} method, which qualitatively detects glucose and other reducing sugars, is characterized by its simplicity and the accessible nature of the used reagents. The objective of the present work is to take advantage of Benedict_{nq} and to establish a new quantitation method, replacing the impractical Benedict_{sq} titration by spectrophotometric detection, where precipitation is achieved by simple centrifugation. Implemented modifications to the protocol allow reductions in the working volume of the original

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assay, providing thus savings on reagents and waste production, concomitant with the possibility to expand the test sample number in shorter time, so much appreciated lately. The protocol proposed in this work (Benedict_q) was validated for reproducibility and accuracy in the evaluation of total reducing sugars. This method is proposed as a low-cost alternative to DNS and phenol-chloroform methods in the food industry, biological research, as well as pharmaceutical and biomedical quality control.

RESULTS AND DISCUSSION

In order to establish the differences between the Benedict_{nq} method and the quantitative method proposed in the present work (Benedict_q), Figure 1 shows a comparative scheme

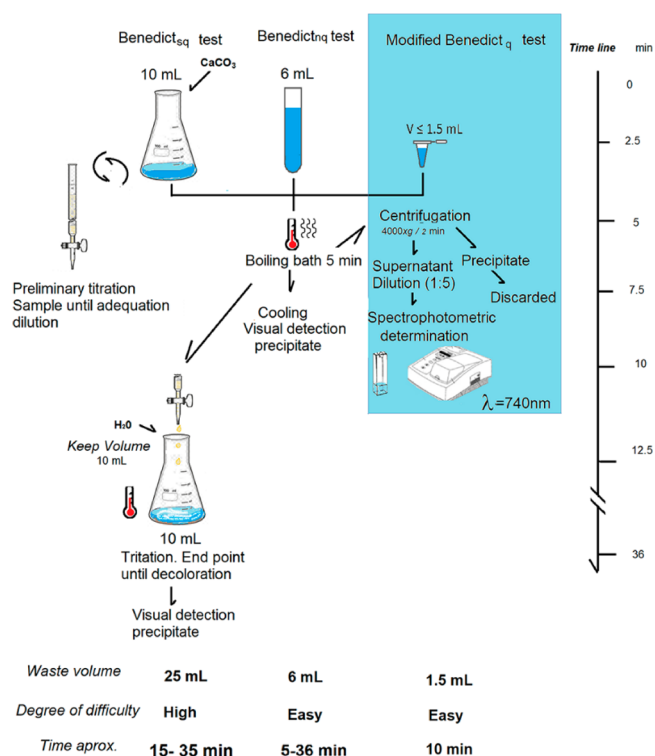


Figure 1. Comparison of the proposed method (blue rectangle) with Benedict_{sq} and Benedict_{nq} methods in terms of practice.

between both. Major differences were the final reaction volume decrement from 6 to 1.5 mL, comprising 1 mL of the reagent and 0.5 mL of the sample. The proposed volume was according to the requirements of the used equipment, so it could be further reduced if the equipment allows it as long as the stoichiometry of the reaction is maintained. The supernatant containing the residual copper sulfate can be immediately detected by a spectrophotometer or immediately stored for many weeks at 4 °C until use. The inclusion of a centrifugation step was implemented to avoid the use of potassium thiocyanate and ferrocyanide and generation of toxic polluting residues, derivatives of the reaction established for Benedict_{sq}, such as cuprous thiocyanate. In general, the thiocyanates, although less harmful than cyanide in humans, are known to affect the thyroid gland.⁹ The exposure to thiocyanate, although found in popular vegetables like Brussels sprouts and collards,¹⁰ decreases thyroidal iodide uptake,¹¹ reducing the gland's ability to produce hormones that are necessary for normal body function.¹²

In order to determine the appropriate wavelength for copper sulfate determination, we performed a spectrophotometric scan from 400 to 890 nm. Water was used for the baseline and 108 mM CuSO₄ as the starting concentration, with further dilutions in distilled water, 1:5 1:10 and 1:100. The Benedict broth base (Bbb) was also included in the analysis to detect whether its components (sodium citrate and sodium carbonate dissolved in distilled water without CuSO₄) affected the absorbance profile. More details could be found in the Methods section.

Direct values of absorbance from the spectrophotometric analysis of copper sulfate dissolved in water revealed a single maximum at 740 nm (λ_{max}). The Bbb did not show any contribution to the absorbance at any wavelength within the analyzed range (Figure 2a). By analyzing the λ_{max} values of different CuSO₄ concentrations in water, we obtained an R^2 of 0.995, so it was feasible to continue with the study (Figure 2b).

After CuSO₄ λ_{max} determination and observation of linearity between absorbance and copper sulfate concentrations between 0 and 108 mM in distilled water, the next step was to analyze whether the response remained unaltered under the conditions proposed by the Benedict_{nq} method (Figure 3a). The concentration of copper sulfate necessary to maintain the reaction stoichiometry proposed by Benedict (108 mM) represented an absorbance of 2, which is not adequate

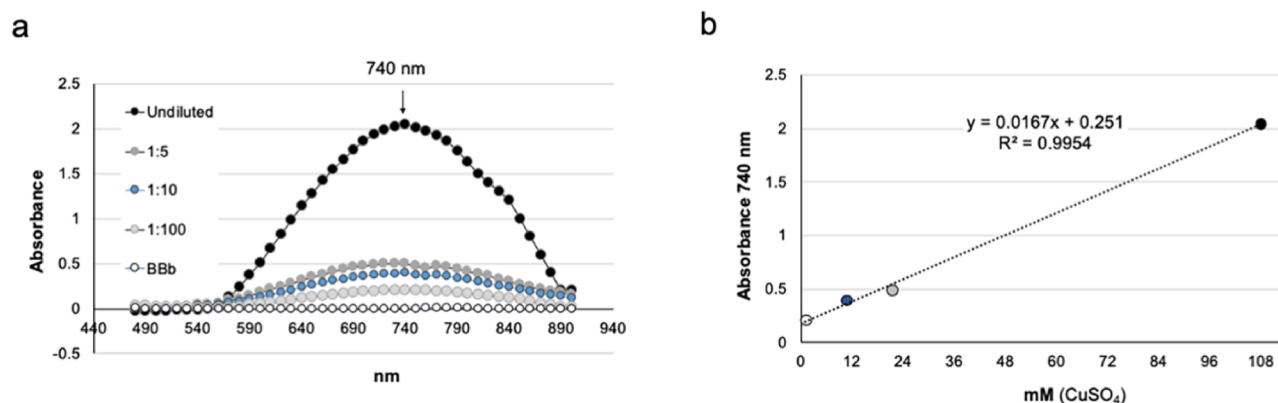


Figure 2. Direct absorbance profiles of the copper sulfate concentration gradient dissolved in water without sugar added. (a) λ_{max} was detected at 740 nm independent to CuSO₄ dilution. The Benedict broth base (Bbb) did not show absorbance within the wavelength range. (b) Relationship between absorbance at 740 nm and CuSO₄ dilutions was linear.

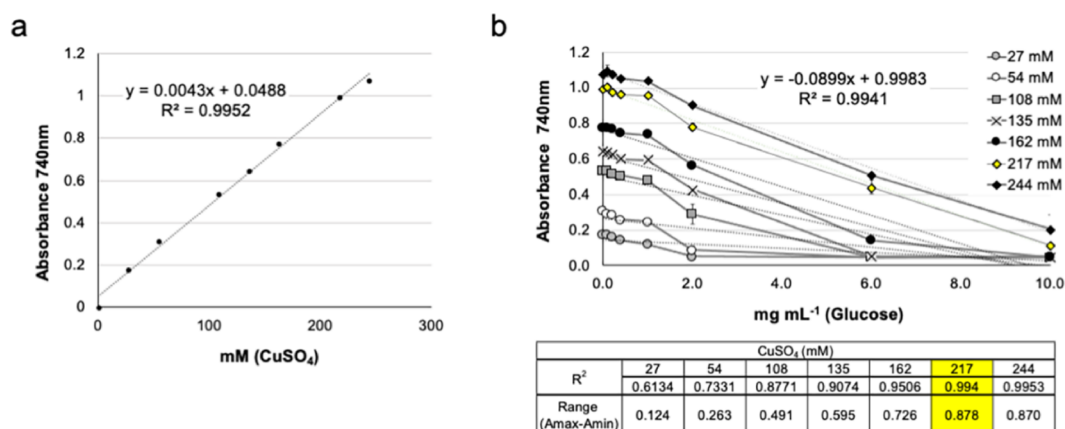


Figure 3. Establishment of an optimal working CuSO₄ concentration range (linear relationship, starting at an absorbance of 1) to determine reducing sugars. (a) Absorbance of different CuSO₄ concentrations without sugar added. (b) Changes in absorbance of CuSO₄ with different concentrations of glucose. The optimal analysis conditions (yellow rhombuses) for glucose are shown. All samples were diluted to 1:5, $n = 3$.

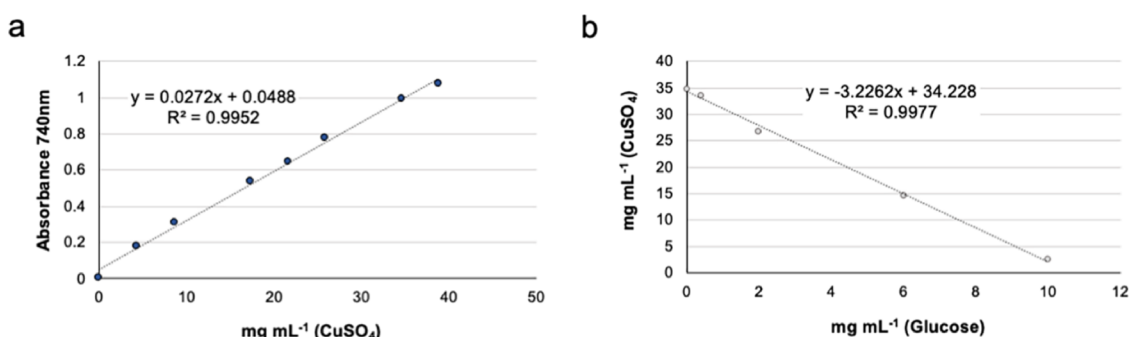


Figure 4. Reduction of CuSO₄ concentration by different glucose amounts. (a) Absorbance of different CuSO₄ concentrations, expressed as mg mL⁻¹, without sugar added. (b) Estimation of the amount of residual CuSO₄ at different glucose concentrations. The slope indicates the amount of CuSO₄ (3.22 mg mL⁻¹) reduced by 1 mg mL⁻¹ of glucose ($n = 3$).

according to the Lambert and Beer law (relationship between different concentrations of a substance and absorbance could only be considered if the absorbance values remain below 1). Therefore, for further analyses, samples were diluted to 1:5 after the reaction and previous to spectrophotometric analysis.

The CuSO₄ concentration in the Benedict reaction established by the author was 108 mM. However, as mentioned before, results were not enough to establish a quantitative stoichiometry. Therefore, to find the optimal concentration range of CuSO₄ to correlate with absorbance values at 740 nm in Benedict's reaction, we analyzed 27, 54, 108, 135, 162, 217, and 244 mM CuSO₄ dissolved in Bbb and diluted to 1:5 or 1:10 after the reaction. However, absorbances at 740 nm for the 1:10 dilutions were always lower than 0.4 (data not shown). Hence, we only worked with the 1:5 dilution (Figure 3). Under these experimental conditions, the absorbance amplitude ranges at 740 nm for different starting CuSO₄ concentrations in Benedict's reaction were obtained (Figure 3a).

In order to optimize the CuSO₄ detection under different concentrations of reducing sugars, we performed a Benedict_q for the established starting concentration range, adding an increasing amount of glucose (Figure 3b). The analysis was performed using a dilution of 1:5 following the Benedict reaction. The results showed different ranges of absorbance, which we designated as the absorbance range, considered as the difference between the absorbance of CuSO₄ at the minimum and maximum concentrations of glucose. Each range

showed different values of linearity (R^2). Linearity is the ability (within a given range) to provide results that are directly proportional to the concentration of the analyte in the samples.¹³ Linearity can be evaluated using the determination coefficient (R^2), which indicates how good the regression model is (Figure 4 and Table 1). Under the conditions of range of absorbance between 0 and 1 (recommended by Lambert and Beer), the best linearity of 0.994 was found for a starting concentration of 217 mM, which as indicated with yellow rhombuses (Figure 3b). Therefore, this concentration was selected for further tests with the method.

In order to determine the amount of CuSO₄ (mg mL⁻¹) that reacted for each mg of glucose in solution, the copper sulfate concentration values, determined according to their proportionality with absorbance at 740 nm, were converted to mg mL⁻¹ and used to estimate the concentration of CuSO₄ without glucose addition (Figure 4a). Subsequently, the absorbance values at 740 nm obtained after the exposure of 217 mM of copper sulfate to different glucose concentrations were used to determine the remaining mg mL⁻¹ CuSO₄ according to the equation of Figure 4a. By this means, the amount of CuSO₄ consumed per mg of glucose was obtained, 3.22 mg mL⁻¹ (value of the slope; Figure 4b). The value of R^2 was 0.997, indicating that CuSO₄ consumption is directly proportional to glucose concentration within the assayed range.

Likewise, the Pearson correlation coefficient was calculated. This coefficient indicates the correlation, strength, and

Table 1. Validation Parameters for the Benedict_q Method^a

glucose	carbohydrate (mg mL ⁻¹)		precision ^a	linearity (R ²)	limit quantification	systematic error	accuracy
	mean	standard deviation					
0.0	-0.09	0.12	0.00	0.998	0.17	-0.09	0.00
0.4	0.43	0.00	0.00			0.02	105.57
2.0	2.37	0.03	1.27			0.37	118.57
6.0	6.45	0.05	0.70			0.45	107.51
10.0	10.05	0.04	0.40			0.05	100.45
maltose							
0.0	-0.20	0.19	0.0	0.998	0.10	-0.20	0.0
0.4	0.28	0.01	2.9			-0.13	68.1
2.0	2.41	0.04	1.8			0.41	120.5
6.0	6.21	0.08	1.3			0.21	103.4
10.0	9.94	0.01	0.1			-0.06	99.4
fructose							
0.0	0.07	0.10	0.0	0.998	0.086	0.07	0.0
0.4	0.48	0.01	2.1			0.08	119.1
2.0	2.20	0.04	1.8			0.20	110.2
6.0	6.16	0.05	0.8			0.16	102.7
10.0	10.30	0.17	1.6			0.30	103.0
lactose							
0.0	0.17	0.16	0.0	0.998	0.09	0.17	0.0
0.4	0.44	0.01	3.2			0.04	108.9
2.0	2.45	0.04	1.7			0.45	122.5
6.0	6.08	0.06	0.9			0.08	101.3
10.0	10.19	0.08	0.8			0.19	101.9
sucrose							
0.0	nd	nd	nd	0.05	nd	nd	nd
0.4	nd	nd	nd		nd	nd	nd
2.0	nd	nd	nd		nd	nd	nd
6.0	nd	nd	nd		nd	nd	nd
10.0	nd	nd	nd		nd	nd	nd

^aData were obtained from three independent replicates in triplicate. The validation parameters for the main reducing sugars are shown, including sucrose as a negative control. ^bPrecision is represented by the coefficient of variation

direction of a linear relationship, as well as the proportionality between two statistical variables. The Pearson correlation between the remaining CuSO₄ concentration and glucose added was 0.999 ($p \leq 0.000$), indicating that the univariate standardization of CuSO₄ concentration according to glucose amounts is optimal.

To validate the performance parameters of the method, after the glucose optimization tests, maltose, fructose, lactose, and sucrose (a non-reducing sugar for the negative control) were used with a CuSO₄ concentration of 217 mM (Table 1). All samples were equally treated, and the R² value was considered to evaluate linearity in data behavior (Figure 5). All reducing sugars showed R² values above 0.99, fitting to the linear regression model, which indicates that there is an inversely proportional relationship between the absorbance at 740 nm (representing remnant CuSO₄) and increasing concentrations of different reducing carbohydrates but not for the non-reducing sucrose.

Accuracy was determined by the recovery percentage values, for which values from 85 to 115% were expected.^{13–15} All sugars had recovery values greater than 90%, except sucrose (negative control). All sugars had good linearity competition and good accuracy, except sucrose, which is the negative control. The lowest quantification limit value corresponded to fructose (0.08 mg /mL), while for glucose and maltose the values were 0.170 and 0.120 mg mL⁻¹, respectively (Table 1). The LOQ value was calculated with the formula $LC = yB +$

10sB, according to the Metrology Center of Mexico (CENAM) as described in the Methods section.

To test the Benedict_q method in a relevant biological sample, the glucose concentration was tested in a glucose-added, medical injectable serum solution (Beplenvax) from Pisa México, sanitary registration 77013 SSA IV. The serum specifications indicated a glucose concentration of 5 g/100 mL (50 mg mL⁻¹), thiamine hydrochloride (10 mg), riboflavin (4 mg), nicotinamide (50 mg), and pyridoxine hydrochloride (5 mg). In addition, as a food sample, the sugary drink Sprite, brand from Coca Cola Company, was tested. The reducing sugar content indicated by the “El poder del consumidor A.C” report was 54 g/600 mL (90 mg mL⁻¹). In both cases, the reaction sample was previously diluted to 1:10 to adjust the glucose values within the detection range of the method. Using the Benedict_q method, it was determined that the glucose serum had a concentration of 49 ± 4 mg mL⁻¹ (once corrected by the dilution) with a % CV of 0.39 and an accuracy of 97%. In the case of the sugary drink Sprite, 68 ± 0.3 mg mL⁻¹ (once corrected by the dilution) was calculated by the method, with a % CV of 0.48 and an accuracy of 99.1%, which shows that the method is reliable for samples of different origins.

The injectable serum solution is added with vitamins, which apparently did not interfere with results, perhaps due to its very low concentration. In order to discard other possible interferences for the copper reaction, the pH was modified for some samples, and for others, protein was added. These

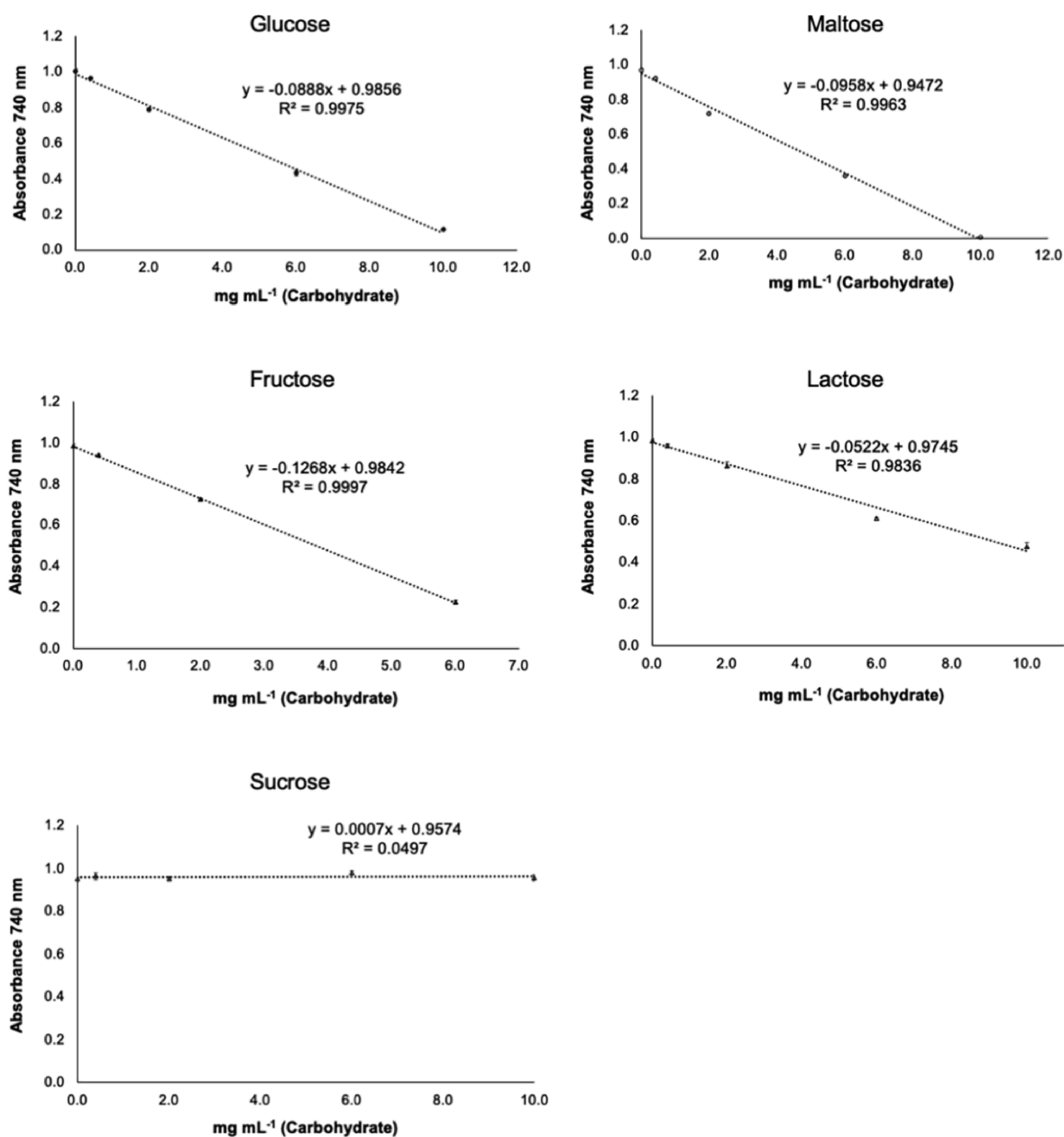


Figure 5. Adjustment to the linear model for standard curves of glucose, maltose, fructose, lactose, and sucrose with the proposed method, Benedict_q.

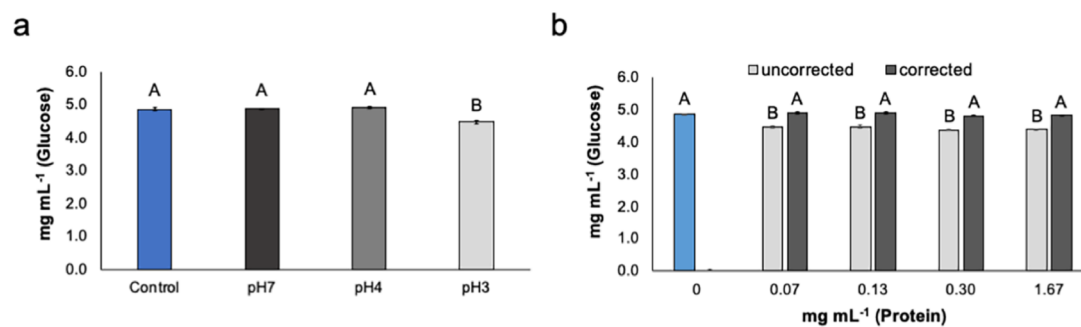


Figure 6. Effect of pH and proteins on the performance of the Benedict_q method. (a) Effect of sample acidification on glucose quantification ($n = 3$). (b) Changes in glucose determination by the presence of protein (BSA). The blue bar corresponds to the previously determined value of glucose concentration. The values represent the concentration in the diluted sample (1:10).

modifications were considered as the most common interferences in biological samples due to their reducing capacity. The glucose content of the medical injectable solution was re-evaluated at modified pH (pH 3, 4 and 7). The samples with pH 4 and 7 did not show significant differences between them, nor with respect to the values previously determined, while for pH 3, there was a significant decrease in the determined glucose concentration (Figure 6a). Also the accuracy dropped from 97 to 84%, while the % CV remained within acceptable values (around 1.3). It is likely that this is due to the fact that the samples with a pH equal to or lower than 3 modify the alkaline environment necessary for the final reaction system to take place.

In order to discard the possible interferences in glucose quantification by the presence of proteins, 0, 0.7, 0.13, 0.3, and 1.67 mg mL⁻¹ bovine serum albumin (BSA) were added to the glucose serum samples. For all the tested concentrations, the effect of the protein translated into an underestimation of the glucose concentration in a non-proportional way with respect to the previous determination. This may be due to the formation of a complex between the peptide bonds that have the CO–NH group and the cupric cations, which would decrease the concentration of remaining copper sulfate. The average of absorbance difference between values obtained with and without BSA was $0.038 \pm .004$, so the simple subtraction of 0.038 returned to the value of previously calculated glucose concentration (Figure 6b, dark gray bars). It is important to highlight that the protein interference was carried out, maintaining constant glucose concentration. Therefore, it would be advisable to rule out protein interference in each sample to be analyzed. Although the assayed interference sources were ruled out, it is possible that, as in any analytical technique, other contaminants, not considered in this document, could interfere with the assay. Such cases, if reported, would deserve further studies.

The systematic measurement error or slant was low for all carbohydrates, which would indicate that the experimental values were very similar to the theoretical ones. Therefore, it was concluded that the method has acceptable values of sensitivity, accuracy, and reproducibility.^{13–15} Additionally, according to its characteristics, determination of reducing sugars by the new method has several advantages over other methods currently used in the industry. First, it requires less volume of the sample and reaction for detection. Second, our new method is very accurate and environment friendly.⁴ In our case, the working volume can be reduced as much as allowed by the spectrophotometer used, being able to work with samples of up to 100 μ L, considering that stoichiometry is maintained. Additionally, samples with pH values above 3 can be analyzed, and the interference by proteins in the method can be easily corrected. Also, in our case, we observed that it is not affected by vitamins and although interference by vitamin C has been reported,¹⁶ interferent levels represent 400% of the recommended dosage. Therefore, we propose that it can be used in clinics and to estimate the content of assimilable carbohydrates in sources of the alternative energy industry. In our laboratory, we have used this method with good results in the determination of reducing sugars during corn seed germination and yeast alcoholic fermentation tests (unpublished data), although its use for human resource training purposes is not ruled out.

We must clarify that the proposed method is a quantification, but not a separation, method. For separation

purposes, high-performance liquid chromatography (HPLC) is the method of choice. The Benedict_q proposed in this work could also be used complementary to physical separation methods such as column chromatography or thin layer chromatography,¹⁷ which would allow to determine the proportion of each of the reducing sugars present in a complex sample.¹⁸ It is also possible to carry out chemical or enzymatic derivatizations in order to improve the specificity as done in HPLC. For example, it is known that when a mixture contains sucrose, it can be hydrolyzed by the action of an acid medium and that the proportion would be 50% glucose and 50% fructose. However, quantifications can be made before and after hydrolysis in order to know more precisely the proportions. It is relevant to comment that, due to the nature of HPLC, the amounts of analyte injected are small, and high dilutions can result in misleading information, which makes it necessary to use internal standards, a wide variety of detectors, and several columns with different conditions to separate each of the carbohydrates. Further, the precision of some methods is very similar to that obtained by us.^{17,18} Finally, it is noteworthy that in the case of some HPLC methodologies, the minimum time necessary comprises the application of linear gradients of buffer, maintenance of the column, washes, and the re-equilibrium of the column representing up to 70 min for each injection.¹⁷

CONCLUSIONS

In conclusion, the proposed method is simple, fast, and very economical, allowing the handling of large number of samples. Copper sulfate has a λ_{max} at 740 nm, providing a reliable spectrophotometric quantification due to a high correlation between absorbance and concentration gradients. The CuSO₄ concentration of 217 mM allows detecting concentrations of reducing sugars between 0.167 and 10 mg mL⁻¹ with an absorbance range between 0 and 1 in a maximum reaction volume of 1.5 mL.

METHODS

All reagents were ACS grade provided by Meyer Chemical Reagents, while carbohydrates were purchased from J.T. Baker. All reagents were weighed on an analytical balance (Mettler Toledo, model XPR105). Reagent spectra were performed on a UV–vis Thermoscientific spectrophotometer, and curves were obtained on a Visible Spectrophotometer VIS 721 spectrophotometer.

Determination of the Absorbance Peak (λ_{max}) of CuSO₄ in Water or Benedict Broth Base. The spectrophotometric baseline was established with distilled water and with these results, it was tested in the Benedict broth base, which contained sodium citrate (670 mM) and sodium carbonate (943 mM), dissolved in distilled water⁷ without CuSO₄. To determine the wavelength at which the λ_{max} of CuSO₄ occurs, this compound was added at 108 mM concentration and mixed. Scanning spectrophotometry was performed, without prior heating of the broth, in a UV–vis spectrophotometer (Genesis Thermoscientific) from 400 to 890 nm, with intervals of 5 nm. Once the peak wavelength (λ_{max}) was determined, and in order to confirm that it does not shift when modifying the copper sulfate concentration, the following dilutions were prepared: 1:5 (21.6 mM), 1:10 (10.8 mM), and 1:100 (1.08 mM). Likewise, the contribution of the

base broth and glucose was discarded (Figure 2a). All determinations were made in triplicate.

Determination of the Optimal Concentration of CuSO₄ in the Benedict Broth. To determine the optimal concentration of CuSO₄, the Benedict broth base (943 mM sodium carbonate and 670 mM sodium citrate) was prepared separately, and different amounts of CuSO₄ were added to obtain the following concentrations: 27, 54, 108, 135, 162, 217, and 244 mM. They were placed in a boiling bath and diluted to 1:5 to read the absorbance at 740 nm on a VIS 721 Spectrophotometer. CuSO₄ concentrations giving absorbance below 1 were chosen in order to conform to the Lambert–Beer law.

Optimization of Glucose Concentration. For each concentration of copper sulfate, a glucose standard curve was made. Aliquots from a 0.1% glucose stock solution were taken and mixed with 1 mL of Benedict's reagent. Distilled water was added to reach a final reaction volume of 1.5 mL. Glucose concentrations were 0, 0.2, 2, 6, and 10 mg mL⁻¹. Reactions were heated in a boiling bath for 5 min and then cooled. The samples were centrifuged for 2 min at 4000 ×g. The supernatant was recovered and diluted to 1:5 with distilled water, and the absorbance at 740 nm was determined with a Spectrophotometer VIS 721. Detections were made in triplicate for each concentration.

Preparation of Different Sugar Calibration Curves. Four reducing sugars were evaluated separately: glucose, maltose, fructose, and lactose from a 0.1% stock solution. Additionally, sucrose was used as a negative control. To validate the analytical method, glucose concentration in a medical injectable solution (Beplonovax from Pisa México sanitary registration 77013 SSA IV) was quantified. For all samples, the mixture was treated as previously described for glucose. The absorbance was determined at 740 nm, and the readings were made in triplicate.

Analytical Validation. The validation parameters of the proposed method were calculated according to what is established by the National Metrology Center and the Mexican Accreditation Entity (CENAM-ema)¹³ COFEPRIS,¹⁴ AMEPRES,¹⁵ and the Bureau International of Weight and Measurements (BIPM).¹⁹ The analytical parameters were as follows:

Precision: percentage of coefficient of variation

$$\%CV = (S/x) \times 100$$

x = sample group mean

S = standard deviation

Acceptance value: $\leq 2\%$

Linearity: fitting the standard curve to the linear model

$$R^2$$

Acceptance value: >0.995

Limit of quantification

$$LC = yB + 10sB$$

where

yB = concentration of the analyte that provides a signal equal to the target signal.

$10sB$ = 10 times the standard deviation of the blank.

Systematic error

$$\text{slant} = x - \mu$$

x = average of the experimental concentration

μ = theoretical or true concentration

Accuracy: degree of agreement between a real value (measured) and a theoretical value (true)

$$\text{recovery\%} = (C_R \div C_v) \times 100$$

C_R = average of the experimental concentration

C_v = theoretical or true concentration

Acceptance value: $\geq 85, \leq 115\%$

Evaluation of Interferences. The glucose concentration of a medical serum (Beplonovax from Pisa México sanitary registration 77013 SSA IV) was evaluated, and on one hand, the interference of the pH was evaluated by modifying it prior to the Benedict reaction with the addition of 0.1 N HCL, adjusting it to values of 3, 4, and 7. On the other hand, the possible interference of proteins was tested with the addition of 0, 0.7, 0.13, 0.3, 1.67 mg/mL bovine serum albumin (BSA) prior to the Benedict reaction.

Statistical Analysis. Tukey tests were performed to determine the significant differences between the corresponding absorbances of each of the copper concentrations using MINITAB (USA). With the absorbance results, the determination coefficient (R^2) and the correlation coefficient (r) were calculated, with respect to a linear regression model for the different concentrations of CuSO₄ analyzed in Excel.

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Author Contributions

A.H.-L. contributed in conception and design of the work, drafting the article, data collection, data analysis and interpretation, and critical revision of the article. D.A.S.-F. did the data collection and data analysis. Z.Z.-S. also did the data collection as well as I.G.-B. T.D.D. contributed in data analysis, drafting the article, and critical revision of the article. A.X.A.-A. did the data collection, analysis, and interpretation and also contributed in drafting the article and critical revision of the article. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Peters, J.-C.; Marker, R.; Pan, Z.; Breen, J.-A.; Hill, J. O. The Influence of Adding Spices to Reduced Sugar Foods on Overall Liking. *J. Food Sci.* **2018**, *83*, 814–821.
- (2) Juturu, V.; Wu, J.-C. Microbial production of lactic acid: the latest development. *Crit. Rev. Biotechnol.* **2016**, *36*, 967–977.
- (3) Bekers, K.-M.; Heijnen, J.-J.; van Gulik, W.-M. Determination of the *in vivo* NAD: NADH ratio in *Saccharomyces cerevisiae* under anaerobic conditions, using alcohol dehydrogenase as sensor reaction. *Yeast* **2015**, *32*, 541–557.
- (4) Zhang, P.; Hai, H.; Sun, D.; Yuan, W.; Liu, W.; Ding, R.; Teng, M.; Ma, L.; Tian, J.; Chen, C. A high throughput method for total alcohol determination in fermentation broths. *BMC Biotechnol.* **2019**, *19*, 30–38.
- (5) Miller, G.-L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428.
- (6) Dubois, M.; Gilles, K.-A.; Hamilton, J.-K.; Roberts, P.-A.; Smith, F. Colorimetric method for the determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356.
- (7) Benedict, R. S. A reagent for the detection of reducing sugars. A rapid method for the quantitative determination of sugar in urine. *J. Biol. Chem.* **1911**, *57*, 1193–1194. Available in: <https://www.jbc.org/content/5/5/485.full.pdf> (accessed 14th June, 2015)
- (8) ACS style American Chemical Society National Historic Chemical Landmarks. *Development of diagnostic test. Strips*; Available in: <http://www.acs.org/content/acs/en/education/whatischemistry/landmarks/diagnosticteststrips.html>. (accessed August 26th, 2020)
- (9) ATSDR Public Health Statement for Cyanide. Public Health Statements July 2006. *Agency for Toxic Substances and Disease Registry*; Department of Health and Human Services: Atlanta, GA: U.S., Available in: <https://www.atsdr.cdc.gov/PHS/PHS.asp?id=70&tid=19>. (accessed November 10, 2020)
- (10) Felker, P.; Bunch, R.; Leung, A.-M. Concentrations of thiocyanate and goitrin in human plasma, their precursor concentrations in brassica vegetables, and associated potential risk for hypothyroidism. *Nutr. Rev.* **2016**, *74*, 248–258.
- (11) Laurberg, P.; Pedersen, I.-B.; Carlé, A.; Andersen, S.; Knudsen, N.; Karmisholt, J. *The Relationship between Thiocyanate and Iodine*. In *Comprehensive Handbook of Iodine*; Preedy, V.-R.; Burrow, G.-N.; Watson, R.; Eds. Academy Press: New York, 2009, p. 275–281, DOI: 10.1016/b978-0-12-374135-6.00028-5.
- (12) Petroski, W.; Minich, D.-M. Is There Such a Thing as "Anti-Nutrients"? A Narrative Review of Perceived Problematic Plant Compounds. *Nutrients* **2020**, *12*, 2929.
- (13) CENAM-ema *Guía técnica sobre trazabilidad e incertidumbre en las mediciones analíticas que emplea la técnica de espectrofotometría de ultravioleta-visible*; México, D.F., 2008.
- (14) COFEPRIS CCAAYAC-CR-03/1: *Criterios para la validación interna y confirmación de métodos fisicoquímicos*. Comisión Federal para la Protección contra Riesgos Sanitarios; 2019, Available in: <https://www.gob.mx/cms/uploads/attachment/file/194517/CCAYAC-CR03.pdf>. (accessed 16th June, 2019)
- (15) AMEPRES *Bolletín AMEPRES*; 2018, <https://www.amepresmexico.mx/wp-content/uploads/2018/12/Boletín-No17>.
- (16) Richard, B.-R.; Guyer, K.-E.; Banks, L.-W., Jr. Urinary Glucose and Vitamin C. *Am. J. Clin. Pathol.* **1977**, *68*, 592–594. Available in <https://ajcp.oxfordjournals.org>
- (17) Templeton, D.-W.; Quinn, M.; Van Wychen, S.; Hyman, D.; Laurens, L. M.-L. Separation and quantification of microalgal carbohydrates. *J. Chromatogr. A* **2012**, *1270*, 225–234.
- (18) Molnár-Perl, I. Role of chromatography in the analysis of sugars, carboxylic acids and amino acids in food. *J. Chromatogr. A* **2000**, *891*, 1–32.
- (19) BIPM (Bureau International des poids and mesures) *About the BIPM*; 2020. Available: <https://www.bipm.org>.