An Automated Flow Injection Analysis Procedure for the Determination of Reducing Sugars by DNSA Method

P. CAÑIZARES-MACÍAS, L. HERNÁNDEZ-GARCIADIEGO, AND H. GÓMEZ-RUÍZ

ABSTRACT: A nonenzymatic spectrophotometric method, coupled to an automatic system of standard additions, based on the reaction with 3,5-dinitrosalicylic acid (DNSA) is described. In a system based on an exhaustive reaction, sample volumes are introduced together with calibration solutions, thus the calibration is performed in a nonsegmented flow system. Homogenization of the sample/standard/carrier takes place in the calibration loop. Later, it is injected in the flow injection analysis system, where the reaction with DNSA occurs, to obtain 3,5-diaminosalicylic acid which is measured spectrophotometrically. With the proposed method, it is possible to eliminate the Rochelle salt, sodium and potassium tartrate, phenol and sodium bisulfite of the principal reagent. Sample throughput was 11 samples/h and precision, expressed as relative standard deviation, was 2.3%.

Keywords: Flow injection; automatic calibration; reducing sugars; DNSA, soft drinks.

Introduction

DETERMINATION OF TOTAL REDUCING SUGARS IS OF GREAT importance in the food industry. During the last decade, analysis innovation of total reducing sugars in foods and pharmaceuticals has been increased by a great variety of methods: enzymatic (Massom and Townshend 1984; Marko-Vargas and Dominguez 1991; Nobutoshi and others 1991), amperemetric (Wang and others 1994; Karyakina and others 1995; Chi and Dong 1995) and chromatographic (Verrette and others 1995; Yang and others 1999). Some of the methods have been adapted to nonsegmented flow configurations, which are coupled with spectrophotometric methods. Many of these methods require working with enzymes, which increases the cost. Generally, nowadays, reducing sugars are determined by the Fehling method (AOAC 1990), but the automation of this method poses many problems because of the many analytical steps, including precipitation and titration.

One of the main problems in the flow injection analysis technique is how to couple it with the pretreatment of the samples. Amine and others (1991) determined glucose in liquid food samples by flow injection analysis (FIA), without pretreatment, but they reported interferences of ethanol when the concentration of it was higher than 1%.

In order to eliminate interferences due to the matrix of the samples, the method of standard additions has been the most suitable. Automated versions of this method are therefore very useful as they allow a wide variety of liquid samples to be directly analyzed in continuous flow systems (Ruzicka and Hansen 1988). Recently, many calibration procedures using configurations of nonsegmented flow to automate this step have been developed, most of which are based on gradients of concentration profiles (Sperling and others 1991; Barón and others 1992). Agudo and others (1992, 1995) have done some work on automatic calibration in nonsegmented flow systems to determine chlorides and nitrates, as well as enzymatic detection of glucose and fructose in food samples.

In this paper, we propose a spectrophotometric method to determine total reducing sugars in different kinds of beverages (soft drinks) based on the reaction proposed by Sumner and Noback (1924). The reagent used by them for the reducing sugar reaction is made up of 3,5-dinitrosalicylic acid (DNSA), phenol, sodium bisulfite, and Rochelle salt.

Miller (1959) adjusted each of these compounds and mainly optimized the Rochelle salt concentration. In subsequent experiments, the same reagent was used to determine reducing sugar (Warwick and others 1982; Bruce and Richard 1982) because the reaction of DNSA with reducing sugars is very simple.

The methods that are being used nowadays have low precision because they need either many steps of pretreatment or require many steps of precipitation and dissolution. In order to increase the quality of analytical results, new methodologies are necessary that do not require the pretreatment of the sample. Automation of those methodologies is the best way to increase the quality of the analytical results.

The proposed automated method is based on this reaction and has the following advantages: the analysis time and costs are reduced; automatic calibration for standard additions avoiding pretreatment of the sample is possible; interferences caused by the sample matrix are eliminated permitting analysis in any liquid matrix.

In a system based on an exhaustive reaction, sample volumes are introduced together with calibration solutions, and thus the calibration can be performed in a nonsegmented flow system. In our study, homogenization of the sample/standard/carrier took place in the calibration loop. Later, it was injected into the flow injection analysis (FIA) system and reacted with DNSA to obtain 3,5-diaminosalicylic acid, which was measured spectrophotometrically at 480 nm.

Materials and Methods

Apparatus and instruments

A Varian Cary 1 UV-VIS spectrophotometer equipped with

a computer and Cary software was used as a detector. A Gilson Minipuls-3 (Villiers-le Bel, France) 8-channel peristaltic pump, 4 Rheodyne 5041 (Rohnert Park, Calif., U.S.A.) low-pressure injection valves (three of them acting as switching valves), a flow cell (Starna Cells Inc., Atascadero, Calif., U.S.A.) (inner volume 18 μL) and PTFE tubing of 0.5 mm i.d. were used to build the hydrodynamic manifold. A Grant W28 thermostat was also employed.

Reagents

All reagents were of analytical grade. A bromocresol green sodium salt (Sigma Chemical Co., St. Louis, Mo., U.S.A.) stock solution was prepared by dissolving 0.10 g of dye in 6.25 mL of ethanol and diluting to 50 mL with 1×10^{-2} M sodium tetraborate solution. A solution 4.35×10^{-3} M DNSA (Sigma) in 0.4 M sodium hydroxide (J.T. Baker, Xalostoc, Mexico) was used. A sodium and potassium tartrate, Rochelle salt, (J.T. Baker, Xalostoc, Mexico), phenol and sodium bisulfite (J.T. Baker) were also used and were mixed with the DNS and NaOH in the reagent reservoir at different concentrations.

A Standard aqueous solution of glucose (Merck, Darmstadt, Germany) was prepared from 0.1 M stock solution.

FIA manifold used for the proposed method

Figure 1A shows the generic manifold used. It combines the automatic preparation of the sample standard mixed solutions for implementation of the standard addition method of FIA determination. The automatic calibration loop is the key part of the manifold. It provides automatic mixing and homogenization of sample, standard, and diluent prior to injection into the FIA system.

An appropriate volume of sample was passed through the selection valve SV_1 (the injection time will depend on the sample's reducing sugar concentration) into the calibration loop (Figure 1B.A). Later, the position of the valve SV_1 was changed and different volumes of standard solution were added (standard of glucose 1 M; Figure 1B.B; one for each sample injection). Once the sample and the standard were in the calibration loop, the position of the valve SV_2 was changed to maintain closure of the system (Figure 1B.C). After 2 min of homogenization, the valve SV_3 was changed and the homogenized mixture (sample/standard/carrier) reached the injection valve IV (Figure 1B.D). Then the homogenized mixture was injected through the valve IV into the distilled water carrier to converge subsequently with DNSA (Figure 1A). The DNSA reduction was carried out along the reactor, r, thermostated at 80 °C. Finally, the sample passed through a flow cell located in the UV-VIS spectrophotometer, where the product of the reducing reaction was measured at 480 nm.

A reproducibility study was carried out using 11 solutions of glucose injected in triplicate. The precision obtained was expressed as relative standard deviation (r.s.d.).

Results and Discussion

Fundamentals of the reaction

The determination of the reducing sugars is based on the reduction of 3,5-dinitrosalicylic acid (DNSA), in alkaline medium and at 80 °C, forming gluconic acid and the 3,5-diaminosalicylic acid that is measured with a spectrophotometer at 480 nm. The stoichiometric ratio between the DNSA and glucose is 1 to 6 and involves 12 interchanged electrons, where 2 nitro groups are reduced to amines due to the exchange of 6 electrons per each nitro group.

Sumner and Noback (1924) were the first to determine the reducing sugars using 3,5-dinitrosalicylic acid. According to them, the reagents phenol, Rochelle salt, and sodium bisulfite are needed to carry out the reaction. The role of these reagents was analyzed in subsequent works. According to Sumner and Noback (1924), phenol has no reducing effect on the DNSA but increases the color produced by the glucose (1 mg of glucose increases intensity of the color 300%). Sodium bisulfite is added to prevent instability of the product after heating. The Rochelle salt (sodium and potassium tartrate) reduces the oxygen concentration before heating,

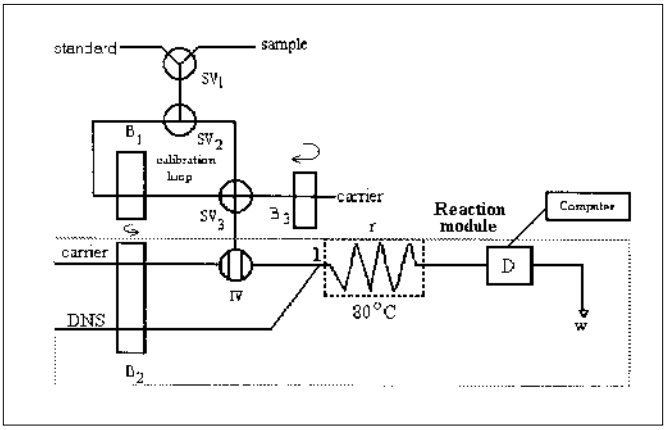

Figure 1A—Generic manifold used combining the automatic preparation of the sample standard mixed solutions for implementation of the addition standard method with FIA determination: SV, selection valve; B, pump; IV, injection valve; r, reactor; w, waste; D, detector

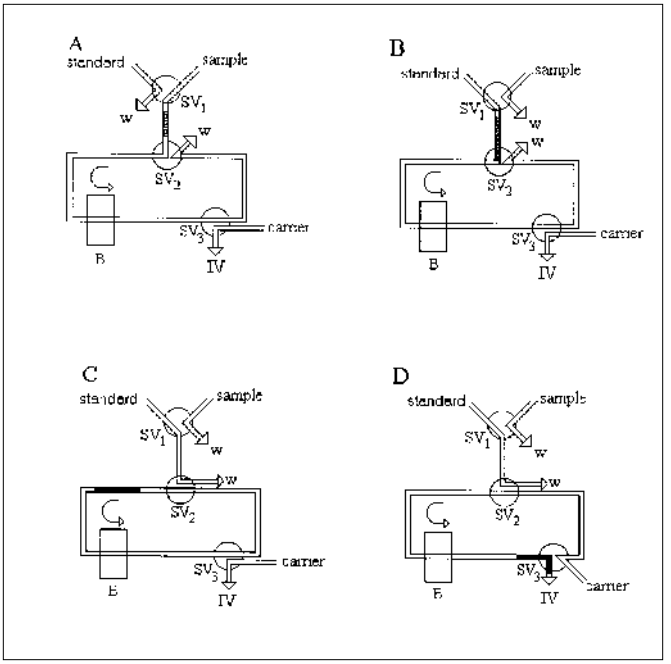

Figure 1B—Schematic diagrams for the implementation of an automatic dilution loop: (A and B) sampling position, (C) Homogenization of the sample/standard/carrier mixture, (D) Injection of the mixture. For details, see text

avoiding degradation of the sugar by the action of the oxygen in alkaline medium; therefore, the intensity of color increases due to higher DNSA reduction.

A key point of discussion in the study of the method to determine reducing sugars by reaction with DNSA was to justify each one of the reagents that takes part in such determination. A series of experiments was done, which allowed researchers to define the degree of importance of the components of the reagents: Rochelle salt, phenol, and sodium bisulfite.

First, an evaluation of the maximum absorption wavelength of the formed compound was made. The wavelength which Sumner and Noback (1924) used was incorrect, as demonstrated by the absorption spectrum where the maximum absorption occurs at 480 nm and not 540 nm. The error is understandable because a filter spectrophotometer was used.

Phenol and sodium bisulfite

The reaction of glucose with DNSA in alkaline medium was carried out with phenol and sodium bisulfite. The results were compared to those obtained without phenol and sodium bisulfite. The signal was not affected by the presence of these reagents. The variation of glucose concentration was done in a range of concentrations from 0.1 to 1.0 mg/mL.

Rochelle salt

The effect of the Rochelle salt in the reaction of glucose with DNSA in alkaline medium was studied and the results were compared to those obtained when the reaction was carried out in the absence of Rochelle salt. The glucose concentration was varied in the same range as for phenol and sodium bisulfite. The Rochelle salt did not interfere in the development of the reaction; with or without salt the spectrum obtained was the same. The intensity of the signal is increased due to the absorbance of Rochelle salt at this wavelength. The increase in the signal did not justify the economic expense that occurs from the use of Rochelle salt, so it was not necessary in the final reagent.

Optimization of the proposed method

The optimization of the method covered the study of a series of variables grouped into chemical characteristics (DNSA concentration), physical (temperature) and due to the dynamic system (volume of injection, flow-rate and reactor length). The variables that affect the calibration loop were optimized too (homogenization time of the sample / standard/carrier mixture, volume of sample and standard introduced, and volume of the calibration loop).

The DNSA concentration was studied over a range of concentrations from 4.35×10^{-4} to 8.7×10^{-2} M. The optimal value found was 4.35×10^{-3} M; above this value, even though the intensity of the signal was greater at the highest signals, double peaks were obtained possibly because of inefficient mixing due to the high reagent concentrations. For less than the optimal concentration, this kind of interference did not exist, but the intensity of the signal was lower.

The length of the reactor was studied over intervals between 60 and 190 cm. For a length of 100 cm or more, the signal was practically constant; therefore, 100 cm was selected as the optimal.

The temperature and the time of contact among the reagent and the reducing sugars were the main aspects in forming the reaction product. A range of temperature from 30 to 85 °C were studied. For higher than 80 °C, the signal obtained did not significantly increase so this (80 °C) was chosen as optimum.

Table 1—Equations calibration curves

Sample	Equation	Regression coefficient	Reducing sugars (mg/ml)
Orange juice	Y= 0.319 + 0.015X	0.9977	91.10 ± 1
Orange soft drink	Y= 0.154 + 0.0140X	0.9999	31.85 ± 1
Sprite	Y= 0.406 + 0.014X	0.9995	162.50 ± 2
Enerplex	Y= 0.207 + 0.144X	0.9990	83.10 ± 1
Cooler	Y= 0.305 + 0.010X	0.9981	128.00 ± 1
Cava Brut	Y= 0.00672 + 0.014X	0.9976	1.40 ± 2
White wine (Carlo Rossi)	Y= 0.0857 + 0.024X	0.9930	15.30 ± 1
White wine (Padre Kino)	Y= 0.0757 + 0.032X	0.9986	13.30 ± 2

The flow rates of the donor stream and the carrier influenced decisively the efficiency of the reaction. At higher flow rates, the time of contact between reagent and sample was very short and the intensity of the signal was lower.

At low flow rates, the signal increased but the analysis time was longer. A flow rate of 0.35 mL/min was selected for both the donor stream and the reagent as a compromise between the signal obtained and the frequency of sampling. The volume of injection (once the sample and the standard have passed through the dilution loop) was studied over a range of 50 to 250 μL. The optimal value was a 50 μL, internal volume of the injection valve loop. At higher volumes, double peaks were obtained.

Optimization of the calibration loop

The performance of the automatic calibration loop was assessed in order to ensure the best precision. For this purpose, the system was optimized and tested by using a stock dye solution (bromocresol green), which acted as both sample and standard, and sodium tetraborate buffer, which functioned as carrier.

The volume of the closed system was also measured and found to be 360 μL. The volume introduced into the closed flow system was controlled by the SV_2 and SV_3 valves, the first one standing at injection position and the second one in filled position. A peristaltic pump, P1, controlled the discharge. Different volumes of samples and/or standards were introduced into the calibration loop. These depended on the flow rate and the injection time, even though the study was done mainly between 40 and 250 μL. The total volume of the calibration loop can be varied to obtain the correct dilution of the sample and the standard and to increase the sensitivity of the method.

To determine the time in which the mixture (sample/standard/carrier) was homogenized, different volumes of sample and standard were introduced into the calibration loop and were injected into the flow injection system at different times. The minimum time required for total homogenization, measured through the absorbance of the dye, was 2.0 min.

Implementation of the automated standard additions method

A glucose standard was used for the calibration curves. The analytical signal of the DNSA with different reducing

sugars is not the same, so the slope of the calibration curves is also different. To many soft drinks are added different amounts of glucose and fructuose syrup, but the addition rate is not always the same, so we used only a glucose standard solution for the standard additions. On the other hand, this method is more precise than the AOAC method where the precision was at 10%, whereas in the proposed method the precision was at 2.3 %.

The implementation of the automated calibration loop, designed for the application of the method of standard additions, was verified using a standard 1 M glucose solution and samples of glucose of known concentration (0.4 and 0.8 M).

Calibration curves were constructed for each glucose solution, introducing to the calibration system different volumes of the standard 1 M glucose solution (41.7, 83.3, 125, 166.7, and 208.3 μL that are equal to 7.5, 15.00, 22.5, 29.9, and 37.4 mg, respectively) and 125 μL of glucose dilutions of 0.4 and 0.8 M (adequate volume for these glucose concentrations) (Figure 2). Each standard addition was done 3 times. It was found that, for the 0.4 M glucose solution, the concentration was 0.41 M, having an error of 3.5%. For the glucose solution of 0.8 M, the error was 2% (0.82 M), proving the reliability of the method.

The precision of the method was studied by repetition and expressed as relative standard deviation for eleven 0.3 M glucose solutions. The value found was 2.3 %.

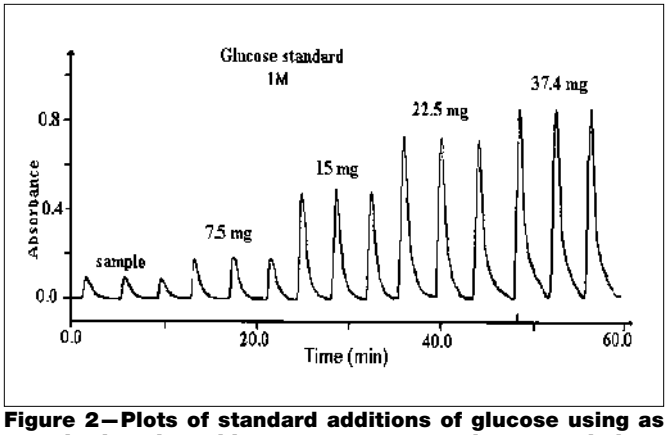

Figure 2—Plots of standard additions of glucose using as sample the wine white; 7.5, 15, 22.5, and 37.4 mg belong to glucose

Applying the proposed method to soft drinks and wines

The proposed method was used to determine the total reducing sugars in liquid samples without pretreatment. A 1 M glucose standard was used and different amounts were introduced into the calibration loop together with the samples. The volume of sample introduced depends on the signal obtained, varying from 30 to 125 μL. For all the samples, more than 5 points were used to construct the calibration curves (Figure 3, A and B).

Table 1 shows the results for soft drinks and wines using the standard additions method and the equations of the calibration curves.

To ensure that ethanol, which is present in the composition of some samples, does not interfere with the reducing sugar determination, an analysis of glucose samples was carried out with different ethanol concentrations (10, 20, 30, and 50% (v/v). The obtained signals are not affected by the ethanol presence. The slopes of the calibration curves in all cases are different because they are influenced by the reducing sugar concentrations of each sample. With wines which contain less reducing sugar concentration, the slope is lower.

Conclusions

FOR DECADES, A METHOD HAS BEEN USED WITHOUT OMITting any reagents. The justification of each component of the original reagent has led to a decrease in the cost of determining total reducing sugars, as it is not necessary to use the Rochelle salt, which was added to the reagent at 30% w/v. Also, the determination of reducing sugars by reaction with 3,5-dinitrosalicylic acid was simplified by using a nonsegmented flow configuration together with an automatic calibration. The analytical procedure allows the direct analysis of liquid samples—without sample treatment—with automated on-line calibration. This allows a throughput of more than 10 samples per hour, which exceeds that of the official AOAC procedure, allowing implementation in a QA/QC program better than what can be achieved with the official procedure.

The results of the study demonstrate a savings of 90% in the cost of the analysis compared with the method of the AOAC, which is of great economic importance to laboratories.

Furthermore, the methodology proposed can be used in the routine determination of total sugars in liquid samples or semisolids by first making a dilution or by dissolving solids in water.

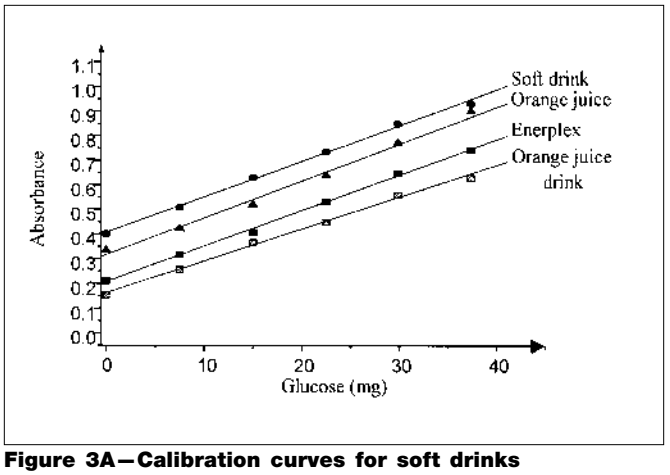

Figure 3A—Calibration curves for soft drinks

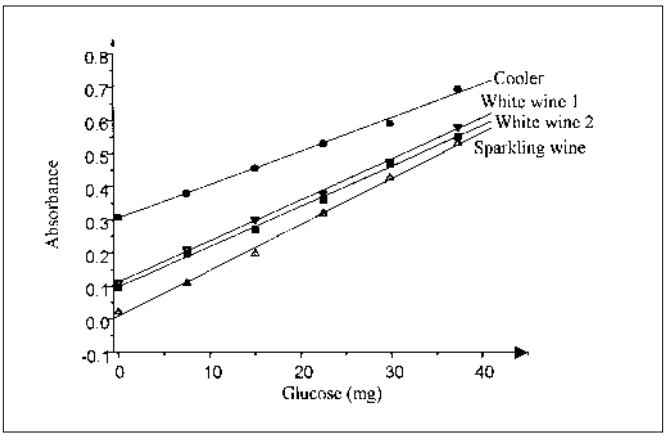

Figure 3B—Calibration curves for wines

References

Agudo M, Rios A, Valcárcel M. 1992. Automatic calibration and dilution in unsegmented flow systems. Anal Chim Acta 264:265-273.
Agudo M, Rios A, Valcárcel M. 1995. Automatic implementation of the method of standard additions in unsegmented flow system. Anal Chim Acta 308:77-84.
Amine A, Marrazza G, Mascin M, Patriarche GJ. 1991. Amperometric determination of glucose in undiluted food samples. Anal Chim Acta 242:91-98.
[AOAC] Association of Official Analytical Chemists. 1995. Official Methods of Analysis, 16th ed. Washington, DC: Association of Official Analytical Chemists.
Barón A, Guzmán M, Ruzicka J, Christian GD. 1992. Novel single standard calibration and dilution method performed by the sequential injection technique. Analyst 117:1839-1844.
Bruce EH, Richard ED. 1982. Glucose measurement errors in enzymic starch hydrolysates at high enzyme-glucose weight ratios. Physil Plant 54:244-248.
Chi QJ, Dong SS. 1995. Amperometric biosensor based on the immobilization oxidases in a Prussian blue film by electrochemical codeposition. Anal Chim Acta 310:429-436.
Karyakina AA, Gitelmacher OV, Karkayina EE. 1995. Prussian blue-based first-generation biosensor: A sensitive amperometric electrode for glucose. Anal Chem 67:2419-2423.
Marko-Vargas G, Dominguez E. 1991. Trends in Anal Chem. Vol. 10:290-297.
Massom M, Townshend 1984. Determination of glucose in blood by FIA and an immobilized glucose oxidase column. Anal Chim Acta 166:111-118.
Miller LG. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426-428.
Nobutoshi K, Furusawa M, Inove Y. 1991. Flow injection system for the fluorimetric determination of fructuose with an immobilized mannitol dehydrogenase reactor. Anal Chim Acta 243:183-186.
Ruzicka J, Hansen EH. 1988. Flow Injection Analysis, 2nd ed. New York: John Wiley and Sons, Inc. p. 60-83.
Sperling M, Fang Z, Welz B. 1991. Expansion of dynamic working range and correction for interferences in flame atomic absorption spectrometry using flow-injection gradient ratio calibration with a single standard. Anal Chem 63:151-159.
Sumner JB, Noback CVJ. 1924. The estimation of sugar in diabetic urine, using dinitrosalicylic acid. J Biol Chem 62:287-
290.
Verette E, Fabrice M, Francois Q. 1995. On-line dialysis with high-performance liquid chromatography for the automated preparation and analysis of sugars and organic acids in foods and beverages. J Chromat A 705:195-203.
Wang J, Liu J, Chen L, Lu F. 1994. Highly selective membrane-free, mediator-free glucose biosensor. Anal Chem 66:3600-3603.
Warwick L, Gray P, Greg J, Quilan M. 1982. Evaluation of the DNS method for analyzing lignocellulosic hydrolisates. J Chem Tech Biotechnol 32:1016-1022.
Yang J, Boyston TD, Powers JR, Weller KM. 1999. Sugars and free amino acids in stored russet Burbank potatoes treated with CIPC and alternative sprout inhibitors. J of Food Sci. 64(4):592-596.
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Authors Cañizares, Hernández and Gómez are with the Deptartment of Analytical Chemistry, Faculty of Chemistry, National University of Mexico, Ciudad Universitaria, C.P. 04510 Mexico D.F., Mexico. Direct inquiries to author Cañizares (email: pilarm@servidor.unam.mx)