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A simple automated microplate method for determining reducing sugars in food extracts and synthetic serum using cupric-neocuproine as reductant

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Abstract: In the present work, a simple automated microplate method based on cupric ion reduction is described for determining total reducing sugars in food extracts and synthetic serum. The reaction of Cu(II)-Nc (cupric-neocuproine) with reducing sugars was performed in alkaline medium in microplates, and the absorbance of the formed highly colored Cu(I)-Nc chelate in a plate reader at 450 nm was recorded. The proposed method was applied to reducing sugars (glucose, fructose, galactose, maltose, and lactose) and their linear calibration curves were constructed. The detection and quantification limits (LOD and LOQ) for glucose were 0.14 and 0.46 μM, respectively. Absorbances of glucose were linear within the concentration range 2.5–54.2 μM and the method showed high linearity (r = 0.9998) over a relatively broad concentration range of analyte. This automated microplate method was validated through linearity, additivity, precision (RSD%, 2.33–6.65), and recovery (101%–103%), revealing that the method is reliable and robust for determining reducing sugars. Total reducing sugar contents of synthetic sugar mixtures, fruit juices, milk, and synthetic serum samples were successfully determined with the proposed method. The results were compared to those of the conventional alkaline Cu(II)-Nc spectrophotometric method. The proposed method offers many advantages when compared to classical methods, such as (sample and reagent) volume reduction (20-fold), simplicity, multiple sample analysis (32 samples in 4 h), and environmental friendliness.

Key words: Reducing sugars, glucose determination, microplate reader, fruit juice, serum

1. Introduction

Carbohydrates (also called saccharides), composed of C, H, and O atoms, constitute an essential macronutrient class synthesized by plants that can be used as a main energy source in human nutrition.[1,2] Saccharides are classified as monosaccharides (glucose, fructose, and galactose), disaccharides (sucrose, lactose, and maltose), oligosaccharides (maltodextrin, cyclodextrin, etc.), and polysaccharides (starch, glycogen, and cellulose). Free-form monosaccharides (simple sugars) and disaccharides are called sugars, and they can also be classified according to their chemical reactions, e.g., reducing and nonreducing sugars.[3,4] The main difference between reducing and nonreducing sugars is that reducing sugars have free hemiacetal or hemiketal groups[5] and can be oxidized by weak oxidizing agents. The reducing sugars include glucose, fructose, and galactose as monosaccharides and lactose and maltose as disaccharides (Figure 1).

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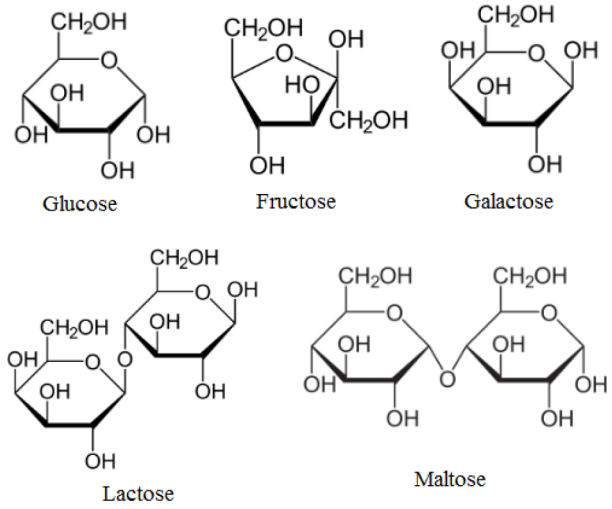

Figure 1. Molecular structures of the reducing sugars.

Identification and quantification of simple carbohydrates is important for the food and beverage industry, because sugars are the indicators of certain food characteristics such as taste, flavor, and naturalness.[6] Reducing sugars are important not only for food, but are an indispensable component of biological samples such as of blood, serum, plasma, and tissue.[7] European legislation controls the sugar concentration of specific foods and beverages.

Determination of total reducing sugars is an integrated step for evaluating other parameters because they are the final products of many biological and enzymatic interactions. Enzymatic hydrolysis of maize straw polysaccharides was investigated for the production of reducing sugars.[8] The reducing sugar content is the limiting factor for color development of potato during the frying process.[9−11] Fruit ripening stage can be evaluated by determining total sugar content. In a modeling study regarding reducing sugar production by enzymatic hydrolysis of cellulose, the produced reducing sugars may inhibit the enzyme in a reversible and competitive manner, rendering their determination important.[12] In the wine industry, the determination of reducing sugars plays an important role. The total reducing sugar content is an important parameter to monitor the fermentation process during wine production. Reducing sugars' determination in wines is also performed for routine quality control and for checking whether the wine meets the legal or commercial criteria of reducing sugars content required for a certain wine type.[13]

In general, sugar determination methods exploit the oxidizability of reducing sugars with suitable reagents. The oldest and most common of these are Somogyi and Nelson (SN),[14,15] Park and Johnson (PJ),[16] 3,5-dinitrosalicylic acid (DNS),[17] copper-bicinchoninate (CuBic),[18,19] and neocuproine (Nc)[20,21] methods. As indicated in the literature, these methods have some disadvantages. For example, the DNS assay required separate calibration curves for each sugar, and sugars with more linkages were overoxidized with the generation of higher absorbance values.[5,22,23] In our previous study, we utilized Cu(II)-Nc reagent in alkaline medium[24] for reducing sugar analysis and compared our findings with the DNS method, showing that the sensitivity and reproducibility of the DNS method are not satisfactory. It has also been reported that the accuracy of the PJ method is affected by the size of reducing sugars[5] and this problem was solved by Hizukuri et al.[25]

Recently, many colorimetric assays have been adapted to microplate format to allow the automation of the assay. With the use of microplate systems, a number of advantages have emerged such as rapid screening

of large numbers of samples that may substantially reduce labor, time, and reagents.[26,27] Nowadays, the consumption of chemicals in large quantities may pose a great threat to natural life protection. The main objective of green analytical chemistry is to minimize the consumption of chemicals (raw materials, reagents, solvents, etc.) by developing new methods and procedures.[28–31] For the determination of sugars in foods and biological samples, many microplate-based methods were developed, most of which were enzymatic and had extra processing steps.[5,32–34] Gonçalves et al. adapted the most common method of reducing sugars determination (using dinitrosalicylic acid: DNS)[35] to a microplate system, which unfortunately required oven heating (at 105 °C) to accelerate the chemical reaction prior to the microplate reading stage. In the mentioned study, it was recommended to use microplates made from crystal polyester to resist this high temperature. The reaction time was 10 min and the absorbances were measured at 540 nm. The linear concentration range for glucose was found to be 0.06–4.00 g L^{-1}. Negrulescu et al. also applied the microplate version of the DNS method.[17] This application differs from the previous one in that the heating process is carried out in a microwave oven. The system used for the reaction consisted of a plastic vessel filled with water, as thermal buffer, containing the microtiter plate placed in a polystyrene support. In this study, the reaction time was 4 min and the absorbances were measured at 540 nm. The linear range for glucose was 0.05–50 mg mL^{-1}. In a recent study, the SN method was adapted to 96-well microplates by Shao and Lin.[5] The reagent used in the study was prepared separately using many chemicals (sodium potassium tartrate, sodium carbonate, sodium bicarbonate, sodium sulfate, copper sulfate, ammonium molybdate, concentrated sulfuric and sodium arsenate dibasic pentahydrate). Furthermore, this multichemical reagent was incubated at 37 °C for 24–48 h. It was emphasized that a heat-resistant polypropylene microplate should be used. After the sample and working reagent were mixed, the microplate was sealed with a silicone mat and taped with a layer of aluminum foil to prevent water vapor from entering the plate, which was kept in a boiling water bath for 20 min. The plate was then placed in a zip-lock bag, cooled with running cold water for 5 min, and absorbances were read at 600 nm 15 min after addition of the color reagent. Selection of 600 nm was explained by the fact that the change in absorbance was smaller than 0.05 and that if a wavelength of 750 nm was chosen color formation would complete in 1.5 h. It was further stated that the linear concentration range of studied sugars (glucose, maltose, maltotriose, maltotetraose, and maltoheptaose) was 0.01–0.6 mM.

In the present study, we adapted the recent alkaline Cu(II)-Nc spectrophotometric method of total reducing sugar assay[24] to an automated microplate-based procedure. The oxidation had to be carried out in alkaline medium, because Singh et al. showed that the rate of oxidation of reducing sugars is directly proportional to the concentration of reducing sugar and free hydroxyl ions and independent of Cu(II) oxidant concentration.[36] The working principle of the alkaline Cu(II)-Nc assay is the reduction of Cu(II)-Nc complex by reducing sugars to the highly colored Cu(I)-Nc charge-transfer chelate in alkaline medium, where the absorbance of the cuprous chelate against a reagent blank was followed at the maximal absorption wavelength of 450 nm.

The proposed method was successfully applied to individual and synthetic mixtures of reducing sugars, commercial fruit juices, milk, and synthetic serum samples. The results were compared and correlated with those of the alkaline Cu(II)-Nc spectrophotometric method. The proposed method not only enables the simultaneous analysis of 96 samples but also offers additional advantages of volume reduction of solvents and reagents, reduction of experimental steps, and simplicity of procedures.

2. Results and discussion

The main purpose of the present study was to adapt the alkaline Cu(II)-Nc spectrophotometric method of reducing sugars assay to a microplate system that may introduce several superiorities over existing colorimetric assays. The microplate assay was optimized with respect to temperature and time, its analytical performance was determined, and it was applied to synthetic mixtures and real samples in comparison to the conventional assay. A separate pH optimization was not conducted, because the conventional assay uses an alkaline medium to enable the enolization of reducing sugars with free hydroxyl ions at the slowest step determining Cu(II) oxidation kinetics of sugars.[36] Polyphenolic interferents to the proposed method were eliminated by solid phase extraction (SPE) prior to color development.

2.1. Assay optimization

Varying temperatures between 30 and 45 °C and time intervals between 1 and 360 min were tested for the optimization of the microplate-based total reducing sugar assay. The microplate reader, being a closed system, cannot be operated at temperatures exceeding 45 °C except with additional processing steps as in some assays.[5,17,32–34,37] The change in molar absorptivity of glucose versus time is summarized in Figure 2 at four different temperatures. As can be seen in Figure 2, the reaction reached saturation within 240 min at 45 °C. The incubation time according to the conventional alkaline Cu(II)-Nc spectrophotometric method was longer due to its lower incubation temperature. On the other hand, as a distinct advantage over the old DNS method,[37] the proposed method required neither elevated temperatures (such as a boiling water bath) nor strong acidity (using hazardous sulfuric acid). The proposed method provides an advantage over the microplate SN assay[5] due to the easy preparation and stability of the reagent solution. In the mentioned assay, concentrated acid was used for reagent preparation and incubated at 37 °C for 24–48 h, followed by a tedious series of processes such as boiling and cooling of the plates. Similarly, microwave heating was applied to accelerate the reaction in the microplate-based DNS method.[17] In addition, the necessity of the use of heat-resistant microplates and of special precautions taken for heating are serious drawbacks. It was also emphasized that the final solution should be filled and drained several times by pipetting to prevent bubble formation after the addition of colorant reagent in the microplate-based SN method.[5]

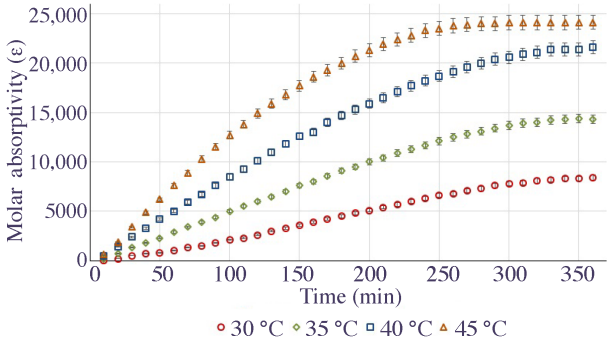

Figure 2. Changes of molar absorptivity (ε, M^{-1} cm^{-1}) of glucose (with respect to the microplate Cu(II)-Nc method) versus time at four different temperatures.

2.2. Analytical figures of merit

The absorbance signals recorded by the proposed method were linearly dependent upon the concentrations of the tested reducing sugars. The linear equation for the calibration graph of glucose drawn at the wavelength of 450 nm with respect to the proposed method was $A_{450} = 2.3 \times 10^4$ C – 0.0071 (r = 0.9998), where C symbolized molar concentration, and the molar absorptivity of glucose was calculated as $\varepsilon = 2.3 \times 10^4$ L mol^{-1} cm^{-1}. The correlation between the absorbance (A) for a tested reducing sugar and its molar concentration (C) was evaluated using five compounds. Standard calibration curves of tested reducing sugars with respect to the proposed method are presented in Figure 3. Table 1 summarizes the linear calibration equations ($A = mC + n$), correlation coefficients (r), linear concentration ranges, limit of detection (LOD), and limit of quantification (LOQ) of tested reducing sugars, defined as LOD = 3 s_{bl} / m and LOQ = 10 s_{bl} / m, respectively (where s_{bl} is the standard deviation of a blank and m is the slope of the calibration line). The LOD and LOQ values for glucose were 0.14 and 0.46 μM, respectively. Absorbances of glucose were linear within the concentration range 2.5–54.2 μM (as final concentrations in solution), and the method showed high linearity (r = 0.9998) over a relatively broad concentration range of analyte. The LOD values and linear concentration ranges of glucose obtained by different methods are compared in Table 2. When Table 2 is examined, the sensitivity of the proposed method is observed to be higher because the LOD value is much lower than those of the other literature methods.

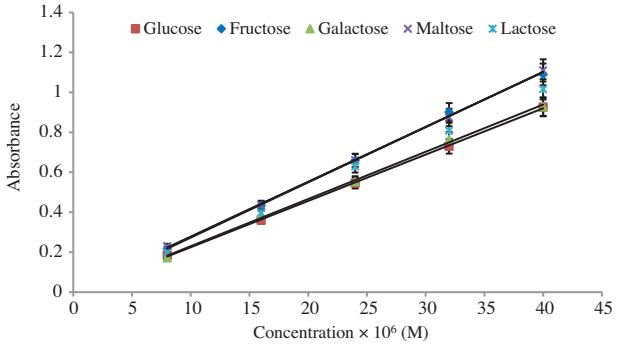

Figure 3. Standard calibration curves of tested reducing sugars with respect to the microplate Cu(II)-Nc method.

The precision and recovery of the microplate Cu(II)-Nc method by using known amounts of glucose as spiking sugar to apple juice and synthetic serum are depicted in Table 3. The precision, which is expressed as the relative standard deviation (RSD, %) in absorbance measurement within the tested concentration range, was approximately 6.4%. The recovery of the method varied from 101% to 103% for individual batches of glucose. The total reducing sugars of the original apple juice and synthetic serum (i.e. without spike) are shown in Table 3 in the units of mg glucose L^{-1}. The recoveries for the spiked glucose were almost quantitative because of the additivity property of glucose levels in accordance with Beer's law. This law was valid within the tested concentration range (of original and spiked glucose) because a single colored product, Cu(I)-Nc, was formed as a result of the quantification reaction and there were no chemical interactions between reaction constituents that would otherwise give rise to chemical deviations from Beer's law.

For statistical comparison of the proposed and conventional Cu(II)-Nc spectrophotometric methods, sugar contents of glucose standards, apple juices, and synthetic serum samples were calculated using glucose calibration curves. The results of Student's t- and F-tests are presented in Table 4, showing that there was no significant difference in accuracy and precision of the two methods at the 95% confidence level.

Table 1. The linear calibration equations, linear concentration ranges, limit of detections (LOD) and limit of quantifications (LOQ) of the tested sugars with respect to the microplate Cu(II)-Nc method.

Reducing sugars	Linear equation and correlation coefficient	Linear range (μM)	LOD (μM)	LOQ (μM)
D-(+)-Glucose	A = 2.3 × 10^4C – 0.007 r = 0.9998	2.5–54.2	0.14	0.46
D-(-)-Fructose	A = 2.8 × 10^4C – 0.0030 r = 0.9994	1.9–44.1	0.05	0.16
D-(+)-Galactose	A = 2.2 × 10^4C – 0.0061 r = 0.9994	2.6–57.9	0.13	0.42
D-(+)-Maltose	A = 2.8 × 10^4C + 0.0028 r = 0.9996	1.7–44.9	0.05	0.16
D-Lactose	A = 2.6 × 10^4C – 0.0054 r = 0.9993	2.1–47.4	0.09	0.31

Table 2. Comparison of the LOD values and linear concentration ranges for different methods of glucose determination.

Methods	LOD (μM)	Linear range (μM)	References
Microplate-based alkaline Cu(II)-Nc	0.14	2.5–54	In this study
Alkaline Cu(II)-Nc	0.6	4–30	24
DNS	80	300–800	37
Microplate-based DNS	83	277–30,751	17
Microplate-based anthrone-sulfuric acid	16	55–666	27
Microplate-based Somogyi–Nelson	-	10–600	5

2.3. Hydrolysis of sucrose

Sugars having acetal or ketal linkages do not directly react with reducing-sugar test reagents because they do not have free aldehyde chains like reducing sugars. After hydrolysis (with dilute hydrochloric acid) of a nonreducing sugar with subsequent neutralization of the acid excess, the products react with the test solutions in the same way as reducing sugars, exemplified by a sucrose sample. Thus, the indirect determination of sucrose becomes possible. By hydrolyzing different quantities of sucrose, the reaction yield was determined. The performance of the hydrolysis procedure was evaluated by applying the proposed microplate and reference methods to the hydrolysate as well as to the possible hydrolysis products of glucose and fructose in a standard mixture. Glucose equivalent sugar contents of sucrose hydrolysate and of a synthetic mixture comprising glucose and fructose are shown in Table 5. These results demonstrate that the microplate-based method can be used accurately for the determination of total reducing sugars in samples containing sucrose hydrolysate or equivalent constituents (Table 5).

2.4. Total reducing sugar content measurement of synthetic mixture solutions

Possible combinations of synthetic sugar mixtures composed of (glucose, fructose, galactose, maltose, lactose) and {sugar + reducing compounds (that are likely to be found in the sample matrix)} were prepared, and

Table 3. Precision and recovery of the microplate Cu(II)-Nc method (N = 3).

		Concentration (mg glucose L^{-1})
Glucose addition to apple juice	Apple juice	11.7
	Added conc.	7.2
	Mean[a]	19.1
	S.D.[b]	0.47
	R.S.D., %[c]	6.35
	REC, %[a,d]	103
Glucose addition to synthetic serum	Synthetic serum	0.75
	Added conc.	0.72
	Mean[a]	1.48
	S.D.[b]	0.017
	R.S.D., %[c]	2.33
	REC, %[a,d]	101

[a] Mean and recovery (%) were calculated on the basis of concentration of total glucose (original + added).
[b] Standard deviation. [c] Relative standard deviation. [d] Recovery.

the suitably diluted solutions were analyzed for total reducing sugar content (as mg glucose L^{-1}) using the microplate Cu(II)-Nc method (Table 6). Polyphenolic compounds were added to synthetic mixtures to see their possible interferences to the proposed method in real sample analysis, and SPE was applied to synthetic mixtures for clean-up of polyphenolics. Recently, the C18 SPE technique has often been used for extraction of sugars and some organic acids from complex solution media.[38] C18 column material is generally used in reversed phase extraction of nonpolar-to-moderately polar compounds (including phenolics). Therefore, the proposed method was repeatedly applied to the synthetic mixtures before and after SPE separation so as to observe the reagent color arising from (sugars + interferents) versus sugars alone. Moreover, SPE was applied to lone sugar mixtures that contained the same type and composition of sugar components as in synthetic mixtures. The results are presented in Table 6. Without a preliminary SPE clean-up procedure, all synthetic sugar mixtures containing interferents gave positive errors in sugar determinations with the proposed method, as expected. However, the recovery values observed after SPE (between 98% and 104%) confirmed that reducing sugars could be quantitatively separated from common interferent compounds and accurately determined in synthetic samples (Table 6).

2.5. Total reducing sugar content measurement of real samples

Table 7 shows the total reducing sugar content values of real samples (fruit juices, milk, and synthetic serum), expressed as g glucose 100 mL^{-1}. The microplate and alkaline Cu(II)-Nc spectrophotometric methods were used for evaluating total reducing sugar contents of real samples. Fruit juices rich in polyphenolic compounds (which may act as interferents in the determination of reducing sugars) have been particularly preferred as real samples. The samples were sequentially passed through two different SPE cartridges (C18 and polyamide) to remove the interference from polyphenolic compounds. The contents determined by both methods were significantly lower than those declared for apricot and cherry juices. Hydrolysis was also applied to these samples by considering

Table 4. Statistical comparison of the proposed and alkaline Cu(II)-Nc spectrophotometric methods' findings of glucose standards (14.4 mg L^{-1}), apple juices (declared sugar content 12.67 g per 100 mL), and synthetic sera (at the 95% confidence level).

Sample	Parameter	The microplate Cu(II)-Nc		Alkaline Cu(II)-Nc
Glucose standards	No. of samples	5		5
	Average	14.9		14.7
	Standard deviation	0.56		0.21
	Variance	0.31		0.04
	Degrees of freedom		8	
	$t_{calc.}$		0.040	
	$t_{crit.}$		2.306	
	$F_{calc.}$		0.002	
	$F_{crit.}$		6.390	
Apple juices	No. of samples	5		5
	Average	12.1		12.1
	Standard deviation	0.29		0.18
	Variance	0.08		0.03
	Degrees of freedom		8	
	$t_{calc.}$		0.614	
	$t_{crit.}$		2.306	
	$F_{calc.}$		0.416	
	$F_{crit.}$		6.390	
Synthetic sera	No. of samples	5		5
	Average	0.763		0.746
	Standard deviation	0.0185		0.0247
	Variance	0.0003		0.0006
	Degrees of freedom		8	
	$t_{calc.}$		1.108	
	$t_{crit.}$		2.306	
	$F_{calc.}$		1.029	
	$F_{crit.}$		6.390	

calc. and *crit.* are abbreviations for the calculated and critical values (at 95% confidence level), respectively, of statistical t- and F-parameters.

that they may contain sucrose. As a result, significant differences were observed in the sugar contents of apricot and cherry juices, which were higher than the previous values. These values are shown in parentheses in Table 7. As can be seen from Table 7, differences between the declared and experimentally found sugar values for fruit juices and milk were max. 8%–12%, respectively. However, the methods employed in determining the reducing sugar contents of these commercial samples were not declared on their packing labels. In addition, there may be an accuracy problem in the declared values. Although the sugar content was not declared for the synthetic serum sample, the values obtained by the proposed and reference methods were compatible with each other.

Table 5. Glucose equivalent sugar contents of sucrose hydrolysates and of synthetic mixtures comprising glucose and fructose (1:1), as determined by the proposed microplate and conventional alkaline Cu(II)-Nc methods.

Sample	The microplate Cu(II)-Nc (mg glucose L^{-1})	Alkaline Cu(II)-Nc (mg glucose L^{-1})
Sucrose[a] hydrolysate	358 ± 6	371 ± 8
(Glucose + fructose)[b]	374 ± 6	362 ± 7
Sucrose[a] hydrolysate	710 ± 10	729 ± 12
(Glucose + fructose)[b]	738 ± 11	721 ± 8

[a] Sucrose concentrations are 360.3 and 720.6 mg glucose L^{-1}, respectively (N = 3).
[b] Synthetic mixtures were prepared as theoretically equivalent to the hydrolysis products of sucrose.

Table 6. Total reducing sugar content (as mg glucose L^{-1}) of synthetic mixtures with respect to the microplate Cu(II)-Nc method.

Sample	Expected	Before SPE	After SPE
Synthetic mixture 1 (glucose, fructose, galactose, bilirubin, catechin, uric acid)	11.4	32.1 ± 1.9 (181.6)[a]	11.9 ± 1.0 (4.4)[a]
Sugar mixture 1 (glucose, fructose, galactose)	11.4	-	11.7 ± 1.2 (2.6)[a]
Synthetic mixture 2 (glucose, maltose, lactose, bilirubin, catechin, uric acid)	12.0	33.7 ± 2.3 (180.8)[a]	12.5 ± 0.4 (4.2)[a]
Sugar mixture 2 (glucose, maltose, lactose)	12.0	-	12.0 ± 1.3 (0)[a]
Synthetic mixture 3 (glucose, fructose, galactose, maltose, lactose, bilirubin, catechin, uric acid)	19.8	37.8 ± 1.8 (90.9)[a]	20.1 ± 1.0 (1.5)[a]
Sugar mixture 3 (glucose, fructose, galactose, maltose, Lactose)	19.8	-	19.4 ± 1.8 (–2.0)[a]

[a] Error % values are given in parentheses.

In conclusion, the use of the microplate Cu(II)-Nc method proposed for total reducing sugar content determination revealed that it is possible to analyze large numbers of samples by using smaller amounts of reagents and samples. Handling of lower volumes of reagents and samples may serve the purpose of green chemistry. While 250 μL of solution was consumed in the proposed microplate based method, the corresponding total volume used in the conventional method was 5 mL. As a result, a 20-fold reduction in solution volume was achieved. Although the analysis time per sample was longer than that of the reference method, the ability to perform the simultaneous analysis of 96 samples saved overall time, owing to the lower temperature employed. In the proposed method, 32 samples (each sample analyzed in triplicate) can be analyzed in 4 h, but the same analysis with the conventional method takes about 11 h. Beer's law was obeyed over a wide concentration

Table 7. Total reducing sugar content (as g glucose 100 mL^{-1}) of studied samples; the declared and found values by the proposed and alkaline Cu(II)-Nc methods.

Sample	The microplate Cu(II)-Nc	Alkaline Cu(II)-Nc	Declared
Apricot juice	10.00 ± 0.10 (14.19 ± 0.50)[a]	10.15 ± 0.15 (13.85 ± 0.66)[a]	14.00
Peach juice	14.28 ± 0.16	14.09 ± 0.29	14.90
Cherry juice	10.36 ± 0.24 (13.28 ± 0.08)[a]	10.39 ± 0.38 (13.11 ± 0.78)[a]	13.50
Apple juice	12.09 ± 0.29	12.10 ± 0.18	12.67
Pomegranate juice	12.25 ± 0.39	11.90 ± 0.15	12.30
Milk	4.06 ± 0.04	4.06 ± 0.08	4.6
Synthetic serum	0.76 ± 0.02	0.75 ± 0.02	-

[a] Results found after hydrolysis are given in parentheses.

range (2.5–54.2 μM for glucose in the microplate assay instead of 4–30 μM in the alkaline Cu-Nc assay). Because the reagent injection and absorbance measurements in the automated system were conducted in a short time, the number of experimental procedures decreased and possible error margin originating from the analyst was reduced in comparison to the conventional method. Sugar determination by simple colorimetry is such a hot topic in analytical sciences that the paper entitled "*Colorimetric method for determination of sugars and related substances*" by Dubois et al. has received more than 27,000 citations, and still is one of the most downloaded articles ever published in the ACS journal *Analytical Chemistry*.[39] In this regard, a microplate Cu(II)-Nc colorimetric method compatible with green analytical chemistry was developed for reducing sugars determination in food and biological matrices that could be used in a simple, reliable, and robust methodology.

3. Experimental

3.1. Chemicals and samples

Commercial fruit juices and UHT milk samples were obtained from local markets.

3.2. Instrumentation

Absorbance measurements for the conventional alkaline Cu(II)-Nc method were recorded using a Varian CARY Bio 100 UV-Vis spectrophotometer (Mulgrave, Victoria, Australia). Quartz cuvettes with 1 cm path length were used for the UV-vis measurements. A BioTek Synergy H1 multimode 96-well microplate reader and Gen5 2.0 software (Winooski, VT, USA) were used for absorbance measurements of the proposed microplate Cu(II)-Nc method. Other necessary laboratory equipment comprised an Elektro-Mag M 4812 P laboratory centrifuge apparatus (İstanbul, Turkey), Hanna HI2002-02 pH-meter using a glass electrode (Woonsocket, RI, USA), Elektro-Mag vortex apparatus (İstanbul, Turkey), Memmert water bath (Schwabach, Germany), Agilent Vac Elut 12 position manifold (Santa Clara, CA, USA) and Isolab vacuum pump (İstanbul, Turkey). Pure water used to prepare and dilute all solutions was obtained from a Millipore Simpak1 Synergy185 (Burlington, MA, USA) ultra-pure water system.

3.3. Preparation of solutions

$CuCl_2$ solution (1.0×10^{-2} M), 0.5 M NaOH alkaline solution containing 2% (w/v) Na_2CO_3, and 0.1 M sodium potassium tartrate were prepared in distilled water. Neocuproine (Nc) solution (1.5×10^{-2} M) and

catechin hydrate standard were prepared in ethanol. All reducing sugar standards were prepared in distilled water. Uric acid and bilirubin were dissolved in (0.1 M) NaOH, and the excess base was neutralized with a suitable volume of (0.1 M) HCl solution.

Synthetic mixture solutions were prepared containing standard reducing sugar and interferent compounds solutions. The mixture components were selected on the basis of sugars and possible interferent compounds found in natural samples. Moreover, these sugar mixtures were prepared without interferent compounds to compare SPE recovery values. They were prepared to contain standards at the final concentrations declared below:

(1) 2×10^{-5} M glucose, fructose, galactose, bilirubin, catechin, uric acid, separately.

(2) 2×10^{-5} M glucose, maltose, lactose, bilirubin, catechin, uric acid, separately.

(3) 2×10^{-5} M glucose, fructose, galactose, maltose, lactose, bilirubin, catechin, uric acid, separately.

The milk sample was prepared by adding 2 mL of 70% (w/v) trichloroacetic acid (TCA) to 2 mL of milk to precipitate the proteins, followed by centrifugation for 5 min at 4500 rpm. One milliliter of supernatant was filtered through the GF/PET microfilter. Commercial fruit juice samples were prepared by filtration through the GF/PET microfilter. The synthetic fetal bovine serum sample was diluted (1:10) with distilled water.

3.4. Analytical methods

3.4.1. Alkaline Cu(II)-Nc spectrophotometric method

The total reducing sugar content was evaluated by alkaline Cu(II)-Nc spectrophotometric method as described by Sözgen Başkan et al.[24] To a test tube were added 1 mL of $CuCl_2$, 1 mL of Nc, x mL of sample, (1 – x) mL of distilled water, 1 mL of alkaline solution, and 1 mL of sodium potassium tartrate solution in this order. The mixture in the stoppered tube was incubated for 20 min in a thermostated water bath at 60 °C. The tubes were cooled to room temperature, and their absorbance values were measured against a blank at 450 nm.

3.4.2. Proposed microplate Cu(II)-Nc method

The alkaline Cu(II)-Nc spectrophotometric method to assess the total reducing sugar content of food extracts and synthetic serum was adapted with some modifications to the 96-well microplate format. The microplate system contained two dispensers. Dispenser (1) was filled with Cu(II):Nc:tartrate (1:1:1, v/v/v) reagent solution and dispenser (2) with alkaline solution. A volume of 50 μL of each sample was micropipetted to each well. The following program (the system being set to a temperature of 45 °C) was run: 150 μL of solution from dispenser (1) and 50 μL of solution from dispenser (2) were added to sample placed well and shaken for 1 min in a flat-bottom 96-well microplate. After 4 h at 45 °C, the absorbance was measured at 450 nm. The experimental set-up of absorbance measurement in the microplate reader system is shown in Figure 4.

3.5. Hydrolysis of sucrose

The hydrolysis process was performed as described elsewhere.[40] To summarize the hydrolysis process, 0.5 mL of concentrated HCl was added to 15 mL of 1.0×10^{-3} M sucrose or real sample solution and incubated for 10 min at 60 °C. At the end of incubation, the hydrolysate was neutralized with 2 M NaOH and diluted to 25 mL.

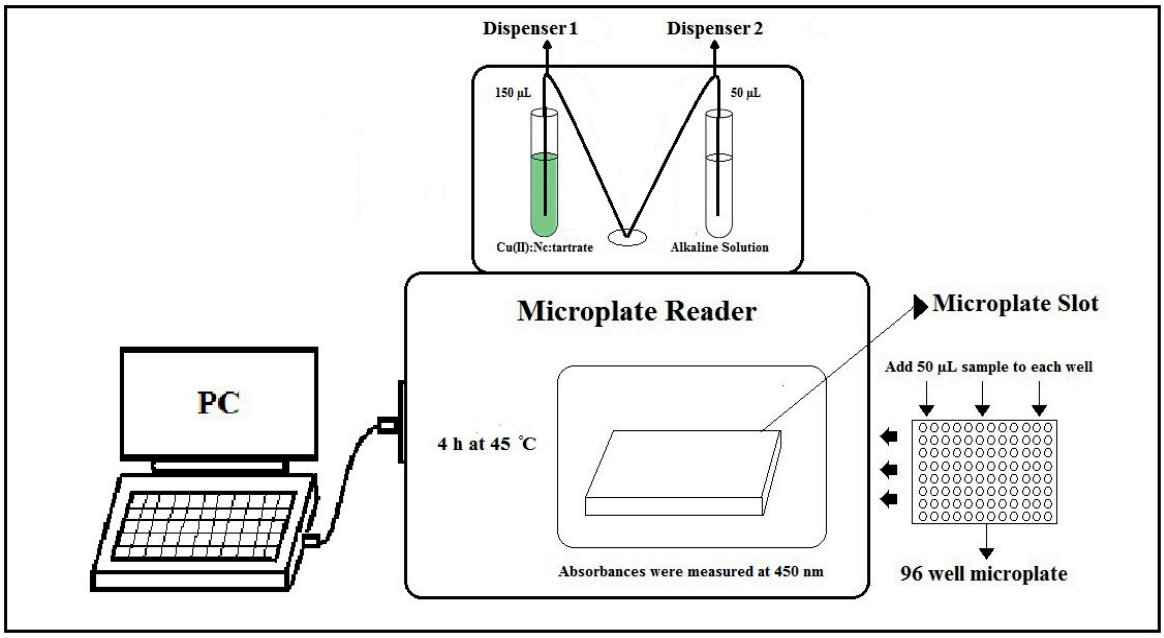

Figure 4. Schematic view of the instrumentation used for absorbance measurements in the microplate reader system.

3.6. Solid phase extraction (SPE) for sample clean-up

SPE cartridges (C18 and polyamide) were used to remove phenolics and other interferents of complex samples to clean up sugars. This process consists of two steps. The first step was performed for removing phenolics (compounds likely to be found in fruit juices), in which 4 mL of sample was passed through a C18 SPE cartridge. The cartridge was then washed with 4 mL of water. Phenolics were retained on the column. The column effluent and wash water were combined. The second step was performed for removing other phenolics not held by the C18 column, in which the combined solution was passed through a polyamide SPE cartridge. Finally, all phenolic compounds were eliminated from sugars after two column sequential separations with respect to polarity differences.

3.7. Statistical analysis

All assays were carried out in triplicate for each sample. Descriptive statistical analyses were performed using Excel software (Microsoft Office 2013) for calculating the mean and the standard error of the mean. The precision and accuracy of two methods were compared by F-test and Student's t-test.

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