SPECTROPHOTOMETRIC DETERMINATION OF REDUCING SUGAR IN WINES EMPLOYING IN-LINE DIALYSIS AND A MULTICOMMUTED FLOW ANALYSIS APPROACH

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ABSTRACT

A multicommuted flow procedure for the spectrophotometric determination of reducing sugars in wine has been developed. A dialysis unit was incorporated in the system to minimize matrix interferences and eliminate the colored compounds of the wine. The oxidation-reduction reaction in an alkaline medium between reducing sugars and the Cu^{2+}-Neocuproine complex was employed. The compound formed was monitored at 460 nm. In the proposed procedure, there was a linear response between 0.25 and 4.0 g L^{-1} reducing sugars (y = (0.36 ± 0.02) + (0.31 ± 0.01) * x, R = 0.9975, n = 6), a coefficient of variance of 2.6% for a reducing sugar solution of 2.0 g L^{-1} (n = 7), a limit of detection estimated at 0.03 g L^{-1}, reagent consumption per determination of 0.330, 0.128 and 0.160 mg for neocuproine, $CuSO_4$ and NaOH, respectively. A sampling throughput of 45 determinations per hour was achieved. The proposed system was applied to wine samples and comparing the results with an AOAC official method no significant difference at a 95% confidence level.

Keywords: Dialysis; Flow injection analysis; Multicommutation; Reducing sugar; Wine.

INTRODUCTION

In the course of the history of humanity, the use of carbohydrates to give taste to foods and beverages or, as a substrate to fermentation, has been a common practice[1]. Glucose and fructose (monosaccharides) are sugars, members of the carbohydrate family, that contain an aldehydic and ketonic group, respectively[2]. In wine, beer and juice production, the quantification of the reducing sugars (RS) is essential. Specifically, the level of RS determines the degree of alcohol in wines[3]. The concentration of glucose, in g L^{-1}, is used for the commercial classification of wines: dry wines, < 5; semi-sweet, 5 to 25; sweet whether fortified or not, 25 to 125.0; and musts and mistelles, > 125[4].

The determination of RS is frequently performed in the wine industry as an important parameter for controlling production and the fermentation process. The determination of the reducing sugars in wines is also used for routine quality control and to verify if the wine contains the legal or commercial reducing sugar content required for a certain wine type. The batch analytical methods to determine RS involves previous steps of sample preparation such as sample clarification and distillation in reflux to remove alcohol in the wine. This procedure is slow and generates a large residue volume. The spectrophotometric batch assay for the total reducing sugars determination based on the reduction of Cu (II) to Cu (I) with reducing sugars in alkaline medium in the presence of neocuproine in various food products was proposed by Başkan et al. (2016)[5]. The interference effect of phenolic compounds in samples was eliminated by a solid phase extraction (SPE) clean-up process. Therefore, are demanding in terms of the chemicals consumed, involve several steps and, thus, much manual work, labor-intensive and time-consuming.

The last decades have demonstrated the important role played by methodologies based on flow analysis for the automation and development of methods based on wet chemistry analysis[6]. These methodologies have been well reported, such as automation tools for handling solutions and pre-treatment of samples[7]. Examples include the coupling of interfaces in flow analysis for pre-treatment of sample such as, gas-liquid (gas diffusion, distillation and hydride generation), liquid-liquid (extraction and dialysis) and liquid-solid (ion exchange, adsorption, precipitation, dissolution and stripping)[8-14].

The indirect determination of RS in of wine employing the classical Fehling's reaction and flow injection coupled to atomic absorption spectrometry (AAS) was reported by Yebra et al. (1993)[15]. The copper(I) oxide is precipitated by injecting the Fehling's solutions into the wine carrier. When Fehling's solutions were injected into the sample stream, there is a decrease in the measured signal AAS owing to Cu(I) precipitate. The difference between the signals yielded the amount of precipitated copper, which was proportional to the reducing sugar concentration in the sample. The sampling frequency of the method is 12 min per analysis, due to interruption of the flow for 10 min, resulting in a high cost of analysis, because the gas consumption excessive for maintaining the AAS connected.

The biosensors and biosensing flow systems for determination of reducing sugars in wines are many varied, and they may be based on amperometry, potenciometry, photometry and fluorimetric detection[16]. However, the methods depend upon the construction of electrode chemically modified or immobilization of material and biomaterial (enzymes) for the molecular recognition.

A procedure based on sequential injection analysis (SIA) with dialysis unit incorporated was described for to spectrophotometric determination of reducing sugars in wine employing reaction of copper (I) with neocuproine, after reduction of copper (II) to copper (I) by reducing sugars. The determination of reducing sugars in two concentration ranges, from 2 to 25 and from 20 to 140 g L^{-1} was applied, yielding sampling-rate of the 14 – 18 samples per hour[17].

Conceptually, the multicommuted flow injection analysis (MCFIA) process includes discretely computer-operated devices strategically positioned in a manifold[18]. The control of these devices by software allows the insertion of an aliquot solution, as well as enablement of individual variable flow rates. Different systems have been proposed for many different types of analytes in different matrices employing MCFIA[19-21]. Other approach uses of the MCFIA have been with micro-pumps, devices that produce a pulsed flow, thus increasing the interactions between the solutions in the MCFIA system[6]. Furthermore, environmentally friendly systems and highly sensitive analyses have been achieved[9,12,22]. Additional advantages of the micro-pumps in a multicommutation system include portability, low energy requirements and the ability to minimize volume reagent consumed and consequently waste generation[23,24].

The association of interface liquid-liquid (extraction or diffusion) with MCFIA has been employed for the determination of molybdenum[25] and lead[26] in plants, copper in water samples[27], anti-hypertensive diltiazem in pharmaceutical samples[28], tartaric acid and potassium in Port and table wines[29] and sulfur dioxide in wines[30].

A MCFIA procedure for simultaneous spectrophotometric determination of nonstructural carbohydrates and reducing sugar in forage materials was proposed by Tumang et al. (2003)[31]. The determination of both analytes was based on the neocuproine method after acid hydrolysis of nonstructural carbohydrates. In the proposed system, ease of operation and robustness were observed, keeping the intrinsic characteristics of the MCFIA.

A multicommutation flow procedure using Cu-4,4'-dipyridyl coordination as the phase-solid reagent for to spectrophotometric determination reducing sugar in juice and coconut water samples was proposed by Leotério et al. (2015)[32]. In the procedure, a linear range from 1.0 to 20.0 g L-1, detection at 0.23 g L-1 and a sampling throughput of 75 determinations per hour were achieved. The system enabled the analysis with simplicity and a significant economy of the reagent and of the waste generation.

In this work, a multicommuted flow procedure based on-line dialysis coupled to micro-pumps for the determination of RS in wine was developed. The dialysis membrane followed by spectrophotometric detection has the advantage of eliminating potential interferences, mainly the color (phenolic compounds) of the wine, requires no sample pretreatment, reducing the amount of reagents consumed, without sacrificing the main analytical properties.

EXPERIMENTAL

Reagents and chemicals

All chemical reagents were of analytical grade and used as purchased without additional purification and all solutions were prepared with deionized water (resistivity > 18 MΩ cm⁻¹) from a Milli-Q system (Millipore Inc., Bedford, MA).

A 100 g L⁻¹ RS stock solution, what consisted of a mixture of fructose (60%, w/w) (Sigma-Aldrich, >99.5%) and glucose (40%, w/w) (Sigma-Aldrich, >99.5%), was prepared by dissolving monosaccharides in 25 mL of water. Working standard solutions with concentrations within the range of 0.25 to 4.0 g L⁻¹, containing 12% (v/v) of ethanol were prepared daily by dilution of the stock solution.

A 0.1 mol L⁻¹ [(Cu-Neo)$_2$]$^{2+}$ solution was prepared daily by dissolving 1.041 g of neocuproine, $C_{14}H_{12}N_2$ (2,9-dimethyl-1,10-phenanthroline, Sigma-Aldrich, >99.5%) in 5 mL of ethanol. This was added to an aqueous solution containing 0.4 g of copper sulphate pentahydrate (Sigma-Aldrich, 99%) and the mixture was diluted to 50 mL with water. The working solutions were obtained by diluting a stock solution into concentrations of 0.0025, 0.005, 0.01, 0.015 and 0.02 mol L⁻¹.

A 1.0 mol L⁻¹ sodium hydroxide stock solution was prepared by dissolving 4.0 g of NaOH (Vetec) in 100 mL water. The working solutions were obtained by diluting a stock solution into concentrations between 0.005 and 0.3 mol L⁻¹.

The wine samples of different types were purchased at local markets. Some wine samples were analyzed after appropriate dilution with water.

Apparatus

The equipment setup included an Ocean Optics USB-4000-UV-VIS spectrophotometer, equipped with optical fiber cables of 50 μm internal diameter to transport the radiation, a tungsten–halogen lamp, a flow cell with a 10 mm optical path (Hellma, Plainview, NY, USA; 80 μL internal volume), solenoid micro-pumps (Biochem Valve™ Inc., Boonton, NJ, USA) of 4 μL (P_1, P_2 and P_3) and 10 μL (P_4 and P_5) nominal volumes, reaction coils and flow lines made of polyethylene tubing (0.8 mm i.d.), acrylic laboratory-made joints used as confluences, a dialysis unit and a thermostatic bath (QUIMIS®).

The dialysis unit consisted of two Perspex blocks that were fixed to each other by four screws. A membrane cellulose nitrate (Sartorius Stedim Biotech, Germany) with a pore size of 0.45 μm was placed between the two blocks, and replaced weekly.

For the control of the micro-pumps and data acquisition, software in language Lab View 8.5® was developed. A homemade electronic interface assembled using an ULN2803 integrated circuit connected to an interface (USB-6009, National Instruments, USA) was employed to drive the micro-pumps. The signal generated by the spectrophotometer was monitored through the computer's USB 2.0 port. The frequencies of the micro-pumps were maintained at 5 Hz, which consisted in equal time intervals switched ON or OFF (0.1s/0.1s).

Flow system and procedure

The design of the flow system was based on multicommutation, and the flow diagram is shown in Figure 1. The flow system was designed with five solenoid micro-pumps (P_1 to P_5) which were assembled to allow the handling of the solutions for the detection. The micro-pumps P_1, P_2, and P_3 were responsible for the introduction of the sample and reagent solutions, respectively, while P_4 and P_5 were used for the introduction of the carrier. In this configuration, all the micro-pumps are switched OFF, thus no solution is flowing through the analytical path.

Initially, the system was activated to fill the flow lines with solutions, which was done following the sequence indicated in Table 1 (steps *a* to *e*).
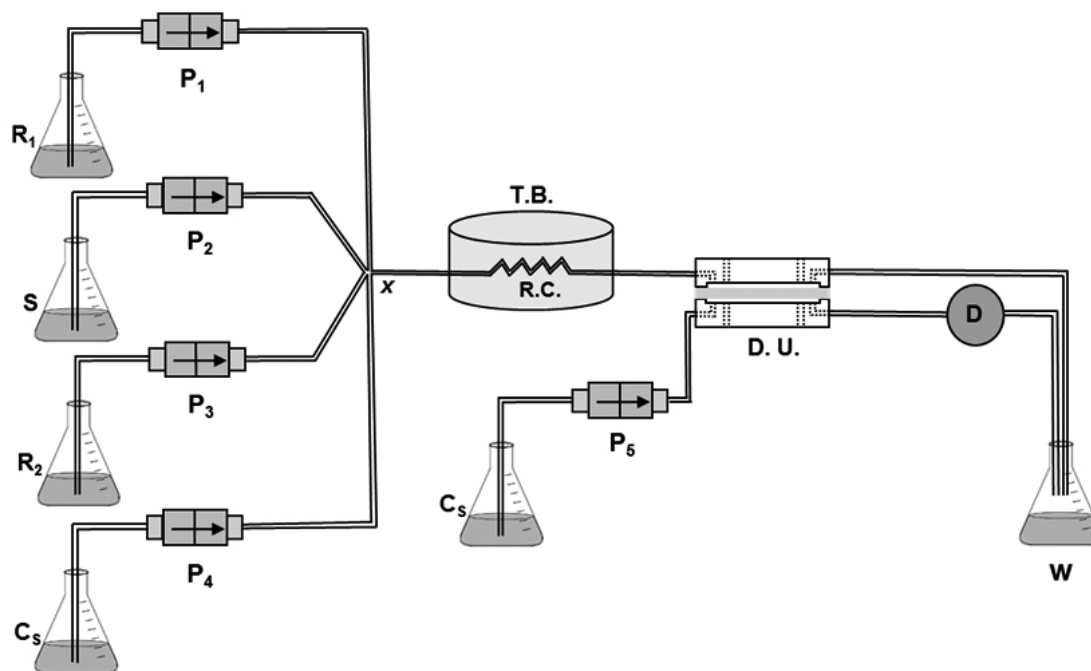

Figure 1. Flow diagram of the multicommuted flow system. S = sample; Cs = carrier solution, water; R_1 = 0.050 mol L⁻¹ NaOH; R_2 = 0.010 mol L⁻¹ [(Cu-Neo)$_2$]$^{2+}$ complex; P_1, P_2, P_3, P_4, P_5 = solenoid micro-pumps; *x* = joint devices machined in acrylic; R.C. = Polyethylene reactor coils (100.0 cm length, 0.8 mm i.d.); T.B. = thermostatic bath; D.U. = dialysis unit. D = spectrophotometric detector at 480 nm; W = waste vessel.

For spectrometer calibration (Table 1, step f), the dark (the tungsten–halogen lamp off) and reference (the tungsten–halogen lamp on) readings were performed. To obtain the relation of absorbance (A) versus time was used the equation $A = -\log((I_2 - I_0)/((I_1 - I_0))$, where I_0 = intensity in the dark, I_1 = intensity with the carrier solution and I_2 = intensity with sample solution. This equation made it possible to calculate the relative absorbance of the sample solution. The absorbance data generated were saved as a file.LVM, which was recognized by the graphic editor program.

Table 1: Switching steps of solenoid micro-pump for determination of reducing sugar.

Step	Event	P_1	P_2	P_3	P_4	P_5	Pulses	Cycles
a	Flow lines filling	0/1	0	0	0	0	50	-
b		0	0/1	0	0	0	50	-
c		0	0	0/1	0	0	50	-
d		0	0	0	0/1	0	50	-
e		0	0	0	0	0/1	50	-
f	Spectrophotometer calibration	0	0	0	0	0	0	-
1	NaOH insertion	0/1	0	0	0	0	8	10
2	Sample insertion	0	0/1	0	0	0	4	
3	$[(Cu\text{-}Neo)_2]^{2+}$ complex insertion	0	0	0/1	0	0	16	
4	Transport, detection and washing	0	0	0	0/1	0/1	100	-

Numbers 0 and 1 in the columns indicate that was switched OFF or ON, respectively. This case, 0.1 s/0.1 s (ON/OFF)

The flow procedure for the determination of reducing sugars in wine sample was performed following the protocol sequence presented in Table 1 (steps 1 - 4). The analytical cycle started with introduction of sodium hydroxide (R_1), sample (S) and $[(Cu\text{-}Neo)_2]^{2+}$ complex (R_2), through the switching of micro-pumps P_1, P_2 and P_3, respectively (steps 1, 2 and 3). The binary sampling pattern concept was adopted [28]. Micro-pumps P_1, P_2 and P_3, were switched ON/OFF sequentially. Thus, a total volume of 0.32 mL NaOH solution (corresponding to 10 cycles of 8 pulses of micro-pump P_1), 0.16 mL sample solution (10 cycles of 4 pulses of micro-pump P_2) and 0.64 mL $[(Cu\text{-}Neo)_2]^{2+}$ complex solution (10 cycles of 16 pulses of micro-pumps P_3) were employed for each measurement.

After the sampling step, micro-pumps P_4 and P_5 were switched ON/OFF simultaneously, and P_4 carried the sample zone to the reaction coil (RC) where it was put in a thermostat bath. The reaction coil was linked the top of the dialysis unit. In the RC, the oxidation-reduction reaction in an alkaline medium, between the $[(Cu\text{-}Neo)_2]^{2+}$ and reducing sugars occurred. The $[(Cu\text{-}Neo)_2]^+$ complex liquid formed diffused to below the dialysis unit and was transported, by the P_5, to the detector (at 460 nm) with the carrier solution. While the analytical signal was processing, the carrier solution was transported to the waste and the analytical run was completed. For the exchange of the sample solution, steps *b* and *d* were carried out, (Table 1), sequentially and with the same four steps (1 − 4) as presented in Table 1.

The determination of the RS was based on a redox reaction with Cu^{2+} in an alkaline medium. For the optimization of the reaction, a study was made ranging the concentrations of the NaOH of 0.005 at 0.3 mol L^{-1} and $[(Cu\text{-}Neo)_2]^{2+}$ of 0.0025 at 0.02 mol L^{-1}. For this study, the concentration of reference solution was fixed as 1.0 g L^{-1} of reducing sugar, a volumetric ratio of 1:1:1 (NaOH:RS:$[(Cu\text{-}Neo)_2]^{2+}$) with 10 pulses for each solution and 10 sampling cycles, maintaining the temperature at 65 °C. Subsequently, the influence of temperature with ranges between 50 and 100 ºC was studied. The volumetric ratio of the NaOH, RS and $[(Cu\text{-}Neo)_2]^{2+}$ complex solutions was established by varying the switching sequential of the micro-pumps P_1, P_2 and P_3. Therefore, the number of pulses of the micro-pumps P_1, P_2 and P_3 varied between 4 and 18, corresponding to variation of 160 at 720 µL, respectively, by sampling cycles presented in Tables 2, 3 and 4. For those studies the number of sampling cycles was set at 10 cycles and analytical curve was constructed by varying the concentration of RS between 0.25 and 4.0 g L^{-1} for each variation in the volumes studied. Following this, a study of the sampling cycles, varying between 2 and 20, was carried out. For all experiments proposed, the reactor length and flow rate was set at 100 cm, and 2.0 mL L^{-1}, respectively.

Once the best operating conditions were established, eleven samples of wine were analyzed in order to prove the usefulness of the proposed system.

Reference method

The results obtained by the procedure proposed were compared with those obtained with the method recommended by AOAC International for RS determination in wines [33]. The recommended procedure consisted of a reaction between reducing sugars with cupric ions in Fehling's solution, reducing them to cuprous ions under the action of heat in an alkaline medium. By reacting with cupric ions, sugars undergo oxidation, while the Cu^{2+} is reduced to Cu^+, forming a red precipitate of cuprous oxide.

RESULTS AND DISCUSSION

General aspects

The theoretical principles of crossflow filtration are based by Fick's law of diffusion [34], where the migration of suspended solids/macromolecules occurs in the sense of filtration surface. The design of a successful crossflow system relies on efficient choice of the membrane type. The physical aspects of the membrane of the relative size exclusion such as type of polymer, the geometry and pore size of the membrane were integral part of process optimization [35] (Pawliszyn, 2012). In preliminary study were tested different membranes types based on their composition and pore size, including cellulose acetate, cellulose nitrate, polyacrilonitrile, polytetrafluoroethylene (Teflon®) and polyvinylidiene fluoride with different porosities. The cellulose nitrate membrane with a pore size of 0.45 µm showed more significant response regarding the percentage recovery being selected to develop the proposed system.

The principle of the method can be expressed by the following chemical equation (1)[36]:

$$[(Cu\text{-}Neo)_2]^{2+} + C_6H_{12}O_6 \xrightarrow{\text{NaOH}} [(Cu\text{-}Neo)_2]^+ + C_5H_{11}O_5COOH \quad (1)$$

Job's method was employed to study the stoichiometric ratio [37], between Cu^{2+} and neocuproine. The solutions were prepared with an equal molar concentration and the volumetric ratio from 1:9 to 9:1 (v/v) was varied. In this study, both solutions were prepared with 0.01 mol L^{-1}. The spectrum wavelength ranging from 400 to 700 nm and the maximum absorption was obtained at 460 nm. The best stoichiometric ratio value estimated between Cu^{2+} and neocuproine was 1:2 (v/v).

Chemical and hydrodynamic parameters of the proposed procedure were studied by the univariate method, considering with pre-established initial conditions, such as concentrations of 1.0 g L^{-1}, 0.01 mol L^{-1} and 0.01 mol L^{-1} for the RS reference, NaOH and $[(Cu\text{-}Neo)_2]^{2+}$ solutions, respectively; 65 ºC for the thermostatic bath; volumetric ratio of 1:1:1 (v/v); with 10 pulses for each solution and 10 sampling cycles, as the starting point. In preliminary studies, the reactor length has not had significant influence on the development of multicommuted system proposed, being the best condition initially established was at steady state condition. In this way, the reactor length was set at 100 cm.

System optimization
Effect of the NaOH and $[(Cu\text{-}Neo)_2]^{2+}$ concentration

The effect of the $[(Cu\text{-}Neo)_2]^{2+}$ and NaOH concentration on the analytical response was investigated maintaining the parameters cited above and using a 1.0 g L^{-1} RS reference solution. The concentration of the NaOH and $[(Cu\text{-}Neo)_2]^{2+}$ varied from 0.005 to 0.30 mol L^{-1}, and from 0.0025 to 0.02 mol L^{-1}, respectively as shown in Figure 2 and Figure 3. As we can see in Figure 2, the magnitude of the analytical signal for the 1.0 mg L^{-1} reference solution increases relative to the increased concentration of the $[(Cu\text{-}Neo)_2]^{2+}$ solution up to 0.01 mol L^{-1} and after that the signal decreases. Given the results obtained, a concentration of 0.01 mol L^{-1} $[(Cu\text{-}Neo)_2]^{2+}$ was established.

The concentration of the NaOH solution might affect the sensibility of the procedure. The study was realized using the same parameters previously

set and using the concentration of the 0.01 mol L⁻¹ [(Cu-Neo)₂]²⁺ solution, yielding the results shown in Figure 3. It is noted in this Figure, that increasing the concentration of the NaOH solution, heightens the magnitude of the analytical signal up to 0.05 mol L⁻¹. Subsequently, there is a decrease in signal due to increased concentration of the NaOH. This fact may be because the decomposition [(Cu-Neo)²]²⁺ complex through the increasing concentration of NaOH, forming the precipitate $Cu(OH)_2$ (Ksp = 2.2 x 10⁻²⁰). The precipitate formed can quickly saturate the cellulose nitrate membrane, making it useless. Thus, the concentration of 0.05 mol L⁻¹ NaOH solution was achieved.
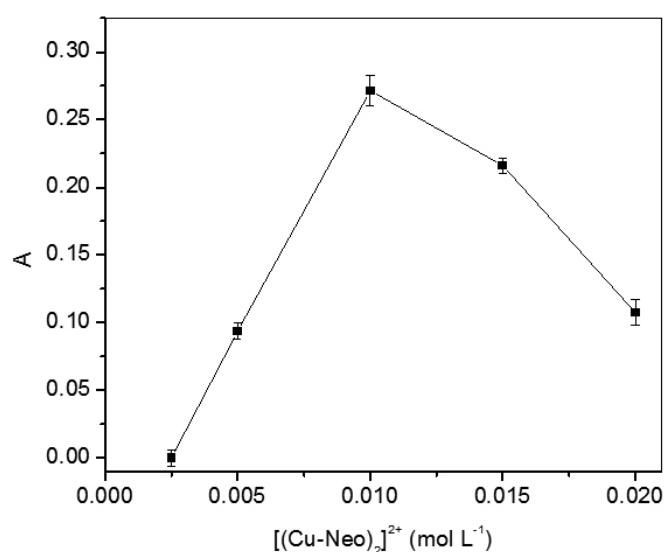

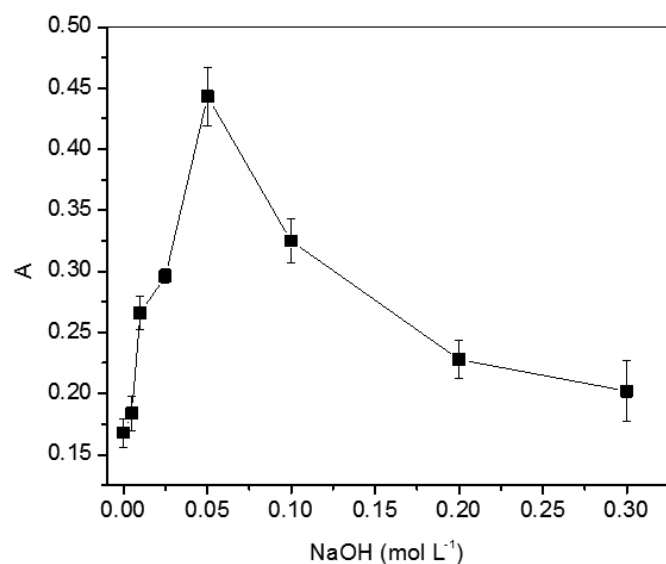**Figure 2.** Effects concerning of the [(Cu-Neo)₂]²⁺ concentration.

Figure 3. Effects concerning of the NaOH concentration.

Effect of the temperature

Temperature influences the reaction kinetics between the [(Cu-Neo)₂]²⁺ complex and reducing sugar. The effect of temperature on the magnitude of the analytical signal varying between 50 at 100 ºC was studied, maintaining the concentration at 1.0 g L⁻¹ of RS. The magnitude of the analytical signal increases as the temperature is increased, up to a temperature of 85 ºC. At temperatures above 85 ºC, an onset of bubbles in the analytical course was noted. This hampered the stability of the flow system. Given this result, a temperature of 85 ºC was achieved.

Effect of the volumetric ratio NaOH/RS/[(Cu-Neo)₂]²⁺

Because the volumetric ratio might affect the sensibility of the procedure, a set experiment was carried out in order to ascertain the optimum conditions. In this study, we used the univariate method.

At first, a study the volume of NaOH solution was carried out by switching ON/OFF micro-pumps P₂ and P₃ ten times and varying the micro-pumping P₁ between four and twelve times for each sampling cycle, for a total of 10 cycles. Total slugs of the NaOH solutions with volumes of 160, 320, 400 and 480 μL were inserted and the volumes of the sample and [(Cu-Neo)₂]²⁺ slugs were maintained at 400 μL, corresponding to a 0.4:1:1, 0.8:1:1, 1:1:1, 1.2:1:1 (v/v) volumetric ratio NaOH/RS/[(Cu-Neo)₂]²⁺, respectively, yielding the results shown in Table 2. As can be seen, when the number of NaOH solution slugs was increased from 160 to 320 μL, the linear coefficient and the slope was improved, thereafter, with the increase of the volume of NaOH solution for 400 and 480 μL, there was a loss of linearity. Given the obtained results, a NaOH volume of the 320 μL, corresponding to 8 pulses of the micro-pump P₁, was achieved.

Table 2: Effect of NaOH solution.

Pulses P₁	NaOH (μL)	Equation	Linear coeficient (R)
4	160	y = (0.2164 ± 0.0406) + (0.3586 ± 0.0330) x	0.9875
8	320	y = (0.1715 ± 0,0479) + (0.4444 ± 0.0389) x	0.9887
10	400	y = (0.2681 ± 0,0621) + (0.4178 ± 0.0505) x	0.9788
12	480	y = (0.2470 ± 0,0705) + (0.3477 ± 0.0575) x	0.9615

To study the volume of the sample (RS) solution, we varied the total number of slugs of the sample solutions between 160 and 480 μL maintaining the volume of NaOH solution and [(Cu-Neo)₂]²⁺ in 320 and 400 μL, respectively. The results are shown in Table 3. This table illustrates how an increase in the sample solution volume of 160 to 480 μL results in a significant loss of linearity. For volumes less than 160 μL, corresponding to 4 pulses per sampling cycle, it was not possible to obtain a significant analytical response due to sensitivity of the proposed method. Given the results, a sample (RS) volume of the 160 μL, corresponding to 4 pulses of the micro-pump P₂ for sampling cycle, was achieved.

Table 3: Effect of sample solution.

Pulses P₂	Sample (μL)	Equation	Linear coeficient (R)
4	160	y = (0.2316 ± 0.0318) + (0.2184 ± 0.0147) x	0.9933
8	320	y = (0.2449 ± 0.0532) + (0.2262 ± 0.0269) x	0.9794
10	400	y = (0.3053 ± 0.1186) + (0.2704 ± 0.0558) x	0.9416
12	480	y = (0.3774 ± 0,0872) + (0.1634 ± 0.0440) x	0.8804

Finally, the total volume of the [(Cu-Neo)₂]²⁺ solution was varied between 160 and 720 μL (corresponding to variation from 4 to 18 pulses) of sample solutions per each sampling cycles and the NaOH and sample solution volumes were made constant at 320 and 160 μL, respectively, yielding the results shown in Table 4. As can be seen, when the volume of the [(Cu-Neo)₂]²⁺ solution slugs was increased from 160 to 640 μL, the linear coefficient and the slope has improved, promoting a significant increase (about 40%) in the sensitivity of the proposed method. The volume of 720 μL showed loss of linearity and slope. This occurred possibly due to dilution of the sample in relation to the excess [(Cu-Neo)₂]²⁺ solution added. Thus, the volume of 640 μL, corresponding to 16 pulses of the micro-pump P₃ for the sampling cycle was fixed to the sequence of studies. Therefore, the volumetric ratio between NaOH, sample (RS) and [(Cu-Neo)₂]²⁺ solution was established as 2:1:4 (v/v/v) after optimization of the proposed procedure.

Table 4: Effect of $[(Cu-Neo)_2]^{2+}$ complex solution.

Pulses P_3	$(Cu-Neo)_2]^{2+}$ (μL)	Equation	Linear coeficient (R)
4	160	$y = (0.1589 \pm 0.1064) + (0.0404 \pm 0.0552)\,x$	0.4766
8	320	$y = (0.1083 \pm 0.0444) + (0.1932 \pm 00224)\,x$	0.9742
10	400	$y = (0,2316 \pm 0,0318) + (0,2184 \pm 0,0147)\,x$	0.9933
12	480	$y = (0,1993 \pm 0,0612) + (0,1861 \pm 0,0309)\,x$	0.9610
16	640	$y = (0,3597 \pm 0,0326) + (0,3079 \pm 0,0169)\,x$	0.9940
18	720	$y = (0,2476 \pm 0,0497) + (0,1959 \pm 0,0251)\,x$	0.9762

Effect of the sampling zone

Considering that the volume of the sample zone might affect the analytical signal and consequently sensitivity, the number of sampling cycles was varied from 2 to 20 cycles, corresponding to a sampling zone between 224 and 2240 μL. As expected, the analytical signal was higher for a higher sampling zone volume. Nevertheless, when the number of sampling cycles was higher than 10, no significant increase in the signal was observed, thus indicating that the system had attained a steady-state condition. Given this, the inserted volumes of NaOH, sample (RS) and $[(Cu-Neo)_2]^{2+}$ solutions of 320, 160 and 640 μL (8 pulses of NaOH, 4 pulses of sample and 16 pulses of $[(Cu-Neo)_2]^{2+\ complex}$), respectively, were selected, which corresponds to 10 sampling cycles.

Proposed procedure performance

After performing the optimization of the proposed system for reducing sugar analysis, an analytical curve was plotted, shown in Figure 4. A linear response was observed between 0.25 and 4.0 g L^{-1} with the equation, $A = (0.36 \pm 0.02) + (0.31 \pm 0.01) * C$ ($R = 0.9975$, $n = 6$, where $A =$ absorbance and $C = RS$ concentration in g L^{-1}). The limit of detection (3σ criterions) and quantification (10σ criterions) were estimated at 0.03 g L^{-1} and 0.1 g L^{-1} respectively, sampling throughput of the 45 determinations per hour and effluent generation of 3.120 mL per determination were estimated. Under optimized conditions, the system showed a stable baseline and a satisfactory repeatability of analytical signals with a relative standard deviation of 2.6% ($n = 7$) to consecutive insertion of the 2.0 g L^{-1} RS solution. Given the above, it can be said that the proposed flow system for reducing sugar determination is feasible for analytical applications.
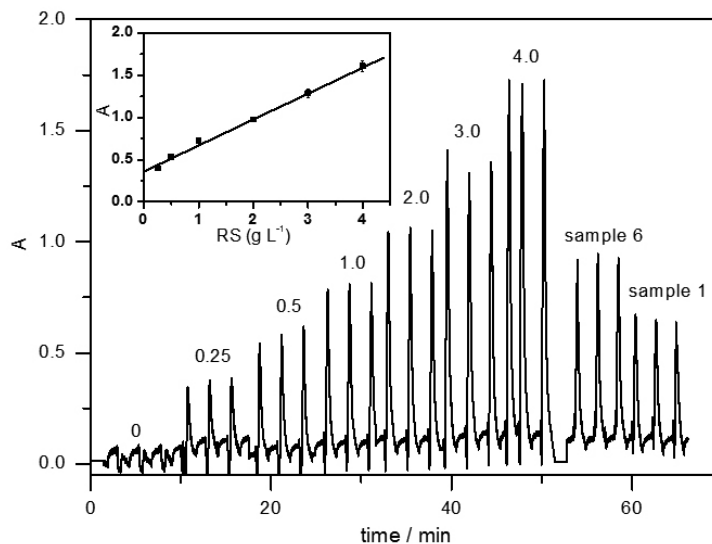

Figure 4. Transient signals obtained with the flow manifold in Fig.1.Numbers indicate RS concentrations in g L^{-1} and letters indicate the consecutive insertion of the samples. The inset shows the corresponding analytical curve graph.

The results, summarized in Table 5, were presented in order to optimize the proposed system.

Table 5: Optimized parameters of the multicommuted flow system for determination of reducing sugars in wine.

Parameter	Range	Selected value
NaOH concentration (mol L^{-1})	0.005 - 0.3	0.05
$[(Cu-Neo)_2]^{2+}$ complex concentration (mol L^{-1})	0.0025 - 0.05	0.01
Temperature (°C)	50 - 100	80
NaOH solution volumes (μL)	160 - 640	320
Sample solution volumes (μL)	160 - 640	160
$[(Cu-Neo)_2]^{2+}$ solution volumes (μL)	160 - 720	640
Sampling cycles	2 - 20	10

To assess the performance of the proposed procedure, a comparison with methods described in the literature is summarized in Table 6. The advantages of the proposed procedure include a lower detection limit, when compared to what has been described in the literature[17,38] and less sample volume, waste generation and reagent consumption than what has been reported in standard methods, leading to a significant reduction in the costs of the analysis. These features indicate the feasibility of the proposed system.

Table 6: Analytical features of spectrophotometric procedures for determination of reducing sugars in wine.

Parameters	AOAC (Latimer Jr 2012)	FIA [a] (Maquieira et al., 1987)	FIA [a] (Peris-Tortajada et al., 1992)	SIA [a] (Araújo et al., 2000)	Proposed method [a]
Linear Range (g L^{-1})	-	0.05 - 1.0	1.2 - 7.2	2 - 25 or 20 - 140	0.25 - 4.0
Detection limit (g L^{-1})	-	-	-	1.2 or 11.2	0.03
R.S.D. (%)	-	1.61	-	2.1 or 1.7	2.6
Sample volume consumption (mL) [b]	50	0.0453	0.143	0.120 or 0.340	0.160
Waste volume (mL) [b]	112	3.453	3.593	8.898 or 9.553	3.120
NaOH consumption (mg) [b]	1000	40.32	34.50	4.95	0.160
Neocuproine (mg) [b]	-	0.672	0.690	0.054	0.330
CuSO$_4$ (mg) [b]	692.78	0.336	0.345	0.027	0.128
Throughput (h^{-1})	1	50	40	18 or 14	45

Comparison results

Once the optimal operational conditions were established, the determination of reducing sugar was performed in wine samples in order to test the functionality of the proposed procedure. The AOAC official method[33] was used to test the accuracy of the assessment samples, yielding the results given Table 7.

Table 7: Comparative results obtained from determination of reducing sugars in wine samples.

Sample	Type of wine	Amount found (g L^{-1})[a]	
		AOAC method	Proposed procedure
1	dry red	1.3 ± 0.1	1.2 ± 0.1
2	dry red	8.4 ± 0.3	5.1 ± 0.2
3	dry red	5.7 ± 0.2	4.3 ± 0.3
4	dry red	3.5 ± 0.2	2.34 ± 0.08
5	dry red	4.0 ± 0.3	2.9 ± 0.1
6	dry red	1.8 ± 0.2	1.73 ± 0.09
7	medium dry	13.7 ± 0.1	15.0 ± 0.4
8	medium sweet	81.3 ± 0.4	81 ± 6
9	Liqueur	110.5 ± 0.7	141 ± 10
10	Liqueur	123 ± 2	156 ± 2
11	dry white	3.0 ± 0.2	4.64 ± 0.01

[a] Result average of three consecutive determinations ± the corresponding standard deviation.

The paired t-test between results at the 95% confidence level was applied. The calculated value was t = 1.356, while the theoretical value is t = 1.812, indicating that there was no statistically significant difference between the results.

CONCLUSIONS

We consider the overall performance of the proposed system, including the equipment and setup of the control software, to be simple and easy to operate, with the advantage of eliminating the color of the wine through a dialysis cell, allowing sample introduction of dry red wine without prior dilution. In addition, we obtained economy of reagents and decrease in waste generation compared to the official method.

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