



# An automatic titration setup for the chemiluminometric determination of the copper complexation capacity in opaque solutions



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## ABSTRACT

An automatic titration setup exploiting flow analysis was proposed for the evaluation of the copper complexation capacity of highly opaque substances (milk and humic substances). The binary search approach was implemented in a flow-batch analyzer, in order to add the in-line selected titrant (e.g. copper ions) volumes to the sample. When the titration end-point was surpassed, the free metal ions catalyzed the reaction of luminol with hydrogen peroxide, yielding the chemiluminescence, which was quantified even in solutions of high opacity. Accuracy was assessed through addition/recovery tests involving classical complexing species (EDTA, DTPA and DTTC), and recoveries ranged from 96% to 115%. The proposed system requires low amounts of reagents and samples (0.42 mg of luminol, 82 µg H<sub>2</sub>O<sub>2</sub>, 1.10 mL of sample) per titration run, meaning *ca.* 12 mL of effluent per titration, and yields precise results (5% r.s.d.) at a sampling throughput of 43 h<sup>-1</sup>.

## 1. Introduction

Currently, there is a high demand for rugged, low-cost and practical analytical procedures. Titration, a classical analytical technique, partially fulfills these requirements and is then widely utilized. It is however a time consuming process that usually generates a large volume of waste. This drawback can be minimized by using a burette controlled by an electromagnetic device, as pioneering demonstrated by Ziegel in 1914 [1] in relation to the potentiometric titration of dichromate with ferrous ions. A photometric detector was further used, allowing titrations involving a dye substance as the indicator [2,3] and electronic devices for end-point detection.

Photometric titrations of colored samples are however challenging, because detection of the indicator color variations in samples with a highly colored background is cumbersome. In fact, the incident radiation beam from the external source traverses the sample bulk, and a considerable part is absorbed, impairing the end-point evaluation. With a chemiluminescent chemical reaction used as end-point indicator, the radiation is produced inside the entire sample bulk, thereby minimizing the absorption by the titrand solution. This aspect was initially highlighted by Kenny and Kurtz [4,5], who titrated Fe(II) with Ce(IV), and HCl with NaOH using siloxene and luminol as the chemiluminescent indicators, respectively. In this later case, H<sub>2</sub>O<sub>2</sub> acted as oxidant for luminol and hemoglobin was the catalyst. Concerning

chemiluminometric titrations in highly opaque media, the determinations of thallium, copper and sulfate [6–10] can be selected as illustrative examples, as the authors reported that to carry out titrations in highly colored samples was always a limiting factor. In order to circumvent this drawback, the samples have been usually subjected to conditioning processes such as cleanup, dilution, and/or successive extractions, yet care should be taken to prevent contamination and/or any modification in the analyte structures [11].

Evaluation of complexing capacity (CC) is often required for assessing metal biological availability, for preparing metal-complexes to be used as fertilizers, and as an indicative of metal dynamics, accumulation and mobility in natural waters. This later aspect is useful for remediation of areas subjected to contamination by toxic metallic species, especially aquatic environments. The metal CC is also relevant in analytical procedures for the determination of proteins in milk.

CC of humic substances (HS) can be evaluated by complexometric titration, using a metal ion solution as the titrant. During titration, the metal ions react to form highly stable complexes and, after end-point, their excess is exploited for end-point estimation [12].

HS are intensely colored, impairing the detection of the titration end-point in an ordinary photometric titration. A similar difficulty occurs with samples of effluent containing dyes, such as e.g. organic substances, emulsions or suspensions. Exploitation of an EDTA solution as a chemical descaling agent is usual in industrial processes. After a

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few cycles of reuse, the solution may contain a large number of suspended particles, thus becoming dark/turbid. An end-point search strategy viable even under opaque conditions is then useful [5,7], and automatic flow titrations based on digital images [13–15] are worthwhile in the context.

As titration in opaque media is often required, a strategy for easy handling the titrand and titrant solutions, and for quantifying the signal generated under poor visibility conditions is welcome. Considering that the determination of complexing ability in highly opaque solutions/suspensions is a current challenge, the main purpose of the present work was to develop a novel strategy for evaluating the CC in strongly opaque samples.

As proof of concept, chemiluminometric titrations of milk, HS and colored solutions relying on the luminol oxidation by  $\text{H}_2\text{O}_2$  catalyzed by  $\text{Cu}^{2+}$  ions in alkaline medium [16,17] were developed. Copper complexation by the assayed samples is the predominant reaction, so that emission of radiation practically does not occur while the titration is in progress. After end-point, the free  $\text{Cu}^{2+}$  ions catalyze the luminol oxidation, yielding the emission of electromagnetic radiation ( $\lambda \sim 430$  nm). This allows the titration end-point attainment to be efficiently accomplished, and the binary search approach [23] in a flow-batch setup [19–21] with a homemade photodetector [18] is relevant in the context. All involved steps are managed by an Arduino microcontroller [22].

## 2. Experimental

### 2.1. Reagents and solutions

All solutions were prepared with distilled-deionized water (resistivity  $> 18 \text{ M}\Omega \text{ cm}$ , at  $25^\circ\text{C}$ ) and analytical grade reagents. The  $10.0 \text{ mmol L}^{-1} \text{ Cu}^{2+}$  stock solution (Merck, 99%) was prepared by dissolving the appropriate amount of anhydrous copper sulfate in water. The  $50 \text{ mmol L}^{-1}$  luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) stock solution was prepared by dissolving the solid material (Sigma,  $> 97\%$ ) in a  $100 \text{ mmol L}^{-1}$  NaOH solution (Merck, 99%). This solution was stored in an amber flask, wrapped with aluminum foil, and kept at ca.  $8^\circ\text{C}$ . The luminol working solution was daily prepared in  $100 \text{ mmol L}^{-1}$  NaOH. The  $50 \text{ mmol L}^{-1} \text{ H}_2\text{O}_2$  solution was prepared by water dilutions of a 30% (w/v) solution (Merck), and the  $10.0 \text{ mg L}^{-1}$  MB (methylene blue) solution was prepared in water from a  $200 \text{ mg L}^{-1}$  stock solution. Three  $10.0 \text{ mmol L}^{-1}$  EDTA (2,2',2''-(1,2-ethanediyldinitrilo)tetraacetic acid), DDTC (*N,N*-diethylcarbamodithioate) or DTPA ((2-[bis[2-[bis(carboxymethyl)amino]ethyl]amino]acetic acid)) (Merck,  $\geq 99\%$ ) stock solutions were prepared by dissolving in 25.0 mL of water. The working solutions were daily prepared by dilutions of these stock solutions with water.

### 2.2. Sample preparation

A  $1000 \text{ mg L}^{-1}$  HS (leonardite, CAS number 1415-93-6) solution was prepared in water. Milk samples were purchased from a local market and 10-fold diluted with water. Prior to analyses, 0.00, 0.30 and  $0.60 \text{ mmol L}^{-1}$  EDTA or DDTC were spiked to the humic acid and milk samples, respectively.

### 2.3. Apparatus

The equipment consisted of three basic units: the flow system module, a homemade luminometer, and the Arduino Due board with peripheral interfaces.

The flow system module (Fig. 1) comprised two compact peristaltic pumps (Welco, Tokyo, Japan), three solenoid pumps (P/N120SP1240-5 TV,  $40 \mu\text{L}$  per pulse, Bio-Chem Valve, Boonton NJ, USA), a 12V regulated power supply with a 2 A current intensity, a flow-batch cylindrical ( $40 \text{ mm}$  depth,  $10 \text{ mm}$  diameter) chamber, and accessories.

The chamber was machined in polytetrafluorethylene (PTFE), and contained a PTFE-coated magnetic stir bar. Polyethylene tubing ( $0.8\text{-mm}$  i.d.) was used to build up the transmission lines.

The luminometer (Fig. 1S) comprised two photodiodes (1237-1010BR, Hamamatsu, Japan) with a  $100 \text{ mm}^2$  sensitive area, three OP07 operational amplifiers, a regulated power supplier ( $-12 \text{ V}$ ,  $+12 \text{ V}$ ;  $0.5 \text{ A}$  current intensity). The flow cell was built up by wrapping (10 turns) a 30-cm polyethylene tube on an acrylic plate ( $8 \text{ mm}$  width,  $2 \text{ mm}$  thick,  $30 \text{ mm}$  long). In order to improve the chemiluminescence detection, the photodiodes were assembled as close to the two flat surfaces as possible (Fig. 1, inset).

The Arduino Due board was used for controlling the flow system module, as well as for data acquisition and treatment. The developed program, written in C++, is available as Supplementary Material. As the control signals from the digital output lines presented a maximum value of  $3.2 \text{ V}$ , and the peristaltic and solenoid pumps operated with a potential difference of  $12 \text{ V}$ , an external interface was required for compatibility. The microcontroller was coupled to a homemade digital interface based on the ULN2803 integrated circuit (Fig. 2S). The analog signal generated by the luminometer was converted to a digital one by the Arduino board while performing data acquisition. The user interfaces comprised a liquid crystal display and a parallax data acquisition (PLX-DAQ) platform, add-in Microsoft Excel via the USB serial port [24]. These tools allowed the titration steps to be visually followed in real time.

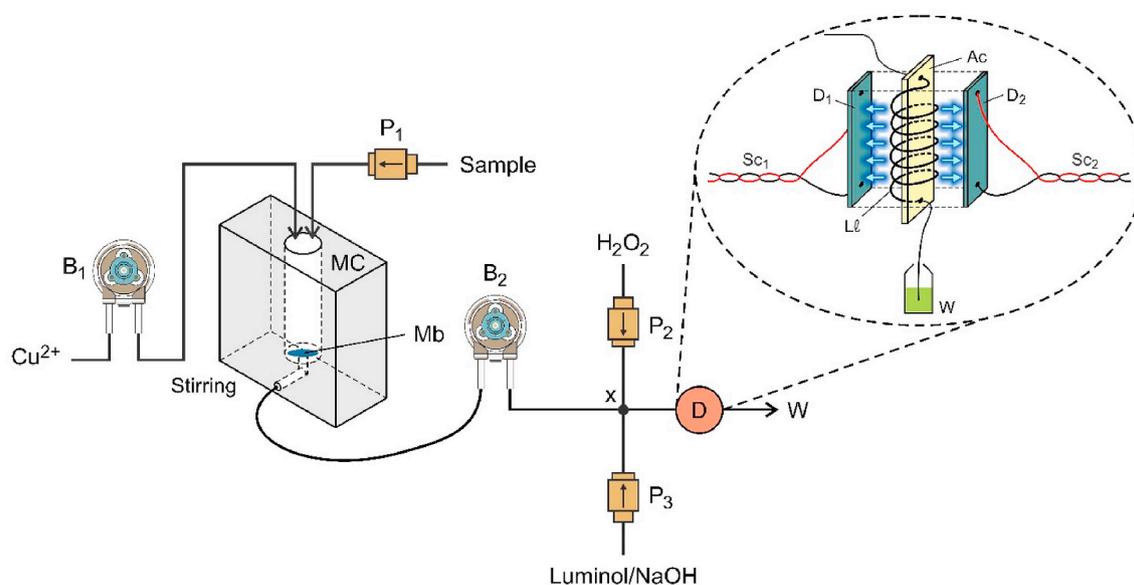
### 2.4. Flow system and procedure

The proposed flow manifold (Fig. 1) permits to exploit binary search for implementing automatic titration in a flow-batch analyzer. The sample and titrant volumes are added into the chamber, and the resulting analytical signal is evaluated to real-time decide the variation of the titrant volume to be inserted in the next attempt. After several attempts, the titration end-point is approached. The system provides then possibilities to perform flow titrations without any operator assistance. During all steps, homogenization of the solution inside the mixing chamber was maintained by continuous stirring ( $300 \text{ rpm}$ ).

The flow system was operated according to Table 1. Initially, a preset volume of sample was inserted into the mixing chamber (MC) by the  $\text{P}_1$  pump. Next, the selected volume of the  $\text{Cu}^{2+}$  solution, defined by the binary search algorithm, was added by the  $\text{B}_1$  pump. After attaining complexation equilibrium, the mixture was transported by the  $\text{B}_2$  pump towards the luminometer, and merged at the x confluence with the  $\text{H}_2\text{O}_2$  and luminol streamed solutions propelled by  $\text{P}_2$  and  $\text{P}_3$  pumps. If the complexation capacity was surpassed, the remaining free  $\text{Cu}^{2+}$  catalyzed the luminol oxidation, generating the chemiluminescence. The emitted radiation from the flow cell was quantified at  $\sim 430 \text{ nm}$  by the  $\text{D}_1$  and  $\text{D}_2$  photodetectors (Fig. 1, inset).

Two signal transducer networks related to the  $\text{A}_1$  and  $\text{A}_2$  operational amplifiers (Fig. 1s) generated electric potential differences (mV) linearly proportional to the intensities of the radiation incident on their sensitive surfaces. The third network, related to the  $\text{A}_3$  operational amplifier, was accountable for signal summation and amplification, whereby the signal delivered by this network was about five-fold the sum of the signals obtained with the  $\text{A}_1$  and  $\text{A}_2$  amplifiers. This analog signal was converted to digital by the Arduino board with a resolution of 12 bits; thus, the measurements were performed within the 0–4096 digital unit range.

Aiming at to simplify the luminometer electronics, no offset adjustment was made. The first analytical run was carried out without adding the  $\text{Cu}^{2+}$  (catalyst) solution, and the value of the generated signal (dark) was used as a reference. The subsequent analytical runs were carried out in the presence of the catalyst, and the net signals were obtained by subtracting the dark value from the current measurements. The analytical signal involved the sum of 20 measurements, which were obtained by reading the signal generated during the pumping semi-



**Fig. 1.** Flow diagram of the proposed module. P<sub>1</sub>, P<sub>2</sub> and P<sub>3</sub>: solenoid pumps; B<sub>1</sub> and B<sub>2</sub>: compact peristaltic pumps; MC: mixing chamber; Mb: magnetic stirring bar; x: four-line joint device machined in PTFE; W: bottle for waste disposal; D: detector. Inset: details of the chemiluminometric detection system [16].

**Table 1**  
Operating sequence.

Step	Event	Device	Action
1	chamber washing	P <sub>1</sub>	10 pulses
2	chamber emptying	B <sub>2</sub>	20 s
3	sample insertion	P <sub>1</sub>	20 pulses
4	Cu <sup>2+</sup> insertion	B <sub>1</sub>	<sup>a</sup> 0–10 s
5	stopping time	–	5–30 s
6	flow line filling	B <sub>2</sub>	1 s
7	signal reading	B <sub>2</sub> + P <sub>2</sub> + P <sub>3</sub>	0.2 s/20 pulses
8	binary search	–	–
9	chamber emptying	B <sub>2</sub>	15 s
1	chamber washing	P <sub>1</sub>	10 pulses

<sup>a</sup> Value depending on the increase or decrease in volume defined by the binary search algorithm (step 8).

cycle (step 7, Table 1) when the B<sub>2</sub>, P<sub>2</sub> and P<sub>3</sub> pumps were switched off.

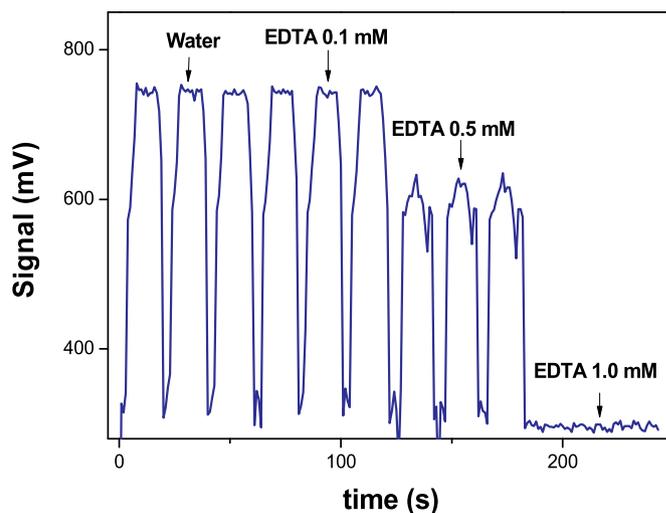
Regarding the analytical sequence (Table 1), the chamber was initially washed with the sample, by sequentially performing steps 1 and 2. Thereafter, aliquots of the sample (step 3) and Cu<sup>2+</sup> solution (step 4) were added into the chamber by sequentially switching the P<sub>1</sub> and B<sub>1</sub> pumps. After the available time interval for complexing reaction development (step 5), the mixture was forwarded by the B<sub>2</sub> pump towards detection. After a 1-s delay time (step 6) for filling the flow line with the sample mixture, the B<sub>2</sub>, P<sub>2</sub>, and P<sub>3</sub> pumps were several times on and off switched (step 7). During this step, data acquisition was performed. If the signal was 10% higher than the analytical blank measurement (reference value), the volume of Cu<sup>2+</sup> solution was reduced to 50% of that related to the previous attempt, and the process was repeated until the analytical signal reached a value lower than the reference signal. In this situation, the volume of copper solution was increased by 50% before the next step. This logic was repeated until the volume variation did not change the magnitude of the signal [23].

### 3. Results and discussion

Cu<sup>2+</sup> was selected as catalyst considering that catalytic effects of Cu<sup>2+</sup> and Cu<sup>+</sup> on the peroxide/luminol reaction are well known [16,25–28], and that this ion presents high affinity with a large number of ligands in both natural and synthetic chemical materials.

In order to demonstrate the applicability of the proposed procedure, model substances (milk, HS, MB) with high opacity were used, along with EDTA, DDTA or DTPA. These complexing agents form highly stable complexes with Cu<sup>2+</sup>, log β<sub>2</sub> values = 18.8, 23.9 and 21.2 [29–31]. A typical response profile obtained by using EDTA aqueous solutions (colorless) as the complexing agent is shown in Fig. 2. This assay involved a 0.30 mmol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>, a 0.30 mmol L<sup>-1</sup> luminol (in 0.1 mol L<sup>-1</sup> NaOH), and a 1.0 mmol L<sup>-1</sup> CuSO<sub>4</sub> solutions. Fig. 2 shows that profiles recorded for water and for 0.10 mmol L<sup>-1</sup> EDTA [Cu-EDTA]<sup>2-</sup> presented no significant differences, because the free Cu<sup>2+</sup> in the titration medium related to 100% or to about 90% of the added one, and the dependence of the emitted radiation intensity with Cu<sup>2+</sup> is asymptotic. This aspect is not a limiting one, as only the portions associated with low free Cu<sup>2+</sup> amounts, near the titration end-point, are usually of interest. For 1.0 mmol L<sup>-1</sup> EDTA, total signal suppression was noted indicating that Cu<sup>2+</sup> was quantitatively complexed.

The analytical signals were characterized by good repeatability, and this is another positive factor towards the robustness of the analytical procedure.



**Fig. 2.** Typical recordings for EDTA standard solutions using a 1.0 mmol L<sup>-1</sup> Cu<sup>2+</sup> solution. Copper solution/complexing reagent volumetric ratio kept constant. For details, see text.

### 3.1. Experimental conditions

The flow analysis module (Fig. 1) was designed to evaluate the catalytic effect of  $\text{Cu}^{2+}$  on the  $\text{H}_2\text{O}_2$ /luminol reaction, thus the efficiency for generating electromagnetic radiation. The concentrations of  $\text{H}_2\text{O}_2$ , luminol and NaOH solutions were defined by the univariate method involving a  $1.0 \text{ mmol L}^{-1}$   $\text{Cu}^{2+}$  solution. The assays were performed by varying the concentrations within the  $0.10\text{--}5.0 \text{ mmol L}^{-1}$  range, except for NaOH solution, which was varied from  $0.01$  to  $0.30 \text{ mol L}^{-1}$ . For  $0.08 \text{ mmol L}^{-1}$  NaOH, increasing the luminol and  $\text{H}_2\text{O}_2$  concentrations increased the analytical signal up to  $3.0 \text{ mmol L}^{-1}$ . Due to the pronounced effect of the alkalinity on chemiluminescence, influence of the NaOH concentration was investigated. An increase in signal intensity was observed up to  $0.10 \text{ mol L}^{-1}$  NaOH with the use of  $3.0 \text{ mmol L}^{-1}$  of luminol and  $\text{H}_2\text{O}_2$ . The  $3.0 \text{ mmol L}^{-1}$   $\text{H}_2\text{O}_2$  and  $3.0 \text{ mmol L}^{-1}$  luminol in  $0.1 \text{ mol L}^{-1}$  NaOH solutions were then selected for further experiments.

### 3.2. Complexing capacity (CC) in a colorless medium and in a MB solution

Formation of copper complexes prevents the catalysis of the luminol oxidation by  $\text{H}_2\text{O}_2$ . When a low quantity of copper is added after the stoichiometric point, luminol oxidation is catalyzed, thus revealing the end-point, which can be indirectly related to the CC of the sample.

Experiments in aqueous media were carried out by using EDTA, DTPA and DDTC standard solutions yielding copper complexes with 1:1, 1:1 and 2:1 stoichiometric ratios, respectively. Standard solution of  $1.0 \text{ mmol L}^{-1}$   $\text{Cu}^{2+}$  and sample volumes of  $1.10 \text{ mL}$  spiked with  $0.5$  and  $1.0 \text{ mmol L}^{-1}$  of the complexing chemical species were used in these trials. The amounts in Figs. 3–5 were calculated by considering the titrand volume ( $1.10 \text{ mL}$ ) and the different titrant ( $\text{Cu}^{2+}$ ) volumes.

The  $\text{Cu}^{2+}$  amounts added to colorless water solutions and to the opaque  $10.0 \text{ mg L}^{-1}$  MB solutions are shown in Fig. 3. For colorless  $0.55$  and  $1.10 \mu\text{mol}$  EDTA solutions, the copper complexing capacities  $[\text{Cu-EDTA}]^{2-}$  in Fig. 3a were  $0.56 \pm 0.01$  and  $1.10 \pm 0.01 \mu\text{mol Cu}^{2+}$ ,

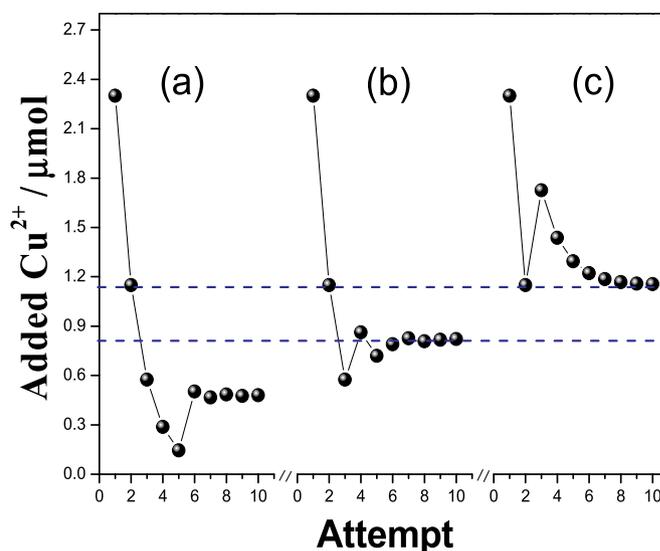


Fig. 4. System response for the complexation of  $\text{Cu}^{2+}$  by leonardite in highly opaque medium. (a): only leonardite; (b, c): leonardite doped with  $0.3$  and  $0.6 \text{ mmol L}^{-1}$  EDTA. Solid and traced lines as in Fig. 3.

which were determined by considering the stoichiometric ratio. These values are consistent with the theoretical ones ( $0.55$  and  $1.10 \mu\text{mol Cu}^{2+}$ ).

As DDTC (Fig. 3b) and DTPA (Fig. 3c) form  $1:2 [\text{Cu-DDTC}]_2$  and  $1:1 [\text{Cu-DTPA}]^{2-}$  complexes with copper, the addition of DDTC ( $0.55$  and  $1.10 \mu\text{mol}$ ) required  $0.27 \pm 0.01$  and  $0.53 \pm 0.01 \mu\text{mol}$  of copper, whereas DTPA ( $0.55$  and  $1.10 \mu\text{mol}$ ) required additions of  $0.56 \pm 0.01$  and  $1.14 \pm 0.01 \mu\text{mol Cu}^{2+}$ .

Fig. 3d shows the responses obtained for the  $10.0 \text{ mg L}^{-1}$  MB solution spiked with  $0.5 \text{ mmol L}^{-1}$  EDTA. In this system,  $1.10 \text{ mL}$  of the (EDTA plus MB) solution were added to the mixing chamber (Fig. 1),

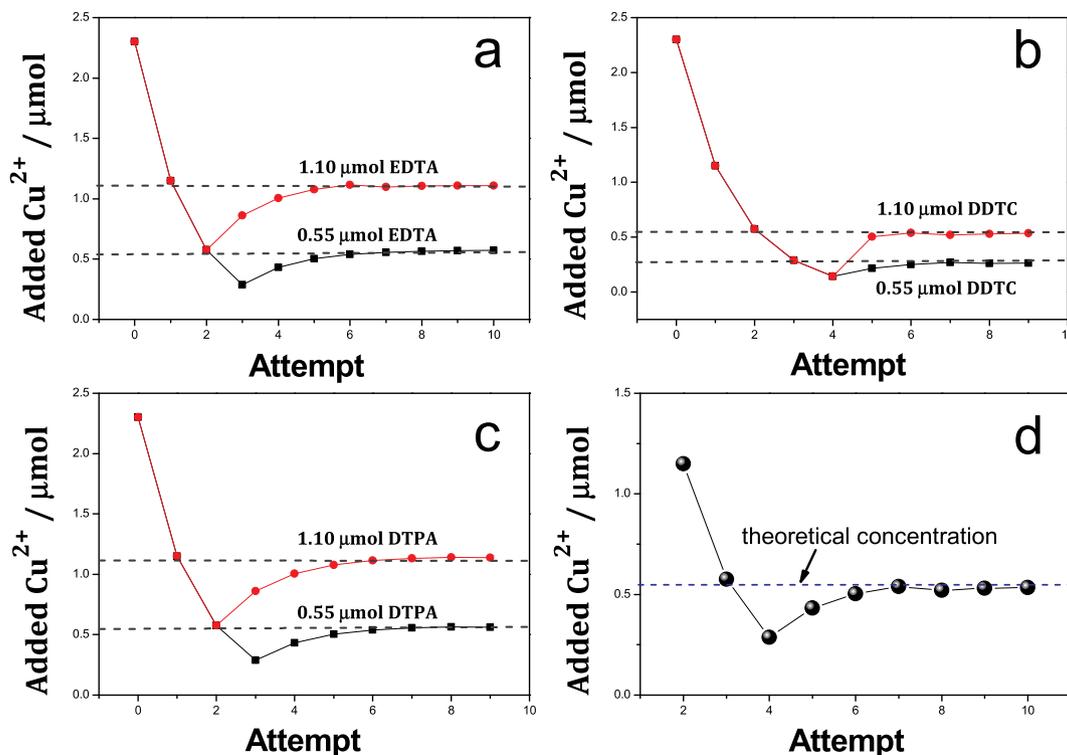


Fig. 3. CC determination of model complexing agents. (a–c):  $0.5$  and  $1.0 \text{ mmol L}^{-1}$  DDTC, DTPA, and EDTA solutions; (d):  $10 \text{ mg L}^{-1}$  MB solution doped with  $0.5 \text{ mmol L}^{-1}$  EDTA. Solid lines: values obtained with the flow system in Fig. 1; horizontal traced lines: theoretical stoichiometric ratios.

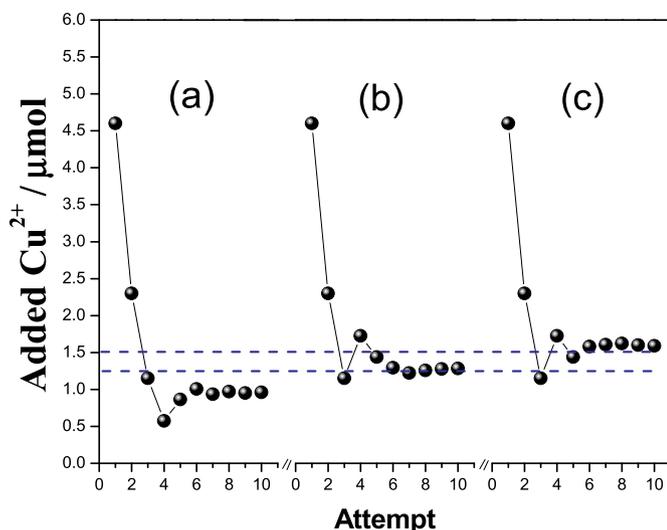


Fig. 5. System response for the complexation of  $\text{Cu}^{2+}$  by milk samples. (a): only milk; (b), (c): milk doped with 0.5 and 1.0  $\text{mmol L}^{-1}$  DDTC. Solid and traced lines as in Fig. 3.

corresponding to  $0.55 \mu\text{mol}$  of the complexant. Under these conditions, the amount of  $\text{Cu}^{2+}$  added for attaining the titration end-point was  $0.53 \pm 0.01 \mu\text{mol Cu}^{2+}$ , demonstrating the viability of this proposal for the CC evaluation of colored solutions.

Compared to the expected theoretical values, the results obtained with the proposed procedure presented a relative deviation of  $< 4\%$ . The values, used to calculate the amount of added  $\text{Cu}^{2+}$ , corresponded to the average of the last three values provided by the flow system.

### 3.3. Evaluation of the copper complexation capacity of humic substances

HS suspensions are highly opaque, thus very difficult to analyze by conventional methods (e.g., UV-Vis and classical titrations relying on color indicator) without resorting from pretreatment steps such as e.g. alkaline extractions. In the present proposal, the HS sample (leonardite) was used without any pretreatment *via* addition and recovery tests involving known amounts of EDTA. The determinations were carried out with a  $400 \text{ mg L}^{-1}$  of leonardite suspension, spiked with 0.00 (no spiking), 0.30 and  $0.60 \text{ mmol L}^{-1}$  EDTA (Fig. 4). Without EDTA, the leonardite sample inside the chamber ( $1.10 \text{ mL}$ ) complexed  $0.48 \pm 0.01 \mu\text{mol Cu}^{2+}$ , meaning a CC of  $1.09 \pm 0.01 \text{ mmol Cu}^{2+}$  per gram of sample. Recoveries were estimated as  $101.6 \pm 2.1\%$  and  $103.1 \pm 1.1\%$  for 0.30 and  $0.60 \text{ mmol L}^{-1}$  EDTA spiking, respectively. To this end, the EDTA-Cu complex stoichiometry was considered, and the amount of complexed copper by the leonardite binding groups were subtracted.

The HS complexing capacity for metallic ions has been often studied, and pronounced variability has been reported, emphasizing the influence of the source material, sample preparation procedure, isolation of the soluble fractions, pH value and ionic strength (Table 2). The humic substances-metal CC values have been usually determined by

Table 2  
Complexing capacities of humic substances.

Methodology	CC ( $\text{mmol g}^{-1}$ )	sample source	Ref.
automatic chemiluminometric titration	$1.09 \pm 0.01$	leonardite	This work
fluorescence quenching	$0.55 \pm 0.2$	leonardite	[32]
potentiometry	$1.18 \pm 0.03$	leonardite	[33]
ultrafiltration/atomic spectrometry	1.12 to $1.99^a$	HS	[34]
total luminescence spectroscopy	1.8 to 3.7	humic acid	[35]

<sup>a</sup>  $\text{mmol g}^{-1}$  of total organic carbon.

adding increasing amounts of  $\text{Cu}^{2+}$ , and assuming a 1:1 metal:ligand stoichiometry, according to the method proposed by Ryan and Weber [32]. For several HS, the CC values were estimated within  $0.21\text{--}1.33 \text{ mmol g}^{-1}$  of humic samples ( $0.55 \pm 0.20$  for leonardite), exploiting the fluorescence quenching method [33]. With potentiometric detection with an ion-selective electrode, CC was estimated within the  $0.73\text{--}1.44 \text{ mmol g}^{-1}$  of humic acids ( $1.18 \pm 0.03$  for leonardite) [34]. For different HS processed by using ultrafiltration system and determination by atomic spectrometry,  $1.12\text{--}1.99 \text{ mmol g}^{-1}$  of total organic carbon values were found [35]. Samples of humic acids extracted from soils presented CC values within the  $1.8\text{--}3.7 \text{ mmol g}^{-1}$  of humic acid, as determined by total luminescence spectroscopy [36].

A literature survey and an analysis of the above-mentioned recovery data reveal that the proposed system is suitable for the determination of CC in highly opaque media. Both the cumbersome sample preparation step, and the large reagent amounts, inherent to previously reported procedures [11,31–35], are not required.

### 3.4. Evaluation of the copper complexation capacity of milk samples

Milk is highly opaque, and some of its constituents may present strong affinity with  $\text{Cu}^{2+}$  due to of the interactions with protein and peptide chains. This aspect is the basis of most methods for protein determination in milk and other matrices (e.g. biuret [37], Lowry [38] and Smith [39] methods). Of note is that the copper complexation with proteins/peptides was recently exploited to identify the adulteration of milk with water, involving measurement of the refractive index of the solution [40].

Initially, the experiments carried out to determine the copper CC of milk involved spiking with  $0.00\text{--}0.60 \text{ mmol L}^{-1}$  EDTA solutions. The recovery values for 2-, 5- and 10-fold water diluted milk samples were as high as 200%, probably because EDTA complexed the  $\text{Ca}^{2+}$  ions present in large amounts in the milk. The available EDTA amounts for complexation with copper ions were then reduced, leading to false positive results. To overcome this drawback, DDTC was used as the model complexing agent. For evaluating the CC of milk, the diluted sample was spiked with 0.00, 0.50 and  $1.00 \text{ mmol L}^{-1}$  DDTC solution. A  $2.0 \text{ mmol L}^{-1}$   $\text{Cu}^{2+}$  solution was added to the mixing chamber of the flow system in Fig. 1. Better recovery values ( $\sim 115\%$ ) were attained for the 10-fold sample dilution, the selected dilution degree.

The milk samples were analyzed in the flow system of Fig. 1, and the concentrations of  $\text{Cu}^{2+}$  complexed by the milk in the presence and absence of complexing agent are shown in Fig. 5. For 0.55 and  $1.10 \mu\text{mol}$  DDTC, additions of  $0.31 \pm 0.03$  and  $0.64 \pm 0.01 \mu\text{mol Cu}^{2+}$  to the mixing chamber were performed, and recoveries of 112 and 116% were attained, by taking into account the 2:1 complex stoichiometry. The unspiked diluted milk inside the mixing chamber was able to complex  $0.96 \pm 0.01 \mu\text{mol Cu}^{2+}$ , thus revealing a CC of  $8.74 \pm 0.02 \mu\text{mol Cu}^{2+}$  per mL of milk. This value was found by setting a 30-s time interval for reaction development (stopping time, in Table 1). The recovery results demonstrate the feasibility of the proposed procedure for the determination of the CC in solutions with high opacity.

### 3.5. System performance

A critical analysis of the recovery values for standard solutions of complexing agents in colorless, highly colored or turbid media (Table 3) demonstrates the applicability of the proposed strategy. Other metal ions that catalyze chemiluminescent reactions (i.e.,  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Cr}^{3+}$ ) could be used as the standard agent [17,41], providing that there is a stoichiometric relation with the model complexing agent.

The proposed procedure presents noteworthy advantages, such as a low consumption of reagents ( $0.42 \text{ mg}$  of luminol,  $81.6 \mu\text{g H}_2\text{O}_2$ , and  $1.10 \text{ mL}$  of sample) for each titration attempt, a coefficient of variation within results estimated as 5%, and a sampling rate of  $43 \text{ h}^{-1}$ .

**Table 3**  
Recovery data for solutions doped with complexing agents.

Medium color	Complexing agent	Recovery (%)	SD (%)
colorless	EDTA	100.3–102.6	0.6–1.3
colorless	DTPA	102.1–103.3	0.8–0.4
colorless	DDTC	96.1–96.3	1.2–1.7
<sup>a</sup> black	EDTA	99.4–104.2	2.1–1.0
<sup>b</sup> white	DDTC	113.5–115.2	4.9–2.9
<sup>c</sup> blue	EDTA	96.1	1.2

<sup>a</sup> Humic substance sample (400 mg L<sup>-1</sup>).

<sup>b</sup> 10-fold water diluted milk sample.

<sup>c</sup> 10 mg L<sup>-1</sup> MB solution.

Moreover, sample preparation and calibration of the detecting system are not needed. The main advantage is that a linear detector response is not required.

#### 4. Conclusions

The proposed microprocessor-controlled (Arduino) analytical module exploiting binary search in a flow-batch system, yields precise results. It is highly versatile, and can be used for fast CC evaluation in media with high opacity. Sample preparation is simple, allowing the CC evaluation without altering the natural state of the sample constituents. This favorable aspect is not extensive to spectrometric techniques, which usually require prior sample preparation, including steps for separating the acid or alkaline soluble fractions, or even ultrafiltration systems through specific membranes. The volume of effluent generated in performing eight attempts to reach the titration end-point is only 12 mL. Use of reagents with low toxicity (e.g. copper and luminol) is an additional positive factor towards attainment of an environmentally friendly procedure, which is advantageous in comparison to conventional methods.

Despite the intense chemiluminescence in alkaline medium, the luminol oxidation by H<sub>2</sub>O<sub>2</sub> catalyzed by Cu<sup>2+</sup> does not yield a linear response, thus has not been exploited for quantitative purposes. Yet, it was exploited in the proposed innovation, and the obtained results indicated that luminol chemiluminescence may become a powerful tool for studies in highly colored or turbid samples with complex matrices (e.g. biological fluids and industrial effluents).

Excellent analytical recovery data were estimated after the addition of complexing agents in media with high opacity and matrix complexity, as verified by using milk and humic substances as proofs of concept. The results encourage one to develop automatic titrations for evaluating the complexing ability of other complex samples.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2019.120530>.

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