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Enrico Prenesti [a], Silvia Berto [a*], Fabio Gosmaro [a1], Paola Fisicaro [b], Marco Bagnati [c], Giorgio Bellomo [c]

[a] *Dipartimento di Chimica, Università degli Studi di Torino, Via Pietro Giuria 5, I–10125, Torino (Italy).*

[b] *Laboratoire National de Métrologie et d'Essais (LNE), Department of Biomedical and Inorganic Chemistry, 1 Rue Gaston Boissier, 75015 Paris (France).*

[c] *Azienda Ospedaliera Maggiore della Carità, Università degli Studi del Piemonte Orientale, Corso Giuseppe Mazzini, 18, 28100, Novara (Italy).*

[*] **Corresponding author**: silvia.berto@unito.it

Abstract

This paper deals with the evaluation of the measurement uncertainty of Total Antioxidant Capacity (TAC) of human plasma estimated by the CUPRAC-BCS method, a photometric method that uses the redox couple Cu(II)/Cu(I) to estimate the reducing capacity of a fluid. The urate is usually used as reference molecule in the clinical chemistry field. In this work, the measurement uncertainty was assessed on TAC values obtained using both urate and Cu(I), as reference species, and two measurement apparatuses i.e. i) a UV-visible spectrophotometer and ii) a routine auto-analyser. A comprehensive list of relevant sources of uncertainty was compiled. Uncertainties estimated are related to the effects of: calibration curve, repeatability, temperature, pH, concentration of the reference stock solutions, electronic exchange of the redox reaction between Cu(II) and urate. The measurements performed with the automatic apparatus associated with the use of Cu(I) ion for the calibration provide the lowest uncertainty, u(TAC)/TAC = 0.0094.

Keywords: Total Antioxidant Capacity, CUPRAC, Human plasma, Measurement uncertainty, Uncertainty budget, Oxidative stress.

[1] Present address: Istituto professionale di Stato per servizi alberghieri e ristorazione G. Giolitti, piazza IV Novembre 12080, Mondovì (CN), Italy

1. Introduction

The term *oxidative stress* identifies the change in the normal physiological balance between oxidant substances of the blood and the antioxidant system of redox detoxification. When pro-oxidant substances increase or antioxidant ones are scarce or ineffective, a situation of oxidative stress ensues leading to molecular damage and tissue injury. Oxidative stress is caused by an excess of ROS - Reactive Oxygen Species - and/or RNS - Reactive Nitrogen Species - in biological fluids that can cause molecular and cell damage. In the course of biological evolution an antioxidant defence system has been developed to protect the human organism against ROS/RNS: in fact, a sophisticated and co-operative array of antioxidant molecules, including endogenous (enzymes and non-enzyme species) and exogenous (as some vitamins and polyphenols) compounds, is found in living organisms [1].

A large number of papers shows the interest of biologists and physicians in this topic, since it is well know that oxidative stress is involved in the pathogenesis and development of many human diseases such as cancer (as lung cancer, prostate cancer) [2], diseases of the cardiovascular system (hypertension, atherosclerosis, ictus, infarct) [3-5], diseases of the central nervous system (Alzheimer's disease, Parkinson's disease, multiple sclerosis) [6-8].

Many analytical methods have been developed to measure the Total Antioxidant Capacity (henceforth, TAC) in a wide range of matrices such as biological fluid, food, beverages, plant extracts and several reviews about them have been discussed [9-13]. The "Trolox Equivalent Total Antioxidant Capacity" (TEAC) [14], the "Ferric Reducing Ability of Plasma" (FRAP) [15], the "Oxygen Radical Absorbance Capacity" (ORAC) [16], the "Total Radical Absorption Potential" (TRAP) [16] and the "CUPric ion Reducing total Antioxidant Capacity" (CUPRAC) [17-21] are some of the most commonly methods used for TAC determination of biological fluids. We chose the CUPRAC method among many others since: i) it works at a physiological pH (the redox reactivity tested corresponds to that of *in vivo* conditions); ii) chemical preparations and

measurements steps are simple and fast; iii) stability of reagents is satisfactory, especially with respects to that of the methods employing radicals; iv) it is inexpensive [18, 22].

This study is aimed to assess the measurement uncertainty of human plasma TAC by way of the CUPRAC-BCS method [11, 17-23]. Two calibrating substances, namely urate and Cu(I), and two testing apparatuses, namely, a UV-visible manual spectrophotometer and a routine auto-analyser of a public hospital (always based on the photometric measurement) were used. The testing method was previously optimized and validated on the auto-analyser ADVIA-2400 [24] in order to assess its analytical performances and adapt it to the automation needs of a hospital laboratory routine. Urate is habitually used as reference molecule to express the TAC in clinical chemistry, because it is the main antioxidant molecule in human plasma. The stability of urate aqueous solutions is limited to few days, as previously verified [24], therefore, the use of Cu(I) as a reference was tested seeking to improve the quality of the measurement.

No systematic studies on the uncertainty evaluation were found in the literature for such method. Since the measurand is not a defined chemical entity, but it corresponds to a redox reactivity under fixed conditions, the method belongs to the empirical ones and provide a method-dependent result [25]. The measurand is hence defined by the method as well as its measurement uncertainty. Therefore, the contribution of significant variables affecting the measurement uncertainty of TAC was considered in order to be able to distinguish analytical variations from clinical ones.

Providing measurement uncertainty of quantities having a clinical meaning is fundamental and the recent literature proves the interest of the scientific community on this topic [26-29]. International organisms for standardization have drawn up guides for the evaluation of the measurement quality in the medicinal laboratories field. Particularly, the International Standard ISO 15189 [30] is focused on the managerial and organizational quality of the medicinal laboratories and, as previously observed by Milinković and co-workers [29], it does not treat the technical aspects of measurement and related uncertainty. At present, ISO is going to develop a technical specifications document for assessing measurement uncertainty in medical laboratories (ISO/NP TS 20914

Medical Laboratories – Practical Guide for the measurement of measurement uncertainty). In this frame, we chose to assess the measurement uncertainty according to the metrological, or bottom-up, approach [31, 32].

2. Materials and Methods

2.1 Chemicals

Uric acid sodium salt (purity ≥ 98 %), bathocuproinedisulfonic acid (BCS, purity ≥ 98 %), Cu(II) sulphate pentahydrate (purity ≥ 98 %), PBS (Phosphate Buffered Saline 0.1 mol L^{-1}) and L-ascorbic acid (purity ≥ 98 %) were purchased from Sigma Aldrich and were of analytical grade. Cu(II) reference solution used for the calibration was by Merck (1000 ± 1) mg L^{-1}.

All solutions were prepared using grade A glassware and ultrapure water from MilliQ apparatus. Uric acid for the calibration was dissolved in 30 mmol L^{-1} NaOH to attain a concentration of 2 mmol L^{-1}. Cu(II) solutions for the calibration were prepared diluting the Cu(II) reference solution so as to obtain concentrations ranging between 0.2 – 2.0 mmol L^{-1}. BCS was dissolved in 10 mmol L^{-1} PBS (pH 7.40) to attain a concentration of 36 mmol L^{-1} (stock solution). Cu(II) sulphate as reagent for samples measurement was dissolved in MilliQ water to reach a concentration of 10 mmol L^{-1} (stock solution). L-ascorbic acid was dissolved in MilliQ water to reach a concentration of 50 mmol L^{-1} (stock solution). Reagent R1 was prepared diluting BCS stock solution to 900 μmol L^{-1} in PBS buffer (pH 7.40). Reagent R2 was prepared diluting Cu(II) sulphate stock solution to 640 μmol L^{-1} in MilliQ water or L-ascorbic acid stock solution to 10 mmol L^{-1} in MilliQ water (see Table 1). pH-metric ready to use buffer solutions: pH 4.01 and 9.00 at 20 °C were from Merck.

Table 1. Composition of the reagents used for the TAC measurement.

Reagent	Reference substance	
	Cu(I)	**Urate**
R1	BCS 900 μmol L^{-1} in PBS[a]	BCS 900 μmol L^{-1} in PBS[a]
R2	L-ascorbic acid 10 mmol L^{-1}	Cu(II) sulphate 640 μmol L^{-1}

[a] BCS = bathocuproinedisulfonic acid; PBS = Phosphate Buffered Saline 0.1 mol L^{-1}

2.2 Plasma sample collection and preparation

Different pools of plasma with different TAC values were used. Blood was collected into tubes containing lithium-heparin. Plasma was obtained by withdrawing supernatants of centrifuged blood at 3500 rpm for 6 min at 15 °C. Each pool was subdivided in aliquots, frozen at –20 ºC and thawed out before use.

2.3 Total Antioxidant Capacity measurement

The TAC is an index of redox reactivity of a fluid in fixed experimental conditions. It does not strict represent the concentration of a specific substance. TAC is detected using a redox test reaction, the result is kinetic dependent and the measurements require the setting of a suitable signal sampling time. Particularly, the CUPRAC-BCS method [11, 17-24] uses Cu(II) ion as an oxidant. This method detects the ability of a blend of antioxidants to transfer electrons to reduce a test redox-active compound under the experimental conditions. The analytical reaction is so represented:

$$\text{Cu(II)-BCS} + S_{rid} \rightarrow \text{Cu(I)-BCS} + S_{ox} \qquad (1)$$

where Cu(II)/(I)-BCS are the copper complexes with BCS and $S_{rid/ox}$ indicates the redox active substances in the sample. The neo-formed Cu(I) ion is detected by the photometric measurement of the complex resulting from the interaction with the selective ligand BCS, whose absorption maximum is in the range 470-500 nm [23]. The extent of formation of Cu(I) reflects the TAC of plasma. To quantitatively express the TAC, it is possible to use both Cu(I) or urate as reference

substances. In the case of plasma, urate is the most abundant antioxidant molecule, therefore it is usually used as reference molecule to express the analytical result of TAC. Urate reduces Cu(II)-BCS to Cu(I)-BCS as reported below [23]:

$$2\ \text{Cu(II)-BCS} + \text{urate} \rightarrow 2\ \text{Cu(I)-BCS} + \text{allantoin} \qquad (2)$$

and the calibration diagram reports the absorbance due to the Cu(I)-BCS formed as a function of the urate concentration. Calibrating with urate or Cu(I) is equivalent, since the stoichiometry of the redox reaction (2) between Cu(II) and urate was previously studied [24]. What might be now of interest is discriminating between the two calibrating substances as to the quality of the results, since the factors affecting the variability of the measurements are different.

2.4 Instruments

Apparatus A: UV-visible spectrophotometer Lambda 25 provided by Perkin Elmer (cuvette optical path-length 10 mm). The time-drive mode at fixed wavelength (working wavelength = 490 nm; henceforth, λ_w) was used to study the kinetic of the reaction. All data were processed with UVlab software (ver. 6.0).

Apparatus B: ADVIA 2400 Chemistry System provided by Siemens. It is a clinical chemistry auto-analyser with 2400 test/h capacity and two reagent trays (R1 and R2). The reaction was monitored by means of a photometer (cuvette optical path-length 6 mm) at 478 nm (henceforth, λ_w) [24], the nearest wavelength available on the automated instrument as to those of interest.

Balance: Mettler MS204S (maximum capacity 220 g; standard uncertainty $6 \cdot 10^{-5}$ g).

pH-meter: Hanna Instruments pH-211 microprocessor.

pH glass electrode: Metrohm, model Porotrode with capillary diaphragm.

Software: all data obtained were presented using the software Origin 6.1. (by OriginLab) and elaborated using SPSS Statistics 17.0 (by SPSS).

2.5 Calibration procedures

The quantification of TAC is achieved through the calibration curve obtained with either urate or Cu(I), in the range of concentrations (henceforth, *C*) 0.2 – 2.0 mmol L^{-1}, as reference species. Experiments were also conducted on two apparatuses: a manual UV-visible spectrophotometer, henceforth Apparatus A, and a routine auto-analyser, henceforth Apparatus B (data in ref. [24]). The two independent calibration procedures were applied on both apparatuses. Hence, we will handle four datasets and four results to be compared.

2.5.1 Calibration with urate. A selected volume of urate solutions, concentrations 0.2, 0.5, 1.0, 1,5 and 2.0 mmol L^{-1}, was added to reagent R1 (final concentration 700 μmol L^{-1}) and incubated at room temperature for 1 min. Absorbance at λ_w was then measured (A_0). Following, reagent R2 (Cu(II) sulphate, final concentration 128 μmol L^{-1}) was added and, after incubation of 3 min [15], the absorbance at λ_w was measured (A_1). The increase of the absorbance value ($\Delta A = A_1 - A_0$) was calculated for each standard and used to build the calibration curve (ΔA *vs* C_{urate}).

2.5.2 Calibration with Cu(I). The calibration curve ΔA *vs* $C_{Cu(I)}$ is achieved reducing the Cu(II) of the reference solutions to Cu(I) using L-ascorbic acid as reducing molecule in large excess. A selected volume of diluted Cu(II) solution (concentrations: 0.2, 0.5, 1.0, 1,5 and 2.0 mmol L^{-1}) was added to reagent R1 (final concentration 700 μmol L^{-1}) and incubate at room temperature for 1 min. The absorbance at λ_w was then measured (A_0). Following, reagent R2 (L-ascorbic acid, final concentration 10 mmol L^{-1}) was added and, after incubation of 3 min [15], the absorbance at λ_w was measured (A_1). The ΔA was calculated for each standard and used to build the calibration curve.

2.6 Sample measurement

A selected volume of sample (plasma) was added to reagent R1 (final concentration 700 μmol L^{-1}) and incubated at room temperature for 1 min. The absorbance at λ_w was then measured (A_0). Following, reagent R2 (Cu(II) sulphate, final concentration 128 μmol L^{-1}) was added and after incubation of 3 min [15] the absorbance at λ_w was measured (A_1). For each sample the ΔA was calculated and reported to the calibration curve obtained with urate or Cu(I) as reference. Particularly, if urate is used for the calibration procedure, TAC is expressed as «μmol L^{-1} of Cu(I)-BCS reducing equivalent» [24]. Instead, if Cu(I) is used, the TAC is expressed as «μmol L^{-1} of Cu(I)-BCS».

3. Results and discussion

3.1 Setting of the photometric measurement on Apparatus A

According on findings achieved on Apparatus B [24], photometric measurements were preliminarily assessed on the Apparatus A. For the formation of the complex, the reagent R1 (final concentration 700 μmol L^{-1}) was added to urate and to the reagent R2 (Cu(II) sulphate, final concentration 128 μmol L^{-1}). Figure 1 shows the kinetic curves recorded for Cu(II) reaction with blank, urate solutions (at five different concentrations corresponding to those used for the calibration curve) or plasma sample. Comparing the signals obtained by the samples and the reference solutions (see trends (c) and (g) in Figure 1) it is possible to confirm that a reaction time of 3 min is suitable to reach a reliable photometric detection, also in the case of Apparatus A.

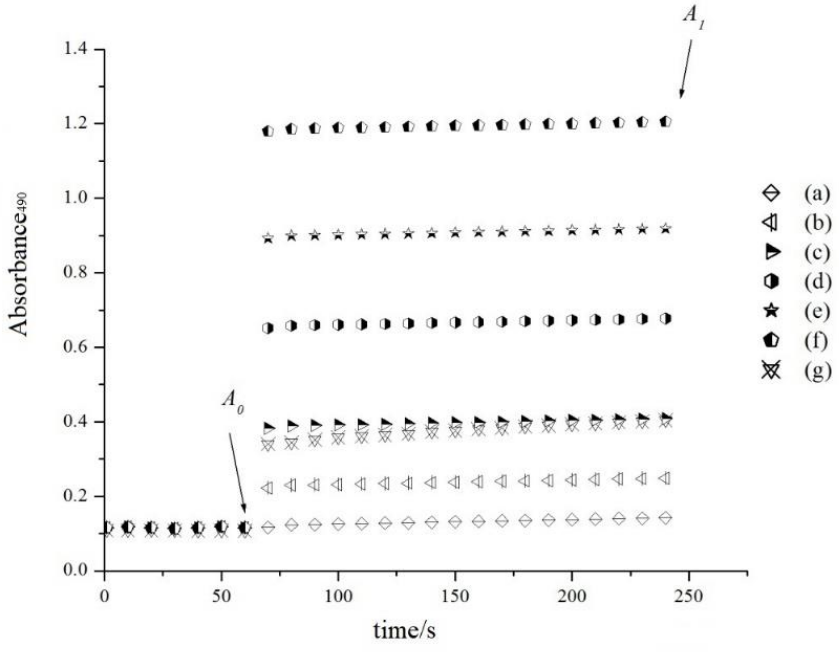

Figure 1. Kinetic curves of Cu(II) reaction in presence of blank (a), urate 0.2 mmol L^{-1} (b), urate 0.5 mmol L^{-1} (c), urate 1.0 mmol L^{-1} (d), urate 1.5 mmol L^{-1} (e), urate 2.0 mmol L^{-1} (f) and a plasma (g). A_0: absorbance reading after 1 min from the R1 addition; A_1: absorbance reading after 3 min from the R2 addition. From 0 to 60 s all curves are superimposed and related symbols are nearly undistinguishable. The measurements were done with Apparatus A.

3.2 Measurement uncertainty evaluation

The quantities significantly affecting the TAC were identified for both calibration procedures and are shown in the comprehensive fishbone diagram of Figure 2. They can be quantitatively expressed as multiplicative factors (*f*) [25]. Being X_o a generic concentration obtained using the inverse regression of the two calibration equations, the TAC value (mmol L^{-1}) is calculated by way of the formulae:

$$TAC = X_o \cdot f_{rep} \cdot f_T \cdot f_{pH} \cdot f_{Cstock\ Cu(II)} \quad \text{from calibration with Cu(I)}$$

$$TAC = 2 \cdot X_o \cdot f_{rep} \cdot f_T \cdot f_{pH} \cdot f_{Cstock\ urate} \cdot f_{RF} \quad \text{from calibration with urate}$$

where:

- 2 is the number of electrons exchanged between Cu(II) and urate [24],
- f_{rep} refers to the repeatability (rep = repeatability),
- f_T refers to the temperature (T, °C),
- f_{pH} refers to the pH,
- f_{Cstock} refers to the concentration of the Cu(II) ($C_{stock\ Cu(II)}$) or urate ($C_{stock\ urate}$) stock solutions,
- f_{RF} refers to the electronic exchange of the analytical redox reaction (RF = redox factor) experimentally defined [24].

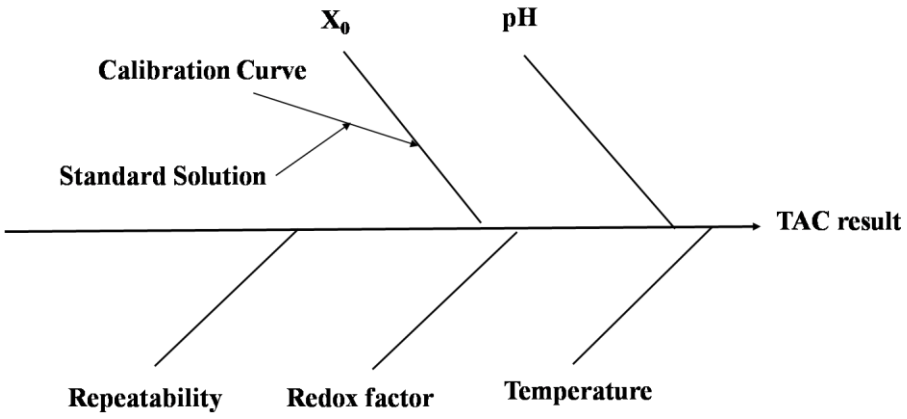

Figure 2. Fishbone diagram: reconnaissance of the parameters affecting the TAC variability in view of the measurement uncertainty evaluation. X_o is a generic concentration obtained using the inverse regression of the calibration equation.

The multiplicative factors f are defined so that their value is 1, but their contribution to the TAC measurement uncertainty have to be taken into account as follows:

- calibration with Cu(I)

$$u(\mathrm{TAC})/\mathrm{TAC} = \sqrt{\left(u(X_\mathrm{o})/\mathrm{X_o}\right)^2 + \left(u(\mathrm{rep})\right)^2 + \left(u(T)/T\right)^2 + \left(u(\mathrm{pH})/\mathrm{pH}\right)^2 + \left(u(C_{\mathrm{stockCu(II)}})/C_{\mathrm{stockCu(II)}}\right)^2}$$

- calibration with urate

$$u(\mathrm{TAC})/\mathrm{TAC} = \sqrt{\left(u(X_\mathrm{o})/X_\mathrm{o}\right)^2 + \left(u(\mathrm{rep})\right)^2 + \left(u(T)/T\right)^2 + \left(u(\mathrm{pH})/\mathrm{pH}\right)^2 + \left(u(C_{\mathrm{stockurate}})/C_{\mathrm{stockurate}}\right)^2 + \left(u(\mathrm{RF})/\mathrm{RF}\right)^2}$$

where the terms $u(x)/x$ indicate the relative standard uncertainty, $u_\mathrm{r}(x)$, of each quantity x. To assess TAC measurement uncertainty (u(TAC)/TAC = u_r(TAC)), calculation of every uncertainty sources is, therefore, a needful prerequisite.

3.2.1 Uncertainty related to the calibration curves. Uncertainty related to the use of the calibration curve was estimated following the guidelines of EURACHEM/CITAC [25]. Separate series of five reference solutions at the concentrations 0.2, 0.5, 1.0, 1.5 and 2.0 mmol L^{-1}, were prepared diluting Cu(II) reference solution or freshly prepared urate solution and analysed in three replicates per day. This work was carried out for six days. The variance related to X_o (var(X_o)) was evaluated by means of the following formula:

$$\mathrm{var}(X_o) = \frac{\mathrm{var}(y_{obs})}{b^2} + \frac{S^2}{b^2}\left(\frac{1}{\sum w_i} + \frac{(X_o - \overline{X})^2}{\sum (w_i X_i^2) - \sum (w_i X_i)^2 \Big/ \sum w_i}\right)$$

where:

- var(y_obs) is the variance related to the observed variable,
- b is the slope of the calibration curve,
- S is the root mean square deviation that indicates the discrepancy between the mean square values of observed data and estimated data values,
- w_i is the weight of y_i,
- X_i is the concentration of the reference solutions used for building the calibration curve,

- $\overline{X}$ is the mean of n concentrations X_1, X_2...X_n of the standards used for building the calibration curve,
- X_o is the concentration obtained using the inverse of the calibration equation.

Table 2 reports the weighted least squares regression parameters obtained for both urate or Cu(I) standard solutions on Apparatus A.

The uncertainty due to the calibration curve was estimated on a sample for a TAC value of 866 µmol L^{-1}. The TAC was measured on a pool of plasma that provided: y_{obs} = 0.267, var(y_{obs}) = $4.575 \cdot 10^{-5}$ obtained from three replicates, and X_o = 0.433 mmol L^{-1} (using urate calibration). The relative standard uncertainty $u_r(X_o)$ of X_o was calculated as follows:

$$u(X_o) = \sqrt{var(X_o)} = 0.0129\ mmol\ L^{-1}$$

$$u_r(X_o) = u(X_o)/X_o = 0.0129/0.433 = 0.0298$$

The same approach was used for X_o derived from the calibration with Cu(I) obtained using the Apparatus B (see ref. 24 for the calibration with urate). Table 3 reports the weighted least squares regression parameters obtained for both urate or Cu(I) standard solutions on Apparatus B. In this case, the uncertainty due to both calibration curves was estimated for a TAC value of 956 µmol L^{-1}.

Table 2. Weighted least squares regression on the 90 data obtained for the Apparatus A and using both urate and Cu(I) as reference solutions.

Table 2A: Coefficients report

	Value	Standard Deviation Error	t-value	Significance
Slope (urate)	0.527	0.002	227.208	0.000
Slope (Cu(I))	0.265	0.002	191.455	0.000
Intercept (urate)	0.026	0.001	27.646	0.000
Intercept (Cu(I))	0.025	0.001	28.140	0.000

Table 2B: ANOVA report

	Sum of squares	Degrees of freedom	Variance	F	Significance
Model (urate)	11.304	1	11.304	51623.536	0.000
Model (Cu(I))	1.876	1	1.876	36655.151	0.000
Prediction Error (urate)	0.019	88	$2.190 \cdot 10^{-4}$		
Prediction Error (Cu(I))	0.005	88	$0.568 \cdot 10^{-4}$		
Total (urate)	11.323	89			
Total (Cu(I))	1.881	89			

Table 2C: Quality of R

	R	R-square	Adj. R-square	ROOT-MSE	N
Model (urate)	0.999	0.998	0.998	0.0148	90
Model (Cu(I))	0.999	0.997	0.997	0.0075	90

Table 3. Weighted least squares regression on the 90 data obtained for the Apparatus B and using both urate and Cu(I) as reference solutions.

Table 5A: Coefficients report

	Value	Standard Deviation Error	t-value	Significance
Slope (urate) [24]	0.461	0.001	346.621	0.000
Slope (Cu(I))	0.232	0.001	251.903	0.000
Intercept (urate) [24]	0.026	0.001	25.140	0.000
Intercept (Cu(I))	0.025	0.001	24.684	0.000

Table 5B: ANOVA report

	Sum of squares	Degrees of freedom	Variance	F	Significance
Model (urate) [24]	9.444	1	9.44	120146.087	0.000
Model (Cu(I))	2.026	1	2.026	63454.930	0.000
Prediction Error (urate) [24]	0.007	88	$0.786 \cdot 10^{-4}$		
Prediction Error (Cu(I))	0.003	88	$0.319 \cdot 10^{-4}$		
Total (urate) [24]	9.451	89			
Total (Cu(I))	2.029	89			

Table 5C: Quality of R

	R	R-square	Adj. R-square	ROOT-MSE	N
Model (urate) [24]	1.000	0.999	0.999	0.0089	90
Model (Cu(I))	0.999	0.999	0.999	0.0056	90

3.2.2 Uncertainty related to the repeatability. The repeatability was evaluated on the sample measurement by studying the precision of the method according to ISO 5725-3:1994 standard [33], under different experimental conditions.

Each pool of plasma was analysed during six days by five independent repetitions *per* day with the aim of determining the within-day repeatability (S_r) and the intermediate-precision ($S_{I(T)}$). The intermediate-precision here considered refers to the repeated measurements during time. Particularly, the data (Table 4) and the calculations reported below refer to Apparatus A using urate as calibrator.

Cochran test to identify variance outliers was conducted on the basis of the following criterion:

$$C_{CAL} = S^2_{rj\max} / \sum_{j=1}^{d} S^2_{rj} \leq C_{TAB(\nu,d)}$$

where C_{CAL} and C_{TAB} are respectively the critical calculate value and the critical tabulate value, $S^2_{rj\max}$ is the highest variance and S^2_{rj} is the variance estimated as follows:

$$S^2_{rj} = [1/(n-1)] \sum_{j=1}^{n} (X_{ij} - \overline{X_j})^2$$

Where the X_{ij} is the value obtained by the single measure and $\overline{X_j}$ mean value of the day *j*. The homogeneity of variances of the results of different groups was then tested:

$$S^2_{rj\max} / \sum_{j=1}^{d} S^2_{rj} = 0.3328 \leq C_{TAB}\,(p = 0.95\%; \nu = 4, d = 6) = 0.4803$$

where ν = n – 1 = 4 are the degrees of freedom in the group of test and d = 6 is the number of groups of tests. The result confirms the homogeneity of variances.

Variance calculation for the within-day repeatability (S^2_r) and intermediate-precision ($S^2_{I(T))}$) were estimated as follow:

$$S^2_r = 1/d \sum_{j=1}^{d} S^2_{rj} = 612\ (\mu mol\, L^{-1})^2$$

and

$$S_r = 25\ \mu mol\ L^{-1}$$

The degrees of freedom (d = 6, n = 5) for the S^2_r are: $\nu_r = d(n-1) = 6(5-1) = 24$.

In order to estimate the intermediate-precision $S_{I(T)}$, first of all it is important to calculate the averages variance $S^2_{IM(T)}$ representing the variation between different groups of measures. The averages variance is estimated from:

$$S^2_{IM(T)} = [1/(d-1)]\sum_{j=1}^{d}(\overline{X_j} - \overline{\overline{X}})^2 = 44\ (\mu mol\ L^{-1})^2$$

where $\overline{\overline{X}}$ is the general mean and the degrees of freedom for the $S^2_{IM(T)}$ are: $\nu_{IM} = d - 1 = 5$. The relationship between $S^2_{IM(T)}$ and S^2_r is:

$$S^2_{IM(T)} = S^2_{IL(T)} + S^2_r/n$$

where $S^2_{IL(T)}$ is the variance representing the variation due to the effect of time between different groups of measures. Before the calculation of $S^2_{IL(T)}$ it is necessary to verify whether $S^2_{IM(T)}$ is greater than S^2_r/n or, rather, to check whether $S^2_{IL(T)}$ is greater than zero. This check was conducted on the basis of the following criterion:

$$S^2_{IM(T)} / \left(S^2_r/n \right) \geq F(p = 1-\alpha; \nu_{IM}, \nu_r)$$

where

$$S^2_{IM(T)} / \left(S^2_r/n \right) = 0.36 \geq F\ (p = 95\%; \nu_{IM} = 5, \nu_r = 24) = 2.62$$

It is possible to observe that $S^2_{IL(T)}$ is not significantly different to zero. Hence, within-day repeatability and intermediate repeatability are indistinguishable. The repeatability of the method, expressed as coefficient of variation, resulted equal to CV = $S_r/\overline{\overline{X}}$ = 25/578 = 0.043 (for the value of TAC = 578 µmol L^{-1} see Table 4).

Then, we considered three pools of plasma with TAC values of 454 (low level), 993 (intermediate level), 1787 (high level) μmol L^{-1} with the aim to verify the dependence between TAC and within-day repeatability. These three levels of activity were tested by way of ten repetitions per level in one day. CV of 0.044, 0.041 and 0.042 were obtained for low, intermediate and high levels respectively. Therefore, a concentration effect on the repeatability of the method in the range tested can be excluded.

For samples measured in replicates, CV must be replaced by CV/√n. Particularly, for the samples measured in triplicate, the uncertainty related to the repeatability was estimated as follows:

$$u(rep) = CV/\sqrt{n} = 0.043/\sqrt{3} = 0.0248$$

Table 5 collects the whole of the results on repeatability. The data related to the Apparatus B are reported in ref. 24.

Table 4. TAC/µmol L^{-1} data of the pool of plasma analysed during six days by five independent repetitions *per* day on Apparatus A using urate as calibrator.

Measure	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	
1	566	566	594	562	579	541	
2	570	562	587	512	608	575	
3	554	587	579	587	562	599	
4	620	587	533	579	595	612	
5	595	575	579	604	591	587	$\overline{\overline{X}}$ [a]
$\overline{Xj}$ [b]	581	575	574	569	587	583	578
S^2_{rj} [c]	704	133	577	1222	302	734	

[a] is the general mean

[b] is the mean of the group

[c] is the variance of the group

Table 5. Relative standard uncertainty and expanded uncertainty of the TAC measurement for the apparatuses A (manual) and B (automated).

Quantity	Relative standard uncertainty	Apparatus A[a]		Apparatus B [b]	
		Cu(I)	Urate	Cu(I)	Urate
X_o	$u_r(X_o)$	0.0297	0.0298	0.0044	0.0041
f_{rep}	u_r(rep)	0.0248	0.0248	0.0075	0.0075 [24]
f_{pH}	u_r(pH)	0.0013	0.0013	0.0013	0.0013
f_T	u_r(T)	0.0033	0.0033	0.0033	0.0033
f_{Cstock} [c]	$u_r(C_{stock})$ [c]	0.0004	0.0122 [24]	0.0004	0.0122 [24]
f_{RF}	u_r(RF)	-	0.0045 [24]	-	0.0045 [24]
	u_r(TAC)	0.0388	0.0410	0.0094	0.0159
	U(TAC) [d] (k = 2)	67	71	18	30

[a] data referred to the pool of plasma having TAC = 866 µmol L^{-1}

[b] data referred to the pool of plasma having TAC = 956 µmol L^{-1}

[c] C_{stock} is alternatively $C_{stock\ Cu(II)}$ or $C_{stock\ urate}$

[d] results of U(TAC) expressed in µmol L^{-1}.

3.2.3 Uncertainty related to the pH. The experiments were carried out in PBS at pH = 7.40. Thus, it is important to consider the effect of the pH on the expanded uncertainty of the TAC measurement. The pH-meter has been calibrated by the two-point calibration method using two reference solutions with pH values 4.01 and 9.00 bracketing the pH under measurement. According to the literature [34], the uncertainty $U(\mathrm{pH(x)})$, can be considered equal to 0.02 (coverage factor $k = 2$). The uncertainty related to the pH reported in Table 5 was estimated as follows:

$$u(pH) = 0.01$$

$$u_r(pH) = u(pH)/pH = 0.0013$$

3.2.4 Uncertainty related to the temperature. Since experimental procedures of TAC measurement were conducted under thermostatic condition of 25 °C it is important to quantify temperature effect on the results. The thermostat used in the experimentation exhibits a precision of 25 ± 0.2 °C (triangular distribution function used). The uncertainty related to the temperature reported in Table 5 was estimated as a follows:

$$u(T) = 0.08165\ ^{\circ}\mathrm{C}$$

$$u_{\mathrm{r}}(T) = u(T)/T = 0.00326$$

3.2.5 Uncertainty related to the concentration of the reference solutions. The uncertainty of the urate reference solution was estimated starting from the evaluation of every input that affects the final concentration of the mentioned solution. Being relationship expressed as follow, $C_{\text{stock urate}} = (m \cdot P)/(MW \cdot V)$, where m, P, MW and V are the symbols for mass, purity, molecular weight and volume, respectively, every of these input were estimated and reported in Table 6.

Table 6. Contributions of uncertainty related to the concentration of the urate reference solution.

Quantity/unit	Estimate	Uncertainty	Distribution factor	*u*(x)	*u*(x)/x
mass/g	0.01901	-	-	$6 \cdot 10^{-5}$ [a]	0.003165
purity	0.98	0.02	$\sqrt{3}$ [b]	0.01155	0.01178
volume/mL	50	0.05	$\sqrt{6}$ [c]	0.0204	0.000408

[a] standard uncertainty specified by the manufacturer of the balance

[b] rectangular distribution

[c] triangular distribution

For $C_{\text{stock urate}} = (m \cdot P)/(MW \cdot V) = 1.96$ mmol L^{-1} the uncertainty was estimated as follows:

$$u_r(C_{stockurate}) = u(C_{stockurate}) / C_{stockurate} = \sqrt{(u_r(m))^2 + (u_r(P)^2 + (u_r(V))^2} = 0.01220$$

With regard to Cu(II) stock solution, the supplier declares a concentration of (1000 ± 1) mg L^{-1}; hence, the triangular distribution function was used to calculate the uncertainty:

$$u(C_{\text{stock Cu(II)}}) = 1/\sqrt{6} = 0.4082 \text{ mg } L^{-1}$$

$$u_r(C_{\text{stock Cu(II)}}) = u(C_{\text{stock Cu(II)}})/C_{\text{stock Cu(II)}} = 0.0004$$

3.2.6 Uncertainty related to the redox factor. Redox factor and its uncertainty were previously estimated and reported in the ref. [24]. The standard deviation associated to the redox factor resulted to be equal to 0.009. This value was used as *u*(RF) and the uncertainty related to the redox factor shown in Table 5 was estimated as follows:

$$u_r(\text{RF}) = u(\text{RF})/\text{RF} = 0.009/1.999 = 0.0045$$

3.3 Discussion

Table 5 reports the parameters significantly affecting the variability of the quantity under measure. A summary of the relative contribution of each quantity on the combined uncertainty is reported in the bar-diagrams of Figure 3. The relative length of the bars shows the weight of each contribution to the measurement uncertainty and it allows identifying what operations deserve further attention to improve the quality of the measurement.

Comparing u_r(TAC) obtained for apparatuses A and B (Table 5), it is possible to observe that lower values for Apparatus B (auto-analyzer) were obtained. Moreover, the comparison between the results obtained on the same instrument, but with different calibrating substances, shows lower values of u_r(TAC) when Cu(I) was employed.

The uncertainties due to the calibration and the repeatability are the main terms discriminating the quality of the measurement derived from apparatuses A and B, being the worse result provided by the manual procedure. This may mainly be due to the repeatability of the dispensed volumes, probably better in the case of automatic system.

The difference between the uncertainties estimated using the two calibration procedures can be attributed to the uncertainty of the stock solution concentration. The $u_r(C_{stock})$ obtained for urate reference solution is higher than that of Cu(II) solution and the value of $u_r(C_{stock})$, in the case of urate, is principally affected by purity of the compound. Therefore, both aspects associated to the use of instruments and chemicals significantly affect the quality of the analytical result and deserve investment to optimize the procedure.

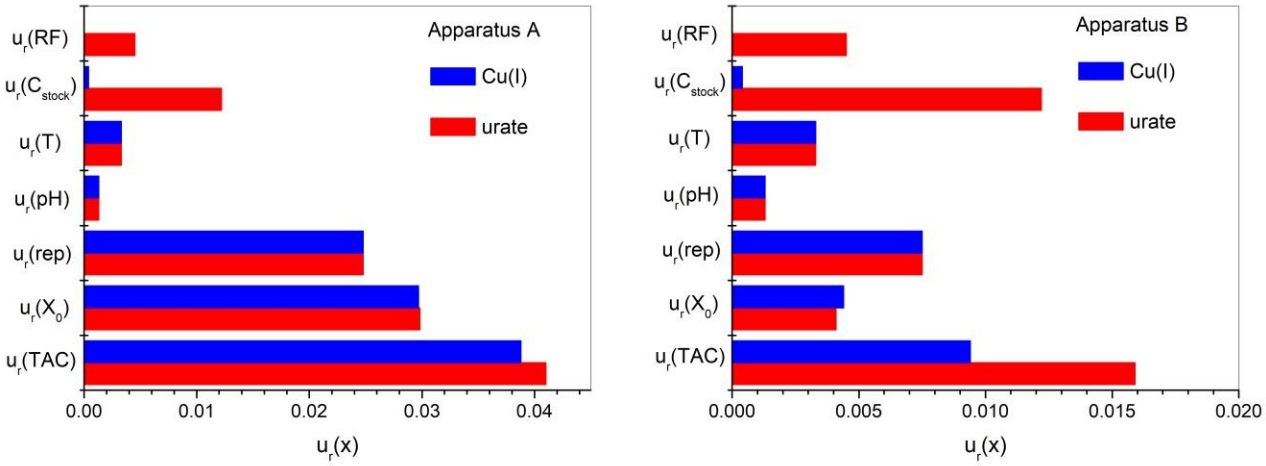

Figure 3. Uncertainty contributions of the TAC measurement for the apparatuses A and B. The values of $u_r(x)$ are taken from Table 5. The meaning of the symbols is explained in paragraph 3.2.

4. Conclusions

The parameters significantly affecting the variability of the quantity under measure have been identified and quantified (Table 5). Their role played on the combined uncertainty was clarified as well. The results allows selecting the best working conditions and could be of help to define the uncertainty acceptance limits for the TAC measurement as well as to identify the reference intervals for target population. Finally, the extent of the method repeatability resulted to be independent of the concentration level in the range tested and the lowest measurement uncertainty is reached employing the auto-analyzer and using Cu(I) for the calibration (u_r(TAC) = 0.0094 resulted).

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