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Uncertainty estimation for total antioxidant capacity measurement of apple juice using main CUPRAC method

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Abstract: The estimation of uncertainty –taking into account all sources of error- is an important tool allowing the identification the influence of each stage of the analytical procedure on the overall quality of the results. Measurement uncertainty - reported with the measurement result - is a parameter that indicates the distribution of the probabilities attributable to the measurement results. The aim of this study is the first time estimation of measurement uncertainty for total antioxidant capacity (TAC) of a Turkish commercial apple juice by the main CUPRAC method that uses Cu(II)-neocuproine (Nc)/Cu(I)-Nc redox couple to measure the TAC levels of various biological fluids and foods. The individual source of uncertainties such as repeatability, calibration curve, concentration of the trolox (TR, reference) solutions, pH, temperature and redox factor in the CUPRAC reaction between TR and Cu(II)-Nc were considered in this work. It was found that the measurement uncertainty was dominantly affected by the total C_{TR} standard solution ($u_r(C_{TR})$ =0.0124). Each uncertainty parameter was evaluated separately, and the relative expanded uncertainty value was calculated as ±3.05% with a 95% confidence interval (k=2).

Keywords: Apple juice; total antioxidant capacity; original CUPRAC method; measurement uncertainty; bottom-up approach.

1. Introduction

Reactive oxygen/nitrogen species (ROS/RNS) generated by the respiratory cycle of oxidative phosphorylation can damage cellular components and related biomacromolecules, such as DNA, proteins and lipids. Unbalanced overproduction of ROS may give rise to alterations in DNA bases or double helix structure resulting in cancerous tumor growth and mutagenic changes [1]. One of the most important tools to prevent oxidative stress-based disorders is generally accepted to be a diet rich in antioxidant foods in the context of preventative medicine and treatment [2]. Showing that food antioxidants may play a major role in the prevention of these important diseases has led to the development of many total antioxidant activity/capacity (TAA/TAC) measurement methods [3]. The main TAA/TAC methods measure peroxyl radical scavenging (ORAC, TRAP), metal ion reducing power (FRAP, CUPRAC), organic radical

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scavenging (ABTS, DPPH), and lipid peroxidation products (TBARS, LDL oxidation assay such as conjugated dienes) [4]. The cupric ion reducing antioxidant capacity (CUPRAC) assay as a simple and widely applicable TAC determination method for plasma antioxidants, flavonoids, food polyphenols, vitamin C and vitamin E was developed by Apak's group using a chromogenic oxidant (Cu(II)-Nc reagent) [5] for oxidizing a majority of food and biological antioxidants. This reagent is stable, inexpensive, readily available, and also responsive to hydrophilic and lipophilic antioxidants. Antioxidants inhibit radical reactions by at least three mechanisms: classical hydrogen abstraction to quench the radical, reduction of a radical to an anion, and binding of metal ions that initiate radical reactions. As opposed to hydrogen atom transfer (HAT) of mixed mode assays, the CUPRAC method is based on the electron-transfer (ET) mechanism [6]. CUPRAC measures the ability of an antioxidant compound to reduce the bis(neocuproine)-chelated copper(II) cation (Cu(II)-Nc) to the orange-yellow-colored bis(neocuproine) copper(I) cation (Cu(I)-Nc) which absorbs at 450 nm. The CUPRAC methodology has evolved into an "antioxidant measurement package" in food chemistry and biochemistry comprising many activity (ROS scavenging activity *etc*.) and capacity (TAC *etc*.) assays, and according to the validated results, these tests have distinct advantages over established classical methods [6].

The uncertainty of measurement is a parameter that characterizes the distribution of values attributed to the measurand and is associated with the measurement result [7]. The measurement uncertainty indicates how well the test result represents the true value. Uncertainty of measurement is also a quantitative indicator of the quality of a test result, but is a fundamental requirement for accredited laboratories. No systematic studies on the uncertainty evaluation were found in the literature for the original CUPRAC method. Since the measurand is not a defined chemical entity but corresponds to a redox reactivity under fixed conditions, the method belongs to the empirical ones and provide a method-dependent uncertainty. The measurand is defined by the method and the uncertainty of measurement. In this study, it is aimed to calculate the measurement uncertainties for the first time in the validated CUPRAC-TAC test.

In the literature, there is only one study about the assessment of uncertainty for TAC. The estimation of measurement uncertainty for TAC of human plasma analyzed with CUPRAC-BCS has been studied by Prenesti et al (2020) [8].The determination and calculation of the measurement uncertainty parameters were carried out in accordance with Quantifying Uncertainty in Analytical Measurement EURACHEM-CITAC Guide [9], ISO-5725 [10] and Guide to the expression of uncertainty in measurement: Developing and using measurement models [11] standards. In this framework, experimental studies were carried out in accordance with ISO/IEC 17025:2017 standards [12] metrology and by determining the uncertainty components in a bottom-up approach.

2. Experimental

2.1. Chemicals and Instruments

Neocuproine (2,9-dimethyl-1,10-phenanthroline, Nc, ≥ 97%) and trolox as a reference standard of antioxidant assays (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, TR, 98%) were purchased from Sigma Aldrich (Steinheim, Germany); copper(II) chloride (99%), ammonium acetate (98%), ethyl alcohol (96%) from E. Merck (Darmstadt, Germany).

All spectrophotometric experiments were performed using an OPTIZEN POP-QX single-beam type UV-Vis spectrophotometer Mecasys (Republic of Korea). Other instruments were Mettler Toledo pH-meter using a glass electrode, Sartorius CP 224 S balance (maximum capacity 120 g) and water bath as a thermostate (GFL-1005).

All statistical analyses and calculations were carried out using Microsoft Excel 2016.

2.2. Main CUPRAC-TAC Assay

The main CUPRAC method is based on the absorbance measurement of the $Cu(Nc)_2^+$ as the CUPRAC chromophore formed as a result of the redox reaction of antioxidant compounds with the $Cu(Nc)_2^{2+}$ as CUPRAC reagent, where the absorbance is recorded at the wavelength of maximum absorbance, λ_{450} nm [5].

The CUPRAC reagent (Cu(II)-Nc) reacts with *n*-electron reductant antioxidants (AOX), according to Eq. 1:

$n\text{Cu(Nc)}_2^{2+}$ + *n*-electron reductant (AOX) ↔ $n\text{Cu(Nc)}_2^{+}$ + *n*-electron oxidized product + $n\text{H}^{+}$ (Eq. 1)

AOXs like α-tocopherol, TR or ascorbic acid are 2 e^{-} reductants, so they generate 2 moles of Cu(Nc)_2^{+} per mole of AOX. This is clearly detected optically. The molar absorptivity of Cu(Nc)_2^{+} alone, about 8.0×10^3 L/mol cm depending on the solvent, increases to about $\varepsilon \approx 1.6\times10^4$ L/mol cm (*i.e*., $2\times(8.0\times10^3)$) when $[\text{Cu(Nc)}_2]^{2+}$ has undergone 2 e^{-} reduction by α-tocopherol, TR or ascorbic acid. AOXs that are n e^{-} reductants should therefore yield ε values of about $n(8\ x10^3)$.

For the CUPRAC-TAC determination, 1 mL of each; 10 mM $CuCl_2$, 7.5 mM Nc, and 1 M NH_4Ac buffer solutions (pH:7.0) were added to a test tube and mixed; (x) mL of apple juice or TR standard solution followed by (1.1 – x) mL of H_2O were added (total volume = 4.1 mL), and the final mixtures were shaken. Absorbance against a reagent blank was recorded at 450 nm after 30 min.

For the normal measurement of antioxidant solution, the scheme is summarized as:

1 mL Cu(II) + 1 mL Nc + 1 mL NH_4Ac + x mL AOX soln. + (1.1 – x) mL H_2O
(total volume= 4.1 mL, A_{450}, t=30 min).

The TAC values (in trolox-equivalents, mM TR) of liquid samples were calculated using (Eq. 2) below:

$$\text{TAC}\ (\text{mM TR}) = \frac{A}{\varepsilon} \times \frac{V_{total}}{V_{sample}} \times \text{df} \times 1000 \qquad \text{(Eq. 2)}$$

A: Sample absorbance measured at 450 nm
Ɛ: The molar absorption coefficient of TR (L/mol cm) with respect to the CUPRAC method
V_{total}: Total volume of CUPRAC measuring solution (4.1 mL)
V_{sample}: Sample volume (mL)
df: Dilution factor

3. Results and Discussion

3.1. Measurement Uncertainty Evulation of the CUPRAC Assay

Uncertainty is a term used to make the analysis results easier to understand and more meaningful. According to the VIM (International Vocabulary for Terms in Metrology), the concept of uncertainty is concisely defined as “measurement uncertainty is non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used” [13]. IUPAC [14] briefly states that a report of a measurement result should contain a statement about what the expected ‘best estimate’for the *true value* is, as well as a statement of the probable interval of possible values specifying the uncertainty.

The parameters of uncertainty for spectrophotometric determination of TAC were identified by using the *fishbone diagram* as shown in Figure 1. This diagram describes the *uncertainty budget* contributed by all sources of uncertainty.

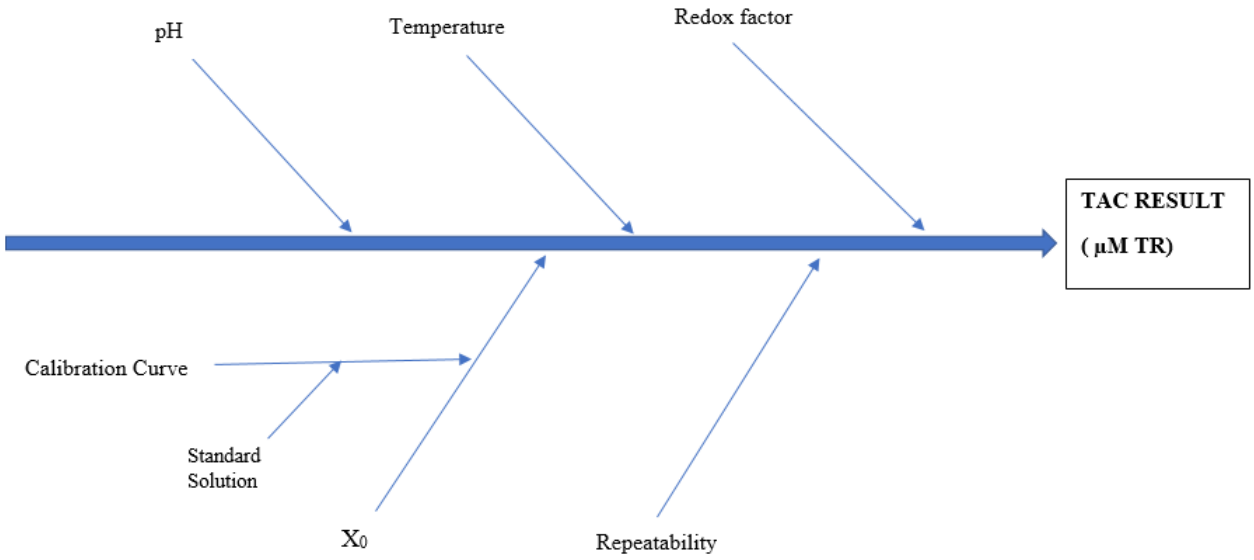

Figure 1. Fishbone diagram of the accumulation of potential sources of uncertainty in TAC measurement with respect to the main CUPRAC method.

$TAC_{Uncertainty}$ value was calculated using the Eq. 3 [8]:

$$TAC_{Uncertainty} = 2 \cdot X_0 \cdot f_{rep} \cdot f_T \cdot f_{pH} \cdot f_{c_{TR}} \cdot f_{RF} \quad \text{(Eq. 3)}$$

a) X_0 : the uncertainty associated with the calibration curve,
b) f_{rep} : the uncertainty associated with the repeatability,
c) f_T : the uncertainty associated with the temperature (T, ◦C),
d) f_{pH} : the uncertainty associated with the pH,
e) $f_{c_{TR}}$: the uncertainty associated with TR standard solution,
f) f_{RF} : the uncertainty associated with electronic exchange of the CUPRAC reaction reflecting redox reactivity under the test conditions (RF = redox factor)
g) 2 is the number of electrons exchanged between Cu(II)-Nc and TR.

3.1.1. Uncertainty Associated with Total C_{TR} of Reference Standard Solution, $u_r(C_{TR})$

The uncertainty components associated with the C_{TR} of reference standard solution can be expressed as a function final concentration of reference solution uncertainties.

The relative standard measurement uncertainty for the preparation of reference solution was evaluated by combining the relative standard measurement uncertainties of purity and volume. The combined reference measurement uncertainty ($u_r(C_{TR})$) value for all elements are given in Table 1.

Table 1. Contributions of uncertainty related to the concentration of the TR reference solution.

Quantity/Unit	Estimate	Uncertainty	Distribution factor	u(x)	u(x)/x
Mass/g	0.0125	-	-	4.2×10^{-5} [a]	0.0034
Purity	0.98	0.02	$\sqrt{3}$ [b]	0.0115	0.0118
Volume/mL	50		$\sqrt{6}$ [c]	-	0.0015

[a]: standard uncertainty of balances associated with the manufacturer's specifications
[b]: distribution of measurement: rectangular
[c]: distribution of measurement: triangular

TR solution was freshly prepared in 96% EtOH at 1 mM concentration prior to measurement according to equation 4:

$$C_{TR} = \frac{(m_{TR} \cdot P)}{(MW_{TR} \cdot V)} = 1.00 \text{ mmol/L} \quad \text{(Eq. 4)}$$

(m: mass, P: purity, MW: molecular weight and V: volume):

For C_{TR}, the uncertainty was estimated using Eq. 5. This equation incorporates all of the uncertainty components used in the calculation of reference standard C_{TR} solution uncertainty.

$$u_r(C_{TR}) = \frac{u(C_{TR})}{C_{TR}} = \sqrt{\left(u_r(m)^2 + u_r(P)^2 + u_r(V)^2\right)} \quad \text{(Eq. 5)}$$

$$\Rightarrow u_r(C_{TR}) = \sqrt{(0.0034)^2 + (0.0118)^2 (0.0015)^2} = 0.0124$$

3.1.2. Uncertainty from the Calibration Curves, $u_r(X_0)$

In this study, the five calibration standards at the concentrations 0.0122, 0.0244, 0.0366, 0.0488 and 0.0610 mmol/L were measured three times each for all elements per day and it was carried out for six days. The measurement uncertainty associated with the calibration curve was estimated according to the guidelines of EURACHEM/CITAC [9]. For the related uncertainty, var(X_0): the variance related to X_0 was calculated using the following formula (Eq. 6):

$$\text{var}(X_0) = \frac{\text{var}(y_{obs})}{b^2} + \frac{S^2}{b^2}\left(\frac{1}{\sum w_i} + \frac{(X_0 - \overline{X})^2}{\sum(w_i X_i^2) - \frac{\sum(w_i X_i)^2}{\sum w_i}}\right) \quad \text{(Eq. 6)}$$

where:

- var(y_{obs}): the variance related to the observed variable,
- b : the slope of the calibration curve,
- S : the root mean square deviation (RMSD) (differences between the mean square values of experimentally found data and estimated data values),

- w_i : the weight of y_i,
- X_i : the concentration of the reference solutions,
- $\overline{X}$: the mean of different standard concentrations for *n* measurements,
- X_0 : the concentration obtained using the inverse of the calibration equation.

$u_r(X_0)$ was calculated as follows using Eq. 7:

$$u(X_0) = \sqrt{\mathrm{var}(X_0)} = 0.000210 \text{ mmol/L}$$

$$u_r(X_0) = \frac{u(X_0)}{X_0} = \frac{0.000210}{0.0434} = 0.0048 \qquad \text{(Eq. 7)}$$

3.1.3. Uncertainty from the Repeatability, $u_r(rep)$

The repeatability has also been considered for the determination of TAC of apple juices using main CUPRAC method. 'Intermediate measurement precision' is defined by IUPAC [14] as the 'measurement precision under a set of intermediate precision conditions of measurement'. The condition of measurement, out of a set of conditions that includes the same measurement procedure, same location, and replicate measurements on the same or similar objects over an extended period of time, may include other conditions involving changes. The changes can include new operators, calibrators, calibrations, and measuring systems. The data set of intermediate precision captures the within laboratory variation, as on different days, or with different equipment or analysts within the same laboratory [15]. TAC of apple juices were determined experimentally by analyzing ten independent determinations during six days. The within-day repeatability (*S*r) and the intermediate-precision (S_I(T)) obtained by CUPRAC and related repeatability has been calculated by using ISO 5725-3:1994 standard [10]. The details are given in Table 2.

Table 2. TAC (μmol/L) of apple juices with respect to the main CUPRAC method (n=5).

Measurement	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	
1	1819.9	1801.0	1814.5	1801.3	1803.1	1805.0	
2	1804.1	1819.2	1798.4	1793.3	1800.5	1810.4	
3	1801.4	1811.4	1811.8	1809.4	1797.9	1799.7	
4	1809.4	1793.2	1795.8	1806.7	1771.8	1810.4	
5	1817.3	1798.4	1817.2	1812.1	1792.9	1789.0	
6	1819.9	1790.6	1814.5	1801.3	1779.7	1818.4	
7	1793.5	1819.2	1790.4	1801.3	1808.3	1794.3	
8	1809.4	1811.4	1817.2	1779.8	1797.9	1802.3	
9	1806.7	1801.0	1798.4	1806.7	1810.9	1807.7	
10	1814.7	1808.8	1806.5	1793.3	1805.7	1802.3	$\overline{X}$ [a]
$\overline{X}$ [b]	1809.63	1805.44	1806.46	1800.53	1796.89	1803.95	1803.82
S^2_{rj} [c]	73.57	102.14	98.68	91.91	155.54	71.61	

[a] refers to general mean
[b] refers to the mean of the group
[c] refers to the variance of the group

The homogeneity of the variance of the dataset was tested with the Cochran test and the result confirms the homogeneity of variances (the number of groups (*d*)= 6 and the degrees of freedom (*v*) = n-1 = 9). Cochran's test statistic is defined as (Eq. 8);

$$C_{\exp} = \frac{S^2_{rj_{\max}}}{\sum_{j=1}^{d} S^2_{rj}} = 0.2621 \le C_{crit.(v,d)}\left(p = 0.95\%; v = 9, d = 6\right) = 0.3584 \qquad \text{(Eq. 8)}$$

where:

- C_{exp}: experimentally found value
- C_{crit} : critical value
- $S^2_{rj\max}$: the highest variance
- S^2_{rj} : the estimated variance

On the other hand, within-day repeatability (S^2_r) (Eq. 9) and intermediate-precision ($S^2_{I(T)}$) (Eq. 10) were calculated as follows:

$$S_r^2 = 1/d \sum_{j=1}^{d} S_{rj}^2 = 98.91(\mu\text{mol/L})^2 \quad \text{(Eq. 9)}$$

$$\Rightarrow S_r = 9.95\ \mu\text{mol/L}$$

$$S_{I(T)}^2 / \left(S_r^2 / n\right) = 1.03 \le F\left(p = 95\%; v_{IM} = 5, v_r = 54\right) = 2.386 \quad \text{(Eq. 10)}$$

The coefficient of variation (CV) (Eq. 11) as the repeatability of the CUPRAC method were calculated as follows:

$$CV = \frac{S_r}{\overline{\overline{X}}} = \frac{9.95}{1803.82} = 0.0055 \quad \text{(Eq. 11)}$$

To estimate the uncertainty of the repeated measurements in the CUPRAC test, the Eq. 12 was used to perform the related uncertainty calculation.

$$u_r\left(rep\right) = \frac{CV}{\sqrt{n}} = \frac{0.0055}{\sqrt{3}} = 0.0032\ \ (n = 3) \quad \text{(Eq. 12)}$$

TAC results of apple juice using the CUPRAC assay in different days are used for the evaluation of the closeness of the data. The individual random effects in the measurement are identified and quantified by one-way ANOVA test of which the results have to be taken into account for the evaluation of uncertainty associated with precision.

Table 3. One-way ANOVA statistics of TAC results of apple juices using CUPRAC assay.

Summary

Groups	Count	Sum	Average	Variance
1	10	18096.31491	1809.63149	73.5692042
2	10	18054.4498	1805.44498	102.138514
3	10	18064.62141	1806.46214	98.6800495
4	10	18005.3195	1800.53195	91.9141029
5	10	17968.85478	1796.88548	155.53993
6	10	18039.46597	1803.9466	71.6133505

ANOVA

Source of Variance	SS	df	MS	F	P_{value}	$F_{criteria}$
Between Groups	1023.09736	5	204.619472	2.06876093	0.08356459	2.38606986
Within Groups	5341.09636	54	98.9091918			
Total	6364.19372	59				

Since $F_{exp.}$=2.06 < $F_{criteria}$=2.38, closeness of the results to each other is proven.

3.1.4. Uncertainty from the pH

The CUPRAC analyses were performed in ammonium acetate (NH_4Ac) buffer (pH = 7.00). To consider the uncertainty associated with pH, a two-point calibration is required, using two standard pH buffers (pH 4 and 9). The uncertainty $U(pH(x))$ was approximately equal to 0.02 when the coverage factor (k)= 2. The uncertainty associated with the pH ($u_r(pH)$) is given by (Eq. 13):

$$u(pH) = 0.01$$

$$u_r(pH) = \frac{u(pH)}{pH} = 0.0014 \qquad \text{(Eq. 13)}$$

3.1.5. Uncertainty from the Measurement Temperature

The CUPRAC analyses were performed under the condition of room temperature. To consider the uncertainty associated with temperature, a water bath with a precision of 25 ± 0.3 ˚C is required. The uncertainty associated with the temperature ($u_r(T)$) is given by (Eq. 14):

$$u(T) = \frac{\Delta T}{\sqrt{6}} = 0.122$$

$$u_r(T) = \frac{u(T)}{T} = 0.00489 \qquad \text{(Eq. 14)}$$

4. Conclusions

The estimate of uncertainty is an essential requirement for quality assurance of analytical measurements, providing reliability of the results. In this work, a bottom up procedure used for estimating the overall uncertainty depends on the CUPRAC data available about the related method performance.

The contributions of all uncertainty sources to the overall budget are summarized in Table 4.

The relative contribution to the overall uncertainty of each of the quantities is shown in Figure 2. The dominant parameter for the uncertainty in the analysis by the CUPRAC method was the reference of C_{TR} ($u_r(C_{TR})$=0.0124). While this uncertainty value we found for C_{TR} in our study was similar to the 0.0122 value in the study by Prenesti et al. [8].

TAC measurement uncertainty with respect to the CUPRAC method ($u_r(TAC)= u(TAC)/TAC$) was calculated by using Eq. 15.

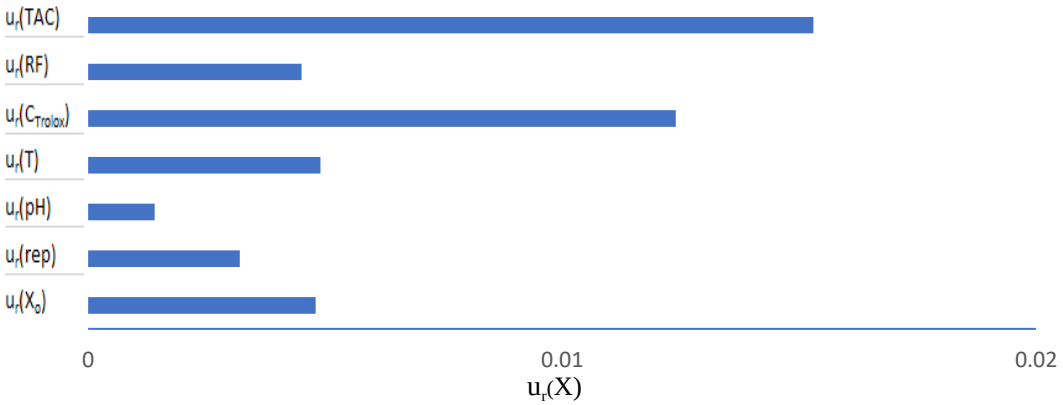

Figure 2. The relative contribution of each of the seven quantities in (Eq.16) to the expanded uncertainty of the TAC measurement with respect to the CUPRAC method

Table 4. The individual uncertainties and the expanded uncertainty of the spectrophotometric CUPRAC-TAC measurements according to the EURACHEM Guide.

Quantity	Relative uncertainty	Uncertainty
X_0	$u_r(X_0)$	0.0048
f_{rep}	$u_r(rep)$	0.0032
f_{pH}	$u_r(pH)$	0.0014
f_T	$u_r(T)$	0.0049
$f_{C_{TR}}$	$u_r(C_{TR})$	0.0124
f_{RF}	$u_r(RF)$	0.0045
	$u_r(TAC)$	0.0153
	$U(TAC)^{a,b}(k=2)$	55

[a] results of *U*(TAC) expressed in µmol/L.
[b] data referred to the apple juice having TAC = 1803.82 µmol/ L

$$\frac{u(TAC)}{(TAC)} = \sqrt{(u_r(X_0))^2 + (u_r(rep))^2 + (u_r(T))^2 + (u_r(pH))^2 + (u_r(C_{TR}))^2 + (u_r(RF))^2} \quad \text{(Eq. 15)}$$

$$U(TAC) = u_r(TAC) \cdot 2 \quad \text{(Eq. 16)}$$

There are very few studies on this subject in the literature. However, measurement uncertainty should be determined for each parameter in antioxidant activity/capacity studies. Evaluation of the analysis results together with the measurement uncertainty will be of great benefit to many antioxidant researchers (dieticians preparing antioxidant-rich diets, food scientists preparing food inventories or classifying food/plant material on the basis of their antioxidant capacity, clinicians in diagnosis and disease prevention).

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