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Comparative evaluation of antioxidant capacities of thiol-based antioxidants measured by different *in vitro* methods

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ABSTRACT

Thiol-type compounds are an important class of strong antioxidants and main determinants of total antioxidant capacity (TAC) of cellular homogenates. The TAC of thiol mixtures and the corresponding TEAC (trolox equivalent antioxidant capacity) values of individual thiols were determined by the CUPRAC (CUPric Reducing Antioxidant Capacity) method, and the results were compared with those found by reference assays for method validation. Synthetic mixtures of thiols were prepared, and the expected and found TAC values (in mM trolox (TR) equivalents) of these mixtures showed a good agreement. The technique of standard additions was performed for thiol mixtures and human serum, and the absorbance results confirmed that apparent chemical deviations from Beer's law were absent in the system. The CUPRAC results were compared with those of reference methods, namely 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)/persulphate and Ferric Reducing Antioxidant Power (FRAP). As being a most important thiol (–SH) peptide at *in vivo* conditions, glutathione (GSH) showed a TEAC value of 0.57 in the CUPRAC method, as opposed to the corresponding value (1.51) in the ABTS/persulphate method. The ABTS/persulphate result was not in accordance with the reversible 1-e oxidation of GSH to the corresponding disulfide that is expected to occur under physiological conditions. FRAP did not give consistent results, and even at relatively high concentrations of GSH, the $TEAC_{FRAP}$ value was only 0.07. The thiol-type antioxidant-bearing pharmaceuticals of Brunac eye drop, Trom and Mentopin effervescent tablets containing N-acetyl-L-cysteine (NAC) were assayed with HPLC for comparison, and the obtained results for NAC were in accordance with those found with CUPRAC.

1. Introduction

Thiol-type antioxidants constituting a class of organic sulfur derivatives (mercaptans) having the sulfhydryl functional groups (–SH) play a crucial role in protecting cells from oxidative damage by interacting with the electrophilic groups of reactive oxygen species (ROS) as a first and major member of the physiological antioxidant defense system. Decreased levels of thiol compounds in the organism have been shown to cause various disorders such as liver failure, coronary artery disease, stroke, and other neurological disorders, and recently, therapy using thiols has been under investigation for these disorders [1].

Biologically derived thiols such as glutathione (GSH), cysteine (CYS), and homocysteine (HCYS) are often called biothiols. The side chain functional group: CH_2–SH of cysteinyl residues serves as an active site for most biologically important thiols (–SH type-antioxidants). On the other hand, disulfide linkages (–S–S–) between two –SH residues (S–S type-antioxidants such as cystine (CYSS), homocystine (HCYSS), and lipoic acid (LA)) are important determinants of protein structures [2]. Thiols as reducing agents have negative standard reduction potentials, i.e., $E^0_{GSSG,GSH} = -0.23$ V, $E^0_{CYSS,CYS} = -0.34$ V, and are relatively redox-stable under physiological conditions. ROS frequently react with cellular thiols under 'oxidative stress' conditions. In the case of a ROS–thiol interaction, the ROS is converted to a relatively less toxic byproduct at the expense of the reducing power of thiol, which itself gets oxidized to a disulfide (R–S–S–R). Thiols generally undergo one electron oxidation with the formation of thiyl radicals (R–S•) by losing the H atom from the –SH group or losing an electron from the sulfur, followed by a proton or two electron oxidation with the generation of sulphenic acid (R–SOH) by specific oxidants. Under physiological conditions of pH, thiyl radicals are unstable and may recombine to form the corresponding disulfide [3].

$$R\text{–}SH \rightarrow R\text{–}S^{\bullet} + H^+ + e^- \quad (1.1)$$

$$R\text{–}SH + H_2O \rightarrow R\text{–}SOH + 2H^+ + 2e^- \quad (1.2)$$

There are various assays of estimating the total antioxidant capacity (TAC) of food and vegetable extracts, beverages and biological fluids. One group of these methods, hydrogen atom transfer

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(HAT)-based assays (ORAC, TRAP), is based on the quenching of free radicals (generally peroxyl radicals) by H-atom donation. Another group of TAC assays, electron transfer (ET)-based assays (CUPRAC, Folin, FRAP, possibly ABTS/persulphate and DPPH), is based on the measurement of the capacity of an antioxidant in the reduction of an oxidant (a fluorescent or chromogenic probe), which generally changes color when reduced [4,5]. While the TAC of blood plasma is mainly accounted for by urate, TAC of cell interior can be expected to depend more on other antioxidants, especially glutathione and protein –SH groups. Alterations in cellular thiol content is the main determinant of changes of TAC of cell homogenates [6]. In spite of being the second largest shareholder contributing to plasma TAC, protein thiols are not efficiently estimated by most methods (especially FRAP, due to the low reactivity of thiols with ferric ions) [7]. Additivity of antioxidant capacities of thiol components is a prerequisite for precise TAC estimation of complex mixtures, but few TAC assays give additive results for thiol antioxidants [8]. On the other hand, there has been no conclusive study describing the antioxidant properties of various thiol-type compounds: –SH type antioxidants such as glutathione, cysteine, homocysteine, S–S type antioxidants such as cystine, lipoic acid, homocystine and other antioxidants (methionine) measured by widely used spectrophotometric TAC assays. Therefore this work is a first attempt for the systematic evaluation and comparison of TAC values of –SH and S–S type (S-containing) antioxidants – individually and in admixtures – using CUPRAC [9], ABTS/persulphate [10], FRAP [11] and Ellman [12] assays with HPLC validation.

2. Experimental

2.1. Chemicals and instruments

The following chemical substances of analytical reagent grade were supplied from the corresponding sources: neocuproine (2,9-dimethyl-1,10-phenanthroline) (Nc), glutathione reduced ethyl ester (GSHEE), (±)-α-lipoic acid (LA), lipoic acid reduced (DHLA), DL-cystine, DL-homocystine: Sigma (Steinheim, Germany); L-ascorbic acid (AA): Aldrich (Steinheim, Germany); potassium persulphate ($K_2S_2O_8$), glutathione (reduced, GSH), 1,4-dithioerythritol (DTE): Sigma–Aldrich (Steinheim, Germany); tris(hydroxymethyl) aminomethane (Tris), glycine, tri-sodium citrate 5,5-hydrate, urea, sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), copper(II) chloride dihydrate, ammonium acetate (NH_4Ac), iron(III) chloride hexahydrate, hydrochloric acid, glacial acetic acid, sodium acetate trihydrate, sodium dihydrogen phosphate dihydrate, methanol (MeOH), ethanol (EtOH), sodium hydroxide, phosphoric acid (85%) and zinc powder: Merck (Darmstadt, Germany); 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (Ellman's reagent), N-acetyl-L-cysteine (NAC), cysteamine, glutathione (oxidized, GSSG), L-cysteine, DL-homocysteine, DL-methionine, ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) (kept at +4 °C), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ): Fluka (Buchs, Switzerland). The reagents were 'analytical reagent' grade unless otherwise stated. Brunac (Acetylcysteine 5%) eye drop was purchased from Arz Ilaclari San. Tic. Ltd. Sti. (Ankara, Turkey), Acetylcystein 600 Trom effervescent tablet from Adeka Ilac Kimyasal Urunler San. Tic. A.S. (Samsun, Turkey), and Acetylcystein 600 Mentopin effervescent tablet from Vitamed Ilac Tic. Ltd. Sti. (Istanbul, Turkey).

The spectra and absorption measurements were recorded in matched Helma quartz cuvettes using a Varian CARY Bio 100 UV-vis spectrophotometer (Mulgrave, Victoria, Australia). For validation of the proposed assay against HPLC on a drug sample (containing NAC), a Perkin Elmer Series 200 HPLC chromatographic instrument equipped with an analytical stainless-steel column packed with Hamilton H × Sil C18 sorbent (250 mm × 4.6 mm, 5 μm) (Reno, NV, USA) was used in conjunction with a UV–vis detector (Perkin Elmer Series 200), running a mobile phase consisting of 40% MeOH + 60% phosphoric acid aqueous buffer (v/v) mixture at a flow rate of 1.0 mL min^{-1}. The mobile phase for HPLC analysis with gradient elution was prepared from primary phosphate ($H_2PO_4^-$) buffer and MeOH. This buffer was prepared by dissolving 0.78 g of sodium dihydrogen phosphate dihydrate in 500 mL of bidistilled water. The pH of the mobile phase thus prepared was adjusted to pH 2.5 with phosphoric acid.

2.2. Preparation of TAC assay solutions

For the CUPRAC test of TAC, the following solutions were prepared: $CuCl_2$ solution, 1.0×10^{-2} M, was prepared by dissolving 0.4262 g $CuCl_2 \cdot 2H_2O$ in water, and diluting to 250 mL. Ammonium acetate buffer at pH = 7.0, 1.0 M, was prepared by dissolving 19.27 g NH_4Ac in water and diluting to 250 mL. Neocuproine (Nc) solution, 7.5×10^{-3} M, was prepared daily by dissolving 0.039 g Nc in absolute ethanol, and diluting to 25 mL with ethanol. For the ABTS test of TAC, the chromogenic radical reagent ABTS, at 7.0 mM concentration, was prepared by dissolving 0.1920 g of the compound in water, and diluting to 50 mL. To this solution was added 0.0331 g $K_2S_2O_8$ such that the final persulphate concentration in the mixture was 2.45 mM. The resulting ABTS radical cation solution was left to mature at room temperature in the dark for 12–16 h, and then used for TAC assays. The FRAP solutions were prepared as follows: a suitable mass of $FeCl_3 \cdot 6H_2O$ was weighed so that the final concentration of Fe(III) in solution would be 2.0×10^{-2} M; 1 mL of 1 M HCl solution was added, dissolved in some water and diluted to 50 mL with H_2O. A suitable mass of TPTZ was weighed such that its final concentration would be 1.0×10^{-2} M, dissolved in absolute EtOH, and diluted to 50 mL. In order to prepare 0.3 M CH_3COOH/CH_3COONa buffer solution at pH 3.6, 3.1 g of $CH_3COONa \cdot 3H_2O$ was weighed and 16 mL glacial acetic acid was added, diluted with water to 1 L. The FRAP reagent was prepared as follows: the pH 3.6 acetic acid buffer, 1.0×10^{-2} M TPTZ solution, and 2.0×10^{-2} M $FeCl_3 \cdot 6H_2O$ solution were mixed in this order at a volume ratio of 10:1:1. The FRAP reagent was prepared and used freshly.

The standard solutions at 1.0×10^{-3} M concentration of NAC, DTE, cysteamine, and LA; at 2.0×10^{-3} M concentration of DHLA were all prepared in absolute EtOH. The standard solutions at 1.0×10^{-3} M concentration of GSH, GSSG, and methionine; at 1.0×10^{-2} M concentration of GSHEE were all prepared in water.

For converting the –S–S– type antioxidants (GSSG, CYSS, LA, and HCYSS) to the CUPRAC- and ABTS-responsive forms (i.e., the corresponding thiols), the procedure originally developed by Tütem and Apak for cystine–cysteine conversion [13] was slightly modified. Briefly, an aliquot of 20 mL withdrawn from 1 mM –S–S– type (disulfide) antioxidant solution was transfered into a flask; 1 mL concentrated HCl and 0.5 g Zn powder were added, and the flask was kept in a microwave furnace for 60 s to aid reductive conversion of the –S–S– functionality to the corresponding –SH groups. The pH of the final solution was roughly adjusted to pH 3 with 2 M NaOH, and the solution was diluted to 50 mL.

2.3. Preparation of protein assay solutions and buffers

Four different buffers were used in the experiments, all originating from the standard tris-buffer. Urea buffers prepared in standard tris-buffer solution had three different pH: 6.8, 7.0, and 8.0. The standard tris-buffer at pH 8.0 contained 0.086 M tris(hydroxymethyl)aminomethane, 0.09 M glycine, and 4 mM citrate; the final pH was adjusted to 8.0 with the addition of 2 M HCl and used as diluent. Urea buffer at pH 7.0 (a suitable amount of

urea was dissolved in standard tris-buffer, 6 M HCl was added to adjust the pH to 7.0, and diluted with distilled water so as to yield a final total urea molarity of 8 M) was also prepared. The protein dissolution buffer (pH 6.8) contained 50 mM tris, 2% SDS, and 8 M urea (the final pH of the solution was adjusted to 6.8 with 2 M HCl) [8].

DTNB solution at 4 mg mL^{-1} was prepared with standard tris-buffer. Urea buffer at pH 8.0 (the standard tris-buffer containing sufficient urea was adjusted to pH 8.0 with the addition of 6 M HCl, and diluted with distilled water so as to yield a final total urea molarity of 8 M) was also prepared for Ellman assay [12].

2.3.1. Preparation of protein samples

2.3.1.1. Egg white (EW). The egg white was completely separated from the yolk, weighed (36.80 g), and suspended in distilled water to make a 250 mL final solution. A 5 mL aliquot was withdrawn, and 5 mL of 5% TCA (w/v) was added into it. The mixture was centrifuged for 10 min, the upper liquid phase was decanted, and the precipitate was washed twice with distilled water. The isolated protein fraction was dissolved in 10 mL of protein dissolution buffer.

2.3.1.2. Whey proteins (WP). The whey liquids separated from the curd was passed through a glass fibre (GF)/polyethyleneterephtalate (PET) 1.0/0.45 μm filter, and diluted as required with the standard tris-buffer prior to analysis.

2.3.1.3. Gelatin (GL). Appropriate weight (1.0076 g) of bovine gelatin supplied from the herbalist was dissolved in distilled water and diluted to 10 mL. Gelatin solution was diluted at 1:1 ratio with the standard tris-buffer prior to analysis.

2.4. CUPRAC assay of total antioxidant capacity

2.4.1. Normal (N) sample measurement

The CUPRAC method, as described by Apak et al. [9], is based on the reduction of a cupric neocuproine complex (Cu(II)–Nc) by antioxidants to the cuprous form (Cu(I)–Nc). To a test tube were added 1 mL each of Cu(II), Nc, and NH_4Ac buffer solutions. Antioxidant standard (or neutralized reduction products of disulfides with Zn/HCl), synthetic mixture or real sample solutions (x) mL and H_2O ($1.1 - x$) mL were added to the initial mixture so as to make the final volume: 4.1 mL. The tubes were stoppered, and after 1/2 h, the absorbance at 450 nm (A_{450}) was recorded against a reagent blank. The standard calibration curves of each antioxidant compound was constructed in this manner as absorbance vs. concentration, and the molar absorptivity of the CUPRAC method for each antioxidant was found from the slope of the calibration line concerned. The scheme for normal measurement of antioxidants is summarized as:

1 mL Cu(II) + 1 mL Nc + 1 mL buffer + x mL antioxidant soln. + ($1.1 - x$) mL H_2O; total volume = 4.1 mL, measure A_{450} against a reagent blank after 30 min of reagent addition.

2.4.2. Modified CUPRAC method applied to human serum

Serum samples separated from the bloods of healthy adult volunteers (of age 25–30) were kept at 4 °C in a refrigerator just prior to analysis; samples were freshly used, and EDTA was not added as preservative. A volume of 0.4 mL of 1.0×10^{-2} M $CuCl_2 \cdot 2H_2O$, 0.4 mL of 7.5×10^{-3} M Nc, and 0.8 mL of pH 7.0 urea buffer were mixed. To this mixture were added ($0.4 - x$) mL of pH 8 standard tris-buffer and (x) mL whole serum sample (1:5 diluted with pH 8 standard buffer) or standard antioxidant solution or a mixture of both. The final mixture at 2.0 mL total volume was let to stand at room temperature for exactly 30 min, and the absorbance at 450 nm was recorded against a reagent blank.

2.4.3. Incubated (I) sample measurement

The mixture solutions containing sample and reagents were prepared as described in 'normal measurement'; the tubes were stoppered and incubated for 20 min in a water bath at temperature 50 °C. The tubes were cooled to room temperature under running water, and their A_{450} values were measured.

2.4.4. Hydrolyzed (H) sample measurement

A suitable mass of the thiol compound was weighed such that the final antioxidant concentration of the methanolic solution would be 1 mM. Each standard was dissolved in a suitable volume of 50% MeOH. In a 100 mL flask, sufficient hydrochloric acid was added to each solution until the final HCl molarity was 1.2 M, and diluted to the mark with 50% MeOH. This solution was decanted to a distillation flask into which a few pieces of boiling stone were added, and refluxed at 80 °C for 2 h. The flask was cooled to room temperature under running tap water. The hydrolyzate was neutralized with 1 M NaOH. The neutralized solution was then subjected to 'normal measurement'.

2.4.5. Hydrolyzed and incubated (H and I) sample measurement

The neutralized hydrolyzate was subjected to incubation at 50 °C in a water bath for 20 min. The A_{450} of running water-cooled samples were 'normally measured' as described in Section 2.4.1.

2.5. ABTS/persulphate assay of total antioxidant capacity

The ABTS/persulphate method [10] was followed. Briefly, the volumes of ($4.0 - x$) mL EtOH and (x) mL sample solution were taken. The reagent blank was prepared with 4 mL EtOH. One mL amount of 1:10 diluted ABTS radical cation solution was added to each mixture at 15 s intervals, and well mixed (total volume = 5.0 mL). The absorbance of the reagent blank (A_0) diminished in the presence of antioxidants, the absorbance decrease (ΔA) being proportional to antioxidant concentration. The decrease in absorbance (ΔA) caused by antioxidants, recorded at 734 nm against ethanol at the end of 6th minute, reflected the $ABTS^{\bullet+}$ radical cation scavenging capacity and was plotted against the concentration of the antioxidant. The $TEAC_{ABTS}$ value of a given antioxidant represents the ratio of the slope of the ΔA vs. concentration line of that antioxidant to that of trolox measured under the same conditions of the ABTS decolorization assay. The TEAC coefficient, being a slope ratio, is unitless.

2.6. FRAP assay of total antioxidant capacity

FRAP assay was carried out by the method of Benzie and Strain [11] with minor modifications. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) by antioxidants to the ferrous form (Fe^{2+}-TPTZ). Antioxidant solution (x) mL and ($0.4 - x$) mL H_2O were added to 3 mL of the FRAP reagent (final volume 3.4 mL), and the increase in absorbance (ΔA) at 595 nm was measured after 6 min. The $TEAC_{FRAP}$ value of a given antioxidant represents the ratio of the slope of the ΔA vs. concentration line of that antioxidant to that of trolox measured under the same conditions of the FRAP assay.

2.7. HPLC assay of individual constituents and TAC of synthetic thiol mixtures

The chromatographic separation of NAC in some pharmaceuticals was carried out on a Hamilton H × Sil C18 column (250 mm × 4.6 mm, 5 μm) (Reno, NV, USA) using an injection volume of 25 μL. The mobile phase consisted of methanol and 10 mM of pH 2.5 phosphoric acid buffer in varying proportions. The gradient elution program was run such that the initial composition

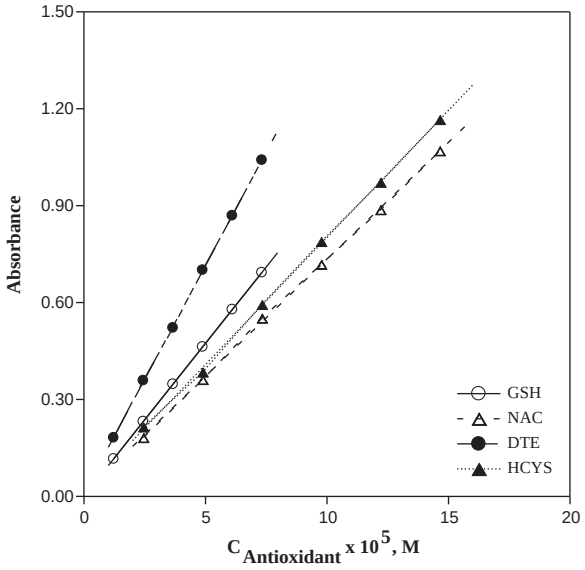


Fig. 1. The calibration curves of GSH, NAC, DTE, and HCYS with respect to the normal CUPRAC method.

of the mobile phase as 40% MeOH and 60% phosphoric acid aqueous buffer was linearly transformed into a final composition of 60% MeOH and 40% phosphoric acid buffer mixture within the program period of 10 min. The flow rate was adjusted to 1.0 mL min^{-1}, and the UV detection wavelength was 215 nm.

The HPLC assay of total antioxidant capacity (TAC) of synthetic binary and ternary mixtures of thiol antioxidants was performed as follows: the thiol constituents were identified with the aid of their retention times, their concentrations were found through peak area calibration, and the theoretical TAC of the mixture was expressed as the sum of the products of HPLC-calculated concentrations with the TEAC coefficients of individual thiols.

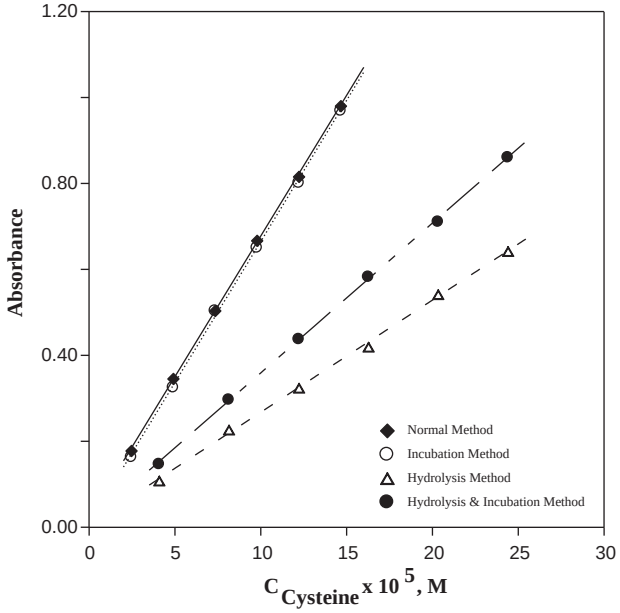


Fig. 2. The calibration curves of cysteine (CYS) with respect to the four types of CUPRAC methods.

2.8. Standard addition of thiols to protein fractions with modified CUPRAC method – protein TAC assay

To investigate the "additivity of absorbances of mixture constituents" when thiol compounds are added to protein matrices, the modified CUPRAC method – protein TAC assay was used [8]. One milliliter of 1.0×10^{-2} M $CuCl_2 \cdot 2H_2O$, 1 mL of 7.5×10^{-3} M Nc, and 2 mL of pH 7.0 urea buffer were mixed. To this mixture were added $(1.0 - x)$ mL of pH 8 standard buffer and (x) mL protein fraction sample or standard thiol solution or a mixture of both. The final mixture at 5.0 mL total volume was let to stand at room temperature for exactly 30 min, and the absorbance at 450 nm was recorded against a reagent blank. It should be noted that the users of the original CUPRAC method [9] should revert to the pH 7.0 urea buffer defined in this work, and should not use the ammonium acetate buffer therein, which would otherwise cause the precipitation of proteins. The pH 7.0 buffer used here is the one pertaining to Ellman's method [12] of thiol determination, but adjusted to pH 7.0 instead of pH 8.0.

2.9. Standard addition of thiols to protein fractions with Ellman's method

A volume of (x) mL sample + $(1.0 - x)$ mL standard tris-buffer at pH 8 + 2.0 mL pH 8.0 urea buffer + 30 μL Ellman's reagent were added in this order to obtain a mixture of 3.03 mL final volume. The absorbance at 412 nm was recorded after 2 min of mixing the sample with reagents [12].

2.10. Statistical analysis

Descriptive statistical analyses were performed using Excel software (Microsoft Office 2002) for calculating the means and the standard error of the mean. Results were expressed as the mean ± standard deviation (SD). Using SPSS software for Windows (version 13), the data were evaluated by two-way ANalysis Of VAriance (ANOVA) [14].

3. Results and discussion

The basic aim of this study is to apply the CUPRAC (cupric ion reducing antioxidant capacity) assay [9] originally developed in our laboratories to biochemically important thiol-type antioxidants and compare the results with those of other ET-based assays (ABTS/persulphate and FRAP methods) and also HPLC method. Additionally, the addivity of TAC values found by the modified CUPRAC procedure (for proteins) was tested for thiol antioxidants alone and in admixtures with proteins.

3.1. CUPRAC assay results

The CUPRAC method, using a cupric neocuproine (2,9-dimethyl-1,10-phenanthroline) chelate (Cu(II)–Nc) as the chromogenic oxidant, is based on the redox reaction with antioxidants producing the cuprous–neocuproine chelate (Cu(I)–Nc) showing maximum light absorption at 450 nm [9]. The reaction equation with thiol-type antioxidants can be formulated as:

$$2Cu(Nc)_2^{2+} + 2R\text{–}SH \leftrightarrow 2Cu(Nc)_2^{+} + R\text{–}S\text{–}S\text{–}R + 2H^{+} \quad (1.3)$$

Thiol-type antioxidants such as reduced glutathione, oxidized glutathione, cysteine, cystine, homocysteine, homocystine, N-acetyl cysteine, lipoic acid, dihydrolipoic acid, cysteamine, glutathione ethyl ester, DTE, and methionine, were used in standard solutions, and assayed using the normal (at room temperature), incubated (at 50 °C), hydrolyzed (at 80 °C), and hydrolyzed and

Table 1
The trolox equivalent antioxidant capacities (TEAC) of various thiol-based antioxidants calculated with respect to the ABTS, FRAP, and CUPRAC methods.

Antioxidants	$TEAC_{CUPRAC}$				$TEAC_{ABTS}$	$TEAC_{FRAP}$
	$TEAC_N$	$TEAC_I$	$TEAC_H$	$TEAC_{H\&I}$		
–SH type antioxidants						
N-acetyl cysteine (NAC)	0.43	0.40	0.43	0.41	0.53	0.48
1,4-Dithioerythritol (DTE)	0.84	0.68	0.17	0.21	0.76	0.86
Cysteamine (CYSA)	0.37	0.48	0.05	0.17	0.13	ND
Glutathione, reduced (GSH)	0.57	0.57	0.19	0.24	1.51	0.07
Glutathione reduced ethyl ester (GSHEE)	0.35	0.33	0.35	0.47	0.46	0.02
Cysteine (CYS)	0.39	0.35	0.16	0.20	1.28	0.14
Lipoic acid, reduced (DHLA)	0.48	0.48	0.87	0.79	0.29	0.36
Homocysteine (HCYS)	0.47	0.46	0.56	0.76	1.38	0.04
S–S type antioxidants						
Glutathione, oxidized (GSSG)	ND (0.58)	0.02 (0.54)	0.05	0.21	ND (0.74)	ND (ND)
Cystine (CYSS)	ND (0.82)	ND (0.74)	0.04	0.15	ND (0.64)	ND (ND)
Lipoic acid (LA)	0.22 (0.82)	0.40 (0.78)	0.01	0.06	0.11 (0.94)	0.06 (ND)
Homocystine (HCYSS)	0.04 (0.77)	0.23 (0.78)	0.34	0.91	ND (0.87)	ND (ND)
Other						
Methionine (M)	ND	ND	ND	ND	ND	ND

ND: not detected.
TEAC coefficients in the parantheses represent reduction products with Zn/HCl for the mentioned antioxidants.

incubated (at 50 °C) CUPRAC methods [9] against trolox as the standard reference compound. The same antioxidant solutions were cross-assayed with ABTS/persulphate and FRAP assays as the reference spectrophotometric methods. The linear calibration equations of the tested antioxidants (as absorbance in a 1-cm cell vs. molar concentration) gave the molar absorption coefficient (ε) as the slope. The molar absorption coefficient of the tested thiol-type antioxidants divided by that of trolox under the same conditions gave the trolox equivalent antioxidant capacity, or TEAC coefficient, of that antioxidant (Table 1). Among the thiol-type antioxidants, DTE shows the highest capacity, and CUPRAC-TEAC coefficients (in parentheses) decrease in the following order: DTE (0.84) > reduced GSH (0.57) > DHLA (0.48) > HCYS (0.47) > NAC (0.43) > CYS (0.39) > CYSA (0.37) > GSHEE (0.35).

Table 2
Comparison of theoretical and experimental CUPRAC antioxidant capacities of synthetic mixture solutions of thiol-type compounds (as mM TR equivalents).

Method	Composition of mixture	$TAC_{component}$ (mM TR-equiv.)	$TAC_{theoretical}$ (mM TR-equiv.)	$TAC_{experimental}$ (mM TR-equiv.)
Normal CUPRAC	0.1 mL 1 mM NAC	1.05×10^{-2}	4.24×10^{-2}	$(4.41 \pm 0.08) \times 10^{-2}$
	0.1 mL 1 mM HCYS	1.14×10^{-2}		
	0.1 mL 1 mM DTE	2.05×10^{-2}		
Incubated CUPRAC	0.1 mL 1 mM NAC	0.98×10^{-2}	3.76×10^{-2}	$(4.01 \pm 0.11) \times 10^{-2}$
	0.1 mL 1 mM HCYS	1.12×10^{-2}		
	0.1 mL 1 mM DTE	1.66×10^{-2}		
Normal CUPRAC	0.1 mL 1 mM CYSA	0.90×10^{-2}	2.90×10^{-2}	$(2.90 \pm 0.07) \times 10^{-2}$
	0.1 mL 1 mM CYS	0.95×10^{-2}		
	0.2 mL 1 mM LA	1.07×10^{-2}		
Incubated CUPRAC	0.1 mL 1 mM CYSA	1.17×10^{-2}	3.97×10^{-2}	$(4.03 \pm 0.04) \times 10^{-2}$
	0.1 mL 1 mM CYS	0.85×10^{-2}		
	0.2 mL 1 mM LA	1.95×10^{-2}		
Normal CUPRAC	50 μL 1 mM GSH	0.70×10^{-2}	3.13×10^{-2}	$(2.93 \pm 0.06) \times 10^{-2}$
	0.1 mL 1 mM NAC	4.24×10^{-2}		
	0.1 mL 1 mM CYS	4.24×10^{-2}		
Incubated CUPRAC	50 μL 1 mM GSH	0.70×10^{-2}	2.53×10^{-2}	$(2.77 \pm 0.02) \times 10^{-2}$
	0.1 mL 1 mM NAC	0.98×10^{-2}		
	0.1 mL 1 mM CYS	0.85×10^{-2}		

Samples were analyzed in triplicate.
TAC values (significantly different); ($P = 0.05$, $F_{exp} = 1.527$, $F_{crit\,(table)} = 6.608$, $F_{exp} < F_{crit\,(table)}$).

Table 3
Statistical comparison of the results obtained using the CUPRAC and HPLC methods for NAC in pharmaceutical samples.

Sample	Method	Mean concn. (mM)	Std. dev. (s)	$S^{a,b}$	$t^{a,b}$	$t_{table}{}^{b}$	F^{b}	$F_{table}{}^{b}$	Recovery (%)
Brunac eye drop	CUPRAC	1.02	0.03	–	–	–	–	–	102.00
	HPLC	1.11	0.05	0.04	2.76	2.78	2.78	19.00	111.00
Trom acetylcystein 600	CUPRAC	3.33	0.06	–	–	–	–	–	90.73
	HPLC	3.74	0.13	0.10	5.02	2.78	4.69	19.00	101.90
Mentopin acetylcystein 600	CUPRAC	3.63	0.08	–	–	–	–	–	98.91
	HPLC	3.58	0.06	0.07	0.87	2.78	1.77	19.00	97.55

[a] $S = \left[\left((n_1-1)s_1^2 + (n_2-1)s_2^2\right)/(n_1+n_2-2)\right]^{1/2}$ and $t_{exp} = (\bar{a}_1 - \bar{a}_2)/S((1/n_1)+(1/n_2))^{1/2}$ where S is the pooled standard deviation, s_1 and s_2 are the standard deviations of the two populations with sample sizes of n_1 and n_2, and sample means of $\bar{a}_1$ and $\bar{a}_2$, respectively, and $t = (n_1 + n_2 - 2)$ degrees of freedom ($n_1 = n_2 = 3$).
[b] Statistical comparison made on paired data produced with the proposed and reference methods; the results given only on the row of the reference method.

Table 4
Slope and intercept forms of calibration equations of the modified-CUPRAC method (protein assay) applied to various thiol-based antioxidants alone and in complex matrices such as the solutions of egg white, whey, and gelatin.

Thiol-type antioxidants	Matrix: alone[a]	Egg white	Whey	Gelatin
N-Acetyl cysteine (NAC)	$A = 7.46 \times 10^3\ c + 0.02\ r = 0.9999$	$A = 7.31 \times 10^3\ c + 0.389\ r = 0.9999$	$A = 7.48 \times 10^3\ c + 0.14\ r = 0.9999$	$A = 7.49 \times 10^3\ c + 0.269\ r = 0.9899$
1,4-Dithioerythritol (DTE)	$A = 1.43 \times 10^4\ c + 0.076\ r = 0.9977$	$A = 1.38 \times 10^4\ c + 0.411\ r = 0.9907$	$A = 1.32 \times 10^4\ c + 0.297\ r = 0.9910$	$A = 1.48 \times 10^4\ c + 0.299\ r = 0.9949$
Cysteamine (CYSA)	$A = 6.05 \times 10^3\ c + 0.044\ r = 0.9975$	$A = 6.03 \times 10^3\ c + 0.375\ r = 0.9910$	$A = 5.74 \times 10^3\ c + 0.197\ r = 0.9980$	$A = 5.82 \times 10^3\ c + 0.191\ r = 0.9980$
Glutathione (GSH)	$A = 8.18 \times 10^3\ c + 0.044\ r = 0.9999$	$A = 8.10 \times 10^3\ c + 0.378\ r = 0.9968$	$A = 7.76 \times 10^3\ c + 0.226\ r = 0.9960$	$A = 8.27 \times 10^3\ c + 0.091\ r = 0.9970$
Cysteine (CYS)	$A = 7.23 \times 10^3\ c - 0.055\ r = 0.9840$	$A = 7.06 \times 10^3\ c + 0.318\ r = 0.9899$	$A = 7.26 \times 10^3\ c + 0.111\ r = 0.9890$	$A = 6.60 \times 10^3\ c + 0.081\ r = 0.9982$
Homocysteine (HCYS)	$A = 7.81 \times 10^3\ c + 0.027\ r = 0.9999$	$A = 7.53 \times 10^3\ c + 0.322\ r = 0.9999$	$A = 7.92 \times 10^3\ c + 0.246\ r = 0.9982$	$A = 7.82 \times 10^3\ c + 0.24\ r = 0.9919$

[a] Equations in the column represent calibration lines for mentioned antioxidant alone.

Table 5
Slope–intercept forms of calibration equations of the modified CUPRAC method applied to thiol compounds alone and in whole serum samples.

Thiol compound	Thiol compound alone	In whole serum (WS)
Cysteine (CYS)	$A = 0.79 \times 10^4\ c + 0.0086\ r = 0.9969$	$A = 0.77 \times 10^4\ c + 0.2139\ r = 0.9984$
Glutathione (GSH)	$A = 0.80 \times 10^4\ c + 0.1163\ r = 0.9982$	$A = 0.78 \times 10^4\ c + 0.3728\ r = 0.9995$
Homocysteine (HCYS)	$A = 0.99 \times 10^4\ c + 0.0431\ r = 0.9985$	$A = 1.08 \times 10^4\ c + 0.3652\ r = 0.9977$

The calibration curves of selected thiol-type antioxidants, GSH, NAC, DTE, and HCYS are shown in Fig. 1. Fig. 2 shows the calibration curves of cysteine with respect to the four types of CUPRAC methods. In this figure, although ε_N and ε_I values are close to each other for cysteine, there was significant decrease in ε value (possibly as a result of partial decomposition) with respect to the hydrolyzed method.

Among the thiol-type antioxidants tested by the CUPRAC method, glutathione ethyl ester (GSHEE) had relatively lower ε value ($\varepsilon_N = 5.86 \times 10^3\ L\,mol^{-1}\,cm^{-1}$) than the two –SH bearing 1,4-dithioerythritol (DTE) ($\varepsilon_N = 1.40 \times 10^4\ L\,mol^{-1}\,cm^{-1}$). Structural properties of thiol-type antioxidants would normally dictate that two –SH bearing DTE and dihydrolipoic acid should exhibit higher TEAC coefficients than one –SH bearing GSHEE [15]. For thiol-type antioxidants having the same number of –SH groups, the $TEAC_{CUPRAC}$ coefficients of DTE and DHLA were found as 0.84 and 0.48, respectively. The antioxidant properties of these compounds are naturally affected not only by the number of –SH groups but also by the overall extent of conjugation in the molecule, accessibility of –SH groups with the chromogenic reagent, and the presence of additional substituents in the vicinity of –SH groups. Disulfide forms of GSH and cysteine having the –S–S– groups, namely GSSG and cystine, respectively, could not be detected by the CUPRAC assay. Among the disulfide compounds, lipoic acid and homocystine had considerably lower ε values, and when these compounds were assayed with the CUPRAC method following Zn/HCl reduction [13,16], the obtained ε values were approximately twice as much of the corresponding simple –SH compounds, namely reduced lipoic acid and homocysteine, respectively (Table 1). Thus, one molecule cystine was reduced to two molecules cysteine ($Cystine + 2H^+ + 2e^- \rightarrow 2\ Cysteine$).

The relatively higher CUPRAC antioxidant capacities of LA than other disulfides such as oxidized glutathione and cystine are probably related to the position of the two sulfur atoms in the 1,2-dithiolane ring, which results in a high electron density in this five membered ring [17], showing a weak reactivity toward the CUPRAC reagent (Table 1).

3.2. Comparison of the CUPRAC and ABTS/persulphate assays

As reference methods for comparison, the TAC measurement methods of ABTS/persulphate and FRAP were used. The ABTS method is based on the scavenging of $ABTS^{\bullet+}$ radical cation by hydrogen atom-donating antioxidants, resulting in an absorbance decrease of the chromophoric radical. As being a most important thiol (–SH) peptide at *in vivo* conditions, glutathione showed a TEAC value of 0.57 in the CUPRAC method, and has proven to be a 1-e reductant. Since 'trolox equivalent antioxidant capacity' (TEAC) is defined as the ratio of the molar absorptivity of the tested antioxidant to that of trolox, and trolox having a CUPRAC molar absorptivity of $\varepsilon_{TR} = 1.67 \times 10^4\ L\,mol^{-1}\,cm^{-1}$ is a 2-e transfer agent, glutathione having a CUPRAC molar absorptivity of $\varepsilon_{GSH} = 9.47 \times 10^3\ L\,mol^{-1}\,cm^{-1}$ is a $n = 2 \times 9.47 \times 10^3 / 1.67 \times 10^4 = 1.13 \approx$ 1-e transfer reductant in the CUPRAC assay. On the other hand, the $TEAC_{ABTS}$ value of GSH was 1.51. The ABTS result was not in accordance with the reversible 1-e oxidation of GSH to the corresponding disulfide ($2GSH \leftrightarrow GSSG + 2H^+ + 2e^-$) that is expected to occur under physiological conditions. The most significant of the multiple roles of thiol compounds *in vivo* may be their critical function as cellular redox buffers, regulating protein thiol/disulfide composition. Disulfide bonds are not only essential in maintaining the structural stability of soluble proteins, but additionally, reversible disulfide formation is involved in many enzymatic and transport processes [18]. By similar reasoning, the $TEAC_{ABTS}$ coefficients of cysteine and homocysteine were found as 1.28 and 1.38, respectively, not in agreement with their physiological redox reactions, e.g., ($2Cysteine \leftrightarrow Cystine + 2H^+ + 2e^-$,

Table 6
Slope and intercept forms of calibration equations of the Ellman assay applied to various thiol-based antioxidants alone and in egg white and whey.

Thiol-type antioxidants	Matrix: alone[a]	Egg white	Whey
N-Acetyl cysteine	$A = 1.27 \times 10^4\ c + 0.0191\ r = 0.9992$	$A = 1.19 \times 10^4\ c + 0.363\ r = 0.9954$	$A = 1.20 \times 10^4\ c + 0.2040\ r = 0.9992$
1,4-Dithioerythritol	$A = 2.39 \times 10^4\ c - 0.0188\ r = 0.9952$	$A = 1.99 \times 10^4\ c + 0.3749\ r = 0.9986$	$A = 2.74 \times 10^4\ c + 0.232\ r = 0.9997$
Cysteamine	$A = 1.08 \times 10^4\ c + 0.0158\ r = 0.9992$	$A = 1.14 \times 10^4\ c + 0.308\ r = 0.9944$	$A = 0.98 \times 10^4\ c + 0.136\ r = 0.9950$
Glutathione	$A = 1.47 \times 10^4\ c + 0.0337\ r = 0.9964$	$A = 1.52 \times 10^4\ c + 0.6253\ r = 0.9937$	$A = 1.52 \times 10^4\ c + 0.205\ r = 0.9973$
Cysteine	$A = 1.17 \times 10^4\ c + 0.038\ r = 0.9996$	$A = 1.18 \times 10^4\ c + 0.4366\ r = 0.9946$	$A = 1.17 \times 10^4\ c + 0.254\ r = 0.9984$
Homocysteine	$A = 1.29 \times 10^4\ c + 0.014\ r = 0.9996$	$A = 1.20 \times 10^4\ c + 0.336\ r = 0.9942$	$A = 1.35 \times 10^4\ c + 0.189\ r = 0.9890$

[a] Equations in the column represent calibration lines for mentioned antioxidant alone.

2Homocysteine ↔ Homocystine + $2H^+$ + $2e^-$). The abnormally high TEAC coefficients of GSH, CYS, and HCYS as measured by the ABTS/persulphate method (Table 1) suggest that these thiols are irreversibly oxidized by the $ABTS^{\bullet+}$ radical cation to higher products such as sulphinic ($-SO_2H$) or sulphonic ($-SO_3H$) acids through sulphenic acid (–SOH) intermediates that is not in accordance with the physiological defensive roles of the mentioned thiols [19,20]. Such higher oxidations are less likely '*in vivo*'. The CUPRAC method treats the thiol compounds at nearly physiological pH as opposed to the ABTS method which optimally acts at more acidic pH.

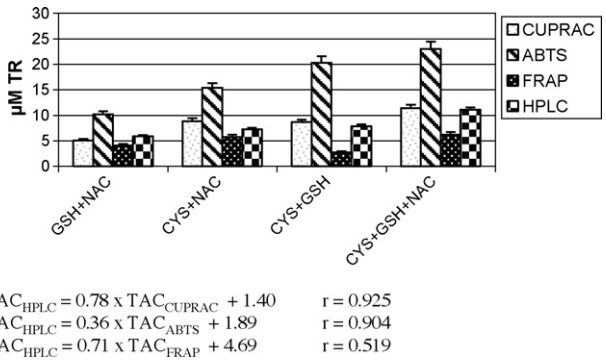
Cystine, lipoic acid, and homocystine gave no or weak direct response to both $CUPRAC_N$ and ABTS antioxidant assays, but when these compounds were subjected to Zn/HCl reduction and neutralized, their CUPRAC responses reached 1.7–2.1 times the corresponding values measured with cysteine, dihydrolipoic acid and homocysteine, respectively, whereas ABTS/persulphate gave erratic ratios (0.5–3.1) under the same conditions (Table 1). These findings confirm that, as a result of Zn/HCl reduction of the disulfide to thiol, CUPRAC measures nearly twice as much molar absorptivity for the reduced disulfide as that for the corresponding thiol, accurately revealing the number of electrons transfered (as a simple whole number of two) during disulfide reduction. In other words, the number of electrons transfered in the CUPRAC redox reaction can be measured as reasonable whole numbers, because CUPRAC reactions are complete (within the protocol time of the assay) and reproducible for well-defined oxidation products.

3.3. Comparison of the CUPRAC and FRAP assays

The FRAP method is based on the reduction of Fe(III)–tripyridyltriazine complex to Fe(II)–tripyridyltriazine (Fe(II)–TPTZ) at low pH by e-donating antioxidants, resulting in an absorbance increase at 595 nm due to the formation of a blue-colored ferrous chelate. The FRAP method did not give consistent results, and even at relatively high concentrations of GSH, the $TEAC_{FRAP}$ value was only 0.07 (in other words, the FRAP method does not sufficiently respond to thiol-type antioxidants such as glutathione). Seemingly, cysteine (0.14), homocysteine (0.04), and GSHEE (0.02) had considerably lower ε values, whereas cysteamine was not detectable with the FRAP method at all.

In order that a chromogenic oxidizing reagent be useful for the determination of thiol-type antioxidants, it should meet both thermodynamic and kinetic criteria. Thermodynamic favourability requires a positive electromotive force (ΔE^0) of the redox reaction between the oxidizing reagent and the antioxidant, giving rise to a large negative Gibbs free energy change (ΔG^0) via the equation $\Delta G^0 = -nF\Delta E^0$, where n is the number of transfered electrons and F is the Faraday constant. For the oxidized and reduced forms of glutathione: GSSG/2GSH, i.e., an important redox couple reflecting the cellular redox status, the standard potential at pH = 0 is +0.180 V, and therefore the formal potential at pH = 7, $\Delta E^{0\prime}$, is −0.240 V [21,22]. Thus, for the $Cu(Nc)_2^{2+}$/GSH redox equilibrium, the electromotive force at pH = 7, $\Delta E^{0\prime}$, would be 0.84 V, providing a great thermodynamic favourability.

However, thermodynamic favourability of a reaction does not always guarantee that it would occur with fast kinetics, i.e., that it would actually take place within the protocol time of a redox-based spectrophotometric assay. The CUPRAC assay advantageously responds much faster than FRAP to certain thiol-type antioxidants. Thiols are not effectively oxidized within the protocol time of the FRAP method. The possible reason for this observation with respect to electronic configurations is the kinetic inertness of high-spin d^5-Fe(III) having half-filled d-orbitals, while CUPRAC utilizing d^9-Cu(II) oxidant involves faster kinetics [9]. In relation to this, Gorinstein et al. [23] studied the antioxidant activity of raw and processed garlic sample extracts, and showed that FRAP values were significantly lower than CUPRAC values; the obtained data verified the advantage of CUPRAC over FRAP in reflecting thiol antioxidant content of food, because thiol-type antioxidants rich in garlic responded to CUPRAC but not to FRAP assay. Besides, FRAP reaction runs at acidic pH (pH = 3.6) and thus does not give a realistic estimate of *in vivo* antioxidant capacity of a sample. At more acidic conditions than the physiological pH, the reducing capacity may be suppressed due to protonation on antioxidant compounds, yielding a lower total antioxidant capacity.



$TAC_{HPLC} = 0.78 \times TAC_{CUPRAC} + 1.40$ r = 0.925
$TAC_{HPLC} = 0.36 \times TAC_{ABTS} + 1.89$ r = 0.904
$TAC_{HPLC} = 0.71 \times TAC_{FRAP} + 4.69$ r = 0.519

Fig. 3. Comparison of the experimentally found trolox (TR)-equivalent antioxidant capacities (in µM TR units) of synthetic mixtures of thiol-type antioxidants as measured by four different methods.

Mazor et al. [24] measured the trolox (TEAC)-, CUPRAC-, and Fe(II)-equivalents (FRAP) antioxidant capacity of some antioxidants, i.e., bucillamine (BUC), N-acetyl cysteine (NAC), glutathione (GSH). The reduction yield of the CUPRAC reagent was proportional to the antioxidant concentrations, and doubled for the 2-e reducing agents like BUC (containing 2 –SH groups), compared to the 1-e reducing agents, NAC and GSH (containing 1 –SH group). On the other hand, the widely used FRAP method, although being capable of detecting BUC and NAC, was unable to detect the 1 –SH bearing tripeptide: GSH [25].

3.4. TAC measurement of synthetic mixture solutions

Synthetic mixtures obeyed Beer's law fairly well, as depicted in Table 2. Synthetic mixtures of thiol-type antioxidants exhibited the theoretically expected antioxidant capacity within ±9.0%, meaning that chemical deviations from Beer's law essentially did not exist and the CUPRAC absorbances of constituents were additive. The two-way ANalysis Of VAriance (ANOVA) comparison by the aid of *F*-test of the mean-squares of 'between-treatments' (i.e., theoretically expected capacity with respect to CUPRAC method and experimentally found capacities of different mixtures in Table 2) and of residuals [14] for a number of real samples (consisting of synthetic mixtures of thiol-type antioxidants) enabled to conclude that there was no significant difference between treatments. In other words, the experimentally found capacity results and theoretically expected capacity calculations were alike at 95% confidence level ($F_{exp} = 1.527$, $F_{crit} = 6.608$, $F_{exp} < F_{crit}$ at $P = 0.05$). Thus, the proposed methodology was validated. On the other hand, there was significant difference between samples with respect to composition of mixtures (i.e., the 'residual' mean-square was much greater than 'between-sample' mean-square at 95% confidence level).

In another experiment, possible binary or ternary mixtures of antioxidants were analyzed for antioxidant capacity using the CUPRAC, ABTS/persulphate, FRAP and HPLC assays (Fig. 3). The expected and experimentally found antioxidant capacities were generally in accordance with each other. The highest correlation of HPLC was obtained with CUPRAC ($r = 0.925$).

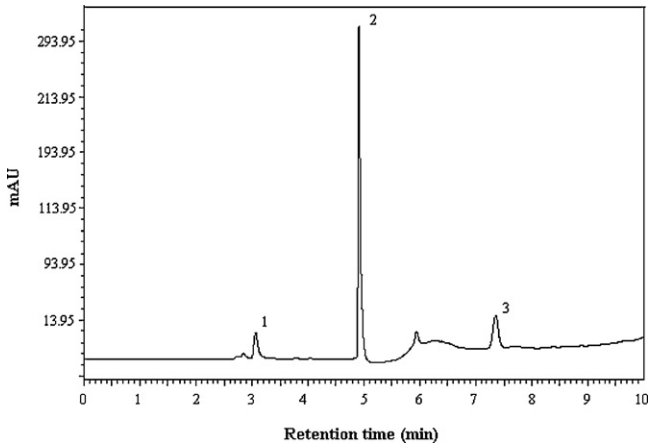


Fig. 4. The chromatogram of a synthetic mixture of thiol-type antioxidants. (1) Cysteine; (2) glutathione; (3) N-acetyl cysteine.

3.5. Comparison of HPLC and CUPRAC assays

Fig. 4 gives the chromatogram of a standard mixture composed of three thiol-type antioxidants including cysteine, glutathione, and N-acetyl cysteine. Fig. 5 shows the chromatogram of Brunac eye drop containing NAC. For $N = 3$ different pharmaceutical samples containing NAC analyzed by CUPRAC and HPLC procedures, the experimental values (i.e., $|t|$ calculated from the pooled-estimate of standard deviation, and F from the ratio of variances) did not exceed the critical ones, indicating that there were no significant difference in accuracy and precision between either synthetic mixtures or methods of determination (Table 3).

3.6. Standard addition of thiols to protein fractions and human serum followed by modified CUPRAC and Ellman assays

The slope–intercept forms of calibration equations of the modified CUPRAC method applied to various thiol-type antioxidants alone and in complex matrices such as egg white, whey, and gelatin are tabulated in Table 4. Regression analysis of the results shown in Table 4 revealed that absorbance vs. concentration relationships with the modified CUPRAC procedure were perfectly linear (r ranged between 0.984 and 0.999) for the thiol-type antioxidants tested. Urea – in combination with SDS – as a buffer component used in the analysis of protein solutions significantly increase the reactivity of thiols and disulfides that may be buried within the protein matrix [26]. In this work, presence of 8 M urea is thought to partly denaturate proteins and to lower the reduction potential of disulfide/thiol couples in peptides/proteins enhancing thiol oxidizability [27].

Since the corresponding slopes of the calibration lines of plasma thiols (CYS, GSH, and HCYS) were very close to each other in individual and "in whole serum" determinations (as seen in a row of Table 5), it can be deduced that the modified CUPRAC method was interference-free for these thiols in serum, i.e., it did not show chemical deviations from Beer's law that may otherwise arise from interactions among thiols and serum constituents.

Similar experiments were repeated using Ellman's assay (Table 6) which uses the thiol-specific reagent (DTNB) capable of reacting with a thiol group to release 5-thio-2-nitrobenzoate (TNB^-), which ionizes to the TNB^{2-} dianion (yellow chromophore) in water at neutral and alkaline pH. However, it should be noted that DTNB basically responds to thiols and not to other classes of antioxidants including polyphenols.

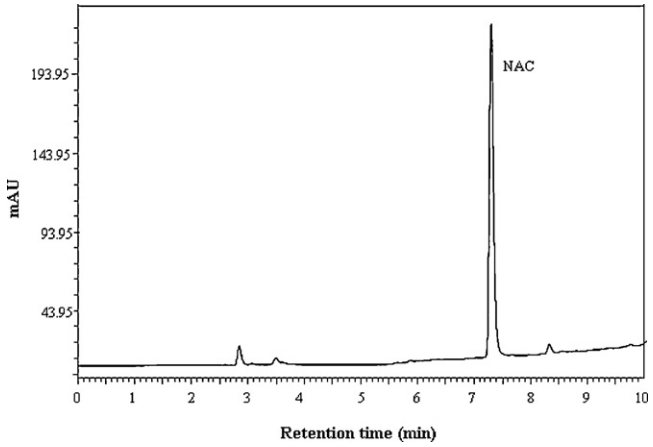


Fig. 5. The chromatogram of Brunac eye drop containing NAC (initial concentration of NAC = 0.3 M).

4. Conclusions

This work reports for the first time the TAC assay of various thiol-type antioxidants using CUPRAC and other spectrophotometric methods. The calibration curves, molar absorptivities, and linear concentration ranges of each antioxidant were established in the proposed and reference systems. The antioxidant properties of these compounds are governed by the number of –SH groups, the overall extent of conjugation in the molecule, accessibility of –SH groups with the chromogenic reagent, and the presence of additional substituents in the vicinity of –SH groups. Synthetic mixtures comprised of thiol-type antioxidants gave the theoretically expected CUPRAC antioxidant capacities (TAC values), indicating that chemical deviations from Beer's law were basically absent, and the observed CUPRAC absorbances were additive. The findings of this study demonstrate that CUPRAC, unlike FRAP, is capable to assay thiol-type antioxidants alone, in mixtures, and in admixtures with other antioxidants including proteins. Moreover, disulfide-type antioxidants can be similarly assayed after a Zn/HCl preliminary conversion to the corresponding thiols, and the CUPRAC results accurately reflect the 2-e reduction processes of disulfide–thiol conversion as opposed to ABTS/persulfate.

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