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# Spectrophotometric total protein assay with copper(II)–neocuproine reagent in alkaline medium

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**Abstract**

Total protein assay was made using copper(II)–neocuproine (Nc) reagent in alkaline medium (with the help of a hydroxide-carbonate-tartarate solution) after 30 min incubation at 40 °C. The absorbance of the reduction product, Cu(I)–Nc complex, was recorded at 450 nm against a reagent blank. The absorptivity of the developed method for bovine serum albumin (BSA) was 0.023 l $mg^{-1}$ $cm^{-1}$, greater than that of Lowry assay (0.0098), and much greater than that of Cu(II)–bicinchoninic acid (BCA) assay (0.00077). The linear range of the developed method (8–100 mg $l^{-1}$ BSA) was as wide as that of Lowry, and much wider than that of BCA (200–1000 mg $l^{-1}$ BSA) assay. The sensitivity of the method was greater than those of Cu-based assays (biuret, Lowry, and BCA) with a LOD of 1 mg $l^{-1}$ BSA. The within-run and between-run precisions as RSD were 0.73 and 1.01%, respectively. The selectivity of the proposed method for protein was much higher than those of dye-binding and Lowry assays: Most common interferents to other protein assays such as tris, ethanolamine, deoxycholate, CsCl, citrate, and triton X-100 were tolerated at 100-fold concentrations in the analysis of 10 mg $l^{-1}$ BSA, while the tolerance limits for other interferents, e.g., $(NH_4)_2SO_4$ and acetylsalicylic acid (50-fold), SDS (25-fold), and glycerol (20-fold) were at acceptable levels. The redox reaction of Cu(II)–Nc as an outer-sphere electron transfer agent with the peptide bond and with four amino acid residues (cystine, cysteine, tryptophan, and tyrosine) was kinetically more favourable than that of Cu(II) alone in the biuret assay. Since the reduction product of Cu(II) with protein, i.e., Cu(I), was coordinatively saturated with Nc in the stable $Cu(Nc)_2^+$ chelate, re-oxidation of the formed Cu(I) with Fenton-like reactions was not possible, thereby preventing a loss of chromophore. After conventional protein extraction, precipitation, and redissolution procedures, the protein contents of the minced meat (veal and turkey), sardine, various milk products, and egg white were analyzed with the proposed and Lowry methods, and the results correlated appreciably ($r = 0.98$). The method was validated by Kjeldahl analyses of the tested samples; the data sets of complex samples assayed by Cu(II)–Nc and Lowry correlated to the findings of Kjeldahl yielded correlation coefficients $r = 0.96$ and 0.97, respectively, with slopes being close to 1. Interferences of glucose and thiol compounds at relatively low concentrations could be compensated for by selecting a lower alkaline pH (i.e., pH 10) at a cost of slightly reduced sensitivity and adding an identical amount of interferent to the reagent blank, respectively, since the absorbances due to BSA and interferent were additive. Thus a novel spectrophotometric method for total protein assay using a stable reagent and chromophore, which was simple, rapid, sensitive, flexible, and relatively selective, was developed, and applied to a variety of food products.


*Keywords:* Protein assay; Copper(II)–neocuproine; Spectrophotometry; Lowry assay

## 1. Introduction

Since proteins are the most characteristic chemical compounds in the living cell and constitute a significant proportion of our diet, determination of proteins with rapid and reliable methods is of great importance in analytical biochemistry, nutritional chemistry and food research. A protein macromolecule is composed of approximately 20 different amino acids linked to each other in a large number. Commonly used methods for determining total nitrogen in proteins are Kjeldahl digestion followed by microtitration [1,2], Dumas combustion [3,4], and modified Lassaigne [5] procedures with their modifications, based on nitrogen liberation as ammonia or elementary nitrogen, or thiocyanate conversion of the fused N-content followed by spectrophotometry. The best established procedure is the Kjeldahl method which is highly reproducible, and suitable for protein assay in solid and semi-solid samples. However, phospholipids, nucleic acids and amino sugars, which contain low levels of N, may cause

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positive errors in routine assays [6]. Despite the simplicity of nitrogen-based assays, they have reduced sensitivity and are accompanied with numerous side reactions that cause errors. Common problems encountered in Kjeldahl analysis include sample size and type incompatibility with acid, salt, or catalyst; contaminated samples, standards, or reagents; inadequate or excessive digestion time; inadequate or excessive distillation; foaming or bumping during digestion; uneven digestion; precipitation of salt or caking of digested sample; instrumental leaks, and NaOH carryover. Spectrophotometric methods of total protein assay generally have distinct advantages over other procedures in terms of simplicity, rapidity, and sensitivity [7–11]. High sensitivity of protein assays may be required in applications of forensic sciences (e.g., detection of protein residues in blood strains), pharmaceutical sciences (e.g., detection of protein contaminants in drugs), and in a number of other applications.

Spectrophotometric procedures work either in the UV or visible range. UV spectrometry applied at 210 nm [12] is not so selective as many foreign substances absorb light at this wavelength. The UV method of Vakaleris and Price [13] is based on the absorbance of aromatic amino acids such as tyrosine and/or tryptophan at about 280 nm, which may show high protein-to-protein variability. Colorimetric protein assay techniques which have been reviewed by Sapan et al. [14] may be broadly classified into two major categories: a chromophore appears either at the end of a dye-binding reaction or a redox reaction with proteins.

The dye-binding spectrophotometric methods depend on the reaction of positively-charged amino acid residues in proteins with an acid dye (e.g., amido black 10B, acid orange 12, and orange G) in acidic medium to form an insoluble complex, and the absorbance of the centrifugate is inversely proportional to protein concentration in solution [15,16]. Coomassie brilliant blue G250 (abbreviated as CB, a triphenylmethane dye belonging to the magenta family) or erythrosin B binds to proteins to cause a batochromic shift in absorption maxima, giving rise to Bradford [17] or Soedjak [18] assays. Since the original Bradford assay [17] has a poor linearity, Zor and Selinger [19] have suggested the use of the ratio of absorbances at 590 and 450 nm to improve the linearity of the CB assay system. Sedmak and Grossberg demonstrated that peptides with a molecular mass less than 3000 Da did not form a complex with CB [20]. The high dependence of the Bradford assay on protein composition (e.g., the importance of lysine and arginine residues on protein) presents a major problem to the broad use of CB binding as a quantitative protein assay [14]. The Bradford assay was also criticised for not working well on samples containing lipids and non-ionic detergents [21]. The dye-binding assays were also tried to be improved by the use of other dyes such as bromophenol blue [22] or eosin B [23], or by the incorporation of metal ions such as pyrogallol red-molybdate(VI) [24–26], pyrocatechol violet-molybdate(VI) [27], and tetrachlorogallein-molybdate(VI) [28], forming a metal-dye ternary complex with protein.

The redox spectrophotometric methods essentially make use of either a heteropoly-molybdenum blue or a copper-based reaction. The heteropoly-blue reactions using the Folin-Ciocalteau reagent (composed of phosphomolybdic-tungstic mixed acids) [29] gave rise to Hull [30] and Lowry [9] procedures, the latter being an extension of the biuret reaction (i.e., Cu(II) is a part of the reagent mixture) which was later modified on various occasions [31–33]. Protein-to-protein variability of the Lowry assay [32,34] is thought to reflect the contributions of specific amino acids (tyrosine, tryptophan) on colour development with the Folin reagent. Various components of the protein solution [35] such as carbohydrates [36], as well as N-containing buffers [37,38] may interfere with the Lowry assay, nevertheless it has been widely used over the years for a variety of protein samples. Copper-based redox methods primarily consist of the biuret [39] and bicinchoninic acid (BCA) [40] assays. The biuret reaction is based on the reduction of Cu(II) with protein in alkaline medium to produce Cu(I), which binds to protein forming a Cu(I)–peptide complex of purplish-violet colour. Besides being not sufficiently sensitive for certain applications, solution constituents such as Tris-buffer, ammonium ions, sucrose, primary amines, and glycerol may interfere with the biuret reaction [39]. The BCA reagent, 2,2′-biquinoline-4,4′-dicarboxylic acid also known as bicinchoninic acid, may be used to detect the cuprous ion produced as a result of biuret-type reaction to give a purple-coloured Cu(I)–BCA complex absorbing at 562 nm [41]. The BCA assay has proven to be superior over other analogic methods in terms of its ease of use, enhanced flexibility, compatibility with ionic and non-ionic detergents, stability, broad linear range, and sensitivity, but is prone to interferences from reducing agents such as thiols [42], copper chelators such as EDTA [14], protein stabilizers such as glucose [43,44], hydrogen peroxide [45], or phospholipids [46]; these interferences were partly overcome by protein precipitation–separation from soluble reducing agents, and the use of anionic detergents such as SDS [47].

The relatively low absorptivity of the BCA method, i.e., 0.77 absorbance units for each mg bovine serum albumin (BSA) per mililiter, as given in the SIGMA ‘BCA protein assay kit’ [48], needs to be increased thereby enhancing sensitivity with a suitable chromogenic reagent selective for Cu(I). Instead of allowing for an extended reaction of protein to reduce Cu(II) to Cu(I) first, and then complexing the in situ formed Cu(I) with a chromogenic ligand, we preferred to use the copper(II)–neocuproine (2,9-dimethyl-1,10-phenanthroline) reagent as an outer-sphere electron transfer agent in alkaline medium where copper reduction and cuprous ion complexation occurs simultaneously. The copper(II)–neocuproine reagent, introduced for various reducing agents as a mild oxidant [49], was previously used by our research team to determine the biochemically important reductants, such as cysteine [50] and Vitamin E [51]. It has recently been used for ascorbic acid assay in foods and beverages [52], and for flavonoids as a total antioxidant capacity assay of food materials [53]. It should be noted here that the Fe(III)-based antioxidant assay reagents such as ferric reducing antioxidant potency (FRAP) [54] cannot oxidize proteins, and therefore cannot be used for the same purpose. The FRAP reagent, Fe(III)-tripyridyltriazine, only functions at acidic pH, and does not oxidatively damage albumin or protein thiols to an appreciable extent [53]. This work aims to make use of the copper(II)–neocuproine reagent to oxidize proteins in alkaline

medium (under slightly elevated temperature incubation) and to record the absorbance of the resulting Cu(I)–neocuproine chelate at 450 nm, to compare the protein findings statistically with those of the conventional Lowry assay for standard BSA solutions, and to apply the developed method to the analysis of real protein samples.

## 2. Experimental

### 2.1. Chemicals and solutions

Neocuproine (2,9-dimethyl-1,10-phenanthroline) hydrochloride, bovine serum albumin, casein, copper chloride dihydrate, sodium carbonate, NaOH, HCl, sodium potassium tartrate, copper(II) sulfate pentahydrate, tris(hydroxymethyl)aminomethane (Tris), glycerol, *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB), D-(+)-glucose monohydrate, and ethanolamine were purchased from E. Merck; Folin-Ciocalteau's phenol reagent (2N), 1,4-dithioerythritol (DTE), 2-mercaptoethanol, glutathione, sodium dodecyl sulfate (SDS), and 1-cetylpyridinium bromide (CPB) monohydrate from Sigma; triton X-100, urea, cesium chloride from Fluka, trichloroacetic acid (TCA) from Riedel, and aspirin (acetylsalicylic acid) from Bayer. All individual amino acids tested, as well as heavy metal salts of $FeCl_3{\cdot}6H_2O$, $FeSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}7H_2O$, and $NiSO_4{\cdot}6H_2O$, were supplied from E. Merck. All chemicals were of analytical reagent grade unless otherwise stated. Tubulin (isolated from cow brain) and lactate dehydrogenase (LDH) (isolated from bacillus stearothermophillus) were kindly supplied by Molecular Biology & Genetics Department of Faculty of Science and Arts, Istanbul Technical University.

The following stock solutions (prepared weekly or daily) were used for the copper(II)–neocuproine assay: BSA (200 mg $l^{-1}$), $CuCl_2{\cdot}2H_2O$ ($1.0 \times 10^{-2}$ M), neocuproine ($1.5 \times 10^{-2}$ M in EtOH), alkaline solution as a mixture of 2.0% $Na_2CO_3$ + 0.1 M NaOH, and $NaKC_4H_4O_6$ (0.1 M).

The following solutions were used for the Lowry assay. Lowry A: alkaline solution as a mixture of 2.0% $Na_2CO_3$ + 0.1 M NaOH, Lowry B: 0.5% $CuSO_4{\cdot}5H_2O$ in 1.0% $NaKC_4H_4O_6$, Lowry C: 50 ml Lowry A + 1 ml Lowry B. Folin reagent was used in 1/3 dilution ratio. The exact composition of Folin reagent (a commercial product of Sigma) is not described in the literature.

Protein extraction, precipitation, and redissolution, was performed as recommended by Peterson [32]. The protein extraction buffer solution was composed of 5% (w/v) SDS, 15% (w/v) glycerol, 0.175 M Tris-buffer at pH 8.8, and 0.1 M dithioerythritol (DTE). The protein precipitation solution was 70% TCA. The precipitation-separated proteins were redissolved using a buffer composed of 1% SDS (w/v), 9 M urea, 0.025 M Tris-buffer at pH 6.8, and 0.7 M 2-mercaptoethanol. Since thiol compounds interfered with the developed method as well with established methods in the literature, 2-mercaptoethanol was excluded from the redissolution buffer when necessary. As an alternative to processing certain samples, the TCA-precipitated protein fraction was taken up with 1 M NaOH solution.

### 2.2. Instruments and apparatus

A Varian Cary 1E UV–vis spectrophotometer was used with a pair of matched quartz cuvettes for Cu(II)–neocuproine and Lowry absorbance measurements at the wavelengths of 450 and 750 nm, respectively. The incubations were conducted in a Clifton thermostatic water bath. A Mistral 2000 (MSE) centrifuge apparatus was used for the separation of proteins after TCA precipitation, and a Bransonic 221 ultrasonic bath was used for protein extractions. The Kjeldahl assays of complex samples were carried out with a Buchi Kjeldahl nitrogen system (containing a Buchi 430 digestion unit and Buchi 321 distillation unit).

### 2.3. Procedures

#### 2.3.1. Proposed and reference methods

Copper(II)–neocuproine spectrophotometric assay of protein (recommended procedure): To a test tube, add 1 ml of $CuCl_2{\cdot}2H_2O$, 1 ml of neocuproine, ($x$) ml sample, ($1.0 - x$) ml $H_2O$, 1 ml alkaline solution, and 1 ml of $NaKC_4H_4O_6$ such that the total volume is 5.0 ml. Incubate the stoppered tube in a thermostated water bath of temperature 40 °C for 30 min. Read the absorbance at 450 nm against a reagent blank containing all constituents but sample. Find the standard protein (BSA) or BSA-equivalent sample protein content by means of a calibration curve.

Lowry assay of protein: To a test tube, add ($x$) ml sample ($1.0 - x$) ml $H_2O$, 2.5 ml Lowry C solution and after exactly 10 min, add 0.25 ml diluted Folin reagent such that the total volume is 3.75 ml. Stopper the tube, mix, and measure the absorbance against a reagent blank after 30 min.

#### 2.3.2. Preparation of real samples for analysis

Milk: One mililiter milk sample was precipitated with 50% (w/v) $(NH_4)_2SO_4$. The protein fraction was isolated by centrifugation, redissolved in 5 ml buffer, and diluted to a final sample volume of 50 ml with water. This stock solution was rediluted 10-fold for analysis.

Egg white: The white part of an egg was weighed (35.263 g), and dissolved in 250 ml water. Protein assay was made on 200-fold diluted sample.

Meat and meat products (sausage, ham, sardine, minced meat of veal, mutton, and turkey): The sample was grinded using a mortar and pestle, and defatted by extraction with two successive 5 ml-portions of diethyl ether (the extracts not being retained). The dried meat product was sampled into 0.100–0.300 g specimens, and extracted with two successive 5 ml-aliquots of a protein extraction buffer (see Section 2.1) in an ultrasonic bath for 15 min. The decanted buffer extracts were combined, and the protein content was precipitated with 70% TCA solution. The precipitated proteins were separated by centrifugation, and redissolved with the suitable buffer (see Section 2.1) not containing 2-mercaptoethanol. Alternatively, the residue was taken up with 5 ml of 1 M NaOH, and diluted to 50 ml with distilled water. The final solution was appropriately diluted for protein assay.

Mutton with DTE used in redissolution buffer: A separate sample of mutton (approximately 0.200 g) that underwent the above preliminary operations (the ultrasonic bath extraction period was 40 min) was tested using the same buffer (i.e., the one containing 0.1 M dithioerythritol (DTE) as the protein reducing agent) in both extraction and redissolution of proteins in order to observe whether the interfering effect of DTE in Cu(II)–neocuproine assay can be compensated for. After protein precipitation and centrifugation, the protein fraction was redissolved with 5 ml of the protein extraction buffer composed of (5% SDS, 15% glycerol, 0.175 M Tris-buffer at pH 8.8, and 0.1 M DTE), and diluted to 50 ml with water. The 1/10 diluted (i.e., 5–50 ml) extraction buffer was taken as reagent blank. Both sample and reagent blank solutions were simultaneously subjected to Cu(II)–neocuproine asssay using distilled water in the reference cuvette, and the absorbance difference of sample and reagent blank at 450 nm was used to calculate the protein content.

Milk products, yoghurt, and cream cheese: The sample was defatted by extraction with two successive 5 ml-portions of diethyl ether (defatting was not applied for milk powder and yoghurt), dried, a 0.3–0.5 g specimen was taken. The sample was subjected to protein extraction, precipitation, centrifugation, and redissolution with 5 ml of 1 M NaOH, and dilution to 50 ml with distilled water, as stated above for meat products. The protein assay was performed on a suitably diluted solution.

Control serum: The control serum (catalog no. ODC0004 with lot no. 016) contained all the conventional constituents (e.g., glucose, creatinine, uric acid, Na- and K-chloride, Ca, P, alkaline phosphatase, bilirubin, triglyceride, cholesterol (HDL, LDL, VLDL), Fe, Mg, fructosamine, total protein, and albumin) in normal ranges. The serum content was dissolved in 25 ml $H_2O$, diluted at 1/50 ratio, and assayed using both the developed and Lowry methods.

## 3. Results and discussion

### 3.1. Temperature and time dependency

The proposed Cu(II)–neocuproine assay was tested at varying water bath temperatures for an incubation period of 30 min (Fig. 1). Although absorbances slowly increased above 40 °C, there was also the possibility of protein degradation. Then the incubation time at 40 °C was optimized for varying periods (Fig. 2), and the start of the plateau region (30 min) was adapted for further tests. The current method does not require high temperatures such as that of the erythrosin method [18], where the proteins may decompose at the prespecified temperature of the assay (90–95 °C). After heating in the recommended protocol, the colour due to Cu(I)–neocuproine complex remains stable for at least 1 h.

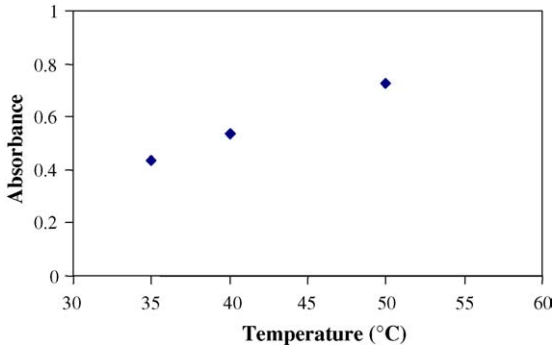


Fig. 1. The change of absorbance with temperature in the Cu(II)–Nc assay (BSA concentration: 20 mg $l^{-1}$, incubation period: 30 min).

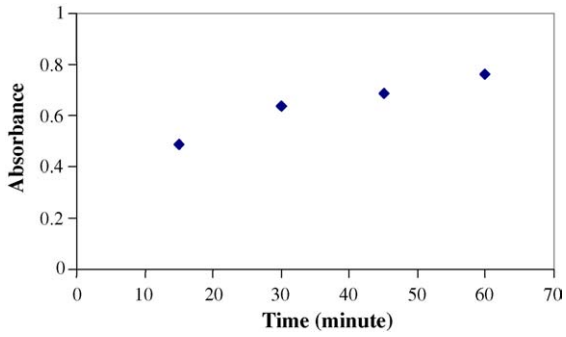


Fig. 2. The change of absorbance with incubation period in the Cu(II)–Nc assay (BSA concentration: 20 mg $l^{-1}$, water bath temperature: 40 °C).

Among the various chromogenic ligands capable of complexing with Cu(I) so as to promote the biuret reaction, i.e., protein reduction of Cu(II), the substituted phenanthrolines that can selectively stabilize the lower oxidation state of copper need to be preferred. Bicinchoninic acid, bathocuproine and neocuproine are useful ligands in this regard. Since neocuproine shifts the Cu(II)/Cu(I) standard potential of 0.153 V to a much higher value of 0.603 V, the lower oxidation state of copper is selectively stabilized with this ligand [49], facilitating the oxidation of certain amino acid residues on protein in alkaline medium. As in the BCA assay, proteins first react with Cu(II) to produce Cu(I), and then the formed Cu(I) reacts with bicinchoninic acid to give a purple complex, the BCA method is faced with kinetic problems which may be partially solved with borate buffer acceleration [55]. Compared to the inner-sphere electron transfer mechanism of biuret and possibly BCA assays, the coordinatively saturated copper(II)–neocuproine chelate is an outer-sphere electron transfer agent, and consequently the current method is faster. The copper(II)–neocuproine assay is accomplished at 40 °C bath temperature for 1/2 h, compared to the recommended 60 °C temperature incubation of the BCA assay [56]. When sample digestion, protein precipitation (if separation from ammonium salts and other amines is required), and distillation steps of Kjeldahl assay are considered as a whole, the proposed assay is not lengthier than Kjeldahl.

### 3.2. Analytical performance

From the analytical performance data (compared to those of the Lowry assay) depicted in Table 1, it is apparent that the developed method is highly sensitive and precise, has a low detection limit and a wide linear range. The LOD and LOQ were about 1 and 3 mg $l^{-1}$, respectively, as BSA equivalent. The slope of the calibration line, expressed as reciprocal concentration of

Table 1
Comparison of figures of merit of the proposed (Cu(II)–Nc) and reference (Lowry) methods tested on BSA as standard

| Parameter | Cu(II)–Nc | Lowry |
|---|---|---|
| Linear range (mg $l^{-1}$) | 8–100 | 11–100 |
| Limit of detection (mg $l^{-1}$)[a] | 1 | 3 |
| Limit of quantitation (mg $l^{-1}$)[b] | 3 | 10 |
| Calibration equation[c] | | |
| *m* (slope) | 0.023 | 0.0098 |
| *n* (intercept) | 0.044 | 0.093 |
| Correlation coefficient ($r^2$) | 0.9995 | 0.9993 |
| Within-run precision[d], R.S.D. (%) | 0.73 | 1.16 |
| Between-run precision[d], R.S.D. (%) | 1.01 | 4.78 |

[a] LOD = 3 s $m^{-1}$.
[b] LOQ = 10 s $m^{-1}$.
[c] Linear equation between absorbance and concentration (mg $l^{-1}$); Abs = *m* (concentration) + *n* for casein, Abs = 0.018 (concentration) + 0.072 ($r$ = 0.9999); for tubulin, Abs = 0.029 (concentration) + 0.041 ($r$ = 0.9995). All the tested proteins showed linearity of absorbance versus concentration approximately over an order of magnitude.
[d] For 20 mg $l^{-1}$ BSA, $n$ = 5 measurements.

BSA per unit cell thickness (1 mg $^{-1}$ cm $^{-1}$), was 0.023, corresponding to a molar absorptivity of $1.56 \times 10^6$ l mol$^{-1}$ cm$^{-1}$ for bovine serum albumin. This is much higher than those of both Lowry and BCA assays. Naturally as the sensitivity increases, an analyst will gain a better chance to remove interferences by dilution of sample. The linear working range of the BCA assay is rather narrow (over a 5-fold concentration range, between 200 and 1000 μg BSA ml$^{-1}$) and the sensitivity is low (0.00077 l mg$^{-1}$ cm$^{-1}$) [48], while the linear range of the developed method is wider (approximately over an order of magnitude concentration range, between 8 and 100 mg $l^{-1}$ BSA) and the sensitivity is much higher, and consequently the limit of protein quantitation is much lower. As is apparent from Table 1, all the tested proteins, i.e., BSA, casein, and tubulin, showed close slopes, excellent linearity, and a wide linear range. Although the exact concentration of LDH could not be estimated, it also exhibited a wide linear range.

The current method is advantageous over dye-binding methods in that the absorbance due to formed Cu(I)–neocuproine is directly proportional to protein concentration, whereas in the dye-binding methods based on insoluble protein-dye adduct formation [15,16], the absorbance of the centrifugate is inversely proportional to protein concentration. Moreover, the dye may slowly precipitate during reagent storage. The bromophenol blue dye-binding assay has a relatively narrow linear range (10–80 μg of protein) not passing through the origin [22]. Unpublished correlation results of Jewett et al. using the Lowry assay between native bovine superoxide dismutase standard concentrations and absorbances (at 660 nm) could only be linearized with the aid of a double-reciprocal plot, pointing out to the inherent nonlinearity of the Lowry assay [57].

### 3.3. Interferences

A list of the effects of most frequently encountered interferents in protein assays together with their tolerance limits in the proposed method is given in Table 2. As seen from this table, tris, ethanolamine, deoxycholate, CsCl, citrate, and triton X-100 are tolerated at 100-fold concentrations in the analysis of 10 mg $l^{-1}$ BSA, while the tolerance limits for other common intereferents, e.g., $(NH_4)_2SO_4$ and acetylsalicylic acid (50-fold), SDS (25-fold), and glycerol (20-fold), were at acceptable levels. Twenty-fold levels of free amino acids, i.e., glutamic acid, phenylalanine, aspartic acid, methionine, leucine, isoleucine, arginine, valine, alanine, threonine, glycine, serine, and lysine did not interfere. Among the few metal ions tested, 5-fold Fe(III), Zn(II), and Ni(II) did not interfere, higher concentrations interfering by hydrolytic precipitation. However iron(II), being a reducing agent, interfered. The current Cu(II)–neocuproine assay is free from most interferences influencing other assays. On the other hand, the CB (Bradford) assay does not work well with non-ionic detergents [21]. Phenol, Na-SDS, and triton X-100 interferes with the CB (Bradford) assay [22]. The high anionic detergent (SDS) tolerance of the developed method is also an advantage for the elimination of phospholipid interference by SDS addition, as recommended in the BCA assay using a similar reagent [47].

Table 2
The tolerance limits of some common interferents in protein assays to the proposed Cu(II)–Nc method in the analysis of 10 mg $l^{-1}$ BSA (the concentration yielding an absorbance change up to 5% of the expected value is taken as the tolerance limit)

| Interferent | Tolerance limit (mg $l^{-1}$) |
|---|---|
| EDTA | Interferes |
| Tris | 1000 |
| Ethanolamine | 150 |
| Triethanolamine | Interferes |
| Dithioerythritol (DTE) | Interferes |
| 2-Mercaptoethanol | Interferes |
| Glutathione (SH-containing peptide) | Interferes |
| Cystine, cysteine, tyrosine, tryptophan | Interfere |
| Deoxycholate | 1000 |
| Starch | 1000 |
| CsCl | 1000 |
| Citrate | 1000 |
| Cetyltrimethyl ammonium bromide (CTAB) | Causes turbidity |
| Cetylpyridinium bromide (CPB) | Causes turbidity |
| Sodium dodecyl sulfate (SDS) | 250 |
| Triton X-100 | 1000 |
| Glycerol | 200 |
| Free amino acids: glutamic acid, phenylalanine, aspartic acid, methionine, leucine, isoleucine, arginine, valine, alanine, threonine, glycine, serine, lysine | 200 |
| Acetyl salicylic acid (Aspirin) | 500 |
| Glucose[a], fructose[a] | 100 |
| Fe(III), Zn(II), Ni(II) | 50 |
| Fe(II) | Interferes |
| $(NH_4)_2SO_4$ | 500 |

[a] The tolerable concentration of glucose yielding minimal absorbance change at pH 10, using 0.1 ml of the alkaline solution in the 'recommended procedure'.

The developed method is not adversely affected from most common cations and anions (unless they actively participate in redox reactions, such as dithionite, sulfide, thiosulfate, thiocyanate, iodide, etc. which may reduce Cu(II)–neocuproine

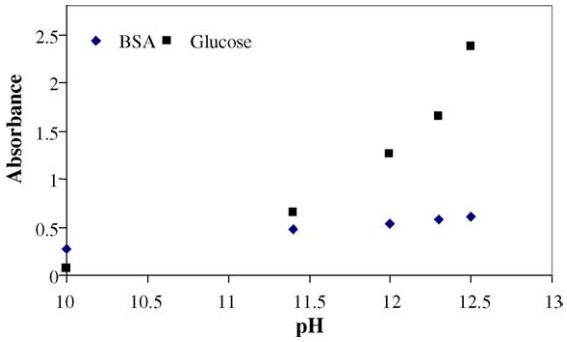


Fig. 3. The absorbance change of BSA (20 mg $l^{-1}$) and glucose (100 mg $l^{-1}$) as a function of pH.

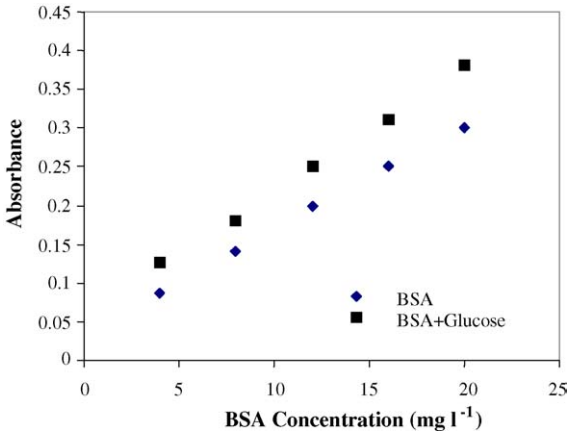


Fig. 4. The calibration line of BSA alone and in the presence of 100 mg $l^{-1}$ glucose at the optimal pH of 10.

to the coloured Cu(I)–neocuproine chelate), ammonium salts, common glassware detergents (non-ionic and anionic surfactants), buffer constituents, glycerol, starch, and most common solvents such as acetone, acetonitrile, DMF, DMSO, ethanol, and methanol. Calcium(II) even at mM range is a serious interferent in the Lowry assay, the errors of which may only be reduced with oxalate spiking of protein samples [58], which is a drawback with respect to the developed method. In comparison to the Lowry assay, copper-based assays such as BCA are generally more interference free [40]. Such chemicals as CsCl, citrate, diethanolamine, thiol compounds (cysteine, DTT, mercaptoethanol), EDTA, deoxycholate, salicylate, Tris-buffer and triton X-100 were reported to interfere with the modified Lowry assay [59]. Another advantage of the coordinatively-saturated Cu(II) in the bis-(neocuproine)copper(II) chelate is that this reagent does not complex with ammonium-containing buffers and primary amines normally interfering with the biuret assay [39] using the sole Cu(II) ion (which is naturally open to the complexing effect of N-donor Lewis bases). Since the high redox potential of neocuproine-chelated Cu(II)–Cu(I) couple is due to selective stabilization of Cu(I) in a tetrahedral environment, Cu(II) chelators decreasing the redox potential, such as EDTA and triethanolamine, interfered. Protein assays have been reported to undergo serious interferences from the presence of drugs, such as aspirin interference to biuret and Lowry assays [60]. The non-interference of aspirin to the copper(II)–neocuproine assay is an advantage over biuret and Lowry methods.

Experiments were designed to observe whether the interferences due to glucose and thiol compounds in protein assay by the proposed method may be compensated for by any means. The influence of glucose as a common interferent to most protein assays was attempted to be eliminated by decreasing the pH of the medium such that glucose oxidation with Cu(II)–Nc was not extensive to produce the coloured Cu(I)–Nc chelate. Fig. 3 shows that glucose has minimum absorbance at pH 10 where the sensitivity of BSA determination is slightly reduced. The calibration line of BSA alone and in the presence of 100 mg $l^{-1}$ glucose at the optimal pH of 10 is shown in Fig. 4. The parallelism of these calibration lines indicate that the absorbances due to BSA and glucose are additive at this pH, and glucose interference can be compensated for by adding glucose at the same level to the reagent blank. Table 2 shows that up to 10-fold glucose may be tolerated at this pH (i.e., achieved by adding 0.1 ml of the alkaline carbonate-hydroxide solution to the colour development medium instead of the normally applied 1.0 ml mentioned in the 'recommended procedure').

Since biologically important thiols acting as reducing agent for proteins adversely affect almost all spectrophotometric assays, it was first established that the presence of 0.7 M mercaptoethanol in the protein extraction buffer ruled out the possibility of protein assay with the developed method. Then this buffer was diluted as appropriate to yield a final mercaptoethanol solution of 4.4 mg $l^{-1}$, and the calibration lines of BSA alone and in the fixed mercaptoethanol solution were observed to be parallel (Fig. 5), indicating the additivity of absorbances and enabling the compensation of the absorbance due to mercaptoethanol by adding a similar amount of thiol compound to the reagent blank in protein assays. Additivity of absorbances in a complex mixture means that the solution constituents do not chemically interact among each other so as to cause a quenching or intensification of the expected absorbance, thereby making the above compensation feasible. Separation of protein from biochemically important reducing thiols such as dithiothreitol (DTT), glutathione (GSH), and 2-mercaptoethanol used as a component of protein extraction buffers was accomplished with trichloroacetic acid (TCA) precipitation, as recommended for interference removal in the

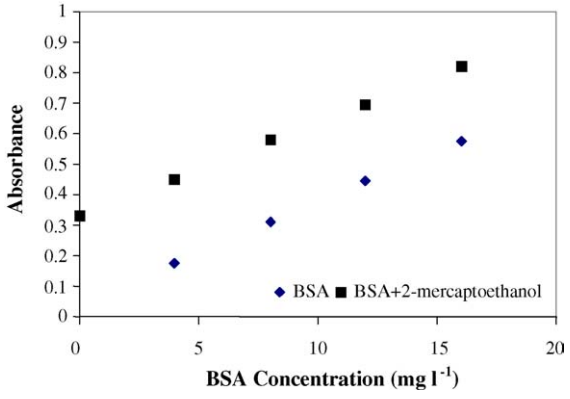


Fig. 5. The calibration line of BSA alone and in the presence of 4.4 mg $l^{-1}$ 2-mercaptoethanol using the recommended method.

BCA assay [61]. Since DTT and 2-mercaptoethanol exist in protein extraction buffer formulations, it may also be important to eliminate their interference by other means. For example, in the BCA assay, Wiechelman et al. have attempted without success to compensate for the adverse effect of such thiols by using a reagent blank containing an identical concentration of the interfering thiol compound [42]. As these authors point out, the observed colour was probably not the sum of the contributions of individual colour-forming constituents of the mixture [42], whereas in the developed method, the additivity of absorbances due to protein and thiol (2-mercaptoethanol), though at a low concentration of the latter, was shown to be valid. Thus an identical low amount of thiol placed in the reagent blank solution should – in principle – compensate for the interfering effect of thiol in protein assay by the current method. An additional benefit of the TCA precipitation–separation of protein is that it also accomplishes the task of removal of other soluble reductant compounds such as ascorbic acid and uric acid, which are known interferents of the BCA assay [55].

### 3.4. Analysis of standard protein samples

Protein extraction, precipitation, and redissolution procedures, as described in the experimental section, were performed on various meat, milk, and egg products, and the findings (as BSA equivalent percentage) of the proposed, AOAC reference (Kjeldahl), and Lowry methods are listed in Table 3. Protein findings with Cu(II)–Nc and Lowry assays did not necessarily lead to the declared amounts by the producers (though milk, cheese, egg white, and sausage products yielded close analytical findings to those claimed by producers), as the protein dissolution buffer did not contain the thiol component at the prespecified high level. The important point here was that the developed and Lowry assays yielded similar results for the real sample protein extracts that correlated appreciably ($r = 0.98$). The method was validated by Kjeldahl analyses of the tested samples; the data sets of complex samples assayed by Cu(II)–Nc and Lowry correlated to the findings of Kjeldahl yielded correlation coefficients $r = 0.96$ and 0.97, respectively, with slopes being close to 1. The protein findings of the proposed and reference methods for the samples of ham (100% veal), sardine, turkey, milk powder, yoghurt, and egg white revealed that the means of the two populations were not essentially different (null hypothesis accepted using *t*-tests at 95% significance level) [62], but these results could not be extended to other samples. Mutton extract analysis in the presence of DTE in redissolution buffer (see Section 2) by compensating for DTE in the reagent blank yielded the protein findings of 8 and 5% using the proposed and reference methods, respectively. The protein content of the control serum was found as 5.54 and 5.45 g $dl^{-1}$ using the proposed and Lowry methods, respectively. It should be noted here that total protein findings of different methods generally do not agree much in literature. Total protein contents of real samples (of saliva) found with the aid of different methods have been reported to vary significantly [63]. Correlation of protein findings with HPLC and BCA method, or with HPLC and 210 nm-UV absorbance method were rather low, with $r = 0.75$ [64]. The Lowry results for water-soluble nitrogenous compounds in cheddar cheese were not identical with those of Kjeldahl, but instead, these two result sets could be correlated with a linear correlation coefficient of 0.97 [6], which was considered quite high. Recent studies have shown that a traditional nitrogen-to-protein conversion factor of 6.25 may not be suitable for different types of protein samples [65]. As for spectrophotometric protein assays carried out with chromogenic redox reagents, each reagent having a different redox potential and coordination chemistry aiming at different amino acid residues and peptide backbone of the protein molecule is expected to show different extents of oxidation, rendering assay results not identical but comparable. This behaviour is also seen among total antioxidant capacity assays of food materials carried out with different reagents [53]. Thus the correlation achieved in this work between Cu(II)–Nc and either Lowry or Kjeldahl assays can be considered to be satisfactory.

Table 3
Protein determination (as BSA equivalent, %) in the protein extracts of various products

| Food product | Cu(II)–Nc[a,b] | Lowry[a,b] | Kjeldahl[a,c] |
|---|---|---|---|
| Sausage (100% turkey) | 11.0 ± 0.9 | 11.4 ± 0.6 | 14.7 ± 0.5 |
| Sausage(100% veal) | 17.0 ± 1.7 | 13.6 ± 1.3 | 14.1 ± 0.4 |
| Ham (100% veal) | 13.0 ± 1.0 | 13.6 ± 1.7 | 14.1 ± 0.1 |
| Turkey (minced) | 20.1 ± 0.6[d] | 24.0 ± 0.9[d] | 22.6 ± 0.8 |
| Veal(minced) | 15.5 ± 0.5[d] | 18.4 ± 0.4[d] | 21.0 ± 0.5 |
| Mutton (minced) extract | 7.1 ± 1.0 | 6.8 ± 1.1 | – |
| Sardine (canned) | 22.1 ± 1.0[d] | 23.2 ± 1.0[d] | 22.4 ± 0.5 |
| Milk (g/100 ml) | 2.0 ± 0.3[d] | 2.0 ± 0.4[d] | 2.9 ± 0.1 |
| Milk powder (g/100 g) | 1.9 ± 0.4[d] | 2.1 ± 0.5[d] | 2.2[e] |
| Yoghurt (g/100 g) | 3.3 ± 0.2[d] | 3.0 ± 0.2[d] | 3.0[e] |
| Cream (g/100 g) | 2.0 ± 0.1[d] | 1.9 ± 0.1[d] | 2.3[e] |
| Cheese (cream) | 15.0 ± 1.6[d] | 18.0 ± 1.2[d] | 13.3 ± 0.3 |
| Egg white | 12.1 ± 0.4 | 12.7 ± 0.5 | 11.8 ± 0.3 |

The correlations of the data set (Cu: copper–neocuproine method, Lw: Lowry method, Kj: Kjeldahl method) were as follows: 1.025 Cu + 0.50 = Kj ($r = 0.96$); 0.93 Lw + 0.90 = Kj ($r = 0.97$); 1.09 Cu − 0.38 = Lw ($r = 0.98$).

[a] $x = \bar{x} \pm \frac{t_{0.95}s}{\sqrt{N}}$.

[b] Sample size, $N = 5$ or 7.

[c] Sample size, $N = 3$.

[d] TCA-precipitated protein fraction taken up with 5 ml of 1 M NaOH.

[e] Declared amount.

### 3.5. Postulated mechanism of copper(II)–neocuproine assay

There is considerable information in the literature describing how proteins react with copper(II). Copper catalyzed oxidation of proteins is described as a site-specific process restricted to specific amino acid residues (i.e., His, Arg, Lys, Pro, Met, and Cys) on the protein molecule [66], and the reduced copper(I) bound to protein may participate in Fenton-like reactions [67]. Site-specific damage of copper(II) to DNA as strand breakage was inhibited by neocuproine acting as a Cu(I) chelator. The extensive DNA base damaging reactions of copper-mediated Fenton reactions have been reported [68]. The in situ formed Cu(I) may be subsequently re-oxidized in a Fenton-like reaction with hydrogen peroxide or oxygen leading to reactive oxygen

species [69]. Similarly, malondialdehyde (MDA), a product of lipid peroxidation, formed a synergistic couple with Cu(II) producing damage to human fibroblasts, and a part of the generated Cu(I) could be reoxidized during redox cycling of Cu(II)/Cu(I) in the course of Cu(II)–MDA reaction [70]. On the other hand, Cu(I) entrapped in the Cu(I)–neocuproine complex could not be lost by reoxidation, as Almeida et al. showed that Cu(I) complexed with neocuproine would not be reactive toward oxygen or hydrogen peroxide [71]. Moreover, Cu(I) formation as a result of Cu(II)–MDA reaction was much higher in the presence of bathocuproine sulfonate acting as Cu(I) scavenger in the incubation medium than in its absence [70], pointing out to the favourable shift in the redox potential of Cu(II)/Cu(I). Combining these facts for the current protein assay, in order not to lose any Cu(I) in reoxidation, to shift the redox potential favourably, and to reduce the undesired side-reactions, Cu(II) as a protein oxidant should be used along with neocuproine such that the bis-(neocuproine)copper(II) chelate would oxidize the relevant amino acid moieities and the peptide bond of protein with a neat reaction.

By a similar reasoning associated with the mechanism of the BCA assay, the structurally similar Cu(II)–neocuproine reagent may be assumed to attack similar sites of protein [42]: the macromolecular structure of the protein, the peptide bond, and the presence of four amino acid moieities (cysteine, cystine, tryptophan, and tyrosine) may be held responsible for colour formation in the developed method. The slow increase in colour intensity with increasing temperature of incubation is most probably a result of incomplete oxidation of tryptophan, tyrosine, and the peptide bond at lower temperatures, as revealed by electrochemical studies in the BCA assay [42].

## 4. Conclusions

The copper(II)–neocuproine method of total protein assay eliminates the need for precisely-timed reagent additions and vortexing inherent in the widely used Lowry method, providing a flexibility of application. The developed method utilizing a stable reagent and a chromophore, is simple, rapid, reproducible, sensitive and is not influenced by common glassware detergents, solvents, and ammonium-containing buffers and is flexible enough to change the analysis protocol when required. The method is more sensitive than the Cu(II)–BCA assay depending on a similar principle. The linear working range is wider, and the detection/quantitation limit is lower than those of most protein assays. The method can be rendered selective to proteins by precipitation/separation from other soluble reductants in solution. If the interferents are at a low concentration with respect to the analyte (protein), high sensitivity enables dilution removal of interferents as an alternative to separation of protein fraction with TCA precipitation. The developed method was applied to a variety of protein-containing food products, and the results correlated well with those found by the Kjeldahl and Lowry assays. Since the developed assay is based on the oxidative colour formation of Cu(II)–neocuproine with oxidizable amino acid residues of protein, this method is in a way an extension of the CUPRAC (cupric reducing antioxidant capacity) assay of antioxidant compounds in foods [53] and human plasma [72], with the exception of adapting a strongly alkaline medium for facilitating protein oxidation.

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