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Spectrophotometric determination of ascorbic acid using copper(II)–neocuproine reagent in beverages and pharmaceuticals

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Abstract

The proposed method for ascorbic acid (Vitamin C) (AA) determination is based on the oxidation of AA to dehydroascorbic acid with a Cu(II)-2,9-dimethyl-1,10-phenanthroline (neocuproine (Nc)) reagent in ammonium acetate-containing medium at pH 7, where the absorbance of the formed bis(Nc)-copper(I) chelate is measured at 450 nm. This chelate was formed immediately and the apparent molar absorptivity for AA was found to be 1.60×10^4 dm^3 mol^{-1} cm^{-1}. Beer's law was obeyed between 8.0×10^{-6} and 8.0×10^{-5} M concentration range. The relative standard deviation for 90 μg AA was 3%. The Cu(II)-Nc reagent is a milder and therefore more selective oxidant than the conventional Fe(III)-1,10-phenanthroline (phen) reagent used for the same assay. This feature makes the proposed method superior for real samples such as fruit juices containing weak reductants such as citrate, oxalate and tartarate that otherwise produce positive errors in the Fe(III)-phen method when equilibrium is achieved. The developed method was applied to a number of commercial fruit juices, pharmaceutical preparations containing Vitamin C, and red wine. The meta-bisulfite content of wine was removed with an anion exchanger at pH 3 prior to analysis, and a difference extractive–spectrophotometric method of AA assay in wine was developed so as to suppress the interferences caused by wine anthocyanins and polyphenols. The findings of the developed method for fruit juices and pharmaceuticals were also statistically compared with those of HPLC so as to establish it as a reliable novel method.
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Keywords: Ascorbic acid; Spectrophotometry; Copper(II)-neocuproine reagent; Fruit juice; Pharmaceuticals; Red wine

1. Introduction

Ascorbic acid (AA), commonly known as Vitamin C is one of the most important water-soluble vitamins in the human diet, because it helps the body in forming connective tissue, bone, teeth, blood vessel walls, and assists the body in assimilating iron and amino acids [1]. A diet deficient in Vitamin C may cause a person to develop scurvy. Vitamin C lowers the incidence of and mortality from two of the most prevalent diseases, cardiovascular disease and cancer [2]. Plants and some animals make their own Vitamin C, but humans do not. For this reason, humans need to seek it from other sources. Vitamin C is present naturally in a wide range of foods, particularly fruits and vegetables. But AA has limited stability and may be lost from foods during storage, preparation and cooking. In some foods, it is purposely added to attract consumers and to act as an antioxidant (e.g., to wines [3]) to prolong the shelf-life of the commercial product. Pharmaceutical preparations have AA to add a supplementary source of Vitamin C in human diets [1].

In view of the great importance and wide use of AA, numerous analytical techniques have been proposed for its determination in different matrices and at different levels. These include titrimetry [4], voltammetry [5–7], potentiometry [8,9], fluorometry [10–12], flow injection (FI) analysis [13–16], spectrophotometry [17–22] and chromatography [23–28]. Especially chromatographic methods are very efficient in AA assay of complex materials such as fruit juices, vegetables and beverages. Also, a few atomic absorption

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spectrometric methods [29,30] are available. These methods for the determination of Vitamin C have been reviewed in two articles (as photometric [31] and non-spectrophotometric [32] methods) by Arya et al., and in an article of flow injection methods [33] by Yebra-Biurrun. A distribution chart of FI determination of AA according to the detection technique reveals that the highest proportion (39.4%) of the determinations are spectrophotometric [33]. Among these methods, direct spectrophotometry in UV region requires the elimination of matrix effects, since many compounds present in samples also absorb in UV region. These elimination efforts usually make the method more complicated and laborious by requiring secondary measurements or pretreatment (e.g., ion-exchange) procedures [34,35]. On the other hand, spectrophotometry-based FI methods of AA assay in the visible region involve redox reactions in which a colored compound is formed or decomposed as a result of a redox reaction. Spectrophotometric methods are particularly attractive because of their speed and simplicity. Most spectrophotometric methods are based on the reducing effect of AA. In many of them, the reduction of Fe(III) to Fe(II) with AA was accompanied by the complexation of reduced Fe(II) with different reagents such as 1,10-phenanthroline [17], 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (Br-PADAP) [18], ferrozine [19] and 4-(2-pyridylazo)resorcinol (PAR) [20]. Another reducable ion, Cu(II), was also used for the determination of AA, followed by the complexation of reduced Cu(I) with different reagents, such as 5-(4-hydroxy-3-methoxybenzilidene) rhodanine (HMBR) [21] and 2,9-dimethyl-1,10-phenanthroline (neocuproine (Nc)) [22].

The proposed method is based on the oxidation of AA to dehydroascorbic acid with the Cu(II)-Nc reagent [36] that was previously used by the authors to determine the biochemically important reductants such as cysteine [37] and Vitamin E [38] in ammonium acetate containing medium at pH 7, where the absorbance of the formed bis(2,9-dimethyl-1,10-phenanthroline)-copper(I) chelate is measured at 450 nm. The original Fe(III)-phenanthroline method reported in 1987 claims the non-interference at 10-fold concentrations of reducing agents such as nicotinic acid, urea, thiourea, sugars, aspartic acid, tartaric acid and citric acid; this is presumably due to the very short time (1–2 min.) needed for analysis [17]. Phenolic antioxidants such as flavonoids in fruit juices may protect the Vitamin C content from oxidative degradation [39], and consequently, oxidation of AA may not be complete within 1–2 min. However, mild reducing agents that would not be expected to pose a problem in the Cu(II)-Nc method will inevitably produce the colored Fe(II)-phenanthroline chelate if sufficient time is allowed for the redox reaction to reach equilibrium, and would inevitably, give rise to interference in the latter method.

Complex mixtures such as wine pose two distinct analytical problems in AA assay: anthocyanin pigments cause high blank signals at the analytical wavelength (450 nm), and polyphenols which may amount to 1720 ± 546 mg l^{-1} in red wines [40] may also react with the Cu(II)-Nc reagent. The former problem may be solved with extractive photometry, as applied in the spectrophotometric determination of iodide by solvent extraction as bis(neocuproine)-copper(I) triiodide ion pair [41], and the latter with oxidative degradation of ascorbic acid [42] in a complex mixture of polyphenols by controlled air oxidation so as to develop a difference spectrophotometric method for AA assay. Another requirement is the removal SO_2 from wine [43] so as not to interfere with the subsequent color development, and this may be accomplished by anion exchange separation of HSO_3^- from diluted wine at a pH higher than the first acidity constant (pK_{a1}) of sulfurous acid. Thus, a difference extractive–spectrophotometric method for AA assay using the Cu(II)-Nc reagent was developed so as to overcome the interference effects due to anthocyanins, polyphenols and SO_2 contents of complex mixtures such as wine.

2. Experimental

2.1. Materials and instrumentation

All chemicals were of analytical reagent grade. The fruit juice commercial samples were purchased from the Turkish market, Istanbul. The red wine sample was supplied from Doluca Bağcılık&Şarapçılık A.Ş. (Turkey). Supradyn (Roche) and Sedergine with Vitamin C (UPSA) as pharmaceuticals were purchased from pharmacies in Istanbul.

Two distinct AA solutions at an equimolar concentration of 5.0×10^{-4} M were prepared, one in de-aerated distilled water by bubbling N_2, and the other in 80 ml glacial acetic acid + 30 g metaphosphoric acid + water mixture in a final solution volume of 1000 ml [10]. 3.0×10^{-3} M Nc solution in ethanol, 1.0×10^{-2} M Cu(II) aqueous solution, and 1 M ammonium acetate aqueous solution were used for the determination of AA with Cu(II)-Nc reagent. For wine analysis, 5% sodium benzoate buffer (prepared by dissolving benzoic acid in 0.1 M NaOH) brought to a final pH of 7.0 was used instead of ammonium acetate.

1,10-Phenanthroline-Fe(III) colour reagent was prepared by mixing 0.198 g of 1,10-phenanthroline monohydrate, 2 ml of 1 M hydrochloric acid and 0.16 g of ferric ammonium sulphate, and finally diluting with water to 100 ml [17].

The absorbances were measured with a Varian Cary 1-E UV–Vis spectrophotometer using quartz cuvettes of 1 cm thickness.

The chromatograph was from Cecil Instruments (Cambridge, UK), and consisted of a pump (CE 1100 HPLC pump), a 20 μl injection valve (Model 7125, Rheodyne, Cotati, CA, USA), an analytical stainless-steel column packed with ThermoQuest Hyperbond C18 (300 mm × 3.9 mm, 10 μm) from Hichrom (Berkshire, UK) and a variable-wavelength UV detector (CE 1220). The mobile phase for HPLC analysis was a phosphate–acetate buffer prepared as a modified version of Lykkesfeldt [28]: A mixture of 7.8 g $NaH_2PO_4 \cdot 2H_2O$ + 6.8 g CH_3COONa was dissolved in 490 ml bidistilled water;

10 ml methanol was added and the final pH was adjusted to 5.4 with the addition of H_3PO_4. The flow rate of the mobile phase was 0.8 ml min^{-1}. Detection wavelength was chosen as 240 nm. Chromatograms were evaluated using an integrator (HP 3395 from Hewlett-Packard). Injections were made with a 25 μl syringe from Hamilton (Reno, NV, USA).

2.2. Recommended procedure for AA determination

One millilitre of Cu(II) solution was placed in a test tube and then 2.5 ml of Nc, 1 ml of ammonium acetate, $(3 - x)$ ml of water and x ml AA solutions were added by mixing in this order. After 2 min, the absorbance at 450 nm was recorded against a reagent blank.

For AA aqueous solutions prepared in the mixture of glacial acetic acid–metaphosphoric acid, it is necessary to add 0.5 ml of 3 M NaOH to the final solution mentioned above so as to bring the final pH to 7.

In the case of wine analysis, AA was assayed by difference spectrophotometry after anion exchange as described in Section 2.6.

2.3. 1,10-Phenanthroline-iron(III) procedure for AA determination

The procedure of Besada was applied [17]. An aliquot of sample solution (containing 20–120 μg of AA) was transfered to a 25 ml standard flask, then added 1 ml of the 1,10-phenanthroline-iron(III) (iron(III)-phen) reagent, mixed and let stand for ca. 1 min. The mixture solution was diluted to the mark with water, and the absorbance of the solution at 510 nm was recorded against a reagent blank. In the case of wine analysis, AA was assayed by difference spectrophotometry after anion exchange as described in Section 2.6.

2.4. Interference studies

The interference effects of 1000-fold (as mole/mole) concomitant species commonly found in fruit juices to the determination of 5.0×10^{-4} mmol AA in de-aerated aqueous solution using both spectrophotometric methods were studied.

2.5. AA determination in fruit juices and pharmaceuticals

Lemon, apple and grape juices and lemon powder purchased from the market and two pharmaceuticals with Vitamin C were analyzed spectrophotometrically using both Cu(II)-Nc and Fe(III)-phen reagents and HPLC method by only proper dilution and filtration after dissolution for powder sample and pharmaceuticals, without any pretreatment processes.

2.6. Recommended procedure for AA assay in wine

The filtered red wine sample was adjusted to pH 3.0 with the addition of a few drops of 1 M HCl, passed twice through a Dowex 1 × 8 Cl$^-$ anion exchanger column (BDH) placed in a burette (Ø = 1 cm) on a glass wool plug at a resin height of 10 cm. After the total removal of sodium meta-bisulfite (added as preservative antioxidant) from the wine, it was diluted at a ratio of 1:100 with water to produce the 'wine sample solution'.

In a test tube were placed 1 ml of Cu(II), 1 ml of Nc (7.5×10^{-3} M in EtOH), 1 ml sodium benzoate buffer (at pH 7.0), x ml of wine sample solution (x usually taken as 0.4 ml), y ml standard AA solution, and finally $(2 - x - y)$ ml H_2O (y was taken between 0.1 and 0.4 ml with 0.1 ml increments in the method of standard additions). The aqueous phase (of 6 ml total volume) was extracted with 3 ml chlorobenzene, organic phase separated, and its absorbance at 450 nm measured. A linear calibration curve for the standard addition method was drawn between absorbance and concentration.

In order to selectively oxidize the AA content of the wine sample solution, it was brought to pH 8 with the addition of NaOH, and O_2 was bubbled through this solution for 1.5 h. From this final solution − of which the AA content was oxidized − another 0.4 ml aliquot was taken and re-subjected to the method of standard additions as described above. The linear calibration curves for the two wine sample solutions (i.e., with original ascorbic acid content retained and oxidized) were let to intersect the horizontal (concentration) axis, and the difference in concentration between the two intersection points was recorded as the AA content of the wine sample solution.

3. Results and discussion

The Cu(I)-Nc chelate, the spectrum of which is given in Fig. 1 (reference: distilled water), was formed immediately from the reduction of Cu(II)-Nc with AA, and was stable for at least 2 h. The spectrum of the Cu(I)-chelate using the

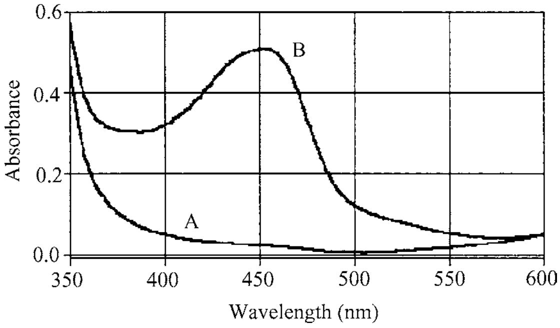

Fig. 1. Spectra of (A): Cu(II)-Nc and B: Cu(I)-Nc complexes. Spectrum (B) was formed with the oxidation of 4.0×10^{-5} M AA with the Cu(II)-Nc reagent (reference: distilled water).

Cu(II)-chelate-containing reagent blank in the reference cell gave an absorbance maximum at 450 nm.

The linear equation for the calibration graph drawn at the wavelength of 450 nm as described in the section 2.2 was

$$A_{450} = 1.60 \times 10^4 C - 0.0596 \quad (r = 0.999)$$

where C is the molar concentration of AA. The LOQ ($10s_{bl}$/slope of linear equation) was 2.9 μM.

The magnitude of the molar absorptivity for AA is typical for 2-electron reductants in the Cu(II)-Nc method [36–38] and ascorbic acid is most probably oxidized to dehydroascorbic acid. Beer's law was obeyed between 8.0×10^{-6} and 8.0×10^{-5} M concentration range, roughly covering a decade of concentration rendering the method useful for routine determinations.

The relative standard deviation in the determination of 90 μg AA was 3%.

The proposed method was compared with some existing spectrophotometric methods in Table 1 in terms of some relevant figures of merit (not containing possible interferences).

The Cu(II)-neocuproine procedure gave similar results for both AA solutions, one in de-aerated distilled water and the other in metaphosphoric acid–glacial acetic acid–water mixture which may provide the most efficient AA extraction from food samples [10,26]. On the other hand, the Fe(III)-phen procedure could not applied to AA determination in an equilibrated mixture of metaphosphoric acid–glacial acetic acid–water because of the interference due to HPO_3. Metaphosphoric acid preferentially stabilized the higher oxidation state of iron (i.e., Fe(III)) by forming stable complexes and thereby reducing the oxidizing power of the Fe(III)-phen reagent.

For both methods, the interference effects of 1000-fold (as mole/mole) concomitant species commonly found in fruit juices and pharmaceuticals on the determination of 5.0×10^{-4} mmol AA in de-aerated aqueous solution are shown in Table 2.

In the presence of 1000-fold (by mole) citrate, oxalate and tartarate ions, 17.6 μg AA can be accurately analyzed with Cu(II)-Nc reagent. However, these ions interfered with the determination of AA in equilibrated solution using the Fe(III)-phen reagent. In this case, the absorbances recorded in the latter method were not constant but increased very rapidly with time. This interference has probably resulted from the fact that the E^0_{red} for [Fe(III,II)-(phen)$_3$] (1.06 V in 1.0 M H_2SO_4 solution) was higher than the E^0_{red} for [Cu(II,I)-(Nc)$_2$] (0.603 V), the former oxidizing weak reductants when given sufficient time. In addition, many methods using 1,10-phenanthroline need either background correction by Cu(II)-catalyzed oxidation or the addition of NH_4F as the inhibitor of the light reduction of Fe(III)-phen complex [20]. Citric acid, widely found in plants and therefore in fruit juices [44] and other natural foods and beverages, is such an important interferent in the Fe(III)-based spectrophotometric assays, i.e., Fe(III)-phen method, that with visible or UV light, Fe(III) is photochemically reduced by citric acid to Fe(II), which reacts with *o*-phenanthroline to form the reddish orange $Fe(phen)_3{}^{2+}$ complex [45]. Thus, when sensitivity and selectivity criteria are evaluated simultaneously for the developed method, the Cu(II)-Nc method is less sensitive but more selective than the ferric-based methods. A further advantage over the ferric-based colorimetric procedures is that the developed method utilizes a neutral pH (i.e., pH 7.0) instead of the acidic pH of ferric procedures where most oxyanions can be easily oxidized.

The determination of AA was carried out in liquid (grape and lemon juices) and solid (lemon powder) fruit juices and pharmaceuticals by both spectrophotometric methods and HPLC using de-aerated water as the solvent for dissolution and dilution, because metaphosphoric acid aqueous solutions recommended in the literature to extract AA from such matrices [10,26] interfered with the Fe(III)-phen method (See Table 3). For the Fe(III)-phen procedure, the absorbances measured (2 min after reagent addition) were used to calculate AA quantities in fruit juices because of the rise of absorbances with time due to oxalate, citrate and tartarate ions, i.e., Fe(III)-phen is capable of oxidizing these substances. In order to observe possible interferences, the 20 min measurements were also made, but not reported in Table 3. Chromatographic results for AA were evaluated with the following linear calibration equation—peak area = $4.3 \times 10^{10}\, C_{AA} - 1.1 \times 10^5$ (C_{AA} = molar concentration of AA).

The problems associated with AA assay in wine were mainly the necessity for the removal of sodium meta-bisulfite

Table 1
Comparison with some existing spectrophotometric methods

Reagent	Working wavelength (nm)	Apparent molar absorptivity (ε, $dm^3\ mol^{-1}\ cm^{-1}$)	Linearity range	LOD ($ng\ ml^{-1}$)	Ref.
Fe(III)-phen	510	2.2×10^4	20–120 μg	–	[17]
Fe(III)-5-Br-PADAP	560	1.31×10^5	0.049−1.20 $\mu g\ ml^{-1}$	15	[18]
Fe(III)-5-Br-PADAP	748	5.69×10^4	0.145−2.40 $\mu g\ ml^{-1}$	44	[18]
Fe(III)-ferrozine	562	2.8×10^4	0.25−0.4 $\mu g\ ml^{-1}$	–	[19]
Fe(III)-PAR	710	1.7×10^4	up to 5.5 $\mu g\ ml^{-1}$	–	[20]
Cu(II)-HMBR	473	2.6×10^4	6.25×10^{-6}–3.75×10^{-5} M	–	[21]
Cu(II)-Nc	450	1.5×10^4[a]	2-20 μg	–	[22]
Cu(II)-Nc	450	1.6×10^4	8.0×10^{-6}–8.0×10^{-5} M	–	this work

[a] Though this value was not reported in the original reference, it was calculated from Fig. 2 of the same source by dividing the absorbance at 450 nm to molar concentration (l = 1 cm).

Table 2
Interferences of various species with the determination of 17.6 μg AA

Interferent	Interferent/AA mole ratio	Proposed method (Cu(II)-neocuproine)	Reference method (Fe(III)-phen)
Oxalate	1000	−	+
Tartarate	1000	−	+
Citrate	1000	−	+
Glucose	1000	−	−
Tannic acid	10	+	+
Glutamic acid	10	+	+
Nitrite	10	−	−
Riboflavin	10	+	+
Pyrogallol	10	+	+
Acetyl salycilic acid	10	−	−
Uric Acid	10	+	+

−: Indicates the absence of interference; +: indicates the presence of interference.

deliberately added to wine as antioxidant preservative, and the interference due to wine anthocyanins and polyphenols, the latter normally causing positive interference in all spectrophotometric assays used. Original wine was passed through the anion exchanger column twice at pH 3 (1.2 pH units higher than the first acidity constant, pK_{a1}, of H_2SO_3) so as to completely retain the HSO_3^- species, which would otherwise interfere with the final color development. Since the ascorbic acid content of wine is in molecular form at this pH, AA was not retained. The high blank values which would be expected in the presence of wine anthocyanins were eliminated by extracting the ion pair of Cu(I)-Nc (formed as a result of AA oxidation with Cu(II)-Nc) and benzoate, i.e., an ion pair formed from a large univalent cation and a large univalent anion with negligible water of hydration, into chlorobenzene in which the effective molar absorptivity of AA did not decrease. In this case, a pH 7 benzoate buffer was used instead of ammonium acetate so as to provide the large anion necessary for extraction. Finally, the problem due to wine polyphenols (which would normally react with the Cu(II)-Nc reagent) was solved by developing a difference extractive–photometric method for AA. The method of standard AA additions was applied to the diluted wine sample, and once more after the controlled O_2-oxidation of the original AA content at pH 8. The optimal time of AA oxidation in the presence of wine polyphenols was 1.5 h. If AA oxidation with O_2 was carried out in the presence of a catalyst such as Cu(II), it was discovered that some polyphenols were also oxidized along with AA. Again, when the pH of the oxidation medium was raised extensively, some polyphenols did oxidize even in the absence of a catalyst. Thus O_2-oxidation of AA was optimized at pH 8 for a period of 1.5 h without the use of a catalyst. When the absorbances (at 450 nm) of the chlorobenzene extracts were recorded against the number of mmoles of AA added, the equations of the linear calibration curves for the original diluted sample (i.e., retaining its AA content) and the O_2-oxidized sample (i.e., that has lost its AA content) were as follows, respectively:

$$A_{450} = 0.141 + 2.63 \times 10^3\,\text{mmol AA added}\quad\text{(before oxidation)}$$

$$A'_{450} = 0.132 + 2.71 \times 10^3\,\text{mmol AA added}\quad\text{(after oxidation)}$$

Table 3
AA quantities of different samples found by the developed and reference methods (the percentage recoveries of spiked AA samples from some of the selected matrices are shown in parantheses)

Sample[a]	Cu(II)-Nc method	Fe(III)-phen method	HPLC method
Red wine	215 ± 5[b]	232 ± 6	–[c]
Apple juice	25.6 ± 0.4 (104%)	15.7 ± 0.5 (90%)	21.5 ± 0.3 (102%)
Grape juice	6.2 ± 0.2 (104%)	6.0 ± 0.3[d] (104%)	6.5 ± 0.3 (93%)
Lemon juice	20.5 ± 0.3 (98%)	29.4 ± 0.5[d] (92%)	20.7 ± 0.3 (99%)
Lemon powder	62.7 ± 0.3	71.8 ± 0.4	64.7 ± 0.3
Supradyn	39.5 ± 0.2	38.3 ± 0.3	40.2 ± 0.3
Sedergine[e]	187.4 ± 0.3	185.6 ± 0.3	191.2 ± 0.3

[a] The AA concentrations of liquid juice samples are given in the units of mg/100 ml, of lemon powder in mg/100 g, and of pharmaceuticals in mg/tablet.

[b] Red wine sample with dilution was analyzed by extractive–photometric version of the Cu(II)-Nc method with standard additions. Ascorbic acid was decomposed by controlled air oxidation at pH 8 in the presence of wine polyphenols, and the difference in absorbance was measured.

[c] Diluted samples suitable for Cu(II)-Nc and Fe(III)-phen assay methods did not fall within the measurable range of HPLC, and therefore could not be analyzed with this technique.

[d] Grape juice and lemon juice yielded much higher values of AA with the Fe(III)-phen method after 20 min of reagent addition, indicating the possible oxidation of other species.

[e] Aspirinated Vitamin C.

The difference in the abscissa (concentration axis) intercepts of these lines was about 4.89×10^{-6} mmol AA standard, yielding an effective AA content around 215 ppm in the original red wine. The reasoning of difference spectrophotometry was applied to both the developed and reference methods, of which the findings are reported in Table 3.

F-test [46] was applied to compare the results for lemon juice obtaining the developed method and reference methods. The calculated *F* values for Cu(II)-Nc–Fe(III)-phen and Cu(II)-Nc – HPLC methods were 1.2 and 2.8, respectively. Since these figures were less than the tabulated *F* value (9.28) at 95% confidence level for three of degrees of freedom of each applied method, it was concluded that there is no significant difference in the precision of these methods. Likewise, since the standard deviation of the developed method was not seriously different from that of the reference methods, the Student's *t*-test carried out using a pooled value of standard deviation (i.e., the developed method was compared with HPLC on different number of measurements; N_1 and N_2 where degrees of freedom: $df = N_1 + N_2 - 2$, yielding the means reported in Table 3) showed that, with the exception of apple juice, the mean AA contents of all real matrices were not significantly different from each other at 95% confidence level. For red wine analysis, the developed method gave comparable means with the Fe(III)-phen procedure. Thus, a validation of the developed method was achieved on different complex matrices reported in Table 3.

Although redox-active colorimetric reagents generally have limited selectivity – depending on their formal standard potential and reaction kinetics – toward biologically important substances, the relative selectivity of the Cu(II)-Nc reagent as a chromogenic oxidizing agent [36] was verified by its lack of attack on citric acid and reducing sugars. Two authors of this work previously used this reagent for the individual assay of Vitamin E (α-tocopherol) [38] and for the simultaneous assay of cystine and cysteine [37], but it should be born in mind that the oil-soluble Vitamin E – if present along with AA – could be separated from the latter with prior solvent extraction using diethyl ether [38], and cystine would not react with the reagent unless subjected to reductive acidic hydrolysis [37]. Certainly, when all biologically important reducing agents in a close standard potential range are present in the same solution of a real matrix and no extra measure is taken (such as controlled air oxidation of AA combined with difference spectrophotometry), then the developed method would measure total antioxidant capacity rather than individual AA assay of the concerned mixture. On the other hand, 5-(4-hydroxy-3-methoxybenzilidene) rhodanine with Cu(II), i.e., a reagent previously used by two of the authors as a chromogenic oxidizing agent for the same purpose [21], is quite inferior to Cu(II)-Nc in AA assay because of the commercial inavailability of the former and its limited aqueous solubility (actually the former reagent is soluble in 3:2, v/v, dioxane–water).

4. Conclusion

So far, spectrophotometric methods based on the reduction of Fe(III) to Fe(II) with AA, followed by the complexation of Fe(II) with different reagents, especially 1,10-phenanthroline (phen) [17], have found extensive use in AA determination. The Cu(II)-Nc reagent is a milder and therefore more selective oxidant than the Fe(III)-phen reagent. This feature makes the proposed method superior for real samples such as fruit juices containing weak reductants such as citrate, oxalate and tartarate [44] that otherwise produce positive errors in the Fe(III)-phen method when equilibrium is achieved. Due to the non-interference of acetyl salicylic acid, Vitamin C-added aspirin formulations may be safely analyzed with the proposed method. Due to the interference of metaphosphate, the Fe(III)-phen method can not be applied to the determination of AA in samples prepared in the metaphosphoric acid–glacial acetic acid–water mixture solution which was cited in relevant literature to dissolve and stabilize the samples containing AA [10,26]. In the proposed method, the coloured Cu(I)-Nc chelate is an immediate product of the 2-electron reduction of the reagent with AA, while the existing literature method of AA assay [22] based on the reduction of Cu(II) to Cu(I) with AA followed by Nc chelation of the formed Cu(I) requires a much longer time of 1 h for full colour development. The linearity of the absorbance/concentration curve over a wide range is also an indication of the completeness of the redox reaction concerned. Another contribution of this work is to modify the developed procedure to AA assay in a very complex matrix such as wine, where the meta-bisulfite content is removed with an anion exchanger prior to analysis, and the interferences due to wine anthocyanins and polyphenols eliminated by the use of difference spectrophotometry with solvent extraction. Thus, a reasonably rapid and low-cost spectrophotometric method for AA assay in complex matrices was developed to meet the needs of conventional analysis laboratories that are not equipped to highly sophisticated levels. The developed method is also applicable to FI analyses with the exception of red wine samples.

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