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Cupric Ferricyanide Reaction in Solution for Determination of Reducing Properties of Plant Antioxidants

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Abstract To develop an antioxidant assay, the oxidation of phenolics and ascorbic acid by cupric ferricyanide in solution was estimated. Solutions (pH 6.6) were kept clear with citrate and gelatin. Reaction kinetics of the color formation at 480 nm indicated further slow increase after initial fast reaction with a sample. With the active phenolics (tannic acid, quercetin, rutin, chlorogenic acid), sufficiently stable endpoint was reached at 35 °C by 90 min. Calibration showed deviation of 5–10 % from the linearity. Antioxidant mixtures of tannic, chlorogenic, and ascorbic acids indicated small positive interaction between tannic and chlorogenic acids. Extracts from forest trees and green tea responded in the same way as individual phenolics to the oxidation. Common plant osmolytes in excess did not interfere with the analysis of tannic acid for its reducing activity. Due to a poor endpoint with ascorbic acid, a method for stabilizing the endpoint was looked further. Borate, molybdate, and lower pH greatly inhibited the reaction with ascorbic acid during the slow phase. By the presence of copper ions, it was possible that the autoxidation of ascorbic acid produced hydrogen peroxide during the slow phase. However, hydrogen peroxide gave substantial color response. Solvent *N*-methylpyrrolidone at low level inhibited or decreased the color formation for several analytes, suggesting that an analyte-dependent activation of oxygen could cause decolorization of cupric ferrocyanide. Current findings indicated that the cupric ferricyanide antioxidant reaction can be potentially developed to an assay for estimating reducing activity in extracts from woody plants or green tea.

Keywords Antioxidant assay · Ascorbic acid · Colloid · Copper · Phenolics

Introduction

Antioxidant substances are important in forest trees and plant-derived food (Riedl and Hagerman 2001; Sakihama et al. 2002; Lavola et al. 2003; Boyer and Liu 2004). Phenolics tend to accumulate during stress in woody plants. Beneficial health effects of plant and food polyphenols are ascribed to their ease of oxidizability (low reduction-oxidation potential) and quick scavenging of free radicals. In this way, polyphenols protect vital cellular components from the free-radical generated oxidative stress.

Poplar and tea leaves accumulate phenolic compounds by 15 % or more of the leaf biomass (Donaldson et al. 2006; Kerio et al. 2013). Among various polyphenols, hydrolyzable tannins are abundant in the biomass of tree leaves (Salminen et al. 2004). Quercetin and rutin accumulate in small concentrations, but they have high antioxidant activity and are poorly soluble in water.

Several methods have been developed to measure different aspects in the antioxidant activity of samples. The protection from oxidative stress is quantified by radical scavenging, peroxide inhibition, hydrogen transfer, electron transfer, total reducing capacity, etc. (Pisoschi and Negulescu 2012). By using the ligand effects in the reaction (Itabashi et al. 1990), antioxidant tests for reducing capacity have been developed (Apak et al. 2007; Campos et al. 2009; Özyürek et al. 2011). The reduction-oxidation potential of the parent oxidant increases due to the stabilization of the reduced reagent in a complex.

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Ferric ferricyanide method uses the oxidation of samples in the presence of ferricyanide and ferric ions (Berker et al. 2007). In copper reduction methods, where the oxidant is a cupric-chelate, the reduction-oxidation potential is determined by the strong and stable complex between reduced copper ion and ligand (Apak et al. 2007; Campos et al. 2009; Özyürek et al. 2011).

As antioxidant mechanisms of individual compounds and mixtures vary, their responses are specified by using concurrently assays with different chemistry. In comparison with the ferric ferricyanide reducing capacity assay (Berker et al. 2007), the current reaction by formation of copper ferrocyanide has lower reduction-oxidation potential. Electrochemical oxidation shows that antioxidants with lower potentials are better in decreasing the peroxidation of lipids, while antioxidants with higher potentials tend to have prooxidant activities (Simić et al. 2007). To understand the specific response of antioxidant in a particular assay, the development of a test for reducing activity was motivated by the possible importance of easily oxidizable antioxidants and the small number of reducing capacity tests available for the low potential antioxidants.

One advantage of copper ferrocyanide is its stability at physiological pH values, whereas the colored product in the ferric ferricyanide assay is persistent only at acid pH values (Garjonyte and Malinauskas 1999). Since traces of copper as impurities from the particular chemicals in the reaction environment affect the oxidation of reducing compounds with ferricyanide (Bridgart et al. 1973; Bridgart and Wilson 1973), inclusion of a definite amount of copper may improve the repeatability of the results.

Ferricyanide is a one-electron oxidant, and the oxidation of AH_2 (polyphenol and ascorbic acid) proceeds by a free-radical intermediate:

$$AH_2 + K_3\left[Fe(CN)_6\right] + K^+ \rightarrow HA^{\cdot} + K_4\left[Fe(CN)_6\right] + H^+$$
$$HA^{\cdot} + K_3\left[Fe(CN)_6\right] + K^+ \rightarrow A + K_4\left[Fe(CN)_6\right] + H^+$$
$$Cu^{2+} + K_4\left[Fe(CN)_6\right] \rightarrow K_2C\ u\ \left[Fe(CN)_6\right] + 2\ K^+$$

Summary reaction for the two electron-oxidation is given by the following equation:

$$AH_2 + 2K_3\left[Fe(CN)_6\right] + 2Cu^{2+} \rightarrow A + 2K_2Cu\left[Fe(CN)_6\right] + 2H^+ + 2K^+$$

Copper could also oxidize the reducing compound but still gives cupric ferrocyanide. Cubic coordination polymer is formed (de Tacconi et al. 2003). The final structure of copper hexacyanoferrate is not clear, and the ratio of Cu to Fe in the product may range from 1:1 to 2:1 (Koyama et al. 1988; Düssel et al. 1996; de Tacconi et al. 2003; Ishizaki et al. 2011). Base cations with smaller hydrodynamic radii move within the polymer in the zeolitic structure for the charge balance (Düssel et al. 1996; de Tacconi et al. 2003). The cupric ferrocyanide particles may exist as a negatively charged colloid of a clear reddish color (maximum absorbance at about 480 nm), which is caused by the charge transfer between valences of the transition metals in the metal ferrocyanide.

Even before the reduction-oxidation in the reagent solution, cupric ions and ferricyanide give an insoluble salt or coordination compound. Therefore, the reagent solution may turn turbid, which influences the absorbance measurement. Previous studies have reported that clear reagent solution of cupric ions and ferricyanide is obtained by using a soluble copper complex (Kerpel-Fronius and Hajós 1968). In addition to keeping the solution clear by ligand, relative stabilities of the copper complex and copper ferrocyanide influence the reduction-oxidation potential of the assay. The coordination of Cu^{2+} should be of a medium strength, which corresponds to the association constant (log k) of around 10. The copper complex should not substantially compete for the copper ions with the color forming copper ferrocyanide.

The base of an assay reaction and the interactions between reagents have been largely established. Further development of a method would consist of the optimization of reaction conditions and specifying the responses of antioxidant samples to the possible assay. The aim of this study was to optimize the reagents and their concentrations in the oxidation of phenolics, ascorbic acid, and plant extracts by cupric ferricyanide in solution.

Materials and Methods

Chemicals

Acetone, 4-aminoantipyrine, ascorbic acid, betaine, citric acid, ferulic acid, fructose, gelatin (Type B, Bloom strength of 225 g), mannitol, phloroglucinol, poly(ethylene glycol) 6000, polyvinylpyrrolidone, potassium ferricyanide, potassium pyrophosphate, L-proline, quercetin, rutin, sodium thiosulfate, sorbitol, and 2-thiobarbituric acid were purchased from Sigma-Aldrich (Germany). Chlorogenic acid, choline chloride, dimethyl sulfoxide, histidine, quinic acid, succinic acid, and tetraglyme (tetraethylene glycol dimethyl ether) were obtained from Fluka (Germany). *N*-Methyl-2-pyrrolidone and tannic acid (deca-galloyl glucose) were purchased from Riedel-de Haën (Germany), poly(ethylene glycol) 400 and surfactant Triton X-100 from BDH (UK), boric acid, glucose, glycerol, KH_2PO_4, $MnSO_4$, NaH_2PO_4, oxalic acid, sodium molybdate, and sucrose from Reachim (Estonia), copper sulfate and tartaric acid from Lach-Ner (Czech Republic), potassium hydroxide and sodium hydroxide from Lachema (Czech Republic), hydrogen peroxide (3 % with a stabilizer) and paracetamol from pharmacy, L-arginine and L-glycine from SF Protein (Finland), and DL-malic acid was a gift from

Algol Chemicals (Estonia). Paracetamol was purified from tablets by extraction and recrystallization with acetone; otherwise, chemicals were used as received.

Plant Material

Material from trees was collected near Tallinn in summer 2005–2009. Green tea and lemon was obtained from a local supermarket. Raw leaves and needles from trees, willow bark and lemon peel, were initially heated in a microwave oven at 800 W for 15–60 s. Thereafter, the material was completely dried in a drying oven at 60 °C. All plant material was ground to a fine powder in steel jars with a ball mill MM200 (Retsch, Germany).

Instruments

Absorbancies of solutions were read in cuvettes of a solvent resistant plastic Plastibrand (Brand, Germany) with a UV-Vis spectrophotometer Heliosα (Unicam, UK). Reaction mixtures were warmed in a small oven UNB 100 (Memmert, Germany). Stock solutions and plant extracts were dissolved and homogenized with a vortex mixer SA3 (Bibby Stuart Scientific, UK). The pH of solutions was measured with a gel electrode PH-100 ATC (Voltcraft, Germany) attached to a pH-meter MP220 (Mettler-Toledo, UK).

Stock Solutions

Stock solution of 61 mmol L^{-1} ferricyanide was made by dissolving 1.2 g $K_3[Fe(CN)_6]$ in 60 mL of water. The ferricyanide solution was kept in a dark bottle in a refrigerator. Phosphate buffer with equal concentrations of Na and K was made by dropping the solution of KOH + NaOH (6+6 mol L^{-1}) to KH_2PO_4+NaH_2PO_4 at the approximate concentration of the buffer. A 125 mmol L^{-1} solution of copper ions was made by dissolving 312 mg $CuSO_4 \cdot 5H_2O$ in 10 mL water in a glass tube. For cupric ions, the ligand (citric acid, oxalic acid, or pyrophosphate) was dissolved at 125 or 250 mmol L^{-1} concentration in 10-mL volume, and the pH was adjusted approximately to the assay pH of 6–7.

Gelatin gel (2 %) was prepared by adding 10 mL water to 0.2 g gelatin at room temperature, mixing immediately and waiting for about 30 min until the gelatin granules were swollen. The swollen gelatin was dissolved by heating shortly to about 60 °C and mixing for about 10 min. The 2 % gelatin was stored as a gel in a refrigerator. Before using the gelatin solution, the tube with the gel was heated in hot water to obtain a fluid solution. The gelatin solution was kept warm at the assay temperature of 35–45 °C until the pipetting of the gelatin to a reaction mixture.

Tannic acid (2 mmol L^{-1}), quercetin (5 mmol L^{-1}), rutin (5 mmol L^{-1}), chlorogenic acid (10 mmol L^{-1}), phloroglucinol (30 mmol L^{-1}), ferulic acid (30 mmol L^{-1}), and ascorbic acid (40 mmol L^{-1}) all in 60 % ethanol, and $MnSO_4$ (20 mmol L^{-1}) in water was used for further dilution. Suspensions of quercetin and rutin were dissolved in a tube by heating with hot water. The stock solutions of analytes were kept in a refrigerator until diluted with 60 % ethanol before use.

Plant Extracts

Plant material was extracted with 3 mL of 60 % ethanol at 60 °C for 30 min. Water-ethanol solvent at 60 % ethanol concentration was chosen because of a sufficient extraction of phenolic compounds in this solvent (Sripad et al. 1982; Turkmen et al. 2007). The water-ethanol solvent ensures low solubility of polysaccharides (Defloor et al. 1998). Polysaccharides interact with gelatin (Schmitt et al. 1998) and may interfere with the colloid stabilization by the gelatin in the assay. To obtain clear solutions from plant material, extracts were further kept in a refrigerator overnight and finally centrifuged with a bench centrifuge Universal 16A (Hettich, Germany) at 1900*g* for 10 min.

Assay

To find suitable reagent concentrations, at first, 50 μL of copper sulfate solution was pipetted to the cuvette. Copper sulfate was followed by water in a volume which was precalculated to give the total volume of 1500 μL. Then, 50–150 μL of a ligand solution at about neutral pH was added. Further, 100–200 μL of buffer, 100 μL of gelatin, 200 μL of potassium ferricyanide, and 200 μL of sample solution were sequentially added. Finally, the solution in cuvette was mixed with a polystyrene spatula. The absorbance was monitored at 480 nm at intervals of 30–60 min. Between readings, cuvettes were kept covered in the dark at the reaction temperature, which was usually 35 °C. Temperatures above 50 °C were not applied because of the accelerated gelatin hydrolysis by heating (Van den Bosch and Gielens 2003).

Suitable concentrations of citrate, copper, and ferricyanide were determined from preliminary tests. Citrate concentration of two to three times of copper ions was sufficient for relatively small increases in blank readings and for the color formation in a reaction with tannic acid at pH around 6.5. Copper concentration of 4 mmol L^{-1} was chosen to ensure 3- to 5-fold excess for the color formation at the largest concentration of tannic acid of 0.027 mmol L^{-1}, giving final absorbance of about 1.6 absorbance units (AU) in the assay mixture. With a leeway, it was assumed that 30 electrons were transferred to an oxidant from the tannic acid molecule, which has ten gallic acid residues in its composition. For each of the electron, one to one-and-a-half Cu^{2+} and one ferricyanide were assumed in the color forming cupric ferrocyanide compound (Koyama

et al. 1988; Düssel et al. 1996; de Tacconi et al. 2003). Ferricyanide above 8 mmol L^{-1} level did not substantially increase the color formation with tannic acid, while this ferricyanide level was appropriate for relatively small blanks of about 0.04 AU.

After finding more-or-less appropriate reaction conditions, a fixed time reaction procedure was used to test analytes, plant extracts and interfering substances. Before analysis, a separate solution was made from copper sulfate, water, ligand, buffer, and gelatin. The solution was gently heated, mixed, cooled to the assay temperature, and kept about 30 min. When the period was completed, the ferricyanide stock solution was added, and the reagent solution was mixed. Aliquots from the obtained mixture of copper ions, ligand, buffer, gelatin, and ferricyanide were pipetted to 1500 μL polypropylene vials each of which contained 200 μL of sample solution and optionally 200 μL of an interferent solution.

Statistical Analysis

With individual substances, linear relationship between absorbance and different concentrations was estimated by intercept, slope, and coefficient of determination (R^2), which were calculated with a spreadsheet program LibreOffice Calc version 4.2.7 (LibreOffice contributors). Effects of tannic acid, chlorogenic acid, and ascorbic acid at the same time in solution were tested with a multiple regression using statistical software R version 3.0.2 (The R Foundation for Statistical Computing). Interactive terms were added to the regression model to test for an interaction between the substances. Adjusted R^2 was used to estimate the improvement in the fit of model.

Results and Discussion

Reagent Concentrations

Keeping other variables unchanged, pH of the reagent was altered at constant concentration of buffer components. In the pH range from 6 to 7, the results of tannic acid oxidation varied only slightly (Fig. 1a). Above neutral pH, a reddish pigment was found on the cuvette wall. Absorbance was greatly reduced at the largest pH of 7.8. Near pH of six blanks increased slightly faster (across time the averages were 5–8 mAU h^{-1} as opposed to 2 mAU h^{-1} near pH of 6.6). The variation here appears to support that pH of about 6.5 was best suited for an cupric ferricyanide assay with tannic acid.

The association of copper ion with citrate is much pH-dependent (Piispanen and Lajunen 1995). The pH dependence of the copper complex formation could greatly affect the reaction rate. By calculation with MaxChelator (version WebMaxc Standard, maxchelator.stanford.edu) for the citrate complex at pH 6.6, the concentration of free copper ions was around 1 μmol L^{-1}. With the oxidation of tannic acid, the relatively small effect of pH on color formation at endpoint could be explained by the contrasting effects of pH on the sensitivity of phenolics to oxidation (Schweigert et al. 2001; Mersal 2009), on one hand, and on the association of cupric ions with citrate which may affect the reduction-oxidation potential of the reagent or an influence of free copper ions, on the other hand.

Increasing the pH above neutral was not suitable with these settings. In alkaline solutions above pH of about 8, copper ferrocyanide is unstable and does not form (Egorin and Avramenko 2012; Chen et al. 2012). The conditional association constant of copper with citrate may surpass that of the copper ferrocyanide. There is also risk of oxidation of citrate and gelatin at higher pH values (Mirsky and Anson 1936; Arribas et al. 2005). There are several pH-dependent reasons why the haze in solutions could be observed. Turbidity or precipitates may indicate cross-linking of gelatin molecules by oxidative coupling (Singh et al. 2002), by tannin hydrogen bonding (Calderon et al. 1968), or by coordination bridges (Shiba 2010).

By altering the ethanol concentration between 8 and 20 %, little effects were found on the absorbance (Fig. 1b). Small increasing tendency was observed by increased ethanol concentration. Also, an increase in blanks with time was larger at ethanol concentration of 14 and 20 %, where the absorbance change rate was 14 mAU h^{-1}, while at 8 and 11 % ethanol, the rate was 6 mAU h^{-1}. To keep the solvent concentration and blanks in minimum, 8 % ethanol concentration was selected for further testing.

The oxidation-reduction potential of ferricyanide is known to decrease substantially by the increase in the percentage of a solvent in the water mixture (Rao et al. 1978). The rate of cysteine oxidation by ferricyanide in a pH 3 buffer was decreased by increasing ethanol percentage (Abu-El-Halawa et al. 1996). Large concentration in the range over 40 % of the non-solvent causes desolvation, aggregation, and precipitation of gelatin in water (Mohanty and Bohidar 2003). Electrostatic interactions are increased, while hydrogen bonding interactions are decreased by increasing ethanol concentration in gelatin solution (Siebert et al. 1996; Mohanty and Bohidar 2003; Cook et al. 2007). Small concentration of ethanol less than about 30 % may stabilize protein solutions (Asakura et al. 1978). In a poor solvent for the protecting polymer, the increased stability of the surface-protected ferrocyanide nanoparticles (Uemura et al. 2004) suggests that an increase in the stability of the gelatin and copper ferrocyanide associate could enhance the color formation during the oxidation.

Gelatin concentration from about 1 mg mL^{-1} was sufficient for the colloid stabilization. The variation of gelatin up to 2.3 mg mL^{-1} had only small effect on the color formation (Fig. 1c). Gelatin levels below 1 mg mL^{-1} gave slight haze.

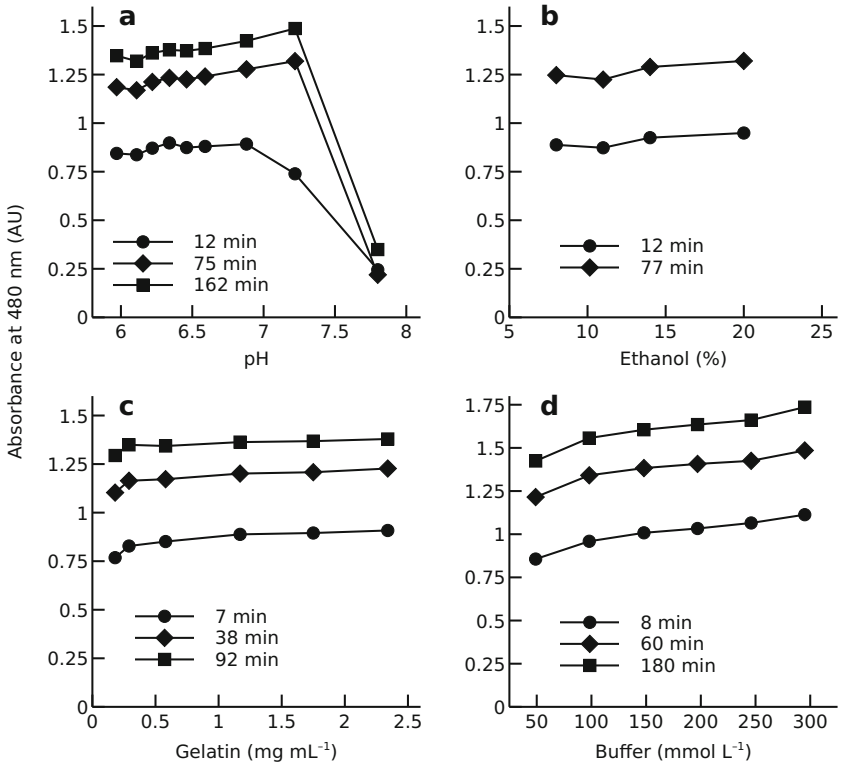

Fig. 1 Influence of variation in pH (**a**), concentrations of ethanol (**b**), gelatin (**c**), and buffer (**d**) on the absorbance during oxidation of tannic acid by cupric ferricyanide (at 35 °C after the first occasion) for the period of time indicated. Initial concentration in the mixture: $CuSO_4$=4 mmol L^{-1}, citrate= 12 mmol L^{-1}, phosphate buffer= 49–15 mmol L^{-1}, gelatin= 1.3 mg mL^{-1}, $K_3[Fe(CN)_6]$= 8 mmol L^{-1}, ethanol=8 %, tannic acid=27 μmol L^{-1}

Gelatin concentration of about 1.3 mg mL^{-1} was selected to obtain clear-colored solutions. Taking the average molar weight of 50,000, the molar concentration of gelatin was 26 μmol L^{-1}. The level was in agreement with the colloid stabilization and adsorbtion of gelatin on a metal ferrocyanide nanoparticles, which were prepared from a 10 mmol L^{-1} ferricyanide in solution, but in acid pH, below the isoelectric point of gelatin (Shiba 2010). In accordance with the present results, previous studies have demonstrated that above its isoelectric point, gelatin also protects the negatively charged colloid against the coagulating (Nemeth and Matijević 1968; Hu et al. 2002).

By testing several buffer concentrations at constant pH, the final absorbance was larger in a more concentrated buffer (Fig. 1d). Buffer concentrations of 150–200 mmol L^{-1} gave possibly slightly increased robustness in results.

Relatively large buffer concentrations are advantageous with acidic and alkaline samples, which may influence the pH in the reaction mixture. By altering buffer concentration, a change in potassium concentration and ionic strength is also accompanied. Larger potassium concentration increases the reduction-oxidation potential of ferricyanide (Krulic et al. 1998). There was probably no inhibition from sodium ions as copper hexacyanoferrate-based modified electrode has shown good electrocatalytic activity even in the sodium containing electrolytes (Baioni et al. 2008). The results of current study indicated that the concentration of phosphate buffer or potassium ions affected reaction rate or color formation during the cupric ferricyanide reaction with tannic acid.

Temporal Changes

Temporal changes in the absorbance showed that a plateau was not reached with any of the substances tested during 240 min (Fig. 2a, b). Tannic acid, quercetin, and rutin reacted most rapidly. Absorbance with quercetin and rutin was almost stable after 50 min. Phloroglucinol gave slowest response of the phenolics. Slight haze was observed with phloroglucinol after 90 min. Ascorbic acid and $MnSO_4$ did not stabilize. After 30 min of the reaction with $MnSO_4$, formation of haze increased the absorbance, but most of it was caused by the buildup of a transparent color.

Extracts from green tea and trees reacted relatively fast, resembling the color formation with phenolics (Fig. 2c, d). Extract from lemon peel was reacting slowly during the initial stage, the deceleration appeared delayed. These analyses with individual substances and plant material suggested a compromise in the reaction time around 100 min for an assay at these reaction conditions.

Compared with other antioxidant methods, the current rates of the completion of color formation were slow (Apak et al. 2007; Berker et al. 2007; Campos et al. 2009). Initial fast reaction during the first seconds and further slow kinetics is found with a copper chelate reduction assay for antioxidant testing in plasma and urine (Campos et al. 2009). Current findings suggest that the completion of the reaction with phenolic antioxidants and ascorbic acid was not substantially catalyzed by copper. Also, a relatively strong Cu-citrate complex

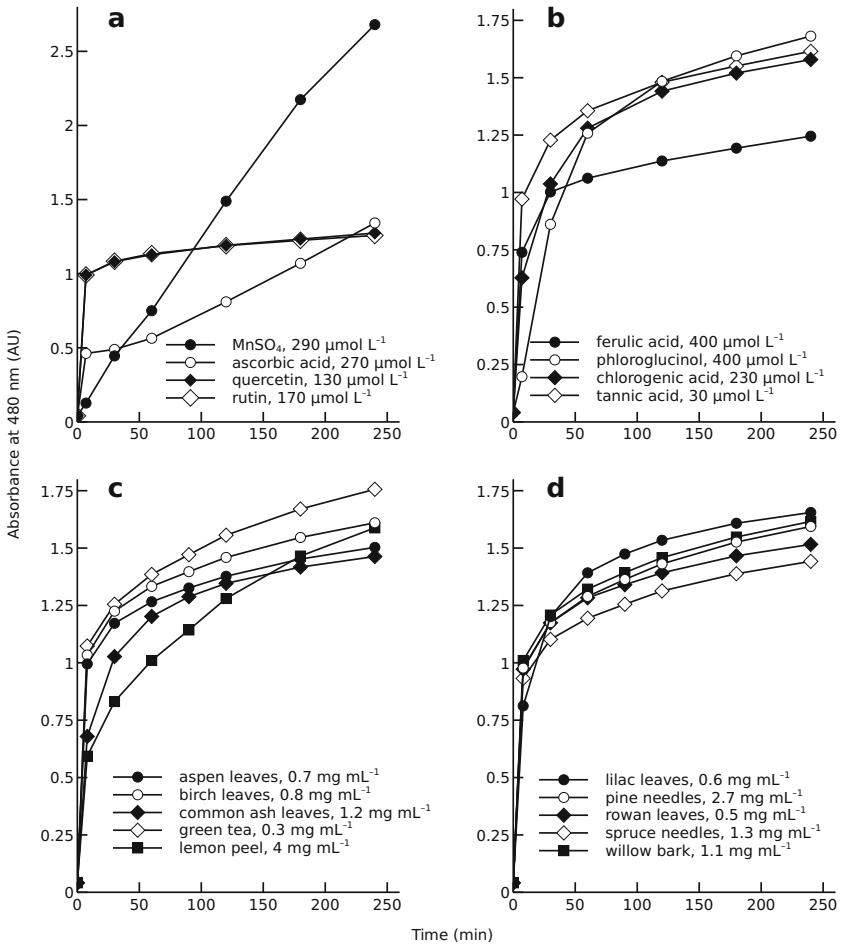

Fig. 2 Time curves of the absorbance in the oxidation by cupric ferricyanide of analytes (**a**, **b**) at 35 °C after 7 min and plant extracts (**c**, **d**) at 35 °C. Initial concentration of analyte or extract is indicated in the key. Initial concentration of reagents in the mixture: $CuSO_4$=4 mmol L^{-1}, citrate=12 mmol L^{-1}, phosphate buffer=150 mmol L^{-1}, gelatin= 1.3 mg mL^{-1}, $K_3[Fe(CN)_6]$= 8 mmol L^{-1}, ethanol=8 %, pH= 6.6

could be assumed; it is likely therefore that the ligand effect of cupric ferrocyanide did not much elevate the reduction-oxidation potential of this ferricyanide reagent.

In addition, binding of polyphenols to gelatin (Calderon et al. 1968) as another cause for the decrease in the rate of polyphenol oxidation should be considered. Arts et al. (2002) argue that a decrease in ABTS radical scavenging by tea polyphenols is larger in the presence of proteins with high capability to form hydrogen bonds with phenolic hydroxyls. For a longer period of time, however, the influence of gelatin is small on the antiradical activity of a condensed tannin (Riedl and Hagerman 2001). In a copper reduction assay at physiological pH, gelatin has little influence on oxidation of phenolics because of relatively weak association between the polyphenol and gelatin (Çekiç et al. 2009).

Calibration

A five-point regression indicated approximately linear dependence of the absorbance on the concentration of ascorbic acid, phenolics, and extracts from the plant material (Table 1). Intercepts from regression statistics were slightly larger than the blank readings but generally in a reasonable agreement. In the dependence on concentration, absorbance at particular level was often less the sum of absorbancies at the half smaller concentrations. For individual substances, the ratio of an absorbance to the sum of absorbancies was on average 0.97 (range from 0.87 to 1.11) and that for extracts was 0.96 (range from 0.86 to 1.06).

Reduced proportion of absorbance at larger concentrations could be obtained by a small amount of catalyst when the reaction rate is saturated by substrate (Lin and Wu 2005; Jia-Qing et al. 2006). The further slow increase after the assay time may support only small effects of catalysis on the calibration results. Linearity of a nanoparticle-based assay depends on the way how the particles are formed and on the size distribution of particles (Özyürek et al. 2012). Uemura et al. (2004) demonstrated clear effects of the protective polymer as well as the solvent on the optical properties and stability of the obtained dispersions of surface-protected nanoparticles. For the current assay, further development is required to evaluate the contribution of the size distribution of ferrocyanide nanoparticles to the calibration. Another possible explanation for the slight deviation from the linearity was that the surface of gelatin-protected particles was modified by the phenolic oxidation products.

Table 1 Linear regression parameters for the dependence of absorbance on sample concentration

Material	Maximum concentration (mmol L^{-1})	Blank (AU)	Maximum absorbance (AU)	Intercept	Slope	R^2
Ascorbic acid	0.27	0.034	0.699	0.035	2.50	0.9965
Ferulic acid	0.40	0.046	1.145	0.062	2.73	0.9992
Chlorogenic acid	0.23	0.035	1.455	0.060	6.13	0.9989
Rutin	0.17	0.034	1.193	0.047	6.78	0.9996
Tannic acid	0.027	0.035	1.442	0.045	46.51	0.9996
Aspen leaves[a]	0.40	0.040	1.563	0.062	3.74	0.9993
Birch leaves[a]	0.46	0.039	1.545	0.060	3.23	0.9992
Spruce needles[a,b]	0.76	0.037	1.557	0.039	1.99	0.9988
Pine needles[a,b]	1.61	0.039	1.576	0.052	0.94	0.9993
Willow bark[a,b]	0.65	0.050	1.569	0.056	2.31	0.9996

Sample dilutions were 0 (blank), 0.125, 0.25, 0.5, and 1 (maximum concentration). Initial concentrations of reagents in the mixture for the cupric ferricyanide oxidation at 35 °C for 90 min: $CuSO_4$=4 mmol L^{-1}, citrate= 12 mmol L^{-1}, phosphate buffer=150 mmol L^{-1}, gelatin=1.3 mg mL^{-1}, $K_3[Fe(CN)_6]$=8 mmol L^{-1}, ethanol= 8 %, pH=6.6

[a] Biomass in tannic acid units (mmol L^{-1})

[b] Slight haze at maximum concentration

Ratios of the slopes (Table 1) relative to rutin—0.37 for ascorbic acid, 0.4 for ferulic acid, and 0.9 for chlorogenic acid—were in reasonable agreement with these ratios of 0.38, 0.47, and 0.96, respectively, in previous cupric reducing antioxidant capacity assay (Apak et al. 2007). The quercetin to rutin ratio of about 1.3 according to Fig. 2a was smaller than the value in the range of 1.7 to 2.5 found with the established ferric or cupric reducing capacity methods (Berker et al. 2007). The ratio of quercetin to rutin in the present study better agreed with that of about 1.1 in a silver nanoparticle-based assay by Özyürek et al. (2012). The ratio of slopes between a tree material and tannic acid gave realistic phenolic concentrations of the largest around 8 % for aspen leaves and the smallest of 2 % for pine needles.

Tests with the mixture of tannic, chlorogenic, and ascorbic acids in solution with various proportions indicated small synergistic effect between tannic and chlorogenic acids. The combination of tannic acid and chlorogenic acid gave 3–9 % larger absorbance than the sum of absorbancies of compounds separately. In the multiple regression without interactive terms, adjusted R^2 was 0.994–0.998 at about 20 degrees of freedom. By adding first-order interactions, it was increased to 0.997–0.999 and the term for interaction between tannic acid and chlorogenic acid was significant at the statistical probability (p) less than 0.001–0.01. The present findings seem to be consistent with other research which found positive interactions between phenolics in a ferric reducing assay at 593 nm (Valerga et al. 2013).

Interferences

Interfering substances were selected primarily by their large concentration in plant tissues and reducing or chelating properties. Soluble carbohydrates and organic acids at 150-fold excess concentration had little influence on the reaction with tannic acid (Table 2). Differences in the final absorbancies were less than 2 % whether an additive was used or not. Larger effect of 3–5 % increase in the absorbance was observed by amino acids arginine and histidine. Arginine, glycine, histidine, and oxalate increased visibly the rate of color formation during first seconds and minutes of the assay.

Table 2 Influence of interferents (2 mmol L^{-1}) on the absorbance at 480 nm obtained with tannic acid (13 μmol L^{-1}) by the cupric ferricyanide oxidation at 35 °C for 90 min

Interferent	% of control
Arginine[a,b]	105
Glycine[b]	101
Malate	100
Oxalate[b]	99
Quinate	100
Succinate	98
Tartrate	102
Betaine	98
Fructose	99
Glucose	98
Histidine[a,b]	103
Mannitol	100
Proline	99
Sucrose	100

Initial concentrations in the mixture: $CuSO_4$=4 mmol L^{-1}, citrate= 12 mmol L^{-1}, phosphate buffer= 150 mmol L^{-1}, gelatin=1.3 mg mL^{-1}, $K_3[Fe(CN)_6]$=8 mmol L^{-1}, ethanol= 8 %, pH=6.6

[a] With 1 mmol L^{-1} malate

[b] Hue appeared faster

Plants may contain high levels of common osmolytes which tend to accumulate during a stress (Jouve et al. 2004). The possible osmolytes sugars, sugar alcohols, and proline did not interfere in excess. Because the stronger complexing of Cu^{2+} by adding amino acids or oxalate should decrease the rate of color formation, the increase in the absorbance by histidine and arginine could be due to a radical processes (Uchida and Kawakishi 1988) or interaction of these amino acids with the copper ferrocyanide (Kamaluddin et al. 1990).

Reaction with Ascorbic Acid

One of the main obstacles was the slow increase in absorbance during the ascorbic acid oxidation (Fig. 2a). This did not well agree with the known pronounced reducing activity of ascorbic acid. Ascorbic acid should be oxidized fast because it reduces phenolic oxidation products even for such a strong reductant as quercetin (Jovanovic et al. 1996; Boots et al. 2003). Only during sample addition and mixing the color formation was fast with ascorbic acid, but there was no stable endpoint. Therefore, the reaction with ascorbic acid was studied further to find conditions for reaching a stable endpoint for the assay.

Alteration of pH

During the slower phase, the rate of a copper ferricyanide reaction with ascorbic acid was considerably affected by the pH of the reaction environment (Fig. 3). In the pH range from 5.6 to 7.2, the color formation was larger at the higher pH values during 260 min. At pH 6.6–7.2, about twice as large absorbance was found as at pH of 5.6, and a reaction velocity started to decelerate at the highest pH by 260 min. After keeping the solutions overnight, absorbance of 1.4 AU was possible to obtain with 160 μmol L^{-1} ascorbic acid. The reaction at pH values of 6.9 and 7.2 was less stable as indicated by the smaller analyte readings and the haze formation in blanks by the end of the total period of 1020 min.

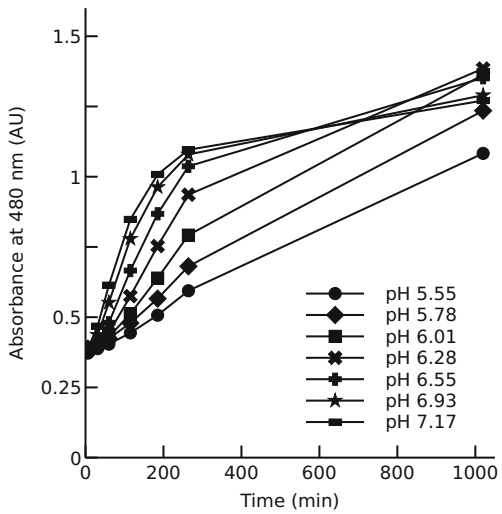

Fig. 3 Time curves of the color formation from the oxidation of ascorbic acid by cupric ferricyanide at different pH values. Initial concentration in the mixture: $CuSO_4$=4 mmol L^{-1}, citrate=4 mmol L^{-1}, oxalate=8 mmol L^{-1}, phosphate buffer=233–107 mmol L^{-1}, gelatin=1.3 mg mL^{-1}, $K_3[Fe(CN)_6]$=8 mmol L^{-1}, ethanol=8 %, ascorbic acid=160 μmol L^{-1}

The effect of pH on ascorbic acid oxidation was relatively large in the studied pH range. At this range, the ascorbic acid molecule is half dissociated (Moya and Coichev 2006). As the dissociation of ascorbic acid does not appreciably increase from pH 5.5 to 7 (Moya and Coichev 2006) and roughly half or more color was formed during the slow phase, therefore, pH influenced probably a further autoxidation of ascorbate. The effect of pH on the oxidation of ascorbate radical was unlikely because it is considered fast with ferricyanide (Van Duijn et al. 1998; Shukla et al. 2008).

Acceleration in the color formation indicated autocatalysis during the slow phase of the reaction. Autocatalysis is possible by accumulation of hydroperoxides during linoleate oxidation (Haase and Dunkley 1969). At pH values of less than about 7, the final color formation by 1020 min was not markedly affected. This suggested that oxygen did not consume some of the reductant without color formation. The pH dependence of a copper-catalyzed oxidation (Haase and Dunkley 1969) may explain the effect of pH on the ascorbate oxidation in the present study. In contrast, with the increase in pH, the concentration of free copper ions is reduced by binding to the citrate complex (Piispanen and Lajunen 1995). Previous studies have explained the sigmoidal kinetics by a build-up of catalyst or by a consumption of an inhibitor (Haase and Dunkley 1969; Ohta et al. 2000). In the current study, it seems possible that the copper citrate complex or small amounts of copper ions had a pH-sensitive influence, which enhanced oxidation of ascorbate or some intermediate products in the reaction between ascorbic acid and ferricyanide when the pH was increased toward neutral values.

Buffer Composition

Buffer composition had marked effect on the color formation with ascorbic acid (Table 3). By using the borate-sorbitol-phosphate buffer instead of the phosphate buffer, smaller absorbance was found with the buffer containing borate and sorbitol. Differences in the absorbancies were 2- to 3-fold and were increasing with time for the test with ascorbic acid. Absorbancies with rutin were not affected by the buffer composition. In the borate-sorbitol-phosphate buffer, larger values were found for quercetin, while the values were smaller for tannic acid during the oxidation. The differences between the effects of buffers were smaller for phenolics than for ascorbic acid. The differences for phenolics were about equal at 106 and 265 min of the reaction time.

Table 3 Absorbance obtained with different buffer composition during the oxidation of ascorbic acid and phenolics by cupric ferricyanide at 45 °C

Analyte	Concentration (μmol L^{-1})	Reaction time (min)	Phosphate (AU)	Borate-sorbitol-phosphate (AU)
Ascorbic acid	267	106	0.9	0.5
		265	1.6	0.5
Quercetin	133	106	1.2	1.4
		265	1.3	1.5
Rutin	173	106	1.3	1.3
		265	1.4	1.4
Tannic acid	27	106	1.5	1.4
		265	1.8	1.6

Initial concentration in the mixture: phosphate=66 mmol L^{-1} or borate-sorbitol-phosphate=100-100-33 mmol L^{-1}, $CuSO_4$=4 mmol L^{-1}, citrate=14 mmol L^{-1}, gelatin=1.3 mg mL^{-1}, $K_3[Fe(CN)_6]$=8 mmol L^{-1}, ethanol=8 %, pH=6.8–6.9

Borate is known to form esters with a diol group containing molecules, such as sorbitol, ascorbic acid, quercetin, and rutin (Dembitsky et al. 2002). Borate esters hydrolyze easily to parent compounds at acid and neutral pH; therefore, large excess of borate is required for the ester formation at the current pH around neutral values. The borate esters could be less susceptible to oxidation because of the borate stabilizing the reduced form of the diol (Mochizuki et al. 2002). Prior studies have noted that in alkaline solutions, borate inhibits autoxidation of catechins, whereas their oxidation in the presence of 20 μmol L^{-1} copper is not affected by borate (Mochizuki et al. 2002). Different strength of the borate esters may affect the inhibition of oxidation of polyphenols and ascorbic acid. Also, it can be suggested that differences in the stabilities of copper complexes with the substrates for the oxidation could vary the effects of borate.

Borate and Molybdate

As found above, the color formation was quick in the beginning of the test reaction with ascorbic acid during few minutes (Fig. 4). Afterward, ascorbic acid showed slow sigmoidal kinetics. Borate decreased the color formation during the slower stage of the reaction, which was tested for 400 min. Without borate, relatively stable color was obtained during the last 60–100 min of the test period.

Somewhat surprisingly previous studies have reported that the oxidation of dehydroascorbate by copper ions is inhibited by borate in alkaline solutions (Militzer 1945). The earlier finding of accelerated decomposition of dehydroascorbate to diketogulonic acid by borate (Huelin 1949) is in contrast to the observed inhibition of ascorbate oxidation since the removal of dehydroascorbate would rather favor the irreversible oxidation. Borate complexes may interact with copper ions and thus affect the influence of copper on the oxidation of ascorbic acid. However, this idea was not supported by previous studies since Lyman et al. (1937) find that borate does not substantially inhibit a copper-catalyzed ascorbate oxidation by oxygen.

Molybdate in the reaction mixture with ascorbic acid decreased the final absorbance, but it was possible to reach a relatively stable endpoint at 45 °C within about 80 min (Fig. 5). Without sorbitol, the differences between the assays were small during the first 35 min. Afterwards, assays without molybdate showed large increase in the absorbance. Differences between the effects of molybdate at 34 and 68 mmol L^{-1} were relatively small. Sorbitol as an additive to the assay without molybdate did not have marked influence. In contrast, sorbitol with molybdate gave larger absorbancies than molybdate alone already at 35 min of the reaction time. When molybdate was used in the reaction solution, a sigmoidal kinetics was not observed during the test period.

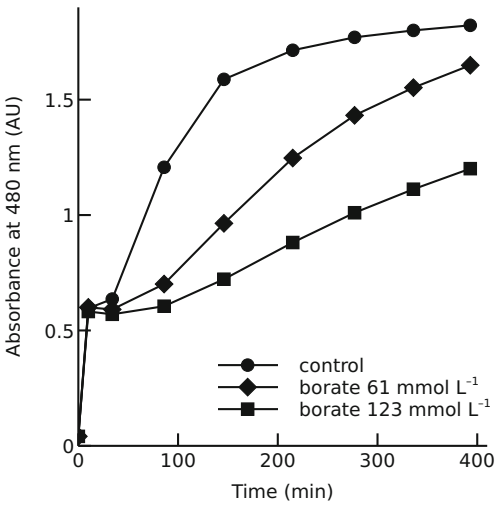

Fig. 4 Time curves of the color formation from the oxidation of ascorbic acid by cupric ferricyanide at different borate concentrations (at 45 °C after 10 min). Borate was included with sorbitol (246 mmol L^{-1}). Initial fixed concentration in the mixture: $CuSO_4$=4 mmol L^{-1}, citrate=8 mmol L^{-1}, pyrophosphate=12 mmol L^{-1}, phosphate buffer=112 mmol L^{-1}, gelatin=1.3 mg mL^{-1}, $K_3[Fe(CN)_6]$=8 mmol L^{-1}, ethanol=8 %, *N*-methylpyrrolidone=1.6 %, ascorbic acid=375 μmol L^{-1}, pH=6.9

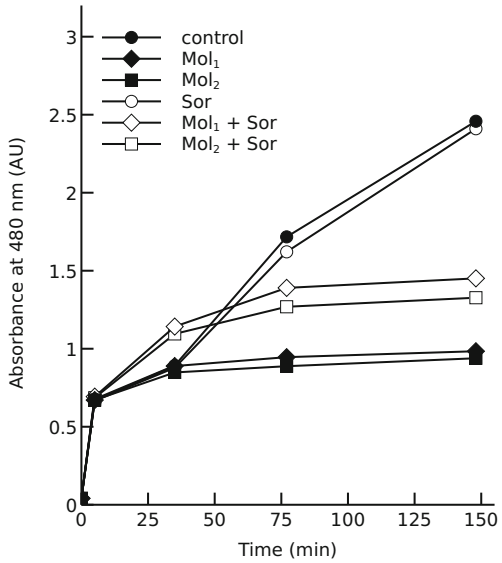

Fig. 5 Effect of molybdate (Mol_1=34 mmol L^{-1}, Mol_2=68 mmol L^{-1}) and sorbitol (Sor=271 mmol L^{-1}) on the time curves of color formation from the oxidation of ascorbic acid by cupric ferricyanide (at 45 °C after 5 min). Initial fixed concentration in the mixture: $CuSO_4$=4 mmol L^{-1}, citrate=4 mmol L^{-1}, pyrophosphate=12 mmol L^{-1}, phosphate buffer= 112 mmol L^{-1}, gelatin=1.3 mg mL^{-1}, $K_3[Fe(CN)_6]$=8 mmol L^{-1}, ethanol=8 %, ascorbic acid=406 μmol L^{-1}, pH=6.8–7.0

It is known that the reaction of oxygen with ascorbic acid is strongly catalyzed by Cu^{2+}, and the catalytic oxidation may produce hydrogen peroxide as an intermediate (Barron et al. 1936). Molybdate and borate could influence the assay reaction with ascorbate by interacting with the intermediate hydrogen peroxide. During the initial fast phase, some ascorbate could remain unoxidized because of the decrease in the reaction rate due to the consumption of substrate. During the slow phase, the oxidation by oxygen with copper ions and ascorbic acid may generate free-radical mediated reduction-oxidation (Nappi and Vass 2000; Hofer 2001). Possibly, the free-radicals may catalyze the reduction of cupric ferricyanide at the expense of organic reagents (ethanol, citric acid, or gelatin) and dehydroascorbic acid degradation products.

Solvents

Of the solvents tested as additives to the reaction conditions, 13 % *N*-methylpyrrolidone gave the largest effect on the color formation during the oxidation of ascorbate (Table 4). With *N*-methylpyrrolidone, the absorbance was smaller in the reaction run for 150 min (the effect was 55 % of control). Since acetone, dimethyl sulfoxide, and *N*-methylpyrrolidone increased the pH of solution, the larger absorbancies with only acetone and dimethyl sulfoxide were attributed to the effect of increased pH on the reaction with ascorbic acid. Glycerol, poly(ethylene glycol) 400, and polyvinylpyrrolidone increased absorbancies because of color formation also in blank cuvettes with these chemicals.

Table 4 Effect of additives on the color formation after oxidation of ascorbic acid by cupric ferricyanide at 35 °C for 150 min

Additive	Absorbance at 480 nm (AU)
None	1.37
Acetone 13 %	1.57
Dimethyl sulfoxide 13 %	1.51
Glycerol[b] 6.6 %	1.45
N-methylpyrrolidone 13 %	0.75
Polyethylene glycol 400[b] 4.3 %	1.01
Polyethylene glycol 6000 2 %	1.30
Polyvinylpyrrolidone[b] 2 %	1.23
Tetraglyme[a] 13 %	1.46
Surfactant TX-100 2.6 %	1.24

Initial concentrations in the mixture: $CuSO_4$=4 mmol L^{-1}, citrate= 6 mmol L^{-1}, oxalate=8 mmol L^{-1}, phosphate buffer=150 mmol L^{-1}, gelatin=1.3 mg mL^{-1}, $K_3[Fe(CN)_6]$=8 mmol L^{-1}, ethanol=8 %, ascorbic acid=260 μmol L^{-1}, pH=6.7–7.1

[a] Slight haze in blank after 150 min

[b] Larger increase in blank

N-Methylpyrrolidone at relatively low solvent concentration of 3 % decreased considerably the absorbance (Table 5). Both ascorbic acid and tannic acid were influenced about equally in their results of the oxidation by copper ferricyanide. The final absorbancies with *N*-methylpyrrolidone were about 70 % of the absorbancies with additives that gave about equal values. Thiobarbituric acid was apparently oxidized at a slow rate both in blank and in the reaction solutions containing ascorbic acid and tannic acid.

Further tests of the influence of *N*-methylpyrrolidone on the oxidation of other reagents gave inconsistent results (Fig. 6). During the oxidation of aminoantipyrine, hydrogen peroxide, and paracetamol, smaller absorbancies were found (Fig. 6a, b, d). In contrast, with the oxidation of thiobarbituric acid and thiosulfate, larger values were found in the presence of *N*-methylpyrrolidone in the reaction mixture (Fig. 6e, f). For manganese sulfate, only negligible effects were observed during the first 44 min (Fig. 6c). Later comparable differences by about 0.5 absorbance units appeared, but the reaction with manganese sulfate proceeded at a steady rate during the test period for 350 min. Slight haze was observed during the manganese sulfate oxidation at relatively high absorbance at the end of the test period. When aminoantipyrine was oxidized in the presence of *N*-methylpyrrolidone, even a decrease in the absorbance was found over time. The decrease was not explainable by changes in blanks. Blanks with *N*-methylpyrrolidone were increasing by 5 mAU h^{-1}, while without the solvent, the respective figure was 11 mAU h^{-1} by the final time of 350 min. Among reagents, for hydrogen peroxide, the shape of the kinetic curve was less affected by *N*-methylpyrrolidone.

Table 5 Effect of additives on the absorbance at 480 nm after the oxidation of ascorbic (260 μmol L^{-1}) and tannic (13 μmol L^{-1}) acids by cupric ferricyanide at 45 °C for 184 min

Additive	Ascorbic acid (AU)	Tannic acid (AU)
Choline chloride 67 mmol L^{-1}	0.38	0.45
Pyrophosphate (pH 7) 4 mmol L^{-1}	0.38	0.46
Molybdate (pH 7) 8 mmol L^{-1}	0.38	0.46
N-methylpyrrolidone 3.3 %	0.26	0.32
Polyethylene glycol 400[a] 2.2 %	0.40	0.47
Polyethylene glycol 6000 1 %	0.38	0.45
Polyvinylpyrrolidone[a] 1 %	0.39	0.47
Thiobarbituric acid[a] 0.67 mmol L^{-1}	0.40	0.56
Surfactant TX-100 1.3 %	0.36	0.47

Initial concentrations in the mixture: $CuSO_4$=4 mmol L^{-1}, citrate=14 mmol L^{-1}, phosphate=33 mmol L^{-1}, borate=100 mmol L^{-1}, sorbitol=100 mmol L^{-1}, gelatin=1.3 mg mL^{-1}, $K_3[Fe(CN)_6]$=8 mmol L^{-1}, ethanol=8 %, ascorbic acid, tannic acid, pH 6.9–7.0

[a] Larger increase in blank

The finding of color formation with hydrogen peroxide was in agreement with earlier studies, which have shown that in neutral or alkaline conditions, hydrogen peroxide can have considerable reducing activity in reactions where copper or ferricyanide is present (Moffett and Zika 1987; Özyürek et al. 2010; Haghighi et al. 2012).

In current results, manganese sulfate gave a steady slow increase in the absorbance. This effect is explained by the manganese redox cycling. The Mn(II)-citrate complex is oxidized to a strong Mn(III)-citrate complex, which however slowly degrades causing oxidation of citrate, while Mn^{2+} is restored (Klewicki and Morgan 1998; Topolski 2011). The Mn(III)-citrate complex may appear when oxygen is present during the testing (Klewicki and Morgan 1998). But in the current assay, cupric ferricyanide could also oxidize Mn(II)-citrate. The increase in the absorbance of copper ferrocyanide suggested a reduction of copper ions or ferricyanide. With the standard reduction-oxidation potential of 0.58 V for Mn(II)/Mn(III)-citrate (Sanjeeva Reddy 2007), it is possible that the ferricyanide and copper system oxidizes Mn^{2+} in the presence of citrate. The reduction-oxidation potential of Mn(II)/Mn(III)-citrate would be less at near neutral pH in accordance

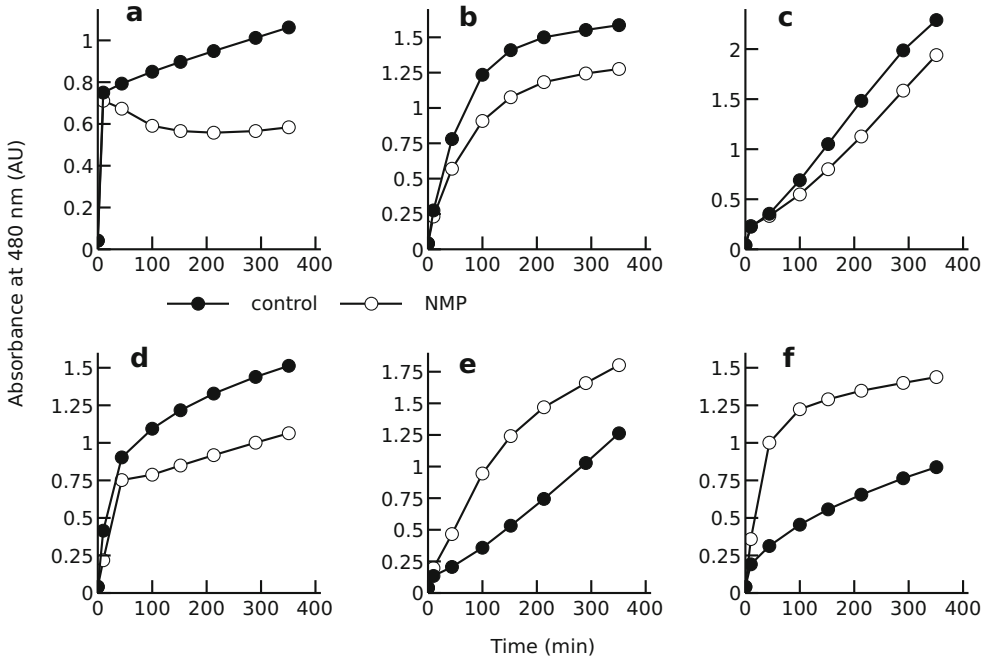

Fig. 6 Influence of *N*-methylpyrrolidone (NMP=3.3 %) on the absorbance obtained from the oxidation of test chemicals by cupric ferricyanide (at 45 °C after 10 min). Initial concentration of the chemical in mixture: aminoantipyrine (**a**)=660 μmol L^{-1}, H_2O_2 (**b**)=2941 μmol L^{-1}, $MnSO_4$ (**c**)=73 μmol L^{-1}, paracetamol (**d**)=260 μmol L^{-1}, thiobarbituric acid (**e**)=3300 μmol L^{-1}, thiosulfate (**f**)=1060 μmol L^{-1}. Initial concentration of reagents in the mixture: $CuSO_4$=4 mmol L^{-1}, citrate=8 mmol L^{-1}, phosphate=33 mmol L^{-1}, borate=100 mmol L^{-1}, sorbitol=100 mmol L^{-1}, gelatin=1.3 mg mL^{-1}, $K_3[Fe(CN)_6]$=8 mmol L^{-1}, ethanol=8 %, pH=6.9

with the diminished degradation of Mn(III)-citrate (Topolski 2011). The standard reduction-oxidation potential of ferricyanide is 0.36 V (Kolthoff and Tomsicek 1934; Rao et al. 1978). With a relatively strong copper citrate complex, the potential of current method would be less than that of the 0.6 V in a cupric reduction capacity assay (Apak et al. 2007) but still larger than the potential of ferricyanide. The finding of continuous increase during manganese oxidation has important implications for using ligands in the proposed assay. The lability of Mn(III)-citrate complex may give erroneous reducing capacity values in the assay with samples containing manganese ions.

The effect of *N*-methylpyrrolidone was not possible to explain by the solvent effects, which decrease the reduction-oxidation potential of ferricyanide (Rao et al. 1978). In contrast to the solvent effect, during the oxidation of thiobarbituric acid and thiosulfate, the rate of color formation increased by *N*-methylpyrrolidone. Also, *N*-methylpyrrolidone was added at a relatively low level of 3 %. At 2 % concentration, *N*-methylpyrrolidone is effective scavenger of radicals and protects the ligand in an iron chelate oxidation of hydrogen sulfide (Hua et al. 2007). At elevated temperatures and with metal catalysts, *N*-methylpyrrolidone and oxygen generate a hydroperoxide radical oxidant, which has been used for oxidative stress testing of chemicals (Drago and Riley 1990; Reynolds et al. 2012). For a lactam oxidation in mild conditions, both a transition metal salt and a free-radical chain initiator have to be present (Minisci et al. 2002). Though precise analysis would be required, current data indicated that *N*-methylpyrrolidone could activate the oxidation of a reducing compound either by oxygen or by copper ferricyanide. The oxidation rate by the copper ferricyanide was increased for a slowly reacting sulfur-based reducing compound, which was used at relatively higher concentration. The effects of adding *N*-methylpyrrolidone therefore need to be interpreted with caution. Current study suggests that due to its complex, substrate-dependent influence and possible oxidative reactivity in the presence of copper ions *N*-methylpyrrolidone was not useful for the endpoint stabilization in the assay.

Conclusions

Comparison of responses by phenolics with these by plant extracts in the reaction with cupric ferricyanide indicated that a reducing activity assay could be developed for woody plants or green tea. There was no interference from the excess of common osmolytes which accumulate in plants. With the oxidation of phenolics, a reasonably stable endpoint was achieved during 90 min. This duration is acceptable for sample output when the analyses are run in parallel. Variation in pH and additives indicated that during longer incubations, the results could be affected by the easy generation of autoxidative processes with ascorbate. By the presence of copper ions, the propensity of ascorbic acid to autoxidation may produce hydrogen peroxide during the slow phase. The results of this research support the idea that significant part of hydrogen peroxide is oxidized with cupric ferricyanide at neutral pH. Molybdate stabilized the endpoint for ascorbic acid during the testing time. However, the suitability of molybdate in the reagent solution requires further evaluation in respect to reaction temperature, polyphenol oxidation, calibration, interferences, and colloid stability. Effect of manganese ions in the sample was complicated because of a steady increase in absorbance due to possible reduction-oxidation cycling with citrate. A solvent *N*-methylpyrrolidone was not appropriate to include as an additive in the current reagent mixture, because of possible activation of oxidation by oxygen and decolorization of the cupric ferrocyanide in the presence of *N*-methylpyrrolidone.

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Conflict of Interest Jaan Klõšeiko declares that he has no conflict of interest. This article does not contain any studies with human or animal subjects.

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