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Direct measurement of total antioxidant capacity of cereals: QUENCHER-CUPRAC method

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ABSTRACT

Polyphenols in cereal samples are distributed as free, soluble-esterified, and insoluble-bound forms either esterified or etherified to cell wall constituents. In the evaluation of total antioxidant capacity (TAC) of cereals, rather difficult and time-consuming acid, alkaline and enzymatic treatments of residue have been applied to complete the extraction of bound phenolic compounds. Thus, this work is aimed to measure the TAC of cereals (*i.e.* barley, wheat, rye, oat) by the 'QUENCHER procedure' (involving forced solubilization of bound phenolics by oxidizing TAC reagents) with the direct use of copper(II)–neocuproine (Cu(II)–Nc) reagent of the CUPric Reducing Antioxidant Capacity (CUPRAC) assay. In this novel 'QUENCHER-CUPRAC' method, reaction time and solvent composition parameters were optimized, and the method was applied to cereal samples with CUPRAC reagent dissolved in 1:1 (v/v) ethanol–water mixture. The antioxidant capacities of soluble and insoluble fractions were simultaneously measured to give a hierarchic TAC order of cereals as: barley > rye > oat > wheat. The TAC values of cereals measured by QUENCHER-CUPRAC were higher than those of original QUENCHER method using $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ reagents. Polyphenolic mixtures in a cellulose matrix gave additive TAC values with QUENCHER-CUPRAC. The proposed method correlated linearly with QUENCHER-$ABTS^{\bullet+}$ ($r=0.956$) and QUENCHER-$DPPH^{\bullet}$ ($r=0.976$).

1. Introduction

Many epidemiological studies have indicated that consumption of whole grain cereal is highly correlated to reduced incidences of chronic diseases [1]. The free radical attack on biomolecules has been suggested as an initial cause of most chronic diseases [2]. The scavenging of reactive species (including radicals) by bioactive phytochemicals such as phenolic compounds rich in whole grains may be a mechanism of protection [3]. Hence, sufficient amounts of phytochemicals from a variety of sources such as fruits, vegetables, and whole grain-based foods are recommended for maximum health benefits [4].

Cereals contain a wide range of phenolic compounds including benzoic and cinnamic acids, anthocyanidins, quinones, flavonols, chalcones, flavanones and amino phenolics [5–9]. Phenolic acids and flavonoids represent the most common form of phenolic compounds found in whole grains that constitute the major and most complex groups of phytochemicals with a number of types that exist as soluble free compounds, soluble conjugates esterified to sugars and other low molecular mass compounds, and insoluble/bound forms [10]. Cereal grains have long been thought to be less important sources of antioxidants than fruit and vegetables [11] although they are actually major dietary components containing a rich variety of antioxidants. The most frequently used methods to measure the *in vitro* antioxidant potential of cereals and their fractions are oxygen radical absorbance capacity (ORAC) [12], 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [13], 2,2′-diphenyl-1-picrylhydrazyl (DPPH) [14] and ferric reducing antioxidant power (FRAP) [15] assays. Several recent articles have reported TAC measurement and constituent analysis of different cereal products [5,16–25]. These articles commonly conclude that cereals possess significant free radical scavenging or antioxidant capacities and may serve as a potential source of natural antioxidants.

It has been reported that an appreciable amount of polyphenols, called nonextractable polyphenols comprised of polymeric polyphenols or low molecular weight polyphenols bound to protein, polysaccharides, or cell walls, can remain in the neglected residual solid masses of solvent extraction processes using aqueous-organic extractants [26,27]. Analytical methodology of polyphenols in cereals, including free and bound forms, generally consists of an extraction with aqueous-organic solvents to recover soluble polyphenols followed by a hydrolysis treatment to obtain free polyphenols from glycosides [28]. By alkaline, acid or enzymatic treatments of residues, significant amounts of polyphenolic compounds may be released from the food matrix, which can

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be analyzed in the corresponding hydrolysates [27,29–31]. Most research on TAC of cereals give limited results due to incomplete extraction of antioxidants depending on the organic solvent used [31]. Besides, some substances cannot be solubilized without modifying their molecular nature by means of chemical or enzymatic treatments which can also affect their measured antioxidant capacity [32]. For many years, it was not clear whether the water-insoluble dietary fiber phenolic fraction could exert any antioxidant action itself, *i.e.* without any chemical hydrolysis [33]. Serpen et al. were the first researchers to demonstrate the concept of antioxidant activity of insoluble material measured in a different way [34]. In reality, the slow and continuous release of dietary fiber phenolics from the insoluble material surviving for a considerable time in the human gastrointestinal tract is known to take place, and in particular, these phenolics may favorably act *in vivo* quenching the soluble radicals that are continuously formed in the intestinal tract [33]. Serpen et al. have shown by the 'QUENCHER approach' (abbreviated for 'QUick, Easy, New, CHEap and Reproducible treatment, involving forced solubilization of bound phenolics by oxidizing TAC reagents) that it is possible to evaluate the antioxidant capacity of compounds without preliminary extraction when they are still bound to the insoluble food matrix, taking advantage from the surface reaction phenomenon between solid (bound antioxidant compounds) and liquid (soluble free radicals derived from TAC reagents) material [34–36]. Using this approach, Serpen et al. measured the TAC of solid matrices containing polyphenols by the extent of quenching of ABTS and DPPH radicalic reagents [34–36] and of the color formation of FRAP reagent [37].

Compared to other chromogenic TAC reagents, the CUPRAC reagent has been shown to be much less dependent on phenolic lipophilicity, steric effects, pH, dissolved oxygen, humidity, and daylight [38,39]. Thus, in the present work, it has been aimed to measure the TAC of cereal products (*i.e.* barley, wheat, rye, oat) by combining the QUENCHER procedure with the conventional CUPRAC assay. This is the first report on the TAC measurement of cereals (including the contribution from bound polyphenols) using the CUPRAC reagent, in which the original CUPRAC assay parameters [38] were successfully adapted to the QUENCHER assay. As a result, the novel 'QUENCHER-CUPRAC' method is proposed here to give a reliable estimate of the actual antioxidant capacity of cereals.

2. Experimental

2.1. Reagents and apparatus

The following chemicals of analytical reagent grade were supplied from the corresponding sources: methanol (MeOH), absolute ethanol (EtOH), copper(II) chloride ($CuCl_2$), ammonium acetate (NH_4Ac), hydrochloric acid, sodium hydroxide, citric acid monohydrate and potassium persulfate were purchased from Merck Chemicals (Darmstadt, Germany); neocuproine (Nc), DPPH, α-cellulose, α-tocopherol (TOC), trolox (TR), vanillic acid (VA), ferulic acid (FRA), caffeic acid (CFA), *p*-coumaric acid (COU) and ethyl acetate were from Sigma-Aldrich Chemicals (Steinheim, Germany); gallic acid (GA), sinapic acid (SPA) and ABTS were from Fluka (Buchs, Switzerland). The cereal samples (barley, rye, wheat, oat) were obtained from local markets (Istanbul, Turkey). All reagents and standard solutions were prepared using Milli Q deionized water (Millipore, Bedford, USA).

The spectra and absorption measurements were recorded in matched quartz cuvettes using a Varian CARY Bio 100 UV–vis spectrophotometer (Mulgrave, Victoria, Australia) having a spectral resolution of ≈1 nm. Other related apparatus were an analytical mill (IKA Labortechnik, Staufen, Germany), sieve (Endecotts Test Sieve, London, U.K.), Biosan Multi Bio rotator, Elektromag centrifuge, and Electromag vortex stirrer (Istanbul, Turkey).

2.2. Preparation of solutions

Samples were ground using an analytical mill during 10 min at room temperature. Ground samples were consecutively passed through a sieve having mesh size of 50 (about 300 μm) to produce a fine powder. Fine powdered samples were stored at −20 °C before analysis, and all analyses were performed in duplicate within a week. $CuCl_2$ solution (10 mM) and ammonium acetate solution (1.0 M, pH=7) were prepared in distilled water and neocuproine solution (7.5 mM) in pure ethanol for the original CUPRAC assay [38]. In this study, CUPRAC reagent was prepared in EtOH:H_2O (1:1, v/v) to analyze TAC of solid samples and in pure ethanol for screening extracts and hydrolyzates at the same concentration. In order to test the solvent effect on TAC of cereals, CUPRAC reagent solutions were prepared in five different volume percentages (100:0; 75:25; 50:50; 25:75; 0:100) of EtOH:H_2O mixtures on the day of analysis. The chromogenic radical reagent $ABTS^{\bullet+}$, at 7.0 mM concentration, was prepared by dissolving this compound in water and adding $K_2S_2O_8$ to this solution such that the final persulphate concentration in the mixture was 2.45 mM. The resulting $ABTS^{\bullet+}$ solution was left to mature at room temperature in the dark for 12–16 h [13]. The $ABTS^{\bullet+}$ solution was diluted with EtOH or EtOH:H_2O (1:1, v/v) to an absorbance of 0.70 (±0.02) at 734 nm prior to use. The DPPH radical solution (at ≈40 mg/L) was prepared in EtOH:H_2O (1:1, v/v) and diluted with the same aqueous ethanol by adjusting the absorbance of the $DPPH^{\bullet}$ solution to 0.750–0.900 AU at 525 nm [40].

2.3. Extraction and hydrolysis procedures

In order to determine the TAC of cereals (containing those of free and bound phenolics), extraction and hydrolysis procedures were performed. One hundred milligrams of ground cereal sample was extracted 3 times with 1.7 mL of water by mixing for 2 min in a vortex mixer. Each extraction step was followed by centrifugation at 5000 rpm for 5 min. Combined clear supernatants were used for TAC measurement. The combined ethanol extract was prepared similarly by extracting the remaining residue 3 times with 1.7 mL of ethanol. In the case of methanol extraction, 100 mg of ground cereal sample was extracted 3 times with 1.7 mL of methanol following the same procedure described above. In order to assess the antioxidant capacity of bound compounds, the final solid residue was hydrolyzed with 10 mL of 4 M sodium hydroxide for 4 h under N_2 by shaking gently in the dark at room temperature, as described by Liyana-Pathirana and Shahidi [41]. After alkaline hydrolysis, the pH of the mixture was adjusted to 3.5 by adding 3 M citric acid monohydrate, and samples were centrifuged at 5000 rpm for 5 min. One milliliter of the supernatant was extracted 3 times with 1 mL of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under a gentle stream of nitrogen at room temperature. The residue was dissolved in a mixture of MeOH:H_2O (1:1, v/v). Free and bound cereal extracts were kept at 4 °C in the dark until analysis.

2.4. Determination of TAC of cereal samples

2.4.1. Original QUENCHER assay

Original QUENCHER assay [37] was performed by measuring the extent of quenching of ABTS and DPPH radicalic reagents as detailed by Serpen et al. [37]. Ten milligram portion of powdered

sample was weighed to an eppendorf, the reaction was started by adding 10 mL of ABTS or DPPH reagent. The mixture was vortexed for 1 min and placed on rotator in the dark. After mixing exactly 30 min for ABTS and 120 min for DPPH reagent, samples were centrifugated at 5000 rpm for 2 min, and filtered through a 0.45 μm micro-filter (Chromafil GF/PET-45/25) before spectrophotometric analysis. The absorbances of clear supernatants were measured at 734 nm or 525 nm for ABTS or DPPH assays, respectively, and decolourization (*i.e.* loss in absorbance) was correlated to antioxidant concentration.

2.4.2. Original CUPRAC assay applicable to solutions

To a test tube were added 1 mL each of Cu(II), Nc, and NH_4Ac buffer solutions prepared in pure ethanol. Extracts (x mL) and pure EtOH ($(1.1-x)$ mL) were added to the initial mixture so as to make the final volume: 4.1 mL. The mixture was vortexed for 20 s, and absorbance measurement was performed exactly after 30 min at 450 nm [38]. The absorbance of the emerging cuprous neocuproine chromophore was correlated to antioxidant concentration. The TAC of cereals was reported as trolox equivalents (mmol TE kg^{-1} solid matter).

2.4.3. QUENCHER-CUPRAC assay applicable to powdered solids

A mass of 10.0 ± 0.1 mg of powdered sample was weighed into a centrifuge tube. The reaction was started by adding to the tube 4.1 mL CUPRAC reagent solution prepared in EtOH:H_2O (1:1, v/v) (containing 1 mL each of Cu(II), Nc and NH_4Ac solutions prepared in EtOH:H_2O (1:1, v/v), and 1.1 mL of the same solvent mixture). For optimization of solvent composition, the CUPRAC reagent solution was prepared in aqueous mixtures containing different percentages of EtOH, but in the 'recommended procedure', EtOH:H_2O (1:1, v/v) was used as solvent throughout. The suspension was vortexed for 1 min, and placed on a rotator for 30 min. Sample was centrifuged at 5000 rpm for 2 min, and filtered through a 0.45 μm micro-filter (Chromafil GF/PET-45/25) before CUPRAC spectrophotometric determination at 450 nm.

2.4.4. QUENCHER-CUPRAC procedure with incubation at elevated temperature

The suspension containing the powdered sample and CUPRAC reagent prepared in EtOH:H_2O (1:1, v/v) was vortexed for 1 min and incubated for 20 min in a shaking water bath at a temperature of 50 °C [38]. The tube was cooled to room temperature under running water, and its A_{450} value was measured. Sample was centrifuged at 5000 rpm for 2 min, and filtered through a 0.45 μm micro-filter (Chromafil GF/PET-45/25) before CUPRAC spectrophotometric determination at 450 nm.

2.5. Statistical analysis

Descriptive statistical analyses were performed using Excel software (Microsoft Office 2002) for calculating the means and the standard error of the mean. Results were expressed as the mean ± standard deviation (SD). Using SPSS software for Windows (version 13), the data were evaluated by two-way ANalysis Of VAriance (ANOVA) [42].

3. Results and discussion

Antioxidants in cereals may be water-soluble, lipid-soluble, insoluble, or bound to cell walls, thus they may not necessarily be available to freely react with chromogenic TAC assay reagents; consequently, due to the slower kinetics involved, the reaction may not go to completion within a reasonable time set by the assay. Since TAC measurement is conventionally limited to liquid extracts, solid samples like cereals and legumes require a preliminary solvent-extraction step before TAC evaluation. This phase has been considered quite critical, as antioxidants show a great variety of solubilities [32]. Most research on the antioxidant capacity of cereals gives insufficient information due to incomplete extraction of antioxidants depending on solvent type [31]. Although longer extraction times and finer grain sizes of powdered samples would increase extraction yield, the measured TAC or phenolic contents would still be underestimated, because some bound phenolics from insoluble fractions would remain unreacted [4]. Besides, some substances cannot be solubilized without modifying their molecular nature by means of chemical or enzymatic treatments which can also affect their antioxidant capacity. Nevertheless, insoluble components are not necessarily non-reactive. Some functional groups that can be linked to the insoluble fraction of cereal components were reported to show antioxidant activity [34]. An important part of the insoluble antioxidants are linked to the side chains of arabinoxilan of hemicelluloses from the cell walls [40].

3.1. Effect of extraction procedures to TAC analysis

In the present study, the TAC of four cereal samples (*i.e.* barley, wheat, rye and oat) was measured by CUPRAC assay applying two different extraction procedures. In the first procedure, components in the cereal were treated sequentially with H_2O and EtOH to extract free phenolics, and the residue was hydrolyzed to extract bound phenolics. In the second one, MeOH was used to extract free phenolics. According to TAC values given in Table 1, (water+ethanol) extracts of cereal samples showed higher TAC values than methanol extracts, probably due to the higher content of water-soluble antioxidants present in cereals. The TAC_{CUPRAC} order of cereals was: barley > rye > oat > wheat, barley having the highest and wheat the lowest antioxidant capacity, in accordance with literature reports [35,43]. Percentage contribution of bound phenolics to the TAC_{CUPRAC} value of all fractions (*i.e.* obtained by adding up water- and EtOH-soluble and bound fractions, or by MeOH-soluble and bound fractions in Table 1) varied from 6.9 to 16.8 in (water+ethanol) extraction and from 19.3 to 37.1 in methanol extraction (Table 1). These results confirmed that insoluble polyphenols bound to cell wall are significant contributors to the TAC of cereals [4,8,31,35,44].

3.2. Optimization of reaction parameters for QUENCHER-CUPRAC method

Direct analysis of TAC of cereals performed by the QUENCHER approach is based on mixing solid samples with free radicals in

Table 1
Total antioxidant capacities (TAC) of cereals analyzed by two sequential extraction procedures.

Cereals	$TAC_{Water+EtOH\ extraction}$ (mmol TE kg^{-1})			$TAC_{MeOH\ extraction}$ (mmol TE kg^{-1})	
	Water[a]	EtOH[b]	Bound[c]	MeOH[d]	Bound[c]
Barley	18.28 ± 0.99	2.79 ± 0.32	2.58 ± 0.06	11.07 ± 0.17	2.65 ± 0.09
Rye	8.64 ± 0.22	3.51 ± 0.07	2.16 ± 0.07	6.55 ± 0.01	2.19 ± 0.09
Wheat	4.31 ± 0.29	2.18 ± 0.08	1.31 ± 0.09	3.25 ± 0.07	1.92 ± 0.04
Oat	7.51 ± 0.33	1.44 ± 0.01	0.67 ± 0.01	3.83 ± 0.09	1.31 ± 0.02

[a] Water-soluble free phenolics.
[b] Ethanol-soluble free phenolics.
[c] Insoluble bound phenolics.
[d] Water-soluble and insoluble free phenolics. Results were expressed as (mean ± SD), $N=3$.

solution such as $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, followed by spectrophotometric measurement [36]. The working principle of the conventional QUENCHER approach developed by Serpen et al. [35,37] and Gökmen et al. [36] for direct measurement of TAC of cereals was successfully adapted to the CUPRAC assay such that Cu(II)-neocuproine chromogenic reagent replaced the radicalic reagents of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. In the application of the QUENCHER-CUPRAC assay to cereal samples with CUPRAC reagent dissolved in EtOH:H_2O (1:1, v/v), TAC expressed as mmol TE g^{-1} was recorded against reaction time, and the optimal contact time at room temperature was chosen as 30 min (Fig. 1). For the conventional QUENCHER method using $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ reagents, the corresponding reaction times were set at 30 min and 120 min, respectively [35–37], clearly showing the kinetic advantage of QUENCHER-CUPRAC over that of $DPPH^{\bullet}$. In accordance with this finding, Magalhaes et al. [45] reported that the times required for reaching end-point conditions in the conventional CUPRAC and DPPH assays were 30 min and 120 min, respectively [45].

In the original CUPRAC assay [38], the sample and reagent solution were mixed and incubated either at room temperature for 30 min or at 50 °C (in a water bath) for 20 min. The CUPRAC assay with incubated measurement was adapted to the QUENCHER procedure to yield TAC values of cereals increasing in the range of 63–103% as a result of incubation at 50 °C (Table 2). It was previously established that slow reacting antioxidants needed elevated temperature incubation so as to complete their oxidation with the CUPRAC reagent [38,39], but in the QUENCHER approach, both solubilization and oxidation of phenolics are apparently accelerated with temperature.

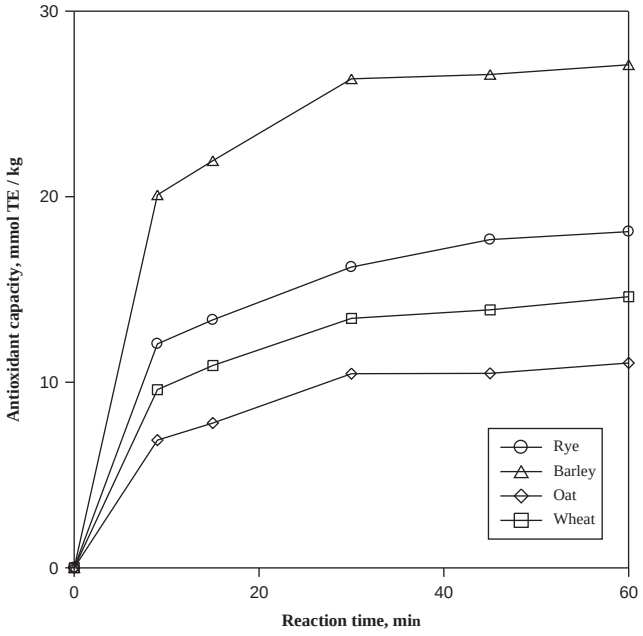

Fig. 1. Rate of increase in TAC of cereals, expressed as mmol of Trolox Equivalent Antioxidant Capacity (TEAC) per kg, with respect to reaction time measured by QUENCHER-CUPRAC assay at room temperature (absorbance measurement at 450 nm).

Table 2
Total antioxidant capacity of cereal products expressed as mmol TE kg^{-1}, measured by the QUENCHER-CUPRAC method carried out at room temperature and incubated (inc) at 50 °C.

Cereals	$TAC_{QUENCHER-CUPRAC}$	$TAC_{QUENCHER-CUPRAC\ (Inc)}$
Barley	26.35 ± 0.61	46.23 ± 0.73
Rye	16.21 ± 0.25	26.40 ± 0.12
Wheat	13.44 ± 0.46	22.00 ± 0.41
Oat	10.46 ± 0.23	21.26 ± 0.86

Data presented as (mean ± SD), $N=3$.

3.3. Solvent composition of CUPRAC reagent

Serpen et al. investigated the effects of solvent composition of different radicalic reagents on the measured TAC of foods by the QUENCHER method, and stated that the solvent composition had a significant influence on TAC values, the highest TAC being measured in 1:1 (v/v) EtOH:H_2O mixture [37]. To test the solvent effect in the QUENCHER-CUPRAC approach, CUPRAC reagent was prepared in EtOH:H_2O mixtures with different volume percentages (0:100, 25:75, 50:50, 75:25, 100:0). As trolox was the reference compound in reporting TAC values, trolox was determined by the CUPRAC assay in different ratio ethanol–water media. Calibration curves of trolox (as absorbance versus concentration) in Fig. 2 demonstrated that solvent composition of the CUPRAC reagent did not affect the slope of trolox more than 5%.

TAC values of cereals were determined by the QUENCHER-CUPRAC method using different ratio EtOH:H_2O solvent mixtures. TAC values were significantly influenced by solvent composition of the CUPRAC reagent (Fig. 3). It was mentioned that water has a critical role on TAC analysis by the direct QUENCHER method. In most cases (including barley, rye and wheat), the highest TAC values with QUENCHER-CUPRAC were observed in 1:1 (v/v) EtOH:H_2O mixture (Fig. 3), as previously reported with $ABTS^{\bullet+}$reagent [37]. The lowest values for all cereals were obtained in bidistilled water (Fig. 3), probably due to insufficient solubilization. For oat, the highest TAC value was measured using the CUPRAC reagent in 3:1 (v/v) EtOH:H_2O mixture (though its performance was close to that of 1:1 mixture). Use of water as a common solvent in the composition of chromogenic reagents was reported to increase TAC values; however, water content above 50% was indicated to decrease the stability of TAC reagent [37].

Rate constants for oxidation of deprotonated phenols (with singlet oxygen) are at least two orders of magnitude greater (*i.e.* at the order of $\geq 10^8\ M^{-1}s^{-1}$) than those of the non-deprotonated species. This means that the anionic forms may

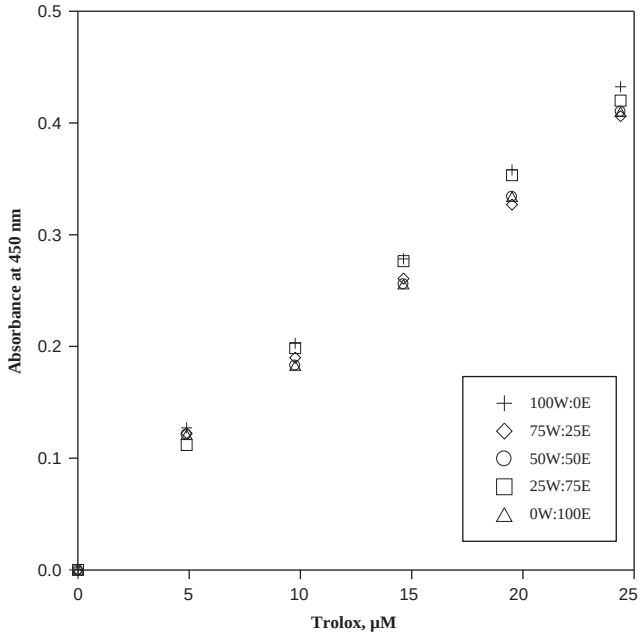

Fig. 2. Absorbance at 450 nm as a function of trolox concentration, using different percentages of ethanol:water (v/v) mixtures in preparation of CUPRAC reagent solution (E: ethanol, W: water).

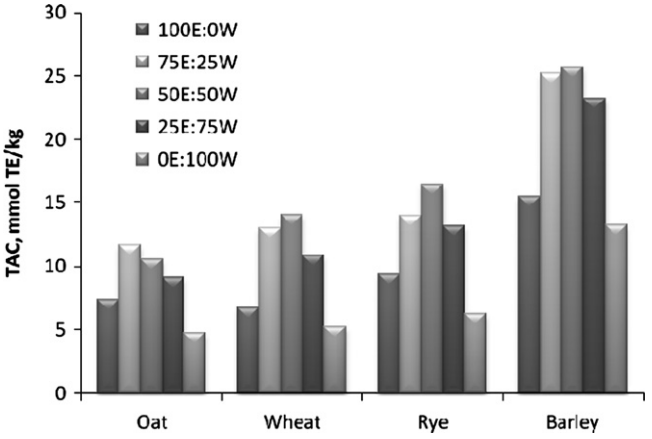

Fig. 3. Effects of different percentages of ethanol:water (v/v) mixtures (as solvent in CUPRAC solution) on measured TAC values of cereal samples (E: ethanol, W: water).

already control the apparent reaction rate at pH values at least two units below the pK_a of the phenol, *i.e.* when the degree of dissociation is $<1\%$ [46]. In an ionizing solvent like water, phenol oxidation is faster than in alcohols. For undissociated phenols, the result is similar; Litwinienko and Ingold have envisaged that hydrogen (H) bond-accepting (HBA) solvents like water can form hydrogen bonds with undissociated phenols creating an H-bonded radical cation more acidic than its neutral parent, resulting in fast proton loss probably concerted with electron transfer (in this case to a TAC reagent), also pointing out to faster oxidation kinetics in water-containing media [47]. As another example of e-transfer acceleration in ionizing solvents, abnormally enhanced rate constants were observed for the oxidation of phenols with $DPPH^{\bullet}$ (diphenylpicrylhydrazyl radical) not only in alcohols but also, for phenols with low pK_a values, in non-hydroxylic, polar solvents like di-*n*-butyl ether, acetonitrile, THF and DMSO, as a result of acidic ionization of phenol into phenoxide anion (ArO^-) followed by fast e-transfer from ArO^- to $DPPH^{\bullet}$ [48]. Applying these ideas to the QUENCHER approach, oxidation of bound phenols requires water for ionization-assisted faster kinetics. On the other hand, alcohol is necessary in the solvent mixture for solubilization of the same compounds. As a compromise between these two factors, a 1:1 (v/v) EtOH:H_2O mixture produces the highest overall yield for combined solubilization-oxidation of bound phenolics with TAC reagents (and specifically with the CUPRAC reagent in the QUENCHER-CUPRAC method) from insoluble food matrices.

3.4. Use of cellulose as a solid matrix diluent in TAC additivity testing of polyphenols by QUENCHER-CUPRAC method

Before applying cellulose as a solid matrix diluent, the fact that cellulose remained an inert matrix (*i.e.* non-responsive to the spectrophotometric assay) in the QUENCHER-CUPRAC method was verified. Then, two homogeneous mixtures of phenolic antioxidants were prepared in cellulose to test the additivity of their TAC values. Theoretically expected and experimentally found TAC values of the two synthetic mixtures in cellulose matrix with respect to the QUENCHER-CUPRAC method are tabulated in Table 3. The theoretically expected trolox equivalent antioxidant capacity (expressed in the units of mM TE of extract solution) was calculated by multiplying the TEAC coefficient of each antioxidant constituting the mixture with its final concentration, and summing up the products. The experimental trolox equivalent TAC of the same mixture was calculated by dividing the observed absorbance (A_{450}) to the molar absorptivity of trolox

Table 3
Comparison of the theoretically expected and experimentally found trolox (TR)-equivalent antioxidant capacities (in mM TE units) of the extracts of synthetic mixtures of phenolic antioxidants in cellulose matrix, measured by the QUENCHER-CUPRAC method.

Composition of mixture	$TAC_{component}$	$TAC_{theoretical}$	$TAC_{experimental}$
100 μL of 0.2 mM VA	9.0×10^{-3}	3.91×10^{-2}	$(3.70\pm0.12)\times10^{-2}$
100 μL of 0.2 mM FRA	9.2×10^{-3}		
100 μL of 0.2 mM CFA	13.7×10^{-3}		
100 μL of 0.2 mM COU	7.2×10^{-3}		
100 μL of 0.2 mM TOC	6.3×10^{-3}	5.03×10^{-2}	$(4.79\pm0.18)\times10^{-2}$
100 μL of 0.2 mM GA	17.7×10^{-3}		
100 μL of 0.2 mM CFA	13.7×10^{-3}		
100 μL of 0.2 mM SPA	12.6×10^{-3}		

Table 4
Total antioxidant capacities (in mmol TE kg^{-1}) of cereal samples measured by QUENCHER procedure combined with CUPRAC, ABTS and DPPH reagents in 1:1 (v/v) EtOH:H_2O solvent mixture.

Cereals	CUPRAC method	ABTS-persulphate method	DPPH method
Barley	26.35 ± 0.61	18.20 ± 0.48	19.43 ± 0.14
Rye	16.21 ± 0.25	11.60 ± 0.30	7.90 ± 0.32
Wheat	13.44 ± 0.46	11.70 ± 0.48	6.73 ± 0.07
Oat	10.46 ± 0.23	7.90 ± 0.54	4.83 ± 0.42

The ANOVA comparison between CUPRAC and ABTS-persulphate assays: $P=0.05$, $F_{exp}=8.91$, $F_{crit\ (table)}=10.13$, $F_{exp}<F_{crit\ (table)}$.
Data presented as (mean ± SD), $N=3$.

under the specified conditions. Then the theoretically calculated TACs were compared to the experimentally found ones to test the applicability of Beer's law (*i.e.* the principle of additivity of individual absorbances of constituents making up a mixture), and were found to agree within 5% (Table 3). Validity of Beer's law for a mixture implies that the observed absorbance is the sum of the individual absorbances of constituents not chemically interacting among each other and also with the cellulose matrix.

3.5. Comparative evaluation of TAC values of cereals measured by QUENCHER-combined CUPRAC, ABTS and DPPH methods

As shown in Table 4, TAC values of cereal samples were measured by QUENCHER method using CUPRAC, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ reagents prepared in a 1:1 (v/v) mixture of EtOH:H_2O. The QUENCHER-CUPRAC method results were found to be higher than those of other reagents. The thermodynamic driving force for the simultaneous solubilization and reaction of antioxidants in the QUENCHER approach is the high equilibrium constant for the redox reaction with TAC assay reagents 'coupled' to that of physical dissolution of antioxidants from partly insoluble matrices [37]. In thermodynamics, two or more reactions can sometimes be coupled so that thermodynamically unfavorable (*i.e.* Gibbs free energy change: $\Delta G^\circ>0$) reactions and favorable reactions (*i.e.* $\Delta G^\circ<0$) are combined to drive the overall process in the favorable direction. In the QUENCHER-CUPRAC method, oxidation-equilibrium constants of bound phenolics (in cereal matrix) with CUPRAC reagent are higher than the corresponding solubilization-extraction equilibrium constants. Therefore, solubilization and redox equilibria proceed simultaneously and the overall (coupled) process is facilitated. For example, the formal redox potential (at pH=7.0) of chlorogenic acid at 1 mM concentration was reported to be 0.219 V [49], and the standard potential for the $Cu^{II,I}(Nc)_2$ redox couple is 0.600 V [38,39]. Using these data, the equilibrium constant for CUPRAC oxidation of chlorogenic acid can be calculated using the Nernst

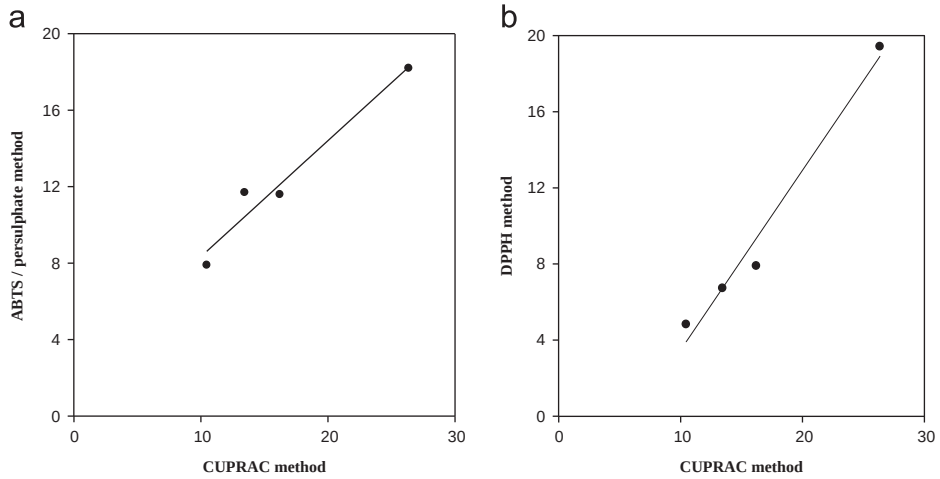

Fig. 4. Correlation of CUPRAC assay results with those of (a) ABTS-persulphate assay ($r=0.956$) and (b) DPPH assay ($r=0.976$) in direct measurement of TAC.

equation: $\Delta E^{o}=0.600-0.219\ V=0.05916\ \mathrm{Log}\ K$. It follows that $K=2.76\times10^{6}$, in other words, the CUPRAC oxidation reaction for chlorogenic acid at pH 7 has a large equilibrium constant, and therefore, the CUPRAC reagent, when present in an extraction medium, may facilitate the solubilization/extraction of chlorogenic acid from a relatively insoluble matrix. This situation explains the higher TAC values found with the CUPRAC assay compared to those with other TAC reagents, as CUPRAC has more favorable kinetics than DPPH [45] and phenolics are partly ionized at the working pH of the CUPRAC assay ensuring easier oxidation. As the favorable redox potential of the CUPRAC reagent is not significantly different from those of ABTS and DPPH, there should be some other mechanism explaining the higher efficiency of the QUENCHER-CUPRAC approach in solubilizing and oxidizing phenols than either of ABTS or DPPH methods. Firstly, the redox potentials should be compared on a conditional basis, *i.e.* polyphenols are oxidized by the CUPRAC reagent at nearly physiological pH, and therefore, the formal aryloxyl radical/phenol reduction potential at pH=7.0 should be taken into account in evaluating phenol oxidizability, as phenols are partly ionized at this pH. Secondly, as opposed to the CUPRAC reagent: $Cu(Nc)_2^{2+}$ being an outer-sphere electron-transfer agent having relatively fast kinetics with little solvent dependency, both ABTS [50] and DPPH radicals [51,52] suffer from substrate accessibility problems due to steric hindrance. Magalhaes et al. [45] stated that among electron transfer assays, the oxidation of a 'standard phenolic mixture' (SPM) with the CUPRAC reagent was faster than with radicalic reagents of DPPH and ABTS, based on a mechanism of sequential proton loss electron transfer (SPLET), which yields an identical net result with hydrogen atom transfer reactions to the free radicals [48,53]. Magalhaes et al. showed that the time periods for stabilization of sensitivity (*i.e.* slope of the calibration line between absorbance and concentration for phenolics) for a SPM with CUPRAC, DPPH and ABTS assays were 60, 120, and 300 min, respectively, clearly showing the kinetic advantage of CUPRAC over radicalic TAC assays [45].

The two-way ANalysis Of VAriance (ANOVA) comparison by the aid of *F*-test of the mean-squares of 'between-treatments' (*i.e.*, CUPRAC and ABTS procedures in Table 4) and of residuals [42] for a number of real samples enabled to conclude that there was no significant difference between treatments. In other words, the TAC values found with the two procedures for a given plant extract were alike at 95% confidence level ($F_{exp}=8.91$, $F_{crit}=10.13$, $F_{exp}<F_{crit}$ at $P=0.05$). Thus, the proposed methodology was validated for real samples.

The results obtained in this work for other cereal samples are generally consistent with literature data. TAC of barley was found to be higher than that of rye, wheat and oat as given in the literature [35,37,43]. Also, TAC of wheat and oat were found to be close to each other [4,43]. For the tested cereals, QUENCHER-CUPRAC correlated linearly with QUENCHER-$ABTS^{\bullet+}$ ($r=0.956$) and QUENCHER-$DPPH^{\bullet}$ ($r=0.976$) (Fig. 4). Generally, the TAC results of cereals correlated well among each other, because all methods were electron transfer-based assays [54,55] having a similar mechanism.

4. Conclusions

The direct measurement of TAC values of cereals without preliminary solvent extraction and hydrolysis procedures was performed by the QUENCHER method using the CUPRAC reagent (*i.e.* essentially cupric neocuproine) for the first time. In this work, original CUPRAC assay parameters [38] were successfully incorporated into the QUENCHER approach for measuring TAC of powdered solid food samples, and this novel method was subsequently named as QUENCHER-CUPRAC to give a reliable estimate of the actual antioxidant capacity of cereals. With this assay, the TAC originating from soluble and insoluble fractions of cereals was measured simultaneously, because some matrix-bound phenolics were forced to solubilize by coupled redox reactions having a higher equilibrium constant. The QUENCHER-CUPRAC method generally produced higher trolox-equivalent TAC values than the corresponding assays with $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ reagents. Possible reasons for this behavior were evaluated as favorable (neutral) pH at which phenolics are partly ionized and therefore more easily oxidized, favorable redox potential capable of oxidizing most phenolics, and favorable kinetics with the outer-sphere electron-transfer agent cupric neocuproine. Additional advantages of the CUPRAC reagent are relative insensitivity to air, sunlight and solvent type (as opposed to DPPH [56]), greater stability and easier accessibility compared to those of radicalic reagents, ABTS and DPPH. In the future, QUENCHER-CUPRAC methodology is believed to be applicable to relatively insoluble food matrices such as fruit and vegetables, pulses, nuts, seeds, fried products, bakery products, proteins, coffee, and a wider spectrum of cereal products, as well as to insoluble cosmetic products, such as cream, balm, and powder. The proposed method may directly measure the TAC of insoluble protein matrix by avoiding preliminary extraction and hydrolysis procedures. As an example from the literature, TAC of defatted soy protein isolate as protein source was determined using a modified ORAC procedure with some overestimation, but the authors discovered that the

temperature of the sample varied throughout the procedure affecting the rate of radical formation, and concluded that the QUENCHER-ORAC$_{FL}$ procedure is suitable for the investigation of anti-radical activities of insoluble materials, but cannot be applied to large sampling for screening purposes [57]. The proposed QUENCHER-CUPRAC method, being less complicated and kinetically favorable, is believed to be potentially advantageous in these cases. Preliminary experiments revealed that the QUENCHER-CUPRAC approach improved the extraction from a defatted soy protein isolate (containing minimum 90% protein) using an urea buffer [58] instead of the conventional ammonium acetate in the CUPRAC assay. The proposed method is easily applicable to conventional laboratories not using highly sophisticated and costly equipment, and therefore expected to find wide use among food scientists for TAC measurement of insoluble food matrices.

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