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Factors Influencing the Antioxidant Activity Determined by the ABTS$^{\bullet+}$ Radical Cation Assay

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This study introduces a simple direct antioxidant assay, based on the reduction of the ABTS$^{\bullet+}$ radical cation, and compares it with the myoglobin/ABTS$^{\bullet+}$ assay. The methods give closely similar results, establishing that the antioxidants studied to date in the latter assay act by scavenging the ABTS$^{\bullet+}$ radical cation and not by inhibiting its formation through reduction of ferryl myoglobin or reaction with H_2O_2.

Keywords: ABTS$^{\bullet+}$ radical cation, total antioxidant activity, flavonoid, ascorbic acid, Trolox

INTRODUCTION

A number of assays have been introduced for the measurement of the total antioxidant activity of body fluids,[1-6] food extracts[7-10] and pure compounds.[11-17] Each involves the generation of a different radical, acting through a variety of mechanisms and the measurement of a range of end points (reviewed in[18,19]). In addition two types of approach have been taken: the inhibition assays in which the extent of the scavenging, by H- or electron-donation, of a pre-formed free radical, relative to that of a standard antioxidant compound (usually Trolox) is the marker of antioxidant activity; several other assays involve the addition of an antioxidant to a system generating the radical.

The purpose of this paper is to compare the antioxidant activities derived from the myoglobin/ABTS$^{\bullet+}$ assay with a decolorisation assay in which the ABTS$^{\bullet+}$ radical cation is generated directly by chemical reduction, in the absence of haem protein and H_2O_2. The principle behind the myoglobin/ABTS assay is the formation of the ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which then oxidizes the phenothiazine compound 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) to produce a radical cation (ABTS$^{\bullet+}$).[5] The question of potential interference from direct interaction of the antioxidant to quench ferryl myoglobin is addressed. The results establish that the antioxidants studied to date act by scavenging the ABTS$^{\bullet+}$ radical cation and not by inhibiting its formation through reduction of ferryl myoglobin.

Materials and methods

Trolox (™ Hoffman-La Roche) (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Aldrich Chemical Co., The Old Brickyard,

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Gillingham, Dorset SP8 4BR, UK) was used an antioxidant standard. 2.5 mM Trolox was prepared in 5mM phosphate buffered saline, pH 7.4, for use as a stock standard as previously described.[5] Fresh working standards were prepared daily by mixing 2.5 mM Trolox with PBS.

Pure antioxidants were dissolved to a concentration of 5 mM; α-tocopherol ($\varepsilon_{mM} = 3.26$ at 292 nm), ferulic acid, caffeic acid and hesperetin were dissolved in ethanol (HPLC grade, Rathburn Chemicals Ltd., Caberston Road, Walkerburn, Peeblesshire, Scotland EH43 6AU); quercetin was dissolved in 70% DMSO (dimethylsulfoxide, Aldrich); uric acid was dissolved in 0.5 g/L lithium carbonate; ascorbic and gallic acids were dissolved in fresh 18 MΩ water. Hesperetin, ferulic, chlorogenic and p-coumaric acids were obtained from Extrasynthese (Extrasynthese, B.P. 62, Z.I. Lyon-Nord, 69726 Genay Cedex, France) and the other pure substances obtained from Sigma-Aldrich (Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset BH12 4QH).

Assay protocol—myoglobin/ABTS assay

The principle behind the myoglobin/ABTS assay is the formation of the ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which converts the phenothiazine compound 2,2′-azinobis-(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS) to produce a radical cation ($ABTS^{\bullet+}$).[5] This blue-green chromogen displays characteristic absorption maxima at 645, 734 and 815 nm. In the presence of antioxidants the radical cation is suppressed to an extent and on a timescale dependent on the activity of the antioxidant. The decrease in absorbance at 734nm is compared with that of Trolox, the water-soluble vitamin E analogue, as an antioxidant standard. The final concentrations used were: 2.5 μM metmyoglobin, 150 μM ABTS, 75 μM H_2O_2. Absorbances were read with a Cobas Bio centrifugal analyser 6 minutes after the addition of hydrogen peroxide and a dose-response curve for Trolox over the range of 0–21μM (final concentrations) derived, using a logit/log 4 plot. Intra-assay precision was in the range 98.5–99.5% and inter-assay precision was 97.5%.

Assay protocol—decolorisation assay

$ABTS^{\bullet+}$ was prepared by reacting a 5 mM aqueous solution of ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) (Aldrich) with the oxidizing agent, manganese dioxide (Sigma-Aldrich) on a Whatman no. 5 filter paper (Whatman International Ltd., St. Leonard's Road, Maidstone, Kent ME 16 OLS, UK).[14] Excess manganese dioxide was removed by filtration through a 0.2 μM Whatman PVDF syringe filter. This solution was then diluted in 5mM phosphate buffered saline (PBS), pH 7.40, to an absorbance of 0.700 (± 0.020) at 734nm in a 1 cm cuvette and incubated at 30°C. 1.0 ml of $ABTS^{\bullet+}$ solution ($A_{734nm} = 0.700 \pm 0.020$) was added to aliquots of Trolox standards or antioxidant samples and vortex-mixed for 30 seconds; an absorbance reading was taken in a temperature-controlled spectrophotometer cuvette at 30°C exactly 1 minute after initial mixing (test). PBS blanks and appropriate solvent blanks were run in each assay. Triplicate determinations were made at each dilution of the standard and samples (Trolox final concentrations 0–25 μmol/L) and the absorbance plotted as a percentage of the blank value. The activity of antioxidants was estimated at a minimum of three different concentrations within the range of the dose-response curve and the mean value derived as the TEAC (Trolox Equivalent Antioxidant Capacity) value. Each compound was assayed on a minimum of 3 separate days (i.e. at least 27 different determinations).

RESULTS

The relationship of the inhibition of absorbance at 734nm with concentration of the Trolox, ascorbate and urate is shown in Figure 1 for the myoglobin/$ABTS^{\bullet+}$ assay, in comparison with the

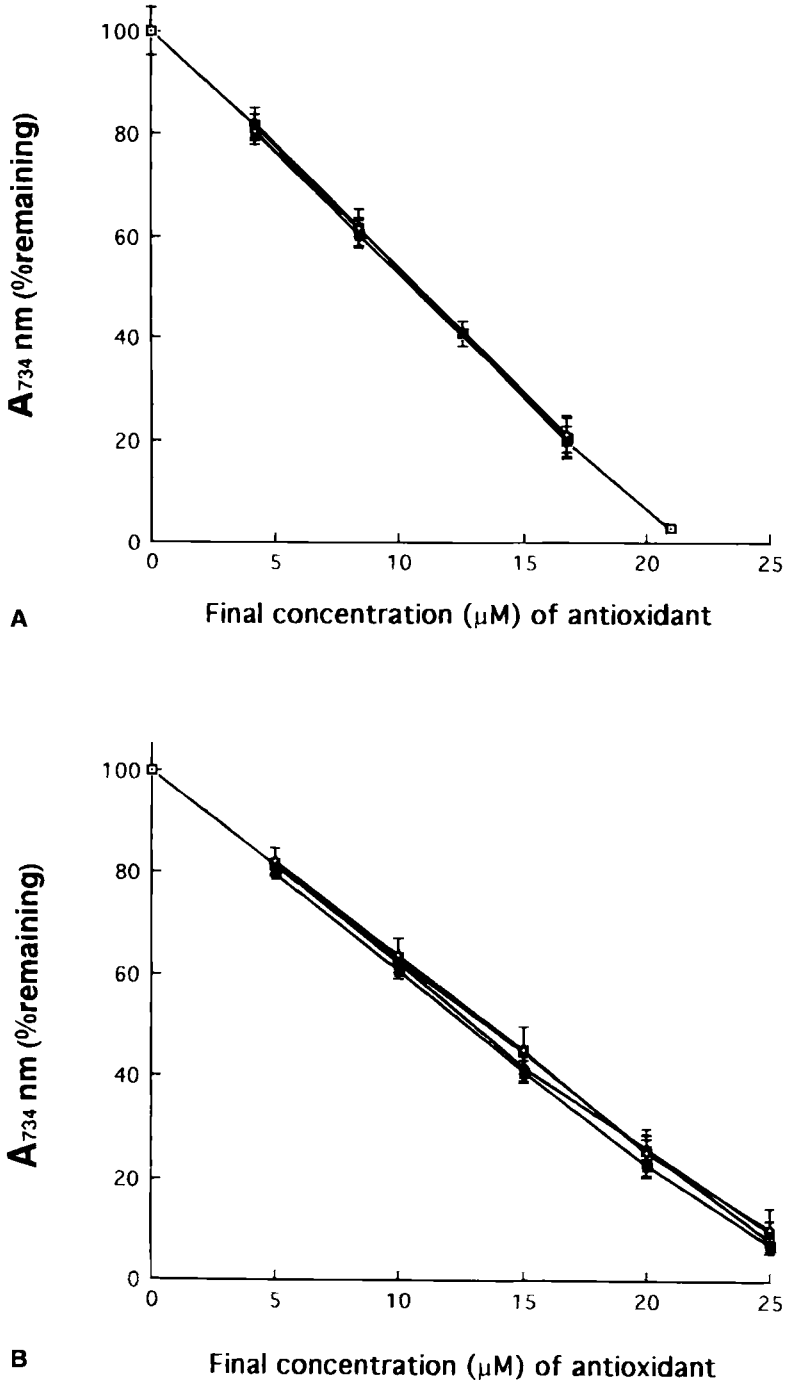

FIGURE 1 Standard curves for the interaction of ABTS$^{\bullet+}$ with Trolox ▣, ascorbic acid ◇, uric acid ■, α-tocopherol ◆. (A) The myoglobin/ABTS assay, (B) the decolorisation ABTS$^{\bullet+}$ assay. The y axis represents the % of the maximal absorbance at 734nm remaining [100% = 0.70].

$ABTS^{\bullet+}$ decolorisation assay. The results show that the reduction of the $ABTS^{\bullet+}$ radical cation follows the same course with Trolox as with ascorbate and urate, and that the response in the two assays for the concentration-dependency of Trolox is closely similar. Comparison of the antioxidant activities of a range of flavonoids compared with the physiological antioxidants α-tocopherol, ascorbic acid and uric acid in the myoglobin/$ABTS^{\bullet+}$ assay, and the $ABTS^{\bullet+}$ decolorisation assay are shown in Table I. The results from the two approaches are highly consistent whether the assay involves pre-formed $ABTS^{\bullet+}$ radical cation or the assay in the presence of activated myoglobin, with the exception of ferulic, p-coumaric and caffeic acids where the variation is ca. 8–10% across the two methods. This variation is not due to a reaction of these compounds with myoglobin/H_2O_2 since the rate constants for the reaction of the hydroxycinnamates with ferryl myoglobin have been shown to be relatively low.[20]

DISCUSSION

The studies described here demonstrate that the scavenging of the $ABTS^{\bullet+}$ radical cation is the mechanism of antioxidant activity in both forms of the assay (at least for the compounds tested here) and that the presence of a peroxidase substrate such as myoglobin (an alternative approach to generation of $ABTS^{\bullet+}$) does not alter the results.

Ascorbic acid is a very efficient reductant of ferryl myoglobin radical.[21-23] Its identical standard curve with that of Trolox in the ferryl myoglobin/$ABTS^{\bullet+}$ assay underlines its action in this assay as a reductant of $ABTS^{\bullet+}$ radical cation rather than direct reduction of ferryl myoglobin. This is substantiated by the similarity of results obtained for the antioxidant activities of the antioxidants described whether in the presence or absence of the myoglobin/H_2O_2 system.

Both these ABTS methods are based on the extent of reduction of the $ABTS^{\bullet+}$ radical cation at a fixed time point and not on the rate of reduction. Thus the time-point selected (in relation to the concentration of myoglobin, hydrogen peroxide, ABTS) is a crucial feature of the assay. A measurement point is selected such that the values for the range of antioxidants (whether in body fluids, food extracts or pure substances etc.) to be studied fall within the appropriate part of the Trolox standard curve. Clearly, a system involving excess antioxidant would not be suitable for this assay. Methods dependent on the measurement of delay time or lag phase to oxidation take both the extent and rate of reduction of the radical into account.[1,4,]

The results obtained with both the $ABTS^{\bullet+}$ decolorisation assay and the ABTS/myoglobin assay for a large family of flavonoids as radical

TABLE I TEAC values determined for antioxidant substances, compared to previously reported values.

Substance	TEAC ± 1 sd [n] ($ABTS^{\bullet+}$ decolorisation method)	TEAC ± 1 sd [n] (myoglobin/ABTS)
ascorbic acid	0.99 ± 0.03 [11]	0.99 ± 0.04 [9]
uric acid	1.02 ± 0.02 [5]	1.00 ± 0.06 [5]
α-tocopherol	1.00 ± 0.03 [3]	0.97 ± 0.01 [3]
gallic acid	3.00 ± 0.04 [6]	3.01 ± 0.05 [7]
p-coumaric acid	2.07 ± 0.14 [3]	2.22 ± 0.06 [7]
chlorogenic acid	1.14 ± 0.05 [4]	1.24 ± 0.02 [6]
ferulic acid	2.17 ± 0.04 [3]	1.90 ± 0.02 [9]
caffeic acid	1.13 ± 0.03 [3]	1.26 ± 0.01 [3]
quercetin	4.91 ± 0.22 [4]	4.72 ± 0.10 [6]
hesperetin	1.32 ± 0.05 [3]	1.37 ± 0.08 [3]

scavengers are consistent with the chemistry of these compounds[12] in terms of the structural characteristics underlying the delocalisation of electrons across the structures of the resulting antioxidant radical and the number and positions of the hydroxyl groups.

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