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# The Antioxidant Activities of Seasonings Used in Asian Cooking. Powerful Antioxidant Activity of Dark Soy Sauce Revealed Using the ABTS Assay

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Scavenging of the ABTS (2,2′-azinobis[3-ethylbenzothiazoline-6-sulphonate])-derived nitrogen-centred radical cation ($ABTS^{\bullet+}$) was used to compare the total antioxidant activities of several seasonings used in Asian cooking. The results were expressed as Trolox equivalent antioxidant capacity (TEAC). The TEAC activities of dark soy sauces were found to be exceptionally high. In evaluating the TEAC of commercial products, attention must be paid to the addition of preservatives by manufacturers to the seasonings tested. Sodium benzoate (a preservative added to several seasonings) did not react significantly with $ABTS^{\bullet+}$, but the sulphite content of certain white wines may have led to an over-estimation of their TEAC.

*Keywords:* ABTS, nitrogen-centred radical, wine, flavonoid, phenolic compounds, soy, soy sauce, hydrogen peroxide, sulphite, preservatives, antioxidants

## INTRODUCTION

There has been considerable recent interest in the beneficial health effects of drinking certain beverages, including wines, green and black teas and even beer.[1–5] Many of these proposed health benefits have been attributed to the antioxidants present in these beverages; usually the focus has been on phenolic compounds such as ferulic acid, catechins, and other flavonoids.[1–7] For example, red wines have high total antioxidant activity (TAA) *in vitro*, of which at least 25% can be accounted for by the major phenolic constituents.[7] Similarly, ~78% of the antioxidant activity of green tea was accounted for by the catechins and catechin-gallate esters.[7] White wines have, in general, lower TAA than red wines.[7]

In the present paper, we have determined the total antioxidant activity of seasonings used in Asian cooking. As an assay of TAA, we used the ABTS method.[8] This method is simple, reliable, reproducible and has advantages over several other methods, such as those involving DPPH (1,1-diphenyl-2-picrylhydrazyl) radical.[7–9] In general, results obtained using the ABTS assay are in agreement with chemical predictions and

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with the results of other assays such as effects on the peroxidation of low-density lipoproteins (reviewed in Ref. [7]) Essentially, persulphate is used to oxidize ABTS (2,2′-azinobis[3-ethylbenzothiazoline 6-sulphonate]) to a nitrogen-centred radical cation, $ABTS^{\bullet+}$, whose reaction with antioxidants can be followed spectrophotometrically. The assay is calibrated with the water-soluble $\alpha$-tocopherol analogue Trolox, and results expressed in terms of Trolox equivalent antioxidant capacity (TEAC).[10] We also discovered that "antioxidants" added by manufacturers as preservatives can sometimes confound the determination of TAA on beverages – an example being sulphites in white wine.

## MATERIALS AND METHODS

All chemicals were obtained from Sigma-Aldrich Pte-Ltd., Singapore, except for ethanol and $HgCl_2$ (Merck, Germany), $H_2SO_4$ (JT Baker, USA), $Na_2SO_3$ and KOH (Fluka, Switzerland) and $H_2O_2$ (BDH, UK). The seasonings used were sweet dark soy sauce (manufactured in Malaysia; no preservatives indicated on label), Kung Bo sauce (Sin Hwa Dee Foodstuff Industries, Singapore; containing soya bean and benzoic acid), HP sauce (HP Foods Ltd., UK; no preservatives indicated but stated to contain some soy sauce), tomato sauce (Maggi, Malaysia, no preservatives indicated), black vinegar (Great Wall Brand, Tianjin Foodstuffs, PR China, no preservatives indicated), Chinese cooking wine (18% (v/v) ethanol; Shao Hsing Hua Tiao Chiew Lam Hock Leong, Singapore), Chinese rice wine (SSH Marketing, Singapore, 12.6% (v/v) ethanol), dark soy sauces (Tiger brand, Singapore; Woh Hup, Singapore; and Tai Hua, Singapore, all contain sodium benzoate), sweet soy sauce (ABC Central Food Industry, Indonesia), chili sauce (Indofoods, Singapore; imported from Indonesia, contains sodium benzoate and chili), oyster and plum sauces (both Woh Hup Food Industries Pte Ltd, Singapore, no preservatives indicated for either), hoisin sauce (Lee Kum Kee Hong Kong; contains soy beans and chili pepper), sweet flour sauce (Sin Ngee Seng, Malaysia, contains sodium benzoate), soup for SOBA noodles (SOBA sauce; Yamasa Corporation, Japan; contains soy sauce) and sesame oil (Chee Seng Oil, Pagoda brand, Singapore, no preservative indicated).

### ABTS Assay

This was carried out essentially as described in Ref. [10], using Trolox as a reference standard. A single time point (1 min) was selected for determination of antioxidant activity unless otherwise stated. ABTS was dissolved in water to a final concentration of 7 mM and potassium persulphate added to a final concentration of 2.45 mM. The mixture was allowed to stand at room temperature overnight ($\geq 12$ h) in the dark. The $ABTS^{\bullet+}$ solution was diluted to an absorbance of 0.70 at 734 nm in phosphate-buffered saline and 10 μl of antioxidant compound or Trolox standard added to 1 ml of $ABTS^{\bullet+}$ solution. Absorbance was measured 1 min after initial mixing. Controls without $ABTS^{\bullet+}$ were used to allow for any absorbance of the seasonings themselves. One ml of PBS (instead of the $ABTS^{\bullet+}$ solution) was mixed with the seasoning at the same dilution and the absorbance at 734 nm read after 1 min. The most-concentrated dark soy sauce preparation (20-fold dilution) gave an absorbance of $\sim 0.03$ at 734 nm. Absorbances at 500 nm were $\sim 0.12$ for the most-concentrated preparation used.

### ABTS Assay on Sesame Oil

The $ABTS^{\bullet+}$ solution was diluted to an absorbance of 0.70 at 734 nm in ethanol instead of the phosphate-buffered saline. Dilutions of the sesame oil and of the Trolox standard were also in ethanol.

*Sulphite* was measured by the fuschin-formaldehyde method.[11] Fuchsin reagent was prepared fresh before use by adding 1.6 ml of a 3% (w/v) basic fuchsin solution in ethanol (left stirring overnight at room temperature) to 93.6 ml de-ionized water containing 4.4 ml concentrated $H_2SO_4$. After thorough mixing, 0.43 ml 37% (v/v) formaldehyde was added. The solution was decolourized by addition of 400 mg activated charcoal. After repeated shaking over a period of 15 min, the solution was filtered.

To a 2.5 ml sample of beverage, 0.5 ml 1% (w/v) KOH (in ethanol) was added and mixed thoroughly. One ml of a saturated aqueous solution of $HgCl_2$ was then added and mixed. After centrifuging at 3000 rpm (2000 *g*) for 10 min, 1.0 ml of the clear supernatant was mixed with 4.0 ml fuchsin reagent. After 15 min at room temperature, the absorbance of each sample was measured at 580 nm. A standard curve was prepared using $Na_2SO_3$ solution (80–480 nmol).

*Hydrogen peroxide* was measured by the FOX assay as described previously.[12] Controls in the presence of added catalase[12] were carried out to correct for any absorbance of the beverages themselves.

## RESULTS

### Total Antioxidant Activity (Trolox Equivalent Antioxidant Capacity, TEAC) of Red Wine

The mean TEAC of red wines is quoted as 12–24 mM, and there is a considerable variation from wine to wine.[7] In agreement with this, we found TEAC values of $14.93 \pm 0.91$ (mean $\pm$ SD, $n = 6$) for one red wine ("Turning Leaf" Cabernet Sauvignon, 1995, California, USA) and 22.2 (mean of duplicate determinations) for another (French Bordeaux).

### Total Antioxidant Activity of Seasonings

We then applied the ABTS method to examine the TEAC of seasonings used in Asian cooking, plus a few other commonly-used seasonings for comparison. The TEAC values were, in general, less than that of the red wines tested (Table I). Several different amounts of each sauce were tested, with appropriate controls to allow for any absorbance due to the sauce itself, and TEAC values were calculated from ranges where the fall in absorbance was proportional to the amount of sauce added. Dilutions of 20–400-fold were usually used for the dark soy sauces, and all three dark soy sauces tested had extremely high TEAC values (Table I). In fact, these values may be underestimates since the data in Table I were based on measurements of $ABTS^{\bullet+}$ depletion at a 1 min time point. Reaction with Trolox was complete by then (Figure 1). However, $ABTS^{\bullet+}$ depletion continued with both the red wine and the dark soy sauce (Figure 1). For example, in one experiment the TEAC of the "Turning Leaf" red wine was 15.3 at 1 min, but 19.5 at 5 min and 26.8 at 50 min. Similarly, the dark soy sauce (Tai Hua Brand) assayed in parallel increased to a TEAC of 200 at 5 min and 318 at 50 min.

TABLE I Trolox equivalent antioxidant capacities of seasonings

| Seasoning type | TEAC (mM) (mean $\pm$ SD, $n = 3$) |
|---|---|
| HP sauce | $9.80 \pm 0.53$ |
| Tomato sauce | $3.23 \pm 0.06$ |
| Kung Bo sauce | $9.17 \pm 0.91$ |
| Black vinegar | $10.37 \pm 1.00$ |
| Chinese cooking wine | $6.17 \pm 0.35$ |
| Chinese rice wine | $0.36 \pm 0.13$ |
| Chili sauce | $11.10 \pm 1.51$ |
| **Dark soy sauce (Tiger brand)** | **$147.33 \pm 9.45$** |
| **Dark soy sauce (Tai Hua brand)** | **$127.33 \pm 4.93$** |
| **Dark soy sauce (Woh Hup brand)** | **$47.1 \pm 1.93$** |
| Sweet dark soy sauce (Zara) | $28.73 \pm 2.54$ |
| Sweet soy sauce | $35.43 \pm 1.60$ |
| Oyster sauce | $5.58 \pm 2.75$ |
| Plum sauce | $3.53 \pm 1.27$ |
| Hoisin sauce | $13.60 \pm 2.16$ |
| Sweet flour sauce | $10.40 \pm 0.53$ |
| Soba sauce | $9.27 \pm 0.64$ |
| Sesame oil | $3.27 \pm 1.08$ |

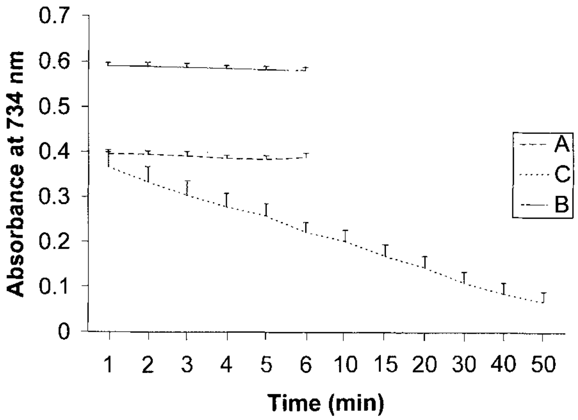


FIGURE 1 Time-course of $ABTS^{\bullet+}$ depletion in the presence of Trolox (B) sulphite (A) or dark soy sauce (Tai Hua Brand) (C). Error bars indicate mean ± SD, $n \geq 3$.

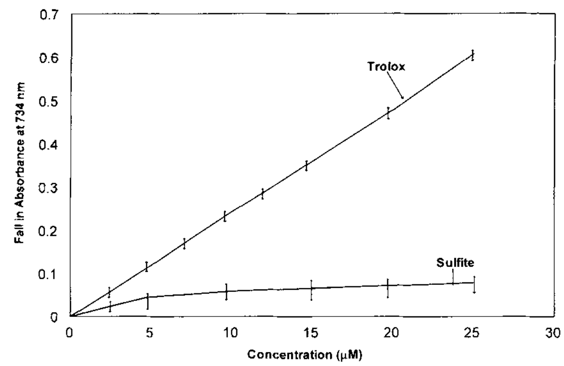


FIGURE 2 Antioxidant activity of sulphite and of Trolox in the ABTS assay. Reagents were added at the concentrations stated and ability to scavenge $ABTS^{\bullet+}$ determined as described in the Materials and Methods section. Data are mean ± SD, $n \geq 3$.

TABLE II Generation of $H_2O_2$ by dark soy sauces

| Sauce brand | $[H_2O_2]$ detected in Fox assay (μM) (mean ± SD, $n = 3$) |
|---|---|
| Tiger | 3.80 ± 0.82 |
| Tai Hua | 4.03 ± 0.38 |
| Woh Hup | 0.58 ± 0.06 |

Controls were performed with added catalase to correct for any absorbance of the dark soy sauces themselves in the FOX assay.[12] For comparison, instant coffee (Nescafe, Nestle, Singapore) generated > 100 μM $H_2O_2$ when assayed in parallel.[12]

## Hydrogen Peroxide Generation

Several beverages have been shown to generate $H_2O_2$ *in vitro*.[12–15] We therefore examined the ability of the dark soy sauces to produce $H_2O_2$, but very little was detected (Table II).

## The Effect of Preservatives

Several agents are added by manufacturers to preserve seasonings and beverages. To avoid misleading conclusions, it is important to check that such agents do not contribute to the TAA activity. High levels of sodium benzoate are added to several seasonings (Materials and Methods section), but this compound had no effect on the $ABTS^{\bullet+}$ radical when tested at concentrations up to 1 mM. This is not surprising since benzoic acid does not have a phenolic –OH group. Similarly, ethanol (present in the cooking wines and the red wines) had no TEAC activity (data not shown), as expected.[8,10] Sulphite, frequently used as a food and beverage preservative,[16,17] was weakly reactive in the ABTS assay (Figure 2) although at concentrations above 5 μM the reactivity with $ABTS^{\bullet+}$ was no longer linear with concentration and the standard deviations were larger than those obtained with Trolox (Figure 1) or with ascorbate (data not shown). The reaction of $SO_3^{2-}$ with $ABTS^{\bullet+}$ was complete at the 1 min time point (Figure 1). However, even this limited antioxidant activity of $SO_3^{2-}$ could be enough to confound determinations of the TAA of some white wines. For example, white wine A (Ernest and Julio Gallo, USA) contained 0.95 mM sulphite, which could have accounted for 5.7% of its measured TEAC (~3 mM). White wine B, (Bordeaux, France) contained 2.82 mM sulphite, which could have accounted for about 12.3% of its TEAC (~4 mM). White wine C (Chardonnay, Chalk Hill, USA) contained 3.36 mM sulphites, which could have accounted for 16.6% of its TEAC (~3.5 mM). By contrast, the measured sulphite content of several red wines examined was found to be too small to affect their TEAC.

## DISCUSSION

Our data show that many seasonings used in cooking or as condiments show some ability to scavenge the $ABTS^{\bullet +}$ radical. This is not surprising because most are of plant origin and plants are rich in antioxidants, especially phenols. However, our most striking results are for the dark soy sauces, which show activities markedly greater than many beverages[5,7] or the other seasonings tested. They appear to contain a complex mixture of antioxidants, many of which react quickly with $ABTS^{\bullet +}$, but others more slowly (Figure 1). Thus 1 ml of the most effective dark soy sauce could have the same "total antioxidant activity" as approximately 10 ml of a red wine. Thus in an Asian meal cooked with 10 ml of soy sauce, the TEAC could be equivalent to that of a 100 ml glass of red wine. Of course, this calculation assumes that cooking does not modify the antioxidant activity.

Much interest has been given to anti-cancer constituents in soybeans, including the isoflavones.[18] Isoflavones are known to show antioxidant activity *in vitro*, including an ability to react with the $ABTS^{\bullet +}$ radical,[19–21] but further experiments are needed to examine their presence in, and the extent to which they could contribute to the antioxidant activities of, dark soy sauce. Pro-oxidants, able to cause DNA strand breakage *in vitro*, have also been identified in soy sauce.[22] However, the dark soy sauces tested contained little $H_2O_2$, as detected by the FOX assay, in contrast with black teas, green teas and coffees.[12,25,26] Of course, further experiments[27] are needed to show that these *in vitro* antioxidant (or pro-oxidant) activities are relevant to effects *in vivo*, although evidence for the absorption of some isoflavonoids is increasing.[18,23]

A second point arising from our data is the importance of considering the antioxidant activities of added preservatives when determining total antioxidant activities of beverages and sauces. For example, ascorbate is often added to fruit juices.[24] Sulphite is added to many foods, including white wines.[16,17] The TEAC of white wines is less, on average, than that of red wines, as is their phenolic content.[7] Our data show that the high levels of sulphite present in some white wines could artificially raise the TEAC values, so that the "correct" values bring white wines further below red wines on the recently described "antioxidant hierarchy" of food servings.[5] On the basis of the *in vitro* $ABTS^{\bullet +}$ assay,[5] the ideal meal would seem to be food cooked with dark soy sauce accompanied by a glass of red wine.

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