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Hydrogen Peroxide Generation in Caco-2 Cell Culture Medium by Addition of Phenolic Compounds: Effect of Ascorbic Acid

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Phenolic compounds have recently attracted special attention due to their beneficial health effects; their intestinal absorption and bioavailability need, therefore, to be investigated and Caco-2 cell culture model appeared as a promising tool. We have shown herein that the addition of a grape seed extract (GSE) to Dulbecco's modified Eagle's medium (DMEM) used for Caco-2 cell culture leads to a substantial loss of catechin, epicatechin and B2 and B3 dimers from GSE in the medium after 24 h and to a production of hydrogen peroxide (H_2O_2). When 1420 μM ascorbic acid is added to the DMEM, such H_2O_2 production was prevented. This hydrogen peroxide generation substantially involves inorganic salts from the DMEM. We recommend that ascorbic acid be added to circumvent such a risk.

Keywords: Cell culture medium; Grape seed extract; Phenolic compounds; Ascorbic acid; Concentration; Hydrogen peroxide

INTRODUCTION

Polyphenolic compounds are present in many fruits, vegetables, and certain beverages[1–3] including tea and wines. Their beneficial effects on health have been reported in coronary heart disease and stroke[4,5] as well as cancer;[6,7] recently, attention has particularly focused on catechin, epigallocatechin, epigallocatechin gallate and quercetin,[8–12] on another flavonoid such as chrysin[13,14] and also on olive oil phenolics.[15,16] In these studies, their metabolism, bioavailability, and antioxidant effects have been examined on cells in culture and the Caco-2 epithelial intestinal cells were generally used as the model system. Elsewhere, many studies have emphasized the antioxidant effects of these molecules;[8–12] they are able to scavenge free radicals and singlet oxygen, but they do not seem to react quickly with hydrogen peroxide (H_2O_2).[17,18] Moreover, phenolic compounds that possess multiple adjacent hydroxyl (–OH) groups linked to their aromatic ring can interact with metal ions to form chelates, or may reduce Fe^{3+} and Cu^{2+} ions to Fe^{2+} and Cu^{+}, respectively, through a redox interaction between the phenol and metal.[19] Thus, phenolic compounds can exhibit antioxidant effects by chelating transitional metals involved in free radical production, and in an other hand, they can be oxidized and can exhibit pro-oxidant effects *in vitro* depending on some reaction conditions[20–22] by reducing metal ions to their lower oxidation states. Our work originated in measuring the bioavailability of phenolic compounds from a grape seed extract (GSE) using Caco-2 cells: these compounds rapidly disappeared after their addition in the culture medium before adding any cells, and we suspected that they were oxidized herein. Consequently, we have investigated the effect of each component of the culture medium for Caco-2 cells, Dulbecco's

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modified Eagle's medium (DMEM). More recently and during this work, Long *et al.*[23] identified an artifact in cell culture media that can affect most phenolic compounds on cells in culture, generating high levels of hydrogen peroxide. This group has also shown that some beverages rich in phenolic compounds, including coffee and tea, can generate significant amounts of H_2O_2 *in vitro*.[24] Such H_2O_2 generation was also measured in this paper.

MATERIALS AND METHODS

Materials

(+)-catechin, (−)-epicatechin and gallic acid, were obtained from Aldrich. Procyanidins dimers B1, B2, B3, B4, were obtained by Dr Teissèdre from grape seeds as detailed below.

Extraction and Isolation of Crude Procyanidins

Grape seeds (*Vitis vinifera*), 150 g, were extracted with methanol as described by Bourzeix *et al.*[25] and by Weinges and Piretti[26] The extract (3 ml, 300 mg) was chromatographed on Fractogel TSK HW-40 (s) (25–40 μm) (450 × 25 mm^2 id) with methanol as eluant, using an ISCO (Lincoln, NE, USA) model UA-5 absorbance monitor set at 280 nm, a peristaltic Miniplus2 pump (GILSON Inc, Middletin, WI, USA) and an ISCO 328 fraction collector. Ten fractions containing procyanidins were collected. Semi-preparative HPLC was performed with a Waters 510 pump (Waters, Miford, MA, USA) a U6K injector, and a Hewlett–Packard (Palo Alto, CA, USA) model 1050 UV–Vis. detector set at 280 nm. The column was a Water RCM Novapak C18 25 × 100 mm^2, 4 μm particle size. Elution was carried out by a linear gradient of 0–500 ml/l methanol with the solvent described below at 2 ml/min.

TLC Analysis

Silica plates (DC Alufolien–Kieselgel 60, 0.2 mm thick, Merk, EM Separation technology, Gibbstown, NJ, USA) were developed with toluene/acetone/formic acid (3:3:1 v/v/v) as described by Lea *et al.*[27] The plates were visualized by spraying with a solution of vanillin (100 g/l) in concentrated HCl.

HPLC Analysis

A Hewlett–Packard, model 1090 with three low pressure pumps and a diode array UV detector coupled to an Hewlett–Packard Chem station was used for solvent delivery system and detection. A Hewlett–Packard column Nucleosil 100 C18, 250 × 4 mm^2, 5 μm particle size was used for the stationary phase with a flow of 0.7 ml/min. The solvents used for separation were: solvent A: 50 mM dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid; solvent B: 20% A with 80% acetonitrile; and solvent C: 0.2 M orthophosphoric acid adjusted with ammonia to pH 1.5; the solvent gradient conditions have been described by Lamuela-Raventos *et al.*[28] Temperature was thermostated at 25°C.

Incubation of Medium

Grape seed extract from La Gardonnenque S.C.A. (Cruviers Lascours, France) was added either to the culture medium or to each component of the medium (Gibco BRL, Cergy, France) at 37°C and at the final concentration of 0.4 mg/ml. The DMEM contained 10% fetal calf serum, 2% L-glutamine, 1% nonessential aminoacids, and 100 U/ml penicillin G and 100 μg/ml streptomycin. Each culture medium component such as inorganic salts, aminoacids, vitamins, fetal calf serum, antibiotics, D-glucose plus phenol red, was separately tested at the same concentration that in the DMEM. GSE was added at the final concentration stated and the mixture was rapidly vortexed; an aliquot was immediately withdrawn (zero time: T_0) and submitted to HPLC analysis. The remaining was allowed to stand at 37°C in a 5% CO_2–95% air atmosphere for 24 h (T_{24}) and then analyzed. The pH was monitored during this experimental period.

Hydrogen Peroxide Measurement

It was performed according to Nourooz-Zadeh *et al.*[29] and Long[23] using the ferrous ion oxidation-xylenol orange (FOX) method. An aliquot of sample (DMEM, DMEM plus GSE at 0.4 mg/ml, or DMEM plus GSE at 0.4 mg/ml plus 1420 μM ascorbic acid) (90 μl) was mixed with 10 μl of methanol and immediately followed by the addition of 0.9 ml FOX reagent,[30,31] vortexed and incubated for 30 min. Solutions were centrifuged at 13,000*g* for 5 min at room temperature and the absorbance was read at 560 nm against a methanol blank.

O_2 Electrode Assay

An oxymeter 9143 (Hanna Instrument, Ronchi, Italy) equipped with a Clark electrode was used. Dissolved O_2 was measured at T_0 and T_{24} in DMEM, in DMEM containing GSE at 0.4 mg/ml and in DMEM containing GSE at 0.4 mg/ml plus 1420 μM ascorbic acid. Oxygen levels are given as μg/ml.

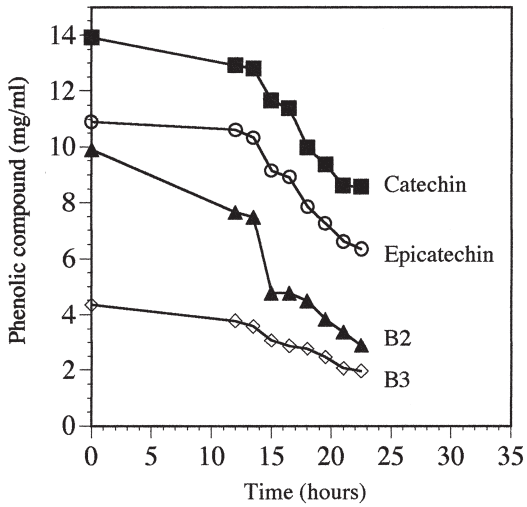

FIGURE 1 Gradual change of phenolics from a GSE mixed (0.4 g/l) with DMEM without adding any cells.

Statistical Analysis

Data are given as mean ± SEM; they were analyzed by one way analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) method using Stat View 4.5 (Abacus Concepts, Inc., Berkeley, CA). A significance level of $p < 0.05$ was adopted for all comparisons.

RESULTS

The GSE contained 4.5% catechin, 4.2% epicatechin, 1.0% B1, 2.8% B2, 1.2% B3 and 0.5% B4. We began by adding this extract at 0.4 g/l to the DMEM at 37°C. The fates of some GSE phenolics added to the medium are reported in Fig. 1. Each phenol assayed in the cell culture medium continuously disappeared: after 24 h, loss of catechin and epicatechin reached about 40%; dimers B2 and B3 were more affected (−69 and −55%, respectively). The influence of each component of DMEM was then studied and results are summarized in Fig. 2. Fetal calf serum, antibiotics, and vitamins were tested separately at the same concentration as in DMEM and exhibited a weak, but significant effect on the disappearance of the five phenolics assayed after 24 h. On the other hand, aminoacids and a mixture of D-glucose plus phenol red (not shown here) had no significant effect. Adversely, inorganic salts mixture triggered off a general dramatic loss of GSE compounds extending to 95% for catechin. There was no effect of salts on B1 concentration. We suspected that phenolic compounds were oxidized in DMEM. In order to prevent such a phenomenon, ascorbic acid was added to the cell culture medium; several concentrations of ascorbic acid ranging from 57 to 2270 μM in DMEM have been tested. Their effect is shown in Fig. 3: ascorbic acid was effective in maintaining the original concentration of each phenolic compound in the cell culture medium as a function of the dose added, the optimal being at 1420 μM. Consequently, dissolved oxygen was measured either in DMEM or in DMEM plus GSE at 0.4 g/l or in DMEM plus GSE at 0.4 g/l supplemented with 1420 μM ascorbic acid (Fig. 4); after 24 h at 37°C, 41% oxygen disappeared in the presence of GSE and this loss amounted 56% in the presence of GSE plus ascorbic acid. Hydrogen peroxide was measured by the FOX assay[23,29] either in DMEM, or in DMEM plus GSE at 0.4 g/l or in DMEM plus GSE at 0.4 g/l supplemented with 1420 μM ascorbic acid (Fig. 5). The culture medium DMEM only contained traces of H_2O_2 between 0 and 24 h at 37°C in a 5% CO_2–95% air atmosphere. When GSE was added to the DMEM, a substantial amount of H_2O_2 was continuously produced, reaching 138 μM after 24 h incubation. Upon addition of ascorbic acid to this mixture, a burst of H_2O_2 production appeared yielding 68 μM H_2O_2 after 2 h incubation; this production was then stabilized at 45 μM between 11 and 24 h, emphasizing a significant H_2O_2-scavenging activity. No gross variation of pH occurred over the 24-h experiment either in the presence or in the absence of ascorbic acid (Fig. 5). A control to see if the GSE can interfere with the FOX assay was performed using known concentrations of H_2O_2 in the presence or in the absence of GSE and results are summarized in Fig. 6: GSE did not induce a difference in absorbance at 560 nm.

DISCUSSION

Our results showed that phenolic compounds from GSE added to the DMEM easily undergo chemical oxidation. This was evidenced by the development of browning appearance when inorganic salts were mixed with GSE, in the absence of phenol red. Addition of phenolic compounds to cell culture media such as DMEM, McCoy's 5A and RPMI 1640 has been recently reported to generate substantial amounts of H_2O_2.[23] The mechanism involved is not yet known. It has also been reported that ascorbate can itself generate H_2O_2 in DMEM[32] and how ascorbate induces H_2O_2 production needs further investigation. Using a crude GSE containing catechin, epicatechin and dimers B1, B2 and B3, we have confirmed the previously reported effects relative to epigallocatechin and epigallocatechin gallate, catechin and quercetin, showing that phenolic compounds are oxidized in cell culture media and lead to H_2O_2 generation;[23] upon addition of catalase, these authors showed that it was really a production of H_2O_2. Here, we have shown that GSE did not interfere with the FOX assay using known

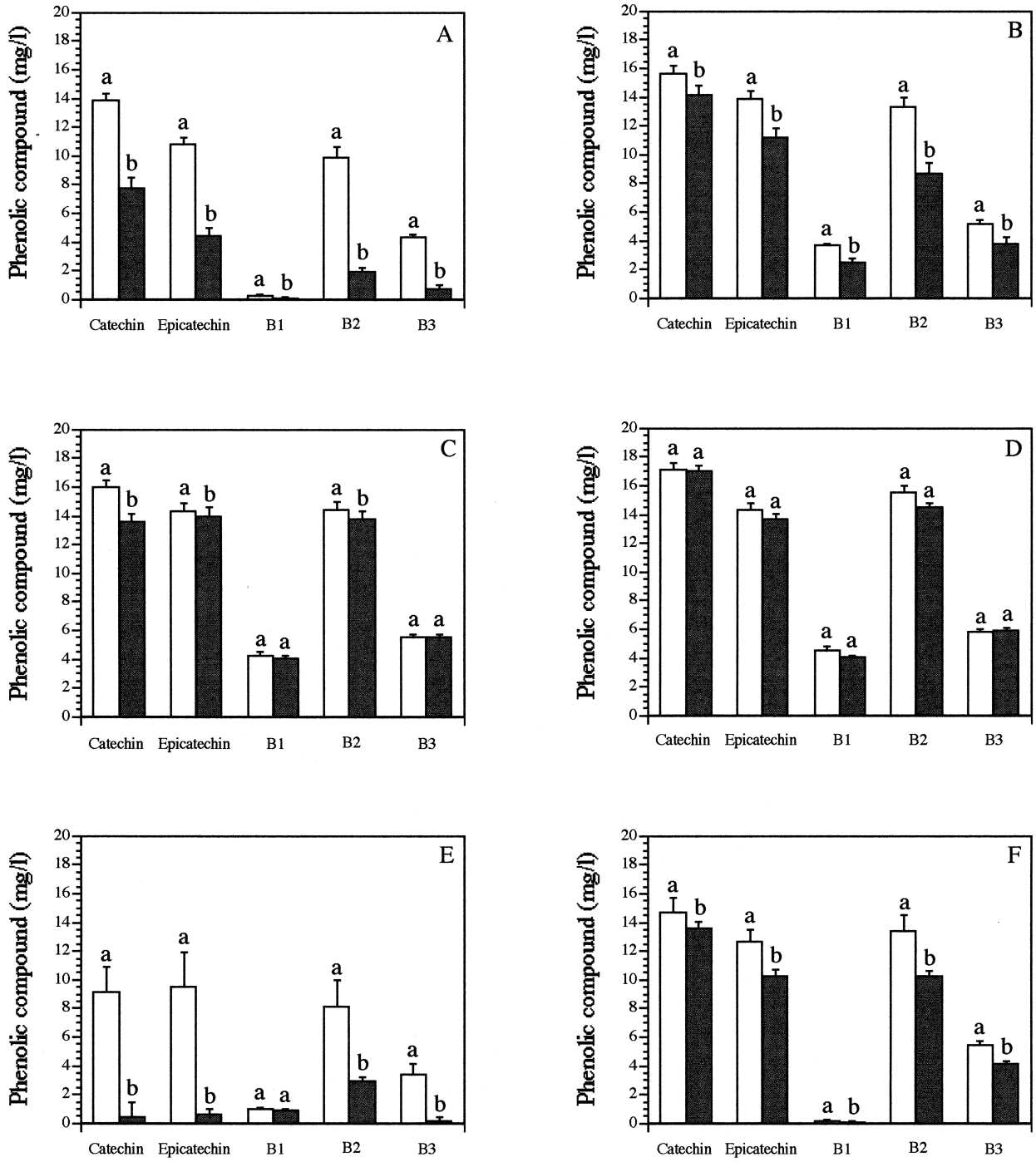

FIGURE 2 Phenolic compounds concentration at 37°C in the medium at zero time (T_0, white bars) and at 24 h (T_{24}, gray bars) after a mixture of GSE (0.4 g/l) with either complete medium culture (A), or fetal calf serum (B), antibiotics (C), amino acids (D), inorganic salts (E) and vitamins (F). Each component B, C, D, E and F taken alone, had the same concentration that in the complete medium culture A. Values are expressed as mean ± SEM ($n = 5$). For each phenolic compound, bars with different letters are significantly different at $p < 0.05$.

concentrations of H_2O_2 (Fig. 6) and that there was no major difference in the pH variation between a medium containing ascorbate and a medium without ascorbate during the 24-h experimental period (Fig. 5). Moreover, we showed that inorganic salts were the major culprits. Avoidance of the changes in the antioxidant capacity of polyphenols upon oxidation is of great interest in order to minimize losses of their health-protecting capacity during cell studies. Polyphenol antioxidant capacity greatly varies depending on its chemical structure, concentration, and oxidation degree.[31,33,34] For instance, changes in the chain-breaking activity and optical density of catechin model systems undergoing chemical oxidation were reported[35] and a notable decrease in chain-breaking activity was associated

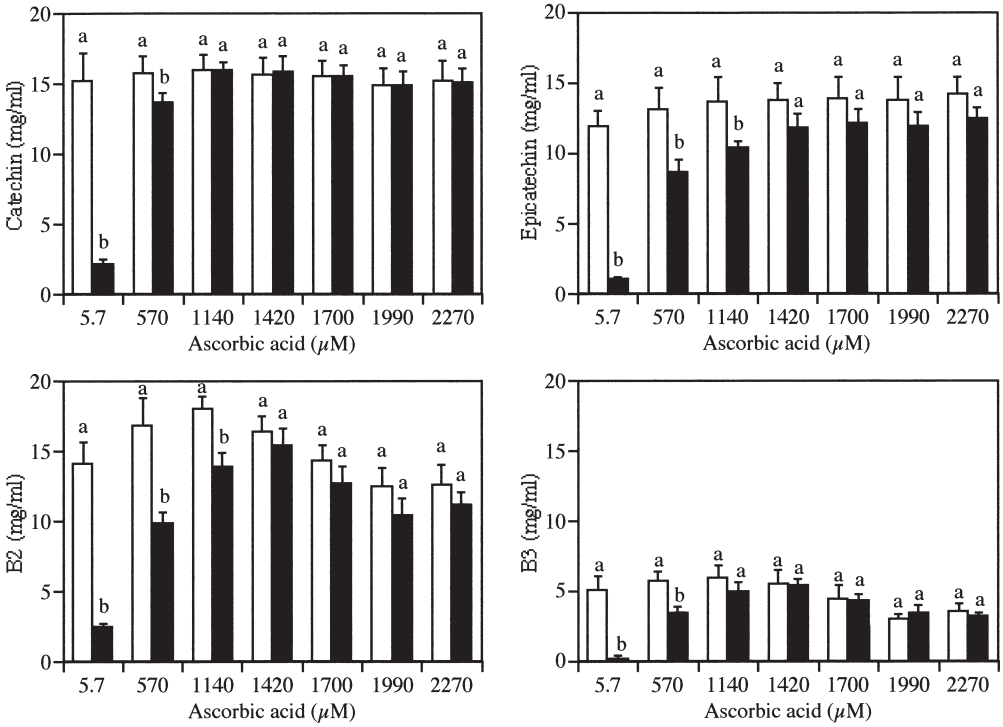

FIGURE 3 Effect of addition of ascorbic acid in the DMEM containing GSE (0.4 g/l) on the evolution of some phenolics of the extract from zero time (white bars) to 24 h (gray bars) at 37°C. Values are expressed as mean ± SEM ($n = 3$). Bars with identical letters are not significantly different ($p < 0.05$).

with the development of browning. We recommend that, in cell culture studies using Caco-2 cells and polyphenols, ascorbic acid be added to the culture medium to avoid oxidation and disappearance of phenolics. Ascorbic acid has also been reported to increase the stability of green tea catechins *in vitro*.[36] Catalase had also been recommended[23] to dispose of H_2O_2 generated. Nevertheless, one should be cautious in interpreting such cell-study data, as the effect of the suggested addition of catalase on cellular effects of polyphenols is not examined. So, in bioavailability studies using cellular models, a balance of phenolic compounds, partially oxidized, cannot be adequately established; therefore, addition of ascorbic acid allows to avoid such analytical device.

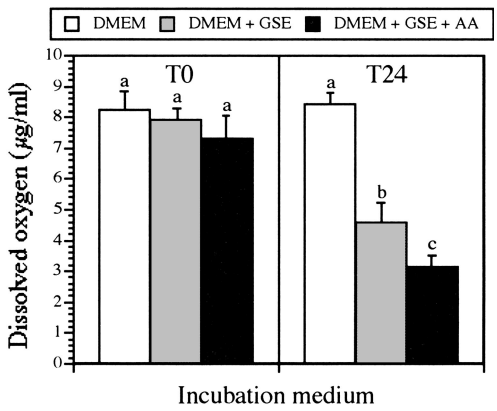

FIGURE 4 Measurement of dissolved oxygen at 37°C and at zero time (T_0) and after 24 h (T_{24}) in the DMEM alone, in DMEM supplemented with 0.4 g/l GSE and in the same mixture plus 1420 μM ascorbic acid (AA). Values are expressed as mean ± SEM ($n = 3$). Bars with identical letters are not significantly different ($p < 0.05$).

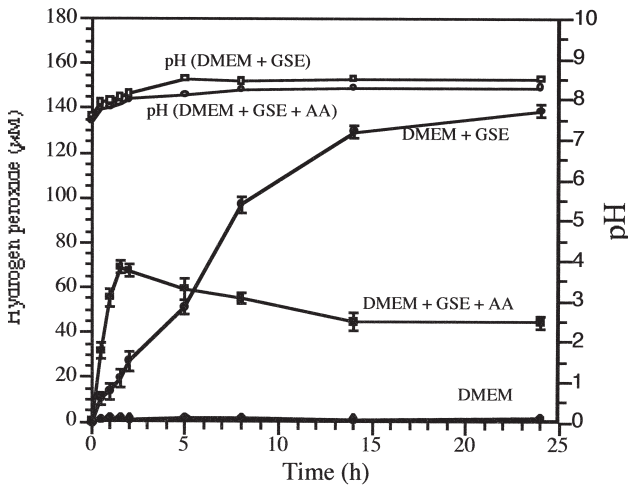

FIGURE 5 Time-dependant measurement of hydrogen peroxide generation at 37° in the DMEM alone, in DMEM supplemented with 0.4 g/l GSE and in the same mixture plus 1420 μM ascorbic acid (AA). Values are expressed as mean ± SEM ($n = 3$). Bars with identical letters are not significantly different.

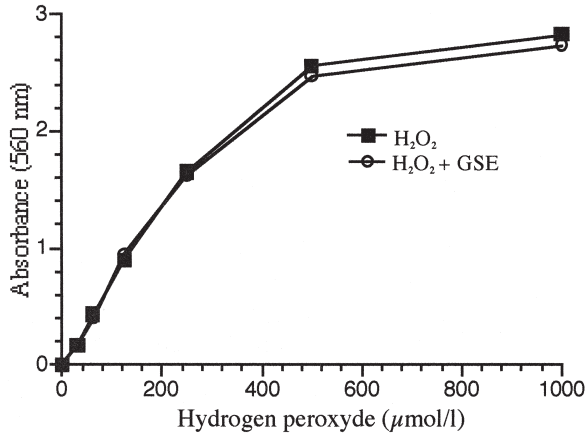

FIGURE 6 Standard curve for ferrous ion oxidation-xylenol orange assay (FOX) using known concentrations of hydrogen peroxide and 0 or 0.4 mg/ml GSE.

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References

[1] Hertog, M.G.L., Hollman, P.C.H. and Katan, M.B. (1992) "Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands", *J. Agric. Food Chem.* **40**, 2379–2383.
[2] Hertog, M.G.L., Hollman, P.C.H. and van de Putte, B. (1993) "Content of potentially anticarcinogenic flavonoids of tea infusions, wine and fruit juices", *J. Agric. Food Chem.* **41**, 1242–1246.
[3] Bravo, L. (1998) "Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance", *Nutr. Res.* **56**, 317–333.
[4] Keli, S.O., Hertog, M.G.L., Feskens, E.J.M. and Kromhout, D. (1996) "Dietary flavonoids, antioxidant vitamins, and incidence of stroke: The Zutphen study", *Arch. Intern. Med.* **156**, 637–642.
[5] Knekt, P., Järvinen, R., Reunanen, A. and Maatela, J. (1996) "Flavonoid intake and coronary mortality in Finland: a cohort study", *Br. Med. J.* **312**, 478–481.
[6] Weber, G., Shen, F., Prajda, N., Yeh, Y.A., Yang, H., Herenyiova, M. and Look, K.Y. (1996) "Increased signal transduction activity and down-regulation in human cancer cells", *Anticancer Res.* **16**, 3271–3282.
[7] Lepley, D.M. and Pelling, J.C. (1997) "Induction of p21/WAF1 and G_1 cell-cycle arrest by the chemopreventive agent apigenin", *Mol. Carcinog.* **19**, 74–82.
[8] Frankel, E.N., Waterhouse, A.L. and Teissèdre, P.L. (1995) "Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins", *J. Agric. Food Chem.* **43**, 890–894.
[9] Rice-Evans, C.A., Miller, N.J. and Paganga, G. (1996) "Structure-antioxidant activity relationships of flavonoids and phenolic acids", *Free Radic. Biol. Med.* **20**, 933–956.
[10] Wiseman, S.A., Balentine, D.A. and Frei, B. (1997) "Antioxidants in tea", *Crit. Rev. Food Sci. Nutr.* **37**, 705–718.
[11] Vinson, J.A. and Dabbagh, Y.A. (1998) "Effect of green and black tea supplementation on lipids, lipid oxidation and fibrinogen in the hamster: mechanisms for the epidemiological benefits of tea drinking", *FEBS Lett.* **433**, 44–46.
[12] Paganga, G., Miller, N.J. and Rice-Evans, C.A. (1999) "The polyphenolic content of fruit and vegetables and their antioxidant activities. What does a serving constitute?", *Free Radic. Res.* **30**, 153–162.
[13] Galijatovic, A., Otake, Y., Walle, U.K. and Walle, T. (1999) "Extensive metabolism of the flavonoid chrysin by human Caco-2 and Hep G2 cells", *Xenobiotica* **29**, 1241–1256.
[14] Galijatovic, A., Walle, U.K. and Walle, T. (2000) "Induction of UDP-glucuronosyl-transferase by the flavonoids chrysin and quercetin in Caco-2 cells", *Pharm. Res.* **17**, 21–26.
[15] Manna, C., Galletti, P., Cucciolla, V., Moltedo, O., Leone, A. and Zappia, V. (1997) "The protective effect of the olive oil polyphenol (3,4-dihydroxyphenyl)-ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco-2 cells", *J. Nutr.* **127**, 286–292.
[16] Manna, C., Galletti, P., Maisto, G., Cucciolla, V., D'Angelo, S. and Zappia, V. (2000) "Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2 cells", *FEBS Lett.* **470**, 341–344.
[17] Bors, W., Heller, W. and Michel, C.T. (1998) "The chemistry of flavonoids", In: Rice-Evans, C.A. and Packer, L., eds, Flavonoids in Health and Disease (Marcel Dekker, New York), pp 111–136.
[18] Jovanovic, S.V., Steenken, S., Simic, M.C. and Hara, Y. (1998) "Antioxidant properties of flavonoids: reduction potentials and electron transfer reactions of flavonoid radicals", In: Rice-Evans, C.A. and Packer, L., eds, Flavonoids in Health and Disease (Marcel Dekker, New York), pp 137–169.
[19] Morel, I., Cillard, P. and Cillard, J. (1998) "Flavonoid–metal interactions in biological systems", In: Rice-Evans, C.A. and Packer, L., eds, Flavonoids in Health and Disease (Marcel Dekker, New York), pp 163–177.
[20] Laughton, M.J., Halliwell, B., Evans, P.J. and Hoult, J.R. (1989) "Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. Effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA", *Biochem. Pharmacol.* **38**, 2859–2865.
[21] Hodnick, W.F. and Pardini, R.S. (1998) "Inhibition of mitochondrial function by flavonoids", In: Rice-Evans, C.A. and Packer, L., eds, Flavonoids in Health and Disease (Marcel Dekker, New York), pp 179–197.
[22] Halliwell, B. (2000) "Antioxidant activity and other biological effects of flavonoids", In: Rice-Evans, C.A., ed, Wake Up to Flavonoids (Royal Society of Medicine Press, London), pp 13–23.
[23] Long, L.H., Clement, M.V. and Halliwell, B. (2000) "Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (−)-epigallocatechin, (−)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media", *Biochem. Biophys. Res. Commun.* **273**, 50–53.
[24] Long, L.H., Lan, A.N.B., Hsuan, F.T.Y. and Halliwell, B. (1999) "Generation of hydrogen peroxide by 'antioxidant' beverages and the effect of milk addition. Is cocoa the best beverage?", *Free Radic. Res.* **31**, 67–71.
[25] Bourzeix, M., Weyland, D. and Heredia, N. (1986) "A study of catechins and procyanidins of grape custers, the wine and other by-products of the wine", *Bull. O.I.V.* **59**, 1171–1254.
[26] Weinges, K. and Piretti, M.V. (1971) "Isolierung des procyanidins B1 aus weintrauben", *Ann. Chem. von Deutschland* **748**, 218–220.
[27] Lea, A.G.H., Bridle, P., Timberlake, C.F. and Singleton, V.L. (1979) "The procyanidins of white grapes and wine", *Am. J. Enol. Vitic.* **30**, 289–300.
[28] Lamuela-Raventos, R.M. and Waterhouse, A.L. (1994) "A direct HPLC separation of wine phenolics", *Am. J. Enol. Vitic.* **45**, 1–5.
[29] Nourooz-Zadeh, J.J., Tajaddini-Sarmadi, J. and Wolff, S.P. (1994) "Mesaurement of plasma hydroperoxide concentrations by the ferrous-oxidation-xylenol orange assay in conjunction with triphenylphosphine", *Anal. Biochem.* **220**, 403–409.
[30] Long, L.H., Evans, P.J. and Halliwell, B. (1999) "Hydrogen peroxide in human urine: implications for antioxidant defense and redox regulation", *Biochem. Biophys. Res. Commun.* **262**, 605–609.

[31] Gordon, M.H. (1990) "The mechanism of antioxidant action *in vitro*", In: Hudson, B.J.F., ed, Food Antioxidants (Elsevier Applied Science, London), pp 1–18.
[32] Clément, M.V., Ramalingam, J., Long, L.H. and Halliwell, B. (2001) "The *in vitro* toxicity of ascorbate depends on the culture medium used to perform the assay and involves hydrogen peroxide", *Antioxid. Redox Signal.* **3**, 157–163.
[33] Nicoli, M.C., Anese, M. and Parpinel, M. (1999) "Influence of processing on the antioxidant properties of fruits and vegetables", *Trends Food Sci. Technol.* **10**, 94–100.
[34] Lu, Y. and Yeap Foo, L. (2000) "Antioxidant and radical scavenging activities of polyphenols from apple pomace", *Food Chem.* **68**, 81–85.
[35] Nicoli, M.C., Calligaris, S. and Manzocco, L. (2000) "Effect of enzymatic and chemical oxidation on the antioxidant capacity of catechin model systems and apple derivatives", *J. Agric. Food Chem.* **48**, 4576–4580.
[36] Chen, Z.-Y., Zhu, Q.Y., Wong, Y.F., Zhang, Z. and Chung, H.Y. (1998) "Stabilizing effect of ascorbic acid on green tea catechins", *J. Agric. Food Chem.* **46**, 2512–2516.