Food Chemistry 115 (2009) 720–726

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Sensitive cell-based assay using DCFH oxidation for the determination of pro- and antioxidant properties of compounds and mixtures: Analysis of fruit and vegetable juices

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A R T I C L E I N F O

Article history:
Received 21 March 2007
Received in revised form 28 November 2008
Accepted 1 December 2008

Keywords:
Vegetables
Fruits
Antioxidant
Pro-oxidant
ORAC assay
Cell-based assay
DCFH-DA
Carrot
Broccoli
α,β-Carotene

A B S T R A C T

Reactive oxygen species play a critical role in cardiovascular diseases, inflammatory diseases, neurodegenerative disorders, cancer and aging. Diets rich in foods containing antioxidants, such as fruits and vegetables, could help prevent these pathologies. It is therefore important to properly assay the antioxidant potentials of these antioxidant foods in order to have a guideline for their proper use. Actual *in vitro* methodologies are often very specific for one mode of action and do not necessarily reflect the normal biological context in which they are used. In this work, we have developed a cell-based assay using 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), a useful indicator of reactive oxygen species (ROS), in order to determine the antioxidant properties of foods, extracts and molecules. Results show a dose-dependent antioxidant activity for pure compounds (in decreasing order of activity: quercetin > caffeic acid > gallic acid > α-tocopherol) and fruit juices (in decreasing order of activity: strawberries > highbush blueberries > kiwis > peaches). These results are in good agreement with results obtained using the $ORAC_{FL}$ assay. However, the cell-based assay detected a pro-oxidant effect with broccoli and carrot juices which was not observed using the $ORAC_{FL}$ assay. Mixed isomers of β,α-carotene isolated from carrots were also found to oxidize DCFH about 212% above control-level. Interestingly, the boiling of broccoli and carrot juices inhibits this pro-oxidant effect and restores the antioxidant properties of the juices. Moreover, the boiling of the β,α-carotene mixed isomers causes their partial degradation and significantly inhibits DCFH oxidation about 68%, suggesting that carotenoids present in broccoli and carrot juices are, in part, responsible for their pro-oxidant effects.

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1. Introduction

Reactive oxygen species (ROS) are involved in such pathologies as cardiovascular diseases (atherosclerosis), inflammatory diseases (asthma, rheumatoid arthritis, allergies), neurodegenerative diseases (Parkinson and Alzheimer diseases), cancer, and aging (Stohs, 1995). The principal ROS formed in biological systems are superoxide anions, hydrogen peroxide, peroxyl radicals and hydroxyl radicals (Bergendi, Benes, Durackova, & Ferencik, 1999). ROS can react with and damage many cellular components (proteins, lipids and DNA) (Halliwell, 1996). The cell has evolved many antioxidant enzymatic systems in order to protect itself against ROS. Principal antioxidant enzymes include superoxide dismutase (SOD), which dismutes the superoxide anion into hydrogen peroxide, glutathione peroxidase (GPx) and catalase (CAT) which eliminates hydrogen peroxide (Mates & Sanchez-Jimenez, 1999). Many small molecules are also involved in ROS detoxification, such as glutathione (GSH), α-tocopherol (vitamin E) and ascorbic acid (vitamin C) (Sies, 1997). In a normal healthy cell, equilibrium is maintained between the generation of ROS and their elimination by the antioxidant system. However, an unbalance can occur when ROS production is greater than the antioxidant defence capacities of the cell, or when the normal antioxidant defences of the cell are inhibited. This unbalance between the oxidant and antioxidant systems, also called oxidative stress, can lead the cellular system towards a pathological state (Sies, 1997).

Diets rich in foods containing antioxidant compounds, such as fruits and vegetables, could help prevent pathologies caused by oxidative stress (Lampe, 1999). Therefore, it is important to evaluate the antioxidant potential of fruits and vegetables in order to be certain of their quality and to develop methods for their optimal conservation. Several *in vitro* methods such as the ORAC assay (oxygen radical absorbing capacity), the TRAP method (total radical-trapping antioxidant parameter) and the TEAC method (trolox equivalent antioxidant capacity) are used for the evaluation of the antioxidant activities of fruit and vegetable extracts (Moreno-Sanchez, 2002; Pellegrini et al., 2003; Proteggente et al., 2002; Wu et al., 2004). *In vitro* tests such as the ORAC assay do not however take into account the physiological conditions of the cell, the

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doi:10.1016/j.foodchem.2008.12.002

bioavailability of the antioxidant molecule as well as general cellular metabolism (Liu & Finley, 2005). Moreover, antioxidant molecules present in fruits and vegetables are heterogenous and multifunctional (Frankel & Meyer, 2000). Many factors can affect their antioxidant potential, such as the affinity of the molecules for the aqueous or lipid phase, the oxidation conditions in the cell as well as the nature of the oxidizable substrate used in the assay (Frankel & Meyer, 2000). Therefore, a one-dimensional assay protocol cannot be used alone to test all relevant parameters (Frankel & Meyer, 2000). A complementary method used to evaluate the antioxidant activities of fruit and vegetable extracts directly in live mammalian cells could be useful. Unfortunately, very few studies use cell culture models to evaluate antioxidant potential. In this work, we have developed a rapid cell-based assay using 2′,7′-dichlorofluorescin (DCFH) oxidation to assess the pro- and antioxidant potential of various pure compounds, fruits and vegetables juices.

2. Materials and methods

2.1. Chemicals

Fluorescein sodium salt (FL), 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), 2′,7′-dichlorofluorescin (DCFH), 2′,7′-dichlorofluorescein (DCF), *tert*-butyl hydroperoxide (*t*-BuOOH), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), quercetin, α-tocopherol, β-carotene, α,β-carotene (1:2 mixed isomers from carrot), caffeic acid, gallic acid and 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) were all purchased from Sigma–Aldrich (Oakville, ON).

2.2. Sample preparation

Fruits and vegetables were purchased at a local grocery store and refrigerated at once. Fruit and vegetable juices were extracted using a juice extractor. Juices were then vacuum filtered with a 0.22 μm filter, frozen at −80 °C and then lyophilized. Dry matter was solubilized in water at 400 mg/ml for the cell-based assay and 100 mg/ml for the ORAC assay. Pure chemicals were dissolved in the appropriate solvents. In order to avoid solvent toxicity in the cell-based assay, the final concentration of solvent in the culture medium was maintained at 0.25% (volume/volume). The sample concentrations tested ranged from 0.5 to 12.5×10^3 μg/ml for the ORAC assay and from 1 to 1000 μg/ml for the cell-based assay.

2.3. $ORAC_{FL}$ assay

The procedure was performed as described by Ou, Hampsch-Woodill, and Prior (2001) with some modifications. Briefly, the ORAC assay was carried out in black round bottom 96-well microplates (Costar) on a Fluoroskan Ascent FL™ plate reader (Labsystems) equipped with an automated injector. Four concentrations of Trolox (the control standard) were used (1.56; 3.13; 6.25 and 12.5 μM) in quadruplicate, and a gradient of 16 concentrations of the samples was prepared without replication. The experiment was conducted at 37.5 °C and in pH 7.4 phosphate buffer, with a blank sample in parallel. The fluorimeter was programmed to record the fluorescence (λ ex.: 485 nm/em.: 530 nm) of fluorescein every minute after addition of 375 mM 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), for a total of 35 min. The final results were calculated using the net area under the curves of the sample concentrations for which decrease of at least 95% of fluorescence was observed at 35 min and which also presented a linear dose–response pattern. ORAC values were expressed in micromoles of Trolox equivalents (TE) per gram (μmol TE/g).

2.4. Cell culture

The L-929 murine fibrosarcoma cell line (ATCC #CCL-1) was obtained from the American Type Culture Collection (Manassas, USA). The cells were cultured in minimum essential medium containing Earle's salts and supplemented with 10% fetal calf serum (Hyclone, Logan, USA), vitamins (1X), sodium pyruvate (1X), non-essential amino acids (1X), penicillin (100 IU) and streptomycin (100 μg/ml) (Mediatech Cellgro®). Cells were incubated in a humidified atmosphere at 37 °C in 5% CO_2.

2.5. Fluorescence quenching assessment

The sensitivity of the fluorimeter and the curve linearity of DCF are determined with growing concentration of DCF in Hank's buffered salt solution (HBSS) ranging from 60 pM to 125 nM (Fluoroskan Ascent FL™, Labsystems; ex.: 485 nm em.: 530 nm). The DCF fluorescence quenching of each sample is previously tested by measuring fluorescence of increasing concentrations of sample ranging from 0.05 to 12.5 mg/ml with 25 nM DCF in HBSS. Intrinsic fluorescence of each sample is also measured in HBSS (without DCF). The cell-based assay is performed in concentrations of samples which are not fluorescent and which do not quench DCF fluorescence.

2.6. Cell-based assay

Pro- and antioxidant activities were evaluated using DCFH-DA probes. L-929 cells were plated in transparent flat bottom 96-well microplates (BD Falcon) at 10,000 cells per well and incubated for 24 h at 37 °C and 5% CO_2. The cells were washed with 150 μl of pH 7.4 phosphate buffer saline (PBS) and incubated for 30 min with 100 μl HBSS (pH 7.4) containing 5 μM DCFH-DA (Sigma–Aldrich, Oakville, ON). The cells were then washed again with 150 μl PBS. To assess antioxidant activity, the cells were incubated for 1 h with growing concentrations of pure compounds or extracts in the absence or presence of 200 μM *tert*-butylhydroperoxide (*t*-BuOOH). Fluorescence was measured immediately after *t*-BuOOH administration and also 90 min later on the automated plate reader (Fluoroskan Ascent FL™, Labsystems), using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. IC_{50} were calculated using the logarithmic regression of the dose–response curve after subtraction of both blank and intrinsic sample fluorescence values. In all cases, the coefficients of determination of the regression (R^2) were greater than 0.95. IC_{50} are the means ± standard deviations of three determinations.

2.6.1. Statistical analysis

The values were expressed as means ± standard deviation of three determinations. The results were analyzed by the Kruskal–Wallis test followed by a Student–Newman–Keuls' or Dunn's post hoc test. P values of 0.05 or less were considered as statistically significant.

3. Results and discussion

The principal objective of this work was to use a cell-based assay to measure the antioxidant potential of natural compounds and mixtures such as fruit and vegetable juices. Some researcher groups have reported the use of 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) in biological systems for the evaluation of natural antioxidants. Takamatsu et al. (2003) reported the screening of some flavonoids for their antioxidant properties on HL-60 cells using a DCFH-DA assay. However, in their assay, (−)-epigallocatechin and quercetin (both positive standards) did not show apparent

antioxidant activities. In another study, Lu et al. (2004) used a DCFH-DA assay to assess the antioxidant activities of procyanidins from grape seeds. Unfortunately, the DCFH-DA cell-based assay used by Lu et al. (2004) was not described. Moreover, Eberhardt, Kobira, Keck, Juvik, and Jeffery (2005) used a cellular assay to evaluate the antioxidant activities of broccoli extracts. However, the positive control used to validate the method was not shown. Recently, Wolfe and Liu (2007) developed a new cellular antioxidant activity assay (CAA), but the method is not very sensitive with EC_{50} ranging from 3 to 63 mg/ml for various fruit extracts. Consequently, the lack of standardization, uniformity and sensitivity of the DCFH-DA assays in the literature prompted us to develop our cell-based assay conditions to assess the antioxidant activities of natural compounds and mixtures.

3.1. Evaluation of pure antioxidant compounds both by a cell-based assay using DCFH oxidation and by the ORAC assay

DCFH-DA is a useful indicator of reactive oxygen species (ROS) and oxidative stress. The nonpolar and non-ionic DCFH-DA crosses cell membranes and is hydrolyzed by intracellular esterases to non-fluorescent 2′,7′-dichlorofluorescin (DCFH). In the presence of ROS such as hydrogen peroxide (H_2O_2), lipid hydroperoxides and peroxinitrite, DCFH is oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF). The oxidation is amplified by intracellular ferrous iron (Fe^{2+}). The reaction of H_2O_2 with Fe^{2+} can generate hydroxyl radicals ($HO^{\cdot}$) which can oxidize DCFH. Superoxide anions do not directly oxidize DCFH, however, in biological systems, superoxide dismutase (SOD) transforms superoxide anions into H_2O_2 which can then oxidize DCFH. In addition, DCFH can be oxidized by intracellular oxidases and oxidants formed during the reduction of H_2O_2. Altogether, these observations indicate that the oxidation of DCFH may be derived from several ROS intermediates (LeBel, Ischiropoulos, & Bondy, 1992; Wang & Joseph, 1999). Therefore, DCFH is useful to indirectly measure the effect of intracellular antioxidant activities in scavenging the ROS and in protecting the DCFH from the oxidation. However, to assess the potency of a compound to inhibit DCF formation, it is important to measure the sensitivity of the fluorimeter to avoid the saturation of the detector and DCF fluorescence quenching induced by vegetable and fruit juices. Therefore, the relative fluorescence of growing concentrations of DCF was measured first, in order to both test the sensitivity of the fluorimeter and confirm curve linearity. In Fig. 1A, results show that relative DCF fluorescence for concentrations ranging from 60 to 1950 pM is linear ($R^2 = 0.9995$). Moreover, a relatively high concentration of DCF (125 nM) does not saturate the detector (data not shown). To test DCF quenching induced by various fruit and vegetable juices, DCF was incubated in the presence of growing concentrations of carrot, broccoli, peach, lemon, strawberry or blueberry juices ranging from 0.05 to 12.5 mg/ml. The results presented in Fig. 1B show that none of the juices significantly quenches DCF fluorescence at concentrations lower than 2 mg/ml except for the lemon juice which quenches about 32% of fluorescence at 0.4 mg/ml. However, at 6.25 mg/ml and more, all tested juices significantly quench DCF fluorescence. Moreover, the higher quenching values at 12.5 mg/ml were obtained with fruit juices including lemon (63%), strawberry (55%), peach (51%) and blueberry (48%) in comparison with vegetable juices such as carrot (19%) and broccoli (10%). These results indicate that a determination of DCF quenching is important before testing the antioxidant potency of fruit and vegetable juices or extracts. Indeed, at high concentrations of fruit juices or extracts a part of the decreasing of DCF fluorescence can be due to DCF quenching and not to antioxidant scavenging.

In the literature, concentrations of DCFH-DA ranging between 5 and 100 μM were used to detect intracellular ROS produced in the

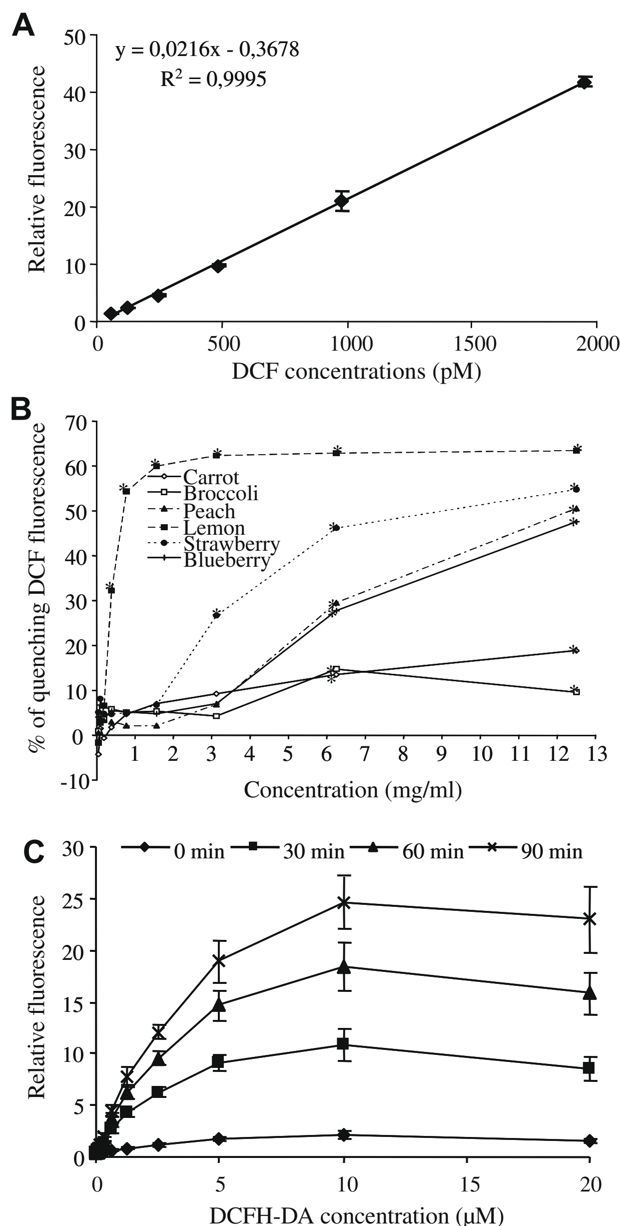

Fig. 1. (A) Standard curve of fluorescent DCF (oxidized product of DCFH) in HBSS (200 μl); (B) effect of fruit and vegetable juices on quenching of DCF fluorescence; (C) measurement, using growing concentrations of DCFH-DA (0.04–20 μM), of ROS induced after incubation of L-929 cells in *t*-BuOOH (200 μM) for 0, 30, 60 and 90 min. *Significantly different from fluorescence DCF without fruit and vegetable juice; $p < 0.05$.

presence of various oxidants. In our cell-based assay, *tert*-butylhydroperoxide (*t*-BuOOH) was chosen as the intracellular oxidizing agent. The optimal concentration of DCFH-DA used to detect intracellular ROS induced by 200 μM *t*-BuOOH was next evaluated on the L-929 cell line. Cells were first loaded for 30 min with growing concentrations of DCFH-DA (0.04–20 μM), then cells washed to eliminate unloaded DCFH-DA and treated for 30, 60 or 90 min with 200 μM *t*-BuOOH. Results presented in Fig. 1C show that the incubation of L-929 cells with 5 μM DCFH-DA permits the optimal detection of ROS induced by *t*-BuOOH. At this concentration, relative fluorescence is not significantly different from that observed when using 10 or 20 μM DCFH-DA.

The antioxidant activity of Trolox, a water-soluble vitamin E analogue, was then evaluated using both the cell-based assay

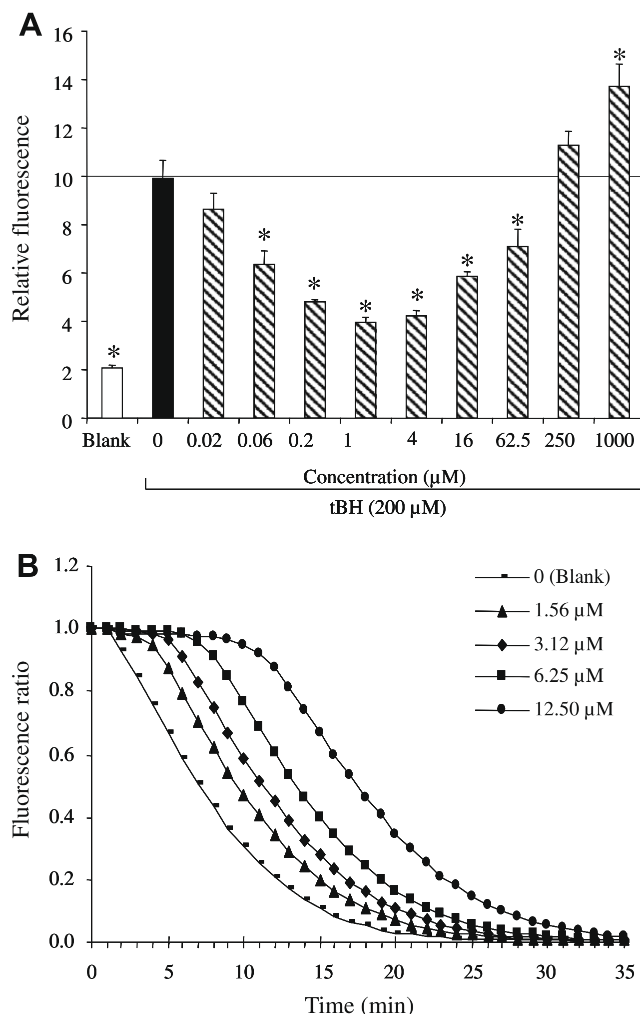

Fig. 2. Evaluation of antioxidant properties of Trolox against treatment with *t*-BuOOH for 90 min using the cell-based assay (A) and against treatment with AAPH using the $ORAC_{FL}$ assay (B). *Significantly different from *t*-BH (200 μM); $p < 0.05$.

(Fig. 2A) and the $ORAC_{FL}$ assay (Fig. 2B). In Fig. 2A, L-929 cells were pre-treated for 60 min with growing concentrations of Trolox (0.02–1000 μM) and were incubated for 90 min in the presence of 200 μM *t*-BuOOH. Results show that the dose–response curve to Trolox is biphasic. In the first phase, Trolox decreases *t*-BuOOH-induced DCFH oxidation in a dose-dependent manner at doses ranging from 0.02 to 1 μM. In the second phase, Trolox increases *t*-BuOOH-induced DCFH oxidation at concentrations above 250 μM. Antioxidant and pro-oxidant properties of Trolox have already been reported during the Cu^{2+}-induced oxidation of low-density lipoproteins (Albertini & Abuja, 1999). The cell-based assay allows the detection of significant antioxidant activity for Trolox concentrations as low as 0.02 μM. In comparison, the $ORAC_{FL}$ assay detect Trolox antioxidant activity at doses higher than 1.56 μM (Fig. 2B). These results show that the cell-based assay is a sensitive assay which allows to detect the anti- and pro-oxidant properties of Trolox. Similar results were obtained with other cell lines including A549, DLD1, HepG2 and WS1 (data not shown).

The activities of pure antioxidant compounds such as quercetin (Fig. 3A), caffeic acid (Fig. 3B), gallic acid (Fig. 3C) and α-tocopherol (Fig. 3D) were then assessed using the cell-based assay. Results show that all these antioxidant standards inhibit ROS induced by *t*-BuOOH in a dose-dependant manner. In order to compare the antioxidant activities of these standard compounds, concentrations inhibiting DCFH oxidation by 50% (IC_{50}) were calculated for each molecule. Regression coefficients (R^2) calculated for each logarithmic curve were superior to 0.95. In Table 1, results indicate that the antioxidant potentials (IC_{50}) of these standards are classified as such: quercetin (0.11 ± 0.08 μM) > caffeic acid (0.9 ± 0.3 μM) > gallic acid (2.6 ± 0.6 μM) > α-tocopherol (119 ± 36 μM). A similar antioxidant potential classification was obtained using the $ORAC_{FL}$ assay. With regard of quercetin's IC_{50}, our cell-based assay is about 50 folds more sensitive in comparison with CAA developed by Wolfe and Liu (2007). Furthermore, the antioxidant activity of α-tocopherol was detected using the cell-based assay, suggesting that DCFH is also present in the cell membranes. Indeed, a NMR chemical shift-polarity study has previously indicated that DCFH-DA and DCFH are located within the lipid bilayer (Afri, Frimer, & Cohen, 2004). This suggests that DCFH can also be used to detect the antioxidant activities of hydrophobic compounds.

3.2. Evaluation of the antioxidant potential of fruit and vegetable juices

The antioxidant activities of highbush blueberry (*Vaccinium corymbosum*), kiwi, peach and strawberry juices were evaluated using the cell-based assay. The L-929 cell line was pre-treated for one hour with increasing concentrations of fruit juices (16–1000 μg/ml) and then incubated for 90 min in the presence of *t*-BuOOH. Results presented in Fig. 4A show that a low concentration of highbush blueberry juice (16 μg/ml) significantly inhibits DCFH oxidation by about 12.6 ± 0.3%, while kiwi, peach and strawberry juices are slightly pro-oxidant at this concentration. However, this result is not statistically significant in comparison with untreated cells. All fruit juices tested have antioxidant properties at concentrations ranging from 62.5 to 1000 μg/ml. In Table 1, IC_{50} values calculated from the logarithmic curves indicate that the antioxidant activity of strawberry juice (52 ± 8 μg/ml) is the greatest, followed by highbush blueberry juice (116 ± 15 μg/ml), kiwi juice (214 ± 30 μg/ml) and finally peach juice (1119 ± 303 μg/ml). The antioxidant potential of fruit juices was also assessed using the $ORAC_{FL}$ assay. The $ORAC_{FL}$ values for strawberry, highbush blueberry, kiwi and peach juices are respectively of 190 ± 3, 125 ± 9, 70 ± 10 and 26 ± 4 μmol of TE/g of dry matter. These results are consistent with those obtained with the cell-based assay. However, comparison of these results with those from the literature is not easy because $ORAC_{FL}$ values for fruits vary with seasons, regions and cultivars (Wang & Lin, 2000; Wu et al., 2004). For example, $ORAC_{FL}$ values obtained by Wang and Lin (2000) for strawberry juice ranged from 82 to 153 μmol of TE/g of dry matter, depending on the developmental stage of the fruit (Wang & Lin, 2000). Moreover, a study on the antioxidant capacities of 30 different *Vaccinium* extracts including highbush blueberries showed that $ORAC_{FL}$ values ranged from 19 to 131 μmol of TE/g (Moyer, Hummer, Finn, Frei, & Wrolstad, 2002).

The antioxidant activities of two vegetable juices (broccoli and carrot) were also evaluated using both antioxidant assays. The $ORAC_{FL}$ values for broccolis and carrots are of 320 ± 20 and 70 ± 10 μmol of TE/g of dry matter, respectively (Table 1). These results are in good agreement with those reported in the literature. Indeed, the antioxidant capacities of different freeze-dried vegetable samples including 59 carrot samples and 130 broccoli samples were analyzed by Ou et al., and the $ORAC_{FL}$ values obtained ranged between 25–99 μmol of TE/g for carrot samples and 23–208 μmol of TE/g for broccoli samples (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002). Surprisingly, the results obtained with the cell-based assay show that broccoli and carrot juices are strongly pro-oxidant when tested at doses ranging between 2 and 500 μg/ml (Fig. 4B). When compared to the level of DCFH oxidation normally induced by *t*-BuOOH alone, DCFH oxidation was increased

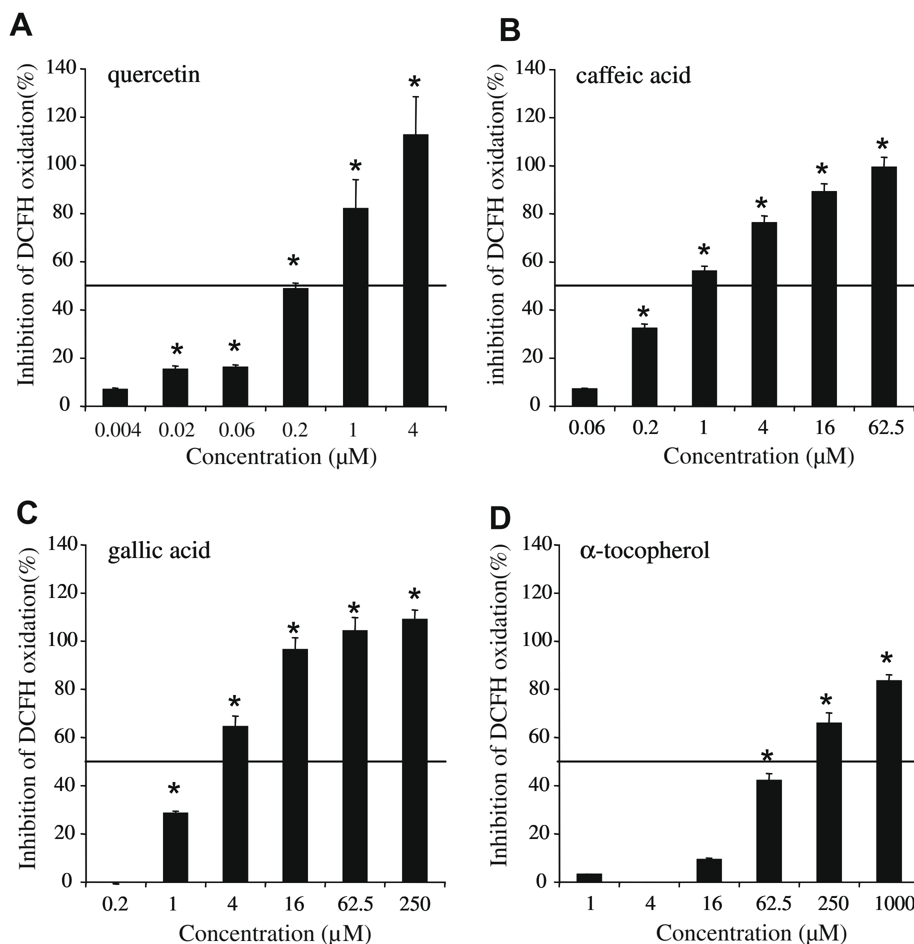

Fig. 3. Evaluation of the antioxidant properties of quercetin (A), caffeic acid (B), gallic acid (C) and α-tocopherol (D) after absorption (60 min) by cells and subsequent exposition (90 min) to 200 µM *t*-BuOOH, using the cell-based assay. The horizontal line is set at 50% inhibition. Data shown are the means ± standard deviation for three determinations and are representative of three different experiments. *Significantly different from *t*-BH (200 µM); $p < 0.05$.

Table 1
Antioxidant effects of compounds, fruit and vegetable juices using the $ORAC_{FL}$ assay and the cell-based assay.

Samples	IC_{50}[a] (µg/ml)	$ORAC_{FL}$ (TE µmol/g)
α-Tocopherol	49.6 ± 15 (119 ± 36)[b]	160 ± 20
Caffeic acid	0.16 ± 0.05 (0.9 ± 0.3)[b]	8000 ± 1000
Gallic acid	0.4 ± 0.1 (2.6 ± 0.6)[b]	6300 ± 300
Quercetin	0.03 ± 0.02 (0.11 ± 0.08)[b]	13000 ± 4000
Blueberries	116 ± 15	125 ± 9
Strawberries	52 ± 8	190 ± 3
Peaches	1119 ± 303[c]	26 ± 4
Kiwis	214 ± 30	70 ± 10
Broccoli	(–)[d]	320 ± 20
Broccoli (boiled)	14 ± 4	210 ± 90
Carrot	(–)[d]	70 ± 7
Carrot (boiled)	57 ± 37	72 ± 6

[a] Concentration inhibiting DCFH oxidation induced by *t*-BuOOH by fifty percent.
[b] IC_{50} value in micromolar (µM).
[c] Extrapolated value obtained from logarithmic curve.
[d] Pro-oxidant activity.

to 204 ± 3% in cells treated with carrot juice (125 µg/ml) and to 179 ± 4% in cells treated with broccoli juice (125 µg/ml). The effect of heat on these pro-oxidant activities was then tested by boiling both juices for 30 min at 100 °C. After boiling, both vegetable juices were found to be strongly antioxidant, with IC_{50} values of 14 ± 4 µg/ml for broccoli juice and 57 ± 37 µg/ml for carrot juice. These results suggest that the pro-oxidant compounds are heat-sensitive. Results presented in Table 1 show that the $ORAC_{FL}$ assay did not detect the pro-oxidant activity of vegetable juices and that the boiling of both samples did not significantly affect their $ORAC_{FL}$ values. The pro-oxidant effects of carrot and broccoli juices were also confirmed on other human cell lines including normal human fibroblasts (WS-1), a human lung adenocarcinoma cell line (A-549) and a human colon carcinoma cell line (DLD-1) (data not shown).

It is known that carrot and broccoli contain carotenoid compounds such as α- and β-carotene (Cortes, Esteve, Frigola, & Torregrosa, 2004; Kurilich et al., 1999). Moreover, it is known that β-carotene exhibits pro-oxidant properties in cultured cells (Palozza, Serini, Di Nicuolo, Piccioni, & Calviello, 2003). The presence of α- and β-carotene in our carrot juice was confirmed by thin layer chromatography using a carotene standard (data not shown). The pro-oxidant effects of carrot juice, β-carotene and a mix of β- and α-carotene isomers isolated from carrots were then evaluated, both before and after boiling, on L-929 cells untreated with *t*-BuOOH. DCFH oxidation in cells untreated with *t*-BuOOH was used as control. Results presented in Fig. 5 show that 4 and 16 µg/ml of unboiled carrot juice increased DCFH oxidation by respectively 24 ± 2% and 191 ± 7% above the control-level. In comparison, 200 µM *t*-BuOOH increased control-level DCFH oxidation by 384 ± 4%. Boiled carrot juice was also evaluated and did not possess pro-oxidant properties. Increasing concentrations of β-carotene (boiled or not) were then tested on the L-929 cell line for their antioxidant properties. The results indicate that at low concentrations (4 and 16 µg/ml) β-carotene is not pro-oxidant. However, 62.5 µg/

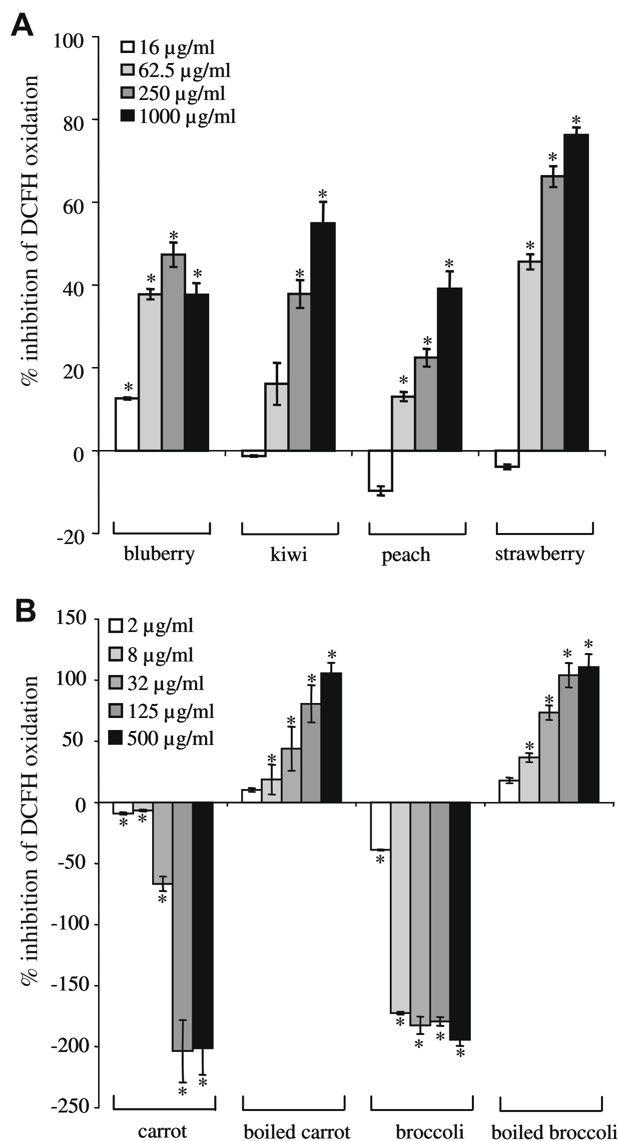

Fig. 4. Evaluation of the antioxidant properties of different fruit juices (A) or vegetable juices (boiled or not) (B) after absorption for 60 min and subsequent exposition (90 min) to 200 μM *t*-BuOOH, using the cell-based assay. Negative values indicate a pro-oxidant effect (DCF fluorescence levels above that of control cells treated with *t*-BuOOH only), whereas positive values indicate an antioxidant effect. Data shown are the means ± standard deviation for three determinations and are representative of three different experiments. *Significantly different from *t*-BH (200 μM); $p < 0.05$.

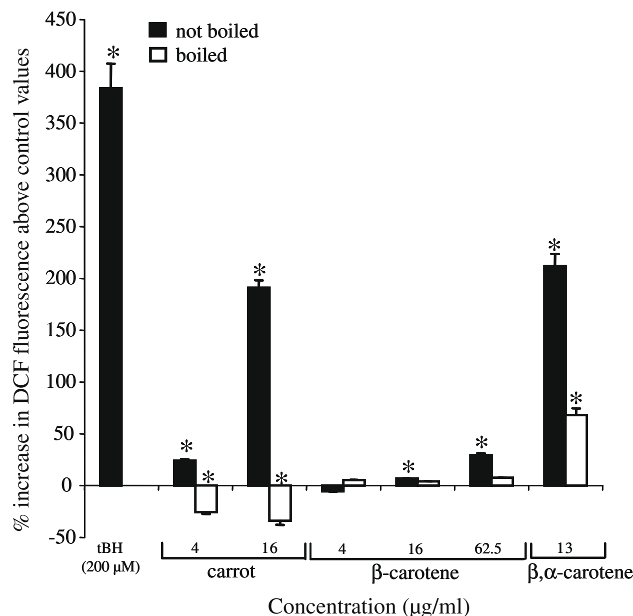

Fig. 5. Prooxidant effect after exposition to carrot juice, β-carotene or mixed isomers of β,α-carotene (2:1) isolated from carrots, using the cell-based assay. No subsequent treatment of cells with *t*-BuOOH was performed. DCFH oxidation by untreated cells were used as control (zero). Values greater than zero indicate a prooxidant effect, while negative values indicate an antioxidant effect. Data shown are the means ± standard deviation for three determinations and are representative of three different experiments. *Significantly different from blank (200 μM); $p < 0.05$.

ml of β-carotene increased control-level DFCH oxidation by 29 ± 2%. As was observed with boiled carrot juice, the boiling of β-carotene significantly reduces (74%) its pro-oxidant properties. Moreover, a mixture of β- and α-carotene isomers (2:1) isolated from carrots was evaluated on L-929 cells. The mixed isomers (13 μg/ml) strongly increased DCFH oxidation (212 ± 12% above control-level), suggesting that α-carotene is mostly responsible for the pro-oxidant effects of carrot juice and/or that the pro-oxidant effect is due to synergistic interactions between β- and α-carotene. As was observed for boiled β-carotene, the boiling of the isomer mixture decreased DCFH oxidation from 68 ± 7%. It is known that β-carotene is sensitive to thermal degradation (Mader, 1964). Thermal degradation of β-carotene and of the mixed isomers (α,β-carotene) isolated from carrots were evaluated using thin layer chromatography. Results indicate that both the β-carotene and the mixed isomers are, in part, degraded after 30 min of boiling (data not shown). Altogether, these results suggest that β- and α-carotene participate in the pro-oxidant effects of carrot juice and that their thermal degradation possibly restores the antioxidant activity of the juice.

The pro-oxidant effects of broccoli and carrot juices on cultured cells and animal or human models have not been reported. Palozza (2005) stated that the administration carotenoid-containing fruits and vegetables have never been associated with procarcinogenic effects due to the carotenoids, possibly because the carotenoids are stabilized by other antioxidant nutrients. However, it is important to note that very few studies on fruit and vegetable juices were realized on cultured cells. Recently, Eberhardt et al. (2005) assessed the antioxidant activities of lipophilic and hydrophilic extracts from broccoli using the HepG2 cell line and the $ORAC_{FL}$ assay. $ORAC_{FL}$ values obtained for the hydrophilic extracts were similar to the results obtained in our study. In contrast, Eberhardt et al. found that both extracts were antioxidant on HepG2 cells. This difference could be explained by the cell line used as well as their culture conditions. Indeed, it has been demonstrated that different chemical and biological environments can modify the redox properties of carotenoids (Palozza et al., 2003). For example, β-carotene becomes pro-oxidant at a high oxygen pressure (Palozza et al., 2003). Therefore, vegetable juices containing carotenoids could be either antioxidant or pro-oxidant, depending on the test conditions. In this work, the cell culture conditions favored the pro-oxidant properties of the vegetable juices.

In conclusion, we have developed a sensitive cell-based assay to assess the pro- and antioxidant properties of compounds and mixtures such as fruit and vegetable juices. This cell-based assay allows the evaluation, in a biological system, of the antioxidant activities of both hydrophobic and hydrophilic compounds. Altogether, our results show that antioxidant activities measured with the cell-based assay are, in general, in good agreement with values obtained using the $ORAC_{FL}$ assay. However, in contrast to the $ORAC_{FL}$ assay, the cell-based assay allowed us to reveal the pro-oxi-

dant effect of broccoli and carrot juices and to identify β,α-carotene as the molecules possibly responsible, in part, for this effect. Finally, this work shows that a cell-based assay can bring important additional information to results obtained with the $ORAC_{FL}$ assay.

Acknowledgments

We thank Angélique Longtin for her help and suggestions. The authors would like to thank Vakhtang Mshvildadze for TLC experiments. This work was supported by Action Concertée FQRNT – Fonds de la recherche forestière du Saguenay – Lac-Saint-Jean.

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