An Improved, Rapid in Vitro Method To Measure Antioxidant Activity. Application on Selected Flavonoids and Apple Juice

Addie A. van der Sluis, Matthijs Dekker,* Ruud Verkerk, and Wim M. F. Jongen

Integrated Food Technology Group, Department of Agrotechnology and Food Sciences, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

A rapid in vitro method for measuring antioxidant activity is presented, which enables the evaluation of health claims and the optimization of product development with respect to health protecting compounds. Antioxidant activity is assessed in a system in which lipid peroxidation is induced in male rat liver microsomes by ascorbic acid and $FeSO_4$. This method has been significantly improved by enabling the use of microtiter plates and an ELISA reader. Large numbers of samples can be analyzed with good reproducibility, which is necessary when dealing with microsomes possessing biological variability. An objective mathematical procedure has been developed to translate data obtained from the lipid peroxidation assay into a value describing the antioxidant activity. As an illustration the method has been applied to measure antioxidant activity of individual flavonoids and apple juice.

Keywords: *Antioxidant activity; flavonoids; assay; apple juice*

INTRODUCTION

The increasing awareness among consumers about the relation between diet and health is a sign for food producers to pay more attention to the possibilities of health protecting properties in new product development. Product characteristics such as sensory properties (taste, color, texture), microbiological safety, nutritive value, and keepability have always been regarded as the only important quality attributes in food product development. Nowadays interest is growing for compounds that have been considered as nonnutritive, but which may play a physiological role in the human body. These compounds might be important in maintaining human health and are referred to as "bioactive compounds". Examples are flavonoids, glucosinolates, carotenoids, organosulfides, sterols, and peptides (Steinmetz and Potter, 1996; Tanaka, 1994).

The concentrations of many bioactive compounds have been analyzed in raw materials (Lister et al., 1994; Hertog et al., 1992). However, before consumption, fruits and vegetables in which bioactive compounds are present may undergo different forms of processing. The effect of processing on the concentration and bioactivity of these compounds has not yet been investigated thoroughly. This paper is part of a project that studies the effects of processing on flavonoids in apple and apple products (Van der Sluis et al., 1999).

In determining the health protecting capacity of a product, not only the concentration of the components of interest is important, but also the bioavailability and the bioactivity. If healthiness is to be considered as a quality attribute in food product development, it has to be measurable and quantifiable. Therefore, good measurement systems are needed which correspond to a possible functional claim. It is also important to set up a measurement system that is able to quantify bioactivity in a complex matrix, as is the case in food products. This will enable the evaluation of functional claims associated with health foods. Because of the fact that flavonoids as present in apple are strong antioxidants, and may function as antioxidants in preventing aging diseases in humans, we use antioxidant activity as a measure of bioactivity for apple and apple products.

According to the broad definition by Halliwell and Gutteridge (1989), antioxidants are compounds which, while present in low concentrations compared to those of an oxidizable substrate, prevent or delay that substrate from being oxidized. In the course of lipid peroxidation they can act as oxygen quenchers, radical scavengers (quenching initial radicals such as hydroxyl radicals as well as quenching intermediate radicals such as peroxy and alkoxy radicals), or metal ion chelators.

A distinction must be made between water-soluble and fat-soluble antioxidants. Examples of water-soluble antioxidants are ascorbic acid (vitamin C) and B-vitamins (e.g., thiamin and riboflavin), while tocopherols (such as vitamin E) and carotenoids (e.g., β-carotene and lycopene) are fat-soluble antioxidants. Antioxidants in the polyphenol and flavonoid groups vary in their hydrophilic−lipophilic properties.

In the literature a lot of methods to assess antioxidant activity of samples can be found (e.g., Gutteridge, 1988; Heinonen et al., 1998; Miller et al., 1993; Vinson and Hontz, 1995a; Wayner et al., 1987). In these methods oxidation is induced in single lipids, mixtures of various lipids, or complex mixtures. Sometimes biological model systems are used to mimic an in vivo situation. These systems may consist of biological membranes, such as liver microsomes, erythrocyte membranes, or liposomes. After induction of oxidation in these systems, antioxidant activity can be assessed by comparing the extent of oxidation after addition of the antioxidant compound of interest with the extent of oxidation occurring in a blank or a reference compound.

* Author to whom correspondence should be addressed. E-mail: Matthijs.Dekker@ift.fdsci.wag-ur.nl. Phone: +31 317 482520. Fax: +31 317 483669.

10.1021/jf000156i CCC: $19.00
Published on Web 08/11/2000

Most of these methods are limited to the measurement of pure compounds, single or mixed with other components. Normally these components are dissolved in a liquid solution, which is added to the reaction medium. But in food products antioxidants are present in a complex matrix and the effect of the product matrix on antioxidant activity is not known yet. It is possible that synergism or antagonism between antioxidants or between antioxidants and other compounds present in the product matrix occurs.

To measure antioxidant activity, we chose rat liver microsomes as an oxidative system, because it is close to the in vivo situation where both an aqueous phase and a lipid phase are present. The extent of lipid peroxidation (LPO), after chemical induction by radical formation, is monitored by the thiobarbituric acid (TBA) test. This is a commonly used combination. Microsomes are a complex and not well-defined substrate, they are composed of centrifuged cell plasma membranes and endoplasmatic reticulum, and they are subject to biological variability. They do not only consist of pure membranes; cytostolic enzymes may also be included in the membranes (Gutteridge, 1988). Because of the fact that most flavonoids are not completely water-soluble antioxidants, this system with a combination of aqueous and lipid phases seems to provide good possibilities to serve as a very general system to measure antioxidant activity.

Given the fact of the natural variation in the components of the assay and the large variation obtained in the results with some of the existing antioxidant assays, there is a need to develop an assay that enables many replications of samples in a fast and convenient way. In this paper we describe the development of such a rapid procedure by adapting an assay based on the LPO of microsomes in such a way that it can easily be performed in microtiter plates. The way in which the antioxidant activity of compounds is expressed is not consistent in the literature. We propose a mathematical method, which enables an objective quantification of this antioxidant activity based upon data covering a wide range of concentration of either a single component or a complex food sample. By using this wide range of concentrations and three or more replications of each concentration, it is possible to reproducibly determine the antioxidant activity of a sample. As an illustration of this improved method, the assessment of the antioxidant activity of several individual flavonoids and of apple juice is given.

MATERIALS AND METHODS

Chemicals. Kaempferol, myricetin, quercetin dihydrate, and rutin trihydrate were purchased from Fluka, chlorogenic acid, phloridzin, (±)-catechin, and (−)-epicatechin from Sigma, and quercetin-3-arabinosid, hyperosid, isoquercitrin, quercitrin, and ideainchlorid from Roth. Quercetin-3-arabinofuranoside and (−)-epigallocatechin were obtained from Apin Chemicals, and reynoutrin was obtained from Plantech. L-(+)-Ascorbic acid and iron(II) sulfate heptahydrate were obtained from Merck. The enzyme Rapidase BE Super was supplied by Gist-Brocades. All other chemicals were of analytical or HPLC grade purity.

Sample Preparation. Flavonoid standards were dissolved in methanol. Two different types of apple juice were prepared from Jonagold apples. Apple pulp was pressed immediately by straight pressing. Pulp-enzymed juice was prepared after the addition of pectolytic enzymes (200 ppm Rapidase BE Super) to apple pulp, which was left for 2 h at room temperature, under continuous stirring before pressing. Another apple juice was bought in an outlet of a nationwide supermarket chain. The apple cultivars that are used in the commercially available apple juice is unknown. Before HPLC analysis and antioxidant activity determination the apple juice samples were diluted to 50% with methanol.

Quantification of Quercetin Glycosides by HPLC. Quercetin glycosides were determined in the apple juices by an adaptation of the method described by Lister et al. (1994). This method was adjusted to the use of a Merck Lichrosorb RP18 (4 × 250 mm, 5 μm) analytical column with a guard column. A Spectra FOCUS scanning UV−vis detector, a Spectra System P2000 solvent programmer, and a Spectra System AS3000 autosampler from Spectra Physics were used. Integrator software was TSP version 3.0.

Eluents (A) 10% acetic acid in water and (B) acetonitrile were degassed by an All-Tech degassing system. After filtration through a 0.45 μm Millipore filter, samples (100 μL) were injected on the column which was maintained at 35 °C. A linear 20 min solvent gradient from 0 to 21% acetonitrile, with a 10 min hold at the final concentration, was used. The column was returned to the initial solvent composition over 1 min and reequilibrated for 10 min before the next injection. The flow rate was 1.0 mL/min.

Quercetin glycosides were monitored at 350 nm and identified and quantified by comparison with standard solutions of known concentrations, and if necessary by comparison of spectra.

Preparation of Rat Liver Microsomes. Male Wistar rats (CKP, Wageningen), receiving normal diets and weighing 200−220 g, were killed by decapitation after overnight starvation. Livers were removed and homogenized (1:2, w/v) in ice-cold phosphate buffer (100 mM, pH 7.4) containing 1 mM EDTA and 0.9% NaCl. The homogenate was centrifuged (10000*g*, 20 min, 4 °C), and the supernatant was collected and centrifuged (100000*g*, 75 min, 4 °C). The pellet was suspended by pottering (1 g of liver/mL) in the phosphate buffer and centrifuged again (100000*g*, 60 min, 4 °C). To be stored, the pellet was suspended (0.5 g of liver/mL) in ice-cold phosphate buffer (100 mM, pH 7.4) containing 0.1 mM EDTA and 20% glycerol. Microsomal protein concentrations were determined by the Biuret method with bovine serum albumin used as standard, and the microsomes were diluted with the latter phosphate buffer to 5 mg/mL protein before storage in 1 mL aliquots in liquid nitrogen.

Antioxidant Activity. Antioxidant activity measurements are based on a method to determine lipid peroxidation in rat liver microsomes (Haenen, 1983). It was optimized to be able to use microtiter plates, a multichannel pipet, and an ELISA reader, which makes it possible to analyze large numbers of samples in a run.

Microsomes (5 mg of protein/mL) were thawed on ice, diluted 5-fold with Tris−HCl buffer (50 mM, pH 7.4), containing 150 mM KCl, and centrifuged (100000*g*, 60 min, 4 °C). The pellet was resuspended in the Tris buffer and diluted to the concentration needed (final concentration 0.5 mg/mL protein unless otherwise stated).

The microsomes (aliquots of 240 μL) were preincubated in a 48-well plate for 5 min at 37 °C. As a test sample 30 μL of a known concentration of an antioxidant or blank (corresponding with the solvent for the antioxidant, e.g., methanol or water) was added. LPO was induced by adding 15 μL of ascorbic acid (4 mM) and 15 μL of $FeSO_4$ (0.2 mM). After incubation for 60 min at 37 °C the reaction was stopped by addition of 0.5 mL of 0.83% thiobarbituric acid dissolved in TCA−HCl (16.8% w/v trichloroacetic acid in 0.125 N HCl). LPO was assessed by measuring thiobarbituric acid reactive species (TBARS) after the plates were heated for 15 min at 80 °C and subsequent centrifugation (2500 rpm, 15 min). A 250 μL sample of each incubation was transferred to 96-well plates, and absorption was read at 540 nm (color) vs 620 nm (turbidity correction) by an ELISA reader.

During the first period of this research, the ELISA reader was equipped with a standard 550 nm filter. Because of the fact that the TBA reaction gives a higher extinction at

somewhat lower wavelengths, a filter of 540 nm later on replaced this filter. It was found that measuring at this wavelength increased the accuracy of the method, but that no significant differences in IC_{50} values were obtained. Therefore, results obtained by both filters are given.

Calculations. The percentage of inhibition produced by a sample at a given concentration can be calculated from the absorbance readings. The percentage of inhibition is expressed as the inhibition of lipid peroxidation of that sample compared to the lipid peroxidation in a blank (eq 1). % I = percentage of inhibition, A_{blank} = absorbance of the blank ($A_{540} - A_{620}$), and A_{sample} = absorbance of the sample ($A_{540} - A_{620}$).

$$\% I = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \quad (1)$$

The dependence of the percentage inhibition on the concentration of the antioxidant can be described by a sigmoid curve (eq 2). This equation is a general description of a sigmoid curve. The parameters describing the inhibition are fitted to the data using the solver option in Excel. From these parameters, the concentration at which 50% inhibition of lipid peroxidation occurs (IC_{50}) can be calculated (eq 3). a and b = reaction constants specific for each compound/food, and c = concentration (μM, mg/L or mL/L). The IC_{50} value is expressed in the same units as c.

$$\% I = 100 \times \frac{(1 - e^{-ac})}{(1 + be^{-ac})} \quad (2)$$

$$IC_{50} = \left(\frac{-1}{a}\right) \ln \frac{0.5}{(1 + 0.5b)} \quad (3)$$

The fit error of the calculated IC_{50} can be described by eq 4, corresponding to the statistical method of least squares.

$$\text{fit error (\%)} = \left(\frac{\sum(\% I_{calcd} - \% I_{measd})^2}{n}\right)^{1/2} \quad (4)$$

% I_{measd} = measured percentage of inhibition of a certain antioxidant concentration, % I_{calcd} = calculated percentage of inhibition of the same antioxidant concentration, n = total number of percent inhibition determinations on which the IC_{50} value is based (in the case of six triplicate concentrations, n = 18).

RESULTS AND DISCUSSION

Color Formation in TBARS Reaction. Determination of the antioxidant activity of a sample or compound is often performed in test tubes or Eppendorfs. It is possible to do more incubations by simultaneously using multititer plates and a multichannel pipet. Faster and more automated absorbance readings can be made using an ELISA reader together with multititer plates. It was possible to obtain absorbance readings which fall within the accuracy of the instrument by adjusting the concentration of microsomes in the system or by changing the volume of TBA/TCA−HCl which is added to stop the LPO reaction. An incubation in which no antioxidant is added results in maximum TBARS formation, which is represented in the development of pink color. The pink color produced by TBARS is best read at 532 nm (Gutteridge, 1988), but it also proved possible to use an ELISA reader with a filter for reading at 550 or 540 nm.

To a methanol blank with a total incubation volume of 0.3 mL and a microsome concentration of 1 mg/mL was added 2.0 mL of 0.42% TBA/TCA−HCl in test tubes. After centrifugation 1 mL was transferred to a cuvette and read by a spectrophotometer at 535−600 nm. This condition resulted in an absorbance reading of 1.2. Adaptations were made that fitted both the well volume and the filter present in the ELISA reader to be able to use 48-well plates with 1.5 mL wells. By using the same microsome concentration and the same incubation volume, the addition of 1.0 mL of 0.83% TBA/TCA−HCl resulted in an absorbance reading of 0.5 with a 550 nm filter. For the 540 nm filter in the ELISA reader, addition of 0.5 mL of 0.83% TBA/TCA−HCl solution and a microsome concentration of 0.5 mg/mL resulted in absorbance readings of 1.2. By lowering the microsome concentration, less TBARS is formed, which is compensated by the use of a 540 nm filter which gives higher absorbance readings than a 550 nm filter.

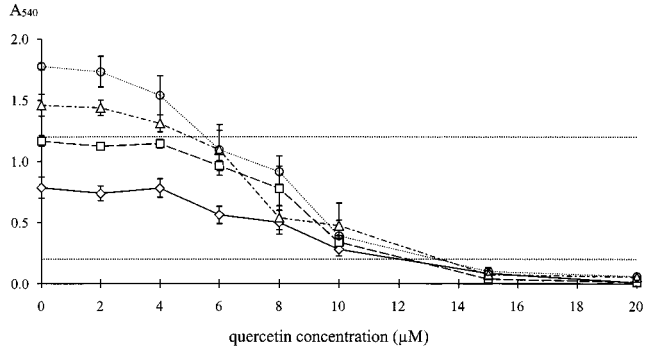

Figure 1. TBARS formation monitored by an ELISA reader. Comparison of color formation produced by microsome concentration 0.3 mg/mL (−◇−), 0.5 mg/mL (- - □ - -), 0.7 mg/mL (- • - △ - • -), or 1.0 mg/mL (•••○•••). Accuracy limits of absorbance measurement (•••). Quercetin was used as an antioxidant. A 0.5 mL portion of 0.83% TBA/TCA−HCl was added. Means and SDs of triplicate analysis.

Effect of Microsomal Concentration. The amount of lipid material present in the incubation medium (microsomal concentration) also determines the color formation after incubation with a blank or antioxidant. Different microsome concentrations were compared using quercetin as an antioxidant, and the color formation has been monitored by a spectrophotometer and ELISA reader. In Figure 1 the absorbance readings by the ELISA reader are presented. The absorbance readings of the same samples by a spectrophotometer at 540 nm show the same pattern, but at slightly higher absolute values.

Using eq 1 and the data from Figure 1, the percentages of inhibition of the various quercetin concentrations were calculated. Methanol was used as a blank. The percentages of inhibition plotted against the quercetin concentration are presented in Figure 2.

As can be seen in Figure 1 changing the microsome concentration has a considerable effect on the level of absorbance, but this effect is much smaller after calculation of the corresponding percent inhibition (Figure 2), because they are compared with a blank in which the antioxidant concentration is zero.

The parameters a and b, describing the sigmoidal dependence of the occurring inhibition on the quercetin concentration, were calculated by data fitting using eq 2. From these parameters the concentration at which 50% inhibition of lipid peroxidation occurs (IC_{50}) was calculated as described in eq 3. The resulting IC_{50} values and fit errors are given in Table 1. Lowering the microsome concentration has the advantage of the possibility to perform more antioxidant activity measurements with one batch of microsomes. The variation in IC_{50} values caused by changing the microsome

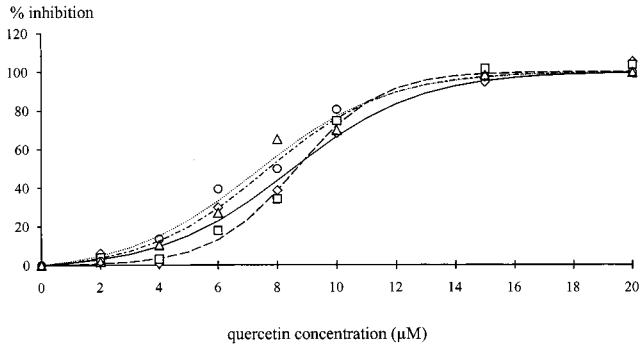

Figure 2. TBARS formation monitored by an ELISA reader. Percentage of inhibition of LPO by quercetin with microsome concentration 0.3 mg/mL (−◇−), 0.5 mg/mL (- - □ - -), 0.7 mg/mL (- • - △ - • -), or 1.0 mg/mL (•••○•••). Quercetin was used as an antioxidant. A 0.5 mL portion of 0.83% TBA/TCA−HCl was added. Means of triplicate analysis. Data from Figure 1.

Table 1. Calculated IC_{50} (μM) from Parameters *a* and *b* Describing Quercetin Inhibition Curves and Fit Errors, Obtained from Triplicate Determination of Eight Different Quercetin Concentrations[a]

microsome concn (mg/mL)	IC_{50} (μM)	fit error (%)	microsome concn (mg/mL)	IC_{50} (μM)	fit error (%)
0.3	8.5	9	0.7	7.7	9
0.5	8.7	7	1.0	7.4	7

[a] Data from Figure 2. An ELISA reader with a 540 nm filter was used. Mean ± SD = 8.1 ± 0.6.

concentration is 7.5%. Regression analysis showed that there is no significant trend present in the data (*p* value 0.093), which indicates that within the microsome concentration range tested, IC_{50} values were independent of the microsomal concentration in the test system. For the accuracy and efficacy of using the microsomes, 0.5 mg/mL was chosen as the standard microsome concentration in the assay.

Reproducibility of Antioxidant Activity Determination. Microsomes were prepared from male rat livers. Because of the fact that the microsomes are of animal origin, they are subject to biological variability. To determine a possible effect of this biological variability on the blanks in the antioxidant activity determinations, in a one year period six different microsome batches were prepared. The protein content of the isolated microsomes was assessed by the Biuret method. The protein estimation of the isolated microsomes (0.5 g of liver/mL) was 7.85 ± 2.60 mg/mL. After isolation and protein content determination, the rat liver microsomes were diluted to 5 mg/mL protein for standardization and stored in liquid nitrogen.

The mean absorbance reading ($A_{550-620}$) of the methanol blanks in the first four batches was 0.472 ± 0.097. This means a coefficient of variation of 21% in 90 repetitions, collected on 19 experimental days. The mean absorbance reading ($A_{540-620}$) of the methanol blanks in the last two batches was 1.197 ± 0.158, causing a variation of 13% in 87 repetitions, collected on 9 experimental days. In both cases the variation in absorbance of the blanks within a batch was about 10%.

To see if there is an effect on antioxidant activity determinations caused by possible biological variability of microsomes, in Table 2 the IC_{50} values (μM) of three different flavonol aglycons are presented. Each IC_{50} value was based on triplicate determination of six or seven different antioxidant concentrations, covering a range from no inhibition to full inhibition. Mean IC_{50} values ± standard deviations were calculated per batch microsomes. For quercetin, single factor analysis of variance was performed, and no difference between IC_{50} values obtained in the four batches was indicated (*p* value 0.763). The variation within batches was larger than the variation between batches. In the case of the two myricetin batches, also no difference between IC_{50} values was observed (*p* value 0.060). This indicates that it is possible to use different batches of microsomes in antioxidant activity determinations, provided that the rats which are used for microsome isolation have lived on the same diets, and are of the same gender, strain, and age.

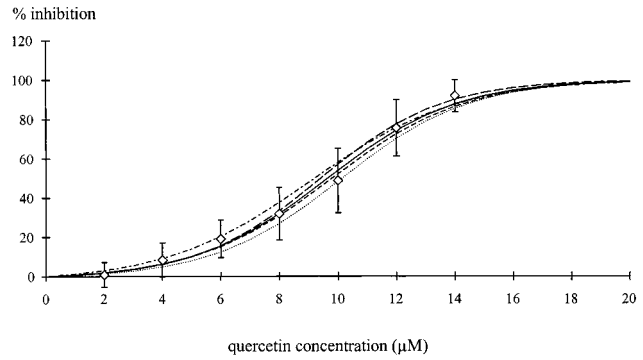

Figure 3. Comparison of the inhibition curves of quercetin analyzed in different microsome batches: mean ± SD (◇), fit batch A (•••), fit batch B (− − −), fit batch C (- • -), fit batch D (- - -), fit all data (—).

Table 2. IC_{50} (μM) of Three Flavonol Aglycons (Dissolved in Methanol), Analyzed in Four Different Microsome Batches, Based on Absorbance Readings by an ELISA Reader ($A_{550-620}$)

flavonoid aglycon	microsome isolation	IC_{50} (μM) (mean ± SD)	*n*[a]	fit error (%)
myricetin	batch A	8.3 ± 0.8	6	12
	batch B	6.7 ± 1.1	2	12
	batches A and B	7.9 ± 1.1	8	13
quercetin	batch A	10.0 ± 1.2	6	12
	batch B	9.4 ± 1.0	7	11
	batch C	9.8 ± 1.6	3	14
	batch D	9.2 ± 1.2	3	11
	batches A−D	9.6 ± 1.1	19	12
kaempferol	batch B	12.4 ± 0.04	2	12

[a] *n* = number of triplicate determinations of six or seven antioxidant concentrations. Fit errors are obtained from fitting on all data sets together.

The IC_{50} values for quercetin given in Table 2 were derived from Figure 3, using eq 3. As an example, Figure 3 shows how close the inhibition curves of quercetin analyzed in the four different microsome batches overlay. The mean percent inhibition ± standard deviation points were obtained from 19 repeated determinations. For analysis of standard compounds in this method, a variation of 10−15% in the obtained IC_{50} value was observed.

In Table 3 the antioxidant activity of selected flavonoid standards is presented. All compounds were dissolved in methanol, unless stated otherwise. Every IC_{50} value was again determined by data fitting on triplicate determinations of six antioxidant concentrations, and the mean IC_{50} ± SD was determined of the replications. The IC_{50} value was also determined by data fitting on all data sets together including the fit error that occurs. Both data fitting on separate data sets and data fitting on combined data sets provide good results.

Table 3. IC_{50} (μM) of Quercetin Glycosides, Chlorogenic Acid, Phloridzin, Catechins, and Ideain[a]

flavonoid compound (dissolved in methanol, unless stated otherwise)	IC_{50} (μM) (mean ± SD)	n	fit error (%)
Q (quercetin)	9.7 ± 1.9	6	14
Q-3-Ga (hyperin)	16.0 ± 2.8	2	11
Q-3-Gl (isoquercitrin)	17.3 ± 3.6	2	15
Q-3-Ar (guaijaverin, foeniculin)	17.2 ± 2.1	2	9
Q-3-Arf (avicularin)	15.1	1	5
Q-3-Rh (quercitrin)	20.8 ± 2.0	2	8
Q-3-Ru (rutin)	22.3± 1.7	3	9
Q-3-Xy (reynoutrin)	11.2	1	4
CA (chlorogenic acid)	124.5 ± 36.0	2	8
CA in water	128.0	1	4
P (phloridzin)	1925.5 ± 199.0	2	10
c (catechin)	15.6	1	8
ec (epicatechin)	12.2 ± 1.9	2	8
egc (epigallocatechin)	8.3	1	4
ecg (epicatechin gallate)	4.8	1	6
egcg (epigallocatechin gallate)	3.5	1	6
Cy-Ga (ideainchlorid)	25.5	1	3

[a] Comparison of values based on absorbance readings by an ELISA reader ($A_{540-620}$). n = number of triplicate determinations of six antioxidant concentrations. Fit errors are obtained from fitting on all data sets together. Ar = arabinopyranoside, Arf = arabinofuranoside, Cy = cyanidin, Ga = galactoside, Gl = glucoside, Rh = rhamnoside, Ru = rutinoside, Q = quercetin, Xy = xyloside.

The quercetin aglycon is a stronger antioxidant than its glycosides. Of the quercetin glycosides the disaccharide rutin is the weakest antioxidant. The other quercetin glycosides possess about the same potency, reynoutrin excluded, which is only a slightly weaker antioxidant than quercetin aglycon. Catechin and epicatechin are less potent antioxidants compared to quercetin, but the catechin gallates, which possess more hydroxyl groups, are stronger than quercetin. The anthocyanidin ideain is a weaker antioxidant than the catechins and quercetin glycosides. From chlorogenic acid (a phenolic acid) the antioxidant activity was determined after the compound was dissolved in methanol or in water. It did not affect the antioxidant capacity of the compound in the test system. The dihydrochalcone phloridzin was the weakest antioxidant tested.

The IC_{50} values as determined here are in a qualitative way consistent with other methods that are described in the literature. A difficulty for quantitative comparison is the existence of a broad variety of methods and description of the data obtained by those methods. Miller and co-workers (1995) reported the same order of antioxidant potency for quercetin, rutin, catechin, and epicatechin. But they only observed a factor of 2 difference between chlorogenic acid and phloridzin, with phloridzin as the less antioxidative compound. They used the Trolox equivalent antioxidant capacity value, which gives the millimolar concentration of a Trolox solution having the same antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation. In this assay the antioxidants' capacity to scavenge the $ABTS^{\bullet+}$ radical in an aqueous phase is measured. Vinson and co-workers (1995b,c) also find the same order in antioxidant activity for quercetin and rutin. But in their test system epicatechin is more active than quercetin, and chlorogenic acid and cyanidin are almost as active as quercetin. Their test system for antioxidant activity was lipoprotein, and cupric ion was used as oxidant. The differences mentioned are probably caused by the hydrophilic or lipophilic properties of the different test systems and the polarity of the antioxidant

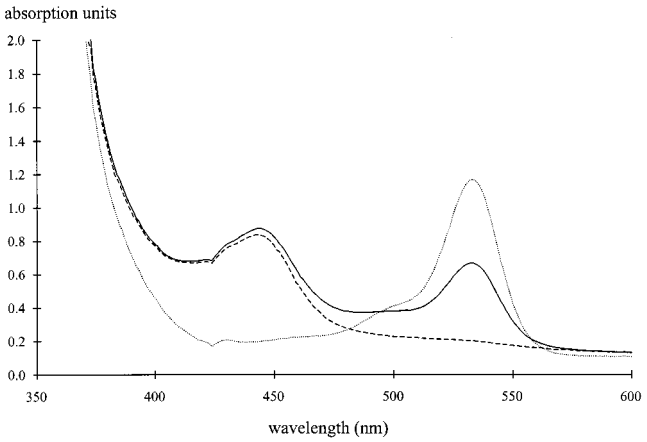

Figure 4. Absorption spectra after TBARS formation monitored by a spectrophotometer: a blank showing a pink color after 80 min of microsomal incubation at 37 °C (···), a commercially available apple juice after 0 min of incubation showing a yellow color (- - -), and the same apple juice after 80 min of incubation showing an orange color (—).

itself. Therefore, it is very important to test the standard compounds of interest and the food products in which they are present in one and the same antioxidant test system. This enables the use of such an assay in product development.

Possible Complications in the Assay Caused by the Food Matrix. The purpose of this research is to measure antioxidant activity in food products to be able to evaluate health claims and the optimization of product development with respect to health protecting compounds. For that reason it is important to know the behavior of other compounds that are present in the food matrix in the antioxidant activity assay. Macronutrients present in apple juice are carbohydrates (123 g/L) and up to 0.5 g/L protein (Schobinger et al., 1978). The main organic acids in apple juice are chlorogenic acid (up to 85 mg/L) and phloridzin (up to 67 mg/L), with concentrations depending on the production process. Caffeic acid, *p*-coumaric acid, and ferulic acid can be present in lower concentrations (up to 11 mg/L) (Schols et al., 1991).

In Figure 4 absorption spectra of a blank and a commercially available apple juice sample after microsomal incubation and TBARS formation are given. The blank shows a pink color after 80 min of incubation at 37 °C, and the apple juice sample after the same 80 min of incubation shows an orange color. The same apple juice sample after 0 min of incubation shows a yellow color after the TBARS reaction. The yellow color was also observed upon heating of apple juice with TBA (without the presence of microsomes). The yellow color is caused by the reaction of sugars that are present in the apple juice with TBA. Wilbur and co-workers (1949) also found a yellow color produced by a reaction between TBA and sugars, with a maximum absorption at 450 nm. In the apple juice sample that had been incubated with the microsomes, TBARS was formed, but the present sugars also reacted with TBA, so the yellow color together with the pink color produced an orange appearance. From Figure 4 the conclusion can be drawn that the yellow color has its absorption maximum at 443 nm and does not influence the measurement of the pink color produced by TBARS at 535 nm. Antioxidant activity measurement of apple juice is possible with this method, but when other products are measured in this

Table 4. IC_{50} Values and Quercetin Glycoside Content of Differently Produced Apple Juices[a]

	straight pressing ($n = 4$)	pulp-enzymed juice ($n = 2$)	commercially available juice ($n = 3$)
[Q-gly] in juice (mg/kg)	6.9 ± 1.6	5.7 ± 0.6	3.5 ± 0.1
IC_{50} of juice (g of fresh juice/L)	57 ± 38	130	59 ± 22

[a] Fit errors <10%.

assay, good controls (choice of blanks and testing of the product in the TBA test in the absence of microsomes) should be taken into account to be sure that no interference of protein, lipids, organic acids, or colored compounds originating from the product itself with the reaction products of the assay occurs.

Antioxidant Activity of Apple Juice Samples. In Table 4 IC_{50} values and the quercetin glycoside content of differently produced apple juices are shown. A commercially available apple juice is compared to Jonagold apple juice prepared by straight pressing and Jonagold apple juice that is prepared by pulp enzyming. Pulp enzyming is often used in industry to obtain a higher juice yield and to facilitate the pressing.

The three different apple juices all have a low quercetin glycoside content, compared to that present in apple, which is 36 ± 19 mg/kg (expressed as quercetin aglycon) (Hertog et al., 1992). Recalculated to quercetin glycosides (using a mean molecular weight of 445.37 for the quercetin glycosides), this would be 53 ± 28 mg/kg.

Apple juice prepared by straight pressing and the commercially available juice have the same antioxidant activity. Apple juice prepared by pulp enzyming has a lower antioxidant activity compared to the other two. The IC_{50} value of the pulp-enzymed juice is obtained by extrapolation (by the data solver) because at the highest concentration possible to test in the antioxidant assay (52 g of fresh juice/L assay for this sample) only 11.4 ± 3.6% inhibition was measured. In this case it was not possible to have antioxidant concentrations that covered a range from no inhibition to full inhibition of LPO.

During the stirring that takes place in the pulp-enzyming process, enzymatic oxidation of compounds (other than quercetin glycosides) occurs, which is the possible cause of the decreased antioxidant activity of pulp-enzymed juice.

The concentration of quercetin glycosides that is present in apple indicates the existence of a big variability (coefficient of variation 53%) in the raw material. The concentration of quercetin glycosides in the apple juices is about 10 times lower. The variation obtained in the antioxidant activity determination is also big (38% and 67% for the commercially available juice and the straight pressed juice, respectively). But the fit errors for the individual antioxidant activity determinations each were lower than 10%.

The possibility to analyze antioxidant activity of differently processed apple juices enables the choice for the product with the highest activity and to optimize apple juice processing with respect to the antioxidant activity of the product.

CONCLUSIONS

In these experiments antioxidant activity was determined by measuring the inhibition of LPO by a range of six different concentrations of a certain antioxidant. For each concentration the percent inhibition was measured after 60 min of incubation. The concentrations were chosen in such a way that the lowest concentration provided no inhibition of LPO and the highest concentration gave full inhibition of LPO. From these data it was possible to calculate the IC_{50} value by an objective mathematical procedure. This makes it possible to evaluate antioxidant activity in a quantitative way, which is not feasible when the inhibition of LPO by a certain concentration of an antioxidant is compared in time courses. In that case only qualitative conclusions can be drawn.

The reproducibility of this method is good, the variation in IC_{50} values that are obtained when standard antioxidant compounds are applied is 10−15%. Within the microsome concentration range tested, IC_{50} values were independent of the microsomal concentration in the test system. However, it is advised to use a standardized microsome concentration (0.5 mg/mL) in the assay. The use of different batches of microsomal isolates did not show an effect on the antioxidant activity determinations.

Antioxidant activity measurement of a sugar-rich food product such as apple juice is possible with this method, but when other products are measured in this assay, good controls should be taken into account to be sure that no interference of proteins, lipids, organic acids, or colored compounds originating from the product itself with the reaction products of the assay occurs.

If interest lies in studying underlying mechanisms regarding antioxidant activity, it is necessary to compare radical-generating systems different from those described in this method, and to test the difference in behavior of antioxidants and products in such assays.

The possibility to compare differently processed food products in one method, such as shown here for apple juices, with regard to their antioxidant activity enables the choice for the product with the highest activity and the optimization of processing with respect to the antioxidant activity of the product.

ACKNOWLEDGMENT

We thank Margot van Soelen, Annemarie Renkens, and Andrea Mertens for their technical assistance.

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Received for review February 4, 2000. Revised manuscript received May 23, 2000. Accepted May 23, 2000.

JF000156I