JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Fenton-Dependent Damage to Carbohydrates: Free Radical Scavenging Activity of Some Simple Sugars

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The antioxidant properties of simple carbohydrates were studied in a chemical system. Hydroxyl radicals generated by a Fenton reaction induce damage on simple carbohydrates with a consequent free radical scavenging activity. Carbohydrate activities were measured by different methods as spin-trapping of hydroxyl radical and electron paramagnetic resonance detection and 1,1-diphenyl-2-picrylhydrazyl quenching. Carbohydrate damage was evaluated in a Fenton system by measuring the reactive substances to thiobarbituric acid, by their decreased detection with an HPLC test, and by a gas chromatographic determination of formic acid from sugar oxidation. Different intensities of damage and scavenging were found according to molecular structure, and some hyphotheses on the carbohydrate action against free radicals were attempted. The assayed disaccharides were shown to be more active toward and less damaged by hydroxyl radical than monosaccharides.

KEYWORDS: Simple carbohydrates; antioxidant properties; hydroxyl radical; free radical scavenging activity

INTRODUCTION

Fresh and processed fruits and vegetables show a progressive quality loss mainly caused by the oxidation of biomolecules such as lipids, pigments, phenols, proteins, and carbohydrates. These oxidations are caused by atmospheric oxygen, opportunistically activated by its one-electron reduction to the superoxide radical anion $^{\bullet}O_2^-$, to hydrogen peroxide H_2O_2, and to the hydroxyl radical $^{\bullet}OH$. Generation of these reactive oxygen species (ROS) beyond the antioxidant capacity of a biological system gives rise to oxidative stress (*1*).

The well-established role of fruits and vegetables is that some components of them, such as vitamins and polyphenols, may be effective in the prevention of diseases associated with free radicals (*2*).

The antioxidant defense system against oxygen toxicity is fully efficient when living cells stand in an omeostatic condition, where oxidation and antioxidation are about at equilibrium. In altered equilibrium conditions, which are manifested, for example, in aerobic organisms exposed to oxygen concentrations up to 21%, pathological states can occurr, because the antioxidant system does not seem to be able to control the increased oxidative processes (*3*). Analogously, senescence processes in fruits and vegetables compromise the biosynthesis of new antioxidant molecules, determining disequilibrium toward oxidation.

One-electron reduction of molecular oxygen can occur by catalytic action of ferrous cation with superoxide radical anion production (*4*):

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} - {}^{\bullet}O_2^- \rightarrow Fe^{3+} + {}^{\bullet}O_2^-$$

However, in biological systems Fe or Cu ions cannot be found in free solution. Normally, they are bound to proteins, membranes, nucleic acids, and chelating agents of low molecular weight (for example, ATP, ADP, and citrate) (*5*).

It is more probable that, in living cells, reduction of molecular oxygen is carried out by strong reducing agents involved in mitochondrial electron transport chains, as reduced flavins and hydroquinones (*6*).

Another important reducing function of molecular oxygen is done by metalloenzymes, to which O_2 is first bound and successively reduced by the metal (normally iron or copper) in the active site (*7*).

The one-electron reduction of $^{\bullet}O_2^-$ to hydrogen peroxide is catalyzed by superoxide dismutase, but it can occurr also spontaneously:

$$2{}^{\bullet}O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide is a compound with a moderate chemical reactivity, but it is essential for the formation of the most

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10.1021/jf030172q CCC: $25.00
Published on Web 11/07/2003

powerful free radical known: the hydroxyl radical (•OH). In fact, H_2O_2 is decomposed to the hydroxyl radical and hydroxyl ion in the presence of transition metals (for example, Fe, Cu, and Cr) according a Fenton-type reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

Besides, the hydroxyl radical can be generated by the direct reaction of superoxide radical with hydrogen peroxide, in the presence of Fe catalyst (*8*, *9*):

$${}^{\bullet}O_2^- + H_2O_2 \rightarrow OH^- + {}^{\bullet}OH + H_2O$$

The highly reactive hydroxyl radical •OH is known to damage almost all biomolecules, including carbohydrates. This fact can be gleaned from the paper of Anbar and Neta (*10*), with reaction constants (*k* value) in the range of 10^8-10^{10} M^{-1} s^{-1}.

The biomolecules are damaged in vivo by the reaction with •OH. This damage can be reversible for the action of repair enzymes (*11*). Hence, repair enzymes can be considered as tertiary antioxidants, being the first-line defense against the ROS constituted by the enzymes superoxide dismutase, catalase, and glutathione peroxidase (*12*) and the second line constituted by molecular species widely present in fruits and vegetables involved in "chain-breaking' processes (*13*).

It is clear that the presence of Fe is essential to generate •OH and that the metal must be in a chelated state. The effect of chelating agents such as EDTA has been widely investigated (*14*).

If, for different causes, free radical species production exceeds the defensive capacity of the antioxidant system, the free radicals react with the molecular cell components.

The indiscriminate reactivity of hydroxyl radical toward the biomolecules implies the following: every biomolecule functions as scavenger at the moment of reaction with •OH; the biomolecule, after the reaction with •OH, becomes itself a free radical; the secondary free radical obtained from the reaction is generally less reactive than •OH, but it can induce damage in other molecules; the biomolecules are subjected to oxidative damage after reaction with •OH.

In plant cells, carbohydrates represent a class of compounds of primary importance, both as plastic and reserve material and as transient metabolic forms.

In the presence of an excess of •OH, carbohydrates can be damaged. This reaction is well evidenced by the production of malondialdehyde, detectable from the reaction with thiobarbituric acid (TBA value), giving a chromogen with absorbance at 532 nm (*15*). Different types of carbohydrates have different reactivities with TBA and, hence, different amounts of damage by •OH.

A very TBA-reactive carbohydrate was deoxyribose, and its reactivity can be used to detect the •OH and, for example, the effect of some free radical scavengers in a Fenton system (*16*, *17*).

However, the direct role of simple carbohydrates in the suppression of free radicals (*18*, *19*) has not been widely discussed in the literature with respect to other types of molecules such as polyphenols or unsaturated lipids.

Recent literature from IVTPA has pointed out that different carbohydrates added to complex systems, such as frozen strawberry juices, differently affect the juice stability, presuming that a higher protective effect of some sugars with respect to others could be related to a higher free radical scavenging activity (*20*).

Many different methods are employed in assays to evaluate the effectiveness of free radical scavenger activities on different substrates such as whole and partially fractionated plant extracts and purified compounds (*21*). The scavenging effect on •OH can be measured in a direct way by detecting the presence of it with electronic paramagnetic resonance (EPR) spectroscopy, using a spin-trapping method (*22*−*24*), so the suppression by a scavenger molecule of this free radical can be easily measured.

The aim of this work is to contribute to the evaluation of the damage of some simple carbohydrates after reaction with •OH generated by a Fenton reaction and their free radical scavenger effect by different methods.

The following methods were used: TBA test to evaluate the reactive substances to thiobarbituric acid produced by sugars in the presence of •OH; EPR to detect the presence of •OH; an HPLC test able to detect the sugar reduction after damage of •OH; a GC headspace test to evaluate the formic acid (HCOOH) produced by the •OH-damaged sugars [formic acid is produced from sugar oxidation by periodic acid, a chemical method used to determine sugar structure; periodic acid is a specific oxidixing agent for vicinal glycols (*25*), and the reaction of sugars with free radicals (for example, •OH) under oxidative conditions also produces formic acid (*26*, *27*); hence, the quantitative analysis of this acid to evaluate the action of •OH on the sugars could be important]; and a spectrophotometric method to detect the extinction of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) by single sugars.

The tested compounds were the more diffused simple carbohydrates in fruits and vegetables, such as maltose, sucrose, glucose, fructose, and deoxyribose and a reduced sugar such as sorbitol.

MATERIALS AND METHODS

Maltose, sucrose, 2-deoxy-D-ribose, D-(+)-glucose, D-(−)-fructose, and sorbitol were the sugars assayed purchased from Merck (Darmstadt, Germany). The sugar stock solutions at a concentration of 52 mM were made in a 0.1 M phosphate buffer solution, pH 7.4 (PBS). These solutions were 6.5-fold diluted with PBS to a final concentration of 8 mM for all experiments except for the TBA test, for which the concentrations used were 0.002, 0.005, 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 mM for each sugar.

2-Thiobarbituric acid (TBA) was obtained from Merck.

Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were purchased from Fluka Chemie AG (Buchs, Switzerland).

All other chemicals used were from Sigma-Aldrich (St. Louis, MO).

For each dissolution or dilution, PBS and all other solutions were previously deaereated by bubbling with pure N_2.

Fenton Reaction. Active oxygen species, such as hydroxyl radical (•OH), are formed through a one-electron reduction of hydrogen peroxide (H_2O_2). The •OH is generated by a process known as redox cycling or Fenton reaction and is catalyzed by transition metals such as Fe^{2+}:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

The reagents of the Fenton reaction were prepared in two different concentrations: less concentrated (weak conditions) and more concentrated (drastic conditions).

For the "weak" Fenton system, a stock solution of $FeSO_4 \cdot 7H_2O$ 10 mM in 12 mM 2NaEDTA and a stock solution of 10 mM H_2O_2 were freshly prepared in PBS.

For the "drastic" Fenton system, a stock solution of $FeSO_4 \cdot 7H_2O$ 20mM in 24 mM 2NaEDTA and a stock solution of 100 mM H_2O_2 were freshly prepared in PBS.

These solutions were all diluted 6.5-fold with PBS to obtain the Fenton reaction mixtures at the following final concentrations:

Fenton reagent	weak conditions	drastic conditions
$FeSO_4 \cdot 7H_2O$	1.54 mM	3.08 mM
2Na EDTA	1.85 mM	3.70 mM
H_2O_2	1.54 mM	15.40 mM

The reaction was prepared by mixing the Fe-EDTA solution with the scavenger or pure PBS for the reference and then started by adding the H_2O_2 solution.

Weak Fenton reaction conditions were used for all tests; in addition, drastic Fenton conditions were used for HPLC and HCOOH tests (see below).

TBA Test. The adopted TBA test method was taken from Halliwell and Gutteridge (*16*) with some modifications.

The weak Fenton reaction mixture and the blank, in a total volume of 1.3 mL, were as follows:

	blank	test
PBS	1.1 mL	0.7 mL
sugar solution	0.2 mL	0.2 mL
Fe-EDTA		0.2 mL
H_2O_2		0.2 mL

The sugar concentrations ranged from 0.002 to 0.8 mM.

The reaction mixtures were incubated at 37 °C for 1 h, and then were added in succession 1 mL of 1% (w/v) TBA dissolved in 50 mM NaOH and 1 mL of 2.8% (w/v) trichloroacetic acid in water. The resulting mixtures were heated at 100 °C for 20 min and cooled, and their absorbances were read at 532 nm against the blank. Each assay was six times replicated.

A plot absorbance versus sugar concentration was prepared for each sugar, and the slopes of the regression lines were calculated.

The rate constant (k) for the reaction of deoxyribose with •OH was obtained from the literature (*28*), value 3.10×10^9 M^{-1} s^{-1}. For other single sugars, an approximated k value in the absence of competition was obtained by the equation

$$k_{approx} = 3.10 \times 10^9 \times slope_{sug}/slope_{deox}$$

where $slope_{sug}$ was obtained from the plot absorbance at 532 nm versus sugar concentration and $slope_{deox}$ was obtained from the plot absorbance at 532 nm versus deoxyribose concentration (**Figure 1**).

EPR Test. The weak Fenton conditions were used for this test, and hydroxyl radical produced in the reaction mixture was trapped with DMPO, according to the method described in detail by Morelli et al. (*29*), with modifications. The resultant adduct DMPO−•OH, consisting of a quartet resonance composed of resonances with 1:2:2:1 relative intensities composed of a doublet of triplet resonances (**Figure 2**), was detected by an X-band EPR spectrometer Varian E-line Century series. The triplet resonance, with relative peak intensities of 1:1:1, arises from free radical coupling with the $I = 1$ ^{14}N, the more abundant isotope. The doublet structure superimposed on the triplet structure arises from free radical coupling with the β vinylic proton ($I = {}^1/_2$).

For •OH measurement, the mixture without the scavenging compounds (blank) contained 0.7 mL of PBS, 0.2 mL of Fe-EDTA, 0.2 mL of 50 mM DMPO in PBS, and 0.2 mL of H_2O_2.

The assayed sugars were diluted in PBS at 52 mM concentration and then were mixed with the Fenton reagents to a 8 mM final concentration. The mixture with the test scavenging compound contained 0.5 mL of PBS, 0.2 mL of scavenger (sugar solution in PBS), 0.2 mL of Fe-EDTA, 0.2 mL of 50 mM DMPO in PBS, and 0.2 mL of H_2O_2.

These solutions were accurately mixed in a glass tube assay and successively placed in the EPR probe, a capillary tube of 100 mm length and 0.7 mm internal diameter. EPR spectra were recorded after exactly 2 min.

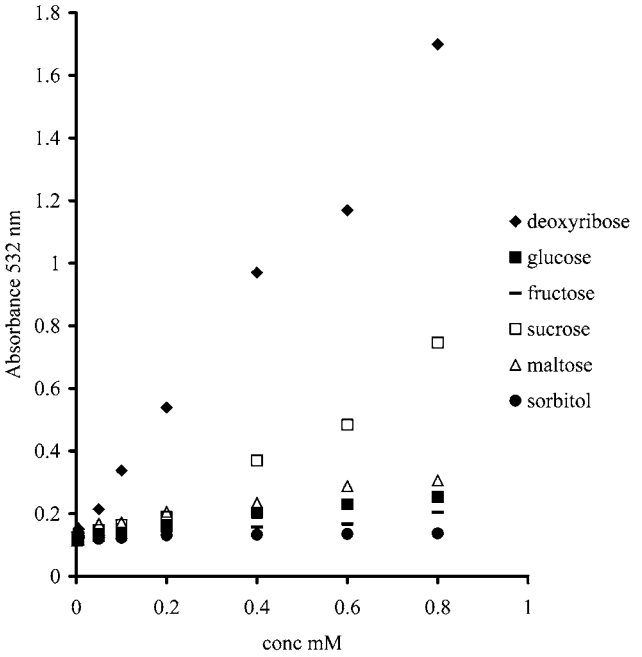

Figure 1. Average values of absorbance versus concentration at 532 nm of deoxyribose and some single sugars in a Fenton system and after reaction with thiobarbituric acid.

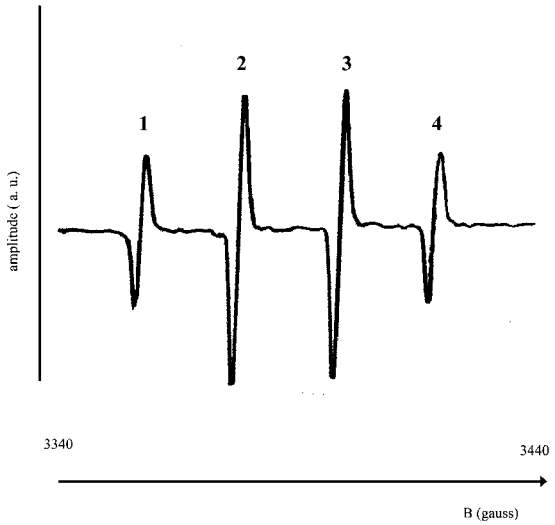

Figure 2. Example of EPR spectrum of a DMPO–•OH adduct after hydroxyl radical generation by Fenton reaction with Fe^{2+} chelated with EDTA.

The instrumental parameters were as follows: frequency, 9.26 GHz; power, 5 mW; field set, 3390 G; scan time, 64 s; time constant, 0.5 s; gain, 16000; modulation, 1 G.

The scavenger activity percent of the test compound for •OH was expressed as the formula

$$I = 100(1 - h_x/h_0)$$

where I was scavenger activity and h_0 and h_x were the relative heights of line 2 (mm) of the DMPO−•OH adduct spectra (**Figure 2**) in a reaction mixture without and with the scavenger compound, respectively. Each assay was repeated 10 times.

HPLC Test. The evaluation of Fenton-dependent damage to sugars was made with the identification of single sugars by their retention time in a PBS solution and by the percent reduction area calculation in sugars subjected only to chelated Fe^{2+} and to Fenton conditions, that is, the •OH.

Three types of experiments were set up: (1) weak conditions, 2 min at room temperature; (2) drastic conditions, 2 min at room temperature; (3) very drastic conditions, 20 min at 100 °C.

The assayed sugars were diluted in PBS at 52 mM concentration and then were mixed with the Fenton reagents to an 8 mM final concentration.

Preliminarly, tested compounds were mixed only with chelated Fe^{2+} to check their stability in the following ways for each type of experiment:

	blank	test
PBS	2.2 mL	1.8 mL
sugar solution	0.4 mL	0.4 mL
Fe-EDTA		0.4 mL

Successively, tested compounds were mixed with Fenton reagents:

	blank	test
PBS	2.2 mL	1.4 mL
sugar solution	0.4 mL	0.4 mL
Fe-EDTA		0.4 mL
H_2O_2		0.4 mL

Just after the reaction time, the mixtures were added to 0.1 mL of 0.5 N HCl and soon injected into the chromatograph (20 μL).

The HPLC system consisted of an isocratic pump Jasco model 880-PU, a refractive index detector Jasco RI-930, and an Jones Chromatography oven. The chromatograms were registered on a Shimadzu C-R3A recorder.

The column used was a HyperRez XP Ca^{2+}, 8 μm, 300 × 7.7 mm, and the mobile phase consisted of bidistilled deareated water at 0.6 mL/min; elution was carried out at 80 °C.

In these conditions, the peaks of tested compounds were well resolved from other signals and showed the following retention times: maltose, 8.83 min; sucrose, 9.08 min; glucose, 11.13 min; fructose, 13.45 min; deoxyribose, 14.15 min; sorbitol, 21.76 min.

Sugar damage was calculated by the percent area ratio test versus blank. Each assay was made in quadruplicate.

HCOOH Test. For this test, the same sugar solutions in PBS, weak and drastic Fenton conditions of HPLC test, were prepared, doubling the used volumes. The mixtures were placed in 20 mL headspace vials, heated at 80 °C for 1 h, and GC analyzed for their HCOOH content in the headspace.

The GC used was a DANI model 3800 with a FID, equipped with a DANI model 3950 headspace automatic sampler. The chromatograms were registered on a Shimadzu C-R3A recorder.

GC separation was performed on a DB-Wax 60 m wide-bore column, internal diameter = 0.53 mm, and film thickness = 1.0 μm; the carrier gas was ultrapure He with a head pressure on column of 1.6 bar. The makeup gas was N_2 at 1.0 bar; air and H_2 for the FID were set at 1.0 bar.

The retention time of HCOOH in these conditions was 3.8 min.

The HCOOH amount was calculated by interpolation of HCOOH peak area of sugar degradation headspace and a calibration curve obtained by diluting standards of pure HCOOH dissolved in the same conditions of assayed sugars.

Each assay was five times replicated.

DPPH• Test. This test was made according to the method of Brand-Williams et al. (*30*) with some modifications and was chosen for its widespread use for the assessment of the antioxidant capacity of foods (*31*−*34*). In its radical form DPPH• absorbs at 517 nm, but upon reduction of a hydrogen donor its absorption decreases.

A final volume of 10 mL of 0.2 mM DPPH• ethanol solution was used. The assayed sugars were diluted in a 60:40 ethanol/water solution at 80 mM concentration and then were mixed with the DPPH• solution to an 8 mM final concentration. The reference solution was made by adding to the DPPH• solution the same volume (1 mL) of pure 60:40 ethanol/water solution.

Table 1. Values of R^2, Slope of the Straight Line in a Plot of Abs 532 nm against Sugar Concentration (mM) and Derived Rate Constants (k_{approx}) for TBA Products from Reaction of Hydroxyl Radicals with Some Simple Sugars

sugar	R^2	slope[a]	$k_{approx}/10^9$ M s
deoxyribose	0.99	1.894e	3.10[b]
sucrose	0.97	0.743d	1.22[c]
maltose	0.98	0.199c	0.33[c]
glucose	0.98	0.171c	0.28[c]
fructose	0.95	0.091b	0.15[c]
sorbitol	0.84	0.025a	0.04[c]

[a] Different letters are for statistically significant differences ($p > 0.05$). [b] Calculated by pulse radiolysis tecnique. [c] Derived by the slope of straight-line deoxyribose plot.

The bleaching of DPPH• was calculated by subtracting the absorbance at 517 nm immediately after the mix of the solutions from the absorbance after 60 min in the dark.

Each assay was three times replicated.

Statistical Analysis. Data were analyzed by one-way analysis of variance in combination with the Tukey multiple-comparison test using the Statgraph package (Manugistic Inc., Rockville, MD).

RESULTS

TBA Test. Hydroxyl radicals, generated in a Fenton system, attack the deoxyribose and other simple carbohydrates, including the sorbitol, a polyalcohol, and set off a series of reactions that result in the formation of TBA-reactive substances, measured as a pink chromogen by its absorbance at 532 nm.

Figure 1 shows an experiment in which the deoxyribose at various concentrations was exposed to hydroxyl radicals for 1 h at 37 °C; a regression line (absorbance vs sugar millimolar concentration) was obtained from the experimental points with a very high value of R^2. Regression lines with high values of R^2 were also obtained (**Figure 1**) in similar experiments from the plots of assayed carbohydrates and sorbitol.

As was expected, these experiments gave evidence for the highest reactivity of the deoxyribose toward the •OH, with the statistically significant highest slope (**Table 1**). The value of the rate constant of this sugar for this reaction (3.10×10^9 M^{-1} s^{-1}) was found in the literature calculated by extensive pulse radiolysis studies using both the thiocyanate and iodide competition methods (*28*, *35*). This value was used for the calculation of k_{approx} related to the other sugars.

The carbohydrates assayed by the TBA method gave significantly lower slopes than the deoxyribose; in decreasing order there were sucrose, maltose, glucose, fructose, and sorbitol. The latter had the lowest value (0.025), indicating a very low reactivity to •OH. Maltose and glucose did not show a statistically significant difference. The differences in slopes reflect the values of k_{approx} to the calculated value of k for deoxyribose.

The same ranking of reactivity for sucrose, glucose, and fructose was also found by Gutteridge (*15*).

EPR Test. In this test, the hydroxyl radicals are generated exactly in the same way as in the TBA test, with the addition of DMPO to form a stable adduct with •OH. The adduct is clearly detectable in an EPR system, with a spectrum formed by four lines with a 1:2:2:1 intensity ratio (**Figure 2**). The spectral intensity (arbitrary units) is proportional to the •OH amount; the residual presence of it in the samples with the addition of scavengers and the consequent inhibition of •OH are given in percent with respect to a blank in the absence of scavenger (**Table 2**). The experimental conditions used in this

Table 2. Averages of Scavenging Activity of Hydroxyl Radicals by Some Sugars Evaluated by EPR[a]

sugar	height (mm)	•OH res %	% inhibition[b]	free OH residues
blank[c]	180.9	100.0	0.0a	
maltose	45.4	25.1	74.9e	8
sucrose	48.4	26.8	73.2e	8
fructose	60.0	33.2	66.8d	5
glucose	62.2	34.4	65.6d	5
deoxyribose	73.9	40.9	59.1c	3
sorbitol	97.3	53.8	46.2b	6

[a] The height of the second line of the EPR spectrum of the adduct DMPO–•OH is considered as the •OH residual amount, given in percent with respect to blank. The % inhibition represents the scavenging of •OH by single molecules with respect to blank. [b] Different letters are for statistically significant differences ($p > 0.05$). [c] Blank is intended absence of scavenger.

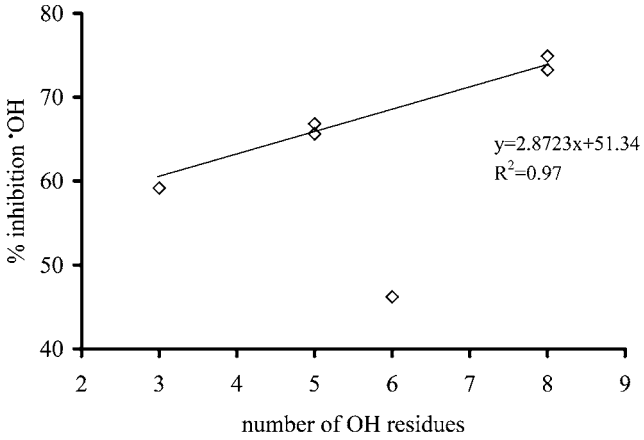

Figure 3. Percent inhibition of Fenton-generated •OH evaluated by EPR, as a function of free OH residues of assayed simple carbohydrates. Maltose and sucrose, 8 OH; sorbitol, 6 OH; glucose and fructose, 5 OH; deoxyribose, 3 OH. The regression line does not include the sorbitol data.

test were chosen after many preliminary trials in order to evaluate the stability and the possible interference by the Fenton reaction components with the DMPO−•OH adduct detection (*29*).

The activities of quenching by single sugars were very significant at the scavenger concentrations (8 mM) used in this Fenton system.

The statistically significant greatest scavenging activities were shown by the disaccharides maltose and sucrose, and the lowest was given by sorbitol; deoxyribose was in a middle range of •OH inhibition. The scavenging activity ranking of deoxyribose with respect to other sugars was in contrast with its activity in forming TBA-reactive substances (**Figure 1**; **Table 1**).

An interesting relationship was found between the number of alcoholic hydroxyl groups and the scavenging activity toward •OH. The relationship (**Figure 3**) was strictly linear for the "true" sugars ($R^2 = 0.97$), with an increasing activity with increased number of OH residues. This fact was not true for sorbitol; in fact, it is not a "true" sugar but a polyalcohol by reduction of the carbonyl function of glucose: it possesses six OH residues with a relatively lower scavenging activity (46.2%) than was expected by its number of OH residues. Sorbitol, plotted together the other sugars (**Figure 3**), drastically lowered the R^2 value for the tendency line (0.25).

HPLC Test. The sugar damage, as reaction of •OH with some simple carbohydrates, was evaluated by the decrease of HPLC signal in different experimental conditions. This test gave the

Table 3. Percent Reduction of Peak Areas Detected by RI-HPLC by Some Simple Sugars[a]

sugar	1.5 mM Fe^{2+} [b]	3.1 mM Fe^{2+} [b]	3.1 mM Fe^{2+}, 20 min, 100 °C[b]
maltose	0.12a	1.57b	0.94a
sucrose	0.34a	0.35a	3.27b
glucose	0.22a	3.06c	9.74d
fructose	0.12a	1.72b	19.62e
deoxyribose	0.10a	1.70b	5.18c
sorbitol	0.21a	7.02d	6.80c

[a] Sugar damage was evaluated in the presence of chelated Fe^{2+}. Blank was considered the sugar dissolved in buffer at pH 7.4. [b] Different letters in the same column are for statistically significant differences ($p > 0.05$).

Table 4. Percent Reduction of Peak Areas Detected by RI-HPLC by Some Simple Sugars in a Fenton System[a]

sugar	1.5 mM Fe^{2+}/ 1.5 mM H_2O_2[b]	3.1 mM Fe^{2+}/ 15.4 mM H_2O_2[b]	3.1 mM Fe^{2+}/ 15.4 mM H_2O_2, 20 min, 100 °C[b]
maltose	1.54a	1.42a	42.02a
sucrose	3.04ab	2.24a	42.39a
glucose	1.74a	32.14b	56.79b
fructose	3.28ab	31.78b	53.01b
deoxyribose	4.80b	34.39b	44.11a
sorbitol	4.45b	63.27c	80.86c

[a] Blank was considered the sugar dissolved in phosphate buffer at pH 7.4 in the presence of chelated Fe^{2+}. [b] Different letters in the same column are for statistically significant differences ($p > 0.05$).

percent reduction in the HPLC signals for a Fenton system related to a blank with an RI detection of the single sugars only in the presence of chelated Fe^{2+}.

Three different experiments were set up. The first was at the same conditions of the TBA and EPR tests. The second and third were performed in more drastic conditions, increasing the Fe^{2+} and H_2O_2 amounts and further adding the action of the temperature, respectively (**Tables 3** and **4**).

It has to be noted that the more drastic the Fenton reaction conditions, the more some peaks, with different retention times from assayed sugars, appeared in HPLC chromatograms.

Preliminarly, the relative stability of sugars was checked only in the presence of chelated Fe^{2+} (**Table 3**).

The action of 1.5 mM Fe^{2+} produced negligible damage to sugars: all data are between 0.1 and 0.3% reduction. The 3.1 mM Fe^{2+} gave some damage, with a low value for sucrose and a relatively high for sorbitol. The temperature at 100 °C for 20 min generally gave high reduction values: they were significantly low for the disaccharides maltose and sucrose and high for glucose and fructose.

The results of sugar damage subjected to the Fenton reaction are shown in **Table 4**.

In the first experiment, sugar reductions were generally low, with a slightly significant high percentage of reduction for deoxyribose and sorbitol. In the second experiment, the differences were more pronounced than in the first: the disaccharides were significantly more stable (∼2% reduction); glucose, fructose, and deoxyribose were between 32 and 34%; the sorbitol was the less stable product to •OH action with a percent reduction of 63.3. The third experiment generally gave high reductions and confirmed the last trend, except for the deoxyribose, that resulted in the group at high stability together with the disaccharides.

HCOOH Analysis. Another important marker of sugar damage under oxidative conditions is the production of HCOOH.

Table 5. Average Values of HCOOH (mM) Evaluated by GC Headspace from Sugar Fenton Degradation in Different Conditions

sugar	PBS, pH 7.4[a]	1.5 mM Fe^{2+}/ 1.5 mM H_2O_2[a]	3.1 mM Fe^{2+}/ 15.4 mM H_2O_2[a]	free OH residues
maltose	5.06a	4.63a	9.82c	8
sucrose	4.55a	4.60a	10.56c	8
fructose	4.44a	4.24a	6.41a	5
glucose	4.45a	4.91a	7.71b	5
sorbitol	4.39a	4.49a	7.62b	6
deoxyribose	5.16a	4.63a	8.21b	3

[a] Different letters in the same column are for statistically significant differences ($p > 0.05$).

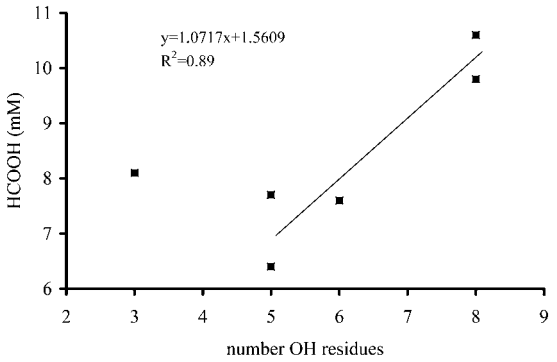

Figure 4. Fenton-dependent formic acid production evaluated by GC by simple carbohydrates, as a function of free OH residues. Maltose and sucrose, 8 OH; sorbitol, 6 OH; glucose and fructose, 5 OH; deoxyribose, 3 OH. The regression line does not include the deoxyribose data.

This product was evaluated by GC headspace, conditioning the vials with the reaction mixtures at 80 °C for 1 h.

HCOOH is produced from sugars at 80 °C without any Fenton reagent (PBS, pH 7.4); the amount was ~4.67 mM (**Table 5**) for all tested compounds. The Fenton conditions used for the EPR test (1.5 mM Fe^{2+} and 1.5 mM H_2O_2) did not give statistically significant differences among the sugars and in the absence of reagents, whereas more drastic conditions (3.1 mM Fe^{2+} and 15.4 mM H_2O_2) gave significant differences in the HCOOH production, with significantly higher amounts only for the disaccharides.

Another interesting relationship was found between the number of hydroxyl groups and HCOOH production. The relationship (**Figure 4**) was good ($R^2 = 0.89$) for all tested compounds excluding deoxyribose, with an increasing amount of HCOOH with the increased number of OH residues. Deoxyribose, plotted together the other sugars, lowered the R^2 value for the regression line (0.51), giving a higher production of HCOOH than was expected from its number of OH residues.

DPPH• Test. The assayed sugars (8 mM) generally showed a very low scavenging activity on the DPPH•. Sugars are weak hydrogen donors with respect to other molecules such as phenols and chlorogenic acid, which was ~10-fold more active in the same concentration (data not shown).

Chlorogenic acid was chosen as a model compound because it is known in the literature as a phenolic compound present in many plant species (*36*) and is widely recognized to be a strong antioxidant (*37*, *38*). The disaccharides, active in other tests, were not distinguished from the reference. Glucose, fructose, and sorbitol showed little significant activity, whereas the only sugar that stood out for its DPPH• bleaching was deoxyribose, reflecting the high reactivity to TBA test (**Table 6**).

Table 6. Average Values of Absorbances (Abs) at 517 nm, after 1 h in the Dark and ΔAbs Evaluated from 0.2 mM DPPH• Quenching by Sugar Idroethanolic Solutions (8 mM)

sugar	Abs at $t = 0$	Abs at $t = 60$ min	ΔAbs[a]
reference[b]	1.488	1.485	0.003a
maltose	1.480	1.480	0.000a
sucrose	1.484	1.482	0.002a
fructose	1.510	1.502	0.008b
glucose	1.505	1.490	0.015b
sorbitol	1.508	1.497	0.011b
deoxyribose	1.492	1.466	0.026c

[a] Different letters in the same column are for statistically significant differences ($p > 0.05$). [b] Reference was a DPPH• solution in the absence of sugars in the same ethanol–water solution of the samples.

DISCUSSION

The ability of deoxyribose to form TBA-reactive substances with respect to other carbohydrates is confirmed. Among the other tested compounds, sucrose had the best activity and sorbitol the least. Maltose and glucose, sugars with a reducing group, show significantly different activities from sucrose and sorbitol, nonreducing sugars. The differences are opposite: sucrose and sorbitol show higher and lower TBA-reactive substance production than maltose and glucose, respectively. It seems by these observations that the presence of reducing groups is not essential in the reaction with •OH.

A very important goal for the experiments of •OH quenching by EPR was the significant and well-detectable activity of •OH inhibition given by simple sugars or reduced ones.

The EPR results differ from the results of TBA test data; the deoxyribose could have had a high index of •OH quenching, but it showed an intermediate activity in good relation with its number of free OH residues.

The highest activities in •OH inhibition were given by the disaccharides maltose and sucrose, whereas the lowest was for sorbitol, the only nonsugar. The low scavenging activity of sorbitol reflected the low TBA reactivity. The high activity of maltose and sucrose (~74%) was matched with the activity of a well-known free radical scavenger molecule, chlorogenic acid (*39*), at the same molar concentration: the •OH inhibition activity of chlorogenic acid was quite similar to that of disaccharides in the same EPR experimental conditions used for the present study (71% of •OH inhibition, data not shown).

The antiradical activities of sugars were also assayed by a DPPH• test. Sugars generally showed a lower reactivity if compared to the responses in the EPR test. The antiradical activity of deoxyribose, assayed by DPPH• quenching, gave the highest response with respect to other sugars and was much lower than the activity of chlorogenic acid at the same molar concentration (data not shown). It can be argued that the EPR test is very much more sensitive than the DPPH• test for the evaluation of antiradical properties related to simple sugars.

Two other experiments were set in order to evaluate the damage of tested compounds in a Fenton system: an HPLC test to measure the remaining amount of the tested compound and a GC headspace test to measure the HCOOH from the oxidative damage of the carbohydrates.

Both of these tests, carried out at the same weak Fenton conditions of EPR, did not evidence good significant differences among the tested compounds. It is evident the better sensitivity and resolution are obtained with the EPR test.

More drastic Fenton conditions were tried, and the differences in sugar reactivities came out. The deoxyribose was positioned in a middle range for both of these tests: its specificity for the

reaction with TBA is clear. The best stability, evaluated by RI-HPLC peak area retention, was for the disaccharides maltose and sucrose, the lowest was for the sorbitol, and the other compounds were intermediate: it has to be noted that the sugar damage amount is reverted if compared with the EPR-detected •OH inhibition. The more a sugar is active in •OH quenching, the more stable (low percent of area reduction) it seemed in drastic Fenton reaction conditions. It could be speculated by this test that the mechanism of •OH scavenging might be linked to the presence of stable free radical intermediates and not strictly linked to the sugar damage. The first step in the reaction of •OH with a hydroxylated compound like a sugar involves abstraction by •OH of a weakly bonded hydrogen atom from the substrate with the production of water and a free radical (*27*, *40*):

$$(H-C-OH)_n + {}^{\bullet}OH \rightarrow ({}^{\bullet}C-OH)_n + H_2O$$

If the sugar involved in this reaction is able to regenerate itself with a hydrogen atom kept from the medium, the result could be a higher stability of the sugar and a more efficient quenching of the •OH given by the regeneration of the scavenging molecule.

Instead, the sugar damage investigated by HCOOH production and analyzed by GC headspace gave a positive response with the EPR activity: the disaccharides, in a Fenton system, produced a higher amount of HCOOH with respect to other sugars. In this test, it seemed that the interaction of •OH with sugars could be attributed to an oxidation similar to the periodate action (*25*) and that the amount of HCOOH is related to the number of OH residues. This fact was not true for deoxyribose; in fact, it did not produce a low amount of HCOOH as was expected by its number of OH residues, because it does not possess the OH residue in the 2-position, and two vicinal OH residues (for example, in the 2- and 3-positions) are essential for the periodate oxidation and the subsequent HCOOH production, giving an amount close to that of disaccharides (**Table 5**).

CONCLUSIONS

The activity and specificity of deoxyribose in forming TBA-reactive substances under Fenton conditions was confirmed, but TBA test conditions of detection are widely different from EPR, DPPH•, HPLC, and GC experiments.

The ranking of sugar activity toward the •OH evaluated by HPLC and sugar stability, evaluated by HPLC, revealed that maltose and sucrose were very active and stable in the Fenton conditions used at room temperature, and, if subjected to a temperature of 80 °C, produced higher amounts of HCOOH than other assayed sugars. The difference of free radical scavenging activity, stability, and HCOOH production between disaccharides and monosaccharides is evident, and the number of OH residues seems to be essential in the reactions of •OH quenching and HCOOH production.

ACKNOWLEDGMENT

This paper is dedicated to Dr. Franco Pizzocaro, the intellect behind the idea of this research, who unfortunately died before the work was completed.

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Received for review March 10, 2003. Revised manuscript received September 11, 2003. Accepted September 12, 2003.

JF030172Q