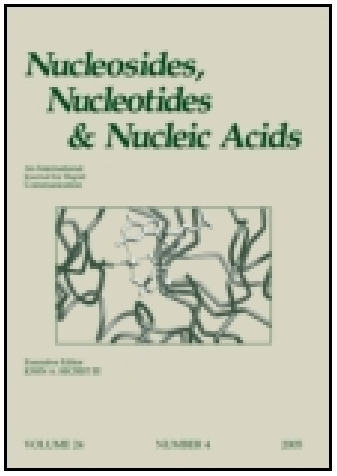

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TIME AND CONCENTRATION DEPENDENCE OF FENTON-INDUCED OXIDATION OF dG

Carl Elovson Grey and Patrick Adlercreutz □ *Department of Biotechnology, Lund University, Lund, Sweden*

□ *The influence of incubation time and Fenton reagent concentrations was investigated on the oxidation of 2′-deoxyguanosine. The compounds identified and quantified, through use of an LC-MS/MS system, were 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8,5′- cyclo-2′-deoxyguanosine (8,5′cyclodG) and the secondary oxidation products guanidinohydantoin and dehydro-guanidinohydantoin. 8-oxodG and 8,5′cyclodG formed very quickly, reaching a maximum rapidly, but with 8-oxodG a rapid decline occurred thereafter due to its further oxidation into the secondary products, which formed more slowly. Due to the better stability, 8,5′cyclodG correlated better with the general level of oxidation than 8-oxodG. The results emphasize the advantages of measuring other oxidation adducts than 8-oxodG alone.*

Keywords Fenton reaction; 2′-Deoxyguanosine; Hydrogen peroxide; Iron sulfate; 8-Oxo-7,8-dihydro-2′-deoxyguanosine; LC-MS/MS; 8,5′-Cyclo-2′-deoxyguanosine; Guanidinohydantoin; Dehydro-guanidinohydantoin; Modified nucleosides; Oxidative damage; Free radicals; Quantification of modified nucleosides; Hydroxyl radical

INTRODUCTION

DNA damage caused by reactive oxygen species (ROS) such as the highly reactive hydroxyl radical (·OH) is believed to be involved in such diseases and disorders as age-related cancer [1,2] and cellular aging.[3,4] Hydroxyl radicals are generated in vivo by reduction of hydrogen peroxide (H_2O_2), formed by dismutation of superoxide anions (O_2^-) produced in cellular metabolism. If Fe^{2+} serves as the reductant, the reaction is known as a Fenton reaction. If some other reducing species such as superoxide anion, ascorbate, or NADH are present, the reaction can become cyclic through the reduction of Fe^{3+} to Fe^{2+}. Only a catalytic amount of iron is then needed to sustain the production of radicals. Substantial levels of H_2O_2 (0.1 mM and above) have been detected in vivo, in human urine, and in ocular tissues.[5] Because of the strong damage potential of the hydroxyl radical, iron is normally kept

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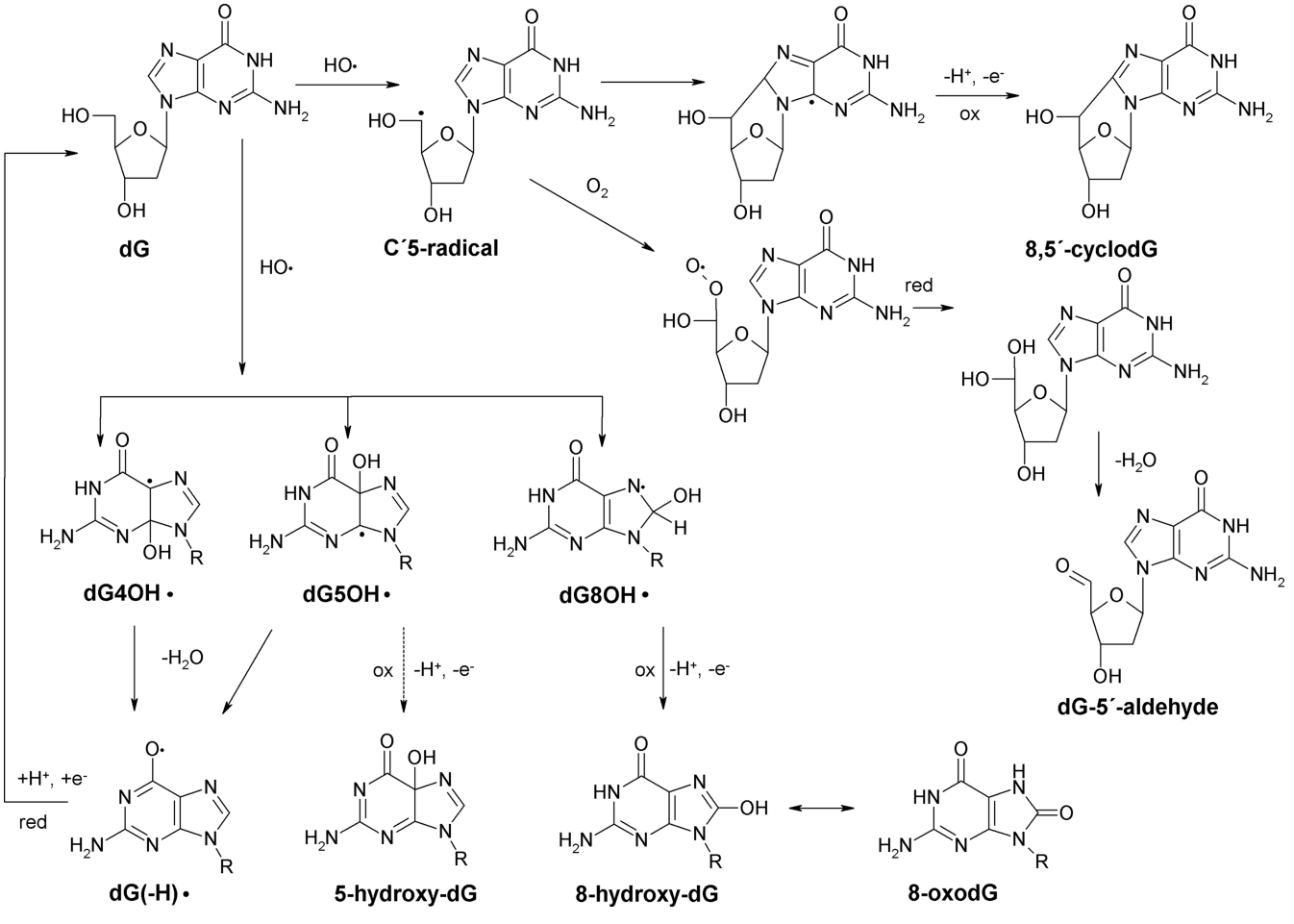
safely bound within the cells in storage proteins such as ferritin. However, reductants such as ascorbate and superoxide anion have the ability to release Fe^{3+} from ferritin.[6,7] Although, as reviewed by Breen et al. and Henle et al.,[6,7] it is not known what oxidizing species are produced in the Fenton reaction; it has been suggested that the ferryl ion (FeO^{2+}), [8] ferryl oxo and peroxo complexes,[9] rather than the hydroxyl radical, are the major oxidants involved. Regardless of what the oxidizing species may be, the two most common modifications of organic molecules by Fenton oxidants are hydrogen abstraction and hydroxylation.[6]

Comprehensive information concerning the mechanisms and structures involved in hydroxyl radical-mediated DNA oxidation is reviewed by Breen et al., Dizdaroglu et al., Burrows et al., and Cadet et al.[2,7,10,11] The identity of 70 different oxidatively modified nucleosides and nucleobases has been determined thus far.[12] The most easily oxidized nucleotide in DNA has been shown to be the electron-rich purine base guanine,[13–15] which generates the well-known oxidatively generated biomarker 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). It has been shown that 8-oxodG is actually more susceptible to one electron oxidation than dG, because of its forming several secondary oxidation products.[12,15]

In various earlier DNA adduct-formation studies, the iron-mediated Fenton reaction was used to generate oxidative conditions. In one study, Henle et al.[16] investigated the adduct formation of 2′-deoxyguanosine (dG), 2′-deoxyguanosine 3′-monophosphate 3′-dGMP, 5′-dGMP, dGpG, and double-stranded DNA under both aerobic and anaerobic conditions, first separating the products on an HPLC column and then quantifying and identifying them by use of UV, mass spectrometry, and NMR. They were able to identify 16 out of 20 products, corresponding to approximately 80% of the observed damage. In another study, by Frelon et al.,[17] oxidation in the case of γ-irradiated and Fenton- oxidized DNA was compared, five oxidation products being quantified by use of LC-MS/MS. However, there is a lack of data concerning the quantification of 8-oxodG and its secondary oxidation products where the reaction time has been varied, especially when the Fenton reaction is used.

In the present article, the influence of the concentration of the Fenton reagents (Fe^{2+} and H_2O_2) and the reaction time on the formation of four primary and secondary oxidation products of dG was investigated by use of LC-MS/MS. The advantage of LC-MS is that it enables several different oxidation products to be measured simultaneously, whereas it could be misleading to measure only one biomarker, such as 8-oxodG. The different oxidation products were identified by their molecular mass, their product ion spectrum, and sometimes their retention time order in the column. The primary oxidation products quantified were 8-oxodG and (5′R)-8,5′-cyclodG, as shown in Scheme 1, using a method recently developed for measurement of the two latter products in DNA.[18] The secondary oxidation products of 8-oxodG were two diastereomers of guanidinohydantoin (Gh) and an additional oxidized



SCHEME 1

species dehydro-guanidinohydantoin (Gh^{ox}) (Scheme 2). Gh is a major secondary oxidation product obtained under acidic conditions.[12] It was earlier believed to exist in dynamic equilibrium with the diastereomer iminoallantoin (Ia) but more recent data have ruled out that possibility.[15,19] The origin of Gh^{ox} is not known for sure—it could be an oxidation product of Gh.[15] Another possibility is that Gh^{ox} is formed through decarboxylation of the peroxide 5-hydroperoxy-8-oxo-7,8 dihydro-2′-deoxyguanosine (5-OOH-8-oxodG) that has been reported to occur in singlet oxygen oxidation of 8-oxodG.[11,20]

MATERIALS AND METHODS

Chemicals

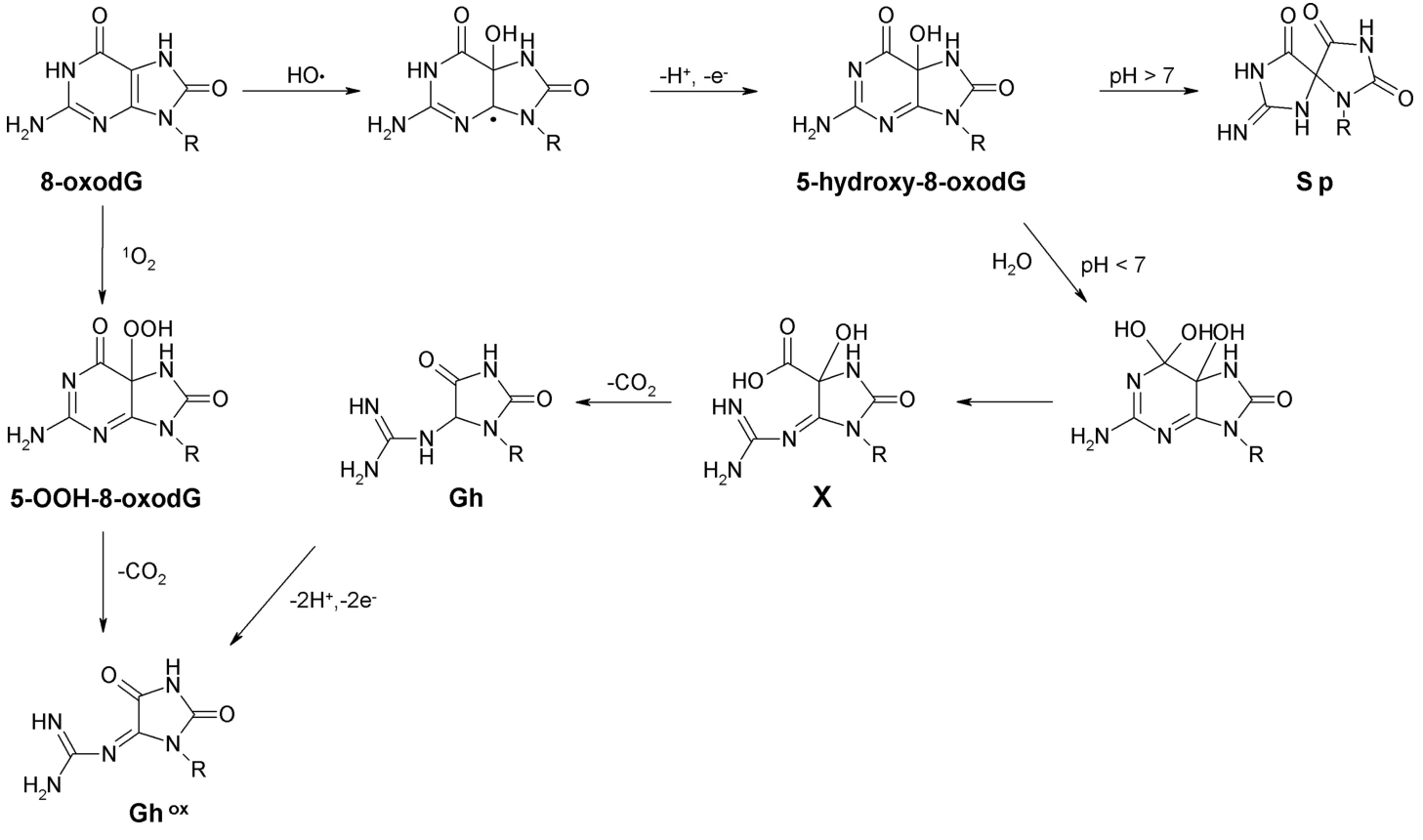
The nucleosides 2′-deoxyguanosine (dG) and 8-oxo-7,8 dihydro-2′-deoxyguanosine (8-oxodG) were purchased from Sigma (St. Louis, MO). Hydrogen peroxide (30%) and acetonitrile were obtained from Merck (Darmstadt, Germany). Acetic acid and ammonium acetate were bought from Fluka (Buchs, Swizerland). Iron(II) sulphate ($FeSO_4$) was purchased from Aldrich (Milwaukee, WI). All water used was of Millipore quality (Millipore Corp., Bedford, MA).

Fenton Oxidation of dG

Stock solutions of dG, $FeSO_4$, H_2O_2, and 8-oxodG were prepared, typically in concentrations 10 times higher than the final concentration. The $FeSO_4$ and H_2O_2 solutions were freshly prepared just prior to the experiments. All solutions were in equilibrium with the atmosphere. To avoid too high a concentration of the Fenton reactants, the samples were mixed in the order water, dG, $FeSO_4$, and H_2O_2 to a final volume of 1 mL. The samples were shaken and incubated, either at room temperature or when the incubation time was longer than 2 min in a water bath held at 37°C. The samples were then filtrated immediately and injected into the HPLC. Standard samples of dG and 8-oxodG were analyzed the same day to obtain an accurate standard curve and for identification purposes.

LC/MS Analysis

The compounds in the oxidation mixture were separated on a PE series 200 HPLC (Perkin Elmer Instruments LLC, Shelton, CT) with a Supelco C_{18} reverse-phase column (Supelcosil LC-18S, 250 mm $*$ 2.1 mm and 5 μm particle size). The mobile phase consisted of 5% acetonitrile in water with a 10 mM ammonium acetate buffer at pH 4.8. A constant flow of 200 μL/min under isocratic conditions was employed.



SCHEME 2

Each of the eluted compounds was detected using a quadrupole time-of-flight (Q-TOF) hybrid tandem mass spectrometer (API Qstar, MDS Sciex, Ontario, Canada) equipped with an electrospray ionization source (Turboion Spray®) set in positive mode. The response was first optimized for dG dissolved in mobile phase through direct injection using a Harvard model 22 syringe pump (Harvard Apparatus Inc, Holliston, MA). The ion source voltage was set to 5500 V and the drying gas had a temperature of 350°C. The same settings were used in both the MS and in MS/MS mode, except for the collision energy, which was set to 10 and 20 eV when MS/MS spectra were obtained. Argon was used as the collision gas.

The mass spectrometer was used for quantification. The peak area of a narrow mass range was integrated to obtain an optimal signal-to-noise ratio. 8-oxodG and dG were quantified using external calibration, whereas the other compounds were compared relatively. Ten-point calibration curves for 8-oxodG and dG were obtained. Linear regression could be employed, since the response was reasonably linear in the concentration range investigated. Although the isotope-dilution quantification method is the method of choice due to its high stability and precision, the external standard method meets the precision requirements of the present study well.

Statistical Analysis

The standard error was estimated using triplicates of three representative samples. It was assumed that the relative standard deviation was constant in the range investigated. This assumption, tested by use of the two-tailed F-test, could not be rejected at a $p = 0.05$ significance level for any of the products that were measured. The pooled relative standard deviation was calculated and served to determine the 95% confidence interval with use of the Student's t-distribution.

RESULTS AND DISCUSSION

Identification of Oxidation Products

The formation of oxidation products was investigated using several preliminary Fenton-oxidation experiments with different incubation times and concentrations of dG, H_2O_2, and $FeSO_4$. Several masses were found in addition to the characteristic dG masses of $m/z = 268$ $[M+H]^+$ and $m/z = 152$ $[B+2H]^+$, which correspond to the protonated molecular ion and to the intense base fragment ion, respectively (Figure 1). The base fragment is formed by protonation of the base followed by cleavage of the N-glucosidic bond and hydrogen transfer from the sugar. This loss of 2-deoxyribose (−116 a.m.u.) is typical for nucleosides when analyzed in the positive ESI mode.[21] If the molecular ion is fragmented in the product ion scan, the sugar moiety

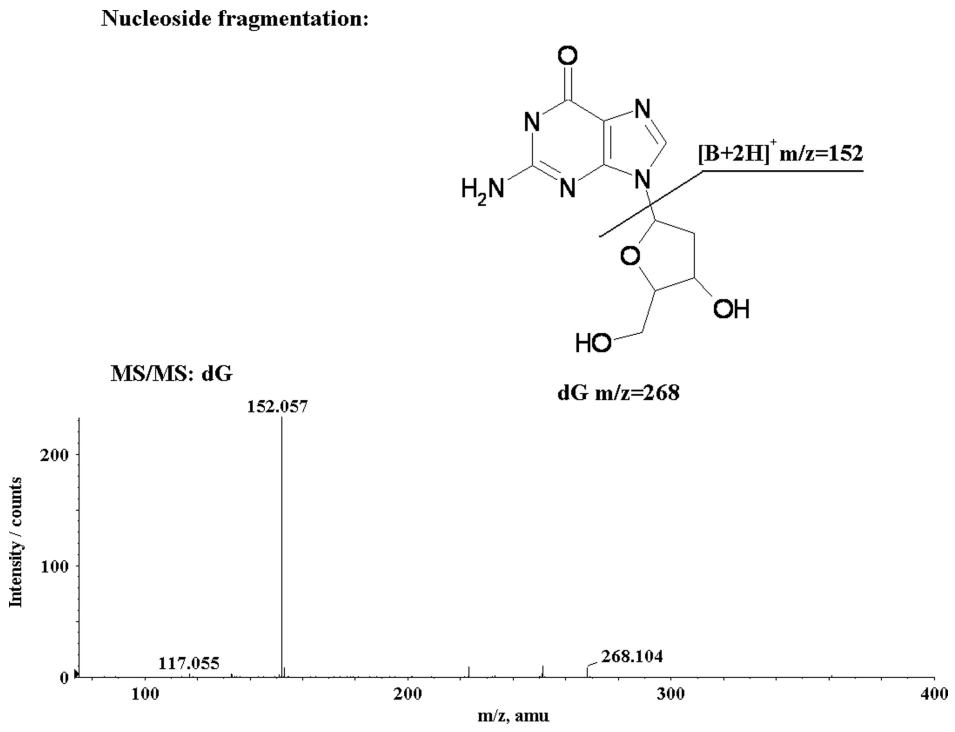


FIGURE 1 Fragmentation and product ion spectrum of dG.

ion S^+ of m/z = 117 also becomes visible, although its signal is much less intense than that of the base fragment ion.

The mass of each of the compounds identified is shown in Table 1, together with their retention times, their fragments as found in MS/MS and comparisons between the predicted and measured masses. 8-oxodG, with a mass of m/z = 284, eluted at 8.25 min. The retention time and fragmentation in MS/MS were identical to those of the standard, the characteristic fragment of m/z = 168 being found (Figure 2A). In addition, the fragment ion of m/z = 168 also appeared in the MS spectrum at the same retention time but at much lower intensity. This is due to spontaneous fragmentation in the MS. The parameters were optimized to maximize the signal for the pseudomolecular ion, although others have obtained a strong signal for the fragment ion and used it, rather than the pseudomolecular ion for quantification.[22]

As can be seen in the chromatogram, shown in Figure 2A, the other four substances with a mass of m/z = 284 are less abundant but are still detectable. The first of these elutes after 3 min with an identical mass and fragmentation profile as 8-oxodG in MS/MS. Since the base fragment ion was of the same mass as 8-oxodG, the oxidation was assumed to have occurred within the base. Thus, the base fragment ion (m/z = 168) was chosen for the product ion scan, an additional fragment of m/z = 113 that was absent in

TABLE 1 Products Observed, Retention Times on the Column, Expected and Observed Pseudomolecular $(M+H)^+$ Ions and Fragments Found in Ion Product Scan. The Identity of Compounds Followed by a Question Mark is Speculative

m/z	Compound	Ret. time/ min	Expected m/z	Observed m/z	Fragments in MS/MS
168	5-HydroxyG?	3.00	168.0516	168.052	140.05, 113.04
	8-oxoG	8.25	168.0516	168.052	140.05
266	Unknown	3.50	—	266.090	248.06, 230.06. 180.05
	(5′R)-8,5′-CyclodG	4.20	266.0884	266.085	248.07, 230.06, 202.07, 180.05
	5′-Aldehyde-dG?	5.00	266.0884	266.086	152.05
	(5′S)-8,5′-cyclodG	6.35	266.0884	266.092	248.07, 230.06, 202.07, 180.05
	Unknown	7.40	—	266.090	248.07, 81.03
	3′-Ketone-dG?	8.75	266.0884	266.087	152.05
268	dG	6.50	268.104	268.101	152.05
272	Gh-ox	3.25	272.0989	272.099	156.05, 114.03
	Unknown	4.50	—	272.097	168,05, 158.07, 115,05
274	Gh	3.20	274.1146	274.116	184.08, 158.07, 117.05
	Gh	4.00	274.1146	274.112	229.09, 184.08, 158.07, 117.05
284	5-HydroxydG?	3.00	284.0989	284.095	168.05
	Hydrated 8,5′-cyclo-dG	4.25	284.0989	284.094	284.29, 267.09, 266.08, 249.08, 248.08, 231.07, 230.06, 181,05, 180,05
	Unknown	5.05	—	284.095	152.05
	Unknown	5.75	—	284.096	152.06
	8-oxo-dG	8.25	284.0989	284.096	168.05, 117.06
300	Sp or 5-OH-8-oxo-dG?	8.25	300.0939	300.092	184.05
318	X (See Scheme 2)?	4.00	318.1044	318.102	202.06, 158.07, 115.06, 98.04

the 8-oxodG peak being found in the peak at 3 min. Although speculative, the radical dG5OH could perhaps oxidize in a manner similar to dG8OH, although much less favorably, to form 5-hydroxy-dG (Scheme 1). The two $m/z = 284$ diastereomers at 5.05 and 5.75 min, respectively, give rise to an abundant fragment at $m/z = 152$ in MS/MS that most likely corresponds to the $[B+2H]^+$ fragment of dG (Figure 2A). Accordingly, these compounds could perhaps be hydroxylation products of one of the carbons in the 2-deoxyribose moiety. The C-4′ carbon has the weakest C-H bond and is perhaps the most likely candidate, because it is the one most susceptible to hydrogen abstraction.[7] The last substance with a mass of $m/z = 284$ that was found eluted at 4.25 min, together with a compound later identified as (5′R)- 8,5′-cyclodG having a mass of $m/z = 266$. The similarity does not end here since both of them also share the same fragments in MS/MS. The mass $m/z = 284$ also fragmented into $m/z = 266$ at the same retention time as (5′S)-cyclo-dG, although much more weakly (not shown). This was expected, since the (5′S) diastereomer signal was only 1/6 times as intense as that of the

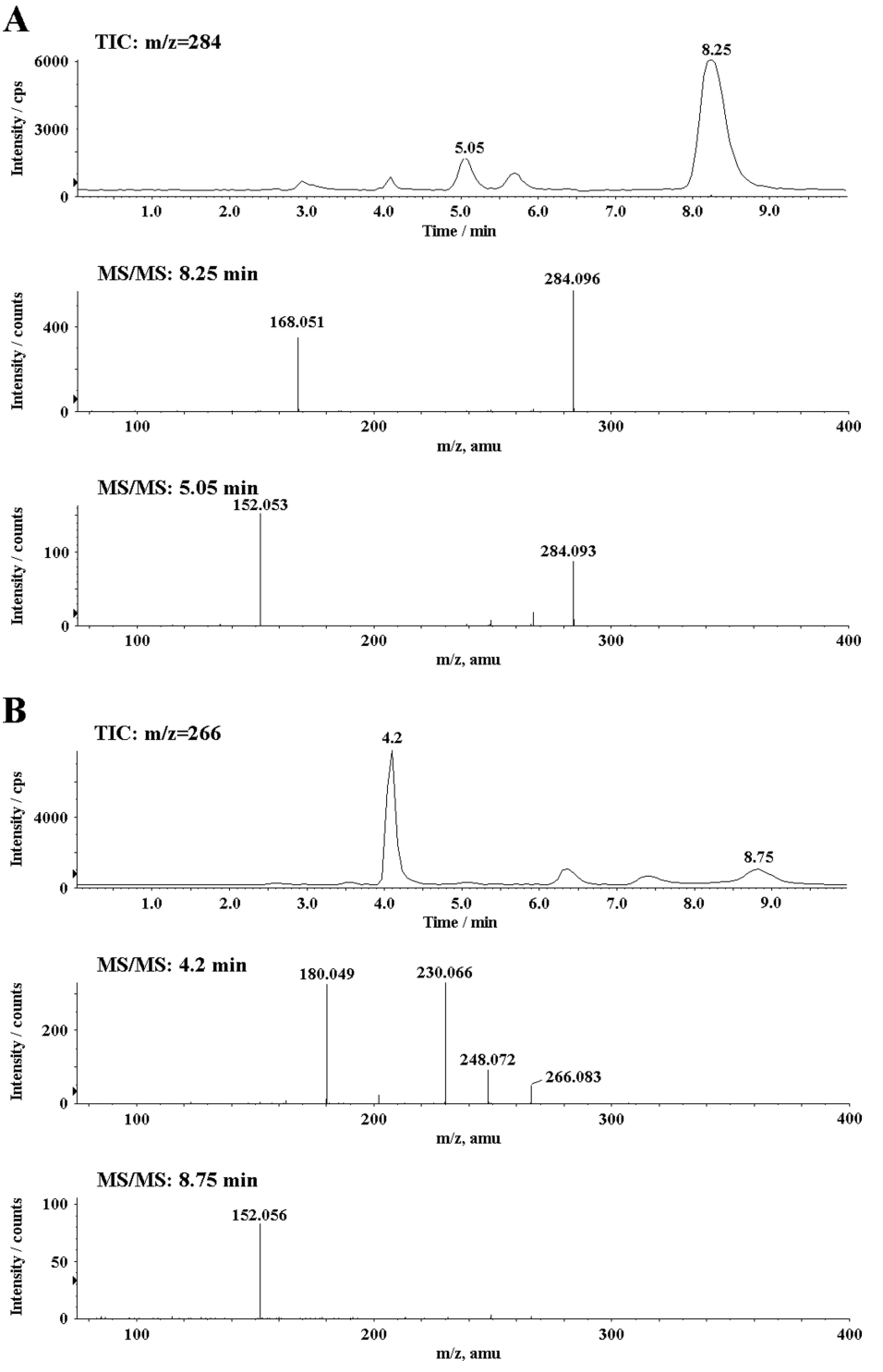


FIGURE 2 Total ion chromatogram (TIC) and product ion spectra of A: m/z = 284, B: m/z = 266, C: m/z = 274, D: m/z = 272 with gradient elution and E: m/z = 318.

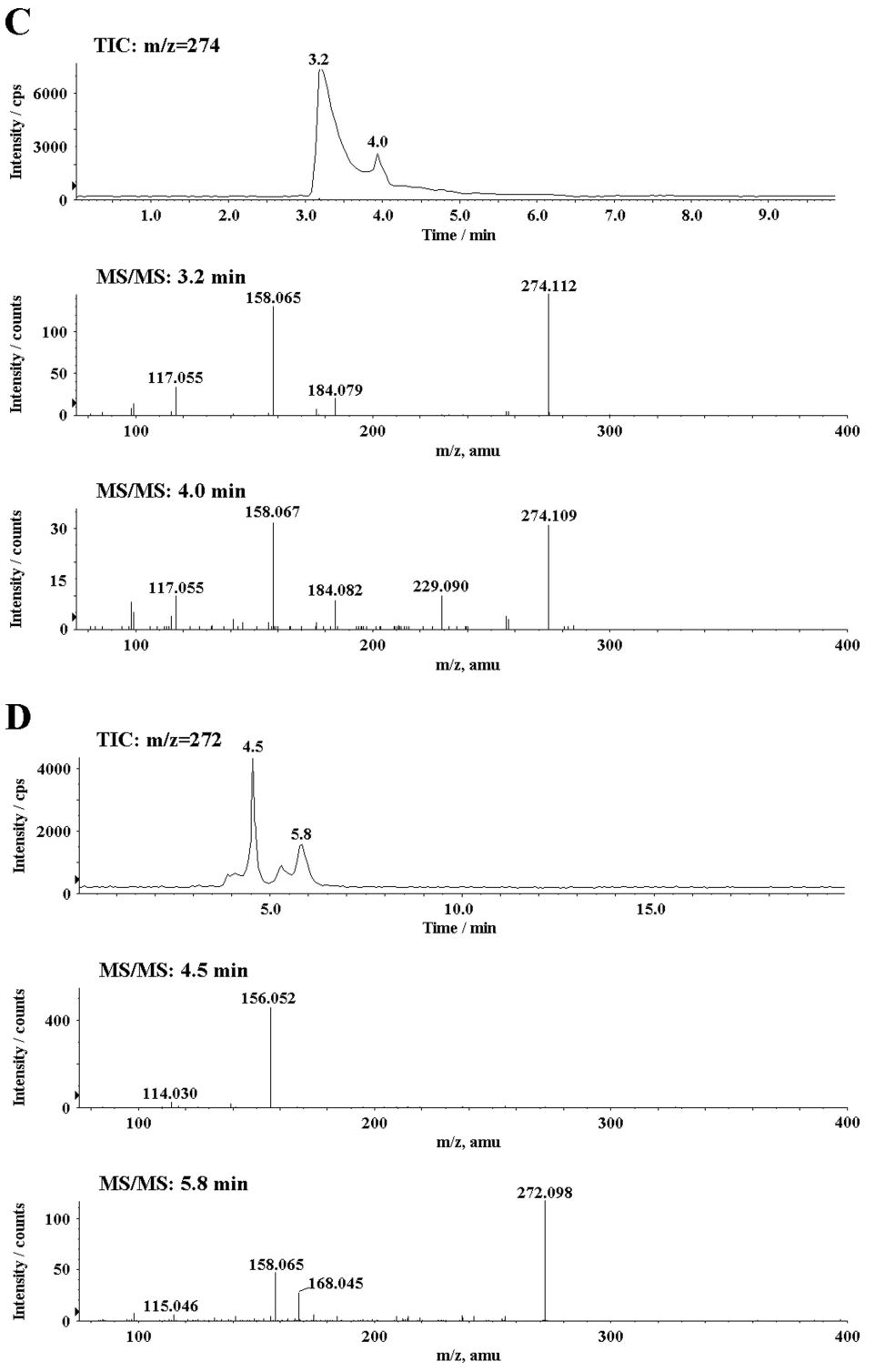


FIGURE 2 (*Continued*).

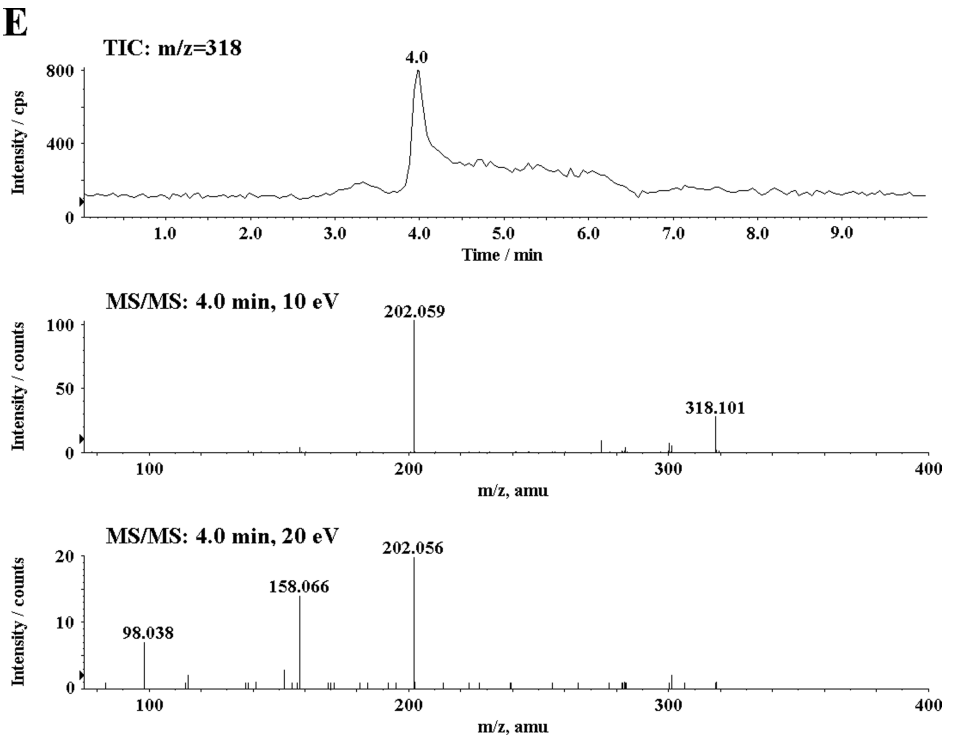


FIGURE 2 (*Continued*).

(5′R) diastereomer. The most logical explanation for the appearance of these substances would appear to be that they are hydrated 8,5′-cyclodG formed in the ionization process in the MS.

Both the (5′R) and the (5′S) diastereomers of 8,5′cyclodG were identified using their molecular weight and retention time order and also by the characteristic ion fragment of m/z = 180 described by Jaruga et al.[18] Another interesting detail, which has not been noted before, is that these compounds fragment in a series of 18 a.m.u., corresponding to the neutral loss of water from 266, 248, and down to 230; see Figure 2B. In addition to (5′R)- and (5′S)-8,5′cyclodG, there were four other substances of identical mass. Two of these, which elute after 5 and 8.75 min, fragment in MS/MS into guanine m/z = 152, the same mass as the $[B+2H]^+$ fragment of dG. This indicates that the base of dG is intact, whearas the 2-deoxyribose has lost two hydrogen atoms. The earlyeluting compound is assumed to be 5′-aldehyde-dG-, since Henle et al.[16] identified this substance with a similar retention order on a reverse-phase column (Scheme 1). The compound at 8.75 min could possibly be 3′-ketone-dG.

The diastereomers of Gh were identified using the molecular mass m/z = 274 and fragment ions of m/z = 158 and 117 (Figure 2C). Henle et al. also observed a compound with m/z = 274 under similar oxidizing

conditions, although it was not identified.[16] The two diastereomers were not completely separated, due to heavy tailing, even when a gradient elution with lower starting concentration of acetonitrile was tried out. This made it difficult to properly quantify them separately. Nevertheless, it was concluded that the early eluting Gh-diastereomer was produced to a much greater extent than the later one. The fragment patterns of the two diastereomers of Gh in MS/MS were similar but the later eluting diastereomer had an additional fragment of $m/z = 229$.

A candidate for Gh^{ox} $m/z = 272$ was found eluting slightly after Gh at 3.25 min, which is fairly similar with the relative small difference in retention time found by Chworos et al. for the corresponding dinucleoside monophosphate.[23] Another diastereomer of m/z = 272, not completely separated from Gh^{ox}, eluted at 4 min. A gradient run, shown in Figure 2D, was made in MS/MS mode, its being shown that the early eluting diastereomer fragmented very easily into the expected fragment of $m/z = 156$ (−116 a.m.u.). Although less abundant, the spectrum also contained a $m/z = 114$ fragment, corresponding to an additional loss of 42 a.m.u. that has previously been reported as a characteristic fragment of Gh^{ox}.[20,24] Hence it was concluded that the $m/z = 272$ diastereomer eluting at 3.25 min was Gh^{ox}. It was also discovered that the later eluting peak consisted of a pair of diastereomers with identical fragments of $m/z = 158$ and $m/z = 168$ in MS/MS.

An unknown nucleoside with $m/z = 318$ eluting at 4 min was found. It fragmented easily into $m/z = 202$ in MS/MS (−116 a.m.u.). In addition, a fragment of $m/z = 158$ was found when a higher collision energy was applied (Figure 2E), which is the same mass as the Gh-base fragment. The fragment is formed through the neutral loss of CO_2 (−43.98 a.m.u.). A possible nucleoside with $m/z = 318$ was proposed by Luo et al. as an intermediate in the formation under acidic conditions, of Gh from 5-hydroxy-8-oxodG [15] (X in Scheme 2). It was also suggested that Gh forms directly from the $m/z = 318$ nucleoside by decarboxylation, which supports the substance found being the proposed intermediate.

Due to the acidity of the Fenton reaction, most of the 8-oxodG ended up as Gh rather than as Sp [12,25] (Scheme 2). Nonetheless, some traces were found of a substance at 8.25 min having the same mass as Sp and 5-OH-8-oxodG ($m/z = 300$), one that fragmented into $m/z = 184$ (−116 a.m.u.). A more intense $m/z = 300$ diastereomer fragmented into $m/z = 152$, the same mass as guanine. A possibility here is that the 2-deoxyribose is oxidized by two hydroxyl groups. An additional compound for which $m/z = 300$ having a fragment of $m/z = 168$, was also found, indicating it to have an extra hydroxyl group both in the 2-deoxyribose and in the base.

Influence of Oxidative Conditions

In order to investigate the influence of the Fenton-reagent concentrations, the reaction time in a set of experiments was held constant at 60 s and

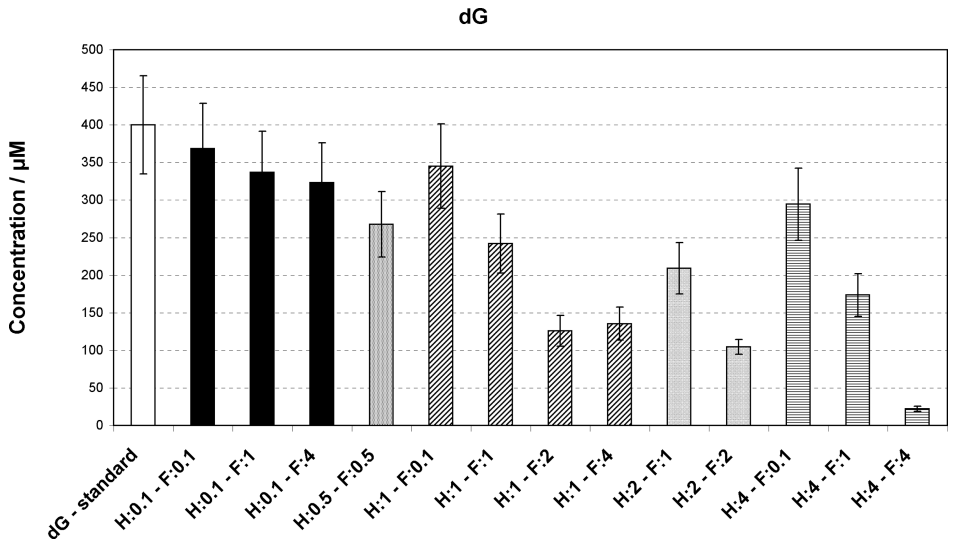


FIGURE 3 Concentration of dG remaining after 60 s reaction showing the different concentrations (mM) of H_2O_2 (H) and $FeSO_4$ (F) employed.

the initial dG concentration was 400 μM. The concentration of the remaining dG, which indicates the overall oxidation, is shown in Figure 3. The trend is clear: a higher concentration of both H_2O_2 and $FeSO_4$ resulted in greater oxidation and less dG that remained. This is to some extent in contrast with the previous observation by Henle et al.,[16] who found that a higher concentration of $FeSO_4$ than 1 mM did not result in either a greater amount of oxidation products or a higher consumption of dG. Our results do indicate, however, that the effect of $FeSO_4$ was as at least as important as that of H_2O_2.

Figure 4 shows how the formation of 8-oxodG varies with the concentrations of H_2O_2 and $FeSO_4$, higher concentrations of these reagents generally resulting in a greater amount of 8-oxodG. However, changes in concentration of H_2O_2 and of $FeSO_4$ differed in the effects they had on the results. An increase in $FeSO_4$ resulted in more 8-oxodG, with the exception that at the highest concentration of 4 mM H_2O_2 almost no 8-oxodG could be detected. It is known that such reducing species as Fe^{2+} can increase the formation of 8-oxodG at the expense of other products.[26] On the other hand, an increase in H_2O_2-concentration had no effect at the lowest level of 0.1 mM $FeSO_4$, the amount of 8-oxodG always being low. At higher concentrations of $FeSO_4$, the influence of H_2O_2 was clearly stronger, since 8-oxodG first increased up to 1 mM of H_2O_2 and then declined when the concentration was increased further. A similar trend, with a maximum at 2 mM, was seen when the concentrations of H_2O_2 and $FeSO_4$ were altered simultaneously.

In Figure 5 the influence of Fenton-reagent concentration on the formation of (5′R)-8,5′cyclodG is shown. The (5′S)-diastereomer behaved in a similar fashion but was produced in lesser amounts (data not shown). In general,

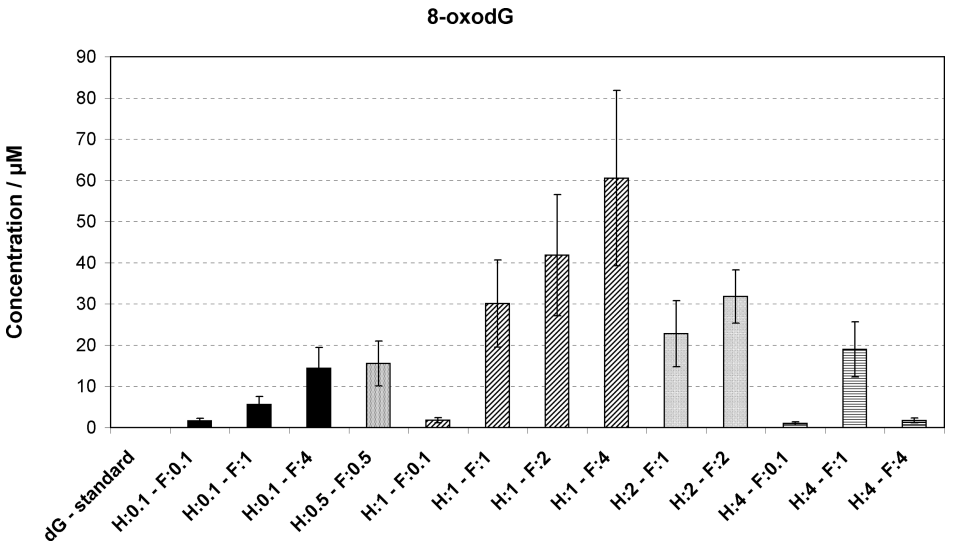


FIGURE 4 Concentration of 8-oxodG after 60 s reaction showing the different concentrations (mM) of H_2O_2 (H) and $FeSO_4$ (F) employed.

higher concentrations of H_2O_2 and $FeSO_4$ resulted in greater amounts of 8,5′cyclodG. The concentration of H_2O_2 had a strong effect in the 0–1 mM range when the concentration of $FeSO_4$ was at 1 mM or higher. When the concentrations of H_2O_2 and $FeSO_4$ were equal, an increase in 8,5′cyclodG was observed up to 2 mM. In most cases, the amount of produced 8,5′cyclodG

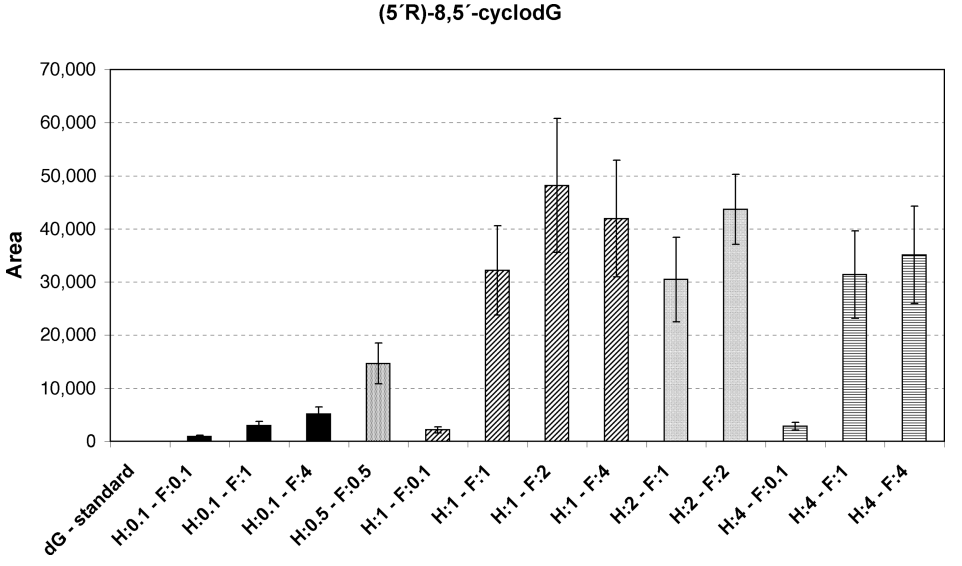


FIGURE 5 Amount of (5′R)-8,5′cyclodG after 60 s reaction showing the different concentrations (mM) of H_2O_2 (H) and $FeSO_4$ (F) employed.

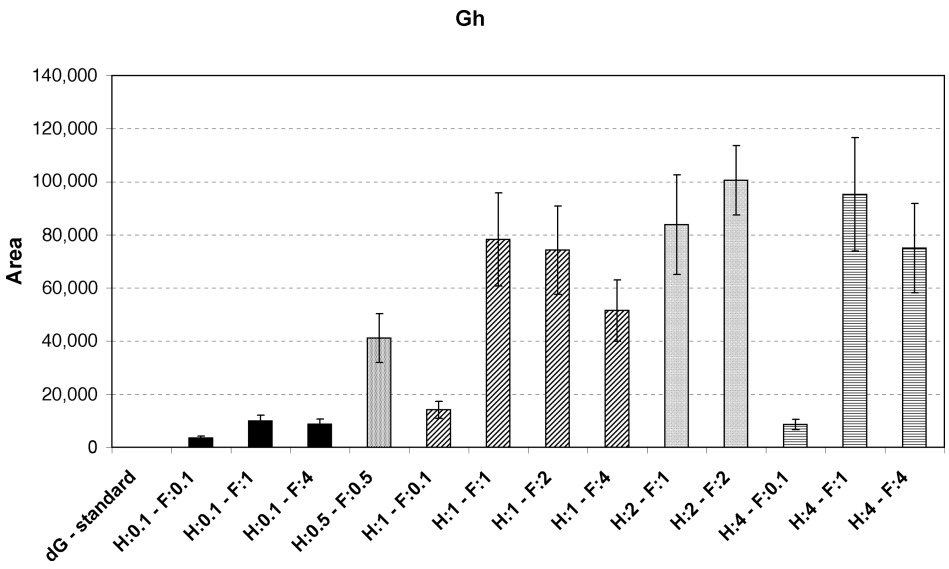


FIGURE 6 Amount of Gh after 60 s reaction showing the different concentrations (mM) of H_2O_2 (H) and $FeSO_4$ (F) employed.

closely followed the overall oxidation. However, concentrations of more than 2 mM $FeSO_4$ and 1 mM H_2O_2 yielded only a small increase or none at all, although the amount of dG remaining indicated that oxidation was greatest at 4 mM (Figure 3). It is possible that the formation of 8-oxodG and its secondary oxidation products is favored by high concentrations of the reagents, which is in agreement with Ravanat et al., who showed that when 8-oxodG is present together with dG it is consumed first, protecting dG in this way.[27]

Gh, the more abundant of the secondary oxidation products, behaved differently than the primary oxidation products did (see Figure 6), a higher concentration of H_2O_2 together with $FeSO_4$ resulting in an increase in the formation of Gh, up to at least 2 mM. In contrast with the primary oxidation products, there was in most cases a decrease when the $FeSO_4$ concentration was increased at a given H_2O_2 concentration. This could be due either to the overall oxidation having decreased, or to $FeSO_4$ somehow decreasing the oxidation of 8-oxodG. Nevertheless, when the $FeSO_4$ concentration increased, the amount of dG remaining was either the same or decreased, indicating that the major effect is probably the latter one. It was concluded that these adducts are favored by high concentrations of H_2O_2 and $FeSO_4$, especially when H_2O_2 is either in excess or at equal concentration.

The other secondary oxidation product Gh^{ox} is shown in Figure 7. Unlike the other quantified adducts, it increased over the entire range of concentrations of H_2O_2 and $FeSO_4$, especially when the concentrations were increased simultaneously. Gh^{ox} behaved in a manner similar to Gh: an equal or excess

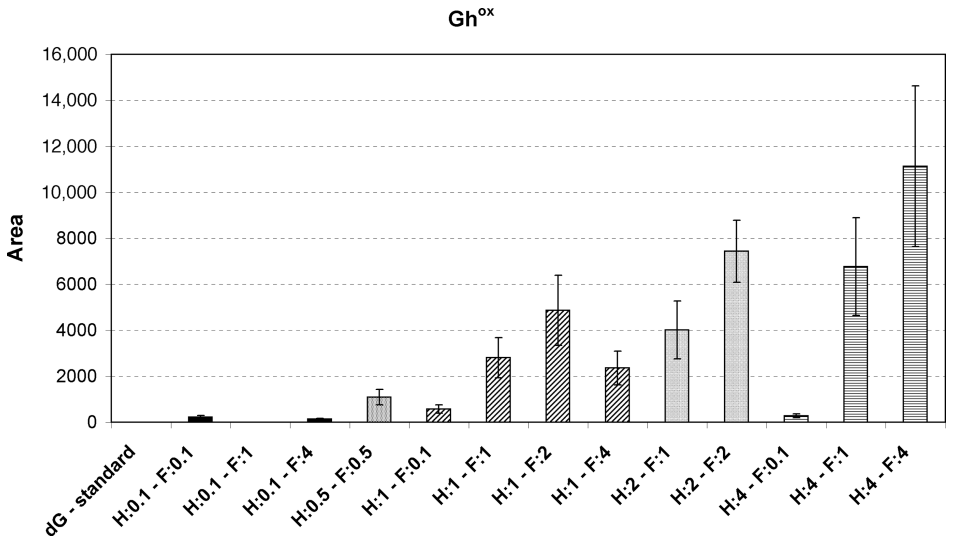


FIGURE 7 Amount of Gh^{ox} after 60 s reaction showing the different concentrations (mM) of H_2O_2 (H) and $FeSO_4$ (F) employed.

concentration of H_2O_2 resulted in the highest levels obtained. When there was a large difference in concentrations; e.g., 4 mM H_2O_2 and only 0.1 mM $FeSO_4$ or vice versa, the degree of oxidation was very low.

Influence of Reaction Time

Figure 8 shows how the amounts of dG and of the different quantified oxidation products vary with incubation time. Basically, both of the primary oxidation products reached their maximum concentrations already after 30 s, the earliest point measured. In the case of 8-oxodG, a strong decrease was seen thereafter. It appeared that 8-oxodG was formed and was further oxidized in a matter of only a few minutes. In contrast, 8,5′-cyclo dG also formed quickly but was more stable and did not oxidize further to any great extent, since after 30s the concentration was rather constant. In another study, White et al. showed that the 8-oxodG concentration oscillated, when ds-DNA was oxidized with the Fenton reaction. It was partly due to the further oxidation of 8-oxodG into Gh.[28] Thus, the reaction time is a very important parameter when the Fenton oxidation reaction is used. Interestingly, these results are not consistent with a study by Qi et al. using chromium(III) as Fenton reagent.[29] The authors observed a steady increase of 8-oxodG formation in DNA up to 90 min, using similar reagent concentrations (0.5 mM). Also, in Cu(II)-dependent Fenton oxidation of DNA Frelon et al. came to the conclusion that the oxidizing species was singlet oxygen rather than the hydroxyl radical.[30] Thus, the oxidizing mechanism of the Fenton reaction seems dependent on the metal used.

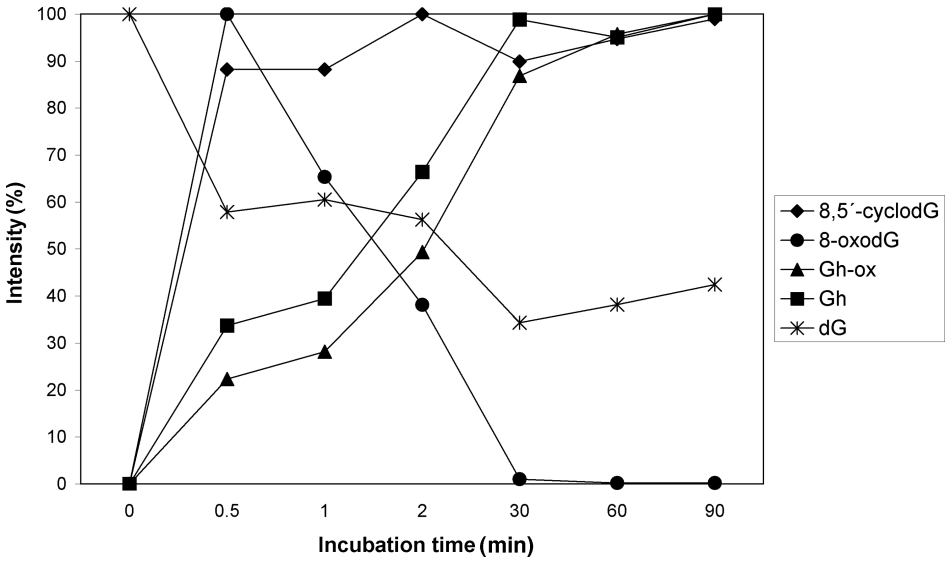


FIGURE 8 Relative amounts dG and oxidation products 8-oxodG, (5′R)-8,5′cyclodG, Gh and Gh^{ox} at 1 mM concentration of H_2O_2 and of $FeSO_4$ at different reaction times. N.B. The maximum amount of each compound is set at 100%, and therefore no quantitative comparisons between the reaction products should be made.

The secondary oxidation products behaved differently. The amounts of Gh and Gh^{ox} increased more slowly with incubation time up to 30 min, after which they leveled off. It appeared that Gh^{ox} was formed somewhat more slowly than Gh was. The observed difference in kinetic properties of the different adducts clearly indicates that the secondary oxidation occurs, at least partly, through another oxidizing species than the hydroxyl radical.

If one considers again the Fenton-concentration effect on the formation of 8-oxodG together with the secondary products Gh and Gh^{ox}, it is not surprising that the level of 8-oxodG decreased when the H_2O_2 and $FeSO_4$ concentrations increased to above 1 mM, the reason being that when the concentrations of H_2O_2 and $FeSO_4$ were high, oxidation was very rapid, a high concentration of 8-oxodG being achieved very quickly. The formation rates of Gh and Gh^{ox} (and of other potential products) then become higher than the rate of formation of 8-oxodG, resulting in the net concentration of 8-oxodG decreasing. Indeed, the results show that at the highest concentration of H_2O_2 and $FeSO_4$, almost no 8-oxodG could be detected, whereas the levels of Gh and Gh^{ox} were high and the amount of intact dG remaining was very low. At 4 mM, the amount of Gh^{ox} in particular was very high, yet the amount of Gh was in fact not higher than at 1 mM. Although far from being conclusive, this and the slower formation rate fit with the hypothesis that Gh^{ox} is formed from Gh.

In studies of the oxidative damage of DNA, 8-oxodG is often used as the sole marker of the level of oxidative stress. The present study clearly shows

that in the Fenton oxidation of dG, which is in free solution, further oxidation of 8-oxodG is a reaction that needs to be taken into account in order to obtain an adequate measure of the overall level of oxidation. This was illustrated by Suzuki et al., who found that the antioxidant epigallocatechin enhanced the formation of 8-oxodG, but this was shown not to be due to increased oxidation but rather due to inhibition of the following oxidation of 8-oxodG.[31] Similarly, in the photooxidation of dG that occurs through a type I photosensitization mechanism, 8-oxodG is not directly detectable because of further oxidation occurring rapidly, although transient 8-oxodG formation has been shown in isotope labeling experiments.[27] Further oxidation of 8-oxodG is highly relevant, not only in the oxidation of dG in solution, but also in DNA oxidation. Since the overall level of DNA oxidation in living cells is relatively low, it appears unlikely that the same site would be oxidised twice. However, it has been reported that oxidative damage can migrate over long distances in DNA, so that readily oxidized lesions, such as 8-oxodG, can act as "sinks" for the trapping of electron holes.[15] Oxidation at other sites in the DNA molecule can thus cause further oxidation of 8-oxodG residues. In quantifying oxidative DNA damage it is probably better, therefore to measure both 8-oxodG and its secondary oxidation products, and insofar as possible other oxidation products as well.

As a consequence of the extensive secondary oxidation the correlation between the overall oxidation and 8-oxodG is not very good, even if the result at 4 mM of both H_2O_2 is neglected. A more stable oxidative marker, such as 8,5′cyclodG, could therefore be beneficial. 8,5′cyclodG is a chemically stable adduct.[32] and the data presented herein also show that it can withstand quite harsh conditions, generated by the Fenton reaction. In addition, the cyclic purines (8,5′cyclodG and 8,5′-cyclodA) are formed in DNA and are believed to be of biological importance. Unlike many other oxidative adducts, they can not be repaired by the base excision repair (BER) system, due to the extra covalent bond between the base and sugar moiety. They also block gene expression by inhibiting DNA-polymerases.[32,33] Indeed, as a single oxidative marker 8,5′cyclodG reflected the oxidation of dG far better than 8-oxodG and was thus found to be more suitable.

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