Regular Article

ANALYTICAL
AND BIOANALYTICAL
CHEMISTRY
RESEARCH

Published by the
Iranian Chemical Society

***Anal. Bioanal. Chem. Res.**, Vol. 10, No. 4, 403-409, September 2023.*

Determination of 8-OHGua by LC-MS/MS after Acid Hydrolysis of Oxidative Damaged DNA

Dua Danjolli-Hashani[a,*], Saffet Celik[b] and Sebnem Selen-Isbilir[c]

[a]*Trakya University Institute of Natural and Applied Sciences, Chemistry Department, 22030, Edirne, Turkiye*

[b]*Technology Research and Development Application and Research Center, Trakya University, 22030, Edirne, Turkiye*

[c]*Trakya University, Faculty of Science, Department of Chemistry, 22030, Edirne, Turkiye*

(Received 2 March 2023, Accepted 6 May 2023)

A variety of influences can cause DNA damage to the genome. The hydroxyl radical attacks the C-8 atom of guanine, forming 8-hydroxyguanine (8-OHGua) or 8-hydroxy-2ˈ-deoxyguanosine (8-OH-2'dG) which are important indicators of oxidative damage on DNA. Determining these damaged base products can be accomplished through measurement by LC-MS/MS after enzymatic hydrolysis or measurement by GC-MS/MS after chemical hydrolysis. In this study, it was aimed to hydrolyze DNA using various strong acids and to measure 8-hydroxyguanine by LC-MS/MS. In the first stage of the study, the nucleoside 8-hydroxy-2'-deoxyguanosine was treated with HCl (2 M and 6 M), TCA (10%), TFA (10%), o-phosphoric acid (2 M), methanesulfonic acid (2 M), and formic acid (80%). The amounts of 8-hydroxyguanine were determined by LC-MS/MS. It has been identified that formic acid with the highest yield (70%) hydrolyzes the β-glycosidic linkage between the base and the sugar. Subsequently, oxidative damage was induced on calf thymus DNA by producing hydroxyl radicals via the Fenton reaction. The resulting oxidative damaged DNA was hydrolyzed using formic acid. The amount of 8-hydroxyguanine was then determined using LC-MS/MS. Based on the results obtained, it was observed that the acidic hydrolysis applied was effective in breaking the N-glycosidic bond, but not effective in breaking the phosphodiester bond of oxidatively damaged DNA.

Keywords: 8-OHG, 8-OH-2'dG, LC-MS/MS, Acidic hydrolysis, Thymus DNA

INTRODUCTION

Oxidative stress is a pathological event that occurs due to free radicals occurring in the body through natural metabolic pathways. It is associated with cancer, lung diseases, liver diseases, eye diseases, inflammation, neurodegenerative diseases, diabetes, and skin diseases. Hydroxyl radical ($OH^{\bullet}$) is the most damaging radical species to biological systems. The hydroxyl radical reacts with components of the DNA macromolecule and, it hydroxylates purine and pyrimidine bases, or damages the phosphodiester backbone between deoxyribose phosphates. The $OH^{\bullet}$ radical formed by the Fenton reaction causes oxidative DNA damage by forming 8-OH-2'dG [1].

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

8-Hydroxyguanine (8-OH-Gua) or 8-hydroxy-2' deoxyguanosine (8-OH-2'dG), the most general biomarkers of oxidative damaged DNA, are measured by analytical methods such as immunological detection, ^{32}P-postlabelling, HPLC, GC-MS/MS, and LC-MS/MS methods. However, these biomarkers may not be measured by some analytical measurement methods due to reaction conditions [2]. To measure 8-OHGua or 8-OH-2'dG, firstly oxidatively damaged DNA has to be hydrolyzed. Both 60% and 88% formic acid have been used for DNA hydrolysis in other studies [3-5], but the DNA hydrolysis methods in these

*Corresponding author. E-mail: dua_danjolli@hotmail.com

researches vary according to the measuring device used. Collins hydrolyzed DNA bases using formic acid (60-88%, 130-150 °C) and were measured 8-oxo-dG by derivatizing with bis(trimethylsilyl) trifluoroacetamide [3], while Douki *et al.* detected FapyGua by GC-MS after acidic hydrolysis with formic acid (88%, 140 °C) [4]. In another study by Aybastier, DNA base damage products were measured by GC-MS/MS after acidic hydrolysis with formic acid (60%, 130 °C) [5].

Researchers have generally reported the use of formic acid hydrolysis in measurements with GC-MS/MS and enzymatic hydrolysis (alkaline phosphatase, DNase I, phosphodiesterase I, and phosphodiesterase II) in measurements using LC-MS/MS [6-9]. To determine the 8-OH-2'dG as an oxidative damage marker, measurement with HPLC-ECD was performed after enzymatic hydrolysis (nuclease P1, alkaline phosphatase) [10], while measurement by LC-MS was carried out after acidic hydrolysis (formic acid, trimethylsilylated) [11]. Some researchers have determined 8-OH-2'dG by HPLC after enzymatic hydrolysis [12-14]. It has also been reported that the products of oxidative DNA damage were determined by GC-MS/MS method under acidic hydrolysis conditions using formic acid [5,8].

Enzymatic hydrolysis is generally used to measure DNA damage products 8-OH-Gua and 8-OH-2'dG by LC-MS/MS [6,15-18]. Because enzymes are expensive, require longer reaction times, and have low analyte recoveries compared to acid hydrolysis [19], this study aimed to determine the level of 8-OH-2'dG formed on thymus DNA after formic acid hydrolysis by LC-MS/MS. Additionally, the study aimed to investigate the levels of 8-OH-Gua by LC-MS/MS, after chemical hydrolysis with formic acid, HCl, TCA, TFA, o-PA, and MSA on 8-hydroxy-2'-deoxyguanosine nucleoside.

EXPERIMENTAL

All chemicals in this study are of analytical purity. Lyophilized Type XV deoxyribonucleic acid (DNA) and 8-hydroxy-2'-deoxy guanosine (8-OH-2'dG) from calf thymus were purchased from Sigma. 8-Hydroxyguanine (8-OH-Gua) hydrochloride was obtained from BIOSYNTH Carbosynth.

Acidic Hydrolysis of 8-OH-2'dG

In the first part of the study, hydrolysis of 8-OH-2'dG nucleoside with formic acid (80%), HCl (2 M and 6 M), ortho-phosphoric acid (2 M), methanesulfonic acid (2 M), TCA (10%) and TFA (10%) acids were performed. In the second part, oxidative damage was induced on calf thymus DNA by the Fenton reaction (H_2O_2 and Fe^{2+}) [8], and then formic acid (80%) was used to hydrolyze oxidatively damaged DNA, as it gave the best results in the initial study. The biomarkers of oxidative damage resulting 8-OH-Gua and 8-OH-2'dG were measured using the Agilent Technologies 6460 Triple Quadrupole LC-MS/MS. The device was calibrated with 8-OH-Gua and 8-OH-2'dG standard compounds (Fig. 2 and Fig. 3).

Measurement of 8-OH-Gua and 8-OH-2'dG by LC-MS/MS

The DNA damage markers 8-OH-Gua and 8-OH-2'dG were measured by LC-MS/MS. Chromatographic separation was performed on a Poroshell EC-C18 (3.0 × 150 mm, 3.5 microns) column. While mobile phase A used ultra-pure water + 0.1% acetic acid, mobile phase B used methanol + 0.1% acetic acid. The injection volume of the sample was set to 10 µl, and the flow rate was set to 0.6 ml min^{-1}. Ionizations were detected by ESI. Other parameters are shown in Table 1.

According to the results obtained from the acidic hydrolysis process applied on 8-hydroxy guanosine; of the

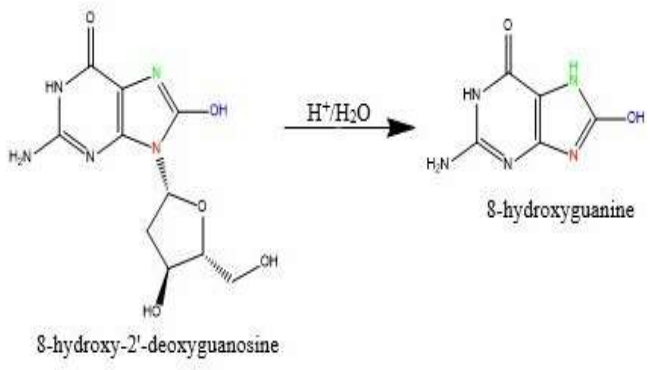

Fig. 1. Conversion of 8-OH-2'dG to 8-OH-Gua by acidic hydrolysis.

Table 1. LC-MS/MS Parameters Used in the Measurements

Analysis Parameters	Conditions
Mobile phase A	Ultra-pure water + 0.1% Acetic acid
Mobile phase B	Methanol + 0.1% Acetic acid
Column temperature	35 °C
Sampler temperature	4 °C
Column	Poroshell EC-C18 (3.0 × 150 mm) 3,5 micron
Injection volume	5 µl
Analysis time	11.00 min
Flow rate	0.600 ml min^{-1}
Ion source	Electrospray ionization (ESI)
Ionization type	Pozitif
Capillary voltage	3000 V
Source temperature	275 °C
Nitrogen gas temperature	275 °C
Nitrogen gas flow	10 l min^{-1}
Delta EMV (+)	0 V

six types of acids used (6 M HCl, 2 M HCl, 80% FA, 2 M o-PA, 2 M MSA, 10% TFA, 10% TCA), only formic acid was found to be effective in breaking the N-glycosidic bond with 70% efficiency. Acid-catalyzed hydrolysis of the N-glycosidic linkage has been reported to be slower for 8-oxo-2'-deoxyguanosine than for 2'-deoxyguanosine [20]. The N-glycosidic linkage of 7,8-dihydro-8-oxo-2′-deoxyguanosine is extremely stable in acid [21]. However, after acidic hydrolysis of DNA with sulfuric acid (0.1 M, 100 °C, 35 min), formic acid (67%), and diphenylamine (2%, 30 °C, 18 h), the purine glucosidic bonds were completely hydrolyzed [22]. Zoltewicz *et al.* [23] reported the C-O cleavage in acid-catalyzed hydrolysis of some purine nucleosides and reported that the mechanism proceeds by protonation of the nucleobase followed by cleavage of the N-glycosidic bond (Fig. 1). Hydrolysis of the N-glycosidic bond is strongly catalyzed by guanine protonation by a grouping of nucleobases [24]. In this study, the N-glycosidic linkage in 8-OH-2'dG was broken by formic acid with 70% efficiency.

In the continuation of the study, the hydrolysis power of formic acid on DNA was investigated. Although the mass changes as the structure destroys in LC-MS/MS measurement, it was determined that formic acid was not very effective on oxidatively damaged DNA. Due to the high stability of the phosphodiester bonds in the DNA backbone, studies of DNA cleavage are rare [25]. The energy $\Delta G^{\circ\prime}$ -5.3 kcal mol^{-1} is required for the hydrolysis of the DNA phosphodiester bond [26]. It is thought that formic acid is not effective in breaking the phosphodiester bond, since proper energy cannot be provided by acidic hydrolysis.

Mass spectrometry (MS), unlike other techniques, provides structural evidence for an analyte and the correct quantification of stable isotope-labeled analyte analogues (by internal standards). The use of MS based on liquid chromatography (LC-MS) or gas chromatography (GC-MS) is a convenient and fast method that enables measurements in complex mixtures [7]. There is the availability of Fpg enzyme from *Escherichia coli* and several glycosidases in enzymatic hydrolysis to measure the DNA damage products [27]. Aybastier *et al*., performed the quantification of bases by GC-MS/MS, preferring acidic hydrolysis to liberate all oxidative damaged DNA bases, since there is no excision enzyme in some of the damage products in their study [28]. The cleavage of the phosphomono(di)ester linkage(s) by acidic hydrolysis with hydrogen fluoride, occurs simultaneously with the hydrolysis of the N-glycosidic linkage. It was stated that deamination that occurred under acidic hydrolysis conditions with formic acid was not detected under acidic hydrolysis conditions with hydrogen fluoride. Quantification of the levels of modified nucleobases in DNA and nucleosides was attempted by testing the acidic hydrolysis of modified DNAs and nucleosides induced by radiation and light. In the experiment including 2'-deoxyguanosine, it was found that cleavage of the corresponding free base portion of the nucleosides occurs quantitatively in a minute. In addition, detectable degradation of the 8-hydroxylated derivative of guanine has been reported under longer exposure to HF (up to 30 min) [29]. Time-dependent analyses of the standards used in the calibration are used to test nucleobase stability under hydrolysis conditions. Lowenthal *et al*., in their study, achieved better results with acidic hydrolysis using formic acid among the three types of acids they tried. They reported that nucleobases have been degraded by trifluoroacetic acid

Table 2. Measurement of 8-OH-Gua and 8-OH-2'dG by LC-MS/MS after Acidic Hydrolysis

Acid	Biomarker (100 ppb)	Retention time (min)	[M-H-]/Fragment (m/z)	ppb (ng ml^{-1})
6 M HCl	8-OH-Gua	4.704	168.0/139.8	1.0142
	8-OH-2'dG	5.250	284.0/168.0	0.4356
2 M HCl	8-OH-Gua	4.736	168.0/139.8	23.1778
	8-OH-2'dG	5.250	284.0/168.0	19.483
80% FA	8-OH-Gua	4.720	168.0/139.8	70.4204
	8-OH-2'dG	5.242	284.0/168.0	7.8919
2 M o-PA	8-OH-Gua	4.720	168.0/139.8	14.6793
	8-OH-2'dG	5.242	284.0/168.0	10.5889
2 M MSA	8-OH-Gua	4.720	168.0/139.8	16.9088
	8-OH-2'dG	5.242	284.0/168.0	8.961
10% TFA	8-OH-Gua	4.704	168.0/139.8	2.9330
	8-OH-2'dG	5.250	284.0/168.0	7.629
10% TCA	8-OH-Gua	4.664	168.0/139.8	2.3423
	8-OH-2'dG	5.250	284.0/168.0	0.0910

(TFA) within <1 h at all temperatures used (at 60°, 120°, and 140 °C). By testing hydrolysis with hydrochloric acid (HCl) at 120° and 140 °C (8 and ~2 M), they reported that compare to purines, the nucleobase pyrimidines degraded rapidly (<1 h). It has also been reported that hydrolysis of ~0.2 M HCl within 2-4 h results in significant deamination of cytosine to uracil and incomplete pyrimidine release. Hydrolysis using formic acid (HCOOH), the third acid tested, has been reported to preserve purines at 140 °C [30].

Similarly, in our study, it was seen that formic acid provided the highest yield for the stability of the purine (guanine) base and 8-OH-Gua biomarker of guanine, compared to the other acids used. After hydrolysis with 80% formic acid, the amount of 8-OH-Gua was found to be 70.4204 ng ml^{-1}, and the amount of 8-OH-2'dG was 7.8919 ng ml^{-1} (Table 2). In a study, it was reported that λDNA could be measured by LC-IDMS method using formic acid hydrolysis [31]. Using the MALDI MS method to investigate the acid hydrolysis process of DNA, Liu *et al*., reported that the cleavage of terminal nucleotides by acid hydrolysis differed from the cleavage of purines (at both terminals) in the MALDI MS spectrum from those in the middle. After acid hydrolysis, the dissociation of damaged nucleotides was found to be different at the 5-terminal compared to that at the 3-terminal in the MALDI MS spectrum [32]. With their newly developed DI-MS platform, global DNA methylation (mC) and hydroxymethylation (hmC) levels were fast, unbiased, and sensitive. It has been reported that it can measure in some way and provide data comparable to LC-MS [33].

There are differences between acidic hydrolysis and enzymatic hydrolysis, and between measurements, depending on the method applied and the laboratory studied [34]. The SIM mode of LC-MS may not provide the sensitivity that is required to measure the 8-OH-Gua in vitro and *in vivo* [35-36]. On the contrary, in the studies of Dizdaroglu *et al*., it was observed that LC-MS was used to measure the 8-OH-Gua and 8-OH-dA in DNA [6,37]. According to our study results, it was observed that the required sensitivity for in vitro 8-OH-Gua measurement could not be achieved with LC-MS/MS.

According to the literature survey, there are various studies in which DNA hydrolysis is carried out by enzymatic hydrolysis; some for measurement by HPLC-EC by using nuclease P1 and alkaline phosphatase [38], by HPLC measurement after nuclease P1 and alkaline phosphatase; or

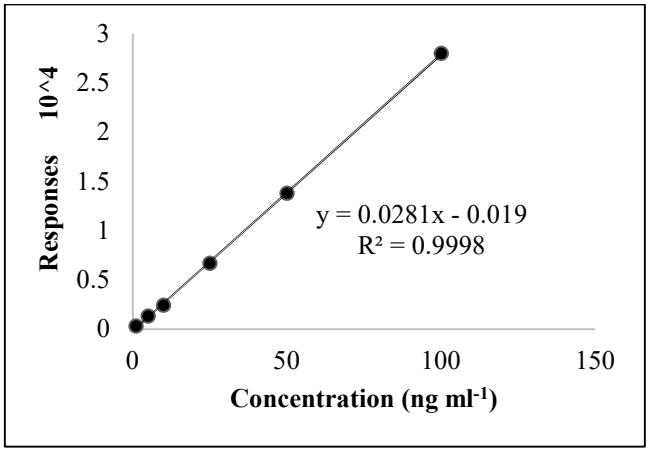

Fig. 2. Calibration curve of 8-OH-Gua.

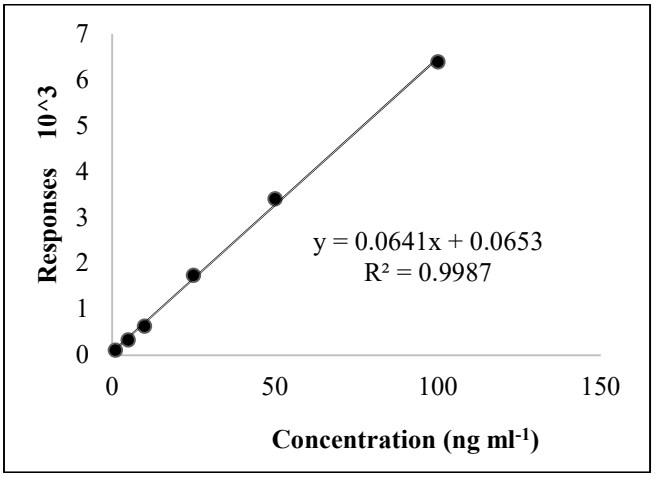

Fig. 3. Calibration curve of 8-OH-2-dHG.

with alkaline phosphatase, phosphodiesterase I, phosphodiesterase II and deoxyribonuclease I hydrolysis [3], for LC-MS/MS measurement after nuclease P1 and alkaline phosphatase [18], for LC-MS measurement after nuclease P1, venom phosphodiesterase and alkaline phosphatase hydrolysis [39], for LC-MS measurement by using DNase I, alkaline phosphatase, phosphodiesterase I and phosphodiesterase II [6], for measurement by LC-MS/MS using various nuclease enzymes (nuclease P1, phosphodiesterase I and alkaline phosphatase) [16]. However, it has been stated that the amount of enzyme and the incubation time in the enzymatic hydrolysis of DNA may cause different results by changing the hydrolysis of deoxynucleoside [40]. On the other hand, it has been reported that the GC/MS method overestimates the level of 8-oxodG due to the severe acidic conditions and long derivatization step required for DNA hydrolysis [41]. In another study, acidic hydrolysis and enzymatic hydrolysis methods were compared, the 8-OH-dGua level was measured by using enzymatic hydrolysis by LC/IDMS-SIM, and the 8-OH-Gua level was evaluated by using acidic hydrolysis with formic acid or hydrolysis with Fpg protein by GC/IDMS-SIM. It has been reported that there is no statistical difference between GC/IDMS-SIM and the levels are similar [6]. According to the result obtained by using formic acid, the enzyme does not cut intact bases. For this reason, it is thought that there is no possibility of 8-OH-Gua artifacts in the hydrolysis of DNA with Fpg protein [6].

In this study, which measured 8-OHGua and 8-OH-2'dG using the LC-MS/MS method, it is thought that formic acid is effective in breaking the N-glycosidic bond but not the phosphodiester bond. A disadvantage of both LC-MS/MS and GC-MS methods is the possibility of oxidation of solid bases during DNA isolation [42]. Therefore, instead of acidic hydrolysis, specific enzymatic hydrolysis may be more beneficial in breaking the strong phosphodiester bond. It has been observed that acidic hydrolysis with formic acid is not suitable for measuring the amounts of 8-OHGua and 8-OH-2'dG in oxidatively-damaged DNA by LC-MS/MS.

CONCLUSIONS

According to the results obtained, although there are measurement methods after acidic hydrolysis in the literature for the measurement of in vitro oxidative damage with Fenton reaction on thymus calf DNA with LC-MS/MS device, enzymatic hydrolysis seems to be more ideal. Therefore, further studies on the optimization of experimental conditions for determining the damaged products of DNA after acidic hydrolysis is recommended.

Acknowledgments

This study was financially supported by Trakya University Scientific Research Fund (TUBAP-2019/159). The authors thank the Technology Research Development Application and Research Center (TUTAGEM) for the use of LC-MS/MS.

ACKNOWLEDGEMENTS

This research has been orally presented at the 3rd International Conference on Engineering and Applied Natural Sciences on 14-17 January in 2023 at Konya/Turkiye.

REFERENCES

[1] W. Qi, R.J. Reiter, D. X. Tan, L.C. Manchester, A.W. Siu, J.J. Garcia, J. Pineal Res. 29 (2000) 54.
[2] M. Dizdaroglu, Mutat. Res. 275 (1992) 331
[3] A. Collins, Free Radical Research 32 (2000) 333.
[4] T. Douki, R. Martini, J.L. Ravanat, R.J. Turesky, J. Cadet, Carcinogenesis 18 (1997) 2385.
[5] Ö. Aybastıer, Journal of Traditional Chinese Medicine 41 (2021) 293.
[6] M. Dizdaroglu, P. Jaruga, H. Rodriguez, Nucleic Acids Res. 29 (2001) e12.
[7] M. Dizdaroglu, E. Coskun, P. Jaruga, Free Radic. Res. 49 (2015) 525.
[8] Ö. Aybastıer, S. Dawbaa, C. Demir, J. Chromatogr. B Analyt Technol Biomed Life Sci. 1072 (2018a) 328.
[9] M. D'Errico, E. Parlanti, M. Teson, B.M.B. de Jesus, P., Degan, A. Calcagnile, E. Dogliotti, EMBO J. 25 (2006) 4305.
[10] M. Murata, M. Mizutani, S. Oikawa, Y. Hiraku, S. Kawanishi, FEBS Lett. 554 (2003) 138.
[11] P. Jaruga, M. Birincioglu, H. Rodriguez, M. Dizdaroglu, Biochemistry 41 (2002) 3703.
[12] M. Dizdaroglu, in: O.I. Aruoma, B. Halliwell (Eds.), DNA and Free Radicals: Techniques, Mechanisms and Applications, OIC International, Saint Lucia, 1998.
[13] H.J. Helbock, K.B. Beckman, B.N. Ames, in: M.N. Kojima, M.I. Johnson, H. Sies (Eds.), Methods in Enzymology, vol. 300, 1999.
[14] M. Nakajima, T. Takeuchi, K. Morimoto, Carcinogenesis 17 (1996) 787.
[15] Y.-M. Shih, M.S. Cooke, C.-H. Pan, M.-R. Chao, C.-W. Hu, Redox Biol. 20 (2019) 556.
[16] P. Jaruga, E. Coskun, K. Kimbrough, A. Jacob, W.E. Johnson, M. Dizdaroğlu, Environ. Toxicol. 32 (2017) 2144.
[17] F. Zhang, W.T. Stott, A.J. Clark, M.R. Schisler, J.J. Grundy, B.B. Gollapudi, M.J. Bartels, Rapid Commun. Mass Spectrom. 21 (2007) 3949.
[18] Y. Hua, S.B. Wainhaus, Y. Yang, L. Shen, Y. Xiong, X. Xu, R.B. Breemen, J. Am. Soc. Mass Spectrom. 12 (2001) 80.
[19] P. Bermejo-Barrera, J. Capelo, A. Mota, Y. Madrid, C. Cámara, TrAC Trends in Analytical Chemistry. 23 (2004) 654-663. 10.1016/j.trac.2004.06.007.
[20] K. Bialkowski, P. Cysewski, R. Olinski, Z. Naturforsch. 51 (1996) 119.
[21] R.S. Das, M. Samaraweera, M. Morton, J.A. Gascón, A.K. Basu, Chem. Res. Toxicol. 25 (2012) 2451.
[22] A. Jones, J. Tittensor, R. Walker, Nature. 209 (1966) 296.
[23] J.A. Zoltewicz, D.F. Clark, T.W. Sharpless, G. Grahe, J. Am. Chem. Soc. 92 (1970) 1741.
[24] R. Rios-Font, L. Rodríguez-Santiago, J. Bertran, M. Sodupe, J. Phys. Chem. B (2007) 6071.
[25] S. Mikkola, T. Lönnberg, H. Lönnberg, Beilstein J. Org. Chem. 14 (2018) 803.
[26] K.S. Dickson, C.M. Burns, J.P. Richardson, J. Biol. Chem. 275 (2000) 15828.
[27] M. Dizdaroglu, Mutat. Res. Mol. Mech. Mutagen. 591 (2005) 45.
[28] Ö. Aybastıer, S. Dawbaa, C. Demir, O. Akgün, E. Ulukaya, F. Arı, Mutat Res-Fund Mol. M. 808 (2018b) 1.
[29] M. Polverelli, M. Berger, J.-F. Mouret, F. Odin, J. Cadet, Nucleosides Nucleotides Nucleic Acids 9 (1990) 451.
[30] M.S. Lowenthal, E. Quittman, K.W. Phinney, Anal. Chem. 91 (2019) 14569.
[31] S. Shibayama, S. Fujii, K. Inagaki, T. Yamazaki, A. Takatsu, J. Chromatogr. A 1468 (2016) 109.
[32] D. Liu, X. Zou, L. Zhong, Y. Lou, B. Yang, Y. Yin, Int. J. Mass Spectrom. 374 (2014) 20.
[33] Y. Sun, S. Stransky, J. Aguilan, M. Brenowitz, S. Sidoli, MethodsX. 8 (2021) 101585.
[34] A. Collins, Carcinogenesis 23 (2002) 2129.
[35] J. Cadet, T. Douki, J.L. Ravanat, Free Radic Biol Med. 49 (2010) 9.
[36] J. Cadet, J.R. Wagner, in: E.C. Friedberg, S.J. Elledge, A.R. Lehmann, T. Lindahl M. Muzi-Falconi (Eds.),

DNA Repair, Mutagenesis, and other Responses to DNA Damage, Cold Spring Harbor Laboratory Press, 2014.

[37] P. Jaruga, H. Rodriguez, M. Dizdaroglu, Free Radic Biol. Med. 31 (2001) 336.
M.K. Shigenaga, J.-W. Park, K.C. Cundy, C.J. Gimeno, B.N. Ames, in: J.N. Abelson, S.P. Colowick, M.I. Simon, H. Sies (Eds.), Methods in Enzymology, Vol. 186, Academic Press, 1990.

[38] P.F. Crain, in: J.N. Abelson, M.I. Simon, J.A. McCloskey (Eds.), Methods in Enzymology, vol. 193, Academic Press, 1990.

[39] B. Yokuş, D.U. Çakır, T. Klin, J. Med. Sci. 22 (2002) 535.

[40] J.-L. Ravanat, R.J. Turesky, E. Gremaud, L.J. Trudel, R.H. Stadler, Chem. Res. Toxicol. 8 (1995) 1039.

[41] E. Atmaca, A. Aksoy, YYU Vet Fak Derg. 20 (2009) 79.