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Optimization and validation of ultrasensitive GC–MS/MS method to measure oxidatively induced DNA damage products and role of antioxidants in oxidation mechanism

Önder Aybastıer, Cevdet Demir*

Bursa Uludag University, Faculty of Science and Arts, Department of Chemistry, 16059, Bursa, Turkey

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ABSTRACT

Oxidation of DNA due to exposure to reactive oxygen species (ROS) is a major source of DNA damage. ROS induced damage to DNA plays an important role in some diseases such as various cancers, aging and neurodegenerative diseases. The detection of DNA oxidation products plays a major role in assessing the mutagenicity potential of specific exposure. The GC–MS/MS method was developed for the ultrasensitive determination of individual DNA damage products. The validation results revealed that the proposed method was reliable and sensitive. Multiple response surface methodology (MRSM) was used to optimize derivatization conditions of oxidatively DNA base damage products before GC–MS/MS analysis. The optimum derivatization conditions were determined as 40 min for derivatization time, 120 °C for derivatization temperature and 1.4 for BSTFA/pyridine ratio under nitrogen atmosphere. The effects of thymol, carvacrol and thymoquinone as antioxidants were investigated on oxidative DNA damage. The determination of the oxidatively induced DNA damage products was performed after adding DNA and antioxidants with different concentrations under oxidative stress. Eighteen DNA base damage products were analyzed simultaneously using GC–MS/MS. This study showed a significant decrease in the amount of DNA base damage products when the antioxidants were present in the medium.

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1. Introduction

Reactive oxygen species (ROS) are formed by normal cellular metabolism in aerobic organisms and by exogenous sources such as UV radiation, ionizing radiations, some drugs and harmful chemicals [1]. ROS which is involved in several biological functions may damage cellular constituents such as DNA, protein and ribosome in high concentrations. Among them, much interest has been focused on DNA, the macromolecule containing the genetic information in all organisms. Damage to DNA may induce cell death or mutation that could give rise, several cellular divisions later, to cancer or different diseases [1,2]. The measurement of DNA damage products is of fundamental importance in assessing the potential carcinogenic effects of specific exposures and understanding their mechanisms of action [3]. Therefore, there is an increasing interest in using oxidative DNA damage products as in vivo biomarkers of oxidative stress [4].

ROS is generated by all aerobic cells and is known to participate in a wide variety of biological processes. Hydrogen peroxide is a major ROS in living organisms, and its homeostasis can have diverse pathological and physiological consequences. Besides, H_2O_2 can produce reactive hydroxyl radicals by the Fenton or Fenton-like reaction [5,6]. ROS can attack DNA bases, leading to more than 20 oxidized base damage products [7]. As a consequence of oxidation, DNA may have base modifications, DNA-DNA and DNA-protein crosslinking and strand breaks [8]. Oxidative DNA damage caused by free radicals and other oxidizing agents is thought to play an important role in disease formation [9]. Hydroxyl radical is the most reactive free radical and reacts with DNA by addition to double bonds and abstraction of hydrogen atoms [10]. Therefore, ROS might be connected to human diseases such as cancer, neurodegenerative and cardiovascular diseases. The Fenton reaction has great importance in living biological system. [5,11].

ROS is essential to the defense system being involved in many biological functions. However, when overproduced, or when the levels of antioxidants become severely depleted, these ROS become highly harmful, causing oxidative stress through the oxidation of biomolecules such as DNA and protein [5,6,11]. Oxidatively-induced damage to DNA can initiate mutagenic processes, various

* Corresponding author.
E-mail address: cevdet@uludag.edu.tr (C. Demir).

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diseases and early aging. Various in vivo pathways exist to repair DNA damage. DNA repair is essential for the maintenance of genomic stability and plays a major role in the prevention of genetic diseases and risk factors for carcinogenesis [9–13].

Oxidized DNA bases can be detected by various analytical methods. The two most commonly used methods are high-performance liquid chromatography with electrochemical detection (HPLC/ECD), and gas chromatography/mass spectrometry with selective ion monitoring (GC/MS-SIM) [14]. In HPLC/ECD, DNA is enzymatically digested into 2'-deoxyribonucleosides before HPLC analysis, while for GC/MS-SIM, acidic hydrolysis is carried out to yield intact and damaged bases that are further derivatized [7,15]. Artefactual oxidation of DNA bases could be induced during derivatization for GC/MS. Unlike acidic hydrolysis, enzymic hydrolysis only cleaves the damaged nucleotides from DNA backbone. The enzymic hydrolysis method does not induce artifacts in the analysis [14,16]. Studies also show that acid hydrolysis does not cause any artifacts once the DNA sample is processed under the nitrogen atmosphere [16]. Because enzymes are significantly more costly than formic acid and take a longer time, acid hydrolysis can replace enzymic digestion. However, HPLC/ECD cannot provide spectroscopic support for structural identification of adduct. In contrast, GC/MS with electron ionization (EI) provides structural evidence for identification and accurate quantification of oxidation products. However, this method requires a clean matrix to avoid the interference of unwanted ions [17,18]. On the other hand, GC–MS/MS enables the elimination of matrix components in the sample, determines the DNA oxidation products in high sensitivity and gives spectral information with better selectivity for 18 products as applied to different matrices in our previous studies [19,20]. In this study, thyme oil and thyme water in addition to standard antioxidants of carvacrol, thymol and thymoquinone, which are present in thyme, were investigated for their ability to inhibit the formation of oxidative DNA damage products in malignant lung cells compared to the control group.

The sensitivity of GC/MS-SIM is 1–2 fmol for 8-hydroxyguanine, whereas the sensitivity of LC/MS is ~30 fmol and LC–MS/MS is 10–20 fmol [21,22]. GC/MS-SIM can provide accurate quantification and identification with high selectivity and sensitivity. In selective ion monitoring mode, GC/MS can analyze a wide range of bases in a single run with high sensitivity and selectivity of detection [14,19].

In this study, a GC–MS/MS triple quadrupole system was applied for the separation of DNA damage products. Determination of product ion, precursor ion and optimal collision energy and analysis method was developed for the simultaneous analysis of eighteen damage products. Derivatization conditions required before GC–MS/MS analysis were optimized using multiple responses central composite design. The DNA bases were analyzed in a single run with high sensitivity and selectivity by GC–MS/MS selected reaction monitoring (SRM) mode. The effects of thymol, carvacrol and thymoquinone as antioxidants were investigated on the quantity of oxidized DNA bases of the calf thymus DNA against Fenton reaction.

2. Materials and methods

2.1. Chemicals and reagents

Iron (II) sulfate-7-hydrate, pyridine anhydrous, absolute ethanol, acetonitrile, 4,6-diamino-5-nitropyrimidine (46D5NP), 5,6-dihydrouracil (56DHU), 5-(hydroxymethyl)uracil (5HMU), 8-hydroxyguanine (8 H G) and deoxyribonucleic acid (DNA) from calf thymus were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trimethylchlorosilane (TMCS), formic acid and methanol were purchased from Merck Chemicals (Darmstadt, Germany). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and hydrogen peroxide were purchased from Fluka (Buchs, Switzerland). 4,6-diamino-5-(formylamino)pyrimidine (46D5FP) from Santa Cruz Biotechnology (Texas, USA). Alloxan (Alx) was from Titan Biotech (Delhi, India). 5-formyluracil (5FU) was from IS Chemical Technology (Shanghai, China). 2-hydroxy adenine (2HA), 5,6-dihydrothymine (56DHT), 5-hydroxyhydantoin (5HH), 2-hydroxy-5-methylhydantoin (5H5MH), 2,8-dihydroxyadenine (28DHA) and 5-(hydroxymethyl)cytosine (5HMC) were purchased from Toronto Research Chemicals (Toronto, Canada). 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), thymine glycol (TG), 5-hydroxyuracil (5HU), 8-hydroxyadenine (8HA), 5-hydroxycytosine (5HC) and 5,6-dihydrouracil (56DHU) were from the National Institute of Standards and Technology (MD, USA).

2.2. Experimental design for DNA base damage products derivatization

The response surface methodology (RSM) is a collection of mathematical and statistical techniques that can be employed to determine a relationship between input parameters and output response [23]. It uses multivariable data obtained from designed experiments to resolve multivariable responses simultaneously.

The experimental design with actual values is shown in Table S1. Eighteen experiments were performed to optimize the parameters. Fourteen experiments were conducted with four replications at the center points to evaluate the pure error.

The investigated parameters were: derivatization time (14.4–135.6 min), derivatization temperature (68–172 °C) and derivatization reagent (BSTFA)/solution (pyridine) ratio (0.362–1.805 v/v). Derivatizations of DNA base products were performed under nitrogen atmosphere to minimize the artefactual oxidation. The derivatization mixtures were added under nitrogen atmosphere and the reaction vial screw caps were closed prior to derivatization. The GC–MS/MS peak area of DNA base damage products of 56DHT, 5FU, 5HMU, 46D5NP, FapyAde and 8 H G were selected as the multiple responses of the design experiments (y).

Second-order polynomial Eq. (1) which includes all interaction terms was used to calculate the predicted response:

$$y = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3} b_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ij} x_i x_j \quad (1)$$

where y is a response; b_0 is the offset term, b_i is the linear effect, b_{ii} is the squared effect, b_{ij} is the interaction effect and x_i and x_j are independent variables.

The experimental results of the central composite design were fitted with a second-order polynomial equation by a multiple regression method. The data were analyzed using the Design Expert program (7.0.0 version). The coefficients were interpreted using the F test and optimum conditions were determined for the derivatization of oxidative DNA base damage products.

2.3. DNA preparation

DNA from calf thymus was purchased in lyophilized form and 5 mg of DNA was dissolved in 100 mL ultra-pure water and incubated at 4 °C overnight [24]. The concentration of DNA was measured at 260 nm by spectrophotometer (Varian Cary 50, Australia). The DNA concentration was calculated using the following equation (2).

$$\mu gDNA = A_{260} \times 50 \quad (2)$$

2.4. Oxidatively induced DNA damage

The required oxidative stress environment was created using the Fenton reaction [25]. Hydrogen peroxide, Fe (II), carvacrol, thymol and thymoquinone stock solutions were prepared.

A total of fourteen samples were prepared to study the antioxidant effects on DNA oxidation. Each sample volume was 2500 μL and contained 100 μg DNA in addition to the other compounds described as follows: (1) only 100 μg DNA, (2) 400 μM carvacrol, (3) 400 μM thymol, (4) 400 μM thymoquinone, (5) 40 μM carvacrol, (6) 40 μM thymol, (7) 40 μM thymoquinone, (8) Fenton (200 μM H_2O_2 and 100 μM Fe (II)), (9) Fenton and 400 μM carvacrol, (10) Fenton and 400 μM thymol, (11) Fenton and 400 μM thymoquinone, (12) Fenton and 40 μM carvacrol, (13) Fenton and 40 μM thymol, (14) Fenton and 40 μM thymoquinone. Each sample preparation begins with adding 100 μg of DNA, pure water, antioxidant solution, H_2O_2 and Fe (II) consecutively.

After properly mixing the contents of the samples, they were well-closed and kept in an incubator at 37 °C for 20 min. The samples then were frozen at lower than −20 °C, then was lyophilized (Free Zone Labconco, MO, USA) freeze dryer with a – 85 °C cooling system at a vacuum of 0.1 mbar. Hydrolysis of the samples was achieved by the addition of 1 mL 60 % (v/v) formic acid in water to each sample and heating them in an oven at 130 °C for 30 min, the hydrolysis process releases the DNA damage products as free bases that can be detected in GC–MS/MS [7,19,24]. Finally, the samples were frozen and lyophilized at the same conditions before derivatization for GC–MS/MS analysis.

2.5. GC–MS/MS analysis

GC–MS/MS measurements were carried out on a Thermo 1300 GC system using a triple quadrupole mass detector TSQ8000 Evo (Thermo Fischer Scientific, Waltham, MA USA). We used a DB-5MS capillary column (5%-phenyl)-methylpolysiloxane (12 m ×0.20 mm ×0.33 μm, Agilent Technologies, Waldbronn, Germany). For electron impact ionization, a 70 eV electron beam was used followed by mass analysis using the first quadrupole (Q1). Characteristic daughter ions were produced as a result of the collisions of the argon as an inert gas with the analyte at the optimum collision energy. Thus, several collision energies between 0 and 30 eV and manifold scan times were tested to obtain the best results. Upon exiting the collision cell, the fragmented ions then travel onto the second quadrupole (Q3), where m/z selection occurred again. A volume of 2 μL sample was injected in the split mode (split ratio: 5) at 250 °C. Carrier gas flow was helium at 1.0 mL/min. The temperature gradient was 130 °C held for 2 min, 8 °C/min up to 230 °C and 20 °C/min up to 280 °C. Temperatures of the ion source and MS transfer line were 250 and 280 °C, respectively. Selected reaction monitoring (SRM) was carried out with optimized settings for each analyte. Each GC–MS/MS run required 17 min, 2 runs for each sample were achieved. Calibrations were achieved for DNA base damage products using different concentrations of standards in pyridine.

3. Results and discussion

3.1. Multiple response optimization of derivatization conditions

The derivatization parameters were optimized using multiple response central composite design combined with response surface methodology. The GC–MS/MS peak areas were selected as the response due to its high derivatization yield for 3 purine (5,6-dihydrothymine, 5-formyluracil, 5-hydroxymethyl uracil) and 3 pyrimidine (4,6-diamino-5-nitropyimidine, fapyadenine, 8-hydroxyguanine) DNA base damage products. The effects of each factor and their interactions were calculated using a Design Expert program (version 7.0.0). By applying multiple regression analysis on the experimental data and the response variables are related by the following second order polynomial equations:

$$\text{56DHT} \quad \begin{aligned} y = {} & 1,432.10^7 - 1,223.10^6 x_1 - 3,909.10^6 x_2 \\ & + 8,535.10^5 x_3 - 1,634.10^6 x_1 x_2 \\ & - 1,906.10^6 x_2^2 - 1,074.10^6 x_3^2 \end{aligned} \tag{3}$$

$$\text{5FU} \quad \begin{aligned} y = {} & 2,766.10^7 - 1,097.10^6 x_1 - 1,993.10^6 x_2 \\ & + 4,765.10^6 x_3 - 1,545.10^6 x_1 x_2 \\ & - 1,660.10^6 x_3^2 \end{aligned} \tag{4}$$

$$\text{5HMU} \quad \begin{aligned} y = {} & 6,078.10^7 + 1,761.10^6 x_1 + 1,957.10^6 x_2 \\ & + 3,155.10^6 x_3 + 1,544.10^6 x_2 x_3 \\ & - 7,185.10^5 x_2^2 - 2,648.10^6 x_3^2 \end{aligned} \tag{5}$$

$$\text{46D5NP} \quad \begin{aligned} y = {} & 2,821.10^7 + 1,005.10^6 x_1 + 1,279.10^6 x_2 \\ & + 2,612.10^6 x_3 - 1,127.10^6 x_3^2 \end{aligned} \tag{6}$$

$$\text{FapyAde} \quad \begin{aligned} y = {} & 1,034.10^8 - 3,681.10^6 x_2 \\ & + 7,886.10^6 x_3 - 4,071.10^6 x_2^2 - 4,914.10^6 x_3^2 \end{aligned} \tag{7}$$

$$\text{8HG} \quad \begin{aligned} y = {} & 2,093.10^6 + 7,573.10^4 x_1 + 1,771.10^5 x_2 \\ & + 3,614.10^5 x_3 - 2,297.10^5 x_3^2 \end{aligned} \tag{8}$$

The variance analysis (ANOVA) results are given in Table S2. The F-test carried out on experimental data makes it possible to estimate the statistical significance of the proposed models. The second order polynomial equations also designate that the respective parameters are considered statistically significant at the 95 % confidence level. The regression coefficients (R^2) showed that the models were compatible with the experimental results.

Optimum conditions for the derivatization of DNA base damage products were determined to obtain the maximum peak area of the products. Second order polynomial models developed in this study were utilized for each response to obtain specified optimum conditions. The optimum derivatization conditions from the model were determined as 40 min for derivatization time, 120 °C for derivatization temperature and 1.4 for BSTFA/pyridine ratio within the experimental region by multiple response optimizations. Reddy et al. [26] used 30 min for derivatization time, 120 °C for derivatization temperature and 1.0 for BSTFA/pyridine ratio during derivatization. The peak area of DNA base damage products was compared with reported derivatization conditions. The change of GC–MS/MS peak area for DNA base damage products is shown in Table 1. In our optimum derivatization conditions, a greater peak area was obtained from the reported conditions. Only 12.6 % smaller was observed in the peak area of alloxan. There are considerable differences in the level of DNA base damage products between different studies, particularly for 8-OH-guanine [18]. This is probably due to the difference in the quantity of oxidation products, types of derivatization, derivatization conditions, solvents used and air excluded during the derivatization process. In this study, the derivatization was performed under the nitrogen atmosphere to avoid the artefactual formation of compounds from free DNA bases during the derivatization of acid hydrolysis of DNA. Pyridine was used as a solvent in the derivatization mixture and thus may prevent oxidation of some of the intact bases in acid hydrolysates during derivatization. DNA base damage products were analyzed

Table 1
The change of peak areas for DNA base damage products between the conditions in the literature (time: 30 min., temperature: 120 °C, BSTFA/pyridine ratio: 1.0) [26] and developed method (time: 40 min., temperature: 120 °C, BSTFA/pyridine ratio: 1.4).

DNA base damage product	Change of peak area (%)
56DHT	+24.3
56DHU	+22.9
5H5MH	+16.9
5HH	+ 9.9
5FU	+29.5
5HMU	+21.0
Alx	−12.6
46D5NP	+20.2
FapyAde	+17.3
2HA	+76.4
8 H G	+30.1

56DHT: 5,6-dihydrothymine, 56DHU: 5,6-dihydrouracil, 5H5MH: 5-hydroxy-5-methylhydantoin, 5HH: 5-hydroxy hydantoin, 5FU: 5-formyluracil, 5HMU: 5-(hydroxymethyl)uracil, Alx: alloxane, 46D5NP: 4,6-diamino-5-nitropyrimidine, FapyAde: 4,6-diamino-5-(formylamino)pyrimidine, 2HA: 2-hydroxyadenin, 8 H G: 8-hydroxyguanine.

more sensitively after multiple response modeling optimizations by GC–MS/MS.

3.2. Optimization of GC–MS/MS conditions

SRM is a highly sensitive and specific mass spectrometry technique that can selectively quantify compounds within complex mixtures. The GC–MS/MS uses a triple quadrupole mass spectrometer that firstly targets the ion corresponding to the compound of interest with subsequent fragmentation of that target ion to produce a range of daughter ions. One or more of these fragment product ions can be selected for quantitative analysis. The compounds that meet both these criteria, i.e. specific precursor ion and specific product ions corresponding to the mass of the molecule of interest are isolated within the mass spectrometer.

The optimized GC–MS/MS SRM acquisition parameters of the targeted molecules showed in Table S3. The precursor ion scan mode was established with a scanning range from 50 to 550 (m/z). The precursor ions of 18 oxidative DNA base damage products were selected for the second collision of ions with different voltages. The selected transitions were optimized in SRM mode under different collision energies in the range 0–30 eV. Then, a signal value and the highest percentage collision value of the two ions and voltages were selected. Finally, the retention time and highest value for the quantitative ion were determined followed by the confirming ion. 5,6-dihydrouracil (56DHU) and 5,6-dihydrothymine (56DHT) were the first to appear, they had approximately the same retention time but were differentiated by different precursor and product ions. The retention times of 5-(hydroxymethyl)uracil (5HMU) and alloxan (Alx) had almost the same condition, but with the difference between their retention time values being slightly wider. The DNA base damage products were eluted almost perfectly with clear recognizable peaks by GC–MS/MS.

3.3. Validation of analytical parameters

The validation study was carried out after the optimization of the GC–MS/MS method and derivatization conditions. The standard calibration curve was constructed by plotting the GC–MS/MS peak area against analyte concentrations. Each concentration level was repeated in duplicate. The results revealed that good linearity was achieved for the analyte concentration and the response in the corresponding ranges, indicated by the determination of the regression coefficient (r^2>0.99, excluding 2HA). The graphics have a wide linear range. The results are summarized in Table S4.

Limit of detection (LOD) and limit of quantification (LOQ) are fundamental parameters used to evaluate the sensitivity of the method. The LODs were determined by the injection of the standard solutions in the minimum concentration in the calibration graphs. LODs were calculated until corresponding to a standard deviation ratio of three. Besides the limits of quantitation (LOQs) were used to assess the sensitivity by the injection of samples until corresponding to a standard deviation ratio often. In addition, the LOQs were calculated in the fmol unit. Jaruga et al. [24] reported the level of sensitivity of GC–MS amounts to ∼1 fmol of the FapyAde and FapyGua. However, in our study, these values were found to be 0.26 and 0.59 fmol, respectively. It is concluded that the developed GC–MS/MS method is sensitive at the optimum ionization and the derivatization conditions of DNA base oxidation products.

Table 2
Validation parameters for GC–MS/MS analysis of DNA base damage products.

Molecule	LOD (ng/L)	LOQ (ng/L)	Sensitivity (fmol)	Repeatability (RSD, %)				% Recovery
				Inter-day (n = 3)		Intra-day (n = 15)		
				2 μg/L	10 μg/L	2 μg/L	10 μg/L	
56DHT	76	254	0.78	0.52	0.30	7.21	6.93	105
56DHU	69	232	0.88	1.92	1.17	6.02	5.66	105
5H5MH	37	125	0.77	0.79	0.38	9.02	8.39	109
5HH	28	93	0.34	1.61	1.57	3.04	3.20	108
5FU	145	485	1.43	0.51	0.53	9.94	10.07	77
5HU	17	58	0.78	4.42	4.83	11.40	11.48	103
5HMU	8	26	0.14	4.95	5.92	7.02	7.49	118
Alx	11	35	0.28	2.88	2.63	10.90	11.84	98
5HC	25	83	0.31	1.68	1.31	8.86	8.07	106
46D5NP	89	297	1.29	1.97	1.30	5.15	4.40	104
TG	16	52	0.25	2.15	1.83	9.87	9.54	113
5HMC	24	80	0.28	1.29	1.22	8.12	7.76	118
FapyAde	31	102	0.26	2.01	1.28	6.10	5.71	103
8HA	28	92	0.26	5.94	5.68	9.22	8.26	103
2HA	76	253	0.66	5.60	5.71	11.35	11.61	129
FapyGua	26	86	0.59	2.55	2.40	8.97	8.69	118
28DHA	142	472	1.20	4.11	4.06	5.88	6.10	83
8 H G	30	99	0.24	3.45	3.37	7.91	7.27	82

LOD: Limit of detection, LOQ: Limit of quantitation, 56DHT: 5,6-dihydrothymine, 56DHU: 5,6-dihydrouracil, 5H5MH: 5-hydroxy-5-methylhydantoin, 5HH: 5-hydroxy hydantoin, 5FU: 5-formyluracil, 5HU: 5-hydroxyuracil, 5HMU: 5-(hydroxymethyl)uracil, Alx: alloxane, 5HC: 5-hydroxycytosine, 46D5NP: 4,6-diamino-5-nitropyrimidine, TG: thymine glycol, 5HMC: 5-(hydroxymethyl)cytosine, FapyAde: 4,6-diamino-5-(formylamino)pyrimidine, 8HA: 8-hydroxyadenine, 2HA: 2-hydroxyadenin, FG: 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 28DHA: 2,8-dihydroxyadenine, 8 H G: 8-hydroxyguanine.

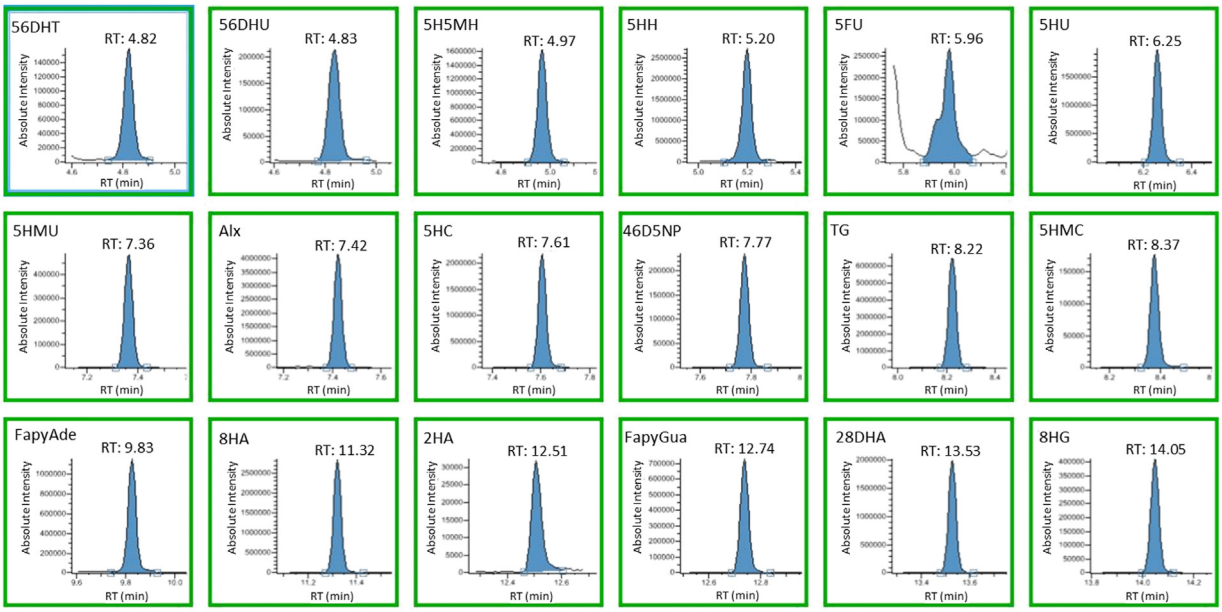

Fig. 1. GC–MS/MS chromatograms of DNA base damage products of 10 μg/L standards.

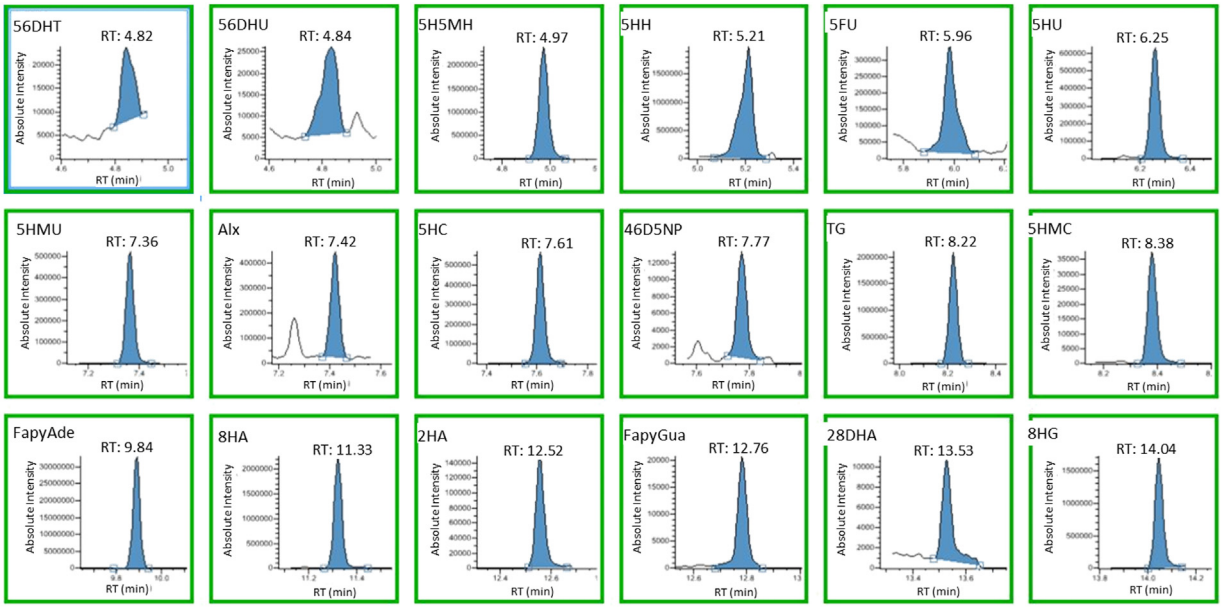

Fig. 2. GC–MS/MS chromatograms of DNA base damage products in sample containing DNA, Fenton and 40 μM carvacrol.

GC/MS applications for the measurement of oxidized DNA bases have been included the use of isotope dilution as an internal standard [12,24,27]. Several studies are known where measurement of a single DNA base product can give misleading results [17]. Improvement of measurement conditions for GC–MS/MS is warranted because of the ability of this technique to measure a range of DNA base damage products.

Intra-day and inter-day repeatability studies were carried out by analyzing DNA base oxidation product solutions at 2 and 10 μg/L concentrations. Intra-day repeatability was assessed by three replicates per concentration in one day (n = 3), while inter-day repeatability was measured for three replicates per concentration on five consecutive days (n = 15). The repeatability and relative standard deviation (RSD) results are shown in Table 2. The RSD values of intra-day precision were in the range of 3.04 %–11.84 % and for inter-day precision, the RSD values were in the range of 0.30 %–5.94 %. The results indicate that repeatability is good enough.

A standard mixture solution of DNA base damage products (10 μg/L) was analyzed using the GC–MS/MS method after derivatization for the determination of recoveries.

The recoveries (R) were calculated using the following equation:

$$R = C_{found}/C_{real} \times 100 \quad (9)$$

Where C_{found} and C_{real} are the concentration of analyte which was determined by GC–MS/MS and the concentration of an analyte in the sample, respectively. The recoveries of the eighteen DNA base damage products ranged from 82 % to 129 %, which shows that the developed method is feasible in the application of analysis. The recoveries of all analytes were tested under the optimized conditions, and satisfying results were observed. The chromatograms

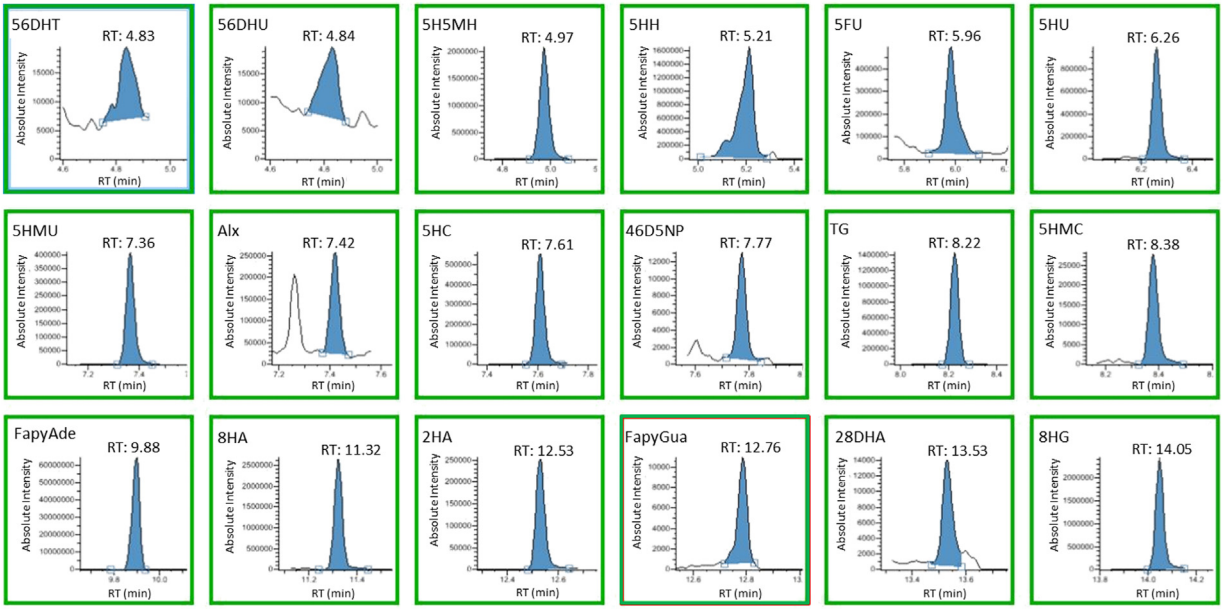

Fig. 3. GC–MS/MS chromatograms of DNA base damage products in sample containing DNA, Fenton and 400 μM thymol.

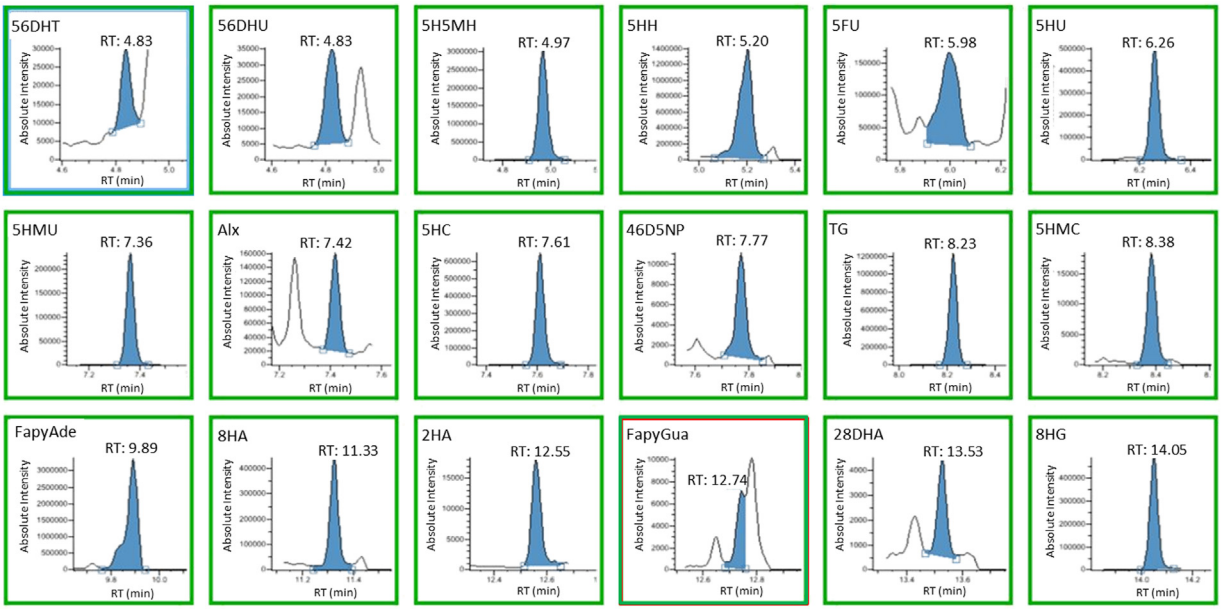

Fig. 4. GC–MS/MS chromatograms of DNA base damage products in sample containing DNA, Fenton and 400 μM thymoquinone.

of GC–MS/MS analysis of 10 μg/L standards of DNA base damage products are shown in Fig. 1.

3.4. The effect of antioxidants on DNA base damage products

The protective effects of carvacrol, thymol and thymoquinone against oxidative DNA base damage were determined by examining the ability of the antioxidants to inhibit DNA base damage. This was achieved by measuring the amounts of DNA base damage products. The chromatograms of GC–MS/MS analysis of the calf thymus DNA with added antioxidants, 200 μM of H_2O_2 and 100 μM of Fe (II) are shown in Figs. 2–4.

Levels of DNA base damage products were measured under the oxidative stress and without oxidative stress environment in the presence of antioxidants of carvacrol, thymol and thymoquinone. The low level of damage products may occur in the control DNA sample (Table 3) during acid hydrolysis and derivatization process [28]. The results show that there is no significant change in the level of damage products in the control DNA sample and the presence of antioxidants at concentrations 40 μM and 400 μM.

Exposure of DNA to the H_2O_2/Fe (II) system significantly increased the levels of all the DNA base damage products in the control sample (Table 4). Under oxidative stress, the oxidative damage products of 5-hydroxy-5-methylhydantoin (5H5MH), 5-hydroxyuracil (5HU), 5-(hydroxymethyl)uracil (5HMU), 2-hydroxyadenin (2HA) and 8-hydroxyguanine (8 H G) exhibited significantly greater levels. In contrast, 5,6-dihydrothymine (56DHT), 4,6-diamino-5-nitropyrimidine (46D5NP), 4,6-diamino-5-(formylamino)pyrimidine (FapyAde) and 2,8-dihydroxyadenine (28DHA) occurred at the lowest level under the Fenton reaction. The most prominent DNA base damage product of 8 H G as a marker molecule has been mostly measured in biological samples [12]. Our

Table 3
Oxidative DNA base damage products (ng/mg DNA) in control samples (without Fe(II) and H_2O_2).

Molecule	Control	400 μM Carvacrol	400 μM Thymol	400 μM Tq	40 μM Carvacrol	40 μM Thymol	40 μM Tq
56DHT	<LOD[a]	0.65 ± 0.21	0.23 ± 0.07	0.32 ± 0.01	<LOD[a]	0.18 ± 0.07	0.35 ± 0.02
56DHU	<LOD[a]	0.83 ± 0.19	0.47 ± 0.14	0.84 ± 0.16	1.18 ± 0.19	0.61 ± 0.13	0.68 ± 0.13
5H5MH	1.51 ± 0.01	5.18 ± 0.03	3.59 ± 0.04	2.80 ± 0.04	1.48 ± 0.11	18.85 ± 1.31	5.62 ± 0.07
5HH	8.27 ± 0.34	8.13 ± 0.23	8.14 ± 0.06	5.80 ± 0.13	6.68 ± 0.13	16.01 ± 0.41	8.07 ± 0.10
5FU	4.62 ± 0.32	15.14 ± 1.61	21.42 ± 1.66	24.91 ± 2.72	23.17 ± 2.27	3.21 ± 0.18	12.76 ± 1.20
5HU	0.19 ± 0.08	2.73 ± 0.28	4.86 ± 0.34	0.81 ± 0.14	0.76 ± 0.16	2.48 ± 0.04	0.45 ± 0.06
5HMU	0.49 ± 0.03	6.63 ± 0.72	4.67 ± 0.89	2.87 ± 0.11	3.86 ± 0.31	5.28 ± 0.41	3.16 ± 0.36
Alx	0.31 ± 0.07	0.60 ± 0.05	0.29 ± 0.01	0.22 ± 0.02	0.30 ± 0.01	1.19 ± 0.04	0.42 ± 0.01
5HC	1.84 ± 0.19	6.52 ± 0.27	2.36 ± 0.37	2.10 ± 0.16	4.09 ± 0.39	3.03 ± 0.14	4.92 ± 0.15
46D5NP	18.81 ± 2.91	1.28 ± 0.14	0.62±.0.02	0.99 ± 0.14	1.09 ± 0.11	0.22 ± 0.02	1.77 ± 0.02
TG	0.17 ± 0.02	0.42 ± 0.02	0.39 ± 0.01	0.36 ± 0.01	0.12 ± 0.01	0.93 ± 0.10	0.42 ± 0.01
5HMC	3.17 ± 0.16	4.62 ± 0.27	1.16 ± 0.08	1.84 ± 0.07	4.89 ± 0.17	2.14 ± 0.06	3.03 ± 0.04
FapyAde	2.25 ± 0.22	2.40 ± 0.18	2.09 ± 0.44	0.66 ± 0.07	1.76 ± 0.17	<LOD[a]	0.46 ± 0.14
8HA	1.95 ± 0.37	5.52 ± 0.89	1.85 ± 0.41	1.92 ± 0.32	3.05 ± 0.35	0.08 ± 0.01	1.07 ± 0.12
2HA	7.05 ± 0.33	6.36 ± 0.86	2.56 ± 0.55	5.44 ± 0.27	6.04 ± 0.22	1.20 ± 0.02	2.05 ± 0.08
FapyGua	0.16 ± 0.03	0.14 ± 0.03	<LOD[a]	0.11 ± 0.02	0.08 ± 0.01	<LOD[a]	0.48 ± 0.02
28DHA	0.35 ± 0.05	0.88 ± 0.07	0.60 ± 0.02	1.61 ± 0.04	1.13 ± 0.03	0.46 ± 0.01	0.50 ± 0.02
8 H G	21.66 ± 2.17	21.36 ± 2.01	14.38 ± 0.95	14.28 ± 1.21	22.12 ± 1.10	15.02 ± 1.37	21.20 ± 1.05
Total	72.80 ± 3.71	89.39 ± 3.02	69.68 ± 2.32	67.88 ± 3.03	81.80 ± 2.64	70.89 ± 2.01	67.41 ± 1.67

[a] less than limit of detection. Tq: thymoquinone, 56DHT: 5,6-dihydrothymine, 56DHU: 5,6-dihydrouracil, 5H5MH: 5-hydroxy-5-methylhydantoin, 5HH: 5-hydroxy hydantoin, 5FU: 5-formyluracil, 5HU: 5-hydroxyuracil, 5HMU: 5-(hydroxymethyl)uracil, Alx: alloxane, 5HC: 5-hydroxycytosine, 46D5NP: 4,6-diamino-5-nitropyrimidine, TG: thymine glycol, 5HMC: 5-(hydroxymethyl)cytosine, FapyAde: 4,6-diamino-5-(formylamino)pyrimidine, 8HA: 8-hydroxyadenine, 2HA: 2-hydroxyadenin, FG: 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 28DHA: 2,8-dihydroxyadenine, 8 H G: 8-hydroxyguanine.

Table 4
Oxidative DNA base damage products (ng/mg DNA) in samples under oxidative stress (with Fenton medium: 200 μM H_2O_2 and 100 μM Fe (II)).

Molecule	Control	400 μM Carvacrol	400 μM Thymol	400 μM Tq	40 μM Carvacrol	40 μM Thymol	40 μM Tq
56DHT	2.05 ± 0.34	2.35 ± 0.37	1.89 ± 0.20	2.55 ± 0.38	1.57 ± 0.42	1.34 ± 0.06	0.93 ± 0.04
5.6DHU	8.59 ± 0.82	9.04 ± 0.25	1.73 ± 0.01	4.76 ± 0.76	2.85 ± 0.48	2.83 ± 0.27	2.78 ± 0.30
5H5MH	546.64 ± 35.92	83.54 ± 1.31	40.09 ± 1.24	58.16 ± 1.24	56.13 ± 4.05	58.25 ± 1.02	57.44 ± 0.86
5HH	140.01 ± 4.14	31.21 ± 0.19	18.65 ± 0.80	14.52 ± 0.18	19.66 ± 0.17	20.33 ± 1.06	23.07 ± 1.16
5FU	38.28 ± 3.07	30.37 ± 3.89	36.56 ± 1.79	24.65 ± 1.21	26.20 ± 1.18	34.56 ± 2.93	41.77 ± 1.53
5HU	521.81 ± 51.06	13.71 ± 0.32	11.72 ± 1.85	6.03 ± 0.37	5.85 ± 0.24	7.58 ± 0.61	13.22 ± 1.11
5HMU	187.47 ± 6.26	43.07 ± 0.56	27.64 ± 0.38	15.22 ± 0.26	43.79 ± 3.28	47.47 ± 1.73	38.49 ± 1.55
Alx	17.93 ± 1.14	3.84 ± 0.03	1.61 ± 0.11	0.94 ± 0.06	2.71 ± 0.18	3.55 ± 0.11	2.02 ± 0.13
5HC	18.13 ± 0.20	8.71 ± 0.15	6.15 ± 0.14	6.90 ± 0.10	5.75 ± 0.35	7.00 ± 0.30	8.39 ± 0.18
46D5NP	1.16 ± 0.23	0.43 ± 0.04	0.43 ± 0.06	0.26 ± 0.03	0.47 ± 0.02	0.64 ± 0.04	0.50 ± 0.01
TG	45.27 ± 3.13	8.49 ± 0.14	5.37 ± 0.20	4.24 ± 0.18	7.51 ± 0.10	7.76 ± 0.09	10.31 ± 0.14
5HMC	21.38 ± 0.62	12.68 ± 0.52	5.16 ± 0.20	3.36 ± 0.18	7.58 ± 0.10	8.65 ± 0.59	8.53 ± 0.76
FapyAde	1.70 ± 0.16	2.03 ± 0.21	1.81 ± 0.14	0.11 ± 0.02	1.20 ± 0.16	1.78 ± 0.28	1.50 ± 0.14
8HA	126.92 ± 9.34	44.29 ± 1.31	25.86 ± 0.17	22.87 ± 2.35	31.53 ± 1.43	36.23 ± 1.24	41.38 ± 1.41
2HA	171.93 ± 11.52	90.81 ± 7.84	69.08 ± 5.71	61.11 ± 5.10	69.29 ± 4.82	71.37 ± 5.41	77.94 ± 2.77
FapyGua	3.37 ± 0.05	0.11 ± 0.01	<LOD	<LOD	<LOD	<LOD	<LOD
28DHA	2.57 ± 0.08	1.32 ± 0.13	1.15 ± 0.10	0.55 ± 0.04	0.91 ± 0.05	1.45 ± 0.14	1.85 ± 0.11
8 H G	97.21 ± 9.18	42.34 ± 2.37	71.82 ± 5.86	35.46 ± 2.93	68.35 ± 4.55	62.90 ± 6.27	77.58 ± 5.40
Total	1952.42 ± 65.42	428.34 ± 9.31	326.72 ± 8.72	261.69 ± 6.65	351.35 ± 8.67	373.69 ± 9.21	407.70 ± 6.90

[a] less than limit of detection. Tq: thymoquinone, 56DHT: 5,6-dihydrothymine, 56DHU: 5,6-dihydrouracil, 5H5MH: 5-hydroxy-5-methylhydantoin, 5HH: 5-hydroxy hydantoin, 5FU: 5-formyluracil, 5HU: 5-hydroxyuracil, 5HMU: 5-(hydroxymethyl)uracil, Alx: alloxane, 5HC: 5-hydroxycytosine, 46D5NP: 4,6-diamino-5-nitropyrimidine, TG: thymine glycol, 5HMC: 5-(hydroxymethyl)cytosine, FapyAde: 4,6-diamino-5-(formylamino)pyrimidine, 8HA: 8-hydroxyadenine, 2HA: 2-hydroxyadenin, FG: 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 28DHA: 2,8-dihydroxyadenine, 8 H G: 8-hydroxyguanine.

results clearly showed that the measurement of one DNA base damage product such as 8 H G only does not necessarily prove the DNA damage in biological systems. Formation of 8 H G and other prominent oxidation products was observed with H_2O_2/Fe (II) system and oxidation inhibiting effects of catechin and cyanidin as antioxidants were observed in our previous work [19].

Two different concentrations of carvacrol, thymol and thymoquinone were tested for their ability to inhibit oxidative DNA base damage. In comparison to the DNA control sample, the antioxidants inhibit the damage by a concentration-dependent proportionality. The results show that carvacrol, thymol and thymoquinone protected the DNA at both concentrations of 40 μM and 400 μM. The highest level of 5H5MH and 5HU decreased by above 90 % compared to the control sample when exposed to the three antioxidants. Thymoquinone which has the highest antioxidant capacity [29] compared to carvacrol and thymol at 400 μM reduced the level of 5HMU from 187.47 ng/mg to 15.22 ng/mg (92 % reduction) whereas at 40 μM reduced to 38.49 ng/mg (80 % reduction). The lowest level of 8 H G among other prominent oxidation products decreased by about 30 % at all concentrations of carvacrol, thymol and thymoquinone. The total values of oxidation products show that thymoquinone has the highest protective effect on DNA oxidation by about 87 % at 400 μM.

The present results show that phenolic compounds present antioxidant/prooxidant activities depending on the dose [30]. It can be seen from the total value of DNA base damage products is that carvacrol exhibits a protective effect at 40 μM, whereas a prooxidant effect at 400 μM concentration in oxidative stress. Similar results were obtained for thymol and thymoquinone. Thus, carvacrol, thymol and thymoquinone can protect the DNA against oxidant agents such as hydroxyl radical and H_2O_2, and they can also damage the DNA previously induced by such agents. A study by Ozkan and Erdogan [31] found that carvacrol at higher concentrations increased the level of 8 H G in lung cancer cells causing

Table 5
Statistics by ANOVA.

Variables	*p* value
DNA DNA + Fenton	0.012
DNA DNA + 400 μM Carvacrol	0.648
DNA DNA + 400 μM Thymol	0.932
DNA DNA + 400 μM Thymoquinone	0.898
DNA DNA + 40 μM Carvacrol	0.823
DNA DNA + 40 μM Thymol	0.959
DNA DNA + 40 μM Thymoquinone	0.881
DNA + Fenton DNA + 400 μM Carvacrol + Fenton	0.041
DNA + Fenton DNA + 400 μM Thymol + Fenton	0.029
DNA + Fenton DNA + 400 μM Thymoquinone + Fenton	0.024
DNA + Fenton DNA + 400 μM Carvacrol + Fenton	0.032
DNA + Fenton DNA + 400 μM Thymol + Fenton	0.034
DNA + Fenton DNA + 400 μM Thymoquinone + Fenton	0.038

DNA damage. However, the protective abilities of these compounds are stronger than their pro-oxidant effects. The protective activity could be related to their antioxidant ability as investigated in our previous work [29].

Analysis of variance was performed to assess the significance of the results obtained by GC–MS/MS (Table 5). In comparing the DNA and Fenton oxidized DNA samples, small *p*-values were obtained which indicates that the variables are different. Thus, the formation of DNA damage is significant between the two samples. No difference was observed when the carvacrol, thymol and thymoquinone of 40 and 400 μM added DNA samples were compared with the DNA sample. Therefore, adding antioxidants alone has no effect on DNA damage. When the DNA and Fenton oxidized DNA containing antioxidants were compared, *p*-values were found between 0.024 and 0.041. DNA base damage was significantly reduced in the presence of antioxidants.

3.5. The effect of antioxidants on DNA base damage products of malignant lung cells

The effects of carvacrol, thymol, thymoquinone, water-methanol extract of thyme (*Origanum vulgare* L.) and thyme oil were investigated on the inhibition of oxidative DNA damage in malign lung cells (A549). Oxidative DNA damage products were measured in malign lung cells culture medium in presence of 40 μM carvacrol, 80 μM thymol, 10 μM thymoquinone, thyme extract and 20 μM H_2O_2. The lowest total amount of DNA damage products was found in the presence of thymoquinone and water-methanol (1:1, v/v) extract of *Origanum vulgare* L. Similar protective effects were observed for DNA damage products in malign lung cells (A549) and in samples under oxidative stress (Table 4). The lowest amount of 5H5MH (0.04 ng/mg DNA) was detected at a concentration of 10 μM thymoquinone and in water-methanol extract in malign lung cells, whereas 5 HU was not detected in these samples. The amount of 13 DNA damage products in malign lung cells are between 0.03 and 4.34 ng/mg in presence of 10 μM thymoquinone and water-methanol extract, other 5 products were not detected. Carvacrol and thymol also showed a high protective effect on DNA damage products with values as low as 0.03–5.95 ng / mg DNA. It can be concluded that the three antioxidants and the water-methanol extract of *Origanum vulgare* L. have the potential to protect cells from oxidative stress. The experimental details can be found in our previous work [29].

4. Conclusions

GC–MS/MS was applied for the separation of products of oxidative damage to DNA bases. Product ion, precursor ion and optimum collision energy were determined. A rapid and sensitive analysis method was developed for the simultaneous analysis of eighteen DNA base damage products. Multiple responses central composite design methodology was successfully employed to optimize derivatization conditions of DNA base damage products. Optimized conditions for maximum peak area were determined including derivatization time, derivatization temperature and BSTFA/pyridine ratio, which were identified as controlling factors. A significant increase in peak areas was obtained according to reported derivatization conditions. Damaged DNA bases were analyzed in a wide range in a single run with superior sensitivity and selectivity with the developed method by GC–MS/MS. The method was validated in terms of sensitivity, linearity, accuracy and repeatability, and the results were found to be within the required limit. The effects of thymol, carvacrol and thymoquinone were investigated on the levels of damaged DNA bases of the calf thymus DNA against Fenton reaction. The antioxidants showed different abilities to inhibit oxidative DNA base damage. The formation of oxidative DNA base damage products was decreased by studied antioxidants, apparently with different antioxidant powers. Therefore, other antioxidants could also prove efficacy in decreasing the production of DNA base damage products. The cancerous and precancerous tissues or cancer cell lines have been shown to contain oxidatively induced DNA damages at elevated levels when compared to surrounding cancer-free tissues or normal cell lines [14]. The early diagnosis of oxidative DNA base damage could be achieved using these molecules as biomarkers if DNA base damage products are determined at lower concentrations.

CRediT authorship contribution statement

Önder Aybastıer: Methodology, Investigation, Validation, Visualization, Writing. **Cevdet Demir:** Conceptualization, Investigation, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

[1] A. Phaniendra, D.B. Jestadi, L. Periyasamy, Free radicals: properties, sources, targets, and their implication in various diseases, Indian J. Clin. Biochem. 30 (1) (2015) 11–26.
[2] J.E. Klaunig, L.M. Kamendulis, B.A. Hocevar, Oxidative stress and oxidative damage in carcinogenesis, Toxicol. Pathol. 38 (2010) 96–109.
[3] P.W. Villalta, S. Balbo, The future of DNA adductomic analysis, Int. J. Mol. Sci. 18 (2017) 1870–1889.
[4] T.B. Kryston, A.B. Georgiev, P. Pissis, A.G. Georgakilas, Role of oxidative stress and DNA damage in human carcinogenesis, Mutat. Res. 711 (2011) 193–201.
[5] X. Mao, C. Gu, D. Chen, B. Yu, J. He, Oxidative stress-induced diseases and tea polyphenols, Oncotarget 8 (46) (2017) 81649–81661.
[6] F. Berg, J. Wilken, C.A. Helm, S.J. Block, AFM-Based quantification of conformational changes in DNA caused by reactive oxygen species, Phys. Chem. B 119 (2015) 25–32.
[7] M. Dizdaroglu, E. Coskun, P. Jaruga, Measurement of oxidatively induced DNA damage and its repair, by mass spectrometric techniques, Free Radic. Res. 49 (2015) 525–548.
[8] J. Cadet, K.J.A. Davies, Oxidative DNA damage & repair: an introduction, Free Radic. Biol. Med. 107 (2017) 2–12.
[9] E.C. Friedberg, G.C. Walker, W. Siede, R.D. Wood, R.A. Schultz, T. Ellenberger, DNA Repair and Mutagenesis, second ed., ASM Press, Washington D.C, 2005.
[10] C.V. Sonntag, Free Radical Induced DNA Damage and Its Repair, Springer, Heidelberg, 2006.
[11] B. Halliwell, Free radicals and antioxidants – quo vadis? Trends Pharm. Sci. 32 (2011) 125–130.
[12] D. Ceylan, G. Tuna, G. Kirkali, Z. Tunca, G. Can, H.E. Arat, M. Kant, M. Dizdaroglu, A. Özerdem, Oxidatively-induced DNA damage and base excision repair in euthymic patients with bipolar disorder, DNA Repair 65 (2018) 64–72.
[13] T. Helleday, E. Petermann, C. Lundin, B. Hodgson, R.A. Sharma, DNA repair pathways as targets for cancer therapy, Nat. Rev. Cancer 8 (2008) 193–204.
[14] M. Dizdaroglu, Oxidatively induced DNA damage and its repair in cancer, Mutat. Res. 763 (2015) 212–245.
[15] M. Costello, T.J. Pugh, T.J. Fennell, C. Stewart, L. Lichtenstein, J.C. Meldrim, J.L. Fostel, D.C. Friedrich, D. Perrin, D. Dionne, S. Kim, S.B. Gabrial, E.S. Lander, S. Fisher, G. Getz, Discovery and characterization of artefactual mutations in deep coverage targeted capture sequencing data due to oxidative DNA damage during sample preparation, Nucleic Acids Res. 41 (2013) e67.
[16] S. Liu, Y. Wang, Mass spectrometry for the assessment of the occurrence and biological consequences of DNA adducts, Chem. Soc. Rev. 44 (2015) 7829–7854.
[17] T.G. England, A. Jenner, O.I. Aruoma, B. Halliwell, Determination of oxidative DNA base damage by gas chromatography-mass spectrometry. Effect of derivatization conditions on artifactual formation of certain base oxidation products, Free Radic. Res. 29 (1998) 321–330.
[18] A. Jenner, T.G. England, O.I. Aruoma, B. Halliwell, Measurement of oxidative DNA damage by gas chromatography–mass spectrometry: ethanethiol prevents artifactual generation of oxidized DNA bases, Biochem. J. 331 (1998) 365–369.
[19] Ö. Aybastıer, S. Dawbaa, C. Demir, Investigation of antioxidant ability of grape seeds extract to prevent oxidatively induced DNA damage by gas chromatography-tandem mass spectrometry, J. Chromatogr. B 1072 (2018) 328–335.
[20] Ö. Aybastıer, S. Dawbaa, C. Demir, O. Akgün, E. Ulukaya, F. Arı, Quantification of DNA damage products by gas chromatography tandem mass spectrometry in lung cell lines and prevention effect of thyme antioxidants on oxidative induced DNA damage, Mutat. Res. Fund. Mol. Mech. Mutagen. 808 (2018) 1–9.
[21] M. Dizdaroglu, P. Jaruga, H. Rodriguez, Measurement of 8-hydroxy-2'-deoxyguanosine in DNA by high-performance liquid chromatography-mass spectrometry: comparison with measurement by gas chromatography-mass spectrometry, Nucleic Acids Res. 29 (3) (2001) e12.
[22] P. Jaruga, M. Birincioglu, H. Rodriguez, M. Dizdaroglu, Mass spectrometric assays for the tandem lesion 8,5′-cyclo-2'-deoxyguanosine in mammalian DNA, Biochemistry 41 (2002) 3703–3711.
[23] B. Davoodi, B. Eskandari, Tool wear mechanisms and multi-response optimization of tool life and volume of material removed in turning of N-155 iron–nickel-base superalloy using RSM, Measurement 68 (2015) 286–294.
[24] P. Jaruga, G. Kirkali, M. Dizdaroglu, Measurement of formamidopyrimidines in DNA, Free Radic. Biol. Med. 45 (2008) 1601–1609.
[25] A. Babuponnusami, K.J. Muthukumar, A review on Fenton and improvements to the Fenton process for wastewater treatment, Environ. Chem. Eng. 2 (2014) 557–572.
[26] P. Reddy, P. Jaruga, T. O'Connor, H. Rodriguez, M. Dizdaroglu, Overexpression and rapid purification of *Escherichia coli* formamidopyrimidine–DNA glycosylase, Protein Expr. Purif. 34 (2004) 126–133.
[27] P. Jaruga, E. Coşkun, K. Kimbrough, A. Jacob, W.E. Johnson, M. Dizdaroğlu, Biomarkers of oxidatively induced DNA damage in dreissenid mussels: a genotoxicity assessment tool for the Laurentian great lakes, Environ. Toxicol. 32 (2017) 2144–2153.
[28] S. Eghbaliferiz, M. Iranshahi, Prooxidant activity of polyphenols, flavonoids, anthocyanins and carotenoids: updated review of mechanisms and catalyzing metals, Phytother. Res. 30 (2016) 1379–1391.
[29] Ö. Aybastıer, C. Demir, Antioxidant activity assay of phenolic compounds isolated from *origanum onites* L. aromatic water by high performance liquid chromatography, Ann. Nutr. Disord. Ther. 4 (3) (2017) 1048–1051.
[30] M.L.R. Cabello, D.G. Praena, M. Puerto, S. Pichardo, A. Jos, A.M. Camean, In vitro pro-oxidant/antioxidant role of carvacrol, thymol and their mixture in the intestinal Caco-2 cell line, Toxicol. In Vitro 29 (2015) 647–656.
[31] A. Özkan, A. Erdogan, A comparative study of the antioxidant/prooxidant effects of carvacrol and thymol at various concentrations on membrane and DNA of parental and drug resistant H1299 cells, Nat. Prod. Commun. 7 (2012) 1557–1560.