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Corresponding Author:	Ana Paula Loureiro Universidade de Sao Paulo Faculdade de Ciencias Farmaceuticas São Paulo, BRAZIL
Corresponding Author's Institution:	Universidade de Sao Paulo Faculdade de Ciencias Farmaceuticas
Corresponding Author E-Mail:	apmlou@usp.br
Order of Authors:	Tiago Franco de Oliveira
	Antonio Anax Falcão de Oliveira
	Miriam Lemos
	Mariana Veras
	Paulo Hilário Nascimento Saldiva
	Marisa Helena Gennari de Medeiros
	Paolo Di Mascio
	Ana Paula Loureiro
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TITLE:

Quantification of Three DNA Lesions by Mass Spectrometry and Assessment of their Levels in Tissues of Mice Exposed to Ambient Fine Particulate Matter

AUTHORS AND AFFILIATIONS:

Tiago Franco de Oliveira[1,2*], Antonio Anax Falcão de Oliveira[1*], Miriam Lemos[3], Mariana Veras[3], Paulo Hilário Nascimento Saldiva[3,4], Marisa Helena Gennari de Medeiros[5], Paolo Di Mascio[5], Ana Paula de Melo Loureiro[1]

[1]Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, Brazil
[2]Departamento de Farmacociências, Universidade Federal de Ciências da Saúde de Porto Alegre, Rio Grande do Sul, Brazil
[3]Laboratório de Poluição Atmosférica Experimental – LIM05, Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil
[4]Instituto de Estudos Avançados, Universidade de São Paulo, São Paulo, Brazil
[5]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil

Corresponding Author:
Ana Paula de Melo Loureiro
apmlou@usp.br

Email Addresses of Co-authors:

Tiago Franco de Oliveira	(oliveira@ufcspa.edu.br)
Antonio Anax Falcão de Oliveira	(anax.antonio@gmail.com)
Miriam Lemos	(mirlemos@usp.br)
Mariana Veras	(verasine@usp.br)
Paulo Hilário Nascimento Saldiva	(pepino@usp.br)
Marisa Helena Gennari de Medeiros	(mhgdmede@iq.usp.br)
Paolo Di Mascio	(pdmascio@iq.usp.br)

*Contributed equally to this work

KEYWORDS:

Mass spectrometry, DNA adducts, DNA oxidation, genotoxicity, oxidative stress, fine particulate matter

SUMMARY:

We describe here methods for sensitive and accurate quantification of the lesions 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), 1,N^6-etheno-2'-deoxyadenosine (1,N^6-εdAdo) and 1,N^2-etheno-2'-deoxyguanosine (1,N^2-εdGuo) in DNA. The methods were applied to the assessment of the effects of ambient fine particulate matter ($PM_{2.5}$) in tissues (lung, liver and kidney) of exposed A/J mice.

ABSTRACT:

DNA adducts and oxidized DNA bases are examples of DNA lesions that are useful biomarkers for the toxicity assessment of substances that are electrophilic, generate reactive electrophiles upon biotransformation, or induce oxidative stress. Among the oxidized nucleobases, the most studied one is 8-oxo-7,8-dihydroguanine (8-oxoGua) or 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), a biomarker of oxidatively induced base damage in DNA. Aldehydes and epoxyaldehydes resulting from the lipid peroxidation process are electrophilic molecules able to form mutagenic exocyclic DNA adducts, such as the etheno adducts 1,N^2-etheno-2'-deoxyguanosine (1,N^2-εdGuo) and 1,N^6-etheno-2'-deoxyadenosine (1,N^6-εdAdo), which have been suggested as potential biomarkers in the pathophysiology of inflammation. Selective and sensitive methods for their quantification in DNA are necessary for the development of preventive strategies to slow down cell mutation rates and chronic disease development (e.g., cancer, neurodegenerative diseases). Among the sensitive methods available for their detection (high performance liquid chromatography coupled to electrochemical or tandem mass spectrometry detectors, comet assay, immunoassays, ^{32}P-postlabeling), the most selective are those based on high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-ESI-MS/MS). Selectivity is an essential advantage when analyzing complex biological samples and HPLC-ESI-MS/MS evolved as the gold standard for quantification of modified nucleosides in biological matrices, such as DNA, urine, plasma and saliva. The use of isotopically labeled internal standards adds the advantage of corrections for molecule losses during the DNA hydrolysis and analyte enrichment steps, as well as for differences of the analyte ionization between samples. It also aids in the identification of the correct chromatographic peak when more than one peak is present.

We present here validated sensitive, accurate and precise HPLC-ESI-MS/MS methods that were successfully applied for the quantification of 8-oxodGuo, 1,N^6-εdAdo and 1,N^2-εdGuo in lung, liver and kidney DNA of A/J mice for the assessment of the effects of ambient $PM_{2.5}$ exposure.

INTRODUCTION:

Some reactive oxygen species (ROS) are able to oxidize carbon double bonds of DNA bases and some carbons in the deoxyribose moiety, generating oxidized bases and DNA strand breaks[1]. As a negatively charged molecule rich in nitrogen and oxygen atoms, DNA is also a target for electrophilic groups that covalently react with the nucleophilic sites (nitrogen and oxygen), giving products that are called DNA adducts[2]. DNA adducts and oxidized DNA bases are examples of DNA lesions that are useful biomarkers for the toxicity assessment of substances that are electrophilic, generate reactive electrophiles upon biotransformation, or induce oxidative stress[1,2]. Although the modified DNA bases can be removed from DNA by base or nucleotide excision repair (BER or NER), the induction of an imbalance between the generation and removal of DNA lesions in favor of the former leads to a net increase of their levels in DNA overtime[3]. Outcomes are the increase of DNA mutation rates, reduced gene expression, and diminished protein activity[2,4-7], effects that are closely related to the development of diseases. DNA mutations may affect diverse cellular functions, such as cell signaling, cell cycle, genome integrity, telomere stability, the epigenome, chromatin structure, RNA splicing, protein

homeostasis, metabolism, apoptosis, and cell differentiation[8,9]. Strategies to slow down cell mutation rates and chronic disease development (e.g., cancer, neurodegenerative diseases) pass through the knowledge of the mutation sources, among them, DNA lesions and their causes.

ROS generated endogenously in excess, due to pollutant exposure, persistent inflammation, disease pathophysiology (e.g., diabetes), etc., are important causes of biomolecule damage, including DNA and lipid damage[1]. As an example, the highly reactive hydroxyl radical (•OH) formed from H_2O_2 reduction by transition metal ions (Fe^{2+}, Cu^{+}) oxidizes the DNA bases, DNA sugar moiety and polyunsaturated fatty acids at diffusion-controlled rates[10]. Among the 80 already characterized oxidized nucleobases[3], the most studied one is 8-oxo-7,8-dihydroguanine (8-oxoGua) or 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo, **Figure 1**), a lesion that is able to induce G→T transversions in mammalian cells[10,11]. It is formed by the mono electronic oxidation of guanine, or by hydroxyl radical or singlet oxygen attack of guanine in DNA[1]. Polyunsaturated fatty acids are other important targets of highly reactive oxidants, such as •OH, which initiate the process of lipid peroxidation[1,12]. It gives rise to fatty acid hydroperoxides that may decompose to electrophilic aldehydes and epoxyaldehydes, such as malondialdehyde, 4-hydroxy-2-nonenal, 2,4-decadienal, 4,5-epoxy-(2*E*)-decenal, hexenal, acrolein, crotonaldehyde, which are able to form mutagenic exocyclic DNA adducts, such as malondialdehyde-, propano-, or etheno adducts[1,12,13]. The etheno adducts 1,N^2-etheno-2'-deoxyguanosine (1,N^2-εdGuo, **Figure 1**) and 1,N^6-etheno-2'-deoxyadenosine (1,N^6-εdAdo, **Figure 1**) have been suggested as potential biomarkers in the pathophysiology of inflammation[14,15].

[Place Figure 1 here]

Studies carried out in the early 1980s allowed the sensitive detection of 8-oxodGuo by high performance liquid chromatography coupled to electrochemical detection (HPLC-ECD). Quantification of 8-oxodGuo by HPLC-ECD in several biological systems subjected to oxidizing conditions led to the recognition of 8-oxodGuo as a biomarker of oxidatively induced base damage in DNA[1,16]. Although robust and allowing the quantification of 8-oxodGuo in the low fmol range[17], HPLC-ECD measurements rely on the accuracy of the analyte retention time for analyte identification and on the chromatography resolution to avoid interferences of other sample constituents. As the electrochemical detection requires the use of salt (e.g., potassium phosphate, sodium acetate) in the mobile phase, the maintenance of adequate analytical conditions needs routine column and equipment cleaning time.

Alternatively, the use of the bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase (FPG) and, afterwards, human 8-oxoguanine glycosylase 1 (hOGG1), for detection and removal of 8-oxoGua from DNA, emerged as a way for the induction of DNA alkali labile sites. The alkali labile sites are converted to DNA strand breaks and allow the very high sensitive indirect quantification of 8-oxoGua by alkaline single cell gel electrophoresis ("comet assay"). The high sensitivity and the accomplishment of the analyses without the need of cellular DNA extraction are the main advantages of this type of assay. It gives the lowest steady-state levels of 8-oxoGua in DNA, typically 7-10 times lower than the levels obtained by bioanalytical

methods based on HPLC. However, it is an indirect measurement of 8-oxoGua and some drawbacks are the lack of specificity or the unknown efficiency of the repair enzymes used[1,16,18]. Immunoassays are other set of methods used for the detection of 8-oxoGua[1] and exocyclic DNA adducts, such as 1,N^6-εdAdo and 1,N^2-εdGuo[12]. Despite the sensitivity, a shortcoming of the use of antibodies for detection of DNA lesions is the lack of specificity due to cross-reactivity to other components of biological samples, including the normal DNA bases[1,12]. The exocyclic DNA adducts, including 1,N^6-εdAdo and 1,N^2-εdGuo, may also be detected and quantified by highly sensitive ^{32}P-postlabeling assays[12]. The high sensitivity of ^{32}P-postlabeling allows the use of very small amounts of DNA (e.g., 10 μg) for detection of about 1 adduct per 10^{10} normal bases[19]. However, the use of radio-chemicals, lack of chemical specificity and low accuracy are some disadvantages[19,20].

A shared limitation of the methods cited above is the low selectivity or specificity for the detection of the desired molecules. In this scenario, HPLC coupled to electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS and HPLC-MS^3) evolved as the gold standard for quantification of modified nucleosides in biological matrices, such as DNA, urine, plasma and saliva[1,19,20]. Advantages of HPLC-ESI-MS/MS methods are the sensitivity (typically in the low fmol range) and the high specificity provided by i) the chromatographic separation, ii) the characteristic and known pattern of molecule fragmentation inside the mass spectrometer collision chamber, and iii) the accurate measurement of the selected mass to charge ratio (m/z) in multiple reaction monitoring mode[1,19]. The use of isotopically labeled internal standards adds the advantage of corrections for molecule losses during the DNA hydrolysis and analyte enrichment steps, as well as for differences of the analyte ionization between samples. It also aids in the identification of the correct chromatographic peak when more than one peak is present[1,12,19,20].

Several methods based on HPLC-ESI-MS/MS have been used for quantification of 8-oxodGuo, 1,N^6-εdAdo and 1,N^2-εdGuo in DNA extracted from different biological samples[12,15,20-29]. Fine particles ($PM_{2.5}$) carry organic and inorganic chemicals, such as polycyclic aromatic hydrocarbons (PAHs), nitro-PAHs, aldehydes, ketones, carboxylic acids, quinolines, metals, and water-soluble ions, which may induce inflammation and oxidative stress, conditions that favor the occurrence of biomolecule damage and disease[30-33]. We present here validated HPLC-ESI-MS/MS methods that were successfully applied for the quantification of 8-oxodGuo, 1,N^6-εdAdo and 1,N^2-εdGuo in lung, liver and kidney DNA of A/J mice for the assessment of the effects of ambient $PM_{2.5}$ exposure[34].

PROTOCOL:

Four week old male A/J mice, specific pathogen free, were obtained from the Breeding Center of Laboratory Animals of Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil, and were treated accordingly to the Ethics Committee of the Faculty of Medicine, University of São Paulo (protocol nº 1310/09).

1. Collection of mice tissues

1.1. Anesthetize the animal with xylazine and ketamine. For a mouse with 30 g of body weight, inject a solution (no more than 2 mL) containing 2.63 mg of ketamine and 0.38 mg of xylazine, intraperitoneally.

1.2. Collect blood (0.5 – 1.5 mL) for complementary analyses (e.g., antioxidant enzyme activity, malondialdehyde levels).

1.3. Shave the abdominal hair from the pelvis to the xiphoid process. Make an incision in a vertical middle line in the hairless area. Make incisions in horizontal lateral lines in order to expose the abdominal organs.

1.4. Cut the abdominal aorta to promote exsanguination and to euthanize the animal.

1.5. Remove the tissues of interest (in this case, liver, kidneys and lungs).

1.5.1. To remove the liver, cut the inferior cava vein and portal hepatic vein.

1.5.2. To remove the kidneys, section the renal veins and arteries.

1.5.3. To remove the lungs, make an incision in the diaphragm extremities and circumference close to the thoracic wall. Break the clavicles by opening a scissor in the interior of the thoracic cavity. Cut the extern bone from the xiphoid process toward the trachea, in order to expose the lungs and heart.

1.5.3.1. Hold the lung with a forceps, section the trachea and the ligaments around the lungs. Remove carefully the block lungs plus heart. To remove the lungs out of the block, hold the heart with a forceps and cut all vessels in its base.

1.6. Wash the isolated tissues immediately in cold saline solution (0.9% NaCl), transfer to cryogenic tubes, and immediately dip the tubes into liquid nitrogen. After completing the work, store the tubes at -80 °C.

CAUTION: Liquid nitrogen in direct contact with the skin, mucosa or eyes causes burns. Use proper individual protection to avoid contact. Work in a ventilated laboratory to avoid asphyxia due to liquid nitrogen vapor.

2. DNA extraction

2.1. Transfer the tubes containing the tissues to dry ice.

2.2. Use a culture plate placed on ice as a base to cut a piece of tissue with a scalpel. Weight 1 g for immediate use. The remaining tissue should be kept on dry ice until it returns to storage at -80 °C.

NOTE: It is important to avoid thawing of the remaining tissue to prevent the formation of artifacts if repetitions of the analyses are needed.

2.3. To each 1 g of tissue in 50 mL capped tubes, add 10 mL of the commercial cell lysis solution containing 0.5 mM deferoxamine and keep on ice.

NOTE: Add deferoxamine to the volume of solution for immediate use. For each 100 mL of solution, add 0.0328 g of the deferoxamine mesylate salt.

2.4. Homogenize the tissues using a Potter or a tissue glass Dounce homogenizer until a homogeneous solution without tissue fragments is obtained. Keep the tube cold (on ice) during homogenization. Use a low speed to avoid heating.

2.5. Add 150 µL of proteinase K solution (20 mg/mL) to each homogenized sample. Shake the tubes by inversion and keep them at room temperature overnight.

2.6. Add 40 µL of ribonuclease A solution (15 mg/mL), shake by inversion, and keep the tubes at room temperature for 2 h.

NOTE: Prepare ribonuclease A solution in sodium acetate buffer 10 mM, pH 5.2 to avoid precipitation. Heat the solution at 100 °C for 15 min before use to obtain a solution free from deoxyribonuclease.

2.7. Add 5 mL of the commercial protein precipitation solution, vortex vigorously, and centrifuge at 2,000 x *g*, 4 °C, for 10 minutes.

2.8. Transfer the supernatants to 50 mL capped tubes containing 10 mL of cold isopropanol. Invert the tubes gently several times until the observation of the precipitated DNA.

NOTE: The protocol can be paused here, keeping the tubes at -20 °C.

2.9. Collect the precipitated DNA using a Pasteur pipette closed at the end. Transfer it to tubes containing 4 mL of 10 mM Tris buffer, 1 mM deferoxamine, pH 7.0.

2.10. After the DNA is completely dissolved in the above solution (do not vortex), add 4 mL of a chloroform solution containing 4% of isoamyl alcohol.

2.11. Invert the tubes 10 times for homogenization, centrifuge at 2,000 x *g*, 4 °C, for 10 minutes to separate the two phases, and transfer the upper phase to a new tube.

2.12. Repeat the steps 2.10 and 2.11 two more times.

2.13. Add 8 mL of absolute ethanol and 0.4 mL of a 5 M NaCl solution to precipitate the DNA.

2.14. Collect again the precipitated DNA and transfer it to 3 mL of 70% ethanol. Repeat this step one more time.

2.15. Discard the ethanol solution with caution and invert the tubes containing the precipitated DNA on absorbent paper to remove the excess of the solution.

2.16. Add 200 µL of 0.1 mM deferoxamine solution to dissolve the DNA. Maintain the tubes at 4 °C until the DNA is completely rehydrated (overnight).

2.17. Determine the DNA concentration by measuring the absorbance at 260 nm and its purity by the 260/280 nm absorbance ratio.

NOTE: To determine the DNA concentration, transfer an aliquot of 10 µL of the DNA solution to 990 µL of ultrapure water (100x dilution). Multiply the absorbance at 260 nm (it should be below 1) by 50 (50 µg/mL is the concentration of double stranded DNA when the absorbance of a 1 cm path length solution at 260 nm is 1) and by the dilution used (100x) to obtain the DNA concentration in µg/mL. If the absorbance at 260 nm is above 1, additional dilutions are necessary. The 260/280 nm absorbance ratio should be equal or above 1.8 for the desired DNA purity, but ratios around 1.6 are acceptable.

3. DNA enzymatic hydrolysis

3.1. Analysis recipe

3.1. 1,N^6-εdAdo and 1,N^2-εdGuo analyses: To an aliquot containing 150 µg of DNA, add 7.5 µL of 200 mM Tris/$MgCl_2$ buffer (pH 7.4), 1.4 µL of the internal standard solution containing 250 fmol/µL of [$^{15}N_5$]1,N^6-εdAdo and [$^{15}N_5$]1,N^2-εdGuo, and 15 units of deoxyribonuclease I. Adjust the final volume to 200 µL with ultrapure water, subtracting the volumes of enzymes to be used on step 3.3.1.

3.2. 8-oxodGuo analyses: To an aliquot containing 80 µg of DNA, add 3.8 µL of 200 mM Tris/$MgCl_2$ buffer (pH 7.4), 2 µL of the internal standard solution containing 1,000 fmol/µL of [$^{15}N_5$]8-oxodGuo, and 8 units of deoxyribonuclease I. Adjust the final volume to 100 µL with ultrapure water, subtracting the volumes of enzymes to be used on step 3.3.2.

NOTE: The internal standards [$^{15}N_5$]1,N^6-εdAdo, [$^{15}N_5$]1,N^2-εdGuo and [$^{15}N_5$]8-oxodGuo can be synthetized and characterized as described[34-36]. The quantities of the internal standards in the injected sample volumes should be the same as those in the injected calibration curve volumes.

3.3. Incubate the samples at 37 °C for 1 hour.

3.3.1. Samples from step 3.1: Add 0.006 units of phosphodiesterase I from *Crotalus atrox* and 15 units of alkaline phosphatase from bovine intestinal mucosa.

3.3.2. Samples from step 3.2: Add 0.0032 units of phosphodiesterase I from *Crotalus atrox* and 8 units of alkaline phosphatase from bovine intestinal mucosa.

3.4. Incubate the samples at 37 °C for 1 hour.

3.5. Centrifuge the samples at 14,000 x *g* for 10 minutes.

3.6. Samples from step 3.3.1: Separate 10 µL of each sample for quantification of the deoxynucleosides (dAdo, dGuo) by HPLC/DAD (step 9). Subject the residual volume to solid phase extraction (step 4).

3.7. Samples from step 3.3.2: Transfer 80 µL of the supernatant to vials for injections of 50 µL (1000 fmol of [$^{15}N_5$]8-oxodGuo) in the HPLC-ESI-MS/MS system. Reserve the remaining 20 µL for quantification of dGuo by HPLC/DAD (step 9).

4. Solid phase extraction for analyses of 1,N^6-εdAdo and 1,N^2-εdGuo

4.1. Load the cartridges (SPE-C18, 30 mg/mL, 33 µm, 1 mL) with 1 mL of the following sequence of solutions: 100% methanol, deionized water, hydrolyzed DNA sample, deionized water, 10% methanol, 15% methanol, and 100% methanol (to be collected).

NOTE: Do not leave the cartridges dry between the applications of the different solutions. Add the next solution immediately after the previous solution enters the cartridge completely.

4.2. Vacuum dry the last elution fraction (100% methanol) containing the adducts.

4.3. Resuspend the dried samples in 83.1 µL of ultrapure water immediately prior to the HPLC-ESI-MS/MS analysis, to obtain 200 fmol of each internal standard in 50 µL of each sample.

5. Preparation of calibration curves

5.1. Prepare at least five points in the interval of 300 to 6,000 fmol of 8-oxodGuo standard, with the fixed amount of 1000 fmol of [$^{15}N_5$]8-oxodGuo in each point. Consider these amounts in the volume injected.

5.2. Prepare at least five points in the interval of 1 to 40 fmol of 1,N^6-εdAdo and 1,N^2-εdGuo, with fixed amounts of 200 fmol of [$^{15}N_5$]1,N^6-εdAdo and [$^{15}N_5$]1,N^2-εdGuo in each point. Consider these amounts in the volume injected.

5.3. Prepare at least five points in the interval of 0.05 – 1 nmol of dGuo and dAdo. Consider these amounts in the volume injected.

6. Preparation of DNA samples for method validation

6.1. 1,N^6-εdAdo and 1,N^2-εdGuo analyses: Add varying amounts of 1,N^6-εdAdo and 1,N^2-εdGuo (e.g., 1.75, 8.75, 17.5, and 35 fmol) and fixed amounts of [$^{15}N_5$]1,N^6-εdAdo and [$^{15}N_5$]1,N^2-εdGuo (350 fmol) to 100 µg of calf thymus DNA and carry out the enzymatic hydrolysis as described in step 3. Process the samples in quadruplicate in two different days. Use the samples for method accuracy and precision assessment.

NOTE: The final volume of the DNA hydrolysates will be 200 µL (step 3), from which 10 µL will be separated for quantification of deoxynucleosides by HPLC/DAD (step 9). The remaining solution (190 µL) will be subjected to solid phase extraction (step 4), the dried fraction will be resuspended in 83.1 µL (step 4.3), from which 50 µL will be injected in the HPLC-ESI-MS/MS system. The amounts of 1,N^6-εdAdo and 1,N^2-εdGuo injected will be 1, 5, 10, and 20 fmol, with 200 fmol of [$^{15}N_5$]1,N^6-εdAdo and [$^{15}N_5$]1,N^2-εdGuo in each sample.

6.2. 8-oxodGuo analyses: Add varying amounts of 8-oxodGuo (e.g., 734, 1468, 2938, and 4408 fmol) and a fixed amount of [$^{15}N_5$]8-oxodGuo (2,000 fmol) to 100 µg of calf thymus DNA and carry out the enzymatic hydrolysis as described in step 3. Process the samples in quadruplicate in two different days. Use the samples for method accuracy and precision assessment.

NOTE: The final volume of the DNA hydrolysates will be 100 µL (step 3), from which 50 µL will be injected in the HPLC-ESI-MS/MS system. The amounts of 8-oxodGuo injected will be 367, 734, 1469, and 2204 fmol, with 1000 fmol of [$^{15}N_5$]8-oxodGuo in each sample.

6.2. Add 13.125 fmol of 1,N^6-εdAdo (to obtain 7.5 fmol in the injection volume) and 35 fmol of 1,N^2-εdGuo (to obtain 20 fmol in the injection volume) to eight samples of 100 µg of calf thymus DNA.

6.2.1. Add the internal standards [$^{15}N_5$]1,N^6-εdAdo and [$^{15}N_5$]1,N^2-εdGuo (200 fmol) to four of the samples. Proceed with the DNA hydrolysis and solid phase extraction of all samples.

6.2.2. Add the internal standards [$^{15}N_5$]1,N^6-εdAdo and [$^{15}N_5$]1,N^2-εdGuo (200 fmol) to the other four samples.

6.2.3. Use the samples to calculate the recovery of the adducts from solid phase extraction.

7. HPLC-ESI-MS/MS analysis of 8-oxodGuo

7.1. Infusing the 8-oxodGuo standard into the equipment, set the ESI-MS/MS parameters for the best detection of its fragmentation pattern by multiple reaction monitoring (MRM): *m/z* 284 $[M+H]^+ \rightarrow$ *m/z* 168 [M – 2'-deoxyribose + H]$^+$.

7.1.1. Use the same parameters for detection of [$^{15}N_5$]8-oxodGuo: *m/z* 289 $[M+H]^+ \rightarrow$ *m/z* 173 [M – 2'-deoxyribose + H]$^+$.

NOTE: Use an equipment equivalent or better than the equipment used in this work (see the **Table of Materials**). The ESI-MS/MS parameters were set as described in **Table 1**.

7.2. Filter (using 0.22 µm porous membranes) and degasify (using a sonicator) all the water based HPLC solvents.

7.3. Use the following chromatography conditions for the analyses, mounting the system as shown in **Figure 2**.

[Place Figure 2 here]

NOTE: Column A is connected to the binary pump. Its eluent is directed to UV detection and waste in the first 16 min and from 32 to 46 min of the chromatography, as shown in **Figure 2A**. This is the column through which the sample is eluted immediately after injection. Column B is connected to the isocratic pump and the mass spectrometer. It receives the eluent of column A only in the 16 – 32 min interval, when the valve is switched to the position shown in **Figure 2B**. The valve switch allows the connection between the two columns, which are eluted by the binary pump gradient. The configuration shown in **Figure 2B** permits further peak separation and narrowing, as well as that only the chromatographic fraction of interest reaches the mass spectrometer, improving sensitivity and selectivity.

7.3.1. Elute a 50 x 2.0 mm i.d., 2.5 µm, C18 column (column A of **Figure 2**) coupled to a C18 security guard cartridge (4.0 x 3.0 mm i.d.) with a gradient of 0.1% formic acid (solvent A) and methanol containing 0.1% formic acid (solvent B) at a flow rate of 150 µL/min and 25 °C.

7.3.1.1. Use the following gradient program for the binary pump: from 0 to 25 min, 0 – 15% of solvent B; 25 to 28 min, 15 – 80% of solvent B; 28 to 31 min, 80% of solvent B; 31 to 33 min, 80 – 0 % of solvent B; 33 to 46 min, 0% of solvent B.

7.3.1.2. Use the switching valve to direct the first 16 min of eluent to waste and the 16 – 32 min fraction to a second column (150 x 2.0 mm i.d., 3.0 µm, C18, column B of **Figure 2**) connected to the ESI source and conditioned by the isocratic pump with a solution of 15% methanol in water containing 0.1% formic acid (150 µL/min).

NOTE: Before using the switching valve program of step 7.3.1.2, check if the 8-oxodGuo standard elutes from the first column after 16 min. It is important to close the valve at 32 min to use the gradient of the binary pump to elute 8-oxodGuo from the second column and get a sharp chromatographic peak. The lesion 8-oxodGuo elutes from the second column at approximately 36 min. Variations of the retention time of the analyte may occur depending on the column and equipment used. Adaptations of the HPLC solvent gradient program may be necessary.

8. HPLC-ESI-MS/MS analysis of 1,N^6-εdAdo and 1,N^2-εdGuo

8.1. Infusing the 1,N^6-εdAdo and 1,N^2-εdGuo standards into the equipment, set the ESI-MS/MS parameters for the best detection of their fragmentation patterns by multiple reaction monitoring (MRM): *m/z* 276 $[M+H]^+ \rightarrow$ *m/z* 160 [M – 2'-deoxyribose + H]$^+$ for detection of 1,N^6-εdAdo and *m/z* 292 $[M+H]^+ \rightarrow$ *m/z* 176 [M – 2'-deoxyribose + H]$^+$ for detection of 1,N^2-εdGuo.

8.1.1. Use the same parameters for detection of [$^{15}N_5$]1,N^6-εdAdo (*m/z* 281 $[M+H]^+ \rightarrow$ *m/z* 165 [M – 2'-deoxyribose + H]$^+$) and [$^{15}N_5$]1,N^2-εdGuo (*m/z* 297 $[M+H]^+ \rightarrow$ *m/z* 181 [M – 2'-deoxyribose + H]$^+$). Set the ESI-MS/MS parameters as described in **Table 1**.

[Place Table 1 here]

8.2. Filter (using 0.22 µm porous membranes) and degasify (using a sonicator) all the water based HPLC solvents.

8.3. Use the following chromatography conditions for the analyses.

8.3.1. Elute a 150 x 2.0 mm i.d., 3.0 µm, C18 column coupled to a C18 security guard cartridge (4.0 x 3.0 mm i.d.) with a gradient of 5 mM ammonium acetate, pH 6.6 (solvent A) and acetonitrile (solvent B) at a flow rate of 130 µL/min and 25 °C.

8.3.1.1. Use the following gradient program for the binary pump: from 0 to 10 min, 0% of solvent B; 10 to 39 min, 0 – 20% of solvent B; 39 to 41 min, 20 – 75% of solvent B; 41 to 46 min, 75% of solvent B; 46 to 47 min, 75 – 0% of solvent B; 47 to 60 min, 0% of solvent B.

8.3.1.2. Use the switching valve to direct the first 15 min of eluent to waste and the 15 – 18 min fraction to the ESI source. Be sure that the adduct standards elute from the column in the set interval (15 – 18 min). Make adjustments if necessary.

9. Quantification of normal 2'-deoxyribonucleosides by HPLC-UV

9.1. Use an equipment similar to the equipment used in this work (see the **Table of Materials**).

9.2. Elute a 250 mm x 4.6 mm i.d., 5 µm, C18 column attached to a C18 security guard cartridge (4.0 x 3.0 mm i.d.) with a gradient of 0.1% formic acid and methanol.

9.2.1. Use the following gradient program: from 0 to 25 min, 0 to 18% methanol; from 25 to 27 min, 18 to 0% methanol; from 27 to 37 min, 0% methanol) at a flow rate of 1 mL/min and 30 °C.

9.2.2. Inject 5 µL of each sample reserved for 2'-deoxynucleosides quantification.

9.2.3. Set the DAD detector at 260 nm for integration of the dGuo and dAdo peaks.

10. Quantification of the DNA lesions

10.1. Integrate the peaks of 8-oxodGuo, [$^{15}N_5$]8-oxodGuo, 1,N^6-εdAdo, [$^{15}N_5$]1,N^6-εdAdo, 1,N^2-εdGuo, and [$^{15}N_5$]1,N^2-εdGuo from the HPLC-ESI-MS/MS analyses.

10.1.1. Calculate the area ratios of 8-oxodGuo/[$^{15}N_5$]8-oxodGuo, 1,N^6-εdAdo/[$^{15}N_5$]1,N^6-εdAdo, and 1,N^2-εdGuo/[$^{15}N_5$]1,N^2-εdGuo for the calibration curves and the samples.

10.1.2. Plot the calibration curves using the area ratios obtained in step 10.1.1 in the y axis and the amounts of analytes present in each point in the x axis.

10.1.3. Calculate the amounts (fmol) of lesions in each injected sample using the ratios calculated in step 10.1.1 and the calibration curves of step 10.1.2.

10.2. Integrate the peaks of dGuo and dAdo from the HPLC-UV analyses.

10.2.1. Plot the calibration curves using the areas obtained in step 10.2 in the y axis and the amounts of analytes present in each point in the x axis.

10.2.2. Calculate the amounts (nmol) of dGuo and dAdo in each injected sample using the areas obtained in step 10.2 and the calibration curves of step 10.2.1.

10.3. Calculate the amounts (nmol) of dGuo and dAdo present in each sample injected in the HPLC-ESI-MS/MS system, considering that the amounts calculated in step 10.2.2 are present in the sample volume of 5 µL, while 50 µL were injected in the HPLC-ESI-MS/MS system.

NOTE: To calculate the amount of dGuo in the samples used for 8-oxodGuo analysis, just multiply the amount (nmol/5 µL) obtained in step 10.2.2 by 10. To calculate the amounts of dAdo and dGuo in the samples used for analyses of 1,N^6-εdAdo and 1,N^2-εdGuo, consider the concentration step after solid phase extraction. The volume of 50 µL injected in the HPLC-ESI-MS/MS system corresponds to 114.32 µL of the original sample. The amounts (nmol/5 µL) obtained in step 10.2.2 should be multiplied by 22.864 to obtain the correct values.

10.4. Calculate the molar fractions 8-oxodGuo/dGuo, 1,N^6-εdAdo/dAdo, 1,N^2-εdGuo/dGuo. The ratio (fmol lesion/nmol normal deoxynucleoside) give the number of lesions per 10^6 normal dGuo or dAdo.

REPRESENTATIVE RESULTS:

The average DNA concentrations (± SD) obtained from mice liver (~ 1 g tissue), lung (~ 0.2 g tissue) and kidney (~ 0.4 g tissue) were, respectively, 5,068 ± 2,615, 4,369 ± 1,021, and 3,223 ± 723 µg/mL in the final volume of 200 µL. A representative chromatogram obtained by HPLC-DAD of the purified DNA is shown in **Figure 3**. The presence of the four 2'-deoxynucleosides, free from the RNA ribonucleosides, which elute immediately before the corresponding 2'-deoxynucleosides, demonstrates the DNA purity.

Representative chromatograms from HPLC-ESI-MS/MS analyses for quantification of 8-oxodGuo, 1,N^6-εdAdo and 1,N^2-εdGuo in mice tissue DNA samples are shown in **Figures 4 to 6**. The chromatogram obtained with UV detection in **Figure 4** shows the four 2'-deoxynucleosides eluting from the first column until ~ 10 min, with a good separation from 8-oxodGuo, eliminating undesired interferences. The normal 2'-deoxynucleosides were not present in the analyses of 1,N^6-εdAdo and 1,N^2-εdGuo, as they were eliminated in the solid phase extraction procedure. Mass spectra of the standards used in this work are shown in **Figure 7**.

Typical linear calibration curves for quantification of 8-oxodGuo, 1,N^6-εdAdo and 1,N^2-εdGuo are shown in **Figure 8**[34]. The methods were accurate and precise, as presented in **Table 2**[34]. The inter-day precision calculated for DNA aliquots supplemented with 367 fmol of 8-oxodGuo was 16.97%, supplemented with 10 fmol of 1,N^2-εdGuo was 14.01%, and supplemented with 1 fmol of 1,N^6-εdAdo was 16.66%. The limits of quantification (S/N = 10) for the standards injected on-column were 25 fmol for 8-oxodGuo, 0.3 fmol for 1,N^6-εdAdo, and 1 fmol for 1,N^2-εdGuo[34].

The methods were applied to the quantification of 8-oxodGuo, 1,N^2-εdGuo and 1,N^6-εdAdo in lung, liver, and kidney DNA samples of A/J mice tissues exposed whole body to ambient air enriched in $PM_{2.5}$, compared to those exposed to in situ ambient air as the study control[34]. The levels found are shown in **Table 3**, and indicate the induction of DNA lesions in lung, liver and kidney by $PM_{2.5}$ exposure[34].

FIGURE AND TABLE LEGENDS:

Figure 1. Chemical structures of the DNA lesions quantified in the present study. dR = 2´-deoxyribose. This figure has been modified from Oliveira et al.[34].

Figure 2. System of two columns used for 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) analyses. A) Configuration used in the first 16 min and from 32 to 46 min of the chromatography; **B**) Configuration used in the interval 16 – 32 min, allowing further separation and peak narrowing in column B prior to elution to the ESI source of the mass spectrometer. This figure has been republished from Oliveira et al.[34].

Figure 3. Chromatogram of the hydrolysate of a DNA sample extracted from mouse lung. The chromatogram was obtained at 260 nm from the HPLC-DAD system. The four 2'-deoxynucleosides are indicated: dC, 2'-deoxycytidine; dA, 2'-deoxyadenosine; dG, 2'-deoxyguanosine; dT, 2'-deoxythymidine.

Figure 4. Representative chromatograms showing the detection of 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-oxodGuo) and the internal standard [$^{15}N_5$]8-oxodGuo by HPLC-ESI-MS/MS, as well as the normal 2'-deoxynucleosides eluting from the first column and diverted to DAD detection (λ = 260 nm) and waste. The DNA sample was extracted from mouse lung. The analyses by HPLC-ESI-MS/MS were performed with multiple reaction monitoring (MRM) using the fragmentations specified in the images.

Figure 5. Representative chromatograms showing the detection of 1,N^6-etheno-2´-deoxyadenosine (1,N^6-εdAdo) and the internal standard [$^{15}N_5$]1,N^6-εdAdo by HPLC-ESI-MS/MS. The DNA sample was extracted from mouse kidney. The analyses were performed with multiple reaction monitoring (MRM) using the fragmentations specified in the images.

Figure 6. Representative chromatograms showing the detection of 1,N^2-etheno-2´-deoxyguanosine (1,N^2-εdGuo) and the internal standard [$^{15}N_5$]1,N^2-εdGuo by HPLC-ESI-MS/MS. The DNA sample was extracted from mouse liver. The analyses were performed with multiple reaction monitoring (MRM) using the fragmentations specified in the images.

Figure 7. Mass spectra of the standards used in this work. The spectra were obtained in MS2 using the collision energy of 20 eV to fragment the $[M+H]^+$ ions.

Figure 8. Calibration curves obtained by HPLC-ESI-MS/MS for quantification of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), 1,N^2-etheno-2´-deoxyguanosine (1,N^2-εdGuo) and 1,N^6-etheno-2´-deoxyadenosine (1,N^6-εdAdo). Relative Area means the area ratios between the lesion and its respective [$^{15}N_5$] internal standard. This figure has been modified from Oliveira et al.[34].

Table 1. Parameters used in the ESI-MS/MS equipment for detection of the DNA lesions. This table has been modified from Oliveira et al.[34].

Table 2. Method accuracy and coefficient of variation (CV) for quantification of 8-oxodGuo, 1,N^2-εdGuo and 1,N^6-εdAdo in DNA. This table has been modified from Oliveira et al.[34].

Table 3. Levels of the DNA lesions in A/J mice tissue samples. The mice were exposed to ambient air and to ambient air enriched in $PM_{2.5}$ ($PM_{2.5}$ concentrated 30 times). Means between the two groups (ambient air and $PM_{2.5}$) were compared using t test. Results were considered statistically significant when P value was less than 0.05. This table has been modified from Oliveira et al.[34].

DISCUSSION:

A major problem encountered in the 8-oxodGuo analyses by HPLC methods is the possible induction of its formation during the workup procedures of DNA extraction, DNA hydrolysis, and concentration of DNA hydrolysates[22,37]. In order to minimize the problem of 8-oxodGuo artifactual formation, it is recommended the addition of deferoxamine to all DNA extraction, storage and hydrolysis solutions, the use of the sodium iodide chaotropic method and avoidance of phenol in DNA extraction, as well as the use of DNA amounts close to 100 µg in the hydrolysis procedure to minimize the contribution of spurious oxidation to the final result[38]. We took into account the recommendations cited above, except the use of the sodium iodide chaotropic method for DNA extraction. Instead, for simplicity, we used commercial solutions for DNA extraction, adding deferoxamine to them before use. In addition, the obtained DNA hydrolysates were directly injected into a first column of the HPLC-ESI-MS/MS system for a

previous separation of 8-oxodGuo from the normal nucleosides. Immediately before the elution of 8-oxodGuo, a switching valve was used to divert the first column eluent to a second column where further separation and peak narrowing were achieved. This approach allowed adequate sensitivity for 8-oxodGuo quantification free from interferences. The most similar approach for quantification of 8-oxodGuo in DNA was described by Chao and coworkers[22], who used a trap column for sample cleanup and 8-oxodGuo retention prior to sample elution into the analytical column, using a switching valve between the columns. Alternatively, a concentration step of 8-oxodGuo collected from fractions eluted from HPLC separations of DNA hydrolysates prior to HPLC-ESI-MS/MS analyses was performed[15], which is much more laborious.

Reported basal levels of 8-oxodGuo in rodent lung tissue, based on HPLC analyses, range from 180 – 450/10^8 dGuo[23,39-42], 1,340 – 2,120/10^8 dGuo[43], or approximately 3,000/10^8 dGuo[44,45], with the lowest values obtained from DNA extraction methods by using sodium iodide. The mean 8-oxodGuo level found here in the lung of mice exposed to ambient air was 2,124/10^8 dGuo. The level increased to 2,466/10^8 dGuo in the animals exposed to ambient air enriched in $PM_{2.5}$ (**Table 3**)[34]. It is possible that the sensitivity for detection of differences between groups could be improved by extracting the DNA with the sodium iodide chaotropic method. In the present study, the mean 8-oxodGuo levels found in control mice lung, kidney, and liver DNA were, respectively, 2.0, 1.8, and 2.7 times higher than the median basal level (1,047/10^8 dGuo) obtained by the European Standards Committee on Oxidative DNA Damage (ESCODD) in an inter-laboratory assessment of 8-oxodGuo in DNA extracted from standard samples of pig liver[37].

The main limitation for detection of 1,N^6-εdAdo and 1,N^2-εdGuo in DNA is the method sensitivity, as these lesions occur at very low levels. The lowest levels of 1,N^2-εdGuo in DNA, quantified by HPLC-ESI-MS/MS, were in the range of 0.87 - 4 lesions per 10^8 dGuo in a human cell line and rat tissues[25,46]. One way to improve the sensitivity and selectivity for their quantification is to concentrate them from large samples of DNA hydrolysates, using solid phase extraction. This cleanup step solves chromatographic troubles that could arise from injections of more than 100 μg DNA hydrolysates into HPLC analytical columns. We used this approach in the validated method presented here. The levels of 1,N^6-εdAdo detected in this study[34] fall within the range obtained in studies employing ultrasensitive immunoaffinity/^{32}P-postlabeling[47-50] and are lower than those described by other groups employing HPLC-ESI-MS/MS[21,23,24]. Similarly, the 1,N^2-εdGuo levels quantified here[34] are consistent with the lowest levels reported by Garcia[25] and Angeli[26] by using HPLC-ESI-MS/MS.

HPLC-ESI-MS/MS systems with higher sensitivity than the equipment used in this study are available. The use of such systems allows the analyses of smaller amounts of DNA, which broadens the applications of the methods presented here for situations in which tissue availability is a limitation. The methods presented here may be adapted for the quantification of other modified deoxynucleosides, depending on the availability of their standards and isotopic standards. Adjustment of the chromatographic conditions would be necessary in order to obtain sharp peaks of all molecules included in the analyses.

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DISCLOSURES:

The authors have nothing to disclose.

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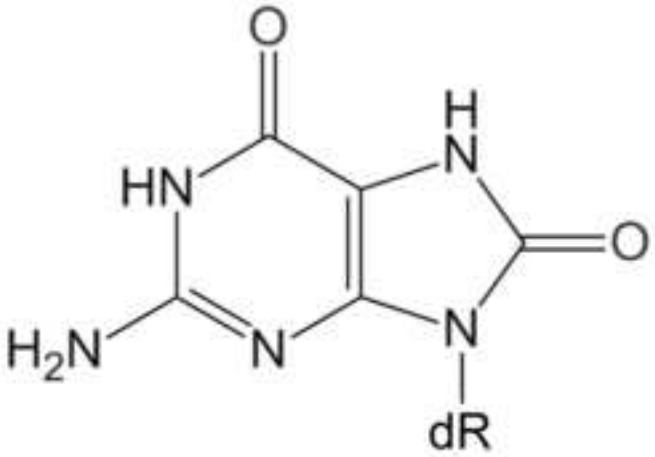
Figure 1



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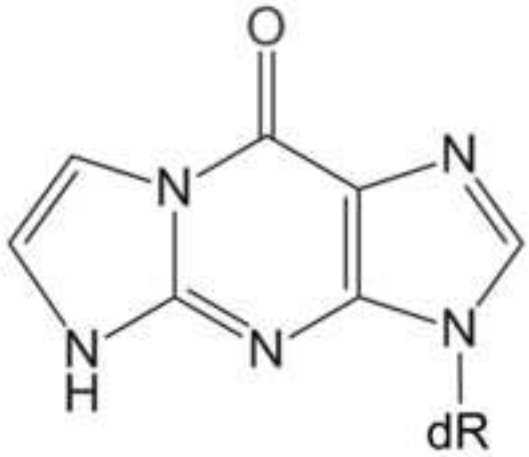

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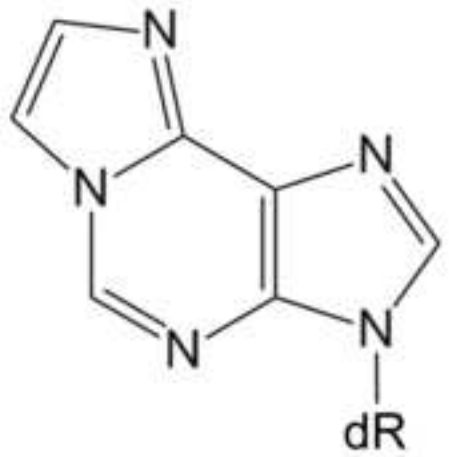

1,N^6-etheno-2'-deoxyadenosine

Figure 2

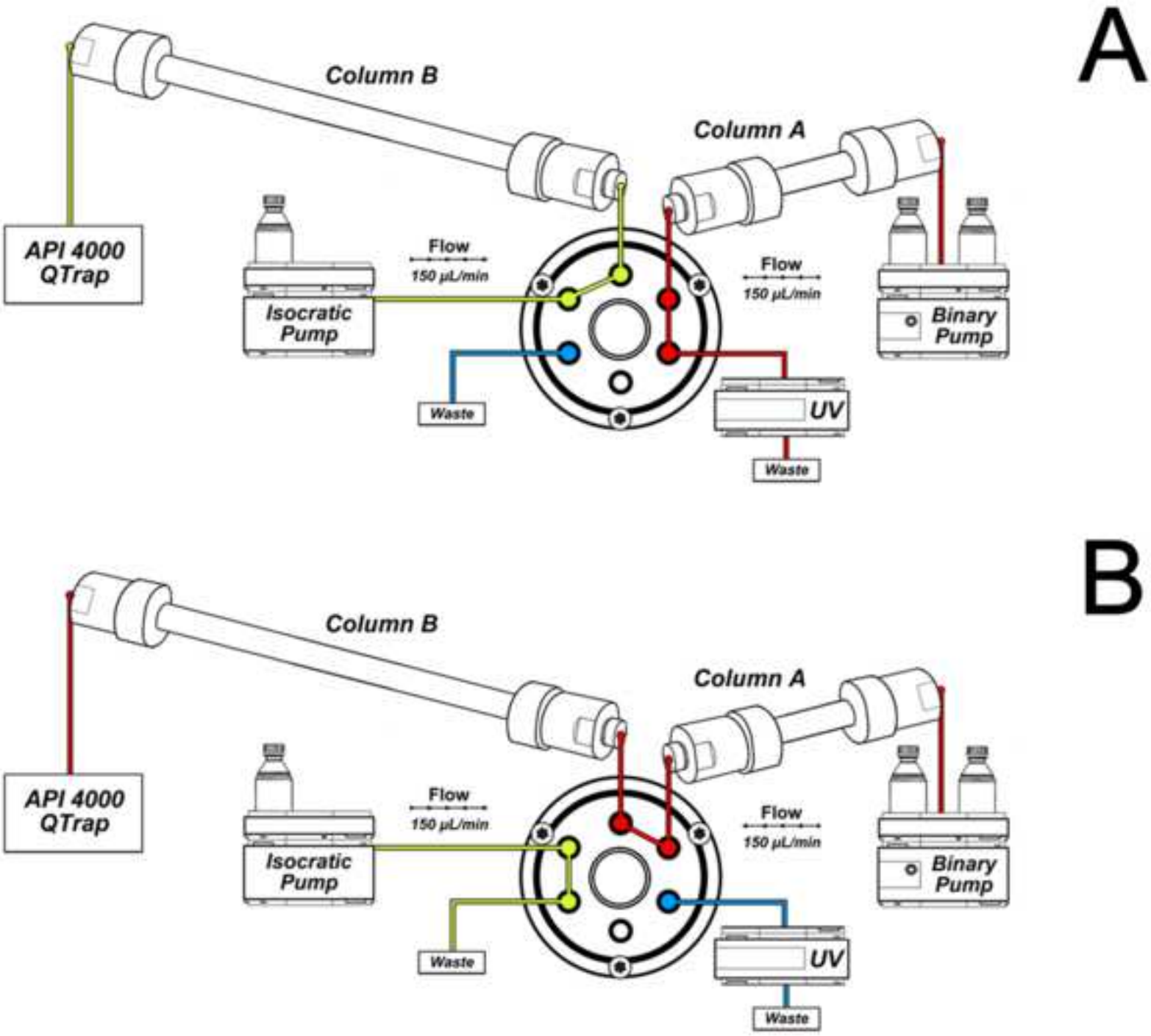

Figure 3

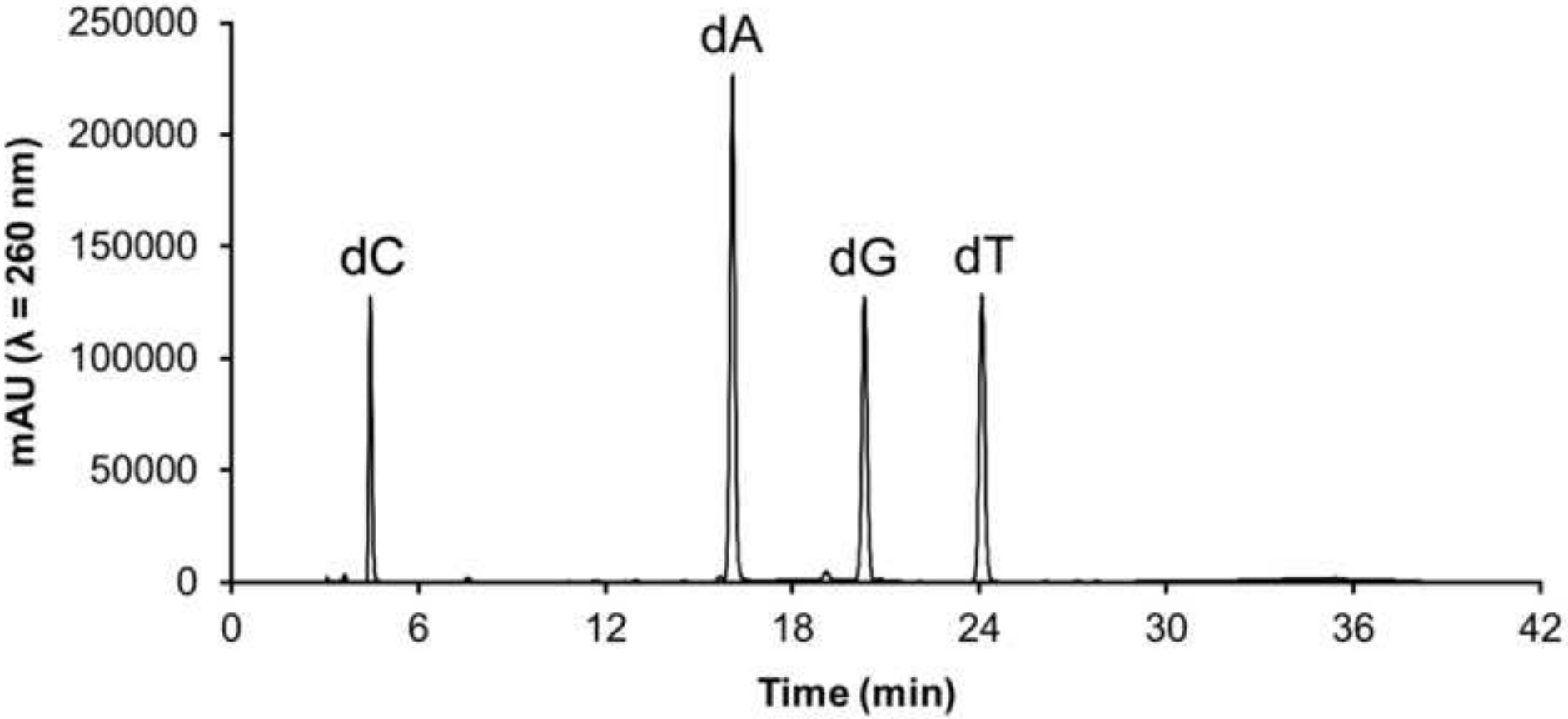

Figure 4

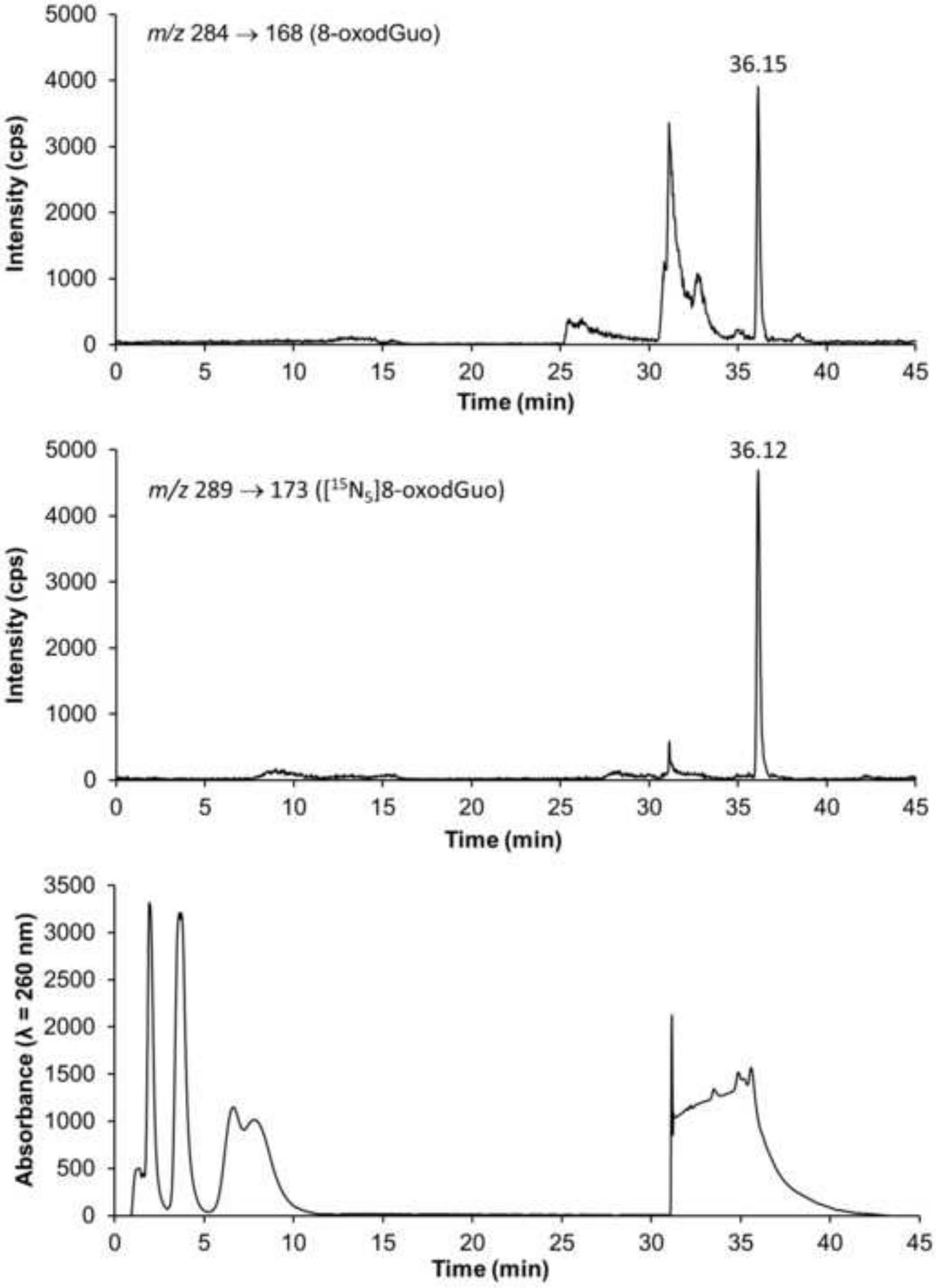

Figure 5

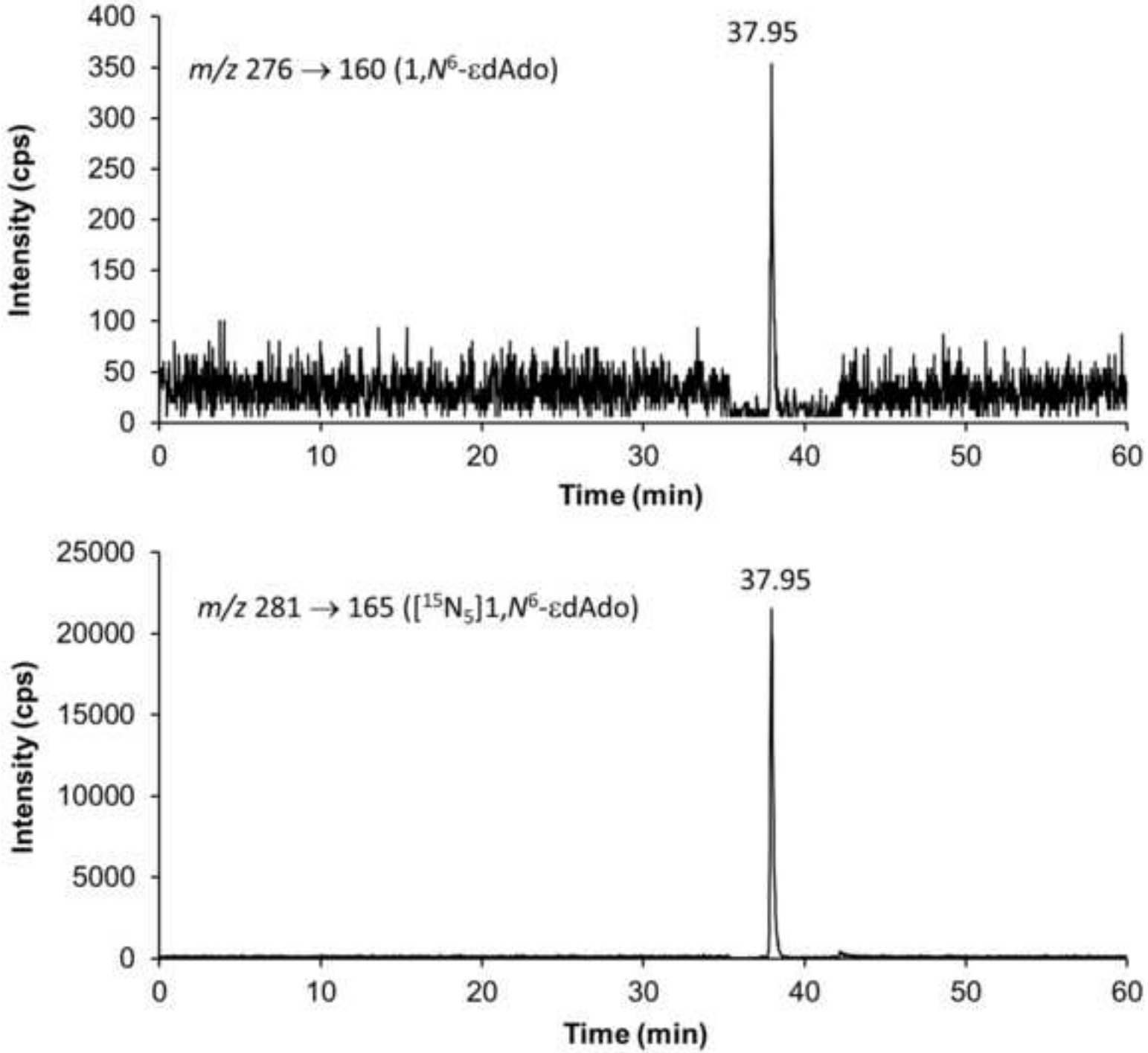

Figure 6

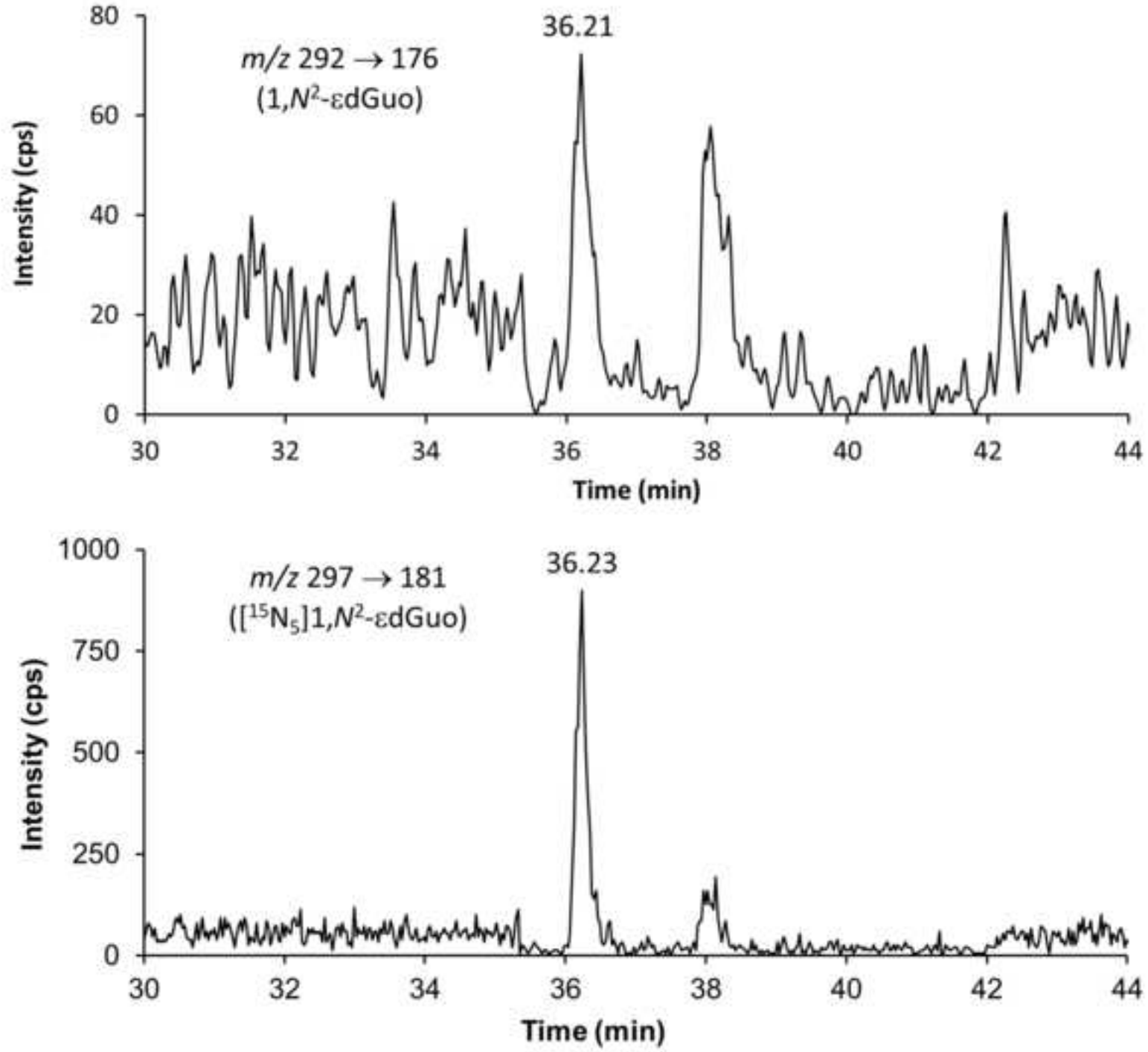

Figure 7

Click here to access/download;Figure;Figure 7.tif

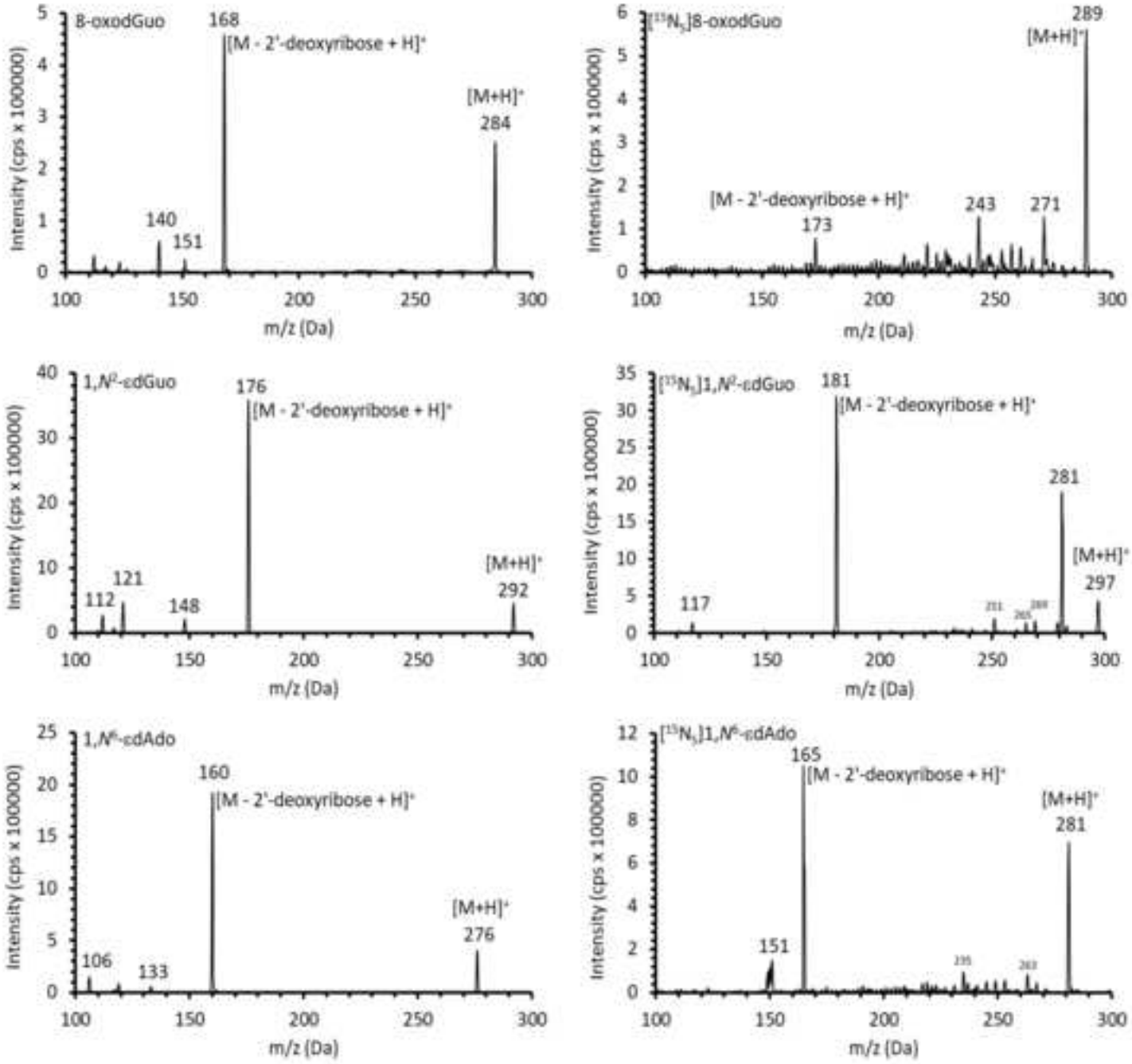

Figure 8

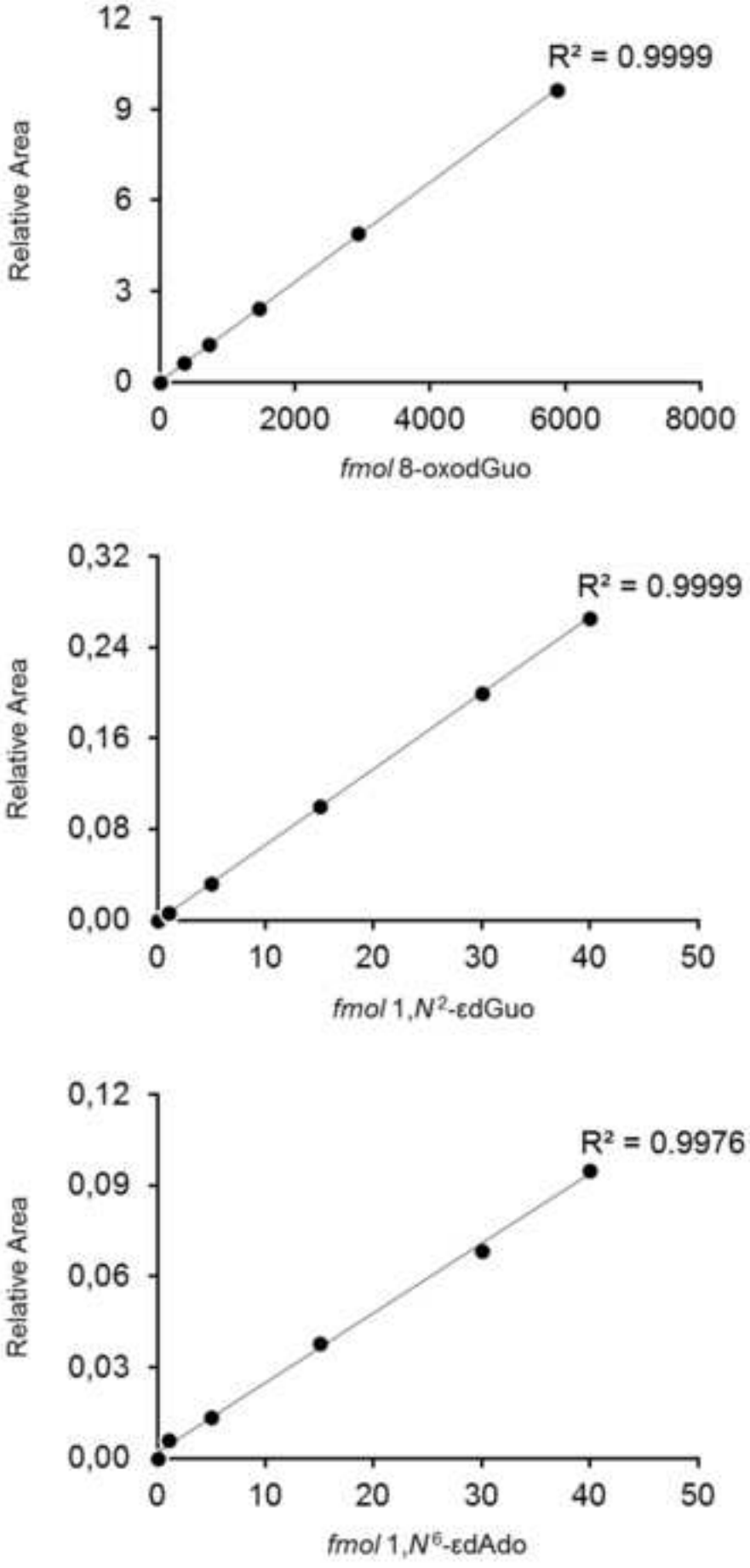

Table 1

ESI-MS/MS parameters

Curtain Gas
Nebulizing Gas
Ion Source Gas
Collision-induced Dissociation Gas
Ion Spray Voltage
ESI Probe Temperature
Declustering Potential
Collision Energy
Collision Cell Exit Potential
Entrance Potential

8-oxodGuo	**Etheno adducts**
20 psi	20 psi
55	50
50 psi	40 psi
Medium	Medium
5000	4500
450	450
31 V, 8-oxodGuo	41 V, 1,N^6-εdAdo
31 V, [$^{15}N_5$]8-oxodGuo	41 V, [$^{15}N_5$]1,N^6-εdAdo
	45 V, 1,N^2-εdGuo
	45V, [$^{15}N_5$]1,N^2-εdGuo
23 eV, 8-oxodGuo	25 eV, 1,N^6-εdAdo
23 eV, [$^{15}N_5$]8-oxodGuo	25 eV, [$^{15}N_5$]1,N^6-εdAdo
	27 eV, 1,N^2-εdGuo
	27 eV, [$^{15}N_5$]1,N^2-εdGuo
16 V, 8-oxodGuo,	8 V, 1,N^6-εdAdo
16 V, [$^{15}N_5$]8-oxodGuo	8 V, [$^{15}N_5$]1,N^6-εdAdo
	16 V, 1,N^2-εdGuo
	16 V, [$^{15}N_5$]1,N^2-εdGuo
10 V	10 V

Table 2

Basal level Average ± SD (fmol)			Added fmol
8-oxodGuo			
373.00	±	2.71	0
373.98	±	4.86	367
374.84	±	5.19	734
357.94	±	15.05	1469
371.07	±	2.43	2204
1,N^2-εdGuo			
0.54	±	0.01	0
0.54	±	0.01	1
0.55	±	0.01	5
0.53	±	0.01	10
0.54	±	0.01	20
1,N^6-εdAdo			
2.08	±	0.10	0
2.05	±	0.04	1
1.99	±	0.06	5
2.03	±	0.07	10
1.97	±	0.03	20

Detected Average ± SD (fmol)			**Detected(-)Basal** Average (fmol)
372.79	±	50.6	-
755.41	±	107.92	381
1069.57	±	108.51	695
1671.67	±	44.27	1314
2272.01	±	40.2	1901
0.54	±	0.09	-
1.47	±	0.16	0.93
5.30	±	0.72	4.76
10.60	±	0.39	10.06
20.20	±	0.93	19.66
2.29	±	0.39	-
3.06	±	0.47	1.01
7.87	±	1.66	5.88
12.43	±	1.25	10.41
22.42	±	3.89	20.46

Accuracy %	CV %
-	13.57
103.93	14.29
94.65	10.14
89.43	2.65
86.25	1.77
-	16.88
93.39	11.17
95.11	13.50
100.63	3.67
98.29	4.60
-	17.05
100.89	15.31
117.60	21.10
104.06	10.06
102.29	17.34

Table 3

	Ambient Air Average ± SEM
Lung	
8-oxodGuo/10^8 dGuo	2124 ± 56.96
1,N^2-εdGuo/10^8 dGuo	ND
1,N^6-εdAdo/10^8 dAdo	1.41 ± 0.23
Liver	
8-oxodGuo/10^8 dGuo	2848 ± 183.5
1,N^2-εdGuo/10^8 dGuo	7.79 ± 2.49
1,N^6-εdAdo/10^8 dAdo	2.82 ± 0.30
Kidney	
8-oxodGuo/10^8 dGuo	1854 ± 87.13
1,N^2-εdGuo/10^8 dGuo	ND
1,N^6-εdAdo/10^8 dAdo	1.09 ± 0.15

(NS, Not Significant; ND, Not Detected)

$PM_{2.5}$ Average ± SEM	N	P value
2466 ± 93.10	6	0.01
ND	-	-
1.44 ± 0.13	7	NS
2949 ± 223.8	6; 5	NS
24.94 ± 5.21	4	0.02
2.18 ± 0.25	6	NS
2363 ± 157.0	6	0.02
ND	-	-
1.52 ± 0.12	7	0.04

Table of materials

Product
[$^{15}N_5$]-2'-deoxyadenosine
[$^{15}N_5$]-2'-deoxyguanosine
acetonitrile
alkaline phosphatase from bovine intestinal mucosa
ammonium acetate
calf thymus DNA
cell lysis solution
chloroform
deferoxamine
deoxyribonuclease I (DNase I)
ethanol
formic acid
HPLC-ESI-MS/MS system

HPLC/DAD system
HPLC column (50 x 2.0 mm i.d., 2.5 µm, C18)
HPLC column (150 x 2.0 mm i.d., 3.0 µm, C18)
HPLC column (250 x 4.6 mm i.d., 5.0 µm, C18)
HPLC C18 security guard cartridge (4.0 x 3.0 mm i.d.)
isoamyl alcohol
isopropyl alcohol (isopropanol)
ketamine
magnesium chloride
magnesium chloride
methanol
phosphodiesterase I from *Crotalus atrox*
protein precipitation solution
proteinase K
ribonuclease A
sodium chloride
SPE-C18 (Strata-X)
tris(hydroxymethyl)-aminomethane

xylazine

Manufacturer
Cambridge Isotope Laboratories
Cambridge Isotope Laboratories
Carlo Erba Reagents
Sigma
Merck
Sigma
QIAGEN
Carlo Erba Reagents
Sigma
Bio Basic Inc
Carlo Erba Reagents
Sigma-Aldrich
HPLC: Agilent 1200 series ESI-MS/MS: Applied Biosystems/MDS Sciex Instruments

Shimadzu
Phenomenex
Phenomenex
Phenomenex
Phenomenex
Sigma-Aldrich
Carlo Erba Reagents
Ceva
Carlo Erba Reagents
Sigma
Carlo Erba Reagents
Sigma
QIAGEN
Sigma-Aldrich
Sigma
Sigma-Aldrich
Phenomenex
Carlo Erba Reagents

Syntec do Brasil

Catalogue number
NLM-3895-25
NLM-3899-CA-10
412413000
P5521
101116
D1501
158908
412653
D9533
DD0649
414542
F0507

HPLC: binary pump (G1312B), isocratic pump (G1310A), column oven with a column switching valve (G1316B), diode array detector (G1315C), auto sampler (G1367C). ESI-MS/MS: Linear Quadrupole Ion Trap mass spectrometer, Model 4000 QTRAP.

Two pumps (LC-20AT), photo diode array detector (DAD-20AV), auto-injector (Proeminence SIL-20AC), column oven (CTO-10AS/VP)
00B-4446-B0
00F-4251-B0
00G-4252-E0
AJO-4287
M32658
A412790010
Commercial name: Dopalen
349377
M2393
L022909K7
P4506
158912
P2308
R5000
S9625
8B-S100-TAK
489983

Commercial name: Xilazin

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Title of Article:	Quantification of three DNA lesions by mass spectrometry and assessment of their levels in tissues of mice exposed to ambient fine particulate matter
Author(s):	Tiago Franco de Oliveira, Antonio Anax Falcão de Oliveira, Miriam Lemos, Mariana Veras, Paulo Hilário Nascimento Saldiva, Marisa Helena Gennari de Medeiros, Paolo Di Mascio, Ana Paula de Melo Loureiro

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CORRESPONDING AUTHOR

Name:	Ana Paula de Melo Loureiro
Department:	Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas
Institution:	Universidade de São Paulo
Title:	PhD
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Manuscript: JoVE59734

Authors: Tiago Franco de Oliveira, Antonio Anax Falcão de Oliveira, Miriam Lemos, Mariana Veras, Paulo Hilário Nascimento Saldiva, Marisa Helena Gennari de Medeiros, Paolo Di Mascio, Ana Paula de Melo Loureiro

Title: Quantification of three DNA lesions by mass spectrometry and assessment of their levels in tissues of mice exposed to ambient fine particulate matter

We would like to thank the pertinent comments and suggestions made by the reviewers. All the changes in the new version of the manuscript are highlighted in red.

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript was proofread and all detected spelling or grammar issues were corrected. The changes are shown in red in the manuscript.

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All company names were removed from the manuscript and added to the Table of Materials and Reagents.

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. If revisions cause the highlighted portion to be more than 2.75 pages, please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The highlighted portion is not more than 2.75 pages.

2. For each step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:

1. 1.2: How much blood? Which analyses?

The required information was added to the protocol step:

"1.2. Collect blood (0.5 – 1.5 mL) for complementary analyses (e.g., antioxidant enzyme activity, malondialdehyde levels)."

2. 1.4: How exactly are these tissues removed?

The following steps were added to the protocol:

"1.3. Shave the abdominal hair from the pelvis to the xiphoid process. Make an incision in a vertical middle line in the hairless area. Make incisions in horizontal lateral lines in order to expose the abdominal organs.

1.4. Cut the abdominal aorta to promote exsanguination and to euthanize the animal.

1.5. Remove the tissues of interest (in this case, liver, kidneys and lungs).

1.5.1. To remove the liver, cut the inferior cava vein and portal hepatic vein.

1.5.2. To remove the kidneys, section the renal veins and arteries.

1.5.3. To remove the lungs, make an incision in the diaphragm extremities and circumference close to the thoracic wall. Break the clavicles by opening a scissor in the interior of the thoracic cavity. Cut the extern bone from the xiphoid process toward the trachea, in order to expose the lungs and heart.

1.5.3.1. Hold the lung with a forceps, section the trachea and the ligaments around the lungs. Remove carefully the block lungs plus heart. To remove the lungs out of the block, hold the heart with a forceps and cut all vessels in its base.

1.6. Wash the isolated tissues immediately in cold saline solution (0.9% NaCl), transfer to cryogenic tubes, and immediately dip the tubes into liquid nitrogen. After completing the work, store the tubes at -80 ºC."

3. 2.4: Please include more detail about homogenization.

The required information was added to the protocol step:

"2.4. Homogenize the tissues using a Potter or a tissue glass Dounce homogenizer until a homogeneous solution without tissue fragments is obtained. Maintain the tube cold (on ice) during homogenization. It is better to use low speed to avoid heating."

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The Table of Materials was updated with the equipment and materials used. Some reagents that were not used in the protocol were removed.

Manuscript: JoVE59734

Authors: Tiago Franco de Oliveira, Antonio Anax Falcão de Oliveira, Miriam Lemos, Mariana Veras, Paulo Hilário Nascimento Saldiva, Marisa Helena Gennari de Medeiros, Paolo Di Mascio, Ana Paula de Melo Loureiro

Title: Quantification of three DNA lesions by mass spectrometry and assessment of their levels in tissues of mice exposed to ambient fine particulate matter

We would like to thank the pertinent comments and suggestions made by the reviewers. All the changes in the new version of the manuscript are highlighted in red.

Reviewer #1:

Manuscript Summary:

The manuscript presents a novel technique for the measurement of DNA lesions in animal tissue employing HPLC with mass spectrometry. I think this a valuable development that will certainly contribute to improving the measurement of oxidation biomarkers in animal models. Regarding manuscript, the protocol is well written, and the content is clearly presented. The technique is properly described and correctly presented step by step. It must be noticed the special attention that has been put to avoid misunderstandings. Therefore, I strongly recommend publishing this manuscript.

Major Concerns:

There are no major concerns.

Minor Concerns:

Line 46: "…of exposed AJ mice." I think "A/J mice" is the correct expression.

We replaced "AJ mice" with "A/J mice" in the manuscript.

Line 152: "…biological samples (12,15,20-29". I think the parenthesis does not correspond.

We removed the parenthesis.

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Reviewer #2:

Manuscript Summary:

Detection of 8-oxo-dGua and two etheno nucleosides by HPLC-ESI-MS/MS with MRM mode of operation.

This includes also the detailed preparation of samples (lungs, liver, kidney of mice).

Major Concerns:

No majors concerns

A study including this method and detection of the oxidized nucleosides has already being published. (ref 30 in the manuscript)

The referee is correct. The invitation to write the protocol was made by the Science Editor of JoVE due to the paper recently published in *Particle and Fibre Toxicology*.

Minor Concerns:

The title should be changed as validated method to determine 8-oxo-dGua and two etheno nucleosides. The multiple reaction method described here is a targeted screening method to quantify a known compound in very low concentration in a biological matrix. "Quantification of DNA lesions" is too general and we could think that a general screening method is used.

The title was changed as shown below:

"Quantification of three DNA lesions by mass spectrometry and assessment of their levels in tissues of mice exposed to ambient fine particulate matter"

In the conclusion, maybe add how this method can be used and modified to screen for unknown/new modified nucleotides.

The following sentence was added to the end of the "Discussion" section:

"The methods presented here may be adapted for the quantification of other modified deoxynucleosides, depending on the availability of their standards and isotopic standards. Adjustment of the chromatographic conditions would be necessary in order to obtain sharp peaks of all molecules included in the analyses."

Review the introduction and "electrophile". Explain why they are responsible for lesions in DNA. Usually electrophile sites of a molecule are attacked by nucleophiles, so I was wondering if you meant that the toxic compounds responsible for DNA lesions were attacking those electrophile sites. Please explain in more details a comprehensive molecular mechanism.

The sentences shown in red were added to the beginning of the "Introduction" section.

"Some reactive oxygen species (ROS) are able to oxidize carbon double bonds of DNA bases and some carbons in the deoxyribose moiety, generating oxidized bases and DNA strand breaks[1]. As a negatively charged molecule rich in nitrogen and oxygen atoms, DNA is also a target for electrophilic groups that covalently react with the nucleophilic sites (nitrogen and oxygen), giving products that are called DNA adducts[1,2]. So, DNA adducts and oxidized DNA bases are examples of DNA lesions that are useful biomarkers for the toxicity assessment of substances that are electrophilic, generate reactive electrophiles upon biotransformation, or induce oxidative stress[1,2]."

What are the components of the fine particle that have this effect on DNA (induce formation of DNA adducts)? Explain the genotoxicity of fine particles.

We modified the last paragraph of the "Introduction" section as shown below:

"Several methods based on HPLC-ESI-MS/MS have been used for quantification of 8-oxodGuo, 1,N^6-εdAdo and 1,N^2-εdGuo in DNA extracted from different biological samples[12,15,20-29]. Fine particles ($PM_{2.5}$) carry organic and inorganic chemicals, such as polycyclic aromatic hydrocarbons (PAHs), nitro-PAHs, aldehydes, ketones, carboxylic acids, quinolines, metals, and water-soluble ions, which may induce inflammation and oxidative stress, conditions that favor the occurrence of biomolecule damage and

disease[30-33]. We present here validated HPLC-ESI-MS/MS methods that were successfully applied for the quantification of 8-oxodGuo, 1,N^6-εdAdo and 1,N^2-εdGuo in lung, liver and kidney DNA of A/J mice for the assessment of the effects of ambient $PM_{2.5}$ exposure[34]."

Line 245: precise that 50 µg.ml-1.cm-1 is the average extinction coefficient of double stranded DNA at 260 nm.

A sentence explaining the meaning of the factor "50" was added to the note, as shown below. Please, note that the factor is not the average extinction coefficient of double stranded DNA at 260 nm.

"Note: To determine the DNA concentration, transfer an aliquot of 10 µL of the DNA solution to 990 µL of ultrapure water (100x dilution). Multiply the absorbance at 260 nm (it should be below 1) by 50 (50 µg/mL is the concentration of double stranded DNA when the absorbance of a 1 cm path length solution at 260 nm is 1) and by the dilution used (100x) to obtain the DNA concentration in µg/mL. If the absorbance at 260 nm is above 1, additional dilutions are necessary. The 260/280 nm absorbance ratio should be equal or above 1.8 for the desired DNA purity, but ratios around 1.6 are acceptable."

Line 299: 83.1 µl. Review the number of significant figures. We want 200 fmol in 50 µl of sample so 80 µl for resuspension volume should be more relevant.

Resuspending the final sample containing 332.5 fmol of internal standards in 80 µL of water would give 207.8 fmol of the internal standards in the injection volume (50 µL). The exact volume for sample resuspension is 83.1 µL in order to have 200 fmol of the internal standards in the injection volume (50 µL).

Line 374: explain the difference between set up A and B also in the text, and how the set up works (how the valves, the pumps and other parts are used and why).

We added the following Note after item 7.3:

"Note: Column A is connected to the binary pump. Its eluent is directed to UV detection and waste in the first 16 min and from 32 to 46 min of the chromatography, as shown in Figure 2A. This is the column through which the sample is eluted immediately after injection. Column B is connected to the isocratic pump and the mass spectrometer. It receives the eluent of column A only in the 16 – 32 min interval, when the valve is switched to the position shown in Figure 2B. The valve switch allows the connection between the two columns, which are eluted by the binary pump gradient.

The configuration shown in Figure 2B permits further peak separation and narrowing, as well as that only the chromatographic fraction of interest reaches the mass spectrometer, improving sensitivity and selectivity."

Remove Lines 411-418 because it is a repeat of lines 360-366.

The lines were removed. Additionally, the information regarding the equipment was moved to the Table of Materials. The new text in place of lines 360-366 is shown below:

"Note: Use an equipment equivalent or better than the equipment used in this work (see the Table of Materials). The ESI-MS/MS parameters were set as described in **Table 1**."

Paragraph lines 360-366 as well as Table 1 and figure 2 should be placed as introduction to items 7 and 8. Maybe add a paragraph announcing the HPLC-MS/MS experiment.

We thought about the addition of the paragraph, but it would break the format of the protocol. We decided to maintain the original places for the text, table and figure.

If possible, show the MS/MS spectra obtained for each standard, and the samples.

We added the mass spectra of the standards in a new Figure (Figure 7). It is not possible to have the mass spectra of the lesions present in the samples, as their concentrations are very low for mass spectra acquisition.

Diagrams of HPLC gradient would be a nice addition to the article.

We decided not to add the diagrams to the protocol due to space limitation (10 pages is the limit for the protocol length). The protocol with figure 2 and table 1 already reaches the 10 page limit.