Supporting Information

Measurement of Oxidatively-Induced DNA Damage in *Caenorhabditis elegans* with High-Salt DNA Extraction and Isotope-Dilution Mass Spectrometry

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The supporting information contains supplementary figures and supplementary tables. Also included are supplementary experimental procedures that describe preparation of DNA extraction buffers, DNA extraction methods, method for reducing protein levels in high salt DNA extracts and GC-MS/MS methods for measuring DNA damage levels in nematodes and human cells.

SUPPORTING FIGURES

Figure S1. Nematode homogenization.
(A) Control nematodes or (B) nematodes homogenized with a handheld mechanical homogenizer for 30 s in mammalian cell lysis buffer. Each image is a bright-field snapshot from 0.2 mL sample in a 24-well plate. The entire well was imaged. Samples were diluted 4X more than samples presented in **Figure 1**, and are therefore included in a separate figure.

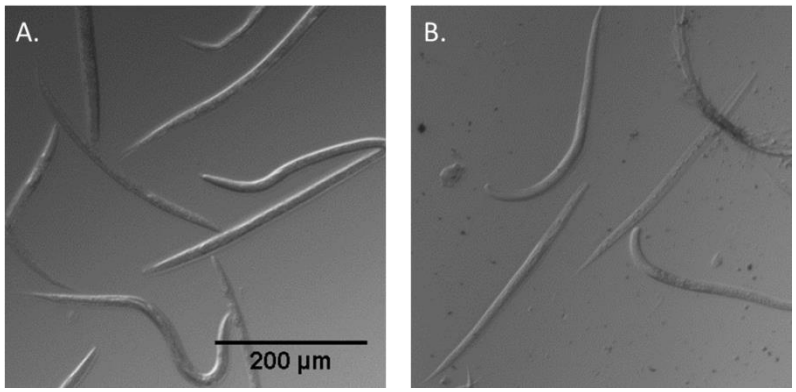
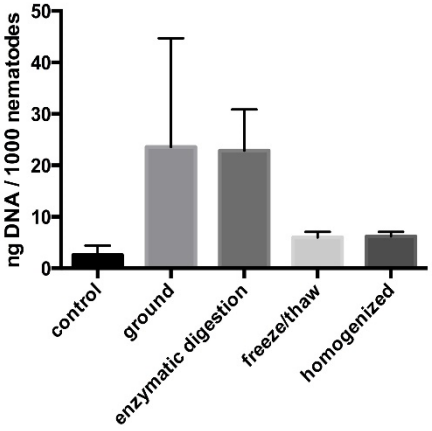


Figure S2. DNA amounts per 1000 nematodes.
DNA from rough nematode extracts was measured with Qubit dsDNA High Sensitivity assay kit. Asterisks indicate statistically significant differences between samples based on the Student's t-test (unpaired, two-tailed). One, two or three asterisks indicate $p \leq 0.05$, 0.01, or 0.001, respectively. NS indicates not significant. All data points represent the mean of 3 to 6 independent measurements. The uncertainties are standard deviations. The control samples were nematode samples that were not subjected to a degradation process.



p-values	ground	enzymatic digestion	freeze/thaw	homogenized
control	ns	0.0004 ***	0.0148 *	0.0022 **
ground	--	ns	ns	ns
enzymatic digestion	--	--	0.0033 **	0.0005 ***
freeze/thaw	--	--	--	ns

Figure S3. Comparison of average DNA yields for different DNA extraction methods. Yield from HS DNA extraction (all media combined) was not significantly different from the phenol extraction yield (all media combined) when analyzed with Student's t-test (unpaired, two-tailed, p-value = 0.2719, N = 18). However, DNA yield from nematodes grown in mCeHR was higher than yield from nematodes grown in SB (analyzed with one-way ANOVA). One or two asterisks indicate $p \leq 0.05$ or 0.01, respectively. All data points represent the mean of 6 to 18 independent measurements. The uncertainties are standard deviations.

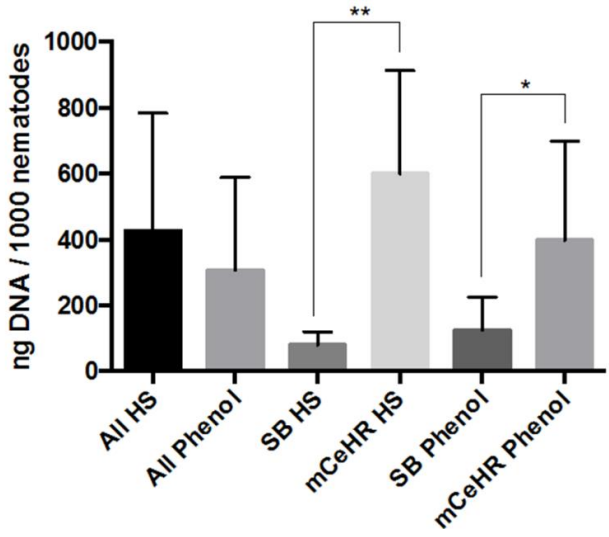


Figure S4. Protein levels in DNA extracts.
HS DNA extracts contained significantly higher levels of protein than phenol extracts, and mCeHR samples contained significantly more protein than extracts from SB samples (500,000 nematodes per sample). Decreasing the number of mCeHR nematodes to 100,000 or 250,000 significantly decreased the amount of protein in DNA extracts. One, two or four asterisks indicate $p \leq 0.05$, 0.01 or 0.0001, respectively. All data points represent the mean of 3 to 12 independent measurements. The uncertainties are standard deviations.

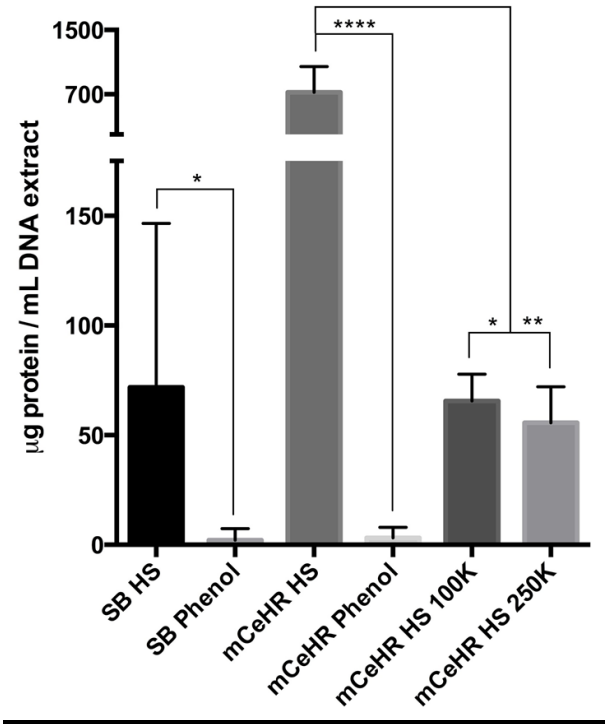
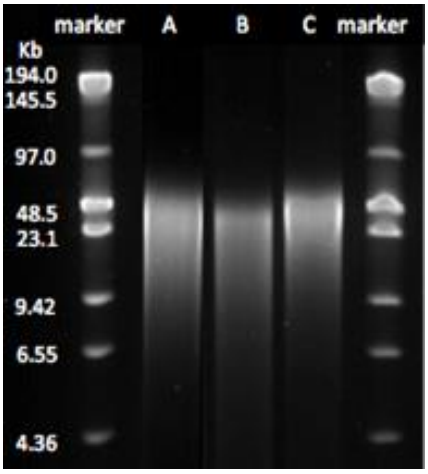


Figure S5. Pulse-field gel image of DNA extracted from *C. elegans*

Representative DNA was extracted with the HS or the phenol extraction protocol from nematodes grown in SB or mCeHR medium. Extracted DNA was similar in size from each extraction type and medium. Samples are as follows: A) phenol extract, SB; B) HS extract, mCeHR; C) HS extract, SB.



SUPPORTING TABLES

Table S1. Description of *C. elegans* cuticle degradation methods.

Ball and grind using mortar and pestle	A glass Pasteur pipette was used to drop thawed nematodes in SB medium into a mortar cooled and filled with liquid N_2. The liquid froze into small balls, which were then ground with a pestle into a fine powder.
Freeze and thaw	Nematode samples were initially thawed at 37 ºC. Samples were then frozen in a bucket of dry ice and thawed at 37 ºC, six times, to break apart the nematodes.
Enzymatic digestion (mammalian cell)	Samples were thawed, mixed with mammalian cell lysis buffer and Proteinase K, and incubated at 37 ºC for 18 h or at 55 ºC for 2 h.
Enzymatic digestion (Schedl)	Samples were thawed, mixed with Schedl cell lysis buffer and enzyme, and incubated at 37 ºC for 18 h or at 55 ºC for 2 h.
Mechanical Homogenization 1	Samples were thawed and homogenized for 10 s to 90 s in SB medium alone.
Mechanical Homogenization 2	Samples were thawed and homogenized for 10 s to 90 s in SB medium with G2 genomic extraction buffer from Qiagen.
Mechanical Homogenization 3	Samples were thawed and homogenized for 10 s to 90 s in SB medium with mammalian cell lysis buffer.

Table S2. Primer sequences used for polymerase chain reaction (PCR).

PCR was used as a metric to measure quality of extracted DNA. Primers used for PCR are listed below with references.

Primer	Sequence	Source
C elegans ACT-1 FWD	GCTGGACGTGATCTTACTGATT	Zhang et al 2012[1]
C elegans ACT-1 REV	GTAGCAGAGCTTCTCCTTGATG	Zhang et al 2012[1]
E. coli dxs FWD	CGCTTCACAATGCCTTTGC	M. Kline, NIST
E. coli dxs REV	ACGCAGTTGGCGTGGAA	M. Kline, NIST

Table S3. Increased lesion levels in DNA extracted from breast cells using phenol compared to DNA extracted with the HS protocol.

Data was analyzed for statistical significance using one-way ANOVA. One, two, three or four asterisks indicate $p \leq 0.05$, 0.01, 0.001 or 0.0001, respectively. ND = not detected. ł = indicates that lesion levels in phenol extracts are lower than in HS extracts. N= 3 independent measurements for the base lesions (FapyAde, FapyGua, 8-OH-Ade, 5-OH-Cyt, 8-OH-Gua) and N = 5 independent measurements for the 8,5'-cyclopurine-2'-deoxynucleoside lesions (*R*-cdA, *S*-cdA, *R*-cdG, *S*-cdG). MCF7: human breast adenocarcinoma cell line; MCF10a: non-tumorigenic human breast cell line.

H. sapiens		
Cell line	Lesion	Ratio (phenol/HS)
MCF7	FapyAde	5.51**
	FapyGua	5.9****
	8-OH-Gua	0.65ł
	5-OH-Cyt	1.06
	8-OH-Ade	ND
	R-cdA	1.07
	S-cdA	1.27
	R-cdG	1.20
	S-cdG	0.97ł
MCF10a	FapyAde	7.72*
	FapyGua	7.58***
	8-OH-Gua	1.46
	5-OH-Cyt	0.92ł
	8-OH-Ade	ND
	R-cdA	0.90ł
	S-cdA	0.80ł
	R-cdG	1.12
	S-cdG	0.83ł

Table S4. DNA extracted from nematodes with phenol has increased lesion levels compared to DNA extracted with the high-salt method.

SB: nematodes cultured in S-basal complete medium; mCeHR: nematodes cultured in modified *C. elegans* Habitation and Reproduction medium; *mCeHR: nematodes cultured in mCeHR medium and irradiated with 10 Gy gamma radiation. Data was analyzed using one-way ANOVA. One, two or three asterisks indicate $p \leq 0.05$, 0.01 or 0.001, respectively. ND = not detected. All data points represent the mean of 3 to 6 independent measurements.

C. elegans

Condition	Lesion	Ratio (phenol/HS)
SB	FapyAde	1.43
	FapyGua	1.73
	8-OH-Gua	2.33*
	5-OH-Cyt	1.12
	8-OH-Ade	4.25
mCeHR	FapyAde	1.48*
	FapyGua	2.71**
	8-OH-Gua	5.27***
	5-OH-Cyt	0.93
	8-OH-Ade	1.15
	R-cdA	ND
	S-cdA	ND
	R-cdG	34.93**
	S-cdG	52.43*
*mCeHR	FapyAde	1.58*
	FapyGua	3.11*
	8-OH-Gua	3.29
	5-OH-Cyt	1.99*
	8-OH-Ade	0.67

Table S5. Oxidatively-induced lesions in calf thymus DNA (ct-DNA).

ct-DNA (Sigma) is run as a control for all mass-spectrophotometric measurements. This table compares lesion levels found in ct-DNA from the previous work reported by Arczewska *et al.*, in 2013.[2] Similar lesion levels between the two studies indicate that other results (levels of lesions in nematode DNA) are not artifacts from processing.

ct-DNA	FapyAde[1]	FapyGua[1]	8-OH-Gua[1]	8-OH-Ade[1]
Arczewska	3.86 ± 0.78	7.79 ± 0.47	20.66 ± 0.33	2.72 ± 0.08
This Work	3.64 ± 1.16	8.08 ± 0.90	14.62 ± 1.45	2.30 ± 0.19
Fold Change	1.06	0.96	1.39	1.20

[1] Measured DNA lesions / 1 x 10^6 DNA bases (mean ± standard deviation). N = 3

SUPPORTING EXPERIMENTAL SECTION

High-salt (HS) DNA extraction method

To extract DNA from aliquots of 250,000 to 500,000 nematodes:

1. Pellet nematode samples by centrifugation at 4300 x g for 5 min at room temperature. Discard supernatant. Wash once with PBS.
2. Add 2 mL of lysis buffer containing 2 mg/mL Proteinase K directly to the pellet and immediately transfer the sample to a labeled 15 mL centrifuge tube rated for high speeds (≥ 15000 x g). Incubate all samples for 2 h at 55 °C. Note: When the lysis buffer is initially mixed with the nematode pellet, the mixture will appear cloudy. The lysis buffer should be transparent prior to use. If the lysis buffer is not transparent, it is likely that the SDS has precipitated out of solution. The SDS can be re-solubilized by gently heating the solution for 30 min at 37 °C prior to experimentation.
3. Remove samples from incubation and immediately place tubes on ice. Add ¼ volume (0.5 mL) of saturated NaCl (6 mol/L) and vigorously shake for 30 s. The solution will become cloudy. Centrifuge sample at 15000 x g for 20 min at 4 °C. Transfer the supernatant into a clean 15 mL high-speed tube. If the supernatant is cloudy or contains particulates, centrifuge a second time and transfer clear supernatant into a new clean tube.
4. Add 2.5 volume cold 96 % ethanol (6.25 mL) to the supernatant and gently invert 20 times [3] until the DNA precipitates.
5. Centrifuge sample at 15000 x g for 10 min at 4 ºC to pellet the DNA, then remove ethanol supernatant. Add 5 mL of 70 % cold ethanol, invert for five seconds, and centrifuge again. Remove EtOH supernatant, and evaporate ethanol for 25 min on a vacuum desiccator containing desiccant. The ethanol needs to be removed, but the sample does not need to dry completely.
6. Re-suspend each sample in 1 mL TE buffer and add RNase A (final concentration = 0.2 mg/mL; 100 µL of Thermo RNase A/T1). Incubate for 1 h at 37 ºC.
7. Add 1 mL lysis buffer containing proteinase K directly to the DNA sample and incubate the sample for 1 h at 55 ºC. Final concentration of Proteinase K: 2 mg/mL.
8. Put sample on ice, add ¼ volume of saturated NaCl (0.5 mL), shake for 30 s, and centrifuge at 15000 x g for 15 min at 4 ºC.
9. Transfer supernatant into a clean 15 mL high-speed centrifuge tube. Centrifuge a second time if supernatant has any white chunks or precipitates.
10. Precipitate DNA by adding 2.5 volume cold 96 % ethanol (6.25 mL). Incubate at -20 ºC if DNA does not readily precipitate. Centrifuge sample at 15000 x g for 15 min at 4 ºC, then remove and discard ethanol supernatant.
11. Wash DNA three times by adding 5 mL of 70 % cold ethanol, inverting for five seconds, and centrifuging at 15000 x g for 10 min at 4 °C. Remove and discard ethanol supernatant.
12. Remove the residual 70 % ethanol in a vacuum desiccator (1/2 h) containing desiccant. Do not over-dry the DNA.
13. Store the DNA at -20 °C.
14. To re-solubilize the DNA, add 0.5 -1 mL of nuclease-free water to the dried DNA and gently rock the sample at room temperature (≈24 °C for 36 h).

Reducing protein levels in HS extracts

In order to improve the protein removal efficiency for the HS DNA extraction method, the number of nematodes utilized per sample was reduced. In this way, the effective concentration of proteinase K enzyme/nematode was increased, leading to more efficient digestion of nematode proteins. DNA extracts from samples with fewer nematodes had significantly decreased amounts of protein (average ± standard deviation, **Figure S5)**. The 260 nm/280 nm ratios were higher in DNA extracted from aliquots with fewer nematodes, indicating lower amounts of protein contamination (1.83 for 250,000 nematode extracts versus 1.6 for 500,000 nematodes).

Phenol/chloroform DNA extraction method

The phenol extraction protocol was adapted from Luke *et al.* (2006).[4] The buffer recipe was originally from Palasterk 1995.[5]

Lysis buffer recipe:
200 mmol/L NaCl
100 mmol/L Tris-HCl, pH 8.0
50 mmol/L EDTA, pH 8.0
0.5 % (mass fraction) SDS
0.1 mg/mL Proteinase K

To extract DNA from aliquots of 250,000 to 500,000 nematodes:

1. Thaw and pellet nematodes described for the HS DNA extraction (centrifuge at 4300 x g for 5 min at room temperature). Discard supernatant. Wash once with PBS.
2. Suspend pellets in 2 mL lysis buffer and incubate at 65 ºC for 2 h in sterile centrifuge tubes. Incubate at 95 ºC for 20 min to 30 min to deactivate the Proteinase K.
3. Add 50 µL RNase A/T1 (final concentration 0.1 mg/mL) and incubate at 37 ºC for 1 h.
4. Inside a chemical fume hood, add 400 µL phenol:chloroform:isoamyl alcohol buffered with Tris (Sigma) and gently mix.
5. Centrifuge at 2750 x g for 5 min at room temperature (20 ºC to 22 ºC).
6. In a fume hood, transfer the aqueous phase into a new tube, avoiding the phenol phase and interface.
7. Repeat the phenol extraction twice more, until no white precipitate is visible at the aqueous/organic interface.
8. Add 40 µL of 3 mol/L sodium acetate and 1 mL of 100 % volume fraction ethanol to each sample. Mix and incubate at 4 ºC for at least one h.
9. Pellet DNA by centrifugation at 15000 x g for 15 min at 4 °C.
10. Wash DNA pellets three times with 70 % volume fraction ethanol.
11. Remove ethanol supernatant and dry under vacuum for 30 min.
12. Freeze at -20 °C or re-suspend DNA in 0.5 mL to 1 mL nuclease-free water and incubate with gentle shaking at room temperature (≈24 °C) for ≈36 h.

GC-MS/MS determination of DNA base lesion profiles

To prepare the DNA for enzymatic digestion, 40 µg to 50 µg DNA samples were dissolved in a buffer consisting of 50 mmol/L sodium phosphate, 100 mmol/L potassium chloride, 1 mmol/L EDTA and 100 µmol/L dithiothreitol (pH 7.4). To this solution, 2 µg each of *E. coli* Fpg and EndoIII were added and each sample was digested at 37 °C for 1 h. The digestion was terminated with the addition of ice-cold absolute ethanol in combination with sample storage at – 20 °C.

Samples were centrifuged at 14000 x g for 30 min, supernatant fractions containing the excised DNA lesions were transferred to glass vials and the solvent was evaporated under vacuum. Samples were solubilized in nuclease-free water, lyophilized, and then trimethylsilylated using bis(trimethylsilyl)trifluoroacetamide)/1 % trimethylchlorosilane in pyridine (120 °C for 30 min). Following derivatization, samples were analyzed by GC-MS/MS.

Oxidatively modified DNA base lesions were identified and quantified using GC-MS/MS with isotope-dilution in the multiple reaction monitoring (MRM) mode. This analysis was conducted based on modifications to a previously developed selected ion monitoring (SIM) mode gas chromatography/mass spectrometry (GC/MS) methodology.[6-8] Mass spectrometry analyses were performed on an Agilent 7000 series triple quadrupole GC-MS/MS system (Agilent Technologies, Santa Clara, CA) operated in positive ion mode with electron ionization. The modular system consisted of a 7693 autosampler, a 7890A GC oven and a 7000 series triple quadrupole mass analyzer set to widest resolution for MS1 and MS2.

In the current MRM mode method, specific reaction transitions for five lesions, namely 4,6-diamino-5-formamido-pyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 8-hydroxyadenine (8-OH-Ade), 5-hydroxycytosine (5-OH-Cyt) and 8-hydroxyguanine (8-OH-Gua), as well as for their stable isotopically-labeled analogues (FapyAde-$^{13}C,^{15}N_2$, FapyGua-$^{13}C,^{15}N_2$, 8-OH-Ade-$^{13}C,^{15}N_2$, 5-OH-Cyt-$^{13}C^{15}N_2$ and 8-OH-Gua-$^{15}N_5$) were utilized. The isotopically-labeled lesion analogues function as internal standards (ISTDs) for lesion quantification. The isotopically-labeled lesion analogues were purchased from Cambridge Isotope Laboratories (Andover, MA) and are part of the National Institute of Standards and Technology Standard Reference Material #2396 (NIST SRM-2396). 8-OH-Gua-$^{15}N_5$ is obtained by hydrolysis of 8-hydroxy-2′-deoxyguanosine-$^{15}N_5$ (8-OH-dG-$^{15}N_5$) in SRM-2396. 8-OH-Gua-$^{15}N_5$ was obtained by hydrolyzing 8-OH-dG-$^{15}N_5$ with 60 % formic acid at 140 °C for 30 min followed by lyophilization. Subsequently, 8-OH-Gua-$^{15}N_5$ was dissolved in 10 mmol/L NaOH before use. The relevant MRM mass transitions were: *m/z* 369 → *m/z* 354 and *m/z* 372 → *m/z* 357.1 for FapyAde and FapyAde-$^{13}C,^{15}N_2$ respectively; *m/z* 457 → *m/z* 442 and *m/z* 460 → *m/z* 445 for FapyGua and FapyGua-$^{13}C,^{15}N_2$, respectively; *m/z* 367 → *m/z* 352 and *m/z* 370 → *m/z* 355 for 8-OH-Ade and 8-OH-Ade-$^{13}C,^{15}N_2$ respectively; *m/z* 343 → *m/z* 342, *m/z* 343 → *m/z* 328 and *m/z* 346 → *m/z* 345, *m/z* 346 → *m/z* 331 for 5-OH-Cyt and 5-OH-Cyt-$^{13}C,^{15}N_2$, respectively and *m/z* 455 → *m/z* 440 and *m/z* 460 → *m/z* 445 for 8-OH-Gua / 8-OH-Gua-$^{15}N_5$, respectively. Final results are reported in terms of the number of lesions quantified / 10^6 DNA bases.

GC-MS/MS determination of 8,5'-cyclopurine-2'-deoxynucleoside lesion profiles

DNA samples (40 μg to 50 μg each) were used for the measurement of 8,5′-cyclopurine-2′-deoxynucleosides, i.e., (5'*R*)-8,5′-cyclo-2′-deoxyadenosine (*R*-cdA) (5'*S*)-8,5′-cyclo-2′-deoxyadenosine (*S*-cdA), (5'*R*)-8,5′-cyclo-2′-deoxyguanosine (*R*-cdG) and (5'*S*)-cyclo-2′-deoxyguanosine (*S*-cdG). DNA samples were supplemented with the aliquots of the stable isotope-labeled analogues of these compounds, namely *S*-cdA-$^{15}N_5$, *R*-cdG-$^{15}N_5$ and *S*-cdG-$^{15}N_5$ as internal standards, which had been synthesized as described previously.[9] The samples were dried in SpeedVac and then dissolved in 50 μL of 10 mmol/L Tris-HCl solution (pH 7.5) containing 45 mmol/L $ZnCl_2$, supplemented with 2.5 μL of 1 mol/L sodium acetate (final pH 6.0). Aliquots of nuclease P1 (2 U), snake venom phosphodiesterase (0.004 U) and alkaline phosphatase (16 U) were added and the samples were incubated at 37 °C for 24 h.

After hydrolysis, the samples were filtered using ultrafiltration membranes with a molecular mass cutoff of 3 kDa by centrifugation at 12000 g for 30 min. Filtered samples were lyophilized and then trimethylsilylated as described.[10]

MRM scans were performed with mass transitions *m/z* 465 → *m/z* 309 for *R*-cdA and *S*-cdA, *m/z* 470 → *m/z* 314 for *R*-cdA-$^{15}N_5$ and *S*-cdA-$^{15}N_5$, *m/z* 553 → *m/z* 397 for *R*-cdG and *S*-cdG, and *m/z* 558 → *m/z* 402 for *R*-cdG-$^{15}N_5$ and *S*-cdG-$^{15}N_5$. These transitions are based on the known mass spectra of the trimethylsilyl derivatives of 8,5′-cyclopurine-2′-deoxynucleosides and their fragmentation patterns, which were reported previously.[11-13] The optimal (maximum) collision energies of the trimethylsilyl derivatives of *R*-cdA, *S*-cdA, *R*-cdG and *S*-cdG were determined as 15 V.

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