Iridium–Schiff Base complex- Based Fluorimetric Turn-Off Sensor and HPLC-DAD Analytical Approaches for Quantitation of 8-Hydroxy-2´-Deoxyguanosine as an Oxidative DNA Damage Biomarker in Biofluids of Healthy and Diabetic Subjects

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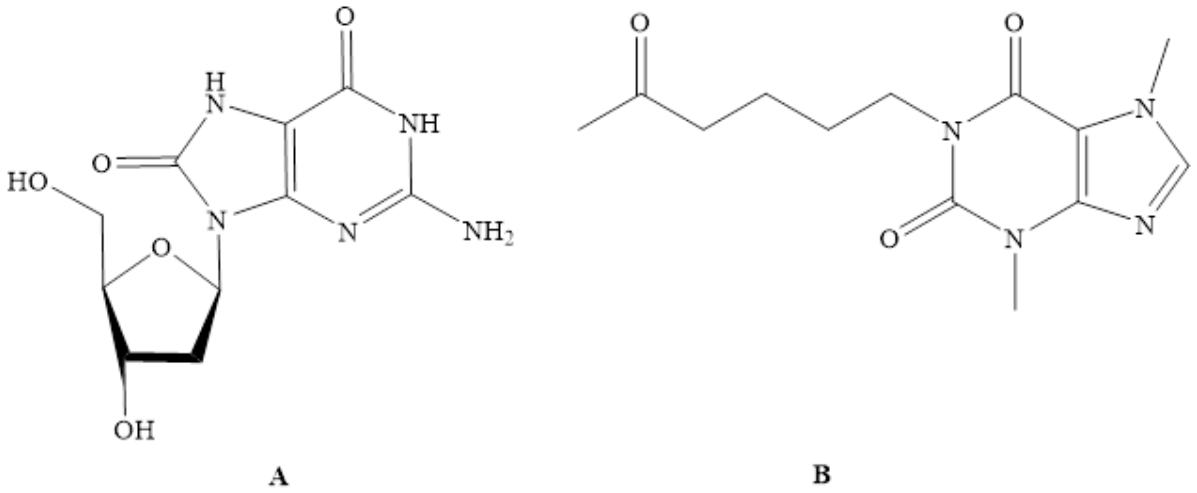

Supplementary scheme 1: chemical structure of (A) 8-OHdG and (B) pentoxifylline

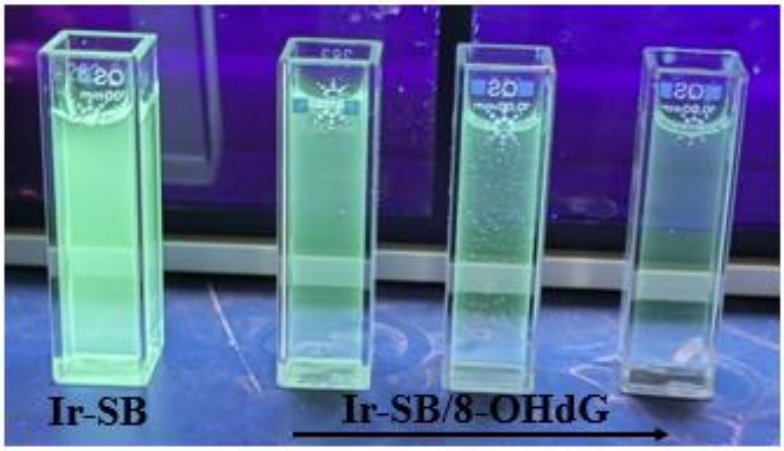

Fig. S1. Image shows the effect of UV excitation at λ= 254 nm on the working system. From right to left: Ir-SB complex, and Ir-SB complex -8-OHdG.

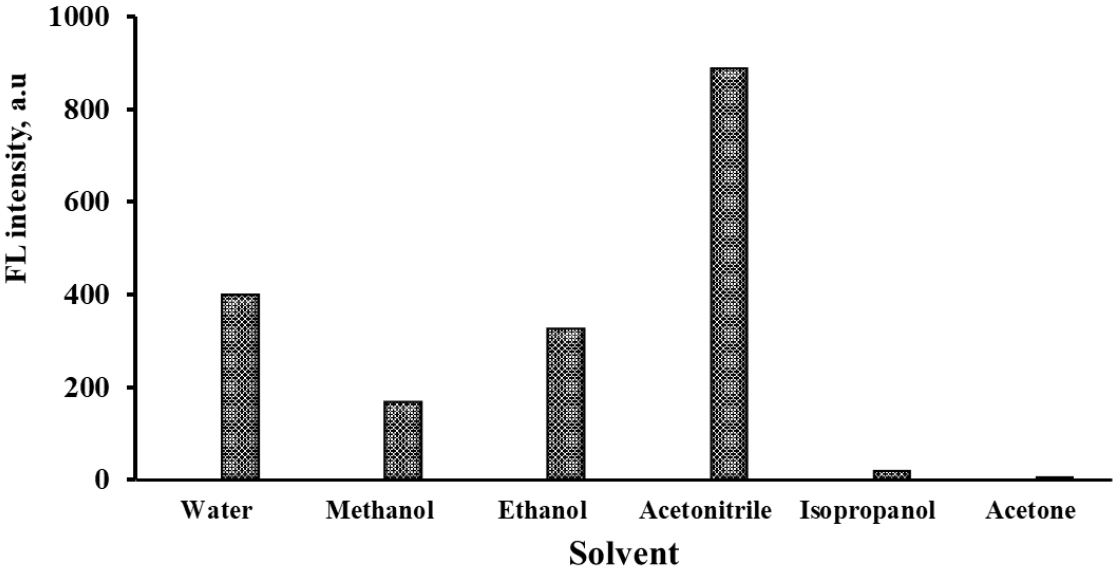

Fig. S2. Fluorescence intensities of Ir-SB complex at λ_{ex} = 292 nm in the presence of different solvents.

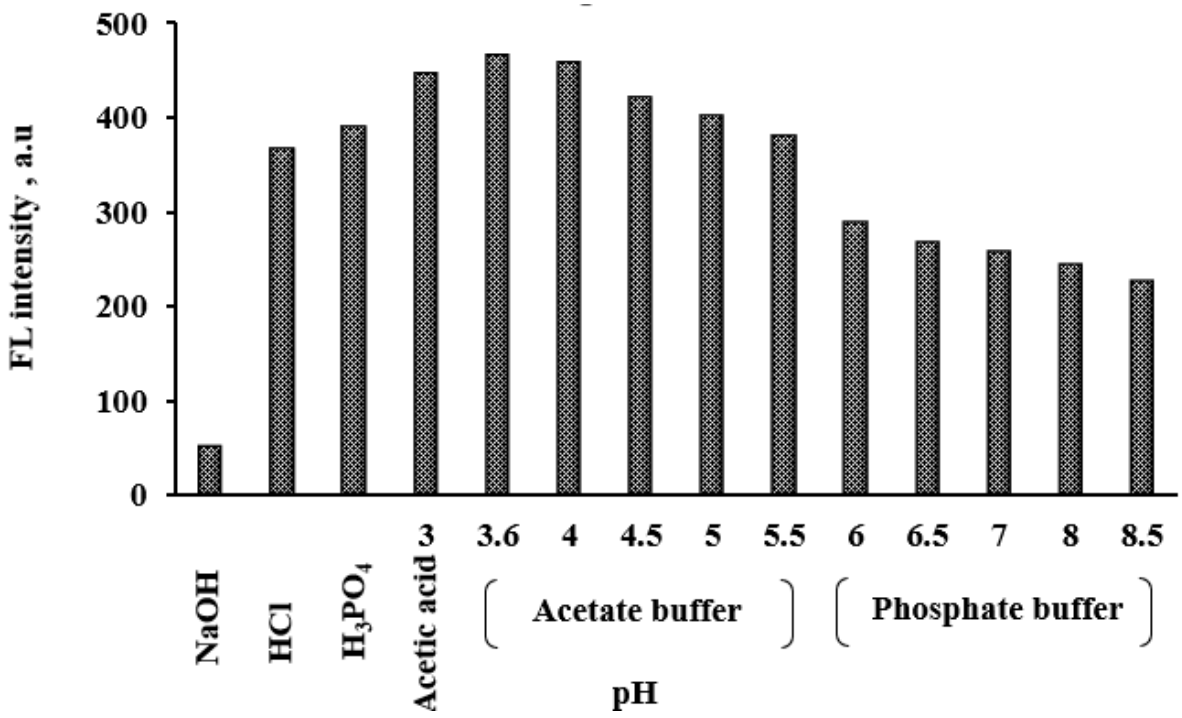

Fig. S3. Fluorescence intensities of the Ir-SB complex fluorophore in the presence of different medium at λ_{ex} = 292 nm.

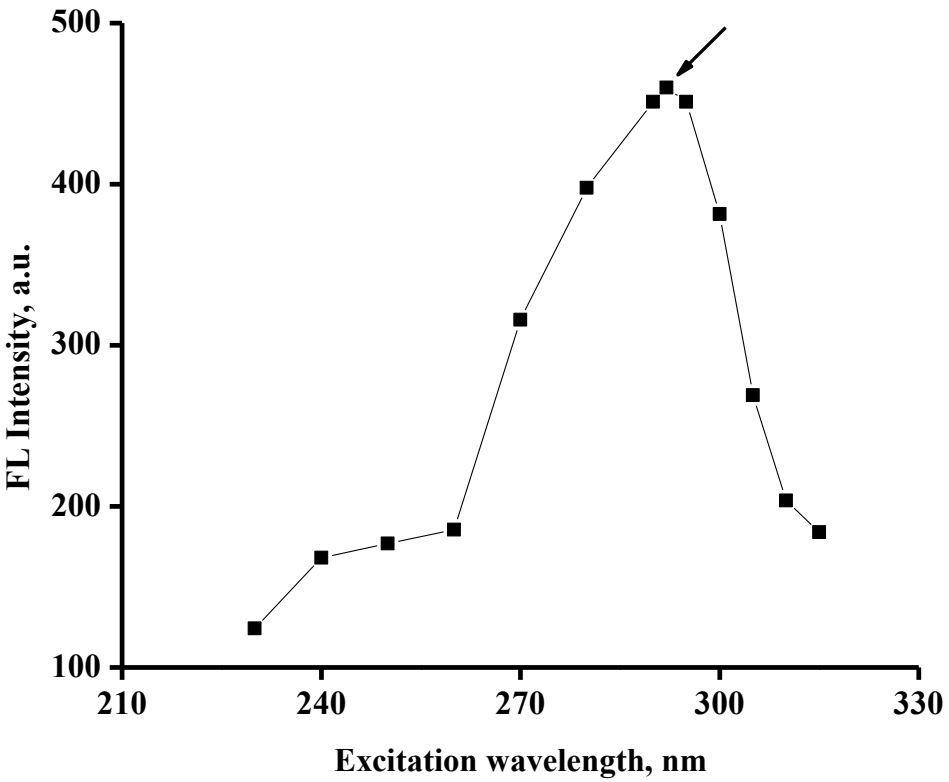

Fig. S4. Fluorescence spectra of Ir-SB complex at different excitation wavelengths.

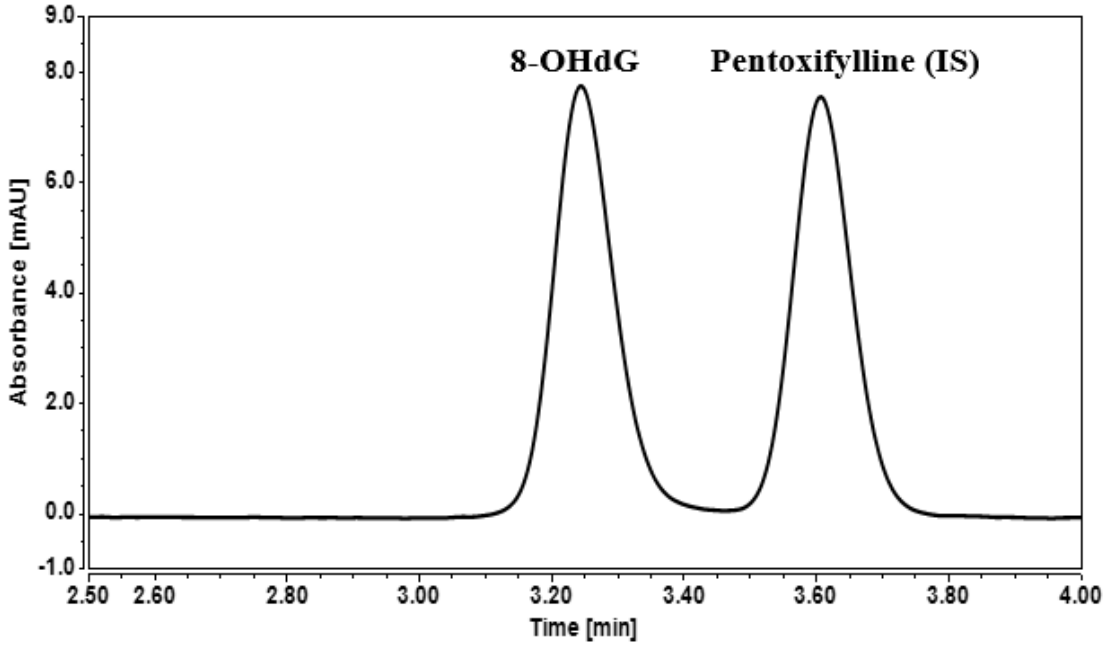

Fig. S5. HPLC-DAD chromatogram of 8-OHdG in presence of pentoxifylline as internal standard

Table S1: System suitability testing parameters of the proposed HPLC method.

Parameters	Obtained value		Reference value [33]
	8-OHdG	pentoxifylline	
Resolution (R_s)	4.36		> 1.5
Selectivity factor (α)	1.11		> 1
Tailing factor(T)	1.02	1.05	< 1.5-2 or <2
Capacity factor(K')	1.70	2.01	1 – 10 acceptable
Column efficiency (N)	18900	19800	Increase with efficiency of the separation
HETP [b]	0.0079	0.0076	The smaller the value the higher the column efficiency

HETP[b] = height equivalent to theoretical plate, (cm / plate)

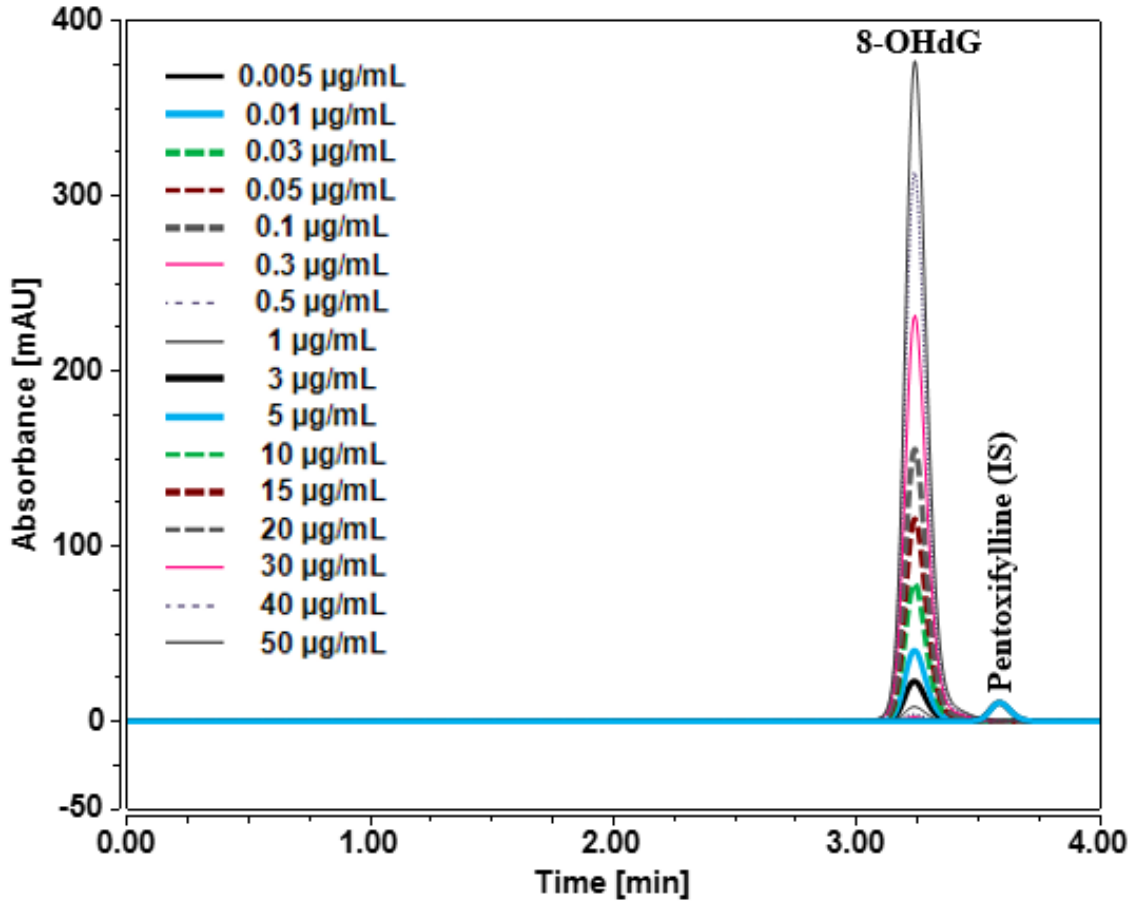

Fig. S6. HPLC-DAD chromatogram of different concentration of 8-OHdG in presence of pentoxifylline as internal standard

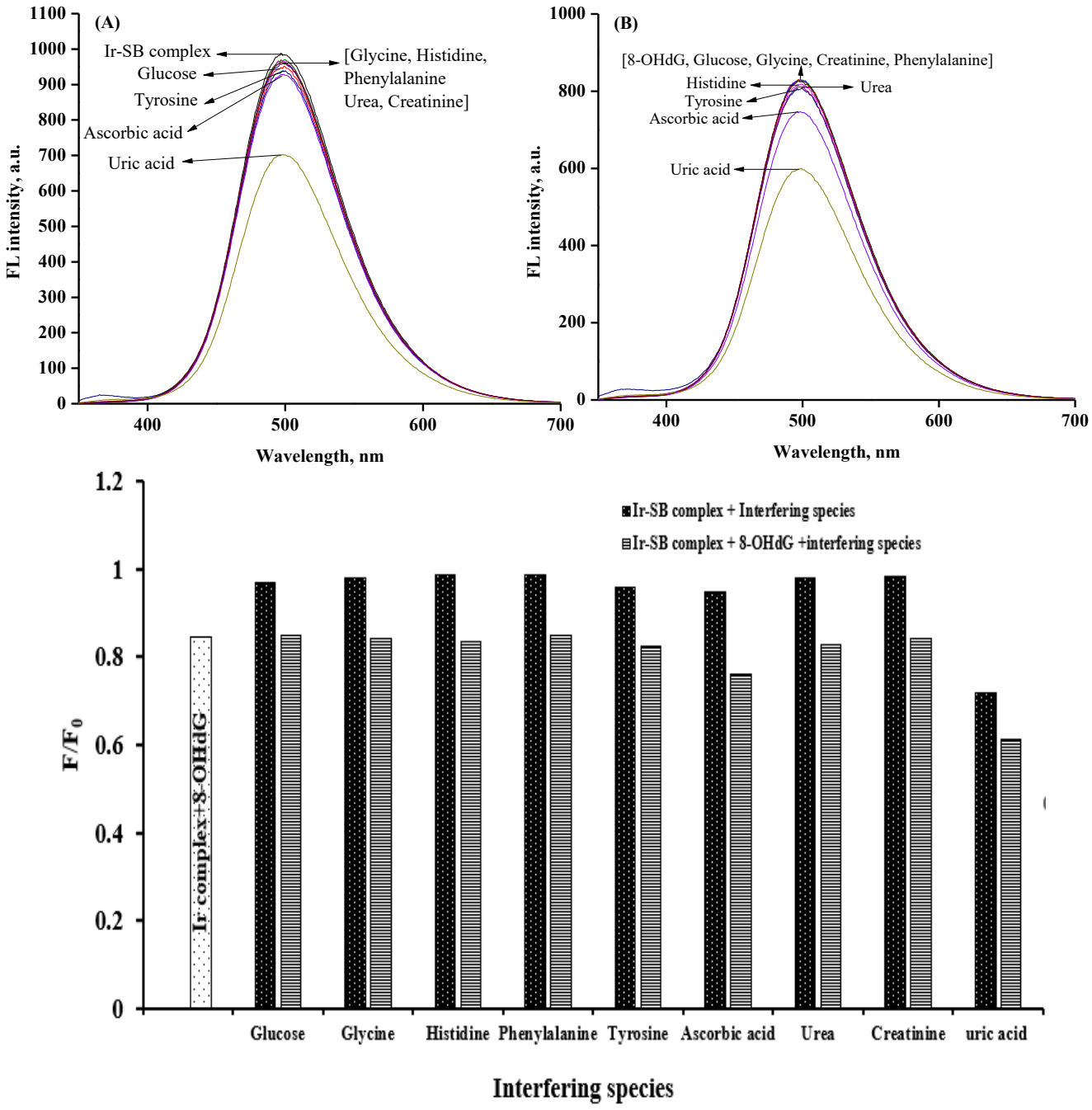

Fig. S7: A) and B) effect of interfering species on the fluorescence intensity of Ir-SB complex and Ir-SB complex in the presence of 8-OHdG. C) The F_0/F ratio of Ir-SB complex (Black bars) and Ir-SB complex -8-OHdG (Grey bars) in the absence and presence of interfering species.The used concentration: Ir-SB complex= 0.215 µg/mL; [8-OHdG] = 5 µg/mL; [Glucose], [Glycine], [Histidine], [Phenylalanine], [Tyrosine], [Ascorbic acid], [Urea], [creatinine]= 40 µg/mL; [uric acid] = 5 µg/mL.

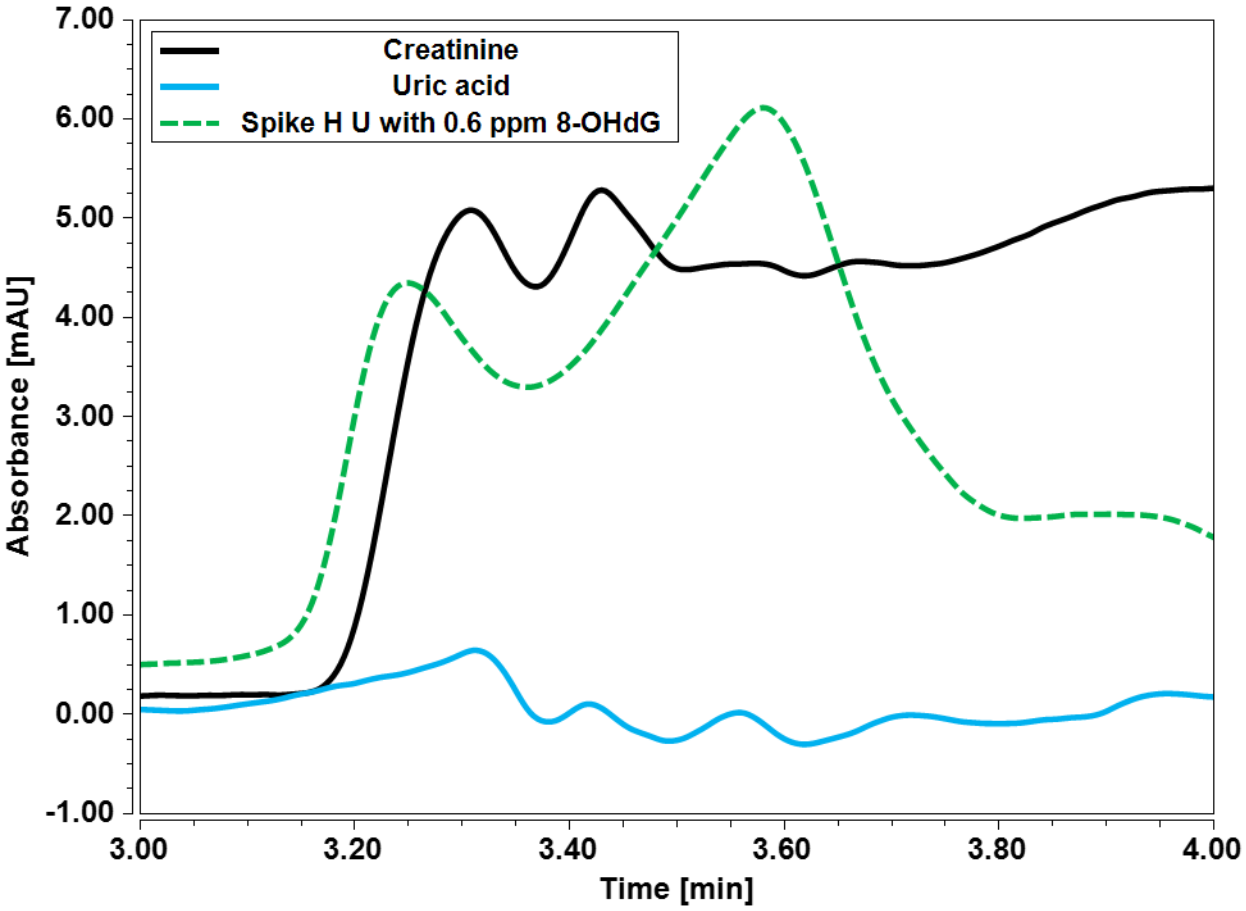

Fig. S8: HPLC Chromatogram of creatinine, uric acid and healthy urine spiked with 0.6 µg/mL 8-OHdG.

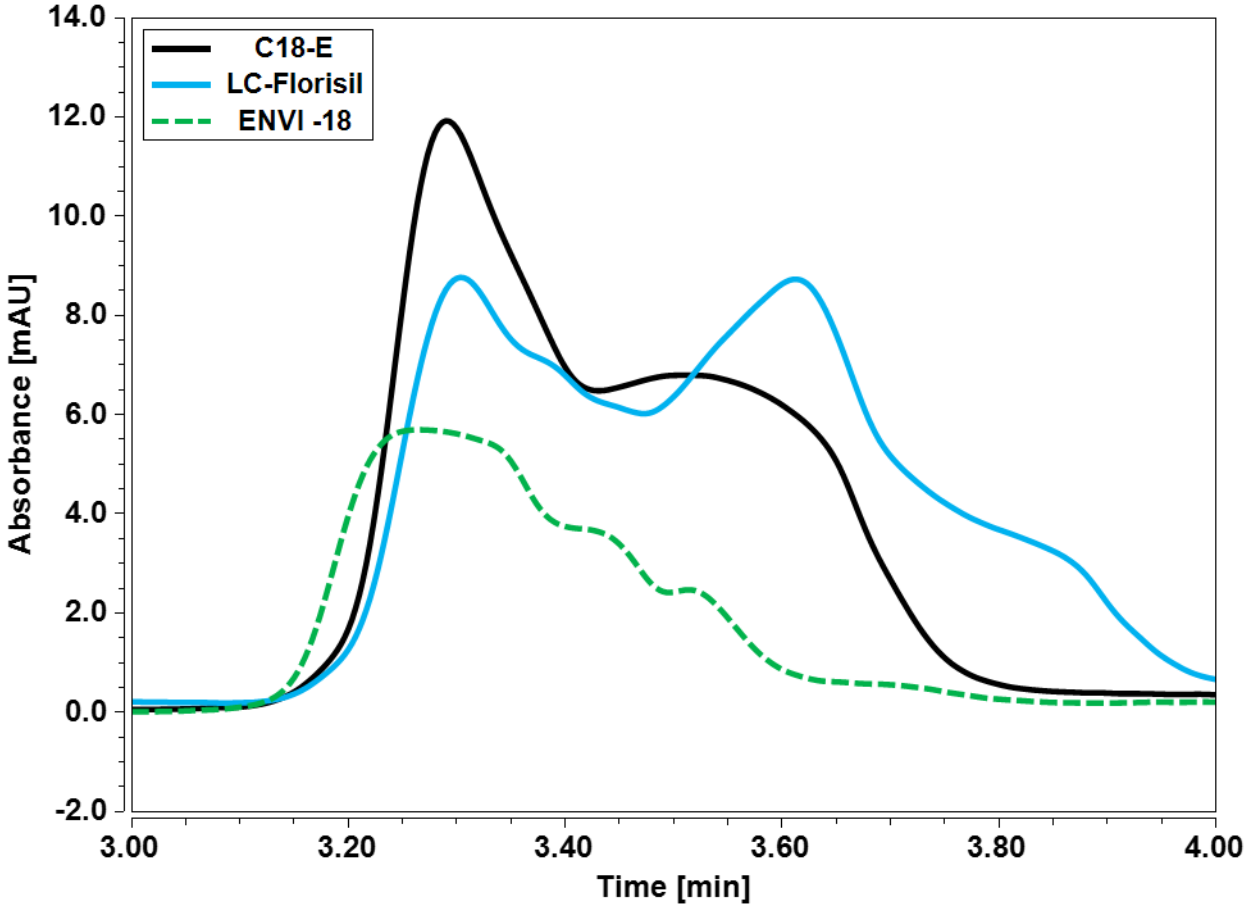

Fig. S9. HPLC chromatogram of extracted 8-OHdG by using different SPE cartridge

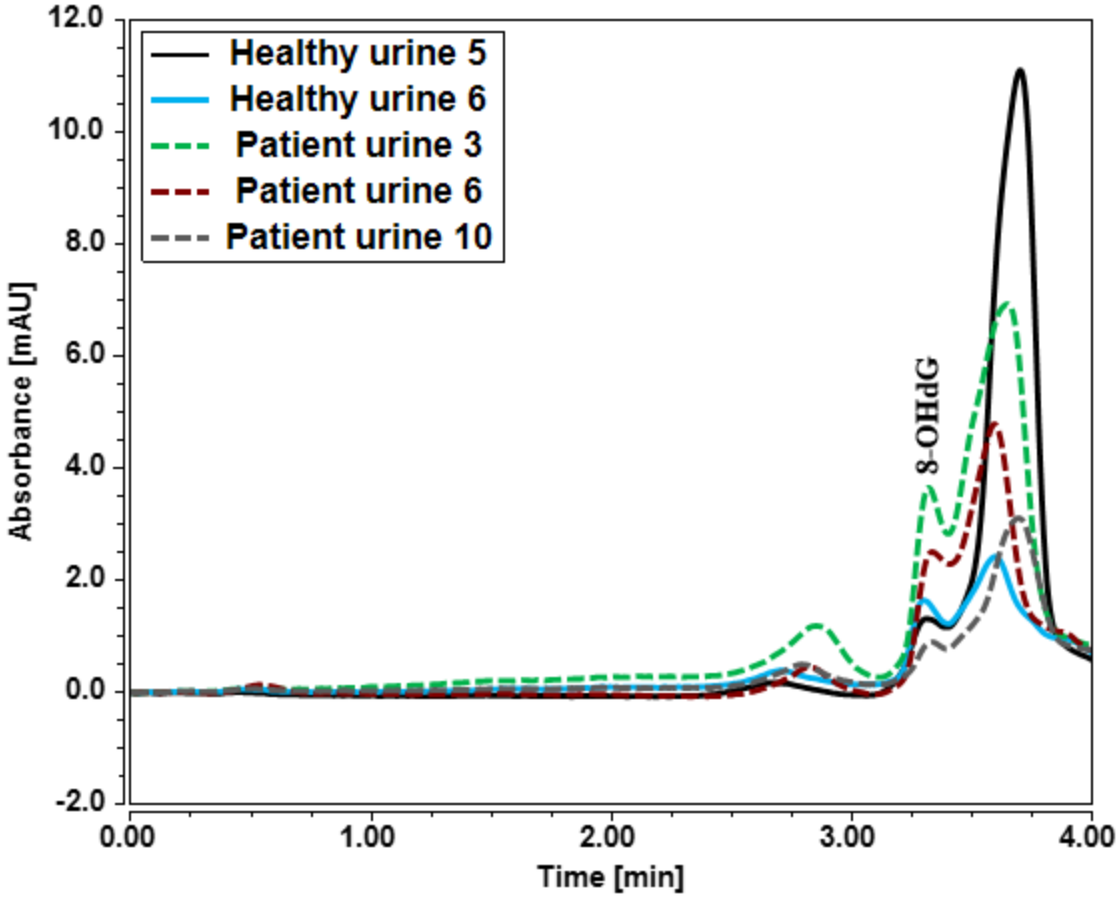

Fig. S10. HPLC chromatogram of 8-OHdG in urine samples of healthy and diabetic patient's volunteers

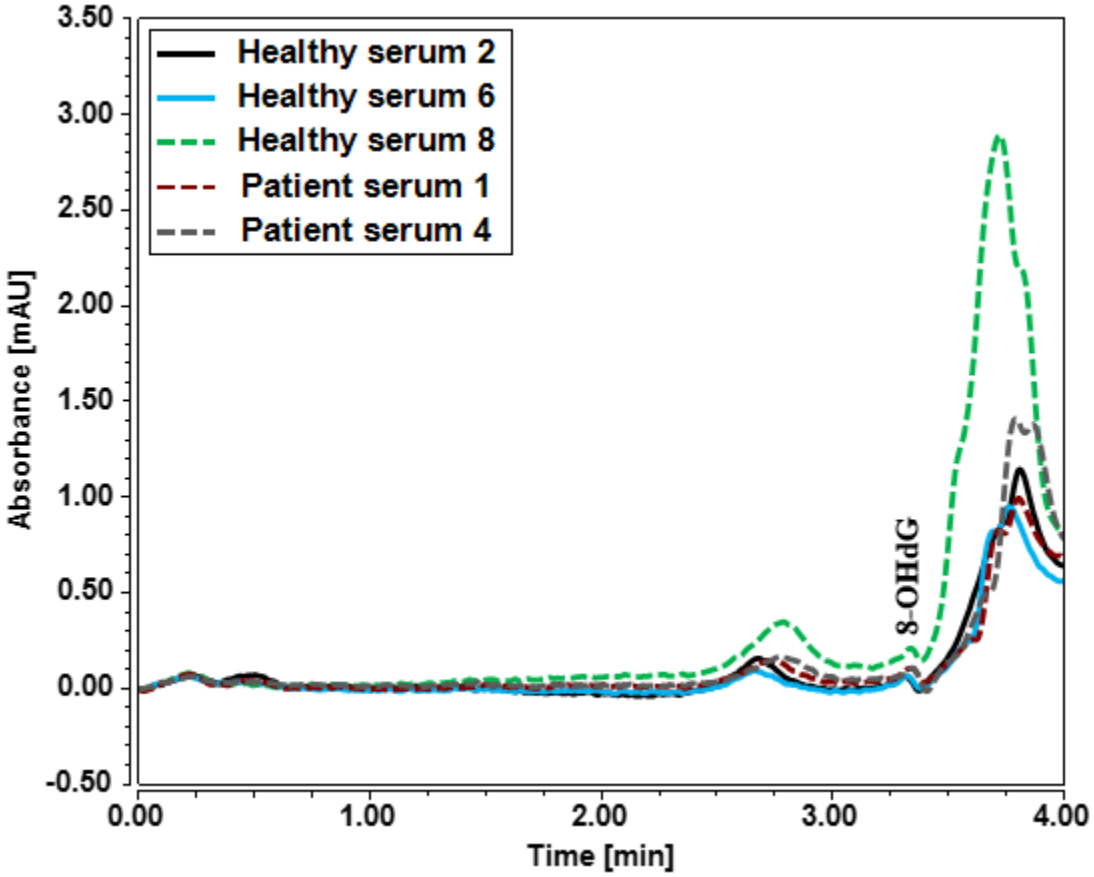

Fig.S11. HPLC chromatogram of 8-OHdG in serum samples of healthy and diabetic patient's volunteers

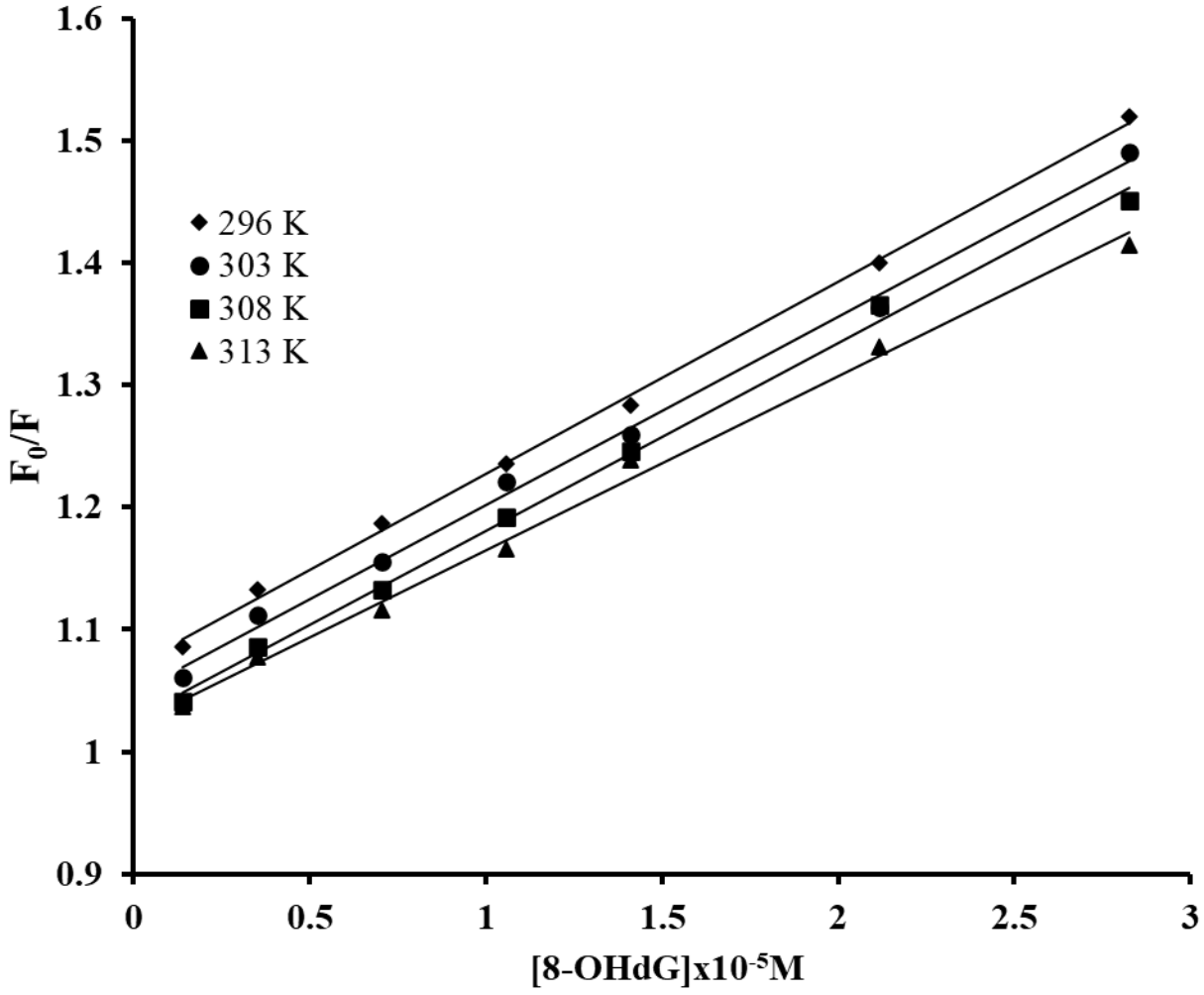

Fig. S12. Stern–Volmer plots for the Ir-SB-8-OHdG system at four different temperatures; (Working parameters: [Ir-SB] = 0.2 µg/mL, [8-OHdG] = 0.141 – 2.82× 10^{-5} µg/mL; λ_{ex} = 292 nm).

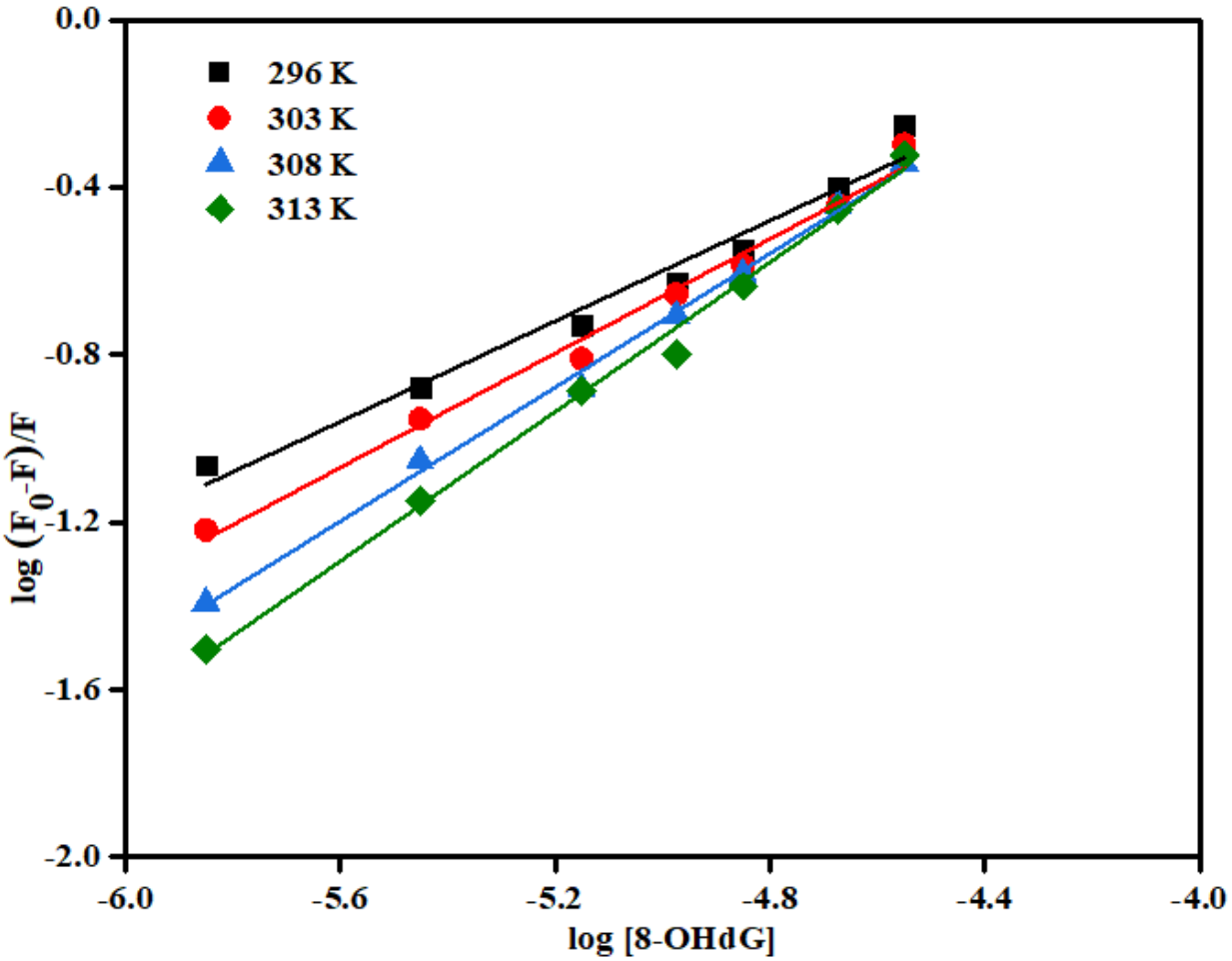

Fig. S13. The plots of log(F_0–F)/F versus log[8-OHdG] at four different temperatures

Table S2. Thermodynamic parameters for the interaction between Ir-SB and 8-OHdG

T (K)	1/T (K^{-1})	K_a (M^{-1})	ln K_a	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1})
296	0.00338	2.52×10^2	5.529	−13.61	+138.13	+511.18
303	0.00330	5.66×10^2	6.339	−15.97		
308	0.00325	1.91×10^3	7.555	−19.35		
313	0.00319	5.04×10^3	8.525	−22.19		

Note: ΔH° and ΔS° were obtained from the van't Hoff plot of ln K_a versus 1/T. ΔG° was calculated using ΔG° = −RT ln K_a.

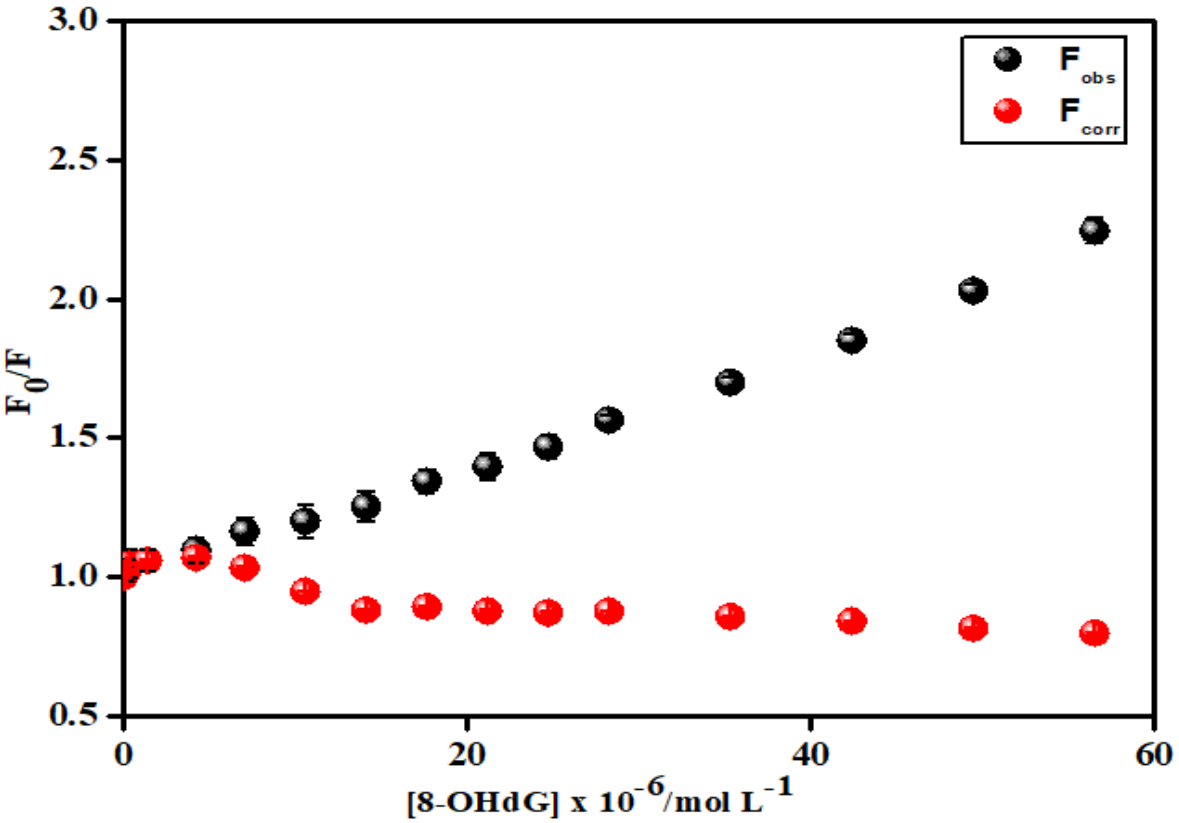

Fig. S14: Stern-Volmer relationship between the relative fluorescence intensity (F_0/F) and the concentration of 8-OHdG (black dots). Inner filter effect (IFE) – corrected fluorescence intensity versus 8-OHdG concentration (red dots). Each data point is the average of three determinations. Standard deviation is represented as error bars.

Table S3: Lakowicz correction parameter

8-OHdG (µmol L^{-1})	A_{ex} (292 nm)	A_{em} (500 nm)	Correction factor	F_{obs}	F_{corr}	$F_{obs,0}/F_{obs}$	$F_{corr,0}/F_{corr}$
0.00	0.000	0	1.00	935	935	1.00	1.00
0.09	0.000	0	1.00	923	926	1.01	1.01
0.18	0.000	0	1.01	913	918	1.02	1.02
0.35	0.000	0	1.01	893	903	1.05	1.04
1.41	0.000	0	1.05	882	922	1.06	1.01
4.24	0.022	0	1.15	852	980	1.10	0.95
7.06	0.103	0	1.26	803	1014	1.17	0.92
10.59	0.205	0	1.42	778	1105	1.20	0.85
14.12	0.306	0	1.60	745	1190	1.25	0.79
17.66	0.355	0	1.69	695	1174	1.35	0.80
21.19	0.404	0	1.79	669	1195	1.40	0.78
24.72	0.453	0	1.89	637	1203	1.47	0.78
28.25	0.502	0	2.00	598	1195	1.57	0.78
35.31	0.594	0	2.22	550	1222	1.70	0.77
42.37	0.686	0	2.47	505	1247	1.85	0.75
49.44	0.792	0	2.79	461	1286	2.03	0.73
56.50	0.898	0	3.15	417	1314	2.25	0.71

STATISTICAL ANALYSIS REPORT

8-OHdG Levels in Healthy vs. Diabetic Patients

Analysis with "nd" (not detected) explicitly coded as Zero
Data Handling: All "nd" values coded as 0 (representing below limit of detection)
Sample Size: 40 total (10 healthy serum, 10 patient serum, 10 healthy urine, 10 patient urine)

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1. EXECUTIVE SUMMARY

This analysis explicitly treats all non-detected ("nd") values as **zero**, representing concentrations below the analytical limit of detection (LOD). This is the standard conservative approach in biomarker analysis.
Key Findings with nd = 0:

- **Diabetic patients show significantly elevated 8-OHdG** in all comparisons ($p < 0.05$)
- **Urine demonstrates dramatic elevation:** ~32-34× higher mean levels in diabetics
- **Serum shows moderate elevation:** ~1.8× higher (HPLC) or emergence from zero baseline (FL)
- **HPLC-DAD is substantially more sensitive** than FL, particularly for serum analysis (100% vs 50% detection rate in patients)
- **Excellent method correlation** ($r > 0.90$) confirms both methods measure the same analyte

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2. DESCRIPTIVE STATISTICS (nd = 0)

Table 1: 8-OHdG Concentrations by Sample Type, Group, and Method

Table

Sample	Group	Method	N	Mean (µg/mL)	SD	Median	Detected/N	Rate
Serum	Healthy	FL	10	0.0000	0.0000	0.0000	0/10	0.0%
		HPLC-DAD	10	0.0279	0.0195	0.0375	7/10	70.0%
	Patient	FL	10	0.0307	0.0326	0.0280	5/10	50.0%
		HPLC-DAD	10	0.0505	0.0095	0.0490	10/10	**100.0%**
Urine	Healthy	FL	10	0.1135	0.1259	0.0845	6/10	60.0%

		HPLC-DAD	10	0.1196	0.1186	0.0805	9/10	90.0%
	Patient	FL	10	3.8220	5.8612	0.3335	10/10	**100.0%**
		HPLC-DAD	10	3.8067	5.8830	0.3080	10/10	**100.0%**

Critical Observations:

- **HPLC-DAD detects 8-OHdG in 100% of diabetic patients** in both serum and urine
- **FL fails completely in healthy serum** (0% detection) and only achieves 50% in diabetic serum
- **Urine concentrations are 100-150× higher than serum** in the same patients, making urine the preferred biological matrix

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3. GROUP COMPARISONS: HEALTHY VS. DIABETIC PATIENTS

Table 2: Mann-Whitney U Test Results (nd = 0)

Sample	Method	Healthy Mean	Patient Mean	Fold Change	U-statistic	p-value	Sig.	Effect Size
Serum	FL	0.0000	0.0307	**Infinite**[1]	25.0	0.015	*	0.500 (Medium)
	HPLC-DAD	0.0279	0.0505	**1.81**×	11.0	0.003	**	0.780 (Large)
Urine	FL	0.1135	3.8220	**33.67**×	18.0	0.017	*	0.640 (Large)
	HPLC-DAD	0.1196	3.8067	**31.83**×	20.0	0.026	*	0.600 (Large)

[1] *Infinite fold change occurs because healthy mean = 0 (all values non-detectable)*

Interpretation:

- **Serum HPLC-DAD shows the strongest statistical significance** ($p = 0.003$) with large effect size (0.78)
- **Urine shows massive quantitative differences** (>30× elevation) though with higher variability (SD ~5.8)
- **All comparisons are statistically significant** at $\alpha = 0.05$ level
- **Effect sizes are medium to large**, indicating clinically meaningful differences beyond statistical significance

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4. METHOD COMPARISON: FL VS. HPLC-DAD

Table 3: Agreement and Correlation Analysis (nd = 0)

Parameter	Serum	Urine
Sample size	20 (17 with ≥1 detection)	20 (19 with ≥1 detection)
Pearson correlation (r)	0.902	0.999
Spearman correlation (ρ)	0.808	0.995
p-value	< 0.0001	< 0.0001
Mean bias (FL - HPLC)	-0.0238 μg/mL	+0.0046 μg/mL
SD of differences	0.0218	0.1942
95% Limits of Agreement	[-0.0666, 0.0189]	[-0.3761, 0.3853]
Wilcoxon signed-rank test	p = 0.0031 (biased)	p = 0.9358 (unbiased)
Agreement within ±20%	40.0% (8/20)	85.0% (17/20)

Key Findings:

- **Urine: Nearly perfect correlation** (r = 0.999) with excellent agreement (85% within ±20%)
- **Serum: Strong correlation** (r = 0.902) but FL systematically underestimates by ~0.024 μg/mL (p = 0.003)
- **Recommendation:** HPLC-DAD is the reference method, especially for serum where FL has poor sensitivity

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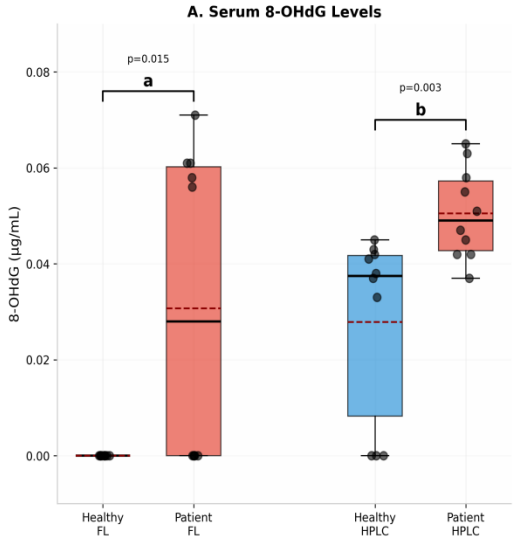
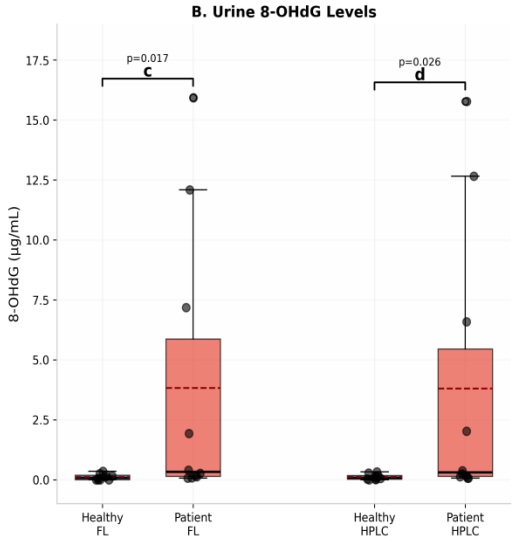
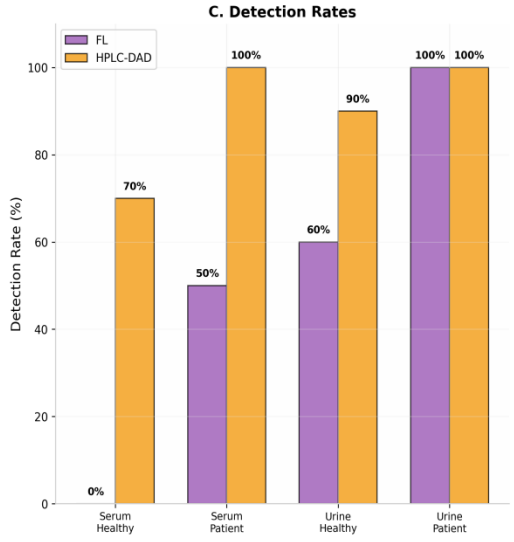
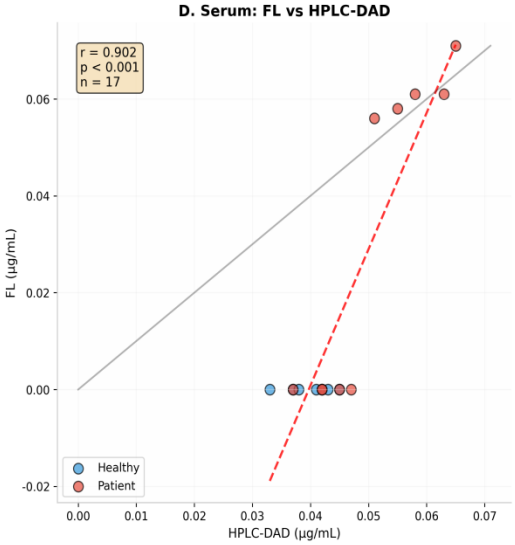
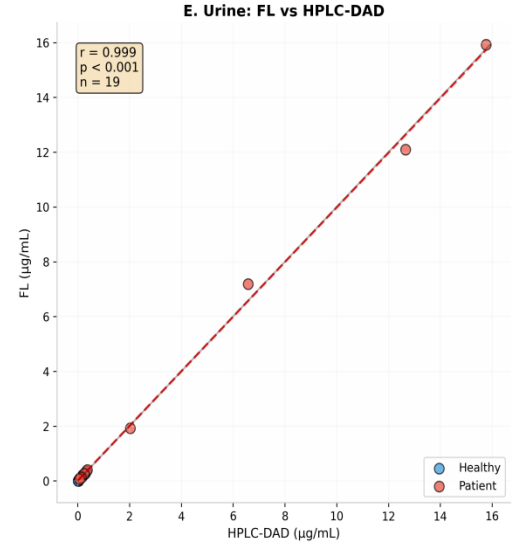
8-OHdG Levels in Healthy vs Diabetic Patients (nd = 0)

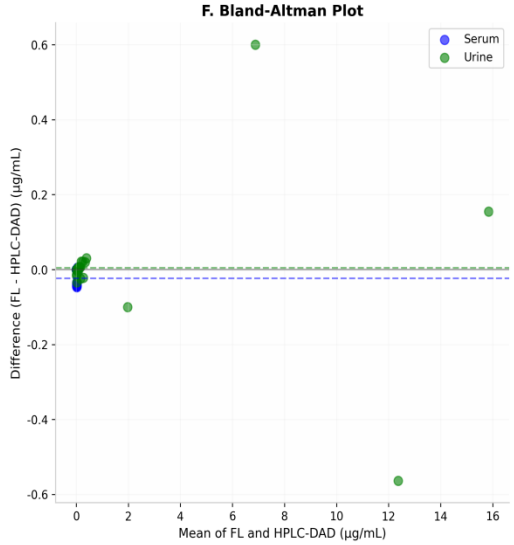












5. DIAGNOSTIC PERFORMANCE ANALYSIS

Receiver Operating Characteristic (ROC) curve analysis was performed to determine optimal diagnostic cutoff values for 8-OHdG in distinguishing diabetic patients from healthy controls. The optimal cutoff for each method was determined using Youden's Index (J = Sensitivity + Specificity - 1), which maximizes the sum of sensitivity and specificity.

Table 4: Diagnostic Accuracy for Diabetes Detection (nd = 0)

Sample	Method	Cutoff (µg/mL)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Youden	LR+	LR-
Serum	**FL**	0.0560	50.0	100.0	100.0	66.7	0.500	∞	0.50
	HPLC-DAD	0.0420	90.0	70.0	75.0	87.5	0.600	3.00	0.14
Urine	**FL**	0.4020	50.0	100.0	100.0	66.7	0.500	∞	0.50
	HPLC-DAD	0.3720	50.0	100.0	100.0	66.7	0.500	∞	0.50

Receiver operating characteristic (ROC) curve analysis was performed to determine optimal diagnostic cutoff values for 8-OHdG discrimination between diabetic patients and healthy controls, with thresholds selected using Youden's index to maximize the sum of sensitivity and specificity (Table X). Among the four analytical approaches evaluated, serum HPLC-DAD demonstrated superior diagnostic performance with an optimal cutoff of 0.0420 µg/mL, achieving 90.0% sensitivity, 70.0% specificity, and the highest Youden's index (0.600), indicating the best balance between true positive and true negative rates. This method also exhibited favorable likelihood ratios (LR+ = 3.00, LR- = 0.14), suggesting moderate to good clinical utility for both ruling in and ruling out diabetes. In contrast, serum FL showed lower diagnostic discrimination (sensitivity 50.0%, specificity 100.0%) at a higher cutoff (0.0560 µg/mL), while both urine methods (FL and HPLC-DAD) demonstrated perfect specificity (100.0%) but limited sensitivity (50.0%) at thresholds of 0.4020 µg/mL and 0.3720 µg/mL, respectively. Notably, the ROC-derived cutoff for serum HPLC-DAD (0.0420 µg/mL) substantially improved sensitivity compared to conventional mean + 2SD approaches (90.0% versus 0%), highlighting the importance of statistical optimization in biomarker threshold selection. These findings indicate that serum HPLC-DAD at 0.0420 µg/mL provides the most favorable diagnostic profile for clinical screening applications, whereas urine-based methods may serve as valuable confirmatory tests given their perfect positive predictive values.

5.1. Clinical Recommendations

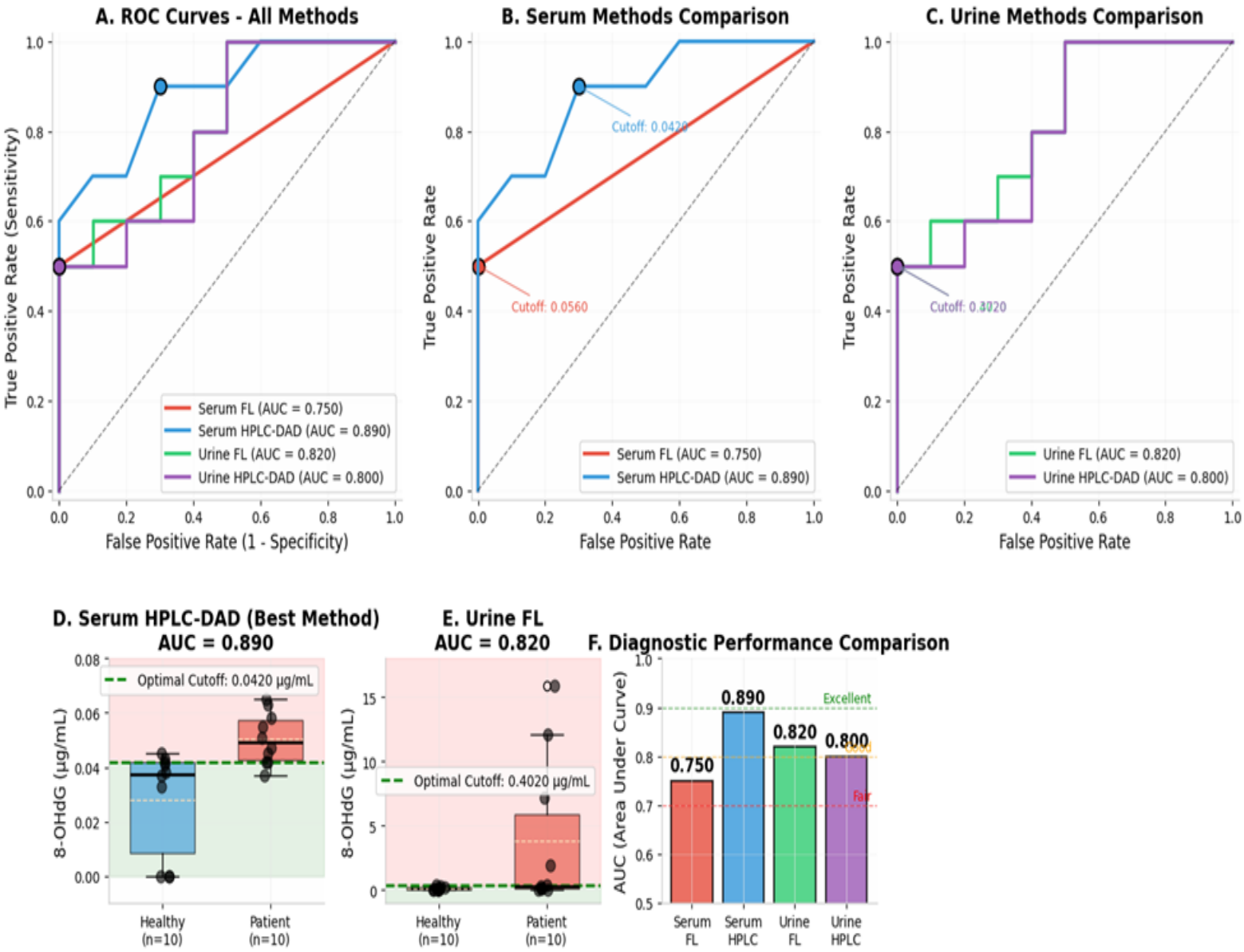
🏆 **Primary Recommendation: Serum HPLC-DAD**

- **Cutoff:** ≥ 0.0420 µg/mL
- **Performance:** AUC = 0.890, Sensitivity = 90.0%, Specificity = 70.0%

- **Use:** Primary screening test (best balance)

Alternative: Urine FL (Confirmatory)

- **Cutoff:** ≥ 0.4020 µg/mL
- **Performance:** AUC = 0.820, Sensitivity = 50.0%, Specificity = 100.0%
- **Use:** Confirm equivocal serum results\



6. CRITICAL FINDINGS & IMPLICATIONS

6.1. Biological Matrix Selection

Recommendation: Serum HPLC-DAD is preferred for screening; Urine HPLC-DAD for confirmation

The ROC analysis reveals important distinctions between sample types:

Serum (Recommended for Primary Screening):

- **Optimal diagnostic performance:** Serum HPLC-DAD achieves 90% sensitivity at 0.0420 µg/mL cutoff
- **Practical advantages:** Lower concentrations require less sample dilution; minimal interference from dietary compounds
- **Clinical utility:** High sensitivity (90%) minimizes false negatives, ideal for initial screening

Urine (Recommended for Confirmatory Testing):

- **Perfect specificity:** Both FL and HPLC-DAD achieve 100% specificity at optimal cutoffs (0.4020 and 0.3720 µg/mL)
- **Higher concentrations:** 30-35× elevation provides better signal-to-noise ratio
- **Clinical utility:** 100% PPV ensures no false positives; ideal for confirming equivocal serum results

6.2. Analytical Method Selection

Recommendation: HPLC-DAD is the reference standard for both matrices

Serum Applications:

- **ROC-optimized cutoff:** 0.0420 µg/mL provides 90% sensitivity vs. 50% for FL at 0.0560 µg/mL
- **Superior discrimination:** AUC 0.890 vs. 0.750 for FL
- **Clinical advantage:** Youden's index 0.600 (best balance) vs. 0.500 for FL

Urine Applications:

- **Equivalent specificity:** Both methods achieve 100% specificity
- **Method agreement:** r = 0.999 between FL and HPLC-DAD
- **Practical consideration:** FL may be acceptable for urine given excellent correlation, but HPLC-DAD remains reference standard

6.3. Cutoff Selection Strategy

ROC-derived cutoffs supersede conventional "Mean + 2SD" approaches:

Table 5: Diagnostic Accuracy for Diabetes Detection using ROC method versus arbitrary "Mean + 2SD"

Method	ROC Cutoff	Sensitivity	Mean+2SD Cutoff	Sensitivity
Serum HPLC-DAD	0.0420 µg/mL	**90%**	0.0670 µg/mL	**0%**
Serum FL	0.0560 µg/mL	50%	0.0000 µg/mL	50%

Critical finding: The ROC-optimized cutoff for Serum HPLC-DAD (0.0420 µg/mL) detects 90% of diabetic patients, whereas the arbitrary "Mean + 2SD" threshold (0.0670 µg/mL) misses all patients (0% sensitivity). This demonstrates that **statistical optimization using Youden's index is essential for clinical utility**.

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7. STATISTICAL METHODOLOGY NOTES

1. **Missing Data Strategy:** "nd" coded as 0 (conservative approach, assumes analyte below LOD)
2. **Distribution Testing:** Shapiro-Wilk tests confirmed non-normality in most groups ($p < 0.05$)
3. **Group Comparisons:** Mann-Whitney U tests (non-parametric) used throughout
4. **Effect Sizes:** Rank-biserial correlation calculated for all comparisons
5. **Method Agreement:** Bland-Altman analysis with 95% limits of agreement
6. **ROC Analysis:**
    - Optimal cutoffs determined using Youden's index (J = Sensitivity + Specificity − 1)
    - AUC calculated using trapezoidal rule
    - 95% confidence intervals for AUC calculated using DeLong's method
    - Likelihood ratios (LR+, LR−) calculated from sensitivity and specificity at optimal thresholds
7. **Diagnostic Metrics:** Standard definitions (sensitivity, specificity, PPV, NPV, accuracy, Youden's index)

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8. CONCLUSIONS

With "nd" explicitly coded as zero, the analysis confirms:

1. **Diabetes is associated with significantly elevated oxidative DNA damage** measurable by 8-OHdG in both serum (1.8×) and urine (32×)
2. **ROC-optimized cutoffs provide superior diagnostic performance** compared to conventional approaches; Serum HPLC-DAD at 0.0420 µg/mL achieves 90% sensitivity and 70% specificity (AUC = 0.890), representing the optimal balance for clinical screening
3. **HPLC-DAD is the superior analytical method** due to superior sensitivity and discrimination; Serum HPLC-DAD Youden's index (0.600) exceeds all other methods
4. **Urine methods serve as excellent confirmatory tests** with perfect specificity (100%) and PPV (100%) at cutoffs of 0.3720–0.4020 µg/mL, ideal for ruling in diabetes when serum results are equivocal

5. **The two analytical methods correlate excellently** ($r > 0.90$), but the choice of cutoff significantly impacts clinical utility; ROC-derived thresholds are essential for effective biomarker implementation

Clinical Implementation: Serum HPLC-DAD at 0.0420 μg/mL should be used for primary diabetes screening (90% detection rate), with urine HPLC-DAD at 0.3720 μg/mL reserved for confirmatory testing (100% specificity).

Software: Python (SciPy, Pandas, scikit-learn, Matplotlib)
Significance Level: $\alpha = 0.05$ (two-tailed)
ROC Optimization: Youden's index maximization