Supporting Information

Analytical Methods

Tip-on-tip micro-solid phase extraction HILIC-LC-MS/MS platform for the determination of urinary methylated nucleosides

C. Mena-Iglesias[a], A. Ballester-Caudet[a], D. García-Gómez[a], M. Bustamante-Rangel[a]*, E. Rodríguez-Gonzalo[a], J.L. Pérez Pavón[a]

[a]Universidad de Salamanca, Department of Analytical Chemistry, Nutrition and Food Science, Faculty of Chemical Sciences, Plaza de los Caídos s/n, Salamanca 37008, Spain

Fax/Phone: +34-923294483

*Corresponding author: E-mail: mbr@usal.es (M. Bustamante-Rangel)

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Section1. Chemical structures and Mass Spectrometric Parameters

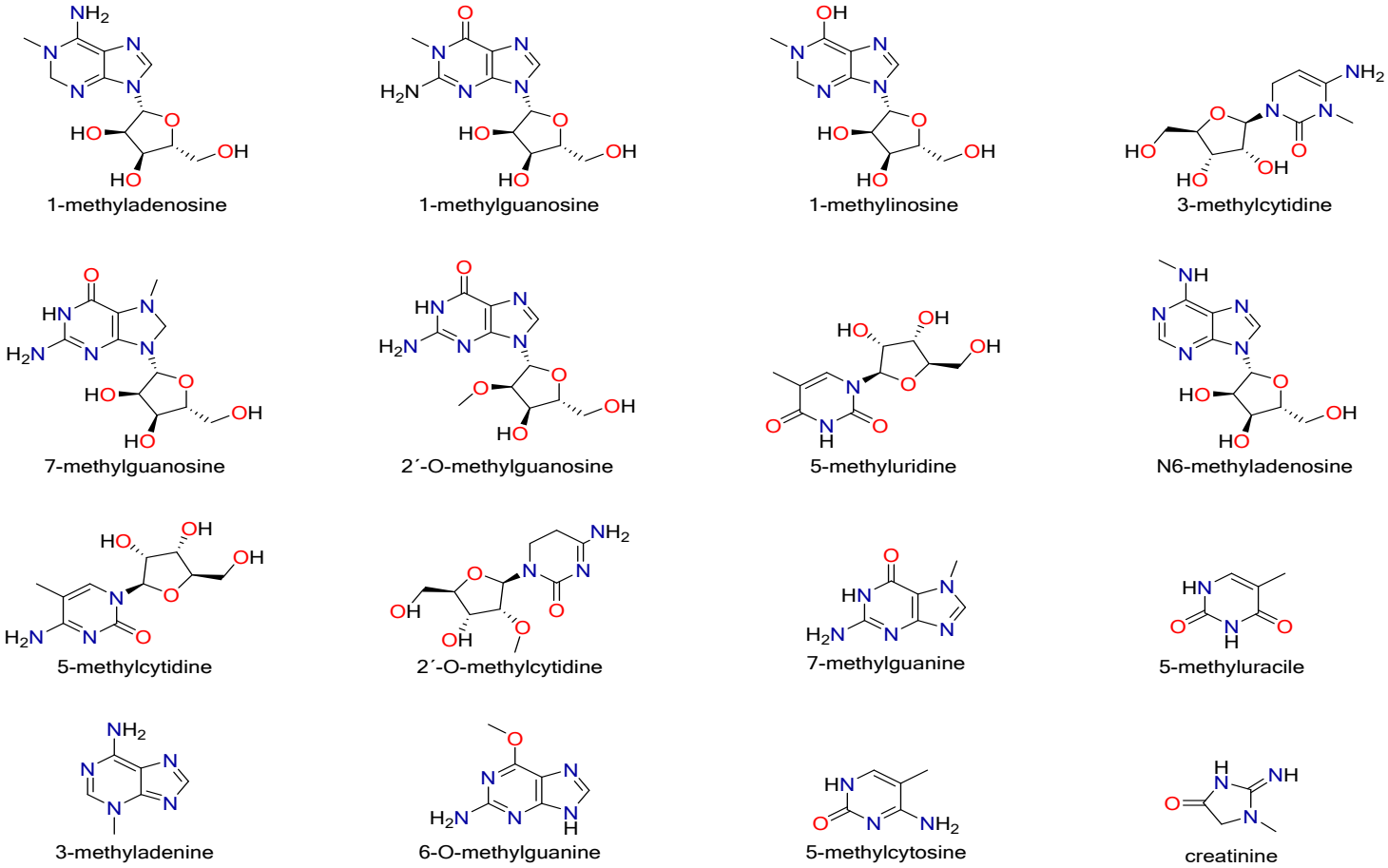

Figure S1. Chemical structures of the targeted methylated nucleosides and creatinine, included in the TOT-μSPE HILIC-MS/MS method developed for the quantification of urinary biomarkers.

Table S1. Optimized mass spectrometric parameters of the studied nucleosides (*Product ion used for quantification).

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision energy (eV)	RT (min)
m^7Gs	299.1	166.1*	81	5	15.1
		41.8		57	
m^1G	298.1	165.6*	81	9	6.5
		134.5		45	
m^1I	283.1	150.6*	81	9	4.8
		282.2		1	
m^6A	282.1	281.3*	121	1	3.8
		93.7		49	
m^1A	282.1	281.3*	121	1	13.6
		132.6		49	
m^5U	259.1	126.6*	81	5	3.3
		55.9		45	
m^3C	258.1	125.6*	81	5	12.5
		94.6		45	
m^5C	258.1	125.6*	81	5	8.9
		82.8		45	
m^7G	166.08	165.6*	121	1	5.2
		41.8		41	
m^6G	166.1	165.6*	121	1	3.1
		66.8		33	
m^3A	150.1	149.6*	121	1	4.2
		41.8		37	
m^5Uc	127.1	126.6*	81	1	2.9
		109.5		13	
m^5Cs	126.1	108.3*	161	17	5.7
		55.8		25	
Cm	258.0	112*	81	45	5.9

		62.8		1	
Gm	298.0	152.2*	81	1	6.2

Section 2. Optimization of chromatographic separation

To evaluate the performance of different stationary phases, several columns with distinct selectivities were tested, including reversed-phase C18, PFP, and HILIC-type columns. Among them, the zwitterionic HILIC column (Z-HILIC, sulfobetaine-modified stationary phase) showed superior performance in terms of retention and resolution of polar nucleosides.

Mobile phase composition was systematically assessed using mixtures of acetonitrile with aqueous ammonium acetate and formic acid solutions under both isocratic and gradient conditions. Gradient elution using acetonitrile and 100 mM ammonium acetate provided optimal separation.

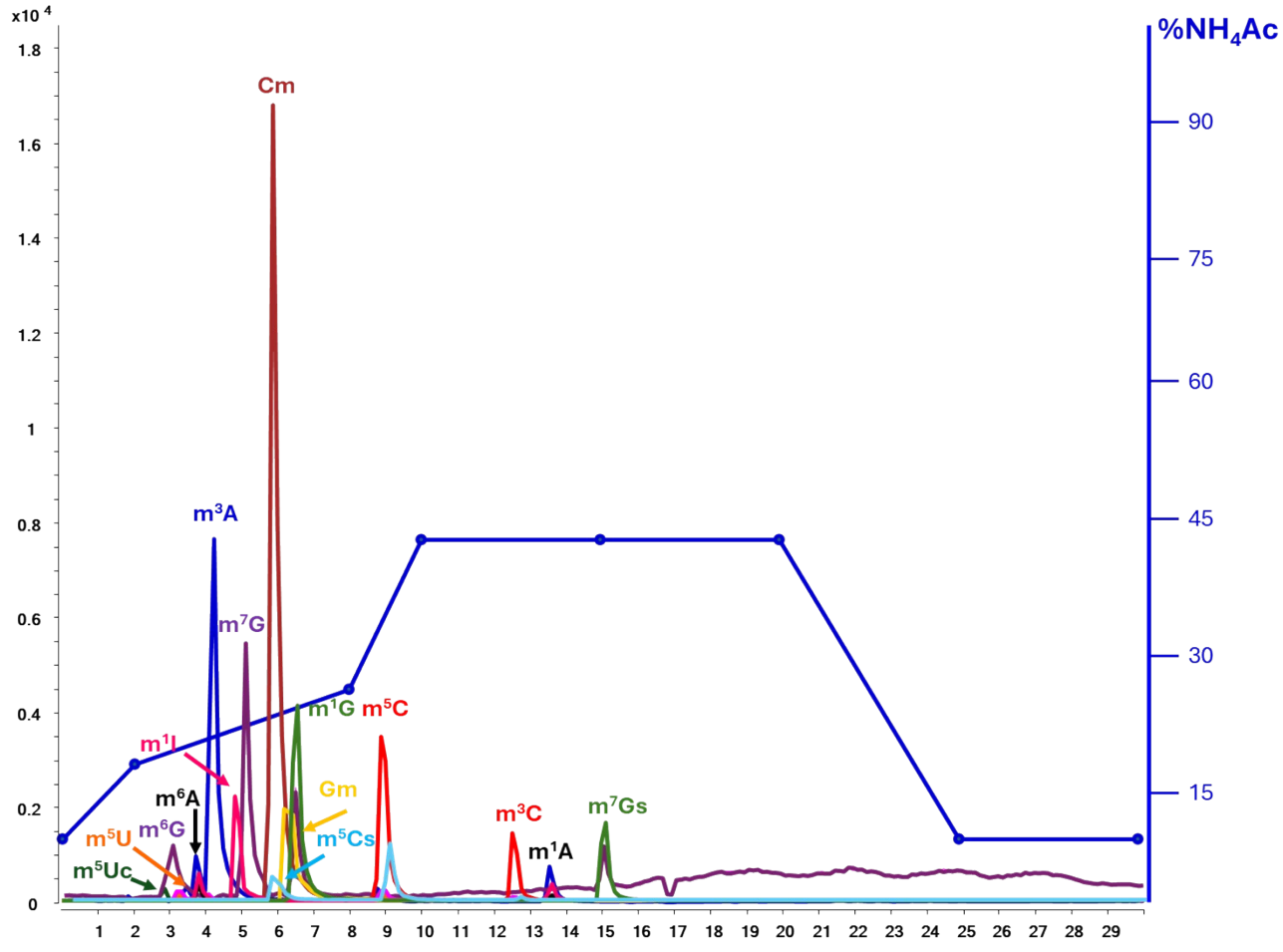

Figure S2. MRM chromatograms of the 15 methylated nucleosides obtained under optimized Z-HILIC conditions using a gradient elution mode.

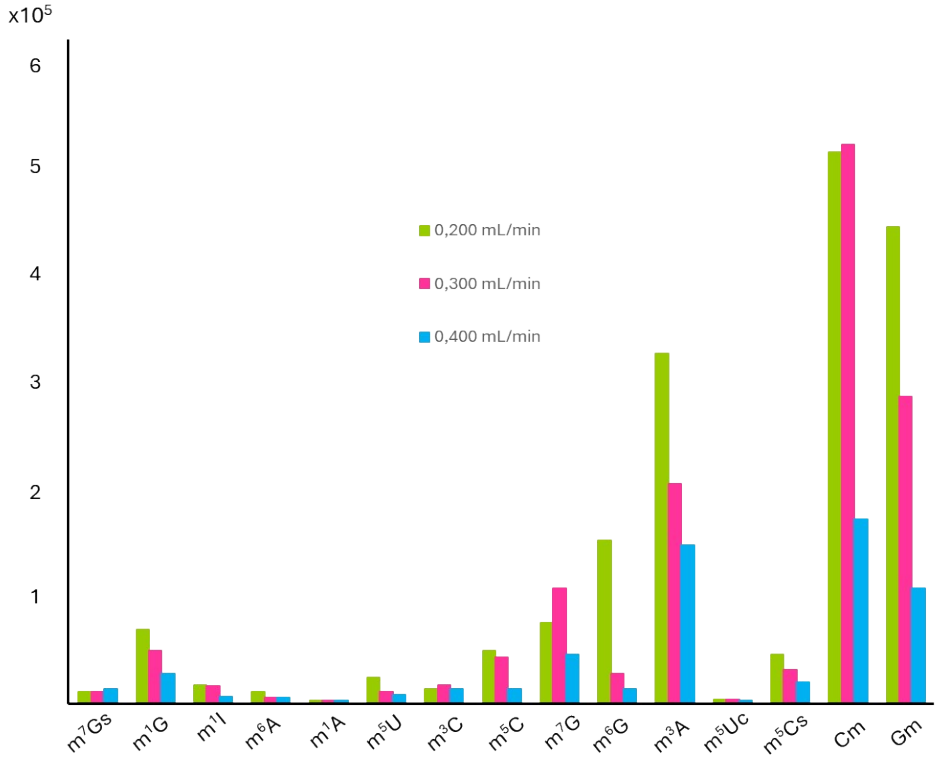

Figure S3. Effect of mobile phase flow rate on signal intensity during chromatographic runs at 0.200, 0.300, and 0.400 mL min^{-1} under identical HILIC-MS/MS conditions.

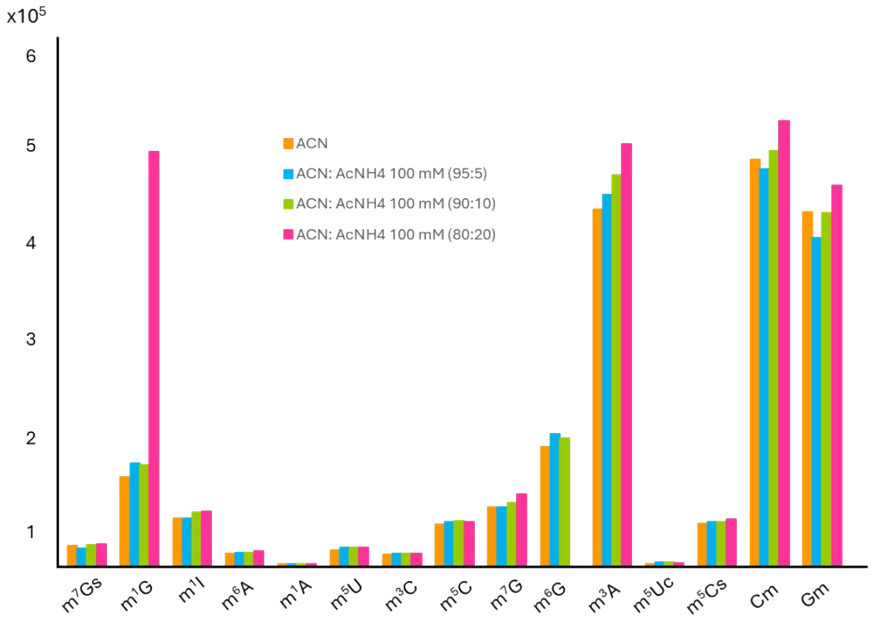

Figure S4. Comparison of signal intensities obtained with different injection media: ACN and ACN:$AcNH_4$ 100 mM at various proportions under identical HILIC-MS/MS conditions.

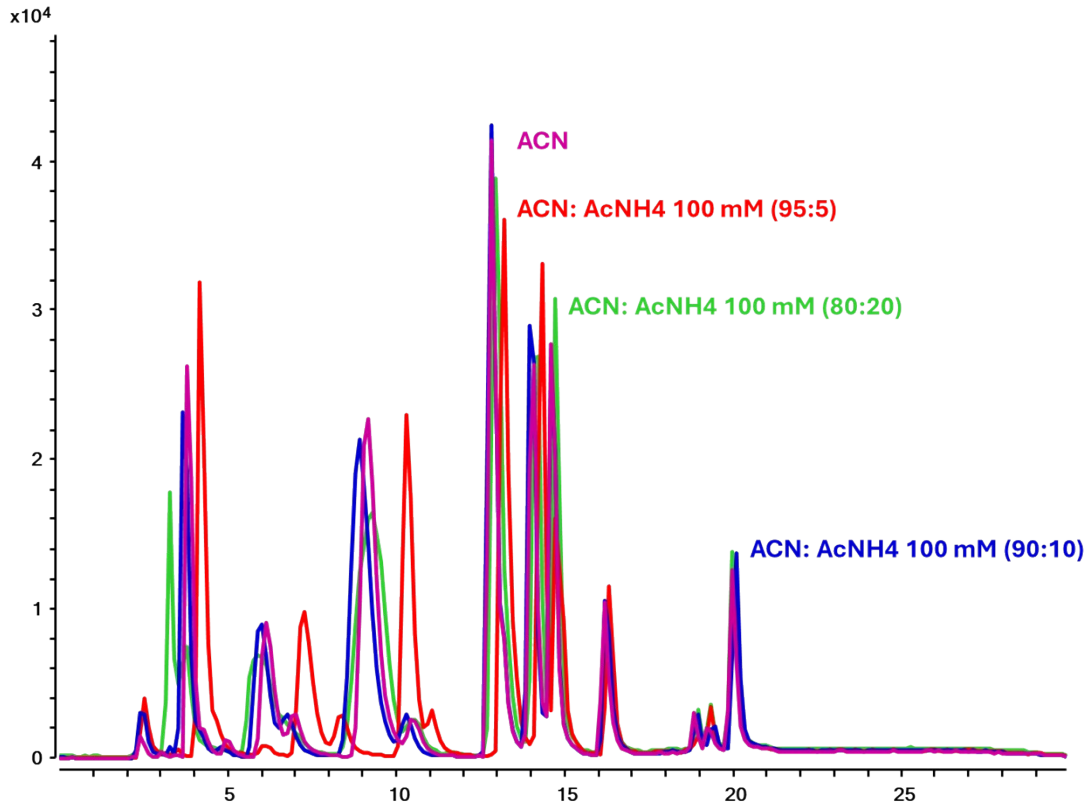

Figure S5. Chromatograms obtained with different proportions of aqueous solvent in the injection medium.

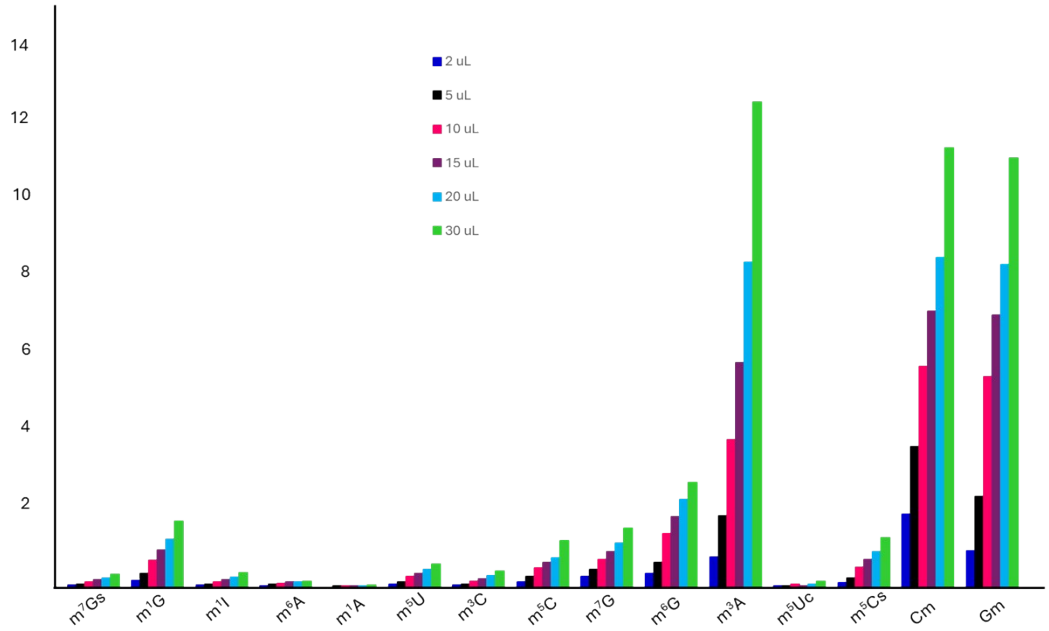

Figure S6. Variation of signal response with increasing injection volume.

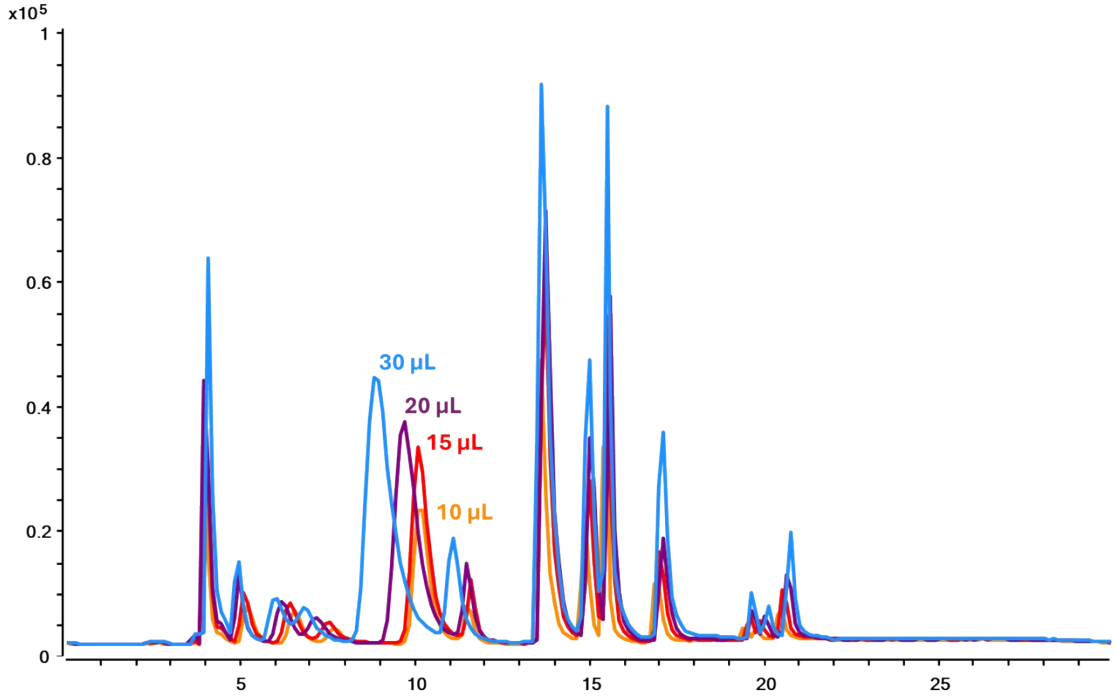

Figure S7. Representative chromatograms showing the effect of increasing injection volume on separation efficiency and peak shape.

Section 3. Optimization of the Tip-on-Tip Micro Solid-Phase Extraction protocol

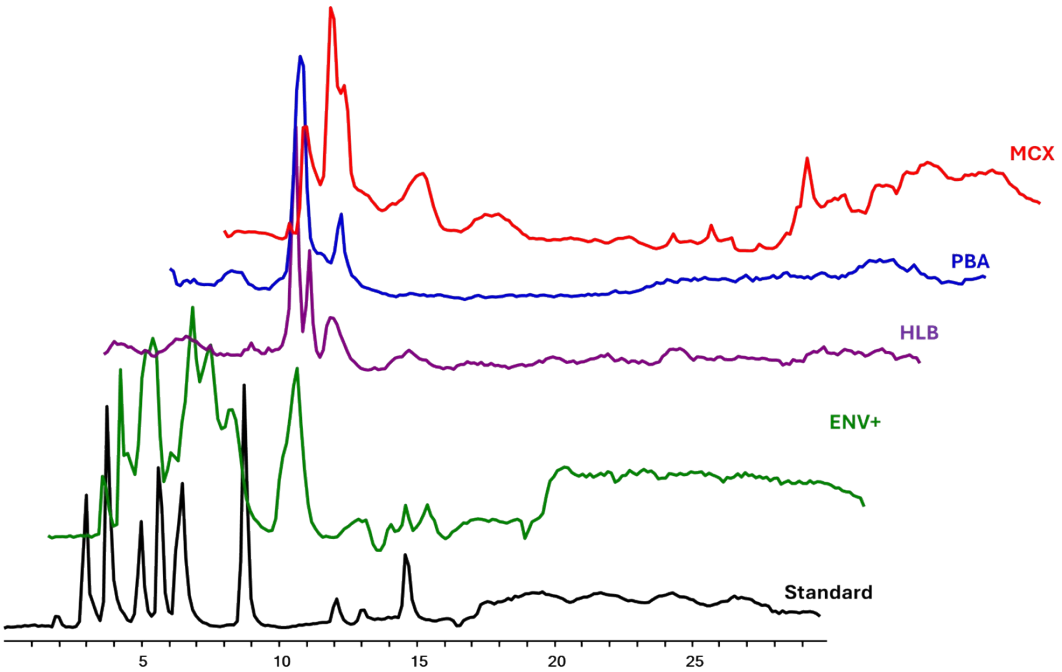

Figure S8. Chromatograms obtained with different sorbent materials in the tip-on-tip micro-SPE configuration.

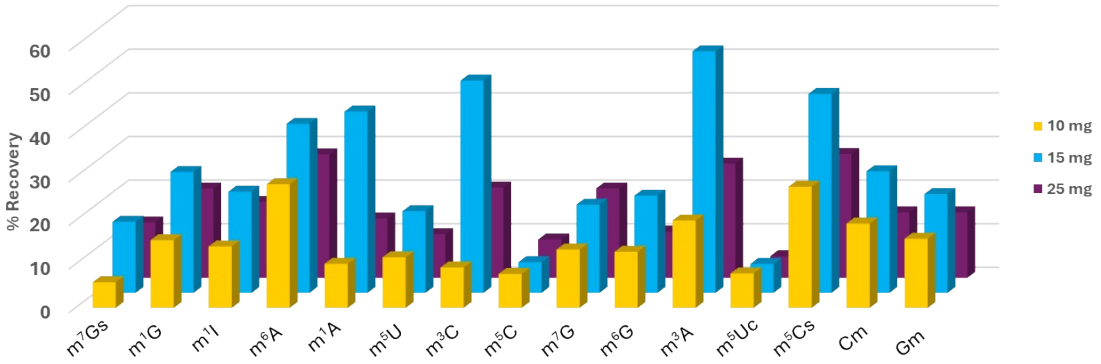

Figure S9. Recovery values (%) of methylated nucleosides obtained with different amounts of ENV+ sorbent.

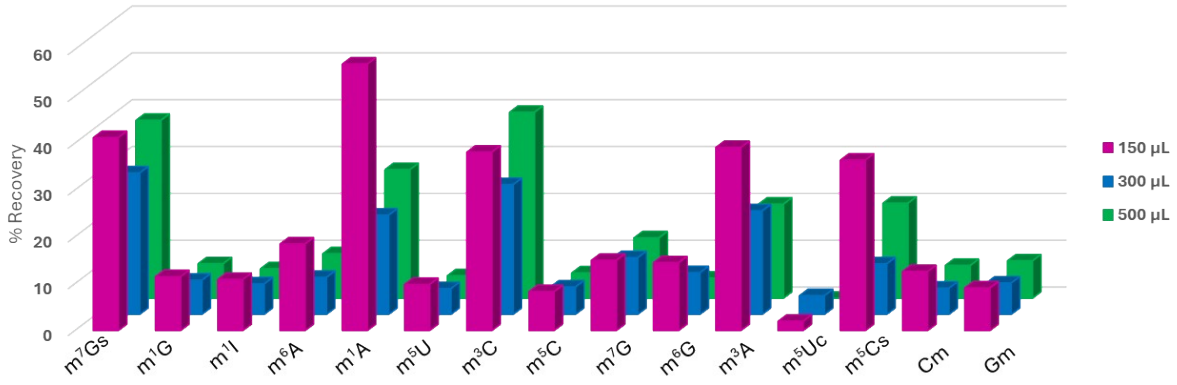

Figure S10. Effect of sample loading volume on the recovery efficiency of methylated nucleosides using the TOT-µSPE.

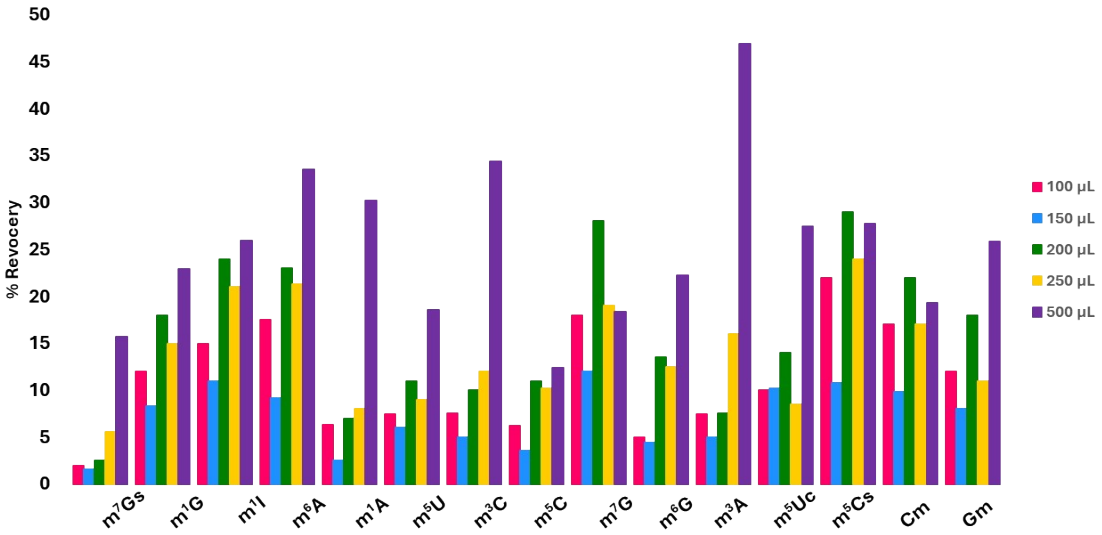

Figure S11. Recovery values obtained with different elution solvent volumes.

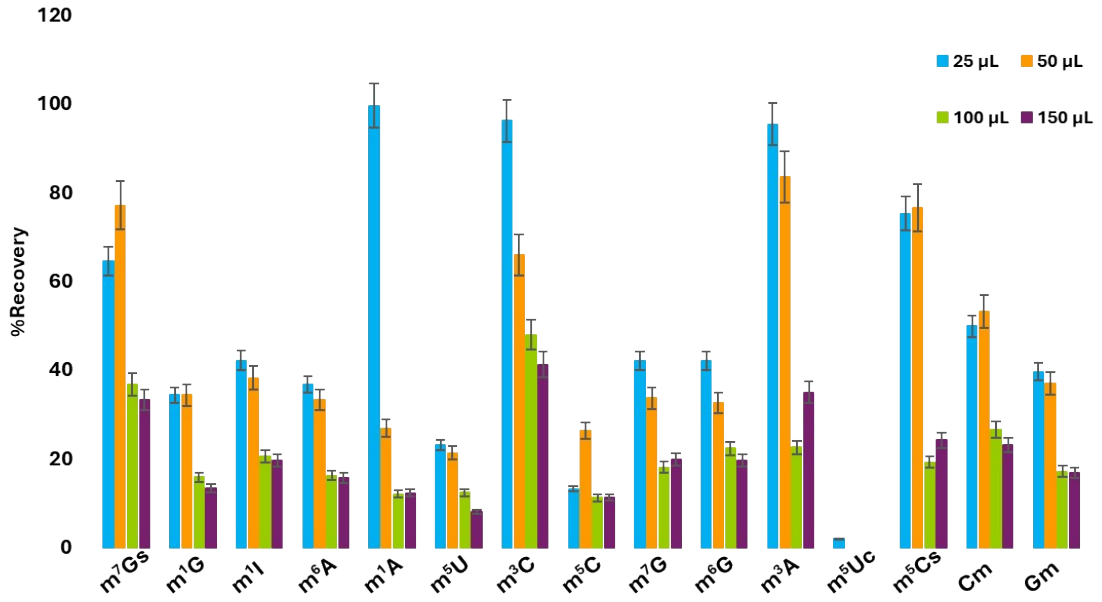

Figure S12. Effect of reconstitution volume on recovery and reproducibility following the elution and evaporation steps of the TOT-µSPE procedure.

Section 4. Quality Control Levels

Three different levels of each analyte were prepared as QC sample. These levels were different for each analyte in each method.

In the dilution method, the concentrations of QC levels were: 10, 40 and 60 µg L^{-1} for m^7Gs, m^6A, m^5U, m^5C, m^3A, m^5Cs and Gm, 50, 300 and 500 µg L^{-1} for m^1G and m^3C, 100, 400 and 600 µg L^{-1} for m^1I, m^1A and m^7G, 150, 600 and 900 µg L^{-1} for m^5Uc and 30, 120 and 180 µg L^{-1} for Cm.

In the TOT-µSPE method, these concentrations were: 200, 400 and 800 µg L^{-1} for m^7Gs, 600, 2400 and 4000 µg L^{-1} for m^1G, 500, 1500 and 3000 µg L^{-1} for m^1I, m^1A, m^3C, m^7G and m^5Uc, 50, 100 and 200 µg L^{-1} for m^6A, m^5U, m^5C and Cm, 150, 300 and 600 µg L^{-1} for m^6G and m^5Cs, 50, 100 and 150 µg L^{-1} for m^3A and 400, 800 and 1200 µg L^{-1} for Gm.