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Rapid LC-MS/MS quantification of cancer related acetylated polyamines in human biofluids

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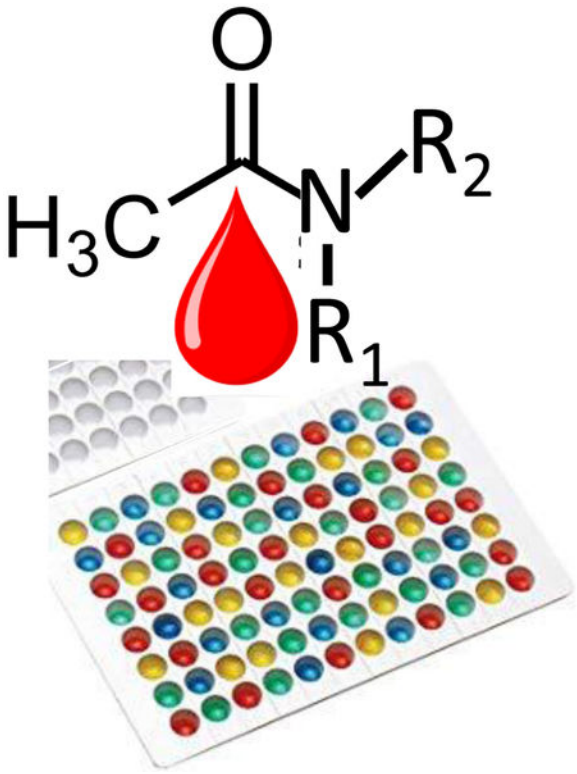
Abstract

Increased urinary acetylated polyamines (APs) are reported as cancer biomarkers in many studies. N^1,N^{12}-diacetylspermine has been proposed as a biomarker indicative of different cancers in urine and plasma. N^1-Acetylspermine has previously been found to be increased in the saliva of patients with breast cancer; however, in plasma this metabolite was too low abundant to be detected by previous analytical methods. In addition, no method has been reported to perform AP analysis on the level of speed, robustness and sensitivity required for daily clinical routines.

Here we describe a high-throughput sample preparation and LC-MS/MS method for the fast, accurate and precise quantification of three APs: N^8-acetylspermidine, N^1-acetylspermine, and N^1,N^{12}-diacetylspermine in plasma, urine and saliva. Stable isotope labeled N^1,N^{12}-diacetylspermine was used as internal standard. Robustness was validated by intra- and inter-day reproducibility. Precision and accuracy of the method were tested at six concentration levels from 0.0375 to 750 ng/mL resulting in less than 15% relative standard deviation and less than 15% percent error in quantification. Using 96-well plates, the assay described herein allows for preparing, analyzing, and quantifying 240 samples per day for a single researcher to quantify three APs commonly related to cancer status.

Graphical abstract:



Keywords

diacetylspermine; acetylated polyamines; polyamines; biomarker; high-throughput; quantitation

1. Introduction

Increased levels of acetylated polyamines (APs) in urine have long been associated with cancer[1]. Specifically, N^1,N^{12}-diacetylspermine (DAS), N^1-acetylspermine (ASP), and N^8-acetylspermidine (ASD) have been found to be elevated in urine of patients with a wide range of cancer types[2-8]. In recent years APs have been found to be elevated in matrices other than urine[9]. For instance, DAS was found to be increased in blood serum of patients 6-12 months prior to lung cancer diagnosis[10]. Similarly, DAS and ASP were found at higher levels in saliva of breast cancer patients[11].

Polyamines have numerous wide-ranging pKa values, and are often at low abundance in common biofluids such as blood plasma and saliva. LC-MS/MS based quantification methods for this class of compounds suffer from relatively wide peaks[10], peak tailing and sample-to-sample carryover. Poor peak shape results in lower signal-to-noise ratios and increased lower limits of quantification (LLOQ), decreasing overall method sensitivity, limiting the type of matrices which can be used, and increasing the amount of sample needed for analysis. Other methods were able to overcome these pitfalls by adding chemical derivatization to the procedure [1, 3, 8, 11]. While chemical derivatization of APs may improve peak shape and sensitivity, it also adds additional steps and a potential source of

error to the process of sample preparation. Enzyme-linked immunosorbent assays (ELISA) are commonly used to quantify DAS in urine[2, 4], but ELISA's are not sensitive enough to quantify concentrations of DAS typically found blood plasma. Furthermore, ELISA's are matrix specific and are marketed only for urinary quantification. The greater abundance of APs in urine, specifically DAS and ASD, certainly contributed to the large number of studies investigating APs in urine in comparison to blood or saliva. Yet, advances in liquid chromatography tandem mass spectrometry (LC-MS/MS) methodology and instrumentation have aided in the study of biofluids containing lower concentrations of APs.

While elevated levels of APs have been associated with a number of cancer types, many cancers types and biofluids have not been characterized. There is a need for a state of the art, high-throughput method capable of accurately and precisely quantifying acetylated polyamines at a wide range of concentrations in a number of diverse biofluids. Here we present a method that is capable of detecting and quantifying APs down to the picomolar range in a fast and precise manner.

2. Experimental

2.1 Chemicals and materials

Reference standards for ASP and ASD were purchased from Sigma-Aldrich (St. Louis, MO). DAS was synthesized in-house and confirmed by liquid chromatography retention time matching and tandem MS/MS spectral matching. Deuterium labeled DAS (DAS-d6) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Formic acid, ammonium formate, LC-MS grade acetonitrile, LC-MS grade water, and LC-MS grade methanol were purchased from Fisher Scientific (Hampton, NH). Chromatographic separation was achieved using an ACQUITY UPLC HSS PFP column (1.8 μm particle size, 2.1 mm, 100 mm) purchased from Waters Corp. (Milford, MA). 1 mL, 96-well extraction plates were purchased from Eppendorf (Hamburg, Germany). 96-well PVDF filter plates were purchased from Agilent Technologies (Santa Clara, CA). 12-[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid (CUDA) was purchased from Cayman Chemical (Ann Arbor, MI). Urine and blood plasma were purchased from BioIVT (Westbury, NY), saliva was provided and used by permission from Dr. Peter Belafsky (UC Davis, M.D, PhD) with appropriate IRB approval.

2.2 Sample extraction

Three commonly assessed biofluids (urine, saliva, and EDTA plasma) were tested and validated using the method described herein. The extraction procedure described below was applied to all three matrices described above. One hundred microliters of sample was added to wells of a 96-well plate. As extraction solvent, a 50/50 mixture of acetonitrile/methanol was spiked with 0.2 ng/mL of DAS-d6 internal standard and cooled to −20°C. 500 μL of this mixture were added to each well via multi-channel pipette to precipitate proteins. Well plates were capped and vortexed for five minutes at speed 6 on a VX-2500 multi-tube vortexer (VWR, Radnor, PA). Samples were subsequently centrifugation for five minutes to pellet proteins. Supernatants were removed and evaporated in a EZ-2 plus centrivap (Genevac, Ipswich, UK). Samples were then re-suspended in 100 μL of 9:1 water:acetonitrile spiked

with 50 ng/mL of CUDA. Plates were capped and vortexed for five minutes and samples were passed through a 0.2 micron polyvinylidene fluoride (PVDF) filter to remove insoluble particulates. Plates were then sealed with aluminum foil via an ALPS 3000 Microplate Sealer (Thermo Scientific, Waltham, MA) and kept at 4°C prior to analysis.

2.3 LC-MS/MS Analysis

A Waters ACQUITY i-class fixed loop ultra-performance liquid chromatography (UPLC) system (Milford, MA) was coupled to a Sciex 6500+ QTRAP (Redwood City, CA) mass spectrometer. Separation of APs was achieved using a Waters ACQUITY high strength silica (HSS) Pentafluorophenyl (PFP) UPLC column (1.8 μm, 2.1 mm × 100 mm) (Milford, MA). Mobile phase "B" consisted of 5% water in acetonitrile, with 0.1% formic acid and 10 mM ammonium formate, mobile phase "A" consisted of water with 0.1% formic acid and 10mM ammonium formate. The flow rate was maintained at 0.4 ml/min and the column compartment was maintained at 45°C. Initial conditions were 10% B, held for one minute, from 1 to 2.2 minutes the gradient uniformly increased until reaching 100% B and was held until 3 minutes. From 3 to 5 minutes the mobile phase composition returned to initial conditions for column re-equilibration.

The mass spectrometer was operated in positive electrospray ionization mode with ion spray voltage set at 5.5 kV, source temperature set at 325°C, and collision gas set to "medium". Multiple reaction monitoring (MRM) mode was used to scan for three metabolites and two internal standards. A full list of transitions, collision energies, declustering potentials, and cell exit potentials can be found in Table 1. Dwell time and entrance potential were 75 milliseconds and 10 volts respectively for all analytes and standards.

2.4 Data processing

Acquired data was processed using Sciex vendor software, MultiQuant version 3.0.2. A 12-point calibration curve was created by 3:1 serial dilution in order to quantify the target analytes. CUDA peak areas were visually inspected to ensure complete injections of desired volumes. A ratio of the analyte's peak area to the DAS-d6 internal standard's peak area was used to construct a calibration curve for each AP target. Calibration curves were all constructed linearly, with the "x" variable weighted as 1/x. Once constructed, the calibration curve was applied to all samples to determine calculated concentrations. The ggplots2 package[12] in R studio[13] was used to create boxplots. All other calculations were performed in Microsoft Excel.

2.5 Method validation

A 4% bovine serum albumin (BSA) in phosphate buffered saline (PBS) solution was used as a blank matrix to test method accuracy and precision with a defined but limited matrix exposure. Polyamine standards were spiked into the BSA solution at six concentrations, ranging from 37.5 pg/mL to 750 ng/mL (ca. 0.1 – 2600 nM), and allowed to equilibrate for 30 minutes at room temperature prior to extraction. BSA samples were extracted and analyzed by the same methods described for plasma samples. Five replicates were extracted and analyzed per concentration. This procedure was repeated over three consecutive days to test inter-day precision and accuracy, giving a total of 90 validation samples analyzed.

Percent relative standard deviation (%RSD), a measure of precision, was calculated as shown in Equation 1, where σ is the standard deviation and μ is the mean.

$$\%\,\mathrm{RSD} = \frac{\sigma}{\mu} * 100\,\% \quad \text{Eq.1}$$

Percent error, a measure of accuracy, was calculated as shown in Equation 2,

$$\text{percent error} = \left| \frac{\#\,\text{experimental} - \#\,\text{actual}}{\#\,\text{actual}} \right| * 100\,\% \quad \text{Eq.2}$$

To validate the precision of the method across a range of common biofluids we analyzed six replicates of each: pooled human blood plasma, human saliva, and human urine.

Extraction recovery was tested by spiking standards into a blank matrix (4% BSA in PBS) at six different concentrations for each analyte with five replicates at each concentration. This was performed by adding known concentrations of each analyte to the blank matrix before extraction and comparing it to the same concentrations added to the blank matrix after extraction.

3. Results and discussion

3.1 Method development

The differences in pKa values adds to the difficulty of assaying APs. ChemAxon's "chemicalize" feature (www.chemicalize.com) predicted the pKa range of all APs in this assay. For example, DAS has six acidic protons at its four nitrogen atoms with predicted pKa values of 4.67, 5.27, 6.69, 7.28, 10.34, and 10.95. To ensure optimal peak shapes, the mobile phase pH should be at least one unit away from any analyte pKa value. We found that adding 0.1% formic acid and 10 mM ammonium formate to our mobile phase gave a pH of 3.35, which was sufficient in preventing multiple peaks or poor peak shapes associated with multiple charge states.

Given the polar nature of APs we anticipated hydrophilic interaction liquid chromatography (HILIC) to be an effective tool. However, our attempts at HILIC-based approaches were hindered by major challenges including high carryover, long run times and wide peak shapes. The most promising HILIC chromatography we found utilized a Waters Atlantis HILIC Silica, 2.1mm × 150 mm, 3 μm particle size (Milford, MA) column, but carryover for all targets, and the peak shape of ASD remained unsatisfying and less reproducible than the final reversed-phase LC method (Supplemental Figure 1).

Given the limitations described above, we next chose to test C18 based columns, but the hydrophobic nature of the columns were unable to retain APs. We chose an alternative approach wherein we used pentafluorophenyl (PFP)-based liquid chromatography. We found the ACQUITY high strength silica (HSS) PFP column able to provide adequate analyte retention while also affording minimal carryover (0.6% by area ratio) and a narrow peak shape (FWHM ≤ 0.07 min) (Figure 1). Using this column we were able to minimize the total

run-time to five minutes including re-equilibration, allowing for high-throughput analysis of 200 samples and an additional 40 quality control, blank or calibration curve injections per 24-hour period. The chromatographic method presented here was ineffective at retaining non-acetylated versions of our targets, spermine and spermidine.

3.2 Method validation

Each target metabolite showed exceptional linearity with R^2 values ≥ 0.99 over a concentration range of about five orders of magnitude (Figure 2). A significant attribute of this method is its accuracy and precision in measuring AP concentrations across a large range which is particularly important for a method designed to assay diverse sample matrices with highly variable AP concentrations. Lower limit of quantification (LLOQ) of all three AP targets was found to be 37.5 pg/mL; injections at 12.5 pg/mL did not yield peaks that were discernible from noise. This LLOQ was found to be sufficiently below the endogenous AP levels found in 100 μl of blood plasma, urine, or saliva with DAS levels ranging from 1 nM (plasma) to 160 nM (urine) (Table 2). Two MRM transitions were quantified for each AP target, the average value was reported for absolute quantification, the individually quantified MRM transitions, and percent difference between the two values, can be found in Supplementary Table 1.

Upon validation, our method showed intra- and interday relative standard deviation and percent error below 15% for all six concentrations tested, including at LLOQ levels (Table 3). A box plot of the percent error of each individual replicate, at all concentrations, for all three targets from the precision and accuracy validation is shown in (Figure 3). Of the 270 individual AP values, 264 of them were quantitated to have an error below 20%, demonstrating a high level of reproducibility of this method.

Recovery ranged from 43-87% for the two mono-acetylated polyamines at all concentrations. Diacetylspermine, however, yielded 55-93% recoveries for the LLOQ concentration and the two highest concentration levels, but surprisingly low recoveries for three concentrations of 20-37% for three mid-point concentration levels (Table 4). Despite low recoveries at some of the DAS concentrations, the precision and accuracy of the method remained superb at those concentrations using five replicate extracts. This recovery paradox illustrates the importance of adding labeled internal standards at the beginning of the sample prep and extraction process, to ensure accurately calculated concentrations.

4. Conclusion

Here we present a high-throughput extraction and LC-MS/MS method capable of preparing and analyzing 200 biofluid samples and 40 quality control samples in a 24 hour period. The proposed method has been shown to be highly reproducible over a wide range of concentrations and a number of commonly researched biofluids. Furthermore, the LLOQ has been shown to be well below normal levels for all targets, making this an ideal method for studies with limited sample volumes. Compared to previous methods, particularly HILC-MS and gas chromatography-MS based methods; the method described here offers a fast, sensitive, and reliable means of measuring three highly researched acetylated polyamines. It can be used in human cohort studies and is well suited for clinical adaptation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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5. References

1. Abdel-Monem MM, Ohno K. Polyamine metabolism III: Urinary acetyl polyamines in human cancer. Journal of pharmaceutical sciences. 1978;67(12):1671–3. [PubMed: 569197]
2. Hiramatsu K, Takahashi K, Yamaguchi T, Matsumoto H, Miyamoto H, Tanaka S, et al. N1,N12-Diacetylspermine as a Sensitive and Specific Novel Marker for Early- and Late-Stage Colorectal and Breast Cancers. Clinical Cancer Research. 2005;11(8):2986–90. doi: 10.1158/1078-0432.ccr-04-2275. [PubMed: 15837752]
3. Seiler N, Graham A, Bartholeyns J. Enhanced Urinary Excretion of N1-Acetylspermidine and the Presence of Tumors. Cancer Research. 1981;41(4):1572–3. [PubMed: 6897929]
4. Umemori Y, Ohe Y, Kuribayashi K, Tsuji N, Nishidate T, Kameshima H, et al. Evaluating the utility of N1,N12-diacetylspermine and N1,N8-diacetylspermidine in urine as tumor markers for breast and colorectal cancers. Clinica Chimica Acta. 2010;411(23):1894–9. doi: 10.1016/j.cca.2010.07.018.
5. Tsuji M, Nakajima T, Sano I. Putrescine, spermidine, N-acetylspermidine and spermine in the urine of patients with leukaemias and tumors. Clinica chimica acta; international journal of clinical chemistry. 1975;59(2):161–7. [PubMed: 1120360]
6. Yamaguchi K, Nagano M, Torada N, Hamasaki N, Kawakita M, Tanaka M. Urine diacetylspermine as a novel tumor marker for pancreatobiliary carcinomas. Rinsho byori The Japanese journal of clinical pathology. 2004;52(4):336–9. [PubMed: 15164602]
7. Nakayama Y, Torigoe T, Minagawa N, Yamaguchi K. The clinical usefulness of urinary N1, N12-diacetylspermine (DiAcSpm) levels as a tumor marker in patients with colorectal cancer. Oncology letters. 2012;3(5):970–4. [PubMed: 22783374]
8. Lee SH, Suh JW, Chung BC, Kim SO. Polyamine profiles in the urine of patients with leukemia. Cancer Letters. 1998;122(1):l–8. doi: 10.1016/S0304-3835(97)00399-6.
9. Fahrmann JF, Hanash SM. N1, N12-Diacetylspermine as a Blood Based Lung Cancer Biomarker. Biochem Anal Biochem. 2016;5(268):2161–1009.1000268.
10. Wikoff WR, Hanash S, DeFelice B, Miyamoto S, Barnett M, Zhao Y, et al. Diacetylspermine is a novel prediagnostic serum biomarker for non–small-cell lung cancer and has additive performance with pro-surfactant protein B. Journal of Clinical Oncology. 2015:JCO. 2015.61. 7779.
11. Tsutsui H, Mochizuki T, Inoue K, Toyama T, Yoshimoto N, Endo Y, et al. High-throughput LC-MS/MS based simultaneous determination of polyamines including N-acetylated forms in human saliva and the diagnostic approach to breast cancer patients. Analytical chemistry. 2013;85(24):11835–42. [PubMed: 24274257]
12. Wickham H ggplot2: Elegant Graphics for Data Analysis: Springer Publishing Company, Incorporated; 2009 216 p.
13. Racine JS. RStudio: A Platform-Independent IDE for R and Sweave. Journal of Applied Econometrics. 2012;27(1):167–72.

Highlights:

- An accurate and precise method of quantifying acetylated polyamines.
- Targets: N^8-acetylspermidine, N^1-acetylspermine, and N^1,N^{12}-diacetylspermine
- A high-throughput LC-MS/MS method capable of analyzing 240 samples per day.
- Validated in three commonly studied biofluids: blood, urine, and plasma
- Lower limit of quantification in the picomolar range for all targets

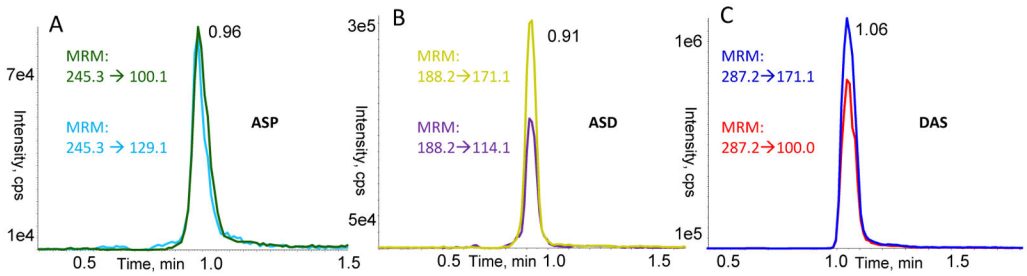

Figure 1.

The chromatographic peaks for all three target metabolite in pooled human saliva. Each target has two MRM transitions. Chromatographic peaks are narrow (FWHM ≤ 0.07 min and show minimal tailing. **A)** Extracted ion chromatogram of two MRM transitions of N^1-acetylspermine (ASP). **B)** Extracted ion chromatogram of two MRM transitions of N^8-acetylspermidine (ASD). **C)** Extracted ion chromatogram of two MRM transitions of N^1,N^{12}-diacetylspermine (DAS).

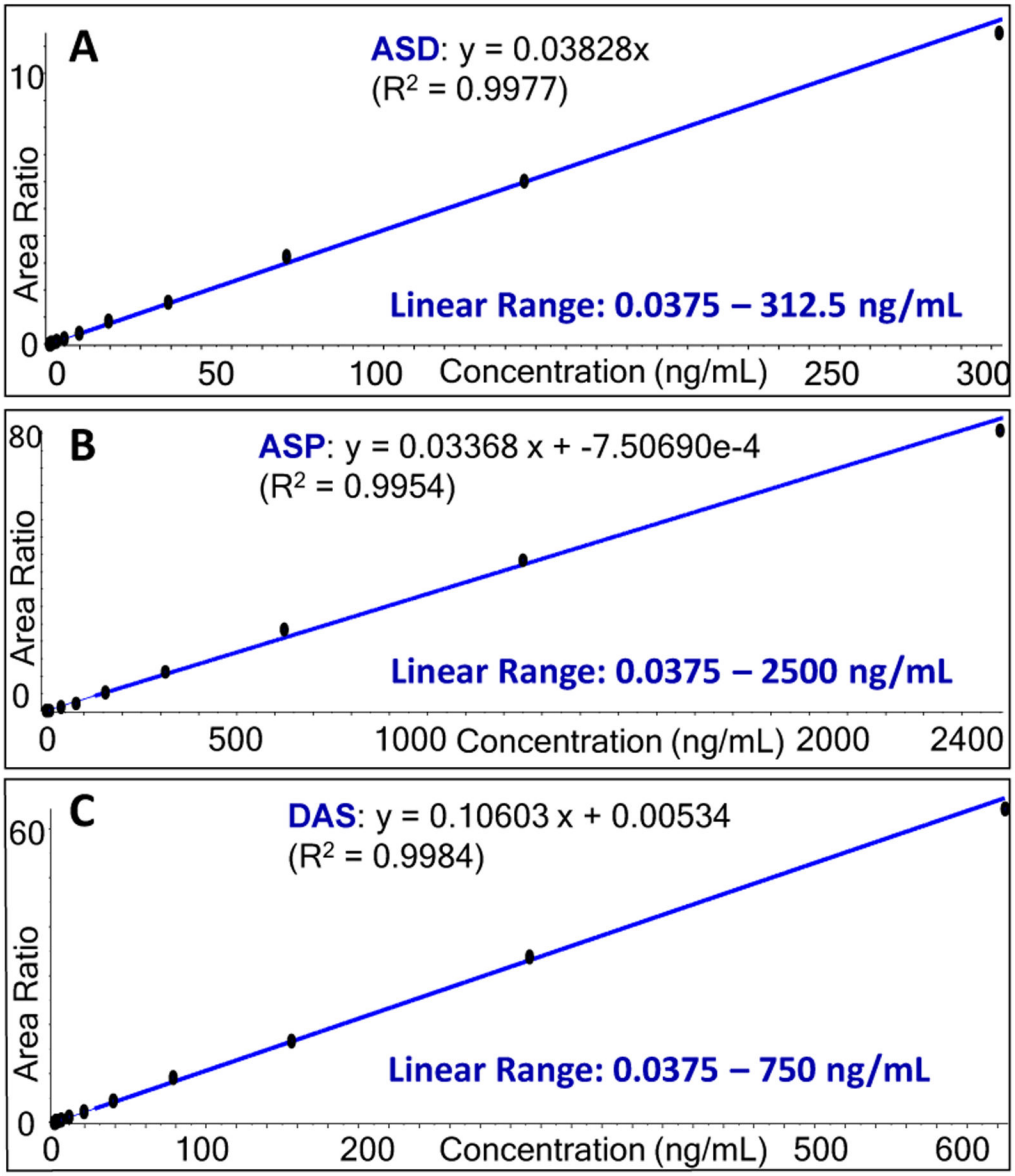

Figure 2.

Each target metabolite showed very good linearity ($R^2 \geq 0.99$) over a large concentration range **A)** Linear range of N^8-acetylspermidine (ASD) from 0.0375–312.5 ng/mL. **B)** Linear range of N^1-acetylspermine (ASP) from 0.0375–2500 ng/mL. **C)** Linear range of N^1,N^{12}-diacetylspermine (DAS) from 0.0375–750 ng/mL.

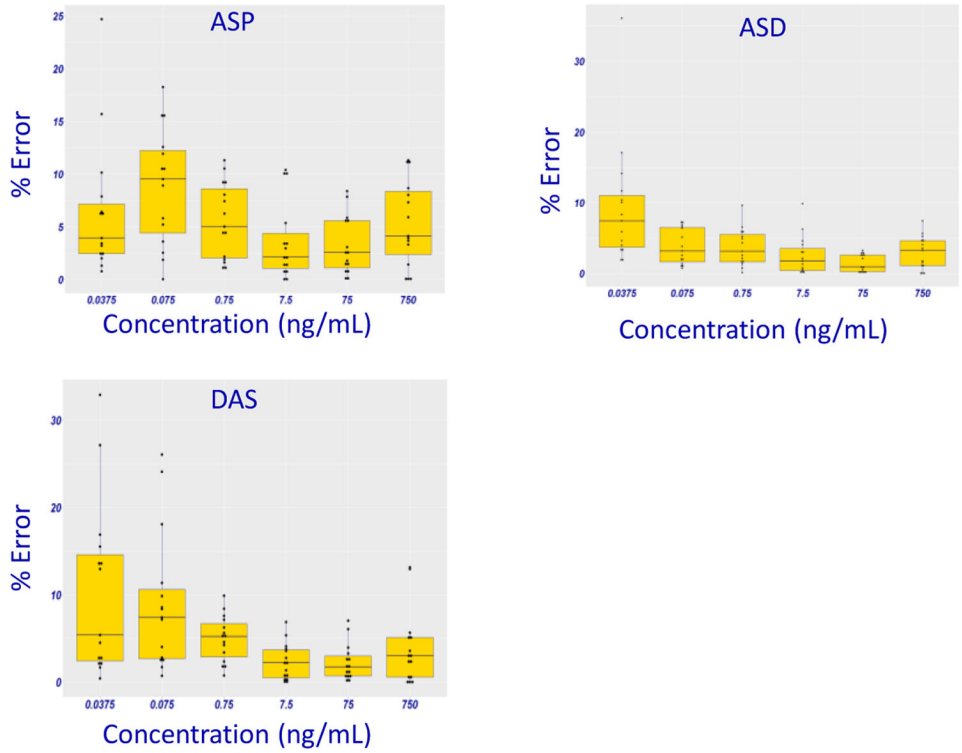

Figure 3.

The absolute percent error (% error) of each individual replicate at six concentrations for each target analyte. Of the 270 individual AP values, only two calculated concentrations yielded a % error greater than 30%. **A)** N^1-acetylspermine had only one of 90 injections with % error greater than 20%. **B)** N^8-acetylspermidine had one of 90 injections with % error greater than 20%. **C)** N^1,N^{12}-diacetylspermine had four of 90 injections with % error greater than 20%.

Table 1.

A complete list of all MRM transitions from the method described herein. Each analyte and class specific internal standard has two MRM transitions. Also included; in order from left to right: retention time (Ret. time), precursor (Q1) and daughter (Q3) ions, declustering potential (DP), collision energy (CE), and cell exit potential (CXP).

Analyte	Abbrev.	Ret. time	Q1 (m/z)	Q3 (m/z)	DP (v)	CE (v)	CXP (v)
N1,N12-	DAS	1.1	287.2	171.1	51	21	18
Diacetylspermine		1.1	287.2	100.0	51	29	12
N1-Acetylspermine	ASP	1.0	245.3	100.1	60	27	4
		1.0	245.3	129.1	60	21	8
N8-Acetylspermidine	ASD	0.9	188.2	171.1	60	21	12
		0.9	188.2	114.1	60	25	18
Standards							
N1,N12-	DAS-d6	1.1	293.3	174.1	56	21	10
Diacetylspermine d6		1.1	293.3	103.0	56	29	12
CUDA	CUDA	2.5	341.3	216.2	155	46	14

Table 2.

Six replicates each of urine, saliva, and plasma were extracted and analyzed by the method described here. The mean of the calculated concentrations for each analyte is shown as well as the relative standard deviations (%RSD). Mean calculated concentrations are the mean of six replicate samples, including the average calculated concentration from both MRM transitions for each target.

Analyte	Matrix	Mean Conc. (ng/mL) n=6	Mean Conc. (nM) n=6	%RSD n=6
DAS	Urine	45.9	160.3	6.1
	Saliva	8.4	29.4	7.8
	Plasma	0.44	1.5	5.1
ASD	Urine	261.8	1399.8	10.7
	Saliva	1.6	8.5	10.1
	Plasma	5.9	31.6	7.5
ASP	Urine	0.63	2.6	9.3
	Saliva	2.4	9.7	14.7
	Plasma	0.13	0.55	11.5

Table 3.

A blank matrix of 4% BSA in PBS was spiked with six different concentrations of N^1,N^{12}-diacetylspermine (DAS), N^1-acetylspermine (ASP), and N^8-acetylspermidine (ASD). Five replicates of each concentration were extracted and analyzed on three consecutive days. Accuracy and precision were calculated for each of three days and also as intra- and inter-day across the three days. Precision (Prec.) was calculated as relative standard deviation, and accuracy (Accu.) was calculated as absolute percent error. Extraction recovery was measured by adding a known concentration of each analyte to a blank matrix (4% BSA in PBS) prior to extraction and comparing it to the same concentration of analyte added to the blank matrix post-extraction.

Analyte	Actual Conc. (ng/mL)	Experimental Concentration [Mean (SD)]	Day 1 Accu. (%) n=5	Day 1 Prec. (%) n=5	Day 2 Accu. (%) n=5	Day 2 Prec. (%) n=5	Day 3 Accu. (%) n=5	Day 3 Prec. (%) n=5	Interday Accuracy (%)	Interday Precision (%)
DAS	0.0375	0.0399 (0.0054)	1.7	8.1	2.8	12.3	14.0	14.0	6.2	12.3
	0.075	0.070 (0.008)	0.2	7.8	4.1	5.3	14.5	14.6	6.3	11.1
	0.75	0.77 (0.04)	3.6	3.1	6.6	2.0	3.7	3.9	2.2	5.2
	7.5	7.48 (0.24)	0.7	2.6	1.2	2.4	0.9	4.4	0.3	3.2
	75	76.0 (2.14)	1.1	3.6	0.7	1.8	2.0	3.2	1.3	2.8
	750	764 (40.77)	1.6	3.3	1.2	3.0	6.1	6.3	1.9	5.3
ASD	0.0375	0.0391 (0.0056)	1.0	11.4	0.4	10.7	10.8	12.8	3.4	12.1
	0.075	0.075 (0.004)	0.2	5.2	0.5	5.4	0.7	4.4	0.1	4.7
	0.75	0.75 (0.035)	5.2	2.8	0.4	1.6	4.7	2.0	0.3	4.6
	7.5	7.4 (0.28)	1.2	3.8	0.2	1.5	3.4	4.3	0.7	3.7
	75	75.0 (1.46)	1.0	1.9	0.7	1.6	0.4	2.2	0.0	1.9
	750	748 (28.54)	3.1	3.7	0.5	2.0	1.8	4.1	0.3	3.8
ASP	0.0375	0.0375 (0.0035)	2.8	4.6	2.5	10.1	5.5	10.7	0.1	9.2
	0.075	0.078 (0.007)	6.8	12.1	5.1	6.2	1.3	9.6	4.4	9.2
	0.75	0.73 (0.047)	2.8	3.4	3.7	5.8	7.1	5.8	2.7	6.4
	7.5	7.4 (0.38)	0.7	5.7	4.6	6.0	0.3	2.0	1.4	5.1
	75	74.7 (3.22)	0.8	3.1	1.9	2.6	1.6	6.2	0.4	4.3
	750	719 (40.47)	5.2	3.8	0.3	4.6	7.6	5.6	4.1	5.6

Table 4.

A blank matrix of 4% BSA in PBS was spiked with six different concentrations of N^1,N^{12}-diacetylspermine (DAS), N^1-acetylspermine (ASP), and N^8-acetylspermidine (ASD). Extraction recovery was measured by adding a known concentration of each analyte to a blank matrix (4% BSA in PBS) prior to extraction and comparing it to the same concentration of analyte added to the blank matrix post-extraction.

Analyte	Spiked Conc. (ng/mL)	Extraction Recovery (%)
DAS	0.0375	54.5
	0.075	28.4
	0.75	20.4
	7.5	37.4
	75	93.1
	750	82.9
ASD	0.0375	43.2
	0.075	82.2
	0.75	79.6
	7.5	87.4
	75	79.2
	750	79.0
ASP	0.0375	82.3
	0.075	82.1
	0.75	58.4
	7.5	53.0
	75	45.0
	750	44.0