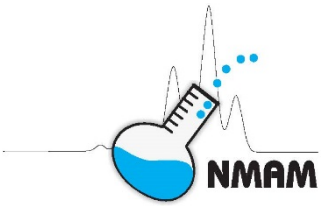


BACKUP DATA REPORT
NIOSH Method No. 8318

Title: 2,5-Hexanedione in urine

Analyte: 2,5 Hexanedione

Author/developer: Corey Downs, James Perkins

Date: November 9, 2015

2,5-HEXANEDIONE in urine: Back-up Data Report

INTRODUCTION AND BACKGROUND

n-Hexane is not a normal constituent of the environment although it can be present in gasoline, paint, glue, and ink. In the workplace, n-hexane is generally present as a vapor where its major form of absorption is via inhalation—although a significant dermal absorption of the liquid is possible as well. Prolonged n-hexane exposure can lead to neural diseases such as peripheral neuropathy [1]. Consequently, monitoring exposure to n-hexane, through urinalysis, continues to be an important part of exposure assessment. A measurement of n-hexane exposure is possible by monitoring the urinary levels of its major metabolite, 2,5-hexanedione [2]. In fact, the neurotoxicity of n-hexane seems to stem from 2,5-hexanedione and its ability to interact with ε-amino groups of lysine in proteins [3]. The production of 2,5-hexanedione is not completely specific to n-hexane exposure; 2,5-hexanedione is also a metabolite of methyl n-butyl ketone. It also has been shown that n-hexane metabolism is inhibited by coexposure to toluene and methyl ethyl ketone [4].

The method for analyzing 2,5-hexanedione evaluated in this report is based on an acid hydrolysis and liquid extraction method of Iwata et al. [4], and Fedtke and Bolt [5], as described by Kawai et al. [6]. Acid hydrolysis can cause more than a tenfold increase in the amount of 2,5-hexanedione detected. n-Hexane is metabolized into several intermediates besides 2,5-hexanedione. Most of these intermediates undergo further transformation and are excreted as glucuronide conjugates. 2,5-Hexanedione, however, is not conjugated; it is referred to as the “free” amount of 2,5-hexanedione. Acid hydrolysis releases n-hexane metabolic intermediates from their conjugation but also converts some of the now freed intermediates (most notably 4,5-dihydroxy-2-hexanone) into 2,5-hexanedione [7]. The conversion of n-hexane intermediates into 2,5-hexanedione, via acid hydrolysis, significantly increases the amount of 2,5-hexanedione detected. The “free” amount of 2,5-hexanedione plus the extra amount gained from conversion of intermediates is deemed the “total” amount of 2,5-hexanedione. This method measures the “total” amount of 2,5-hexanedione.

The reactions for freeing n-hexane intermediates and converting them to 2,5-hexanedione are pH dependent. A pH of ≤ 1 is critical for driving these reactions to completion and thus producing consistent and reproducible results when trying to quantify

the “total” amount of 2,5-hexanedione present in a sample [4]. Acid hydrolysis can also cause an elevated 2,5-hexanedione background level in pooled urine blanks.

Work by Kawai et al. [6] suggests that acid hydrolysis may not be necessary and that more accurate results are obtained when only “free” 2,5-hexanedione is measured by skipping the hydrolysis step. They found a much closer correlation between n-hexane exposure by inhalation (as measured by personal samplers) and 2,5-hexanedione in urine when the acid hydrolysis step was omitted rather than included (correlation coefficient of 0.867 vs. 0.344). Furthermore, the background level of pooled urine blanks was zero in the absence of acid hydrolysis. Because of these findings, guidelines published by various organizations have taken different approaches.

The Biological Exposure Indices (BEI) Committee of the American Conference of Governmental Industrial Hygienists (ACGIH) recommends the Kawai approach and sets the BEI at 0.4 mg/L of “free” 2,5-hexanedione measured without acid hydrolysis [8]. The BEI change from “total” to “free” took place in 2002-2003, shortly after the development of this method. European organizations have continued to use an acid hydrolysis approach but with some modifications. The German recommendation for the biological tolerance value (BAT) set forth by the Deutsche Forschunggemeinschaft (DFG) is to perform an acid hydrolysis and then add the levels of 2,5-hexanedione and 4,5-dihydroxy-2-hexanone. This combined concentration should not exceed 3.5 mg/L [9]. The Swiss (SUVA – Swiss National Accident Insurance Fund) take a similar approach by adding the amounts of 2,5-hexanedione and 4,5-dihydroxy-2-hexanone after hydrolysis, but sets their recommended level at 5 mg/L [10]. Recently the Japan Society for Occupational Health published its recommendation and included values for 2,5-hexanedione both with (3 mg/g creatinine) and without (0.3 mg/g creatinine) hydrolysis [11].

Work by Tondel et al. [12] has recently looked at 2,5-hexanedione levels in the general population. Their studies of 227 persons nonoccupationally exposed to n-hexane showed that the mean urinary concentration for women was 0.36 mg/L and for men was 0.45 mg/L. More studies need to be performed to determine if this is environmental exposure or endogenous production, but background levels should be expected and there are statistical differences noted by gender and by age.

NOTE: Proper safety precautions should always be taken when dealing with any chemical but especially when working with biological fluids such as urine. Manipulating biological samples poses a serious health risk because of the potential transmittance of infectious diseases including hepatitis and HIV. Lab coats, goggles, and gloves (powder-free latex or nitrile) must be worn at all times. Work should be performed in an isolated hood where possible. All waste is required by law to be disposed of in a properly-labeled, autoclavable container.

REAGENTS AND MATERIALS

Presented in Table 1 is the list of reagents and solvents used for this method and its evaluation. 2-Methyl-3-heptanone was selected for use as an internal standard to normalize

TABLE 1. LIST OF CHEMICALS

Chemical	Vendor	CAS #	Purity	Lot #
2,5-Hexanedione	Aldrich	110-13-4	98%	TI 016622 LI
2-Methyl-3-heptanone	Aldrich	13019-20-0	99%	MO 08128 LO
Methanol	Burdick & Jackson	67-56-1	HPLC	BY967
Dichloromethane	Burdick & Jackson	75-09-2	HPLC	CD197
HCl Conc. (36-38%)	JT Baker	7647-01-0	Trace Metal	H39036
Uri-Sub (Synthetic Urine)	CST Technologies, Inc.			US080901Z

values of 2,5-hexanedione determined in the urine samples. Hydrochloric acid was utilized during the acid hydrolysis and methanol was used while preparing the primary stock solutions. Both synthetic and pooled urine were used during the study. Many methods developed for determination of urinary metabolites utilize water as the base matrix due to possible interferences during method development. DCL decided to begin experiments using synthetic urine to more closely study the possible matrix effects of urine and to minimize biological exposure during the early part of the study. Actual urine used in the study was collected from volunteer employees at DataChem Laboratories in Salt Lake City. The urine was collected and pooled as needed. Once pooled, it was stored at 4 °C in 1-L polyethylene screw-top bottles. The stock synthetic urine was always stored at room temperature.

Preparation of Primary Stock and Extraction Solutions

To make the primary stock solutions, 10-mL volumetric flasks were tared on an analytical balance. With a syringe, an appropriate amount of 2,5-hexanedione (25-250 μL) was added to each flask while noting the increase in mass. The contents of the 10-mL volumetric flasks were brought to volume with methanol, mixed, and transferred to a 13 x 100 mm glass culture tube with a Teflon-lined cap. The solution was stored at 4 °C in the dark until needed. This produced stock solutions containing a range of 2-20 mg/mL of 2,5-hexanedione. Depending on the desired range needed for the study, dilutions were made from the appropriate primary stock solution.

The extraction solution was made by adding 5 mg of 2-methyl-3-heptanone into enough dichloromethane to make a total volume of 1 L. This concentration of internal standard in extraction solution was employed because it provided good peak shape, peak area, and reproducibility for the internal standard.

SAMPLE PREPARATION AND INSTRUMENT CONDITIONS

Sample Preparation

For each study, the samples and standards were always prepared in the same manner. Exactly 5.0 mL of urine, synthetic or pooled, was transferred into a 13 x 100 mm glass culture tube. Utilizing a magnetic stirrer and stir bar, the pH of the sample was adjusted to between 0.5 and 1.0 by adding HCl. As noted in the introduction, precise control of the pH is critical for consistent results. The tube was then capped and heated in a water bath for 30 minutes at 100 °C. After cooling, 2 mL of the dichloromethane extraction solution, containing the internal standard (5 μg/mL), was added to the tube and the sample was shaken vigorously for about one minute. After the phases separated, the samples were spun lightly in a centrifuge to aid the separation. A portion of the dichloromethane extraction solution (bottom layer) was transferred to a 2-mL GC autosampler vial for analysis.

Instrument Conditions

All of the samples and standards were run on the same system with the same set of conditions. The system consisted of an HP5890 Series II gas chromatograph equipped with a flame ionization detector and an autosampler. The column was a polyethylene glycol fused silica capillary column (DB-WAX, 30 m x 0.32 mm I.D., 0.50 μm film). Figure 1 shows the

temperature program that was utilized. For dirtier samples, the final hold time was extended to bake-out the column. The head pressure was maintained at 15 psi. The carrier gas consisted of pre-purified helium and the FID was supplied with pre-purified hydrogen and filtered air. The injector and detector temperatures were 200 °C and 250 °C, respectively. A 3-μL aliquot of the dichloromethane extract was injected for each sample. The injection conditions were splitless and the purge was delayed for 0.7 minutes. Table 2 shows the retention times of the analytes given these conditions. The final temperature (220 °C) was maintained until the column appeared to be clean in order to avoid any carryover into the next sample injection.

FIGURE 1. TEMPERATURE PROGRAM

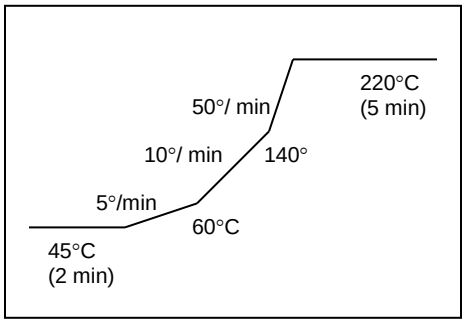


TABLE 2. ANALYTE RETENTION TIMES

Analyte	Retention Time (min)
2-Methyl-3-heptanone	4.73
2,5-Hexanedione	10.55

Calibration

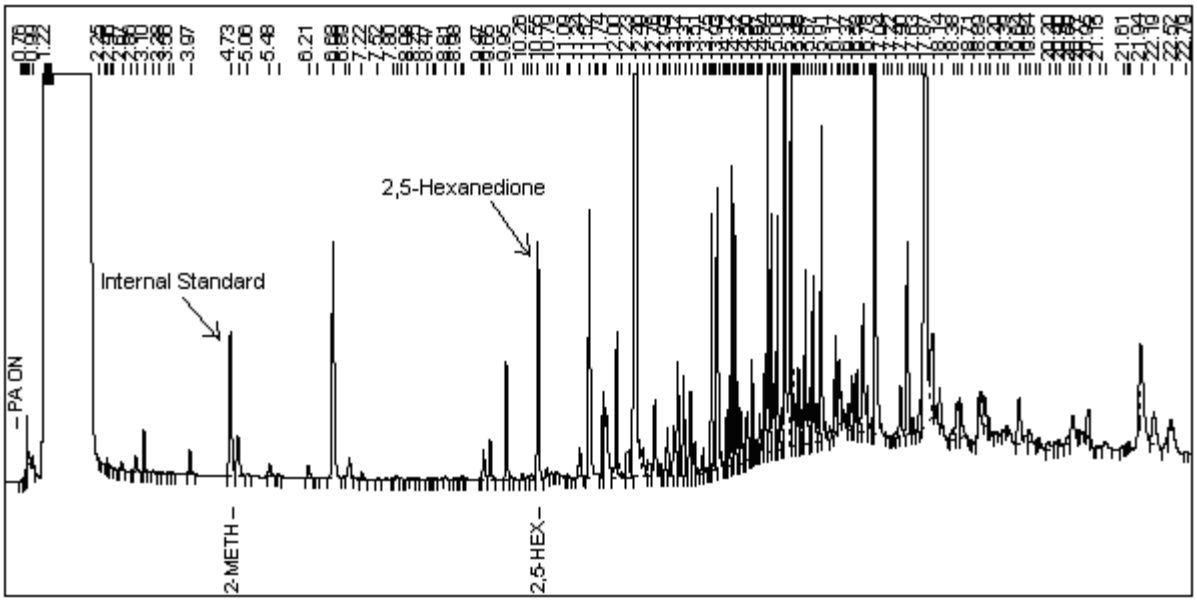
To quantify the 2,5-hexanedione present in samples, a calibration curve was constructed daily with at least six working standards covering the anticipated concentration range of the samples. The working standards were prepared by diluting a known amount of 2,5-hexanedione stock solution into enough pooled urine to make a total of 5.0 mL for each standard. Along with the working standards, at least one pooled urine blank was prepared by transferring 5.0 mL of pooled urine (the same pooled urine used for creating the working standards) into a culture tube. The 5 mL aliquot of each working standard and pooled urine blank was then processed using the same procedure as described previously.

After analyzing the samples, a calibration graph was generated by plotting, for each working standard, the normalized analyte response (peak area of analyte divided by the peak area of the internal standard on the same chromatogram) on the y-axis vs. mg of analyte/L of urine on the x-axis. A linear or quadratic model was utilized in processing the working standard data depending on which model provided a better fit to the data. Because acid hydrolysis would create detectable amounts of 2,5-hexanedione in pooled urine blanks, before plotting the calibration graph it was often necessary to subtract the normalized analyte response of the pooled urine blank from the normalized analyte response of each working standard. The normalized analyte response was then calculated for each sample and the corresponding 2,5-hexanedione concentration was read from the x-axis of the calibration curve (mg/L of urine). If desired, the samples can be further normalized by using their creatinine concentrations.

Chromatogram Comparison/Analysis

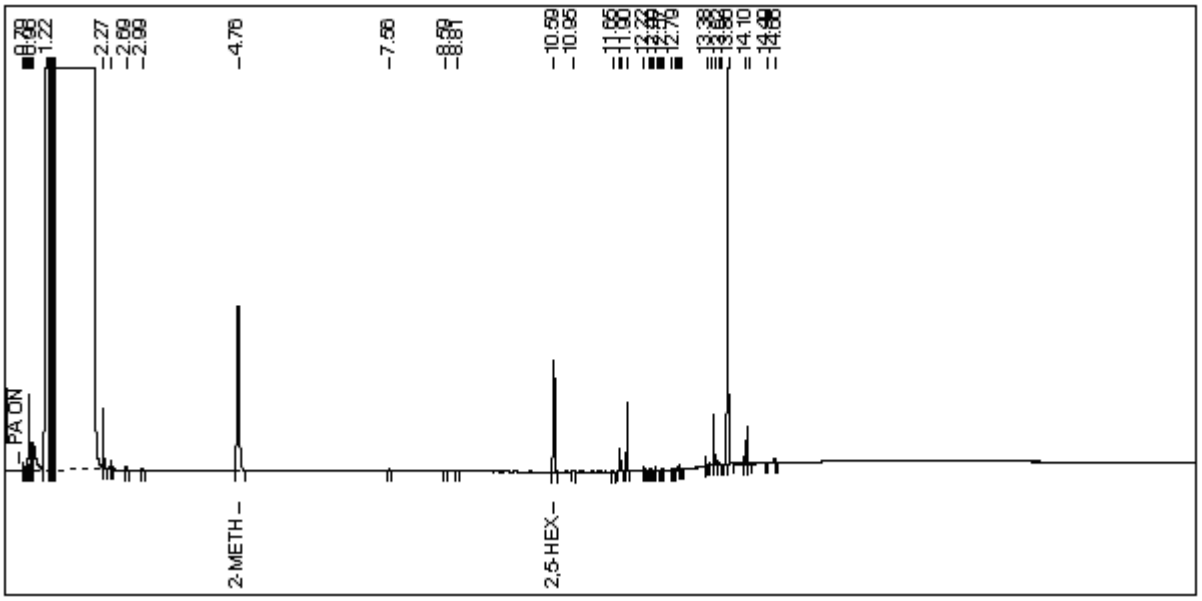
Figures 2 and 3 display typical chromatograms when the previously described conditions are employed with pooled and synthetic urine, respectively. Although, as the figures show, the final analyte elutes after 10 to 11 minutes, an additional 10 to 15 minutes at a high temperature is required to purge the remaining compounds and prevent buildup in the column. 2-Methyl-3-heptanone was chosen as the internal standard because of its availability and because its retention time positioned it in a relatively interference-free portion of the chromatogram of the urine sample. It may appear that the initial temperature program ramp begins too slowly and that higher initial temperatures and ramps would decrease the analysis time. Although this may be the case, it is not recommended. Field samples tended to be quite

FIGURE 2. TYPICAL CHROMATOGRAM (POOLED URINE)



variable. A more aggressive temperature program caused overlapping and interfering peaks in some samples. As expected, the synthetic urine chromatogram contains fewer peaks and the baseline is more stable. Using synthetic urine for making standards allowed much lower levels of the analyte to be quantified as addressed in the studies that follow.

FIGURE 3. TYPICAL CHROMATOGRAM (SYNTHETIC URINE)



Synthetic urine appears to be a viable alternative to pooled urine when preparing standards and quality control samples. Some advantages of using synthetic urine are,

- being less of a health hazard because it is not a biological sample
- allowing for a 40X decrease in the LOD
- eliminating the need for blank subtractions
- producing comparable percent recoveries.

Using pooled urine, however, is advantageous because it most closely resembles the matrix of the field samples and may produce the most accurate results. Ultimately, it may not matter what type of urine is used for standards. The method calls for a comparison of a subject's 2,5-hexandione levels before and after exposure. The method is interested in detecting a relative increase between the two samples. Because the increase is relative, as long as the same type of urine is used as standards for both the before and after samples, the relative result should be the same no matter what type of standards are employed.

LIMIT OF DETECTION AND QUANTITATION STUDY

This study was to determine the limit of detection (LOD) of the method based upon both synthetic urine and pooled control urine spiked with 2,5-hexanedione. The limit of quantitation (LOQ) for both was then calculated as 10/3 times the LOD. The resulting LOQ was then used to determine the target concentrations for all subsequent steps in the method evaluation.

Synthetic Urine Study

A range of standards for analysis was prepared in duplicate by diluting the primary stock solution (described in Reagents and Materials section) and spiking the appropriate volume into 5 mL of synthetic urine. Table 3 lists the range of concentrations used during the study. The samples were then prepared and analyzed as described in Sample Preparation and Instrument Conditions section. The LOD and LOQ were estimated by fitting the data to a quadratic curve followed by applying Burkart's Method to the data [13]. The correlation coefficient was 0.997 for the quadratic curve. The data provided an LOD estimate of 0.005 μg/mL. This equates to a LOQ of 0.02 μg/mL.

TABLE 3. SYNTHETIC URINE STANDARDS

Standard	[2,5-HD] μg/mL
7	0.1183
8	0.0475
9	0.0238
10	0.0190
11	0.0143
12	0.0095
13	0.0048

Pooled Urine Study

For the pooled control urine, a range of solutions for analysis was prepared in duplicate by diluting the primary stock solution (described in Reagents and Materials section) and spiking the appropriate volume into 5 mL of pooled control urine. The samples were then prepared and analyzed as described in Sample Preparation and Instrument Conditions section. Table 4 shows the range of concentrations used during the study. As expected, because of the acid hydrolysis, the blank contained a significant response. It was questionable as to whether standard 8 produced a signal larger than the average blank response. The LOD and LOQ were estimated by fitting the data to a quadratic curve followed by applying Burkart's Method to the data [13]. The correlation coefficient was 0.999 for the quadratic curve. The data was analyzed both with and without including standard 8. The analysis was also performed both with and without blank subtractions. Table 5 contains the results using these various scenarios. The study was repeated several days later with similar results. From the data, it was determined that the LOD for this method, utilizing the conditions specified in Sample Preparation and Instrument Conditions section, is 0.2 μg/mL. The value for the LOQ is 0.7 μg/mL.

TABLE 4. STANDARD CONCENTRATIONS FOR SPIKED POOLED URINE

Standard	[2,5-HD] μg/mL
1	46.6
2	11.8
3	2.37
4	1.19
5	0.466
6	0.118
7	0.047
8	0.024

TABLE 5. LOD/LOQ RESULTS

STD 8 Included	Blank Subtraction	LOD (μg/mL)	LOQ (μg/mL)
Yes	Yes	0.2	0.6
Yes	No	0.1	0.5
No	Yes	0.2	0.8
No	No	0.2	0.6

The LOD is 40 times lower for synthetic urine than for pooled urine. The increase in sensitivity can be attributed to the lack of 2,5-hexanedione in the synthetic urine blank. Acid hydrolysis causes a blank sample of pooled urine to produce a 2,5-hexanedione peak of substantial area. That area, however, can vary as much as 20% between replicate blanks. When small concentrations of 2,5-hexanedione are present, it is difficult to determine whether the increase in area is due to the analyte or to the variation in the blank response. Because a blank sample of synthetic urine, even after acid hydrolysis, does not show a 2,5-hexanedione response, even minute signal increases can be attributed to analyte detection and a lower LOD is possible. With the success of this study and the data obtained from it, it was then possible to perform the spiked pooled urine experiments.

LONG-TERM STABILITY STUDY

This experiment was designed to assess the stability of 2,5-hexanedione in urine under various conditions of storage. The study covered a period of 7 days for samples stored at room temperature (24 °C) and 32 days for samples stored refrigerated (4 °C). The study was performed using pooled urine for both samples and standards. For samples to be considered stable, they must have a loss of less than 10% over a period of seven days [9]. Once at the laboratory, the analysis might be further delayed for various reasons; therefore, the study was extended for 32 days.

Samples were prepared by spiking 15 μL of a 2,5-hexanedione primary stock solution (see Reagents and Materials section) into 5 mL of pooled control urine. The stock solution was prepared so that the final concentration of the analyte in each sample was 7.11 μg/mL (10 x LOQ). A total of 30 samples and 8 blanks were prepared. On each day, the designated samples plus one blank were analyzed as described in Sample Preparation and Instrument

Conditions section. With each sample set, a fresh series of standards were prepared and analyzed to produce a calibration curve. Table 6 shows the schedule for the analysis of the samples stored at room temperature (24 °C), the length of storage, the results for each set of samples, and their recoveries. All of the recoveries were nearly 100%. One sample on Day 7 had a slightly lower recovery (95.5%) but, as noted in the table, HCl was not added to this sample as specified by the method. Given the closeness of this number and the fact that the acid addition should have little effect on recovery of a spiked sample, the value is included in the values calculated at the bottom of the table. Although it appears that the samples were stable for at least seven days at room temperature, it is recommended that the samples always be refrigerated to minimize the amount of bacterial growth and possible analyte degradation.

TABLE 6. LONG TERM STABILITY STUDY AT ROOM TEMPERATURE (24 °C)

Storage Time	Sample #	Storage Temperature	Target Value	Amount Recovered	% Recovered	Comments
1 Day	1	24 °C	7.108	7.098	99.9	
1 Day	2	24 °C	7.108	6.936	97.6	
1 Day	3	24 °C	7.108	7.550	106.2	
1 Day	4	24 °C	7.108	7.163	100.8	
1 Day	5	24 °C	7.108	7.120	100.2	
1 Day	6	24 °C	7.108	7.176	101.0	
7 Days	1	24 °C	7.108	6.960	97.9	
7 Days	2	24 °C	7.108	7.047	99.1	
7 Days	3	24 °C	7.108	7.018	98.7	
7 Days	4	24 °C	7.108	7.304	102.8	
7 Days	5	24 °C	7.108	6.785	95.5	No acid added
7 Days	6	24 °C	7.108	7.016	98.7	
Mean			7.108	7.098	99.9	
Std. Deviation			0	0.195	2.74	

To determine the stability of the urine samples for longer terms of storage, up to 30 days, an experiment was performed as per the recommendations from the "NIOSH Guide for Air Sampling and Method Development and Evaluation" [9]. Five mL aliquots of pooled urine were divided into one group of six and four groups of three. Since the data from the one-day storage at room temperature was already performed in the previous experiment, it was not deemed necessary to repeat the experiment a second time. The eighteen samples were refrigerated at 4 °C. Results from the refrigerated study are presented in Table 7. The group of 6 was analyzed after 7 days. The remaining four groups of 3 samples each were analyzed after 10, 14, 21, and 32 days. Table 7 clearly shows that the analyte is stable for at least 32 days in urine when stored at 4 °C. Although no further studies were conducted, the analyte is most likely stable for much longer than 32 days.

TABLE 7. LONG TERM STABILITY STUDY AT 4 °C

Storage Time	Sample #	Storage Temperature	Target Value	Amount Recovered	% Recovered
7 Days	1	4 °C	7.108	7.077	99.6
7 Days	2	4 °C	7.108	7.052	99.2
7 Days	3	4 °C	7.108	7.037	99.0
7 Days	4	4 °C	7.108	7.260	102.1
7 Days	5	4 °C	7.108	7.043	99.1
7 Days	6	4 °C	7.108	7.400	104.1
10 Days	1	4 °C	7.108	7.007	98.6
10 Days	2	4 °C	7.108	6.955	97.8
10 Days	3	4 °C	7.108	7.222	101.6
14 Days	1	4 °C	7.108	6.976	98.1
14 Days	2	4 °C	7.108	7.003	98.5
14 Days	3	4 °C	7.108	7.178	101.0
21 Days	1	4 °C	7.108	7.142	100.5
21 Days	2	4 °C	7.108	7.238	101.8
21 Days	3	4 °C	7.108	7.218	101.5
32 Days	1	4 °C	7.108	7.455	104.9
32 Days	2	4 °C	7.108	7.252	102.0
32 Days	3	4 °C	7.108	7.097	99.9
Average			7.108	7.145	100.5
Std. Deviation			0	0.143	2.0

PRECISION, ACCURACY, AND BIAS STUDY

A study was performed to determine the precision, accuracy, and bias of the method using both pooled and synthetic urine. The bias for a method must be less than or equal to 10%. The coefficient of variation (CV) must be less than or equal to 0.1. When these conditions are met the method is deemed unbiased and accurate with 95% probability to within ±25% of the actual concentrations [14].

Synthetic Urine Study

For the synthetic urine study, five concentrations ranging from 1 x LOQ (0.02 μg/mL) to 100 x LOQ (2.0 μg/mL) were studied. Five milliliters of synthetic urine were used for each sample. Seven samples at each level (a total of 35 samples) were spiked with a 2,5-hexanedione solution (described in Reagents and Materials section) to produce the desired concentrations. A sample blank was also included at each level. The data were also analyzed in a similar manner. All concentration levels were included in the calculations. Table 8 displays the average recoveries of the analyte over the ranges studied.

TABLE 8. AVERAGE RECOVERIES FOR SYNTHETIC URINE

Level	Rough Target	Actual Spike (μg/mL)	Average % Recovered	CV
1 X LOQ	0.02	0.020	97.9	0.0140
3 X LOQ	0.06	0.062	96.4	0.0314
10 X LOQ	0.20	0.198	94.4	0.0201
30 X LOQ	0.60	0.602	95.1	0.0091
100 X LOQ	2.00	1.995	90.8	0.0128

Table 9 shows the final results after using the pooled coefficients of variation to calculate the overall precision and accuracy.

TABLE 9. PRECISION, BIAS, AND ACCURACY FOR SYNTHETIC URINE

Range (μg/mL)	0.02 – 2.0
Accuracy (%)	8.2
Bias	
Average	-0.0507
Range	-0.0916 to 0.0209
Precision	
Overall ($\hat{S}_{rT}$)	0.0192

Pooled Urine Study

For the pooled urine, five concentrations ranging from 3 x LOQ (2.1 μg/mL) to 300 x LOQ (210 μg/mL) were studied. A 1 x LOQ level was also included in the study to monitor baseline noise, but its data was not included in the final calculations. Five milliliters of pooled control urine were used for each sample. Seven samples at each level (a total of 42 samples) were spiked with a 2,5-hexanedione solution (described in Reagents and Materials section) to produce the desired concentrations. A sample blank was also included at each level. The samples were then prepared and analyzed according to the protocol listed in Sample Preparation and Instrument Conditions section. Table 10 shows the average recoveries of 2,5-hexanedione over the ranges studied. The data were processed to determine the coefficient of variation at each concentration level and whether these levels could be pooled using Barlett's test of homogeneity [14]. The 1 x LOQ level could not be pooled.

TABLE 10. AVERAGE RECOVERIES FOR POOLED URINE

Level	Rough Target (μg/mL)	Actual Spike (μg/mL)	Average % Recovered	CV
1 x LOQ	0.7	0.692	107.9	0.0589
3 x LOQ	2.1	2.14	97.4	0.0230
10 x LOQ	7	7.11	102	0.0187
30 x LOQ	21	21.2	102.6	0.0128
100 x LOQ	70	71.1	103.5	0.0305
300 x LOQ	210	212	101.8	0.0282

The pooled coefficients of variation were then used for calculating the overall precision and accuracy of the method presented in Table 11.

TABLE 11. PRECISION, BIAS, AND ACCURACY FOR POOLED URINE

Range (μg/mL)	0.7 – 212
Accuracy (%)	5.6
Bias	
Average	-0.0189
Range	-0.1924 to 0.0345
Precision	
Overall ($\hat{S}_{rT}$)	0.0235

SUMMARY

All data derived during the method development met all NIOSH criteria for precision, bias, and accuracy in all studies performed [14]. The method proved to be rugged and adaptable to both synthetic urine and human urine samples. The method was successfully used in a Health Hazard Evaluation field study [15].

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APPENDIX

Review of User Check for NMAM Method 8318 (2,5-Hexanedione in urine)

User check samples were prepared by a BHAB researcher (Dr. Clayton B'Hymer) to be analyzed by ALS Environmental using draft NMAM Method 8318. The urine was obtained from personnel in the Taft building at NIOSH and then combined and mixed in the BHAB labs into a single pool of urine from which all samples were prepared. A total of 25 urine samples were prepared. Five samples were left blank. Five samples were prepared containing the analyte at each of the following levels: 2.02 mg/L, 4.09 mg/L, 49.3 mg/L, and 143.6 mg/L. The samples were prepared and shipped frozen to ALS Environmental on April 7, 2015 and arrived there the next day. The samples were analyzed on April 10, 2015. The sample preparation procedure and analytical conditions found in draft method 8318 were used without exception.
For this analysis, the Lower Limit of Quantitation (LLOQ) was determined by ALS to be 0.70 mg/L for the compound of interest. As mentioned above, the spike levels ranged from 2.02 to 143.6 mg/L, which is 3 to 200 times the LLOQ and fall within the method detection range of 0.2 to 212 mg/L.

The table (Table 1) below shows the data obtained from the User Check samples. 2,5-Hexanedione was not detected in any of the blank samples (data not shown), so no corrections were required. A summary table (Table 2) of average recoveries and precision as calculated by relative standard deviation (RSD) follows.

Table 1

Spike ID	Target concentration (mg/L)	Concentration found (mg/L)	Recovery (%)
5	2.02	1.80	89.11
11	2.02	1.90	94.06
16	2.02	0.37	18.32
20	2.02	1.84	91.09
24	2.02	1.88	93.07
4	4.09	3.78	92.42
8	4.09	3.71	90.71
15	4.09	4.00	97.80
18	4.09	3.92	95.84
21	4.09	3.88	94.87
1	49.3	48.1	97.57
2	49.3	48.6	98.58
10	49.3	49.9	101.22
13	49.3	48.3	97.97
22	49.3	48.8	98.99
3	144	134	93.06
7	144	136	94.44
12	144	126	87.50
14	144	174	120.83
25	144	132	91.67

Table 2

Spiked amount mg/L	Recovery (%)	RSD (%)
2.02	77.1	42.7
4.09	94.3	2.97
49.3	98.9	1.44
143.6	97.5	13.6
Overall	92.0	20.3

Statistical tests for outlier points (Dixon's Q-test and Grubbs test) were performed on the data at each concentration level. No outliers were found at the three highest concentration levels, but one sample in the lowest concentration level, Sample 16 (18.32%), was determined to be an outlier by both statistical tests. Table 3 gives the summary values for accuracy and precision when this rejected data point has been removed.

Table 3

Spiked amount mg/L	Recovery (%)	RSD (%)
2.02	91.8	2.39
4.09	94.3	2.97
49.3	98.9	1.44
143.6	97.5	13.6
Overall	95.8	7.37

Once this outlier is removed, the accuracy at every concentration level is within ± 9% of the true value, which is well within acceptable values for biological monitoring methods. The relative standard deviation (RSD) for each individual level ranges from 1 to 14 per cent. These precision values (and the overall precision) are also well within acceptable limits. Two of the primary guidelines on bioanalytical method validation state that accuracy and precision should be within ± 15% at each level and within ± 20% at the lowest level [1,2]. The contract lab reported no difficulties understanding the draft method nor in setting it up or analyzing the samples. The method has relatively few analytical steps, is quite straightforward, is sensitive enough to determine occupational exposures, and has been shown to have adequate precision and accuracy. It is recommended that the method, NMAM Method 8318 (2,5-hexanedione in urine) be approved and accepted for inclusion in the NIOSH Manual of Analytical Methods.

Dale Shoemaker, PhD
Research Chemist
November 9, 2015

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