TRICHLOROACETIC ACID IN URINE

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BACKUP DATA REPORT

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I. INTRODUCTION

Trichloroacetic acid (TCAA) in urine has been documented to be from several sources. It is one of several metabolites found due to exposure to trichloroethylene (TCE) [1-3] either from inhalation of trichloroethylene vapors or ingestion of trichloroethylene contaminated water [4]. TCAA is also one of the metabolites of other chlorinated compounds, such as methyl chloroform and tetrachloroethene.

TCE is produced in the United States by two companies, the Dow Chemical Company and PPG Industries. In 1998, demand in the United States was about 171 million pounds, of which about 15 million pounds was imported. Approximately 84 million pounds were exported. TCE is a stable, non-flammable solvent which is used as a degreaser for metal parts, in dry cleaning, as a paint and lacquer thinner, as an extraction solvent and as an intermediate in the production of hydrofluorocarbon refrigerants [5]. Its human carcinogenicity, while studied extensively, has proved inconclusive [2-6].

Chlorinated drinking water is another source of TCAA [7-10]. In the case of chlorinated drinking water, TCAA is one of several disinfection by-products as opposed to being formed in vivo. Chloroform, TCAA, and chlorophenols have been detected in treated water [11].

TCAA has been classified as a possible human carcinogen [12]. Even with this possible carcinogenicity, solutions containing TCAA as an ingredient are used for cosmetic treatments, such as chemical peels, tattoo removal, and the treatment of warts, including genital warts [13].

Caution should be used in the evaluation of urinary TCAA levels. TCAA is a non-specific metabolite of several compounds. Urinary TCAA levels reflect exposure to any and all of these precursors. Background TCAA was detected in 76% of urine samples in a US general population sample with a median concentration of 3.3 µg/L…approximately 300 times lower than the range of this method [10].

A number of methods have been used to quantify TCAA. The Fujiwara Method was used by Bernauer [2] and Raaschou-Nielsen, *et al.* [3]. This method, which dates to 1914, is based on the reaction between pyridine and TCAA in an alkaline medium followed by spectrophotometric detection at 530 nm. Isotope-dilution high-performance liquid chromatography-electrospray ionization tandem mass spectrometry was used by the Kuklenyik/Calafat group [8, 10]. Methylation procedures, using either boron trifluoride (BF_3) /methanol [1], diazomethane [4, 11], or a methanol/sulfuric acid esterification procedure [7, 9], all followed by gas chromatography, have also been employed. The use of a dynamic headspace method with gas chromatography/mass spectrometry has been investigated [14]. An excellent review article of analytical methods used to determine TCAA and other metabolites of TCE more thoroughly covers work in the field [15]. In this method, the procedure used by O'Donnell [1] with some modifications will be used.

II. REAGENTS

Reagent	Vendor	Grade/purity	Lot #
Toluene	Burdick & Jackson	HPLC	CM156
Sodium trichloroacetate	Aldrich	97%	11827HB
Methyl trichloroacetate	Aldrich	99 %	06830TB
Boron trifluoride/methanol 14%	Sigma	N/A	104K5321
Sodium sulfate, anhydrous	Fisher	Reagent	046962RW

Sodium trichloroacetate was used instead of TCAA for all phases of this method development as well as in the preparation of standards. TCAA is very hygroscopic; the salt is much less so.

Conversion factor of sodium trichloroacetate to the acid:

MW sodium trichloroacetate = 185.37 g/mol. MW TCAA = 163.39 g/mol.

Conversion factor = 163.39/185.37 = 0.8814.

III. MATERIALS

1. Polypropylene centrifuge tubes, 15-mL, with screw cap. VWR Cat. # 21008-089 or equivalent.
2. Disposable Pasteur pipettes, 6" & 9".
3. Test or culture tubes, ~8-mL (13 mm x 100 mm), screw top with PTFE-lined caps.
4. Gas chromatograph with electron capture detector (GC-ECD), autosampler and data collection system.
5. Microliter syringes for making standard solutions and GC injections.
6. Various glass volumetric flasks for preparing standards.
7. Adjustable pipettor, 1-5-mL, and tips.
8. Adjustable pipettor, 100-200 µL, and tips.
9. Glass wool
10. Vortex mixer.
11. Oven capable of maintaining 60 °C.

IV. SAMPLE PREPARATION

Into an 8-mL glass test tube with a PTFE-lined cap, 200 µL sample and 500 µL BF_3/methanol are combined. The mixture is heated to 60 °C. The tube is cooled and 2 mL toluene is added and vortexed for ~60 seconds. After the phases separate, an aliquot of the top,

organic layer is passed through a Pasteur pipette containing approximately a 1 cm deep bed of anhydrous sodium sulfate. The effluent is collected in a 2-mL GC vial for analysis.

V. INSTRUMENTATION AND ANALYSIS CONDITIONS

Instrument: Hewlett-Packard Model 5890 gas chromatograph with autosampler and electron capture detector (GC-ECD). Makeup gas was nitrogen at 40 mL/min.

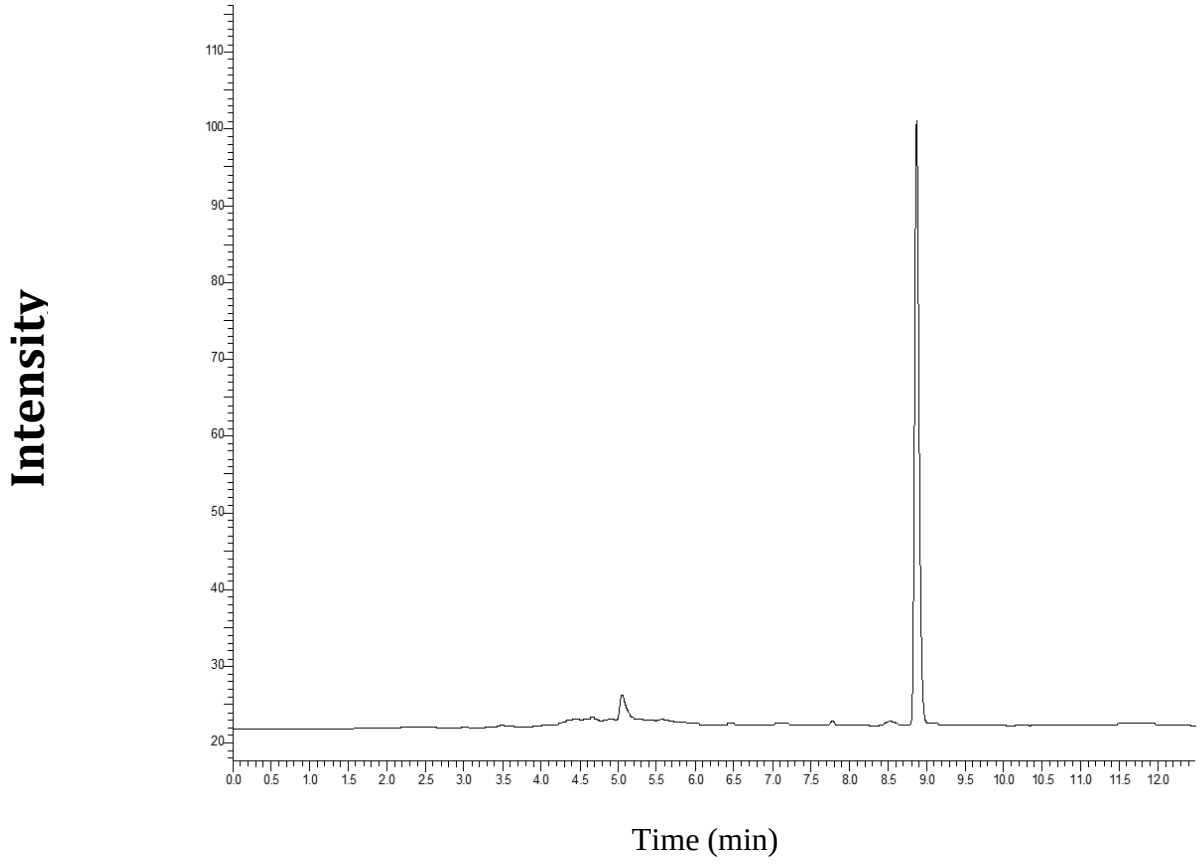
Column: DB-624 fused silica capillary (75 m x 0.53 mm, 3 μm film thickness)

Oven Conditions: 80 °C held for 0.5 minutes, ramped to 180 °C at a rate of 20 °C per minute, held for 7 minutes. Total run time, 12.5 minutes.

Injector: One microliter injected. Injector temperature 250 °C. Splitless for 0.5 minutes.

While only one major peak appears in the chromatogram (Figure 1), toluene appears to extract other non-volatile material from the urine which, in a short time, causes the injection syringe to seize up. The combination of syringe rinse solvents found to alleviate this problem was to first rinse with 1:3 acetic acid:water, followed by a second rinse with 1:1 methanol:acetone between each sample injection.

Figure 1. Chromatogram of trichloroacetic acid methyl ester



VI. PERCENT COMPLETION OF THE DERIVATIZATION REACTION

The percent completion of the derivatization reaction, or derivatization efficiency, was determined by comparing the GC peak response for TCAA carried through the derivatization process to that of a liquid standard of authentic TCAA methyl ester (also known as methyl trichloroacetate.

In the first step, the response factor for pure TCAA methyl ester was determined. A liquid standard of TCAA methyl ester was prepared by diluting 10.69 mg of the 99% pure ester in 7.0 mL toluene to give 1512 µg/mL after correcting for the purity. A 1:10 dilution of this was prepared, giving a 151.2 µg/mL solution. Finally, a 2 µL aliquot of this diluted solution was added to 2 mL toluene to give a solution containing 0.1512 µg/mL as TCAA methyl ester.

An analysis of this diluted solution according to the GC conditions given in Section V gave a GC peak with an area of 24289.5. The response factor was calculated by dividing the observed peak area by the concentration of the liquid standard:

$$RF = \frac{\text{GC Peak Area}}{\text{Conc, µg/mL}} = \frac{24289.5}{0.1512} = 160645$$

In the second step, the derivatization efficiency was calculated from an analysis of a simulated sample at the 10× limit of quantitation (LOQ) level using TCAA. The concentration of a 10× LOQ sample was 3.0355 µg/mL as TCAA. A 200 µL aliquot of a 10× LOQ sample was taken for analysis. Theoretically it contains 0.6071 µg as the amount of TCAA that is will be subjected to derivatization (at 60 °C and 180 minutes reaction time):

3.0355 µg/mL × 0.2 mL = 0.6071 µg.

Extracting the derivative into 2.0 mL toluene gives a final theoretical concentration of 0.30355 µg/mL as TCAA. To convert to the methyl ester, the ratio of the molecular weights (MW) was used:

MW TCAA methyl ester = 177.4145 g/mol
MW TCAA = 163.3877 g/mol

$$0.30355\mu g/mL \times \frac{177.4145}{163.3877} = 0.3296\ \mu g/mL \text{ as TCAA methyl ester.}$$

In other words, 0.3296 µg/mL of TCAA methyl ester should be observed if derivatization is complete.

However, the typical GC peak area for a 10× LOQ sample was only about 27500. Using the response factor calculated in step 1 for pure TCAA methyl ester (160645), the concentration of a GC peak with an area of 27500 is only 0.1712 µg/mL as the TCAA methyl ester, according to the following calculation:

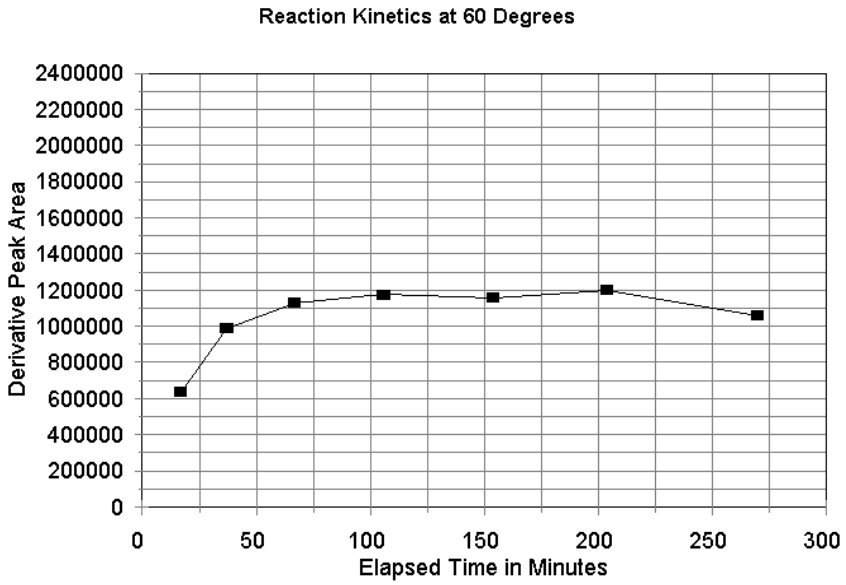
$$\text{Concentration acid in } \mu g/mL = \frac{27500}{160645} = 0.1712\ \mu g/mL \text{ as methyl ester.}$$

In other words, only 0.1712 µg/mL is observed instead of the theoretical 0.3296 µg/mL. Therefore, the percent derivatization efficiency is 52%, (0.1712 / 0.3296 x 100 = 52%). The consequence of this finding is that the calibration standards should be prepared from serial dilutions of TCAA that are subsequently derivatized, as opposed to preparing serial dilutions of the pure methyl ester.

VII. REACTION KINETICS

A series of tubes was prepared with identical spikes of sodium trichloroacetate in water. To each tube, 500 µL BF_3/methanol was added, mixed and placed in a 60 °C oven. Periodically, a tube was removed, cooled, extracted with 2 mL of toluene and analyzed. The results are plotted in the graph below.

Figure 2. Boron Trifluoride/Methanol/Trichloroacetate Reaction Kinetics:



O'Donnell [1] reacted the BF_3/methanol with sodium trichloroacetate for 30 minutes. It appears from this experiment that the response would improve with a longer reaction time. Consequently, incubation times for the method will be a minimum of 90 minutes.

VIII. LIMIT OF DETECTION/LIMIT OF QUANTITATION (LOD/LOQ) DETERMINATION FOR METHOD DEVELOPMENT SPIKING LEVELS:

The LOD and LOQ were determined from a calibration curve as follows. Working standards were prepared in water covering the range 21.08 µg/mL to 0.02108 µg/mL. A 200 µL aliquot of each standard was placed in an 8-mL test tube and mixed with 500 µL BF_3/methanol. After capping, the tubes were heated at 60 °C for 2.5 hours. The tubes were cooled, extracted into 2.0 mL toluene, and analyzed as described previously.

The LOD and LOQ were determined by Burkart's [16] method. Using all the standards, the LOD was calculated to be 0.08 µg/mL and the LOQ was 0.3 µg/mL. All standards exhibited a peak in the appropriate retention time window. Spiking levels for the balance of the method development were calculated as follows:

Table 1. Method Spiking Levels:

	Spiking Levels in µg/mL of Sample			
Analyte	**3× LOQ**	**10× LOQ**	**30× LOQ**	**100× LOQ**
TCAA	0.9	3	9	30

IX. EXTRACTION EFFICIENCY AT 3×, 10×, 30×, AND 100× LOQ

Six replicate samples at each of the four spiking levels described above were prepared in urine. Samples were mixed, derivatized, and analyzed as described previously. Results are tabulated below.

Table 2. Extraction Efficiency at Four Levels, All Data:

100X LOQ

	Recovery	Target	% Recovery
1	29.0572	30.355	95.72
2	27.8472	30.355	91.74
3	29.2032	30.355	96.21
4	29.6468	30.355	97.67
5	29.5373	30.355	97.31
6	31.4651	30.355	103.66
		Average	97.05
		Rel. Std Dev	3.87
		% Std. Dev.	3.98

10X LOQ

	Recovery	Target	% Recovery
1	3.2685	3.0355	107.68
2	3.1190	3.0355	102.75
3	3.1460	3.0355	103.64
4	2.9962	3.0355	98.71
5	2.9375	3.0355	96.77
6	3.1623	3.0355	104.18
		Average	102.29
		Rel. Std Dev	3.95
		% Std. Dev.	3.86

30X LOQ

	Recovery	Target	% Recovery
1	9.6047	9.0644	105.96
2	9.7867	9.0644	107.97
3	10.1900	9.0644	112.42
4	9.8796	9.0644	108.99
5	9.7732	9.0644	107.82
6	10.8144	9.0644	119.31
		Average	110.41
		Rel. Std Dev	4.85
		% Std. Dev.	4.39

3X LOQ

	Recovery	Target	% Recovery
1	0.8693	0.90644	95.90
2	0.8382	0.90644	92.47
3	0.8799	0.90644	97.07
4	0.8657	0.90644	95.51
5	0.8562	0.90644	94.46
6	0.8446	0.90644	93.18
		Average	94.76
		Rel. Std Dev	1.73
		% Std. Dev.	1.83

X. PRECISION AND ACCURACY

Precision and accuracy calculation results are given in Appendix 1. No Grubbs outliers were found. All six replicates for all four concentration levels were used in the calculations. All combinations passed the Bartlett's test for homogeneity, but omitting the 3× LOQ level gave the lowest Chi squared (0.0853, see Line 2 in Section B of Table 2 in Appendix 1).

When all concentration levels are used, the Chi^2 value was found to be 3.4493, and the overall precision (S_{rt}) was 0.03655 (see Option#1 in Section 1 of Table 2 in Appendix 1). The bias was 0.01128 (see Option#1 in Section 1 of Table 2 in Appendix 1). Accuracy was calculated to be 7.5 % from these values [17].

However, the F' test passed only when the 30× LOQ level was omitted. Doing so gives a higher Chi squared and a negative mean bias, but not much change in the overall accuracy (7.65% instead of 7.50%). Nevertheless, recoveries at all levels were excellent (94.76%, 102.29%, 110.41%, and 97.05% for the 3×, 10×, 30×, and 100× LOQ levels respectively) in spite of failure to pass the F' test for all other combinations of concentration levels [17].

XI. LONG TERM STORAGE

Urine in 25-mL volumetric flasks was spiked with sodium trichloroacetate at 10×, 30×, and 100× LOQ. The volumetrics were brought to volume with urine as needed and mixed. Several milliliters of each concentration were placed in 15-mL polyethylene centrifuge tubes and frozen at -17 °C for 7, 14, 21, 30, and 46 days. At the conclusion of the allotted time, a tube of each concentration was removed, allowed to thaw, and mixed by vortexing. Six 200-µL aliquots were placed in 8-mL tubes and derivatized as described earlier. The methyl derivatives were stored at -17 °C until analysis. Quantitation was against derivatized TCAA standards prepared from aqueous solutions. Results are plotted below and tabulated in Tables 3-5 following.

Figure 3. Trichloroacetic Acid in Frozen Urine Long Term Storage

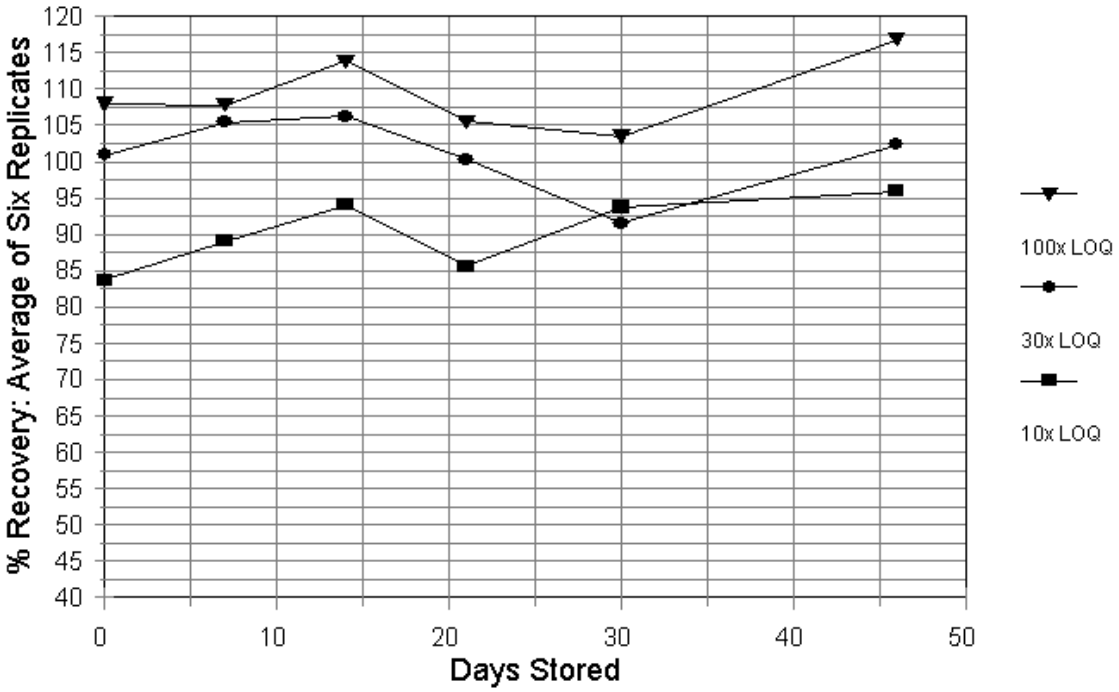


Table 3. Long Term Storage at 100× LOQ; All Data.

		Recovery	Target	% Recovery
Day 0				
	1	33.0212	30.355	108.78
	2	32.6073	30.355	107.42
	3	32.8886	30.355	108.35
	4	32.3048	30.355	106.42
	5	32.9763	30.355	108.64
	6	32.8553	30.355	108.24
			Average	107.97
			Rel. Std Dev	0.90
			% Std. Dev.	0.83
7 Day				
	1	32.2666	30.355	106.30
	2	32.3122	30.355	106.45
	3	32.9304	30.355	108.48
	4	32.8526	30.355	108.23
	5	32.6574	30.355	107.58
	6	33.4021	30.355	110.04
			Average	107.85
			Rel. Std Dev	1.40
			% Std. Dev.	1.30
14 Day				
	1	34.7413	30.355	114.45
	2	34.8563	30.355	114.83
	3	34.8963	30.355	114.96
	4	34.8731	30.355	114.88
	5	33.7627	30.355	111.23
	6	34.1619	30.355	112.54
			Average	113.82
			Rel. Std Dev	1.56
			% Std. Dev.	1.37

		Recovery	Target	% Recovery
21 Day				
	1	32.1331	30.355	105.86
	2	32.2564	30.355	106.26
	3	31.8995	30.355	105.09
	4	32.0005	30.355	105.42
	5	31.7873	30.355	104.72
	6	32.2372	30.355	106.20
			Average	105.59
			Rel. Std Dev	0.62
			% Std. Dev.	0.59
30 Day				
	1	30.6243	30.355	100.89
	2	31.4171	30.355	103.50
	3	30.556	30.355	100.66
	4	31.555	30.355	103.95
	5	31.1005	30.355	102.46
	6	33.1914	30.355	109.34
			Average	103.47
			Rel. Std Dev	3.17
			% Std. Dev.	3.07
46 Day				
	1	35.6597	30.355	117.48
	2	35.7261	30.355	117.69
	3	35.7543	30.355	117.79
	4	35.1567	30.355	115.82
	5	35.0608	30.355	115.50
	6	35.3923	30.355	116.59
			Average	116.81
			Rel. Std Dev	0.99
			% Std. Dev.	0.85

Table 4. Long Term Storage at 30× LOQ; All Data.

		Recovery	Target	% Recovery
Day 0				
	1	9.1795	9.0644	101.27
	2	9.2067	9.0644	101.57
	3	9.1776	9.0644	101.25
	4	9.1578	9.0644	101.03
	5	8.967	9.0644	98.93
	6	9.2359	9.0644	101.89
			Average	100.99
			Rel. Std Dev	1.05
			% Std. Dev.	1.04
7 Day				
	1	9.9995	9.0644	110.32
	2	9.3242	9.0644	102.87
	3	9.4308	9.0644	104.04
	4	9.4994	9.0644	104.80
	5	9.6864	9.0644	106.86
	6	9.3696	9.0644	103.37
			Average	105.38
			Rel. Std Dev	2.80
			% Std. Dev.	2.65
14 Day				
	1	9.9311	9.0644	109.56
	2	9.8919	9.0644	109.13
	3	9.4446	9.0644	104.19
	4	9.3159	9.0644	102.77
	5	9.8473	9.0644	108.64
	6	9.4009	9.0644	103.71
			Average	106.33
			Rel. Std Dev	3.09
			% Std. Dev.	2.90

		Recovery	Target	% Recovery
21 Day				
	1	9.0472	9.0644	99.81
	2	9.2436	9.0644	101.98
	3	9.0834	9.0644	100.21
	4	9.1373	9.0644	100.80
	5	8.9584	9.0644	98.83
	6	9.0868	9.0644	100.25
			Average	100.31
			Rel. Std Dev	1.05
			% Std. Dev.	1.04
30 Day				
	1	8.4667	9.0644	93.41
	2	8.276	9.0644	91.30
	3	8.496	9.0644	93.73
	4	8.3936	9.0644	92.60
	5	8.1175	9.0644	89.55
	6	8.0507	9.0644	88.82
			Average	91.57
			Rel. Std Dev	2.04
			% Std. Dev.	2.23
46 Day				
	1	9.0197	9.0644	99.51
	2	9.4457	9.0644	104.21
	3	9.0964	9.0644	100.35
	4	9.5737	9.0644	105.62
	5	8.7439	9.0644	96.46
	6	9.78	9.0644	107.89
			Average	102.34
			Rel. Std Dev	4.28
			% Std. Dev.	4.18

Table 5. Long Term Storage at 10× LOQ; All Data.

		Recovery	Target	% Recovery
Day 0				
	1	2.6346	3.0355	86.79
	2	2.5863	3.0355	85.20
	3	2.4804	3.0355	81.71
	4	2.4738	3.0355	81.50
	5	2.5493	3.0355	83.98
	6	2.5162	3.0355	82.89
			Average	83.68
			Rel. Std Dev	2.07
			% Std. Dev.	2.47
7 Day				
	1	2.5186	3.0355	82.97
	2	2.7647	3.0355	91.08
	3	2.7156	3.0355	89.46
	4	2.6999	3.0355	88.94
	5	2.7645	3.0355	91.07
	6	2.7521	3.0355	90.66
			Average	89.03
			Rel. Std Dev	3.10
			% Std. Dev.	3.48
14 Day				
	1	2.868	3.0355	94.48
	2	2.882	3.0355	94.94
	3	2.8437	3.0355	93.68
	4	2.8659	3.0355	94.41
	5	2.7261	3.0355	89.81
	6	2.932	3.0355	96.59
			Average	93.99
			Rel. Std Dev	2.27
			% Std. Dev.	2.41

		Recovery	Target	% Recovery
21 Day				
	1	2.5345	3.0355	83.50
	2	2.7531	3.0355	90.70
	3	2.323	3.0355	76.53
	4	2.793	3.0355	92.01
	5	2.4243	3.0355	79.86
	6	2.7613	3.0355	90.97
			Average	85.59
			Rel. Std Dev	6.57
			% Std. Dev.	7.67
30 Day				
	1	2.63	3.0355	86.64
	2	2.7325	3.0355	90.02
	3	2.8801	3.0355	94.88
	4	2.9906	3.0355	98.52
	5	3.002	3.0355	98.90
	6	2.8394	3.0355	93.54
			Average	93.75
			Rel. Std Dev	4.80
			% Std. Dev.	5.12
46 Day				
	1	3.1129	3.0355	102.55
	2	2.9117	3.0355	95.92
	3	2.7957	3.0355	92.10
	4	2.9487	3.0355	97.14
	5	2.904	3.0355	95.67
	6	2.805	3.0355	92.41
			Average	95.96
			Rel. Std Dev	3.81
			% Std. Dev.	3.97

XII. CONCLUSIONS

TCAA is easily methylated by BF_3/methanol. While the reaction does not go to completion, it appears to be reasonably reproducible and independent of concentration. As with any multi-step derivatization/extraction procedure, experimental errors can be compounded. These errors may explain why the variation in recoveries causes difficulties with the F' test in the statistical evaluation of the method. Other precision and accuracy criteria appear to be met. TCAA is stable in urine for at least 46 days when stored frozen at -17 °C.

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APPENDIX 1. Precision and Accuracy for Trichloroacetic Acid:

TABLE 1. RECOVERY FROM SPIKED URINE

ANALYTE: **Trichloroacetic Acid**
MEDIA: **Urine**
INT STD = **none**

Method = Derivatization/Extraction
Instrument: GC-ECD
Derivatizing agent = boron trifluoride/methanol

			Concentration Level							
			3X LOQ		10X LOQ		30X LOQ		100X LOQ	
			Applied in ug/mL 0.90644		Applied in ug/mL 3.03550		Applied in ug/mL 9.06440		Applied in ug/mL 30.35500	
Replicate			Found in ug/mL	Percent Recovery	Found in ug/mL	Percent Recovery	Found in ug/mL	Percent Recovery	Found in ug/mL	Percent Recovery
1			0.8693	95.90	3.2685	107.68	9.6047	105.96	29.0572	95.72
2			0.8382	92.47	3.1190	102.75	9.7867	107.97	27.8472	91.74
3			0.8799	97.07	3.1460	103.64	10.1900	112.42	29.2032	96.21
4			0.8657	95.51	2.9962	98.71	9.8796	108.99	29.6468	97.67
5			0.8562	94.46	2.9375	96.77	9.7732	107.82	29.5373	97.31
6			0.8446	93.18	3.1623	104.18	10.8144	119.31	31.4651	103.66
7			none	none	none	none	none	none	none	none
average =			0.8590	94.76	3.1049	102.29	10.0081	110.41	29.4595	97.05
std dev =			0.01572	1.7343	0.11982	3.9473	0.43969	4.8508	1.17393	3.8674
CV 2i=			0.01830	inlier CV	0.03859	ok	0.04393	ok	0.03985	ok
Bias i =			-0.05236	ok	0.02287	ok	0.10411	>10%	-0.02950	ok
N =	0	0	6		6		6		6	

TABLE 2. CALCULATION of ACCURACY, OVERALL PRECISION, and MEAN BIAS

Analyte = Trichloroacetic Acid
Medium = Urine
Int Std = none

Instrument = GC-ECD
Derivatizing agent = boron trifluoride/methanol
Range studied = 0.90644 to 30.35500 ug/mL

Section 1 NOTES:	OPTION	LEVELS OMITTED for BARTLETT'S TEST (See Note)	LEVELS OMITTED for F' TEST (See Note)	FINAL OVERALL VALUES Pooled CV = Overall Srt	Calculated, not using nomogram Accuracy	MEAN BIAS	Range of Bias From =	Range of Bias To =	Continued below in Section 2 on LINE:
1, 2	#1			**0.03655**	**7.50**	**0.01128**	-0.05236	0.10411	A
1, 3	#2	3X LOQ	3X LOQ	**0.04085**	**9.97**	**0.03249**	-0.02950	0.10411	B
1, 4	#3	10X LOQ	10X LOQ	**0.03584**	**7.17**	**0.00742**	-0.05236	0.10411	C
1, 5	#4	30X LOQ	30X LOQ	**0.03373**	**7.65**	**-0.01966**	-0.05236	0.02287	D
1, 6	#5	100X LOQ	100X LOQ	**0.03538**	**8.31**	**0.02487**	-0.05236	0.10411	E

Section 2	Homogeneity of individual group CVs.						Homogeneity of individual group biases.					
		Bartlett's Criteria for 4, 5, or 6 conc. levels			Pass Bartlett's?			F' Theoretical for 4, 5, or 6 conc. levels			PASS F' test? Is F' < theoretical?	
		Percentile of X^2 dist.		df	Percentile of X^2 dist.					df		
LINE:	Chi sq'd	0.95	0.975		0.95	0.975	F' =	at a=0.05	at a=0.025		at a=0.05	at a=0.025
A	3.4493	7.81	9.35	3	YES	YES	7.36684	3.09839	3.85870	3	no	no
B	**0.0853**	5.99	7.38	2	YES	YES	4.89806	3.68232	4.76505	2	no	no
C	3.3270	5.99	7.38	2	YES	YES	8.34891	3.68232	4.76505	2	no	no
D	2.8437	5.99	7.38	2	YES	YES	**1.83301**	3.68232	4.76505	2	YES	YES
E	3.2642	5.99	7.38	2	YES	YES	7.12855	3.68232	4.76505	2	no	no

NOTES:

1 Group CVs are poolable for all possible combinations of 4 and 3 concentration levels. Chi^2 is lowest for Option #2, but Options #1, #3, #4, and #5 conserve the lowest concentration level making the applicable range extend to a lower concentration level.
2 Group biases are non-homogenous for 4 concentration levels (3X, 10X, 30X, 100X LOQ).
3 Group biases are non-homogenous for 3 concentration levels (10X, 30X, 100X LOQ).
4 Group biases are non-homogenous for 3 concentration levels (3X, 30X, 100X LOQ).
5 Group biases ARE homogenous for 3 concentration levels (3X, 10X, 100X LOQ).
6 Group biases are non-homogenous for 3 concentration levels (3X, 10X, 30X LOQ).

APPENDIX 2. User Check Results and Review:

User check samples were prepared by a CEMB researcher (Dr. Dale Shoemaker) to be analyzed by ALS Environmental using draft NMAM Method 8322. A total of 25 urine samples were prepared. 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. Five samples were left blank. Five samples were prepared containing the analyte at each of the following levels: 1.01 mg/L, 5.03 mg/L, 20.11 mg/L, and 100.6 mg/L. 60 mL of each level was prepared and then equally aliquotted into the five samples. The samples were prepared and shipped frozen to ALS Environmental on November 20, 2013 and arrived there the next day. The samples were analyzed on November 27, 2013. The sample preparation procedure and analytical conditions found in draft method 8322 were used with one minor change to the chromatographic temperature program. A higher final temperature and hold time were found to be necessary by ALS to ensure adequate cleaning of the chromatographic column. This change was approved by NIOSH ahead of time.

For this analysis, the Lower Limit of Quantitation (LLOQ) was determined by ALS to be 0.10 mg/L for the compound of interest. As mentioned above, the spike levels ranged from 1.01 to 100.6 mg/L which is 10 to 1000 times the LLOQ and fall within the method detection range of 0.9 to 100 mg/L.

The table (Table 1) below shows the data obtained from the User Check samples. TCAA was not detected in any of the blank samples (which is to be expected; 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 (%)
3	1.01	0.80	79.21
8	1.01	1.30	128.71
12	1.01	1.30	128.71
18	1.01	1.30	128.71
24	1.01	1.10	108.91
6	5.03	2.70	53.68
13	5.03	5.60	111.33
15	5.03	5.70	113.32
16	5.03	5.60	111.33
22	5.03	5.30	105.37
1	20.1	20.0	99.50
5	20.1	13.0	64.68
17	20.1	22.0	109.45
20	20.1	20.0	99.50
21	20.1	19.0	94.53
2	100.6	98.0	97.42
7	100.6	110.0	109.34
10	100.6	100.0	99.40
14	100.6	110.0	109.34
23	100.6	110.0	109.34

Table 2

Spiked amount mg/L	Average recovery (%)	RSD (%)
1.01	114.85	18.9
5.03	99.01	25.8
20.1	93.53	18.2
100.6	104.97	5.7
Overall	103.09	18.7

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 lowest and highest levels by either test, but each of the two middle concentrations contained a point that was determined to be an outlier by both statistical tests (sample 6 at the 5.03 mg/L level (53.68%) and sample 5 at the 20.1 mg/L level (64.68%)). Table 3 gives the summary values for accuracy and precision when these two rejected data points have been removed.

Table 3

Spiked amount mg/L	Average recovery (%)	RSD (%)
1.01	114.85	18.9
5.03	110.34	3.1
20.1	100.75	6.2
100.6	104.97	5.7
Overall	107.97	11.7

As can be seen, the precision for the two middle levels and for the data set as a whole improve when these two samples are rejected. The accuracy at every concentration level is within ± 15% of the true value, which is acceptable for biological monitoring methods. The relative standard deviation (RSD) for each individual level ranges from 3 to 6% except for the lowest concentration level where it is 19%. 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 8322 (Trichloroacetic acid in urine) be approved and accepted for inclusion in the NIOSH Manual of Analytical Methods.

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March 26, 2014

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Addendum – January 30, 2015

Comments were added to the Evaluation of Method section on the recommendation of external reviewers. Concerns about outliers consisting 10% of the User Check samples were noted. Recommendation to include some levels of duplicate injections as well as duplicate analyses were added so these types of errors would be noticed if they occur.