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NIST-TRACEABLE NMR METHOD TO DETERMINE QUANTITATIVE WEIGHT PERCENTAGE PURITY OF NITROGEN MUSTARD HN-3 FEEDSTOCK SAMPLES

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14. ABSTRACT
A NIST-traceable method for using nuclear magnetic resonance (NMR) with proton detection is described to determine the weight percent purity of feedstock samples containing nitrogen mustard (HN-3).

15. SUBJECT TERMS

HN-3	Purity determination
Nitrogen mustard	Nuclear Magnetic Resonance

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PREFACE

The work described in this report was authorized under contract no. W911SR-10-D-0004. This work was started in January 2012 and completed in May 2012.

This report was published through the Technical Releases Office; however, it was edited by the Technical Information Specialist, Toxicology, Toxicology and Obscurants, Research and Technology Directorate, U.S. Army Edgewood Chemical Biological Center (ECBC).

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CONTENTS

FIGURES

TABLES

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NIST-TRACEABLE NMR METHOD TO DETERMINE QUANTITATIVE WEIGHT PERCENTAGE PURITY OF NITROGEN MUSTARD HN-3 FEEDSTOCK SAMPLES

1. INTRODUCTION

This procedure is based on published Technical Report procedures for using Nuclear Magnetic Resonance (NMR) instruments for determining the purity of CW agent samples.[1,2,3,4,5] The procedure utilizes an internal standard to establish an absolute weight percentage for the analyte of interest. Identifying the structures of other components in the mixture is not necessary. All that is necessary is to know the NMR chemical shifts of the major analyte, the internal standard, and the molecular weights. The weight percent calculations are not negatively affected by the presence of undetectable components in the sample (for example, inorganic salts, insoluble solids, etc.).

The procedure has been adapted to use a NIST standard material. An internal standard was purchased from Sigma Aldrich that has a NIST-traceable purity. A balance calibrated with NIST traceable weights was also used. These modifications make the method NIST-Traceable.

To determine the purity of nitrogen mustard-3 (HN-3), proton NMR was used for detection.

2. PROCEDURE

2.1 Supplies

The following supplies can be used for the procedure. Equivalent supplies may be available from other vendors.

The internal standard was 1,2,4,5-tetramethylbenzene, purchased from Fluka (Sigma Aldrich), Part Number 74658-5G, CAS No. 95-93-2, as a TraceCERT® certified reference material (CRM) standard for quantitative NMR.

The following supplies were purchased from Wilmad, (1172 NW Boulevard Vineland, NJ 08360, phone 800-220-5171 (http://www.wilmad-labglass.com/ordering/index.jsp):

Item	Part Number
5 mm dia. 8" long NMR tube	WG-1000-8-50

Teflon inserts	6005
pasteur pipets, 9"	C-7095B-9

The following supplies were purchased from Sigma Aldrich (http://www.sigmaaldrich.com/chemistry.html):

Item	Part Number
chloroform, 99.9% D	23,689-6

For the Precision and Accuracy testing, a JEOL ECS-400 Nuclear Magnetic Resonance spectrometer with a 400 MHz (9.8 T) magnet and 5 mm liquid analysis probe was used. A Sartorius Cubis balance (Model MSA6.6S-000-DM, precision 1 µg) was used after installation in a fume hood and calibration using NIST-traceable weights. NMR systems and balances from other vendors should give comparable results, if the operators have the appropriate training.

Other common laboratory equipment will be used, including a vortex mixer, spatulas, and volumetric pipets. This equipment is not critical to the accurate performance of the method.

2.2 Sample Preparation

This procedure was performed under proper engineering controls, in accordance with surety and safety regulations, equipment validations, and SOPs approved by the Safety and Health Office. The balance must be calibrated using NIST-traceable weights.

a. Tare a screw-cap vial with cap on the balance. Transfer 10-20 mg of neat internal standard, the NIST traceable 1,2,4,5-tetramethylbenzene, into the vial. This compound is a solid material, so it is transferred with a spatula. Replace the cap and determine the weight of the internal standard to an accuracy of 0.01 mg. Tare the balance after recording the weight.
b. Add 5-35 mg of feedstock agent sample to the vial. The liquid agent can be measured with a pipet (4 to 30 µl of liquid). (A precision and accuracy test of this method has been done over this range of agent amounts, see Appendix I.) Record the weight to an accuracy of 0.01 mg in a laboratory notebook. Appropriate agent accountability documentation is used to record the consumption.
c. Add 0.5 ml of reagent-grade deuterated chloroform ($CDCl_3$).
d. Vortex or mix the sample for at least 15 s to dissolve both compounds in the solvent.

e. Transfer the solution into a PTFE NMR tube insert. (Optional: A glass 4mm insert tube or capillary tube may be used, and flame sealed, if desired)
f. Place the insert into a 5 mm glass NMR tube and push it to the bottom of the tube. Cap the insert with a PTFE stopper. Cap the NMR tube with a cap.

2.3 Obtaining an NMR spectrum

Operators of the NMR must have sufficient training to understand the general operational principles and to use the instrument computer control to perform the required tasks. To validate the NMR is functioning correctly, a manufacturer sample such as 0.01% ethylbenzene in deuterated acetone can be analyzed to check the signal response. The analysis of this sample can be done periodically as part of the instrument QC validation. Detailed QC specifications are not included in this method.

a. Place the NMR tube into the spinner using a depth gauge to orient the tube at the correct position relative to the detection coils. Activate the lift air supply, place the sample on top of the magnet, and deactivate the lift air supply to lower the sample into the magnet bore. (Note: The doubly-contained NMR tube that contains agent will be outside of engineering controls.)
b. Lock the instrument on the deuterium signal from the $CDCl_3$.
c. Shim the magnet to maximize the lock signal.
d. Tune and match the probe. (Tune for the optimal signal response, and impedance match the probe to the sample. On some instruments, this operation is done automatically by the instrument software and autotune equipment. On older instruments, it must be done using manual adjustments on the NMR probe.)
e. OPTIONAL: Determine the T_1 relaxation time of the analytes in the sample solution. Use the instrument console to load the data file or instrument parameters for an inversion recovery experiment for proton detection. Perform the experiment with at least six delay times. Process the data to plot the recovery curve for each analyte peak, and determine the T_1 relaxation time from the data plot. Identify the longest T_1 value for all the peaks. The relaxation delay time for the quantitative purity measurement is calculated to be at least 10 times the longest relaxation time. This procedure to determine the T_1 relaxation time should be done if there is an inconsistency in the purity determination, if a new instrument is being used, or if it is necessary to minimize the experiment acquisition time.
f. Load instrument parameters to acquire a 1D proton spectrum. If the T_1 relaxation time is not d (step e is not performed), then set the relaxation time to 40 s. (This is typically 20 times longer than the longest T_1 in the solvent.) Do not use Nuclear Overhauser Enhancement (NOE), decoupling, or water peak suppression.

g. Open a new data file on the NMR computer with a unique filename, the sample information, and notebook reference. Load the parameters for proton acquisition. The following parameters are used. (Actual parameter names will vary depending on the make and model of the NMR and can be found in the NMR documentation.):

   Relaxation time: 40 s or as determined in step e.
   Excite pulse: 90º pulse (determining the time for this pulse should be found in the NMR instrument documentation)
   Number of data points: 64K
   Sweep width: 15 ppm
   Center frequency: 5 ppm
   Decoupling: off
   NOE: off
   Automatic gain determination: on

h. Acquire data.
i. A total of seven or more replicate runs are acquired for statistical determination of the NMR variability, signal to noise ratio, and integration errors. Several samples can be prepared to determine the weighing statistical errors (see Appendix I).

2.4 Data Processing

a. Apply a window function (exponential multiplication). This may be done using a line broadening parameter of 0.5 to 2 Hz, which can be adjusted to enhance the signal to noise ratio. A larger line broadening produces wider peaks, which can degrade the resolution between peaks. The same value of line broadening must be used for all the data files.
b. Fourier transform (FFT) to convert data from time to frequency domain and to produce the NMR spectrum. A sample spectrum is shown in Figure 1.
c. Phase all peaks in the spectrum and correct the baseline if necessary.
d. If necessary for reporting, reference the chemical shift against the internal standard.
e. Integrate the relevant peaks in the spectrum to obtain the areas. A sample integrated spectrum is shown in Figure 2 with an expanded y-scale. Some data systems will perform automatic integration of peaks. It is necessary for the operator to examine the integration to make sure that the correct parts of the peak are included in the integration. If the integration is incorrect, the spectrum can be manually integrated. In particular, Figure 2 shows that each peak has two ^{13}C satellite peaks on each side of the main peak. These peaks are produced by molecules that have a natural abundance of ^{13}C isotopes, and they each represent 0.55% of the center peak. The satellite peaks should be included in the integration of the central peak. (If the magnet is not well shimmed, the satellite peaks may not be resolved.)

2.5 Purity Calculation

The weight percent of the analyte (Wt% A) in the sample is calculated using the following formula, where analyte A is the HN-3, and IS is the internal standard:

$$\text{Wt\% A} = \frac{\text{Area under A peak}}{\text{Area under IS peak}} \times \frac{\text{MW of A}}{\text{MW of IS}} \times \frac{\text{Weight IS}}{\text{Weight A}} \times \frac{\text{No. identical H(IS)}}{\text{No. H (A)}} \times 100\%$$

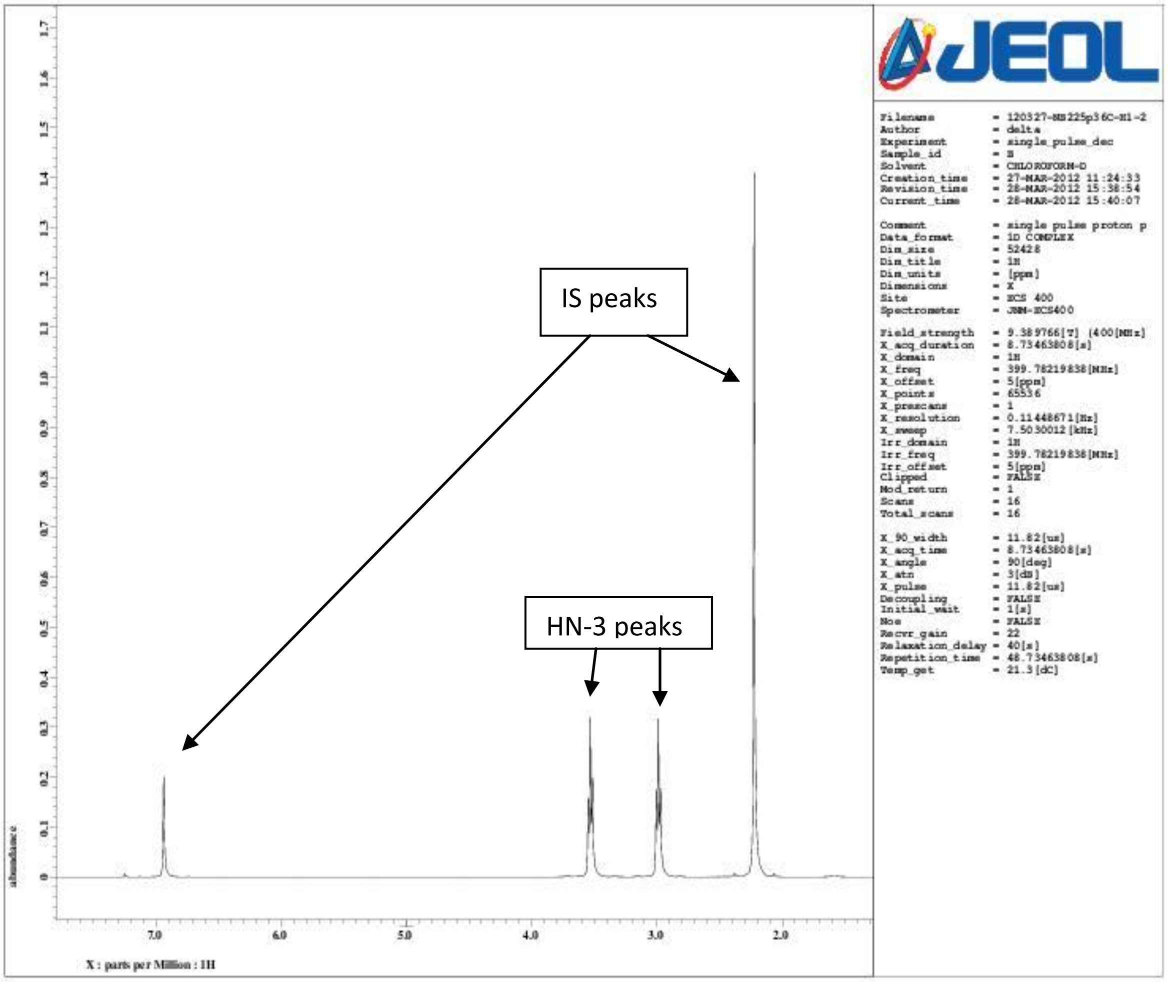
Area under A peak = total sum of the area of the two triplet peaks at 3.0 and 3.5 ppm and the ^{13}C satellite peaks that are associated with them;
Area under IS peak = total area of the singlet peak at 2.2 ppm and the ^{13}C satellite peaks;
MW of A = average molecular weight of HN-3, which is 204.54 D;
MW of IS= average molecular weight of the internal standard, which is 134.22 D;
Weight IS=balance recorded weight of internal standard in the vial;
Weight A=balance recorded weight of feedstock HN-3 sample in the vial;
No. identical H (IS)=the number of identical protons in the internal standard, which is 12;
No. H (A)=the number of protons in the integrated peaks of the analyte, which is 12 when both triplet peaks at 3.0 and 3.5 ppm are added.

The purity of the internal standard is assumed to be 100%, but if the documentation from the NIST traceable internal standard indicates that a different purity is appropriate, then that purity value can be used.

If the analytical statistical accuracy is reported, the calculated weight percentages for each replicate run can be averaged to find a mean (average) and standard deviation. For seven replicates, the mean ± 2 standard deviations provide the 95% confidence range.

3. CONCLUSION

By using the NIST-traceable internal standard, and the balance that is calibrated with NIST-traceable weights, the purity of the CW agent feedstock HN-3 is determined using a NIST-Traceable method.



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Sample_id         = B
Solvent           = CHLOROFORM-D
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Revision_time     = 28-MAR-2012 15:38:54
Current_time      = 28-MAR-2012 15:40:07

Comment           = single pulse proton p
Data_format       = 1D COMPLEX
Dim_size          = 52428
Dim_title         = 1H
Dim_units         = [ppm]
Dimensions        = X
Site              = ECS 400
Spectrometer      = JNM-ECS400

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Scans             = 16
Total_scans       = 16

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Initial_wait      = 1[s]
Noe               = FALSE
Recvr_gain        = 22
Relaxation_delay  = 40[s]
Repetition_time   = 48.73463808[s]
Temp_get          = 21.3[dC]
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FIGURE 1: Proton NMR spectrum of HN-3 agent and the internal standard 1,2,4,5-tetramethylbenzene.

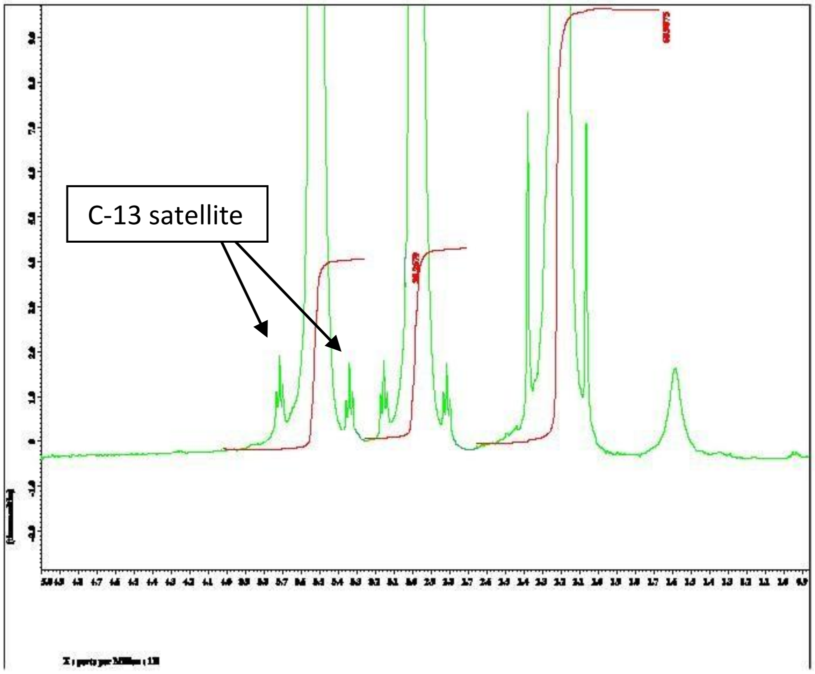

FIGURE 2: Plot showing the spectrum in Figure 1 with an expanded y-scale. The small ^{13}C satellite peaks next to the central peaks are shown. Integrals are shown in red.

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APPENDIX I: Precision and Accuracy Testing Data for the HN-3 Method

I-1. APPROACH

The HN-3 purity determination method was validated using a variation of the protocol used in a Class I Precision and Accuracy (P&A) test. This kind of test is typically used for validation of air monitoring methods. The requirements are not exactly applicable to an NMR purity determination test. A four-day test was used. On each day of the test, 10 samples and two blanks were prepared. The 10 samples were prepared with amounts of HN-3 of 0.2Z, 0.5Z, 0.8Z, 1.0Z, and 1.5Z, each sample in duplicate, where Z = 25 mg of HN-3. As a result, the purity method was validated for a quantity of agent from 5 mg to 37.5 mg.

This testing was not in strict accordance with a normal P&A test. First, NMR is not a trace detection method, and the purpose of the method is not to detect low amounts of agent for safety purposes, as it is for air-monitoring applications. For a typical Class I P&A, the amount of agent is measured in nanograms, while the NMR method is measured in milligrams.

The data from a P&A test is typically processed using a program called Certify (latest version is version 6.0). Certify contains statistical criteria for the acceptance of data or the test method within acceptable measurement limits. Certify does not apply to the NMR purity determination very well, however. The target Z levels (where Z is the required detectable amount) are set in the program to be the same for all replicates from the four-day test. The approximate target amount is measured using an adjustable pipet, and this approximate amount was chosen according to the target Z levels. For the NMR purity method, the target levels are determined by the weight of the agent taken from the NIST-traceable balance. The accurate target amount is found from the weight of the agent in the vial. The accurate amount cannot be entered into the Certify program as an x-coordinate.

The data that is obtained from this test easily passes the Certify pass/fail criteria of ±10%, which is much less tight than a purity determination requires. But because of the way the data is entered, Certify is testing the accuracy of the pipeting. The accuracy of the weighing and NMR determination is better than the accuracy of the pipeting. The actual accuracy of the data from weighing and NMR determination is much better than the Certify calculations suggest, so using Certify to quantify the P&A results in this case does not accurately indicate the method performance. As a result, the results are reported in terms of standard deviations and correlation coefficients of the data.

The P&A test was performed on a JEOL ECS-400 Nuclear Magnetic Resonance Spectrometer, installed on July 2011 to meet the manufacturer’s specifications. Data was collected and processed by operators who have received software and hardware training from JEOL representatives.

Weighing was done using a Sartorius Cubis balance, barcode 9804. The balance was calibrated by the ECBC Calibration Team on 30 Nov 11 (expires 29 Nov 12) by Reese (W959QC), identification number 27102674.

The internal standard was 1,2,4,5-tetramethylbenzene, purchased from Fluka (Sigma Aldrich), Part Number 74658-5G, CAS No. 95-93-2, as a TraceCERT® certified reference material (CRM) standard for quantitative NMR. The lot number is #BCBC1486, Pcode number 100975857, expiration June 2012. The standard was received with a certification of analysis of 99.95% (g/g), and uncertainty of 0.08% (g/g).

The T_1 for the solutions (see Section 2.3 step e) was determined to be 1.93 s for the HN-3 peaks, and 2.23 s for the standard peak. As a result, 40 s was used as the NMR relaxation delay time since it is greater than 10 times the T_1 time.

I-2. RESULTS

Tables I-1 to I-4 show the data sets collected on each day of the four-day P&A test. Figure I-1 shows the data plotted together with the regression lines and correlation coefficients.

Table I-1: Data from Day 1.

Target Z (wt agent/25 mg)	Area of Analyte (agent)	Area of Standard (TEP)	Wt. Of Standard	Sample Weight	Found Z	
1.5380	80.9512	53.5569	16.4000	38.4500	1.5110	
1.5880	85.9070	48.7781	14.5400	39.7000	1.5610	
1.0592	58.0405	54.676	16.1700	26.4800	1.0463	
1.0680	56.9870	41.7849	12.6000	26.7000	1.0475	
0.8632	41.6541	38.5118	12.5000	21.5800	0.8241	
0.8160	43.3314	43.6611	13.2900	20.4000	0.8040	
0.6440	36.2046	47.1015	13.5400	16.1000	0.6344	
0.5896	31.7929	53.5087	16.1300	14.7400	0.5842	
0.2148	11.7654	52.042	15.4200	5.3700	0.2125	
0.2428	14.4832	51.0918	13.8600	6.0700	0.2395	
0.0000	0.0000	1	12.9600	0.0000	0.0000	
0.0000	0.0000	1	15.8200	0.0000	0.0000	
0.8632[a)]	46.6019	41.7236	12.5000	21.5800	0.8510	
					correlation	0.99990

a) This row of data was a remeasurement of the same sample with better shimming, which slightly improved the resulting found Z.

Table I-2: Data from Day 2.

Z (wt agent/25 mg)	Area of Analyte (agent)	Area of Standard (TEP)	Wt. Of Standard	Sample Weight	Found Z
1.4360	84.9107	56.0931	15.9800	35.9000	1.4745
1.5956	93.9077	54.6169	14.9400	39.8900	1.5658
1.0384	62.1455	55.6028	14.9200	25.9600	1.0165
1.0664	61.6724	59.6639	16.6200	26.6600	1.0472
0.8584	51.3326	48.3202	13.0900	21.4600	0.8477
0.8388	50.7061	54.3117	14.6500	20.9700	0.8337
0.5920	36.0909	51.6591	13.6400	14.8000	0.5809
0.5560	32.9266	58.5863	16.0600	13.9000	0.5502
0.2560	16.0369	64.0023	17.0800	6.4000	0.2609
0.2528	15.1923	59.1811	15.8800	6.3200	0.2485
0.0000	0.0000	1	14.1200	0.0000	0.0000
0.0000	0.0000	1	16.4200	0.0000	0.0000

correlation 0.99948

Table I-3: Data from Day 3.

Z (wt agent/25 mg)	Area of Analyte (agent)	Area of Standard (TEP)	Wt. Of Standard	Sample Weight	Found Z
1.6268	98.8407	55.1482	14.7000	40.6700	1.6060
1.4780	85.2285	58.9585	16.8000	36.9500	1.4804
0.9916	60.3796	75.8574	20.2500	24.7900	0.9825
1.0160	62.0782	43.1736	11.5700	25.4000	1.0141
0.8300	50.6648	57.5644	15.4000	20.7500	0.8262
0.8368	50.4028	55.0457	14.9200	20.9200	0.8328
0.5900	35.2934	54.5066	14.7800	14.7500	0.5834
0.5744	34.7625	54.6726	14.7400	14.3600	0.5713
0.2100	12.9107	54.8143	14.5200	5.2500	0.2085
0.2196	13.5240	52.0666	13.7100	5.4900	0.2171
0.0000	0.0000	40.3935	10.5900	0.0000	0.0000
0.0000	0.0000	46.8453	12.2600	0.0000	0.0000

correlation 0.99995

correlation 0.99995

Table I-4: Data from Day 4.

Z (wt agent/25 mg)	Area of Analyte (agent)	Area of Standard (TEP)	Wt. Of Standard	Sample Weight	Found Z
1.5872	93.7219	74.4285	20.5900	39.6800	1.5804
1.4700	85.6474	67.7452	19.1900	36.7500	1.4789
1.0396	60.6869	68.8901	19.2500	25.9900	1.0337
1.0320	61.0960	63.3159	17.5400	25.8000	1.0317
0.8168	48.1967	62.8602	17.3700	20.4200	0.8118

0.8268	48.2507	69.3331	19.4200	20.6700	0.8238
0.5388	32.3323	59.3855	16.0700	13.4700	0.5333
0.5896	35.0091	42.0767	11.4800	14.7400	0.5822
0.2188	13.0287	64.8029	17.6500	5.4700	0.2163
0.2112	12.8665	53.2979	14.2600	5.2800	0.2098
0.0000	0.0000	47.7711	12.4500	0.0000	0.0000
0.0000	0.0000	63.1748	16.6600	0.0000	0.0000

correlation 0.99997

Correlation coefficients for all the days between the target Z (as a weight) and the found Z are >0.999.

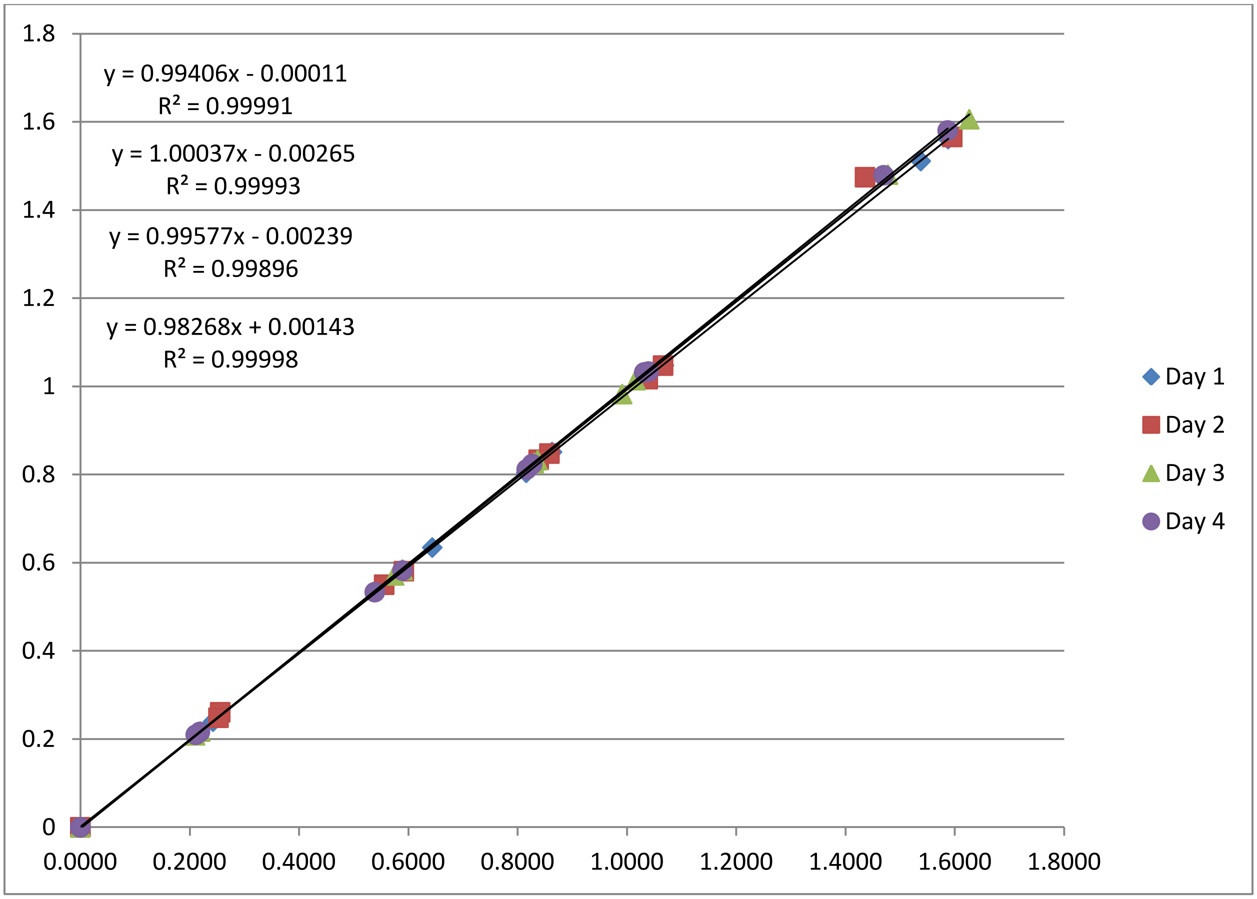

Figure I-1: Plot of the data from four days, Found Z vs. Target Z.

To do the calculation for the Found Z, the formula from Section 2.5 was used, except it was normalized to 1Z = 25 mg instead of using the actual Weight A. Using this method, the purity of the HN-3 sample can be determined from the slopes of the curves from Figure I-1. Averaging all four slopes gives an average purity of 99.3 wt%.

The typical way to determine the purity is simply to calculate purity for each run using the formula in Section 2.5. Table I-5 shows the calculations for Day 1 data, excluding the blank runs. The resulting average purity is 98.25 wt%, with a standard deviation of 1.0%. The 95% confidence limit is 2.05%.

Table I-5: Data from Day 1, used to calculate purity for each run.

Area of Analyte (agent)	Area of Standard (TEP)	Wt. Of Standard	Sample Weight	Weight %
80.9512	53.5569	16.4000	38.4500	98.25
85.9070	48.7781	14.5400	39.7000	98.30
58.0405	54.676	16.1700	26.4800	98.78
56.9870	41.7849	12.6000	26.7000	98.08
41.6541	38.5118	12.5000	21.5800	95.47
43.3314	43.6611	13.2900	20.4000	98.53
36.2046	47.1015	13.5400	16.1000	98.51
31.7929	53.5087	16.1300	14.7400	99.08
11.7654	52.042	15.4200	5.3700	98.93
14.4832	51.0918	13.8600	6.0700	98.64

Average	98.2572
Standard Deviation	1.0262
Confidence Limits	2.0524

To minimize the amount of sample preparation, it is possible to prepare only one sample and rerun it multiple times. This approach minimizes the hazard from handling neat agent and minimizes the consumption of agent and generation of waste. However, the repetitions include only the error that is generated by the NMR data acquisition and integration, and not errors from weighing and sample preparation. Table I-6 shows data from repeated runs of the 1Z sample from Day 1. The error shown by the standard deviation is smaller.

Table I-6: Data from repeated runs of one prepared sample.

Area of Analyte (agent)	Area of Standard (TEP)	Wt. Of Standard	Sample Weight	Weight %
57.9411	54.5232	16.1700	26.4800	98.89
59.7900	56.3442	16.1700	26.4800	98.75
59.8997	56.4393	16.1700	26.4800	98.76
59.9236	56.4048	16.1700	26.4800	98.86
59.7463	56.3664	16.1700	26.4800	98.64
59.4996	56.125	16.1700	26.4800	98.65
59.3066	55.9259	16.1700	26.4800	98.68
59.2736	55.9288	16.1700	26.4800	98.62

Average	98.7330
Standard Deviation	0.1023
Confidence Limits	0.2045


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