Laboratory Procedure Manual

Analyte: **4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)**

Matrix: **Urine**

Method: **MIP Column and LC API MS/MS**

Method No:

Revised: **October 8, 2009**

as performed by:

Emergency Response & Air Toxicants Branch
Division of Laboratory Sciences
National Center for Environmental Health

contact:

Dr. John T. Bernert
Phone: 770-488-7911
Fax: 770-488-0181
Email: JTB2@cdc.gov

Dr. Eric J. Sampson, Director
Division of Laboratory Sciences

Important Information for Users

The Centers for Disease Control and Prevention (CDC) periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

Data File Name	Variable name	Description
NNAL_E	URXNAL	NNAL

1 SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

a. *Analyte.*

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol. NNAL. $C_{10}H_{15}N_3O_2$; Mol Wt 209.12.

b. *Clinical Relevance.*

The tobacco-specific nitrosamine NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) is a significant component of tobacco and tobacco smoke. In the smoker's body, NNK is rapidly reduced to its metabolite, NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanonol). A significant portion of NNAL may also exist in the glucuronide form NNAL-Gluc (NNAL-N-Gluc and NNAL-O-Gluc) in the urine. Both NNK and NNAL are potent lung carcinogens in rodents (1), and they are likely involved in the increased lung cancer risk in smokers (2-4). The NNAL metabolite has similar carcinogenicity to NNK (5). NNAL (either free and/or total forms) may be used as a biomarker for exposure to NNK among active smokers, and also among nonsmokers following exposure to secondhand smoke (SHS).

c. *Assay Principle.*

NNAL is measured by using liquid chromatography linked to tandem mass spectrometry (LC/MS/MS). For "total" NNAL assays, the urine sample is fortified with an NNAL-$^{13}C_6$ internal standard, and then hydrolyzed using β-glucuronidase in incubations for at least 24 hours. The samples are then extracted and cleaned up on a specially-designed solid-phase molecularly-imprinted polymer (MIP) column, after which the analyte is eluted and analyzed by LC/MS/MS, monitoring the m/z 210->180 native, and m/z 216->186 internal standard transition ions. NNAL concentrations are derived from the ratio of the integrated peaks of native to labeled ions by comparison to a standard calibration curve. Free NNAL measurements are conducted in a similar manner, but with the omission of prior enzymatic hydrolysis. Bound NNAL (i.e. NNAL-Gluc) may be estimated from the difference of (Total NNAL – Free NNAL). This method has been described previously (6)

2 SAFETY PRECAUTIONS

Safety glasses, gloves and clothing must be worn during the extraction and processing of samples by this method.

a. ***Reagent Toxicity / Carcinogenicity.*** Some of the reagents used in this procedure are toxic, and NNAL itself is believed to be a potent human carcinogen. Universal safety precautions must be taken to avoid inhalation or dermal exposure to assay reagents or analytical standards.

b. ***Radioactive Hazards.*** This procedure does not use radioactive materials and there are no radioactive hazards associated with it.

c. ***Biological Hazards.*** This assay involves human urine samples. Universal precautions must be followed. Analysts working directly with the specimens must use proper technique and avoid any direct contact with the sample. Lab coats, gloves and protective eyewear (as required) should be worn while handling the specimens.

d. ***Chemical Hazards.*** Reagents and solvents are used in this study including those listed below in section 6.a. MSDSs for these chemicals are readily accessible on the internet (e.g., http://www.msdssearch.net/MSDSSearch.asp, or http://msds.ehs.cornell.edu/msdssrch.asp). Hardcopies are maintained on file.

e. ***Mechanical hazards.*** There are no unusual mechanical hazards associated with this method. Analysts should know and follow the manufacturer's recommendations concerning the safe handling of instruments and other equipment. High voltages are found within certain areas of the mass spectrometer and care must be taken when working in those areas. Safety interlocks on instruments such as the mass spectrometer, LC autosampler and centrifuge covers, etc. should not be defeated during normal operations.

f. ***Protective equipment.*** Standard safety precautions should be followed when performing this procedure including the use of a lab coat/ disposable gown, safety glasses, appropriate gloves, and the use of biological safety cabinets and chemical fume hoods as needed. Refer to the laboratory Chemical Hygiene Plan and standard CDC / DLS safety policies and procedures guidelines for details related to specific activities or reagents.

g. ***Training.*** Formal training in the use of tandem mass spectrometry is required. All analysts must be CLIA-certified and demonstrate proficiency in the analysis before handling samples. Educational and specific training information is maintained for all analysts certified to work on this method.

h. ***Disposal of Wastes.*** All waste disposal must be in compliance with DLS policy. Discard solvents and other waste reagents into an appropriate container marked for waste handling and store it in a chemical fume hood. Place all disposable items that come in contact with biological specimens in a biohazard autoclave bag which is maintained in an appropriate covered container until autoclaved. Unshielded needles, pipet tips and disposable syringes with attached needles must be placed in a sharps container and autoclaved when the container is full. Wipe down all surfaces potentially exposed to biological samples with a freshly prepared bleach solution (10% dilution of commercial sodium hypochlorite or the equivalent) each day. Non-disposable

glassware or other equipment that comes into contact with biological samples must be rinsed with bleach before cleaning and reuse.

3 COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. ***Software and knowledge requirements.*** This method has been validated using a MIP column solid-phase extraction (SPE) sample preparation procedure, followed by liquid chromatography electrospray-ionization tandem mass spectrometry using a Sciex API 5000 mass spectrometer controlled by Analyst version 1.4.1 or up software. Sample analyte concentrations are calculated in Analyst and the results with additional information (retention times, area counts, etc) are exported and merged with cleanup information using custom programs. The final results and QC evaluation are performed using SAS programs. Knowledge of, and experience with these software packages is required in performing these functions.

b. ***Sample information.*** All samples are analyzed in runs of, typically, 24 samples including blanks, QC and unknowns. Each run is prepared as an "NNAL Sample Preparation" file that contains sample ID, analyst, dilution, date of analysis and other information. A program generating the database containing this information has been developed by this laboratory and is maintained on the intranet. This program is used to prepare background and cleanup information about samples in each run, which should be entered into the database at the time of preparation of each run.

c. ***Data maintenance.*** Following the LC/MS/MS analysis, the standards and samples are processed online using Analyst software, and information individually documented in the Sciex Analyst database, which includes sample file number, sample I.D., date and time assayed, integrated peak area counts, retention times, quantitated results, etc is copied *via* a transfer ("*.AQ") file. These files are processed by another custom program ("NNAL AQ.EXE") which checks the data and merges the results with the corresponding information for the matched sample drawn from the Sample Preparation file. The resulting merged data are then stored in *.DAT files which are further processed and analyzed using SAS programs and procedures.

d. ***Information security.*** Information security is provided at multiple levels. The data systems used in this work are accessed via computers that require individual login and passwords and that default to locked conditions during extended periods of nonuse. Sensitive portions of custom software are protected with additional password requirements. In addition, all systems and equipment are located on the Chamblee campus of CDC which has restricted access with security personnel approving all entry. Furthermore, the individual laboratory building has multiple levels of controlled access including the requirement for card keys to access the building itself, and also the individual floors where the equipment is located. Confidentiality of the results is protected by use of blind coded ID numbers only (no personal identifiers are ever used).

4 COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. ***Special requirements.*** There are no special requirements such as fasting or adherence to special diets for this assay.

b. ***Sample collection.*** The specimen for these analyses is human urine. Samples are collected in standard urine collection cups (*note*: cups and all other materials contacting samples should be pre-screened and approved by this laboratory before they are used to avoid background contamination issues). Samples should be refrigerated as soon as possible after collection, and frozen for longer term storage. The sample should be well-mixed, and placed in an appropriate vial (e.g. Corning 50 mL polypropylene tubes, #430290), and the tubes capped securely. Be careful not to overfill the tube (maximum volume of approximately 45 mL) to allow for expansion in the freezer.

c. ***Sample handling.*** Specimen handling conditions for urine samples including general collection and transport requirements are outlined in the DLS protocol for urine collection and handling (available from this laboratory or the DLS specimen handling activity). In general, urine samples should be frozen at –20^{o}C and shipped with dry ice by overnight air. A packing list must be included with the samples, and the laboratory (or the specimen handling group) should be notified before shipment. Unless special arrangements are made, shipment schedules should avoid having samples arrive at CDC on the weekends or holidays since sample handling at those times may not be appropriate. After receipt, samples are stored frozen at –20^{o}C, or in some cases at –70^{o}C for long-term storage.

d. ***Sample quantity.*** A minimum of 10 – 20 ml of urine is needed depending on the nature of the analysis (e.g. whether both free and total measurements will be made). The optimum volume is approximately 30 – 40 ml of urine.

e. ***Unacceptable specimens.*** Criteria for defining a sample as unacceptable include (1) use of improper collection materials or techniques leading to elevated background contamination; (2) sample volumes less than the required minimum; or (3) improper shipment or storage of samples leading to thawing, leaking or similar problems. All samples are logged in at receipt and problems with storage or shipment are identified at that point; inadequate volumes will generally be identified when the samples are thawed for analysis. If a sample must be rejected as unacceptable, a description of the problem must be entered into the database and associated with that sample.

f. ***Long-term stability.*** Long-term stability results are currently under investigation for this analyte. However, literature reports and preliminary work including accelerated stability studies (Appendix A) in this laboratory suggest that urine samples are stable for <u>total</u> NNAL analyses for at least 6-12 months and probably longer when stored in appropriate containers at low temperatures (-20^{o}C or less). The stability of the glucuronide forms during storage is also under investigation, but is probably not as great as that for total NNAL.

5 PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6 PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

Note: Class A glassware such as pipets and volumetric flasks are used unless otherwise stated.

a. ***Solvents and reagents. Identification and handling.***

(1) 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol - this is a suspected lung carcinogen, and suitable protective clothing, gloves and eye/face protection must be utilized. It can be harmful if inhaled, swallowed or absorbed through the skin, and should only be used in a chemical safety hood. If contact occurs, flush area immediately with copious amounts of water.

(2) Potassium Hydroxide – this is a very caustic base, corrosive to all tissues. It is used to adjust the pH of phosphate buffer. It generates considerable heat when mixed with water or an acid. It is nonflammable but would be harmful if inhaled or swallowed. Safety glasses and gloves must be worn while working with this reagent.

(3) Ammonium Acetate – this is used to make the LC mobile phase. It may be harmful by inhalation, ingestion or skin absorption. Inhalation may cause irritation to mucous membranes and the upper respiratory tract. Safety glasses and gloves must be worn while working with this reagent.

(4) Formic Acid - this is used to adjust the pH of the LC mobile phase. It will bum skin tissue and is harmful if inhaled or swallowed. If exposure occurs, flush the area with copious amounts of water. Always wear protective clothing and safety glasses when working with this reagent.

(5) Toluene - this is a flammable liquid, and also may form explosive vapors. Remember that the vapor is heavier than air and may travel some distance to an ignition source. As a liquid it is a skin irritant, and may be absorbed through the skin. Large volumes of toluene should be handled with gloves in a chemical fume hood.

(6) Methylene chloride - this solvent is chemically stable and relatively unreactive. It poses a relatively low hazard. It is not flammable, but the vapor can be irritating to the eyes, nose and throat, and skin or eye contact with the liquid should be avoided. Flush copiously with water if any contact should occur. Evaporation of significant volumes of this solvent must be performed in the Savant evaporator, or in a chemical fume hood.

(7) Methanol - this solvent is used to pre-condition SPE columns. It is toxic by ingestion, inhalation and skin absorption. It may cause acidosis, blindness and death. It is also flammable. Evaporation of significant volumes of this solvent must be performed in the Savant evaporator or in a chemical fume hood. Safety glasses and gloves must be worn when handling this solvent.

(8) Acetonitrile - used as a mobile phase for LC. It is toxic by ingestion, inhalation and skin absorption, and can be a source of cyanide toxicity. It is also flammable. Evaporation of significant volumes of this solvent must be performed in the Savant evaporator, or in a chemical fume hood. Wear appropriate protective clothing to prevent skin exposure.

(9) Sodium Hydroxide – this is a very caustic base, corrosive to all tissues. It is used to adjust the pH of phosphate buffer. It generates considerable heat when mixed with water or an acid. It is

nonflammable but would be harmful if inhaled or swallowed. Safety glasses and gloves must be worn while working with this reagent.

b. ***Stock reagent preparation***

(1) 0.5 M Phosphate Buffer (pH 6.4). KH_2PO_4, potassium phosphate monobasic, FW 136.09, stored at room temperature in the chemical cabinet in 103/3117. To a 2000-mL volumetric flask, add 136.09 g KH_2PO_4. Stir until dissolved. Continue stirring while adding KOH pellets to pH 6.4. PO4 buffer is made on a weekly basis.

(2) β-glucuronidase. β-glucuronidase type IX-A from E. Coli, Sigma, 1,724,400 units/g. A 20,000 units/ml solution in pH 6.4 phosphate buffer is made according to usage. For example, to make a 10-ml solution of 20,000 units/ml using enzyme with this activity, 0.1160 g β-glucuronidase power is dissolved in 10 ml of pH 6.4 phosphate buffer.

c. ***Calibration materials***

One complete set of NNAL calibration standards was prepared for use in September, 2003 and stored frozen. The standard curve is run four times at the beginning of each sample run to establish the standard curve. A total of 10 standards were prepared ranging from 0 to 250 ng/ml. The NNAL-$^{13}C_6$ internal standard spiking solution was prepared as a 100 ng/ml solution in water, and is stored in refrigerator in 103/3121.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). Toronto Research Chemicals M-325740, 99.9+%, $C_{10}H_{15}N_3O_2$, Mol Wt 209.12.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol - 1,2',3',4',5',6'-$^{13}C_6$ (NNAL-$^{13}C_6$). Cambridge Isotope Laboratories,99.9+%, Mol Wt 215.2.

Water. TEDIA, CAT # WS-2211.

Original stocks:

Stock Native O: dissolve 112.24 mg of (99.9+%) NNAL in 50 ml water. 2,245,000 ng/ml

Stock IST: dissolve 3.01 mg of (99.9+%) NNAL-$^{13}C_6$, in 100 ml water. 30,100 ng/ml

Working Stocks:

Level A: dilute Stock Native O with water (11.137 ml of Stock Native O q.s. to 100 ml); 250,000 ng/ml.

Level B: dilute Level A 1:10 with water (10 ml of Level A q.s. to 100 ml); 25,000 ng/ml.

Level C: dilute dilute Level B 1:5 with water (20 ml of Level B q.s. to 100 ml); 5,000 ng/ml.

Level D: dilute C 1:10 with water (10 ml of Level C q.s. to 100 ml); 500 ng/ml.

Level E: dilute D 1:10 with water (10 ml of Level D q.s. to 100 ml); 50 ng/ml.

Level ISA: dilute Stock IST with water (42.13 mL of Stock ISA q.s. to 500 ml); 2,500 ng/ml.

ISTD Spiking Solution:

100 ng/ml NNAL-$^{13}C_6$ in water. Take 20 ml of Level ISA (2500 ng/ml) and q.s. to 500 ml with water in a volumetric flask. Spiking solution was stored at 4^{o}C in the walk-in refrigerator in 103/3117.

Below is the complete list of NNAL standards. Currently 1:25 diluted standard 1, 4-12 are used for calibration curve daily.

STD #	Ng/20µl	Ng/ml	Final Volume (ml)	Total (ng)	Stock	Vol. of Stock (ml)	Vol. of ISA (ml)	ISTD ng/20µl
1	0	0	500	0	Level E	0	20	2
2	0.001	0.05	500	25	Level E	0.5	20	2
3	0.002	0.1	500	50	Level E	1	20	2
4	0.005	0.25	500	125	Level E	2.5	20	2
5	0.01	0.5	500	250	Level E	5	20	2
6	0.02	1	500	500	Level E	10	20	2
7	0.05	2.5	500	1250	Level D	2.5	20	2
8	0.2	10	500	5000	Level D	10	20	2
9	1	50	500	25,000	Level C	5	20	2
10	5	250	500	125,000	Level B	5	20	2
11	10	500	500	250,000	Level B	10	20	2
12	50	2500	500	1250 µg	Level A	5	20	2
13	100	5000	500	2500 µg	Level A	10	20	2
14	500	25000	500	12.5 mg	Native O	50568	20	2

15	**1000**	**500000**	**500**	**25 mg**	**Native O**	**11.136**	**20**	**2**

d. ***Controls.***

(1)Quality control materials. There are seven quality control pools for the urinary NNAL assay. Pools QC1, QC2 and QC3 represent the high, medium and low NNAL quality control pools, respectively. Pools QC1 (high NNAL) and QC3 (low NNAL) were prepared in house by spiking blank urine with NNAL standards. QC2 (medium NNAL), QC4, QC5, QC6 and QC7 were received smokers urine. Pools were pooled, mixed well and 5.5 ml aliquots were dispensed into appropriate sample tubes and frozen at -70°C (freezer #70666).

(2)Proficiency testing materials. These materials are prepared from nonsmokers urine spiked at known levels with NNAL. Three pools are prepared at known concentration levels with target amounts of approximately 50, 500 and 2,000 pg/ml. The pools are blind-coded and stored at –70^{o}C. Four aliquots, including one level duplicated at random, are analyzed by the standard procedure at least twice a year. The coded results are reviewed by DLS personnel not involved in the analysis to confirm acceptable method performance.

e. ***Major Instrumentation and Other Equipment.***

Hybrid Liquid Chromatograph, a Shimadzu SCL-10A system controller, two Shimadzu LC-10AD liquid chromatographs, a Shimadzu DGU-14A degasser, an Agilent 1100 autosampler and an Agilent 1100 column oven, (CDC #141261).

Mass Spectrometer. PE Sciex API 5000 triple quadrupole mass spectrometer, (CDC #168304); equipped with both Turbo Ionspray and APCI ionization source.

Data System. Dell Computer Pentium 4, Windows XP (CDC #168305).

Water bath/shaker. Precision, Jouan Inc. Model Shallow Form Bath

Solid Phase Extractor. Varian Cerex SPE Processor.

Vacuum Evaporater. Savant SpeedVac SC200 (CDC #70583), equipped with a VP190 high vacuum pump, RT 4104 refrigerated trap and a VaporNet VN100 auxiliary trap.

Centrifuge. (Two) IEC Centra 7R centrifuge (CDC #16815) Sorvall RC5C superspeed refrigerated centrifuge. (CDC #100547)

7 CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. ***Cleaning and calibration***

The inlet is removed and the system front end is cleaned each morning before the day's runs. Periodically (typically once per month) the system calibration is confirmed by infusing an appropriate

calibrator solution. Overall performance and complete mass calibrations with PPGs are conducted at each preventive maintenance, following significant repairs or other changes in the instrument, and on other occasions as indicated.

b. ***Calibration Curve***

A calibration curve for this assay is based on the analysis of the standard set described in section 6.c. A set of ten standards ranging in value from zero to 250 ng/ml is analyzed in duplicate in the forward and the reverse direction prior to the start of each sample run. After adjusting for dilution, the calibration range for this method corresponds to a range of values from the limit of detection (LOD) to 50:1 (ISTD : native area ratio).

c. ***Verification***

Initial. The initial accuracy of this method was established by analyzing a series of pure standards prepared as described above. The ratio of native and labeled NNAL area counts was regressed on concentration using 1/X weighting using Sciex system software. The resulting calibration curves were linear to a concentration level of at least 100 ng/ml, and R-squared values were typically > 0.999.

Daily. Prior to assaying each run of unknowns, the results from standard analyses are reviewed for acceptable accuracy, precision and instrument sensitivity. The results from the 20 calibration standards analyzed prior to each run are reviewed daily. Acceptable back-calculated values for standards above the detection limit are typically in the range of nominal concentration ± 10%.

8 PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

Analytical runs are set up using the NNAL Sample Prep program and the samples are racked in their appropriate order. Each run consists of 24 samples. The 01 position is a blank. The 02 and in some cases the 03 positions are QCs. The remaining positions, 04 thru 24 are filled with unknown samples which may include one or more "blind" QC samples. The runs and samples are numbered sequentially by run number and sample position as: NBXXX-001 to NBXXX-024. For example, the first run is NB001 and the next run would be NB002, etc.

a. ***Sample Preparation***

Free NNAL measurement:

(1). Preparation of Chem Elut column

a. Prepare fresh 0.1 N KOH.
b. Set up vacuum system and large column plate.
c. Apply 5 ml 0.1 N KOH to each column. Allow to stand for 3 minutes.
d. Elute 2 x 10 ml with methylene chloride. Discard methylene chloride.

e. After second elution, leave under N2 flow for 20 min at ca 10 psi.
f. Dry in oven for 48 hours.

(2). Liquid-liquid Extraction by methlyene chloride using Chem Elut Columns

a. Remove samples from freezer and allow them to thaw completely. Let ISTD come to room temperature.
b. Pipet 20 µl ISTD (2 ng/20 µl C^{13} NNAL) into each16 x 125 mm tube.
c. Pipet 5 ml urine sample into each tube.
d. Pipet 100 µl 10N NaOH (freshly made daily, 2 grams in 5 ml water) to each sample. Vortex to mix well.
e. Apply the entire sample to a pre-washed 5 ml Chem Elut column.
f. Allow to dry on the column for 3-4 min.
g. Add 7 ml of methylene chloride to the column. Allow to elute by gravity (NO N2 pressure). Collect methylene chloride fraction by 16 x 125 mm tubes.
h. Repeat with a second 7 ml aliquot of methylene chloride.

(3). Liquid-liquid extraction by HCl

a. Add 3 ml 0.1 N HCl into methylene chloride in each 16 x 125 mm tube and cap the tube.
b. Place the tube on the rotator for 10 min.
c. Centrifuge the tube for 10 in.
d. Transfer HCl layer by transfer pipet in 13 x 100 mm tube. Be careful not to draw any methylene chloride layer.
e. Add 30 µl 10N NaOH into each 13 x 100 mm tube to neutralize.
f. Add 3 ml PO4 buffer and follow MIP SPE procedure.

(4). MIP SPE procedure

a. Assemble the extractor and turn the Nitrogen gas on to about 20 psi.
b. Condition the MIP columns with 1 ml methylene chloride, followed by 1 ml methanol and 1 ml water.
c. Vortex the sample and load sample on MIP column. Adjust the pressure so that the sample is drawn through the column at a rate of about 0.5 ml/min.
d. Once all of the samples have eluted, allow the columns to dry under nitrogen at 20 psi for 5 min.
e. After drying, the columns are washed at 15 psi with 1 ml water, followed by 1 ml toluene and 1 ml toluene/methylene chloride (9:1).
f. The analyte is eluted with 3 x 1 ml aliquots of methylene chloride which are combined.
g. Load samples onto sodium sulfate columns. After sample is drained, add another 0.5 ml methylene chloride.
h. The elutent is dried down to about 200 µl using a vacuum centrifuge (SPD2010 SppedVac, Savant Inc. Farmingdale, NY), which takes about 35-40 min.

i. Transfer the samples to LC sample vials. The samples are then dried in the LC vials and the residue is reconstituted in 20 µl water. 10 µl is injected into the LC for analysis.

Total NNAL measurement:

a. Remove samples from freezer and allow them to thaw completely. Let ISTD come to room temperature.
b. Pipet 20 µl ISTD (2 ng/20 µl C^{13} NNAL) into each16 x 125 mm tube.
c. Pipet 5 ml urine sample into each tube.
d. Pipet 0.5 ml of β-glucuronidase solution (20,000 u/ml) into each tube.
e. Place tubes in the water bath that is set to 37°C for 24 hours to hydrolyze. Leave the shaking function on (at setting = 40 rpm) during the incubation.
f. After the hydrolysis, remove the tubes from the water bath and continue the procedure from step 2. d. (Liquid-Liquid Extraction by methlyene chloride using Chem Elut Columns) as given above in the section for free NNAL measurement.

b. ***LC/MS/MS Analysis***

The LC mobile phase A contains 0.5 g ammonium acetate/liter, formic acid (adjusting pH to 5). Mobile phase B is 100% acetonitrile. Prepare the mobile phase A stock as needed and store it at 4 ^{0}C. LC pump A is primed by mobile phase A prior to each sample run.

Operate the turbo-ionspray ionization source of API 5000 mass spectrometer at a source temperature of 600^{0}C with a spray voltage of 1500 volts. Record all LC/MS/MS data in MRM (multiple reaction monitoring) mode collecting data for transition ions at m/z 210.0/93.0 (NNAL confirmation), m/z 210.0/180.0 (NNAL quantitation), and m/z 216.1/186.1 (NNAL ISTD). Optimize the compound related mass spectrometric parameters for each individual ion transition. Optimize the collision gas and curtain gas for the m/z 210.0/180.0 transition. Set the analytical quadruple to unit resolution.

HPLC separation is achieved using an XTerra® RP18 column (5 µm, 3x150 mm) purchased from Waters (Milford, MA). Maintain the column temperature at 40^{0}C and the eluant flow rate at 0.600 ml/min.

The LC gradient program is as follows:

Time (min)	Mobile phase A	Mobile phase B	System Controller
0.01			start
0.02	85%	15%	
2.60	85%	15%	
2.61	0%	100%	
2.80	0%	100%	

2.81	85%	15%	
4.00			stop

Prior to performing a run, inject a NNAL standard to determine accurate system operation and suitable sensitivity.

Immediately after the run, wash the LC column and place the system in standby.

c. ***Data Processing***

(1)The peak integration is automatically performed by the quantitation program in Analyst. Typical method parameters are as follows:

Noise Threshold	20
Bunching Factor	2
Separation Width	0.20
Separation Height	0.01
Exp. Peak Ratio	5.00
Exp. Adjusted Ratio	4.00
Exp. Valley Ratio:	3.00

(2)After the sample is quantitated by the Analyst software, check each integration manually and reintegrate manually where it is needed. Save the quantitation result file (.rdb) and access for review.

(3)After the quantitation result file is generated, export the file as a text file (.aq). NNAL standards and sample results are processed and stored separately for further analysis by the NNAL AQ program. The calibration curve is generated using a weighted regression method (weighted using 1/X) where the ratio of the area of standard and the area of ISTD is plotted vs. concentrations of standards.

(4)Periodically download the files and directories to CD-ROM or external hard drive for review and storage.

d. ***Calculations***

The results are reported in ng/20 µl which correlates to the amount of NNAL in the original 5.0 ml of urine. This can then be converted to ng/ml urine by dividing by 5; or it can be expressed relative to the urine creatinine value when available: NNAL (ng/5 ml)/Creatinine (mg/5 ml) = NNAL ng/mg creatinine.

9 REPORTABLE RANGE OF RESULTS

a. *Linearity Limits*

Samples are obtained from both smokers and non-smokers. Therefore, a broad range of urine NNAL levels can be expected, extending from below the LOD to greater than 500 ng/ml urine. If the value of a sample exceeds the highest point on the standard curve (50:1 as ISTD : native area ratio), the sample is repeated using a smaller sample aliquot and re-analyzed.

b. *Limit of Detection*

An initial estimate of the limit of detection was determined by calculating $3*S_o$ by the extrapolated limiting standard deviation method (7). That provided an estimate of ca. 0.6 pg/mL.

c. *Precision*

Short-term precision was estimated by the repetitive analysis of three levels of spiked urine samples with concentrations of 5, 50 and 200 pg/mL. Two runs with 5 replicates each resulted in relative standard deviations of 12%, 6% and 3%, respectively. Inter-day precision was estimated over a period of 1 week with daily analyses of the same urine pools. Two replicates at each level were analyzed each day for 5 days with an observed day-to-day precision of 24.9%, 14.1% and 3.9%, respectively.

d. *Accuracy*

The initial accuracy of this method was established by analyzing a series of fortified nonsmokers urine samples with known amounts of NNAL as part of method validation procedures. Those results have been described (6), and may be summarized as follows (all N=4):

Target Conc. pg/ml	Observed Mean	Std Dev.	95% CI	% Bias
10	10.4	0.576	9.4 – 11.3	3.6
20	20.4	0.961	18.9 – 22.0	2.3
50	52.0	3.370	46.7 – 57.4	4.1
100	97.9	2.161	94.5 - 101	-2.1
200	214	5.679	205 - 223	6.9
5000	5,118	161.8	4800 - 5400	2.4

e. *Analytical Specificity*

Analytical specificity in this method is mainly conferred through the use of tandem mass spectrometry which is a very specific technique. Further assurance of the identity of the analyte in

unknowns is provided by comparison of retention times during HPLC of the unknowns with that observed with standards; by coelution of the analyte with the labeled internal standard; and by the calculation of appropriate confirmation ion ratios.

f. ***Carryover***

Carryover effects were investigated in urine samples at NNAL concentrations up to 1,000 pg/mL. No carryover was observed between samples in these evaluations.

g. ***Freeze-Thaw and Storage Stability***

Thirty samples from a single urine pool were examined over a period of 7 days in three groups: group (1) was kept frozen at -20^{o}C throughout the study; in group (2) the samples were subjected to one freeze-thaw cycle each day, and two samples selected for analysis each day; whereas in group (3) all samples were kept at room temperature with two samples again selected for analysis each day. All samples remained stable under these conditions including those completing up to four freeze-thaw cycles and those kept at room temperature (ca. 23^{o}C) throughout the study. Our results with QC pools stored at -60^{o}C also are consistent with long-term sample stability when the samples are kept at low temperature during storage.

10 QUALITY Assessment and Proficiency Testing

There are several human urine pools (both fortified and unfortified) that are used for QC in this analysis. All pools were subjected to an initial characterization run series with at least 20 replicates over a 2-week period. The preparation of these pools was described previously in section 6.3. In addition, water blanks are included in all sample runs.

a. *CONTROL (QC) PROCEDURES*

(1)The water blanks for NNAL are typically negative or below LOD for the method (non-detectable). If a calculated concentration of any amount is obtained, the blank value will be subtracted from the sample value.

(2)For the bench QC, the run is rejected if any pool is outside of the 3-sigma limits of the mean, or if any two pools are outside of 2-sigma limits in the same direction. All data are periodically batched and analyzed by using the Division of Laboratory Science SAS-QC program incorporating standard critieria for analysis. Any run failing the DLS SAS QC analysis is repeated if sufficient sample volumes exist; otherwise, no quantitative results for the samples analyzed in that run are reported.

(3)NNAL concentrations are checked to make certain the values are within the range of the method. The actual measured concentration must be no greater than the highest standard value (50:1 as ISTD : native area ratio); if above that limit, the sample must be diluted and reanalyzed if sufficient volume exists. In addition, expected ion ratios for confirmation and quantitation ions, the expected retention times, etc are checked for each sample.

b. *Proficiency Testing*

(1). Proficiency testing is performed at least semi-annually. Currently, no external source of PT materials is available. Therefore, PT assays are conducted using nonsmokers' urine spiked with known amounts of NNAL. In addition, split sample comparisons with an external reference laboratory are conducted periodically to help confirm our results.

(2) Analytical PT results are reviewed by the analyst and the supervisor. Acceptable results require that > 80% of the results agree with the target value ± 10%. If the assay fails PT, all analyses are stopped and the source of error is investigated. No assays will resume until the problem has been resolved and a repeat PT assay has been passed.

11 REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. *Calibration.*

System calibration and general readiness is assessed on a daily basis from a review of the instrument's operating conditions, the values for the water blanks and the results of the pre-run standard (e.g. Internal standard area counts and calculated concentration). When corrective actions are indicated, they are performed and the system is re-evaluated with additional standards until acceptable results are obtained before unknowns are analyzed.

b. *Quality Control.*

If the results from analysis of QC samples are outside the acceptable limits and a reason is identified for the apparent problem, it is indicated and the run is scheduled for repeat sample preparation and analysis for samples that have sufficient quantity. If the problem is not identified, sample preparation and analysis is suspended until the problem or problems are discovered and corrected. Any questionable sample identified by QC or individual sample evaluation that cannot be confirmed by repeat analysis is not included in the reportable database of results.

12 LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Some plastic materials, solvents, air and water may provide trace amounts of NNAL, which could contribute as a contaminant to the level measured in the urine sample. However, by monitoring reagents, using clean glassware and incorporating the solid phase extraction columns into the assay, contamination by an outside source or interference is eliminated. In addition, the specificity of detection by LC/MS/MS helps to avoid background chemical interferences with the quantitation ion. To assure the absence of such interference, all collection and storage devices are pre-screened before they are used.

13 REFERENCE RANGES (NORMAL VALUES)

Study populations typically include both smokers and non-smokers, therefore, a large range of

urinary NNAL levels are expected. No defined reference range currently exists. Previous literature values reported for active smokers have typically been greater than 100 pg/mg creatinine for free NNAL, with geometric mean values for total NNAL of approximately 600 pg/mg creatinine. Among nonsmokers exposed to SHS, the values are much lower, with total NNAL levels typically less than 10-20 pg/mg creatinine. For our method, expected levels for smokers, non-smokers and SHS-exposed and non-exposed non-smokers are currently under investigation.

14 CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable for this procedure.

15 SPECIMEN STORAGE AND HANDLING DURING TESTING

Samples are received frozen and stored frozen at -70°C until analysis. After samples are aliquoted, the remainder of the urine is returned to freezer at -70°C until duplicate analysis is completed or for repeat analysis if required.

16 ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

If a problem with the method exists, samples are held in the freezer until the problem can be resolved. If necessary, filtered and extracted samples ready for analysis can be stored, well-sealed, at -70°C for at least 1 month before they are assayed.

17 TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable at this time.

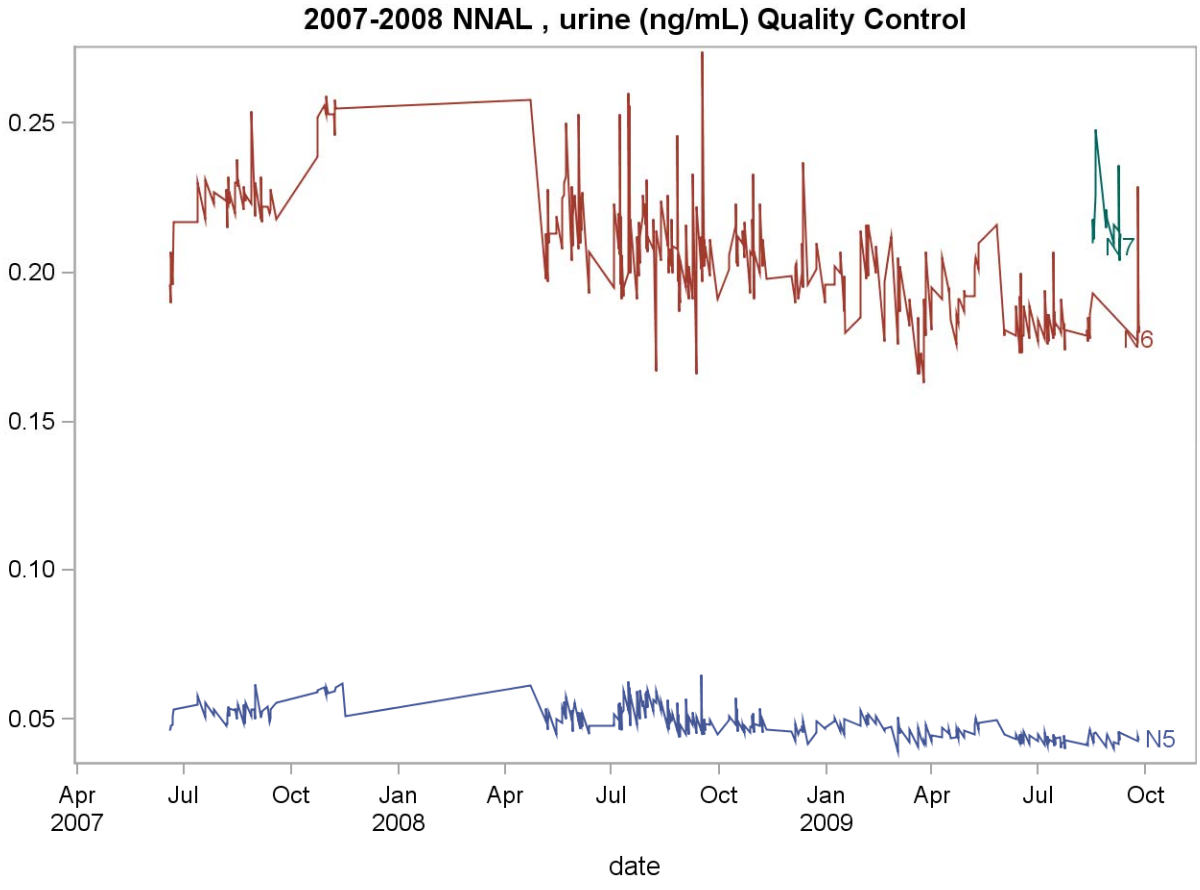
18 TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND ATRACKING

Following analysis, residual samples, if available, are held in storage at -70°C in Chamblee building 103, freezer #16474.

19. SUMMARY STATISTICS AND QC GRAPHS

Summary Statistics for NNAL

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
N5	436	19JUN07	25SEP09	0.04852	0.00488	10.1
N6	410	19JUN07	25SEP09	0.20492	0.01899	9.3
N7	17	17AUG09	10SEP09	0.21776	0.01083	5.0



REFERENCES

1. Smith C. J. and Hansch C. The relative toxicology of compounds in mainstream cigarette smoke condensate. ***Food and Chemical Toxicology***, *38*:637-646, 2000.

2. Hecht S. S. and Hoffmann D. Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. ***Carcinogenesis***, *9*: 875-884, 1988.

3. Hecht, S. S. Biochemistry, biology and carcinogenicity of tobacco-specific N-nitrosamines. ***Chem. Res.Toxicol***., *11*: 559-603, 1998.

4. Preston-Martin, S. N-nitroso compounds as a cause of human cancer. ***IARC Sci. Publ.***, *84*: 477-84, 1987.

5. Hecht, S. S. Human urinary carcinogen metabolites: biomarkers for investing tobacco and cancer. ***Carcinogenesis***, *23*: 907-922, 2002.

6. Xia Y, McGuffey JE, Bhattacharyya S, Sellergren B, Yilmaz E, Wang L, and Bernert JT. Analysis of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in urine by extraction on a molecularly-imprinted polymer column and liquid chromatography / atmospheric pressure ionization tandem mass spectrometry. ***Anal Chem.*** 77:7639-7645, 2005.

7. Taylor JK. **Quality Assurance of Chemical Measurements,** Lewis Publishers, Chelsea, Michigan. 1987.

Appendix A

NNAL Accelerated Stability Study

Objective: This study was conducted in support of a planned analysis of stored, frozen urine samples for total NNAL. Preliminary information from the literature suggests that NNAL is stable for at least 6 months when stored frozen. In addition, our experience with frozen urine pools used for QC purposes indicates that the samples remain stable for at least 2 years when maintained frozen. That study is ongoing as the pools continue to be stored and assayed over time. However, since the proposed new study samples from NHANES will have been frozen for from 2-4 years before analysis, this accelerated stability study was undertaken to obtain additional information on sample stability.

Approach: Two smoker urine pools at two concentration levels were prepared and labeled A and B. Aliquots of the pools were then stored at six temperatures including: room temperature (ca. 22^{o}C), two heated temperatures of 37^{o}C and 55^{o}C, in a refrigerator at 4^{o}C, and frozen at -20^{o}C and -70^{o}C. Periodically, aliquots were analyzed for both free NNAL (without prior hydrolysis with β–glucuronidase), and total NNAL including hydrolysis. Both assays were performed for the first 4 weeks (28 days), and then only total NNAL assays were performed for the remaining study period up to 85 days of incubation. Assays were made on MIP columns with LC API MS/MS analysis as described above.

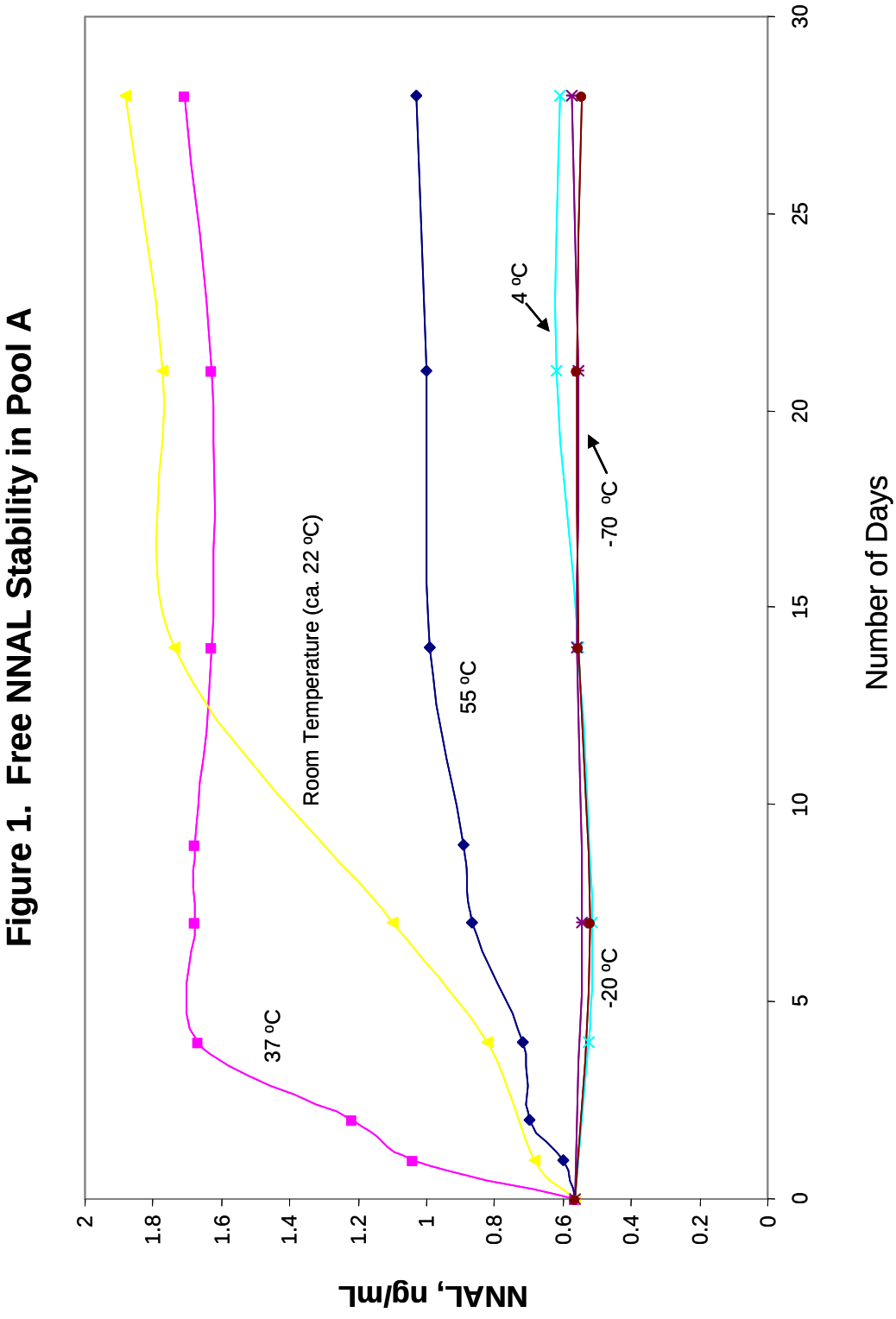
Results: As expected, the glucuronides were not completely stable during incubation. Free NNAL concentrations for pools A and B are given in Figures 1 and 2, respectively. In both pools, there was a gradual rise in free NNAL levels at room temperature; for pool A the hydrolysis seemed to be complete after the first 2 weeks, whereas for pool B increasing levels were seen for the entire 4-week period. The rate of increase was substantially greater at 37^{o}C, consistent with endogenous enzymatic activities being involved, whereas both the rate and extent of hydrolysis was lower at 55^{o}C. The latter results may have reflected partial protein inactivation at this temperature. Whether this represents endogenous enzyme content, or

perhaps more likely, bacterial contamination in the samples, was not determined, but no unusual effort was made to maintain sterility in these samples; rather, they were handled in the usual way throughout.

By contrast, neither pool A nor pool B showed any consistent changes in concentration at any temperature when total NNAL was measured (Figures 3 and 4). Even at 37^{o}C and at 55^{o}C the content of total NNAL remained stable in these pools.

Conclusion: These results suggest that NNAL measurements in stored, frozen urine samples would be expected to remain valid for an extended period of time. Even the free NNAL measurements remained stable in these studies for at least 4 weeks when stored in the refrigerator, or at either of the two freezer temperatures. Furthermore, our QC pool measurements of both free and total NNAL have remained stable for about 2 years so far. This suggests that free NNAL would probably be measurable in the stored samples, and total NNAL certainly would be. Our plans are to measure total NNAL specifically, and these results, in conjunction with prior information, are consistent with the validity of such measurements in frozen urine samples, even after storage for several years.

Figure 1. Free NNAL Stability in Pool A



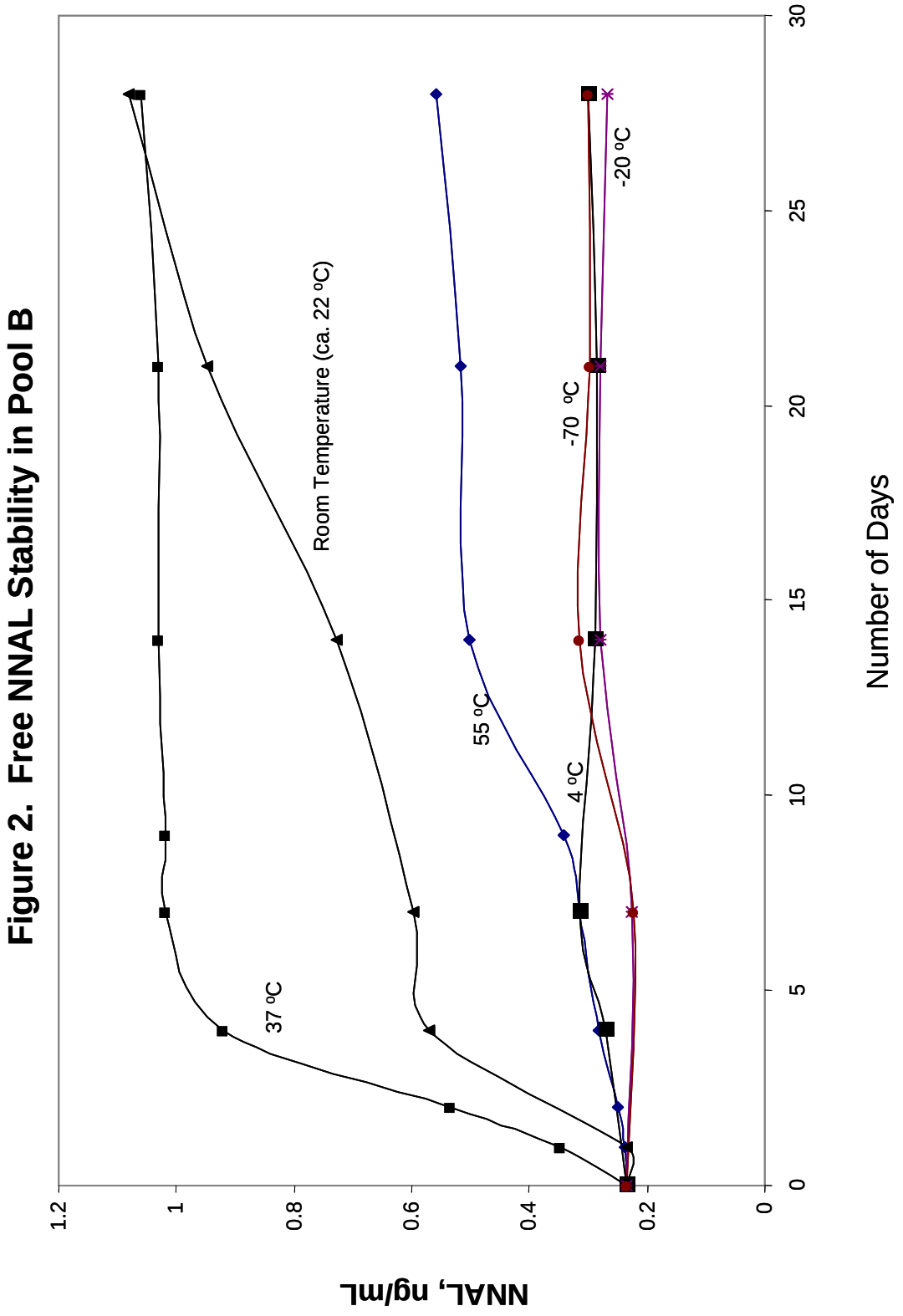


Figure 2. Free NNAL Stability in Pool B
NNAL, ng/mL
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
Number of Days
37 ºC
Room Temperature (ca. 22 ºC)
55 ºC
4 ºC
-70 ºC
-20 ºC

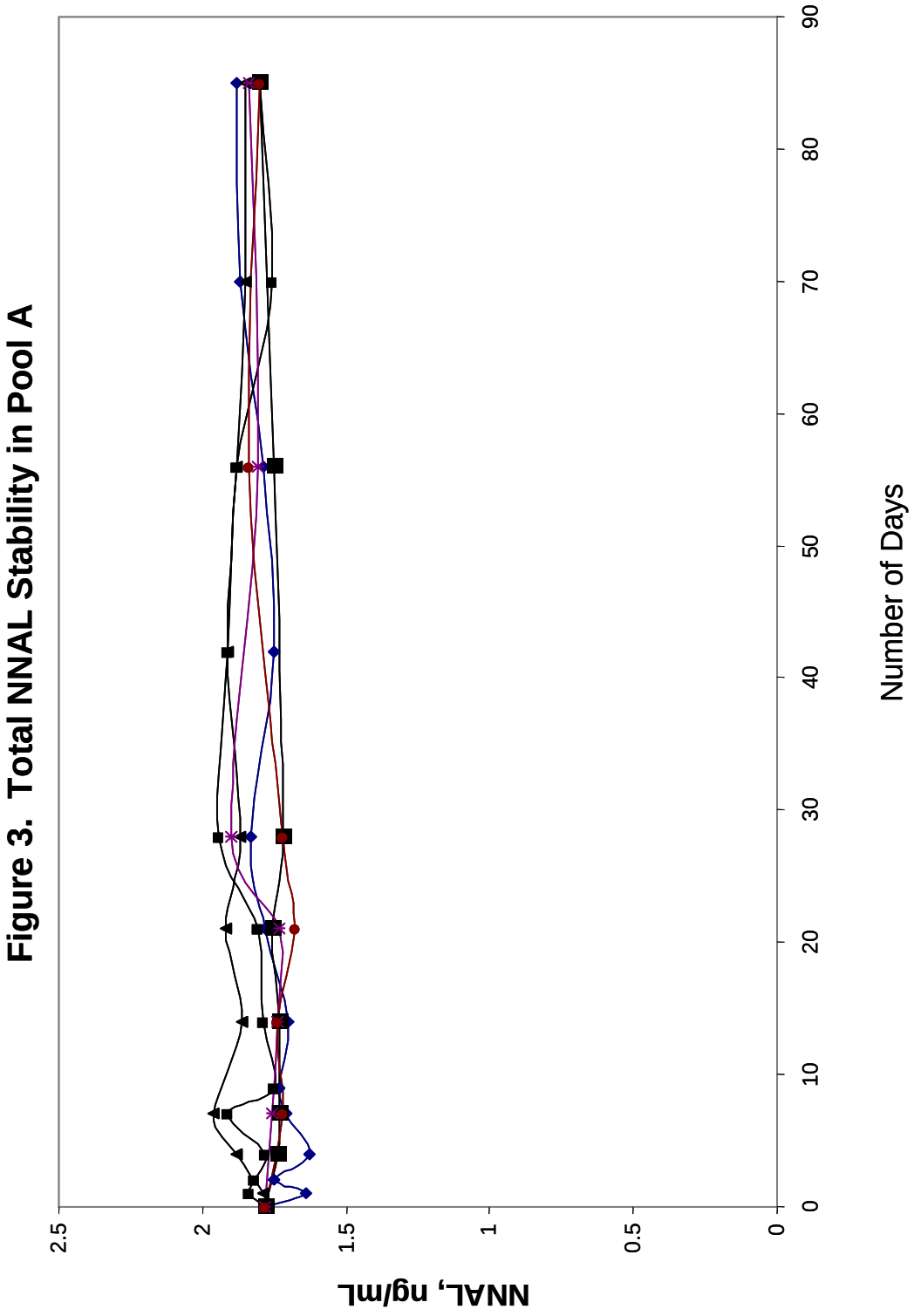


Figure 3. Total NNAL Stability in Pool A

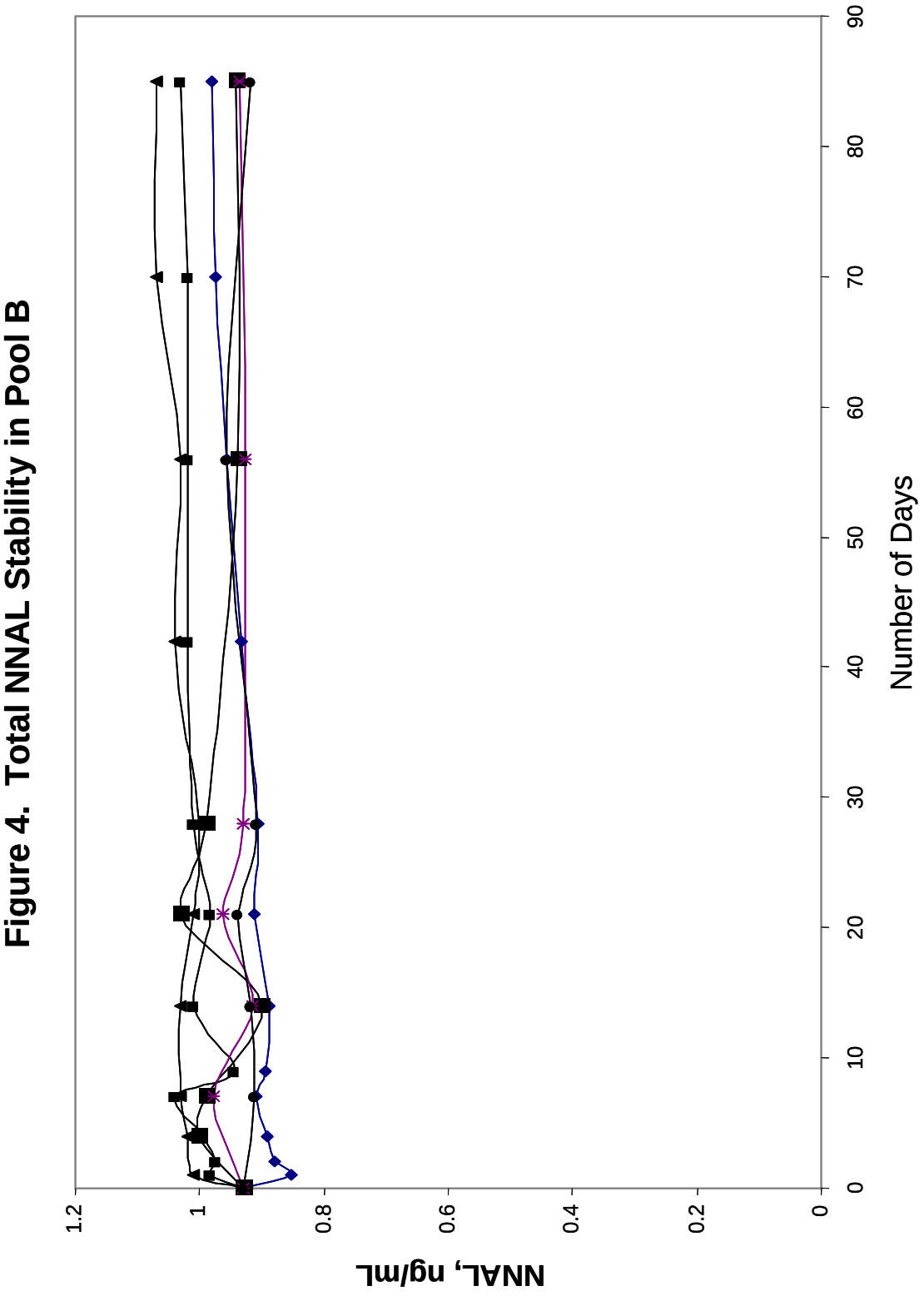


Figure 4. Total NNAL Stability in Pool B

Appendix B

Use of Chem Elut column

The initial method for NNAL in urine used a standard liquid-liquid extraction with methylene chloride conducted in glass tubes. This required extended mixing and additional centrifugation steps. Therefore, the method has been modified to conduct liquid-liquid extraction using Chem Elut columns (Varian CE 12198006) to provide a solid-phase support during the extraction process; enabling this extraction to be completed in a single step, and providing a quicker and more efficient process. A description of this revised procedure is on page 13.

To validate this update, we conducted additional accuracy and precision evaluations with the results as given in the following tables:

Chem Elut Accuracy

Target Conc ng/ml	Calc Conc ng/ml	Accuracy %
0.05	0.0490	-2.00
0.5	0.5053	+1.05
2.00	2.0275	+1.38

Chem Elut Precision

Lab ID	NQ4 Calc Conc ng/ml	Mean	STD	CV %
NB049-002	0.0465	0.0470	0.0016	3.44
NB050-002	0.0500			
NB055-002	0.0463			
NB056-002	0.0477			
NB058-002	0.0462			
NB060-002	0.0465			
NB061-002	0.0490			
NB063-002	0.0453			
NB064-002	0.0453			

Appendix C

Ruggedness Validation

New DLS policies require ruggedness validation in method development. The ruggedness validation requires testing of final method for variables levels of the five most influential factors influencing accuracy. For urinary NNAL LC/MS/MS assay, six factors are identified to influence accuracy the most:

1. Glucuronidase reaction concentration for hydrolysis. The final method uses 10,000 units per 5 ml of sample.
2. Glucuronidase reaction time in hydrolysis. The reaction time is 24 hours in the final method.
3. Dichloromethane elution volume on Chem Elut column. The Volume is 7 ml in the final method.
4. pH for MIP column elution. The pH is adjusted to 6.4 by using buffer in the final method.
5. Loading/eluting rate on MIP. The rate is set to 0.5 ml/min in the final method.

The results of ruggedness testing are displayed in the tables below. The variations had no significant effect on the calculated results of NNAL.

Table 1. Glucuronidase reaction concentration

Glucuronidase concentration (u/ml)	Calculated NNAL (ng/ml)	Recovery %
8000	0.2653	42.02
10000	0.2666	42.20
12000	0.2679	42.38

Table 2. Glucuronidase Reaction Time

Time of hydrolysis (hours)	Percent of Maximum
5	89.97
24	100
48	100

Table 3. Dichloromethane elution volume

Dichloromethane volume (ml)	Calculated NNAL (ng/ml)	Recovery %
5	0.2600	40.31
7	0.2666	42.20
9	0.2631	44.08

Table 4. pH for MIP column elution

pH	% NNAL Quan Area Counts
6.03-6.15	96.31
6.44-6.46	100
6.89-7.00	76.32

Table 5. Loading/Eluting rate on MIP

Loading/Eluting Rate (ml/min)	NNAL Quan Area Counts	Recovery %
0.25	0.2695	46.45
0.5	0.2666	42.20
0.75	0.2636	37.95