



Laboratory Procedure Manual

Analyte: N-terminal hemoglobin adducts of acrylamide and glycidamide

Matrix: Red Blood Cells/Whole Blood

Method: Liquid Chromatography Tandem Mass Spectrometry

Method No:

Revised:

as performed by:

Clinical Chemistry Branch
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Important Information for Users

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.

This document details the Lab Protocol for NHANES 2003-2004.

A tabular list of the released variables follows:

Data File Name	Variable Name	SAS Label
106AGE_C	LBXACR	Acrylamide (pmoL/G Hb)
	LBXGLY	Glycidamide(pmoL/G Hb)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

A. Clinical relevance

Acrylamide was identified as being neurotoxic to animals and humans (i,ii), mutagenic to male germ cells (iii) and probably genotoxic (iv). It is classified as probably carcinogenic to humans by the International Agency for Research on Cancer, and as a potential occupational carcinogen by the Occupational Safety and Health Agency. Research on non-occupational human exposure to acrylamide, were initially driven to elucidate unexplained background biomarker concentrations in control subjects of occupational studies. These studies identified tobacco smoke (v,vi,vii,viii) and food (ix,x,xi,xii) as sources of acrylamide exposure in the general population. The finding that the general population is being exposed to acrylamide created the need for biomonitoring of acrylamide exposure. The assessment of human exposure in the general population will define the extend of exposure and the evaluation of possible associations between exposure to acrylamide and health effects.

Glycidamide, the primary metabolite of acrylamide, has a higher reactivity towards nucleophilic reagents than acrylamide and, in contrast to acrylamide, forms DNA-adducts. Further, results from animal studies suggest that genetic damage in somatic and germ cells is dependent upon the metabolism of acrylamide to glycidamide by CYP2E1 (xiii,xiv,xv). The metabolism of acrylamide to glycidamide seems to depend on exposure dose and exposure path. Because of the concentration and exposure path dependent metabolism of acrylamide and the differences in toxicity of acrylamide and glycidamide, measurement of acrylamide as well as glycidamide adducts are needed to obtain comprehensive information about acrylamide exposure and possible risks associated with this exposure.

B. Test principle

This procedure describes a method to measure hemoglobin adducts of acrylamide and its primary metabolite glycidamide in human whole blood or erythrocytes. Specifically, the reaction products with the N-terminal valine of the hemoglobin protein chains (N-[2-carbamoyl-ethyl]valine and N-[2-hydroxycarbonyl-ethyl]valine for acrylamide and glycidamide adducts, respectively) are measured. This method is based on modified Edman reaction, which uses the effect of N-alkylated amino acids being able to form Edman products in neutral or alkaline conditions without changing the pH to acidic conditions required in conventional Edman reaction procedures (xvi).

It was first described for N-terminal hemoglobin adducts of ethylene oxide, propylene oxide and styrene oxide (xvii) and later optimized to increase yield of Edman products of these adducts (xviii). This optimized method was then successfully applied to adducts produced by other chemicals such as acrylamide, glycidamide and acrylonitrile (xix,xx,xxi,xxii). This optimized method was further refined and modified in-house to increase sensitivity and enable automation (xxiii) The procedure described here consists of 4 parts:

1) Preparation of the specimen for measurement of hemoglobin adducts of acrylamide and glycidamide; 2) Total hemoglobin measurement in the sample solution used for hemoglobin adduct measurements; 3) Modified Edman reaction in the sample solution and isolation of Edman products; 4) Analysis of Edman products by HPLC/MS/MS and results processing.

Because results are reported in pmol adduct per g hemoglobin, the amount of hemoglobin used for the modified Edman reaction needs to be known. Therefore, this procedure includes a measurement procedure for total hemoglobin. It is a commercial assay kit based on a well-established procedure commonly used in clinical chemistry. Quantitation of the acrylamide and glycidamide hemoglobin adduct is performed using octapeptides with the same amino acid sequence as the N-terminal of the beta-chain of hemoglobin and with acrylamide and glycidamide attached at the valine (AA-VHLTPEEK, GA-VHLTPEEK) and the corresponding stable isotope labeled AA-Val(13C5 15N)-HLTPEEK and GA-Val(13C5 15N)-HLTPEEK as internal standards. Total hemoglobin measurement is performed using calibrators provided with the manufacture's assay kit.

2. SAFETY PRECAUTIONS

A. General Safety

Consider all specimens as potentially positive for infectious agents including HIV, hepatitis B and hepatitis C. We recommend the hepatitis B vaccination series for all analysts working with whole blood and /or serum.

Observe all safety regulations as detailed in the Division (DLS) Safety Manual. Additional information can be found in your lab's chemical hygiene plan.

Observe universal precautions; wear protective gloves, lab coat, and safety glasses during all steps of this method. Discard any residual sample material into auto cleavable bags.

Place all disposable plastic, glassware, and paper (pipette tips, autosampler vials, gloves etc.) that are in contact with blood or blood products in a biohazard autoclave bag and keep these bags in appropriate containers until sealed and autoclaved. Use disposable bench diapers during sample preparation and serum/ blood handling and discard after use. Also wipe all contaminated work surface with 10% bleach solution when work is finished.

Acetic acid: Handle with extreme care. It is caustic and toxic; avoid contact with skin and eyes. Do not store with inorganic acids.

Organic solvents: Handle only in well-ventilated areas or as required under a fume hood.

Isopropyl Ether: Forms explosive peroxides on prolonged storage. Keep container in well ventilated place.

Pentafluoropenyl isothiocyanate: Do not breathe vapor. Do not get in eyes, on skin, or clothing. Handle only in well-ventilated areas.

Formaldehyde: Toxic to reproduction, wear appropriate personal protective equipment, clothing, safety goggles, and gloves. Avoid contact with skin or eyes. Use adequate ventilation. Store away from acids.

Reagents and solvents used in this study include those listed in Section 6. Material safety data sheets (MSDSs) for all chemicals are readily available in the MSDS section as hard copies in the laboratory. MSDSs for other chemicals can be viewed at <http://www.ilpi.com/msds/index.html> or at <http://intranet.cdc.gov/ohs>.

B. Waste Disposal

Operators of this method should take the CDC-OHS Hazardous Chemical Waste Management Course (initial and yearly refreshers).

1. Waste to be placed into Biohazard Autoclave Bags & Pans
All biological samples, pipette tips, all disposable plastic well plates used during sample preparation, papers which contact blood or red blood cells, gloves, etc.).
2. Waste to be placed into Sharps Containers
Glass pipette tips, broken glass, cuvettes for HemoCue measurement, etc. Large broken glass which will not fit in the sharps container should be placed in a separate autoclave pan from other waste and labeled as “broken glass” (see the “Autoclaving” section of the CDC safety policies and practices manual located in the laboratory).

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT RESULT ENTRY

- A. For NHANES, data is transmitted electronically Westat's ISIS computer system and transferred from there to NCHS. Abnormal values are confirmed by the analyst, and codes for missing data are entered by the analyst and are transmitted as part of the data file to the Westat ISIS computer, and are eventually forwarded to NCHS. Westat also prepares the abnormal report notifications for the NCHS Survey Physician.
- B. Files stored on the network or CDC mainframe are automatically backed up nightly by DLS LAN support staff and CDC Data Center staff, respectively. Backup of the daily data containing all raw data files and result files for each run are the responsibility of the analyst. Typically these files are backed up once a week onto a floppy disk or a CD-ROM using a CD writer.
- C. Documentation for data system maintenance is contained in printed copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

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

A. General specimen requirements

No fasting or special time of day for specimen collection is required. Specimens for acrylamide- and glycidamide hemoglobin adducts analysis may be fresh or frozen erythrocytes or EDTA-whole blood. This procedure was evaluated with EDTA-whole blood and erythrocytes obtained from EDTA-whole blood. The applicability of this method for other coagulants was not tested.

A 1.0 mL sample of whole blood or 0.5 mL packed red blood cells is preferable to allow for repeat analyses; a sample volume of 550 μ L of whole blood or lyzed red blood cells is required for analysis. More sample is needed if blood clots are present in the tube. The appropriate amount of whole blood or red blood cells is dispensed into a 2.0-mL cryogenic vials with the external screw cap. These cryovials should be labeled in accordance to CDC and NCEH/DLS policies and regulations.

If there are analytes other than acrylamide and glycidamide to be measured, the sample needs to be divided; the appropriate amount of blood or lyzed red blood cells should be transferred into a sterile cryovial labeled in accordance to CDC and NCEH/DLS policies and regulations.

Other specimen handling conditions are outlined in the Policies and Procedures Manual of DLS (copies are available in the Protein Biomarker Laboratory and the electronic copy of this file is located at Q:\SHARE\DLS CLIA Folder).

B. Specimen Storage

Specimens collected in the field can be shipped refrigerated at 5°C or frozen on dry ice. Specimens can be kept refrigerated for 3 days. For long term storage samples are stored at -70°C. Samples are stable for at least 5 years if stored at -70°C. Two to three freeze-thaw cycles did not show any changes in values. However, multiple freeze-thaw cycles of diluted whole blood samples possibly increases the formation of blood clots, which complicates the analysis of the samples. Therefore, diluted whole blood samples should not undergo more than 3 freeze-thaw cycles.

C. Specimen Storage and Handling During Testing

Specimens are allowed to reach room temperature during preparation. Once the samples are ready to be analyzed by HPLC/MS/MS, they are placed into the autosampler tray. The unused portion of the patient specimen is returned to the freezer. Diluted samples (see section 2 can be stored refrigerated over night.

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

Not applicable for this procedure

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

A. Sources for equipment and instrumentation

1. Reagent preparation

- a. Accumet AB 15 pH meter (Fisher scientific, Suwanee, GA) with Orion Micro-Combination pH electrode, pH range 0-14, temperature range 0-100 °C. (Thermo Electron Corp., Bellefonte, PA).
- b. Sartorius Analytical Balance (Accurate “0.00 mg” Digital Scales and Electronic Balances, Edgewood, NY.)

- c. Sartorius Chemical Balance (Electronic “0.000 g” Balances, Edgewood, NY.)
- d. Fisherbrand Desiccator Cabinets, Tall (Fisher scientific, Suwanee, GA). the Dessicant, 6-8 Mesh 100% (EMD Chemicals, Gibbstown, NJ).

2. Sample processing

- a. Tecan (Tecan US., Research Triangle Park, NC). Model Genesis Freedom 200 with Model Pos ID V2200 barcode reader
- b. Matrix (Matrix Technologies Corporation, Hudson, NH). Plate Mate 2X2
- c. Lab rotator Barnstead with flat surface rotor (Lab-Line, Melrose Park, IL)
- d. Vortex- Genie 2 with well plate adapter (Scientific Industries Inc, Bohemia, NY)
- e. Precision Oven (Thelco Laboratories, Torrance, CA).
- f. SPE 215 system with 8 needle arm (Gilson Inc., Middleton, WI)
- g. Eppendorf Centrifuge 5810 R V4.2 with A-4-62 rotor (GMI, Ramsey, MN)
- h. GeneVac EZ-2 Evaporation System with Side Bridge holders and universal rotor (GeneVac Inc., Valley Cottage, NY)
- i. Hamilton Dispenser Microlab 500 Series with 2- 25.0 ml syringes (Hamilton Co., Reno, NV)
- j. Eppendorf 8-channel pipette 50-1200 μ l, (Eppendorf, Westbury, NY)
- k. Gilson 1000 μ l pipette (Gilson Inc., Middleton, WI)
- l. Gilson 200 μ l pipette, fixed (Gilson Inc., Middleton, WI)
- m. Gilson 10 μ l pipette (Gilson Inc., Middleton, WI)
- n. Eppendorf repeater pipette (Eppendorf, Ramsey, MN)
- o. Hand held scanner (Symbol, Technologies. Inc, Bohemia, NY.)
- p. Transfer pipettes (Samco scientific, San Fernando, CA).
- q. Fisherbrand 20” Flexible-Arm lamp (Fisher scientific, Suwanee, GA) with halogen bulb.
- r. Boekel Orbitron Rotator, model II (Fisher scientific, Suwanee, GA).
- s. Microman, 10 μ L positive-displacement pipette (Gilson, Inc, Middleton, WI).
- t. Fisherbrand Octagonal stirring bars, 1”L x 5/16” D (Fisher scientific, Suwanee, GA).
- u. Amber Glass Bottles 60 mL(Fisher scientific, Suwanee, GA).

3. Sample measurement

- a. Power Wave 200 Spectrophotometer (Bio-Tek Instruments, Winooski, VT).
- b. Finnigan TSQ Quantum Ultra™ with atmospheric pressure chemical ionization unit (Thermo Electron, San Jose, CA).
- c. Surveyor HPLC with autosampler and photodiode array detector (Thermo Electron, San Jose, CA).
- d. HemoCue (HemoCue Inc., Lake Forest, CA.)

B. Sources for General Laboratory Equipment and Consumables

1. Reagent preparation

- a. 1 mL, 2mL, 3mL and 4mL volumetric glass pipettes
- b. Various glass beakers, 2L volumetric flask, four 500mL and two 100mL graduated cylinders, and Teflon seal 1L and 2L glass bottles, silanized volumetric flask, silanized volumetric pipettes, class A glassware
- c. Pyrex Culture Tubes, (Corning 16x100 mm, 13X100mm)
- d. BD Vacutainer K3 EDTA (Fisher scientific, Suwanee, GA)
- e. 15.0 ml plastic falcon tube (KSE, Durham, NC).
- f. Plastic Tube (Fisher scientific, Suwanee, GA)

2. Sample processing

- a. 48 well, 5 mL, rectangular well, pyramid bottom, natural polypropylene plate (Innovative Microplate, Chicopee, MA).
- b. 96 well, microtiter plate, Nonbinding Surface (NBS*) (Fisher Scientific, Suwanee, GA).
- c. 48 well, 7.5 ml fritted plate, with 25 μM PE frit, Long drip (Innovative Microplate, Chicopee, MA).
- d. 48 well, 7.5 mL, rectangular well, pyramid bottom, natural polypropylene (Innovative Microplate, Chicopee, MA).
- e. 96 well, 2 ml, deep well plate (VWR International, West Chester, PA).
- f. Bulk Isolute Sorbent HM-N (Biotage, Charlottesville, VA).
- g. 1000 μl, clear pipetting tips for Tecan (Molecular Bio Products, San Diego, CA).
- h. 1000 μl, conductive pipetting tips for Tecan (Molecular Bio Products, San Diego, CA).
- i. 200μl, conductive pipetting tips for Tecan (Molecular Bio Products, San Diego, CA).
- j. 300 μl, clear extended length with filter tips for Matrix (Fisher scientific, Suwanee, GA)
- k. 2 mil PE 96-well pattern sealing film (Bio Tech Solutions, Mt. Laurel, NJ).

- l. Arctic sealer silicon PTFE film (Arctic White LLC, Bethlehem, PA).
 - m. 96 well, 1mL filter plate, with 0.2 μ M GHP (Pall Corporation, Ann Arbor, MI).
 - n. Nalgene 2mL cryovials with ext-tread (fisher scientific, Suwanee, GA).
 - o. Microcuvettes for Hemocue (Aura Tech, Greensboro, NC).
 - p. CP10ST (0.5 μ L-10 μ L) M10 pipette tips for Microman (Gilson, Inc, Middleton, WI).
 - q. Aluminum Foil (Fisher scientific, Suwanee, GA)
3. Sample measurement
- a. HPLC column: Termo Hypersil GOLD, 50 x 2.1 mm, 1.9 μ , (Termo Electron, Holbrook, NY)
 - b. Guard column: Krudkatcher filter (Phenomenex, Torrance, CA)

C. Sources for Chemicals and Standards

1. Reagent preparation
 - a. Cyanmethemoglobin Diluent Powder (CMH Solution), (Fisher Scientific, Suwanee, GA).
 - b. Cyanmethemoglobin Standard (CMH Standard),(Fisher Scientific, Suwanee, GA).
 - c. Mili-Q Water (Aqua Solutions, Jasper, GA)
 - d. Acrylamide Octapeptide (Bachem Bioscience Inc., King of Prussia, PA).
 - e. Glycidamide Octapeptide (Bachem Bioscience Inc., King of Prussia, PA).
 - f. Labeled Acrylamide Octapeptide (Bachem Bioscience Inc., King of Prussia, PA).
 - g. Labeled Glycidamide Octapeptide (Bachem Bioscience Inc., King of Prussia, PA).
 - h. Acetic Acid ACS grade (J. T.Baker, Phillipsburg, NJ)
 - i. Ammonium acetate (Fisher scientific, Suwanee, GA)
 - j. Hemo Point H2, Hemoglobin Control, 6 vials (Stanbio Labortory, Boerne, TX).
2. Sample processing
 - a. Formamide ACS grade (USB, Cleveland, OH).
 - b. Pentafluorophenyl Isothiocyanate 96 %(Alfa Aesar, Ward Hill, MA).
 - c. Isopropyl Ether ACS grade (EMD, Gibbston, NJ).

- d. Toluene ACS/HPLC grade (Burdick and Jackson, Muskegon, MI).
 - e. Ethylacetate HPLC grade (EMD, Gibbston, NJ).
3. Sample measurement
- a. Methanol HPLC grade 99.9 % (Caledon Laboratories, Ontario, Canada).
 - b. Deionized water with resistance to at least 15 megaOhm-cm and filter before use, using 0.45 μm nylon filters
4. Calibrators and internal Standards

The following calibrators and internal standards were obtained from Bachem and used as custom synthesis products:

- a. Acrylamide Octapeptide (AA-VHLTPEEK):
Labeled as (-)-AA-VHLTPEEK,
FW=1022.5 g/mol,
Chemical formula: C₄₅H₇₄N₁₂O₁₅
Chemical purity: 71 %
- b. Glycidamide Octapeptide (GA-VHLTPEEK):
Labeled as (-)-GA-VHLTPEEK,
FW=1038.5 g/mol,
Chemical formula: C₄₅H₇₄N₁₂O₁₆
Chemical purity: 71 %
- c. Labeled Acrylamide Octapeptide [AA-Val(13C₅,15N)-HLTPEEK]:
Labeled as (-)-AA-V*HLTPEEK,
FW=1028.2 g/mol,
Chemical purity: 80 %
Isotopic purity: >99%
- d. Labeled Glycidamide Octapeptide [GA-Val(13C₅,15N)-HLTPEEK]
Labeled as (-)-GA-V*HLTPEEK,
FW=1045.2 g/mol,
Chemical purity: >99%
Isotopic Purity: >99%

D. Preparation of Reagents and Materials

Prepare all reagents using deionized water with resistance to at least 18 megaOhm-cm. and filter before use, using 0.45 μm nylon filters.

1. Reagents used for total hemoglobin measurement

Preparation Cyanmethemoglobin (CMH Solution)

According to the manufacturer's procedure, this solution can either be purchased already diluted in liquid form or can be prepared from a premixed reagent powder.

Prepare CMH Solution from reagent powder by reconstituting 4 packages of CMH Diluent Powder in a 2.0-L volumetric flask using Mili-Q water according to the manufacturer's recommendation. The CMH Diluent Powder consists of (0.05 g KCN, 0.20 g potassium ferricyanide, 1.00 g sodium bicarbonate).

2.0-L CMH solution is sufficient for approximately 768 samples including controls. CMH Solution is also used as the calibrator dilution solvent and used as blank in the calibration curve.

Store CMH solution in a dark amber bottle (or bottle wrapped in aluminum foil) at room temperature (18-26 °C) for no longer than 2 years.

Note: Wash thoroughly after handling. Remove contaminated clothing and wash before reuse. Minimize dust generation and accumulation. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale. Acids should not be used around sodium cyanide or potassium cyanide unless absolutely necessary and then only after careful planning. Use only with adequate ventilation or respiratory protection.

2. Reagents used for Edman reaction and purification of Edman products

- a. 1.58 M Acetic Acid Solution for adjusting the pH of the blood samples.

This solution is used for adjusting the pH of the blood samples to be between 5.5 and 6.5.

Pipette 2 mL of concentrated acetic acid (use a borosilicate disposable 1.0 mL glass pipette) in 20 mL of deionized water in a Pyrex culture tube and mix thoroughly. Solution needs to be prepared fresh at the day of use. 12 mL of 1.58 M acetic acid solution is sufficient for (4) 48-wellplates.

- b. 0.52 M Acetic Acid Solution

This solution is used for adjusting the pH of the 400 mM ammonium acetate solution.

Pipette 290 μ L of concentrated acetic acid in 10 mL of deionized water (use a borosilicate disposable 1.0 mL glass pipette) in a Pyrex culture tube and mix thoroughly. Prepare solution fresh as needed.

- c. 400 mM Ammonium acetate buffer solution

This solution is used for adjusting the pH of the calibrator solutions.

Dissolve 1.52 g ammonium acetate (use Sartorius chemical balance) in 40 mL of water using a 100 mL tube. Adjust the pH of the solution by titrating with 0.52 M acetic acid solution to pH 5.0 (± 0.2 pH). Adjust the solution to a final volume of 50 mL.

Prepare solution every four months or as needed.

d. 6 mM K3EDTA saline solution

This solution is used to dilute the samples 1:0.33 (v/v) to facilitate automated pipetting.

Dissolve 0.0175 g K3EDTA in 7 mL using saline solution using a Pyrex culture tube. Prepare solution as needed.

e. Liquid-liquid extraction solution

Add 500 mL isopropylether, 400 mL of ethylacetate and 100 mL toluene in a 1-L glass bottle, use separate graduated cylinders for each solvent. Close the bottle and mix thoroughly (Isopropylether/Ethylacetate/Toluene - 50/40/10 v/v/v).

This solution needs to be prepared fresh at the day of use. 2L of this solution is sufficient for four 48-well plates.

CAUTION! Isopropylether, Ethylacetate, Toluene are volatile organic compounds. Wear gloves, safety glasses, lab coat and/or apron, and work only inside a properly operating chemical fume hood.

f. Preparation of extraction plate

Fill the extraction filter plate (48-well 7.5-mL filter plate with 25 μm filter and long drip) with Isolute Sorbent HM-N (diatomaceous earth). The plate should be filled up to 0.5 cm or $\frac{1}{4}$ inch to the top. Tap all sides of the plate to get a uniform packing. Plates can be prepared the day before and stored in a desiccator.

Note: Perform packing of the plate in the hood to avoid inhaling of sorbent dust.

3. Reagents used for analysis of Edman products by HPLC/MS/MS

Solvent C methanol

Solvent D (methanol/water 50:50 v/v): Add 500 mL methanol and 500 mL water in 1-L glass bottle. Use separate graduated cylinders for each solvent. The HPLC mobile phase is made up daily or as needed.

4. Standards and Calibrators

a. Preparation of Calibrators for acrylamide and glycidamide adduct measurement

All glassware that is in contact with the octapeptide calibrators must be silanized otherwise losses due to adsorption of the peptides to the glass are observed resulting in inaccurate calibrators.

The calibrators for acrylamide and glycidamide adduct measurements are prepared using acrylamide and glycidamide octapeptides (AA-VHLTPEEK and GA-VHLTPEEK). Seven different concentrations are prepared with concentrations of 1.25, 2.5, 5.0, 10, 20, 40 and 80 nM octapeptides of acrylamide and glycidamide. This concentration range covers acrylamide and glycidamide adduct values observed in the general population, non-smoking, smoking and non-occupationally exposed people.

- (1) Acrylamide Octapeptide (AO) stock solution of 72.9 μM
Dissolve 10.5 mg of acrylamide octapeptide (use Sartorius Analytical Balance) in 100 mL deionized-water in a silanized, volumetric 100-mL flask.
- (2) Glycidamide Octapeptide (GO) stock solution of 75.6 μM
Dissolve 2.77 mg of glycidamide octapeptide (use Sartorius Analytical Balance) in 25 mL deionized-water in a silanized, volumetric 25-mL flask.

Note: if new batches of calibrators with different purities are used, adjust the amount of octapeptide appropriately.

- (3) Calibrator stock solution 1000 nM
Pipette 1370 μL of the 72.9 μM acrylamide octapeptide stock solution and 1320 μL of the 75.6 μM glycidamide octapeptide stock solution in a 100-mL volumetric flask and add water to the 100 mL. Mix the solution thoroughly.
- (4) Calibrator solutions
Prepare 80, 40, 20, 10, 5.0, 2.5 and 1.25 nM calibrator solution from the 1000 nM solution by serial dilution. All pipettes used need to be silanized volumetric pipettes. From this solution, prepare the calibrator solutions as described in Table 1.
Take 50 mL of the 1000 nM solution into a 500 mL volumetric flask and dilute to the mark to obtain a 100 nM standard.

Concentration of calibrator solution	Volume to take from calibrator solution	Dilute to final volume	Obtained concentration of calibrator solution
100 nM	160 mL	200 mL	80 nM
80 nM	100 mL	200 mL	40 nM

40 nM	100 mL	200 mL	20 nM
20 nM	100 mL	200 mL	10 nM
10 nM	100 mL	200 mL	5.0 nM
5.0 nM	100 mL	200 mL	2.5 nM
2.5 nM	100 mL	200 mL	1.25 nM

Table 1: Dilution scheme for acrylamide and glycidamide calibrator solutions

Aliquot 0.9 mL of each calibrator level into a 2.0 mL cryovials and store calibrators at -70 °C. One cryovial 0.9 mL is sufficient for 4 analytical runs (4 x 48-wellplates), volumes described are sufficient for a total of 500 analytical runs.

5. Preparation of Internal standard solutions for acrylamide and glycidamide adducts measurement
 - a. Labeled acrylamide octapeptide stock solution (LAO) 66 μ M
Dissolve 0.84 mg of labeled acrylamide octapeptide (use Sartorius Analytical Balance) in 10 mL deionized-water bottle using a 10-mL silanized, volumetric flask.
 - b. Labeled glycidamide octapeptide stock solution (LGO) 164 μ M
Dissolve 1.69 mg of labeled glycidamide octapeptide (use Sartorius Analytical Balance) in 10 mL deionized-water using a 10 mL silanized, volumetric flask.
 - c. Internal Standard Solution
This solution contains 33 nM labeled acrylamide octapeptide and 82 nM labeled glycidamide octapeptide.

Pipette 500 mL of the LAO and 500 mL LGO stock solution into a 1000 mL volumetric flask (use silanized volumetric glassware only). Store the solution in 5 mL aliquots in a 10 mL falcon tubes at -70 °C. 1000 mL is sufficient for 10,000 analytical samples.

Note: Diluted standards are stable for at least 4 years when stored at -70 °C

6. Preparation of Calibrators for Hemoglobin Measurement
Calibrators are prepared according to the Stanbio manufacturer's procedure recommendation that is provided as package insert with the reagents and chemicals.
Dilute the CMH standard A (20 g/dL) provided by the manufacturer with CMH solution prepared in section 0 using the dilution scheme shown in Table 2. The following concentrations of calibration solutions are prepared: 20, 15, 10, 5, and 0 g/dL.

Concentration of CMH standards solution A	Volume to take from calibrator solution	Dilute with CMH solution to final volume	Obtained concentration of calibrator solution
20 g/dL	4 mL	0 mL	20 g/dL
20 g/dL	3 mL	4 mL	15 g/dL
20 g/dL	2 mL	4 mL	10 g/dL
20 g/dL	1 mL	4 mL	5 g/dL
20 g/dL	0 mL	4 mL	0 g/dL

Table 2: Dilution scheme for total hemoglobin calibrator solutions

A new calibration curve must be prepared with each sample set.
See manufacturer's recommendation for stability of the hemoglobin standards

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Higher order reference materials are currently not available for these analytes or measurements. Exchange of calibrators with other laboratories measuring the same analyte with different methodologies has been performed and the concentration of the calibrators has been confirmed.

Results of in-house recovery studies showed a mean (\pm SD) recovery of 96.0% \pm 1.9 for acrylamide- and glycidamide hemoglobin adducts added externally to whole blood and red blood cells at different levels (0.5-20 pmol /200 μ L)

In-house studies showed that aqueous calibration gave same results as calibration in serum. Daily calibrations will thus be performed in aqueous solutions.

All necessary calculations are performed in an Excel spreadsheet after copying the result tables containing the integrated areas, calculated using the quantitation settings on the Finnigan Xcalbur into Excel. After calculating area ratios between the analytes and their internal standards, a linear calibration standard curve, not forced through zero calculated on the basis of single analysis is generated. The zero point calibrator available has only internal standard. The concentrations are calculated from the regression equation based on area ratios between analyte and internal standard.

At the end of each run, the calibration curve is reanalyzed as unknowns. The measured concentrations of these calibrators must agree within the assigned values of their set values for acrylamide and glycidamide respectively.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Daily Analysis of Samples

1. Preparation of the MS/MS system

All instrument tuning and mass calibration is performed according to manufacturer's instructions and recommendations. The tuning and mass calibration of the Finnigan Quantum is performed by infusion either running the instrument in Auto-Tune or using Manual Tune using a solution of polytyrosin. The instrument settings defined in this tuning and calibration step are saved on the local hard drive (Usually tuning and calibration is performed after regular preventive maintenance twice a year or after cleaning the Q0 unit).

All other method related instruction materials and manuals (such as daily required maintenance files) are kept in the laboratory next to the instrument.

The corona discharge needle is checked every day for cleanliness. If discharge needle is not clean (indicated by dark coloration of the needle), it is cleaned by wiping the needle with a polishing cloth.

Sample tube is flushed with methanol daily. Q 0 is cleaned approximately every two months.

The pump oil in the rough pump is changed approximately every six months. The oil level is checked once a week.

2. Preparation of the HPLC system

A Krudkatcher that acts as a pre-column is replaced approximately every 300 injections or when pressure increases profoundly.

The injection port is periodically checked for clogging by doing a syringe and a needle wash before every sequence. The liquid should flow freely through the waste line.

Assure that solvents are full and that the waste is emptied every day and that there are no air bubbles in the line. LC column is replaced approximately every 1500 injections, if separation performance is insufficient or pressure gets too high (> 290 bars).

3. Preparation of the Tecan system
Before using the TECAN instrument flush lines with 25 ml of 5 % methanol and tighten all eight cones on the tips, also tighten all diluter syringe connections.

Assure that no air bubbles are in the Teflon lining. Assure that the rinse solvent bottle has sufficient solutions and empty waste bottle as needed.

4. Preparation of the Gilson system
Before using the Gilson instrument, flush lines with 5 ml of elution solvent two times. 8.0 ml of mix solvent will not overflow in the plate since the packed plate absorbs at least 1.0 ml of solvent.

Assure that no air bubbles are in the Teflon lining. Empty the waste bottle as needed.

5. Preparation of the Matrix system
Insert 96 tip magazines into the 96 channel pipetting head. Assure pipettor or tips are 96 5 μ L-300 μ L long tips (5536+5537).

B. Preparation of Samples for Analysis

The descriptions described here are setup to process (4) 48-wellplates, with each plate having its own calibrators, QCs and run ID. Less than four plates can be processed by making appropriate adjustments for reagents and samples.

1. Preliminaries
This section contains a description on how samples are diluted, homogenized, assessed for blood clots and registered for tracking of sample ID throughout the analysis process.

The dilution of the samples will reduce the viscosity of the sample and enable accurate and precise pipetting of the sample using automated pipetting systems. Labels are printed using a Sato label printer for all the plates and necessary tubes for proper identification.

Blood clots can occur especially in frozen samples. They prevent the accurate and precise pipetting of the sample. Therefore, samples need to be tested for blood clots visually and either removed manually or settled on the bottom of the

sample vial by centrifugation to minimize interfere with the pipetting process.

Because samples are transferred from the vial to the well plate, it is important to know the sample ID of each cell in the well plate. Using the Tecan instrument with a defined program for sample transfer enables the reliable tracking of samples in the well plate. Certain vial positions relate to certain cells in the well plate

2. Sample preparation procedure

Allow frozen samples, quality controls, buffer, internal standard and calibrators to reach ambient temperature. Homogenize all samples, quality controls and standards by gently mixing the vials for about 15 minutes using a rotator.

Dilute blood samples using Tecan program entitled: Dilution 0.3 task from cryovial to cryovial. 1:0.33 (v/v) with 6 mM K3EDTA saline solution and homogenize gently using laboratory rotator.

Centrifuge blood samples and quality control materials at 15 °C and 4000 rpm for 10 min using the centrifuge. This step is to move blood clots at the bottom of the cryovials. After centrifugation, visually inspect each sample tube for air bubbles; remove them if present to facilitate automated pipetting.

Place all sample vials, quality control vials, calibrators and reagents on the Tecan instrument according to the scheme depending on the tasks being performed. All bar-coded vials need to be aligned in a manner that the barcode reader installed in the Tecan instrument can read it properly. Therefore, face barcodes to the right-hand side of the 16 position racks and confirm that they can be scanned.

Scan all vials and reagents using the barcode reader installed on the Tecan (execute program: Task 1: HB-measure 4_Plates.gem). Manually scan vials that could not be read with the Tecan instrument using the hand held scanner attached to the instrument computer. If the barcode cannot be read by the scanner installed on the Tecan instrument, the program will prompt to an option to manually enter IDs using the keypad or to scan the tube and rack number using a hand held scanner that can be read with the Tecan barcode reader. After the scanning is successfully completed, a file is created ("barcode

ID file”) that contains the sample ID from the barcode, the position of the vial and the position of the reagents on the Tecan instrument.

Assure that the scan results are saved as “.gem”-file on the instrument computer at [C drive:// Program files/Gemini/ Output/Archive]. A special file management program (“Lab Rat”) automatically copies this file to the network drive (Q:\CCB 8), while renaming it using the current date, time and seconds format.

After scanning the vials, the program “Task 1: HB-measure 4_Plates.gem “ executes pipetting of 550 μ L of diluted blood samples using 1000 μ L conductive tips to 48-well 5 mL plates. This plate is named “Dilution Plate” The program will pause with a wait prompt to allow assessing samples in the 48-well 5 mL plate for blood clots using a lamp. If a blood clot is observed, it is removed by the operator using a pipette tip. If blood clot is large, sample is removed from the cell and the same sample is pipetted by hand to the plate without blood clots. After plate was assessed and corrective actions have been taken as needed place Dilution plate on the Matrix Plate Mate 2X2 instrument one dilution plate at a time.

Refer to section 8C of this document for the hemoglobin measurement procedure, followed by the Edman procedure in section d.E. Note the hemoglobin measurement procedure has to be performed first before continuing with the Edman procedure.

If samples are manually pipetted, scan the barcode of each vial individually and note the position of each individual sample in the well plate and transfer samples manually..

Note: Barcodes from NHANES cover the entire cryovial disabling the Pos ID to detect whether a tube is present. Therefore, cryovials need to be placed on the Tecan rack and raised by 1 centimeter by placing a small cap below the tube. This will allow the barcode reader to sense that there is a tube present to be scanned.

C. Hemoglobin Measurement

1. Preliminaries

The hemoglobin measurement provides information about the hemoglobin concentration in the diluted sample solution used for modified Edman reaction. With the concentration of the sample solution and the specific volume used for modified Edman reaction, the amount of hemoglobin used for modified Edman reaction is calculated. This result is later used to calculate the reported results for acrylamide and glycidamide hemoglobin adducts in pmol adduct per g hemoglobin. This step is necessary, because the dilution of the specimens with EDTA saline solution 1:0.33 v/v cannot be performed accurately and precisely with the Tecan instrument because of the high viscosity of whole blood and erythrocytes.

The measurement procedure performed in this protocol is based on a commercial clinical assay. In principle, the hemoglobin and its derivatives, except sulfohemoglobin, are oxidized to methemoglobin by ferricyanide in the presence of alkali. Cyanmethemoglobin is formed from methemoglobin by reaction with cyanide (CMH solution). The resulting red colored complex has peak absorption at 540 nm, the value of which is proportional to total hemoglobin concentration.

2. Hemoglobin measurement procedure

- a. Pipette 2.5 mL of the CMH Solution using the Hamilton pipettor into a 5 mL 48-well pyramid bottom well plate. Place plate with CMH solution and dilution plate with blood samples on the Matrix worktable.
- b. Manually add 10 µL of the hemoglobin QC pools using positive displacement pipette in the 2.5 mL CMH solution to the appropriate cells in each 48 well plate to the following positions:

	Plate 1	Plate 2	Plate 3	Plate 4
low QC to cell:	1f	2f	3f	4f
medium QC to cell:	1g	2g	3g	4g
high QC to cell:	1h	2h	3h	4h

Note: the batch ID and expiration date of the QC materials.

- c. Transfer 20 µL of diluted blood sample (transfer is performed using two tips using 10 µL volume in each tip) prepared in section 2. to the plate containing the CMH solution and mix thoroughly by running Matrix program “Task1. HB-measurement”. Each Dilution Plate containing 550µL diluted

blood for the CMH measurement is placed on the Matrix separately, after the first plate CMH measurement is performed then the second Dilution Plate is placed on the Matrix Plate Mate 2X2. 96 channel pipettes are used. Each sample is being measured in duplicates, but later the two cells are averaged from the duplicate measurements. See Table **Error! Reference source not found.** for sample transfer to the 96 well microtitor plate (MTP)

- d. Read the absorption of the empty 96-well MTP
Create a new folder one time on the C drive to store the raw data from the UV/VIS plate reader. (i.e., HB_reading_AA folder)

Open the Powerwave software (KC4) on the computer and load program “HB_reading_AA.

- e. Place the 96-well MTP on Matrix instrument and transfer 200 μ L sample solution in duplicate measurements prepared in step 2) to the 96-well MTP uses the Matrix program “Task 1. HB_Measurement”.

One 96-well microtiter plate will have duplicate measurements of one sample. Using a 200 μ L fixed-volume Gilson pipette, pipette the hemoglobin calibrators to the 96 well MTP manually to the following positions:

HB calibrators	1st 96-well MTP	2nd 96-well MTP	3rd 96-well MTP	4th 96-well MTP
Blank	1a and 2a	3a and 4a	5a and 6a	7a and 8a
5 g/dl calibrator	1b and 2c	3b and 4b	5b and 6b	7b and 8b
10g/dl calibrator	1d and 2e	3c and 4c	5c and 6c	7c and 8c
15g/dl calibrator		3d and 4d	5d and 6d	7d and 8d
20g/dl calibrator		3e and 4e	5e and 6e	7e and 8e

- f. Insert the 96-well microtiter plate with the sample solutions back in Powerwave 200 plate reader and select “OK” to measure the absorbance at 540 nm of the sample solution. Each MTP is measured individually once the final absorbance is

measured for the first MTP, the second, third and fourth MTP can be measured thereafter one at a time.

- g. After reading the absorption in step 5, the instrument software automatically subtracted the absorbance of the empty plate (obtained in step 3) from the absorbance (obtained in step 5) and stores the results as text-file on the local C drive (HB_reading_AA folder). The software automatically prompts to save raw absorbance data as a text file. The file is imported in Excel and saved as file using the date and the run IDs of the plates being measured (HB_raw_[Date][ID].xls) in the appropriate folder in q-drive.

3. Hemoglobin Measurement Data Processing

The absorbance of the calibrators and the known concentration of the calibrators are used to calculate a calibration curve using linear regression. The x-axis represents the concentration while the y-axis represents the absorbance. The total hemoglobin content in the samples is calculated using the calibration curve.

Note: All calculations can be performed using an MSEXcel template named “HB_Calc_2006_Plate 1 and 2.xls and HB_Calc_2006_Plate 3 and 4.xls”. Use the appropriate template according to the plate 1 and 2 or plate 3 and 4 to assure proper sample assignment. The template assumes the samples to be arranged in the plate as described in Table **Error! Reference source not found.**

- a. Open MSEXcel and import the data created in 0
- b. Open file “Hb_Calculations_2006.xls”
- c. Copy all absorption data from HB_raw_[Date][ID].xls to file “Hb_Calc_2006_Plate_X and X.xls depending which plate you are working with”, sheet “Plate R. Raw 1” for the first plate 48-well plates and to “Plate R. Raw 2” for the second 48-well plates to cells A4 through M11 using the “Past Special Link” function.

Note: All absorption data can be copied and pasted in one step for each 96 well plate. The designated area in the Hb_Calc_2006_Plate_X and X.xls file is setup for the data as they were produced by the Powerwave Software.

- d. Save the modified “Hb_Calc_2006_Plate_X and X.xls”-file under the appropriate name using the current date and corresponding run ID in the q-drive. Once the instrument readings are inserted in the template file, the following calculations are performed automatically:

- From the duplicate samples and calibrator absorbance measurements the average absorbance is calculated.
 - The average absorbance of the calibrators is plotted against the total hemoglobin concentration.
 - A linear regression is performed using the “linreg” function in MSExcel.
 - The total hemoglobin content for each samples and QCs is calculated by using the obtained calibration curve.
- e. Open the appropriate file containing Barcode Reading Data generated by the Tecan Pos ID mark the first column to sort all values A to Z and copy the information in the renamed template file HB_calc_2006_Plate X and X.xls into worksheet “Barcode R. Raw”. The total hemoglobin measurement results are then automatically assigned to the correct sample ID.

4. Hemoglobin Measurement Data Evaluation

- a. Assess the calibration curve data for any apparent unusual data.
- b. Compare the obtained QC values against the manufacturers established values.
- c. Assure that the total hemoglobin concentration in the sample solution is not less than 7.6 g/dL and no more than 14.2 g/dL to assure that there is any pipetting error. Note this concentration is for a diluted blood samples 1:0.3 v/v if it would be undiluted it would be out side of the normal hemoglobin physiological range.
- d. If values for QCs are within the limits and the total hemoglobin concentration is within the desired range, continue with modified Edman reaction. Otherwise repeat measurements using the HemoCue device.
- e. If concentration values deviate from CMH to HC values use the HC value and manually type in concentration into the worksheet “Barcode & abs. to AA calc.”
- f. Barcode & abs. to AA calc worksheet automatically calculates weather additional volume is required to be added to the samples to have at least 0.025 g of hemoglobin.
- g. Make appropriate adjustments such as using more sample volume for Edman reaction if necessary.

Note: The template is setup to highlight total hemoglobin values that are not within the desired range using the conditional formatting function. If hemoglobin values are too low, the template automatically calculates the additional volume that needs to be added on top of the 350 μ L volume necessary for the Edman reaction in section d.D.

D. Modified Edman reaction and analyte purification

In this step the N-terminal valine containing the acrylamide and glycidamide is cleaved from the hemoglobin protein chains with the Edman reagent. The resulting pentafluorophenyl thiohydantoin derivatives are isolated from the reaction mixture by liquid-liquid extraction on diatomaceous earth and prepared further for HPLC/MS/MS analysis.

1. Modified Edman reaction and analyte purification procedure
Add calibrators, internal standards, reagents and supplies on Tecan according to Tecan layout for “Task 2. Calibrators 4 Plates.gem” for the addition of reagent and amounts.

To prepare the calibrators in each plate load program “Task 2. Calibrators 4 Plate.gem” and run program. The program performs the following tasks:

- a. Pipette 200 μL calibrators using a 1000 μL conductive tips in new 48-well 5-mL plate called “Edman Plate” see Article XV.
 - b. Pipette 200 μL deionized water as blank in the assigned well.
 - c. Add 100 μL internal standard solution using 200 μL conductive pipette tip in wells containing the calibrators and the blank.
 - d. Pipette 80 μL of 400 mM Ammonium Acetate Buffer solution to the calibrators to adjust pH to 7.0 \pm 0.5.
2. To transfer exact volumes of the blood samples from each plate to a new plate load Matrix program “Task2. Blood transfer 350 μL ” and run program. The program performs the following task.
 - a. Pipette 350 μL samples and QCs using 300 μL extended length clear tips with filter using the Matrix Plate mate 2x2 from the Dilution Plate 1 described in section 2 to the Edman Plate 1. Perform each plate individually by loading / starting the program as many times as plates are available.
 3. To add formaldehyde solution to the Edman plate use 300 μL extended length clear tips with filter using the Matrix Plate mate 2x2, perform the formaldehyde addition one plate at a time. This requires loading / starting the program as many times as plates there are to add formaldehyde too. Load program “Task 3. Adding FA” and run program. The program performs the following tasks.
 - a. Pipette 1.5 ml of formaldehyde using 300 μL clear pipettes in all wells.

- b. The 96 channel tips use two tips to transfer 600 μL of volume in one cell at a time and repeat the process and changes volume addition up till 1.5 ml total volume is reached.
 4. To prepare the samples for each plate, load Tecan program “Task 3. Edman 4 Plate.gem” and run program. The program performs the following tasks:
 - a. Pipette 100 μL internal standard solution using 200 μL conductive pipette tip in all wells containing sample.
 - b. Pipette 55 μL 0.22 M Acetic Acid using 200 μL conductive tips in all wells containing samples to adjust pH to 7.0.
 - c. Pipette 10 μL Edman reagent using a 200 μL conductive tips in all wells of the plate.
 - d. Seal plates and transfer them into the oven.
 - e. Incubate samples at 55 $^{\circ}\text{C}$ for 2 hours. Assure samples are shaken during that time using a laboratory shaker (Lab Rotator in the oven is set to dial 9).
 - f. Let sample cool to room temperature and remove seal.
 5. To transfer samples to the extraction plate for liquid-liquid extraction, arrange plates and supplies on Matrix and load and run program “Task 4. Blood transfer 2.5mls”. This program transfers all sample solutions to the filter plates using 300 μL extended length, clear pipette tips with filter.
 - a. Transfer the derivatized blood samples to the 7.5 ml 48 well filter plates filled with Isolute material.
 - b. Wait 5 minutes for samples to properly penetrate in sorbent material.
 - c. Transfers filter plates containing the samples to the Gilson instrument. Arrange extraction plates / collection plate in a fashion that the extraction plate is on the bottom in respect to the filter plate (7.5-mL 48-well plates).
 - d. Load and run program “Task 1. CE 8.0 mL elution.Elute”. The program dispenses 8.0 mL of a solution of Isopropylether/Ethylacetate/Toluene in portions of 1 mL (2 times 0.5 mL) per well.
 - e. Remove elution solvents by placing the collection plates containing the extraction solutions in a GeneVac EZ-2 concentrator. Instrument setting is set on “low boiling and no heat”.
 - f. After solvent has been removed. Wash the walls with ethylacetate solution to recover the analytes on the side of the walls. Use the repeater pipette with a 10 mL tip and set the dial on 1 (corresponds to 200 μL dispense volume). Add 200 μL of

ethylacetate to each wall of collection plate. Then, add an additional 200 μL to the wider walls of the wells (total wash volume: 1,200 μL).

- g. Vortex plate on level 4 for 30 seconds.
6. Arrange collection plates containing ethylacetate and supplies on Tecan instrument. Use Tecan instrument by loading and running program “Task 5. Wall wash transfer 4 Plates.gem”.
 - a. Transfer samples recovered from the wall wash step to a new 96-well 2-mL plate called “Reconstitution Plate”
 - b. Remove solvents by placing the Reconstitution Plates in a GeneVac EZ-2 concentrator. The instrument is set to “low boiling point” and no lamp setting.
 - c. Dry Reconstitution Plate for 4 hours, during this time the solvent evaporated completely.
 7. To reconstitute the samples load Matrix program “Task5. Reconstitution Step” and run program. The program performs the following tasks.
 - a. Arrange plates according to Figure 19
 - b. Reconstitute samples with methanol and water by loading and running program “Task 5. Reconstitute Step”. This program adds 80 μL of methanol and 120 μL of water to each well.
 - c. Mixes plate using 96 channel Matrix tips.
 - d. Transfer sample to filter plate (GHP 0.2 μm filters) containing a new 96 well 2 ml collection plate called “ Final Plate” below the filter plate. This program transfers the samples in the filter plate. The filter plate and Final Plate then is transferred to the centrifuge manually. Two rubber bands are placed on the plates before centrifuging. The samples are filtered by centrifuging for 10 min at 3700 rpm and 15 $^{\circ}\text{C}$.
 - e. After filtration is finished the Final Plate is sealed and transfer for analysis by HPLC/MS/MS.

E. Analysis of Edman products by HPLC/MS/MS

In this section, a run sequence is created using the information obtained from scanning the barcodes of the sample vials (section 2). The runs sequence is then used by the HPLC/MS/MS software to analyze the samples in the wellplates.

1. Creating a run sequence

A run sequence consists of (2) 48-well plates combined in on 96-well plate and additional samples such as water and instrument

controls that are added to assess carry-over and instrument function. The sequence is created by combining the Barcode ID file with a sequence template file.

- a. Load template file “Sequence_1 and 2_2006.XLS or Sequence_3 and 4_2006.XLS” depending on which plates you are working with.
- b. Load Barcode ID file, sort all IDs using the A to Z short function
- c. Copy information from Barcode ID file to “Sequence_X and X_2006.XLS” into worksheet “Barcode ID” file using the “Paste Special Link” function. After copying the information, the sequence information is automatically compiled.
- d. Save sheet “Export Sequence” as “CSV” file using the date and RunIDs as name (i.e., 061206_P_255_256). Use the date when sample preparation was started.
- e. Open Finnigan Xcalibur software and activate “Sequence Setup”.
- f. Import the CSV file and add additional information such as injection volume, path or instrument method as needed.
- g. Save sequence using the same name as used for the CSV file.
- h. Insert Shutdown method to shut down the instrument at the end of the run if no more plates will be run.
- i. Preliminary instrument setup and performance check
 - (1) Make sure that all the solvent reservoirs are full or have enough solvent to run the sequence. Check the Argon gas pressure. It should be about 20 psi. Make sure the tank is not empty.
 - (2) Empty the waste solvent reservoir of the LC system into the LC waste 4L bottle labeled for acrylamide project located under the sink.
 - (3) Turn the flow on the LC on and wait until pressure is stable and record the HPLC pressure before each sequence run in the appropriate log book.

F. Chromatographic and mass spectrometric conditions (for additional information see page 52)

HPLC system: ThermoFinnigan Surveyor HPLC system (San Jose, CA)

HPLC column: Thermo Hypersil GOLD, (50 x 2.1 mm, 1.9 μ , Termo Electron, Holbrook, NY)

Column temperature: 50 °C

Eluent: Methanol and water (63/37 v/v) C methanol, D methanol water 1:1

Flow rate: 400 μ L/min

Injection conditions: 50 μ L (no waste option) with needle wash and syringe flush after each injection.
MS system: ThermoFinnigan TSQ Quantum Ultra (San Jose, CA)
Ionization conditions: Atmospheric pressure chemical ionization (APCI), positive ion mode at 4.5 μ A, and 375°C vaporizer temperature

Detection conditions: single reaction monitoring (SRM) at 10 eV collision energy of transitions

m/z 396 \rightarrow m/z 379 for AA-Val-PFPTH,

m/z 402 \rightarrow m/z 385 for AA-Val(13C5,15N)-PFPTH,

m/z 412 \rightarrow m/z 395 for GA-Val-PFPTH and

m/z 418 \rightarrow m/z 401 for GA-Val(13C5,15N)-PFPTH

For confirmatory purposes the transitions

m/z 396 \rightarrow m/z 337 for AA-Val-PFPTH and

m/z 412 \rightarrow m/z 357 GA-Val-PFPTH are monitored.

G. Final instrument setup and sample sequence run

1. Load the sequence created in section 8.d.1.
2. Place the sample plates that correspond to the sequence loaded in the auto sampler of the LC system. Make sure cell A1 of the plate is at the left back position and the plates are positioned on the rack corresponding to the sequence.
3. Run the sequence to analyze samples.

H. Processing of HPLC/MS/MS data

1. Transfer raw data files obtained from the HPLC/MS/MS measurements to the corresponding folder on the Network (Q-drive).
2. Open Finnigan Xcalibur and activate Sequence set up, make sure the files are in the right directory.
3. Perform automated data integration using the XCalibur processing program "021006 AAGA conf ions short column". Save file reprocessed.

4. Assess automated integration for correctness and adjust integration as needed manually.
5. Save integration results as xqn-file using the same file name as used for the corresponding sequence file.
6. Create a hardcopy of each integrated peak using the report template named “AA report template.doc”.
7. Export integration results as MSEXcel file (use option “Short Excel Report” in Xcalibur Quan Browser). For saving the file, use the same file name as used for the corresponding sequence file.
8. Open MSEXcel and load template file “AA_calculation_2006_CC Data for plate 1&2.xls or AA_calculation_2006_CC Data for plate 3&4.xls” and load the MSEXcel export file created with Finnigan Xcalibur.
9. Copy sheets of “AA_calculation_2006_CC Data for plate 1&2.xls or AA_calculation_2006_CC Data for plate 3&4.xls” to export file. Close excel sheet and save current file using the same file name as used for the corresponding sequence file with plate number the addition of the word “data” at the end of the file name.
10. In the “Summary” sheet of the file, update all links to refer to the sheets in the current file.
11. Open file containing the corresponding hemoglobin measurement data (see section: 0) and insert results from worksheet “Barcode & abs. to AA calc” from columns A,B and C to the Summary sheet to AA calculation_2006_CC Data for plate X&X.XLS depending which plate you are working on to columns P,Q and R.
12. Assess if sample IDs from hemoglobin measurement match sample IDs of adduct measurements. If IDs match, the correct reportable result is calculated automatically. If sample IDs do not match, assess the reasons and make appropriate adjustments.
13. After links have been updated, calibration curves, acrylamide and glycidamide adduct values are calculated automatically.

Note: The template automatically performs an ID match test and highlights sample IDs that do not match. Further, the template automatically highlights data that are outside the calibration curve.

9. REPORTABLE RANGE OF RESULTS

This method is linear for acrylamide and glycidamide adducts in the range 1.25 – 80 nmol/L. Assuming 25 mg of hemoglobin were used for adduct measurements, the linear range would translate to 40 pmol/g hemoglobin and 20 pmol/g hemoglobin and 125 pmol/g hemoglobin and 350 pmol/g hemoglobin for glycidamide- and acrylamide adducts respectively. Samples outside this range are re-analyzed for confirmation before results are released. Sample above this range will be diluted further with water. Samples below this range will be processed by

diluting them less or by using higher sample volumes for the Edman reaction.

10. QUALITY CONTROL (QC) PROCEDURES

A. Bench QC Materials

The bench QC pools used in this method comprise three levels of concentration spanning the “low-normal” to “high-normal” ranges of the analyte of interest. The intent of bench QC is for the analyst to evaluate the performance of the analytical system. The analyst inserts the bench QC specimens in each sample set (set of samples that are processed together in one batch).

Pending on the sample specimen being used for analysis three levels of either erythrocyte controls, or whole blood controls are analyzed in duplicate in each run as bench quality control materials. Hemoglobin adduct ranges of 25 - 50 pmol/g hemoglobin (low QC pool), 50 – 150 pmol/g hemoglobin (medium QC pool) and 150 –400 pmol/g hemoglobin (high QC pool) are targeted. Erythrocytes or whole blood (1000 µL) is aliquot into 2.0 mL cryovials, capped, and frozen. The QC pools are stored at -70 °C and are stable for at least 4 years.

Means plus range limit for all pools are established by analyzing duplicates for at least 20 consecutive runs. The low QC pool is prepared by selecting and pooling blood from non-smoking individuals. Smoking status can be determined by using cotinine measurements using test such as TobacAlert from Nymox Corp (www.tobacalert.com). The medium QC pool is prepared by mixing a high QC pool with a low QC pool 1:1 (v/v). The high QC pools are obtained from people actively smoking tobacco products.

The quality control materials for total hemoglobin measurements provided by the assay manufacturer are used and assessed according to the manufacturer’s recommendations.

B. Blind QC Materials

Blind QC samples are QC materials placed in vials, labeled, and processed so that they are indistinguishable from the subject samples handled by the analyst. The supervisor decodes and reviews the results of the blind specimens without the analyst knowing of their presence in the runs. At least one low-normal concentration and one high-normal concentration QC material should be run in the laboratory for this purpose.

C. Calibration and Calibration Verification Procedures

Assessment of runs using QC materials

The results from the QC pools are checked after each run. The system is declared “in control” if all three QC results are within 2s limits and the run is accepted. If one of the three QC results is outside the 2s limits then apply rules below and reject if any condition is met - the run is then declared “out of control”:

- * 13s: Any of the three QC results are outside the 3s limit
- * 2x2s: Two of the three QC results in the run are outside the 2s limit (same side of mean)
- * R4s: Sequential QC results (either within the run or across runs) are outside the 2s limit on the opposite sides of the mean
- * 10x: Ten sequential QC results (across pools and across runs) are on the same side of the mean

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared “out of control” for that analyte as assessed by internal (bench) QC.

The initial limits are established by analyzing pool material in 20 consecutive runs and then are re-evaluated quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, electronic copies of the QC results from each run are stored in the analyte-specific folder. Electronic copies of the tracking of the QC results over time are stored in the analyte-specific folder. A hardcopy of the QC results from each run is also kept by the analyst.

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

Assess whether the hardware is functioning properly. Make sure the MS calibrations are proper. Check the instrument calibration.

Check the autosampler and UV signal for proper sample injections.

Look for sample preparation errors, specimen, right volume of buffer etc.

Check the calibrations of the pipettes.

If the steps outlined above do not result in correction of the “out of control” values for QC materials, consult the supervisor for other appropriate corrective actions.

Do not report analytical results for runs not in statistical control.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

No known interferences exist with this method for the detection and quantitation of acrylamide and glycidamide adducts. However, other unknown compounds are detected with this method. Poor chromatographic separation may cause interferences with these compounds.

Acrylamide and glycidamide adducts are formed due to exposure coming from food, smoking and certain occupational activities related to handling of acrylamide. The adduct concentrations measured with this method do not provide any information about the exposure source(s).

Though adduct values of people exposed to tobacco smoke are higher than in groups not exposed to tobacco smoke, data from individuals cannot be used to assign it to one or the other group, because of the high inter-individual variability and the overlap of adduct values in both groups.

13. REFERENCE RANGES (NORMAL VALUES)

Reference ranges have not been established yet. An in-house assessment using a convenience sample from 96 individuals found the following adduct values: the median globin adduct values are 129 pmol/g globin (range: 27 - 453 pmol/g globin) and 97 pmol/g globin (range: 27 – 240 pmol/g globin) for AA-Hb and GA-Hb, respectively. Higher values have been reported in the literature and were related to occupational exposures.

14. CRITICAL CALL RESULTS (“PANIC VALUES”)

Currently, no information is available linking hemoglobin adduct data to certain health outcomes.

15. Test Result Reporting System; Protocol for Reporting Critical Calls

Not applicable

16. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

The Microsoft Access database is used to keep records and track specimen's results. If analyses are used for smaller, non-NHANES studies, records are kept on file on the CDC network and on CDs or DVDs.

Records including related QA/QC data are maintained for 10 years after completion of the study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual specimen from these analyses for non-NHANES studies may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at -70°C . The specimen ID is read off of the vial by a barcode reader attached to the computer used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the DIF file containing the electronic copy of the results is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for keeping a notebook containing the ID numbers of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies.

17. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

Because the analysis of hemoglobin adducts of acrylamide and glycidamide is inherently complex and challenging, there are no acceptable alternative methods of analysis in the laboratory. If the analytical system fails, we recommend that the specimens be stored at -70°C until the analytical system is restored to functionality.

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

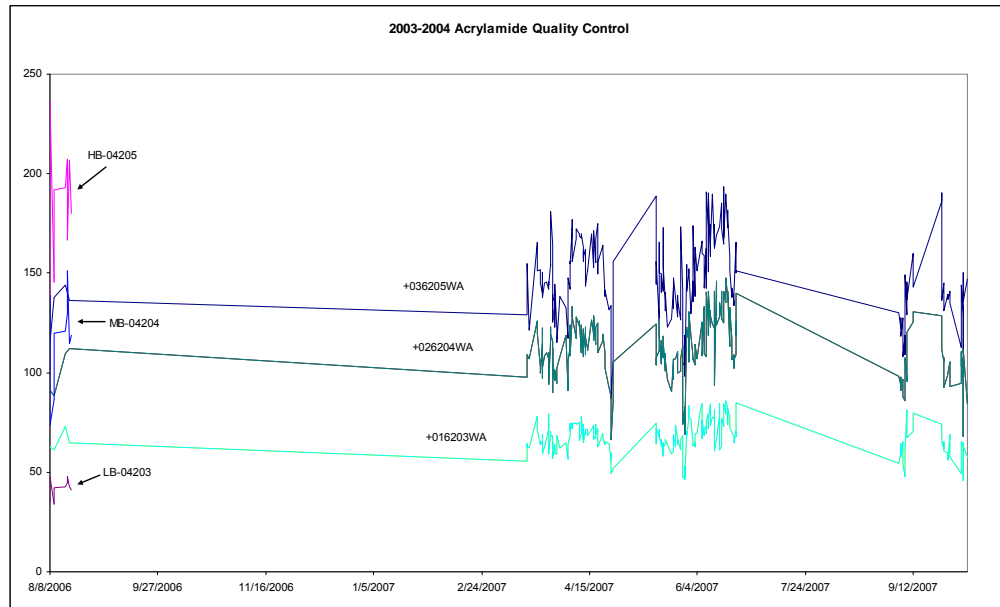
All shipments are recorded on the NHANES Shipping Log upon receipt. Actions taken during the course of analysis, result reporting, and specimen retention are also recorded on the log.

19. Summary Statistics and QC Graphs

A. Acrylamide

Summary Statistics for Acrylamide by Lot

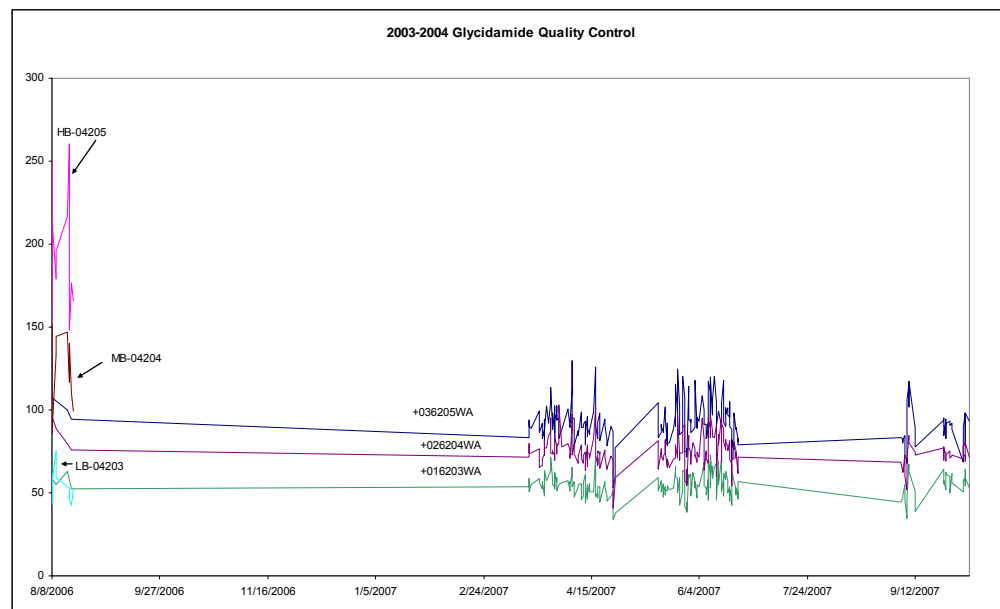
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LB-04203	11	8/8/2006	8/18/2006	41.04	4.87	11.9
+016203WA	210	8/8/2006	10/7/2007	67.31	7.91	11.8
+026204WA	211	8/8/2006	10/7/2007	111.45	15.06	13.5
MB-04204	12	8/8/2006	8/18/2006	114.63	21.41	18.7
+036205WA	209	8/8/2006	10/7/2007	148.27	19.57	13.2
HB-04205	12	8/8/2006	8/18/2006	182.19	32.66	17.9



B. Glycidamide

Summary Statistics for Glycidamide by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LB-04203	11	8/8/2006	8/18/2006	53.34	9.84	18.4
+016203WA	212	8/8/2006	10/7/2007	54.63	7.02	12.8
+026204WA	216	8/8/2006	10/7/2007	74.7	9.06	12.1
+036205WA	214	8/8/2006	10/7/2007	92.28	11.48	12.4
MB-04204	12	8/8/2006	8/18/2006	124.82	21.08	16.9
HB-04205	12	8/8/2006	8/18/2006	193.12	38.02	19.7



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