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Title: Analysis of Nitrosamines using Supercritical Fluid Extraction		
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Contents

A. INTRODUCTION

1. Theory

This procedure extracts nitrosamines from fried bacon samples using supercritical fluid extraction (SFE). The nitrosamines are retained on a silica gel trap, which allows most of the co-extracted lipids to pass through and be discarded. Much of the fatty material retained on the column is washed off using a dichloromethane/pentane solution, and a mixture of ethyl ether in dichloromethane elutes the retained nitrosamines. The resulting eluate is concentrated to a small volume and analyzed by gas chromatography using a thermal energy analyzer (TEA) for detection of the nitrosamines.

2. Applicability

The procedure is applicable to the volatile nitrosamines nitrosodimethylamine (DMNA), nitrosodiethylamine (DENA), nitrosodipropylamine (DPNA), nitrosodibutylamine (DBNA), nitrosopiperidine (NPIP), nitrosopyrrollidine (NPYR), and nitrosomorpholine (NMOR) in bacon.

B. EQUIPMENT

Note: Equivalent instrumentation or apparatus may be substituted.

1. Apparatus

a. Beaker - 100 mL.

b. Mortar and pestle - glass, 5 - 6 inch diameter.

c. Extraction vessel - 24 mL capacity, Keystone Scientific, Inc.

d. Tamping rod for extraction vessel - Applied Separations.

e. Polypropylene wool - Applied Separations.

f. Funnel - 60 mm, to fit into extraction vessel.

g. Wrenches open end - 15/16 inch and 1 1/16 inch.

h. SPE cartridge - 6 mL, polypropylene, Applied Separations.

i. Polyethylene frits for 6 mL cartridge - Applied Separations.

j. Food processor or grinder with 11/32" grinder plate - Waring.

k. Volumetric flask - 200 mL, Class A.

l. Concentrator tubes - 10 mL

m. Micro Snyder column.

2. Instrumentation

   a. SFE Extraction apparatus. Spe-ed SFE Model 7010, with 680 bar air driven pump. Applied Separations.

   b. Refrigerated circulating bath (used to cool SFE CO_2 pump head). VWR brand model 1162 VWR Scientific.

   c. Thermal Energy Analyzer. TEA Model 543 Analyzer. Thermedics, Inc. Analytical Instruments.

   d. Gas Chromatograph: Suitable instrument requires packed column capability and replaceable liner. Oven design must allow direct insertion of a 2 cm diameter TEA interface to depth of 7 - 10 cm, and connection of GC column to interface by means of a short, inert transfer line.

   e. GC Column: Glass, 2.5 - 3.0 m by 3 mm ID, packed with 10% Carbowax 20M + 5% KOH on Chromosorb PAW, 100/120 mesh.

   f. Strip chart recorder with 10 mV input.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents or solutions may be substituted.

1. Reagents

   a. Carbon Dioxide (liquid) - SFC grade with dip tube.

   b. Hydromatrix - Varian, Inc.

   c. Propyl Gallate

   d. Silica Gel 60, acid washed - EM Reagents distributed by Brinkmann Instruments, Inc. Sieve to 70-150 mesh and wash with dichloromethane. Dry in oven at 100 °C for 24 hours.

   e. Dichloromethane (DCM) - Burdick & Jackson distilled in glass.

   f. Pentane - EM Omnisolve.

   g. Diethyl ether, anhydrous - Baker Analyzed ACS Reagent.

2. Solutions

   a. 25% Dichloromethane in pentane:

      Add 50 mL dichloromethane to a 200 mL volumetric flask, and dilute to volume with pentane.

b. Ether in dichloromethane:

Add 60 mL diethyl ether to a 200 mL volumetric flask, and dilute to volume with dichloromethane.

D. STANDARDS

1. Source: Stock standard solutions of the internal standard (ISTD) DPNA and the external standard mixture of DMNA, DENA, DPNA, DBNA, NPIP, NPYR, and NMOR may be obtained from Chem Service.

2. Preparation: Make appropriate serial dilutions of both solutions so that the nominal concentration of the DPNA spiking standard is 40 ng/mL, and the nominal concentration of NPYR in the mixed standard is 60 ng/mL.

3. Storage and Stability (As per manufacturer's information)

E. SAMPLE PREPARATION

Randomly selected bacon slices should be fried at 340 - 345 °F (171 - 174 °C) for three minutes on each side for each individual strip. Begin timing when first strip is laid in skillet. At the end of exactly 3 minutes each strip is flipped over in the same sequence they were placed in the skillet. After frying, excess fat should be removed by blotting with paper towels. Freeze and then grind sample, using a 11/32" grinder plate, or Waring blender-type food processor, or equivalent.

F. ANALYTICAL PROCEDURE

1. Prepare Sample for Extraction

   a. Seal one end of 24 mL extraction vessel and label as top.

   b. Invert vessel, place a plug of polypropylene wool into vessel and tamp down into top using a tamping rod.

   c. Weigh 5 - 6 g hydromatrix into a 50 - 100 mL beaker. Optimum amount will fill vessel to within 2 cm of the top using procedure described below, and can be determined experimentally. Do not use less than 5 grams.

   d. Weigh 5.00 $\pm$ 0.05 g fried bacon on top of hydromatrix.

   e. Add 0.25 g of propyl gallate to mixture.

   f. Dump beaker contents into glass mortar. Blend for 20 - 30 seconds using pestle, until bacon is thoroughly broken up and is incorporated into the hydromatrix.

   g. Place funnel into open-end extraction vessel.

h. Pour about one-third of total sample mixture into vessel, and then tamp it down securely with a tamping rod. Use forces equivalent to dropping rod from a height of 7 - 10 cm two or three times. Do not over-compress.

i. Pour half of the remaining mixture into vessel, and tamp down with rod. Spike with 1.0 mL of external standard for recovery or 1.0 mL of ISTD for all the other samples at this point.

j. Add last of remaining mixture to vessel and tamp down with rod.

k. Fill tube with increments of additional hydromatrix and tamp, if necessary, until level of mixture is approximately 2 cm from top of vessel.

l. Wipe down the beaker, mortar, and pestle with a plug of wool. Place plug into the extraction vessel and tamp down so that no fibers protrude from the end of the vessel.

m. Carefully wipe off sealing surface of the extraction vessel, and then apply end cap. Tighten securely.

2. Set Up the SFE

a. Check liquid level in chiller bath and verify that it is within 1 - 2 inches of the top. Turn on chiller cooling and circulation and allow bath temperature to cool to at least -14 °C before attempting to use air pump. Cool down can require 1 - 1.5 hours, depending on ambient temperature.

b. Turn on power to SFE extractor and pump module. Make sure that all manual valves on the extractor are closed and the oven chamber is empty.

c. Set oven temperature to 40 °C and valve temperature to 115 °C and turn on power to the valve heater.

d. Flush pump head with CO_2 as follows: Before attaching extraction cells to SFE, open the inlet valve for one of the cells. Adjust air pressure regulator on pump so that the pump begins to cycle. Allow pump to cycle at least five times if used within the last 24 hours, or 15 times if dormant for a longer period, to expel all air from the system. Close the inlet valve.

3. Install Extraction and Collection Vessels

a. Prepare collection tube as follows: Seat one fritted disc at the bottom of an empty 6mL-collection tube. Add 1.0 g sieved silica gel, then seat second fritted disk on top of silica gel layer.

b. Slide collection tube over collar of assembly. Connection must be tight, since escaping CO_2 will pressurize the collector.

c. Attach electronic flow meter to lower end of collection tube. Turn meter on and set it to record collection volume.

d. Place vessel in holding clamp. Open oven door and attach filled extraction vessel(s). Orient the vessel so end labeled top is up. First vessel should be attached to position 1 in oven. Securely finger tighten compression nuts and locking caps. (Refer to manufacturer's manual) Tape thermocouple securely to side of vessel in position 1.

e. Adjust CO_2 pressure to 6000 psi or 414 bar by turning regulator valve on pump. Open inlet valve to cell and allow cell to fill with CO_2. Observe fittings and check for leaks. If leaks are detected, close inlet and re-tighten the problem connection. Re-test for leaks until none are detected.

f. Increase CO_2 pressure to 8500 - 9000 psi or 621 bar by turning regulator valve, and continue to check for leaks. If leaks appear, close inlet and re-tighten, then open inlet and re-examine fittings. Continue until no leaks are observed.

4. SFE Extraction

a. Close oven door. Turn on oven heater and allow system to heat until vessel temperature (not oven temp) reaches 40 °C. This typically takes 10 - 15 minutes.

b. Adjust CO_2 pressure to 10000 $\pm$ 100 psi or 690 bar.

c. Verify that restrictor valves have been reset to limit flow to 2 - 3 L/minute. Slowly open outlet valves and adjust flow to 2.5 $\pm$ 0.3 L/minute using the needle valve restrictors. (CAUTION! Never attempt to shut off flow using needle valves, since this will eventually ruin them. Take great care when adjusting flows with these valves, since a very small turn of the wheel can make a large change in flow rates.

d. Re-adjust air pump and needle valves when necessary during course of extraction to maintain initial parameters. Conditions should be re-checked at least once every three minutes.

e. Continue extraction until a total of 50 L of gas have passed through the collection tubes. At the optimum flow rate, this should take about 20 minutes.

f. After 50 L have been collected, close off inlet valve to that vessel.

Note: Immediately before closing off inlet to last vessel, turn down pressure on air pump so CO_2 pressure is less than 8000 psi or 552 bar, or pump is likely to over pressurize when inlet valve is closed.

g. Record position of valve needed to maintain regulator flow. Depressurize extraction vessel by bleeding down through the needle valves, opening valves periodically to maintain a flow of 2.5 - 3.5 L/minute. When flow drops to zero, open needle valve an additional full turn. Disconnect flow meters from collection tube.

h. After all vessels are emptied of residual CO_2, turn off oven, and loosen top and bottom end caps and fittings.

i. Remove the extraction vessel from the oven. Attach syringe with appropriate fitting to outlet line and flush line and needle valve with 0.5 mL hexane, allowing rinse to deposit in the collection tube. Remove collection tube and flush with an additional 2 mL hexane, collecting output in a waste vial. Empty lines by blowing out with a syringe full of air. Carefully return needle valve to original position recorded in step 4.g above. DO NOT OVERTIGHTEN. Wipe off any fat that may adhere to needle valve outlet tube and collar.

j. Close outlet valve. Re-check that all mechanical valves are closed at this point. De-pressurize CO_2 pump by resetting air pressure to 0, then bleed off residual CO_2 pressure in the pump by slowly opening one of the inlet valves. Close valve when pressure reading on pump drops below 1000 psi or 69 bar.

5. SPE Elution and Concentration

a. Add 4 mL 25% DCM/Pentane to the SPE cartridge.

b. When first rinse just reaches top of silica gel layer, add a second 4 mL 25% DCM/Pentane rinse and allow to drain. Discard rinses.

c. Place a 10 mL concentrator tube under the SPE cartridge. Elute cartridge with 4 mL of 30% ethyl ether/DCM. Save eluent.

d. Repeat SPE cartridge elution with an additional 4 mL of 30% ethyl ether/DCM. Save combined extracts.

e. Add one Hengar granule to the tube, attach a micro Snyder column, and immediately place in a 65 °C water bath (do this in a fume hood). Concentrate to < 0.5 mL.

f. Before injection on a gas chromatograph, adjust volume of solution to 1.0 mL with DCM.

Note: Replace GC inlet liner every 6 - 8 injections of sample to remove dissolved lipids from SFE extraction process.

6. Instrumental Settings

a. Typical SFE Operating Parameters

Note: The following parameters may be adjusted to insure optimal instrument performance.

Pump Coolant Temperature:	-14 °C
Oven Temperature:	40 °C
Operating Pressure:	10,000 psi or 690 bar
Valve Temp.:	115 °C

b. Typical GC/TEA Operating Parameters

Note: The following parameters may be adjusted to ensure optimal instrument performance.

GC oven temperature:	180 °C for 5 minutes. Ramp at 10 °C/minute to 190 °C and hold for 13 minutes.
GC injector temperature:	200 °C
Carrier gas:	Nitrogen at 20 mL/minute
TEA interface temperature:	250 °C
TEA furnace temperature:	500 °C
TEA trap	Cooled by liquid nitrogen, trap inserted to 10 cm depth.

7. Chromatography

Note: SFE extracts contain dissolved lipids, which collect in the inlet port of the chromatograph and can interfere with quantitation after accumulation of appreciable levels.

a. Before attempting GC quantitation, make sure that a new inlet liner has been installed. Replace liner after every 6 - 8 injections of extract onto the system.

b. Adjust GC and TEA operating parameters for optimum sensitivity and resolution.

c. Inject a constant volume (5 µL) of standard until consistent response is achieved.

d. Inject the ISTD used for spiking adjacent to at least one of the standards used for quantitation.

e. Inject no more than two sample extracts between injections of standards. Adjust sample volumes immediately before injection (5.e), and record sample and injection volumes.

f. If injection goes off scale, dilute with DCM to a larger volume (record volume for later verification), and re-inject using a constant volume.

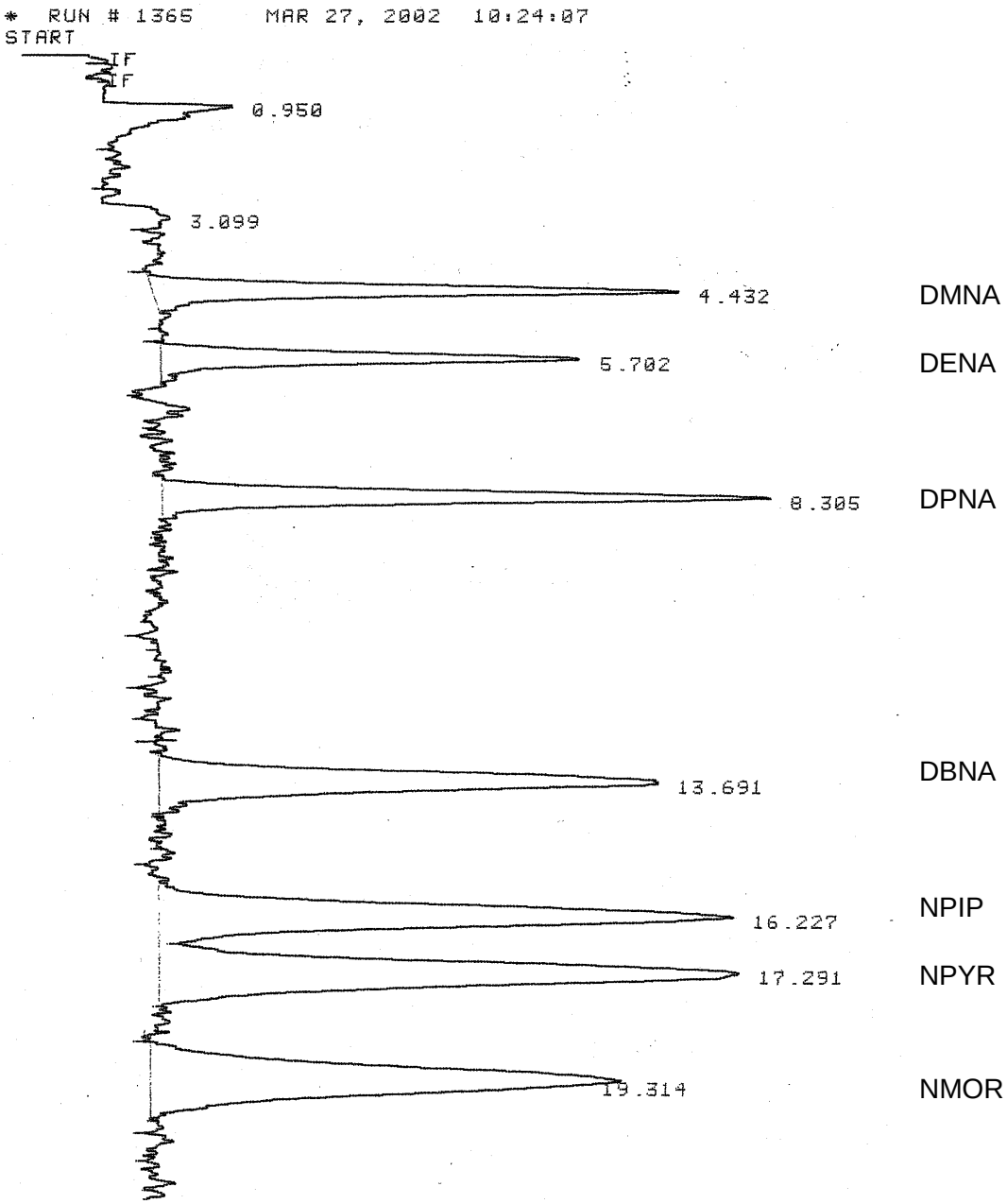
8. Re-Running Samples

Experience has shown that recoveries of many nitrosamines tend to decrease with increasing concentrations of fat in the sample. The effect is most pronounced with DBNA, followed by DPNA, but it does affect other nitrosamine recoveries as well.

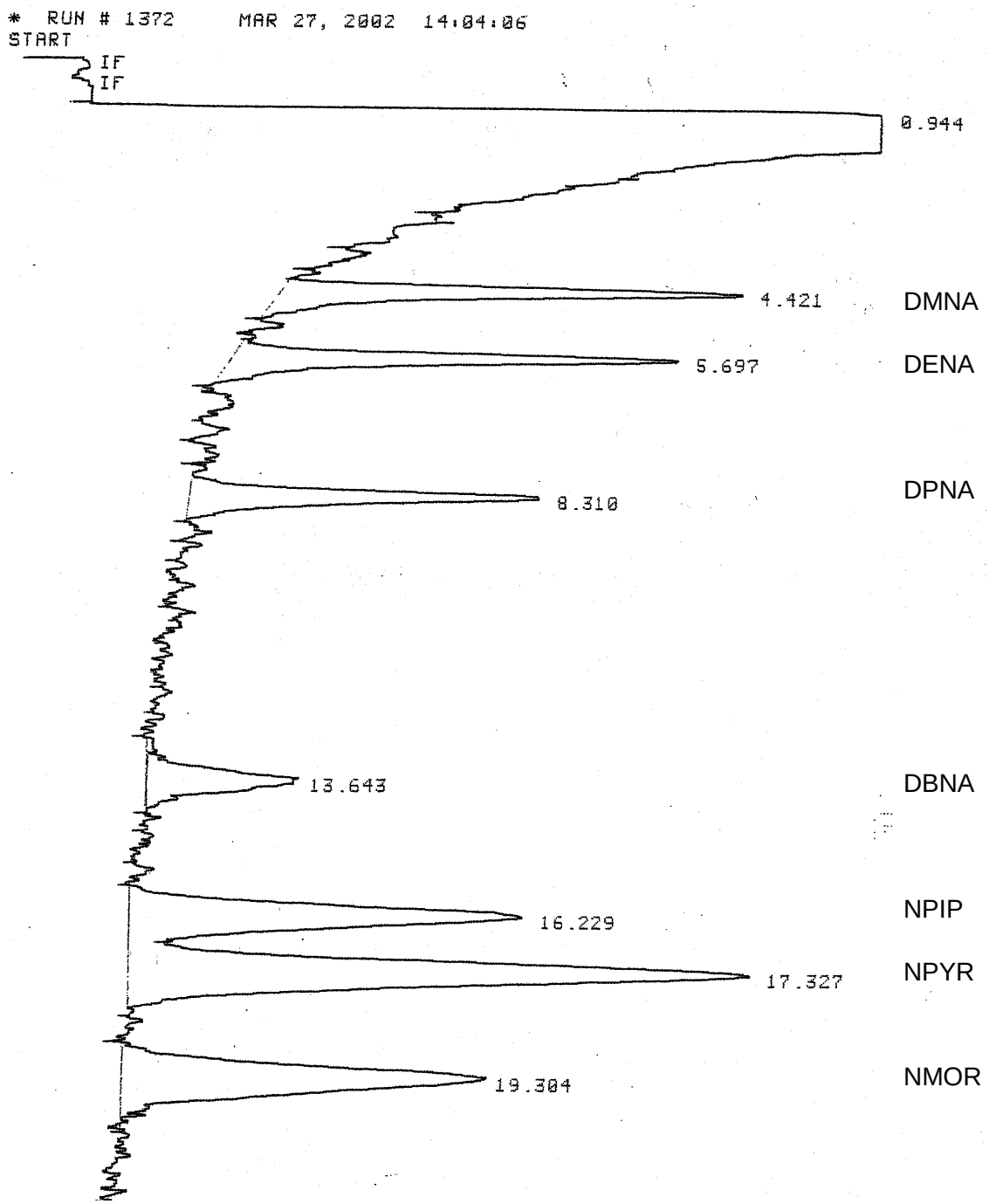
Samples that produce DPNA recoveries lower than 75% are likely to have reduced recoveries of NPYR as well. Such samples should be re-analyzed using either a halved sample size or twice the normal amount of silica gel in the trap. If the latter approach is taken, wash and elution volumes should be increased to 12 mL.

9. Sample Chromatograms

   a. Mixed Nitrosamine Standard 10 ppb



b. Mixed Nitrosamine Recovery 10 ppb



G. CALCULATIONS

a. Measure peak heights of all nitrosamines in standards and samples.

b. If all standards during the course of a day's run agree within 10%, it is permissible to average all of them as the basis of calculation. Otherwise average the two standards closest to the sample in question for quantitation.

c. Calculate DPNA recovery in each sample using the formula:

$$\% \text{ Recovery} = \frac{(PK.HT.ISTD)(CONCSTD)}{(Pk.Ht.std)(CONCISTD)}$$

Where:

Pk.Ht.ITSD and PK.HT.STD=	peak heights for DPNA in the sample and standard chromatograms
CONCSTD =	DPNA concentration in the mixed standard
CONCISTD =	DPNA concentration in the ISTD spiking solution.

d. Calculate concentration for each nitrosamine in samples using the formula:

$$CONC(ppb) = \frac{(IVSTD)(PKHT.Sample)(CONCSTD)(Vsample)}{(Ivsample)(PKHT.STD)(Wtsample)}$$

Where:

IVSTD and Ivsample =	injection volumes, in µL, of standard and sample solutions.
PKHTSample and PKHT.STD =	analyte peak heights in sample and standard chromatograms.
CONCSTD =	concentration of analyte of interest in the mixed STD, in ng/mL.
Vsample =	final volume of the sample extract, in mL.
Wtsample =	initial sample weight, in grams.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety glasses, protective gloves, lab coat.

2. Hazards

Procedure Step	***Hazard***	***Recommended Safe Procedures***
Hydromatrix	none	Good laboratory practices
Propyl Gallate	Skin and respiratory sensitizer. May cause irritation to the skin, eyes, or respiratory tract.	
Silica Gel	May cause irritation to the skin, eyes, or respiratory tract.	
Dichloromethane	Harmful if swallowed, inhaled, or absorbed through the skin. Suspect cancer hazard.	
Pentane	Flammable liquid and vapor. Harmful if swallowed or inhaled. Causes irritation to the skin, eyes, and respiratory tract.	
Diethyl Ether	Flammable liquid and vapor. After long standing or after exposure to air or light it may form explosive peroxides that are sensitive to mechanical impact and static discharge. Harmful if swallowed, inhaled, or absorbed through the skin. Inhalation of vapors may cause dizziness and unconsciousness.	Protect from exposure to air. Do not evaporate to near dryness.
Standards	The nitrosamines are potent carcinogens and the solvent itself is suspected of promoting tumors.	This dilution must be done in a fume hood being cautious not to contaminate the area.

3. Disposal Procedures

Procedure Step	***Recommended Safe Procedures***
Sample waste Nitrosamine solution	Store in a prominently labeled container until disposal by contractor
Hydromatrix, Propyl gallate, Silica Gel	Disposal of in accordance with local, state, and Federal regulations.
Dichloromethane	Store with chlorinated waste until disposal by contractor or in-house specialist
Pentane, Diethyl ether	Collect waste in tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range (ppb)*	*Acceptable Recovery (%)*	*Acceptable Repeatability CV (%)*
DMNA	2 - 20	70 -110	< 15
DENA	4 - 20	70 -110	< 15
NPIP	5 - 20	70 -110	< 15
NPYR	5 - 20	70 -110	< 15
NMOR	5 - 20	70 -110	< 15
DPNA	10 (ISTD)	70 -110	< 15
DBNA	5 - 20	45 110	< 15

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample Preparation	Bacon must be thoroughly ground and mixed into hydromatrix to form a homogeneous sample.
b. Extraction Pressure	Keep between 9,800-10,100 psi
c. CO_2 Flow Rate	Keep between 2.2 - 2.8 L/minute (try to maintain 2.5 L/minute average
d. CO_2 Volume	Minimum 50 L
e. Skillets	Calibrated when used, to produce a minimum temperature of 340 - 345 °F (171 - 174 °C) in the oil. Written records to be kept for each skillet. Readjust and calibrate skillet until required temperature is obtained.

3. Readiness To Perform

a. Familiarization

i. Phase I: Standards- Standard curves are not prepared in this analytical procedure

ii. Phase II: Fortified samples- Three levels. 5, 10, 20 ppb, three acceptable replicates each, with blanks; for a minimum of three days.

iii. Phase III: Check samples for analyst qualification

8 blind samples, 7 fortified between 5 and 20 ppb and one of which should be at the none detected level if possible.

iv. Samples submitted by the Quality Assurance Manager (QAM) or Supervisor.

v. Authorization from QAM is required to commence official analysis.

b. Acceptability criteria.

Refer to section I.1 above.

4. Intralaboratory Check Samples

   a. System, minimum contents.

      i. Frequency: One per week per analyst when analyses are run.

      ii. Fortified blank prepared by supervisor.

      iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory Quality Assurance Manager (QAM) for:

         (a) All replicate findings.

         (b) Running average difference between replicates.

         (c) All % recoveries.

         (d) Running average, standard deviation, and CV for recoveries.

   b. Acceptability criteria.

      Refer to I. 1.

      If unacceptable values are obtained, then:

      i. Stop all official analyses by that analyst.

      ii. Take corrective action.

5. Sample Acceptability and Stability

   a. Matrices: Bacon, sliced (18 - 24 slices to the lb.), slab (cut 10 slices to the inch).

   b. Sample Receipt Size: Minimum 1 pound.

   c. Condition on Receipt: 25 - 60 °C

   d. Sample storage:

      e. Time: Up to six months (after frying).

      Ii. Condition: Frozen

6. Sample Set

   a. External standard for quantitation

   b. Recovery (blank tissue fortified with mixed standard of nitrosamine of interest.

   c. Unknown samples to be analyzed.

   d. Blank

7. Sensitivity

   Minimum proficiency level (MPL): 5 ppb.

J. WORKSHEET

Nitrosamine Standards

Date Extracted:
Date Analyzed:
Analyst:

Standard Id:

Bacon Id:

NA7 Std

Analyte	Conc. ng/ml	Height								
		NA7 Std1	NA7 Std2	NA7 Std3	NA7 Std4	NA7 Std5	Avg. 1&2	Avg. 2&3	Avg. 3&4	Avg 4&5
DMNA										
DENA										
DPNA										
DBNA										
NPIP										
NPYR										
NMOR										

DPNA Std

Conc. ng/ml	Height						
	Std1	Std2	Std3	Std4	Avg. 1&2	Avg. 2&3	Avg. 3&4

K. APPENDIX

Reserved.

L. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Division.