CHAPTER 5

Chemical Evaluation Methods for Quality and Safety

This section contains information on the official methods for testing mercury and histamine. It also contains information on analysis of histamine by HPLC and new indicators of freshness, putrescine and cadaverine, using DFO (Canadian) and USFDA methodology and modifications to the methods used by Thailand.

The HPLC method for histamine is experimental, not recognized by the AOAC. Our experience is that it does not give good results with fresh fish. Results with canned tuna are good except when the tuna is canned in oil.

The chemical indices for the product are in somewhat of a flux*:

CANADA methods:

Histamine	<100ppm (average of 5 cans), no one (1) can >300ppm
Putrescine	>0.8ppm early stages of decomposition (Note: not official limits)
Cadaverine	>0.5ppm early stages of decomposition (indices used for comparative tests with sensory)

USFDA methods:

Histamine	<50 ppm (≥ 1 can/12 cans for decomposition, no one (1) can >500 ppm for adulteration
Putrescine	0.5 - 0.7 ppm decomposed (Note: not official limit - studies in progress)
Cadaverine	0.6 - 0.7 ppm decomposed (Note : not official limit - studies in progress)

This section contains the following information:

1. Determination of mercury in seafood - official method
2. Determination of histamine in seafood - official method - fluorometric method
3. Determination of histamine in canned fish by HPLC
4. Putrescine and cadaverine in canned tuna - DFO (Canada)
5. Putrescine and cadaverine in canned tune - USFDA
6. Putrescine and cadaverine in canned tuna - Thailand modification
7. Examples of some results for putrescine and cadaverine

* It should be noted that critical limits enforced by countries, may change based on scientific evidence and risk assesment.

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CHAPTER 1 - CONTAMINANTS
SECTION 1 : MERCURY TOTAL

1. SCOPE AND APPLICATION

1.1 This methos is applicable to fish and fish products as well as other biological tissues.

2. PRINCIPLE OF THE METHOD

2.1 A prepared liquid sample with mercury in the divalent form (Hg ++) enters the system and is mixed with a reducing agent (usually $SnCl_2$ to form elemental mercury vapor). The mixture flows into a liquid-gas separator where argon or nitrogen is introduced to carry the mercury vapor through a drying tube for water vapor removal.

The dry vapor then enters one path of a double path optical cell which has been optimized for fast response time (small diameter) and sensitively (long length). A mercury source, powered by a constant current power supply, delivers a stable source of emission at 254 nm. Absorbance by mercury cold vapor is measured using a solid state detector with a wide dynamic range. The resulting signal is referenced to the simultaneous absorbance of the pure carrier gas flowing through the second optical path under identical conditions.

3. SAMPLING PROCEDURE AND STORAGE

3.1 Commercial shipment: Take representative sample from the product lot and store as to maintain sample integrity.

3.2 Survey samples: Fish may be either pooled or individual. For species normally greater than 30cm in length, and individual fish may be used as a sample. For species less than 30cm length, a pooled sample is required. Store as to maintain sample integrity.

4. SAMPLE PREPARATION

4.1 Commercial shipment: Sample preparation should take into account the type of product and how it is used and prepared by the consumer.

4.1.1 For fish and fish products that contains no free liquid: comminute the sample until homogeneous.

4.1.2 For products that are packed in water, brine or similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate size sieve for 1 to 1.5 min. Comminute the part of the sample retained by the screen until a homogeneous blend is obtained.

4.13 For products that are packed in a medium that may be or is normally used by the consumer, e.g. fish canned in its own juice or oil: transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.

4.2 Survey Samples

4.2.1. For individual fish: weigh and measure the fork-length, i.e. from the nose to the fork of the tail, for size correlation.

4.2.2 For a pooled sample: determine the average values for length and weight of the fish.

4.2.3 Pass the skinned fillets through a commercial meat grinder a sufficient number of times to obtain a homogeneous blend (e.g three times).

4.3 Collect the homogenized sample into a thoroughly cleaned, sealable plastic pot of glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before use.

5. APPARATUS

5.1 Mercury Analyzer, equipped with a mercury vapour lamp, auto-sampler, proportioning pump, and reagent manifold.

5.2 Microwave Digestion Unit: The digestion programs are as follows:

Step 1	250 Watts	5 minutes
Step 2	400 Watt	5 minutes
Step 3	600 Watt	5 minutes
Vent		1 minute

5.3 Digestion vessels; TFM vessel (P/N 33802).

5.4 Polypropylene bottle (30 ml).

6. REAGENTS

6.1 Nitric acid (HNO_3).

6.2 Hydrogen peroxide (H_2O_2) 30-40%.

6.3 Stannous Chloride $SnCl_2$ (10%).

6.4 Magnesium perchlorate anhydrous. Leeman Labs, Inc. Cat No. 0240-1119.

6.5 Hydrochloric acid (10%).

6.6 Standard Mercuric(II) Nitrate.

Stock Standard Solution (1.0mg Hg/ml).

High Intermediate Standard Solution (10μg Hg/ml). Dil. 1.0ml Stock soln to 100ml with 1N HCl (3).

Low Intermediate Standard Solution (1.0μg/ml. Dil. 10.0ml. high intermediate std soln to 100ml with 1N HCl.

Working Standard Solution Dil. 0.5, 1.0, 2.0, 3.0 and 4.0ml low intermediate std soln to 100ml with 1N HCl (0.005, 0.01, 0.02, 0.03 and 0.04μg (Hg/ml, resp.).

7. PROCEDURE

7.1 Place the TFM vessel (P/N 33802) directly on the balance plate and set to zero. Weigh the sample 0.5g into this vessel.

7.2 Add 5ml of HNO_3 conc. and 1ml of H_2O_2 (30%) to the vessel.

7.3 Put the vessel into the protection shield, cover and then put the vessels onto the polypropylene rotor body.

7.4 Place the polypropylene rotor body to the Microwave Digestion Unit and set the digestion program as follows:

Step 1	250 Watt	5 minutes
Step 2	400 Watt	5 minutes
Step 3	600 Watt	5 minutes
Vent		1 minutes

7.5 Place the polypropylene rotor body in the water bath with running water to cool the temperature of the vessel down to room temperature.

7.6 Take the vessels out of the polypropylene rotor body, remove the protection shield and the cover.

7.7 Transfer the solution to a graduated beaker, washing repeatedly the inside of the vessel with distilled water.

7.8 Filter the solution with cotton wool and transfer to 25ml volumetric flask. Adjust the volume to 25ml with distilled water.

7.9 Transfer to 30ml polypropylene bottle for mercury determination by mercury Analyzer.

7.10 Set up the Mercury Analyzer according to the manufacturer's instructions.

7.11 Place the sample, standards and blank, and check samples on the auto-sampler tray. A prepared standard and liquid sample with mercury in the divalent form (Hg^{++}) enters the system and is mixed with a reducing agent (usually $SnCl_2$ to form elemental mercury vapor. The mixture flows into a liquid-gas separator where argon or nitrogen is introduced to carry the mercury vapor through a drying tube for water vapor removal.

The dry vapor then enters one path of a double path optical cell which has been optimized for fast response time (small diameter) and sensitivity (long length). A mercury source, powered by a constant current power supply, delivers a stable source of emission at 254nm. Absorbance by the mercury cold vapor is measured using a solid state detector with a wide dynamic range. The resulting signal is referenced to the simultaneous absorbance of the pure carrier gas flowing through the second optical path under identical conditions.

8. CALCULATION

8.1 Prepare a calibration curve of absorbance versus nanograms Hg in the standards.

8.2 Determine the mercury concentration in the sample by comparing the sample absorbance to the calibration curve, taking into account the sample weight and the dilution factor. Express the result in terms of total mercury on a wet basis (ppm).

9. REFERENCES

9.1 MLS-1200 MEGA Microwave Digestion System with MDR Technology, MILESTONE s.r.l. Sorisole(BG), Italy.

HISTAMINE IN CANNED FISH BY HPLC

1. Reagent

   1.1 Histamine dihydrochloride
   1.2 Methanol
   1.3 Benzoyl chlorine
   1.4 Diethyl ether
   1.5 6% Trichloric acid
   1.6 2M Sodium hydroxide
   1.7 Preparation of standard amine solution

   Histamine dihydrochloride was dissolved in 10 ml deionized water. The final concentration was 10 mg/ml solution.

2. Benzoylation Of Standard Amine Solution

The benzoyl derivatives of amines were prepared according to the method of Redmond and Tseng (1979) with minor modification. To the standard amine solution (50μl) 2 M sodium hydroxide (1 ml) was added, followed by 10μl of benzoyl chloride, mixed on a vortex mixer and allowed to stand 20 min. Saturates sodium chloride solution (2 ml) was added, followed by extraction with 4 ml diethyl ether. After centrifugation, the upper organic layer was transferred into a clean tube and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 500μl of methanol and 5μl aliquots were injected for HPLC analysis.

3. Sample Preparation and Amine Extraction

After removal of oil, the fish was ground in a Waring Blender 3 min. Twenty grams ground sample was transferred to a 250ml centrifuge tube, and homogenized with 50ml 6% trichloric acid in a Polytron type 10-35 homogenizer 3 min. The homogenate was centrifuged (12000 rpm, 10 min, 4°C) to allow precipitation, and filtered through Whitman No. 1 filter paper. The filtrate was placed in a volumetric flask and made up to 100 ml. Each extract (2ml) was derivatized with benzoyl chloride as described. Recoveries of amines in augmented samples were determined by adding 25-50ppm amines in canned fish.

4. Chromatographic Conditions

Isocratic and gradient elution systems were used. For isocratic systems the mobile phase was methanol - water 55:45 v/v) at 1.5ml/min. at room temperature. The gradient elution program was set at 1.1ml/min, starting with a methanol-water mixture (55:45, v/v) for 2.5 min. The program proceeded linearly to methanol-water 88:22 (v/v), with flow rate increasing from 1.1 ml/min to 1.3 ml/min over 3.5 min. This was followed by the same composition and flow-rate for 2 min, then decreased over 7 min to methanol-water (55:45 v/v) at 1.1ml/min.

Ref: **Gow-Chin Yen and Chiu-Luan Hsieh (1990) Simultaneous Analysis of Biogenic Amines in Canned Fish by HPLC, Journal of Food Science, Vol. 56**

HISTAMINE DETERMINATION BY HPLC

Sample 20G + 60ml 6% TCA.

Homogenize for 3 mins.

Centrifuge for 10 mins and filter.

Adjust the volume to 100ml.

Take 2ml extract to test tube.

Add 1ml 2M NaOH, 10 μl Benzoyl Chloride. Mix on vortex mixer and stand for 20 mins.

Add 2ml Sat. NaCl and 4ml Diethyl Ether, centrifuge.

Transfer upper organic layer to test tube and evaporate to dryness by N_2 gas.

Dissolve residue in 1ml MeOH and inject 20μl for HPLC analysis.

DETERMINATION OF HISTAMINE IN SEAFOOD FLUOROMETRIC METHOD

1. SCOPE APPLICATION

1.1 Histamine-like substances" are the principle compounds implicated as causing scombroid poisoning, an allergy-like condition caused predominantly by consumption of toxic fish of the sub-order Scombroidea which includes the tuna, bonita, kingfish, and mackerel. This method is suitable for the analysis of "histamine-like substances" in the above species, fish products utilizing these species, and mahi mahi (dolphin fish). The level of "histamine-like substances" can be used as both an indicator of spoilage and as an indicator of substances of public health significance.

2. PRINCIPLE OF THE METHOD

2.1 The "histamine-like substances" are extracted from the sample with methanol, interferring compounds are removed by anion exchange chromatography, and the purified histamine is then derivatized with orthophthalaldehyde (OPT) to form a fluorophore. Its intensity is measure by fluorometry. The results are reported as equivalent histamine levels.

3. INTERFERENCES

3.1 In fish flesh, large amounts of the amino acid, histidine, may be present and this compound interferes with the determination of histamine as it also forms a fluorophore with OPT. Several other homologues of histidine and other polyamides may also react with OPT; however, they are normally present at low levels and are not a major problem. The anion exchange procedure should minimize these problems.

4. SAMPLING PROCEDURE AND STORAGE

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. SAMPLING PREPARATION

5.1 Upon receipt of the sample by the laboratory, the sample information should be checked and recorded in the laboratory records. The condition of the sample should be checked to ensure that fresh samples have been properly refrigerated and that frozen samples are still frozen. It is essential that a sample has been handled and stored in a manner that ensured its original quality has been maintained.

5.2 Fresh raw samples should be prepared immediately upon receipt but in no instance should a delay longer than 3 hours under refrigeration be permitted, otherwise the sample should be quick frozen upon receipt. Store other samples to maintain their integrity taking into account the type of product and how it is stored commercially.

5.3 Sample preparation should take into account the type of product and how it is used and prepared by the consumer. Samples that are too large or have a texture that is too tough for homogenization should be treated in a food processor or passed through a food grinder a sufficient number of times to ensure a uniform mix.

5.3.1 Fresh raw samples should be analysed immediately after grinding but if this is impossible, they should be quick frozen to ensure that decomposition does not proceed.

5.3.2 Products packed in a medium that may be or is normally used by the consumer, e.g. fish canned in its own juice or oil: transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.

5.3.3 Products packed in water, brine or similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate size sieve for 1 to 1.5 minutes. Comminute the part of the sample retained by the sieve until homogeneous blend is obtained.

5.3.4 Processed products containing no separable liquid: thaw in the package (if frozen) and pass the sample through a food grinder a sufficient number of times to obtain a uniform mix. Thorough grinding and mixing is extremely important if the product is made up of several distinct components such as fish dinners.

5.4 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before analysis.

A. **Apparatus**

(a) Chromatographic tube - 200 x 7 (id) mm polypropylene tube (Chromaflex, Kontes Glass Co. No. K-420160 or equiv.) fitted with Kontes No. K-422372 Kel-F Hubs and ca 45cm Teflon tubing. Control flow rate at >3ml/min by ***adjusting ht of column*** relative to tubing outlet. Alternative, use 2-way valve in place of tubing.

(b) Photofluorometer - Perkin-Elmer Model 203 or 204 with medium pressure Hg lamp, or equiv. instrument with excitation at 350 nm and measuring emission at 444nm.

© Repipets - 1 and 5ml (Labindustries Inc., 620 Hearst Ave, Berkely, CA 94710, or equiv.).

B. **Reagents**

(a) Ion exchange resin - Bio-Rad AG 1-X8, 50-100 mesh (Bio-Rad

Laboratories, 1414 Harbour, South, Richmond, CA 94804) or Dowex 1-X8, 50-100 mesh. Convert to -OH form by adding ca 15ml 2N NaOH/g resin to beaker. Swirl mixture and let stand <30 min. Decant liq. and repeat with additional base. Thoroughly wash resin with H_2O, slurry into fluted paper (S&S No. 588, or equiv.), and wash again with H_2O. Prep. Resin fresh weekly and store under H_2O.

Place glass wool plug in base of tube, (a), and slurry in enough resin to form 8cm bed. Maintain H_2O level above top of resin bed at all times. Do not regenerate resin in packed column; rather, use batch regeneration in beaker when necessary. Wash column with ca 10ml H_2O before applying each ext.

(b) Phosphoric acid - 3.57N. Dil 121.8ml 85% H_3PO_4 to 1 liter. For other concn H_3PO_4, vol. required for 1 L 3.57N acid = 17493/(density H_3PO_4). Standardize 5.00ml by titrn with 1.00N NaOH to phthln end point, and adjust concn if necessary.

(c) o-Phthalicdicarboxaldehyde (OPT) solution - 0.1%. Dissolve 100mg OPT (Adrich Chemical Co., Inc., No. P3,9400, or equiv.) in 100ml distd-in-glass MeOH (Burdick & Jackson Laboratories, Inc., or equiv.). Store in amber bottle in refrigerator. Prep. fresh weekly.

(d) Histamine std solution - Store in refrigerator. (1) Stock solution. - 1mg/ml as free base. Accurately ca 169.1mg histamine dihydrochloride (98%, Aldrich Chemical Co., Inc., No. 11,2607, or equiv.) into 100ml vol. flask, and dissolve and dil. to vol with 0.1N HCl. Prep fresh weekly. (2) Intermediate solution - 10μg/ml. Pipet 1ml stock solution into 100ml vol. flask and dil. to vol. with 0.1N HCl. Prep fresh weekly. (3) Working solutions - 0.5, 1.0, and 1.5μg/5ml. Pipet 1, 2 and 3ml intermediate solution into sep 100ml vol. flasks, and dil. each to vol. with 0.1N HCl. Prep fresh daily.

C. Preparation of Standard Curve

Pipet duplicate 5ml aliquots of each working std solution into sep. 50ml glass or polypropylene erlenmeyers. Pipet in 10ml 0.1N HCl to each flask and mix. Pipet in 3ml 1N NaOH and mix. Within 5 min, pipet in 1ml OPT solution and mix immediately. After exactly 4 min, pipet in 3ml 3.57N H_3PO_4 and mix immediately. It is important to mix thoroughly after each addition and at least once during OPT reaction. (Run 6-10 OPT reactions simultaneously by adding reagents to erlenmeyers in set order) Prep. blank by substituting 5ml 0.1N HCl for histamine solution. Within 1.5 hr, record fluorescence intensity (I) of working std solutions with H_2O in ref. cells, using excitation wavelength of 350nm and emission wavelength of 444nm. Plot I (corrected for blank) against μg histamine/5ml aliquot.

D. **Determination**

Weigh 10g sample, add approx. 50ml MeOH, blend in polytron homogenizer and transfer to 100ml vol. flask, rinsing with MeOH and adding rinsing to flask. Heat in water bath to 60°C and let stand 15 min at this temperature. Cool to 25°C, dilute to volume with MeOH, and filter thru folded paper. Alcohol filtrate may be stored in refrigerator several weeks. Pass 4-5ml H_2O thru solumn, (a), and discard eluate. Pipet 1ml ext onto column and add 4-5ml H_2O. Immediately initiate column flow into 50ml vol. flask contg 5.00ml 1.00N HCl. When liquid level is ca 2 mm above resin, add ca 5ml H_2O and let elute. Follow with H_2O in larger portions until ca 35ml has eluted. Stop column flow, dil. to vol. with H_2O, stopper, and mix. Regenerate eluate.

Pipet 5ml eluate into 50ml erlenmeyer, and pipet in 10ml 0.1N HCl. Proceed as in preparation of calibration curve beginning "Pipet in 3ml 1N NaOH...".

If sample contains >15mg histamine/100g fish, pipet 1ml sample - OPT mixture into 10ml beaker contg exactly 2ml blank-OPT mixt, and mix thoroughly. Read fluorescence of new solution. Dilute and mix aliquots with blank-OPT mixture as needed to obtain measurable reading. This approximation indicates proper dilution of eluate required prior to second OPT reaction needed for reliable quantitation of sample. Alternatively, use sensitivity range control of fluorometer (if instrument has one of aliquot of eluate with 0.1N HCl, and proceed as in preparation of calibration curve beginning "Pipet in 3ml 1N NaOH..."

E. **Calculation**

Plot of I (measured by meter deflection or recorder response and corrected for blank) against μg histamine/5ml solution should be straight line passing thru origin with slope = m = [(Ia/1.5) + Ib + 2Ic]/3.

mg Histamine/100g fish = (10)(F)(1/m)(Is)

Where Is, Ia, Ib, and Ic = fluorescence from sample, 1.5, 1.0, and 0.5μg histamine stds resp., and F = dilution factor = (ml eluate + ml 0.1N HCl)/ml eluate. F = 1 for undild eluate.

If calibration plot is not linear, use std curve directly for quantitation. Each subdivision on abscissa should be 0.1μg histamine/5ml solution. Read all values from curve to nearest 0.05μg histamine/5ml solution.

mg Histamine/100g fish = (10) (F)(W)

Where W = μg histamine/5ml solution as detd std curve.

Ref : JAOAC 60, 1125, 1131 (1977)

PUTRESCINE AND CADAVERINE IN CANNED TUNA
DFO (CANADA)

1. SCOPE AND APPLICATION

The diamines putrescine and cadaverine, which are formed from the amino acids ornithine and lysine, are the products of bacterial decomposition of fish tissue. These diamines can be used as indices of decomposition in fish and shellfish.

2. PRINCIPLE OF THE METHOD

Putrescine and cadaverine are extracted with methanol, and internal standard hexane diamine is added, and a dry residue of their hydrochloride salts is prepared. The salts are derivatized with pentafluoropropionic anhydride, and then separated from excess reagent on an alumina column. The derivatives are then injected into a gas chromatograph with an electron capture detector to determine the levels of putrescine and cadaverine.

3. SAMPLING PROCEDURE AND STORAGE

Take a representative sample from the product lot and store so as to maintain sample integrity.

4. SAMPLE PREPARATION

4.1 Upon receipt of the sample by the laboratory, the sample information should be checked and recorded in the laboratory records. The condition of the sample should be checked to ensure that fresh samples have been properly refrigerated and that frozen samples are still frozen, it is essential that a sample has been handled and stored in a manner that ensures its original quality has been maintained.

4.2 Fresh raw samples should be prepared immediately upon receipt but in no instance should a delay longer than 3 hours under refrigeration be permitted, otherwise the sample should be quick frozen upon receipt. Store other samples to maintain their integrity taking into account the type of product and how it is stored commercially.

4.3 Sample preparation should take into account the type of product and how it is used an prepared by the consumer. Samples that are too large or have a texture that is too tough for homogenization should be comminuted in a food processor or passed through a food grinder a sufficient number of times to ensure a uniform mix.

4.3.1 Fresh raw samples should be analysed immediately after grinding; but if this is impossible, they should be quick frozen to halt decomposition.

4.3.2 Products packed in a medium that may be or is normally used by the consumer, e.g. fish canned in its own juice or oil: transfer the entire contents of the package into a homogenizer and blend for one minute or until a homogeneous mix is obtained.

4.3.3 Products packed in water, brine or similar medium that is normally discarded by the consumer: open the package and drain the product on an appropriate sieve for 1 to 1.5 minutes. Comminute the part of the sample retained by the sieve until a homogeneous blend is obtained.

4.3.4 Processed products containing no separate liquid: thaw in the package (if frozen) and pass the sample through a food grinder a sufficient number of times to obtain a uniform mix. In the case of products that are made up of several separate distinct components such as fish dinners, only the fish component would be analysed.

4.4 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separated from the sample, thoroughly reblend before analysis.

.5. REAGENTS

5.1 Pentafluoropropionic (PFP) anhydride : Refrigerate and protect from moisture

5.2 Ethyl acetate : Distilled in glass

5.3 Toluene : Distilled in glass

5.4 Methanol : Distilled in glass

5.5 **Stock solution** (1mg/ml Putrescine, 1mg/ml Cadaverine)
Dissolve 100mg each of putrescine and cadaverine in 0.1N HCL and dilute to 100ml in volumetric flask.

Intermediate solution (10μg/ml)
Dilute 1ml stock solution into 100ml volumetric flask.

Working solution (1μg/ml)
Dilute 10ml intermediate solution into 100ml volumetric flask.

5.6 **Hexane Diamine stock solution** (1mg/ml)

Dissolve 100mg hexane diamine in 0.1N HCL and dilute to 100ml in a volumetric flask.

Working Solution (10μg/ml)
Dilute 1ml stock solution to 100ml in a volumetric flask.

6. PROCEDURE

6.1 Extraction

Weigh 10g sample, add approx. 45ml MeOH, blend in polytron homogenizer and transfer to 100ml volumetric flask. Adjust to volume with MeOH, shake and let settle.

6.2 Derivatization

6.2.1 For standards, pipet the following into 100ml round bottom flask:

Working solution.	ml used	diamine in sample (μg)
B	1	1
B	5	5
A	1	10
A	2	20

For sample, pipet 10ml extract into flask.

6.2.2 Pipet 1ml hexane diamine working solution (internal std) into each flask.

6.2.3 Add 0.5ml 1N HCL and evaporate to dryness on a rotary evaporator at 50°C.

6.2.4 To residue, add 1ml ethyl acetate 250μl pentafluoropropionic acid anhydride (PFPAA).

6.2.5 Stopper flask loosely, swirl contents to mix thoroughly and place in a 50°C water bath for 30 mins, swirling occasionally (If the reaction has not cleared within 15 min add an additional 250μl PFPAA, if reaction mixture remains cloudy then no reaction took place).

6.2.6 After reaction, evaporate at 50°C the solvent/reaction mixture to a glass with nitrogen.

6.2.7 Dissolve the resulting glass in 2ml of 30:70 ethyl acetate:toluene.

6.2.8 Prepare alumina column (80 x 9mm bed volume) with 3% moisture deactivated alumina, with 1 cm anhydrous sodium sulphate.

6.2.9 Condition column with 10ml hexane, collect effluent, and total derivatized extract to column bed.

6.2.10 Rinse flask with further 3ml 30:70 and add to column when initial extract absorbed onto bed surface.

6.2.11 Repeat with 18ml 30:70, and add to column. Collect entire column effluent, mix by swirling.

6.3 Clean Up

Prepare alumina column

ALUMINA : Heat alumina 2 hours at 125°C, stopper and let cool to room temperature. Adjust activity by adding 3g water to 97g alumina. Equilibrate for a minimum of 4 hrs with shaking.

For each sample, pack a 24 x 10.5mm column to a height of 8cm. Cover with 1cm of anhydrous sodium sulphate.

6.4 Chromatography Condition

Set up chromatography as follows:
Megabore Capillary Column DB-225 (30m x 0.53mm ID)

- Oven Temperature -iso-	180°C
- Injector Temperature	200°C
- ECD Detector Temperature	280°C
- Carrier Gas N_2/He	10ml/min
- Make-up Gas N_2	50ml/min
- Run-End Time	15ml/min

7. CALCULATIONS

If an integrator - data handling system, is not used, calculate the ratio of the standard peak height (putrescine or cadaverine) to the peak height of the hexane diamine. From the standard ratios, plot a calibration curve (this should be linear from 1μg/g to 20μg/g). The concentration of putrescine and cadaverine can then be calculated.

$$Concpu = \frac{Concps \times Hiss \times Hpu}{Hps \quad Hisu}$$

where : pu = putrescine in unknown
ps = putrescine in standard
iss = internal standard in standard
isu = internal standard in unknown

8. REFERENCE

8.1 STARUSZKIEWITCZ W.F. AND BOND, J.F., Gas Chromatography Determination of Cadaverine, Putrescine, and Histamine in Foods. JAOAC (V.64 No. 3 1981).

8.2 FARN, G AND SIMS, G., Chemical Indices of Decomposition in Tuna, Proceedings of an International Symposium on Seafood Quality Determination. Elsevier Science Publishers, B.V. Amsterdam 175-183 (1986).

Putrescine and Cadaverine - Fish Quality Indices

Sample Extraction

Weigh 10.0g of sample into a 150ml beaker.

Add 60ml MeOH and homogenize with a polytron homogenizer.

Transfer homogenate (rinsing with MeOH) to 100ml volumetric flask, make up to volume.

Mix volumetric flask contents thoroughly, allow sample residues to settle, producing a clear extract.

Pipet 10ml of the clear extract into a 125ml round bottom flask.

Add 1.00ml of 5ppm hexane diamine working solution and 0.50ml 1N HCl to the 10ml of extract.

Evaporate to complete dryness on a vacuum rotary evaporator at 50°C.

Derivatization Process and Sample Clean-Up

To the dried sample add 1ml ethyl acetate & 250μl pentafluoropionic acid anhydride (PFPAA).

Stopper flask loosely, swirl contents to mix thoroughly, and place in a 50°C water bath for 30 minutes, swirling occasionally. (If the reaction has not cleared within 15 min add an additional 250μl PFPAA, if reaction mixture remains cloudy then no reaction took place).

After reaction, evaporate at 50°C the solvent/reaction mixture to a glass with nitrogen.

Dissolve the resulting glass in 2ml of 30:70 - ethyl acetate:toluene.

Prepare alumina column (80x9mm bed volume) with 3% deactivated alumina, with 1cm anhydrous sodium sulphate.

Condition column with 10ml hexane, collect effluent, add total derivatized extract to column bed.

Rinse flask with further 3ml 30:70 and add to column when initial extract absorbed into bed surface.

Repeat with 18ml 30:70, and add to column.

Collect entire column effluent, mix by swirling.

Chromatographic Conditions

Packed Column OV-225 (6ft x 0.25"OD x 4mm ID)	
- Oven Temperature - iso-	180°C
- Injector Temperature	200°C
- ECD Detector Temperature	280°C
- Carrier Gas N_2/He	60 ml/min
- Make-up Gas N_2	----
- Run-End Time	20 minutes

Megabore Capillary Column DB-225 (30m x 0.53mm ID)	
- Oven Temperature -iso-	180°C
- Injector temperature	200°C
- ECD Detector Temperature	280°C
- Carrier Gas N_2/He	10 ml/min
- Make-up Gas N_2	50 ml/min
- Run-End Time	15 minutes

PUTRESCINE AND CADAVERINE IN CANNED TUNA
USFDA

Modifications have been made to the GLC method for the determination of putrescine and cadaverine developed by Staruskiewicz and Bond to reduce the number of steps and shorten the time it takes to analyse a sample. The original method involved four steps - extraction of the sample, making a fluorinated derivative, putrifiction of the reaction by column chromatography, and detection of the diamines by GLC. Modifications have been made in the extraction procedure (using 75% methanol in water instead of 100% methanol) and by replacing the column chromatography with solid phase extraction (SPE).

Materials and Methods

Apparatus

1. Gas Chromatograph (electron capture detector) : Varian Model 3700 or equivalent, with model 20-21 Ni 63 pulsed capture detector. Representative operating conditions: temperature (°C) : injection port 210, detector 320, column 165; gas flow (ml/min): Nitrogen 25; electrometer range 10 - 10amp full scale. Detector makeup purge gas as needed (e.g. 40ml/min).

2. GLC column (for putrescine and cadaverine derivatives): Glass column 1.8m (6ft) x 2 mm id, packed with 3% OV -225 on 100 - 120 mesh Gas Chrom Q. Condition with gas flow at room temperature for 2 hrs., increase temperature gradually (ca 6°C/min) to 240°C, and hold for 16 hrs.

   Retention times of PFP derivatives were 6, 10, 12 min, respectively, for putrescine, cadaverine, and hexane diamine.

Reagent

1. Pentafluoropropionic (PFP) anhydride. : Refrigerate and protect from moisture.

2. Methanol: Distilled in glass.
   75% methanol : To 750ml MeOH in a 1 litre flask, add distilled water with swirling to volume.

3. SPE Tubes : SUPELCLEAN LC - Alumina-N SPE tubes, 3ml size Cat # 5-7086, Supelco Inc., Bellefonte, PA 16823-0048 USA.

4. Hexane diamine standard solution:

   Stock Solution : Dissolve 163mg hexane diamine dihydrochloride in 0.1N HCl and dilute to 100ml in vol. flask with 0.1N HCl.

   Intermediate Solution : 20μg/ml. Dilute 2ml stock solution to 100ml in vol. flask with 0.1N HCl.

Working Solution : 5μg/m. Dilute 25 ml Intermediate solution to 100ml in vol. flask with 0.1N HC1.

5. Standard solution of diamines. :
Stock Solution: Dissolve 91.4mg of putrescine dihydrochloride and 171.3mg of cadaverine dihydrochlorine in 0.1N NCl and dilute to 100ml in vol. flask with 0.1N HC1.

Intermediate Standard Solution : (10μg/ml cadaverine and 5μg/ml putrescine as free base): Dilute 1 ml stock solution to 100ml in vol. flask with 0.1N HC1.

Working Standard Solution : (1μg/ml cadaverine and 0.5μg/ml putrescine as free base) Dilute 10ml Intermediate solution to 100ml in vol. flask with 0.1N HC1.

Calibration

Pipet 1ml of internal standard solution plus indicated volume (Table 1) of diamine standard solution into 100ml round-bottom 24/40 flask. Add ca. 0.5ml 1N HC1 and evaporate to dryness on rotary evaporator at ca. 50°C (or steam bath under nitrogen). To residue add 1ml ethyl acetate and 300μl PFP anhydride, stopper, mix and heat at 50°C for 30 min. Swirl solution at least once during reaction.

Add 2ml to toluene to the reaction mixture. Add 2ml of hexane to each SPE tube (3ml from Supelco) and let flow through by gravity. Discard the hexane. Add 150μl of the diluted reaction mixture to the top of each tube. Start collecting effluent when the sample is added. Add 3 or 4 drops of 30% ethyl acetate in toluene to the tube. After the sample passes into the frit, add 2ml of 30% ethyl acetate in toluene. Add an additional 6ml for a total of 8ml of 30% ethyl acetate in toluene and collect all the effluent. Inject 102μl into the GLC system. Calculate ration r_c = peak height PFP cadaverine/peak height PFP hexane diamine vs. cadaverine derivated (w). Repeat calculation for r_p values for putrescine.

Determination

Fishery products : Extract product with 75% methanol as follows:

Transfer 10g prepared sample to blender bowl and add approx 60ml 75% methanol. Blend for ca 2 min at high speed. Transfer to 100ml vol. flask, rinsing lid and blender jar with 75% methanol and adding rinsing to flask. Heat in water bath to 60°C and let stand at this temperature for 15 min. Cool to room temperature and dilute to volume with 75% methanol. Filter thru folded filter paper.

Pipet 10ml extract into round bottom flask, add 1ml internal standard solution and ca 0.5ml 1N HCl, and evaporate to dryness on rotary evaporator at ca 50°C. Continue as in Calibration, sentence 3, beginning "to residue....".

If values obtained for putrescine and cadaverine are above the most concentrated calibration standards then a smaller volume will be injected in order to quantify the sample content. However, no injection will be acceptable if the internal standard is less than 5% of full scale. If the sample is so concentrated that this approach is unacceptable then the sample may be qualified through adjustment of the instrument attenuation.

Table 1. Diamine Calibration Standard

Calibration Solution	Std Solution	ml used	Equivalent μg cadeverine/g sample	Equivalent μg putrescine/g sample
I	B	0.5	0.5	0.25
II	B	1	1	0.5
III	B	5	5	2.5
IV	A	1	10	5
	If required for Higher Levels			
V	A	2	20	10
VI	A	3	30	15
VII	A	5	50	30
VII	A	10	100	30

A = Intermediate solution
B = Working solution

Notes

1. Adjust GC to give full scale recorder response for injection of 2μl of calibration solution IV.

2. Full calibration of GC using duplicate injections of each of the calibration solution is required only at the beginning of study unless the instrument is shut down during analyses. On succeeding days when samples are analysed, rechromatograph Calibration Solution II to insure that calibration of instrument is stable.

3. A calibration solution beyond IV is required if amine levels the calibration range. For example, if 85μg cadaverine/g is found in a sample, run a calibration solution at a level of ca 100μg/g to extend the calibration line, employing the same analytical technique used for the sample extract analysis, e.g., attenuation factors or a reduction in the volume injected into the GC.

   In each case in which the sample values are higher than the most concentrated calibration standard a new calibration standard higher than the determined values will be prepared and analysed in similar fashion.

   Alternatively, carry a smaller aliquot of the 75% methanol extract (eg. 1 ml) through the procedure and multiply by the appropriate factor to calculate μg/g values.

Calculation

For each sample calculate r_c and r_p from the chromatogram. Determine W_c and W_p from the calibration graph.

μg diamine/g sample = (W) (F)

Where F = attenuation (or dilution) factor, if required.
All calculations will be carried out to 0.1μg/g.

Results and Discussion

Elution profiles of cadavarine and putrescine from 3ml SPE tubes were determined using standard solutions as shown in figure 1 and 2. A putrescine recovery of 98% was obtained with 8ml of eluent using concentrations of 1μg to 30μg. A cadaverine recovery of 100% was obtained with 8 ml of eluent using concentrations of 2μg to 60μg. Satisfactory results were obtained with the SPE columns with varying samples sizes up to 200μl and varying concentrations of eluent from 25% to 35%.

Table 2 shows a comparison of several brands of SPE columns and the effect on the recovery of the diamines from the column. The larger column containing 1.7g alumina (Waters) and 1.6g alumina (Supelco) has a larger sample capacity but also required a larger elution volume. A quantitative recovery was obtained on all the columns containing 1g of alumina by adjusting the concentration of the eluent. The column containing 0.5g of alumina did not have a large enough capacity to separate the diamines from the excess reagent.

The Fisher PrepSep was eliminated because the mixture from PFP reaction would have to be diluted with 5 times the volume of toluene for an effective clean-up which would cause problems in detection int he GLC system. The Baker column was eliminated because of the extra peak that was present in the final chromatogram. The Supelco column was selected due to the smallest elution volume without a large increase in the dilution factor.

The use of SPE columns reduced the sample clean-up time prior to GLC analysis from 1 hr. to 10 mins. The modified method requires less space, is more convenient, eliminates the washing of glass columns, and reduces the amount of solvent used by 2/3.

Table 2 *Comparison of SPE Column*

Brand names	Sample size (μl)	wt. of alumina (g)	conc. of eluent	% recovery
Waters SepPak	250	1.7	12ml 100% EtOAC	100
Fisher PrepSep	100	1.0	4ml 20% EtOAC	100
Benton & Jackson	100	0.5	2ml 15% EtOAC	no separation
Supelco 6ml tube	200	1.6	16ml 30% EtOAC	100
Baker 6ml tube	100	1.0	8ml 30% EtOAC	100 w/extra peak
Supelco 3ml tube	100	1.0	8ml 30% EtOAC	100

DETERMINATION OF PUTRESCINE AND CADAVERINE

Sample Extraction

Weigh 10g of sample into a 150ml beaker.

Add 60ml of 75% MeOH and homogenate with a polytron homogenizer.

Transfer homogenate (rinsing with 75% MeOH) to a 100ml vol. flask, heat in water bath at 60°C for 15 min.

Cool and dilute to column with 75% MeOH.
Filter thru Whatman No. 1.

Pipet 10ml of the clear extract into a 125ml RBF.

Add 1ml of 5 ppm hexane diamine working solution and 0.5ml 1N HCl to the 10ml of extract.

Evaporate to complete dryness on a vacuum rotary evaporator at 50°C.

Derivatization Process and Sample Clean-Up

To the dried sample add 1ml ethyl acetate & 300μl PFPAA.

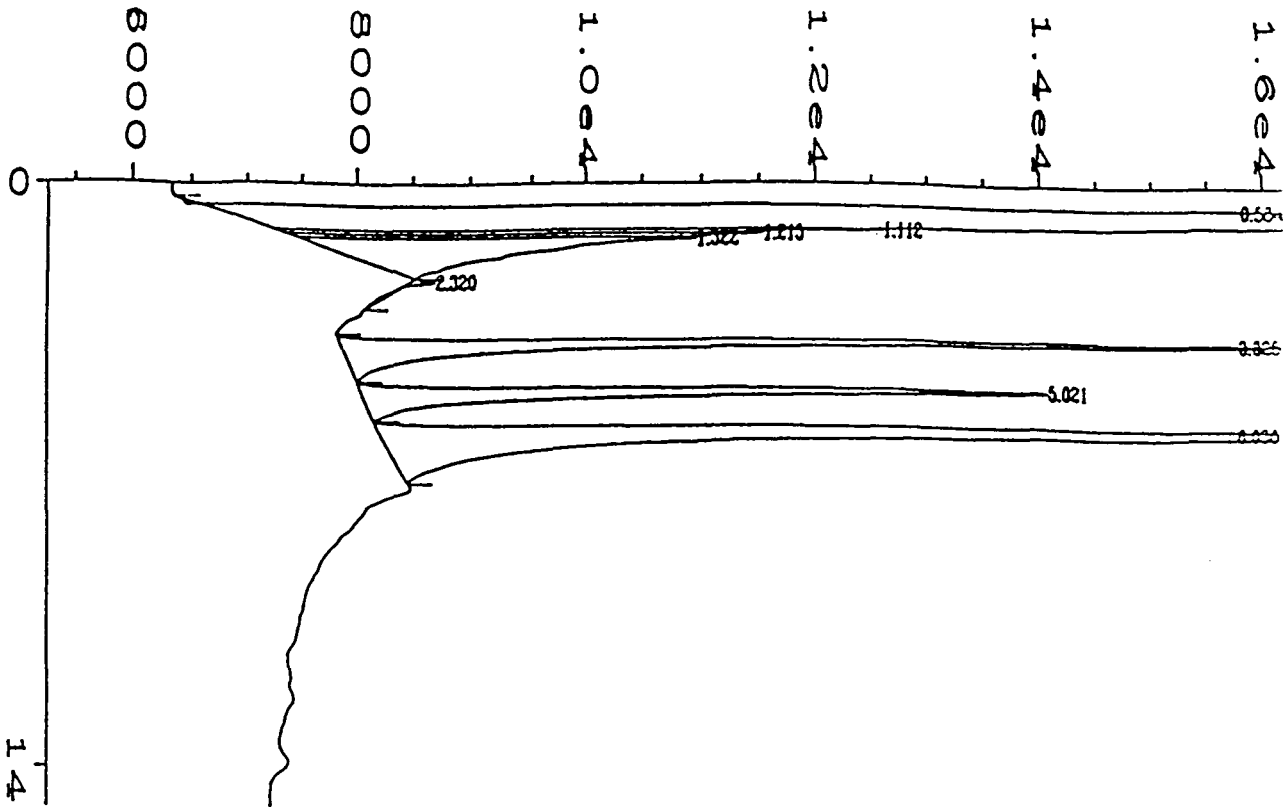
Stopper flask loosely, swirl contents to mix thoroughly, and place in a 50°C water bath for 30 min, swirling occasionally.

Add 2ml of toluene to the reaction mixture.

Add 2ml of hexane to SPE tube and let flow through by gravity. Discard the hexane.

Add 150μl of the diluted reaction mixture to SPE tube, following with 3 or 4 drops of 30% ethyl acetate in toluene. Start collecting effluent when the sample is added.

Add 2ml of 30% EtOAC, then additional 6ml for a total of 8ml and collect all the effluent.



Internal Standard Report

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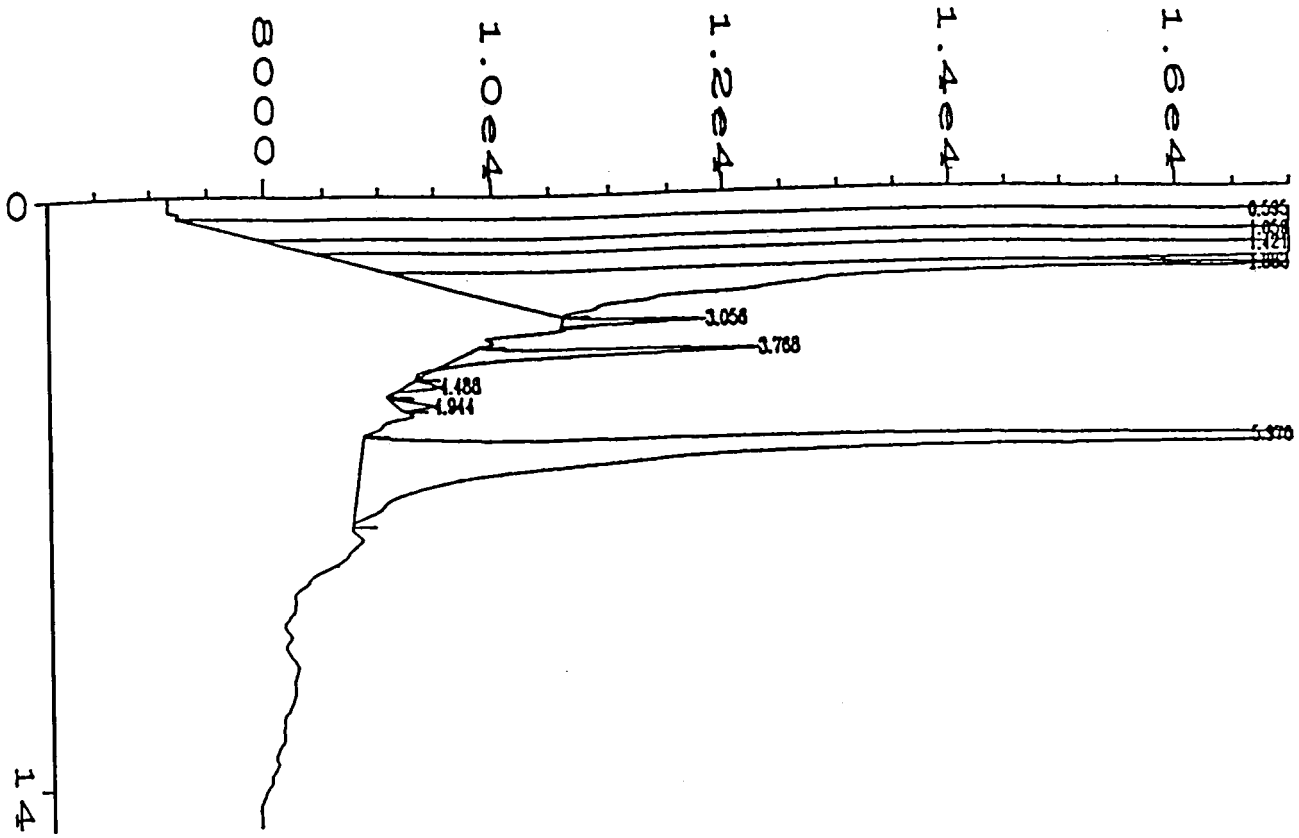
Instrument	:	INSTRUMEN	Vial Number	:	1
Sample Name	:	pcstd	Injection Number	:	
Run Time Bar Code	:		Sequence Line	:	
Acquired on	:	15 Jul 95 10:15 AM	Instrument Method	:	ECD.MTH
Report Created on	:	15 Jul 95 10:31 AM	Analysis Method	:	ECD/MTH
Last Recalib on	:	15 Jul 95 10:05 AM	Sample amount	:	1
Multiplier	:	1	ISTD Amount	:	1

Sig. 1 in C:\HPCHEM\1\DATA\PC\001F0139.D

Ret Time	Area	Type	Width	Ref #	Amount %	Name
3.825	112482	BB	0.177	1	223.626	putrescine
5.021	86359	BB	0.214	1	221.776	cadaverine
6.036	230896	BB	0.287	1-IR	100.000	hexane diamine

Time Reference Peak	Expected RT	Actual RT	Difference
3	6.085	6.036	-0.8%



Internal Standard Report

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Sample Name	:	PFI 15	Injection Number	:	
Run Time Bar Code	:		Sequence Line	:	
Acquired on	:	15 Jul 95 12:49 PM	Instrument Method	:	ECD.MTH
Report Created on	:	15 Jul 95 01:06 PM	Analysis Method	:	ECD/MTH
Last Recalib on	:	15 Jul 95 10:05 AM	Sample amount	:	1
Multiplier	:	1	ISTD Amount	:	1

Sig. 1 in C:\HPCHEM\1\DATA\PC\009F0145.D

Ret Time	Area	Type	Width	Ref #	Amount %	Name
3.768	23855	BB	0.143	1	41.731	putrescine
4.944	3063	BB	0.141	1	6.922	cadaverine
5.976	262408	BB	0.331	1-IR	100.000	hexane diamine

Time Reference Peak	Expected RT	Actual RT	Difference
3	6.085	5.976	-1.8%

Determination of Putrescine and Cadavarine
Modified from USFDA and DFO (Canada)

Sample Extraction

Weigh 10g of sample into a 150ml beaker.

Add 60ml of 75% MeOH and homogenize with a polytron homogenizer.

Transfer homogenate (rinsing with 75% MeOH) to a 100ml vol. flask, make up to volume.

Mix volumetric flask contents thoroughly, allow sample residues to settle, producing a clear extract.

Pipet 10ml of the clear extract into a 125ml round bottom flask.

Add 1ml of 5ppm hexane diamine working solution and 0.5ml 1N HCl to the 10ml of extract.

Evaporate to complete dryness on a vacuum rotary evaporator at 50°C.

Derivatization Process and Sample Clean-Up

To the dried sample add 1ml ethyl acetate and 300μl PFPAA.

Stopper flask loosely, swirl contents to mix thoroughly, and place in a 50ml water bath for 30 min, swirling occasionally.

Add 2ml of toluene to the reaction mixture.

Add 2ml of hexane to SPE tube and let flow through by gravity, discard the hexane.

Add 150μl of the diluted reaction mixture to SPE tube, following with 3 or 4 drops of 30% ethyl acetate in toluene. Start collecting effluent when the sample is added.

Add 2ml of 30% EtOAC, then additional 6ml for a total of 8ml and collect all the effluent.

Inject 1μl to GC.