DETERMINATION OF PHYTOSTEROLS IN WASTEWATER TREATMENT PLANT INFLUENTS AND BIOLOGICALLY TREATED EFFLUENTS FROM PULP AND PAPER MILLS BY GAS CHROMATOGRAPHY/FLAME IONIZATION DETECTION

NCASI
West Coast Regional Center
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This method is included in the NCASI methods manual as a proposed method. The purpose of including proposed methods in the NCASI methods manual is to make it known that a method is under development and to solicit comment regarding the technical merit and applicability of the method. This method should not be construed as having been rigorously validated on any matrix and therefore, those electing to apply the method are strongly encouraged to conduct rigorous QA/QC or validation such that the quality of the data generated can be evaluated. Please note that proposed methods are not suitable for use as regulatory monitoring or compliance methods.

DETERMINATION OF PHYTOSTEROLS IN WASTEWATER TREATMENT PLANT INFLUENTS AND BIOLOGICALLY TREATED EFFLUENTS FROM PULP AND PAPER MILLS BY GAS CHROMATOGRAPHY/FLAME IONIZATION DETECTION

1.0 Scope and Application

1.1 This method is designed to determine phytosterols, specifically campesterol, β-sitosterol, stigmastanol, and stigmasterol (Section 17, Table 1) in wastewater treatment plant influents and biologically treated effluents. This method involves liquid/liquid extraction of the analytes followed by trimethylsilyl derivatization of the analytes, clean-up by silica chromatography (as required), and quantification by gas chromatography/flame ionization detector (GC/FID). Procedures for confirmational analyses using gas chromatography/mass spectrometric detector (GC/MS) are described.

1.2 This method has been validated at the single laboratory level in wastewater treatment plant influents from kraft, kraft/recycle, kraft/groundwood, and sulfite pulp and paper mills, and in biologically treated effluents from kraft, thermomechanical, thermomechanical/groundwood, recycle, and sulfite pulp and paper mills, and is a proposed method. Demonstration of extraction efficiency and method performance for specific matrix types is recommended.

1.3 The estimated method detection limits were determined as specified at 40 CFR 136 Appendix B (Federal Register 1984), using a biologically treated final effluent sample from a kraft mill producing unbleached softwood pulp. The calculated method detection limits are listed in Section 17, Table 2. The lower instrument calibration limit for the target analytes is approximately 1.5 μg/L.

1.4 The GC/FID portions of this method are for use only by analysts experienced with capillary GC/FID or under the close supervision of such qualified persons. The GC/MS portions of this method are for use only by analysts experienced with capillary GC/MS or under the close supervision of such qualified persons.

2.0 Summary of Method

2.1 Biologically treated effluents

Place a 100-mL aliquot of pH 2 preserved effluent into a beaker and fortify with cholesterol as the surrogate. Add potassium carbonate solution to bring the solution to pH 7, and add a pH 7 buffer to maintain this pH during extraction. Extract the solution with methyl-t-butyl ether, concentrate, and exchange into hexane. Convert the phytosterols to their trimethylsilyl derivatives by the addition of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA). Utilize silica gel chromatography to clean up sample extracts that have elevated chromatographic baselines. Add dotriacontane as an internal standard and analyze the extract by GC/FID. Use GC/MS for confirmation of the target

analyte when previous characterization of the sample will not ensure proper identification.

2.2 Wastewater treatment plant influents

Place a 50-mL aliquot of pH 2 preserved influent into a beaker, fortify with cholesterol as a surrogate, and add 50 mL of reagent grade water to adjust the final volume to 100 mL. Add a sufficient amount of potassium carbonate solution to bring the solution to pH 7, and add a pH 7 buffer to maintain this pH during extraction. Extract the solution with methyl-t-butyl ether, concentrate, and exchange into hexane. Convert the phytosterols to their trimethylsilyl derivatives by the addition of BSTFA. Utilize silica gel chromatography to clean up sample extracts that have elevated chromatographic baselines. Add dotriacontane as an internal standard and analyze the extract by GC/FID. Use GC/MS for confirmation of the target analyte when previous characterization of the sample will not ensure proper identification.

2.3 Quantitative analysis

Perform quantitative analysis by GC/FID, employing an internal standard technique. Perform identification of target analytes (qualitative analysis) by comparing the relative retention time of the analytes detected to that of an authentic standard. A target compound is identified when its relative retention time meets the criteria described in Section 12 and the absolute retention time of the internal standard meets the criteria determined in Section 10.4. Additional confirmation using GC/MS is recommended to ensure proper analyte identification unless previous characterization of the sample will ensure proper identification.

2.4 Quality assurance

Assure quality through reproducible calibration and testing of the extraction and GC/FID system. Analyze a method blank with each sample set (samples started through the extraction process on a given day, to a maximum of 20), along with a sample duplicate and a matrix spike to ensure quality data. Fortify each sample with a surrogate and calculate the surrogate recovery to assist in assessing data quality. A complete description of quality control procedures, calculations, and method performance criteria are listed in Section 9.

3.0 Definitions

3.1 These definitions are specific to this method, but conform to common usage as much as possible.

3.1.1 µg/L–micrograms per liter

3.1.2 Silylation–derivatization of a polar hydrogen group with a trimethylsilyl ($Si(CH_3)_3$) group

3.1.3 May–this action, activity, or procedural step is neither required nor prohibited

3.1.4 May not–this action, activity, or procedural step is prohibited

3.1.5 Must–this action, activity, or procedural step is required

3.1.6 Should–this action, activity, or procedural step is suggested, but not required

3.1.7 GC/FID–gas chromatograph with a flame ionization detector

3.1.8 GC/MS–gas chromatograph with a mass spectrometric detector

4.0 Interferences

4.1 Solvents, reagents, glassware, and other sample processing hardware may contribute analytical interferences resulting in misinterpretation of chromatograms. Run method blanks initially and with each subsequent sample set to demonstrate that the solvents, reagents, glassware, and other sample processing hardware are free from interferences under the conditions of the method. Specific selection of reagents and purification of solvents by distillation in all-glass systems may be required.

4.2 The flame ionization detector (FID) is a non-selective detector. There is a potential for non-target compounds present in the samples to interfere with the analyses. Therefore, GC/MS confirmation is recommended to ensure proper analyte identification, unless previous characterization of the sample will ensure proper identification.

4.3 Interferences co-extracted from samples will vary considerably from source to source, depending on the diversity of the site being sampled.

4.4 The surrogate compound, cholesterol, has been detected in some effluent samples that have contributions from sewage treatment facilities. It has also been reported as a wood extractive in pine bark (Rowe 1965). Therefore, samples from new sources should be analyzed without the addition of the surrogate to determine if cholesterol is present. In the event that cholesterol is native to the sample, a sample-specific matrix spike experiment should be performed instead of surrogate recovery using cholesterol to assess the accuracy of the method for that sample.

4.5 The silylating agent, BSTFA, must remain in a water-free environment in order to effectively derivatize the analytes.

4.6 Contamination by carryover can occur when samples containing high concentrations of the target analytes are analyzed in sequence with low concentration samples. Whenever unusually concentrated samples are encountered, they should be followed by injection of a solvent blank to check for cross contamination prior to the analysis of additional samples.

5.0 Safety

5.1 The toxicity or carcinogenicity of each compound or reagent used in this method has not been precisely determined; however, each chemical compound should be treated as a potential health hazard. Exposure to these compounds should be reduced to a level protective of human health. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of data handling sheets should also be made available to all personnel involved in these analyses.

5.2 Methyl-t-butyl ether is a flammable liquid which may be harmful if inhaled or absorbed through the skin. Use it in a laboratory fume hood or wear an approved respirator, and avoid contact by wearing chemical-resistant gloves, eye protection, and other protective clothing.

5.3 As with all samples, precautions should be taken to avoid exposure to potentially toxic, caustic, or nuisance odor compounds, and samples should be handled with gloves and opened in a fume hood.

6.0 Equipment and Supplies

6.1 Brand names and suppliers are cited for illustrative purposes only. No endorsement is implied.

6.2 Do not use glassware with any star fractures, cracks, or severe scratches. All fittings should be snug, and clamps and springs should be in good working order. All glassware should be washed with detergent, rinsed with tap water, then rinsed with reagent-grade water. If blank contamination is observed, the glassware may be solvent rinsed and baked prior to use.

6.3 Sampling equipment

6.3.1 It is recommended that glass containers and Teflon™ tubing be utilized during sample collection. Use amber glass bottles equipped with Teflon™-lined screw caps to store all samples.

6.3.2 Automatic sampling equipment which comes in contact with a sample should be constructed of glass, Teflon™, or stainless steel.

6.4 Equipment for sample extraction (per sample)

6.4.1 One 250-mL (or larger) beaker, equipped with a Teflon™-coated stir bar

6.4.2 One 250-mL separatory funnel with ground glass stopper and Teflon™ stop-cock

6.4.3 One 50-mL centrifuge tube with cap

6.4.4 One 100-mL graduated cylinder

6.4.5 One 25-mL graduated cylinder

6.4.6 One magnetic stir plate

6.5 Equipment for sample concentration and silica gel clean-up (per sample)

6.5.1 15-mL graduated concentrator tube (part number 8080 Pyrex™ or equivalent); a ground-glass stopper may be used to prevent evaporation of extracts

6.5.2 250-mL evaporation flask

6.5.3 ½-inch springs

6.5.4 Three-ball macro Snyder column

6.5.5 Micro Snyder column

6.5.6 One glass column, 20 cm x 8.0 mm o.d. x 6.0 mm i.d. with a tapered end

6.5.7 15-mL culture tube with Teflon™-lined screw cap

6.5.8 One 2-mL glass autosampler vial with Teflon™-lined crimp top

6.5.9 Teflon™ boiling chips

6.5.10 Analytical filter pulp (No. 289 Schleicher and Schell or equivalent)

6.6 Other apparatus

6.6.1 Hot water bath in a hood, capable of ±5°C temperature control, preheated to a minimum of 90°C

6.6.2 A pH meter calibrated using a two-point calibration procedure at pH 2 and pH 7 using pH 2 and pH 7 buffer solutions

6.6.3 Magnetic stirrer

6.6.4 Nitrogen evaporation apparatus

6.6.5 Balances–an analytical balance capable of weighing to the nearest 0.1 mg with an accuracy of ±0.1 mg, and a top-loading balance capable of weighing to the nearest 10 mg with an accuracy of ±10 mg

6.6.6 Gas Chromatograph–must be equipped with a flame ionization detector and a splitless injection port for capillary column, and have the capacity of running the

temperature program and performance specifications outlined in Sections 9.2 and 10.1

6.6.7 Gas Chromatographic Column–30 ±5 m x 0.25 ±0.02 mm ID x 0.25 µm, 5% phenyl, 94% methyl, 1% vinyl silicone bonded phase fused silica capillary column (DB-5 or equivalent)

6.6.8 Mass Spectrometer (alternate confirmation method)–70 eV electron impact ionization; must repetitively scan from 42 to 420 AMU in 0.95 to 1.00 second, and must produce a unit resolution (valley between m/z 441-442 less than 10% of the height of the 441 peak), background corrected mass spectrum from 50 ng decafluorotriphenylphosphine (DFTPP) introduced through the GC inlet; spectrum must meet the mass intensity criteria listed in Section 9.3 and Section 17, Table 3; mass spectrometer must be interfaced to the GC via a directly coupled column with a heated transfer line per the manufacturer's specifications; all portions of the column which connect the GC to the ion source must remain at or above the oven temperature during analysis to preclude condensation of less volatile compounds; data system should collect and record the MS data, store the ion intensity data, process GC/MS data, generate reports, and compute and store response factors

6.6.9 The gas chromatograph data system should collect and record the GC data, process and store GC/FID data, generate reports, and compute and record response factors.

7.0 Reagents and Standards

7.1 Solvents

7.1.1 Hexane, methyl-t-butyl ether (MTBE), and acetone supplied by Burdick & Jackson, or equivalent high purity solvent suitable for gas chromatography and pesticide residue analysis

7.1.2 Organic-free reagent water in which the compounds of interest and interfering compounds are not detected by this method; all organic-free water and buffer solutions should be stored in glass to prevent the leaching of contaminants from plastic containers; containers must have tightly-fitting Teflon™-lined caps

7.2 Standards

7.2.1 β-Sitosterol, campesterol, cholesterol, and stigmastanol can be purchased from Sigma or an equivalent supplier. Use standards of the highest purity available. If standards have a chemical purity of <98%, correct all calculations, calibrations, and matrix spikes for the difference in purity.

7.2.2 Stigmasterol can be purchased from Fluka or an equivalent supplier. If standards have a chemical purity of <98%, correct all calculations, calibrations, and matrix spikes for the difference in purity.

7.2.3 Prepare primary standards of β-sitosterol and campesterol in methanol at a concentration of 2 mg/mL ±0.1 mg. Place the solutions into amber glass vials with Teflon™-sealed caps. Store the tightly-sealed standard stock solutions at 4°C.

7.2.4 Prepare primary standards of the stigmasterol and stigmastanol in acetone at a concentration of 2 mg/mL ±0.1 mg. Place the solutions into amber glass vials with Teflon™-sealed caps. Store the tightly-sealed standard stock solutions at 4°C.

7.2.5 Prepare a working stock of β-sitosterol, campesterol, stigmasterol, and stigmastanol by diluting 1 mL of each primary stock (Section 7.2.3 and 7.2.4) into a 50-mL volumetric flask with acetone, yielding a final concentration of approximately 40 µg/mL.

7.2.6 Prepare the primary standard of cholesterol (surrogate) in methanol at a concentration of 2 mg/mL ±0.1 mg. Place the solution into an amber glass vial with a Teflon™-sealed cap. Store the tightly-sealed standard stock solution at 4°C.

7.2.7 Prepare the working stock of cholesterol by diluting 1 mL of the primary stock (Section 7.2.6) into a 50-mL volumetric flask with acetone, yielding a final concentration of approximately 40 µg/mL.

7.2.8 Dotriacontane, 97% pure, is available from Aldrich or an equivalent supplier. Prepare a working stock solution of 1 mg/mL in hexane.

7.2.9 Prepare a six-point calibration curve encompassing the sample concentration range of approximately 1.5 to 380 µg/L for a 100-mL sample in the following manner. Place ~100 µL of hexane into a 2-mL autosampler vial. Spike 5 µL of the analyte working stock solution (Section 7.2.5) and 5 µL of the surrogate working stock solution (Section 7.2.7) into the hexane. Adjust the final volume to dryness using nitrogen blowdown. Add 250 µL of hexane, 250 µL of acetone, and 100 µL of BSTFA. Allow the reaction to proceed at room temperature for a minimum of one hour. Add 10 µL of 1.0 mg/mL dotriacontane and proceed with GC/FID or GC/MS sample analysis as described for the samples in Section 11.8 or 11.9. Repeat this procedure using 10, 50, 100, 400, and 800 µL of the analyte working stock solution and the surrogate working stock solution to result in a six-point calibration curve.

7.2.10 Decafluorotriphenylphosphine (DFTPP) for GC/MS confirmation analyses can be purchased from Supelco or an equivalent supplier as a 25,000 µg/mL solution in dichloromethane. Prepare a working stock solution in hexane at a concentration

of 50 µg/mL. Store in the dark in autosampler vials with Teflon™-seal crimp caps prior to use. This standard is required if GC/MS is utilized to confirm compound identification.

7.2.11 Stock solutions of all standards should be checked for signs of concentration or formation of precipitates prior to the preparation of calibration or performance test standards. Replace the stock solutions if a change in concentration is indicated by the inability to meet the criteria specified in Sections 9.2 and 10.5.

7.3 Reagents for sample preservation and pH adjustment

7.3.1 Sulfuric acid, reagent grade, 6N in organic-free reagent grade water for sample preservation

7.3.2 Potassium carbonate, ACS reagent grade, for use in adjusting sample pH during extraction; prepare a 4.3 M solution in reagent grade water by dissolving 602 grams in one liter of reagent grade water

7.3.3 Buffer capsules, certified at pH 7.00 ±0.02 at 25°C, can be purchased as Metrepak pHydrion buffers from Fisher or a comparable supplier.

7.4 Reagents for silica gel column clean-up of extracts

7.4.1 Sudan I (1-phenylazo-2-naphthol), dye content ~97%, can be purchased from Aldrich or another supplier.

7.4.2 Azulene, 99% pure, can be purchased from Aldrich or a comparable supplier.

7.4.3 Prepare the indicator solution used during silica gel clean-up procedures by adding 10 mg of Sudan I and 150 mg of azulene to 20 mL of hexane.

7.4.4 Silica gel, grade 62, 60 to 200 mesh can be purchased from Aldrich or a comparable supplier; activate at 130 to 135°C for a minimum of 16 hours.

7.5 Reagent for extract drying

Sodium sulfate, 10 to 60 mesh, granular, can be purchased from Aldrich or a comparable supplier; dry overnight at 130 to 135°C prior to use.

7.6 Reagents for derivatization

The N,O-bis(trimethylsilyl)trifluoroacetamine (BSTFA), 99+% pure, can be purchased from Supelco or another supplier.

8.0 Sample Collection, Preservation, and Storage

8.1 Sample collection

Collect grab samples in glass containers with Teflon™-lined screw caps. Composite samples may be collected using automatic sampling equipment. The parts of the automatic sampling equipment that come in contact with the sample should be constructed of glass, Teflon™, or stainless steel. Composite samples should be refrigerated during the sampling period.

8.2 Sample preservation

Preserve all samples in the field by acidification to pH 2 to pH 3 using sulfuric acid, then refrigerate. This should be done as soon as possible after sample collection. Ship samples in iced containers as quickly as possible.

8.3 Sample and extract storage

Samples may be stored for up to 30 days in the refrigerator (4°C). Maintain extracts at 4°C prior to analysis. Analyze the extracts within 30 days of extraction.

9.0 Quality Control

9.1 Each laboratory that uses this method should operate a formal quality assurance program. The minimum requirements of this program consist of an initial demonstration of laboratory capability, and ongoing analyses of standards and blanks as a test of continued performance. Laboratory performance is compared to established performance criteria to determine if the results of analyses meet the performance characteristics of the method.

9.2 GC/FID performance and calibration verification

9.2.1 Conduct a calibration check to determine that the GC/FID system is operating within acceptable parameters before each set of samples (samples started through the extraction process on a given day, to a maximum of 20) is analyzed. The calibration check involves reanalyzing one of the extracts used in the calibration curve (Section 7.2.9 and 10.3). Evaluate the calibration check by calculating the relative response factor for each analyte based on the concentration of internal standard and its response. If the relative response factor determined from the calibration verification analysis varies by less than ±15% from the initial relative response factor determined for that point in the curve, the initial calibration curve is considered valid. These analytes may be sensitive to GC/FID instrument conditions such as contamination of the injection port, detector, and/or column. If the calibration check fails to meet the ±15% acceptance criterion, appropriate GC/FID maintenance is necessary. Reanalyze the calibration verification upon completion of all necessary instrument maintenance. If all recommended

instrument maintenance fails to correct all calibration verification difficulties, the calibration curve should be reprepared and reanalyzed.

9.2.2 Verify the ability of the GC/FID system to resolve β-sitosterol and stigmastanol for each set of samples analyzed. The resolution of β-sitosterol and stigmastanol must be greater than 1.5 when calculated using the following equation.

$$Resolution = [2(T_a - T_b)/(W_{ba} + W_{bb})]$$

where:

T_a = *retention time of compound a*
T_b = *retention time of compound b*
W_{ba} = *peak width at the base for compound a*
W_{bb} = *peak width at the base for compound b*

A chromatogram of the 50 µg/L calibration standard showing acceptable resolution is presented in Figure A1.

9.2.3 The relative retention times of all target analytes and surrogates in the calibration verification standard analyzed at the beginning of each sample set must fall within the relative retention time windows in Section 12.1. The absolute retention time of the internal standard must meet the criteria determined in Section 10.4. If the retention time of any analyte in the standard does not fall within the ±3 x SD (standard deviation) window, a new initial calibration is necessary unless system maintenance corrects the problem.

9.3 GC/MS performance

9.3.1 Verify the GC/MS by performing a DFTPP tune prior to analyzing any samples, blanks, or standards. Analyze the tune check just prior to the calibration standard analyses, and confirm that it meets the specifications listed in Section 17, Table 3.

9.3.2 Determine that the GC/MS system is operating within acceptable parameters by conducting a calibration check before each set of samples (samples started through the extraction process on a given day, to a maximum of 20) is analyzed. The calibration check involves reanalyzing one of the extracts used in the calibration curve (Section 7.2.9 and 10.3). Evaluate the calibration check by calculating the relative response factor for each analyte based on the concentration of internal standard and its response. If the relative response factor determined for the calibration verification point analyzed varies by less than ±15% from the initial relative response factor determined for that point in the curve, the initial calibration curve is considered valid. These analytes may be sensitive to GC/MS instrument conditions such as contamination of the injection port, detector, and/or column. If the calibration check fails to meet the ±15% acceptance criterion, appropriate GC/MS maintenance is necessary. Reanalyze the calibration

verification upon completion of all necessary instrument maintenance. If all recommended instrument maintenance fails to correct all calibration verification difficulties, the calibration curve should be reprepared and reanalyzed.

9.4 Blanks

9.4.1 Demonstrate that the analytical system is free of contamination by preparing and analyzing a blank with each sample set. Prepare a method blank using the same procedure as a regular sample (Section 11.0).

9.4.2 If any of the compounds of interest (Section 17, Table 1) or any potentially interfering compounds are found in the blank at greater than 10% of the method detection limit or lowest calibration limit (assuming a response factor of one relative to the internal standard dotriacontane for compounds not listed in Section 17, Table 1), analysis of samples is halted until the source of contamination is eliminated and a blank shows no evidence of contamination at this level.

9.5 Surrogate recovery spikes

Spike all samples with the surrogate compound to monitor surrogate recovery. Compute the recovery of the surrogate compound as the ratio of concentration found to concentration spiked, using the following equation.

$$\textit{Percent recovery} = \frac{\textit{Concentration found} \times 100}{\textit{Concentration spiked}}$$

Performance criteria for acceptable surrogate recovery as determined during a single laboratory validation of this method is presented in Section 17, Table 4. The criteria were determined by calculating the average recovery ± two times the standard deviation of the recoveries for biologically treated effluent samples. If the recovery is greater or less than the acceptable criteria, action should be taken to resolve the problem and the samples should be reextracted and reanalyzed. Analyze samples from new sources without the addition of the surrogate to determine if cholesterol is present. In the event that cholesterol is native to the sample, a matrix spike of the sample should be done to assess accuracy of the method in the sample, instead of surrogate recovery using cholesterol.

9.6 Matrix spikes

9.6.1 Assess the accuracy of the method by analyzing a matrix spike with each set of samples. Wastewater treatment plant influents and biologically treated effluents contain variable levels of analytes; for samples with a high ratio of non-detects, a duplicate matrix spike may be appropriate. Demonstrate performance throughout the working concentration range of the method by varying the spike level of the target analyte working stock (Section 7.2.5) added to the sample prior to pH adjustment and extraction (Section 11.3). Adjust the amount of working stock added to the sample to give a final concentration in the sample that is a minimum

of twice the native level present. Prepare the matrix spike sample in exactly the same manner as a regular sample, using the pH adjustment and buffering, extraction, concentration, derivatization, and silica gel clean-up procedures outlined in Section 11.0.

9.6.2 Compare the recovery of the spiked compounds with the single laboratory matrix spike recovery data reported in Section 17, Table 5. If the levels determined are outside the control limits (the average recovery ± three times the standard deviation), repeat the extraction and analyses of the sample. If the results are outside the warning limits (the average recovery ± two times the standard deviation), the analyst should review the analytical data and procedure for possible degradation of standards or other analytical problems.

9.7 Sample and duplicate precision

Analyze a sample and duplicate with each set of samples to assess the precision of the analyses. For effluent and influent samples that may contain low levels of analytes or a high frequency of non-detects, a duplicate matrix spike may be used to assess precision. Calculate the relative percent difference in concentration for each sample and duplicate pair using the following equation.

$$\text{Relative Percent Difference} = \frac{(\text{Highest concentration} - \text{Lowest concentration}) \times 100}{\text{Average concentration of the sample and duplicate}}$$

A summary of the precision determined in a single laboratory is provided in Section 17, Table 6 for wastewater treatment plant influent and biologically treated effluent samples.

9.8 Field replicates and field spikes

Depending on specific program requirements, field replicates and field spikes of the analytes of interest into samples may be required to assess the precision and accuracy of the sampling and sample transporting techniques.

10.0 Calibration and Standardization

10.1 Assemble the GC/FID and establish the operating conditions outlined below. Optimize the GC conditions for analyte separation as specified by the criteria outlined in Section 9.2 by adjusting the linear velocity of the carrier gas. Once the operating conditions are optimized, use the same operating conditions to analyze all samples, blanks, calibration curves, calibration verification samples, and matrix spikes.

GC-FID Operating Conditions for NCASI Method STER-97

Injector Temperature:	290°C
Splitless Valve Time:	0.2 min

Carrier Gas:	Hydrogen @ 30 cm/sec & 23°C
Injection Volume:	1 µL
Temperature Program °C:	
Initial:	130 for 1 min
Ramp:	130 to 280 @ 15°C/min
Post Run:	280 for 15 min
Oven Equilibration:	0.50 min
Run Time:	26.0 min
FID Temperature:	320°C

10.2 For confirmation using GC/MS, assemble the GC/MS and establish the operating conditions outlined below. Optimize the GC conditions for analyte separation and verify that the system can meet the criteria specified in Section 9.3. Once the GC system is optimized, the same operating conditions must be used to analyze all samples, blanks, calibration curves, calibration verification samples, and matrix spikes.

GC-MS Operating Conditions for NCASI Method STER-97

Injector Temperature:	290°C
Splitless Valve Time:	0.8 min
Carrier Gas:	Helium @ 30-35 cm/sec & 130°C
Injection Volume:	1 µL
Temperature Program °C:	
Initial:	130 for 1 min
Ramp:	130 to 280 @ 15°C/min
Ramp 2:	280 to 320 @ 4°C/min
Post Run:	320 for 3 min
Oven Equilibration:	0.50 min
Run Time:	24.0 min
Interface Temperature:	290°C
MS Conditions:	
Scan Start Time:	8.00 min
Scan Range:	50 to 550 AMU
Scans/Sec:	1.5

10.3 Internal standard quantitation

10.3.1 Analyze the calibration standards (Section 7.2.9) using the procedure described in Section 11.8. Compute the relative response factors using the following equation.

$$RRF = [(A_S/A_{IS}) \times (C_{IS}/C_S)]$$

where:

A_S = *area of the target compound in the calibration standard*
A_{IS} = *area of the internal standard in the calibration standard*
C_{IS} = *concentration of the internal standard in the calibration standard*
C_S = *concentration of the target compound in the calibration standard*

10.3.2 If the average of the relative response factors (RRF) calculated across the calibration range is constant, i.e., within the control limit expressed in Section 17, Table 7, the calibration is acceptable and the average RRF can be used in all target analyte quantifications; otherwise, evaluate the problem, undertake the appropriate remedial action, and reanalyze the calibration curve extracts. If remedial actions and reanalysis fail to produce a constant RRF, prepare new calibration curve extracts and analyze. The statistics for response factors determined during the single laboratory validation of this method are included in Section 17, Table 7.

10.4 Retention time windows are established to compensate for minor shifts in absolute retention times as a result of sample loadings and normal chromatographic variability. Prior to establishing the absolute retention time window for the internal standard, verify that the chromatographic system is operating reliably and confirm that the system can meet the criteria in Section 9.2 and 10.3. Using the midpoint calibration standard, establish the retention time window by analyzing the standard once every 24 hours over a 72-hour period and calculating the mean and standard deviation of the absolute retention times for the internal standard for the three replicates. The width of the absolute retention time window for the internal standard is defined as the midpoint standard absolute retention time ± three times the standard deviation of the mean absolute retention time established during the 72-hour period. Retention times and retention time windows calculated in a single laboratory validation of the method are listed in Section 17, Table 8.

10.5 Verify calibration prior to the analysis of each set of samples (Sections 9.2 and 9.3). Analyze one of the calibration standards (Section 7.2.9) prior to the analysis of each set of samples. It is recommended that the selected calibration standard vary over time in order to verify the calibration over the calibration range of the method. Recalibrate if the relative response factor for the target compounds in the analyzed calibration verification point differ by ±15% of the relative response factor determined for that calibration point in the current calibration curve. Calculate the percent difference between the calibration curve and the calibration verification relative response factors using the following equation:

*Percent Difference = [(RRF_{AVG} - $RRF_{V)}$/ RRF_{AVG}] * 100*

where:

RRF_{AVG} = the average relative response factor from the initial calibration curve
RRF_V = the relative response factor from the calibration verification

10.6 Process a blank with the curve to confirm that the glassware, reagents, and other components are free from contamination. Prepare the blank using the procedure used to prepare the calibration standards, omitting the addition of the target analytes (Section 7.2.9).

10.7 Demonstrate that the target analytes are detectable at the minimum levels using the lowest level calibration curve standard.

11.0 Procedures

11.1 This section includes the procedures used to extract, concentrate, derivatize, and clean up treatment plant influent and biologically treated effluent samples. The extraction, concentration, and derivatization procedures are used for all types of samples and method blanks. Silica gel clean-up may be required for some extracts as indicated by elevated baselines in the chromatograms or detection of interferences, and is described in Section 11.5.

11.2 Remove the sample, surrogate working stock (Section 7.2.7), internal standard working stock (Section 7.2.8), and the appropriate analyte working stock solution (Section 7.2.5) from the refrigerator and bring to room temperature.

11.3 Extraction of effluent samples

11.3.1 Shake the sample to ensure homogeneity and immediately measure a 100-mL portion of the biologically treated effluent sample into a 250-mL beaker using a graduated cylinder. For method blanks, measure 100 mL of reagent grade water.

11.3.2 Spike with 200 µL of approximately 40 µg/mL surrogate working stock (Section 7.2.7). For matrix spikes add a 200-µL spike of the target analyte working stock (Section 7.2.5).

11.3.3 Adjust to pH 7 by the addition of 4.3 M potassium carbonate, then add approximately one half of a pH 7 buffer capsule and stir until the buffer dissolves completely.

11.3.4 Quantitatively transfer the beaker contents into a 250-mL separatory funnel and extract once with a 25-mL portion of MTBE. Allow the phases to separate for a minimum of ten minutes, then drain the aqueous phase into the 250-mL beaker and transfer the MTBE layer to a centrifuge tube. Centrifuge the MTBE layer and any emulsions after each extraction.

11.3.5 Repeat the extraction three times with 20 mL of MTBE. Combine the four MTBE extracts in a Kuderna-Danish (KD) apparatus.

11.4 Concentration and drying of the extract

11.4.1 Confirm that the water bath temperature is at a minimum of 90°C. Add a clean boiling chip to the KD apparatus, and attach a three-ball Snyder column. Pre-wet the Snyder column by adding 1 mL of MTBE to the top of the column. Place the KD apparatus in the water bath and concentrate the extract until the apparent volume of liquid reaches 3 to 5 mL.

11.4.2 Perform a solvent exchange by cautiously adding ~20 mL of hexane to the top of the Snyder column. Further concentrate the extract to a volume of ~2 mL by slowly placing the KD apparatus into a 90°C hot water bath. Rinse down the Snyder column and 250-mL KD flask with hexane and remove them. Place a micro-Snyder column on the thimble and add one more boiling chip. Continue boiling off the solvent to a volume of 0.5 mL. Do not boil to dryness.

11.4.3 Construct a drying column using a 5 ¾-inch Pasteur pipette with a plug of filter pulp in the tip that has had 5 cm of dried sodium sulfate tapped into the column. Dry the extract by loading it onto the top of the sodium sulfate column followed by three 1-mL rinses of hexane. Place each rinse of hexane on the column when the surface begins to dry. Fill the column reservoir with a 2- to 3-mL aliquot of hexane to ensure that the extract is carried through the column. Collect all transfers and rinses (total volume 3 to 5 mL) in a 15-mL screw-capped culture tube. Add a boiling chip to the culture tube and concentrate the extract to ~0.5 mL in a 90°C hot water bath.

11.4.4 Transfer the final extract with two hexane rinses to a 2-mL autosampler vial. Concentrate the extract to approximately 250 µL using nitrogen blowdown. Add a 250-µL aliquot of acetone to the extract to adjust the volume to 500 µL. Rinse any sample residue from the sides of the vial during the acetone addition.

11.5 Silylation of the extract

Derivatize the sample by adding 100 µL of the BSTFA derivatizing reagent to the 2-mL autosampler vial. Cap the autosampler vial with a Teflon™-lined septa and sonicate for 30 to 60 seconds to ensure thorough mixing. Allow the derivatization reaction to proceed at room temperature for a minimum of one hour.

11.6 Silica gel clean-up of extracts

Silica gel clean-up of the sample extracts may be necessary for samples containing high background levels of non-target analytes in the retention time intervals of the target analytes, surrogate, and internal standard. It is also recommended for samples that yield elevated baselines in the GC/FID or GC/MS chromatograms. If silica gel clean-up is

required, the following procedure is recommended. Proceed to Section 11.7 if silica gel clean-up is not required.

11.6.1 Preparation of silica gel columns

Gently push a small plug of analytical filter pulp to the bottom of a 20 cm x 8.0 mm o.d. x 6.0 mm i.d. glass column with a tapered end. Mark the column using a marking pen at 4 and 5 cm above the filter plug. Dry pack the column with 4 cm of activated silica gel (grade 62, 60 to 200 mesh), tapping the sides to ensure tight packing. Add 1.0 cm of dried sodium sulfate to the top of the silica gel. The column can be used immediately or stored in a drying oven at 130°C. It is recommended that columns be used within one week of preparation.

11.6.2 Extract clean-up

Allow the column prepared in Section 11.6.1 to come to room temperature. Using nitrogen blowdown reduce the extract to dryness. Add 500 µL of hexane, cap and sonicate for 30 seconds to redissolve the sample. Add 20 µL of the indicator solution (Section 7.4.3) to the derivatized sample extract. Pre-elute the column by rinsing with approximately 1 to 2 mL of hexane; discard this rinse. Place a 15-mL screw-cap culture tube as a receiver under the column. Just prior to exposure of the sodium sulfate layer to air (~500 µL), quantitatively transfer the sample to the column using two rinses of 0.2 to 0.3 mL of hexane. Take care to focus the sample on the column by allowing the sample to soak down onto the column to the point where the sodium sulfate is almost exposed to air before the hexane rinses are added. After the hexane rinses, add 0.2 to 0.3 mL 95:5 hexane:MTBE to the autosampler vial, and then add it to the column just prior to the exposure of the sodium sulfate layer to air. Fill the column reservoir approximately half full with the 95:5 hexane:MTBE solution. Just before the azulene (purple-blue band) reaches the bottom of the column, remove the 15-mL screw-cap culture tube and replace it with a graduated conical centrifuge tube. Collect 2 mL of the eluant in the centrifuge tube prior to the elution of the Sudan I (yellow-orange band). This is the sample extract. Concentrate the sample extract to 0.5 mL using nitrogen blowdown. Transfer the final extract with two hexane rinses to a 2-mL autosampler vial. Concentrate the extract to approximately 250 µL using nitrogen blowdown. Add a 250-µL aliquot of acetone to the extract to adjust the volume to approximately 0.5 mL. Rinse any sample residue from the sides of the vial during the acetone addition.

11.7 Internal standard addition

Add 10 µL of the internal standard spiking solution (dotriacontane) to the derivatized extract. Cap the vial with a Teflon™-lined crimp top. If the extracts are not analyzed immediately, store at 4°C. Always allow the extract to come to room temperature prior to GC/FID or GC/MS analysis.

11.8 GC/FID analysis

11.8.1 The GC/FID conditions should be set according to the criteria described in Section 10.1.

11.8.2 Bring the calibration verification check solution to room temperature. Perform the GC/FID calibration check as outlined in Section 9.2.

11.8.3 Bring the method blank extract to room temperature and verify that any precipitate has redissolved. Inject a 1-µL volume of the method blank extract, using splitless injection. Verify that the analytical system is free of contamination.

11.8.4 Bring the sample extract or standard to room temperature and verify that any precipitate has redissolved. Inject a 1-µL volume of the standard solution or extract, using splitless injection.

11.9 GC/MS analysis (alternative confirmation)

11.9.1 The GC/MS conditions should be set according to criteria described in Section 10.2.

11.9.2 Bring the DFTPP tune solution to room temperature. Perform the DFTPP tune as outlined in Section 9.3.1.

11.9.3 Bring the daily calibration solution to room temperature. Perform the daily calibration verification as outlined in Section 9.3.2.

11.9.4 Bring the sample extract or standard to room temperature and verify that any precipitate has redissolved. Inject a 1-µL volume of the standard solution or extract, using splitless injection.

12.0 Data Analysis

12.1 GC/FID data analysis

An analyte is identified by comparison of the relative retention time of the sample with the relative retention time of an authentic standard of the target compound analyzed using the same analytical conditions. Refer to Section 17, Table 8 for a list of the retention times and relative retention times for the target analytes. Identification of a compound is confirmed when the following criteria are met.

12.1.1 The sample component relative retention time (RRT) must fall within the relative retention time window described in Section 17, Table 8.

12.1.2 The absolute retention time of the internal standard must fall within the absolute retention time window calculated in Section 10.4.

12.2 GC/MS data analysis

An analyte is identified by comparison of the sample mass spectrum with the mass spectrum of a standard of the suspected compound which has been previously stored in a mass spectral library. Refer to Section 17, Table 9 for a list of the characteristic ions. Identification of a compound is confirmed when the following criteria are met.

12.2.1 The RRT should be assigned by using EICPs for ions unique to the component of interest.

12.2.2 The sample component RRT must fall within ±0.06 RRT units of the RRT of the standard component.

12.2.3 Verify that the selected ions specified in Section 17, Table 9 are present and maximize within the same two consecutive scans.

12.2.4 The relative percent abundance of the ions designated in Section 17, Table 9 must agree within ±20% of those observed for the mid-point calibration curve standard during the most current calibration curve analysis.

12.2.5 The m/z's present in the mass spectrum from components in the samples that are not present in the reference spectrum should be accounted for by contamination or background ions. If the experimental mass spectrum is contaminated, or if identification is ambiguous, an experienced spectrometrist must determine the presence or absence of the compound.

12.3 Internal standard quantitation

The dotriacontane internal standard is used to quantitate the corresponding phytosterols. Calculate the concentration of the target compound in the sample according to the following equation.

Concentration of target (µg/L) = $[(A_S \times C_{IS})/(A_{IS} \times RRF_{Ave})]$

where:

A_S = *area of the compound being measured*
C_{IS} = *concentration (µg/L) of the dotriacontane internal standard in the sample*
A_{IS} = *area of the internal standard*
RRF_{Ave} = *averaged relative response from the initial calibration curve*

12.4 Data review requirements

12.4.1 Review the data for accuracy of the identification, GC problems, interferences, and bias. Correct any problems prior to reporting the analytical results.

12.4.2 Manually review the chromatograms to confirm internal standard and analyte identification and area integrations. As part of this review, assess the need for sample/extract dilutions or clean-up. The procedure for conducting extract dilution and reanalysis is described in Section 12.5. The silica gel clean-up procedure is described in Section 11.6.

12.4.3 Visually inspect the total ion chromatogram for obvious problems which might result in poor internal standard recoveries or false negatives/false positives. The presence of non-target species can become apparent from this review.

12.4.4 Resolve any inconsistencies between duplicate analyses (i.e., if a compound shows up in one replicate but not the other), and attempt to determine the reason.

12.4.5 Generate a GC/FID report that includes the retention time of the compound, area of the compound, width of the peak, and calculated concentration of the target compound detected. If review of the data shows any problems which could affect subsequent analyses, analyses are discontinued until the problems are resolved.

12.5 Results outside the calibration range

If the calculated concentration of any of the target analytes exceeds the concentration of the highest calibration point, dilute an aliquot of the extract with hexane to bring the concentration within the calibration range of the method, and reanalyze. A maximum dilution of 1 to 10 is allowed in order to maintain sufficient internal standard concentrations in the extracts.

12.6 Comparison of results from different detectors

When sample results are confirmed using two different detectors (GC/FID and GC/MS), the agreement between the quantitative results should be evaluated after the qualitative identification has been confirmed. Calculate the relative percent difference (RPD) between the two results using the following formula:

$$RPD = [| C_1 - C_2 | / (C_1 + C_2 / 2)] \times 100$$

where:

C_1 = *concentration resulting from the GC/FID analysis*
C_2 = *concentration resulting from the GC/MS analysis*

The vertical bars in the above formula indicate the absolute value of the difference between the two concentration results.

Compare the calculated relative percent difference with the single laboratory validation data reported in Section 17, Table 10. If the levels determined are outside the control limits (the average relative percent difference ± three times the standard deviation) check the chromatograms for anomalies, review the chromatographic conditions, correct any problems, and repeat the analyses of the sample. If the results are outside the warning limits (the relative percent difference ± two times the standard deviation), the analyst should review the analytical data, procedure, and chromatographic conditions and take steps to correct any problems.

13.0 Method Performance

13.1 Single laboratory performance for this method is detailed in Section 17, Tables 2, 4, 5, 6, and 7. Acceptance criteria were established from single laboratory use of the draft method.

13.2 A chromatogram of a calibration standard from the GC/FID is shown in Figure A1.

14.0 Pollution Prevention

Pollution prevention approaches have not been evaluated for this method.

15.0 Waste Management

15.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

15.2 Instructions for sample and waste handling and disposal

15.2.1 Store all flammable waste solvents in a metal safety can labeled FLAMMABLE until proper disposal can be accomplished.

15.2.2 Neutralize the potassium carbonate solution and pour it down the drain with copious amounts of water.

15.2.3 Pour the aqueous portion of the extracted sample aliquot down the drain with copious amounts of water.

15.3 For further information on waste management, the Environmental Protection Agency suggests you consult "The Waste Management Manual for Laboratory Personnel," and "Less is Better: Laboratory Chemical Management for Waste Reduction." Both are available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC, 20036.

16.0 References

Federal Register, Vol. 49, No. 209. October 26, 1984. *Appendix B to Part 136-Definition and procedure for the determination of the method detection limit-revision 1.11.*

Rowe, J.W. *The sterols of pine bark.* Phytochemistry, Vol. 4., 1965, pp 1 to 10.

17.0 Tables, Diagrams, Flowcharts, And Validation Data

Table 1. Compounds Determined by GC/FID Using NCASI STER-97

Compound	CAS Registry Number
Campesterol	474-62-4
Stigmasterol	83-48-7
β-Sitosterol	83-46-5
Stigmastanol	19466-47-8

Table 2. Method Detection Limits Assessed in Biologically Treated Effluents Using NCASI STER-97 by GC/FID

Compound	MDL[a] (µg/L)
Campesterol	0.41
Stigmasterol	0.43
β-Sitosterol	0.47
Stigmastanol	0.44

[a] Method Detection Limit determined using 40 CFR 136 Appendix B, Federal Register 1984.

Table 3. DFTPP Criteria for NCASI STER-97 GC/MS: Confirmation Analyses

m/z	Ion Abundance Criteria
51	8-82% of mass 198
68	< 2% of mass 69
69	11-91% of mass 198
70	< 2% of mass 69
127	32-59% of mass 198
197	< 1% of mass 198
198	Base peak, 100% relative abundance
199	4-9% of mass 198
275	11-30% of mass 198
441	44-110% of mass 443
442	30-86% of mass 198
443	14-24% of mass 442

Table 4. Surrogate Recovery in Biologically Treated Effluents During NCASI STER-97 Method Validation Studies by GC/FID[a]

Compound	Spike Concentration (µg/L)	Recovery Range (%)	Average Recovery (%)	Standard Deviation	RSD (%)	n
Cholesterol	82	51 - 105	87.5	15.5	17.7	20

[a] Data is not available for influents due to a change in surrogate selection during the initial method development experiments.

Table 5. Matrix Spike Recovery for Compounds During Method Validation Studies Using NCASI STER-97 by GC/FID

Compound	Spike Concentration Range	Recovery Range	Average Recovery	Standard Deviation	RSD	Matrices Analyzed[a]
	µg/L	(%)	(%)		(%)	n
Effluents						
Campesterol	67 - 281	59 - 99	74	10	13	12
Stigmasterol	75 - 272	60 - 99	74	10	13	13
β-Sitosterol	47 - 285	62 - 104	81	12	15	13
Stigmastanol	76 - 262	61 - 101	32	10	13	13
Influents						
Campesterol	67 - 281	28 - 125	84	23	28	10
Stigmasterol	75 - 272	25 - 117	78	24	31	11
β-Sitosterol	47 - 285	60 - 133	92	22	24	10
Stigmastanol	76 - 262	19 - 100	76	23	30	11

[a] Samples were collected from n different mills

Table 6. Single Laboratory Precision for Campesterol, β-Sitosterol, Stigmasterol and Stigmastanol Using NCASI Method STER-97 by GC/FID

Compound	Native Concentration Range	Relative Percent Difference Range	Mean RPD	n
	(µg/L)	(%)	(%)	
Effluents				
Campesterol	1.1 - 42	0.1 - 23	7	35
Stigmasterol	1.2 - 108	0.7 - 28	8	36
β-Sitosterol	2.0 - 471	0.9 - 25	8	38
Stigmastanol	2.5 - 55	0.2 - 28	8	36
Influents				
Campesterol	3.0 - 166	6.2 - 37	17	6
Stigmasterol	1.3 - 120	17 - 27	22	6
β-Sitosterol	32 - 725	2.3 - 27	11	6
Stigmastanol	6.8 - 254	6.4 - 31	17	6

Table 7. Response Factor Statistics For NCASI STER-97 Compounds

Compound	Response Factor Range[a]	Average Relative Response Factor[b]	Average Relative Standard Deviation[c]	Standard Deviation[d]	Warning Limit[e]	Control Limit[f]
			(%)			
Campesterol	0.56 - 0.88	0.75	5.9	3.6	13.2	16.8
Stigmasterol	0.67 - 1.09	0.91	5.3	2.7	10.7	13.4
β-Sitosterol	0.46 - 0.67	0.62	6.2	3.0	12.2	15.2
Stigmastanol	0.81 - 1.13	0.93	4.1	1.7	7.5	9.2
Cholesterol (S)[g]	0.91 - 1.19	1.02	5.2	1.0	7.2	8.2

[a] The average response factor range observed for eight six-point calibration curves
[b] The average of the relative response factors determined from eight six-point calibration curves
[c] The average relative standard deviation expressed as a percent for the eight six-point calibration curves
[d] The standard deviation of the relative standard deviations for the eight calibration curves
[e] The warning limit is expressed as the average relative standard deviation observed for eight six-point calibration curves plus two times the standard deviation
[f] The control limit is expressed as the average relative standard deviation observed for eight six-point calibration curves plus three times the standard deviation
[g] All data expressed for cholesterol are based on the analysis of three six-point calibration curves.
(S) Surrogate

Table 8. Retention Time Statistics For NCASI STER-97 Compounds

Compound	Retention Time	Average Relative Retention Time[a]	Relative Retention Time Window[b]	Absolute Retention Time Window[c]
Campesterol	16.37	1.081	1.075 - 1.087	
Stigmasterol	18.20	1.114	1.109 - 1.118	
β-Sitosterol	19.27	1.180	1.176 - 1.183	
Stigmastanol	19.50	1.195	1.189 - 1.201	
Cholesterol (S)	16.05	0.980	0.977 - 0.984	
Dotriacontane (IS)	16.40			16.34 - 16.42

[a] The average relative retention time calculated from eight six-point calibration curves
[b] The relative retention time window is the average relative retention time ± 3 times the standard deviation of the relative retention times from eight six-point calibration curves.
[c] The absolute retention time window was determined from seven replicates of a 25-µg/L standard analyzed over a 72-hour period.
(S) Surrogate
(IS) Internal Standard

Table 9. Characteristic Ions for NCASI STER-97 Compounds Using GC/MS Confirmation

Compound	Primary Ion	Secondary Ions
Campesterol	343	382, 472
Stigmasterol	255	394, 355
β-Sitosterol	357	396, 255
Stigmastanol	215	306, 383, 473
Cholesterol (S)	329	368, 328
Dotriacontane (IS)	99	85, 71

(S) Surrogate
(IS) Internal Standard

Table 10. Relative Percent Differences Determine for GC/FID and GC/MS Confirmation Results

Compound	Range of RPDs Observed	Average RPD	Standard Deviation of the RPDs	Warning Limit	Control Limit	n
	(%)	(%)				
Campesterol	0.5 - 28	9.3	6.7	23	29	15
Stigmasterol	0.1 - 22	8.9	6.4	22	28	18
β-Sitosterol	0.1 - 14	6.6	4.3	15	19	15
Stigmastanol	0.2 - 13	5.4	3.9	13	17	15
Cholesterol (S)	1.0 - 21	9.6	5.5	22	28	23

(S) Surrogate

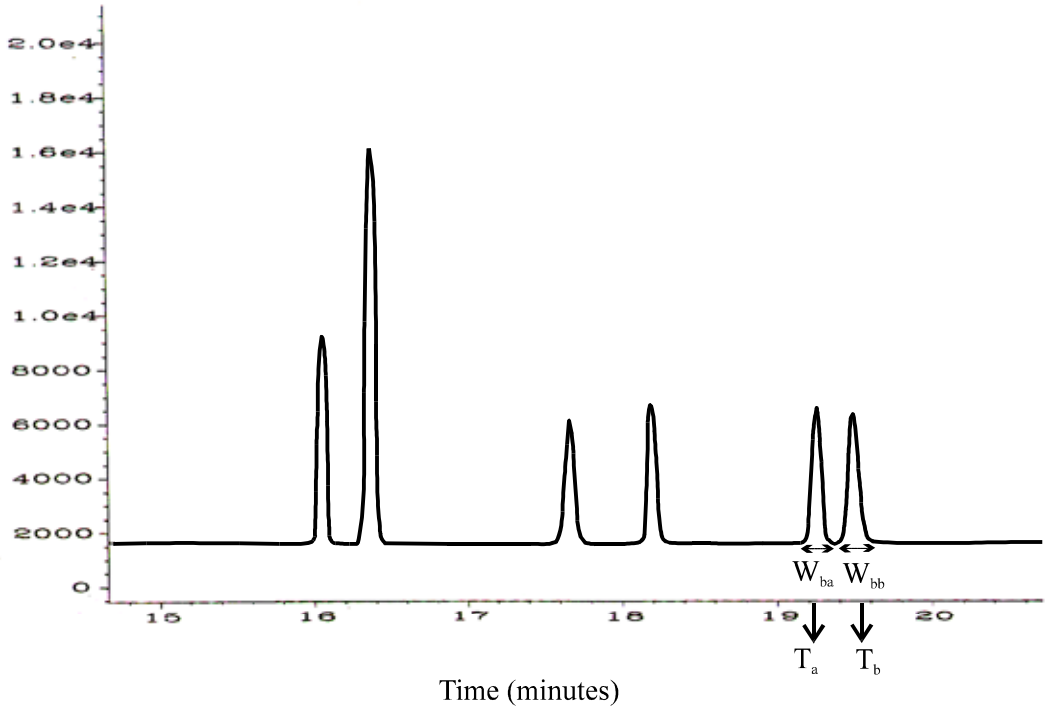

Figure 1. Chromatogram of the 50-µg/L Calibration Standard