IMPLEMENTATION OF A CHEMICAL METHOD
FOR DIFFERENTIATING HUMAN AND ANIMAL FECAL IMPACTS
IN SURFACE WATERS AND SEDIMENTS

ENVIRONMENTAL MONITORING DIVISION

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# Table of Contents

TABLE OF CONTENTS .............................................................................................................. i  
LIST OF FIGURES .................................................................................................................. ii  
LIST OF TABLES .................................................................................................................... ii  
EXECUTIVE SUMMARY ........................................................................................................ iii  
I. INTRODUCTION .................................................................................................................. 1  
II. METHODOLOGY ............................................................................................................... 3  
III. RESULTS ........................................................................................................................ 7  
IV. DISCUSSION ..................................................................................................................... 13  
V. CONCLUSIONS ................................................................................................................ 14  
VI. LITERATURE CITED ...................................................................................................... 15  
APPENDIX I Chromatograms and mass spectral data of the sterols ....................................... 16  
APPENDIX II Standard curves of the sterols ........................................................................ 22  
APPENDIX III Structural conformations of the sterols .......................................................... 29
List of Figures

1. Sterol Ratios, Species; Dog................................................................. 12
2. Sterol Ratios, Species; Horse......................................................... 12
3. Sterol Ratios, Species; Duck .......................................................... 12
4. Sterol Ratios, NIST Sludge Reference Sample .................................. 12
5. Sterol Ratios, Species; Human......................................................... 12
6. Sterol Ratios, Domestic Wastewater Influent ........................................ 12

List of Tables

1. Retention Time Windows................................................................. 8
2. Method Detection Limit Data.......................................................... 9
3. Method Performance Data............................................................. 10
4. Reproducibility Data (municipal digested sludge)............................. 10
5. Sterol Concentrations in Fecal Matter, Domestic Wastewater Influent and Sludge............... 11
EXECUTIVE SUMMARY

This report concerns the development of a chemical method for investigating elevated bacterial levels in Broward County waterways. The proposed method involves the extraction and analysis of fecal sterols from environmental samples from areas suspected to be impacted by human sanitary waste and the assessment of the source of the sterols based upon concentration ratios. The precision and accuracy of the method is evaluated and a limited amount of data from the analysis of environmental samples is presented.

Certain areas of county waterways are adversely affected by high levels of fecal coliform bacteria, a common indicator of fecal contamination in surface water. Fecal coliform bacteria are common in the gut of warm-blooded animals and man. These bacteria contaminate surface waters by a variety of avenues including stormwater runoff, septic tank seepage, discharges from vessel sewage holding tanks and assorted bird and marine mammals. Knowing whether the elevated bacteria levels have a human or non-human source is important since bacteria from human waste presents a risk of disease transmission.

The Environmental Monitoring Division (EMD) has been investigating test methods that can be used to identify sources of fecal contamination. Studies have shown that cholesterol is metabolized differently in each animal species as a result of the characteristic intestinal flora of the species. EMD has focused on four fecal “sterols” that are metabolites of cholesterol. The fecal sterols studied in this method are coprostanol, epicoprostanol, cholestanol and epicholestanol. These sterols are “stereoisomers”, chemically identical and differing only in the geometric orientation of their molecular components (see Appendix III). A method was developed to analyze these sterols in surface water, feces and sediments. The method involves the separation of the sterols from the samples followed by measurement using gas chromatography-mass spectrometry (GC-MS).

The method described in this report provides for the preparation of trimethylsilyl derivatives of the fecal sterols which greatly improves chromatography. Standard curves over the range of 10 to 1,000 picograms (on-column) were found to fit the form of a quadratic function. A “cleanup” step using column chromatography is also described for the elimination of interference from petroleum hydrocarbons.

Method detection limits were estimated to be between 10 and 50 nanogram per liter for environmental water samples (based on a one liter sample) and 10 to 50 nanogram per gram for sediment samples (based on a one gram sample). The water detection limits may not be adequate to detect very low levels of coprostanol unless significant quantities of water (greater than four liters) are processed or unless the water sample contains an adequate amount of particulate matter (greater than 100 mg/l). Since the fecal sterols bind to particulate matter that settle into canal sediments, sediment sterol concentrations are much greater and thus are expected to fall within the method’s detection limit. The method was found to recover between 80 and 95 percent of the sterols applied to glass fiber filters with relative standard deviations of less than 6 percent.

Using the method described in this paper, it was shown that it was possible to tell the difference between fresh fecal samples of human and non-human origin based upon the concentration ratios of two of the fecal sterols. The coprostanol/cholesterol concentration ratio was shown to be greater than 1.0 in human feces and less than 1.0 in non-human feces. If this relationship can be shown to be preserved in samples of suspended particulate matter or sediment in the environment, this analysis may be used to indicate the presence of human fecal material. Prior research has shown that when fecal material is discharged to surface waters, the sterol components accumulate on particles that settle into bottom sediments where they are stable due to oxygen-poor conditions. Sediments in waterways found to contain elevated bacteria and a coprostanol/cholesterol concentration greater than 1.0 would suggest that the bacteria have a human source while a ratio of less than 1.0 may suggest a mixed or predominantly non-human source.
I. INTRODUCTION

A. BACKGROUND

Fecal coliform bacteria are widely used as indicators of sewage contamination in surface waters. Since fecal coliform are common to both humans and other animals, ascertaining the source of bacteria found in surface waters is important because the potential to transmit human disease is greater if the source of the bacteria is human sanitary waste. The shortcomings of fecal coliform bacteria as an indicator of sanitary waste provided the impetus for the Environmental Monitoring Division (EMD) of the Department of Natural Resource Protection (DNRP) to seek another, more specific indicator of human sanitary waste. Research by others (Hatcher & McGillivary, 1979; Venkatesan, 1992; Hawkins-Writer et al., 1995; Murtaugh and Bunch, 1967; McCorquodale and Burney, 1996) suggest that certain fecal sterols, metabolites of cholesterol, may be useful for this purpose.

The subject of this paper is the development and implementation by the EMD laboratories of a gas chromatographic-mass spectrometric (GC-MS) method for the detection and quantitation of a group of fecal sterols for the purpose of differentiating between human and non-human bacterial impacts on surface waters. One of these sterols, coprostanol, is formed in the gut of humans and higher mammals by enzymatic hydrogenation of cholesterol or by stereo-specific bacterial reduction of cholesterol (MacDonald et al., 1983). Coprostanol is a compound of moderate polarity and reactivity. Its solubility, and that of the other fecal sterols, in water is negligible. Cholesterol, the parent compound, is also found in the feces of man but is also common in aquatic environments making it less useful as a human-specific sewage indicator. Coprostanol, on the other hand, has been shown to be useful as a sewage tracer even when fecal coliform populations have diminished due to water chlorination or the toxic effects of industrial effluents (LeBlanc et al., 1992).

The non-polar, non-ionic, and water-insoluble nature of the fecal sterols result in their association with fine-grained particles and sediments (Hawkins-Writer et al., 1995). The primary theory for the association of non-ionic, non-polar contaminants such as the fecal sterols with particulate matter is a partitioning interaction with organic coatings on these smaller particles (Hawkins-Writer et al., 1995). Mechanisms of organic matter binding are highly contingent on surface interactions. Smaller particulates have a higher affinity for organic matter due to the large ratio of surface area to volume. Studies indicate that coprostanol-sediment concentration is affected by the percent of fine-grained sediments. Researchers have recently demonstrated a positive correlation between fecal sterol concentrations, particulate size, and fraction of organic carbon in sediments. Between 84 and 95% of the fecal sterols discharged from sewage-treatment plants is associated with particulate matter and is quickly assimilated into the finer bed sediments of the aquatic environment. The use of a sterol ratio has been suggested to remove any bias induced by particle size and percent carbon (Hawkins-Writer et al., 1995).

Besides coprostanol, several other sterols have been observed in water and sediment samples, including cholesterol, cholestanol, epicholestanol and epicoprostanol and their presence has been employed in evaluating impacts from sewage (Hatcher & McGillivary, 1979). Solubilities, degradation rates, and binding affinities are similar. Coprostanol concentrations
in marine sediments have been shown to be highest in areas affected by “human” pollution (Hatcher & McGillivary, 1979). Results from these studies indicate that large proportions of the fecal sterols, coprostanol and cholesterol, are associated with particulates and sediments. Previous studies of coprostanol in sediment have documented that the relative amount of cholesterol in sediment is greater than the amount of coprostanol (Hawkins-Writer et al., 1995).

Cholesterol is produced in the marine environment and also constitutes 9.5% of fecal sterols. In contrast, coprostanol is not produced in the marine environment but comprises 50 to 80% of human fecal sterols (Brown & Wade, 1984).

Coprostanol is stable in aquatic environments. Nishimura and Koyama (1977) incubated cholesterol and cholestanol in a lake sediment. Over a period of 450 days at 15 degrees Celsius, the cholesterol and cholestanol added to the sediments exhibited no significant changes in concentration. Therefore, under anaerobic conditions, essentially no decomposition occurred. Additionally, there was no biodegradation of coprostanol in marine sediments (Nishimura and Koyama, 1977).

While coprostanol shows limited biodegradation in oxygen-poor marine sediments, coprostanol may be aerobically degraded by bacteria. Cholesterol has been shown to be biologically hydrogenated into coprostanol and cholestanol in anoxic sediments over a period of 1200 days. The conversion of cholesterol to coprostanol, however, was only 2-3% over the 1200 day time span. Trace amounts of coprostanol (less than 0.010 ug/G) should not be attributed to sewage sources. Fortunately, the sterol ratio, coprostanol/ (coprostanol + cholestanol) may be a useful index for monitoring urban sewage (Grimalt et al., 1990).

B. STUDY OBJECTIVE

One of the largest sources of fecal bacteria to surface waters, the discharge of treated sanitary wastewater, has been reduced to essentially zero in Broward County due to the consolidation of many smaller wastewater treatment plants into regional systems. Nonetheless, elevations continue to be observed in various areas of the County. The possible sources of the bacteria include storm water runoff, septic tank seepage, marine mammals, illegal and/or inadvertent cross-connections between the sanitary and storm sewer systems, and discharges from inhabited marine vessels. The human component of this mix of sources has the greatest potential to spread disease so differentiating between the human and non-human sources of these bacteria is important. The objective of this work was to implement an analytical protocol for detecting and quantifying coprostanol and other associated fecal sterols for the purpose of differentiating between human and non-human sources of fecal bacteria in surface waters. This paper will deal with the extraction of the fecal sterols from water, fecal matter and sediment samples using ultrasonic agitation, followed by their separation by gas chromatography and detection and quantitation by mass spectrometry. A limited amount of data on fecal sterols in water, sediments, and fecal matter of assorted animals is also presented.
II. METHODOLOGY

A. STANDARDS, REAGENTS, SOLVENTS, MATERIALS AND EQUIPMENT

The standards, reagents, solvents, materials and equipment required for this work appear below:

1. Standards:
   o **5α-Cholestane (Internal Standard), #26700, Fluka Chemika-Biochemika Analytika**, Ronkonkoma, N.Y.
   o **5β-Cholestan-3β-ol (Coprostanol), #10873, Research Plus, Inc., Bayonne N.J.**
   o **5β-Cholestan-3α-ol (Epicoprostanol), Research Plus, Inc, #1084-3**
   o **5α-Cholestan-3β-ol (Cholestanol), #26710, Fluka**
   o **5α-Cholestan-3α-ol (Epicolestanol), #1066-3, Research Plus, Inc.**
   o Cholesterol, #26728, Fluka
   (Stock sterols: each prepared in Optima grade pyridine at 1,000 ng/uL)

2. Reagents:
   o Bis(trimethylsilyl)trifluoroacetamide(BSTFA)+trimethylchlorosilane (TMCS), 99:1 Sylon BFT Reference, Supelco Inc., derivatizing reagent
   o Sylon CT, 5%-dimethyldichlorosilane (DMDCS) in toluene (w/v, Supelco), glassware silanizing agent
   o Sodium Sulfate (anhydrous)
   o Alumina (chromatographic grade aluminum oxide, activated at 130 degrees C for 16 hours)
   o Silica Gel-adsorbent (Davidson 923 grade, activated at 130 degrees C for 16 hours)
   o Florisil, activated at 130 degrees C for 16 hours followed by deactivation with 5% water by weight

   o Methylene Chloride, Optima grade
   o Acetone, Optima grade
   o Hexane, Optima grade
   o Toluene, G.C. grade
   o Pyridine, G.C. grade

4. Equipment:
   o Hematology mixer
   o Heat Systems Ultrasonic Processor
   o Vortex-Genie mixer
   o Muffle furnace and drying oven
   o Regulated water bath
   o Hewlett-Packard 5890 II + gas chromatograph, splitless injection
   o Hewlett Packard 5972 MSD- mass spectrometer
   o Ultra-high purity grade helium and nitrogen at 99.999%
5. Materials:
- Gelman A/E glass fiber filters, 1.0 um nominal pore size, pre-ignited at 400 degrees Celsius for 4 hours
- Several large aluminum trays for ice baths
- 30 meter x 0.25mm x 0.25 um HP-5ms GC column
- 47 mm vacuum filter apparatus

6. Glassware:
- heavy-walled beakers, 150-mL
- 10 mL glass syringes w/ valve for chromatographic columns
- TurboVap tubes, 250-mL, 1-mL endpoint
- 15-mL screw cap vials with Teflon-lined caps

B. EXTRACTION

Since fecal sterols in water are primarily associated with the particulate matter, suspended solids were isolated from the water samples by filtration through pre-ignited glass-fiber filters that were pre-rinsed with 3x10 ml volumes of deionized water. The quantity of water filtered varied from 1 to 4 liters, depending upon the amount of suspended particulate (ideally, at least 100 mg). Sediment quantities varied between 10 and 50 grams. The particulate-laden filter or sediment aliquot was then dried in a vacuum desiccator over a 24-hour period.

The extraction procedure was based upon EPA method 3550 for solid waste. A filter or sediment sample was placed in a 150 mL beaker in an ice bath. Fifty mL of methylene chloride-chloroform extractant (1+1) was added to the beaker. Using an ultrasonic agitator, the sample was sonicated for 3 minutes at a 50% duty cycle. The solution was allowed to settle and the supernate decanted through a drying column containing six to eight inches anhydrous sodium sulfate into a TurboVap concentrator tube. This process was repeated two more times and the drying column was rinsed with 50 mL extractant. With the TurboVap set at 53 degrees Celsius and 11 psi, the samples were concentrated to 1.0 mL. The extracts were transferred to 15 mL glass vials with Teflon-lined screw caps.

Extractions were performed on fecal samples from several animal species including human, horse, duck, and dog. The extractions were performed according to the coprostanol method developed for sediments. Two-hundred milligrams of fecal matter were used in place of sediment and extracted according to the method stated above. Column chromatography was then used as necessary to help eliminate interfering substances.
C. CLEANUP

The removal of petroleum hydrocarbons that may interfere with the analysis may be performed if required (Brown & Wade, 1984). The cleanup column consisted of a 10 mL syringe barrel with a Hamilton three-way valve attached. The column contained 1 g of alumina over 1 g of Florisil. The column was prepared for use by sequential rinses with a 3 mL portion of methanol, 6 mL methylene chloride, and 6 mL hexane/toluene mixture (95+5). Prior to charging the extracts to the column, they were brought to near dryness under a stream of nitrogen then brought to 2 mL with of 95+5 hexane/toluene. The extract was transferred from its vial to the column with a 2-mL portion of hexane-toluene. The interfering hydrocarbons contained in the extract were then eluted with 6 mL hexane:toluene mixture (95+5) and discarded. The sterols were eluted from the column with 8 mL of methanol and the eluate collected in a 15-mL vial and reduced to less than 1 mL under a stream of nitrogen in preparation for derivatization.

D. DERIVATIZATION

In order to improve the chromatography of the sterols, derivatization was performed. Sterol-TMS (trimethylsilyl)-ethers were prepared by reacting 1.0 mL of the extract with 100 uL of Sylon BFT (BSTFA:TMCS, 99+1). The extracts were capped tightly (with Teflon-lined caps), mixed on a vortex mixer for 10 seconds and placed in a 55 degree C water bath for 60 minutes. After the time period, they were removed from the water bath and allowed to cool to room temperature. Again the samples were reduced to near dryness with a gentle stream of nitrogen and reconstituted to 1 mL with methylene chloride. To this 1-mL final sample was added 10 uL of the internal standard, alpha-cholestane, at a concentration of 10 ng/uL then transferred to 2-mL Teflon-lined capped autosampler vials. In some extracts, particulates were present. These were allowed to settle or filtered through 0.2 um Teflon-syringe filters after the addition of the internal standard.

E. STANDARD PREPARATION

Calibration solutions of the authentic or “neat” (greater than 98%) sterols were prepared at a concentration of 1.0 ug/uL in pyridine. Pyridine was chosen as the solvent due to the sterols’ high solubility in it. A mixed stock sterol solution was then prepared by placing 1.0 mL of each stock sterol in a 15-mL vial, concentrating to 1.0 mL and derivatizing as detailed above. The mixed stock standard was then diluted in methylene chloride to a concentration of 100 ng/uL and stored at 4 degrees Celsius.

F. GAS CHROMATOGRAPHY/MASS SPECTROMETRY

In addition to coprostanol, cholesterol may be metabolized to the fecal sterols cholestanol, epicholestanol, and epicoprostanol which are stereoisomers of coprostanol (see Appendix III). Since these compounds produce mass spectra that are very similar to each other,
chromatographic conditions must be carefully optimized to achieve satisfactory separation for identification purposes.

The derivatized extracts were analyzed by gas chromatography-mass spectrometry (GC-MS). The system employed a pressure-programmed gas chromatograph and a mass-selective detector operated in selected-ion monitoring (SIM) mode using the Hewlett-Packard DOS Chemstation and Enviroquant software. The injector was operated in splitless mode using a single tapered 4-mm glass inlet liner packed with silanized glass wool. A two-meter uncoated fused silica guard column was attached to the head of the column using a universal press fit connector. The other end was directly introduced into the ion source of the mass selective detector (MSD). The initial oven temperature of 50 degrees C was held for one minute, increased to 210 degrees C at a rate of 4 degrees C per minute, and then to 280 degrees C at a rate of 2 degrees C per minute, then to 300 degrees C at 8 degrees C per minute, where it was held for one minute. The pressure was programmed to run in constant flow mode at 0.90 mL/minute. Two uL of extract were introduced to the inlet via autosampler (HP 7673) that was controlled by the Chemstation software. The MS was operated at a source temperature of 185 degrees C and a pressure of about 10^-5 Torr.

G. CHROMATOGRAPHIC RUNS AND RESPONSES

Chromatograms were produced using selected ion monitoring to improve quantitation and detection limits. The ions used for identification of the TMS ethers of the fecal sterol compounds were the ion, m/z 215, corresponding to the ABC steroid rings (C_{16}H_{23}), m/z 370 corresponding to the cholestane structure (C_{27}H_{46}) and m/z 355, corresponding to the additional loss of a methyl group from the cholestane structure (C_{26}H_{43}). The m/z 215 ion fragment was used as the primary ion for quantitation because it is generally the most intense ion in the mass spectrum thereby enhancing the signal-to-noise ratio. The compounds were further identified by comparing the relative retention times of the primary and qualifier ions to that of the internal standard, 5-alpha-cholestane. Quantitative determination of coprostanol and its analogues was made by establishing the relative response of the primary ion for each sterol in relation to the response of the primary ion of the internal standard (m/z 217).

The areas under the peaks in the chromatograms were integrated using the ChemStation integration software. Calibration curves were plotted for each sterol at 10, 20, 50, 100, 500, and 1,000 picograms. Quality control measures included daily standard checks that were processed along with the sample extracts to ensure that response remained constant. Procedural blanks and standards were run for each analysis to ensure that no contamination or carry over was occurring. The concentration of particulate-associated fecal sterol in water was expressed in nanograms per liter (ng/L). Fecal sterol concentrations in sediments and fecal samples were expressed as nanograms per gram dry weight (ng/G).
H. ASSESSMENT OF METHOD PERFORMANCE

Method performance was estimated by spiking four glass fiber filters with 500 nanograms of each sterol in pyridine. The sonication extraction process proceeded according to the usual method for sample extraction, derivatization and quantitation. A second measure of precision was made by analyzing a National Institute of Standards & Testing (NIST) domestic waste water sludge sample in quadruplicate.

I. ASSESSMENT OF MINIMUM DETECTION LIMIT

The minimum detection limit for the analysis of coprostanol is based on the results of the analyses of the seven glass-fiber filters that were spiked with 10 uL per filter of a mixed sterol standard (100 ng each sterol). The detection limits were calculated according to 40 CFR Part 136, Appendix B (Federal Register, 1984). The actual detection limits, however, are dependent on the volume of water filtered or the mass of sediment. The filters were subjected to the standard sonication and extraction method. The results represent the best case scenario detection limits. When actual samples are employed, the detection limit may increase due to interfering substances and the resulting detector “noise”.

J. ANALYSES OF SAMPLES

Although the application of the method to real world situations will be the subject of a future report, a limited amount of data was generated on real samples for this report. These samples included fecal matter from a dog, a duck, a horse, and a human. In addition, a sample of municipal digested sludge (obtained from the NIST) and a sample of fresh domestic wastewater were subjected to the test protocol.

III. RESULTS

A. GAS CHROMATOGRAMS AND MASS SPECTRA

See Appendix I for representative chromatograms and mass spectra of the sterols. A sample chromatogram of a domestic wastewater influent is also presented.

Table 1 lists the retention time windows of each component in the standard mix. The windows are calculated as the mean ± 3 standard deviations in minutes. The table also presents the relative retention time windows based upon the retention time of the internal standard.
**TABLE 1**
Retention Time Windows

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean RT, min.</th>
<th>RT Window, min.</th>
<th>Mean RRT</th>
<th>RRT Window</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholestane (internal standard)</td>
<td>60.04</td>
<td>60.01-60.06</td>
<td>1.000</td>
<td>0.9996-1.000</td>
</tr>
<tr>
<td>Coprostanol</td>
<td>66.96</td>
<td>66.94-66.98</td>
<td>1.115</td>
<td>1.115-1.116</td>
</tr>
<tr>
<td>Epicholestanol</td>
<td>67.21</td>
<td>67.19-67.23</td>
<td>1.119</td>
<td>1.119-1.120</td>
</tr>
<tr>
<td>Epicoprostanol</td>
<td>67.42</td>
<td>67.39-67.45</td>
<td>1.123</td>
<td>1.122-1.130</td>
</tr>
<tr>
<td>Cholestanol</td>
<td>70.04</td>
<td>70.01-70.07</td>
<td>1.167</td>
<td>1.166-1.167</td>
</tr>
</tbody>
</table>

**B. STANDARD CURVES**

See Appendix II for typical standard curves for the four sterols.

**C. ESTIMATION OF THE METHOD DETECTION LIMIT**

Table 2 presents data from which the method detection limit (MDL) was determined. The MDL is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero and is determined from analysis of a sample in a given matrix containing the analyte (40 CFR Part 136, 1984). The MDLs ranged from approximately 10 pg/μL to 50 pg/μL (in the final extract) for coprostanol and its stereoisomers.
<table>
<thead>
<tr>
<th>Rep. #</th>
<th>Coprostanol (pg/uL) (in final extract)</th>
<th>Epicholestanol (pg/uL) (in final extract)</th>
<th>Epicoprostanol (pg/uL) (in final extract)</th>
<th>Cholestanol (pg/uL) (in final extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99.83</td>
<td>93.69</td>
<td>107.31</td>
<td>110.64</td>
</tr>
<tr>
<td>2</td>
<td>101.09</td>
<td>94.73</td>
<td>106.91</td>
<td>111.57</td>
</tr>
<tr>
<td>3</td>
<td>106.06</td>
<td>98.24</td>
<td>107.57</td>
<td>134.41</td>
</tr>
<tr>
<td>4</td>
<td>109.12</td>
<td>108.44</td>
<td>150.51</td>
<td>107.44</td>
</tr>
<tr>
<td>5</td>
<td>103.32</td>
<td>100.46</td>
<td>114.42</td>
<td>111.60</td>
</tr>
<tr>
<td>6</td>
<td>99.06</td>
<td>92.77</td>
<td>110.69</td>
<td>99.47</td>
</tr>
<tr>
<td>7</td>
<td>104.08</td>
<td>97.84</td>
<td>105.68</td>
<td>108.09</td>
</tr>
<tr>
<td>Actual extract conc’n (pg/uL)</td>
<td>100</td>
<td>109</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Avg. % Recovery</td>
<td>104</td>
<td>90.6</td>
<td>118</td>
<td>116</td>
</tr>
<tr>
<td>Avg. % Std. Deviation</td>
<td>3.8</td>
<td>5.3</td>
<td>18.7</td>
<td>10.7</td>
</tr>
<tr>
<td>MDL (pg/uL)</td>
<td>10.4</td>
<td>15.6</td>
<td>46.7</td>
<td>31.4</td>
</tr>
</tbody>
</table>

**D. METHOD PERFORMANCE**

Table 3 presents the results of the method performance study based upon the replicate analyses of filters spiked with known quantities of fecal sterols. With the exception of epicholestanol (78.3%), the recoveries were in excess of 90%. Precision was good and ranged from 3.9% to 7.1%.
TABLE 3
Method Performance Data
Replicate Analyses of Spiked Filters

<table>
<thead>
<tr>
<th>Spike Value (ng)</th>
<th>COPROSTANOL</th>
<th>EPICHOLESTANOL</th>
<th>EPICOPROSTANOL</th>
<th>CHOLESTANOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1, ng</td>
<td>491.85</td>
<td>458.55</td>
<td>509.83</td>
<td>520.43</td>
</tr>
<tr>
<td>Run 2, ng</td>
<td>460.17</td>
<td>427.25</td>
<td>474.45</td>
<td>467.89</td>
</tr>
<tr>
<td>Run 3, ng</td>
<td>451.78</td>
<td>422.64</td>
<td>466.93</td>
<td>465.65</td>
</tr>
<tr>
<td>Run 4, ng</td>
<td>424.63</td>
<td>399.04</td>
<td>441.33</td>
<td>440.71</td>
</tr>
<tr>
<td>Avg. % Recovery</td>
<td>91.4%</td>
<td>78.3%</td>
<td>94.6%</td>
<td>94.7%</td>
</tr>
<tr>
<td>Rel. Std. Deviation</td>
<td>4.8%</td>
<td>3.9%</td>
<td>4.9%</td>
<td>5.8%</td>
</tr>
</tbody>
</table>

Table 4 presents the results from the replicate NIST sludge analyses. Precision, expressed as relative standard deviation (RSD), was less than 3% for each sterol that was present.

TABLE 4
Reproducibility Data
Replicate Analyses of Municipal Digested Sludge

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>COPROSTANOL</td>
<td>78.9</td>
<td>79.0</td>
<td>74.9</td>
<td>79.1</td>
<td>78.0</td>
<td>2.28</td>
</tr>
<tr>
<td>EPICHOLESTANOL</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>EPICOPROSTANOL</td>
<td>56.7</td>
<td>55.7</td>
<td>52.8</td>
<td>56.3</td>
<td>55.4</td>
<td>2.76</td>
</tr>
<tr>
<td>CHOLESTANOL</td>
<td>31.7</td>
<td>31.1</td>
<td>29.7</td>
<td>31.7</td>
<td>31.1</td>
<td>2.63</td>
</tr>
</tbody>
</table>
E. ANALYSIS OF SAMPLES

The results of analyses appear in Table 5.

**TABLE 5**
Sterol Concentrations in Fecal Matter, Domestic Wastewater & Sludge, ug/G

<table>
<thead>
<tr>
<th></th>
<th>Dog</th>
<th>Horse</th>
<th>Duck</th>
<th>Human</th>
<th>Sludge</th>
<th>Influent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coprostanol</td>
<td>56.0</td>
<td>3.65</td>
<td>72.7</td>
<td>5,420</td>
<td>5.18</td>
<td>1,513</td>
</tr>
<tr>
<td>Cholestanol</td>
<td>166</td>
<td>0.568</td>
<td>95.1</td>
<td>1,120</td>
<td>0.212</td>
<td>355</td>
</tr>
<tr>
<td>Epicholestanol</td>
<td>0.514</td>
<td>ND</td>
<td>ND</td>
<td>4.05</td>
<td>19.4</td>
<td></td>
</tr>
<tr>
<td>Epicoprostanol</td>
<td>6.07</td>
<td>1.26</td>
<td>48.0</td>
<td>ND</td>
<td>1.62</td>
<td>106</td>
</tr>
<tr>
<td><strong>Coprostanol:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cholestanol Ratios</strong></td>
<td>0.33</td>
<td>0.27</td>
<td>0.76</td>
<td>4.8</td>
<td>24.4</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Figures 1-5 below depict the fecal sterol concentrations, relative to coprostanol, in each of the fecal samples. It should be noted that of the four cholesterol metabolites studied, coprostanol is the predominant sterol in the human fecal sample while cholestanol is the predominant sterol in the other animal samples. On page 18, a chromatogram of raw domestic wastewater is presented. Coprostanol was also the predominant fecal sterol in this sample.
FIGURE 1
Sterol Ratios
Species: Dog

FIGURE 2
Sterol Ratios
Species: Horse

FIGURE 3
Sterol Ratios
Species: Duck

FIGURE 4
Sterol Ratios
NIST 176 Reference Sample

FIGURE 5
Sterol Ratios
Species: Human

FIGURE 6
Sterol Ratios
Domestic Navel Orange Peel
IV. DISCUSSION

A. GAS CHROMATOGRAMS AND MASS SPECTRA

As would be expected for stereoisomers, the mass spectra for the derivatized sterols were all similar, however differences in specific ionic abundances were noted. Underivatized sterols are normally difficult to chromatograph, however, the derivatization process was effective in improving the chromatography. The retention times were close together for some of the sterols, e.g., epicholestanol and epicoprostanol differ by less than 0.2 minutes and resolution between coprostanol and epicholestanol differed by 0.3 minutes. The retention time windows for the sterols, however, were mutually exclusive providing a good degree of confidence of identification. Structural conformations of the sterols are depicted in Appendix III.

B. STANDARD CURVES

The response of the sterols from 10 pg/μL to 1,000 pg/μL in the final extracts were best fit to a quadratic curve. All final extracts were diluted to fall within this range. For each standard curve, the “response ratio” is the detector response for the sterol divided by the detector response for the internal standard. Similarly, the “amount ratio” is the concentration of the sterol divided by the concentration of the internal standard.

C. ESTIMATION OF THE METHOD DETECTION LIMIT

The estimated detection limits for sterols found by this method (10 to 50 pg/μL, in the final extract) may not be adequate for surface water samples. These limits equate to approximately 10 to 50 ng/L for a one liter water sample or 10 to 50 ng/G for a one gram sediment sample (the limit varies depending on the amount of interfering substances present).

D. METHOD PERFORMANCE

The procedure used to determine the method performance in this work presents a best case scenario since the sterols would not be expected to adhere tightly to the glass fiber filter. The combination of intense agitation provided by the ultrasonic disrupter and the efficacious solvent system, however, will likely be shown to be effective in future method performance studies involving actual sediment samples fortified with authentic fecal sterols.

E. ANALYSIS OF SAMPLES

In all of the samples that would be expected to contain a fecal matter component that is primarily of human origin (e.g., human feces, domestic wastewater influent and sludge), the coprostanol concentration was found to be greater than the cholestanol concentration. This is
the opposite as is seen for the dog, horse and duck fecal samples where the cholestanol concentration was greater than the coprostanol concentration. This observation forms the basis of the use of this assay for differentiating human from non-human sources of bacteria in environmental samples.

V. CONCLUSIONS

The study provides a precise method for extracting and analyzing fecal sterols in environmental samples. In fresh animal fecal samples, coprostanol was found to be the predominant sterol in human feces while cholestanol was predominant in the other animal samples. The observation of elevated levels of coprostanol relative to cholestanol then forms the basis for differentiating between human and animal fecal sources.

The effects of environmental and microbiologically induced degradative changes have been noted in the literature, however, these changes occur primarily in digested sludge samples where reducing bacteria are present converting cholesterol into coprostanol (McCalley et al., 1981). In anoxic aged sediment beds, however, coprostanol levels have been noted to be extremely stable. Studies have shown that coprostanol will degrade under certain conditions, such as aerobic wastewater treatment processes or when incubated with bacteria isolated from lake water. Under anaerobic conditions common in sediments, however, experiments have shown coprostanol, as well as cholesterol and cholestanol, to be stable. While coprostanol (and similar compounds) are degraded under aerobic conditions, they appear to be persistent in sediments once it is deposited.

Coprostanol appears to be a good tracer for sanitary sewage. This sterol is specific for human waste, as it is the principal human fecal sterol. The coprostanol content in human feces far exceeds the amounts in dog, duck, and horse fecal matter. A high concentration in sediment samples suggests an impact from human sanitary waste in the area.

The insolubility of coprostanol in water coupled with its association with particulate matter (hence, sediment beds) plays a large role in determining its usefulness as an indicator for human pollution. Coprostanol appears to be more resistant to environmental stress than fecal coliform bacteria and is, in many ways, more reliable an indicator of sanitary waste pollution. A disadvantage of the method is the time required to perform the analysis and the expense of the instrumentation involved, however, this work presents a modified and simpler technique than previous methods, with good precision and sensitivity.

As an investigative tool, the analysis of coprostanol and its stereoisomers shows promise for differentiating between human and non-human sources of fecal bacteria in surface waters and other environmental samples.
VI. LITERATURE CITED


APPENDIX I

CHROMATOGRAMS AND MASS SPECTRA
GAS CHROMATOGRAM OF STANDARD STEROL MIX

Abundance

5a-Cholesterol

Coprosterol

Epi-cholestanol

Epi-coprostanol

Cholesterol

Cholestanol

Time→

0 1000 2000 3000 4000 5000 6000 7000 8000 9000 10000 11000 12000 13000

1 2 3 4 5

STD
GAS CHROMATOGRAM OF DOMESTIC WASTEWATER INFLUENT
MASS SPECTRUM OF CHOLESTEROL

Average of 73.568 to 73.674 min.: S101797A.D

Cholesterol, Deriv. (Full Scan)

MASS SPECTRUM OF COPROSTANOL

Average of 70.695 to 70.848 min.: S101797A.D

Coprostanol, Deriv. (Full Scan)
MASS SPECTRUM OF EPICHOLESTANOL

Average of 70.989 to 71.084 min.: S101797A.D

Epicholestanol, Deriv. (Full Scan)

MASS SPECTRUM OF CHOLESTANOL

Average of 71.154 to 71.248 min.: S101797A.D

Epicoprostanol, Deriv (Full Scan)
MASS SPECTRUM OF EPICOPROSTANOL

Average of 63.784 to 63.937 min.: S101797A.D

MASS SPECTRUM OF 5-ALPHA-CHOLESTANE

Average of 73.827 to 73.933 min.: S101797A.D
CHOLESTEROL STANDARD CURVE

Response Ratio

Cholesterol

Amount Ratio

R = 1.84e-003 A^2 + 6.76e-002 A + 0.00e+000
Curve Fit: Quad/(0,0)

Method Name: C:\MPCHEM1\METHODS\COPRSIM2.M
Calibration Table Last Updated: Tue May 21 09:49:06 1996
COPROSTANOL STANDARD CURVE

R = 7.95e-004 A^2 + 3.31e-002 A + 0.00e+000
Curve Fit: Quad/(0,0)

Method Name: C\text{\textsc{HPCHEM11\textsc{METHODS\textsc{COPRSIM2,M}}}}

Calibration Table Last Updated: Tue May 21 09:49:06 1996
EPICHOLESTANOL STANDARD CURVE

\[ R = 2.07 \times 10^{-3} A^2 + 6.76 \times 10^{-2} A + 0.00 \times 0.00 \]

Curve Fit: Quad(0,0)

Method Name: C3HPCHEM11METHODSCOPRSIM2.M
Calibration Table Last Updated: Tue May 21 09:49:06 1996
CHOLESTANOL STANDARD CURVE

Response Ratio

Amount Ratio

\[ R = 1.09 \times 10^{-3} A^2 + 3.91 \times 10^{-2} A + 0.00 \times 10^0 \]

Curve Fit: Quad/(0,0)

Method Name: C3IMPCHEMM1METHDSCIOPRSIM2M

Calibration Table Last Updated: Tue May 21 09:49:08 1996
APPENDIX III
DERIVATIZED STEROL STRUCTURES
AND CONFORMATION
Bold lines signify projection of the substituent above the plane of the paper, dotted, below.

**CHOLESTEROL**

(CH₃)₃SiO

**COPROSTANOL**

(CH₃)₃SiO

**CHOLESTANOL**

(CH₃)₃SiO

**EPICOPROSTANOL**

(CH₃)₃SiO

**EPICHOLESTANOL**

(CH₃)₃SiO
THIS REPORT DESCRIBES A METHOD FOR PERFORMING A CHEMICAL ANALYSIS OF FECAL STEROLS IN SURFACE WATERS AND SEDIMENTS. THE PURPOSE OF THE REPORT IS TO PROVIDE GUIDANCE TO OTHER INDIVIDUALS OR AGENCIES INTERESTED IN APPLYING THE METHOD FOR THE PURPOSE OF INVESTIGATING THE ORIGINS OF FECAL BACTERIA IN SURFACE WATERS.

THE REPORT DESCRIBES A METHOD FOR EXTRACTING FECAL STEROLS FROM ENVIRONMENTAL SAMPLES AND THE SUBSEQUENT ANALYSIS OF THE STEROLS USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY. THE REPORT PROVIDES A WAY TO DIFFERENTIATE BETWEEN HUMAN AND NON-HUMAN SOURCES OF BACTERIA BASED UPON THE RATIOS OF FECAL STEROLS FOUND IN ASSOCIATION WITH BACTERIA IN ENVIRONMENTAL SAMPLES.

THE REPORT INCLUDES ANALYTICAL STANDARD CURVES FOR THE FECAL STEROLS AND METHOD PERFORMANCE MEASUREMENTS INCLUDING PRECISION, ACCURACY AND DETECTION LIMITS.