ncasi

NATIONAL COUNCIL FOR AIR AND STREAM IMPROVEMENT

DEVELOPMENT AND EVALUATION OF A METHOD FOR THE DETERMINATION OF PHYTOSTEROLS IN PULP AND PAPER MILL EFFLUENTS

TECHNICAL BULLETIN NO. 746

OCTOBER 1997

Acknowledgments

This report was prepared by Diana Cook, Senior Research Scientist at the NCASI West Coast Regional Center. Other assistance was provided by Research Assistants Angela Parrish, Nikki Edgar, Dean Hoy, and Jon Jones.

For more information about this research, contact:

Diana Cook
Senior Reseach Scientist
NCASI West Coast Regional Center
P.O. Box 458
Corvallis, OR 97339
(541) 752-8801

Dr. Robert Fisher
Vice President, Health Effects
NCASI
P.O. Box 13318
Research Triangle Park, NC 27709-3318
(919) 558-1989

For information about NCASI publications, contact:

Publications Coordinator
NCASI
P.O. Box 13318
Research Triangle Park, NC 27709-3318
(919) 558-1987

National Council of the Paper Industry for Air and Stream Improvement, Inc. (NCASI). 1997. *Development and evaluation of a method for the determination of phytosterols in pulp and paper mill effluents*. Technical Bulletin No. 746. Research Triangle Park, N.C.: National Council of the Paper Industry for Air and Stream Improvement, Inc.

NCASI's Mission
To serve the forest products industry as a center of excellence for providing technical information and scientific research needed to achieve the industry's environmental goals.

ncasi

serving the environmental research needs of the forest products industry since 1943

PRESIDENT'S NOTE

Aquatic biology research investigations have recently focused on the possible importance of the discharge of phytosterols from pulp mills. A validated method for measuring the levels of these naturally-occurring substances in pulp and paper mill wastewaters has not been published to date. This research was undertaken to develop and validate an analytical technique for measuring phytosterols, specifically campesterol, stigmasterol, β-sitosterol and stigmastanol, in untreated and biologically-treated combined mill wastewaters. The described method involves liquid/liquid extraction of untreated and biologically-treated combined mill wastewaters at pH 7 with methyl-t-butyl ether, followed by derivatization of the phytosterols as trimethylsilyl derivatives, silica gel clean-up, and analysis by gas chromatography using a flame ionization detector. A method for confirmation using gas chromatography and mass spectrometry is also described. The method has a lower calibration limit of 1.5 μg/L and a detection limit of 0.41 μg/L, 0.43 μg/L, 0.47 μg/L, and 0.44 μg/L for campesterol, stigmasterol, β-sitosterol, and stigmastanol, respectively. This report includes information on the single laboratory validation of the method relating to accuracy and precision.

The bulletin also includes data from biologically-treated effluent samples collected at 23 U.S. mills. The method was used to determine the estimated discharge levels of campesterol, stigmasterol, β-sitosterol, and stigmastanol at 22 U.S. mills. Results from this work show median discharge levels of 0.5 g/T, 0.8 g/T, 4.5 g/T, 1.5 g/T for campesterol, stigmasterol, β-sitosterol, and stigmastanol, respectively. Untreated and biologically-treated wastewater samples from nine mills were collected, analyzed, and used to estimate the removal efficiency for the phytosterols across wastewater treatment systems. Removal of campesterol, stigmasterol, β-sitosterol, and stigmastanol during biological wastewater treatment was found to range from 63% to 93% for campesterol, >38% to 95% for β-sitosterol, and >13% to 96% for stigmastanol, with the exception of one mill site. Mill C yielded estimated removal efficiencies of 2% for campesterol, -25% for β-sitosterol, and -22% for stigmastanol. The estimated removal efficiencies determined for stigmasterol were highly dependent on the type of treatment system. The concentration of stigmasterol decreased across activated sludge systems and increased across aerated stabilization basins. The stigmasterol increase may be due to other sources of plants sterols, such as algae (Nishimura and Koyama 1977), that exist in aerated stabilization basins.

The significance of the presence of these compounds in pulp mill effluent is not known at this time. Hypotheses that phytosterols may affect aspects of individual fish reproduction processes have been forwarded and are being investigated in NCASI's aquatic biology program.

Ronald A. Yeske

October 3, 1997

DEVELOPMENT AND EVALUATION OF A METHOD FOR THE DETERMINATION OF PHYTOSTEROLS IN PULP AND PAPER MILL EFFLUENTS

TECHNICAL BULLETIN NO. 746
OCTOBER 1997

ABSTRACT

A method is reported which will measure phytosterols, specifically campesterol, stigmasterol, β-sitosterol, and stigmastanol, in wastewater treatment plant influents and biologically-treated effluents from kraft, kraft/recycle, kraft/groundwood, thermomechanical/groundwood, and sulfite pulp and paper mills. The isolation technique involves liquid/liquid extraction with methyl-t-butyl ether as the solvent, followed by trimethylsilylation, clean-up (as required) using silica gel, and gas chromatography/flame ionization detector analysis. Confirmation using gas chromatography and mass spectrometry is also described. The method has a limit of detection of approximately 0.4 μg/L, depending upon the sample matrix and target analyte. The method's accuracy and precision have been assessed at the single laboratory level in treatment plant influents and biologically-treated effluents. A full description of the method and preliminary validation is presented. Information on the application of the developed method to estimate discharge levels of campesterol, stigmasterol, β-sitosterol, and stigmastanol from 22 U.S. mills is included. The discharge rates of the different mills ranged from 0.2 to 25.2 g/T. Generally, β-sitosterol discharge levels were the highest, but the observed range of discharge rates varied by more than one order of magnitude. Removal efficiencies were determined for nine U.S. mills utilizing biological treatment systems, including activated sludge systems and aerated stabilization basins. Data from activated sludge systems and aerated stabilization basins showed a decrease for campesterol, β-sitosterol, and stigmastanol across the treatment systems for the majority of mill sites assessed. The only exception to these removal efficiencies was observed for stigmasterol. The concentration of stigmasterol appeared to increase during treatment in aerated stabilization basins and decrease in activated sludge systems. The stigmasterol increase may be due to other sources of plants sterols, such as algae (Nishimura and Koyama 1977), that exist in aerated stabilization basins.

KEYWORDS

campesterol, stigmasterol, β-sitosterol, stigmastanol, aerated stabilization basin, activated sludge, discharge levels, phytosterols, removal efficiency, analysis

RELATED NCASI PUBLICATIONS

Special Report No. 96-07 (December 1996). *Proceedings of the 1996 NCASI West Coast Regional Meeting. Endocrine active substances*.

Special Report No. 94-11 (November 1994). *Proceedings of the 1994 NCASI West Coast Regional Meeting. The endocrine disrupter chemicals issue*.

Technical Bulletin No. 501 (August 1986). *Procedures for the analysis of resin and fatty acids in pulp mill effluents*.

CONTENTS

TABLES

FIGURES

DEVELOPMENT AND EVALUATION OF A METHOD FOR THE DETERMINATION OF PHYTOSTEROLS IN PULP AND PAPER MILL EFFLUENTS

1.0 INTRODUCTION

A number of recent investigations have addressed the potential aquatic biological implications of the discharge of phytosterols from pulp mills (Denton et al. 1985; Hunsinger et al. 1988; Knutson et al. 1995; Mellanen et al. 1996). Although the presence of phytosterols has previously been reported in European kraft (Holmbom 1980; Holmbom and Lehtinen 1980; Dahlman et al. 1993; Oikari and Holmbom 1996; Strömberg et al. 1996) and thermomechanical (TMP) pulp and effluents (Ekman and Holmbom 1989; Ekman et al. 1990; Carlberg 1993), information on the levels of sterols in U.S. pulp and paper mill biologically-treated effluents is not readily available.

A review of the methodology reported for measuring phytosterols revealed that gas chromatography is an effective method for their analysis. A majority of the published methods utilize a solvent extraction followed by trimethylsilyl derivatization and gas chromatography/flame ionization detector (GC/FID) or gas chromatography/mass spectrometer (GC/MS) analysis (Ekman and Holmbom 1989; Holmbom 1980; Oikari and Holmbom 1996). An alternative procedure using adsorption/desorption on XAD-2 resin has also been investigated by Junk and coworkers (Junk et al. 1974). Validation information is not readily available for the methods described in the literature.

Because of ongoing studies by NCASI and others to assess the significance of phytosterols in pulp mill effluents, NCASI undertook the task of developing a tool for measuring the levels of phytosterols in pulp and paper mill effluent samples. A method for analysis of phytosterols in pulp and paper mill treatment system influents and biologically-treated effluents was developed and validated by NCASI at the single laboratory level. A full description of the method and supporting validation data are included in this report. During the course of method development and validation, samples from 23 U.S. mills were analyzed and the discharge levels of campesterol, stigmasterol, β-sitosterol, and stigmastanol were determined for 22 of the mills sampled. Estimates of treatment system removal efficiencies were determined for nine mills.

2.0 METHOD DEVELOPMENT

2.1 Target Analyte Selection

A review of the literature was conducted to aid in determining the phytosterols to target with the sterols method. β-Sitosterol and betulinol have been reported by Oikari and Holmbom in final effluent from a European bleached kraft mill pulping hardwood and softwood (Oikari and Holmbom 1996). The mill they studied was equipped with an activated sludge system for biological treatment. In addition, lupeol, cycloartenol, and methylene cycloartanol have previously been reported by Holmbom in effluent from a Finnish pulp and paper mill (Holmbom and Lehtinen 1980). Sitosterol, betulin, lupeol, stigmastanol, campesterol, campestanol, methylene cycloartanol, and cycloartenol have been identified by GC/MS in elemental chlorine free (ECF) and totally chlorine free (TCF) acetone extracts of oxygen bleached pulp (Jansson et al. 1994). Authentic standards for sterols previously identified in pulp and paper mill samples are readily available at acceptable purities for β-sitosterol, stigmasterol, campesterol, betulin, lupeol, and stigmastanol. Preliminary screening of biologically-treated effluent from U.S. pulp and paper mills indicated that β-sitosterol, stigmasterol, campesterol, and stigmastanol were the most commonly identified phytosterols in the effluents sampled. Therefore, a method was development to

determine the levels of these four sterols in treatment plant influents and biologically-treated effluents. The chemical structures of the target analytes are given in Figure 1.

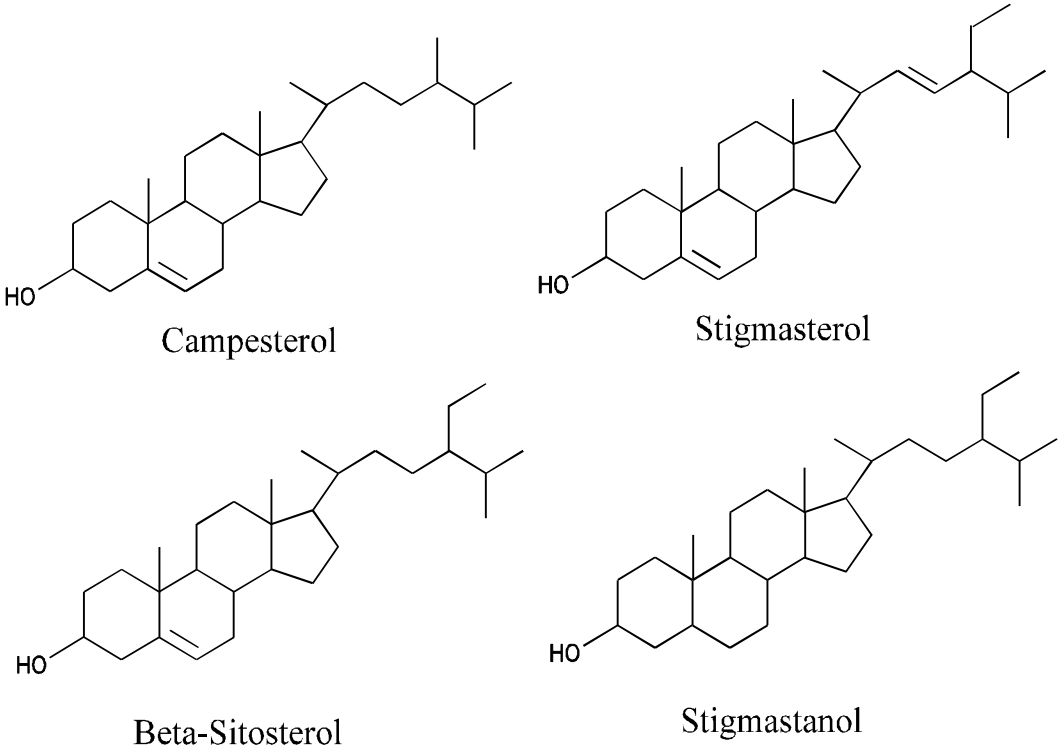


Figure 1. Chemical Structures of the NCASI Phytosterol Method (STER-97) Target Analytes

2.2 Experimental Approaches

Several methods for the isolation of phytosterols from pulp and paper matrices were initially investigated. Experience with a steam distillation approach indicated that the phytosterols do not steam distill effectively. A solid phase extraction approach using C_{18} Empore™ disks was investigated, but experiments resulted in poor recovery and the method was not reproducible. It was hoped that the phytosterols could be analyzed using NCASI Method 85.02 (NCASI 1986) for resin and fatty acids, and that the phytosterols, resin acids (RA), and fatty acids (FA) could be determined with one analysis. This proved to be unsuccessful due to low recoveries of the ethylated phytosterols after the silica gel clean-up employed in the RA/FA method. The extraction of the phytosterols with methyl-t-butyl ether as conducted during the resin and fatty acid analysis method, with minor modifications, proved to be an effective approach for isolating the phytosterols.

2.3 Experiments Conducted to Optimize the Phytosterols Method

A liquid/liquid extraction procedure, similar to the NCASI Method 85.02 for resin and fatty acids, that utilizes methyl-t-butyl ether (MTBE) as the extraction solvent was tested. Initially, spike recovery experiments were conducted to determine the optimum pH for extraction of the phytosterols. Two replicates of 100 mL each of reagent grade water and biologically-treated effluent were adjusted to pH 5, pH 7, and pH 10 and spiked with campesterol, β-sitosterol, stigmasterol, and stigmastanol. In addition, biologically-treated effluent samples that were not fortified with the target analytes were

analyzed to determine background levels for use in calculating recoveries. The samples were extracted once with 25 mL of MTBE, then three times with 20 mL of MTBE. The extracts were concentrated, dried with sodium sulfate, and derivatized with N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) to the trimethylsilyl derivatives. Dotriacontane was added as the internal standard, and the extracts were analyzed by GC/FID. Recoveries of the phytosterol spikes were calculated to assess the optimum pH for extraction of the target analytes. The average recovery for the two replicates at pH 5, pH 7, and pH 10 and the concentration detected are listed in Table 1.

Table 1. Recovery of Phytosterol Spikes from Reagent Grade Water and Biologically-Treated Effluent at pH 5, pH 7, and pH 10 Using a MTBE Extraction

	Campesterol	Stigmasterol	β-Sitosterol	Stigmastanol	Total Sterols
Reagent Grade Water	%	%	%	%	%
pH 5	95	91	94	95	94
pH 7	92	87	90	96	91
pH 10	70	66	68	73	69
Final Effluent	% (µg/L)	% (µg/L)	% (µg/L)	% (µg/L)	%
pH 5	89 (209)	97 (186)	NA (574)	80 (194)	88
pH 7	82 (196)	91 (175)	NA (646)	81 (195)	85
pH 10	86 (205)	94 (181)	NA (677)	85 (203)	88

NA - not available due to an insufficient spike level in the matrix, which had high native levels of β-sitosterol

The results of these experiments indicate that the recovery of phytosterols is slightly higher at pH 5 in reagent grade water than at pH 7, and is lowest at pH 10. The total sterol recovery at pH 7 varied by less than 3% from the total sterol recovery at pH 5, indicating that the phytosterols are effectively recovered at pH 5 or pH 7 from reagent grade water using the procedure described above.

The total sterol recoveries determined in biologically-treated effluent varied by less than 3% at pH 5, pH 7, and pH 10, indicating that the phytosterols can be effectively recovered at pH 5, pH 7, or pH 10 from the biologically-treated effluent sample. Although the percent recovery of β-sitosterol was not determined due to the high native concentration in the sample, comparison of the determined concentrations indicates that it is also effectively extracted at pH 7 and pH 10. Comparison of the GC/FID chromatograms from the effluent samples extracted at pH 5 and pH 7 indicates that the amount of extraneous organic materials is much lower at pH 7. This observation has also been noted by Voss during the extraction of effluent samples for resin and fatty acids with MTBE (Voss and Rapsomatiotis 1985). Therefore, pH 7 was selected as the optimum for sample extraction to avoid the potential for elevated baselines and possible interferences.

2.4 Distribution Experiments

Experiments were conducted to determine the distribution of the target analytes between the solid and aqueous phases of biologically-treated effluents. Table 2 lists the results of these distribution experiments. The results obtained for three of the four biologically-treated effluents tested, using soxhlet extraction of filtered solids in combination with liquid/liquid extraction of the filtrate, varied by

15% or less from the results obtained by liquid/liquid extraction of the whole effluent. Higher variability was observed in the Mill C matrix, which had the lowest total suspended solids content of the four mills tested.

Table 2. Distribution Experiment Results

Mill Code	TSS (mg/L)[a]	Percent in Aqueous[b]	Percent on Solids[c]	Concentration µg/L Aq + Solids	Concentration µg/L Whole Effluent[d]	RPD(%)[e]
B	80	14	86	67	77	15
C	33	58	42	366	555	40
M	281	10	90	244	229	6
W	109	35	65	188	180	5

[a] Total suspended solids, determined using Standard Method 2540D
[b] Determined by MTBE extraction of the filtrate passing through a GF/A-E glass fiber filter
[c] Determined by soxhlet extraction of the filter residue with MTBE
[d] Determined by liquid/liquid extraction of the whole effluent with MTBE
[e] This refers to the relative percent difference between the results obtained using the liquid/liquid extraction method on the whole effluent samples, and the results obtained when adding the concentrations from the aqueous filtrate extraction and solid filter residue extraction.

Isolation and quantitation of phytosterols in whole effluent samples, extracted at pH 7 with MTBE, gave comparable or better results than soxhlet extraction of filtered solids in combination with liquid/liquid extraction of the filtrate. Based on these results, a one-step liquid/liquid extraction procedure using whole effluent samples was adopted.

2.5 Brief Description of NCASI Method STER-97

A brief description of the analytical method is given below. Appendix A contains the entire method and single laboratory method performance results.

2.5.1 *Extraction Procedure*

A 100 mL aliquot of sample is adjusted to pH 7 using 4.7 M potassium carbonate solution. The sample is buffered with monobasic potassium phosphate and dibasic sodium phosphate and extracted once with 25 mL of MTBE, then three times with 20 mL MTBE. The combined solvent extract is concentrated, exchanged into hexane, and passed through a column of sodium sulfate. The sample is then concentrated to a volume of approximately 250 µL.

2.5.2 *Derivatization Techniques*

The 3β-hydroxy group of the phytosterols is reactive enough towards the standard silylating agents to quantitatively form the trimethylsilyl derivatives under relatively mild conditions. The sterols are derivatized as the trimethylsilyl ethers using BSTFA. The procedure followed for the early part of this work involved taking the extract to dryness using nitrogen, suspending the target analytes in 250 µL of BSTFA, and reacting overnight at room temperature (Gleispach 1974). Although this technique provided an effective method for derivatization of the target analytes, it required an overnight reaction

time. An abbreviated procedure was developed that requires an hour at room temperature for effective derivatization of the target analytes. The sample extract is concentrated to a volume of 250 µL and derivatized by the addition of 250 µL of acetone and 100 µL of BSTFA. The derivatization is complete after approximately one hour at room temperature. For samples requiring silica gel clean-up, the derivatized extract is taken to dryness using nitrogen blowdown and resuspended in approximately 500 µL of hexane. This step is included to remove the acetone and the byproducts of the BSTFA derivatizing agent, which are volatile and can alter the elution profile of the phytosterols if present in the extract. Samples that do not require column clean-up are processed as described in Section 2.5.4.

2.5.3 *Silica Gel Clean-up of the Extracts*

Silica gel clean-up of the sample extracts is necessary for samples containing high background levels of non-target analytes in the retention time intervals of the phytosterols, surrogate, and internal standard. Elevated baselines can cause problems with accurate integration of the peaks, and may result in a bias in the concentrations of target analytes reported. Clean-up steps are also recommended for samples containing large quantities of extraneous organics. The clean-ups reduce the frequency of instrument maintenance, such as replacing injection port liners, and preserve the life of the column. If silica gel clean-up is required, the following procedure is recommended. Prepare activated silica gel columns as described in Appendix A, Section 11.6.1. The column clean-up procedure described in full detail in Appendix A, Section 11.6 is briefly summarized and explained in this section.

An indicator solution composed of azulene (blue) and sudan I (yellow/orange) is added to the sample extract and the extract is qualitatively transferred to a column pre-eluted with hexane. The column is eluted with a solvent system made up of 95% hexane and 5% MTBE. This solvent system was selected after running column profile experiments to determine a solvent system that would separate the sterols from non-target analytes and provide the highest phytosterol recoveries. Start collecting the fraction containing the phytosterols just before the azulene (blue band) reaches the bottom of the column. Collect 2 mL of the eluant in a graduated conical tube, prior to the elution of the Sudan I (yellow-orange band). Column profile experiments indicate that the highest recovery of phytosterols results from collection and analysis of this fraction. The extract fraction is concentrated to 500 µL using nitrogen blowdown and transferred to a 2 mL autosampler vial with two hexane rinses. The extract is concentrated to approximately 500 µL, and the internal standard is added as described in Section 2.5.4.

2.5.4 *Addition of the Internal Standard*

The internal standard selected for these analyses was dotriacontane. Dotriacontane is not a chemical constituent of pulp and paper mill wastewaters, and interferences have not been encountered in the wastewaters sampled to date by this laboratory. Dotriacontane has a molecular weight of 450 g/mole, very similar to the derivatized phytosterols which range from 466 to 482 g/mole, and elutes at a retention time just prior to the phytosterols and just after the surrogate using the GC conditions of the STER-97 method.

The dotriacontane is spiked into the extract and a 1 µL injection is analyzed by splitless capillary gas chromatography using GC/FID. Confirmation can be done by gas chromatography/mass spectrometry using the approach described in Appendix A, Section 11.9.

2.5.5 *Analyses - GC/FID and GC/MS*

All GC/FID analyses were performed using a Hewlett-Packard Model 5890 gas chromatograph with a J & W 30 m x 0.25 µm x 0.25 mm I.D. DB-5 capillary column. The GC conditions were: 130°C (1

min. hold) to 280°C @ 15°C/min. (15 min. hold); injector, 290°C; detector, 320°C; carrier gas, hydrogen. The concentration of target analytes was calculated using average relative response factors generated from a six-point calibration curve which spanned the range of 1.5 to 380 µg/L. The extracts of samples with concentrations greater than this range were diluted into the concentration range and reanalyzed.

All GC/MS analyses were performed using a Hewlett-Packard Model 5972 gas chromatograph with a J & W 30 m x 0.25 µm x 0.25 mm I.D. DB-5 capillary column. The GC conditions were: 130°C (1 min. hold) to 280°C @ 15°C/min. (15 min. hold); injector, 290°C; detector, 290°C; carrier gas, helium.

The sterols are qualitatively identified and quantitated based on relative retention times and response factors determined from authentic standards during the GC/FID analyses. During the GC/MS analyses, the sterols are qualitatively identified and quantitated based on relative retention times, ion abundances, and response factors determined from authentic standards. The β-sitosterol (Sigma, 97% pure), campesterol (Sigma, 97% pure), stigmasterol (Fluka, 98% pure), stigmastanol (Sigma, 98% pure), and internal standard dotriacontane (Aldrich, 97% pure) were verified for purity by GC/MS and GC/FID as single component solutions prior to use.

2.6 Selection of the Surrogate

Surrogates are frequently utilized in analytical methods to provide a means of monitoring the extraction efficiency of each sample and the effects the sample matrix may have on the analytical method. Each sample analyzed is fortified with the surrogate compound and analyzed under the same conditions as the actual samples. The recovery of the surrogate compound is determined, giving an indication of the method's effectiveness in that particular matrix. An effective surrogate compound should mimic the behavior of the target analytes, not interfere with the analysis of the target analytes, and not be found in the samples to which the method will be applied. Surrogate compounds were selected for investigation based on their similarity to the target phytosterols with regard to chemical structure, molecular weight, retention time during GC analysis, stability, and availability. The following compounds were investigated during the course of method development as possible surrogates: α-coprostanol, ergosterol, dehydroergosterol, and cholesterol. α-Coprostanol did not effectively derivatize with BSTFA, and its use as a surrogate was not pursued. Ergosterol, though very similar in structure to the phytosterols, is air and light sensitive as well as highly toxic. For these reasons it was not selected as a surrogate. Dehydroergosterol is also air and light sensitive and was found to be unstable in stock spiking solutions stored longer than two weeks, making it an impractical choice as a surrogate.

The use of cholesterol as the surrogate in this method was investigated only after attempts to find an alternate surrogate failed. Cholesterol is a known constituent of wastewater from sewage treatment sources and has been found to be a trace constituent of pine bark (Rowe 1965). Therefore, it is recommended that samples from new sources be analyzed without the addition of cholesterol to determine if it is native to the sample matrix. If the NCASI Method STER-97 surrogate cholesterol is native to the sample, matrix spike and matrix spike duplicate analyses should be conducted in the matrix to assess method performance, in preference to surrogate recovery.

3.0 METHOD VALIDATION INFORMATION AND DISCUSSION

3.1 Instrument Precision, Linearity, and Calibration of the GC/FID Using NCASI Method STER-97

3.1.1 *GC/FID Precision*

The GC/FID instrument precision was verified by the analysis of the midpoint of the calibration curve a total of seven times over an eight-hour period. The results of the GC/FID instrument precision experiment are listed in Table 3.

Table 3. GC/FID Instrument Precision: Results from Seven Replicate Analyses

	Concentration of Midpoint	Average Relative Response Factor	Standard Deviation	Relative Standard Deviation (%)
Campesterol	45.5 µg/L	0.79	0.0017	0.21
Stigmasterol	47.3 µg/L	0.97	0.0012	0.13
β-Sitosterol	46.6 µg/L	0.66	0.0010	0.15
Stigmastanol	43.3 µg/L	1.02	0.0012	0.12

3.1.2 *Linearity*

The linearity of the GC/FID method was assessed by analyzing a six-point calibration curve that ranged from concentrations of 1.5 to 380 µg/L. The calibration criteria are expressed as maximum relative standard deviations (RSD) in the average relative responses (RRF) for the method. During the course of this study, eight calibration curves were prepared and analyzed. A statistical summary of the data from the eight calibration curves analyzed is listed in Appendix A, Table A7. The average relative response factors and average relative standard deviations determined for campesterol, stigmasterol, β-sitosterol, and stigmastanol were 0.75 (RSD 5.9%), 0.91 (RSD 5.3%), 0.62 (RSD 6.2%), and 0.93 (RSD 4.1%), respectively. Figure 2 illustrates the linearity normally observed for a six-point calibration curve. It is a graph of the response (area) versus the concentration in the calibration point for one of the eight curves analyzed.

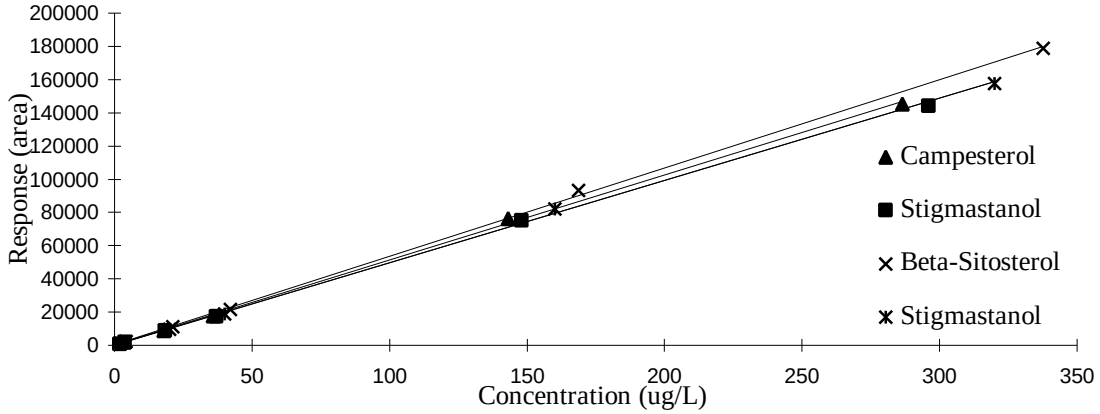


Figure 2. Calibration Curve Linearity

3.1.3 *Calibration Verification Results*

The working method calibration curve was verified with each set of samples analyzed by selecting one of the six points in the calibration curve and reanalyzing the standard, using the same instrument conditions used to prepare the calibration curve, prior to sample analyses. The relative response factors for the point analyzed are compared to the relative response factor determined for that point during the current calibration curve. A summary of the daily method calibration verification results obtained during the process of method validation studies is presented in Table 4. The mean relative percent differences (RPDs) observed for the daily calibration verifications were less than 3.1% for all of the target analytes and the surrogate.

Table 4. Summary of Daily Method Calibration Verification

Compound	Standard Deviation of the RPDs	Mean RPD (%)	Range of RPDs	N
Campesterol	2.1	2.6	0.1 - 8.2	39
Stigmasterol	2.2	2.6	0.1 - 9.5	39
β-Sitosterol	3.7	3.1	0.1 - 17	39
Stigmastanol	2.8	3.1	0.1 - 14	39
Cholesterol (S)	2.2	2.8	0.2 - 7.3	18

(S) Surrogate

Instrument conditions are considered to be steady if the relative response factors of the target compounds deviate by no more than 15% from the relative response factors for the calibration check point in the most recent calibration curve. Based on the single laboratory data described in Table 4, this limit reflects the mean RPD observed plus three times the standard deviation of the RPDs. If this criterion was not met, instrument conditions were assessed and corrections were made to achieve the criterion, and the calibration point was reanalyzed prior to the analysis of sample extracts. In the event that the calibration verification failed again, remedial action was taken, and a new calibration curve was prepared and reanalyzed prior to sample analyses.

3.2 Stability Studies

3.2.1 *Sample Preservation Investigations*

Experiments were conducted to determine a method of preservation for phytosterols in biologically-treated effluents. Standard storage techniques for biologically active samples involve preservation at pH 2 and refrigeration at 4°C. The comparative stability of campesterol, β-sitosterol, stigmasterol, and stigmastanol was assessed in a biologically-treated effluent sample from a kraft mill pulping softwood and equipped with an aerated stabilization basin for biological treatment. The samples were preserved at pH 3.5, pH 7, and pH 10 upon receipt at the laboratory. All samples were stored at 4°C prior to extraction. Three replicates at each pH were analyzed using the NCASI STER-97 method at day 1, day 7, day 14, and day 21. Figure 3 illustrates the general trend observed over the 21 days of this stability study for β-sitosterol (the phytosterol most commonly detected in effluent samples). Figure 3 is a graph of the average concentration of the three replicates preserved at pH 3.5, pH 7, and pH 10 on

each day of the study. Further statistical analyses were done on the data collected for each compound over the 21 days of the stability study.

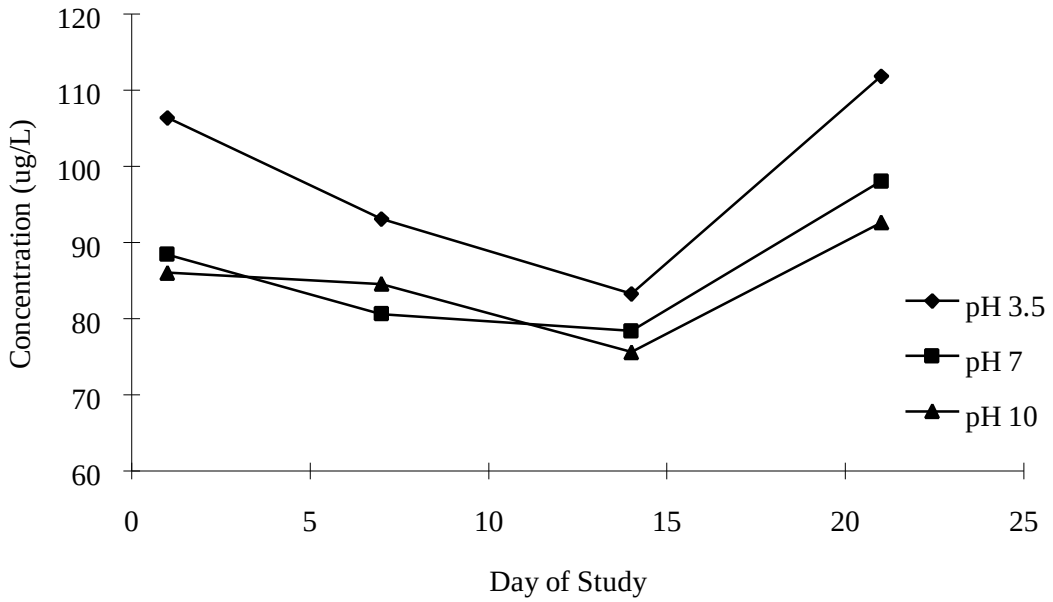


Figure 3. Average Concentration Versus Time for β-Sitosterol at pH 3.5, pH 7, and pH 10

A summary of the definitions and descriptions of statistical terms used throughout this bulletin can be found in Appendix B, Section B1. A linear regression analysis was conducted to determine if the concentrations in the samples preserved at each of the various pHs changed significantly with time. A summary of these results is given in Table 5, and a complete listing can be found in Appendix B, Section B2.

Table 5. Summary of Linear Regression Statistics for Phytosterol Stability in Biologically-Treated Effluent Preserved at pH 3.5, pH 7, and pH 10

Analyte	pH 3.5 P-Value	pH 7.0 P-Value	pH 10.0 P-Value
Campesterol	0.84	0.19	0.53
Stigmasterol	0.78	0.59	0.31
β-Sitosterol	0.86	0.26	0.60
Stigmastanol	0.95	0.12	0.97

The p-values provide a valid indication of the potential for the data to suggest a time-dependent change in concentration. The significance of the change was assessed based on a selected alpha value of 0.05, equivalent to a significance level of 5%. If the calculated p-value was less than 0.05, the change in concentration with time was judged to be significant. Analysis of the calculated p-values suggests that

the concentration of campesterol, stigmasterol, β-sitosterol, and stigmastanol did not change significantly with time when stored at pH 3.5, pH 7, or pH 10 and 4°C. The p-values for the slope of the regression were all greater than 0.05 at pH 3.5. The samples preserved at pH 3.5 showed smaller changes in concentration over time than the samples preserved at pH 7 and pH 10. Based on the results of this experiment, storage at 4°C and sample preservation at pH 2 to pH 3.5 using sulfuric acid were adopted for the preservation of sterols in biologically-treated effluent samples.

3.2.2 *Extract Stability*

Experiments were conducted to determine the stability of the trimethylsilyl derivatives of the phytosterols in the hexane/acetone extracts stored at 4°C over a period of 30 days. The extracts were analyzed by GC/FID on the day they were extracted, and reanalyzed 30 days later. The concentrations determined on day 1 were compared to the concentrations determined on day 30 for two extracts prepared using the method described in Appendix A. Extract A was prepared from a final effluent from a bleached kraft mill pulping softwood and equipped with an aerated stabilization basin for biological treatment. Extract B was prepared from a final effluent from an unbleached kraft mill pulping softwood and equipped with an aerated stabilization basin for biological treatment. The results listed in Table 6 indicate that the trimethylsilyl derivatives of campesterol, stigmasterol, β-sitosterol, stigmastanol, and cholesterol are stable in the hexane/acetone extracts stored at 4°C for a minimum of 30 days.

Table 6. Comparison of Phytosterol Concentrations in Extracts Analyzed on Day 1 and Day 30

Analyte	Extract A			Extract B		
	Day 1 µg/L	Day 30 µg/L	RPD (%)[a]	Day 1 µg/L	Day 30 µg/L	RPD (%)[a]
Campesterol	30.8	27.9	10	7.7	7.4	3
Stigmasterol	8.3	7.9	5	8.1	8.1	0
β-Sitosterol	388	393	1	96.6	96.3	0.2
Stigmastanol	38.6	36.4	6	7.8	7.8	0
Cholesterol (S)	62.5	60.8	3	1.9	1.9	0

[a] Relative percent difference
(S) Surrogate

4.0 METHOD PRECISION AND ACCURACY

4.1 Method Accuracy - Matrix Spike and Surrogate Recovery

The accuracy of the method, as indicated by the recovery of spiked analytes, was determined in biologically-treated effluents from 13 mills and treatment system influents from 11 mills. The results of matrix spike recovery experiments conducted using biologically-treated effluents are presented in Figure 4. A complete summary of the matrix spike experiments in effluents and influents is provided in Appendix A, Table A5. The average percent recovery of campesterol, stigmasterol, β-sitosterol, and stigmastanol from biologically-treated effluents was 74%, 74%, 81%, and 74%, respectively.

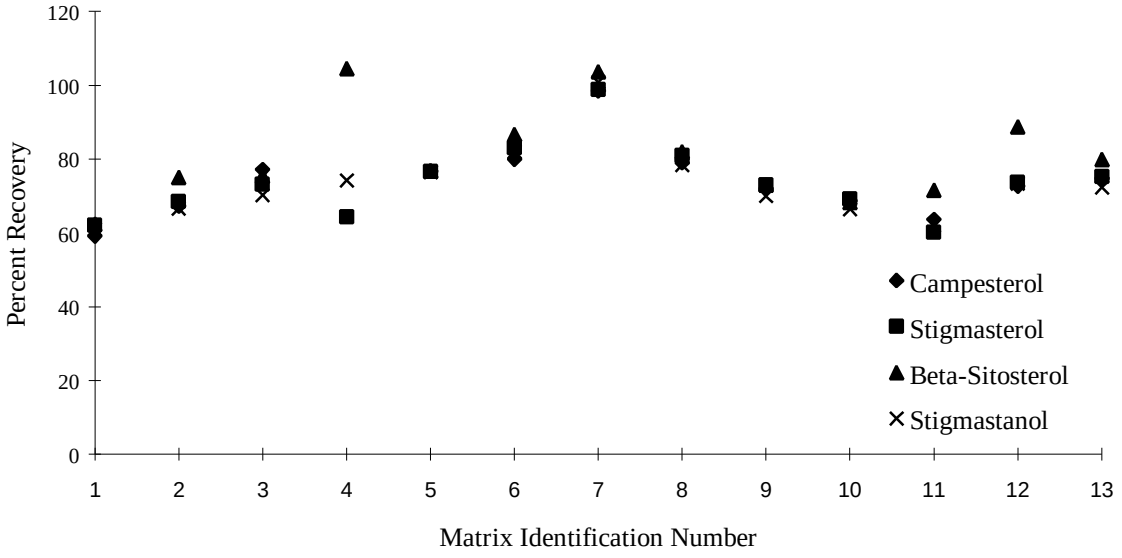


Figure 4. Results of Matrix Spike Experiments Conducted in Biologically-Treated Effluents from Thirteen Mills

The matrix spike recovery experiments conducted in treatment system influents are presented in Figure 5. The average percent recovery of campesterol, stigmasterol, β-sitosterol, and stigmastanol from treatment system influents was 84%, 78%, 92%, and 76%, respectively.

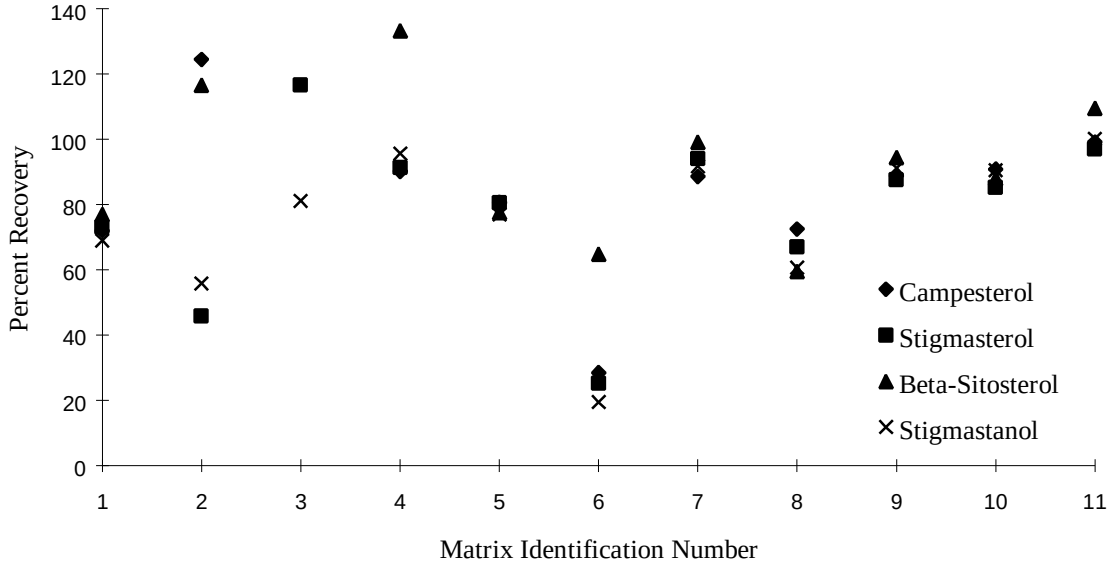


Figure 5. Results of Matrix Spike Experiments Conducted in Treatment System Influents from Eleven Mills

A regression analysis was conducted to examine the relationship between the concentration of the matrix spike and the percent recovered. These data are summarized in Table 7, and a full description of the statistics are given in Appendix B, Section B3.

Table 7. Statistical Results of Correlations Between Spike Concentration and Percent Recovery of Phytosterol Matrix Spikes

Compound	F-Ratio	P-Value
Campesterol	2.74	0.11
Stigmasterol	3.83	0.06
β- Sitosterol	3.66	0.07
Stigmastanol	3.58	0.07

The statistical analysis indicates that for spike concentrations between 67 and 281 μg/L for campesterol, 75 and 272 μg/L for stigmasterol, 47 and 285 μg/L for β-sitosterol, and 76 and 262 μg/L stigmastanol, the p-values ranged from 0.06 to 0.11, suggesting a tendency for the percent recovery to decrease with increasing spike concentration.

The matrix spike experiments were conducted using a combined solution of all the phytosterols; the level at which the samples were spiked was based on the concentration of β-sitosterol native to the sample. The concentration of β-sitosterol was higher than the other phytosterols in all of the matrices analyzed, and an effort was made to spike the target analytes at a minimum of two times the native level during the matrix spike experiments. These data represent only results for matrix spike experiments conducted at the mid to upper concentration range of the method. The relationship between the percent recovery and spike levels for lower concentrations was not investigated in the pulp and paper matrices sampled.

4.2 Method Precision - Replicate Analyses

The precision of the method was characterized and continuously monitored using duplicate analyses with each group of samples analyzed. Single laboratory precision assessments in biologically-treated effluents were based on 35, 36, 38, and 36 experiments for campesterol, stigmasterol, β-sitosterol, and stigmastanol, respectively. Six experiments were conducted to assess the method precision in treatment plant influent samples. These experiments provided information on the precision of the method at a wide range of concentrations for phytosterols native to the samples analyzed. A summary of these results is given in Appendix A, Table A6. The mean relative percent differences calculated in biologically-treated effluents was 7 to 8%, and ranged from 0.1 to 28% depending on the compound and the sample matrix. The mean relative percent differences calculated in treatment system influents were 17%, 22%, 11%, and 17%, respectively, for campesterol, stigmasterol, β-sitosterol, and stigmastanol, and ranged from 2.3 to 37% depending on the compound and the sample matrix.

4.3 Method Detection Limits

Estimates of the method's detection limits and low level precision were determined using a biologically-treated effluent sample from a kraft mill producing unbleached softwood pulp and equipped with an aerated stabilization basin biological treatment system. The sample was spiked with the target analytes to achieve a final concentration ranging from 1.5 to 3.0 μg/L. Seven replicates of the spiked sample were analyzed using the method in Appendix A. The relative standard deviation (RSD) was calculated

in order to allow for precision assessment. From this information, a method detection limit was estimated by multiplying the standard deviation of the seven replicates by 3.143 (Federal Register 1984). From these calculations, estimated detection limits of 0.41 µg/L, 0.43 µg/L, 0.47 µg/L, and 0.44 µg/L were determined for campesterol, stigmasterol, β-sitosterol, and stigmastanol, respectively.

4.4 Analytical Considerations

All the chromatographic data generated during method validation were carefully examined for possible interferences, the presence of non-derivatized target compounds, stability of instrument conditions, and degradation of the standards. The area counts of the dotriacontane internal standard were tracked over time and found to be a valuable indicator of changing instrument conditions. The area counts for dotriacontane were relatively constant, deviating by less than 5%. A decrease in the dotriacontane area counts can indicate degradation of the standard, poor injection of the extract onto the GC column, or changes in instrument conditions.

Examination of the resulting gas chromatographs for peaks with the relative retention times indicated in Table 8 can identify incomplete derivatization of the phytosterols which will result in a low bias of the analytical results. The non-derivatized phytosterols elute from the GC column prior to their derivatized analogs under the GC/FID conditions of the STER-97 method. Throughout the method validation studies, non-derivatized phytosterols were detected when the sample extracts were not sufficiently dried prior to addition of the BSTFA. For this reason, the method was modified to include passage of the extract through a sodium sulfate drying column prior to the final concentration step. After this procedure was adopted, the non-derivatized phytosterols were not observed in the samples or standards analyzed.

Table 8. Relative Retention Times of the Underivatized and BSTFA Derivatized Phytosterols

Compound	Relative Retention Time Underivatized Sterols	Relative Retention Time BSTFA Derivatized Sterols
Campesterol	1.045	1.081
Stigmasterol	1.077	1.113
β-Sitosterol	1.141	1.181
Stigmastanol	1.152	1.194

5.0 STEROL LEVELS IN MILL INFLUENTS AND BIOLOGICALLY-TREATED EFFLUENTS

Using the method detailed above, a survey of plant sterol discharge levels from biological treatment systems representing a cross-section of geographical regions, pulping types, wood furnish, and treatment types was conducted at 22 of the 23 mills listed in Table 9. The sites include major pulp producing regions of the U.S. and represent pulping technologies including bleached and unbleached kraft, bleached sulfite, kraft and recovered fiber, thermomechanical and groundwood, and different types of recycled fiber (deink and non-deink). In addition to estimating discharge levels, the effectiveness of activated sludge and aerated stabilization basins for the removal of phytosterols was assessed at nine mill sites.

Table 9. Mill Sampling Site Descriptions

Mill Code	Treatment System[a]	Pulping Process[b]	Bleaching	Furnish[c]	Geographic Location[d]
A	AS(UNOX)	Kraft/Recycle	Yes	SW, HW, OCC	S[e]
B	ASB	Kraft	Yes	SW	S
C	ASB	Kraft	No	SW	S
D	ASB	Kraft	Yes	SW	S
E	ASB	Kraft	No	SW	S
F	ASB	Kraft/TMP/GW	Yes	SW	S
G	AS	Kraft/GW	Yes	SW, HW	NE
H	AS	Kraft	Yes	SW, HW	W
I	AS	Kraft	Yes	SW	S
J	AS	Sulfite	Yes	SW	C
K	AS	Kraft	Yes	HW	NE
L	ASB	Kraft	Yes	SW, HW	S
M[e]	AS	Recycle/Non-deink	No	OCC	W
N	ASB	Kraft	Yes	SW	S
O	ASB	Kraft	Yes	SW, HW	S
P	ASB	Kraft	Yes	SW	W
Q	ASB	Kraft	No	SW	W
R	AS	Kraft	Yes	SW, HW	NE
S	ASB	Recycle/Non-deink	No	OCC	S
T	AS	TMP/GW	Yes	SW	NE
U	ASB	Recycle/Non-deink	No	OCC	S
V	ASB	GW/TMP/deink	Yes	Recovered fiber/ONP	S
W	ASB	GW/TMP/deink	Yes	Recovered fiber	W

[a] AS, activated sludge; ASB, aerated stabilization basin
[b] TMP, thermo-mechanical pulping; GW, groundwood,
[c] SW, softwood; HW, hardwood; OCC, old corrugated containers; ONP, old newspapers
[d] S, south; NE, northeast; C, central; W, west
[e] Discharge levels were not determined for this mill sample since flow and production rates were not provided. The concentrations in the influents and effluent were utilized to assess treatment system removal efficiency.

5.1 Phytosterol Discharge Levels

Samples for this portion of the study were collected in glass bottles with Teflon™-lined screw caps and preserved at pH 2 in the field using sulfuric acid. Samples were stored at 4°C and extracted within 30 days. Grab samples were collected from aerated stabilization basin treatment systems. Composite sampling schemes were used for mills with activated sludge treatment systems. The estimated discharge levels were determined only when analytes were detected in treated effluent and when sampling episodes involved a minimum of four samples collected over a period of one month.

If less than half of the effluent data points had non-detect concentrations, the lowest calibration point (1.5 µg/L) was substituted for the non-detects and the average concentration was calculated. This representation of the data provides a high biased, or upper limit, estimate of the discharge levels. The average concentrations, effluent flow, and production were determined for each sampling episode. The

averages were used to compute the discharge levels. The estimated discharge levels per air dried ton (10% moisture) of pulp produced for 22 U.S. mills are summarized in Table 10.

Table 10. Estimated Phytosterol Discharge Levels for 22 U.S. Mills (g/T)

Mill Code	Treatment System	Campesterol	Stigmasterol	β-Sitosterol	Stigmastanol	Total Sterols
Kraft						
G	AS	0.8	3.1	10.4	4.9	19.2
I	AS	-	-	0.4	-	0.4
K	AS	-	-	0.2	-	0.2
R	AS	0.5	0.7	6.8	1.3	9.3
B	ASB	0.7	1.0	12.0	1.1	14.8[a]
C	ASB	0.5	0.6	8.1	0.6	9.9[b]
D	ASB	0.4	0.8	4.2	2.8	8.1
E	ASB	1.0	1.0	12.1	2.8	16.8
L	ASB	1.0	0.8	12.8	3.1	17.7
N	ASB	0.2	1.1	4.3	1.0	6.6
O	ASB	0.8	1.0	10.7	1.5	14.0
P	ASB	1.4	1.4	7.3	1.6	11.7
Q	ASB	0.1	0.1	0.5	0.1	0.7
Kraft/GW						
H	AS	1.7	-	3.4	1.8	6.9
Kraft/ Recycle						
A	AS	0.4	0.3	4.6	1.5	6.8
Kraft/GW/ TMP						
F	ASB	1.6	1.6	20.3	1.7	25.2
Sulfite						
J	AS	-	2.8	2.6	1.5	6.9
TMP						
T	AS	0.2	0.7	0.7	0.1	1.8
GW/TMP/ Deink						
V	ASB	0.1	0.3	0.6	0.1	1.1
W	ASB	1.0	0.1	4.9	0.7	6.7
Recycle/ Non-Deink						
S	ASB	0.1	0.1	0.1	-	0.3
U	ASB	0.1	0.3	0.3	0.1	0.7

[a] The estimated discharge levels ranged from 6.9 to 28.6 g/T during a thirteen-month period.

[b] The estimated discharge levels ranged from 5.8 to 22.3 g/T during a thirteen-month period.

The estimated discharge levels for the sterols ranged from 0.2 to 25.2 g/T. β-Sitosterol was the only phytosterol (analyte) quantified in all samples, and its discharge level was generally the highest. The data were evaluated for trends between the discharge levels and location (South, West, Northeast), treatment system (AS or ASB), and bleached or unbleached pulp. The parameters were assessed using analysis of variance between the different groups of discharge levels for campesterol, stigmastanol, β-sitosterol, or stigmastanol. For example, there are two categories of mills, those that bleach and those that do not; therefore, the discharge levels for the mills that bleach were compared to the discharge levels for the mills that did not bleach. Table 11 summarizes the statistical results for the parameters examined. A complete description of the statistical results can be found in Appendix B, Section B4.

Table 11. Statistical Results of Correlations Between Treatment System Type, Mill Location, Bleaching, and Phytosterol Discharge Levels

Dependent Variable	Factor	F-Ratio	P-Value
Campesterol	Treatment System Type	0.08	0.782
Campesterol	Mill Location	1.55	0.243
Campesterol	Bleaching	2.56	0.128
Stigmasterol	Treatment System Type	3.88	0.066
Stigmasterol	Mill Location	NA	NA
Stigmasterol	Bleaching	2.90	0.107
β-Sitosterol	Treatment System Type	2.07	0.166
β-Sitosterol	Mill Location	0.49	0.692
β-Sitosterol	Bleaching	0.53	0.476
Stigmastanol	Treatment System Type	0.74	0.402
Stigmastanol	Mill Location	NA	NA
Stigmastanol	Bleaching	1.17	0.295
Total Sterols	Treatment System Type	1.00	0.330
Total Sterols	Mill Location	0.19	0.903
Total Sterols	Bleaching	0.97	0.336

NA - not applicable due to a statistically significant difference amongst the standard deviations

The results of the single factor analysis of variance indicate that the discharge levels of campesterol, stigmastanol, β-sitosterol, or stigmastanol do not significantly correlate with treatment system type, mill location, or whether the pulp is bleached or unbleached for the 22 mills sampled ($p>0.05$).

A statistical analysis of the relationship between campesterol, stigmastanol, β-sitosterol, or stigmastanol discharge levels and mill process or mill furnish could not be performed due to the lack of a sufficient number of data points representing each process or furnish type. However, some general trends were observed and graphical summaries of campesterol, stigmastanol, β-sitosterol, or stigmastanol discharge levels plotted by mill process and mill furnish are included in Appendix B, Section B4. Generally, mills that use recycled fibers have lower median discharge levels of

stigmasterol, β-sitosterol, and stigmastanol. No conclusions could be drawn based on the mill process and discharge level data.

5.2 Estimated Removal Efficiencies

Conservative estimates of the treatment efficiencies for nine mill sites were determined using the following guidelines. Estimates based on single influent and effluent composite samples were only made for mills with activated sludge treatment systems. Estimates from aerated stabilization basin treatment systems were based on multiple samplings. The estimated treatment system removal efficiencies of sterols for five mills using aerated stabilization basins and for four mills using activated sludge treatment systems are given in Table 12. Treatment efficiencies were calculated based on average influent and average effluent concentrations for the sampling period. When a non-detect was encountered in an effluent sample, the lowest calibration limit was substituted. This procedure results in underestimation of treatment efficiency. Such estimates are flagged with a ">" in Table 12, indicating the treatment efficiency is expected to be higher than the number reported.

Table 12. Summary of Estimated Phytosterol Removal Efficiencies at Nine U.S. Mills

Mill Code	Campesterol	Stigmasterol	β-Sitosterol	Stigmastanol	Total Sterols
ASB	(%)	(%)	(%)	(%)	(%)
B	63	-316	78	62	68[a]
C	2	-367	-25	-22	NA
D	65	-377	62	55	56[a]
E	81	-192	81	54	78[a]
AS					
A	88	88	86	90	88
G	71	66	71	96	70
H	93	>95	95	94	95
I	>85	-	>38	>13	NA
J	-	>61	89	96	89

[a] stigmasterol was omitted from the total sterol removal efficiency calculation
NA - not applicable

In aerated stabilization basin systems, stigmasterol generally increased across the treatment system. The stigmasterol increase may be due to other sources of plants sterols, such as algae and microorganisms (Nishimura and Koyama 1977), that are more likely to contribute phytosterols in aerated stabilization basins with long retention times and quiescent ponds, than in activated sludge systems. The estimated removal efficiencies at mill sites using activated sludge treatment systems ranged from 61 to 96% for the majority of sites. The sterol levels in the influent of Mill I were near the method detection limit, making interpretation of removal efficiencies difficult. The activated sludge treatment systems demonstrated a decrease for all the targeted phytosterols across the treatment system. Biological treatment reduced β-sitosterol in mill wastewaters for eight of the nine sites assessed.

6.0 SUMMARY AND CONCLUSIONS

An analytical method using GC/FID detection (GC/MS confirmation) has been developed and validated at the single laboratory level for campesterol, stigmasterol, β-sitosterol, and stigmastanol in kraft, kraft/recycle, kraft/groundwood, sulfite, kraft/groundwood/thermomechanical, and recycled wastewater treatment system influents and biologically-treated effluents (Appendix A). Validation of the method yielded the following results.

a) The method detection limits determined for the target analytes were 0.41 µg/L for campesterol, 0.43 µg/L for stigmasterol, 0.47 µg/L for β-sitosterol, and 0.44 µg/L for stigmastanol. The lower quantitation limit used during the sample analyses was 1.5 µg/L.

b) Average percent recoveries for campesterol, stigmasterol, β-sitosterol, and stigmastanol ranged from:

   1) 72% to 79% in biologically-treated effluents, and

   2) 69% to 80% in wastewater treatment system influents.

c) Variability in the recovery of the surrogate, cholesterol, of less than 18% (RSD) was observed in all matrices analyzed.

d) An average relative percent difference of 6.7% to 7.7% was observed for campesterol, stigmasterol, β-sitosterol, and stigmastanol native to biologically-treated effluent samples when duplicate experiments were conducted.

e) An average relative percent difference of 11% to 22% was observed for duplicate analyses of treatment system influents.

The results obtained from samples collected at U.S. mills indicate that:

f) β-Sitosterol, detected at all sampling sites, had the highest estimated discharge level, ranging from 0.2 to 20.3 g/T.

g) Generally, mills using recycled fibers had lower estimated discharge levels for the phytosterols.

h) Discharge levels of phytosterols did not correlate with biological treatment type, geographic location of the mill, or bleaching. Additional sampling would be required to identify the principle factors which influence the phytosterol discharge levels.

i) Treatment systems generally remove 63% to 93% of the campesterol, >38% to 95% of the β-sitosterol, and >13% to 96% of the stigmastanol present in the influent. Stigmasterol appears to increase across aerated stabilization basin treatment systems.

REFERENCES

Carlberg, G. 1993. Characterization of effluents from mechanical pulp production. Presented at the *International Environmental Symposium*, EUCEPA, Paris, France.

Dahlman, O., Haglind, I., Mörck, R., De Sousa, F., and Strömberg, L. 1993. Chemical composition of effluents from chlorine-dioxide bleaching of kraft pulps before and after secondary treatment. Presented at the *International Environmental Symposium*, EUCEPA, Paris, France.

Denton, T., Howell, W., Allison, J., McCollum, J., and Marks, B. 1985. Masculinization of female mosquitofish by exposure to plant sterols and mycobacterium smegmatis. *Bulletin of Environmental Contamination and Toxicology*, 35, 627-632.

Ekman, R., and Holmbom, B. 1989. Analysis by gas chromatography of the wood extractives in pulp and water samples from mechanical pulping of spruce. *Nordic Pulp and Paper Research Journal*, **1**, 16-24.

Ekman, R., Eckerman, C., and Holmbom, B. 1990. Studies on the behavior of extractives in mechanical pulp suspensions. *Nordic Pulp and Paper Research Journal*, **2**, 96-102.

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*.

Gleispach, H. 1974. The use of different silylation agents for structure analysis of steroids. *Journal of Chromatography*, **91**, 407-412.

Holmbom, B. 1980. A procedure for analysis of toxic compounds in pulp and paper mill waste waters. *Paperi ja Puu*, **9**, 523-531.

Holmbom, B., and Lehtinen, K-J. 1980. Acute toxicity to fish of kraft pulp mill waste waters. *Paperi ja Puu*, **62**(11), 673-684.

Hunsinger, R., Byram, B., and Howell, W. 1988. Unchanged gonadal morphology of mosquitofish masculinized by exposure to degraded plant sterols. *Journal of Fish Biology*, **32**, 795-796.

Jansson, M., Wormald, P., and Dahlman, O. 1994. Reactions of wood extractives during ECF and TCF bleaching of kraft pulp. International Pulp Bleaching Conference, pp. 61-64.

Junk, G., Richard, J., Grieser, M., Witiak, D., Witiak, J., Arguello, M., Vick, R., Svec, H., Fritz, J., and Calder, G. 1974. Use of macroreticular resins in the analysis of water for trace organic contaminants. *Journal of Chromatography*, 99, 1, 745-762.

Knutson, S., Solomon, K., Van Der Kraak, G., Hodson, P., and Parrott, J. 1995. Effect of sitosterol on fish reproduction. *Second SETAC World Congress*, 16th, (Nov).

Mellanen, P., Petanen, T., Lehtimake, J., Makela. S., Bylund, G., Holmbom, B., Mannila, E., Oikari, A., and Santti, R. 1996. Wood-derived estrogens: Studies *in vitro* with breast cancer cell lines and *in vivo* in trout. *Toxicology and Applied Pharmacology*, **136**, 381-388.

National Council of the Paper Industry for Air and Stream Improvement, Inc. (NCASI). 1986. *Procedures for the analysis of resin and fatty acids in pulp mill effluents.* NCASI Technical Bulletin No. 501. Research Triangle Park, NC: National Council of the Paper Industry for Air and Stream Improvement, Inc.

Nishimura, M., and Koyama, T. 1977. The occurrence if stanols in various living organisms and the behavior of sterols in contemporary sediments. *Geochimica et Cosmochimica Acta.*, **41**, 379-385.

Oikari, A., and Holmbom, B. 1996. Ecotoxicological effects of process changes implemented in a pulp and paper mill: a Nordic case study. *Environmental Fate and Effects of Pulp and Paper Mill Effluents*, Servos, M., Munkittrick, K., Carey, J., and Van Der Kraak, G., (Ed.), St. Lucie Press, Delray Beach, Florida, pp. 613-625.

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

Strömberg, L., Mörck, R., De Sousa, F., and Dahlman, O. 1996. Effects of process changes and external treatment on effluent chemistry. *Environmental Fate and Effects of Pulp and Paper Mill Effluents*, Servos, M., Munkittrick, K., Carey, J., and Van Der Kraak, G., (Ed.), St. Lucie Press, Delray Beach, Florida, pp. 3-19.

Voss, R., and Rapsomatiotis, A. 1985. An improved solvent-extraction based procedure for the gas chromatographic analysis of resin and fatty acids in pulp mill effluents. *Journal of Chromatography*, **346**, 205-214.

APPENDIX A

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 (Table A1) 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. 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 Table A2. 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 Table A3; 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 Table A3.

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 (Table A1) 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 Table A1), 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.

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

Performance criteria for acceptable surrogate recovery as determined during a single laboratory validation of this method is presented in Table A4. 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 Table A5. 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 Table A6 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 Table A7, 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 Table A7.

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 Table A8.

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 Table A8 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 Table A8.

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 Table A9 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 Table A9 are present and maximize within the same two consecutive scans.

12.2.4 The relative percent abundance of the ions designated in Table A9 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.

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

where:

A_S = *area of the compound being measured*
C_{IS} = *concentration (mg/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 Table A10. 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 Tables A2, A4, A5, A6, and A7. 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 A1. 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 A2. 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 A3. 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 A4. Surrogate Recovery in Biologically-Treated Effluents During NCASI STER-97 Method Validation Studies by GC/FID[a]

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

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

Table A5. 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	73	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 A6. 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 A7. 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
(Table notes continued on next page.)

[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 A8. 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	17.73	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 A9. 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 A10. 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

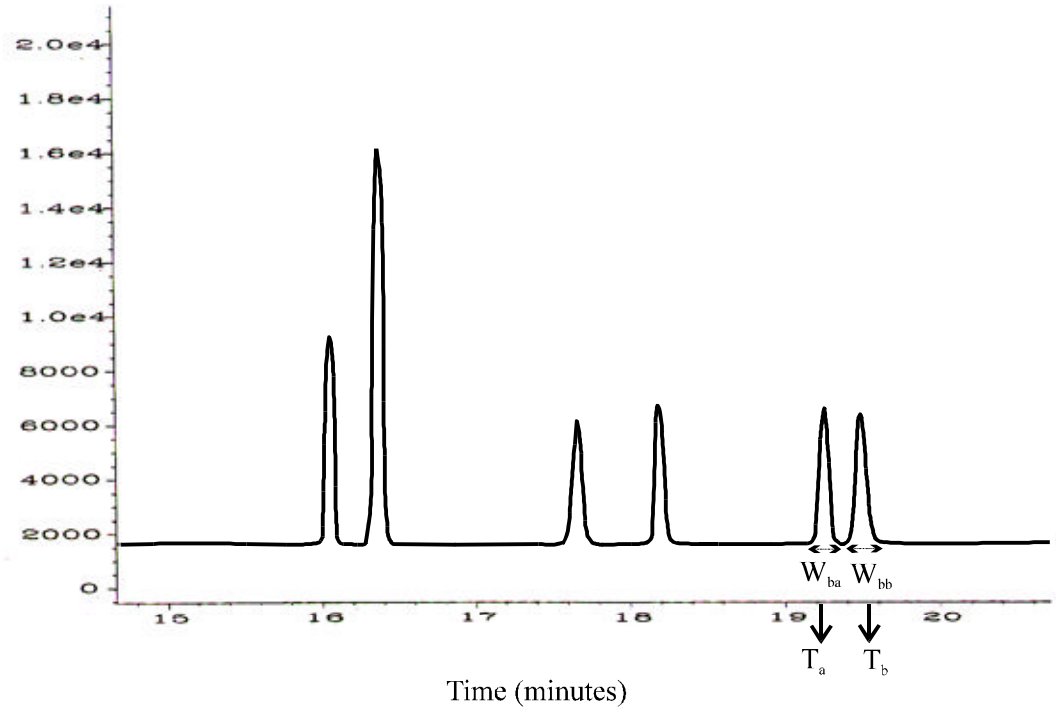


Figure A1. Chromatogram of the 50-µg/L Calibration Standard

APPENDIX B

DATA AND STATISTICAL ANALYSIS RESULTS

B1. DEFINITIONS AND DESCRIPTIONS OF STATISTICAL TERMS

The statistical procedures and tests utilized throughout this technical bulletin are defined as follows.

1. Relative Standard Deviation (RSD) - a measure of the spread or dispersion of data expressed as a percentage
2. ANOVA (Analysis of Variance) - a statistical tool based on F-ratios that measures whether a factor contributes significantly to the variance of a response
3. F-ratio - in one way (ANOVA), a ratio of the variance between groups to the variance within groups; in general, a numeric value that results from the ratio of mean squares
4. Alpha Level (α) - a limit selected by the investigator representing the probability limit beyond which he or she is unwilling to believe a random event has occurred
5. P-value (ANOVA) - the probability that the difference in means is due to random variability of samples pulled from a normal distribution
6. Linear Regression - when one variable, y, is considered a linear function of another variable, x, this function can be expressed in the form of $y = A + Bx$, where y = the dependent variable, x = the independent variable, and A and B are constants for the true linear function
7. P-value (Linear Regression) - the probability that the slope is zero
8. Cochran's Test - a statistical test for homogeneity of variance - The hypothesis is that the variances across different levels are equal; if the significance levels are greater than 0.05, do not reject the hypothesis that the variances are not significantly different.
9. Bartlett's Test - a statistical test for homogeneity of variance - The hypothesis is that the variances across different levels are equal; if the significance levels are greater than 0.05, do not reject the hypothesis that the variances are not significantly different.
10. Hartley's Test - a statistical test for homogeneity of variance - The hypothesis is that the variances across different levels are equal; if the significance levels are greater than 0.05, do not reject the hypothesis that the variances are not significantly different.
11. Kruskal-Wallis Test - a nonparametric method that tests the assumption that the medians of samples are equal
12. Box and Whisker Plot - a graphical summary of the presence of outliers in data for one or two variables - The plot divides the data into four equal areas of frequency; a box encloses the middle 50%, the median is represented by a vertical line inside the box, and the mean is plotted as a plus sign. The "whisker" lines extending from the box show the upper and lower quartiles.

B2. STABILITY STUDY STATISTICAL ANALYSES

Regression Analysis for Campesterol Concentration over a Period of 21 Days

Dependent Variable: Campesterol µg/L pH 3 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	21.97	1.65	13.33	0.0000
Slope	0.026	0.12	0.21	0.8362

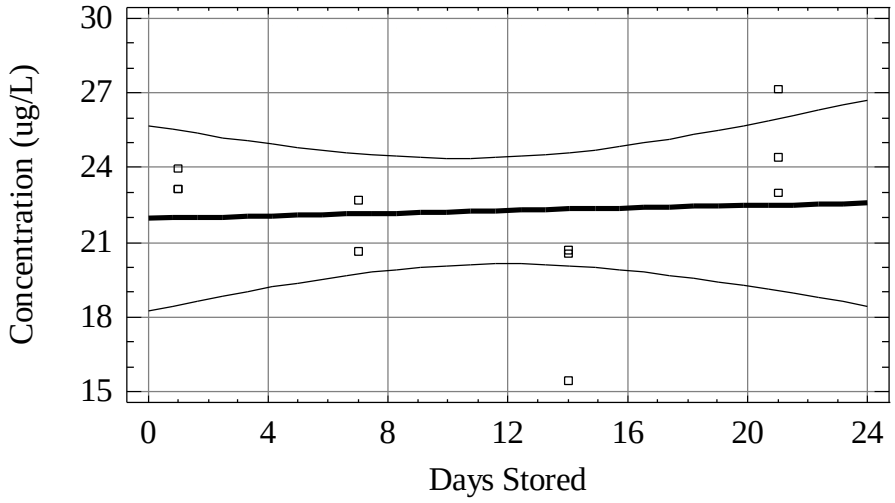
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	0.44	1	0.44	0.05	0.8362
Residual	88.01	9	9.78		
Total (Corr.)	88.46	10			

Correlation Coefficient: 0.07 R-Squared: 0.5 %

Standard Error of Estimate: 3.13

Linear Regression Model Plot



Regression Analysis for Campesterol Concentration over a Period of 21 Days

Dependent Variable: Campesterol µg/L pH 7 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	18.49	0.98	18.89	0.0000
Slope	0.106	0.075	1.42	0.1852

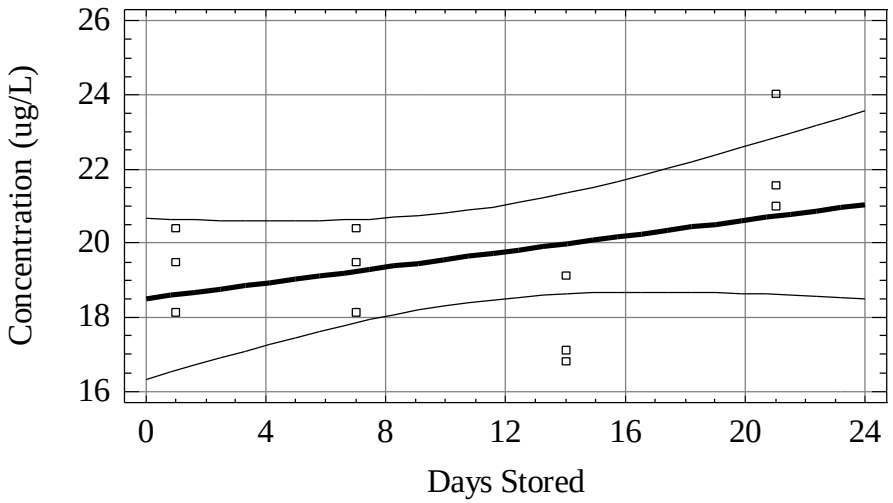
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	7.62	1	7.62	2.02	0.1852
Residual	37.64	10	3.76		
Total (Corr.)	45.27	11			

Correlation Coefficient: 0.41 R-Squared: 16.8 %

Standard Error of Estimate: 1.94

Linear Regression Model Fit



Regression Analysis for Campesterol Concentration over a Period of 21 Days

Dependent Variable: Campesterol µg/L pH 10 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	18.46	0.87	21.24	0.0000
Slope	0.043	0.066	0.64	0.5348

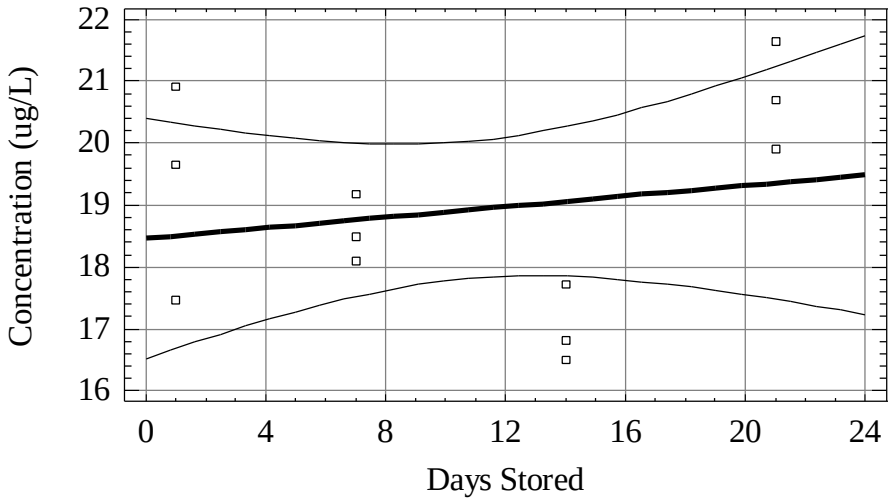
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	1.23	1	1.23	0.41	0.5348
Residual	29.65	10	2.96		
Total (Corr.)	30.88	11			

Correlation Coefficient: 0.20 R-Squared: 4.0 %

Standard Error of Estimate: 1.72

Linear Regression Model Plot



Regression Analysis for Stigmasterol Concentration over a Period of 21 Days

Dependent Variable: Stigmasterol µg/L pH 3 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	28.1	1.90	14.77	0.0000
Slope	0.04	0.14	0.27	0.7812

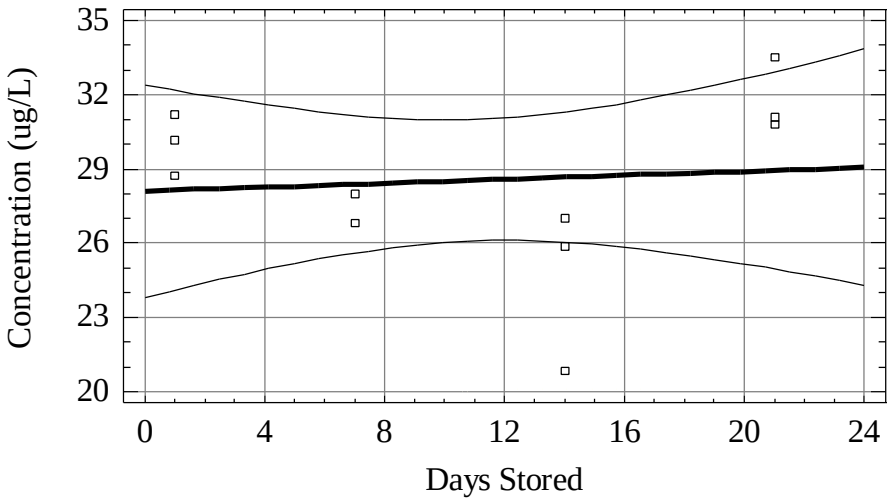
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	1.07	1	1.07	0.08	0.7812
Residual	117.24	9	13.03		
Total (Corr.)	118.31	10			

Correlation Coefficient: 0.09 R-Squared: 0.9 %

Standard Error of Estimate: 3.61

Linear Regression Model Plot



Regression Analysis for Stigmasterol Concentration over a Period of 21 Days

Dependent Variable: Stigmasterol µg/L pH 7 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	23.27	1.15	20.21	0.0000
Slope	0.05	0.09	0.56	0.5862

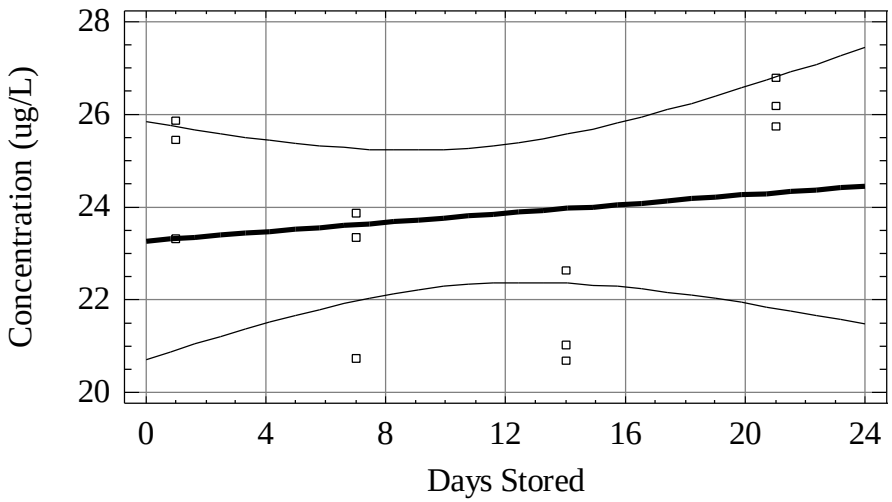
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	1.65	1	1.65	0.32	0.5862
Residual	52.04	10	5.20		
Total (Corr.)	53.68	11			

Correlation Coefficient: 0.18 R-Squared: 3.1%

Standard Error of Estimate: 2.28

Linear Regression Model Plot



Regression Analysis for Stigmasterol Concentration over a Period of 21 Days

Dependent Variable: Stigmasterol µg/L pH 10 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	23.48	1.26	18.68	0.0000
Slope	0.10	0.096	1.06	0.3123

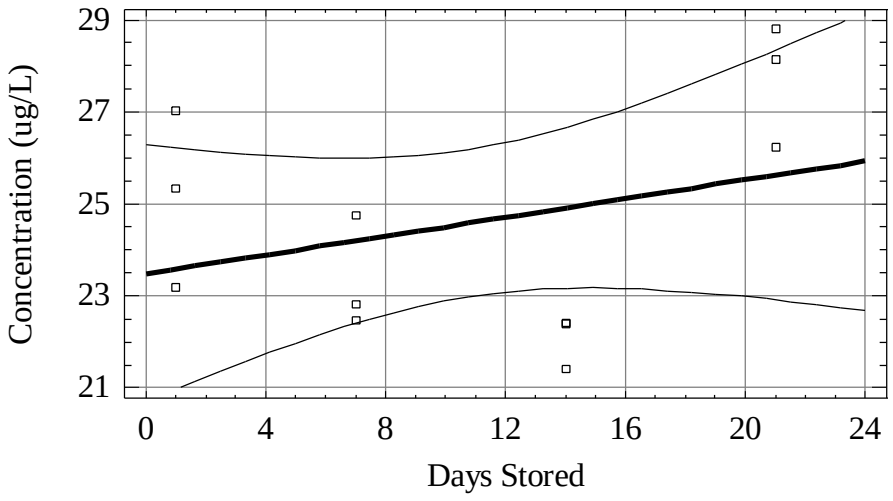
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	7.03	1	7.03	1.13	0.3123
Residual	62.05	10	6.20		
Total (Corr.)	69.07	11			

Correlation Coefficient: 0.32 R-Squared: 10.2 %

Standard Error of Estimate: 2.49

Linear Regression Model Plot



Regression Analysis for β-Sitosterol Concentration over a Period of 21 Days

Dependent Variable: β-Sitosterol µg/L pH 3 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	99.48	5.29	18.82	0.0000
Slope	0.26	0.39	0.66	0.5264

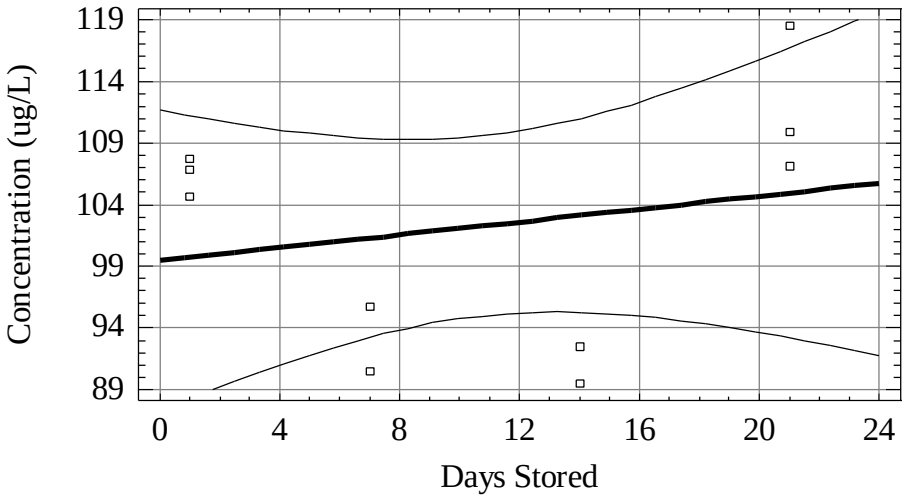
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	43.86	1	43.86	0.44	0.5264
Residual	799.76	8	99.97		
Total (Corr.)	843.62	9			

Correlation Coefficient: 0.23 R-Squared: 5.2 %

Standard Error of Estimate: 10.00

Linear Regression Model Plot



Regression Analysis for β-Sitosterol Concentration over a Period of 21 Days

Dependent Variable: β-Sitosterol µg/L pH 7 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	81.82	4.67	17.52	0.0000
Slope	0.42	0.36	1.19	0.2611

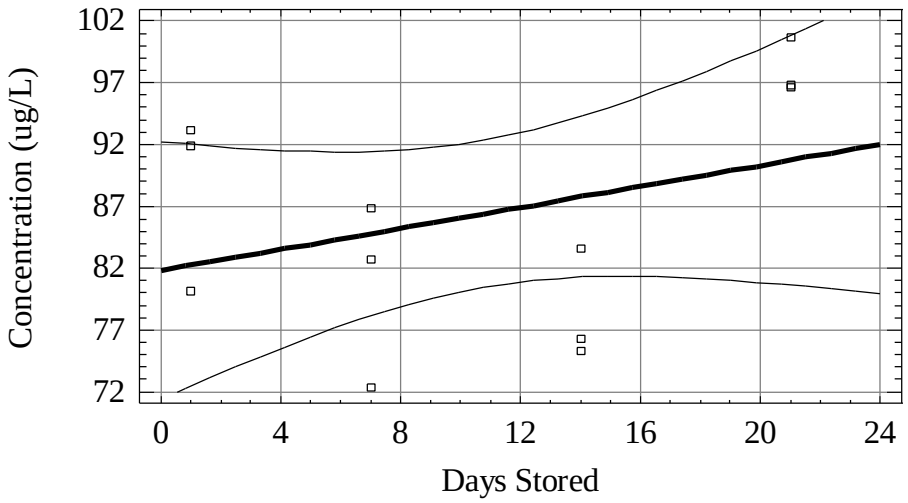
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	121.55	1	121.55	1.42	0.2611
Residual	856.62	10	85.66		
Total (Corr.)	978.17	11			

Correlation Coefficient: 0.35 R-Squared: 12.4 %

Standard Error of Estimate: 9.26

Linear Regression Model Plot



Regression Analysis for β-Sitosterol Concentration over a Period of 21 Days

Dependent Variable: β-Sitosterol µg/L pH 10 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	82.84	4.29	19.29	0.0000
Slope	0.18	0.33	0.54	0.6030

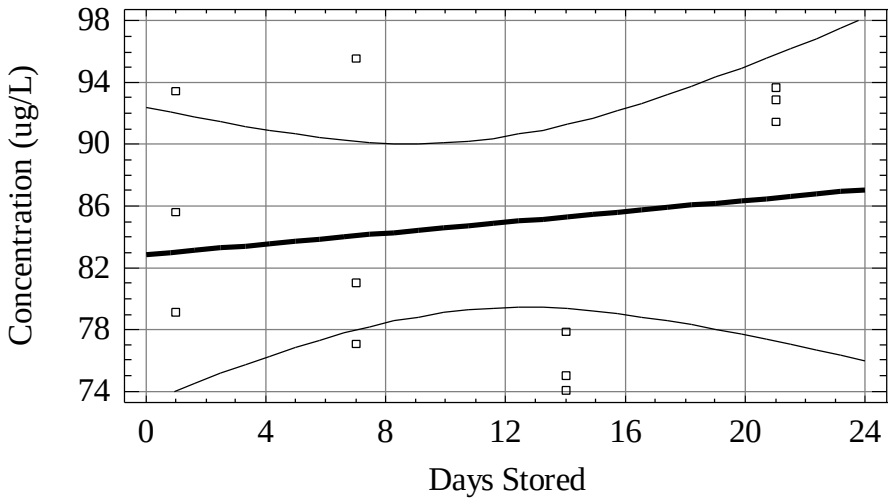
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	20.88	1	20.88	0.29	0.6030
Residual	724.00	10	72.40		
Total (Corr.)	744.88	11			

Correlation Coefficient: 0.17 R-Squared: 2.8 %

Standard Error of Estimate: 8.51

Linear Regression Model Plot



Regression Analysis for Stigmastanol Concentration over a Period of 21 Days

Dependent Variable: Stigmastanol µg/L pH 3 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	22.47	1.81	12.40	0.0000
Slope	0.01	0.13	0.064	0.9501

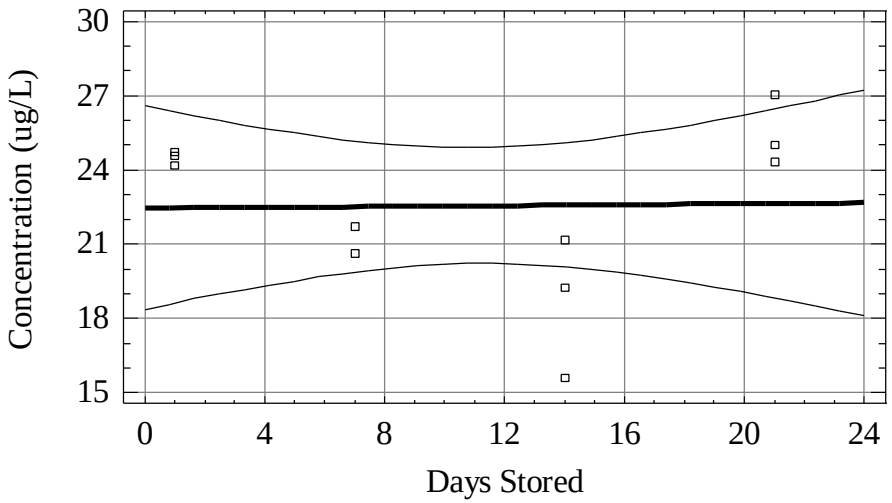
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	0.05	1	0.05	0.00	0.9501
Residual	106.37	9	11.82		
Total (Corr.)	106.42	10			

Correlation Coefficient: 0.021 R-Squared: 0.05 %

Standard Error of Estimate: 3.44

Linear Regression Model Plot



Regression Analysis for Stigmastanol Concentration over a Period of 21 Days

Dependent Variable: Stigmastanol µg/L pH 7 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	18.20	1.10	16.50	0.0000
Slope	0.14	0.08	1.70	0.1198

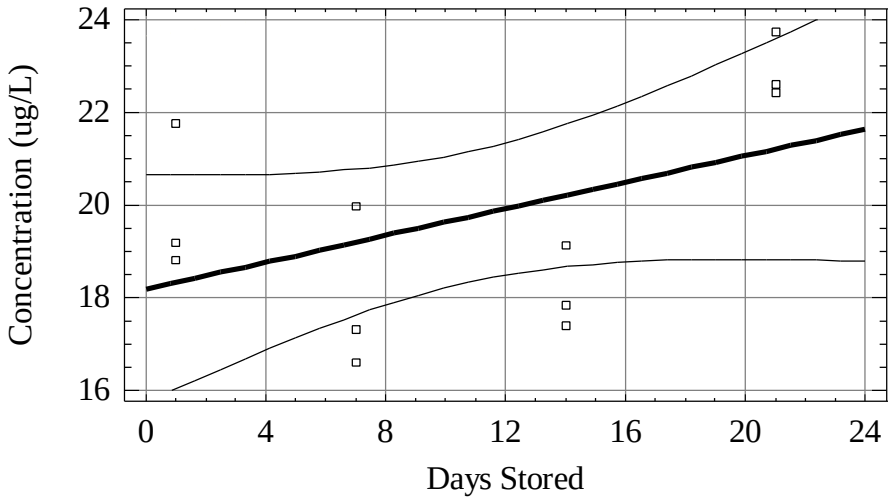
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	13.81	1	13.81	2.89	0.1198
Residual	47.73	10	4.77		
Total (Corr.)	61.55	11			

Correlation Coefficient: 0.47 R-Squared: 22.4 %

Standard Error of Estimate: 2.18

Linear Regression Model Plot



Regression Analysis for Stigmastanol Concentration over a Period of 21 Days

Dependent Variable: Stigmastanol µg/L pH 10 Preservation Independent Variable: Days Stored

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	20.10	1.25	16.02	0.00
Slope	0.004	0.10	0.04	0.97

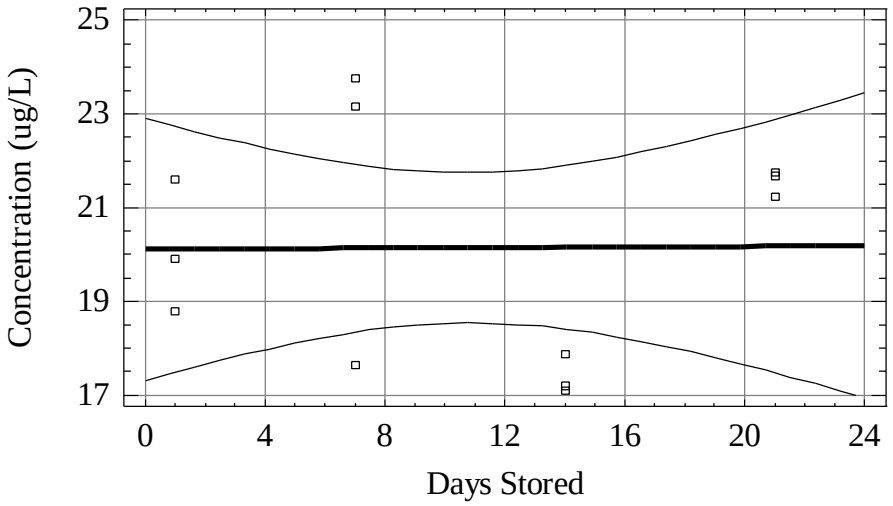
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	0.009	1	0.009	0.00	0.97
Residual	61.84	10	6.18		
Total (Corr.)	61.85	11			

Correlation Coefficient: 0.012 R-Squared: 0.01 %

Standard Error of Estimate: 2.49

Linear Regression Model Plot



B3. STATISTICAL ANALYSIS RESULTS OF CORRELATIONS BETWEEN CONCENTRATION AND PERCENT RECOVERY OF PHYTOSTEROL MATRIX SPIKES

Regression Analysis for Percent Recovery of Campesterol and Matrix Spike Concentration

Dependent Variable: Percent Recovery Independent Variable: Concentration µg/L

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	74.2	3.0	24.7	0.00
Slope	-0.03	0.02	-1.65	0.11

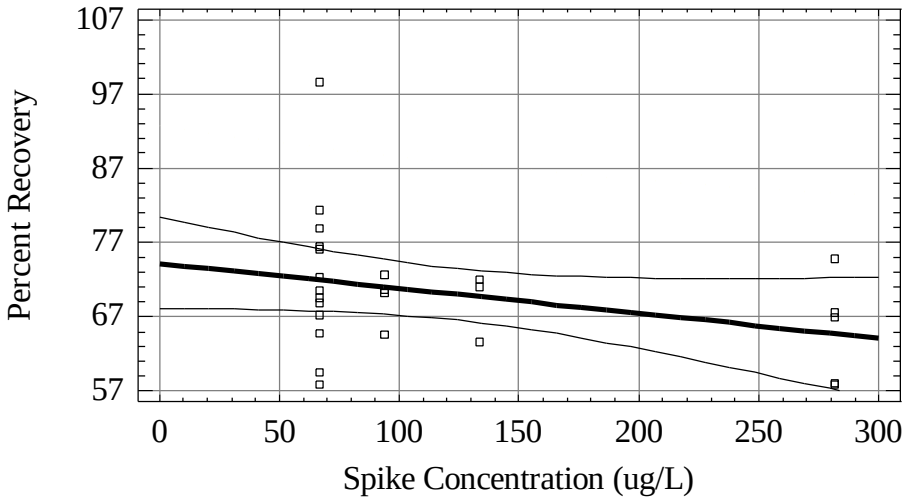
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	192.7	1	192.7	2.74	0.11
Residual	1619.0	23	70.4		
Total (Corr.)	1811	24			

Correlation Coefficient: -0.33 R-Squared: 10.6%

Standard Error of Estimate: 8.4

Plot of Fitted Model



Regression Analysis for Percent Recovery of Stigmasterol and Matrix Spike Concentration

Dependent Variable: Percent Recovery Independent Variable: Concentration µg/L

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	75.9	2.9	26.1	0.00
Slope	-0.03	0.02	-2.0	0.06

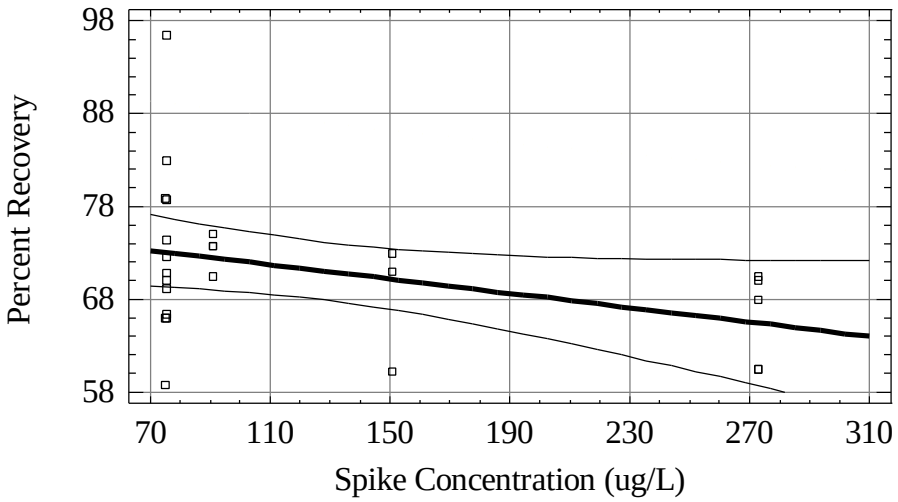
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	220.3	1	220.3	.83	0.06
Residual	1322.4	23	57.5		
Total (Corr.)	1542.8	24			

Correlation Coefficient: -0.38 R-Squared: 14.3%

Standard Error of Estimate: 7.6

Plot of Fitted Model



Regression Analysis for Percent Recovery of β-Sitosterol and Matrix Spike Concentration

Dependent Variable: Percent Recovery Independent Variable: Concentration µg/L

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	79.1	4.3	18.4	0.00
Slope	-0.06	0.03	-1.9	0.07

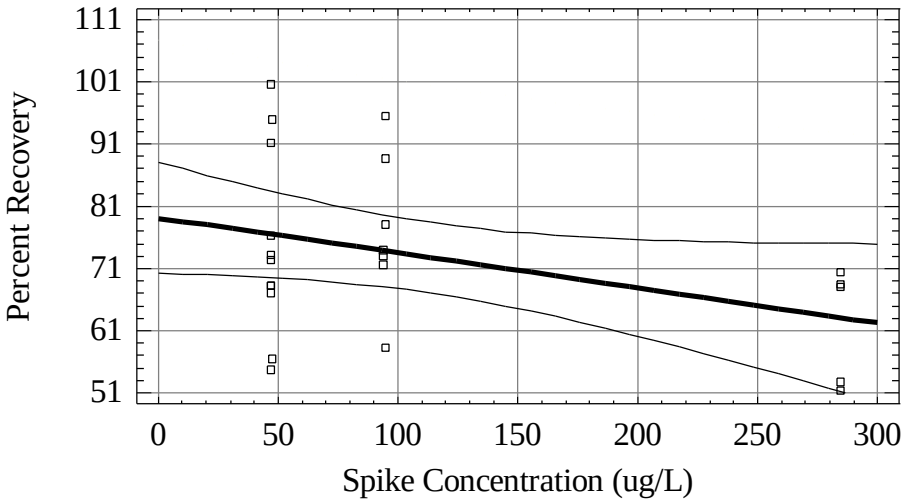
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	623.7	1	623.7	3.66	0.07
Residual	3576	21	170.3		
Total (Corr.)	4199.7	22			

Correlation Coefficient: -0.38 R-Squared: 14.8%

Standard Error of Estimate: 13.0

Plot of Fitted Model



Regression Analysis for Percent Recovery of Stigmastanol and Matrix Spike Concentration

Dependent Variable: Percent Recovery Independent Variable: Concentration µg/L

Parameter	Estimate	Standard Error	T-value	P-Value
Intercept	73.4	3.3	22.4	0.0
Slope	-0.04	0.02	-1.89	0.07

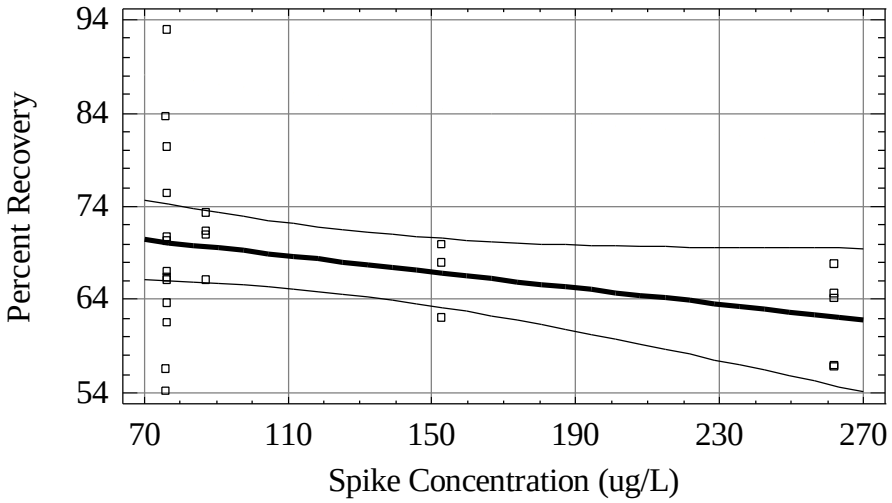
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	247	1	247	3.58	0.07
Residual	1587	23	69		
Total (Corr.)	1834	24			

Correlation Coefficient: -0.37 R-Squared: 13.5%

Standard Error of Estimate: 8.3

Plot of Fitted Model



B4. STATISTICAL ANALYSIS FOR THE DISCHARGE LEVELS OF CAMPESTEROL, STIGMASTEROL, b-SITOSTEROL, AND STIGMASTANOL COMPARED BY TREATMENT SYSTEM TYPE, MILL LOCATION, BLEACHING, MILL PROCESS, AND MILL FURNISH

Campesterol Correlation with Treatment System Type

Statistics Summary

Dependent Variable: Campesterol Level Factor: Treatment System Type

Parameter	Count	Average	Variance	Standard Deviation
AS[a]	5	0.72	0.347	0.589
ASB[b]	14	0.64	0.258	0.508
Total	19	0.66	0.265	0.514

[a] AS = Activated Sludge
[b] ASB = Aerated Stabilization Basin

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	0.022	1	0.022	0.08	0.7826
Within Groups	4.74	17	0.279		
Total (Corr.)	4.76	18			

Variance Check

Cochran's Test: 0.57 P-Value = 0.675
Bartlett's Test: 1.01 P-Value = 0.719
Hartley's Test: 1.34

There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

Stigmasterol Correlation with Treatment System Type

Statistics Summary

Dependent Variable: Stigmasterol Level Factor: Treatment System Type

Parameter	Count	Average	Variance	Standard Deviation
AS[a]	5	1.52	1.742	1.32
ASB[b]	14	0.73	0.242	0.49
Total	19	0.94	0.690	0.83

[a] AS = Activated Sludge
[b] ASB = Aerated Stabilization Basin

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	2.31	1	2.31	3.88	0.066
Within Groups	10.12	17	0.60		
Total (Corr.)	12.42	18			

Variance Check

Cochran's Test: 0.88 P-Value = 0.009
Bartlett's Test: 1.54 P-Value = 0.009
Hartley's Test: 7.19

There is a statistically significant difference among the standard deviations at the 95% confidence level. There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

β-Sitosterol Correlation with Treatment System Type

Statistics Summary

Dependent Variable: β-Sitosterol Level Factor: Treatment System Type

Parameter	Count	Average	Variance	Standard Deviation
AS[a]	8	3.64	12.67	3.56
ASB[b]	14	7.01	36.29	6.02
Total	22	5.79	29.45	5.43

[a] AS = Activated Sludge
[b] ASB = Aerated Stabilization Basin

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	58.1	1	58.05	2.07	0.166
Within Groups	560.5	20	28.02		
Total (Corr.)	618.6	21			

Variance Check

Cochran's Test: 0.74 P-Value = 0.112
Bartlett's Test: 1.11 P-Value = 0.150
Hartley's Test: 2.86

There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

Stigmastanol Correlation with Treatment System Type

Statistics Summary

Dependent Variable: Stigmastanol Level Factor: Treatment System Type

Parameter	Count	Average	Variance	Standard Deviation
AS[a]	6	1.85	2.58	1.61
ASB[b]	13	1.32	1.11	1.05
Total	19	1.50	1.52	1.23

[a] AS = Activated Sludge
[b] ASB = Aerated Stabilization Basin

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	1.14	1	1.14	0.74	0.402
Within Groups	26.24	17	1.54		
Total (Corr.)	27.38	18			

Variance Check

Cochran's Test: 0.699 P-Value = 0.239
Bartlett's Test: 1.08 P-Value = 0.257
Hartley's Test: 2.33

There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

Campesterol Correlation with Mill Location

Statistics Summary

Dependent Variable: Campesterol Level Factor: Mill Location

Parameter	Count	Average	Variance	Standard Deviation
South	12	0.58	0.215	0.463
West	4	1.05	0.483	0.695
Northeast	3	0.50	0.09	0.3
Total	19	0.66	0.265	0.514

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	0.77	2	0.386	1.55	0.243
Within Groups	3.99	16	0.250		
Total (Corr.)	4.76	18			

Variance Check

Cochran's Test: 0.61 P-Value = 0.154
Bartlett's Test: 1.11 P-Value = 0.474
Hartley's Test: 5.37

There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

Stigmasterol Correlation with Mill Location

Statistics Summary

Dependent Variable: Stigmasterol Level Factor: Mill Location

Parameter	Count	Average	Variance	Standard Deviation
South	12	0.74	0.19	0.44
West	3	0.53	0.56	0.75
Northeast	3	1.5	1.92	1.38
Total	18	0.83	0.52	0.72

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	1.70	2	0.852	1.81	0.197
Within Groups	7.06	15	0.470		
Total (Corr.)	8.76	17			

Variance Check

Cochran's Test: 0.72 P-Value = 0.042
Bartlett's Test: 1.57 P-Value = 0.055
Hartley's Test: 10.1

There is a statistically significant difference among the standard deviations at the 95% confidence level. There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

β-Sitosterol Correlation with Mill Location

Statistics Summary

Dependent Variable: β-Sitosterol Level Factor: Mill Location

Parameter	Count	Average	Variance	Standard Deviation
South	13	6.96	39.53	6.29
West	4	4.03	8.10	2.85
Northeast	4	4.53	24.34	4.93
Total	21	5.94	30.40	5.51

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	36.24	2	18.12	0.57	0.575
Within Groups	571.67	18	31.76		
Total (Corr.)	607.91	20			

Variance Check

Cochran's Test: 0.55 P-Value = 0.268
Bartlett's Test: 1.13 P-Value = 0.362
Hartley's Test: 4.88

There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

Stigmastanol Correlation with Mill Location

Statistics Summary

Dependent Variable: Stigmastanol Level Factor: Mill Location

Parameter	Count	Average	Variance	Standard Deviation
South	11	1.48	1.11	1.05
West	4	1.05	0.63	0.79
Northeast	3	2.1	6.24	2.50
Total	18	1.49	1.61	1.27

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	1.89	2	0.95	0.56	0.585
Within Groups	25.49	15	1.70		
Total (Corr.)	27.38	17			

Variance Check

Cochran's Test: 0.782 P-Value = 0.013
Bartlett's Test: 1.36 P-Value = 0.133
Hartley's Test: 9.90

There is a statistically significant difference among the standard deviations at the 95% confidence level. There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

Campesterol Correlation with Bleaching

Statistics Summary

Dependent Variable: Campesterol Level Factor: Bleaching

Parameter	Count	Average	Variance	Standard Deviation
Bleached	14	0.77	0.270	0.520
Unbleached	5	0.36	0.158	0.397
Total	19	0.66	0.265	0.514

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	0.624	1	0.624	2.56	0.1280
Within Groups	4.14	17	0.243		
Total (Corr.)	4.76	18			

Variance Check

Cochran's Test: 0.63 P-Value = 0.451
Bartlett's Test: 1.02 P-Value = 0.546
Hartley's Test: 1.71

There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

Stigmasterol Correlation with Bleaching

Statistics Summary

Dependent Variable: Stigmasterol Level Factor: Bleaching

Parameter	Count	Average	Variance	Standard Deviation
Bleached	14	1.12	0.77	0.878
Unbleached	5	0.42	0.15	0.383
Total	19	0.94	0.69	0.831

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	1.81	1	1.81	2.90	0.1066
Within Groups	10.61	17	0.62		
Total (Corr.)	12.42	18			

Variance Check

Cochran's Test: 0.84 P-Value = 0.026
Bartlett's Test: 1.20 P-Value = 0.095
Hartley's Test: 5.25

There is a statistically significant difference among the standard deviations at the 95% confidence level. There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

β-Sitosterol Correlation with Bleaching

Statistics Summary

Dependent Variable: β-Sitosterol Level Factor: Bleaching

Parameter	Count	Average	Variance	Standard Deviation
Bleached	17	6.24	29.96	5.47
Unbleached	5	4.22	30.83	5.55
Total	22	5.79	29.45	5.43

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	15.88	1	15.88	0.53	0.4763
Within Groups	602.67	20	30.13		
Total (Corr.)	618.54	21			

Variance Check

Cochran's Test: 0.51 P-Value = 0.965
Bartlett's Test: 1.00 P-Value = 0.972
Hartley's Test: 1.03

There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

Stigmastanol Correlation with Bleaching

Statistics Summary

Dependent Variable: Stigmastanol Level Factor: Bleaching

Parameter	Count	Average	Variance	Standard Deviation
Bleached	15	1.65	1.47	1.21
Unbleached	4	0.9	1.66	1.29
Total	19	1.49	1.52	1.23

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between Groups	1.76	1	1.76	1.17	0.2948
Within Groups	25.62	17	1.51		
Total (Corr.)	27.38	18			

Variance Check

Cochran's Test: 0.53 P-Value = 0.867
Bartlett's Test: 1.00 P-Value = 0.899
Hartley's Test: 1.13

There is not a statistically significant difference among the means at the 95% confidence level.

Kruskal-Wallis Test: There is not a significant difference between the medians at the 95% confidence level.

Campesterol Correlation with Mill Furnish

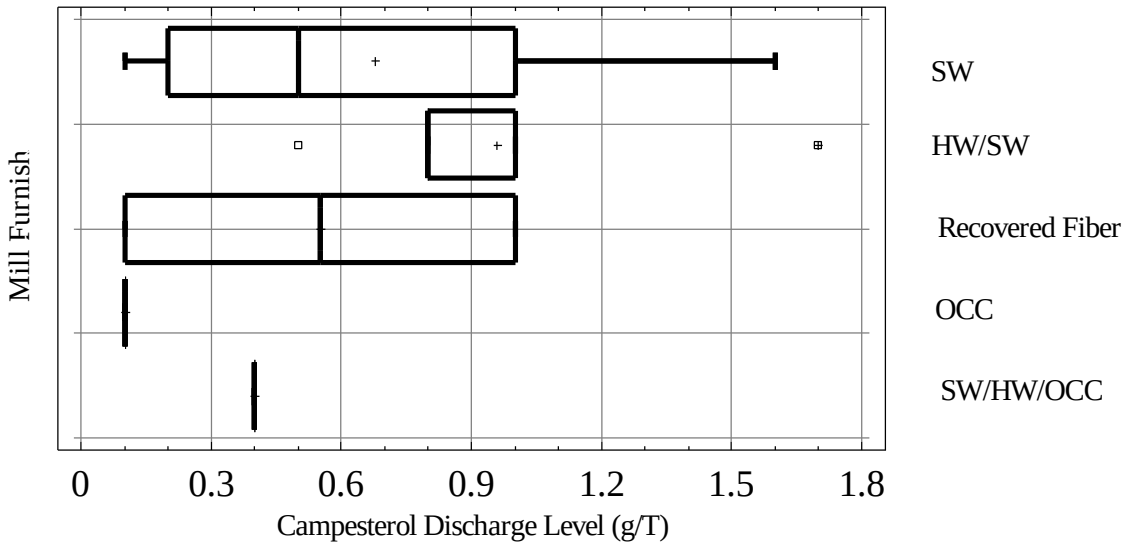
Statistics Summary

Dependent Variable: Campesterol Level Factor: Mill Furnish

Parameter	Count	Average	Variance	Standard Deviation
Softwood	9	0.68	0.30	0.54
HW/SW[a]	5	0.96	0.20	0.45
Recovered Fiber	2	0.55	0.41	0.64
OCC[b]	2	0.1	0.0	0.0
SW/HW/OCC	1	0.4	0.0	0.0
Total	19	0.66	0.26	0.51

[a] = Hardwood and softwood
[b] = Old corrugated containers

Box-and-Whisker Plot



Stigmasterol Correlation with Mill Furnish

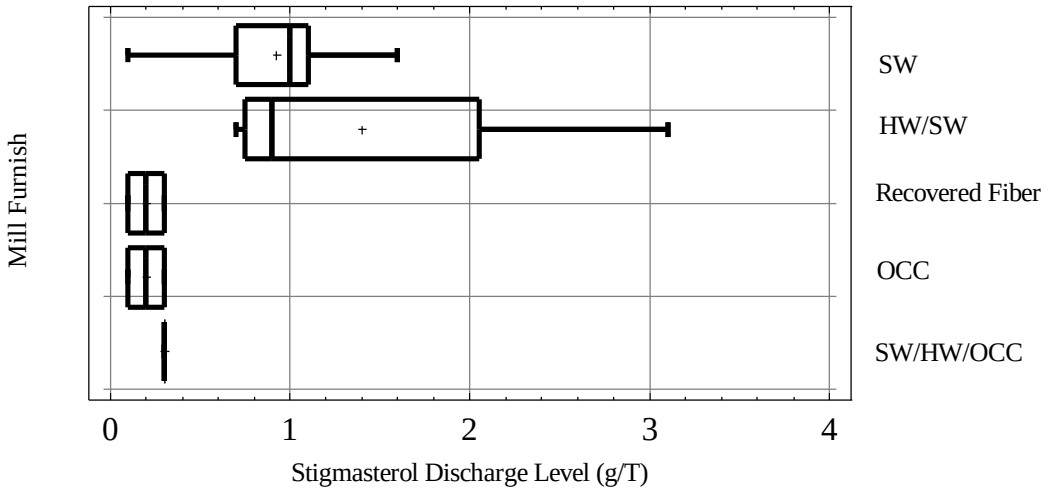
Statistics Summary

Dependent Variable: Stigmasterol Level Factor: Mill Furnish

Parameter	Count	Average	Variance	Standard Deviation
Softwood	9	0.92	0.20	0.44
HW/SW[a]	4	1.40	1.3	1.14
Recovered Fiber	2	0.20	0.02	0.14
OCC[b]	2	0.20	0.02	0.14
SW/HW/OCC	1	0.30	0.0	0.0
Total	18	0.83	0.52	0.72

[a] = Hardwood and softwood
[b] = Old corrugated containers

Box-and-Whisker Plot



β-Sitosterol Correlation with Mill Furnish

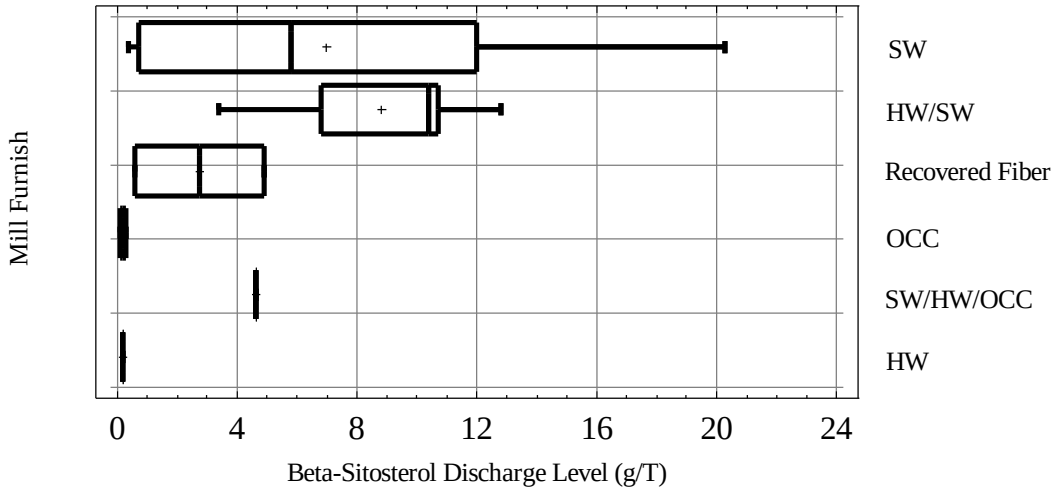
Statistics Summary

Dependent Variable: β-Sitosterol Level Factor: Mill Furnish

Parameter	Count	Average	Variance	Standard Deviation
Softwood	10	7.0	41.1	6.4
HW/SW[a]	5	8.8	13.8	3.7
Recovered Fiber	2	2.8	9.2	3.0
OCC[b]	2	0.2	0.02	0.14
SW/HW/OCC	1	4.6	0.0	0.0
Hardwood	1	0.2	0.0	0.0
Total	21	5.9	30.4	5.5

[a] = Hardwood and softwood
[b] = Old corrugated containers

Box-and-Whisker Plot



Stigmastanol Correlation with Mill Furnish

Statistics Summary

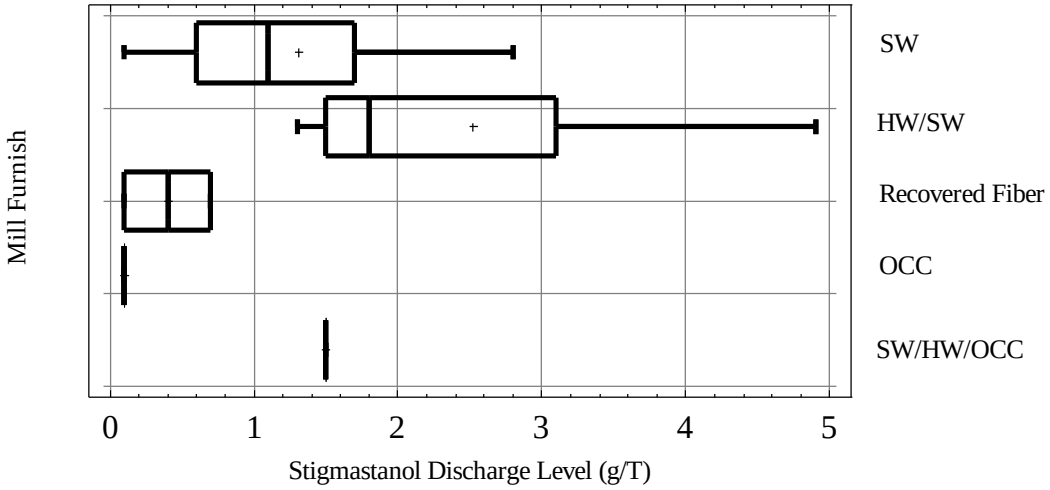
Dependent Variable: Stigmastanol Level Factor: Mill Furnish

Parameter	Count	Average	Variance	Standard Deviation
Softwood	9	1.3	1.0	1.0
HW/SW[a]	5	2.5	2.3	1.5
Recovered Fiber	2	0.4	0.18	0.42
OCC[b]	1	0.1	0.0	0.0
SW/HW/OCC	1	1.5	0.0	0.0
Total	18	1.5	1.6	1.3

[a] = Hardwood and softwood

[b] = Old corrugated containers

Box-and-Whisker Plot



Total Sterols Correlation with Mill Furnish

Statistics Summary

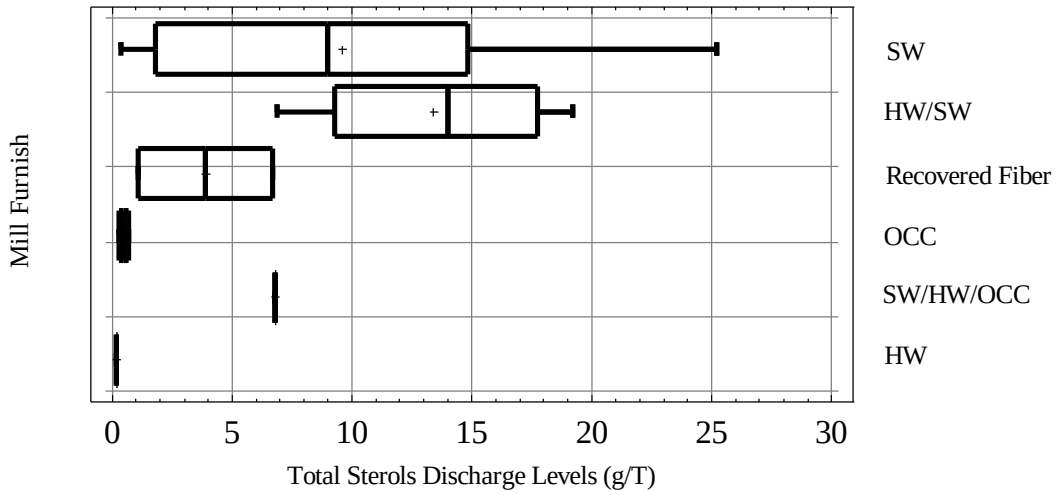
Dependent Variable: Total Sterols Level Factor: Mill Furnish

Parameter	Count	Average	Variance	Standard Deviation
Softwood	10	9.6	62.5	7.9
HW/SW[a]	5	13.4	27.9	5.3
Recovered Fiber	2	3.9	15.7	4.0
OCC[b]	2	0.5	0.08	0.3
SW/HW/OCC	1	6.8	0.0	0.0
Hardwood	1	0.2	0.0	0.0
Total	21	8.5	53.3	7.3

[a] = Hardwood and softwood

[b] = Old corrugated containers

Box-and-Whisker Plot



Campesterol Correlation with Mill Process

Statistics Summary

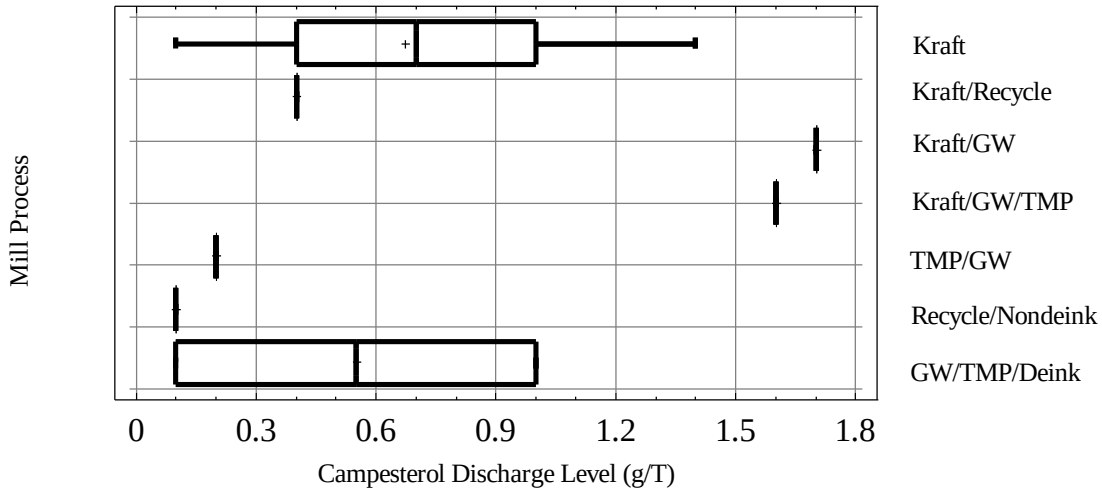
Dependent Variable: Campesterol Level Factor: Mill Process

Parameter	Count	Average	Variance	Standard Deviation
Kraft	11	0.7	0.15	0.38
Kraft/Recycle	1	0.4	0.0	0.0
Kraft/GW[a]	1	1.7	0.0	0.0
Kraft/GW/TMP[b]	1	1.6	0.0	0.0
TMP/GW	1	0.2	0.0	0.0
Recycle/Nondeink	2	0.1	0.0	0.0
GW/TMP/Deink	2	0.6	0.41	0.64
Total	19	0.66	0.26	0.51

[a] GW = Groundwood

[b] TMP = Thermomechanical

Box-and-Whisker Plot



Stigmasterol Correlation with Mill Process

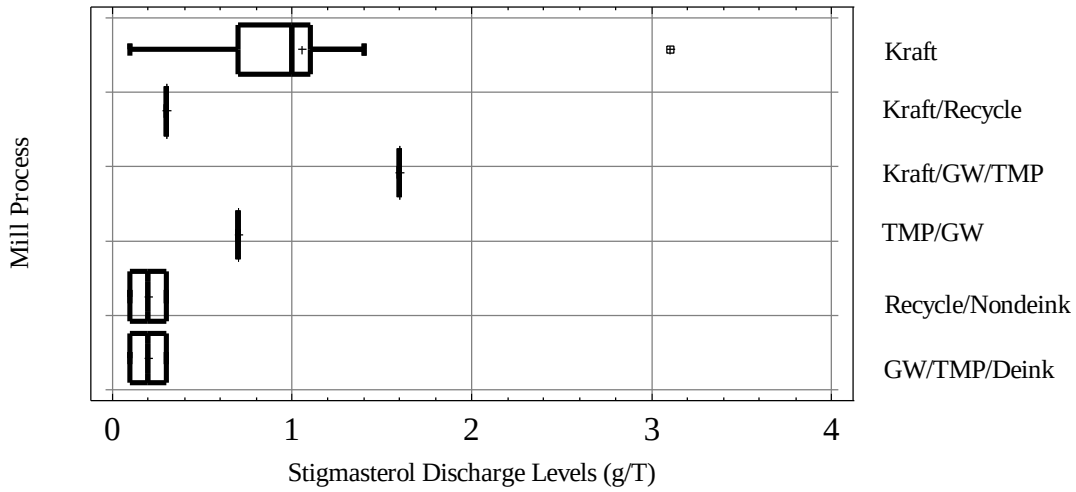
Statistics Summary

Dependent Variable: Stigmasterol Level Factor: Mill Process

Parameter	Count	Average	Variance	Standard Deviation
Kraft	11	1.1	0.6	0.8
Kraft/Recycle	1	0.3	0.0	0.0
Kraft/GW[a]/TMP[b]	1	1.6	0.0	0.0
TMP/GW	1	0.7	0.0	0.0
Recycle/Nondeink	2	0.2	0.02	0.14
GW/TMP/Deink	2	0.2	0.02	0.14
Total	18	0.8	0.52	0.7

[a] GW = Groundwood
[b] TMP = Thermomechanical

Box-and-Whisker Plot



β-Sitosterol Correlation with Mill Process

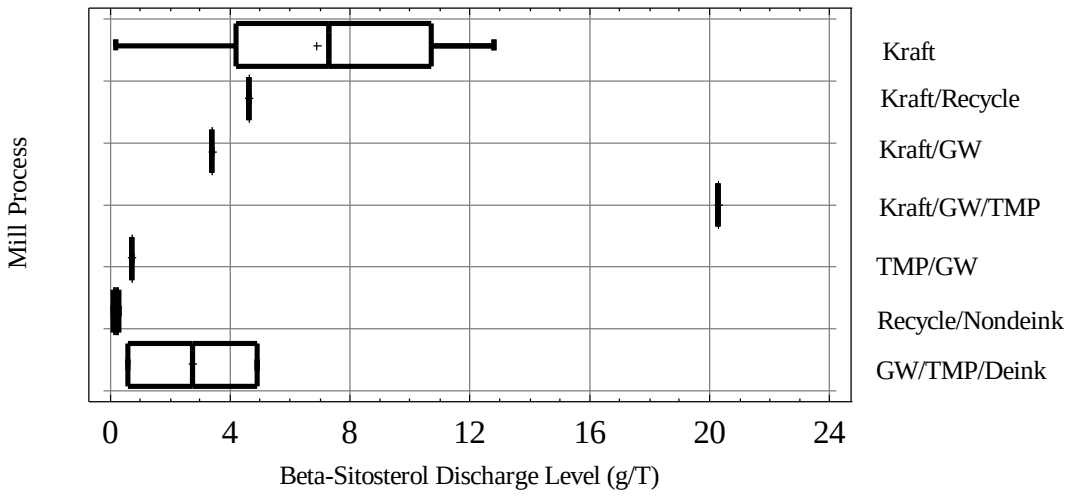
Statistics Summary

Dependent Variable: β-Sitosterol Level Factor: Mill Process

Parameter	Count	Average	Variance	Standard Deviation
Kraft	13	6.9	21.5	4.6
Kraft/Recycle	1	4.6	0.0	0.0
Kraft/GW[a]	1	3.4	0.0	0.0
Kraft/GW/TMP[b]	1	20.3	0.0	0.0
TMP/GW	1	0.7	0.0	0.0
Recycle/Nondeink	2	0.2	0.02	0.14
GW/TMP/Deink	2	2.8	9.2	3.0
Total	21	5.9	30.4	5.5

[a] GW = Groundwood
[b] TMP = Thermomechanical

Box-and-Whisker Plot



Stigmastanol Correlation with Mill Process

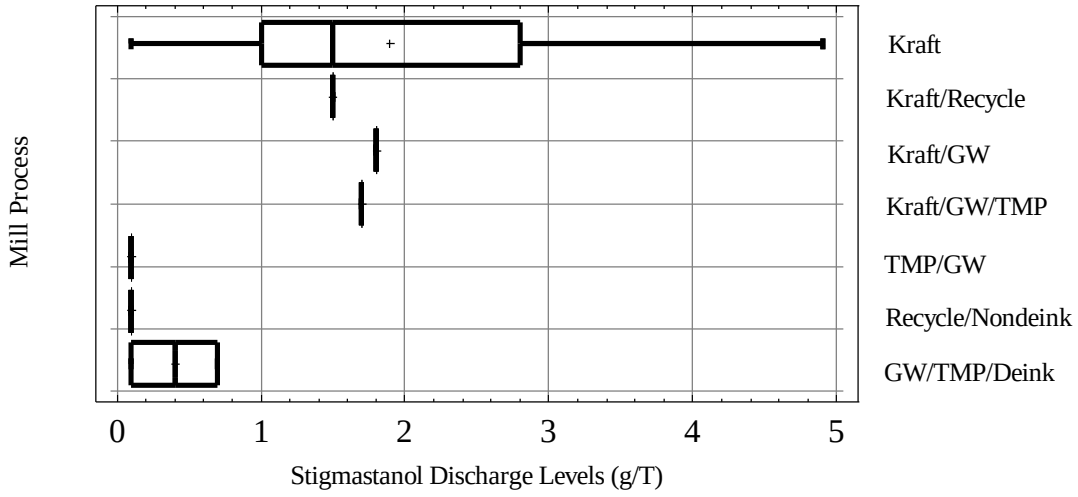
Statistics Summary

Dependent Variable: Stigmastanol Level Factor: Mill Process

Parameter	Count	Average	Variance	Standard Deviation
Kraft	11	1.9	1.9	1.4
Kraft/Recycle	1	1.5	0.0	0.0
Kraft/GW[a]	1	1.8	0.0	0.0
Kraft/GW/TMP[b]	1	1.7	0.0	0.0
TMP/GW	1	0.1	0.0	0.0
Recycle/Nondeink	1	0.1	0.0	0.0
GW/TMP/Deink	2	0.4	0.18	0.4
Total	18	1.5	1.6	1.3

[a] GW = Groundwood
[b] TMP = Thermomechanical

Box-and-Whisker Plot



Total Sterols Correlation with Mill Process

Statistics Summary

Dependent Variable: Total Sterol Level Factor: Mill Process

Parameter	Count	Average	Variance	Standard Deviation
Kraft	13	10.0	43.5	6.6
Kraft/Recycle	1	6.8	0.0	0.0
Kraft/GW[a]	1	6.9	0.0	0.0
Kraft/GW/TMP[b]	1	25.2	0.0	0.0
TMP/GW	1	1.8	0.0	0.0
Recycle/Nondeink	2	0.5	0.08	0.3
GW/TMP/Deink	2	3.9	15.7	4.0
Total	21	8.5	53.2	7.3

[a] GW = Groundwood

[b] TMP = Thermomechanical

Box-and-Whisker Plot