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RAPID METHOD FOR EXTRACTION OF THE FECAL STEROL COPROSTANOL USING SUPERCRITICAL FLUID CARBON DIOXIDE

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ABSTRACT

There has been increased recent interest in the use of chemical markers such as the fecal sterol coprostanol to quantify sewage-derived contamination in estuarine and marine waters. Coprostanol offers advantages over classical microbiological techniques that use *Escherichia coli* which is not unique to human feces, and *Streptococcus fecalis* which is associated with humans but again is not a perfect tracer. Traditional methods measuring sewage-derived contamination using bacterial indicators have posed problems with specificity and time of assay; organisms must be cultured and enumerated, a process that can take up to four days. Chemical techniques using extraction with a chloroform/methanol solvent mixture can take 24 to 36 hours before a result is attainable. We discuss the use of supercritical CO_2 and modifiers for extracting coprostanol as a rapid method for assessment of sewage contamination in environmental samples. Supercritical fluid extraction (SFE) reduces extraction times for environmental and related samples and can produce fractions ready for derivatization in 30 minutes or less. The considerably shorter extraction time potentially enables analysis of environmental and related samples in two to three hours.

INTRODUCTION

There has been increasing recent world wide interest in the use of chemical markers to quantitate sewage-derived contamination in coastal, estuarine and fresh water environments [1,2]. The fecal biomarker coprostanol is gaining recognition as a conservative tracer for sewage contamination in aquatic environments. Coprostanol is formed though microbial reduction of cholesterol in the human intestine. The recognition of the usefulness of coprostanol to trace sewage has developed as traditional microbial indicators come under scrutiny for their reliability, specificity and length of assay. It also has been recently demonstrated how fecal biomarkers can offer new advantages as their use enables environmental scientists and water managers to distinguish between sources of fecal pollution [3, unpublished data].

These findings highlight the advantages that the use of fecal sterols offer over the traditional microbial methods. The use of bacterial indicators such as *Escherichia coli* that is not unique to human feces is currently the accepted standard for water quality guidelines. Similarly *Streptococcus fecalis* which is associated with humans again is not an ideal tracer. Here we report the use of supercritical fluid CO_2 as a rapid extraction method for fecal sterols. Development of this method involved stepwise manipulations of pressure, temperature, modifier type and extraction time, in an attempt to recover equal amounts of the fecal marker as can be obtained by solvent extraction techniques. Furthermore we demonstrate the environmental application of this technique by comparing recovery of fecal sterols by SFE for samples with high coliform counts obtained from a fresh water river system.

METHODS

Primary effluent was collected from the Knoxville wastewater treatment plant and 50 ml aliquots of primary effluent were filtered onto 4.7 cm glass fibre filters (GF/F, Whatman). The filters were immediately frozen and stored at -20' C.

Some of the filters were lyophilised to ascertain the effect of drying the filters. Water samples were collected at the point of discharge of the Knoxville treatment plant in the Tennessee River, Knoxville, TN, 0.5 kilometre upstream from the point of discharge, with subsequent samples collected at 0.5, 1, and 1.5 km downstream of the point source. The samples were analysed for total coliforms using a "m-ColiBlue24TM" kit.

Supercritical fluid extractions were performed with two pumps (model 260D, Isco Inc.) and a model SFX2-10 extractor (Isco, Inc.) The extractor was fitted with a 71 cm long, 0.5 mm internal diameter restrictor (1.5 ml/min). One pump was used to deliver the CO_2 and the other to modify the CO_2 with different amounts of ethanol or ethanol/water mixtures. Filters were placed in 10 ml extraction cartridges and sealed in the extractor. After 1 minute, the system was pressurised. All extractions were performed at 400 ATM and 100°C. Solutes from each extraction were collected for 30 minutes in glass vials containing 20 mL of 1:1 (v:v) chloroform:methanol mixture. The collection solvent was evaporated with a stream of nitrogen at 37°C. The residue was transferred to test tubes with teflon lined caps and derivitized with bis(trimethylsily)trifluoroacetamide to convert the sterols to O-TMSi ethers. Sterols were quantified with a HP5890 gas chromatograph using split/spiltless injection, a 50 m x 0.2 mm x 0.11 μm HP1 (Hewlett-Packard) column and mass spectrometric (Hewlett Packard 5972 mass selective detector) or flame ionisation detection. A short oven temperature ramp was used; conditions have been described elsewhere [4] and a representative GC trace is shown in Figure 1. Peak areas and an internal standard (5α-cholestane) were used to determine the concentration of each sterol.

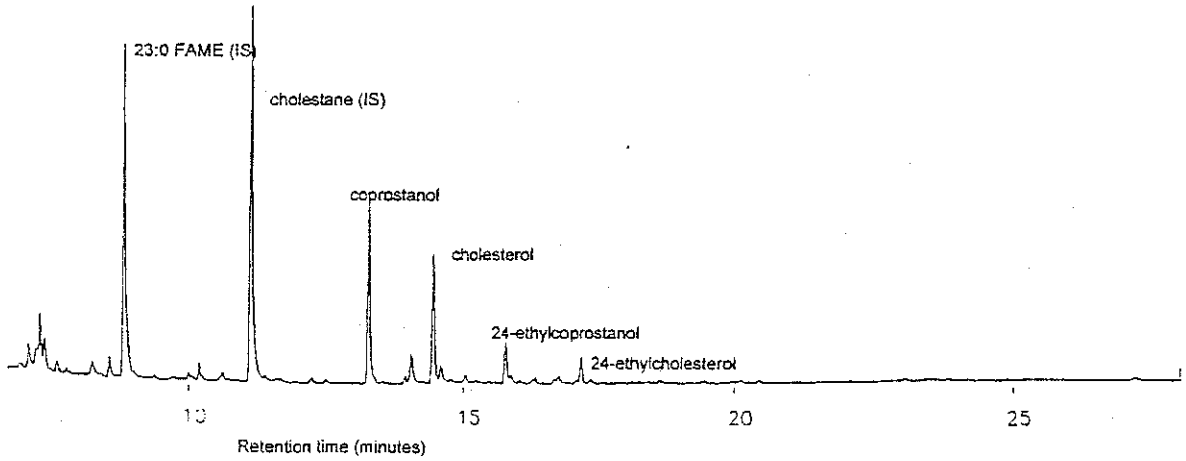

Figure 1. Partial gas chromatogram illustrating separation of fecal sterols obtained from SFE of primary sewage effluent. IS denotes internal standard. FAME denotes fatty acid methyl ester.

Standard solvent extractions of effluent were used to ascertain the percent recovery for each treatment. Briefly, the procedure was as modified [5] from the one phase chloroform/methanol/water Thigh and Dyer method [6]. After phase separation, the sterols were recovered in the lower chloroform layer. The solvents were removed in vacuo and samples were made up to a known volume and stored under nitrogen at -20°C. Total sterols were obtained following alkaline saponification of the total lipid. Products were extracted into hexane-chloroform (4:1, v:v) and stored at -20°C. Sterols were converted to their corresponding trimethylsilyl ethers as described above.

RESULTS AND DISCUSSION

A new method for supercritical fluid extraction of the fecal sterol coprostanol was developed in order to be better able to rapidly quantitate levels of sewage pollution in aquatic systems. The supercritical fluid method described here enables rapid extraction of particulate matter from marine, estuarine or fresh water systems. This will allow local government and public health authorities to potentially quickly assess sewage pollution in areas of public concern. Hawthorn et al. [7] described the advantages of supercritical fluid extraction as compared to traditional solvent extraction. The properties that supercritical fluid displays enable a reduction in extraction time due to increased solute diffusivities and decreased solvent viscosities. The use of supercritical fluid also has waste disposal and health and safety implications with the reduction in use of toxic or chlorinated solvents. The use of these solvents is reduced by two orders of magnitude.

Analysis of standards and primary effluent

Initial SFE experiments were carried out using pure sterol standards, 5α-cholestane and cholesterol. The standards were extracted under a variety of temperature and pressure regimes to optimise extraction conditions. The results from these

initial experiments showed that good recoveries were obtained at 80° C and 200 ATM. In moving from standards to primary effluent samples, the change in matrix sample provided difficulties with recoveries of coprostanol and cholesterol. For primary effluent samples the experimental design covered a series of temperature, pressure and modifier conditions in an effort to emulate recoveries that were comparable to the Thigh and Dyer solvent extraction. Initial effluent samples were analysed with varying degrees of wetness and this posed a problem with variability observed in sterol recovery. After varying temperature and pressure during extraction and the use of two different modifiers, methanol and then ethanol, the conditions that we considered the most suitable for the recovery of the fecal sterols were 100°C and 400 ATM using the modifier ethanol:water 95:5% at an addition rate of 7.5% total flow and an extraction time of thirty minutes. Further experiments using these conditions indicated 20-30 minutes was a suitable extraction period (Figure 2).

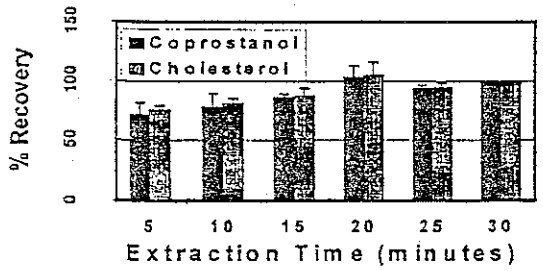

Figure 2. Percent recovery of cholesterol and coprostanol from SFE of primary sewage effluent (100°C 400 ATM; ethanol water 95:5). 100% represents recovery using Thigh and dyer solvent extraction.

Considering the variation due to wetness of the sample filters an experiment was performed to use a series of extraction conditions for both wet filters and lyophilised filters. The results clearly showed the effect that lyophilisation has on the recoveries of coprostanol and cholesterol from SFE as compared to the Thigh and Dyer solvent extraction. These results also showed that the use of the 95:5% ethanol:water at an addition rate of 7.5% total flow gave the best recoveries of the fecal sterols (Figure 3). Laboratory blanks were extracted using both SFE and solvent extraction techniques; coprostanol or cholesterol were not detected.

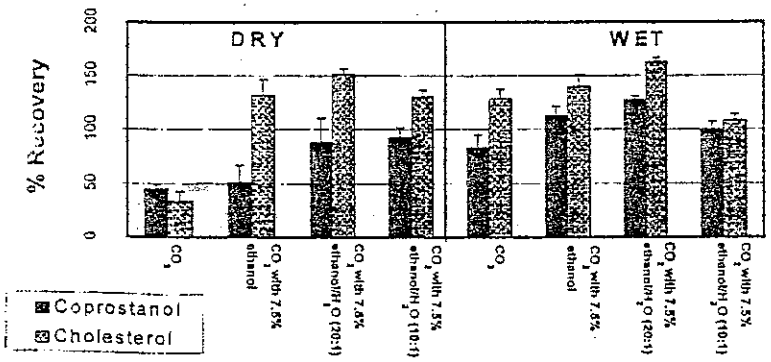

Figure 3. Comparison of recovery of cholesterol and coprostanol from SFE of primary sewage effluent 100% represents recovery using Thigh and Dyer solvent extraction.

Analysis of environmental samples

Application of the SFE technique was undertaken for samples obtained from the Tennessee River. Analyses for the fecal marker coprostanol were carried out using the optimised SFE conditions described above. The concentration of coprostanol varied from 8460 ng/L at the river bank close to the point of discharge, decreasing to 104 ng/L at 0.5 km upstream, then increasing at each subsequent downstream station to 422 ng/L at 1.5 km from the point of discharge. In comparison fecal coliforms varied from 455 cfu/100 ml at the point of discharge decreasing over the two downstream stations to 105 cfu/100 ml and then increasing to 142 cfu/100 ml at 1.5 km from the point of discharge.

Table 1. Bacterial indicator (fecal coliforms, ctu/100 ml) and coprostanol concentrations (ng/L) for the Tennessee River water samples.

Site Number	Distance from Discharge (km)	Fecal coliforms	Coprostanol
1	PE	ND	639*
2	0	455	846
3	0.5	428	104
4	0.5	207	187
5	1	105	273
6	1.5	142	422

n= 2-3 for both assays; ND not determined. * concentration in µg/L, PE = primary effluent. Site 3 upsteam, sites 4-6 downstream from discharge point.

In recent studies of environmental samples by our group correlation has been observed between fecal coliforms and coprostanol [e.g. 8]. Although the number of sampling sites was limited in this instance such a correlation was not observed. Chlorination is known to eliminate fecal coliform, yet coprostanol is largely unaffected. The high coprostanol concentration (8460 ng/L) at the discharge point is indicative of very significant sewage pollution which is not indicated by fecal coliform abundance. The bacterial indicator abundance has been clearly affected by chlorination used at the Knoxville treatment plant. In addition, the increase in coprostanol concentration away from the point of discharge of the treatment plant may reflect input of fecal matter from other sources.

As stated in the methods, all work was carried out using the ISCO extraction unit and pumps. Some of this work was also trialed using the Hewlett Packard SFE unit. This unit has a two step (solid phase trapping followed by solvent extraction) sample collection system compared to the solvent collection used by the ISCO system. Using the HP system we noted variable production of steroidal ketones under the SFE conditions employed. The extraction efficiencies that were achieved using the ISCO system could not be reproduced on the Hewlett Packard system although conditions could not be directly matched as the HP system has a 325 ATM pressure limit. The effect of sample matrix, water content, and instrumental conditions on sterol recovery is clearly significant and we recommend appropriate recovery experiments are performed when using SFE methodology to obtain fecal sterols and/or other components.

CONCLUSION

Recoveries for coprostanol and cholesterol from sewage effluent are compared for SFE and conventional solvent extraction. Results demonstrated that SFE can be used as a rapid extraction method for fecal sterols. SFE provided recoveries of coprostanol and other sterols comparable with liquid solvent extraction; an extraction time of approximately 30 minutes was achieved. Application of the SFE method to environmental samples also has been demonstrated along with comparison to traditional microbiological methods of quantifying sewage pollution in aquatic environments.

The SFE procedure used for fecal sterols offers the capability of interfacing direct to a GC and/or GCMS. In addition, development of sequential extraction procedures may allow other lipid classes and compounds of interest to be obtained from the same sample (e.g. phospholipids, hydrocarbons, PAH, tocopherol). The use of SFE reduces the use of toxic chlorinated solvents therefore decreasing health and waste disposal problems. The development of rapid SFE-based methods for analysing fecal sterols and other signature lipids will enable such compounds to have wider application in urban effluent and wastewater management.

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