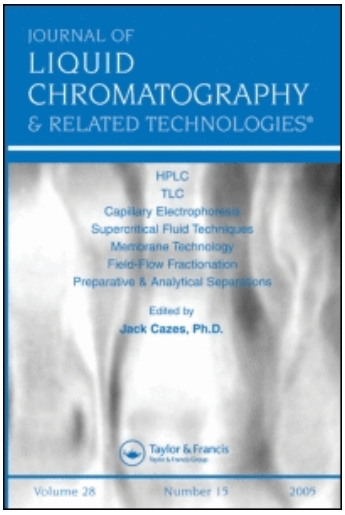

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SOLID PHASE EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTODIODE ARRAY DETECTION OF CHEMICAL INDICATORS OF HUMAN FECAL CONTAMINATION IN WATER

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ABSTRACT

Faster and more sensitive analysis of water that is contaminated by human fecal matter is very important for public health. The current microbiological methods to assess water quality do not meet this need. Alternate non-microbial human fecal indicators have been proposed by various researchers. The high performance liquid chromatography (HPLC) analysis with photodiode array detection (PDA) of three human fecal indicators, caffeine, urobilin, and coprostanol was developed. Both caffeine and urobilin were analyzed simultaneously using a linear gradient protocol and monitored at 270 nm and 480 nm. The analysis gave linearity and limit of detection down to hundredths of μg/mL.

A solid phase extraction (SPE) protocol was also developed for caffeine and urobilin. Coprostanol was analyzed using HPLC with PDA detection with post-column derivatization. The derivatization was achieved by acylation of coprostanol with p-nitrobenzoyl chloride.

INTRODUCTION

The densities of coliform and fecal coliform bacteria have traditionally been used to assess water quality and predict the risk of transmission of waterborne diseases. The reliability of coliforms to accurately ascertain fecal contamination has been questioned. This is primarily due to the extreme variability of coliform survival in varying environmental conditions and poor correlation with the presence of specific pathogens. Furthermore, due to the slow turnaround time to obtain results, utilities, unknowingly, may be releasing substandard water to the public.

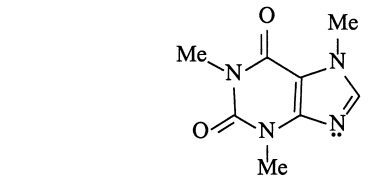
There has been an increased interest in developing supplemental and/or alternate indicators of human contamination to better define water quality and predict the risk of disease outbreaks. Other non-microbial indicators such as caffeine, urobilin, and coprostanol have been proposed and have shown great potential as alternate markers of human contamination.[1-4] These substances are human by-products and are found in feces and urine. The current study uses three of these substances, caffeine, urobilin, and coprostanol, whose structures are shown in Figure 1.

About 5-10% of caffeine, the most widely consumed drug in the world, is excreted unchanged by humans.[5, 6] In sewage, it is only slowly metabolized by a microorganism, *Pseudomonas putida*.[7] The U.S. Geological Survey used caffeine as a sewage tracer along the Mississippi River to assess water quality. The utility of caffeine as a fecal marker, however, is diminished when industries that produce caffeine-containing food and beverages are within the vicinity of a water source being assessed for its water quality.

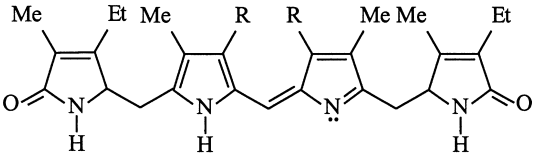
When bacteria in the human gut reduce and hydrolyze the bile pigment, bilirubin, the product is urobilin, a compound which imparts a yellowish tint to urine and fecal matter.[8] It was first used as a fecal indicator by Miyabara and co-workers to estimate the extent of fecal pollution in the urban rivers of Japan.[9] They obtained excellent correlation of levels of urobilin with fecal coliforms in these rivers. Urobilin is an excellent human fecal marker since there are no other major sources of this compound in nature.

Coprostanol (5β-coprostanol) is the most established of the three compounds as a sewage tracer. Numerous studies have been conducted which used it to assess the quality of both marine and fresh water sources.[10-19] This compound is formed when intestinal bacteria reduce cholesterol. Aside from humans, mammals like cats and pigs also produce coprostanol. Thus, its utility as a human fecal indicator is severely limited.

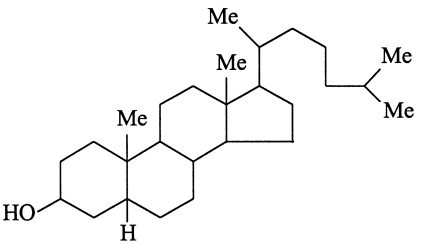
No other study has attempted to analyze the levels of these three compounds in water. The detection of these compounds in water samples can



caffeine



urobilin



coprostanol

Me = Methyl, Et = Ethyl, R=CH_2CH_2COOH)

Figure 1. Structures of the three chemical indicators of human fecal contamination in water used in this study.

strongly suggest that the water is non-potable. In this study, an HPLC method to analyze these three human fecal markers in water is described. Each of these compounds had been analyzed in previous separate studies. Caffeine has been analyzed by a number of methods, foremost of which is reversed-phase HPLC using a variety of detection methods.[20-27] Coprostanol has been analyzed mostly by GC using a variety of detectors.[15-19] A limited number of methods to analyze urobilin in water samples have been published. Two methods described the use of HPLC with UV or fluorescence detection.[8, 28]

In this study, the unique capability of a PDA detector was used to simultaneously analyze caffeine and urobilin using HPLC. Coprostanol was also analyzed using the same method but with an additional step of post-column derivatization in order to introduce a chromophore to the molecule for UV-Vis detection. To extract these compounds in water, a SPE procedure was developed.

EXPERIMENTAL

Instrumentation

The HPLC set-up includes a Waters Alliance 2690 solvent delivery and sample handling module along with a Waters 996 PDA detector (Waters, Milford, MA). Samples in the module were kept at 25°C while the Waters NovaPak C_{18} column (150.0 x 3.9 mm, 4 μm, 60 Å) equipped with a pre-filter and a C_{18} guard column (Upchurch, Oak Harbor, WA) were kept at ambient conditions. Unless noted, the mobile phase flow rate was kept at 1.0 mL/min.

An Eppendorf MR-100 mobile-phase recycler (Brinkmann, Westbury, NY) was used whenever isocratic separation was utilized. Post-column delivery of reagent for derivatization of coprostanol was accomplished using a Dionex (Sunnyvale, CA) Reagent Delivery Module (RDM) - a known pressure of dry helium delivers the reagent at a specific flow rate. Unless noted, the flow of the post-column reagent was 1 mL/min. A Bruker 300-MHz NMR spectrometer was used to determine the spectra of the derivatized coprostanol.

Except for the Dionex RDM module, all stages of the HPLC set-up was controlled by the Waters Millenium Software Version 2.11 using a NEC 466 computer. The same software was used for data acquisition and handling.

The SPE method development was performed on a Waters vacuum manifold using Waters classic C_{18} cartridges. Later on, the SPE of large-volume samples was done using a sample delivery set-up from Supelco (Bellefontaine, PA). Supelco C_{18} syringe cartridges were fitted with covers connected to teflon delivery lines with stainless steel ends dipped in the sample containers. Vacuum was then applied on the manifold forcing the samples through the teflon delivery lines at a controlled flow rate and into the respective SPE cartridges. Samples collected after SPE were then evaporated to dryness at 50°C using a Zymark (Hopkinton, MA) TurboVap LV evaporator with nitrogen gas held at 10 psi to purge the vaporized solvent.

Materials and Reagents

Prior to use, solvents were pre-filtered through a 0.2 μm nylon membrane filter (Millipore, Bedford, MA) and degassed by an online degasser. All sam-

ples were filtered using a 0.2 μm Acrodisc syringe filters (Gelman, Ann Arbor, MI).

HPLC-grade acetonitrile and methanol as well as reagent-grade phosphoric acid and dimethyl sulfoxide were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). The derivatization agent, p-nitrobenzoyl chloride, was also obtained from Sigma-Aldrich as well as, pyridine and ethyl acetate, used to purify the derivatized coprostanol for NMR analysis.

Reagent-grade caffeine, theobromine, theophylline, β-hydroxyethyl-theophylline (BHET), caffeic acid, and coprostanol were obtained from Sigma-Aldrich Chemicals. Urobilin was obtained from Porphyrin Chemicals (Logan, UT). Freshly prepared solutions were used in the analyses.

RESULTS AND DISCUSSIONS

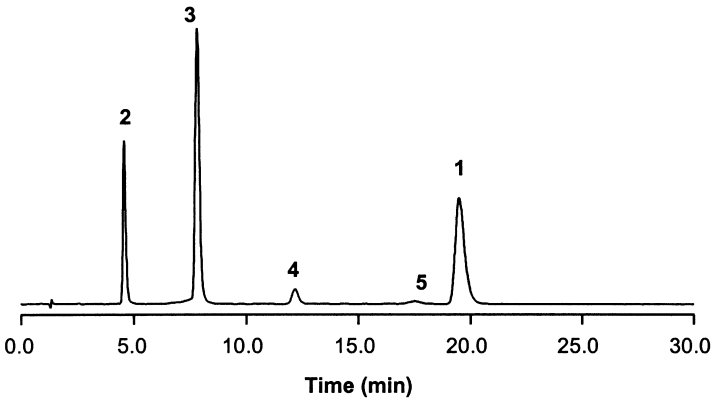
As shown in Figure 1, the three chemical human fecal markers are structurally different from each other. Consequently, the strategy adopted for method development was to develop a method for each compound first, then proceed with the simultaneous detection of at least two of the most nearly-related compounds - caffeine and urobilin. Coprostanol was identified earlier as a difficult compound to analyze by HPLC using PDA detection due to the absence of a chromophore in its structure.

Analysis of Caffeine and Urobilin

A method to analyze caffeine was developed by analyzing it together with other structurally related compounds (theobromine, theophylline, BHET and caffeic acid) each at 100 μg/mL. A chromatogram is shown in Figure 2 with the chromatographic conditions.

A 10 μg/mL solution of urobilin was analyzed using similar conditions used for the analysis of caffeine. However, no peaks were obtained. Finally, a method using a combination of acetonitrile, dimethylsulfoxide (DMSO), and methanol solvents was successful in eluting this large molecule as shown in Figure 3. The appearance of a shoulder on the urobilin peak in these chromatograms confirms the presence of impurities in the urobilin sample. These impurities are urobilin-related compounds.

The absorption maxima of caffeine is at 274 nm while that of urobilin is at 488 nm. PDA detection made possible the simultaneous detection of these compounds. As shown in Figure 4, a linear gradient was used to elute both caffeine (t_R ~ 5 min) and urobilin (t_R ~ 16 min) from a solution containing 200

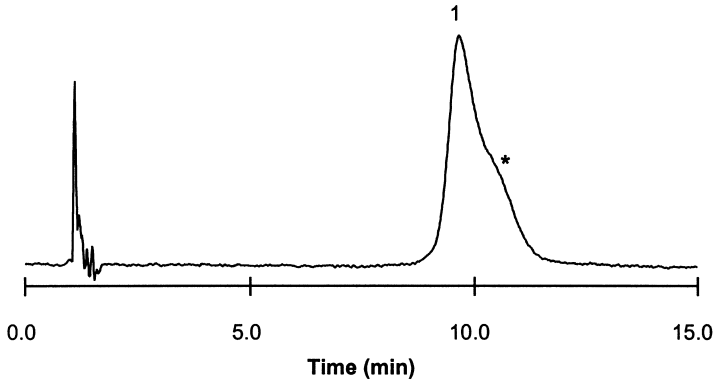


PicoTag™ (C18)
0.05% HAC in 5% ACN
15 µL injection volume
1 mL/min flow rate
PDA, 270 nm

1 caffeine
2 theobromine
3 theophylline
4 BHET
5 caffeic acid

Note: HAC = acetic acid; ACN = acetonitrile; BHET = β-hydroxyethyltheophylline

Figure 2. HPLC analysis of caffeine and its related compounds.



PicoTag™ (C18)
ACN/DMSO/MeOH
25/25/50 (v/v/v)
25 µL, 1 mL/min
PDA, 480 nm

1 urobilin
* impurity

NOTE: DMSO needed to elute urobilin.
(ACN = acetonitrile; DMSO = dimethylsulfoxide; MeOH = methanol)

Figure 3. HPLC analysis of urobilin.

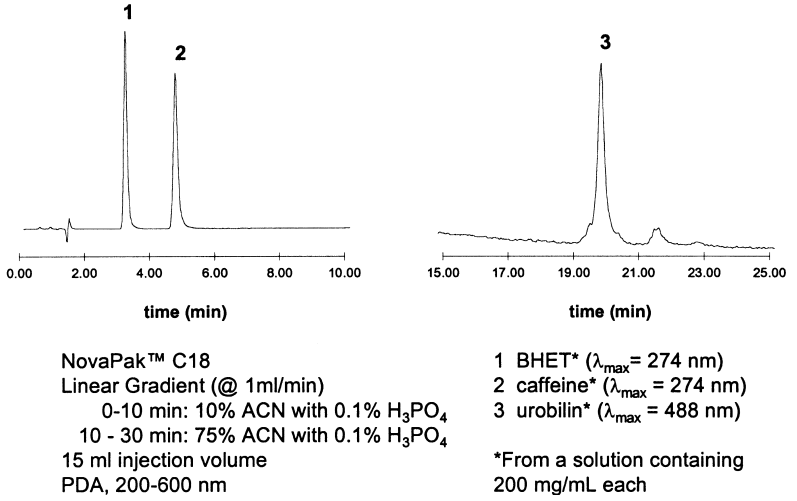


Figure 4. Simultaneous analysis of caffeine and urobilin.

μg/mL of each plus BHET as an internal standard. The extra peak eluting after urobilin is most likely the impurities of the urobilin sample.

With BHET as the internal standard, a series of standard solutions containing caffeine and urobilin were prepared to determine (in several replicates) the linearity of the simultaneous analysis of these compounds. The concentrations of these standard solutions were 0.05, 0.5, 5.0, 25.0, and 50.0 μg for caffeine and BHET and 0.2, 2.0, 10.0, and 20.0 mg/mL for urobilin. The lower concentrations used for the standard solutions of urobilin is due to its limited solubility in water. Figure 5 is an example of a plot of the concentration vs. the peak height of all three compounds.

The linearity of the analysis extends all the way down to hundredths of μg/mL. As shown by the magnitude of the slopes of the fitted line, the method is more sensitive for caffeine than urobilin. Limit of detection was calculated to be ~0.04 μg/mL for caffeine and ~0.10 μg/mL for urobilin at the 95% confidence level.

The SPE of caffeine and urobilin was performed on known concentrations of caffeine and urobilin. As mentioned in the experimental section, C_{18} cartridges were used. Retention times for caffeine and urobilin obtained from the above analysis of these compounds were used in the SPE method development.

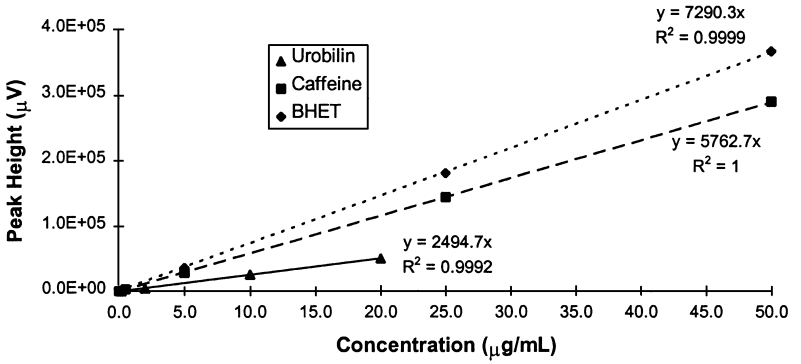


Figure 5. Linearity of the simultaneous analysis of caffeine and urobilin.

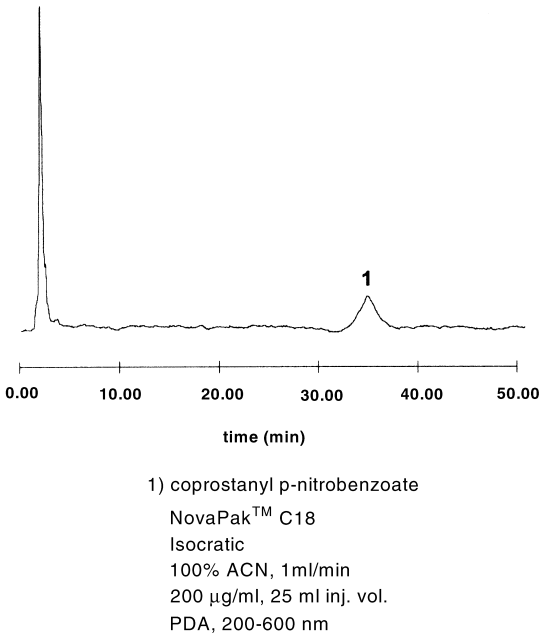


Figure 6. HPLC analysis of coprostanol with post-column derivatization.

The resulting protocol include: 1) cartridge conditioning using acetonitrile and water; 2) sample addition, 3) sample washing with water, 4) sample elution using a series of solvents including 1% HCl, 0.1% H_3PO_4 in 35% acetonitrile, 0.1% H_3PO_4 in 100% acetonitrile. Recoveries ranged from 80 to 97% for caffeine and urobilin.

Analysis of Coprostanol

Coprostanol, devoid of a chromophore, had to be derivatized for it to be analyzed by HPLC with PDA detection. A chromophore, p-nitrobenzoate, was coupled to coprostanol by a simple acylation of the hydroxyl group of coprostanol. This reaction, and the chromatographic analysis were performed in N_2 atmosphere. The resulting product, coprostanyl benzoate, was analyzed by NMR to confirm its identity. As shown in Figure 6, results confirmed that coprostanol can be analyzed using this method.

CONCLUSION

The analysis of caffeine, urobilin, and coprostanol can be accomplished using HPLC with PDA detection. While caffeine and urobilin can be analyzed simultaneously, coprostanol had to be derivatized and analyzed separately. Future studies will include analysis of real samples such as drinking water or sewage water using the methods developed. While an SPE protocol was developed for the extraction of caffeine and urobilin from a water matrix, a method will be developed for the separate extraction of coprostanol.

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Dr. Piocos is currently with the Procter and Gamble Company, Cincinnati, Ohio 45201, U.S.A.

REFERENCES

1. L. B. Barber, II, J. A. Leenheer, W. E. Pereira, T. I. Noyes, G. K. Brown, C. F. Tabor, J. H. Writer, **Contaminants in the Mississippi River,** R. H.

Mead, ed., U.S. Geological Survey Circular 1133, Reston, VA (1995), pp. 1-18.

2. J. Albaiges, F. Casado, F. Ventura, Wat. Res., **20**, 1153-1159 (1986).

3. R. Leeming, P. D. Nichols, Wat. Res, **30**, 2997-3006 (1996).

4. Y. Miyabara, N. Sugaya, J. Suzuki, S. Suzuki, Bull. Environ. Contam. Toxicol., **53**, 77-84 (1994).

5. A. W. Garrison, J. D. Pope, F. R. Allen, **Identification and Analysis of Organic Pollutants in Water**, L. H. Keith, ed., Ann Arbor, MI (1981), pp. 571-576.

6. M. J. Arnaud, **Caffeine, Coffee and Health,** S. Garatini, ed., Raven Press, New York, NY, 1984, pp. 43-95.

7. O. A. Ogunseitan, W. J. Microbiol. Biotechnol., **12**, 251-256 (1996).

8. T. Drake, Wat. Engineer. Man., **9** (1994).

9. R. V. A. Bull, C. K. Lim, C. H. Gravy, J. Chromatogr., **218**, 647-654 (1981).

10. Y. Miyabara, Y. Sakata, J. Suzuki, S. Suzuki, Environ. Pollut., **84**, 117-122 (1994).

11. R. Leeming, A. Ball, N. Ashbolt, P. Nichols, Wat. Res., **30**, 2893-2900 (1996).

12. J. H. Writer, J. A. Leenheer, L. B. Barber, G. L. Amy, S. C. Chapra, Wat. Res., **29**, 1427-1432 (1995).

13. L. A. LeBlanc, J. S. Latimer, J. T. Ellis, J. G. Quinn, Estuar. Coast. Shelf Sci., **34**, 439-458 (1992).

14. C. M. G. Vivian, Sci. Tot. Environ., **53**, 5-40 (1986).

15. I. Perez-Martinez, S. Sagrado, M. J. Medina-Hernandez, Chromatographia, **43**, 149-154 (1996).

16. I. N. Papadoyannis, V. F. Samanidou, K. A. Georga, J. Liq. Chromatogr. Rel. Technol., **19**, 2559-2578 (1996).

17. P. Dobrocky, P. N. Bennett, L. J. Notarianni, J. Chromatogr. B, **652**, 104-111 (1994).

18. C. O. Thompson, V. C. Trenerry, B. Kemmery, J. Chromatogr. A, **694**, 507-514 (1995).

19. P. Campins-Falco, R. Heraez-Hernandez, A. Sevillano-Cabeza, J. Liq. Chromatogr. Rel. Technol., **16**, 1297-1314 (1993).

20. D. W. Mellini, N. E. Caporaso, H. J. Issaq, J. Liq. Chromatogr., **16**, 1419-1426 (1993).

21. J. N. Micelli, W. Chapman, J. Liq. Chromatogr., **13**, 2239-2251 (1990).

22. T. E. B. Leakey, J. Chromatogr., **507**, 199-220 (1990).

23. Y. Miyabara, M. Tabata, J. Suzuki, S. Suzuki, J. Chromatogr., **574**, 261-265 (1992).

24. P. D. Nichols, R. Leeming, M. S. Raymer, V. Latham, J. Chromatogr., **143**, 189-195 (1993).

25. M. Venkatesan, I. R. Kaplan, Envir. Sci. Technol., **24**, 208-213 (1990).

26. M. M. Krahn, C. A. Wigren, L. K. Moore, D. W. Brown, J. Chromatogr., **481**, 263-273 (1989).

27. M. Yde, E. De Wuf, S. De Maeyer-Cleempoel, D. Quaghebeur, Bull. Environ. Contam. Toxicol., **28**, 129-134 (1982).

28. P. G. Hatcher, P. A. McGillivary, Environ. Sci.Technol., **13**, 1225-1229 (1979).

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