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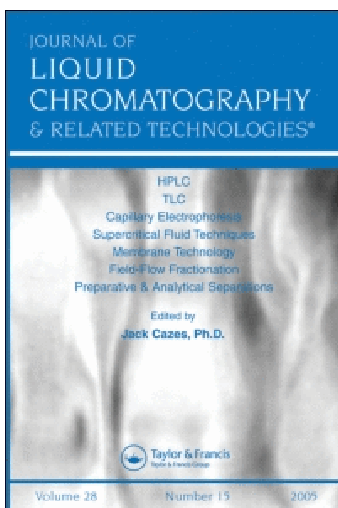

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IDENTIFICATION OF NITROGENOUS ORGANIC COMPOUNDS IN AQUATIC SOURCES BY STOPPED-FLOW SPECTRAL SCANNING TECHNIQUE

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ABSTRACT

The presence of nitrogenous organic compounds in raw water sources for municipal supplies is of environmental concern because many of them exert significant chlorine demand (1), while some produce complex stable mutagenic products upon chlorination (2, 3) or are precursors in haloform formation (1). To assist in assessing the importance of this problem high performance liquid chromatography (HPLC) was utilized to identify trace quantities of N-organic contaminants in concentrated samples of municipal water supplies of northeastern Massachusetts. Chromatographic resolution of complex mixtures was achieved on a reversed phase column (Zorbax C-8, DuPont Co.) using a 0.05 M phosphate buffer (pH = 6.9) or a 0.05 M borate buffer (pH = 8.9) to 50% methanol gradient. Constituents of concentrated samples were identified by the amount of time required for elution from the analytical column (retention position), and the positions of maximum and minimum U.V. absorbances, which were measured by stopped-flow spectral scanning of resolved chromatographic peaks. A fluorometric monitor utilizing fluorescamine and borate buffer revealed groups of primary amine compounds not detectable by U.V. spectroscopy.

INTRODUCTION

Reports (4-6) indicating the widespread presence of micro quantities of numerous organic contaminants in our water resources have evoked concern because of the potential carcinogenic and toxic properties of a number of compounds. Although the composition and extent of hydrocarbon contamination in natural water by hydrocarbons and chlorination derivatives is presently under extensive examination, the identification of nitrogenous organic compounds in these waters has not be significantly pursued. This is because many

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nitrogenous materials are relatively non-volatile, so that their identification and analysis by gas chromatography is not possible without prior formation of volatile derivatives. Some nitrogenous heterocyclic compounds (m-aminophenol, uracil, tryptophan, pyrrole, chlorophyll, alanine, proline, ℓ-hydroxy-proline, and indole) are precursors of haloform formation during the chlorination of water supplies (1). Others (uracil and cytosine for example) produce complex stable mutagenic chlorinated products when allowed to react with chlorine (2, 3).

Recent advances in the field of high performance liquid chromatography have made the detection of many non-volatile nitrogenous compounds analytically feasible. HPLC separations of various mixtures of nitrogenous organic compounds have been previously described (Table 1) (7-52). This study presents the chromatographic methods used to separate and identify N-organic constituents found in natural waters and dilute algal cultures.

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MATERIALS

The chromatographic system consisted of a DuPont (Wilmington, DE) 848 pump with a DuPont 838 programmable gradient accessory, a Schoeffel (Westwood, NJ) SF 770 U.V. monitor with a SFA 339 wavelength drive assembly, a FS 970 spectrofluoro fluorescence monitor with a wavelength drive, a Duplex minipump (Laboratory Data Control, Riviera Beach, FL) and a Schoeffel mm 700 memory module. Both the U.V. and fluorescence monitors were fitted with wavelength drive mechanisms for use in identifying peaks by a stopped-flow spectral scanning technique. The memory module provided baseline correction during spectral scanning. The minipump was used to deliver buffer and reagents for producing fluorescent derivatives to the column effluent before it entered the fluorometer.

Figure 1 is a schematic presentation of the chromatographic system and is similar to that used by Stein et al. (53). All Teflon tubing,

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Table 1

HPLC Separation of Nitrogenous Organic Compounds

Compounds	Column	Mobile phase	Reference
nucleic acid bases	Zorbax CN[1] paired-ion chromatography	propionic acid with heptane sulfonic acid	DuPont Co. (7)
nucleic acid bases	Zipax SCX[1]	0.01 HNO_3	Kirkland (8)
nucleic acid hydrolysis products: purines, pyrimidines, nucleosides, RNA hydrolyzates, nucleotides	XAD-4 support coated with triethylammonium bicarbonate	linear gradient, 0.1-0.4 M triethylammonium bicarbonate	Vematsu and Suhadolnik (9)
nucleosides	Aminex A-28[2]	Na Borate at pH 3-9 with varying molarity	Schneider and Glazko (10)
nucleotides	Spherisorb 10μ[3]	tetra-_n_-butylammonium hydrogen sulfate and 10-camphorsulfonic acid pH 3.9	Hoffman and Liao (11)
nucleosides and bases in serum and plasma	μBondapak C_{18}[4]	KH_2PO_4 pH 5.5 60/40 MeOH/H_2O	Strop _et al_. (12)
nucleosides and their bases	μBondapak C_{18}	.01 F KH_2PO_4 pH 5.5 MeOH-H_2O gradient (80/20)	Hartwick and Brown (13)

(continued)

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Table 1 (continued)

Compounds	Column	Mobile phase	Reference
purine nucleotide, nucleoside and base metabolites from biological extracts	μBondapak C_{18}	.05 M ammonium dihydrogen phosphate buffer	Anderson and Murphy (14)
adenosine in the presence of other nucleic acid components	μBondapak C_{18}	.007 F KH_2PO_4 H_2O/MeOH gradient	Hartwick and Brown (15)
amino acids and amino sugars	Aminex A-6	sodium citrate buffers of varying pH	Hadzija and Keglevic (16)
fluorescamine derivatized amino acids	Durrum DC-1A[5]	citrate buffers pH 3.28 and 4.25	Stein *et al*.(17)
O-phthalaldehyde derivatives of amino acids	Aminex A-6	citrate buffers: pH 3.2, 4.25, and 6.4	Roth and Hampai (18)
amino acids as dansyl derivatives with fluorometric detection	LiChrosorb SI60[6] LiChrosorb RP8	benzene-pyridine-acetic acid mixture	Bayer *et al*. (19)
ninhydrin chromagens of amino acids	Technicon[7] chromobeads, type B	citrate buffers pH 2.88 and 5.00	Ellis and Garcia (20)

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dansyl amino acids	Zipax R[1]	methyl ethel ketone with light petroleum	Frei and Lawrence (21)
amino acids with fluorescamine detection	DC-4A[5]	Na citrate buffers	Georgiadis and Coffey (22)
dansyl amino acids	Micropak MCH-10[8] ion pairing technique	buffered MeOH/H_2O 0.01 M $(CH_3)_4$ NCl counter ion source	Ellis and Garcia (23)
dansyl amino acids	Micropak-NH_4-10[8]	dichloromethane-acetic acid (99:1)/acetonitrile-acetic acid (90:10) gradient	Johnson _et al._ (24)
dansyl amino acids	Particil PAC[9] Poragel PN[4] Vydac polar phase[10]	acetonitrile-water-acetic acid	Hsu and Currie (25)
amino acids	resin coated glass beads Poracil C[4] Corasil II[4]	distilled and deionized water pH = 6	Grushka and Scott (26)
urinary constituents	BioRad A-15[2] BioRad A-27[2]	ammonium acetate-acetic acid buffer pH 4.28 (0.015 - 6 M)	Mrochek _et al._ (27)
urinary U.V. adsorbing metabolites	Zerolit[11]	acetic acid-ammonium acetate (pH 4.4) varying from .015 - 6 M	Geeraerts _et al._ (28)
urine amino acids	Aminex A-7[2]	sodium acetate-acetic acid buffer, pH 4.4; .015 - 6 M	Hamilton (29)

(continued)

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Table 1 (continued)

Compounds	Column	Mobile Phase	Reference
serum and urine components	Aminex A-27	sodium acetate-acetic acid buffer, pH 4.4; .014 - 6 M	Katz et al. (30)
human urine, blood serum, *cerebrospinal* fluid, and amniotic fluid	Aminex A-27	sodium acetate-acetic acid buffer, pH 4.4; .014 - 6 M	Katz et al. (30)
U.V. absorbing constituents of human urine	Aminex BRX[2]	sodium acetate-acetic acid buffer, pH 4.4; .014 - 6 M	Burtis (31)
acidic urinary constituents	LiChrosorb ODS[6]	increasing acetonitrile concentration in dilute acid solution	Molnar and Horvath (32)
U.V. absorbing constituents of human urine	BioRad AGI-18[2]	sodium acetate-acetic acid buffer, pH 4.4; .015 - 6 M	Scott (33)
dansyl *polyamine* derivatives	MicroPak CH-10[8]	water/acetonitrile gradient	Johnson et al. (24)
complex biological mixture	Aminex A-27 Aminex A-6	sodium acetate-acetic acid buffer, pH 4.4; .015 - 6 M	Scott et al. (34)

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aromatic bases	Zipax SCX	water with 0.15 M $NaNO_3$	DuPont Co (35)
cyanopyridines	Zipax SCX	water with 0.10 $NaNO_3$ and 0.1 N H_3PO_4	Talley (36)
Neuroamines, phenethylamines, B-hydroxyphenethyl, amine and indoleamines	Zipax SCX	ammonium phosphate pH 7	McMurtrey (37)
aza arenes	μBondapak C-18 μ Porasil[4]	20-80% CH_3CN in water 1% propanol in hexane	Dong and Locke (38)
aromatic amine carcinogens	Zipax SCX	0.1 ammonium acetate buffer	Mefford et al. (39)
Caffeine in coffee	Zipax SCX	0.01 M nitric acid	Madison et al. (40)
organic constituents in primary and secondary sewage treatment plant effluents	strong cation-exchange column		Jolley et al. (41)
trace organic compounds in municipal sewage effluent	Aminex A-27	sodium acetate-acetic acid buffer, pH 4.4; .015 - 6 M	Pitt et al (42)
organic halogen products in chlorinated municipal sewage effluents	Aminex A-27	sodium acetate-acetic acid buffer, pH 4.4; .015 - 6 M	Jolley et al. (43)

(continued)

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Table 1 (continued)

Compounds	Column	Mobile Phase	Reference
trace organic compounds	C_{18} μBondapak	20-100% acetonitrile in water	Hites and Biemann (44,45)
organic compounds extracted from drinking water by adsorption onto XAD macroreticular resins	μPorasil μBondapak	hexane to chloroform + hexane gradient hexane to ethanol + hexane gradient	Thurston (46)
tryptophan and some of its metabolites in biological fluids	Spherisorb ODS	50% MeOH + 50% paired ion chromatographic solution	Riley et al. (47)
benzamide, benzene-sulfonamide, or 4-methoxybenzamide derivatives or primary and secondary amine compounds	μBondapak C_{18}	mixtures of water and MeOH or acetonitrile	Clark and Wells (48)
dansyl amino acids	μBondapak C_{18} Spherisorb 5ODS[12]	linear gradient formed from acetonitrile and sodium phosphate buffers (neutral pH)	Wilkinson (49)
amino acids and aromatic amino acid derivatives	Amberlite CG-120 Type III[2]	gradient from pH = 3.25 to 4.25 to 7.70 using sodium citrate or Borax	Ohtsuki and Hatano (50)

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amino acids	Durrum DC-6A[5]	citrate buffers (pH 3.2, 3.5, and 4.0)	Kasiske et al. (51)
mono-, di-, and triphosphate nucleotides of adenine, guanine, hypoxanthine, xanthine, uracil, thymine and cytosine	Partisil 10-SAX[9]	.007 F KH_2PO_4 and .007 F KCl (pH 4.0) to 0.25 F KH_2PO_4 and 0.50 F KCl (pH 5.0) gradient	McKeag and Brown (52)

1. DuPont Company, Wilmington, Delaware
2. Bio Rad Laboratories, Richmond, California
3. Spectra Physics, Santa Clara, California
4. Waters Associates, Milford, Massachusetts
5. Durrum Chemical Company, Palo Alto, California
6. Merck, A.G., Darmstadk, Germany
7. Technicon Industrial Systems, Tarrytown, New York
8. Varian Associates, Palo Alto, California
9. Whatman Inc., Clifton, New Jersey
10. Separation Group, Hesperia, California
11. Permutit, London, Great Britain
12. Jones Chromatography Ltd., Llanbradach, United Kingdom

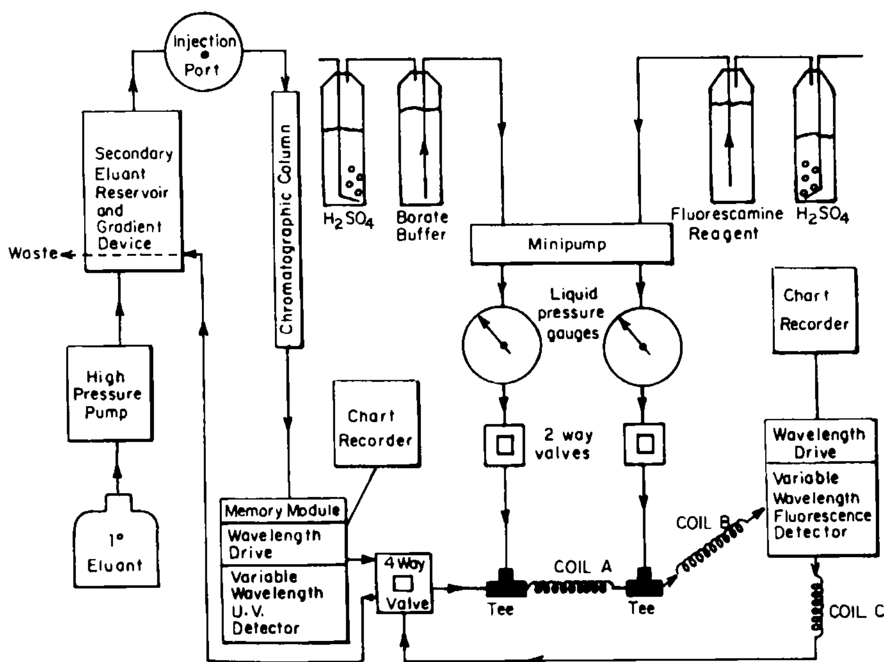

FIGURE 1: Schematic presentation of chromatographic system.

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connectors and tees were purchased from Rainin Instrument Co. (Boston, MA). 250-ml reservoir bottles were placed approximately 3 feet above the minipump and connected to it by 1.5 mm I.D. x 3.0 mm O.D. teflon tubing. Air displacing the fluorescamine and borate buffer was first passed through 50% sulphuric acid in a gas trap to remove traces of primary amines. The exit valves of the pump were connected to 0.8 mm I.D. x 1.5 mm O.D. tubing. Coils A, B, and C were approximately 0.40 m, 1.5 m, and 9.0 m lengths of 0.3 mm I.D. x 1.5 mm O.D. tubing respectively. The coiled pieces of tubing, A and B, were inserted to provide sufficient mixing of the reagents. Coil C was included to induce sufficient back-pressure to prevent gas bubble formation when acetone was mixed with the aqueous buffer at the second tee. A back-pressure of about 40 psi was maintained and was monitored by two liquid pressure gauges.

These gauges also helped to dampen the pulsating flow of reagents produced by the positive displacement reciprocating piston mechanism of the minipump.

METHODS

A 4.6 mm x 25 cm Zorbax C-8 column (DuPont Co.) was used with either a 0.05 M phosphate buffer (pH 6.9) or a 0.05 M borate buffer (pH 8.9) to 50% methanol gradient. A 4.6 mm x 5 cm guard column packed with permaphase ODS (DuPont Co.) was placed between the injection port and the Zorbax C-8 column to prevent fouling of the analytical column by sample or mobile phase contaminants. 0.05 M phosphate buffer (pH 6.9) was made from equal molar concentrations of KH_2PO_4 and Na_2HPO_4. Borax buffer was composed of 0.05 M sodium borate ($Na_2B_4O_7 \cdot 5H_2O$) adjusted with phosphoric acid. The concentration gradient used in the chromatographic separations is shown in Figure 2.

Five to 50 µl samples concentrated 1,000 to 2,000 fold by rotary evaporation and lyophilization, were introduced into the chromatographic system through a Rheodyne (Berkeley, CA) model 7120 syringe-loading injector. Eluted compounds were detected by U.V. and fluorescence

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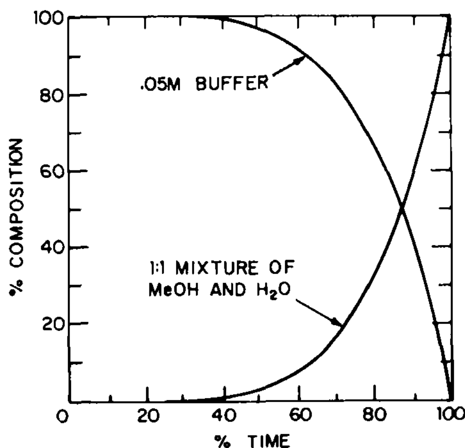

FIGURE 2: Concentration gradient used in chromatographic elution.

spectroscopy with or without fluorescamine derivatization. Fluorescamine (4-phenylspiro-[furan-2(3H), 1'-phthalan]-3,3'-dione) (Hoffman-LaRoche Inc., Nutley, NJ) reacts very rapidly, at room temperature, with primary amines in borate buffer to yield highly fluorescent pyrrolinone derivatives which can be measured at picomole levels (53-56). Borate buffer was prepared by titrating 0.1 M boric acid to pH 9.3 with 4.5 M LiOH · H_2O and was introduced at a flow rate of about 15 ml/hour. The fluorescamine solution consisted of 15 mg/L in acetone and was used at a flow-rate calculated to be less than 25-30% of the total flow (equal to 15 ml/hour).

Identification of unknown resolved chromatographic peaks was achieved by comparison of retention positions and ultraviolet data with those of reference compounds. U.V. data were obtained by stopped-flow spectral scanning of individual chromatographic peaks.

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RESULTS AND DISCUSSION

Separations of mixtures of reference compounds using phosphate and borate buffered mobile phases are shown in Figures 3 and 4 respectively. Although the manufacturer reported the operating pH range for the Zorbax C-8 stationary phase to be between pH 2 and pH 9, it was found that deterioration of this material occurred at pH 6.9 and increased rapidly under more alkaline conditions. This was later explained by silica dissolution arising from use of alkaline eluant.

Extended analytical column life was later achieved using the guard column which was thought to presaturate the mobile phase with dissolved silica, thereby decreasing the rate of silica dissolution in the analytical column.

A typical chromatogram of a concentrated field sample is shown in Figure 5. Identification of chromatographically separated substances was based predominantly upon retention positions and on the positions of maximum and minimum U.V. absorbances of resolved chromatographic

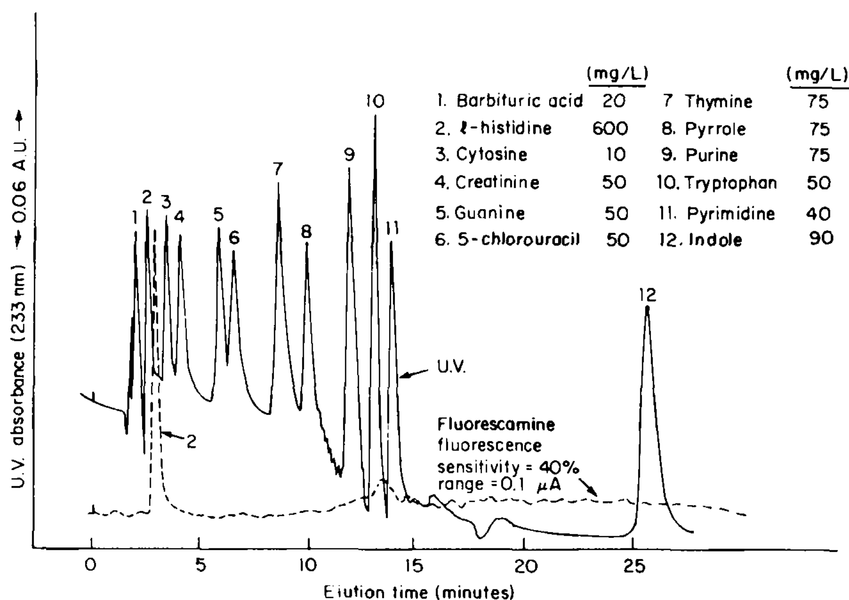

FIGURE 3: Chromatogram of mixture of 12 compounds in water. Column: 25 cm x 4.1 mm Zorbax C-8. Mobile phase: .05 M phosphate buffer (pH = 6.9) to 50% MeOH, 10 minute gradient. Fluorescamine and borate buffer introduced before fluorometer to monitor derivatized primary amine compounds. (excitation = 390 nm; emission = 470 nm).

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peaks measured by stopped-flow spectral scanning. Absorbance ratio values were not used frequently as identification parameters because the presence of coeluting materials altered the relative absorbance magnitudes determined at several U.V. wavelengths of the compounds being characterized. Unknown substances were initially identified with particular N-organic compounds on the basis of retention times. The identities of the compounds were then corroborated by comparing the U.V. spectra of the unknown materials to those of reference substances.

The spectra of two reference nitrogenous organic compounds and two chromatographically separated unknown materials identified with these particular N-organic compounds are shown in Figures 6-9. The figures illustrate the correspondence of positions of maximum and minimum U.V

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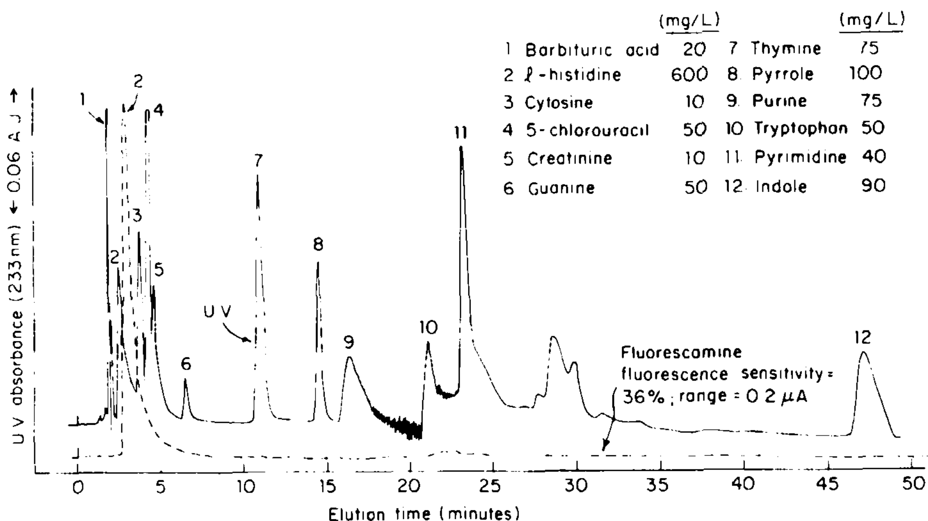

FIGURE 4: Chromatogram of mixture of 12 compounds in water. Column: 25 cm x 4.1 mm Zorbax C-8. Mobile phase: .05 M borate buffer (pH = 8.9) to 50% MeOH, 20 minute gradient. Fluorescamine and borate buffer introduced before fluorometer to monitor derivatized primary amine compounds. (excitation = 390 nm; emission = 470 nm).

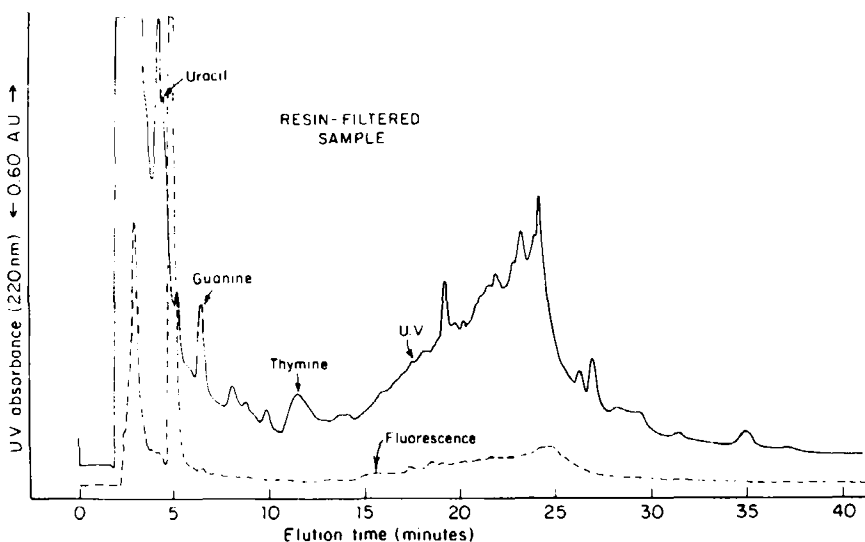

FIGURE 5: Chromatogram of Middleton Pond (Danvers, MA) sample concentrated 1,000 fold. Column: 25 cm x 4.1 mm Zorbax C-8. Mobile phase: .05 M borate buffer (pH = 8.9) to 50% MeOH, 20 minute gradient. Fluorescamine and borate buffer introduced before fluorometer to monitor derivatized primary amine compounds. (excitation = 390 nm; emission = 470 nm).

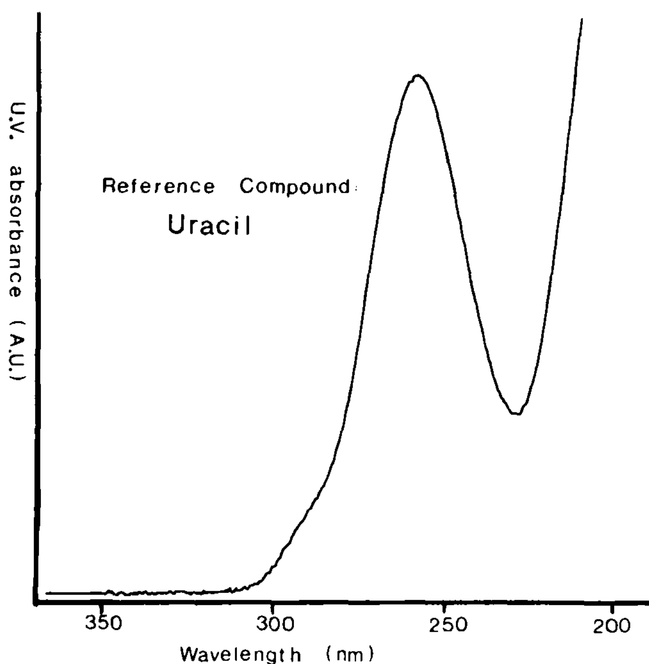

FIGURE 6: U.V. spectrum of uracil.

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absorbances in the spectra of the known and unknown materials. Although U.V. spectra do not provide certain identification, they do, in conjunction with retention data, provide a reasonable degree of confidence in the proposed identities of the compounds.

The large, rapidly eluting peak in Figure 5, was observed in all chromatograms of concentrated field samples. Underivatized fluorescence for this material was substantially less than the corresponding derivatized fluorescence indicating the presence of primary amine substances in this rapidly eluting group of compounds. Many reference amino acid compounds were also shown to elute rapidly from the Zorbax column using either the phosphate or borate buffer.

Figure 5 illustrates the enhanced sensitivity and selectivity of fluorescence detection over conventional absorbance spectroscopy. The

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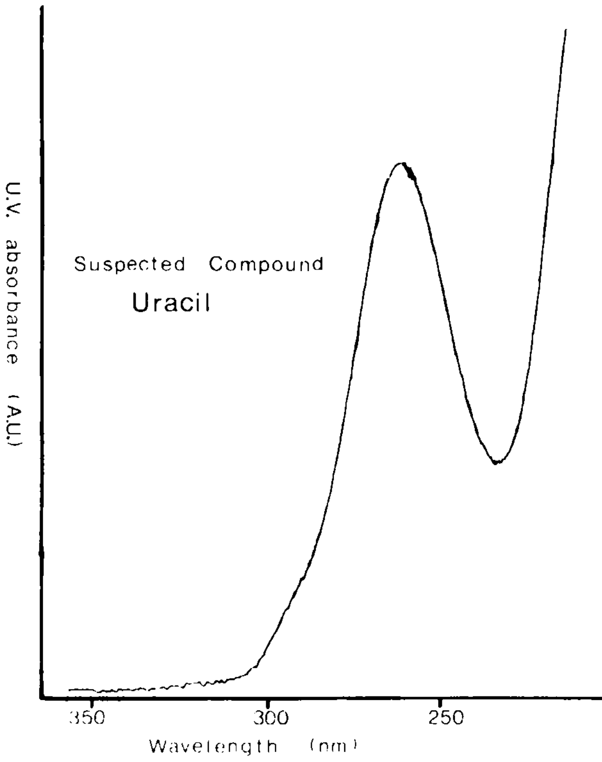

FIGURE 7: U.V. spectrum of compound suspected to be uracil.

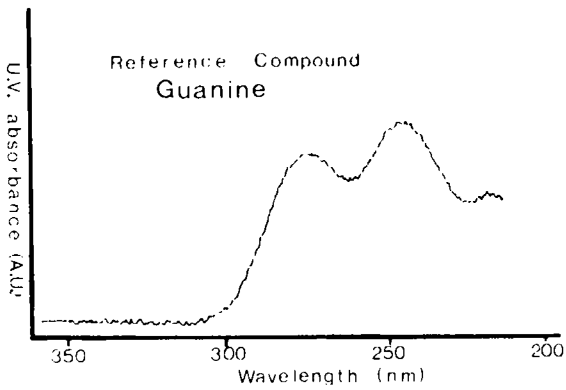

FIGURE 8: U.V. spectrum of guanine.

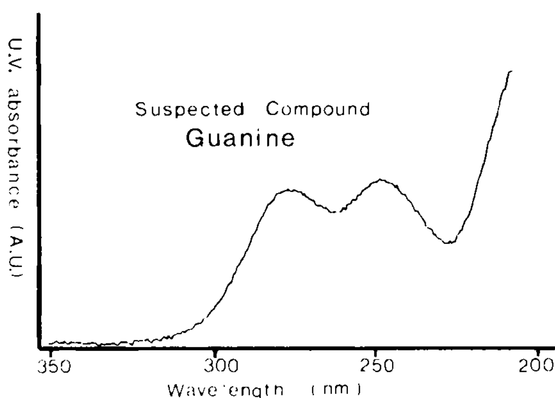

FIGURE 9: U.V. spectrum of compound suspected to be guanine.

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fluorescamine-derivatized trace revealed groups of nitrogenous materials having little or no U.V. absorbance. However, resolution of these groups of derivatized material into identifiable chromatographic peaks was not achieved. Thus, while fluorescence detection using fluorescamine did indicate the presence of primary amine materials in the concentrated raw water sources, it did not provide information on the specific nature of their composition.

A number of N-organic compounds were identified in the concentrated water supplies, including adenine, 5-chlorouracil, cytosine, guanine, purine, thymine, and uracil. The levels found and environmental significance of their presence are discussed elsewhere (57).

CONCLUSION

Non-volatile nitrogenous organic contaminants can be identified in dilute aquatic sources by reversed-phase high performance liquid chromatography and stopped-flow spectral scanning technique. Fluorescence detection with fluorescamine derivatization reveals groups of primary amines and amino acids not detectable by conventional absorption spectroscopy.

ACKNOWLEDGEMENT

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