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QUANTITATIVE DETERMINATION OF NITRILOTRIACETIC ACID AND RELATED AMINOPOLYCARBOXYLIC ACIDS IN INLAND WATERS

ANALYSIS BY GAS CHROMATOGRAPHY

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SUMMARY

Quantitative methods were developed for detecting nitrilotriacetic acid (NTA) iminodiacetic acid (IDA), N-methyl-iminodiacetic acid (NMIDA), glycine (GLY), sarcosine (SARC), and N-oxalyl-iminodiacetic acid (N-oxalyl-IDA) in a variety of water samples and N-nitroso-iminodiacetic acid (N-nitroso-IDA) in river water. NTA, NMIDA, and N-oxalyl-IDA were converted to their corresponding *n*-butyl esters; IDA, GLY, and SARC were converted to N-trifluoroacetyl *n*-butyl ester derivatives; N-nitroso-IDA was converted to the N-trifluoroacetyl *n*-butyl ester derivative of IDA. These volatile derivatives were separated and analyzed on an ethylene glycol adipate gas chromatographic column. For quantitative analysis, the amount of each amino acid injected was 3 to 200 ng, and the amount of each amino acid separated from the water sample was 0.25 to 1000 μg. Response values for NTA, IDA, NMIDA, GLY, and SARC were reproducible and showed no statistical bias for river water sample concentrations of 20 to 0.025 mg/l. Relative standard deviations ranged from 8 to 13%. The response value for N-nitroso-IDA varied with concentration but was reproducible at any given concentration.

Each or all of the above amino acids could be detected in samples of river water, well water, water containing nitrate ions, water containing formaldehyde, primary sewage effluent, and secondary sewage effluent.

INTRODUCTION

Proposed use of nitrilotriacetic acid (NTA, compound 1) as a detergent builder, which would lead to its ubiquitous entry into the environment, has prompted several studies of its biodegradability (for a listing of the literature in this area, see ref. 1) and the development of polarographic[2,3], colorimetric[4–9], and gas chromatographic[10,11] procedures for detection of microgram quantities of NTA[11]. We have extended the

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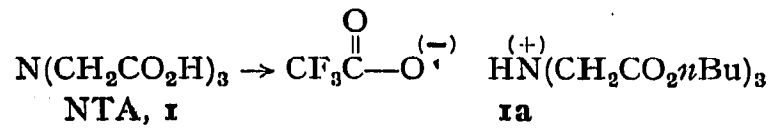
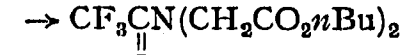
biodegradability studies to mixtures of NTA, iminodiacetic acid (IDA, compound **2**), N-methyl-iminodiacetic acid (NMIDA, compound **3**), glycine (GLY, compound **4**), sarcosine (SARC, compound **5**) and N-oxalyl-iminodiacetic acid (N-oxalyl-IDA, compound **6**) in various media and to N-nitroso-iminodiacetic acid (N-nitroso-IDA, compound **7**) in river water. This extension has led to the development of several new sample preparation and derivatization procedures for detection of submicrogram quantities of the above amino acids.

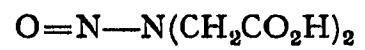
Our basic approach was to spike a 10-ml water sample with glutamic acid (used as an internal standard), freeze-dry the sample, and treat the residue with 3 *N* 1-butanol–HCl. The alcoholic HCl was stripped off, and the residue was shaken with aqueous potassium bicarbonate and extracted with methylene chloride. The methylene chloride extract was treated with trifluoroacetic anhydride and concentrated. This procedure converted amino acids **1** to **7** to *n*-butyl ester derivatives (**1a**, **3a**, and **6a**) and to N-trifluoroacetyl *n*-butyl ester derivatives (**2a**, **4a**, and **5a**).



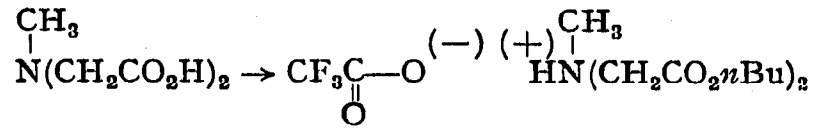
NTA, **1** **1a**

IDA, **2**

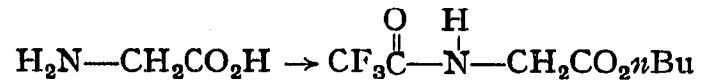
and → 



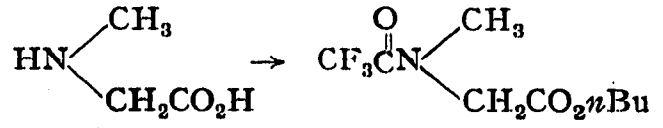
N-nitroso-IDA, **7** **2a**



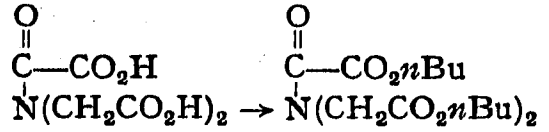
NMIDA, **3** **3a**



GLY, **4** **4a**



SARC, **5** **5a**



N-oxalyl-IDA, **6** **6a**

The decision to use N-trifluoroacetyl *n*-butyl esters was not arbitrary. This derivative system had been refined for the detection of amino acids in HCN polymer hydrolysates[12] and is currently used in many laboratories for the analysis of protein hydrolysates[13]. We found that compounds **1a** to **6a** were sharply resolved on an ethylene glycol adipate column[13], were stable for at least one week, were easily concentrated, and were ideally suited for flame ionization detection.

Compounds **6** and **6a** had not been synthesized before. The synthesis and characterization of these compounds and an improved synthesis of N-nitroso-IDA are presented in EXPERIMENTAL.

EXPERIMENTAL

Apparatus

A Varian Aerograph 2100 dual-column gas chromatograph with flame ionization detectors was used in the dual, differential mode. The signal from the gas chromatograph was fed to an Infotronics CRS-104 digital integrator and then to both channels of a Varian Aerograph, Model 30, dual-pen recorder. For the GC scans presented in this paper, the range of the low-sensitivity pen was 0 to 50 mV, and the range of the high-sensitivity pen was 0 to 1 mV.

Mass spectra were obtained with a GC–MS system composed of a Varian Aerograph 1700 gas chromatograph interfaced with a Varian CH7 mass spectrometer. IR spectra were run on a Beckman IR4 spectrophotometer; NMR spectra were obtained with a Varian T-60.

The reactions were carried out in 20 × 125 mm and 16 × 75 mm screw-capped test tubes fitted with Teflon-lined caps (Corning 9826, available from A. H. Thomas Co., Philadelphia, Pa.).

A Sonogen, Model HD-50, sonic bath was used to agitate the amino acid–BuOH–HCl solutions. The bath was covered with a 1/8-in.-thick Teflon sheet, through which holes had been drilled with a cork borer. Sample tubes were placed through the holes and held in place with rubber O-rings. The temperature of the bath when covered with the sample holder was 75° ± 3°.

A Thelco, Model 81, water bath (Fisher Scientific, 15-475) maintained at 65° ± 0.5° was used to heat the 20 × 125 mm sample tubes. A Lab-Line Temp-Blok containing twelve 16-mm I.D. holes (A. H. Thomas, 6124-C40) was used to heat the 16 × 75 mm sample tubes. The Temp-Blok was heated with a Thermolyne, Model HP-A1G15B, hot plate (Fischer Scientific, 11-494); temperature variation was ±2° in the range of 50 to 100°.

A Büchi all-glass rotary evaporator (Rinco Instrument Company, Model VE 50) was used to remove volatile solvents.

Materials

Butanol–HCl. A weighed amount of dry HCl gas (Liquid Carbonic Corp., 99%) was added to *n*-butanol (Mallinkrodt, AR grade) which had been distilled (100 mm Hg) from calcium hydride. The final normality was adjusted to 3.0 ± 0.1.

Methylene chloride and 1,1,2-trichlorotrifluoroethane (Freon 113). Methylene chloride (Matheson Coleman and Bell, reagent grade) and Freon 113 (Matheson,

99%) were distilled from calcium hydride and stored in solvent generators, which were vented to the atmosphere through $CaCl_2$ drying tubes.

Trifluoroacetic anhydride and trifluoroacetic acid. Both were purchased from Eastman Kodak Company and were used without further purification.

Hydrogen, nitrogen, and air. Hydrogen, 99.92%, and nitrogen, 99.99% (standard grade gases supplied by The Acetylene Gas Company) were fed through Gas-Dry filter traps (Chemical Research Services, Chicago, 120-cc. capacity) which contained indicating Drierite and Linde 4A molecular sieves. Dry compressed air (house air) was fed through a Gas-Dry filter trap (CRS, 120-cc. capacity) which contained 6–16 mesh silica gel (Fisher Scientific, S-155, grade 05) and 8 mesh calcium chloride. No change in the base line was observed for an attenuation of 1×10^{-11} Amp/mV when the above grades of gases were used in place of purer gases.

Glutamic acid, glycine, and sarcosine. L-Glutamic acid (Schwarz Bioresearch, Inc.), glycine hydrochloride (Eastman Organic Chemicals), and sarcosine hydrochloride (Sigma Chemical Company, Sigma grade) were used without further purification.

Iminodiacetic acid and N-methyl-iminodiacetic acid. Iminodiacetic acid (Matheson Coleman and Bell) and N-methyl-iminodiacetic acid (Aldrich) were recrystallized from water and dried *in vacuo* over P_2O_5.

Nitrilotriacetic acid. Nitrilotriacetic acid, recrystallized ten times from water, was obtained from the Monsanto Inorganic Chemical Division. This material was dried *in vacuo* over P_2O_5.

N-Nitroso-iminodiacetic acid (7). To a 500-ml, 4-necked flask—equipped with an overhead stirrer, an addition funnel, and a thermometer to which was connected an I^2R Thermowatch probe (Instruments for Research and Industry, 102 Franklin Ave., Cheltenham, Pa. 19012)—were added 26.6 g (20 mmoles) of iminodiacetic acid and 200 ml of 1 *M* phosphoric acid. The contents of the flask were heated to 50°, and a solution of 27.6 g (400 mmoles) of sodium nitrate in 60 ml of water was added dropwise. The reaction was then stirred for 1 h and extracted with five 100-ml portions of diethyl ether. The ethereal extract was dried ($MgSO_4$) and concentrated to yield a yellowish white residue which after recrystallization from methyl acetate–pentane afforded 13 g (40%) of nitroso acid: m.p. 149–150° (dec.), (lit.[14], 146–148°); NMR (DMSO-d_6) δ 13.5 (s, 2, $CH_2COO\mathit{H}$), 5.8 (s, 2, CH_2COOH), 5.5 (s, 2, CH_2COOH).

Anal. Calcd. for $C_4H_6N_2O_5$: C, 29.63; H, 3.73; N, 17.28. Found: C, 29.49; H, 4.13; N, 17.21.

Chloroglyoxylic acid, n-butyl ester (8). *n*-Butanol (1.15 moles, 85.5 g) was added dropwise to 174.5 g (1.38 moles) of oxalyl chloride. During the addition this reaction was stirred vigorously and cooled with an ice bath which kept the reaction temperature below 10°. After addition was completed, the reaction was stirred for 15 min, excess oxalyl chloride was stripped off, and the chloro-ester was distilled at 1 mm pressure on a Büchi rotary evaporator. Yield: 151 g (80% based on *n*-butanol) of **8**.

Chloroglyoxylic acid, benzyl ester (9). Benzyl alcohol (1.31 moles, 141 g) was added dropwise to oxalyl chloride. An ice bath kept the reaction temperature below 10°. After addition was completed, the reaction was stirred at room temperature for 20 min and distilled, giving 125 g (50%) of acid chloride **9**: b.p. 70–72° (5×10^{-3} mm).

N-Oxalyl-iminodiacetic acid, tributyl ester (6a). Into a four-necked, 1-l flask —equipped with a stirrer, a thermometer, and an addition funnel with a nitrogen

inlet—were placed 27.2 g (165 mmoles) of *n*-butyl chloroglyoxylate (**8**) and 500 ml of ethyl acetate. The stirrer was turned on, and 24.5 g (100 mmoles) of dibutyl iminodiacetate were added dropwise. The reaction mixture was refluxed for 1 h, concentrated, and distilled, giving 20 g (61%) of the keto ester (**6a**): b.p. 160–173° (1×10^{-5} mm); IR (neat between NaCl plates) 2950 cm^{-1}, 3865, 1781, 1681, 1468, 1285, 1183, 1058, 1047; mass spectrum (70 eV) *m/e* (relative intensity) 373(6) 301(7) 299(22) 245(5) 244(29) 243(25) 216(33) 188(7) 187(13) 160(60) 144(67) 88(69) 57(100).

Anal. Calcd. for $C_{18}H_{31}NO_7$: C, 57.89; H, 8.37; N, 3.75. Found: C, 57.70; H, 8.39; N, 3.57.

N-Benzyloxyoxalyl-iminodiacetic acid (10). To a 2-l beaker which contained a magnetic stirring bar were added 39 g (0.2 mole) of iminodiacetic acid disodium salt monohydrate, 500 ml of water, and 84 g (0.3 mole) of sodium bicarbonate. Benzyl chloroglyoxylate (**9**) (49.5 g, 0.25 mole) was added in 5-ml portions over the period of an hour; the reaction mixture was stirred for an additional hour and extracted with two 25-ml portions of ethyl acetate. The aqueous portion of the mixture was returned to the beaker, carefully acidified with 6 M H_2SO_4 to a pH of 1, and extracted with four 100-ml portions of ethyl acetate. The ethyl acetate extract was dried (Na_2SO_4), concentrated, and cooled overnight, giving a white crystalline material; the by-product (benzyloxyoxalic acid) of the reaction was washed off with methylene chloride. The remaining product, N.benzyloxyoxalyl-iminodiacetic acid, 16 g (25 %), m.p. 150–155° (dec), NMR (DMSO-d_6) 11.0 δ (s, 2, CH_2COOH), 7.40 (s, 5, C_6H_5), 5.28 150–155° (dec), NMR (DMSO-d_6) 11.0 δ (s, 2, CH_2COOH), 7.40 (s, 5, C_6H_5), 5.28 (s, 2, $C_6H_5CH_2$), 4.20 (s, 2, CH_2COOH), 4.10 (s, 2, CH_2COOH), was used without further purification.

N-Oxalyl-iminodiacetic acid (6). N-Benzyloxyoxalyl-iminodiacetic acid (**10**) (11.3 g, 38 mmoles) was dissolved into 300 ml of 50% (v/v) acetic acid–water to which 5 g of 5% Pd on charcoal had been added. The solution was shaken for 1.5 h under 40-p.s.i. hydrogen pressure, filtered, and then freeze-dried to yield an amorphous material which was shaken with methyl acetate to yield 1 g (13%) of the free acid. A sample of the free acid was heated for 45 min with 3 *N* BuOH–HCl to give the tributyl ester, which was found to have the same gas chromatographic retention time (EGA column) as **6a**.

Gas chromatographic columns and conditions

Glass U-shaped columns 1.9 m long with 2-mm I.D. (Applied Science, State College, Pa.) were used. The columns were washed with 10% aqueous HF, distilled water, acetone, and trifluoroacetic anhydride and flushed with dry nitrogen. The column packing material—ethylene glycol adipate (Analabs Inc., stabilized grade), 0.65% w/w on acid-washed Chromosorb W, 80/100 mesh—was purchased from Regis Chemical Company (1101 N. Franklin Street, Chicago, Ill. 60610, Code No. 201033) and was sieved through a 60-mesh sieve prior to use.

The packing material was gently vibrated into the column; sections of the column that reached into heated areas of the injector and detector blocks were left unpacked. Only the smallest amount of specially treated (see below) glass wool required to keep the packing in the column was used. The glass wool plug used for the detector end of the column was placed about 7 cm down the column, just below the heated area of the detector block. The column was placed in the oven without a glass

wool plug in the injector end, baked out at 220° (20 ml/min nitrogen carrier gas) for 24 h, and then gently tapped so that the baked-out packing would settle. The carrier gas was turned off, the injector septum was carefully removed, and column packing was added through the injector side until the packing was within 1 cm of the heated portion of the injector block. (This is easily accomplished in the Varian Aerograph 2100, which has a vertical column oven.) A very small plug of glass wool was placed into the very end of the injector side of the column, the septum was replaced, and the column was baked out at 220° for an additional 12 h.

For a typical GC scan the following conditions were used: detection mode, flame ionization; electrometer mode, dual, differential-column; injection size, 1 to 3 μl; sensitivity, 1×10^{-11} Amp/mV; column oven temperature, 80 to 220° at 8°/min; injection block temperature, 230°; detector block temperature, 250°; nitrogen carrier gas, 60 p.s.i., 20 ml/min; H_2, 25 p.s.i., 35 ml/min; air, 10 p.s.i., 400 ml/min.

A high sensitivity range (1×10^{-11} Amp/mV) and the dual, differential-column mode permitted small sample sizes (3 to 1000 ng), which in turn greatly reduced column contamination and increased column life and peak resolution. At this high sensitivity, septum bleed was found to be a severe problem. The CRS "Red Septum" (Chemical Research Services, Inc., Addison, Ill. 60101) was found to give the best results of the commercially available low-bleed septums. These septums were baked out overnight at 230° prior to use. A 1.5-mm-thick Teflon disc was placed between the red septum and the reference column, where septum bleed had caused the formation of negative peaks.

Calculations

The weights and concentrations of NTA, IDA, NMIDA, GLY, SARC, N-oxalyl-IDA and N-nitroso-IDA are expressed in terms of the free amino acid form of these compounds. Relative weight ratio (*RWR*) values were calculated by the following equation:

$$RWR_{IS} = \frac{\text{area aa}_I}{\mu\text{g aa}_I} \times \frac{\mu\text{g IS}}{\text{area IS}}$$

where aa_I = the particular amino acid under consideration and IS = internal standard.

Part per million (p.p.m.) values were calculated by the following equation:

$$\text{p.p.m.} = \frac{1}{RWR_{IS}} \times \frac{\text{area aa}_I}{\text{area IS}} \times \frac{\mu\text{g IS}}{\text{g of sample}}$$

Sample preparation

Procedure 1. For the analysis of solutions of GLY, SARC, IDA, NMIDA, and NTA in the concentration range of 1000 to 0.025 mg/l, a 10-ml water sample was placed into a 20 × 125 mm screw-capped test tube. The sample was spiked with 1 μmole of glutamic acid (147.1 μg) and placed in dry ice in such a way that it froze along the side of the test tube. The cap was loosened, the tube was placed inside a 1200-ml Virtis freeze-dry flask, and the water was removed by freeze-drying (Note 1).

Samples which had been fixed with formaldehyde were not spiked with glutamic acid; instead, they were freeze-dried and then spiked with 0.2 μmole of tetracosane in *n*-butanol.

Procedure 2. Samples which contained nitrite ions were treated in the following way. A 10-ml sample was cooled to 0° in an ice bath, and 100 μl of neat trifluoroacetic acid were added. The sample was immediately frozen and freeze-dried. Tetracosane, 0.2 μmole in *n*-butanol, was added as an internal standard.

Determination of the esterification conditions by a factorial experimental design

A sample consisting of 1 μmole each of all five amino acids and 0.2 μmole of *n*-butyl stearate (added as an internal standard) was treated with 2 ml of *n*-butanol–HCl. The temperature, time, and HCl concentration for each experiment are listed in Table I. The *n*-butanol–HCl was stripped off, and the residue was treated for 30 min at room temperature with 1 ml of 25% trifluoroacetic anhydride in methylene chloride. The trifluoroacetic anhydride was stripped off, and the residue was diluted with 0.5 ml of methylene chloride. Table II lists the *RWR* values for each amino acid for each condition.

Derivatization procedures

Procedure 1: GLY, SARC, NMIDA, IDA, and NTA in distilled water. This procedure works well for solutions containing 1 mg to 0.25 μg of each amino acid. The following internal standards have been used: glutamic acid (1 μmole, 147.1 μg)—1 ml of a 1 μmole/ml aqueous solution was added to the water sample prior to freeze-drying; tetracosane (0.2 μmole, 67.7 μg)—50 μl of a 4 μmoles/ml *n*-butanol solution was added at the esterification stage; and *n*-butyl stearate (100 μg)—100 μl of a 1 mg/ml *n*-butanol solution was added at the esterification stage. Unless special situations prevailed—*e.g.*, the presence of formaldehyde—glutamic acid was routinely used, since it served as an internal standard for the freeze-drying and the derivatization steps.

Into a 20 × 125 mm screw-capped test tube containing the freeze-dried sample was placed 2 ml of 3 *N* *n*-butanol–HCl. The tube was tightly capped, placed in a 75° sonic bath for 5 min, and then placed in a 65° water bath for 45 min. The *n*-butanol–HCl was stripped off at 1-mm pressure on a rotary evaporator (45° bath temperature, Note 2), and 1 ml of 25% v/v trifluoroacetic anhydride in methylene chloride was added. The contents of the tube were thoroughly shaken, transferred to a 16 × 75 mm screw-capped test tube, and allowed to stand at room temperature for 30 min. The acetylating reagent was stripped off at room temperature with a stream of dry nitrogen (test tube block at room temperature); 0.5 ml of Freon 113 was added to the tube, which was then tightly capped and placed into the sonic bath for 10 sec (Note 3). The sample was then ready for injection.

Procedure 2: GLY, SARC, NMIDA, IDA, and NTA in water containing dissolved salts. This procedure works well for 1 mg to 0.25 μg each of the above amino acids. The freeze-dried sample in a 20 × 125 mm screw-capped test tube was esterified according to procedure 1. To the residue obtained after removal of the *n*-butanol–HCl was added 2 ml of 0.5 *M* aqueous $KHCO_3$. The tube was capped and vigorously shaken, and the aqueous bicarbonate solution was extracted with three 2-ml portions of methylene chloride. The methylene chloride extracts were placed into a 16 × 75 mm screw-capped test tube, 10 μl of trifluoroacetic acid were added, the contents were shaken, and the volatile reagents were removed with a stream of nitrogen (test tube block at 40°). Trifluoroacetic anhydride (2 ml, 25% v/v in methylene chloride) was added to the residue. The tube was placed into a sonic bath for 5 sec and then

allowed to stand at room temperature for 30 min. The acetylating reagent was stripped off with a stream of nitrogen (test tube block at room temperature), 0.5 ml of Freon 113 (Note 3) was added to the residue, and the sample tube was placed in a sonic bath for *ca.* 10 sec.

Procedure 3: NMIDA, IDA, and NTA in water containing dissolved salts. This procedure works well for 1 mg to 0.25 μg of each amino acid. The esterification conditions presented in procedure 1 and the extraction conditions presented in procedure 2 were used. During the extraction process the methylene chloride was placed into a 16 × 75 mm screw-capped test tube. Trifluoroacetic anhydride (1.0 ml) was cautiously added to the methylene chloride extract; the mixture was allowed to stand at room temperature for 30 min; the reagents were stripped off with a stream of nitrogen (test tube block at room temperature); and the residue was dissolved into 0.5 ml of Freon 113 (Note 3).

Notes and comments on the derivatization procedures

(1) The freeze-drying proceeded slowly when the sample tubes were placed inside a freeze-dry flask. The process was greatly accelerated by an I²R (Instruments for Research and Industry, Cheltenham, Pa.) IR lamp.

(2) A 20 × 120 mm sample tube was connected directly to the rotary evaporator by means of a 13-mm (plug diameter), 18-mm (cap diameter) rubber serum cap (A. H. Thomas, Philadelphia, Pa., No. 8826). The serum cap was drilled out with a cork borer, the plug end of the serum cap was fitted into the test tube, and the cap end was fitted over the $ 14/20 outer joint of the Büchi evaporator.

(3) Samples containing 1 mg to 10 μg of each amino acid were dissolved in 0.5 ml of Freon 113 or methylene chloride. Samples containing 10 to 0.25 μg of each amino acid were dissolved in 0.30 ml of Freon 113.

Mass spectra

All mass spectra were obtained by rapid magnetic scanning of the gas chromatograph peaks. The ionizing voltage was 70 eV.

Nitrilotriacetic acid tri-n-butyl ester, m/e (relative intensity). 359 (3), 259 (16), 258 (100), 202 (9), 158 (33), 146 (7), 144 (11), 102 (10), 88 (33), 87 (10), 57 (67).

N-Methyl-iminodiacetic acid di-n-butyl ester, m/e (relative intensity). 259 (3), 159 (9.1), 158 (100), 102 (31.4), 74 (18.3), 58 (14.3), 57 (21.1).

N-Trifluoroacetyl-iminodiacetic acid di-n-butyl ester, m/e (relative intensity). 341 (2), 267 (8), 229 (5), 212 (4), 211 (33), 184 (26), 156 (7), 140 (8), 126 (9), 69 (13), 57 (100).

N-Trifluoroacetyl-sarcosine n-butyl ester, m/e (relative intensity). 241 (3), 186 (3), 141 (26), 140 (100), 112 (5), 69 (20), 57 (33).

RESULTS AND DISCUSSION

Derivatization studies

NTA and NMIDA are tertiary amino acids and consequently are not trifluoroacetylated. Instead, they are converted to nonvolatile ammonium salts; *cf.* 1a and 3a. These salts were thermally cleaved to volatile amines in the injector block of the gas chromatograph,

$$(CF_3COO)^{(-)} \quad {}^{(+)}HN(CH_2CO_2nBu)_3 \xrightarrow{230^\circ} CF_3COOH + N(CH_2CO_2nBu)_3$$

and the cleavage reaction was reproducible as long as there was no column packing material in the heated area of the injector. The temperature dependence of the cleavage was as follows: at 150° no cleavage was observed—*i.e.*, no peaks were obtained for NTA and NMIDA; at 180° there was *ca.* 50% cleavage; at 220 to 250° there was complete cleavage; above 260° SARC and NMIDA decomposed.

Our first attempts to convert NTA, IDA, NMIDA, GLY, and SARC to volatile derivatives utilized the reactions presented below and the reaction conditions developed for the protein amino acids[13]. These conditions worked well for IDA,

$$R{-}\underset{\displaystyle NH_2}{\underset{|}{C}}HCO_2H + nBuOH \xrightarrow{3\,N\ HCl} R{-}\underset{\displaystyle NH_3{}^+Cl^-}{\underset{|}{C}}HCO_2nBu \qquad (1)$$

$$R{-}\underset{\displaystyle NH_3{}^+Cl^-}{\underset{|}{C}}HCO_2nBu + (CF_3\overset{\displaystyle O}{\overset{\|}{C}})_2O \longrightarrow R{-}\underset{\displaystyle HN{-}COCF_3}{\underset{|}{C}}HCO_2nBu \qquad (2)$$

glutamic acid (GLU, used as an internal standard), GLY, and SARC but caused partial decomposition of NTA and NMIDA. We thus had to find a new set of conditions.

Reaction 1, the esterification reaction, was very efficiently optimized by use of a factorial experimental design. *RWR* values were measured at two levels of reaction time, reaction temperature, and HCl concentration. Results of the experiment are

TABLE I

ESTERIFICATION OF NTA, IDA, NMIDA, GLY, AND SARC: ASSIGNMENT OF FACTORS

Factors	*Low level*	*High level*
HCl concentration	$C_1 = 1.25\ N$	$C_2 = 3.0\ N$
Reaction temperature	$T_1 = 65^\circ$	$T_2 = 100^\circ$
Reaction time	$t_1 = 10$ min	$t_2 = 45$ min

FACTORIAL DESIGN[a]

	T_1		T_2	
	C_1	C_2	C_1	C_2
t_1	1	2	3	4
t_2	5	6	7	8

[a] Each number represents a particular set of reaction conditions for that observation, *e.g.*, experiment 6 is an esterification reaction at conditions of $C_2t_2T_1$.

TABLE II

ESTERIFICATION OF NTA, NMIDA, IDA, GLY, AND SARC: $RWR_{n\text{-Butyl stearate}}$ VALUES AS A FUNCTION OF REACTION CONDITIONS

	$RWR_{n\text{-Butyl stearate}}$[a]							
Expt. No.[b]	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
SARC	0.50	0.96	0.65	0.73	0.69	1.07	0.73	0.61
GLY	0.86	0.95	0.86	0.95	0.86	1.04	1.00	0.82
NMIDA	0.00	0.60	0.00	0.30	0.00	0.88	0.25	0.00
IDA	0.41	1.12	0.64	0.92	0.64	1.13	1.00	0.77
NTA	0.39	0.77	1.00	1.18	0.89	1.14	1.14	1.10

[a] A reactant mixture composed of 1 μmole each of SARC, GLY, NMIDA, IDA and NTA and 0.1 μmole of *n*-butyl stearate was used for each experiment. Each esterified mixture was treated with trifluoroacetic anhydride at room temperature prior to GC analysis.

[b] Conditions for each experiment are presented in Table I.

presented in Tables I and II. One experimental observation happened to fall directly on the maximum *RWR* point for all the amino acids (experiment 6, Table II). The conditions for this experiment were: temperature 65°; acid concentration, 3 *N*; reaction time, 45 min. Variation of the experimental conditions around this point did not change the *RWR* values. Further analysis of Table II showed that temperature was the most critical variable. An increase in temperature decreased the *RWR* values for SARC, GLY, and NMIDA and increased the *RWR* values for IDA and NTA.

In one experiment, separate solutions of NTA and of NMIDA in 3 *N* BuOH–HCl were heated to 150° for 2 h. NTA underwent cleavage to IDA,

$$n\text{BuOH} + \overset{(+)}{\text{HN}}(\text{CH}_2\text{CO}_2n\text{Bu})_3 \xrightarrow{\text{HCl}} \overset{(+)}{\text{H}_2\text{N}}(\text{CH}_2\text{CO}_2n\text{Bu})_2 + n\text{BuOCH}_2\text{CO}_2n\text{Bu}$$

while NMIDA produced a polymer. At 65°, however, very little cleavage (below 0.5%) of NTA occurred.

Trifluoroacetic anhydride, 25% v/v in methylene chloride, was used for the trifluoroacetylation of GLU, IDA, GLY, and SARC. These compounds were quantitatively acetylated in 30 min at room temperature; the tertiary amines were found to be inert to trifluoroacetic anhydride under these conditions. Longer reaction times, *e.g.*, three weeks at room temperature, caused *ca.* 10% conversion of NTA to IDA. It was therefore important to remove all trifluoroacetic anhydride at the end of the 30-min reaction period.

When satisfactory conditions were found, the structure of each derivative was checked by GC–mass spectrometer. Cracking patterns for compounds **1a** to **6a** are consistent with the assigned structures. The cracking patterns for derivatized IDA and SARC (**2a** and **5a**) and for the free-amine form of derivatized NTA and NMIDA (**1a** and **2a**) have not been published before and thus are included in EXPERIMENTAL.

Sample preparation

The TAB-GC analytical system required an anhydrous sample that was (a) relatively free of polymeric material and inorganic salts and (b) completely free of low-molecular-weight organic materials that had the same GC retention times as

NTA, IDA, NMIDA, GLY, and SARC. In practice, this meant the quantitative separation of amounts as small as 0.25 μg of each amino acid from 10 ml of water which contained a large variety of dissolved matter. We initially investigated a classical ion-exchange separation procedure[15] and a batchwise cation-exchange procedure in which Ca^{2+} and Mg^{2+} salts were exchanged for NH_4^+. Both of these procedures, however, were tedious and extremely difficult to quantify at low sample concentrations.

We decided, therefore, to develop an extraction method based on the fact that the butyl esters of NTA, IDA, NMIDA, GLY, and SARC were soluble in methylene chloride whereas the inorganic salts and most of the polymeric material were insoluble. Thus, a 10-ml water sample was freeze-dried to a white residue, which was then heated with 3 *N* BuOH–HCl. After removal of BuOH–HCl, the sample was shaken with aqueous $KHCO_3$, which converted the hydrochloride salts of the amino acid esters to the corresponding methylene chloride-soluble esters, which were subsequently extracted with methylene chloride.

Unlike the ion-exchange method, which separates the amphoteric fraction from the rest of the sample, an extraction procedure is relatively nonspecific and separates a large variety of low-molecular-weight organics from the aqueous sample. Thus, our chromatograms contain a large number of peaks other than those specifically due to the amino acids of interest; *cf.* Figs. 1, 2, and 3. Fortunately, however, there was almost

TABLE III

THE RELATIVE WEIGHT RESPONSE OF SARC, GLY, NMIDA, IDA, AND NTA: DISTILLED WATER SAMPLES[a]

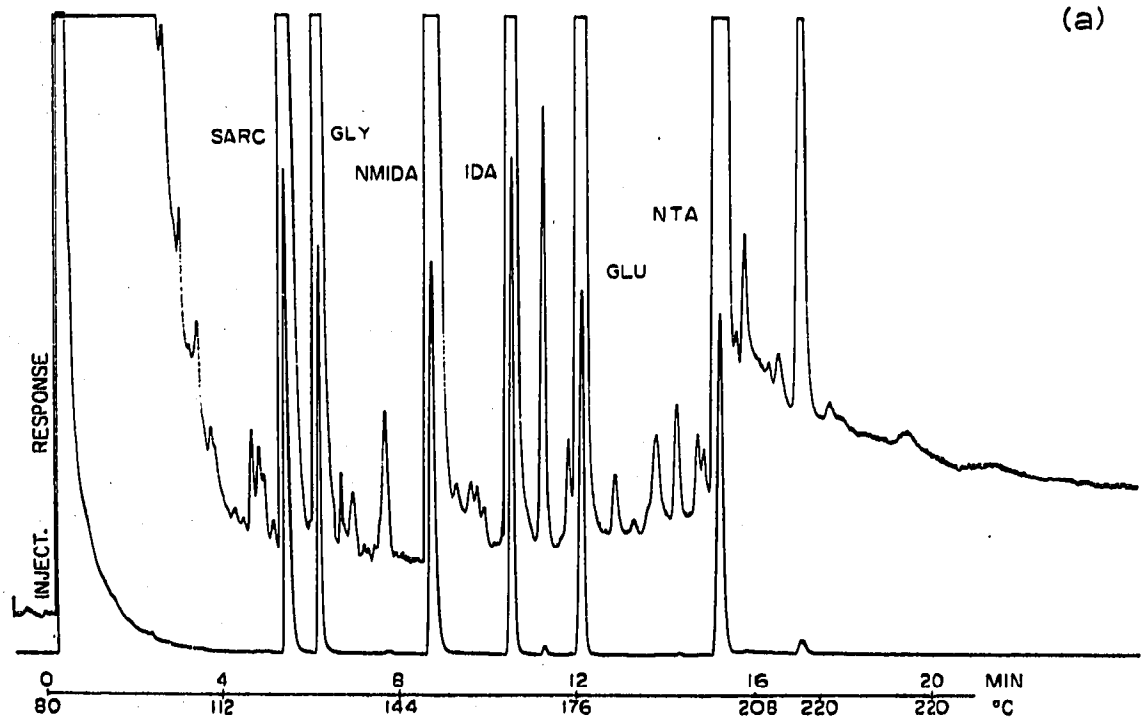
Amino acid	*Amount (µg)*	RWR_{GLU}					*Relative standard deviation*[b]
		1	*2*	*3*	*4*	*Ave.*	
SARC	200	0.94	0.92	0.96	1.01	0.96	4.0
GLY	200	0.93	0.87	0.85	0.88	0.88	3.8
NMIDA	200	0.88	0.89	0.89	0.89	0.89	0.6
IDA	200	1.06	1.06	1.09	1.13	1.09	3.1
GLU	147.1	1.00	1.00	1.00	1.00	–	–
NTA	200	0.83	0.90	0.87	0.83	0.86	3.4

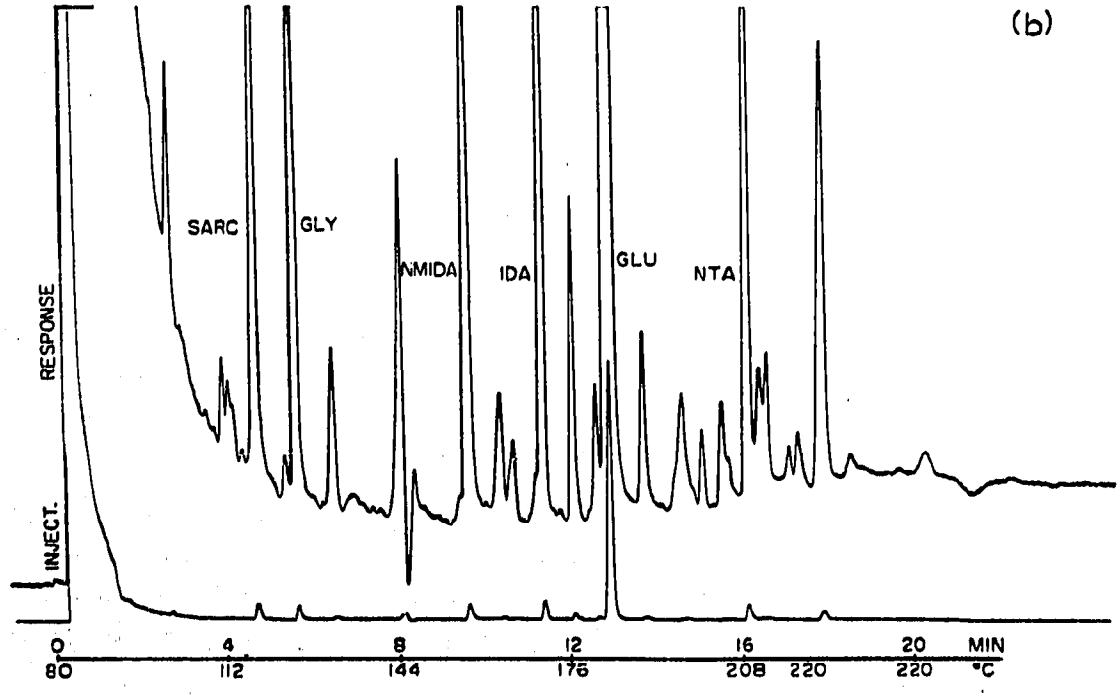
[a] Sample preparation procedure 1 and derivatization procedure 1 were used.
[b] Relative standard deviation = (σ/Ave) × 100, where σ = standard sample deviation.

no impurity peak interference. In some situations, *e.g.*, with Suffolk County, New York, water samples, the peaks due to the organics initially present in the water even served as a fingerprint for the source of the water.

Specific examples

The large variety of water samples analyzed during the course of this work required the development of two sample preparation procedures and three derivatization procedures. Listed below is the specific combination of sample preparation procedure, derivatization procedure, and internal standard required for each application.


(a)
SARC
GLY
NMIDA
IDA
GLU
NTA
RESPONSE
INJECT.
0 4 8 12 16 20 MIN
80 112 144 176 208 220 220 °C


(b)
SARC
GLY
NMIDA
IDA
GLU
NTA
RESPONSE
INJECT.
0 4 8 12 16 20 MIN
80 112 144 176 208 220 220 °C

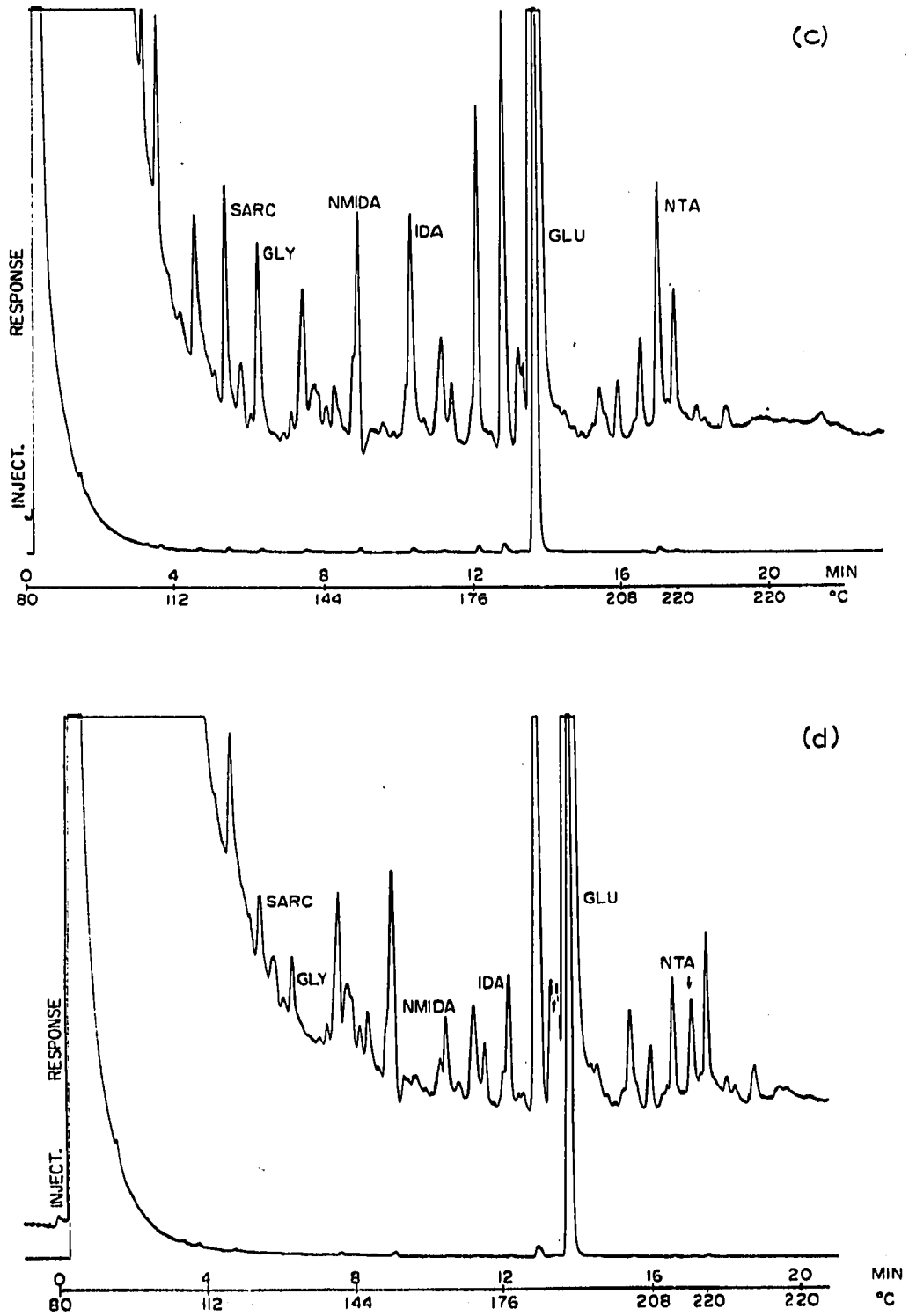
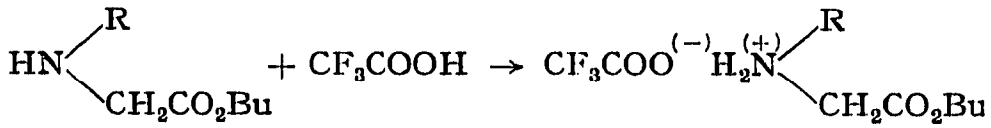


Fig. 1. Chromatograms of *n*-butyl esters of NTA and NMIDA and N-TFA *n*-butyl esters of GLU, IDA, GLY, and SARC. (a) 20 p.p.m. in Detroit River water, 1-μl injection; (b) 1 p.p.m. in Detroit River water, 1-μl injection; (c) 0.1 p.p.m. in Detroit River water, 3-μl injection; (d) 0.025 p.p.m. in Detroit River water, 3-μl injection. GLU, 1 μmole (147.1 g), added to each sample as internal standard; derivatized amino acids in 300 μl of Freon 113. Column: 1.9 m × 2 mm I.D. glass U column packed with 0.6 % w/w stabilized grade EGA on 80/100 mesh a.w. Chromosorb W. Conditions: initial temperature, 80° programmed at 8°/min to 220°; attenuation, upper pen at 1 × 10^{-11} Amp/mV, lower pen at 50 × 10^{-11} Amp/mV.

Determination of NTA, IDA, NMIDA, GLY, and SARC in distilled water. This analysis is similar to the method commonly employed for the protein amino acids[13]. Sample preparation procedure 1 and derivatization procedure 1 were used. Glutamic acid was employed as the internal standard. The *RWR* values for this procedure are presented in Table III.

Determination of NTA, IDA, NMIDA, GLY, and SARC in inland waters. This analysis uses sample preparation procedure 1 and derivatization procedure 2. One problem peculiar to this analysis was that partial loss of GLY and SARC (the two most volatile amino acids) occurred when the methylene chloride extract was concentrated. Addition of 10 μl of trifluoroacetic acid to the methylene chloride extract conveniently solved the problem, converting the volatile amino acid esters to the corresponding nonvolatile ammonium ion salts:



$R = CH_3, H$

This procedure has allowed us to follow the simultaneous biodegradation of all five amino acids in Meramec and Detroit River water. Table IV shows *RWR* values for the concentration range of 20 p.p.m. to 25 p.p.b*.

TABLE IV

THE RELATIVE WEIGHT RESPONSE OF SARC, GLY, NMIDA, IDA, AND NTA: DETROIT RIVER WATER SAMPLES[a]

µg of each amino acid	RWR_{GLU}[b]				
	SARC	*GLY*	*NMIDA*	*IDA*	*NTA*
100	1.02	0.96	0.97	1.02	0.71
50	0.86	0.82	0.97	1.04	0.85
10	0.83	0.78	0.81	0.98	0.86
5	0.89	0.84	0.79	1.01	0.86
2.5	0.72	0.62	0.81	1.09	0.75
1.0	0.83	0.75	0.77	0.99	0.71
0.5	0.75	0.69	0.79	0.88	0.76
0.25	0.90	0.88	0.91	1.05	0.72
Ave.	0.85	0.79	0.85	1.01	0.78
100 × σ/Ave.	10.9	13.6	9.9	6.2	8.7

[a] Each sample consisted of a mixture of all five amino acids dissolved into 10 ml of river water. Thus, the concentration of each amino acid was the following: p.p.m. = 0.1 × μg amino acid.

[b] One μmole (147.1 μg) of glutamic acid was added to each river water sample as an internal standard.

Fig. 1 presents four of the GC scans used to obtain the data for Table IV.

The *RWR* values in Table IV are lower than those in Table III because of the extraction workup required for the Detroit River water samples.

We used the same procedure to analyze qualitatively for N-oxalyl-IDA (6), a

* Throughout this article the American (10^9) billion is meant.

possible biodegradation intermediate of NTA. The synthesis of this compound utilized the following reactions:

$$ClC(=O)C(=O)Cl + BzOH \xrightarrow{0^\circ} BzOC(=O)C(=O)Cl + HCl$$

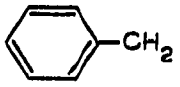
9

$$9 + HN(CH_2CO_2H)_2 \xrightarrow[23^\circ]{NaHCO_3} BzOC(=O)C(=O)N(CH_2CO_2H)_2$$

10

$$10 + H_2 \xrightarrow[23^\circ]{Pd/C} HOC(=O)C(=O)N(CH_2CO_2H)_2 + BzOH$$

6

Bz = 

A sample of tributyl ester (**6a**) was independently synthesized by the following route:

$$BuOH + ClC(=O)C(=O)Cl \xrightarrow{0^\circ} nBuOC(=O)C(=O)Cl$$

8

$$8 + HN(CH_2CO_2nBu)_2 \xrightarrow[Reflux]{EtOAC} nBuOC(=O)C(=O)N(CH_2CO_2nBu)_2$$

6a

A gas chromatogram of all five amino acids and compound **6** is presented in Fig. 2.

Determination of NTA, IDA, and NMIDA in primary sewage effluents, secondary sewage effluents, and inland waters. This analytical procedure used sample preparation procedure 1 and derivatization procedure 3. Both glutamic acid and tetracosane have been employed as internal standards. Derivatization procedure 3 is one step shorter and requires considerably less attention than derivatization procedure 2. The payment for this decrease in time is partial loss of SARC and GLY. Nevertheless, the method has been used to analyze quantitatively for NTA, IDA, and NMIDA and qualitatively for GLY and SARC in river water, well water, sewage-plant effluent, and various samples of municipal drinking water. The RWR_{GLU} values for NTA, IDA, and NMIDA were within 10% of those reported in Table IV.

Of particular importance was the application of this procedure to water samples fixed with formaldehyde. Formaldehyde reacted with glutamic acid to yield a compound that did not elute from an ethylene glycol adipate column. Our control experiments, however, showed that a 1% formaldehyde solution did not react with NTA, NMIDA, or IDA at room temperature.

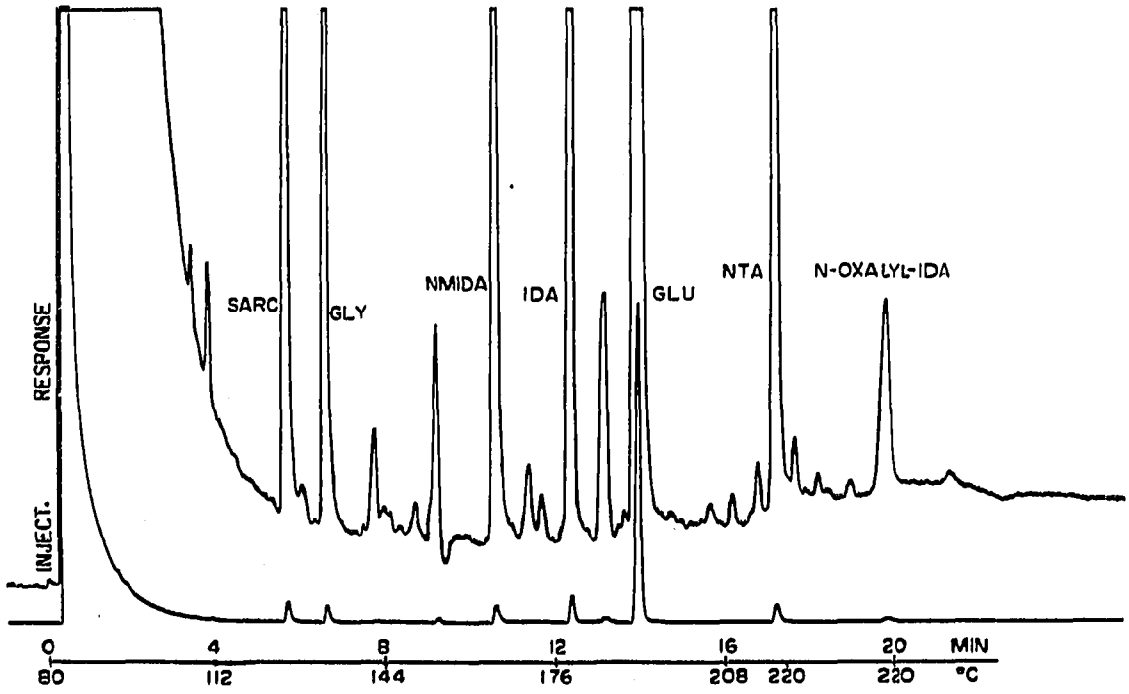


Fig. 2. Chromatogram of *n*-butyl esters of N-oxalyl-IDA, NTA, and NMIDA and N-TFA *n*-butyl esters of SARC, GLY, IDA, and GLU. Sample: N-oxalyl-IDA, NTA, NMIDA, IDA, GLY, and SARC each at the 10-μg level in 10 ml of Meramec River water; GLU, 1 μmole (147.1 μg), added as internal standard; derivatized amino acids in 300 μl of Freon 113, 1 μl of sample injected. See the legend to Fig. 1 for other details.

TABLE V

THE RELATIVE WEIGHT RESPONSE OF NTA IDA, AND NMIDA: SUFFOLK COUNTY, N.Y., WELL WATER[a]

Amino acid	*Amount (μg)*	*No. of determinations*	*RWR Ave.*	*100 × σ/Ave.*
NTA	0.5	6	0.61	10
IDA	0.5	6	0.78	17
Tetracosane	50	6	1.00	—
NMIDA	0.5	6	0.48	18

[a] A 10-ml water sample was used; water was 1% in formaldehyde.

TABLE VI

RELATIVE WEIGHT RESPONSE OF N-NITROSO-IMINODIACETIC ACID

μg of N-nitroso-IDA	*μg of GLU*	*Source of water[a]*	*RWR_{GLU} IDA[b]*
200	147.1	Meramec River	0.83
50	147.1	Meramec River	0.83
50	147.1	Detroit River	0.79
10	147.1	Detroit River	0.73
5	147.1	Detroit River	0.67

[a] Each sample was composed of N-nitroso-IDA in 10 ml of river water.
[b] N-Nitroso-IDA is cleaved to IDA during the derivatization process.

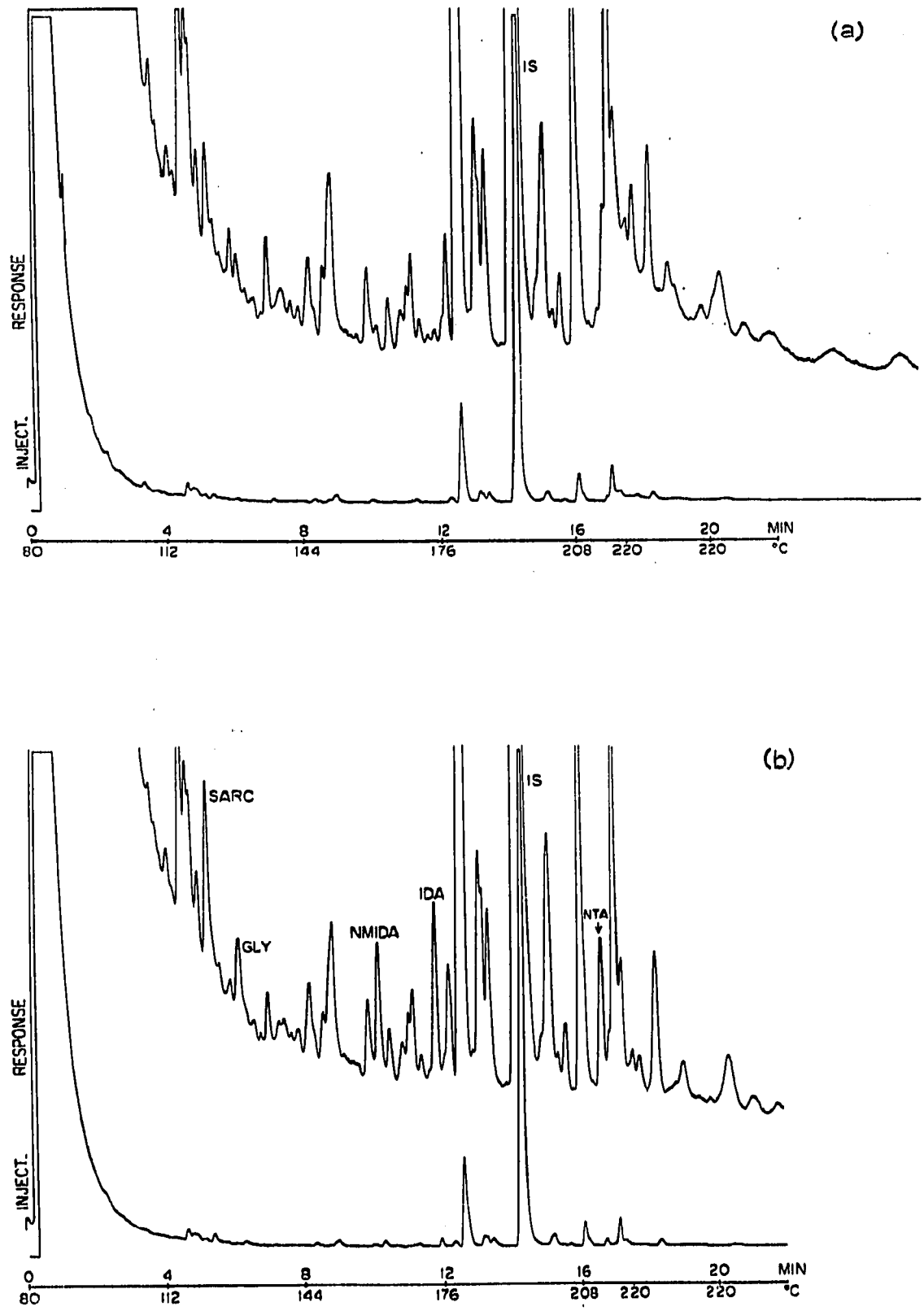


Fig. 3. GC chromatogram obtained for Suffolk County, N.Y., well water. Sample: (a) 10-ml water sample; (b) 10 ml of the same water spiked with 0.5 μg each of NTA, IDA, NMIDA, GLY, and SARC; tetracosane, 50 μg, added to each sample as internal standard; derivatized sample in 300 μl of Freon 113, 2 μl of this solution injected. See the legend to Fig. 1 for other details.

To circumvent the glutamic acid problem, tetracosane was used as the internal standard. In Table V, the $RWR_{\text{Tetracosane}}$ values are presented for NTA, NMIDA, and IDA in Suffolk County, N.Y., well water which was fixed with formadehyde. Two GC scans of this water are presented in Fig. 3.

Analysis of N-nitroso-iminodiacetic acid. Sample preparation procedure 1 and derivatization procedure 3 are utilized for this analysis. Treatment of N-nitroso-IDA with *n*-butanol–HCl not only converted the carboxyl groups to *n*-butyl esters but also cleaved off the N-nitroso group. This cleavage reaction, characteristic of N-nitrosoamines[16], is illustrated below.

$$ON{-}N(CH_2CO_2H)_2 + n\text{BuOH} \xrightarrow{HCl} \overset{(+)}{H_2}N(CH_2CO_2n\text{Bu})_2 + n\text{BuONO}$$

$Cl^{(-)}$

Some decomposition of the N-nitroso compound occurred, in that its RWR_{GLU} values (Table VI) vary slightly with concentration. The *RWR* values, however, are reproducible at any given concentration, and the method is capable of detecting 0.05 p.p.m. of N-nitroso-IDA.

Analysis of NTA, IDA, NMIDA, GLY, and SARC in water containing nitrite ions. This analysis uses sample preparation procedure 3 and derivatization procedures 1 or 3, depending on whether the analysis has to be quantitative for GLY and SARC. The sample preparation procedure was designed to remove the nitrite from the solution so that subsequent reactions between the amino acids and nitrous acid would not occur during the esterification reaction. The sample was cooled in an ice bath, acidified with trifluoroacetic acid, and freeze-dried. These steps converted all nitrite to nitrous acid, which was removed during the freeze-drying process. The RWR_{GLU} values in Table VII show that no interaction between nitrous acid and the amino acids occurred during the sample preparation procedure.

TABLE VII

RWR VALUES FOR NTA, IDA, NMIDA, GLY, AND SARC IN WATER CONTAINING NITRITE IONS[a]

μg of each amino acid	*μg of* $NaNO_2$	$RWR_{\text{tetracosane}}$[b]				
		SARC	*GLY*	*NMIDA*	*IDA*	*NTA*
100	500	0.94	0.85	0.99	1.08	0.93
50	250	0.78	0.69	0.91	1.01	0.94
50	0	0.93	0.84	1.04	1.09	0.99

[a] The sample was composed of amino acids and $NaNO_2$ in 10 ml of distilled water. Sample preparation procedure 3 and derivatization procedure 2 were used for the analysis.

[b] Tetracosane, 0.2 μmole (67.7 μg), was added as an internal standard.

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