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ABSTRACT

An indirect method of simultaneously estimating prokaryotic and filamentous fungal biomasses in marsh grass litter was developed. Spartina alterniflora samples were hydrolyzed in 6N HCl (100°C, 4.5 h). Amino sugars in the hydrolysates were isolated by ion-exchange chromatography (Dowex 50W-8X, 2N HCl fraction) and converted to O-methyloxime acetate derivatives. Ion-exchange chromatographic purification of muramic acid and glucosamine gave better recoveries (>90%) and reproducibility (CV <5%) than thin-layer chromatography. O-methyloxime acetates were easier to prepare than aldonitrile acetates of amino sugars. O-methyloxime acetate derivatives produced responses four times greater than aldonitrile acetates during gas chromatography. Sample derivatives were analyzed by gas chromatography. Five packed columns tested gave incomplete separation of amino sugars. Of the packed columns, 2% DEGA provided the best separations. An OV-101 capillary column completely separated muramic acid and glucosamine from other amino sugars in less time than other columns tested. N-methyl-glucamine remained with the amino sugars during ion-exchange chromatographic purification. It may be added to samples before acid hydrolysis and can be used as an internal standard. Some marsh grass samples were pre-extracted with acetone. Acetone extraction removed 28% of the glucosamine in these samples. Using previously determined conversion factors, the remaining glucosamine suggested that some 7% of the S. alterniflora dry weight could be fungal biomass. This method of measuring muramic acid and glucosamine concentrations is rapid, sensitive, and inexpensive compared to previous gas chromatographic methods.

INTRODUCTION

The inadequacy of methods to determine bacterial and fungal biomasses continues to be a problem for microbial ecologists. Popular methods of estimating bacterial and fungal numbers or biomass include (1) selective plating, (2) direct counts, and (3) biochemical estimates. Beyond providing identification of some of the species present and crude estimates of numbers, plating techniques are of limited value to microbial ecologists (Jones, 1979). Direct-count measures of bacterial and fungal numbers have three drawbacks: (1) incomplete extraction of organisms, (2) incomplete and interfering staining and fluorescence, and (3) the subjectivity of different observers. Cell or cell-fragment volumes and specific gravities must also be estimated when converting counts into biomasses. Both of these factors can also contribute to large variances between replicate counts and among analysts.

Indirect methods of estimating bacterial and fungal biomasses include assaying specific biochemicals or their derivatives. These methods have several advantages over direct methods: (1) extraction efficiencies are usually higher, (2) often samples may be stored longer prior to analysis, and (3) less subjectivity is required of the investigator. Adenosine triphosphate (Holm-Hansen and Booth, 1966), lipopolysaccharide (Watson et al., 1977), and muramic acid (Millar and Casida, 1970) have been used to indirectly estimate bacterial biomass. Chitin, as chitosan (Ride and Drysdale, 1972) and as glucosamine (Cochran and Vercellotti, 1978), as well as ergosterol (Seitz et al., 1979; Lee et al., 1980) have been used to estimate fungal biomasses in complex samples.

This paper focuses on the measurement of amino sugars for estimating bacterial and fungal biomasses. Concentrations of amino sugars have been measured by several methods (colorimetric, enzymatic, amino acid analyzer, and gas-liquid chromatography). Appendix I summarizes the procedures used by various investigators. These methods of estimating biomasses are based upon several assumptions: (1) bacteria and fungi are sole sources of muramic acid and glucosamine, (2) concentrations do not vary with age, size, or nutritional status, (3) interference by other biochemicals is minor or non-existent, and (4) muramic acid and glucosamine from dead biomass do not accumulate.

As with the more direct methods, there are potential disadvantages of the muramic acid and glucosamine procedures for estimating biomasses: (1) assay times are relatively long, (2) cell-wall components are probably more closely correlated with cell surface area than cell volume, (3) concentration of cell-wall molecules per unit biomass does vary between species for prokaryotes and filamentous fungi, and (4) bacterial and filamentous fungal walls are not the only sources of glucosamine in culture or environmental samples. These problems are dealt with in detail below (Discussion, and Appendix II).

The purpose of this paper is to present a gas chromatographic method for simultaneously estimating muramic acid and glucosamine concentrations.

In this way, both prokaryotic and filamentous fungal biomasses can be estimated together. The goals during the development of this method were to: (1) achieve more sensitivity than previous chromatographic methods, (2) abbreviate assay times, and (3) eliminate interference from compounds similar to muramic acid and glucosamine. We compare this method with other purification and analysis methods, and discuss its potential for estimating prokaryote and filamentous fungal biomasses.

METHODS

Materials and Reagents

Muramic acid (3-carboxyethyl-D-glucosamine: Mur), D(+)-glucosamine HCl (GlcN), D-mannosamine HCl (ManN), D(+)-galactosamine HCl (GalN), β-D(+)-glucose (Glc), β-phenyl-D-glucopyranoside (PhGlc), N-methyl-glucamine (MeGluA), and ninhydrin were obtained from Sigma Chemical Co., St. Louis, MO. Hydroxylamine HCl was purchased from Fisher Scientific Co., Fair Lawn, NJ. O-methyl-hydroxylamine HCl was obtained from Pfaltz and Bauer, Inc., Stamford, CT. Ethanolamine and D(+)-cellobiose was purchased from Eastman Kodak Co., Rochester, NY. Pyridine and acetic anhydride were obtained through Baker Chemical Co., Phillipsburg, NJ. Pyridine was stored over KOH pellets. 1-dimethylamino-2-propanol was purchased from Aldrich Chemical Co., Milwaukee, WI. Methanol and chloroform were glass distilled quality. All other chemicals and solvents were reagent grade. Other materials are described below.

Sampling and Analysis

A standard mix was prepared by dissolving 10 mg each of glucosamine, muramic acid, mannosamine, galactosamine, glucose, and N-methyl-glucamine in 10 ml of 0.2N HCl. This mixture was stored frozen. Figure 1 is an overview of the analysis procedure. Standards were added at the hydrolysis (0.3 ml), preliminary purification (0.1 ml), and derivatization (1.0 ml) stages to help quantify losses during the analysis. Culms and leaves of standing dead Spartina alterniflora (tall form) were collected at Sapelo Island, GA (July 1978), freeze-dried, and ground with a Wiley mill to pass a 40 mesh screen. One-tenth gram of the freeze-dried S. alterniflora was used as a natural sample. Natural samples, both spiked and unspiked, were analyzed by several different procedures. Table 1 summarizes the treatments. All treatments were run in triplicate.

Pre-extraction and Hydrolysis

In two treatments (Table 1: VII, VIII), samples were pre-extracted with acetone (Whipps and Lewis, 1980) to eliminate non-structural (i.e. lipid) glucosamine sources. Each sample was washed with shaking (20 min) in a 50 ml glass centrifuge tube. The sample was centrifuged and the acetone was removed. Each sample was then washed three times with 10 ml of distilled water. After removing the final wash, each sample was transferred to a large screw cap hydrolysis tube (25 x 200 mm) with 5 ml of 6N HCl. Samples from other treatments were placed directly in the hydrolysis tubes. Five milliliters of 6N HCl ($\geq$20:1 vol/wt) were then added to each tube.

To test for possible oxidation of amino sugars during acid hydrolysis, some treatment tubes were flushed with N_2 for several minutes prior to hydrolysis. Other treatment tubes were closed with Teflon-lined screw caps without N_2 purging. All samples were hydrolyzed for 4.5 h at 100°C, cooled

and refrigerated overnight. Hydrolysis time and temperature were followed from previous work with muramic acid in detritus samples (King and White, 1977). No further attempt was made to identify optimal hydrolysis conditions.

Cooled hydrolysates and washings (3 x 4 ml distilled water) were filtered under vacuum (680 mm Hg) through pre-weighed glass-fiber filters (Reeve Angel 934 AH) into 300 ml evaporation flasks. Filtrates were refrigerated until they could be evaporated. The filters were dried (60°C, 1 wk) and reweighed to determine residue weights.

All filtrates were evaporated at 67°C under vacuum (680 mm Hg) on a Buchler Flash-Evaporator (Buchler Instruments, Fort Lee, NJ). Samples were evaporated to dryness with or without the addition of 0.6 ml 50% glycerin in ethanol (Table 1). Dawson and Mopper (1978) found less condensation of hydrolysate compounds with the addition of glycerin prior to evaporation. Dried hydrolysates were diluted to 2 ml with distilled water. These dilutions and washings (3 x 0.8 ml) were transferred to volumetric flasks and brought to 5 ml. The solutions were transferred to test tubes and centrifuged to precipitate fine particulate matter. Until preliminary purification by ion-exchange chromatography, the hydrolysate solutions were stored frozen in glass scintillation vials.

Ion-Exchange Chromatography (IEC)

Ion-exchange chromatography (IEC) was performed with Dowex 50W-8X (200-400 mesh) resin. The resin was conditioned according to Boas (1953). A 1:1 (g/ml) suspension of suction-dried resin and distilled water was prepared. Immediately after shaking, 5 ml of the suspension was quickly pipetted into 25 x 0.8 cm chromatography columns (Pierce Chemical Co., Rockford, IL) with attached 50 cm Teflon siphon tubes. These columns had been previously plugged with a layer of glass wool and a layer of sea sand (Merck and Co., Inc., Rahway, NJ). Six columns were prepared and reused for all analyses.

Hydrolysate solutions (1-2 ml) or standards were added to the columns. Ten milliliters of distilled water were then added to each column to elute neutral compounds. Amino sugars were recovered into 16 x 125 cm test tubes with 10 ml of 2N HCl. Each column was treated with 12 ml of 2N NaOH and then regenerated with 15 ml of 2N HCl. When not in use, columns were plugged after receiving 4 ml of 2N HCl. Before columns were used for the next sample, each was washed with 20 ml of distilled water. The gravity-fed chromatography columns produced variable flow rates averaging less than 0.5 ml/min. The siphon tubes attached to the columns prevented the solvents from traveling below the top of the resin bed. The gravity-fed chromatography columns produced variable flow rates averaging less than 0.5 ml/min. The siphon tubes attached to the columns prevented the solvents from traveling below the top of the resin bed.

Amino-sugar fractions were evaporated in a hood overnight at 30-40°C with a stream of dry air. Evaporated samples were stored at 4°C.

Thin-Layer Chromatography (TLC)

Before ion-exchange chromatography was incorporated into the method, standards were separated by thin-layer chromatography (TLC). Two TLC methods were investigated for their ability to separate amino sugars (Fazio et al., 1979; Esser, 1965). Standard solutions (10-100 µg/50 µl distilled water) were streaked 3 cm from the bottom of 20 x 20 cm commercial plates. Samples were dried between applications with a hair dryer. Glucosamine or muramic acid standards (approx. 10 µg) were streaked on each side of the sample to help determine R_f values. All TLC was performed at room temperature in glass tanks lined with filter paper and filled with 200 ml of developing solvent.

Silica gel plates (Sil G-25; Brinkman Instruments, Inc., Westbury, NY) were used with Fazio et al.'s (1979) method. The developing solvent contained 9:1:1 (v/v/v) isopropanol : acetic acid : distilled water. Cellulose plates (Cell 300-10; Brinkman Instruments, Inc., Westbury, NY) were used with Esser's (1965) method. The developing solvent contained 60:45:4:30 (v/v/v/v) butanol : pyridine : acetic acid : water. Single development by both methods took 5-6 h. When the solvent front was within 1 cm of the top, the plate was removed and air dried. Standards along the edge and standard solutions were visualized with ninhydrin (0.1% in isopropanol) after heating (100°C; 10-20 min).

Recovery experiments for glucosamine and muramic acid were performed following Fazio et al.'s (1979) method. After the standards were visualized, an area ± 2 cm from the mean R_f value of the standards was loosened with a razor blade. These scrapings were transferred to a 16 x 125 mm test tube and 5 ml of 0.1N acetic acid was added. The scrapings and acid were mixed and allowed to stand at least 30 min. The suspension was filtered through a glass fiber filter (Reeve Angel 934 AH) followed by three 1 ml washings to the acetic acid solution. Filtrates were collected in 16 x 125 mm test tubes and evaporated at 30-40°C with a stream of dry air. Evaporated samples were refrigerated until volatile derivatives were formed.

Volative Derivative Formation

Two types of derivatives were used: aldonitrile acetates (ANA) and O-methyloxime acetates (OMOA). Both procedures for preparing these derivatives involved two steps, oxime formation and conversion to acetates. Oximes were formed by Fazio et al.'s (1979) method with 0.4 ml of 0.22N hydroxylamine HCl (0.301 g/20 ml) in pyridine. This mixture was modified to also contain 30 µg of β-phenyl-glucose as an internal standard. This oximation reagent was refrigerated between uses and discarded after 30 days. Gas chromatography standards received 600 µg of β-phenyl-glucose. The reaction tubes were sealed with Teflon-lined screw caps, mixed, and heated for 2 h at 55°C. After cooling, the reagents were evaporated with a stream of dry air. One millimeter of acetic anhydride : pyridine (1:1 v/v) was freshly mixed and added to convert the oximes to acetate derivatives. The reaction tubes were heated for an additional 2 h at 55°C after being sealed and mixed. The mixture was then cooled and evaporated to a syrup.

At this point a cleaning procedure was employed before gas chromatographic analysis. The derivatives were dissolved in 1.0 ml of chloroform. One milliliter of 1N HCl was added and mixed. The acid was withdrawn and the chloroform layer was washed three times with 1.0 ml of distilled water. After the last wash, the chloroform layer was transferred with 3 rinses (0.4-0.6 ml) to a smaller test tube (10 x 75 mm) and the chloroform was evaporated with dry air. Derivatives of gas chromatography standards were not evaporated after washing with acid and water; instead, the chloroform layer was transferred to another screw cap test tube and directly injected into the gas chromatograph. Once prepared, derivatives were refrigerated until gas chromatographic analysis.

Oximes for conversion to O-methyloxime acetates by MaWhinney et al.'s (1980) method were prepared with 0.4 ml of an O-methyloximation reagent (1.5 g O-methylhydroxylamine HCl, 5.0 ml methanol, 8.9 ml pyridine, and 1.1 ml of 1-dimethylamino-2-propanol). This reagent was also refrigerated between uses and discarded after 30 days. Samples received this mixture with 32 μg of β-phenyl-glucose, and standards (1.0 ml, evaporated to dryness) received 600 μg of β-phenyl-glucose. The reaction tubes were sealed with Teflon-lined screw caps, agitated, and heated for 20 min at 70°C. Afterwards the reaction mixture was cooled and evaporated. Two milliliters of freshly mixed acetic anhydride : pyridine (3:1 v/v) solution was added to form the O-methyloxime acetate derivatives. After resealing the cap, the reaction tube was again agitated and heated to 70°C for an additional 25 min. The mixture was then evaporated to a syrup. The same clean-up and storage procedures were used for O-methyloxime acetate derivatives as had been used for aldonitrile acetates.

Gas-Liquid Chromatography

Analyses were performed on a Hewlett-Packard HP-5700A gas chromatograph with dual flame ionization detectors. This instrument was modified to accept both packed columns and fused capillary columns. All packed columns were 6 ft x 2 mm ID. One OV-101 fused silica capillary column (Hewlett-Packard: 10 m x 0.21 mm ID) was used for quantitative analyses. Peak retention times and areas were measured with a Spectra-Physics Minigrator integrator (Spectra-Physics, Inc., Santa Clara, CA). Analyses were traced with a strip chart recorder (Linear Instruments Corp. Model 252, Costa Mesa, CA).

Derivatized standards were analyzed under six gas chromatographic conditions. This helped identify procedures which gave the best separations in the least time. Standards were run individually and in mixtures to determine retention times. These conditions are summarized in Table 2. Molar responses of gas chromatographic standards, analyzed on the OV-101 capillary column, were calculated as:

$$KF\ (\text{molar response of compound}) = \frac{\text{Area}_{\text{internal standard}}}{\text{Area}_{\text{compound}}} \times \frac{\text{Moles}_{\text{compound}}}{\text{Moles}_{\text{internal standard}}}$$

Glucosamine results were adjusted by a correction factor (0.831) before molar responses were calculated because glucosamine HCl was used as a standard.

Derivatized samples, IEC and TLC standards were dissolved in 50 µl of chloroform. Each sample was sealed for 15 min but not longer than 2 h prior to analysis. One to 4 µl were injected into the gas chromatograph. The amount of compound in unknown samples was determined by:

$$\text{Moles}_{\text{compound}} = \text{Area}_{\text{compound}} \times \text{KF}_{\text{compound}} \times \frac{\text{Moles}_{\text{internal standard}}}{\text{Area}_{\text{internal standard}}}$$

Concentrations in natural samples were reported in µg/g of sample. Recoveries were determined by analyzing samples spiked with standards.

RESULTS

Data presented in Table 3 indicate that 28% of the glucosamine in the S. alterniflora sample was from non-structural sources. One gram of S. alterniflora (dry) was the practical limit for hydrolysis in large test tubes. Larger samples sometimes produced a plug which would travel up the hydrolysis tube away from the acid. Hydrolysates were evaporated in 20-30 min under the stated conditions with little or no boiling. The addition of 0.6 ml 50% glycerin in ethanol reduced boiling during this step.

Recoveries of muramic acid and glucosamine were determined for all hydrolysis treatments. Tables 3 and 4 summarize the recovery statistics when β-phenyl-glucose and N-methyl-glucamine were used as internal standards. N-methyl-glucamine remained with the amino sugar fraction during ion-exchange chromatography. This property allows it to be conveniently added as an internal standard before acid hydrolysis when hydrolysis is to be followed by ion-exchange clean-up.

Preliminary Purification

Work with S. alterniflora samples indicated that a preliminary purification step was necessary before derivatives were formed from the hydrolysates. Hydrolysate derivatives tended to emulsify during the cleaning procedure without a preliminary purification step.

Tables 5 and 6 give recovery statistics for ion-exchange chromatographic purification of standards. In these tables, different sample volumes applied to the column and different volumes of acid eluting the amino sugars are compared. Initially, 5.0 ml of 2N HCl was used to elute amino sugars. This volume was increased to 6 and 10 ml when poor recoveries for muramic acid and N-methyl-glucamine were noted. The additional acid compensated for the dead space in the column siphon tubes and recoveries of these compounds were increased (Table 5). Table 6 reports recoveries when N-methyl-glucamine was used as the internal standard. Ion-exchange blanks were also run. No glucosamine or muramic acid was detected in these blanks.

Thin-layer chromatography Rf values were calculated for various compounds. Values for both Fazio et al.'s (1979) and Esser's (1965) methods are presented in Table 7. Table 8 is a comparison of recoveries for glucosamine and muramic acid standards by thin-layer and ion-exchange chromatographic purification.

Acetate Derivatives

Aldonitrile acetates (Fazio et al., 1979) took 4 h to prepare, excluding evaporation time, while O-methyloxime acetates (MaWhinney et al., 1980) were prepared in 0.75 h. Figure 2 graphically shows molar responses of muramic acid and glucosamine relative to β-phenyl-glucose. O-methyloxime

acetates of muramic acid and glucosamine yielded a response roughly four times greater than the aldonitrile acetates of the two compounds.

Gas-Liquid Chromatography

Table 9 summarizes retention times of compounds analyzed as aldonitrile and O-methyloxime acetates under different gas chromatographic conditions. The 2% DEGA column provided the quickest and most complete separation of amino sugars using packed columns. Other amino sugars overlapped often with muramic acid and glucosamine when analyzed on packed columns. A chromatograph of standards run on the 3% SP-2100 column (Table 9) is shown in Figure 3. On this column, the mannosamine peak often merged with the muramic acid and galactosamine peak.

The OV-101 fused-silica capillary column provided the best separation of muramic acid and glucosamine from other amino sugars in the least time. All peaks appeared within 25 min on this column. Figures 4 and 5 show capillary-column recordings of aldonitrile and O-methyloxime acetate derivatives of standards. Each peak in both tracings, except internal standards, represents approximately 8 µg before a 49.85:1 split in the injector. Chromatographic conditions for these figures are given in Table 9. Several neutral sugars were analyzed on this capillary column as O-methyloxime acetates. These standards included glucose, galactose, mannose, arabinose, xylose, rhamnose, and fucose. All neutral sugar derivatives gave split peaks. However, major peaks of pentose and hexose sugars were within specific regions.

A capillary column recording of a S. alterniflora sample spiked with amino sugars is shown in Figure 6.

DISCUSSION

Accuracy and Efficiency

The optimal procedure for the analysis of muramic acid and glucosamine by gas chromatography is indicated by the bold line in Figure 1. This procedure allows muramic acid and glucosamine concentrations to be measured simultaneously. Muramic acid and glucosamine were completely separated from other amino sugars. This is important because galactosamine is present in bacterial and fungal polysaccharides (Sharon, 1965). In some bacterial cell walls the molar ratio of galactosamine to muramic acid is 0.7:1 (Stewart-Tull, 1968). In colorimetric methods, galactosamine can interfere with the measurement of muramic acid (Millar and Casida, 1970) and glucosamine (Ride and Drysdale, 1972). An enzymatic method of measuring muramic acid (as lactate) overestimates concentrations for some unknown reason (King and White, 1977). In previous studies, trimethylsilyl ether derivatives of muramic acid (Casagrande and Park, 1978) and glucosamine (Cochran and Vercellotti, 1978) produced split peaks when analyzed by gas chromatography. Concentrations are harder to quantitate when derivatives produce split peaks. The glucosamine peaks merged with the glucose peak in Cochran and Vercellotti's (1978) gas-chromatographic method. Cochran and Vercellotti found that this could be remedied by using an alkaline flame ionization detector or a Coulson specific phosphorus-nitrogen conductivity detector. Unfortunately, galactosamine was still not separated from the secondary peak of glucosamine.

Analysis time for the muramic acid and glucosamine method (this report) was shortened compared to previous methods (Table 10) which measure muramic acid and glucosamine separately. The total time for the muramic acid and glucosamine assay was about 10 hours per sample. Four hours of this time, including portions of all stages of the analysis, required close attention by the analyst. Times for evaporation (after IEC and derivative formation: overnight) and rejuvenation of the ion-exchange columns (1.5 h) are not included in this estimate. However, if six samples were processed simultaneously, the effective time per sample decreased to about 3 hours. This procedure is faster than Fazio *et al.*'s (1979) method, but not as fast as Cochran and Vercellotti's (1978) method (Table 10). However, Cochran and Vercellotti's (1978) gas-chromatographic method has some interference problems that are difficult to overcome.

The gas-chromatographic portion of the present analysis (<30 min) is quicker than amino acid analyzer techniques: 150 (Stahmann *et al.*, 1975) and 210 (Rosan, 1972) min. The total analysis time, however, may be more closely comparable because some amino-acid-analyzer techniques do not include a preliminary purification step. Ride and Drysdale (1972) reported that their colorimetric chitosan assay takes 5 h. Colorimetric techniques may be quicker than gas-chromatographic techniques, but they are less sensitive (Cochran and Vercellotti, 1978) and more prone to interference problems (Millar and Casida, 1970; Ride and Drysdale, 1972).

Sensitivity

The lower detection limit of our technique for muramic acid and glucosamine is about 0.09 nmoles. This was measured after a 50:1 split at the gas-chromatograph injector, and corresponds to 1.15 μg of muramic acid and 0.82 μg of glucosamine before the split. Based on these sensitivities, and the losses and dilutions involved, the initial sample for this analysis should contain between 25-50 μg of these compounds. For comparison, sensitivities of other methods are given in Table 10. These values were calculated from published data and may be crude estimates. In addition, none of the gas-chromatographic methods except the present use split-mode injection during the analysis. In the lactate-cleavage enzymatic method of measuring muramic acid (Moriarty, 1977), 10 ng of lactate is the lower detection limit. This corresponds to 28 ng of muramic acid. If the preferred procedure in Figure 1 could be modified to work with a splitless gas-chromatographic technique, detection of muramic acid and glucosamine would approach the limit of Moriarty's (1977) method. Alternative means of lowering detection levels per unit original sample would be to concentrate these compounds at the hydrolysis stage or add more hydrolysate to the ion-exchange columns.

Recoveries of Cell-Wall Hexosamines

Whipps and Lewis (1980) have shown that hexosamine in acetone-soluble compounds can be a significant portion of the total hexosamine present in natural samples, and that this portion of the hexosamine is a significant contributor to variation in the hexosamine:fungal-biomass ratio. The results in Table 3 confirm the potential existence of this problem in samples of dead *S. alterniflora*. Twenty-eight percent of the glucosamine in the *S. alterniflora* sample was from sources other than chitin and peptidoglycan (i.e., lipids).

Dawson and Mopper (1978) have found that condensation reactions among sugars, amino sugars, and amino acids can occur during rotary evaporation. These reactions can account for substantial losses. This was avoided in the present study by addition of glycerin; higher recoveries of muramic acid and glucosamine in *S. alterniflora* samples were achieved when glycerin was added before evaporation of the hydrolysates (Table 3). Hydrolysis conditions in this method are similar to optimal hydrolysis conditions for fungal mycelium found by Wu and Stahmann (1975) and Stahmann *et al.* (1975). Recovery of glucosamine in *S. alterniflora* samples was 100% or greater (Table 3). Muramic acid recoveries ranged from 85 to 111% in these samples. These recoveries are compared to recoveries achieved by other methods in Table 10.

Recovery of ion-exchange standards was better than 90% for both muramic acid and glucosamine (Table 5). These ion-exchange recoveries are close to those reported by Boas (1953).

Reproducibility

Coefficients of variation for detected quantities of muramic acid and glucosamine in hydrolysis standards and S. alterniflora samples were always less than 10% (Table 3). Yields of muramic acid and glucosamine from ion-exchange columns exhibited coefficients of variation less than 5% (Table 8). These values are smaller than reproducibility values of similar analyses (Table 10).

Comparative Costs

The estimated cost per sample for muramic acid and glucosamine analyses are compared in Table 10. These estimates are for expendable supplies and do not include investigator time. The cost of sample hydrolysis was similar for all methods in Table 10. Several gas-chromatographic methods and one colorimetric method (Millar and Casida, 1970) involve purification of hydrolysates prior to analysis. Ion-exchange chromatography or thin-layer chromatography was used for this purpose. Assuming that ion-exchange columns were used only once, the cost per sample could be as high as $1.50. However, if the columns are rejuvenated and reused, the cost per sample including solvents is decreased to less than $0.15. Thin-layer chromatographic purification is more expensive. This procedure was estimated to cost greater than $1.50 per sample, including TLC plates and developing solvents.

The cost of derivative formation for gas chromatography varied widely. Alditol acetates ($0.02/sample), aldonitrile acetates ($0.02/sample), and O-methyloxime acetates ($0.08/sample) were estimated to be much less expensive to prepare than trimethylsilyl ether derivatives ($1.92/sample) of amino sugars (Casagrande and Park, 1978).

Colorimetric detection expenses were estimated to be about five percent of other detection methods. Gas-chromatographic detection expenses, assuming 500 assays per column, were estimated to be $0.34 per sample for packed-column analysis and $0.49 per sample for capillary-column analysis. The estimated expense for enzymatic detection of muramic acid alone ($0.39/sample) was within this range (Moriarty, 1977).

Potential Applications of the Muramic Acid and Glucosamine Method

Muramic acid (Millar and Casida, 1970; Moriarty, 1977) and glucosamine (Cochran and Vercellotti, 1978) have potential as indicators of prokaryotic and filamentous fungal biomasses. Appendix II summarizes biomass conversion values determined in previous work. If acetone-soluble glucosamine sources are eliminated, one may estimate prokaryotic and fungal biomasses using the method described in this report. Glucosamine contributed by prokaryotes can be accounted for if a ratio of muramic acid:glucosamine in peptidoglycan layers is known or assumed. As a preliminary example, conversion values reported in Appendix II were used to estimate bacterial and fungal biomasses in one set of samples of standing-dead S. alterniflora (culms + leaves).

The S. alterniflora sample (100 mg dry) contained 3082 µg GlcN · g dry weight^{-1} (Table 3). When a larger sample was analyzed, 105 µg Mur · g dry weight^{-1} was detected. Bacterial and fungal biomasses were estimated as a percentage of the sample dry weight using these concentrations (Table 11). The ratio of muramic acid:glucosamine in prokaryotic cell walls (µg:µg) was assumed to be 1:1. After subtracting the estimated prokaryotic glucosamine contribution, the remaining glucosamine was assumed to be of fungal origin. Table 11 presents biomass estimates when the highest and lowest and mean conversion values from Appendix II were used.

It is evident that fungal biomass is overestimated when the lowest reported conversion value for a fungus is used. This indicates the need for use of conversion values for species known to be or to have been actively growing in the natural sample. When microbial biomasses are compared using mean conversion values (Table 11), bacterial biomass in the S. alterniflora sample is less than 7% of the estimated fungal biomass. By accounting for or eliminating extraneous glucosamine sources (i.e., insect or crustacean exoskeletons), this method should be adaptable to estimation of prokaryotic and fungal biomasses in environmental samples.

The method outlined in Figure 1 is rapid, sensitive, and less expensive than previously described gas-chromatographic methods (Casagrande and Park, 1978; Cochran and Vercellotti, 1978; Fazio et al., 1979). The muramic acid and glucosamine technique described herein may be preferable to direct-counting methods when both bacterial and fungal biomasses are desired for plant or detritus samples.

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LITERATURE CITED

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TABLES

TABLE 1: COMBINATIONS OF SAMPLE, STANDARD, AND PRELIMINARY TREATMENTS WHICH WERE TRIED PRIOR TO SAMPLE CLEAN-UP AND GAS CHROMATOGRAPHY

COMBINATIONS*	0.1 g S. ALTERNIFLORA	0.3 ml STANDARDS	ACETONE PRE-EXTRACTION	HYDROLYSIS UNDER N_2	EVAPORATION WITH GLYCERIN
I	X				
II	X	X			
III	X			X	
IV	X	X		X	
V	X			X	X
VI	X	X		X	X
VII	X		X	X	X
VIII	X	X	X	X	X
IX		X		X	X
X**				X	X

* Roman numerals designate the same combinations used in Table 3.

** Blank runs.

TABLE 2: GAS CHROMATOGRAPHIC CONDITIONS TESTED FOR MURAMIC ACID AND GLUCOSAMINE ANALYSIS

1) 3% SP-2100 on 100/120 Supelcoport (Supelco, Bellefonte, PA); 100°C, 2 min.; 100-200°C, 4°C/min.; 8 min. hold; N_2 = 30 ml/min.; H_2 = 30 ml/min.; Air = 240 ml/min.; Injector = 200°C; Detector = 250°C.

2) 3% OV-225 on 100/120 Gas Chrom Q (Applied Sci. Laboratories, Inc., State College, PA); 100°C, 2 min.; 100-200°C, 4°C/min.; 8 min. hold; N_2 = 30 ml/min.; H_2 = 30 ml/min.; Air = 240 ml/min.; Injector = 200°C; Detector = 250°C.

3) 3% QF-1 on 100/120 Gas Chrom Q (Applied Sci. Laboratories, Inc., State College, PA): 100°C, 2 min.; 100-210°C, 4°C/min.; 8 min. hold; N_2 = 30 ml/min.; H_2 = 30 ml/min.; Air = 240 ml/min.; Injector = 200°C; Detector = 250°C.

4) 3% SP-3220 on 100/120 Supelcoport (Supelco, Bellefonte, PA); 230°C isothermal; N_2 = 21 ml/min.; H_2 = 30 ml/min.; Air = 240 ml/min.; Injector = 250°C; Detector = 300°C.

5) 2% diethylene glycol adipate (stabilized grade) on 100/120 Chromosorb W HP (Analabs, Inc., North Haven, CT); 170°C, 2 min.; 170-240°C, 8°C/min.; 4 min. hold; N_2 = 36 ml/min.; H_2 = 31 ml/min.; Air = 219 ml/min.; Injector = 250°C; Detector = 300°C.

6) OV-101 fused silica capillary column (Hewlett-Packard, Avondale, PA); 190°C, 2 min.; 190-230°C, 4°C/min.; 230°C, 16 min.; N_2 = 0.5408 ml/min.; Split ratio = 49.8517:1; make-up gas (N_2) = 46 ml/min.; Injector = 250°C; Detector = 300°C.

TABLE 3: RECOVERIES OF GLUCOSAMINE (GlcN), MURAMIC ACID (Mur), AND N-METHYL-GLUCAMINE (MeGluA) INCLUDING POTENTIAL LOSSES DURING HYDROLYSIS (INTERNAL STANDARD ADDED PRIOR TO GAS CHROMATOGRAPHY: β-PHENYL-GLUCOSE)*

TREATMENT**		GlcN	Mur	MeGluA
I: No N_2; to dryness	$\mu g \cdot g^{-1}$ S. alt.	3651.15	0	-
	SD	126.92	0	-
	CV	3.48	0	-
	% recovery	120.39	84.60	93.80
	g residue·g^{-1} S. alt.		0.4334±0.0148	
III: N_2; to dryness	$\mu g \cdot g^{-1}$ S. alt.	3609.18	0	-
	SD	282.60	0	-
	CV	7.83	0	-
	% recovery	100.30	94.18	93.53
	g residue·g^{-1} S. alt.		0.4797±0.0067	
V: N_2; to dryness w/glycerin	$\mu g \cdot g^{-1}$ S. alt.	4283.08	0	-
	SD	114.16	0	-
	CV	2.67	0	-
	% recovery	117.96	110.73	124.94
	g residue·g^{-1} S. alt.		0.4815±0.0085	
VII: N_2; to dryness w/glycerin; acetone ext.	$\mu g \cdot g^{-1}$ S. alt.	3082.17	0	-
	SD	35.94	0	-
	CV	1.17	0	-
	% recovery	122.10	92.87	115.08
	g residue·g^{-1} S. alt.		0.4286±0.0037	
IX: N_2; to dryness w/glycerin; no S. alt.	μg	294.13	328.25	402.55
	SD	10.93	14.51	20.34
	CV	3.72	4.42	5.05
	% recovery	118.00	103.61	126.11
X: N_2; to dryness w/glycerin; blank	μg	0	0	0
	SD	0	0	0
	CV	-	-	-
	% recovery	-	-	-

* Roman numerals indicate combinations of preliminary sample treatments given in Table 1.

** Number of replicates was 3 in every case in this table.

TABLE 4: RECOVERIES OF GLUCOSAMINE (GlcN), MURAMIC ACID (Mur), AND β-PHENYL-GLUCOSE (PhGlc) INCLUDING POTENTIAL LOSSES DURING HYDROLYSIS (INTERNAL STANDARD ADDED PRIOR TO ACID HYDROLYSIS: N-METHYL-GLUCAM[NE]

TREATMENT		GlcN	Mur	PhGlc
IX* N_2; To Dryness w/ glycerin; No S. alt.	µg	233.60	260.42	27.76
	SD	3.10	7.13	4.51
	CV	1.33	2.74	16.25
	% Recovery	93.71	82.20	86.75
	N	3	3	3

* Roman numeral indicates combinations of preliminary sample treatments given in Table 1.

TABLE 5: RECOVERIES OF GLUCOSAMINE (GlcN), MURAMIC ACID (Mur), AND N-METHYL-GLUCAMINE (MeGluA) INCLUDING LOSSES DURING ION-EXCHANGE CLEAN-UP (AFTER HYDROLYSIS). β-PHENYL-GLUCOSE WAS ADDED AS AN INTERNAL STANDARD PRIOR TO GAS CHROMATOGRAPHY.

TREATMENT		GlcN	Mur	MeGluA
0.1 ml;	µg	93.53	44.36	75.49
5 ml 2N HCl	SD	40.07	28.39	45.09
	CV	42.84	63.99	59.73
	% Recovery	94.52	41.83	70.42
	N	6	6	3
1.0 ml;	µg	95.91	55.42	76.79
5 ml 2N HCl	SD	6.65	12.60	22.40
	CV	6.93	22.73	29.18
	% Recovery	97.27	50.94	71.63
	N	3	3	3
1.0 ml;	µg	96.74	98.39	129.28
6 ml 2N HCl	SD	2.71	4.68	4.88
	CV	2.80	4.76	3.78
	% Recovery	98.11	90.44	120.60
	N	3	3	3
1.0 ml;	µg	85.38	120.49	126.21
10 ml 2N HCl	SD	0.54	2.03	1.41
	CV	0.63	1.68	1.12
	% Recovery	102.76	114.10	118.62
	N	3	3	3

TABLE 6: RECOVERIES OF GLUCOSAMINE (GlcN), MURAMIC ACID (Mur), AND β-PHENYL-GLUCOSE (PhGlc) INCLUDING LOSSES DURING ION-EXCHANGE CLEAN-UP (AFTER HYDROLYSIS). N-METHYL-GLUCAMINE WAS ADDED AS AN INTERNAL STANDARD PRIOR TO ION-EXCHANGE CHROMATOGRAPHY.

TREATMENT		GlcN	Mur	PhGlc
0.1 ml;	µg	194.05	86.45	146.82
5 ml 2N HCl	SD	78.52	9.83	107.26
	CV	40.46	11.37	73.06
	% Recovery	196.80	79.46	393.26
	N	3	3	3
1.0 ml;	µg	140.61	78.42	47.07
5 ml 2N HCl	SD	34.23	5.14	12.12
	CV	24.34	6.55	25.75
	% Recovery	142.60	72.08	147.11
	N	3	3	3
1.0 ml;	µg	80.46	81.74	26.59
6 ml 2N HCl	SD	4.78	1.35	1.00
	CV	5.94	1.65	3.77
	% Recovery	81.60	75.13	83.10
	N	3	3	3
1.0 ml;	µg	72.06	101.58	26.99
10 ml 2N HCl	SD	0.94	0.58	0.30
	CV	1.30	0.57	1.11
	% Recovery	86.73	96.19	84.34
	N	3	3	3

TABLE 7: THIN-LAYER CHROMATOGRAPHY: R_f VALUES

COMPOUND	METHOD	
	A. FAZIO *et al.* 1979	B. ESSER 1965
B-D(+)-Glucose	0.4428 ± 0.0146	
D(+)-Glucosamine HCl	0.2639 ± 0.0136	0.4345 ± 0.0037
D-Mannosamine HCl	0.3073 ± 0.0223	0.4659 ± 0.0052
D(+)-Galactosamine HCl	0.2058 ± 0.0006	0.3878 ± 0.0055
Muramic Acid	0.2957 ± 0.0103	
Ethanolamine	0.1270 ± 0.0017	0.4940 ± 0.0051
Cellobiose	0.3839 ± 0.0083	

A. 9:1:1 ISOPROPANOL : ACETIC ACID : WATER
SILICA GEL G PLATES; 0.25 mm THICK

B. 60:45:4:30 BUTANOL : PYRIDINE : ACETIC ACID : WATER
CELLULOSE PLATES; 0.10 mm THICK

TABLE 8: HEXOSAMINE RECOVERIES: THIN-LAYER vs ION-EXCHANGE CHROMATOGRAPHY

METHOD		GLUCOSAMINE	MURAMIC ACID
TLC*	µg	11.09 ± 4.51	33.13 ± 25.85
ANA derivatives	N	3	3
	CV	40.65%	78.00%
	% Recovery	11.07%	32.39%
TLC*	µg	5.79 ± 2.68	34.04 ± 3.53
OMOA derivatives	N	3	3
	CV	46.29%	10.37%
	% Recovery	6.97%	32.23%
IEC**	µg	96.74 ± 2.71	98.39 ± 4.68
OMOA derivatives	N	3	3
	CV	2.80%	4.76%
	% Recovery	98.11%	90.44%

* Fazio et al. 1979. Appl. Environ. Microbiol. 38:349-350.

** Boas. 1953. J. Biol. Chem. 204:553-556.

TABLE 9: CARBOHYDRATE RETENTION TIMES (Seconds ± S.D.)

COMPOUND	METHOD						
	3% SP-2100[1**]	3% OV-225[2**]	3% QF-1[3**]	3% SP-2330[4*]	2% DEGA[5*]	OV-101 CAP[6**]	OV-101 CAP[7*]
B-D(+)-Glucose	1549 ± 7	1759	1727 ± 25	153 ± 1	345 ± 1	443 ± 1	522 ± 1
D(+)-Glucosamine HCl	1751 ± 9	2344	2045 ± 14	697 ± 3	563 ± 2	681 ± 2	719 ± 3
D-Mannosamine HCl	1843 ± 8	2157	2174 ± 43	855 ± 2	594 ± 1	---	749 ± 5
D(+)-Galactosamine HCl	1870 ± 7	2058	2302 ± 76			---	
Muramic Acid	1883 ± 4	2189	2073 ± 17	1027 ± 5	684 ± 3	863 ± 4	882 ± 7
N-Methyl-Glucamine	---	---	---	1184 ± 4	635 ± 1	927 ± 5	934 ± 6
β-Phenyl-Glucose	---	---	---	787 ± 3	652 ± 1	1065 ± 5	1068 ± 6

* O-Methyloxime Acetates ** Aldonitrile Acetates

1. 3% SP-2100 on 100/120 Supelcoport; 6 ft. x 2 mm ID; N_2 = 30 ml/min.; 100-200°C; 4°C/min.; N = 4.
2. 3% OV-225 on 100/120 Gas Chrom Q; 6 ft. x 2 mm ID; N_2 = 30 ml/min.; 100-200°C; 4°C/min.; N = 1.
3. 3% QF-1 on 100/120 Gas Chrom Q; 6 ft. x 2 mm ID; N_2 = 30 ml/min.; 100-210°C; 4°C/min.; N = 2.
4. 3% SP-2330 on 100.120 Supelcoport; 6 ft. x 2 mm ID; N_2 = 21 ml/min.; 230°C; N = 6.
5. 2% DEGA on 100/120 Chromosorb W HP; 6 ft. x 2 mm ID; N_2 = 36 ml/min.; 170-240°C; 8°C/min.; N = 6.
6. OV-101 Capillary (Fused Silica); 10 m x .21 mm ID; N_2 = 0.5408 ml/min.; 190-230°C; 4°C/min.; N = 15.
7. OV-101 Capillary (Fused Silica); 10 m x .21 mm ID; N_2 = 0.5408 ml/min.; 190-230°C; 4°C/min.; N = 15.

TABLE 10: COMPARISON OF ANALYSIS SENSITIVITIES, RECOVERIES, REPRODUCIBILITIES, AND ASSAY TIME

AUTHOR (METHOD: COMPOUND)**	DETECTION SENSITIVITY	RECOVERY (%)	REPRODUCI-BILITY (%)	COST/ SAMPLE ($)	TIME/ SAMPLE
MILLAR and CASIDA, 1970 (COL: Mur)		79.0	5.1	0.61*	>13 H*
KING and WHITE, 1977 (COL: Mur)	5-20 µg/ml	-	17.0*	1.82*	>12 H*
MORIARTY, 1977 (ENZ: Mur)	0.28 µg/ml*	-	10.0	0.40*	> 8.5 H*
CASAGRANDE and PARK, 1978 (GLC: Mur)	-	83.0*	-	2.51*	> 5 H*
FAZIO et al., 1979 (GLC: Mur)	0.11 µg/injection*	99.8	9.5	2.26*	17 H
RIDE and DRYSDALE, 1972 (COL: Chitosan)	0.1 µg GlcN	100.1*	4.5*	0.08*	5 H
COCHRAN and VERCELLOTTI, 1978 (GLC: GlcN)	0.27 µg/injection*	75.0	-	1.14*	> 3.5 H*
MaWHINNEY et al., 1980 (GLC: GlcN, GalN)	0.10 µg/injection	100.0*	-	0.51*	<10 H*
BENZING-PURDIE, 1982 (GLC: GlcN, GalN)	-	-	-	0.39*	>22 H*
HICKS and NEWELL, 1982 (GLC: Mur)	0.15 µg/injection	93.0	7.8	0.79	10 H
(GLC: GlcN)	0.82 µg/injection	122.0	3.8		

* Estimated from published data.

** GLC: gas-liquid chromatographic
COL: colorimetric
ENZ: enzymatic

Mur: muramic acid
GlcN: glucosamine
GalN: galactosamine

TABLE 11: PRELIMINARY MICROBIAL BIOMASS ESTIMATES IN STANDING-DEAD SPARTINA ALTERNIFLORA USING CONVERSION VALUES REPORTED IN APPENDIX II

	BACTERIA		FUNGI	
	Conversion Value $\mu g\ Mur \cdot mg\ dry\ wt^{-1}$	% of sample dry weight	Conversion Value $\mu g\ GlcN \cdot mg\ dry\ wt^{-1}$	% of sample dry weight
LOW	2.6	4.04	1.8	265.39
MEAN	20.6 (N=47)	0.51	40.6 (N=24)	7.33
HIGH	127.0	0.08	114.0	2.61

FIGURES

FIGURE LEGENDS

FIGURE 1. Flowchart for muramic acid and glucosamine analysis. The bold line indicates the preferred procedure.

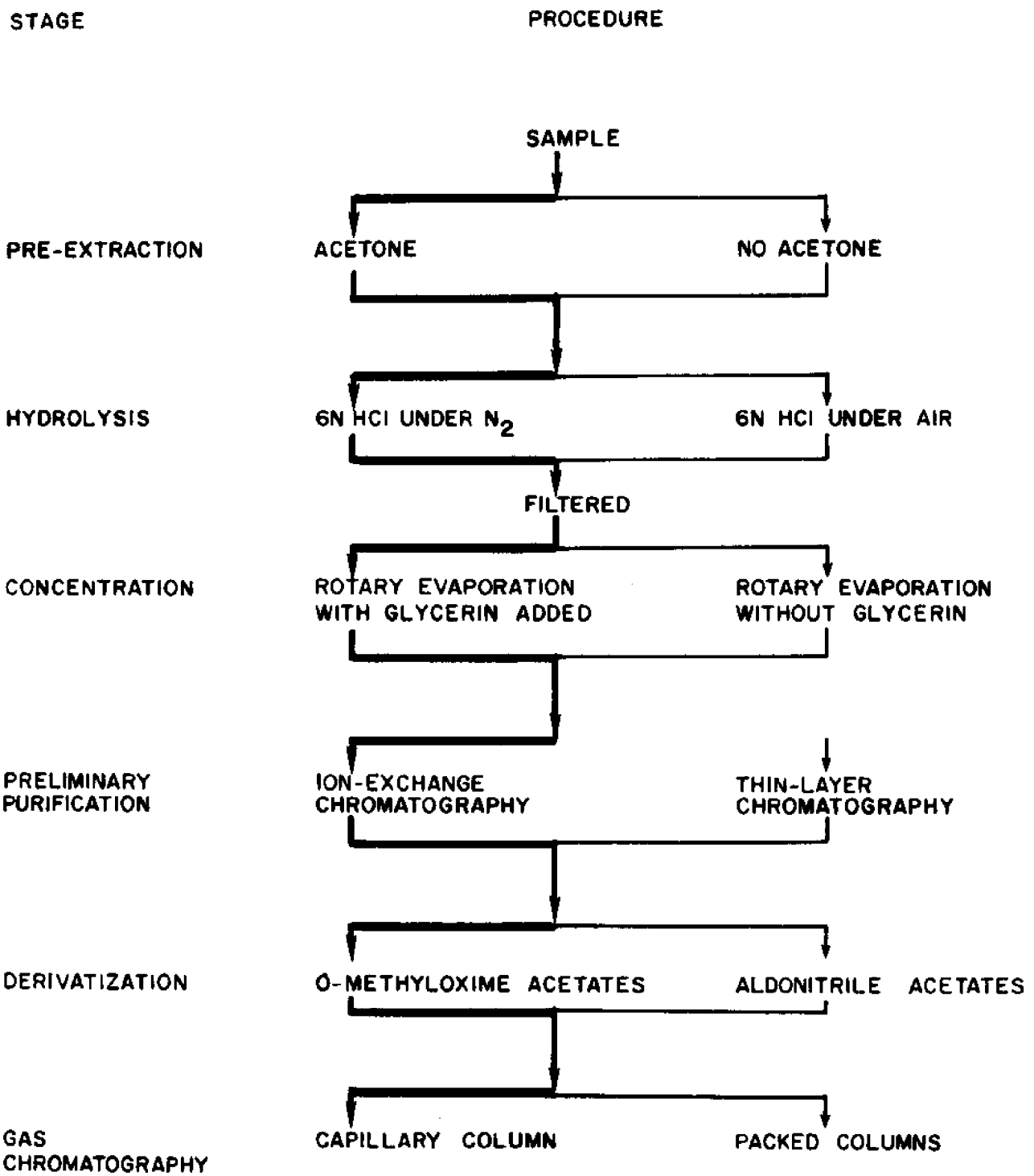
FIGURE 2. Compound concentration vs. molar response relative to β-phenyl-glucose. Aldonitrile and O-methyloxime acetates of muramic acid, glucosamine and N-methyl-glucamine. Molar response is interpreted as the number of moles of compound required for a response equal to one mole of β-phenyl-glucose.

FIGURE 3. Aldonitrile acetates of standard compounds run on 3% SP-2100 on 100/200 Supelcoport. 6 ft. x 2 mm ID; 2 min. hold; 100-200°C, 4°C/min.; 8 min. hold; injector = 200°C; detector - 250°C; N_2 = 30 ml/min.

FIGURE 4. Aldonitrile acetates of standard compounds run on OV-101 capillary column. 10 m x 0.21 mm ID; 2 min. hold; 190-230°C, 4°C/min.; 16 min. hold; injector = 250°C; detector = 300°C; N_2 carrier = 0.5408 ml/min.; split ratio = 49.85:1.

FIGURE 5. O-methyloxime acetates of standard compounds run on OV-101 capillary column (10 m x 0.21 mm ID). Chromatographic conditions same as in Figure 4.

FIGURE 6. O-methyloxime acetates of amino sugars from brown Spartina alterniflora run on OV-101 capillary column (10 m x 0.21 mm ID). Chromatographic conditions same as in Figure 4.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ROTARY EVAPORATION WITH GLYCERIN ADDED
ROTARY EVAPORATION WITHOUT GLYCERIN
PRELIMINARY PURIFICATION
ION-EXCHANGE CHROMATOGRAPHY
THIN-LAYER CHROMATOGRAPHY
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ALDONITRILE ACETATES
GAS CHROMATOGRAPHY
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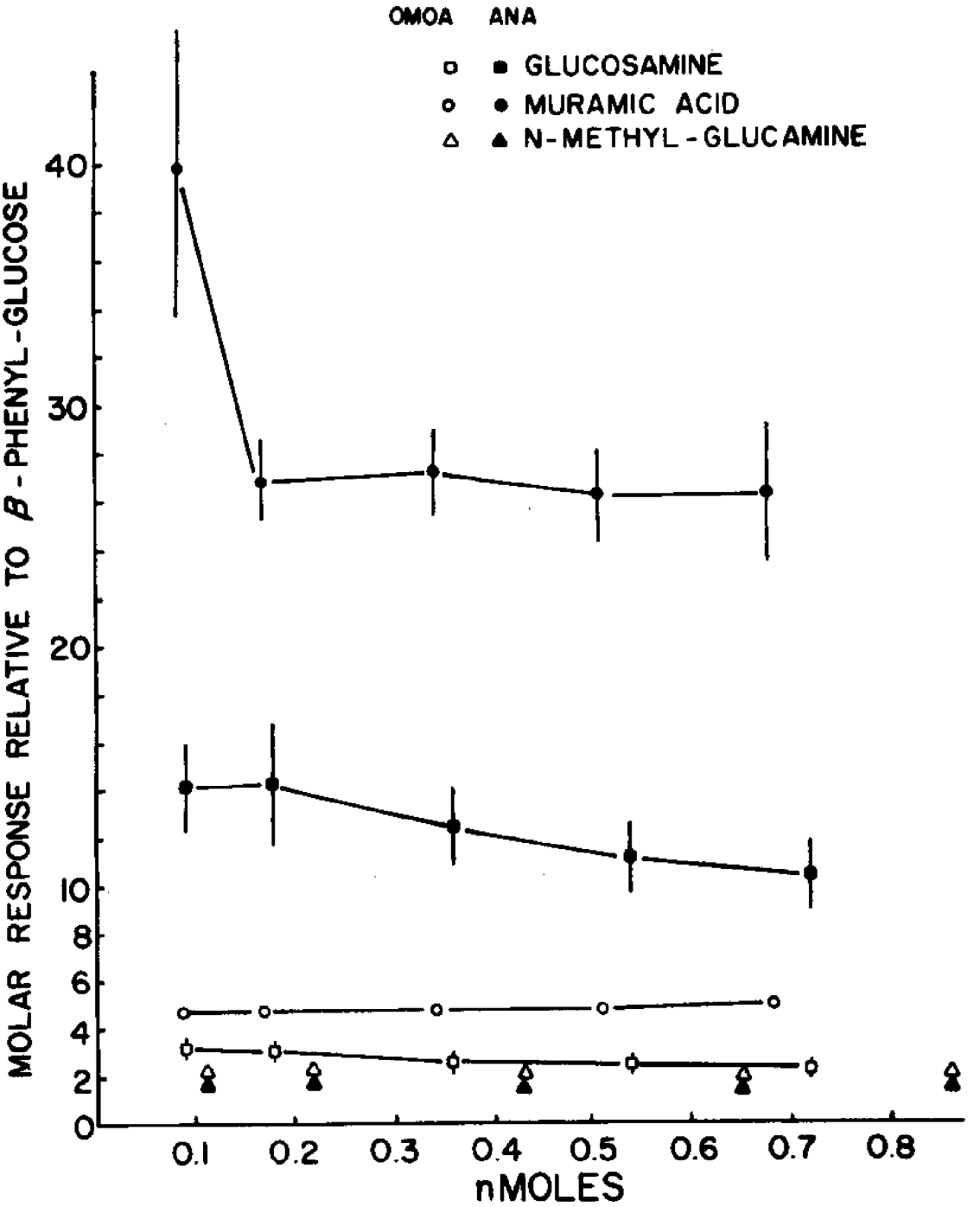
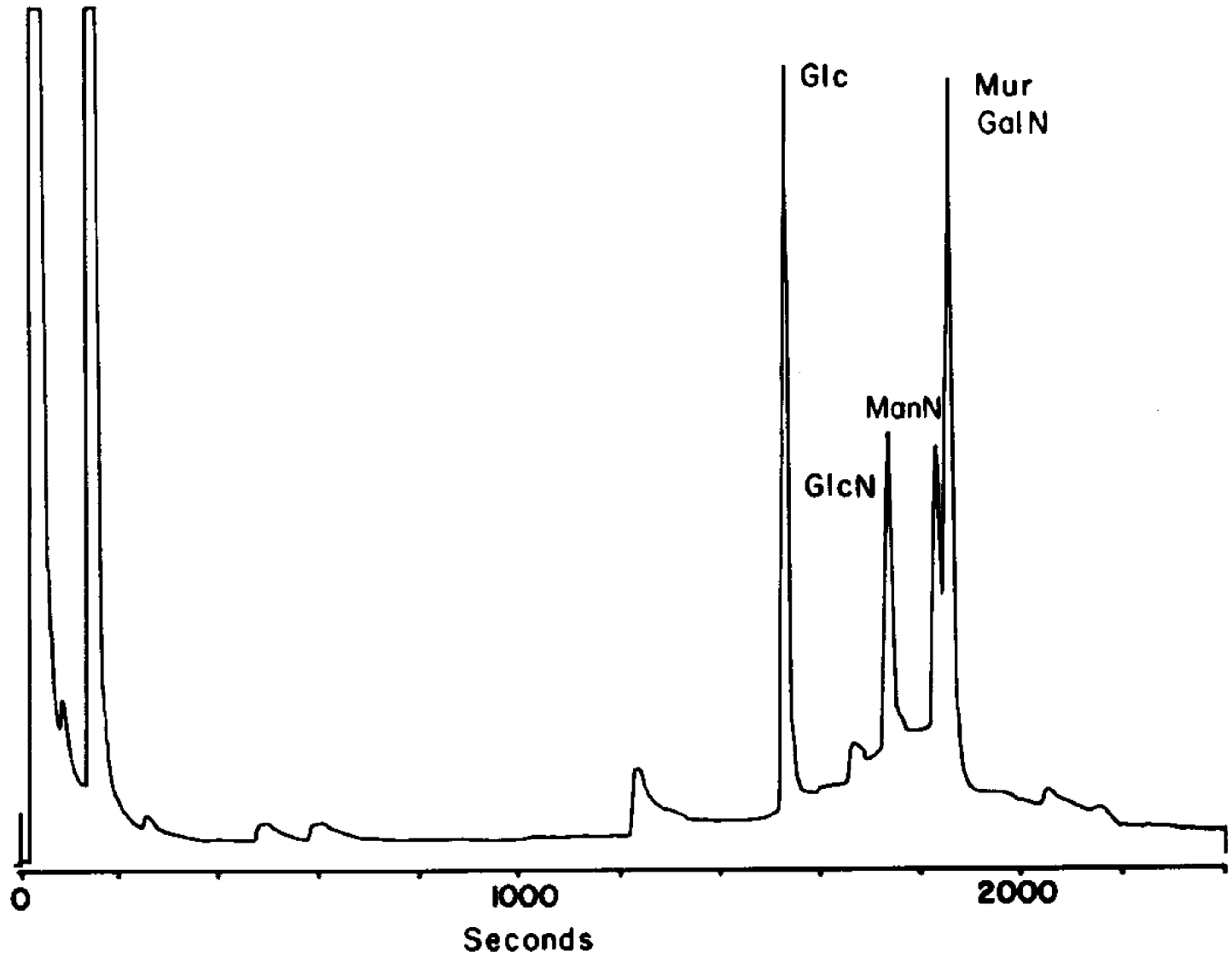

OMOA ANA
GLUCOSAMINE
MURAMIC ACID
N-METHYL-GLUCAMINE
MOLAR RESPONSE RELATIVE TO β-PHENYL-GLUCOSE
40
30
20
10
8
6
4
2
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
nMOLES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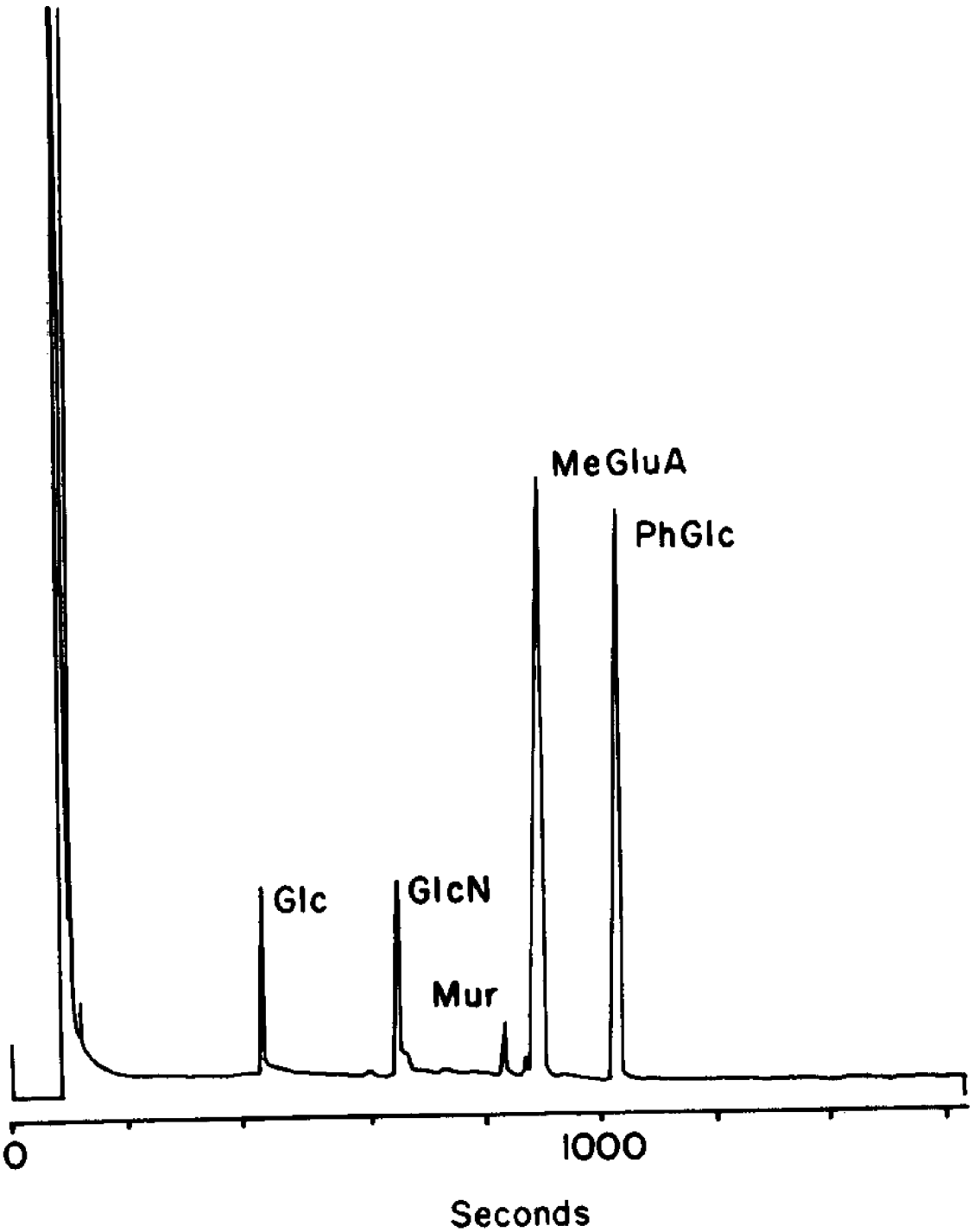
Mur
GalN
ManN
GlcN
0
1000
2000
Seconds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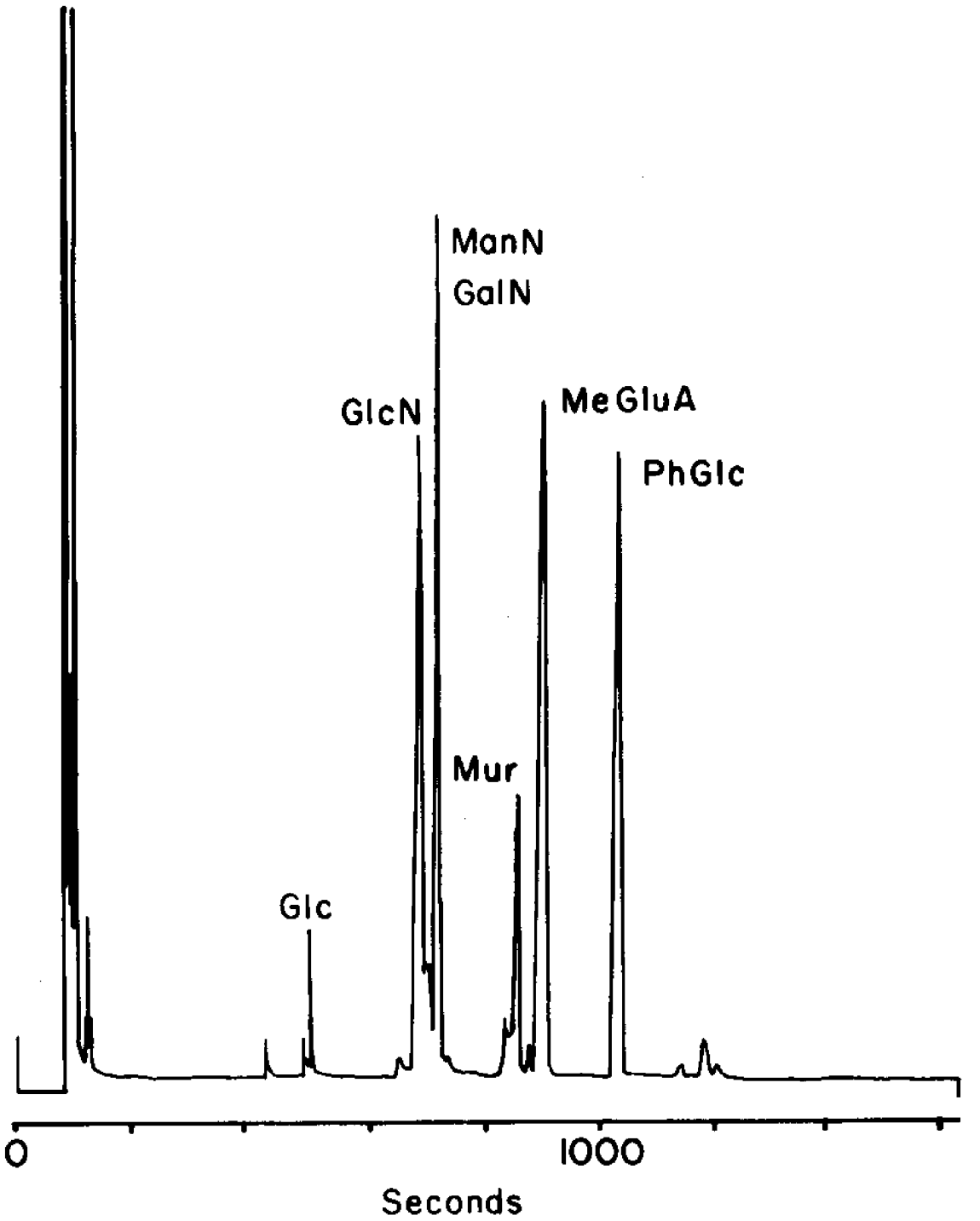
PhGlc
Glc
GlcN
Mur
0
1000
Seconds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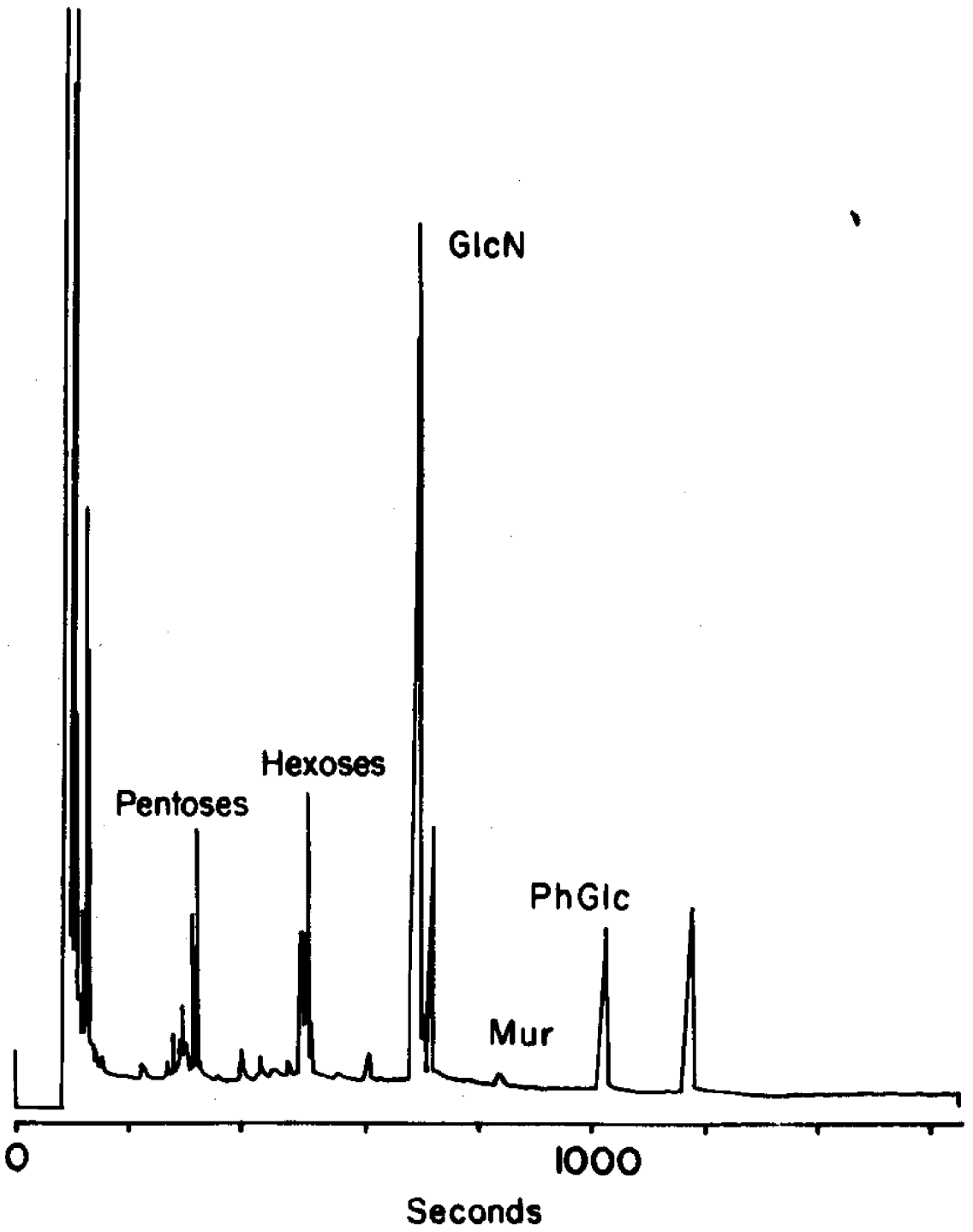
GlcN
Hexoses
Pentoses
PhGlc
Mur
0
1000
Seconds

APPENDICES

APPENDIX I: AMINO SUGAR ANALYSES - METHODS AND NATURAL SAMPLES

AUTHOR	SAMPLE	HYDROLYSIS	INITIAL PURIFICATION	DERIVATIZATION	ANALYSIS	COMPOUNDS REPORTED
Boas, 1953.	animal tissues	6N HCl, 100°C various times	IEC	none	COL	hexosamines
Gardell, 1953.	cornea poly-saccharide	6N HCl, reflux 2, 8, 24 hrs.	IEC	none	COL	GlcN, GalN
Reissig et al., 1955.	standards	none	none	none	COL	N-acetyl amino sugars
Blumenthal and Roseman, 1957.	fungi (25)	6N HCl, 100°C 6 hrs.	none	none	COL	amino sugars
Levvy and McAllan, 1959.	standards	none	none	none	COL	N-acetyl amino sugars
Ashwell et al., 1965.	standards	none	none	none	COL	GlcNAc, GalNAc, ManNAc, N-acetyl amino sugars
Esser, 1965.	standards	none	TLC	none	COL	Mur, GlcN
Günther and Schweiger, 1965.	standards	none	TLC	none	COL	GlcNAc, GlcN GalNAc, GalN
Stewart-Tull, 1968.	bacteria (7) standards	6N HCl, 105°C 18 hrs.	none	none	COL	Mur, GlcN GalN

AUTHOR	SAMPLE	HYDROLYSIS	INITIAL PURIFICATION	DERIVATIZATION	ANALYSIS	COMPOUNDS REPORTED
Tsuji et al., 1969.	standards, muco-polysaccharides	2 N HCl, 100°C 2 hrs.	none	none	COL	amino sugars
Millar and Casida, 1970.	soils (33) bacteria (18)	6N HCl, reflux 4 hrs.	IEC	none	COL ENZ	Mur
Ride and Drysdale, 1971.	fungus (1), fungus infected tomato plants	4-8N HCl, 100°C 18 hrs Enzymatic, 37°C 48 hrs.	AE	none	COL	GlcNAc , GlcN
Ride and Drysdale, 1972.	fungi (5) infected plants	120% KOH 130°C, 1 hr.	AE, P	none	COL	chitosan
Dietrich, 1973.	Oomycetes (7)	5N HCl, 100°C 5 hrs. Enzymatic, 35°C 60 hrs.	PC	none	COL	amino sugars, N-acetyl amino sugars
Swift, 1973.	fungus (1) infected sawdust	6N HCl, 80°C 16 hrs.	IEC	none	COL	hexosamines
Hadzija, 1974.	standards	none	none	none	COL	Mur
Laborda et al., 1974.	fungus (1) conidia	4N HCl, 105°C 8 hrs.	IEC	none	COL	amino sugars
Vladovska-Yukhnovska et al., 1974.	standards	none	TLC	FLUOR	FLUOR	GalN

AUTHOR	SAMPLE	HYDROLYSIS	INITIAL PURIFICATION	DERIVATIZATION	ANALYSIS	COMPOUNDS REPORTED
Harrower, 1977.	fungus (1) infected wheat leaves	120% KOH 130°C, 1 hr.	AE	none	COL	chitosan
King and White, 1977.	bacterium (1) pine and oak leaves, marine sediment	6 N HCl, 100°C 4.5 hrs.	TLC	none	COL ENZ	Mur
Sharma et al., 1977.	fungi (8)	120% KOH, 130°C 1 hr.	P	none	COL	chitosan
Frankland et al., 1978.	fungus (1)	6 N HCl, 80°C 16 hrs.	IEC	none	COL	hexosamines
Swift, 1978.	fungus (1)	6N HCl, 80°C 16 hrs.	IEC	none	COL	hexosamines
Whipps and Lewis, 1980.	fungus (1)	120% KOH, 130°C 1 hr.	AE, P	none	COL	chitosan
Moriarty, 1975.	mullet and prawn gut contents, bacteria	12N HCl, 100°C 6 hrs.	C	none	ENZ	Mur
Moriarty, 1977.	bacteria, blue-green alga, marine sediment	12N HCl, 100°C 6 hrs.	C	none	ENZ	Mur

AUTHOR	SAMPLE	HYDROLYSIS	INITIAL PURIFICATION	DERIVATIZATION	ANALYSIS	COMPOUNDS REPORTED
Moriarty, 1979a.	seawater	4N HCl, 100°C 6 hrs.	none	none	ENZ	Mur
Moriarty, 1979b.	prokaryotic alga	12N HCL, 100°C 6 hrs.	C	none	ENZ	Mur
Moriarty, 1980.	marine sediment	6N HCl, 100°C 12-18 hrs.	C	none	ENZ	Mur
Rosan, 1972.	standards	none	none	none	AAA	Mur, GlcN GalN
Stahmann et al., 1975.	fungi (6), infected plants	6N HCl, 110°C 1, 2, 4, 6, 9 hrs.	F	none	AAA	GlcN
Wu and Stahmann, 1975.	fungus (1), infected plant	6N HCl, 110°C 2-4 hrs.	F	none	AAA	GlcN, GalN
Blanchette and Shaw, 1978.	fungi (3) infected wood chips	6N HCL, 110°C 2 hrs.	F	none	AAA	GlcN
Dawson and Mopper, 1978.	human blood, bacterium marine sediment	1.8N p-toluene-sulfonic acid, 110°C, 22 hrs.	IEC	none	LC (?)	GlcN, GalN, ManN
Moss et al., 1971.	standards	none	none	HFB-n-PE	GLC	Mur

AUTHOR	SAMPLE	HYDROLYSIS	INITIAL PURIFICATION	DERIVATIZATION	ANALYSIS	COMPOUNDS REPORTED
Casagrande and Park, 1977.	standards	none	none	TMS	GLC	Mur
Casagrande and Park, 1978.	peat	4N HCl, ?°C optinum time	none	TMS	GLC	Mur
Cochran and Vercellotti, 1978.	fungi (4)	8N HCl, 95°C 2-3 hrs.	none	TMS	GLC COL AAA	GlcN
Fazio et al., 1979.	marine sediment	6N HCl, reflux 4.5 hrs.	TLC	ANA	GLC	Mur
Casagrande et al., 1980.	peat	4N HCl, ?°C 10 hrs.	NA	TMS	GLC	GlcNAc
Fox et al., 1980.	rat tissue infected with bacterial cell walls	2N H_2SO_4, 100°C 3 hrs.	TLC	AA	GLC	Mur
MaWhinney et al., 1980.	glycoproteins standards	3N HCl, 125°C 45 min.	IEC	OMOA ANA TMS	GLC	GlcN, GalN, ManN
Benzing-Purdie, 1981.	soil	6N and 3N HCl, 105°C 18 hrs.	none	AA	GLC	GlcN, GalN
Bobbie et al., 1981.	aufwuchs on teflon	6N HCl, reflux 4.5 hrs.	IEC	ANA	GLC	Mur, GlcN

AUTHOR	SAMPLE	HYDROLYSIS				COMPOUNDS REPORTED
Pritchard et al., 1981.	Streptococcus spp. (6)	1.4N methanolic HCl, 80°C, 24 hrs.	none	TFA	GLC	MurAc GlcNAc GalNAc
THIS REPORT	standards Spartina	6N HCl, 100°C 4.5 hrs.	IEC TLC	OMOA ANA	GLC	Mur GlcN, GalN, ManN, MeGluA

Abbreviations:

AE - acetone extraction
C - centrifugation
F - filtration
IEC - ion exchange chromatography
NA - N-acetylation
P - precipitation
PC - paper chromatography
TLC - thin layer chromatography
LC - liquid chromatography
AA - alditol acetates
ANA - aldonitrile acetates
FLUOR - fluorescent
OMOA - O-methyloxime acetates
TMS - trimethylsilyl ether
TFA - trifluoroacetates
HFB-n-PE - Heptafluorobutyl-n-propyl esters
COL - colorimetric
ENZ - enzymatic
AAA - amino acid analyzer
GLC - gas-liquid chromatography
GlcNAc - N-acetyl-glucosamine
Mur - muramic acid
GlcN - glucosamine
GalN - galactosamine
ManN - mannosamine
MeGluA - N-methyl-glucamine
MurAc - N-acetyl muramic acid
GalNAc - N-acetyl-galactosamine

APPENDIX II: MURAMIC ACID AND GLUCOSAMINE CONVERSION FACTORS

AUTHOR (METHOD)	ORGANISM	ORIGIN OR STRAIN: SUBSTRATE	CULTURE CONDITIONS	GROWTH PHASE	PRELIMINARY PURIFICATION	ug · mg dry weight^{-1} Mur	GlcN
Stewart-Tull, 1968. (COL)	*E. coli* (-)	8196	S: MEA	-	none	4.41	8.24
	Mic. lysodeikticus (+)	2665	S: MEA	-	none	3.99	3.19
	Myco. phlei (+)	8151	L: SM	-	none	3.38	5.46
	Prot. vulgaris (-)	4175	S: MEA	-	none	3.64	3.83
	Ps. aeruginosa (-)	6750	S: MEA	-	none	3.62	5.64
	Staph. aureus (+)	6571	S: MEA	-	none	4.84	5.51
	Strep. pyogenes (+)	2400	L: HDB	-	none	3.54	2.65
Millar and Casida, 1970. (COL: Lactate)	*Act. humiferus* (+)		L: NZCY	St	none	10.4	
	Aer. aeogenes (-)		L: NZCY	St	none	4.3	
	Agro. ramosus (-)		L: NZCY	St	none	6.4	
	Arth. aurescens (+)		L: NZCY	St	none	11.6	
	Brevi. linens		L: NZCY	St	none	6.6	
	E. coli (-)		L: NZCY	St	none	3.3	
	Lacto. brevis (+)		L: NZCY	St	none	6.4	
	Mic. freudenreichii (+)		L: NZCY	St	none	11.5	
	Prot. vulgaris X19 (-)		L: NZCY	St	none	3.3	
	Ps. aeruginosa (-)		L: NZCY	St	none	2.6	
	Salm. choleraesuis (-)		L: NZCY	St	none	3.2	
	Ser. marcescens (-)		L: NZCY	St	none	3.4	
	Shi. flexneri (-)		L: NZCY	St	none	4.0	
	Strep. faecalis var. *liquefaciens* (+)		L: NZCY	St	none	9.6	
	Strep. spp. (+)		L: NZCY	St	none	10.1	
	Bac. alvei spores		L: NZCY	St	none	30.7	
	Bac. cereus spores		L: NZCY	St	none	42.2	
	Bac. subtilis spores		L: NZCY	St	none	39.8	

AUTHOR (METHOD)	ORGANISM	ORIGIN OR STRAIN: SUBSTRATE	CULTURE CONDITIONS	GROWTH PHASE	PRELIMINARY PURIFICATION	ug · mg dry weight^{-1} Mur	GlcN
Moriarty, 1975.* (ENZ: Lactate)	Arth. globiformis (-)		L: PYB	-	none	189	
	Bac. subtilis (+)		L: PYB	-	none	236	
	E. coli (-)		L: PYB	-	none	44	
	Entero. aerogenes (-)		L: PYB	-	none	44	
	Mic. aurantiacus (+)		L: PYB	-	none	256	
	Prot. vulgaris (-)		L: PYB	-	none	44	
	Ps. aeruginosa (-)		L: PYB	-	none	51	
	Ser. marcescens (-)		L: PYB	-	none	49	
	Strep. venezuelae (+)		L: PYB	-	none	116	
	Isolate No. 1 (-)	M: sed	L: ZM	-	none	244	
	Isolate No. 2 (-)	M: sed	L: ZM	-	none	244	
	Isolate No. 3 (-)	M: sed	L: ZM	-	none	244	
	Isolate No. 4 (-)	M: sed	L: ZM	-	none	133	
	Isolate No. 5 (-)	M: sed	L: ZM	-	none	38	
	Isolate No. 6 (-)	M: sed	L: ZM	-	none	42	
King and White, 1977. (COL: Lactate)	E. coli (-)		L: NB	Ex	none	2.63	
Moriarty, 1977.* (ENZ: Lactate)	Arth. globiformis (+)		L: PYB	Ex	none	60	
	Bac. subtilis (+)		L: PYB	Ex	none	89	
	Entero. aerogenes (-)		L: PYB	Ex	none	22	
	E. coli (-)		L: PYB	Ex	none	22	
	Mic. aurantiacus (+)		L: PYB	Ex	none	127	
	Prot. vulgaris (-)		L: PYB	Ex	none	22	
	Ps. fluorescens (-)		L: PYB	Ex	none	17	
	Ser. marcescens (-)		L: PYB	Ex	none	22	
	Isolate No. 1 (-)	M: sed	L: ZM	Ex	none	22	
	Isolate No. 2 (-)	M: sed	L: ZM	Ex	none	20	
	Isolate No. 3 (-)	M: sed	L: ZM	Ex	none	20	
	Isolate No. 4 (-)	M: sed	L: ZM	Ex	none	16	
	Isolate No. 5 (-)	M: sed	L: ZM	Ex	none	16	
	Isolate No. 6 (-)	M: sed	L: ZM	Ex	none	13	
	Isolate No. 7 (-)	M: sed	L: ZM	Ex	none	11	
	Isolate No. 8 (-)	M: sed	L: ZM	Ex	none	11	
	Isolate No. 9 (+) rod (+ cocci)	M: sed	L: ZM	Ex	none	29	
			L: ZM	St	none	36	

AUTHOR (METHOD)	ORGANISM	ORIGIN OR STRAIN: SUBSTRATE	CULTURE CONDITIONS	GROWTH PHASE	PRELIMINARY PURIFICATION	ug · mg dry weight^{-1} Mur	GlcN
	Isolate No. 10 (-)	M: sed	L: ZM	Ex	none	31	
	Isolate No. 11 (-)	M: sed	L: ZM	Ex	none	98	
	Oscillatoria tenuis (BGA)		S		none	24	
Moriarty, 1979. (ENZ: Lactate)	Prochloron sp. Isolate 1	M: tunicate	none	-	none	1.9	
	Prochloron sp. Isolate 2	M: tunicate	none	-	none	1.8	
	Prochloron sp. Isolate 3	M: tunicate	none	-	none	1.6	
White et al., 1980. (GLC)	E. coli Type B (-)		L: NB	St	LE	0.03^{+}	0.05
Ride and Drysdale, 1971. (COL: Hexosamines)	Fusarium oxysporum f. lycopersici		L: several shaker and stationary	-	none		80
Ride and Drysdale, 1972.*** (COL: Hexosamines Chitosan)	Ascocphyta pisi		L: VM	-	none		47 (20)
	Botrytis fabae		L: VM	-	none		34 (38)
	Fusarium oxysporum		L: VM	-	none		76 (59)
	Mycosphaerella pinodes		L: VM	-	none		49 (25)
	Verticillium albo-atrum		L: VM	-	none		30 (24)
Dietrich, 1973.*** (COL: Hexosamines) (PC)	Achyla flagellata	SPC 31	L: SMSPS	-	ME		24 (20)
	Achlya orion	SPC 32	L: SMSPS	-	ME		31 (10)
	Achlya pseudoradiosa	SPC 30	L: SMSPS	-	ME		38 (10)
	Dictyuchus monosporus	SPC 33	L: SMSPS	-	ME		18 (25)
	Pythium ultimum	SPC 34	L: SMSPS	-	ME		13 (5)
	Pythium sp.	SPC 35	L: SMSPS	-	ME		12 (5)
	Saprolegnia diclina	SPC 36	L: SMSPS	-	ME		18 (-)

AUTHOR (METHOD)	ORGANISM	ORIGIN OR STRAIN: SUBSTRATE	CULTURE CONDITIONS	GROWTH PHASE	PRELIMINARY PURIFICATION	ug · mg dry weight^{-1} Mur	GlcN
Swift, 1973. (COL: Hexosamines)	Coriolus versicolor (L. ex Fr.) Quel		L: GAM	-	none		
			C/N = 450/1				16.5
			C/N = 45/1				12.4
			S: Beech logs				1.82
			S: Chestnut sawdust				2.56
Laborda et al., 1974. (COL: Hexosamines)	Fusarium colmorum (conidial walls)	2148	-	-	-		100
Harrower, 1977.** (COL: Chitosan)	Septoria tritici Desm.	T: wheat	L: BSM	-	-		(34.3)
Sharma et al., 1977.** (COL: Chitosan)	Helicodendron triglitziense (Jaap) Linder		L: GSM	-	none		(18)
			L: BLE	-	none		(53.5)
	H. giganteum Glen-Bott		L: BLE	-	none		(19.6)
	H. conglomeratum Glenn-Bott		L: BLE	-	none		(14.4)
	H. tubulosum (Riess) Linder		L: BLE	-	none		(10.2)
	H. hyalinum Linder		L: BLE	-	none		(9.6)
	H. fuscum (Berk. & Curt.) Linder		L: BLE	-	none		(10.6)
	Helicoon ellipticum (Pk) Morgan		L: BLE	-	none		(9.6)
	Hel. fuscosporum Linder		L: BLE	-	none		(13.5)
Cochran and Vercellotti, 1978. (AAA)	Aspergillus niger	NRRL 6009	L: YEB	-	none		40.8
	Cladosporidium cladosporoides	NRRL 6078	L: YEB	-	none		77.8
	Fusarium moniliforme	NRRL 3197	L: YEB	-	none		113.5
	Penicillium citrinum	NRRL 6010	L: YEB	-	none		84.9

AUTHOR (METHOD)	ORGANISM	ORIGIN OR STRAIN: SUBSTRATE	CULTURE CONDITIONS	GROWTH PHASE	PRELIMINARY PURIFICATION	ug · mg dry weight^{-1} Mur	GlcN
Frankland *et al.*, 1978. (COL: Hexosamines)	*Mycena galopus* (pers. ex Fr.) Kummer		S: ABL	-	none		42.83
Swift, 1978. (COL: Hexosamines)	*Stereum hirsutum*	T: oak branches	S: OB	-	none		11.03
Whipps and Lewis, 1980. (COL: Chitosan)	*Fusarium oxysporum* Schlecht. ex Fr.		L: VM	St	none AE		64.73 39.33
White *et al.*, 1980. (GLC)	*Neurospora crassa*		L: VM	-	LE		0.93

* - values converted from ug Mur/mg C to ug Mur/mg dry weight assuming carbon content is 45% of dry weight (Moriarty, 1977).

** - numbers in parentheses represent chitosan concentration reported as ug GlcN/mg dry weight.

*** - values for cell walls only; numbers in parentheses represent ug GlcNAc /mg cell wall dry weight.

\+ - calculated assuming Mur:GlcN (mole/mole) in *E. coli* is 0.3816 (Stewart-Tull, 1968).

ABBREVIATIONS

Act. - Actinomyces
Aer. - Aerobacter
Agro. - Agromyces
Arth. - Arthrobacter
Bac. - Bacillus
Brevi. - Brevibacterium
E. - Escherichia
Entero. - Enterobacter
H. - Helicodendron
Hel. - Helicoon
Lacto. - Lactobacillus
Mic. - Micrococcus
Myco. - Mycobacterium
Ps. - Pseudomonas
Prot. - Proteus
Salm. - Salmonella
Ser. - Serratia
Shi. - Shigella
Staph. - Staphylococcus
Strep. - Streptococcus

COL - colorimetric
ENZ - enzymatic
AAA - amino acid analyzer
GLC - gas-liquid chromatography
PC - paper chromatography

M - marine
T - terrestrial
L - liquid medium
S - solid medium
St - stationary phase
Ex - exponential phase
AE - acetone extraction
LE - liquid extraction
ME - 5% NaOH in methanol extraction

ABL - ash, beech leaves
BLE - beech leaf extract
BSM - basal salts medium
GAM - glucose, asparagine medium
GSM - glucose, salts medium
HDG - Hartley digest broth
MEA - meat extract agar
NB - nutrient broth
NZCY - N-Z Case, yeast extra t broth
OB - oak branches
PYB - peptone, yeast extract broth
SM - Sarton medium
SMSPS - Seymour;s MSPS medium
VM - Vogel's medium
YEB - yeast extract broth
ZM - Zobell's medium 2216E

Mur - muramic acid
GlcN - glucosamine
GlcNAc - N-acetylglucosamine