RESEARCH SUB-PROGRAM

MEASURING CHANGES IN SOIL MICROBIAL POPULATIONS BY ANALYSIS OF THEIR PHOSPHOLIPID SIGNATURES

March 1998

COESA Report No.: RES/MAN-011/96

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FORWARD

This report is one of a series of **COESA** (Canada-Ontario Environmental Sustainability Accord) reports from the Research Sub-Program of the Canada-Ontario Green Plan. The **GREEN PLAN** agreement, signed Sept. 21, 1992, is an equally-shared Canada-Ontario program totalling $64.2 M, to be delivered over a five-year period starting April 1, 1992 and ending March 31, 1997. It is designed to encourage and assist farmers with the implementation of appropriate farm management practices within the framework of environmentally sustainable agriculture. The Federal component will be delivered by Agriculture and Agrifood Canada and the Ontario component will be delivered by the Ontario Ministry of Agriculture and Food and Rural Assistance.

From the 30 recommendations crafted at the Kempenfelt Stakeholders conference (Barrie, October 1991), the Agreement Management Committee (AMC) identified nine program areas for Green Plan activities of which the three comprising research activities are (with Team Leaders):

1. **Manure/Nutrient Management and Utilization of Biodegradable Organic Wastes** through land application, with emphasis on water quality implications
   - **A.** Animal Manure Management (nutrients and bacteria)
   - **B.** Biodegradable organic urban waste application on agricultural lands (closed loop recycling) (Dr. Bruce T. Bowman, Pest Management Research Centre, London, ONT)

2. **On-Farm Research**: Tillage and crop management in a sustainable agriculture system. (Dr. Al Hamill, Harrow Research Station, Harrow, ONT)

3. **Development of an integrated monitoring capability** to track and diagnose aspects of resource quality and sustainability. (Dr. Bruce MacDonald, Centre for Land and Biological Resource Research, Guelph, C

The original level of funding for the research component was $9,700,000 through Mar. 31, 1997. Projects will be carried out by Agriculture and Agri-Food Canada, universities, colleges or private sector agencies including farm grc

This Research Sub-Program is being managed by the Pest Management Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford St., London, ONT. N5V 4T3.

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INTERPRETATIVE SUMMARY

Microorganisms are the most abundant life form in soil. This soil microbial community consists of a large number of types of organisms - the actual number is so large that it will probably never be known. The biomass of each type present, the microbial community structure, is affected by many factors such as nutrient level and source, temperature, moisture, available oxygen and the presence of other types. These organisms affect many aspects of agriculture-related activities from being essential for plant growth as providers of necessary nutrients, through such processes as organic matter decomposition and nitrogen fixation, to being unwanted pests as the agents of plant diseases. Their universal presence and the sensitivity of their community structure to changes in their environment also makes them good candidates for indicators of changes in soil quality. To use them as indicators, simple, reliable and sensitive methods of measuring changes in the soil microbial community structure must be available. One method under development is based on the chemical composition of the phospholipids present in the cell walls of the microorganisms. Phospholipids are a major cell wall component and their chemical composition differs for different types of organisms thereby providing a link between chemical composition and community structure. In this study we examined the merits of two different measures of phospholipid composition, fatty acid composition and class composition, for detecting changes induced by differences in tillage practice at four paired field sites. Fatty acid composition was determined by gas chromatograph and differences were observed between the paired sites, between the till and no-till treatments and between high and low plot elevations. Class composition was determined by ^{31}P NMR spectroscopy and consistent differences between tillage treatments were also found. The complexity of the differences in the fatty acid composition and the limited state of our knowledge of the composition of most soil organisms restricted the conclusions the could be drawn about the changes in community structure involved.

TECHNICAL ABSTRACT

Well documented, gas chromatography (gc)-based procedures were used to separate the fatty acids derived from the mixture of phospholipids extracted from cultured microorganisms (4 bacteria, 2 fungi and 1 actinomyces) and from 28 soil samples from four paired field sites (8 plots) under conventional tillage and no tillage treatments respectively. Twenty samples were taken from high elevations and 8 samples were taken from low elevations of a single pair. The fatty acid mixtures from the cultured microbes were relatively simple consisting to 3-10 major components which together accounted for 90+% of the total material. Differences between the organisms tested were readily observable. In contrast, the fatty acid mixtures from the soil samples consisted of numerous components at similar low concentrations such that the 20-30 largest components accounted for only 75% of the total material. Differences in these samples were not easily detected by visual comparison. The percent composition of the fatty acid mixture was calculated for each of the soil samples based on the area of the gc response for each component and the total area for all components. A subset of the data with components >0.5% in at least one sample was selected (64 components). The relative concentration of each of these components in the 28 samples was calculated and components which exhibited similar relative concentration among the samples, ie. a high correlation of changes in concentration with treatment, were grouped by hierarchical cluster analysis (6 clusters). Representative components were selected from each cluster and the percent composition data for these representative components in the 28 samples was tested by MANOVA to assess the significance of differences and by discriminant analysis to identify the components (clusters) most responsible and the samples which could be correctly classified based on the differences observed. Significant differences were

found between the four pairs of plots ($P<0.001$), the two tillage treatments ($P<0.001$) and the two elevations within the plots ($P<0.002$). Discriminant functions based on the representative components correctly classified all 28 samples among the four paired sites and between the two tillage treatments. The 16 samples taken at 2 elevations at 1 site also were all correctly classified. Components from all six clusters had large coefficients in the site discriminant function, but only four clusters were strongly represented in the tillage function. The representative components involved were: antiso-15:0, 16:1(9c), 16:0 and cyclo-19:0(9,10) usually associated with bacteria, 18:2(9,12) usually associated with fungi and an as yet unidentified fatty acid.

A ^{31}P NMR-based procedure was adapted to provide high quality spectra of the phospholipids extracted from microbes and soils. Line widths of 3 Hz or less could be obtained even for crude total lipid extracts. The chemical shifts of some phospholipid classes present were found to be both concentration and matrix dependent which made assignment of the signals somewhat difficult when total lipid extracts were analysed. Some of this uncertainty could be removed by separating the phospholipids present by column chromatography and diluting to similar concentrations before recording the spectra. A total phospholipid concentration of ca. 8.3 mg/mL was selected as providing a good compromise between an acceptable matrix effect and a reasonable data acquisition time (4-5 hr). The 25 mg quantity of phospholipids required could typically be obtained from 250-500 g of soil. The spectra of the phospholipids from the fungi and actinomyces examined (4 genera) differed both qualitatively and quantitatively suggesting that phospholipid class composition had potential as a measure of changes in microbial community structure. Lipids were extracted from 8 soil samples from the four paired field sites. ^{31}P NMR spectra of the phospholipids in the total lipid extracts were recorded. Differences were observed in the phospholipid class composition estimated from the spectra. Statistical analysis of the data was made difficult by the fact that two of the four major components were found to be highly correlated in the discriminant analysis by site. Only 6 of the 8 samples were classified correctly when three components were used in the analysis. Analysis based on the three major components and a minor component classified all 8 samples correctly with high probability. The major components were not correlated in the analysis by tillage and the resulting discriminant function classified all 8 samples correctly between the two tillage types with high probability. The treatment related differences observed in phospholipid class composition suggest that it has potential as a tool for detecting changes in soil microbial community structure.

SOMMAIRE

Les micro-organismes sont la forme de vie la plus abondante dans les sols. Ils comprennent un grand nombre d'organismes de différents types, un si grand nombre qu'il ne pourra probablement jamais être déterminé. La biomasse de chaque type d'organisme présent dans le sol - la structure de la communauté microbienne - est influencée par de nombreux facteurs, dont la teneur en substances nutritives et la source de ces substances, la température, le taux d'humidité, la quantité d'oxygène disponible et la présence d'autres types d'organismes.

Ces organismes influent à leur tour sur quantité d'aspects des activités para-agricoles; ainsi, ils jouent un rôle essentiel dans la croissance des végétaux en tant que fournisseurs de substances nutritives, ils agissent dans des processus comme la décomposition des matières organiques et la fixation de l'azote et ils causent des maladies des plantes. De plus, leur présence universelle et la sensibilité de la structure de leur communauté aux changements dans leur environnement peuvent en faire de bons indicateurs des changements dans la qualité des sols. Pour pouvoir utiliser les micro-organismes des sols comme indicateurs, il faut recourir à des méthodes simples, fiables et sensibles de mesure des variations de la structure de la communauté microbienne. Une de ces méthodes, en voie d'élaboration, est fondée sur la composition chimique des phospholipides présents dans la paroi cellulaire des micro-organismes. Les phospholipides sont un important élément de la paroi cellulaire et leur composition chimique varie selon les types d'organismes; de ce fait, ils permettent d'établir une relation entre la composition chimique et la structure des communautés.

Nous avons examiné deux différentes méthodes de mesure de la composition en phospholipides, de la composition en acides gras et de la composition par classe visant à déceler les changements produits par différentes pratiques de travail du sol dans quatre paires de parcelles. La composition en acides gras a été déterminée au moyen d'un chromatographe en phase gazeuse, et des différences ont été relevées entre les parcelles de chaque paire, entre les sols traités et non traités et entre les parcelles à grande et à faible élévation.

La composition par classe a été déterminée par spectroscopie RMN 31P, et des différences constantes ont été observées entre les sols soumis à différents traitements. La complexité des différences dans la composition des acides gras et le caractère limité des connaissances sur la composition de la plupart des organismes du sol limitent les conclusions que nous pouvons tirer quant aux changements dans la structure de la communauté.

RÉSUMÉ TECHNIQUE

Nous avons employé des méthodes de chromatographie en phase gazeuse bien documentées pour séparer les acides gras dérivés du mélange de phospholipides extraits de micro-organismes de culture (4 bactéries, 2 champignons et 1 actinomyces) et de 28 échantillons de sol provenant de quatre paires de parcelles respectivement soumises et non soumises à un travail du sol. Vingt échantillons ont été prélevés dans une parcelle à grande élévation et huit échantillons dans une parcelle à faible élévation d'une même paire. Les mélanges d'acides gras provenant des microbes de culture étaient relativement simples; ils comprenaient de 3 à 10 éléments majeurs qui représentaient au moins 90 % de l'ensemble des matières. Les différences entre les organismes analysés étaient facilement observables. En revanche, les mélanges d'acides gras contenus dans les échantillons de sol comprenaient de nombreux éléments présents à de faibles concentrations, de sorte que les 20 à 30 éléments les plus importants ne représentaient que 75 % de l'ensemble des matières. La comparaison visuelle ne permettait pas de déceler facilement les différences

dans ces échantillons. La composition en pourcentage du mélange d'acides gras a été calculée pour chacun des échantillons de sol d'après l'aire de réaction du chromatographe en phase gazeuse pour chaque élément et d'après l'aire totale pour l'ensemble des éléments. Nous avons sélectionné un sous-ensemble des données correspondant à une concentration des éléments (64 éléments) supérieure à 0,5 % dans chaque échantillon. Nous avons calculé la concentration relative de chacun de ces éléments dans les 28 échantillons, et groupé par classification hiérarchique (en 6 groupes) les éléments dont la concentration relative était semblable d'un échantillon à l'autre (c.-à-d. lorsqu'il existait une étroite corrélation dans les changements de concentration en fonction du traitement). Nous avons choisi des éléments représentatifs dans chaque groupe et vérifié les données sur la composition en pourcentage propres à ces éléments représentatifs dans les 28 échantillons par analyse de la variance à plusieurs variables pour évaluer la signification des différences et par analyse discriminante pour identifier les éléments (groupes) ayant la plus grande influence et les échantillons pouvant être classés correctement selon les différences observées. Des différences significatives ont été décelées entre les quatre paires de parcelles.

Une méthode de spectroscopie RMN 31P a été adaptée pour la production de spectres de grande qualité des phospholipides extraits des microbes et des sols. Des largeurs de ligne de 3 Hz tout au plus ont pu être obtenues et ce, même pour les extraits de lipides totaux bruts. Les variations dans la composition chimique de certaines classes de phospholipides en présence dépendaient de la concentration et de la matrice, de sorte qu'il était quelque peu difficile d'attribuer les signaux lorsque les extraits de lipides totaux étaient analysés. Nous avons pu réduire cette incertitude en séparant les phospholipides présents par chromatographie sur colonne et en diluant à des concentrations semblables avant d'enregistrer les spectres. Nous avons considéré qu'une concentration totale de phospholipides d'environ 8,3 mg/mL constituait un bon compromis entre un effet de matrice acceptable et un temps d'acquisition des données raisonnable (de 4 à 5 h).

En général, il était possible d'obtenir les 25 mg de phospholipides nécessaires à partir d'une quantité de sol allant de 250 à 500 g. Nous avons noté une différence qualitative et quantitative entre les spectres des phospholipides provenant des champignons et des actinomyces (4 genres) examinés, ce qui indiquait que la composition des classes de phospholipides pouvait servir d'indicateur des changements subis par la structure de la communauté microbienne. Des lipides ont été extraits de 8 échantillons de sol prélevés dans les quatre paires de parcelles. Nous avons enregistré 31 spectres RMN 31P des phospholipides dans les extraits de lipides totaux. L'estimation de la composition des classes de phospholipides en fonction des spectres a permis de déceler des différences dans cette composition. L'analyse discriminante par site ayant montré une étroite corrélation entre deux des quatre éléments majeurs, l'analyse statistique des données a été rendue difficile.

Seulement six des huit échantillons étaient classés correctement quand l'analyse portait sur trois éléments, tandis que les huit échantillons étaient classés correctement avec une forte probabilité quand l'analyse portait sur les trois éléments majeurs et un élément mineur. Il n'existait pas de corrélation entre les éléments majeurs selon l'analyse en fonction du travail du sol, et la fonction discriminante qui en a résulté assurait un classement correct avec forte probabilité des huit échantillons entre les deux types de traitement du sol. Les différences liées au traitement du sol observées dans la composition des classes de phospholipides portent à croire que l'analyse des signatures des phospholipides pourrait permettre de déceler les changements dans la structure de la communauté microbienne des sols.

OBJECTIVES

1. To determine the feasibility of using phospholipid fatty acid composition as a means of detecting changes in the soil microbial community structure produced by common agronomic practices.

2. To determine the feasibility of obtaining ^{31}P NMR spectra of the phospholipids in extracts of soil and examine the usefulness of the information on phospholipid class composition that can be derived from the spectra for detecting changes in the soil microbial community structure.

METHODOLOGY

Microbial Culture

The bacteria (*Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*) were grown from stock cultures (Dr. C.M.Tu, AAFC, PMRC, London). All, except the *P. aeruginosa*, were grown in nutrient broth (5 g peptone, 3 g beef extract, 15 g agar per L) at room temperature. The *P. aeruginosa* was grown in tryptic soy broth (15 g Bacto-tryptone, 5 g Bacto-soy, 5 g sodium chloride, 15 g agar per L). The fungi and actinomyces were isolated from one of the soil samples (*Trichoderma* sp., *Paecilomyces* sp.) or were from stock cultures (*Rhizopus stolonifer*, *Streptomyces griseus*) (Dr.J.Traquair, AAFC, PMRC, London). They were grown at room temperature in yeast malt glucose broth (4 g yeast, 10 g malt, 4 g glucose per L) except for the *Trichoderma* sp. which was grown without glucose. For fatty acid analysis by gc, the cells were grown to a density of 1011-1012 cells/mL, harvested by filtration and the filter and microbes used for extraction. To produce the larger amounts of material required for ^{31}P NMR analysis, the fungi and actinomyces were grown in Roux bottles to the point where the mycelium mat covered the surface of the broth, or for *Paecilomyces* sp.and *Streptomyces griseus*, until spores just started to form. Except for *S. griseus*, the mycelia were harvested by filtration using a Buchner funnel, glass fibre filter and vacuum. After separation from the filter the material collected was immersed in methanol. *S. griseus* mycelia were separated from the broth by centrifugation and the pellet was immersed in methanol.

Soil Sources

Soils were sampled in the fall of 1995 at 8 locations (4 paired till (T) and no-till (N) sites) of the Green Plan experiment of Dr. Alan Tomlin, AAFC, PMRC, London. At one pair of sites (T-1, N-1) with appreciable slope, four cores (5 cm diam. x 5 cm deep) were taken at both high and low elevations on the plots. At the other three sites, two cores were taken at a single elevation. This provided 28 samples for phospholipid analysis.

Lipid Extraction

When destined for gc analysis, lipids were extracted from soils and filtered microbes by tumbling the sample (100 g soil) with 100 mL of 7:3 methanol-chloroform for 1 hr. Extracts of soil for NMR analysis were obtained from 250 or 500 g of soil using proportionately larger volumes of solvent. The solids present were separated from the extract by filtration (Buchner funnel, Whatman No. 1 paper and suction). Extracts of microbial mycelia for NMR were obtained by mascerating (Polytron) the harvested material sequentially with methanol, 7:3 methanol-chloroform and chloroform. After each masceration, the extract was separated by filtration and combined with any previous extract.

The extract was evaporated to a small volume on a rotary evaporator (40 C, water aspirator vacuum) and the residual water was removed by repeatedly adding 10 mL of methanol and continuing the evaporation until no water remained.

For NMR analysis involving the total lipid extract, the residue was extracted with 2 x 5 mL of chloroform. The chloroform extract was transferred to screw cap test tubes and the solvent was evaporated at 40 C under a flow of nitrogen.

Phospholipid Separation

To provide a phospholipid fraction for gc analysis or when desired for NMR analysis, the phospholipids were separated from the glyco- and neutral lipids present in the lipid extracts by column chromatography on silica essentially as described by Zelles and Bai (1993). The procedure was modified slightly to incorporate rinsing of the residue not initially transferred to the chromatography column with chloroform using a small volume of acetone and subsequently using a small volume of methanol and transfer of these rinses to the chromatograph column prior to its elution with each of these solvents. For the larger quantities of lipids from 250 or 500 g of soil or from the mycelia, three to five chromatography columns were used. The methanol was evaporated from the phospholipid fractions at 40 C under a flow on nitrogen.

Transesterification of the Phospholipids

To provide the fatty acid methyl esters required for gc analysis, the phospholipid fraction was transesterified as described by Zelles and Bai (1993).

Gas Chromatographic Analysis of Fatty Acid Methyl Esters

The mixture of fatty acid methyl esters derived from the phospholipids was analysed by gas chromatography using the column and temperature program described by Zelles and Bai (1993), except that detection was by FID and the maximum temperature of 280 C was maintained for 30 min to permit elution of all components. Duplicate injections were made for all samples. A qualitative standard containing 28 fatty acid methyl esters was analysed periodically with a series of samples as a check of instrument and column performance. Peak areas and retention times were determined electronically. Components were "identified" with a component number based on their retention times. Consecutive component numbers 1 to 1000 were assigned to each successive 0.075 min interval of the chromatogram

over the 75 min containing the majority of the components eluted. This spacing matched the resolution of the capillary column and successfully identified all significant components (>0.125%) in the samples analysed.

^{31}P NMR Analysis

The procedure used to prepare the samples and obtain the NMR spectra was basically that of Glonek (1994). Tetraphenylphosphonium bromide (ca. 1 mg) was added to the total lipid or phospholipid material to be analysed to provide an internal chemical shift standard. The mixture was dissolved in deuterochloroform (for the phospholipid fraction the volume of deuterochloroform was adjusted to produce a concentration of 8.3 mg/mL) and 3 mL was transferred to a standard 10 mm OD NMR tube. The cesium hydroxide/EDTA reagent was added and the mixture was shaken and then allowed to separate into an upper water-rich and a lower deuterochloroform-rich layer. Phosphorus-31 NMR spectra at 121.42 MHz were recorded for the lower deuterochloroform-rich layer (without removing the upper layer) at ambient temperature (ca. 293 K) using a Varian XL-300 spectrometer system in the Dept. of Chemistry, University of Western Ontario (Dr.P.A.W.Dean). The chemical shift of the internal reference was measured relative to that of the standard reference, 85% phosphoric acid, by sample interchange. Typically the spectrum of a sample containing 25 mg of phospholipids could be measured in 10-15 x 10^3 transients, with a pulse width of 8.6 microseconds, a tip angle of 45 degrees and an acquisition and recycle time of 1.28 seconds. Using these conditions, each spectrum required about 4.5 hr of instrument time. The composition was calculated using the area of the signal assignable to each phospholipid class or mixture of classes in situations in which signals were not totally resolved.

Data Analysis

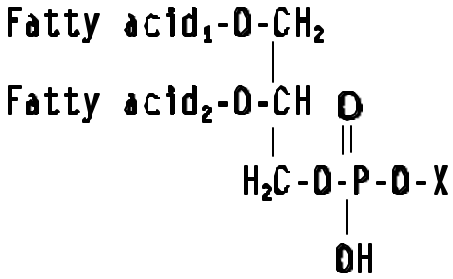
Average fatty acid compositions were calculated for each of the 28 samples using the data from the duplicate gc analyses. A reduced data set of components present at >0.5% in at least one of the samples was selected. It contained 64 components. The relative concentrations of each of these components in the 28 samples was calculated (% of component "n" in sample "k" divided by total of % of component "n" in samples 1-28). The relative concentration data was submitted to hierarchical cluster analysis which yielded six groups of components having similar relative concentrations in all 28 samples. Ten representative components (component no. 256, 260, 309, 317, 336, 392, 394, 397, 442 and 523) were selected from the six groups by choosing the major component of each group and the two next largest components for the two groups with larger numbers of components. The average fatty acid composition data for these representative components was analysed by MANOVA and discriminant analysis.

RESULTS AND DISCUSSION

Introduction

An absolute measure of soil quality is difficult to define. The difficulty is due to the large number of factors and the complex interactions among them that affect what are generally considered as desirable properties of soil. One important factor is the biomass and its activity. This biomass is predominantly microorganisms of literally countless families, genera and species. One aspect of defining soil quality requires that we understand the differences that exist in this microbial community between "good" and "poor" soils. This information would provide criteria for assessing the effects of other factors as we strive to maintain soil quality. The distribution of the biomass among the various families, genera and species, the microbial community structure, is one way in which "good" and "poor" soils may differ. We currently do not know what constitutes a desirable community structure. In fact, the microbial community structure in soil is somewhat difficult to determine. Traditional plate counting methods are of limited value because, a) it is difficult to find a medium on which all of the diverse types of microbes present will grow and grow equally well and b) plate counts of multicellular organisms are difficult to relate to their actual biomass in the soil. More recently developed methodologies involving direct counting using microscopy/image analysis or chemical analysis of suitable signature chemicals overcome some of these difficulties.

A number of signature chemicals or "biomarkers" have been used to study microbial community structure. These include: ergosterol, muramic acid, diaminopemelic acid, glucosamine, teichoic acid components (glycerol and ribitol) and the fatty acids derived from total lipid, lipopolysaccharides or phospholipids (Alef and Nannipieri, 1995). Of these, the fatty acids from phospholipids have the broadest application because of the universal presence of phospholipids in the cell walls of all organisms. In clinical methodology, the fatty acids derived from all the lipids of a cultured organism are routinely used to identify the organism. Total lipid fatty acids have also been used to study soil microbial community structure (Haack et.al., 1995, Cavigelli et.al., 1995). Workers favouring the use of phospholipids argue that the phospholipids degrade very quickly after cell death while other types of lipids my survive of longer periods. Because of this the phospholipids are presumed to give a more accurate "snapshot" of the system at the time of extraction than the total lipids do. Fatty acid analyses based on total lipid in soil are generally much simpler to do, but a comparative study of the use of phospholipids and total lipids has not been done. In the absence of such a study, we decided it would be best to examine the phospholipids.



Chemically, phospholipids are a complex mixture of compounds formed from a variety of long carbon chain carboxylic acids (related to acetic acid) called fatty acids, glycerol, phosphoric acid and a fourth group of chemicals (X in diagram) of more diverse structure, seven of which appear frequently (X= hydrogen, choline, inositol, ethanolamine, serine, phosphatidylglycerol and glycerol). Phosphoslipids are named by adding the term "phosphatidyl" before the name of the X group, eg. phosphatidylcholine (except X= hydrogen, phosphatidic acid and X= phosphatidylglycerol, diphosphatidylglycerol). Variations exist in which a) fatty acid 1 is absent, denoted by prefixing the term "lyso" to the name and b) fatty acid 1 is replaced by a long carbon chain aldehyde denoted by the term "plasmalogen" following the name. The seven basic structures along with the two variations described constitute the 21 common phospholipid "classes". Each class is itself a complex mixture due to the large number of different long chain fatty acids (100's) that can be utilized as fatty acid 1 or 2 and the resulting extremely large number of combinations that are possible as the two fatty acids are frequently different.

To date, studies using phospholipids as signature chemicals have necessarily included a step in which the phospholipids present are degraded to yield a mixture of fatty acids. The composition of this mixture is then determined by gas chromatography using well established procedures. Because of their physiological importance, there is currently a great interest in quantitatively analysing the phospholipids themselves to determine their class composition. Their physical properties preclude using gas chromatography, and liquid chromatography techniques used to date have had limited success because of the wide range of polarity of the materials and the lack of a suitable, relatively inexpensive method of detection. There have been several reports of the use of ^{31}P NMR spectroscopy to determine the class composition of phospholipids isolated from plant and animal sources (London and Feigenson 1979, Marinier et.al. 1988, Meneses and Glonek 1988, Metz and Dunphy 1996) using special techniques to reduce the line broadening effect of the transition metal ions present. This assay is based on the fact that each of the phospholipid classes produces a unique resonance signal. We were interested in determining if these techniques could be used or modified so that analytically useful spectra could be obtained on the phospholipids extracted from a medium of very high metal ion content such as soil. If useful spectra could be obtained, we proposed to compare the value of class composition with the more traditional fatty acid composition as a means of detecting changes in soil microbial community structure.

This report describes the results of our efforts to detect changes in the soil microbial community structure resulting from different tillage treatments using gas chromatography-based analysis of phospholipid-derived fatty acids and 31-P NMR analysis of phospholipid classes.

GC-Based Phospholipid-Derived Fatty Acid Analysis.

It is well known that many species of microbes produce lipids that have a fatty acid composition unique to that species when grown under carefully controlled conditions (Lechevalier 1977, O'Leary 1975, Shaw 1977). Differences in composition between genera and species are often easily detected simply be visual inspection of the output from the gas chromatograph such as is shown in Figures 1 and 2 for a number of cultured microbes.

Although very limited in scope, these chromatograms illustrate some of the general differences in composition observed between the lipids from bacteria and fungi. Bacterial lipids usually contain the otherwise unusual branched chain fatty acids (analysed as methyl 13-methyltetradecanoate (i-15:0, 256), methyl 12-methyltetradecanoate (a-15:0, 260), methyl 15-methylhexadecanoate (i-17:0, 345) and methyl

14-methylhexadecanoate (a-17:0, 349)) and/or cyclopropyl fatty acids (analysed as methyl 9,10-methylenehexadecanoate (cyclo-17:0(9,10), 356) and methyl 9,10-methyleneoctadecanoate (cyclo-19:0(9,10), 442)) which are not common in fungi. Fungal lipids usually contain more highly unsaturated fatty acids (analysed as methyl 9,12-octadecadienoate (18:2(9,12), 392), methyl 6,9,12-octadecatrienoate (18:3(6,9,12), unnumbered component at ca. 37 min in *R. stolonifer* and *S. griseus*) and methyl 9,12,15-octadecatrienoate (18:3(9,12,15), coelutes with methyl cis-9-octadecenoate (18:1(9,cis), 394)) which are not common in bacteria. The *R. stolonifer*- and *Trichodema*-derived methyl esters examined in this study also contained no methyl trans-9-octadecenoate (18:1(9,trans), 397) or other coeluting 18:1 isomers which were present in all the bacteria.

Differences in the fatty acid composition of the phospholipids extracted from the mixed microbial populations present in soils are often not as obvious as can be seen in Figure 3 which shows part of the gas chromatograph output from the analysis of the till and no-till plots from one site in the Green Plan experiment. The presence of the methyl esters of the branched chain fatty acids (212, 256, 260, 345, 349), cyclopropyl fatty acids (356, 442) and the diunsatureated fatty acid 18:2(9,12) (392) indicates that (not unexpectedly) the soil contained both bacteria and fungi. The differences in the relative amounts of the components between the till and no-till soils suggests a change in the community structure involving the relative numbers of bacteria, as well as, the ratio of bacteria to fungi (more 18:2(9,12) (392) in no-till).

To evaluate the strength of the relationship of the fatty acid composition of the 28 samples analysed to the variables (site, tillage or elevation) in the experiment, hierarchical cluster analysis was carried out on a subset of the percent composition data for the samples which included only the 64 components present at >0.5% in at least one of the samples. The result of the cluster analysis based on Euclidean distance and Ward linkage is shown in Figure 4. Other distance and linkage methods gave similar results. Composition was most strongly affected by site as differences in composition between sites was sufficiently large to permit differentiation between the sixteen samples from site 1, the four samples from site 2 and the eight samples from sites 3 and 4. Sites 3 and 4 were not differentiated in this analysis. No indication of a relationship between composition and tillage or elevation was observed.

To further examine the differences at each site, an average composition was calculated for the four sites using data from four samples (2 T, 2 N) for each site. Differences (average - site "n") are shown in Figures 5 - 8 and are in the order site 2 > site 4 > site 3 > site 1. The distribution of the differences among the components was different for each site with the branched chain fatty acids (256, 260, 345 and 349) and cyclopropyl fatty acids (356, 442) usually associated with bacteria and polyunsaturated fatty acids (392, 394) usually associated with fungi being some of the more important. The monounsaturated fatty acids (analysed as methyl cis-9-hexadecenoate (16:1(9,cis), 309) and methyl trans-9-octadecenoate (18:1(9,trans) or other coeluting isomers, 397) were also important contributors to the differences at some sites. Site 1 contained below average (+ difference) numbers of i-15:0 and cyclo-19:0(9.10) producing bacteria and 18:2(9,12) producing fungi and above average (- difference) numbers of i-14:0, a-15:0 and a-17:0 producing bacteria. At site 2 the situation was the reverse of site 1. Site 3 contained below average numbers of i-15:0, i-16:0, cyclo-17:0(9,10) and cyclo-19:0(9,10) producing bacteria and above average numbers of a-15:0 producing bacteria and 18:2(9,12) producing fungi. Site 4 contained below average numbers of cyclo-19:0(9,10) producing bacteria and 18:2(9,12) producing fungi.

To determine if differences due to tillage could be detected, an average no-till composition was

calculated (14 samples) and its difference from the average overall composition (28 samples) (overall average - no-till average) was determined. It is shown in Figure 9. The size of the maximum differences were about 1/3 of those between sites. The differences suggest that below average numbers of i-15:0, a-15:0, i-16:0, i-17:0, a-17:0, cyclo-17:0(9,10) and cyclo-19:0(9,10) producing bacteria and above average numbers of i-14:0 producing bacteria and 18:2(9,12) producing fungi were associated with the no-till treatments.

Likewise, to determine the presence of differences due to elevation the average high elevation composition of site 1 was calculated (8 samples) and its difference from the average composition of site 1 (16 samples) (site 1 average - high site 1 average) was determined. It is shown in Figure 10. Maximum differences were similar in magnitude to those between tillage treatments. The differences suggest that below average numbers of i-14:0, i-15:0 and cyclo-19:0(9,10) producing bacteria and 18:2(9,12) producing fungi and above average numbers of a-15:0, i-16:0, a-17:0 and cyclo-17:0(9,10) producing bacteria were associated with the higher elevation.

To determine if there was any statistical significance associated with these relatively small differences the data was tested by MANOVA. To carry out this analysis it was first necessary to systematically identify and remove variables (fatty acid components) that were highly correlated and reduce the number of variables to the 22 or less acceptable for analysis of a data set with five degrees of freedom associated with the various treatments and only 28 observations. This was accomplished by calculating the relative concentration of each of the 64 components in the 28 samples (% of component "n" in sample "k" divided by the total of % of component "n" in samples 1 to 28). Components with similar relative concentration patterns across the treatments represented in the 28 samples were likely to be associated with one or more organisms whose numbers or activity also varied with the treatments. To group these components, the relative concentration data was submitted to hierarchical cluster analysis (Euclidean distance, Ward linkage). The result is shown in Figure 11. The major components (those >1% and marked * in Figure 11) were distributed unevenly among six clusters. The degree of similarity (based on their relative concentrations) of the components in two of the clusters is shown in Figures 12 and 13. Admittedly, the similarity of some of the components was not as great as others, but cluster analysis was the only method available by which we could group the components in an unbiased way. A better method of pattern recognition would be an asset in grouping components with highly correlated behaviour. The major component in each of the clusters was selected along with two additional large components from the two larger clusters (all marked S in Figure 11) to provide a group of 10 representative components. The results of the MANOVA of the composition data for these 10 components is included in Appendix 1. The results showed that significant differences in fatty acid composition existed between the sites, the tillage treatments and the elevations.

To identify the representative components (clusters) most responsible for the significance of the differences and determine the usefulness of differences of the magnitude observed for classification of the samples the data was submitted to discriminant analysis. The results are included in Appendix 1. All 28 samples were classified correctly according to site with a high degree of certainty as can be seen from the Summary of Classification and Summary of Classified Observation tables. Each of the clusters of components was strongly represented in the coefficients of the discriminant function (cluster 1, 260; cluster 2, 317; cluster 3, 309; cluster 4, 392; cluster 5, 442 and cluster 6, 523) indicating that differences

between the sites was widely distributed over the components of the microbial community represented by the groups. It is reassuring to see that sites that were paired using various criteria at the beginning of the experiment remain paired as one site based on the phospholipid composition suggesting a positive correlation between the factors represented in these criteria and microbial community present. All 28 samples were classified correctly according to tillage with a high degree of certainty for all but two samples (19 and 22). Only three clusters were strongly represented in the discriminant function (cluster 1, 260; cluster 2, 317 and cluster 5, 442). The relatively weak contribution of component 392 (18:2(9,12), characteristic of fungi) to the discriminant function suggests the major difference in the microbial community structure caused by different tillage practices is in the relative numbers of bacterial species rather than the relative numbers of bacteria and fungi. Because of the large site related differences only the 16 samples taken from site 1 (the only site where samples were taken at low elevations) were included in the discriminant analysis for elevation. All samples were classified with a high degree of certainty. Components representing cluster 1 (309) and cluster 2 (336) were the strongest contributors to the discriminant function.

^{31}P NMR-Based Phospholipid Class Analysis.

The reliable data available on the phospholipid class composition of microbes is very limited. This has been due to the lack of a simple, quantitative method for the analysis of the phospholipid classes. The ^{31}P NMR-based method recently developed by Glonek (1994) for phospholipid class analysis of plant and animal tissue extracts provides a potential solution to the analytical problem. To assess the usefulness of this methodology for examining soil microbial community structure we first determined the spectra of phospholipids extracted from the mycelia of *Rhizopus stolonifer*, *Trichoderma* sp., *Paecilomyces* sp. and *Streptomyces griseus*. The spectra are shown in Figures 14 - 17, respectively. The known chemical shift values for various phospholipid classes are listed in Table 2. The spectra of *R. stolonifer* and the *Trichoderma* sp. are most similar. The large signal at a chemical shift of 0.045 ppm is due to phosphatidylcholine (PC) and the second largest signal at 1.05-1.07 ppm is due to diphosphatidylglycerol (DPG). Signals at 0.486, 0.833 and 0.930 in the *R. stolonifer* spectrum correspond most closely to phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE) respectively. In the *Trichoderma* sp. spectrum there are signals at 0.543 and 0.896 ppm which probably are PI and PS also. Changes such as this in the chemical shifts of the signals were one of the problems encountered. The phospholipid resonance signals appear in a very narrow frequency range and their chemical shifts are quite sensitive to concentration changes and the presence of other components in the sample. The signals in question could be positively identified by spiking the sample with a small amount of known phospholipid and redetermining the spectrum. Due to limited resources this was not done. The phospholipids from *Paecilomyces* sp.also contains PC as the major component, but signals at 0.606, 1.014 and 1.108 ppm are different from those present in *R. stolonifer* and the *Trichoderma* sp. The 0.606 ppm signal is likely lysophosphatidylcholine (LPC), but the others are not readily assignable using the known chemical shifts listed in Table 2. Phosphatidyl choline was only a minor component of *Streptomyces griseus* whose phospholipid class composition was obviously very different from the other three microbes. These spectra show that there is considerable diversity in the phospholipid class

composition of these randomly selected microbial species and therefore this type of analysis has potential as a measure of soil microbial community structure.

After modifying the original technique slightly to compensate for the higher metal ion content, useful spectra of the phospholipids present could be obtained routinely using total lipid extracts of soil. The spectra obtained using these extracts from the high elevations of the till and no-till plots at the four sites are shown in Figures 18 - 25. The slightly wider line widths and higher noise levels in these spectra compared to those shown in Figures 14 - 17 are due to the other components in the total lipid extract and lower concentrations of the phospholipids respectively. Although the chemical shifts of the signals varied somewhat among the samples because of concentration and matrix effects, the spectra had a number of easily recognizable common features and some differences.

As it was not economically feasible to positively identify each of the signals in these spectra by spiking the samples and some of the signals were not sufficiently well resolved to permit accurate quantitation, signals appearing in one of twelve limited chemical shift ranges were assumed to be due to the same phospholipid class or mixture of phospholipid classes. The chemical shift ranges selected, the average chemical shift for obvious components and the tentative structural assignment were as follows: 0.039 - 0.130, avg. 0.053 and 0.110, PC + PC plasmalogen; 0.446 - 0.525, avg. 0.480, PI; 0.597 - 0.670, avg. 0.610, LPC; 0.689 - 0.810, avg. 0.710 and 0.810, LPC plasmalogen + unknown; 0.939 - 1.076, avg. 0.98, 1.04 and 1.07, PS + PE + PE plasmalogen); 1.080 - 1.20, avg. 1.11 and 1.20, DPG + PA; 1.31 - 1.36, avg. 1.34, LPE; 1.386 - 1.529, avg. 1.43, PG; 1.48 - 1.64, avg. 1.53, DiLDPG; 1.742 - 1.809, avg. 1.76, LPA and 2.06, avg. 2.06, LPG. The chemical shifts of the signals assigned to PS (0.98) and to PE (1.04) differ most from those listed in Table 2. The signals of these two components were the most sensitive to matrix and concentration changes and these assignments are based on the result of spiking one sample with these materials. The composition of the samples based on these twelve identifiable regions was calculated from the area of each region and the sum of the area of all twelve regions. The results are summarized in Figure 26. The small number of observations limited the statistical treatment of the data. Attempted discriminant analysis according to site, using the four major "components" (PC + PC plas, PS + PE, DPG + PA and PG) revealed that PC + PC plas and PG were highly correlated in this analysis. Replacing PG with one of the other uncorrelated components allowed successful analysis. The resulting discriminant function correctly classified the eight samples among the four sites but it was strongly dependent on the relatively minor replacement component making its value questionable. Analysis using only three major components (PC + PC plas, PS + PE and DPG + PA) correctly classified 7 of the 8 samples according to site but only one with high probability. Analysis by tillage using the four major components provided a discriminant function that correctly classified the 8 samples between the two tillage types with high certainty. Using only the three major components used for analysis by site, weakened the tillage discriminant function to the point where only 6 of the 8 samples were classified correctly and with greatly reduced probability. It is not clear if the analysis is weakened by too few variables or by the absence of PG. These analyses are included in Appendix 2.

The phospholipid class composition contains information that is useful in classifying samples and has potential to help in determining microbial community structure. A larger data set would be desirable to more fully evaluate its potential. The correlation between PC + PC plas and PG is of interest in light of the fact that PG was not a component of the fungi and actinomyces examined. There is a possibility that PG is

produced mainly by bacteria and that PC:PG ratios could provide a measure of fungi:bacteria ratios.

Clearly, we have succeeded in demonstrating that small differences in phospholipid fatty acid composition or phospholipid class composition arise in soils at different sites or when the soils are subjected to different tillage practices and that these differences are consistent enough to permit classification of samples taken from these soils with a high degree of certainty. Our ultimate goal of relating these differences to differences in the microbial community structure has proven somewhat more elusive. Chemical biomarkers are used to overcome the selectivity that is associated with an analysis of community structure based on techniques which include only culturable microorganisms, BUT are then themselves limited by the fact that little is known about the phospholipid composition of these "non culturable" organisms. Conclusions must be reached by extrapolating from known organism/composition relationships. It would appear that the errors which could arise from making assumptions about the "non culturable" organisms based on the phospholipid fatty acid composition of a few cultured organisms could be as large as those associated with the techniques which chemical biomarkers are designed to replace. This is not to say that studies using chemical biomarkers are not worthwhile, but only to say that extreme caution should be used in interpreting the results.

SUMMARY

Maximum differences of ca. 3% from the average content were observed for a few of the many fatty acids derived from the phospholipids extracted from soils from the four sites. Maximum differences from the average content for some of the fatty acids in the till and no-till treatments at the four sites and high and low elevations at one site were ca. 0.5%.

Although small, these differences were sufficiently consistent to establish that the fatty acid composition of the microbial phospholipids at some of the sites was significantly different ($P<0.001$) and that significant differences in the microbial phospholipid fatty acid composition also existed between tillage treatments ($P<0.001$) and between the elevations at one site ($P<0.002$).

Subsequent discriminant analysis revealed that these differences were sufficiently large and consistent to classify all 28 samples correctly with regard to site and tillage and a subset of 16 samples taken at one site with regard to elevation.

Based on general differences in the fatty acid composition known to exist between culturable bacteria and fungi, the differences observed suggest that differences in the bacterial community structure , as well as, in the relative numbers of bacteria and fungi exist at the different sites and elevations and are associated with the different tillage treatments.

^{31}P NMR spectra of the phospholipids in the total lipid extract of the soils, that could be used to determine the phospholipid class composition of the extracts, were found to be routinely obtainable.

Differences in phospholipid class composition observed for 8 soil samples (1 T and 1 N from each site) were sufficiently large and consistent to classify the samples correctly with regard to site and tillage suggesting that this type of analysis also has potential for determining changes in the soil microbial community structure.

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Table 1. Chemical names, common abbreviations and component numbers used for some typical fatty acid methyl esters derived from microbial phospholipids.

Chemical Name	Abbreviation	Component No.
Methyl undecanoate	11:0	110
Methyl 2-hydroxydecanoate	2-OH 10:0	115
Methyl dodecanoate	12:0	145
Methyl tridecanoate	13:0	185
Methyl 2-hydroxydodecanoate	2-OH 12:0	193
Methyl 3-hydroxydodecanoate	3-OH 12:0	205
Methyl 12-methyltridecanoate	i-14:0	212
Methyl tetradecanoate	14:0	228
Methyl 13-methyltetradecanoate	i-15:0	256
Methyl 12-methyltetradecanoate	a-15:0	260
Methyl pentadecanoate	15:0	272
Methyl 2-hydroxytetradecanoate	2-OH 14:0	281
Methyl 3-hydroxytetradecanoate	3-OH 14:0	295
Methyl 14-methylpentadecanoate	i-16:0	300
Methyl cis-9-hexadecenoate	16:1(9,cis)	309
Methyl hexadecanoate	16:0	317
Methyl 15-methylhexadecanoate	i-17:0	345
Methyl 14-methylhexadecanoate	a-17:0	349
Methyl 9,10-methylenehexadecanoate	cyclo-17:0(9,10)	356
Methyl heptadecanoate	17:0	361
Methyl 2-hydroxyhexadecanoate	2-OH 16:0	371
Methyl 9,12-octadecadienoate	18:2(9,12)	392
Methyl cis-9-octadecenoate	18:1(9,cis)	394
Methyl trans-9-octadecenoate	18:1(9,trans)	397
Methyl octadecanoate	18:0	404
Methyl 9,10-methyleneoctadecanoate	cyclo-19:0(9,10)	442
Methyl nonadecanoate	19:0	446
Methyl eicosonate	20:0	487
Methyl heneicosonate	21:0	527
Methyl 2-hydroxydocosanoate	2-OH 22:0	645

Table 2. Chemical names, common abbreviations and chemical shifts of 31-P signals for some common phospholipids.

Chemical Name	Abbreviation	Chemical Shift (ppm)
Phosphatidylcholine	PC	0.05
Phosphatidylcholine plasmalogen	PCplas	0.12
Phosphatidylinositol bisphosphate	DiPPI	0.35*
Phosphatidylinositol monophosphate	PPI	0.51**
Phosphatidylinositol	PI	0.52
Lysophosphatidylcholine	LPC	0.61
Lysophosphatidylcholine plasmalogen	LPCplas	0.69
Dimethylphosphatidylethanolamine	DiMEPE	0.71
Phosphatidylserine	PS	0.82
Lysophosphatidylinositol	LPI	0.89
Phosphatidylethanolamine	PE	0.92
Phosphatidylethanolamine plasmalogen	Peplas	0.96
Monolysodiphosphatidylglycerol	MonoLDPG	1.03***
Diphosphatidylglycerol	DPG	1.07
Phosphatidic acid	PA	1.17
Lysophosphatidylethanolamine	LPE	1.36
Phosphatidylglycerol	PG	1.41
Lysophosphatidylethanolamine plasmalogen	LPEplas	1.42
Phosphatidylglycerol plasmalogen	PGplas	1.44
Dilysodiphosphatidylglycerol	DiLDPG	1.58
Monolysodiphosphatidylglycerol		1.63***
Lysophosphatidic acid	LPA	1.80
Phosphatidylinositol bisphosphate		1.98*
Lysophosphatidylglycerol	LPG	1.99
Phosphatidylinositol monophosphate		2.77**
Phosphatidylinositol bisphosphate		2.87*

Note: *, ** and *** indicate multiple responses due to the presence of more than one phosphorus atom in the same molecule.

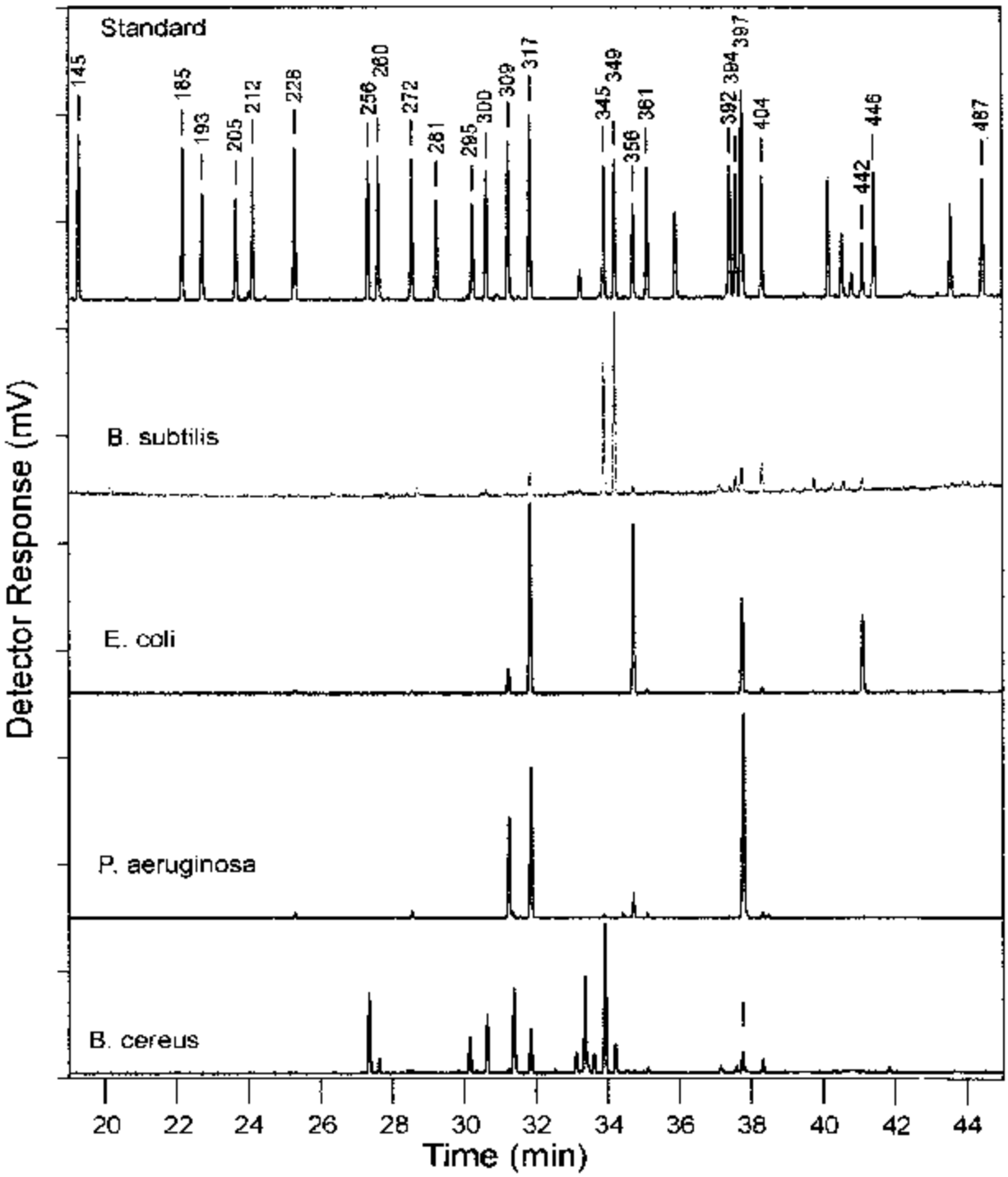


Figure 1. Capillary gc analysis of methyl esters of fatty acids derived from the phospholipids extracted from cultured bacteria.

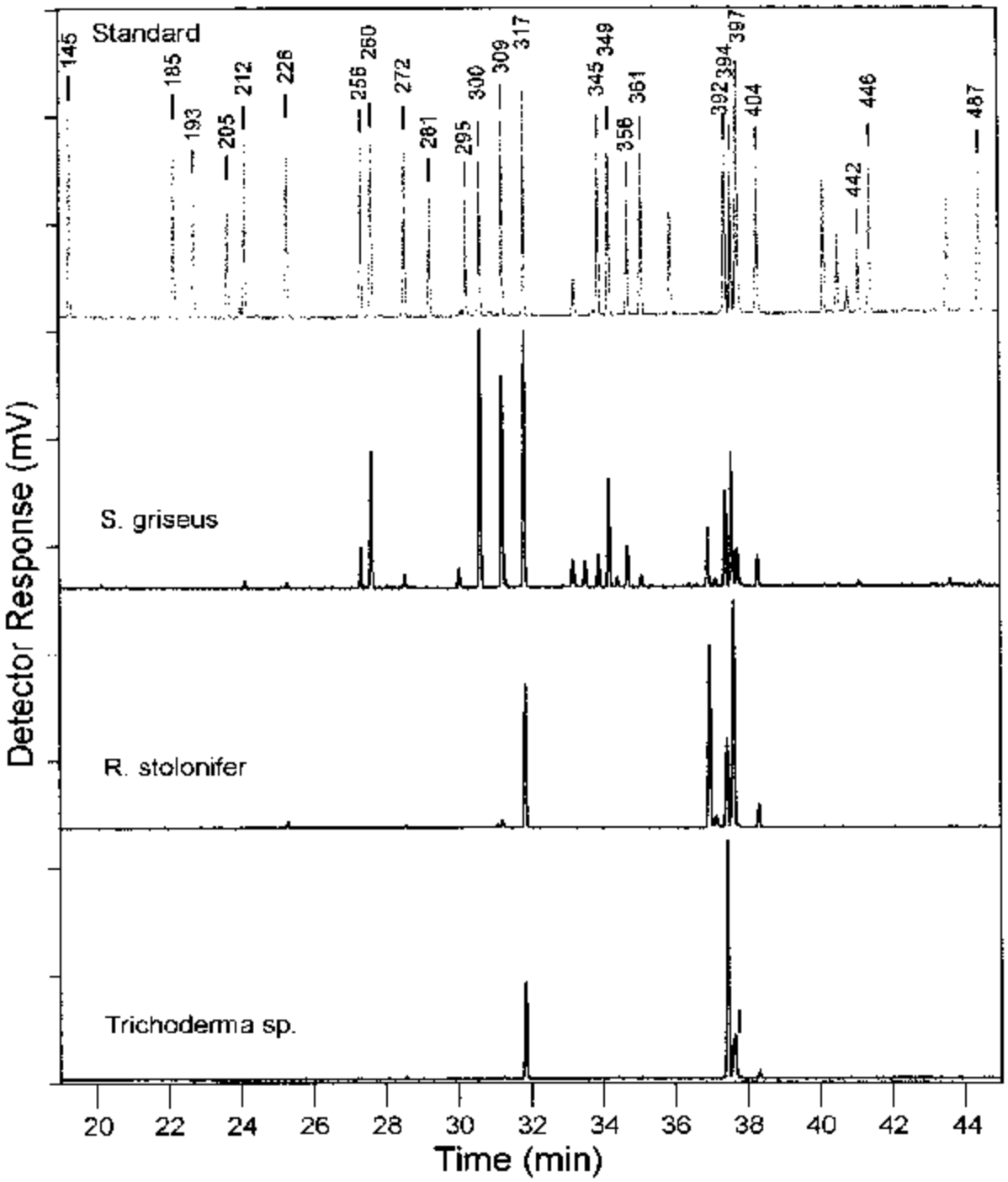


Figure 2. Capillary gc analysis of methyl esters of fatty acids derived from the phospholipids extracted from cultured actinomyces and fungi.

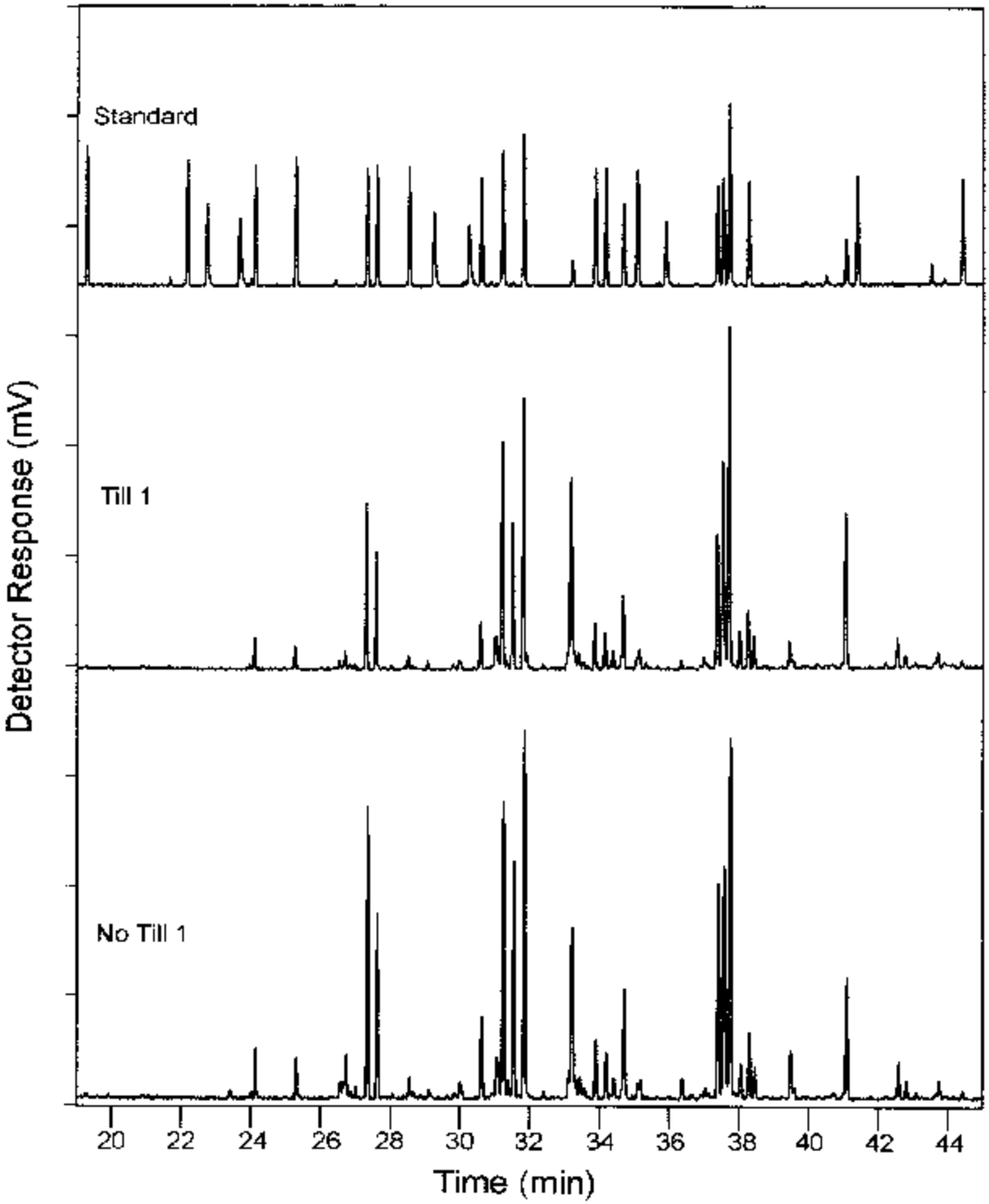
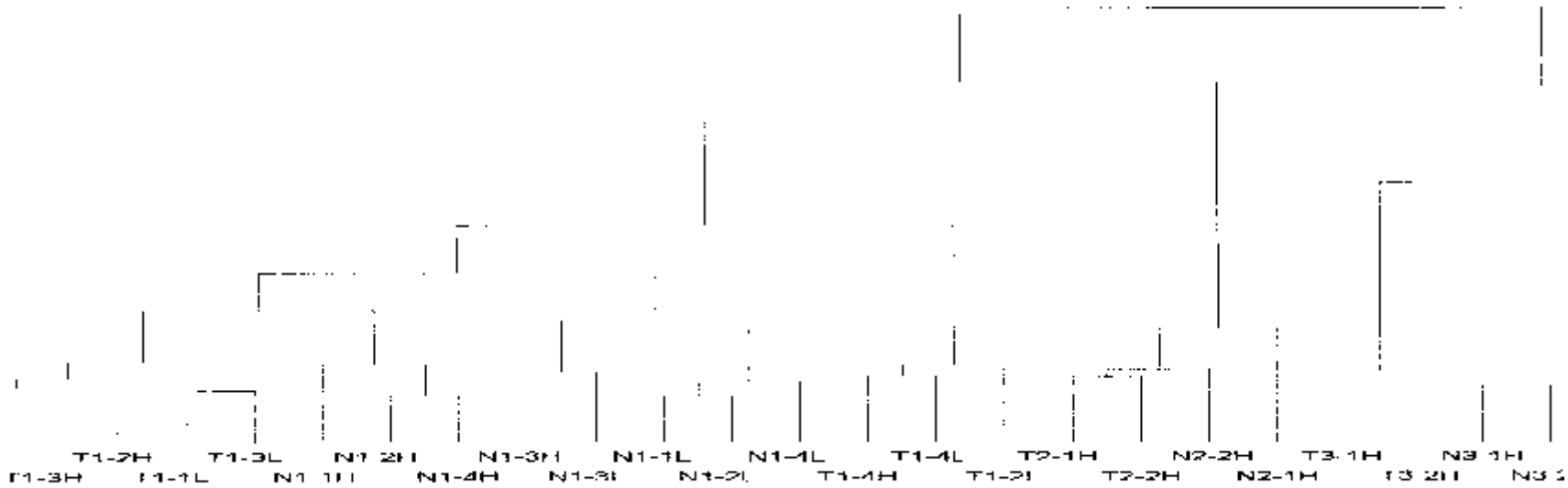


Figure 3. Capillary gc analysis of methyl esters of fatty acids derived from the phospholipids extracted from the till and no-till fields plots at site 1.



rarchical cluster analysis (Euclidean distance, Ward linkage) of fatty acid composition data for the 28 s
(H) and low (L) elevations from till (T) and no-till (N) plots at 4 sites.

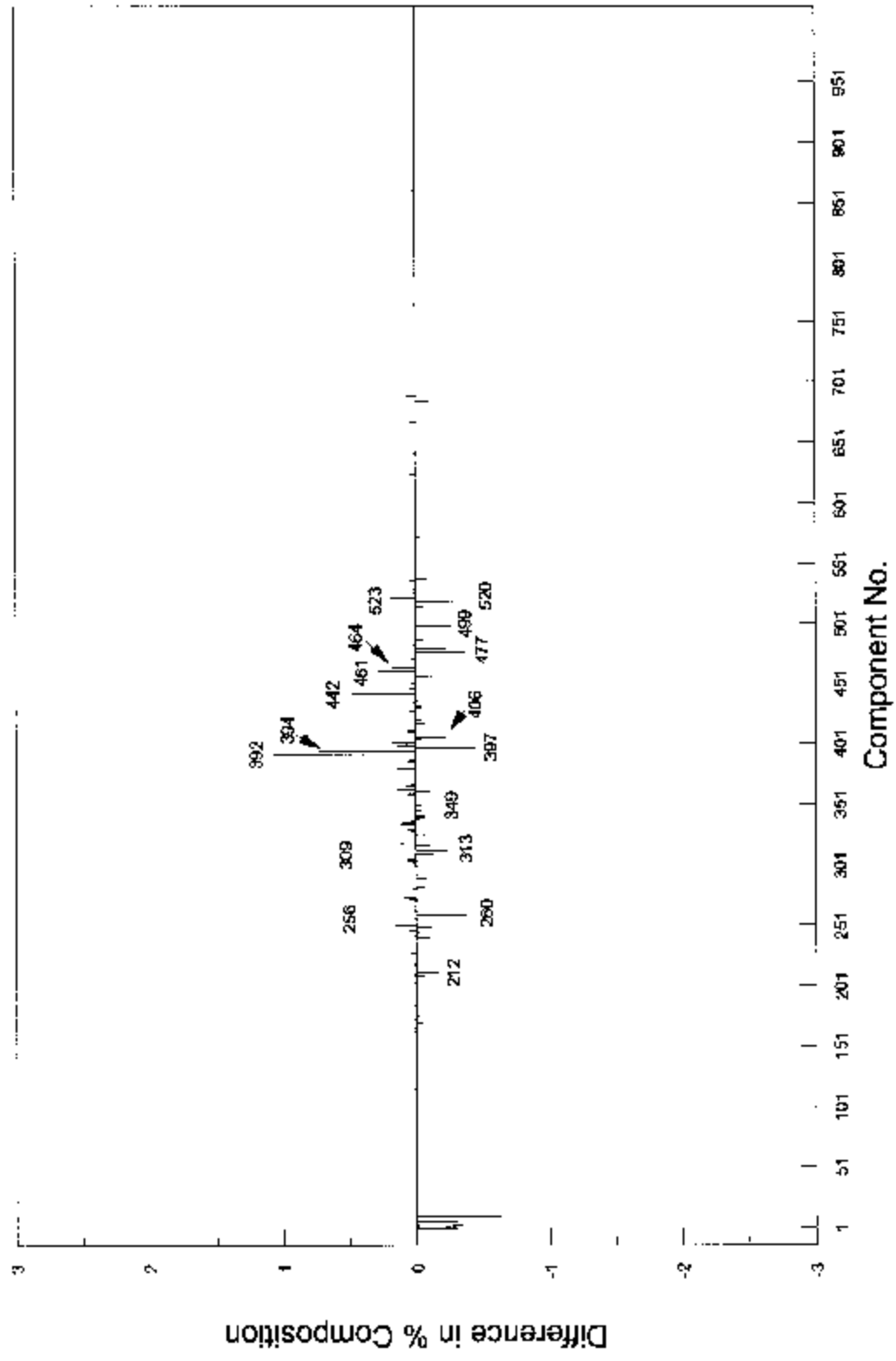


Figure 5. Difference in percent composition: sites average - site 1.

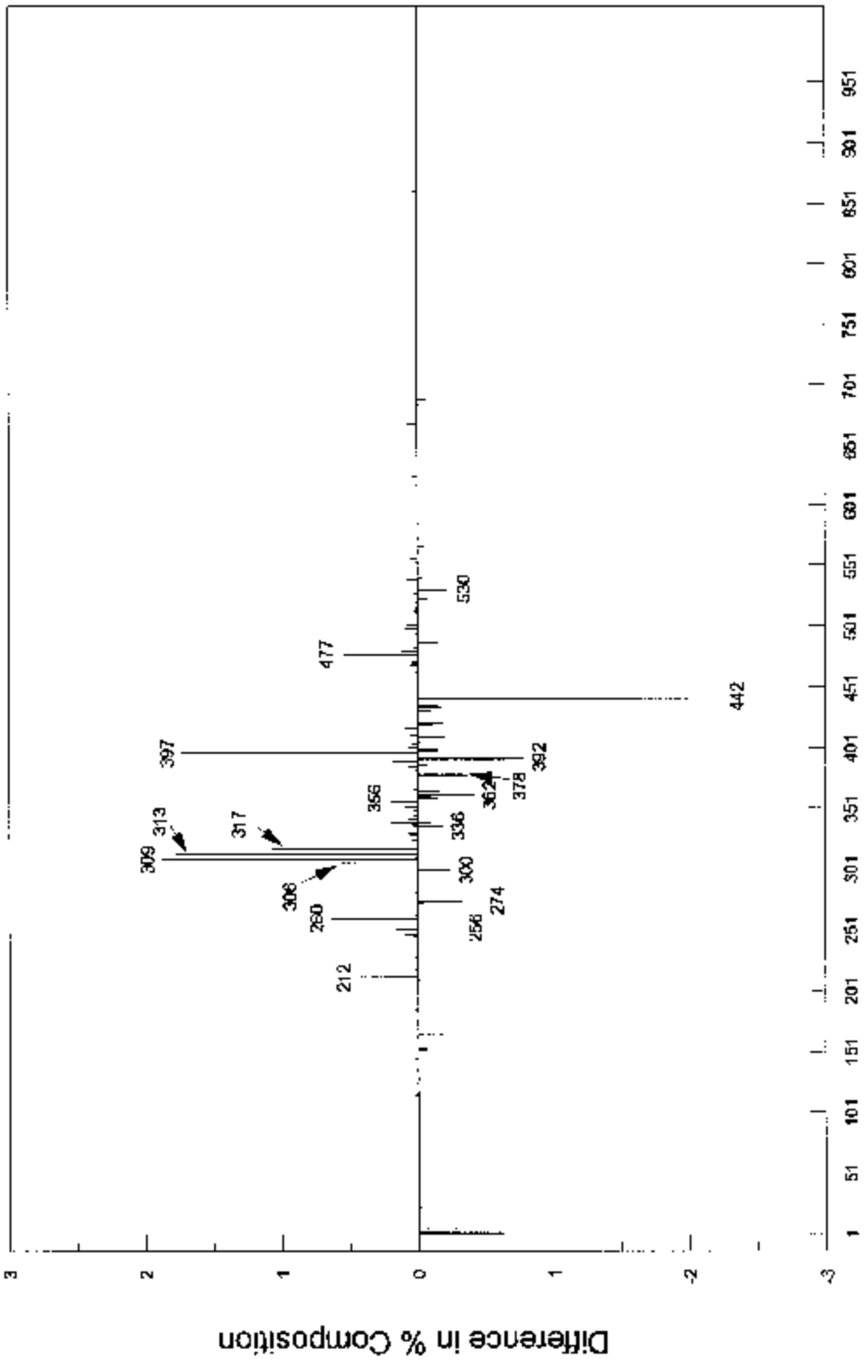


Figure 6. Difference in percent composition: sites average - site 2.

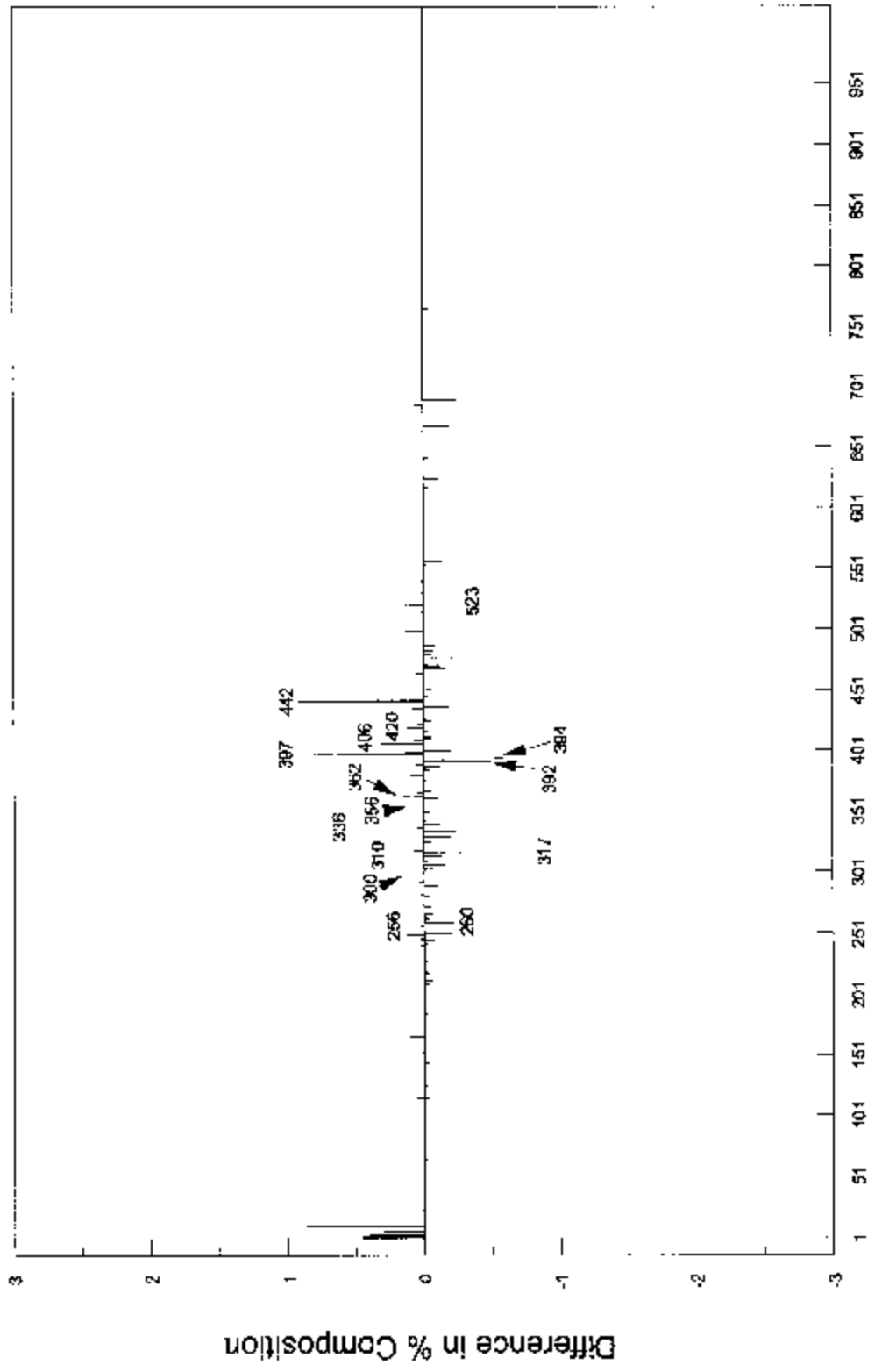


Figure 7. Difference in percent composition: sites average - site 3.

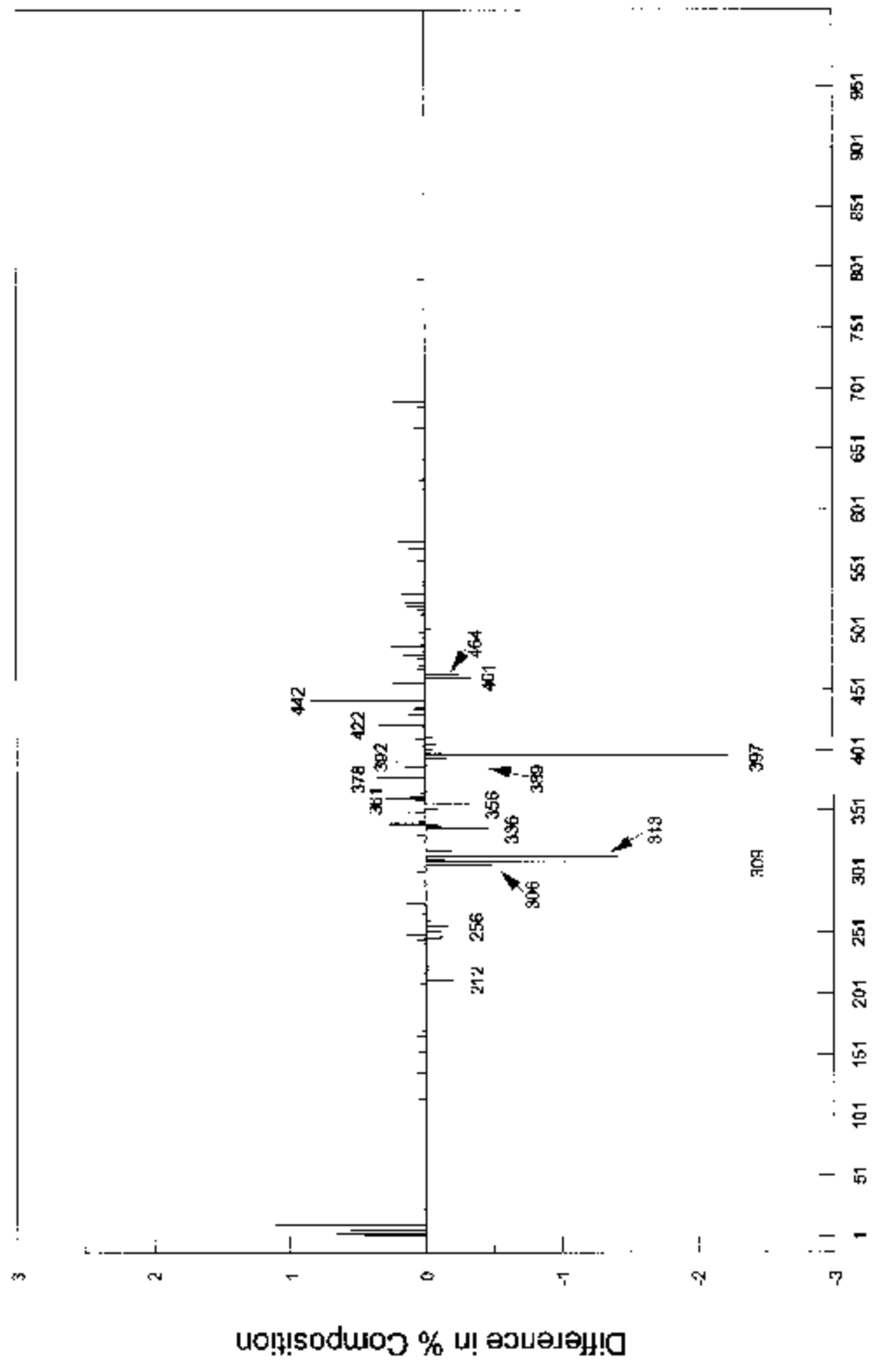


Figure 8. Difference in percent composition: sites average - site 4.

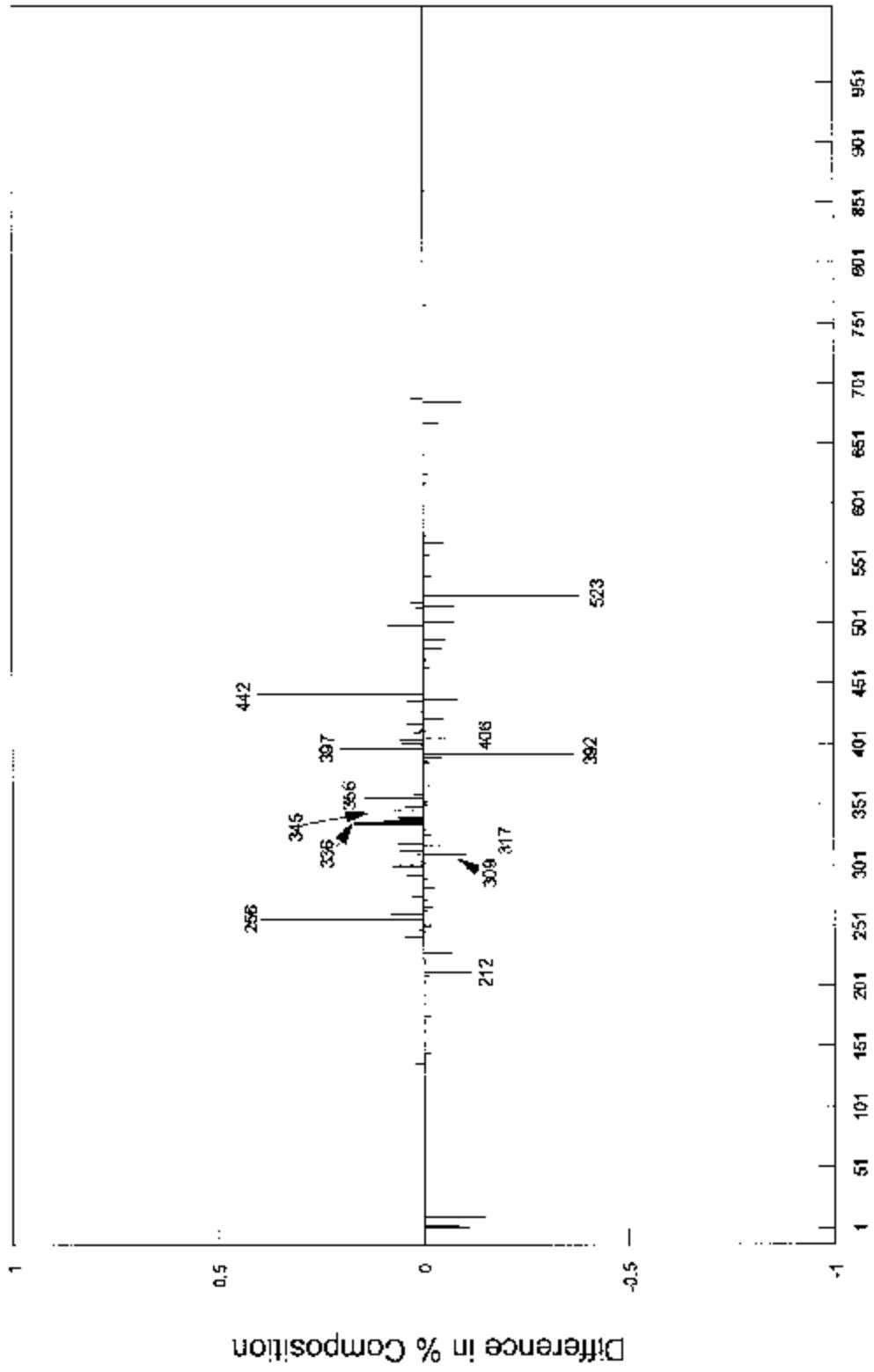


Figure 9. Difference in percent composition: overall average - no-till average.

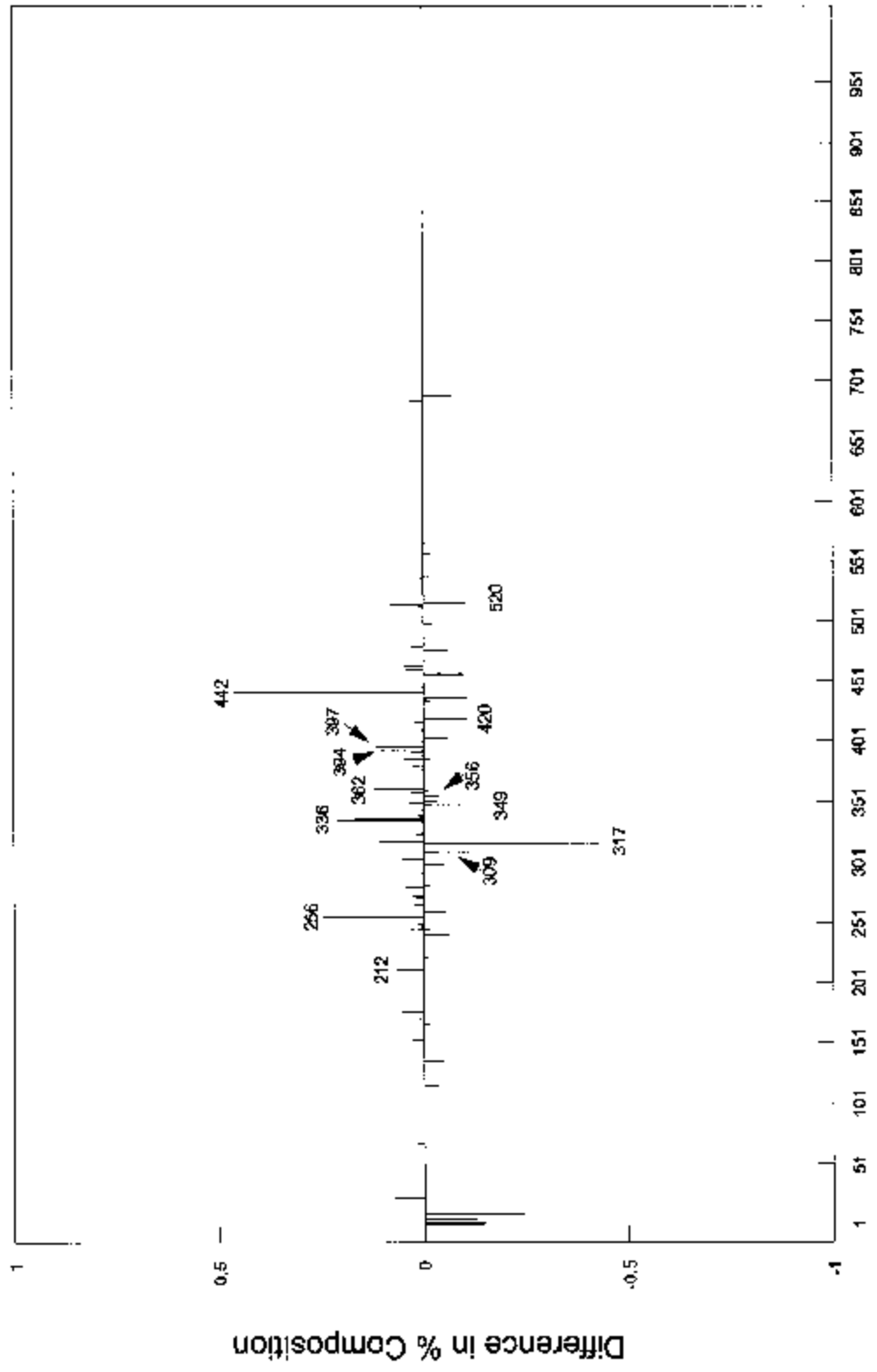


Figure 10. Difference in percent composition: site 1 average - site 1 high elevation average.

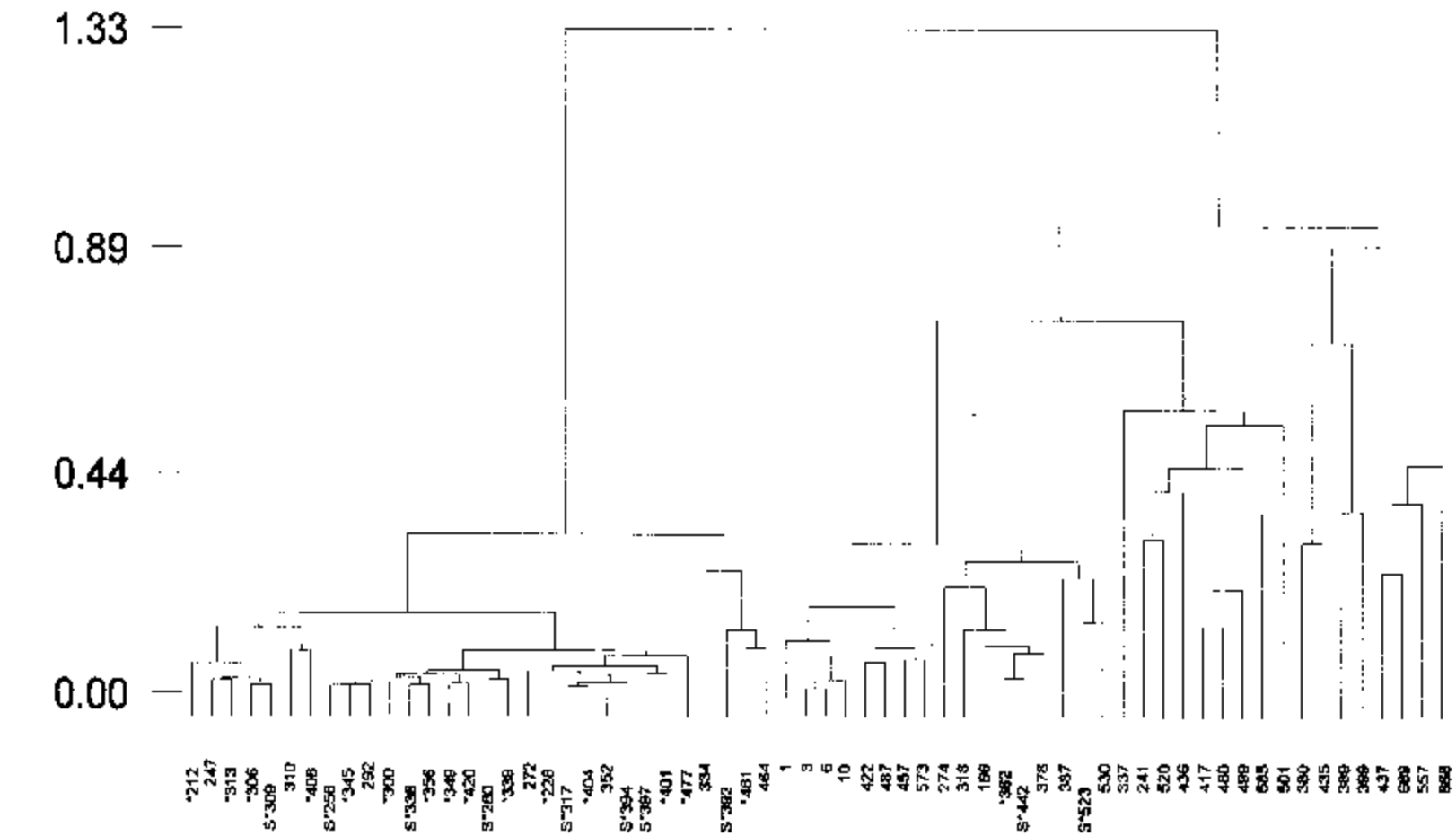


Figure 11. Hierarchical cluster analysis (Euclidean distance, Ward linkage) of relative fatty acid composition data for the 64 components present at >0.5% in the 28 samples. (*indicates a major (>1%) component; s indicates a selected representative component.)

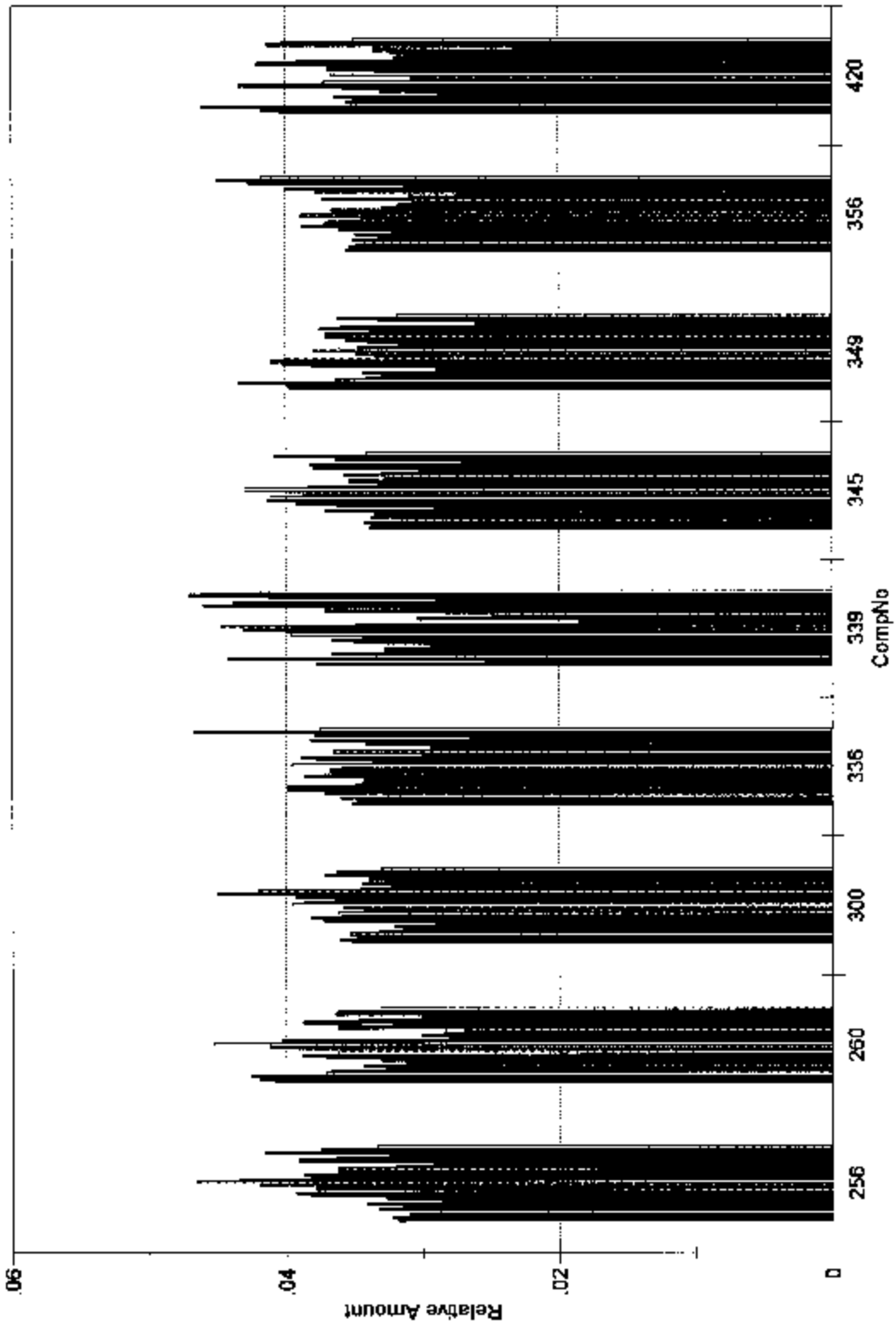


Figure 12. Relative amounts of each of the 9 major (>1%) components of cluster 1 in samples 1 - 28, respectively.

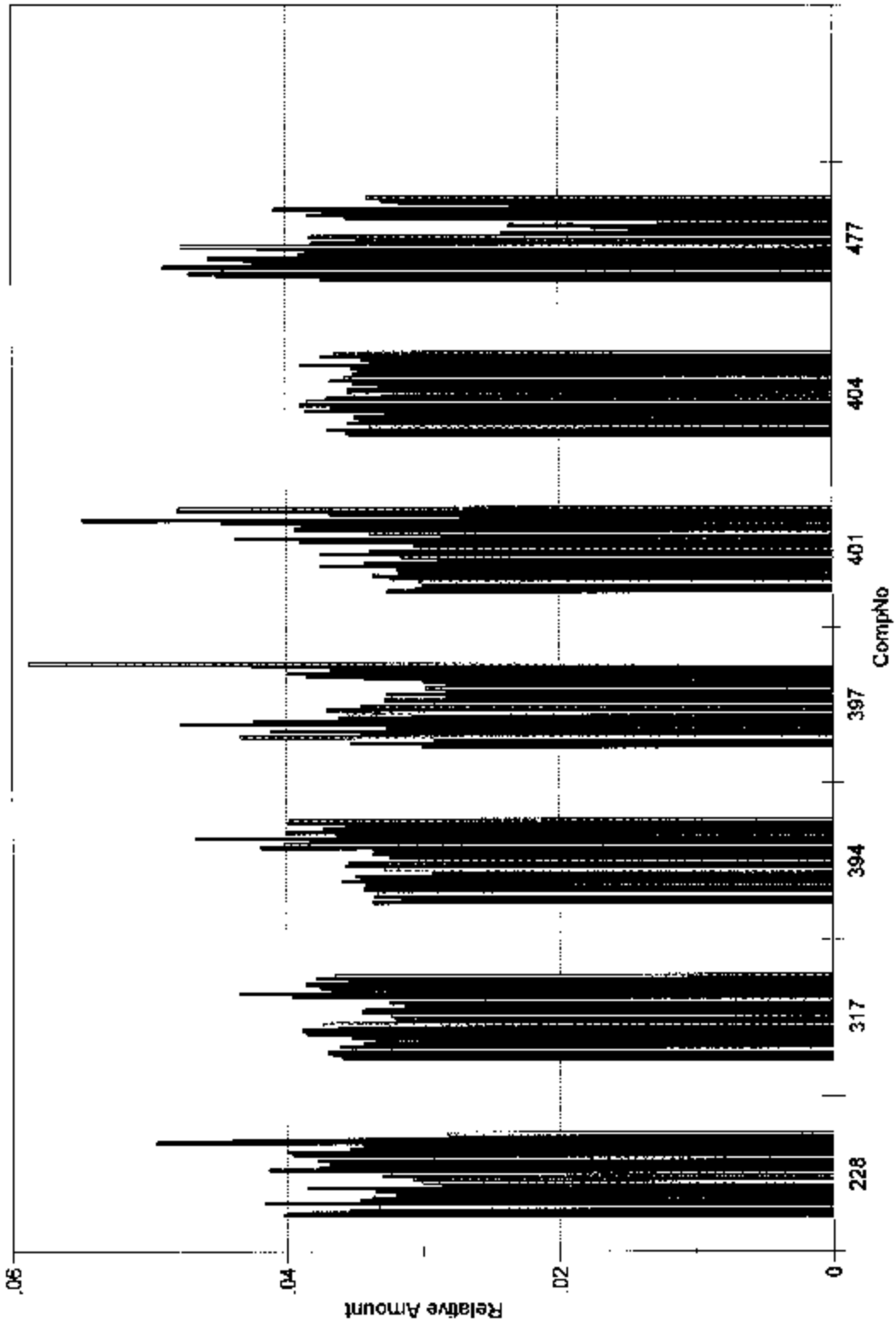


Figure 13. Relative amounts of each of the 7 major (>1%) components of cluster 2 in samples 1 - 28, respectively.

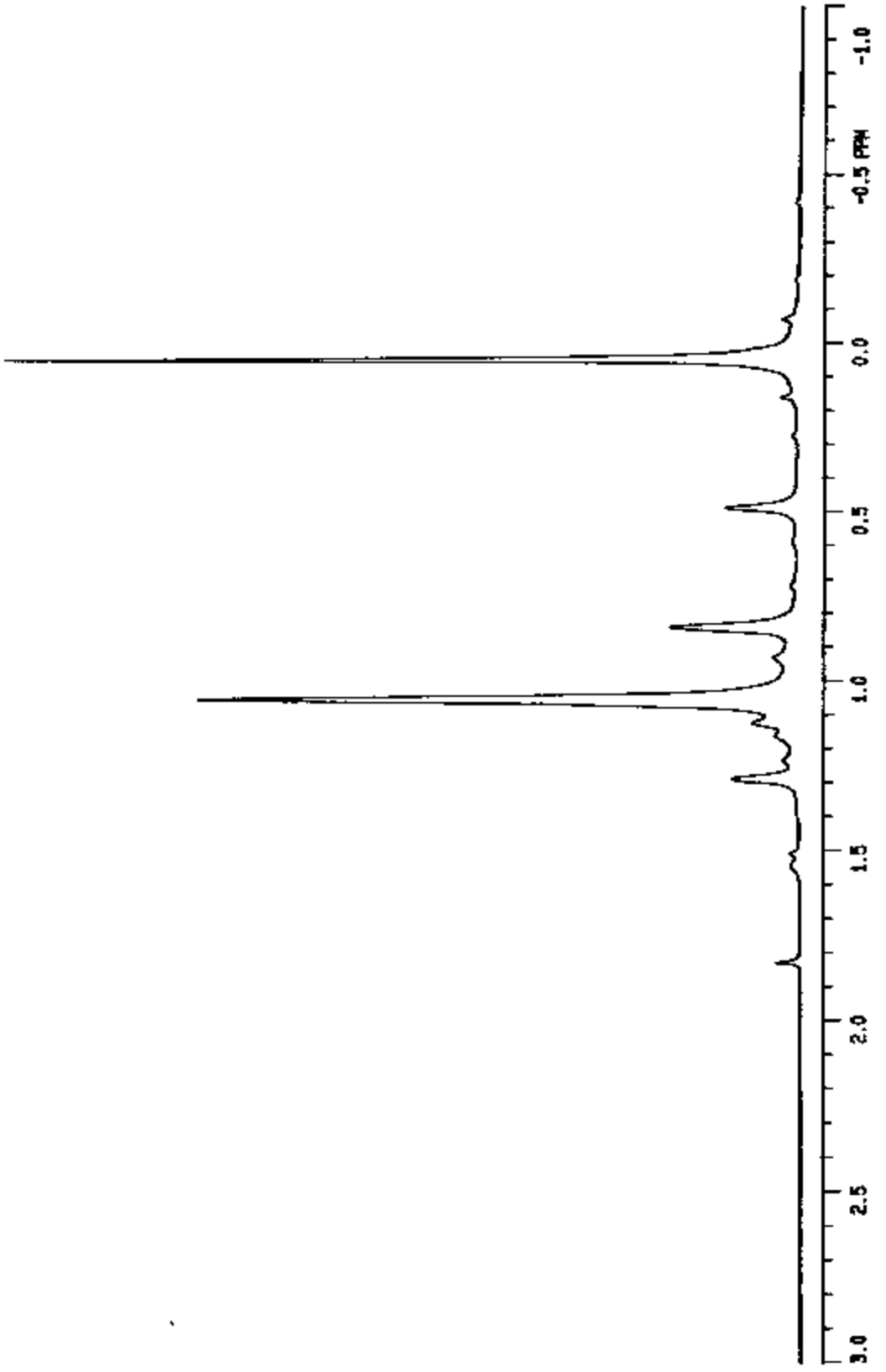


Figure 14. ^{31}P-NMR spectrum of phospholipids from Rhizopus stolonifer mycelia.

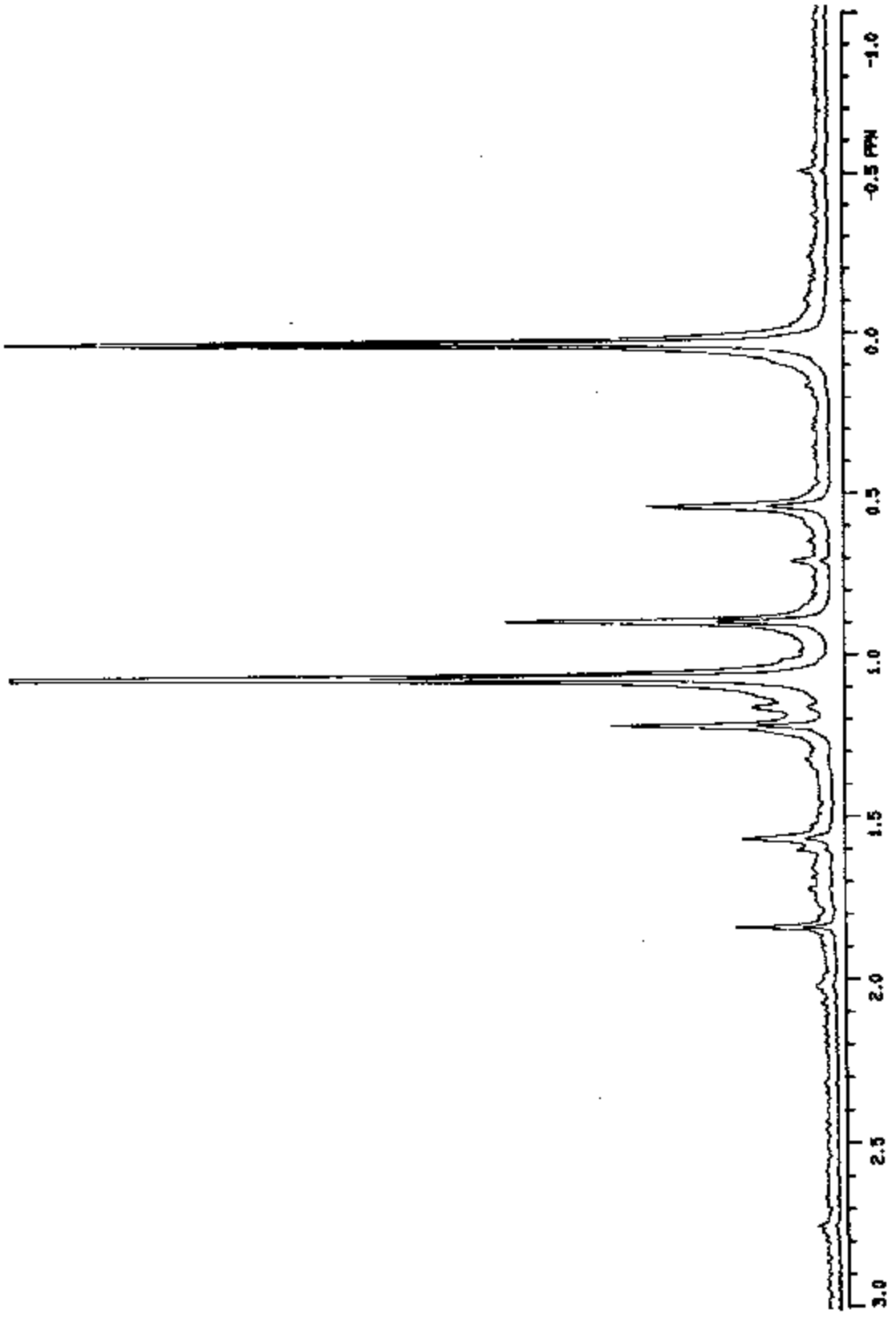


Figure 15. ^{31}P-NMR spectrum of phospholipids from Trichoderma mycelia.

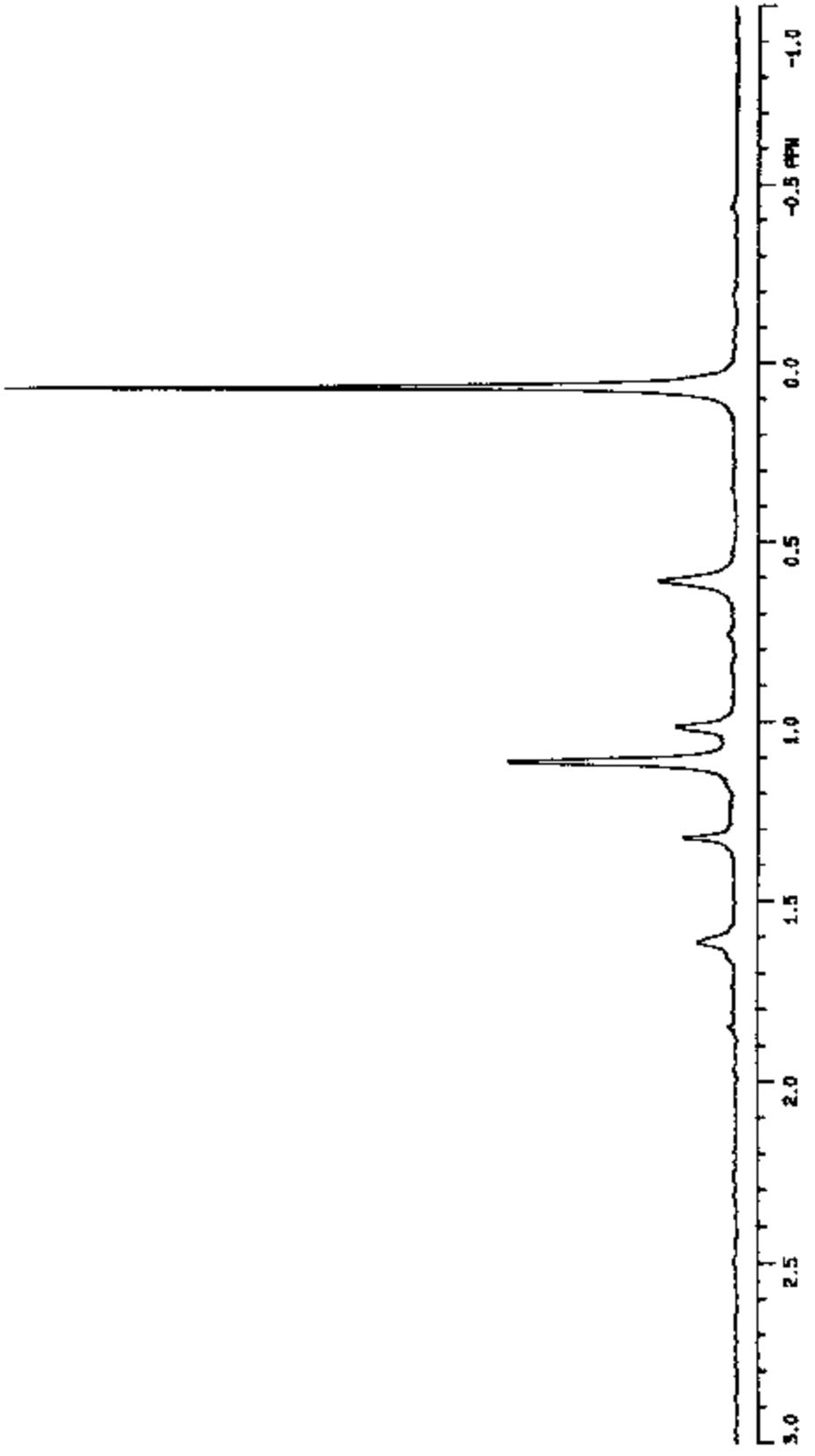


Figure 16. ^{31}P-NMR spectrum of phospholipids from Paecilomyces mycelia.

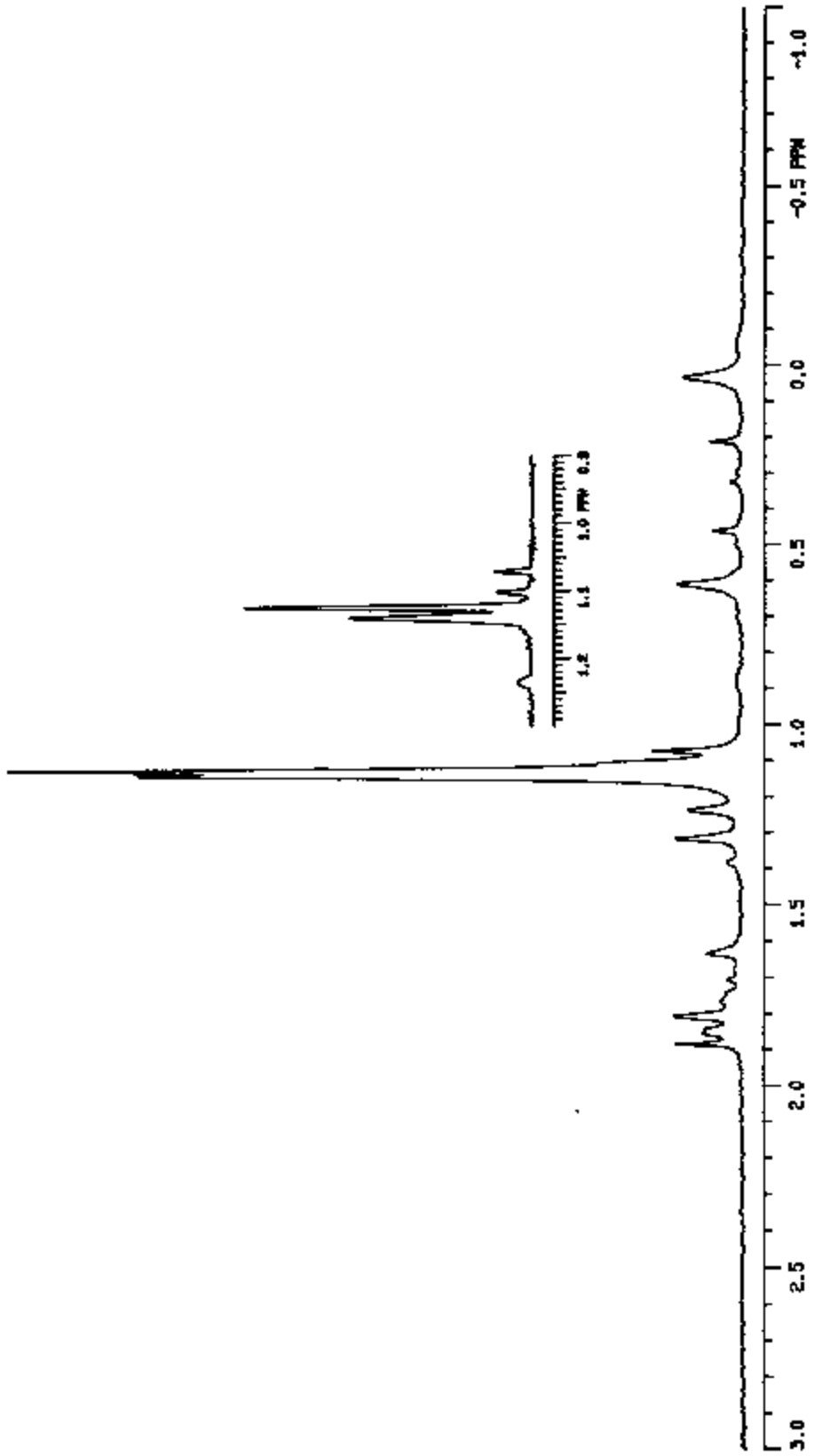


Figure 17. ^{31}P-NMR spectrum of phospholipids from Streptomyces griseus mycelia.

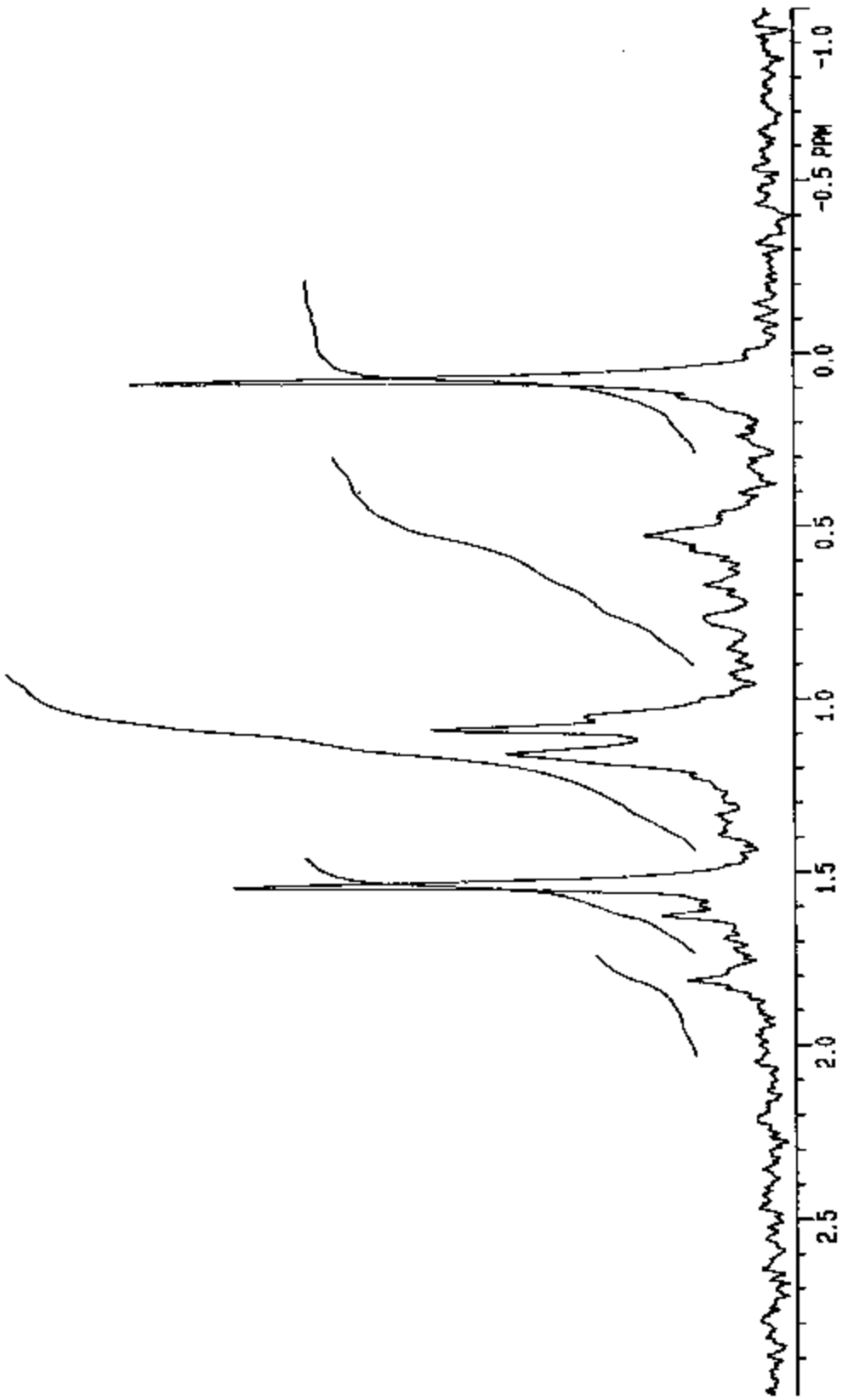


Figure 18. ^{31}P-NMR spectrum of the total lipid extract of plot N-1.

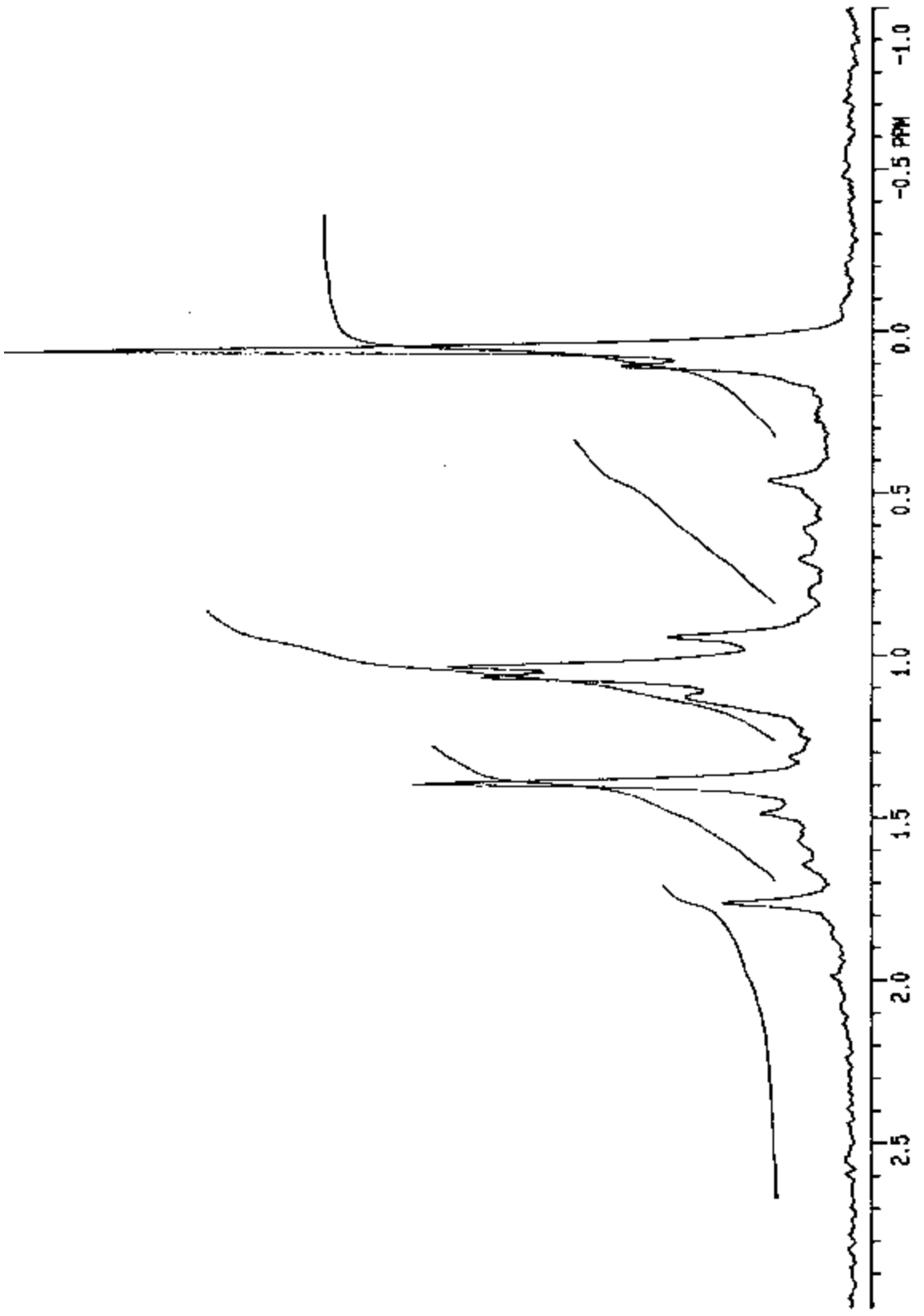


Figure 19. ^{31}P-NMR spectrum of the total lipid extract of plot T-1.

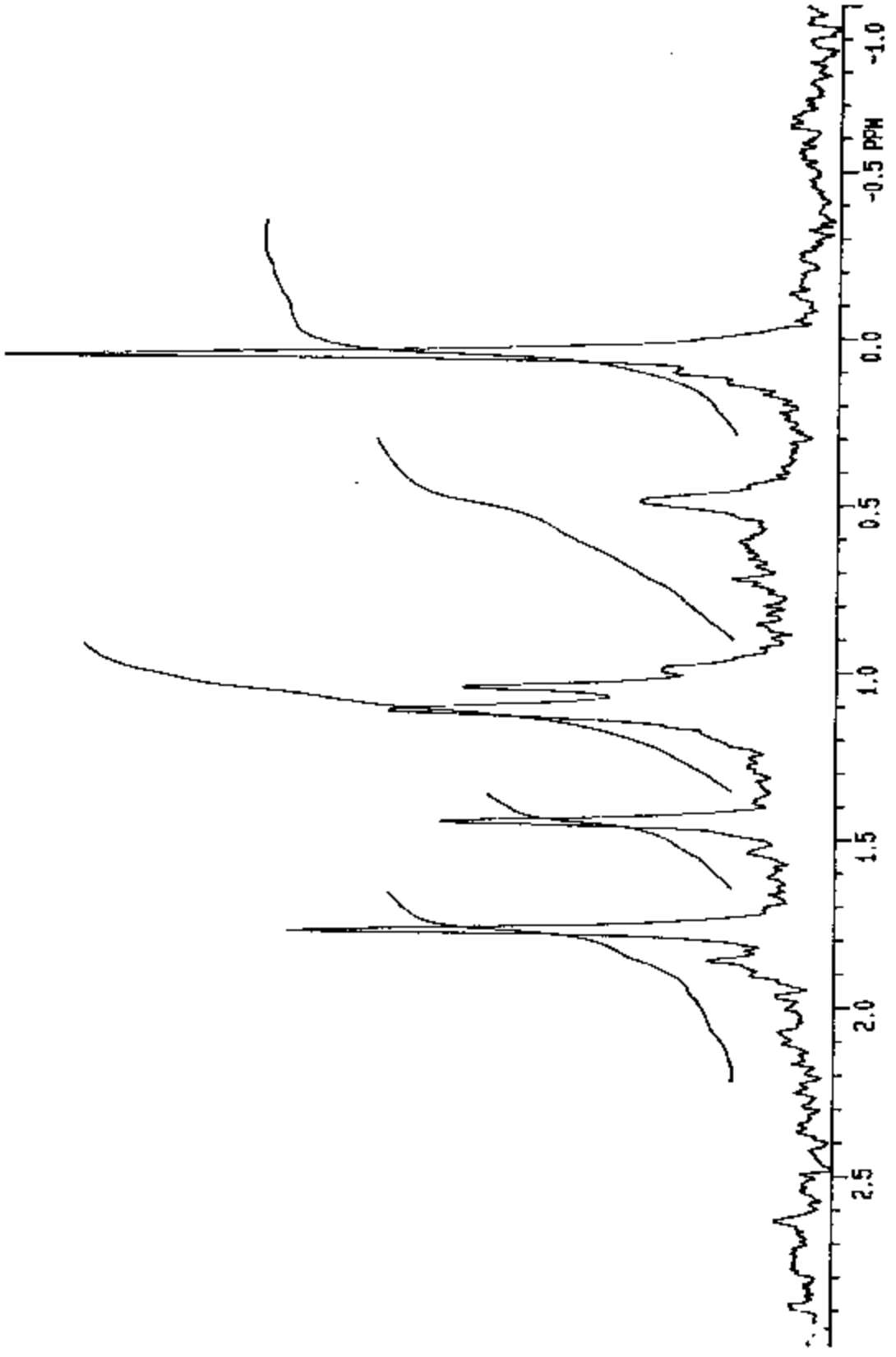


Figure 20. ^{31}P-NMR spectrum of the total lipid extract of plot N-2.

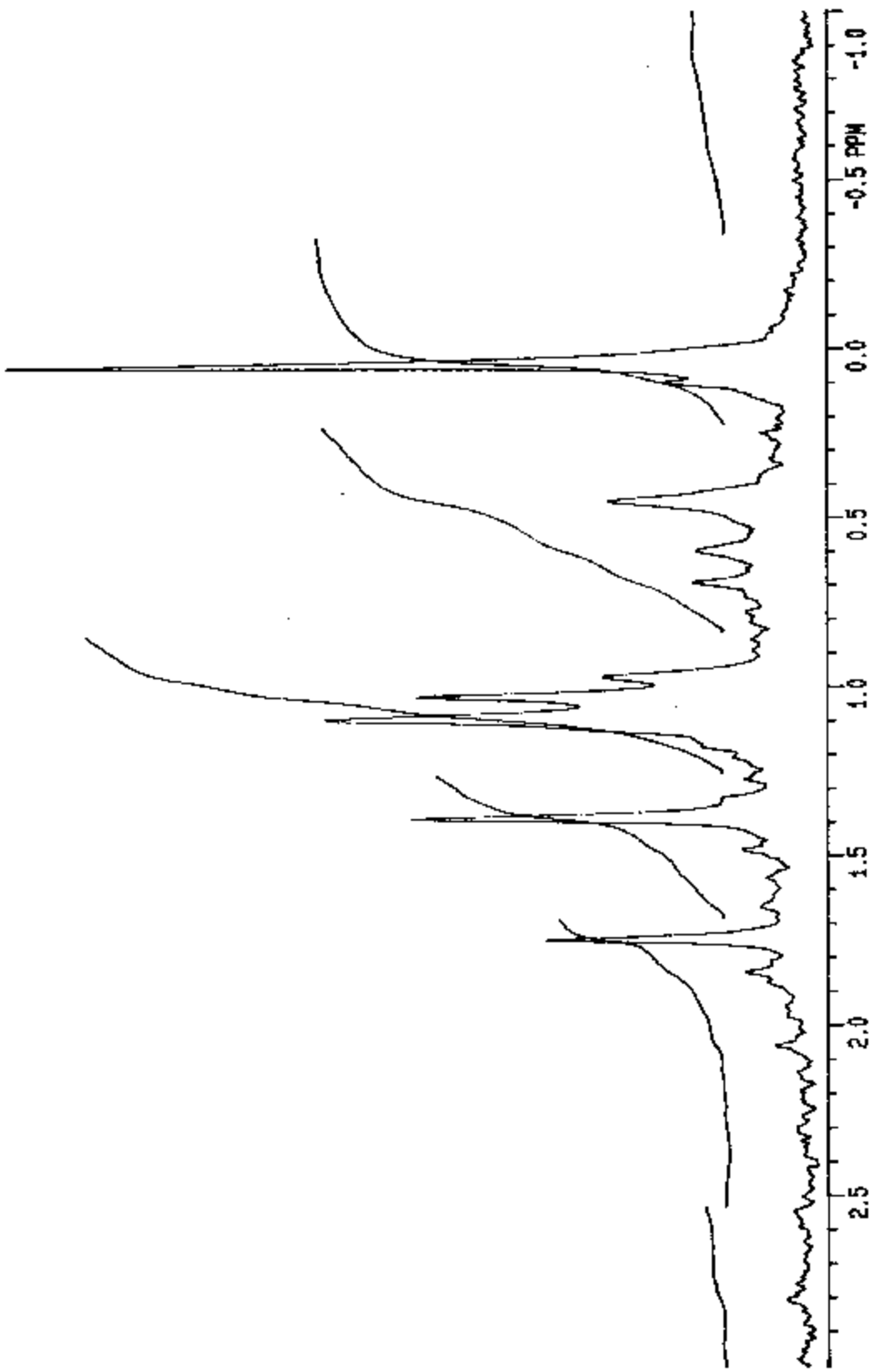


Figure 21. ^{31}P-NMR spectrum of the total lipid extract of plot T-2.

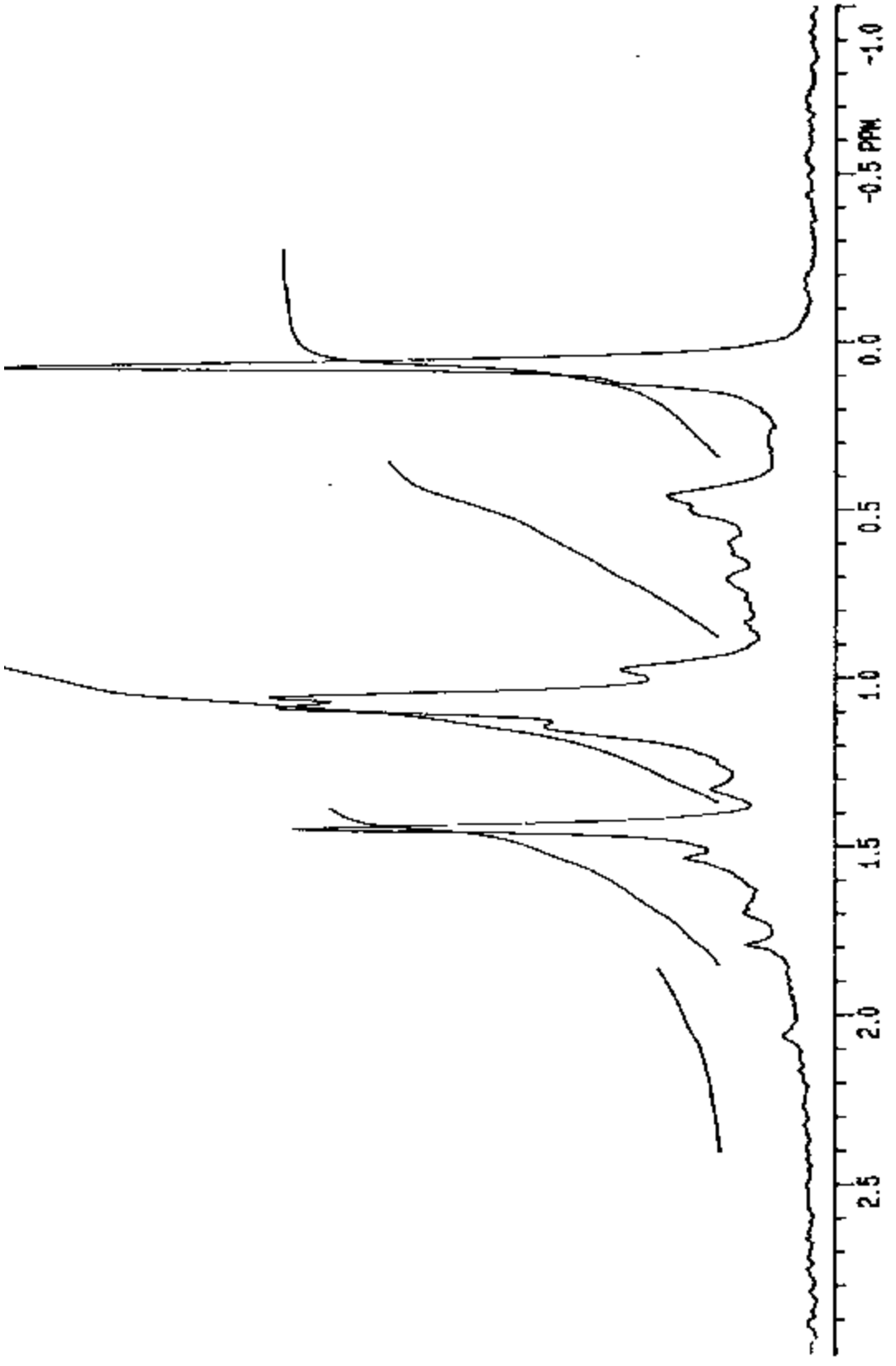


Figure 22. ^{31}P-NMR spectrum of the total lipid extract of plot N-3.

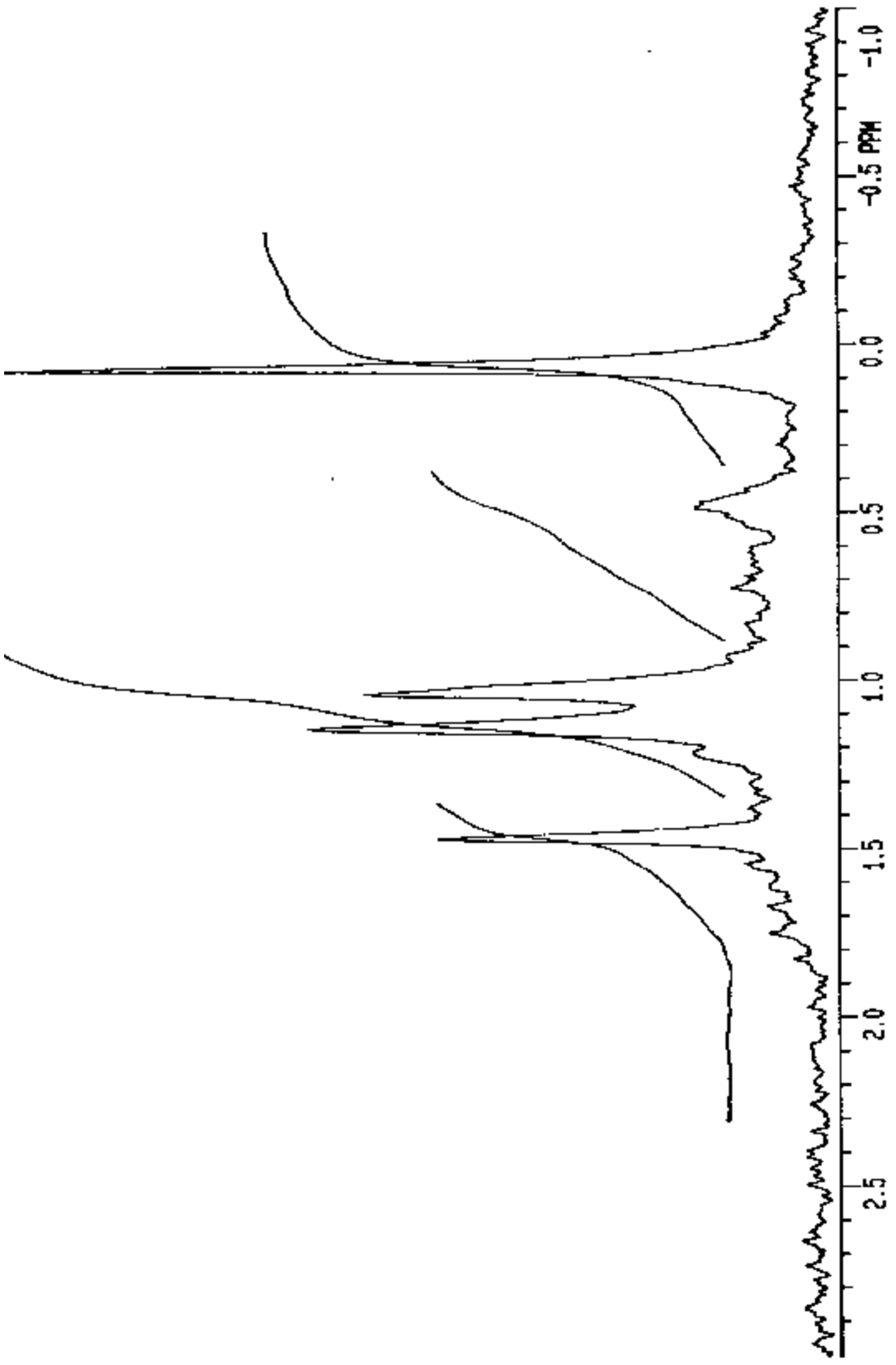


Figure 23. ^{31}P-NMR spectrum of the total lipid extract of plot T-3.

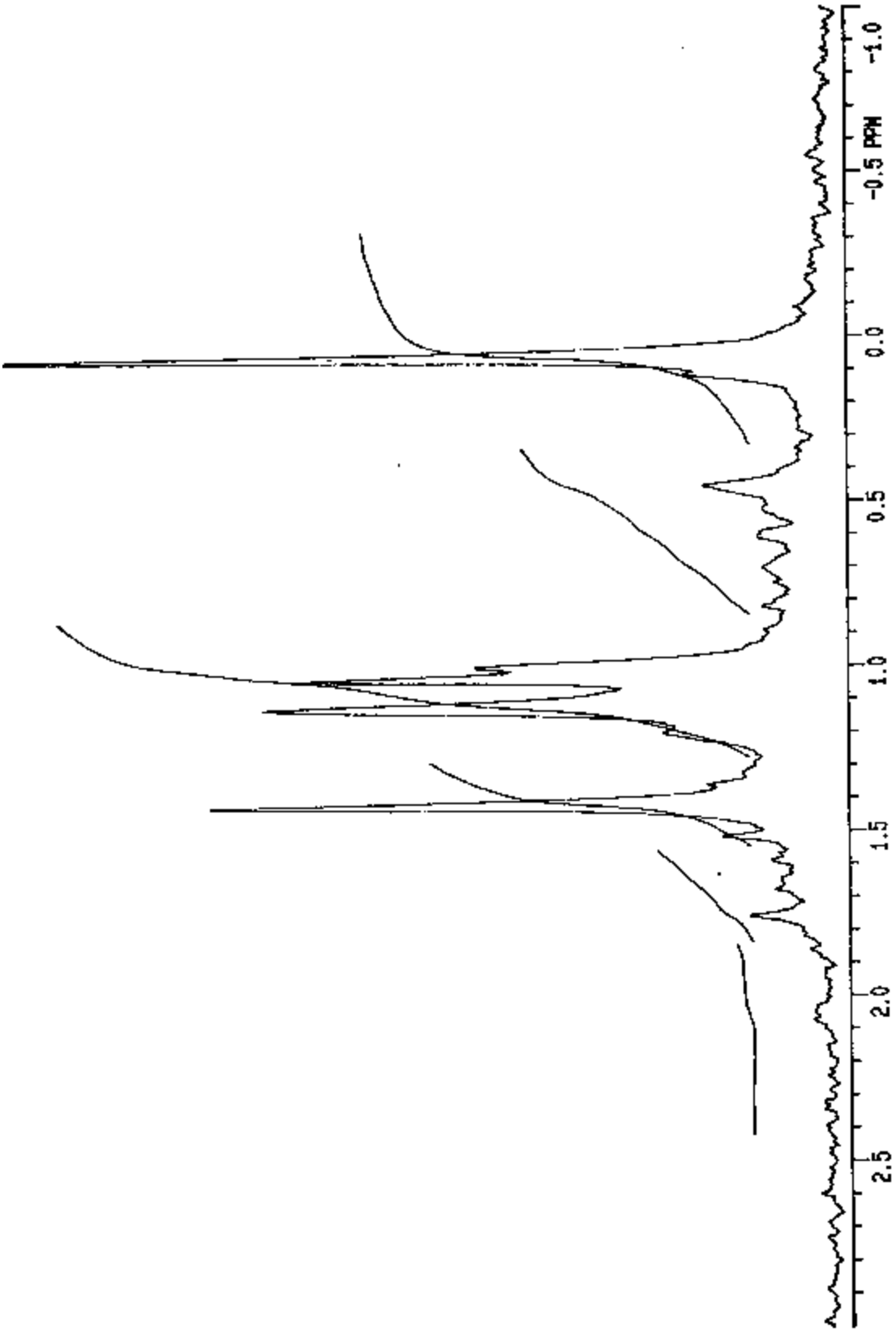


Figure 24. ^{31}P-NMR spectrum of the total lipid extract of plot N-4.

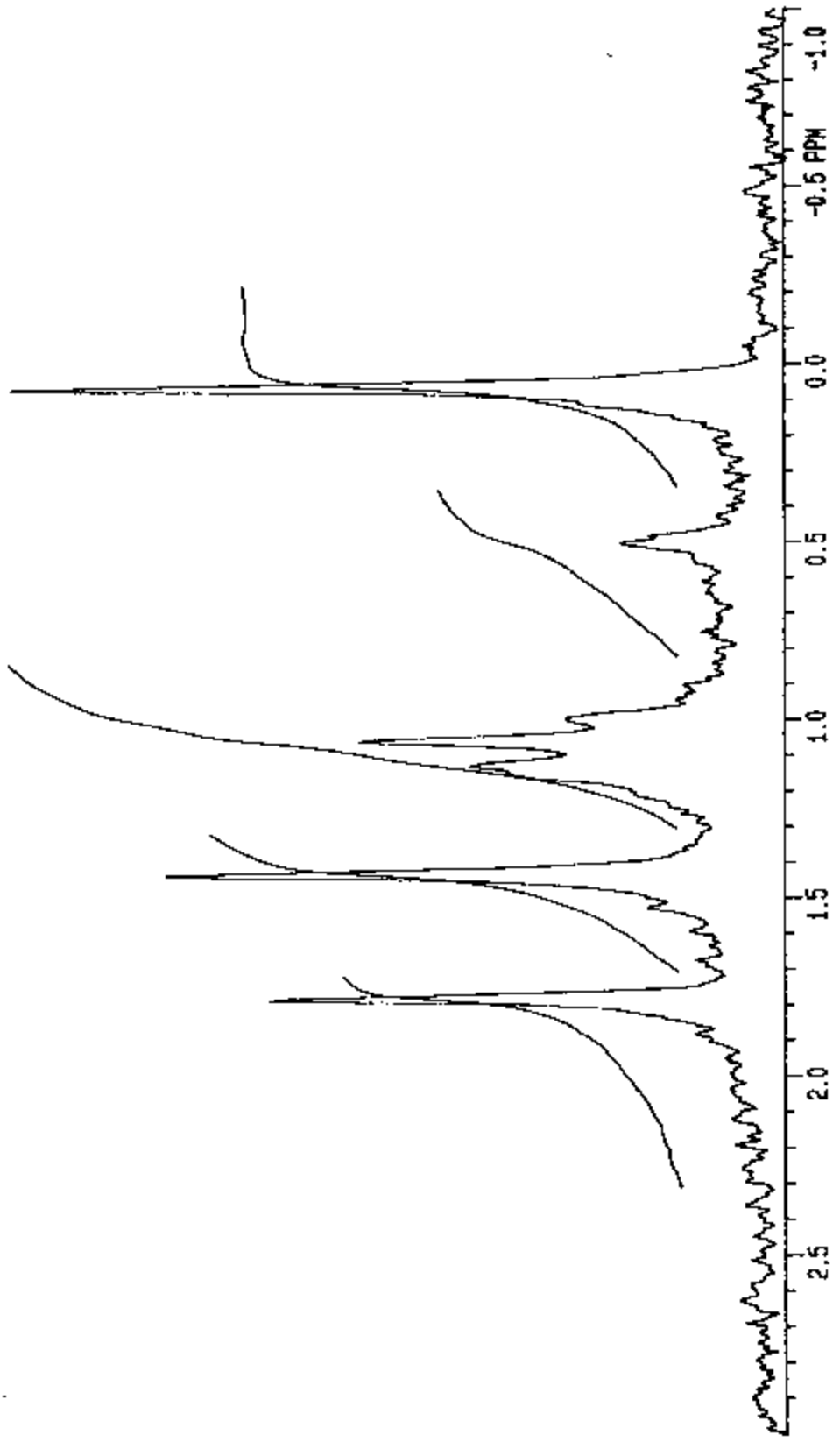


Figure 25. ^{31}P-NMR spectrum of the total lipid extract of plot T-4.

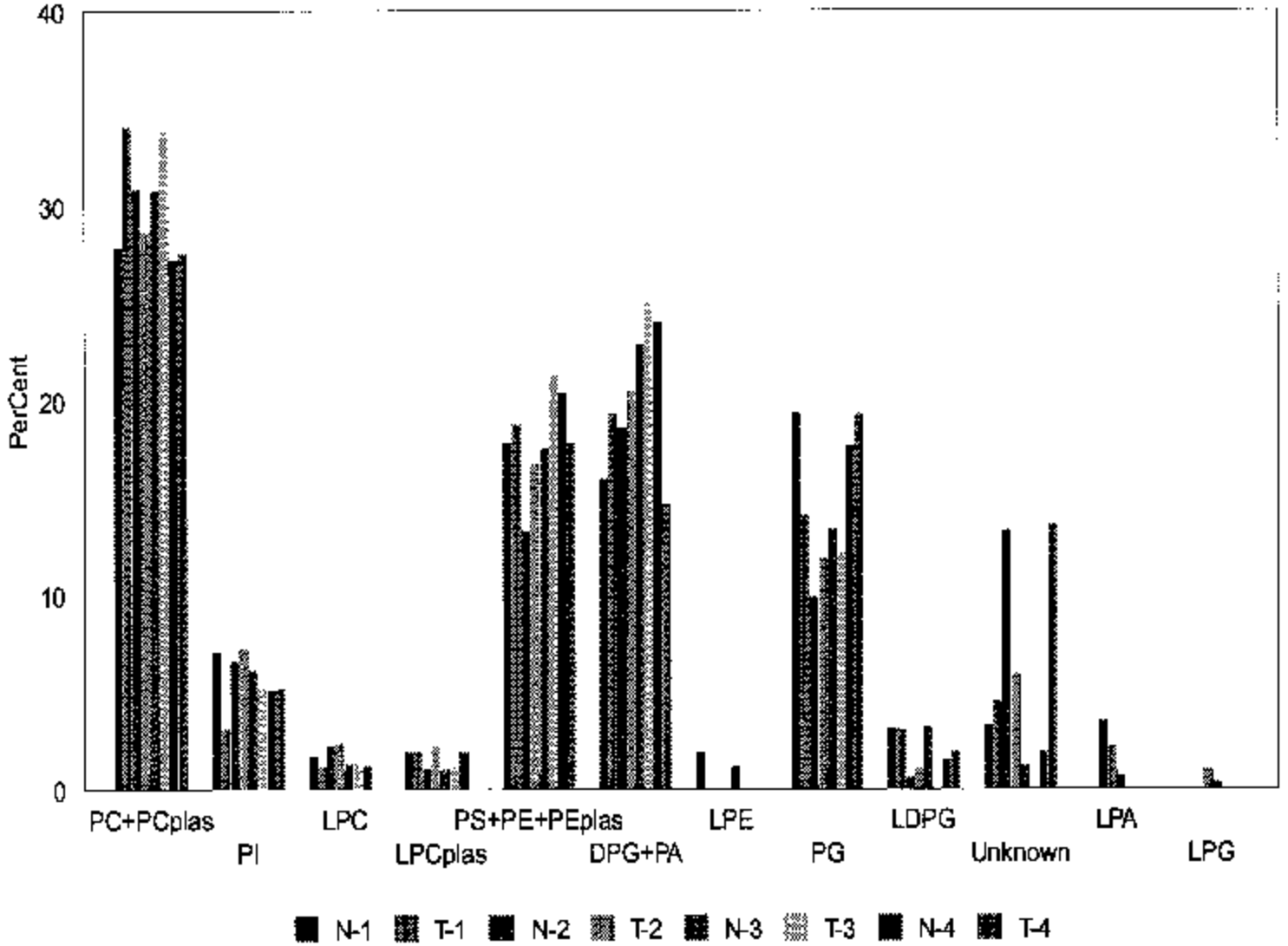


Figure 26. Phospholipid class composition of the no-till (N) and till (T) plots at the 4 sites as determined from the ^{31}P-NMR spectra.

Appendix 1. Statistical analysis of phospholipid fatty acid composition data.

SECTION A
MANOVA - General Linear Model

```
MANOVA for site                 s =  3    m =  3.0    n =     5.5
Criterion        Test Statistic    Approx F            DF       P
Wilk's                 0.00058      15.139   ( 30,    38)  0.000
Lawley-Hotelling      54.21144      21.082   ( 30,    35)  0.000
Pillai's               2.50920       7.669   ( 30,    45)  0.000
Roy's                 31.97561


MANOVA for tillage              s =  1    m =  4.0    n =     5.5
Criterion        Test Statistic           F            DF       P
Wilk's                 0.10231      11.406   ( 10,    13)  0.000
Lawley-Hotelling       8.77374      11.406   ( 10,    13)  0.000
Pillai's               0.89769      11.406   ( 10,    13)  0.000
Roy's                  8.77374


MANOVA for elev                 s =  1    m =  4.0    n =     5.5
Criterion        Test Statistic           F            DF       P
Wilk's                 0.17332       6.201   ( 10,    13)  0.002
Lawley-Hotelling       4.76969       6.201   ( 10,    13)  0.002
Pillai's               0.82668       6.201   ( 10,    13)  0.002
Roy's                  4.76969
```

SECTION B
Discriminant Analysis

```
Linear Method for Response:   site
Predictors:  256  260  309  317  336  392  394  397  442  523

Group        1        2        3        4
Count       16        4        4        4

Summary of Classification

Put into       ....True Group....
Group              1        2        3        4
1                 16        0        0        0
2                  0        4        0        0
3                  0        0        4        0
4                  0        0        0        4
Total N           16        4        4        4
N Correct         16        4        4        4
Proportion     1.000    1.000    1.000    1.000

N =   28     N Correct =   28     Proportion Correct = 1.000
```

Squared Distance Between Groups

	1	2	3	4
1	0.000	48.803	37.747	179.350
2	48.803	0.000	114.845	301.617
3	37.747	114.845	0.000	81.056
4	179.350	301.617	81.056	0.000

Linear Discriminant Function for Group

	1	2	3	4
Constant	-4739.6	-4576.0	-5084.7	-5832.0
256	137.8	132.0	155.9	172.2
260	371.9	359.9	370.1	389.0
309	280.7	274.9	293.4	322.2
317	217.6	214.9	221.8	230.2
336	164.4	159.3	169.6	186.8
392	207.5	207.7	209.1	225.5
394	-4.0	-9.6	10.0	10.4
397	78.5	75.6	80.2	86.6
442	187.8	199.7	180.8	186.1
523	187.5	189.1	199.0	213.2

Variable	Pooled Mean	Means for Group 1	2	3	4
256	5.4536	5.4313	5.7026	5.2191	5.5286
260	4.3238	4.6059	3.4374	4.2952	4.1106
309	6.1952	5.8224	4.6760	6.5781	8.8227
317	9.4479	9.3447	8.5436	10.4009	9.8123
336	7.2516	7.3453	7.2712	6.5571	7.5515
392	2.5799	1.9346	3.8729	3.5847	2.8632
394	6.9596	6.5351	7.2787	7.8659	7.4320
397	9.5993	9.6128	7.9947	8.7931	11.9560
442	4.8831	4.8412	7.0300	3.8544	3.9321
523	0.62990	0.55464	0.73635	0.93430	0.52010

Variable	Pooled StDev	StDev for Group 1	2	3	4
256	0.7272	0.8144	0.1998	0.6790	0.6427
260	0.4190	0.4768	0.1594	0.3323	0.3631
309	0.4692	0.3844	0.2563	0.5969	0.7750
317	0.5656	0.5766	0.3769	0.7849	0.3726
336	0.8323	0.4657	0.8116	0.9894	1.6792
392	1.150	0.634	1.351	1.960	1.703
394	0.5352	0.3645	0.8389	0.8759	0.3960
397	1.561	1.459	0.543	1.241	2.649
442	0.6954	0.6818	0.6515	0.6659	0.8224
523	0.4994	0.5244	0.3344	0.6160	0.3599

Summary of Classified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
1	1	1	1	9.246	1.000
			2	78.835	0.000
			3	39.558	0.000
			4	178.216	0.000
2	1	1	1	8.482	1.000
			2	77.299	0.000
			3	46.425	0.000
			4	198.798	0.000
3	1	1	1	9.611	1.000
			2	68.706	0.000
			3	45.341	0.000
			4	181.756	0.000
4	1	1	1	6.130	1.000
			2	70.497	0.000
			3	49.762	0.000
			4	190.345	0.000
5	1	1	1	2.106	1.000
			2	53.711	0.000
			3	27.243	0.000
			4	159.859	0.000
6	1	1	1	7.737	1.000
			2	41.307	0.000
			3	49.591	0.000
			4	201.006	0.000
7	1	1	1	8.194	1.000
			2	33.383	0.000
			3	43.592	0.000
			4	183.934	0.000
8	1	1	1	12.25	1.000
			2	51.65	0.000
			3	52.30	0.000
			4	166.61	0.000
9	1	1	1	7.880	1.000
			2	64.435	0.000
			3	39.209	0.000
			4	182.638	0.000
10	1	1	1	8.957	1.000
			2	76.155	0.000
			3	26.238	0.000
			4	159.000	0.000
11	1	1	1	13.65	1.000
			2	44.50	0.000
			3	85.79	0.000
			4	261.86	0.000
12	1	1	1	4.443	1.000
			2	48.765	0.000
			3	43.742	0.000
			4	204.019	0.000

13	1	1	1	6.775	1.000
			2	41.175	0.000
			3	51.219	0.000
			4	194.390	0.000
14	1	1	1	5.499	1.000
			2	49.738	0.000
			3	40.243	0.000
			4	180.795	0.000
15	1	1	1	7.927	1.000
			2	57.150	0.000
			3	58.239	0.000
			4	210.819	0.000
16	1	1	1	11.95	1.000
			2	54.37	0.000
			3	36.28	0.000
			4	146.38	0.000
17	2	2	1	62.02	0.000
			2	10.11	1.000
			3	123.17	0.000
			4	296.18	0.000
18	2	2	1	36.801	0.000
			2	7.907	1.000
			3	107.632	0.000
			4	271.993	0.000
19	2	2	1	51.606	0.000
			2	8.179	1.000
			3	106.000	0.000
			4	301.910	0.000
20	2	2	1	78.132	0.000
			2	7.148	1.000
			3	155.921	0.000
			4	369.728	0.000
21	3	3	1	27.268	0.000
			2	94.326	0.000
			3	6.375	1.000
			4	101.901	0.000
22	3	3	1	59.760	0.000
			2	124.273	0.000
			3	9.885	1.000
			4	112.940	0.000
23	3	3	1	35.673	0.000
			2	125.580	0.000
			3	5.984	1.000
			4	87.623	0.000
24	3	3	1	58.003	0.000
			2	144.917	0.000
			3	7.473	1.000
			4	51.475	0.000
25	4	4	1	203.73	0.000
			2	318.13	0.000
			3	104.32	0.000
			4	13.92	1.000

26	4	4	1	190.763	0.000
			2	308.522	0.000
			3	84.005	0.000
			4	9.607	1.000
27	4	4	1	206.47	0.000
			2	336.20	0.000
			3	102.79	0.000
			4	11.61	1.000
28	4	4	1	162.55	0.000
			2	289.72	0.000
			3	79.22	0.000
			4	10.97	1.000

SECTION C
Discriminant Analysis

Linear Method for Response: tillage
Predictors: 256 260 309 317 336 392 394 397 442 523

Group	1	2
Count	14	14

Summary of Classification

Put into	....True Group....	
Group	1	2
1	14	0
2	0	14
Total N	14	14
N Correct	14	14
Proportion	1.000	1.000

N = 28 N Correct = 28 Proportion Correct = 1.000

Squared Distance Between Groups

	1	2
1	0.0000	21.7474
2	21.7474	0.0000

Linear Discriminant Function for Group

	1	2
Constant	-2524.0	-2672.0
256	-4.6	1.3
260	308.2	313.1
309	48.6	47.2
317	192.3	198.1
336	15.2	10.6
392	86.6	82.8
394	-18.5	-9.8
397	41.4	42.8
442	224.8	230.2
523	-13.4	-27.1

Variable	Pooled Mean	Means for Group 1	Means for Group 2
256	5.4536	5.0555	5.8517
260	4.3238	4.2441	4.4035
309	6.1952	6.2982	6.0922
317	9.4479	9.5720	9.3238
336	7.2516	7.0820	7.4211
392	2.5799	2.9464	2.2134
394	6.9596	6.9083	7.0108
397	9.5993	9.3962	9.8023
442	4.8831	4.4768	5.2893
523	0.62990	1.00921	0.25059

Variable	Pooled StDev	StDev for Group 1	StDev for Group 2
256	0.5802	0.5931	0.5670
260	0.5763	0.5568	0.5952
309	1.310	1.346	1.274
317	0.7558	0.7716	0.7397
336	0.8378	0.8944	0.7772
392	1.326	1.412	1.233
394	0.7409	0.7148	0.7660
397	1.885	1.638	2.104
442	1.132	1.238	1.014
523	0.3109	0.3736	0.2317

Summary of Classified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
1	1	1	1	9.089	0.999
			2	23.168	0.001
2	1	1	1	6.737	1.000
			2	37.098	0.000
3	1	1	1	10.75	1.000
			2	45.76	0.000
4	1	1	1	5.475	1.000
			2	32.971	0.000
5	1	1	1	1.362	1.000
			2	17.291	0.000
6	1	1	1	6.451	1.000
			2	30.858	0.000
7	1	1	1	4.272	1.000
			2	31.181	0.000
8	1	1	1	9.288	1.000
			2	27.914	0.000
9	2	2	1	30.471	0.000
			2	7.614	1.000
10	2	2	1	34.786	0.000
			2	5.753	1.000

11	2	2	1	24.098	0.000
			2	8.064	1.000
12	2	2	1	16.787	0.003
			2	5.048	0.997
13	2	2	1	33.705	0.000
			2	5.616	1.000
14	2	2	1	45.003	0.000
			2	6.691	1.000
15	2	2	1	40.491	0.000
			2	8.149	1.000
16	2	2	1	31.434	0.000
			2	7.521	1.000
17	1	1	1	15.79	1.000
			2	31.59	0.000
18	1	1	1	9.710	0.978
			2	17.334	0.022
19	2	2	1	18.61	0.138
			2	14.95	0.862
20	2	2	1	27.71	0.000
			2	10.78	1.000
21	1	1	1	7.461	1.000
			2	45.609	0.000
22	1	1	1	19.02	0.867
			2	22.77	0.133
23	2	2	1	24.644	0.000
			2	6.505	1.000
24	2	2	1	28.609	0.000
			2	4.756	1.000
25	1	1	1	19.43	1.000
			2	42.40	0.000
26	1	1	1	12.82	1.000
			2	36.16	0.000
27	2	2	1	34.35	0.000
			2	17.43	1.000
28	2	2	1	36.12	0.000
			2	13.47	1.000

SECTION D
Discriminant Analysis

```
Linear Method for Response:   selev
Predictors:  s256  s260  s309  s317  s336  s392  s394  s397  s442  s523

Group        1        2
Count        8        8

Summary of Classification

Put into       ....True Group....
```

Group	1	2
1	8	0
2	0	8
Total N	8	8
N Correct	8	8
Proportion	1.000	1.000

N = 16 N Correct = 16 Proportion Correct = 1.000

Squared Distance Between Groups

	1	2
1	0.0000	81.9589
2	81.9589	0.0000

Linear Discriminant Function for Group

	1	2
Constant	-53542	-56042
s256	2748	2828
s260	2554	2599
s309	3839	3934
s317	784	784
s336	3917	4015
s392	1543	1583
s394	137	145
s397	1301	1332
s442	1124	1147
s523	2427	2496

Variable	Pooled Mean	Means for Group 1	2
s256	5.4313	5.1854	5.6772
s260	4.6059	4.6557	4.5562
s309	5.8224	5.9351	5.7097
s317	9.3447	9.7728	8.9165
s336	7.3453	7.1352	7.5554
s392	1.9346	1.9023	1.9669
s394	6.5351	6.4270	6.6432
s397	9.6128	9.4954	9.7301
s442	4.8412	4.3775	5.3049
s523	0.55464	0.60088	0.50841

Variable	Pooled StDev	StDev for Group 1	2
s256	0.8009	0.5904	0.9666
s260	0.4906	0.3910	0.5732
s309	0.3792	0.4397	0.3071
s317	0.3830	0.3314	0.4284
s336	0.4265	0.3564	0.4866
s392	0.6559	0.5015	0.7802
s394	0.3591	0.3523	0.3658
s397	1.505	1.445	1.562

s442	0.5023	0.5256	0.4779
s523	0.5405	0.6159	0.4528

Summary of Classified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
1	1	1	1	8.715	1.000
			2	94.864	0.000
2	1	1	1	7.508	1.000
			2	94.031	0.000
3	1	1	1	10.24	1.000
			2	81.49	0.000
4	1	1	1	8.450	1.000
			2	87.639	0.000
5	2	2	1	41.54	0.000
			2	10.34	1.000
6	2	2	1	101.032	0.000
			2	6.107	1.000
7	2	2	1	116.715	0.000
			2	8.635	1.000
8	2	2	1	85.826	0.000
			2	9.703	1.000
9	1	1	1	9.419	1.000
			2	82.554	0.000
10	1	1	1	8.864	1.000
			2	78.400	0.000
11	1	1	1	10.31	1.000
			2	105.14	0.000
12	1	1	1	7.114	1.000
			2	102.169	0.000
13	2	2	1	99.332	0.000
			2	7.137	1.000
14	2	2	1	76.994	0.000
			2	6.169	1.000
15	2	2	1	101.11	0.000
			2	10.08	1.000
16	2	2	1	102.51	0.000
			2	11.21	1.000

Appendix 2. Statistical analysis of phospholipid class composition data.

SECTION A

Discriminant Analysis

Linear Method for Response: site
Predictors: PC+PCpla LPC PS+PE DPG+PA

Group	1	2	3	4
Count	2	2	2	2

Summary of Classification

Put into	....True Group....			
Group	1	2	3	4
1	2	0	0	0
2	0	2	0	0
3	0	0	2	0
4	0	0	0	2
Total N	2	2	2	2
N Correct	2	2	2	2
Proportion	1.000	1.000	1.000	1.000

N = 8 N Correct = 8 Proportion Correct = 1.000

Squared Distance Between Groups

	1	2	3	4
1	0.000	15.850	35.355	112.679
2	15.850	0.000	75.048	184.144
3	35.355	75.048	0.000	29.040
4	112.679	184.144	29.040	0.000

Linear Discriminant Function for Group

	1	2	3	4
Constant	-416.09	-467.23	-295.17	-195.48
PC+PCpla	24.80	26.82	20.27	15.63
LPC	182.88	202.80	141.88	106.34
PS+PE	13.26	12.21	10.81	9.97
DPG+PA	-25.18	-26.83	-19.16	-15.07

Variable	Pooled Mean	Means for Group 1	2	3	4
PC+PCpla	30.137	31.000	29.800	32.300	27.450
LPC	1.4250	1.4500	2.3500	1.3000	0.6000
PS+PE	17.963	18.300	15.000	19.450	19.100
DPG+PA	20.137	17.650	19.550	23.950	19.400

Variable	Pooled StDev	StDev for Group 1	2	3	4
PC+PCpla	2.559	4.384	1.556	2.121	0.212
LPC	0.4610	0.3536	0.0707	0.0000	0.8485
PS+PE	2.031	0.707	2.404	2.616	1.838
DPG+PA	3.662	2.333	1.344	1.485	6.647

Summary of Classified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
1	1	1	1	2.000	0.997
			2	13.928	0.003
			3	47.179	0.000
			4	126.188	0.000
2	1	1	1	2.000	1.000
			2	21.772	0.000
			3	27.532	0.000
			4	103.170	0.000
3	2	2	1	26.987	0.000
			2	2.000	1.000
			3	95.131	0.000
			4	215.826	0.000
4	2	2	1	8.713	0.034
			2	2.000	0.966
			3	58.964	0.000
			4	156.462	0.000
5	3	3	1	47.395	0.000
			2	85.805	0.000
			3	2.000	1.000
			4	22.635	0.000
6	3	3	1	27.316	0.000
			2	68.290	0.000
			3	2.000	1.000
			4	39.445	0.000
7	4	4	1	112.096	0.000
			2	178.434	0.000
			3	26.109	0.000
			4	2.000	1.000
8	4	4	1	117.262	0.000
			2	193.854	0.000
			3	35.971	0.000
			4	2.000	1.000

SECTION B
Discriminant Analysis

```
Linear Method for Response:   tillage
Predictors:  PC+PCpla  PS+PE  DPG+PA  PG

Group        1        2
Count        4        4
```

Summary of Classification

```
Put into       ....True Group....
Group              1        2
1                  4        0
2                  0        4
Total N            4        4
N Correct          4        4
Proportion     1.000    1.000

N =    8     N Correct =    8     Proportion Correct = 1.000

Squared Distance Between Groups
                1        2
1          0.0000  20.8822
2         20.8822   0.0000

Linear Discriminant Function for Group
                1        2
Constant  -844.01  -697.62
PC+PCpla    38.89    35.45
PS+PE     -102.63   -90.16
DPG+PA      58.92    51.80
PG          74.34    65.97

Variable   Pooled    Means for Group
             Mean        1        2
PC+PCpla   30.137   29.225   31.050
PS+PE      17.963   17.275   18.650
DPG+PA     20.137   20.400   19.875
PG         14.725   15.100   14.350
```

Variable	Pooled StDev	StDev for Group 1	StDev for Group 2
PC+PCpla	2.740	1.893	3.381
PS+PE	2.501	2.941	1.964
DPG+PA	4.006	3.766	4.233
PG	3.890	4.289	3.446

Summary of Classified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
1	1	1	1	3.728	0.982
			2	11.725	0.018
2	2	2	1	18.817	0.000
			2	2.803	1.000
3	1	1	1	3.223	1.000
			2	22.052	0.000
4	2	2	1	23.634	0.000
			2	3.093	1.000
5	1	1	1	1.809	1.000
			2	32.569	0.000
6	2	2	1	36.280	0.000
			2	3.564	1.000
7	1	1	1	2.911	1.000
			2	28.855	0.000
8	2	2	1	17.127	0.001
			2	2.869	0.999

Appendix 3. Phospholipid Fatty Acid Compostion Data

C. No.	T1-1H	T1-2H	T1-3H	T1-4H	T1-1L	T1-2L	T1-3L	T14L	N1-1H	N1-2H
1	0.88	1.20	1.21	0.69	0.61	0.92	0.88	0.34	1.07	0.77
3	1.35	1.35	1.22	1.03	1.18	1.18	1.18	0.86	1.17	1.19
6	1.14	1.08	1.10	0.87	0.95	1.01	0.99	0.73	0.99	1.00
10	2.38	1.97	2.19	1.84	1.85	1.86	2.32	1.54	1.93	1.68
166	0.12	0.34	0.36	0.32	0.21	0.00	0.16	0.14	0.10	0.23
212	1.32	1.06	1.04	1.16	1.38	1.27	0.98	1.14	1.21	0.99
228	0.95	0.94	0.84	0.79	0.98	0.82	0.80	0.76	0.79	0.91
241	0.15	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.20	0.58
247	0.53	0.48	0.48	0.52	0.58	0.48	0.45	0.50	0.61	0.63
256	4.87	4.92	4.74	4.39	5.08	4.82	5.21	4.37	4.99	5.83
260	4.95	5.08	5.15	4.49	4.45	3.97	4.16	3.78	4.00	4.49
272	0.39	0.46	0.40	0.37	0.42	0.38	0.43	0.38	0.40	0.21
274	0.17	0.30	0.11	0.04	0.04	0.09	0.22	0.03	0.00	0.00
292	0.68	0.71	0.61	0.65	0.58	0.58	0.71	0.54	0.68	0.71
300	1.94	2.00	1.92	1.96	1.84	1.74	1.77	1.61	2.06	2.11
306	1.79	1.55	1.55	1.62	1.69	1.59	1.64	1.54	1.75	1.62
309	6.65	5.81	5.86	6.19	6.05	5.58	5.83	5.89	6.37	5.75
310	0.45	0.42	0.36	0.42	0.41	0.35	0.32	0.37	0.46	0.50
313	4.27	3.64	3.43	4.43	3.99	3.58	3.43	4.01	5.45	4.62
317	9.50	9.68	9.78	9.32	9.54	9.09	8.86	9.33	10.20	10.27
318	0.19	0.45	0.12	0.22	0.51	0.53	0.56	0.22	0.06	0.41
334	0.24	0.00	0.00	0.00	0.00	0.00	0.47	0.00	0.25	0.58
336	7.13	7.06	7.31	6.61	7.54	8.10	8.10	7.09	6.97	6.97
337	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
339	0.81	0.54	0.95	0.71	0.78	0.70	0.70	0.63	0.75	0.78
345	1.61	1.60	1.63	1.49	1.60	1.59	1.76	1.38	1.72	1.87
349	1.85	1.85	2.02	1.69	1.58	1.53	1.59	1.34	1.77	1.87
352	0.70	0.75	0.66	0.73	0.67	0.62	0.64	0.65	0.71	0.67
356	2.62	2.61	2.57	2.56	2.59	2.45	2.57	2.37	2.66	2.87
362	0.60	0.58	0.60	0.52	0.70	0.73	0.86	0.58	0.63	0.65
378	0.35	0.38	0.37	0.33	0.39	0.38	0.39	0.35	0.50	0.50
380	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
387	0.09	0.00	0.14	0.17	0.17	0.26	0.14	0.15	0.00	0.00
389	0.09	0.09	0.19	0.19	0.11	0.00	0.00	0.00	0.10	0.09
392	1.63	1.34	2.03	2.53	1.92	1.72	1.92	3.56	1.86	2.57
394	6.58	6.15	6.54	6.49	6.70	6.69	6.66	7.00	6.75	6.80
397	8.09	9.48	7.82	11.65	9.29	11.06	8.76	12.82	11.40	9.73
399	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
401	1.08	1.01	0.99	1.00	1.07	1.11	1.05	1.05	1.24	1.13
404	2.07	2.09	2.16	1.98	2.08	2.02	2.04	1.92	2.26	2.15
406	0.77	1.43	0.81	2.19	0.95	2.04	0.97	2.48	1.18	1.12
417	0.00	0.10	0.13	0.05	0.33	0.36	0.17	0.32	0.35	0.27
420	1.41	1.45	1.61	1.23	1.24	1.22	1.27	1.00	1.15	1.25
422	0.50	0.64	0.75	0.61	0.68	0.77	0.64	0.54	0.52	0.40
435	0.05	0.05	0.23	0.18	0.08	0.00	0.04	0.00	0.00	0.02
436	0.23	0.18	0.00	0.00	0.00	0.10	0.00	0.13	0.09	0.00
437	0.32	0.19	0.46	0.45	0.00	0.15	0.14	0.00	0.08	0.00
442	4.03	3.87	4.16	3.83	4.59	5.05	5.29	4.76	4.59	4.38

457	0.56	0.39	0.55	0.45	0.25	0.22	0.24	0.16	0.17	0.33
461	0.47	0.30	0.37	0.40	0.46	0.36	0.37	0.40	0.24	0.22

C. No.	T1-1H	T1-2H	T1-3H	T1-4H	T1-1L	T1-2L	T1-3L	T1-4L	N1-1H	N1-2H
464	0.29	0.00	0.00	0.21	0.29	0.05	0.17	0.17	0.35	0.08
477	1.38	1.66	1.73	1.64	1.80	1.56	1.59	1.68	1.44	1.42
480	0.29	0.52	0.44	0.55	0.71	0.59	0.41	0.82	0.39	0.39
487	0.35	0.35	0.45	0.48	0.53	0.47	0.49	0.49	0.31	0.42
499	0.12	0.25	0.23	0.00	0.78	0.00	0.90	0.00	0.64	0.72
501	0.23	0.19	0.00	0.26	0.00	0.88	0.00	1.02	0.00	0.00
520	0.59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.70	0.69
523	0.67	1.40	1.48	0.90	0.92	1.01	1.02	0.70	0.00	0.00
530	0.23	0.14	0.28	0.19	0.24	0.24	0.27	0.24	0.00	0.00
557	0.00	0.10	0.00	0.10	0.00	0.00	0.45	0.00	0.00	0.00
573	0.37	0.54	0.54	0.54	0.45	0.31	0.54	0.31	0.34	0.36
668	0.00	0.09	0.00	0.17	0.00	0.00	0.00	0.06	0.00	0.00
685	0.17	0.30	0.23	0.46	0.42	0.34	0.52	0.39	0.00	0.00
689	0.20	0.30	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.08

C. No.	N1-3H	N1-4H	N1-1L	N1-2L	N1-3L	N1-4L	T2-1H	T2-2H	N2-1H	N2-2H
1	1.04	0.40	0.54	0.54	0.95	0.20	1.80	1.26	0.55	0.72
3	1.48	1.03	0.79	0.76	0.99	0.50	1.90	1.37	1.24	1.59
6	1.21	0.87	0.62	0.60	0.92	0.42	1.56	1.11	0.97	1.32
10	2.70	1.72	1.25	1.00	1.73	0.94	3.37	2.33	2.12	2.89
166	0.35	0.28	0.30	0.32	0.38	0.39	0.26	0.38	0.56	0.48
212	0.63	0.91	1.24	1.07	0.97	1.31	0.64	0.49	0.59	0.51
228	0.68	0.71	0.73	0.78	0.77	0.98	0.89	0.87	0.89	0.69
241	0.00	0.25	0.11	0.04	0.00	0.23	0.00	0.00	0.09	0.20
247	0.51	0.53	0.56	0.58	0.54	0.72	0.50	0.53	0.48	0.46
256	6.00	5.75	5.79	6.40	7.11	6.64	5.83	5.91	5.54	5.52
260	4.69	4.38	4.73	4.99	5.49	4.88	3.40	3.65	3.44	3.26
272	0.42	0.40	0.42	0.36	0.42	0.48	0.51	0.48	0.53	0.45
274	0.10	0.18	0.37	0.14	0.23	0.20	0.30	0.32	0.76	0.67
292	0.70	0.71	0.71	0.81	0.85	0.73	0.68	0.81	0.79	0.65
300	1.99	2.00	1.90	1.98	2.19	2.14	2.02	2.17	2.49	2.33
306	1.60	1.56	1.83	1.73	1.65	1.64	1.24	1.29	1.08	1.00
309	5.38	5.46	5.89	5.58	5.06	5.80	4.91	4.88	4.39	4.53
310	0.42	0.45	0.33	0.34	0.33	0.47	0.19	0.31	0.38	0.36
313	3.51	4.33	4.63	4.48	3.54	4.66	2.70	2.89	2.44	2.44
317	9.56	9.87	8.34	8.49	8.56	9.12	9.07	8.28	8.58	8.25
318	0.33	0.29	0.39	0.56	0.68	0.41	0.26	0.54	0.20	0.75
334	0.66	0.26	0.64	0.62	0.68	0.50	0.00	0.24	0.00	0.00
336	7.85	7.19	7.46	7.27	8.04	6.83	7.68	7.90	6.09	7.41
337	0.00	0.00	0.65	0.67	0.77	0.63	0.00	0.00	0.00	0.00
339	0.74	0.85	0.86	0.92	0.96	0.75	0.40	0.65	0.53	0.60
345	1.96	1.95	1.84	1.90	2.04	1.82	1.58	1.68	1.56	1.70
349	1.91	1.86	1.55	1.62	1.76	1.61	1.47	1.58	1.65	1.72
352	0.59	0.67	0.59	0.60	0.54	0.63	0.58	0.59	0.57	0.69
356	2.74	2.72	2.54	2.87	2.65	2.71	2.43	2.35	2.26	2.75
362	0.95	0.71	0.89	1.01	1.39	1.04	1.28	1.31	1.01	1.06
378	0.47	0.46	0.44	0.49	0.52	0.48	0.74	0.61	0.88	0.89
380	0.00	0.00	0.05	0.18	0.06	0.16	0.69	0.36	0.37	0.41
387	0.06	0.15	0.10	0.24	0.15	0.21	0.00	0.55	0.17	0.12
389	0.00	0.07	0.23	0.23	0.09	0.24	0.00	0.00	0.28	0.00
392	1.20	2.06	1.54	1.80	0.85	2.42	2.84	3.27	5.86	3.52
394	5.71	6.39	6.96	6.90	5.91	6.33	6.57	6.55	8.16	7.84
397	8.26	9.54	9.96	9.29	7.84	8.82	7.60	8.76	7.61	8.00
399	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.51	0.47
401	0.95	1.04	1.24	1.12	1.00	1.01	1.29	1.44	0.94	1.12
404	2.28	2.25	2.17	1.93	2.07	2.08	1.94	2.05	2.15	2.09
406	0.85	1.31	0.97	0.98	0.78	0.86	1.09	1.12	1.15	1.24
417	0.55	0.22	0.10	0.38	0.23	0.12	0.00	0.00	0.00	0.00
420	1.51	1.30	0.97	1.07	1.28	1.16	1.29	1.25	1.47	1.36
422	0.84	0.49	0.37	0.37	0.59	0.37	0.50	0.46	0.57	0.58
435	0.00	0.00	0.00	0.00	0.04	0.15	0.61	0.40	0.00	0.00
436	0.32	0.00	0.07	0.20	0.05	0.18	0.00	0.00	0.58	0.26
437	0.31	0.17	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00
442	5.38	4.77	5.95	5.72	5.65	5.43	7.11	6.71	6.40	7.90
457	0.54	0.43	0.20	0.21	0.25	0.27	0.47	0.39	0.32	0.39
461	0.35	0.53	0.74	0.40	0.31	0.49	0.46	0.57	0.63	0.48

C. No.	N1-3H	N14H	N1-1L	N1-2L	N1-3L	N1-4L	T2-1H	T2-2H	N2-1H	N2-2H
464	0.00	0.00	0.62	0.17	0.00	0.22	0.50	0.31	0.31	0.38
477	1.55	1.75	1.40	1.28	1.41	0.89	0.54	0.65	0.87	0.47
480	0.40	0.50	0.30	0.36	0.51	0.23	0.00	0.23	0.18	0.00
487	0.41	0.37	0.31	0.24	0.27	0.29	0.55	0.47	0.42	0.43
499	0.56	1.09	0.21	0.66	0.53	0.19	0.00	0.00	0.43	0.00
501	0.00	0.00	0.09	0.00	0.00	0.19	0.00	0.00	0.00	0.00
520	1.04	0.40	0.16	0.39	0.40	0.29	0.00	0.53	0.00	0.00
523	0.00	0.36	0.12	0.00	0.00	0.29	1.22	0.47	0.67	0.59
530	0.00	0.19	0.06	0.09	0.12	0.18	0.50	0.36	0.31	0.39
557	0.66	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00
573	0.74	0.58	0.21	0.24	0.38	0.37	0.61	0.40	0.45	0.48
668	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
685	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00
689	0.57	0.49	0.17	0.18	0.08	0.33	0.00	0.32	0.21	0.68

C. No.	T3-1H	T3-2H	N3-1H	N3-2H	T4-1H	T4-2H	N4-1H	N4-2H
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.81	0.21	0.30	0.27	0.07	0.21	0.16	0.10
6	0.86	0.15	0.28	0.22	0.05	0.18	0.14	0.08
10	0.95	0.25	0.31	0.32	0.11	0.32	0.27	0.19
166	0.10	0.16	0.08	0.15	0.14	0.21	0.15	0.10
212	1.15	1.23	1.03	0.79	2.00	1.18	0.74	0.83
228	0.94	0.95	0.84	0.81	1.17	1.04	0.67	0.67
241	0.05	0.08	0.05	0.05	0.00	0.05	0.02	0.02
247	0.54	0.56	0.71	0.62	0.95	0.73	0.58	0.63
256	4.88	4.46	5.97	5.56	4.95	6.35	5.71	5.10
260	4.39	3.90	4.69	4.20	3.64	4.41	4.38	4.01
272	0.55	0.53	0.46	0.47	0.56	0.25	0.43	0.41
274	0.13	0.14	0.16	0.06	0.10	0.09	0.00	0.00
292	0.60	0.56	0.69	0.67	0.48	0.63	0.73	0.62
300	1.91	1.79	1.91	1.81	1.88	2.06	2.01	1.83
306	1.77	1.77	2.12	1.95	2.36	2.26	2.19	2.12
309	6.21	5.95	6.94	7.21	9.98	8.39	8.42	8.50
310	0.00	0.00	0.00	0.29	0.58	0.43	0.41	0.38
313	4.51	4.27	4.60	4.69	7.62	5.27	5.07	5.25
317	10.47	11.48	9.72	9.94	10.22	9.38	9.99	9.66
318	0.16	0.00	0.50	0.28	0.00	0.46	0.65	0.41
334	0.50	0.38	0.72	0.61	0.00	0.35	0.82	0.59
336	5.98	5.55	6.94	7.76	5.39	7.70	9.49	7.62
337	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.28
339	0.80	0.68	0.99	0.94	0.62	0.88	1.01	0.89
345	1.56	1.44	1.80	1.81	1.29	1.73	1.94	1.62
349	1.72	1.57	1.74	1.67	1.21	1.54	1.68	1.48
352	0.64	0.75	0.69	0.70	0.79	0.74	0.83	0.81
356	2.29	2.02	2.79	2.95	2.31	3.15	3.32	3.08
362	0.61	0.57	0.63	0.57	0.33	0.80	0.83	0.58

378	0.37	0.38	0.44	0.46	0.00	0.22	0.00	0.00
380	0.04	0.05	0.04	0.02	0.03	0.06	0.02	0.05
387	0.09	0.28	0.34	0.35	0.00	0.00	0.00	0.00
389	0.00	0.25	0.26	0.25	1.95	0.38	0.20	0.00
392	4.76	5.74	1.93	1.92	5.27	2.72	1.33	2.14
394	7.46	9.10	7.09	7.81	7.27	6.95	7.77	7.74
397	7.58	8.05	9.20	10.35	10.71	9.89	11.42	15.82
399	0.00	0.00	0.00	0.00	0.48	0.35	0.27	0.00
401	1.30	1.28	1.48	1.81	0.90	1.22	1.59	1.58
404	2.05	2.03	2.06	2.28	1.98	2.02	2.19	2.13
406	0.65	0.79	0.84	0.99	1.44	1.00	1.06	1.35
417	0.05	0.07	0.19	0.15	0.05	0.04	0.09	0.14
420	1.12	1.06	1.12	1.17	0.81	1.44	1.40	1.22
422	0.38	0.37	0.28	0.14	0.00	0.00	0.00	0.00
435	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
436	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
437	0.59	0.56	0.20	0.19	0.00	0.40	0.00	0.08
442	3.22	3.42	4.10	4.68	2.84	3.79	4.73	4.37
457	0.44	0.38	0.12	0.22	0.00	0.00	0.09	0.08
461	0.68	0.95	0.86	0.73	1.26	0.93	0.89	1.06

C. No.	T3-1H	T3-2H	N3-1H	N3-2H	T4-1H	T4-2H	N4-1H	N4-2H
464	0.36	0.35	0.37	0.28	0.82	0.69	0.38	0.65
477	1.31	1.41	1.37	1.50	0.86	1.16	1.22	1.25
480	0.20	0.24	0.29	0.36	0.10	0.10	0.00	0.00
487	0.42	0.35	0.33	0.42	0.11	0.16	0.00	0.00
499	0.04	0.15	0.03	0.05	0.14	0.30	0.17	0.00
501	0.08	0.15	0.00	0.00	0.00	0.00	0.00	0.47
520	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
523	1.53	1.41	0.39	0.41	0.35	1.06	0.38	0.30
530	0.20	0.22	0.03	0.07	0.00	0.00	0.00	0.00
557	0.51	0.18	0.12	0.00	0.00	0.00	0.00	0.00
573	0.67	0.54	0.41	0.58	0.18	0.00	0.42	0.31
668	0.61	0.33	0.17	0.00	0.00	0.00	0.00	0.00
685	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
689	0.83	0.57	0.55	0.00	0.00	0.00	0.00	0.00

Appendix 4. Phospholipid Class Composition Data

Component	N1	T1	N2	T2	N3	T3	N4	T4
PC+PCplas	27.9	34.1	30.9	28.7	30.8	33.8	27.3	27.6
Pi	7.1	3.1	6.6	7.2	6.1	5.2	5.1	5.2
LPC	1.7	1.2	2.3	2.4	1.3	1.3	1.2	0
LPCplas	1.9	1.9	1	2.3	1	1.1	1.9	0
PS+PE+PEplas	17.8	18.8	13.3	16.7	17.6	21.3	20.4	17.8
DPG+PA	16	19.3	18.6	20.5	22.9	25	24.1	14.7
LPE	1.9	0	0	0	1.2	0	0	0
PG	19.4	14.1	9.9	11.9	13.4	12.1	17.7	19.3
LDPG	3.1	3.1	.6	1.1	3.2	0	1.5	1.9
Unknown	3.3	4.5	13.3	5.9	1.2	0	1.9	13.6
LPA	0	0	3.5	2.2	.7	0	0	0
LPG	0	0	0	1	.4	0	0	0