Identification of Signature Compounds for Breast Cancer Via Nuclear Magnetic Resonance

by

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Via Nuclear Magnetic Resonance

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Abstract

Chloroform methanol extracts from breast tissue were analyzed via ^{31}P, ^{1}H, and ^{13}C NMR. Tissue samples were obtained from the frozen section room at Saint Elizabeth Medical Center, Youngstown, Ohio. The extraction procedure was a chloroform methanol extraction that resulted in two layers, an chloroform layer and an aqueous layer. Via a series of changes in protocol, the chloroform layer revealed the presence of triglycerides of arachidonic acid and linoleic acid. During the many changes in the aqueous phase of the extraction procedure, a couple of peaks appeared via ^{31}P NMR analysis. One of the peaks was confirmed to be phosphoethanolamine and the other more commonly occurring peak was attributed to be inorganic phosphate.

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Appendix A
Glossary of Medical Terms

anesthesia	•To induce loss of feeling or sensation permitting performance of surgery.
atypia	•The condition of being irregular or not conforming to type.
cytotoxin	•A toxin or antibody that has a specific toxic action upon cells of certain organs.
epithelial cells	•Sheets of cells that cover the outer surfaces of the body and line the interior surfaces of body cavities and hollow body organs.
hyperplasia	•An abnormal increase in the number of normal cells.
intercalation	•The insertion between two objects.
localized	•Remaining or restricted to one area.
malignant	•Tendancy to become progressively worse and result in death. Having properties of malignancy and invasion, related to tumors.
menarche	•The onset of menstruation.
menopause	•The cessation of menstruation.
metastatic	•The transfer of disease form one organ or part to another not directly connected.

microcalcifications	•Small masses of calcium deposits.
mortality	•Destined to die.
pleiotropic	•Producing many effects in the phenotype.
postmenopausal	•After menopause.
premenopausal	•Before menopause.
spiculated	•A sharp, needle-like body.
stereotactic biopsy	•A system that uses three dimensional coordinates to locate the site to be operated on.

Appendix B
List of Medical Abbreviations

BSE	breast self examination
CM	chloroform / methanol
CT	computerized axial tomoraphy
DCIS	ductal carcinoma in situ
EIC	extensive intraductal component
ER	estrogen receptors
FBC	familial breast cancer
FFTP	first full-term pregnancy
GPC	glycerol 6-phosphocholine
GPE	glycerol 6-phosphoethanolamine
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
PCA	perchloric acid
PC	phosphocholine
PDE	phosphodiester
PE	phosphoethanolamine
PME	phosphomonoester
PR	progesterone receptors
TRAM	transverse rectus abdominus muscle flap

Appendix C
NMR Abbreviations

ACQ CPU	acquisition computer processing unit
ADC	Analog to digital conversion
β	Beta
B	external magnetic field, applied field
$CDCl_3$	deuterochloroform
DMF	dimethylformamide
DMSO	dimethylsulphoxide
D_2O	deuterated water
FID	free induction decay
kHz	kilohertz
LED	light emitting diode
MHz	megahertz
MRS	magnetic resonance spectroscopy
NMR	nuclear magnetic resonance
ppm	parts per million
PROMS	programmable read only memory
RAM	random access memory
rf	radio frequency
rps	revolutions per second
STM	sum to memory

T_1	longitudinal relaxation time or spin-lattice relaxation
T_2	transverse relaxation time or spin-spin relaxation
T^*_2	effective transverse relaxation time
TMS	tetramethlysilane
γ	gamma, gyromagnetic ratio
μ	mu, magnetic moment
α	alpha
ω_L	Larmor circular frequency
σ	sigma, shielding constant

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Part I. Introduction

Chapter 1 Breast Cancer

Section 1: What is Breast Cancer?

Over 150,000 women in the United States are diagnosed each year with a disease that will leave one third of them dead and the rest scarred physically and emotionally.[1] This extraordinary disease that is particularly prevalent in the Western World and leaves many women consumed with anxiety is breast cancer. Breast cancer not only threatens one's longevity but also physically distorts one's body. The victim has to wake up every day to face either an impending, premature death or living with a mangled body. This no doubt leads to an emotional disturbance that will inadvertently also affect the victim's spouse, family, friends, and co-workers. As a consequence, cancer patients are encouraged to seek counseling and support groups to aid in coping with life.

Breast cancer is a heterogeneous disease with the risk factors targeted at gender and age; most patients are females and are greater than 50 years of age.[1] Those who experience breast cancer at an age less then fifty have a slightly greater mortality rate.[2] The death rate due to these tumors can be greatly reduced, even if not completely eliminated, if the tumor is caught during the final stage of a series of functional and structural changes of epithelial cells.[2]

The progression of these changes start with normal epithelial cells, leading to hyperplasia (an abnormal increase in the number of normal cells), atypia (condition of

being of irregular type), and finally to DCIS (ductal carcinoma in situ). As stated, if the tumor was diagnosed during the DCIS stage, a complete cure would be feasible. However, it can take two to three years before a lump is detected via regular clinical examination or breast self examinations (BSE); and, in the meantime, the tumor may have already spread to other parts of the body. A second and more ideal form of detection may be to identify the tumor before it becomes malignant via tumor markers such as small molecules or proteins. Unfortunately, "No such tumor marker exist at present."[2a]

Two schools of thought exist as to how the tumor spreads. The first is the Halsted theory which states that the cancer originates in the epithelium of the breast, remains localized for a period of time, and then spreads systematically to local and regional targets. These targets are the skin and lymph nodes. Finally, the cancer spreads to distant organs such as the brain, liver, and bone. This theory suggests that a local cure will promote a higher overall cure rate.

The second theory is called the Systemic theory. Within this theory, the primary tumor is thought to spread directly to local and distant organs. The Systemic theory states that the pituitary tumor metastasizes very early in the course of cancer development leading to a low probabitity of recovery.

Based on clinical and experimental evidence, each theory is partially correct.[2] As a result, the form of therapy that is most likely to succeed is determined by the stage of the cancer. For example, a nonmetastatic and non invasive or localized cancer may require only local therapy; where as a metastatic cancer may need systemic therapy.

On a biological level, cancerous tissue offers a few observable characteristics that differ from those of non-cancerous tissue. These biological characteristics are due to the most obvious feature of cancer, being the phenemon of rapidly dividing cells. In fact, the following are biological features that are required for rapid cell division to occur.

First of all, malignant tissue exhibits an increased amount of phosphomonoesters (PME) and phosphodiesters (PDE) compared to benign or normal tissues. The primary phosphodiesters are glycerol 3 - phosphethanolamine (GPE) and glycerol 3 - phosphocholine (GPC). PME are the lipid component of biological membranes. It is believed that the increase in concentration of PME is reflective of membrane turnover.[4,5]

In addition to the increase in the above mentioned phospholipids, there are other compounds which demonstrate a significant increase or decrease in their concentration in cancerous tissues. For example, lactate, a by-product of glycolysis, displays a significant increase in concentration. (see Figure 1.1.1)

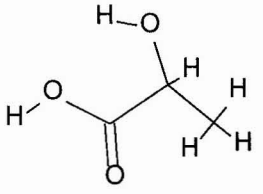


Figure 1.1.1 Diagram of the molecule lactate.

This increase in lactate is due to decreased oxygen availability in the oxygen deprived cancerous tissue mass. It is the increase in anaerobic metabolism which leads to lactate production. The production of lactate is seen in the scheme below.[4,6] (see Figure 1.1.2)

glucose

↓ glycolysis → ATP

2 pyruvate → 2 Lactate (anaerobic)

↓ (aerobic)

2 acetyl CoA

Figure 1.1.2 Lactate Production in Anaerobic Metabolism

Another biological finding in cancerous tissue was the increase in succinate, which is ultimately another by-product of glucose metabolism. The acetyl CoA formed in scheme I reacts with oxaloacetate in the first step of the citric acid cycle. Succinate is formed five steps later.[4,6] (see Figure 1.1.3)

Acetyl CoA

↓

Citrate

↓

Isocitrate

↓

α-ketoglutarate

↓

Succinyl CoA

↓

Succinate

Figure 1.1.3 Formaton of Succinate

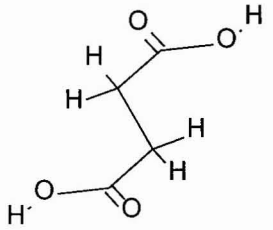


Figure 1.1.4 The molecule succinate, a by-product of glucose.

As expected, due to reduced circulation of oxygen there is an observed decrease in glucose concentration. Again, this is because glucose is the starting material for lactate and also for the citric acid cycle in which succinate is formed. Another finding was a decrease in inositol. Inositol is utilized during the synthesis of acidic phospholipids.[4,6]

In addition to all of the other observable changes in metabolite concentrations, there are the changes in concentrations of the nucleosides. These nucleosides include the following types: nucleoside monophosphate (NMP), nucleoside diphosphate (NDP), and nucleoside triphosphate (NTP). Nucleosides are formed from the joining of purine and pyrimidine bases to ribose-phosphate or deoxyribose-phosphate.[6] Increased nucleotide biosynthesis is necessary for DNA replication and thus cell division, which is more rapid in caner cells.

Section 2: Who is at Risk?

As discussed earlier, the two most important risk factors for breast cancer are age and gender. However, other factors also aid in determining the risk for breast cancer. The first is residence in the Western World. This place of residence bears some correlation with diet. More specifically, the consumption of animal fat found in foods such as butter, cheese, ham, and sausage may by related to the incidence of breast cancer.[3] It has been demonstrated that as ethnic groups migrate from one region to another, they adopt culturally determined habits of their new homes and this alters their incidence of breast cancer. For example, Japanese women who live in Japan are at a lower risk for breast cancer than are American citizens of Japanese ancestry. With this thought in mind, it can be said that genetic predisposition is not the sole factor for determining risk factors.[2,3]

A second factor that places women at higher risk is their mothers' and sisters' histories of breast cancer. If a woman's mother or sister is diagnosed with premenopausal breast cancer, then the individual is at higher risk. On the other hand, women with a family history of postmenopausal breast cancer do not display any increased risk. An increased sensitivity for cells to turn cancerous may possibly be due to heredity. Another contributing factor is environmental exposure to natural radiation, viruses, or chemicals. A last contributing factor is the natural deficiency of certain enzymes that destroy carcinogenic chemicals.[2,3]

A third risk factor for breast cancer is race. Caucasian females greater than forty years of age are more prone to breast cancer than are Blacks of the same age. However, Caucasians and Blacks have a similar risk for breast cancer when they are under the age of forty years. Another, perhaps surprising risk factor, is that those living in a higher socio-economic class are at an increased risk for this disease. For most cancers, lower socio-economic classes are at a higher cancer risk presumably because of insufficient access to health care, lack of knowledge concerning preventive measures, and exposure to carcinogens. However, breast cancer behaves in a contrarien fashion. "How higher socio-economic attainment translates into higher breast cancer risk is not certain."[3a] One suggestion is that animal fat intake is linked to income which causes the link of income to breast cancer. Obesity in women is another risk factor for breast cancer. The incidence for breast cancer in obese women is slightly higher than in lean women. In addition, the mortality rate for obese women is much higher due to the inability to detect the tumor because of the obesity of the women. The last of the more important factors is incomplete breast feeding.[3] A study of women from Hong Kong supports this statement. It was discovered that these women inadvertently breast feed a greater percentage of the time from the right breast; hence the left breast suffered from incomplete breast feeding and years later, usually resulted in left sided breast cancer. Therefore, it has been suggested that lactation has some sort of protection effect.[2]

Some less important risk factors include a delay before the first full-term of pregnancy (FFTP), which may be correlated with income and education, menarche (onset

of menstruation) before twelve years of age, and menopause after the age of fifty five.[3] These factors seem to involve the endocrine system.

The increased presence of breast cancer in women suggests that female hormones play a role in the disease.[3] Ovarian function has been linked with breast cancer based on evidence dealing with the age at onset of menarche and/or menopause. Menarche commences according to critical female body mass proportional to the females height. Females who lose weight due to anorexia neurosa or vigorous atheletic training during college years develop amenorrhoea resulting in fewer ovarian cycles and decreased chance to develop breast cancer. At the other end of the time line, women who begin menopause around the age of forty-five also have a decreased number of ovarian cycles and therefore a decreased risk of development.[2,3]

Even though genetic disposition does not play a major role in the population in general, familial and hereditary breast cancer does exist. Familial breast cancer (FBC) refers to a family whose genetic factors interact with common environmental factors so as to increase the risk of breast cancer.[3] This is observed in first degree relatives, that is, their natural mother and / or sister. Persons who have first degree relatives with familial breast cancer have a three-fold, higher risk factor and also have a greater tendency to develop breast cancer postmenopausal (after menopause). The other, hereditary breast cancer, deals with more than one disease. It is a heterogeneous group of breast cancer prone disorders,[3] meaning it may be associated with other tumors such as brain tumors, breast ovary syndrome and sarcoma, or leukemia.[2] In addition, hereditary breast cancer demonstrates an earlier age of onset. However, after the age of fifty, the risk of breast

cancer is no greater for an individual with hereditary breast cancer than for one without hereditary breast cancer.

All told, for familial and hereditary breast cancer, "it is not possible to make a distinction between hereditary (genetic) and familial (polygenic/sporadic) disease so that risks have been blurred."[2b] This is possible due to the many genes that play a part in hereditary breast cancer.[2]

Section 3: Detection and Treatment

"The main benefit of screening is prolonged life but another anticipated benefit is less costly and less unpleasant treatment."[3b] The American Cancer Society suggests that yearly physical exams should commence at age 40 and that a mammogram should be taken every one to two years between the ages 40 and 50. At age 50 and older, they recommend that a mammogram should be done yearly. Those who are at high risk due to family history should consider mammography screening earlier in life.[1] In addition, the American Cancer Society also suggests that baseline mammograms should be taken at age 35.[2] Statistically, it has been shown that 90% of diagnosed breast cancers were found through the combined efforts of physical exams and mammography. Mammography can detect microcalcifications (150 μm or smaller) and areas of distorted dense tissue. In conjunction, physical exams detect palpable cancers that do not necessarily appear on mammograms, possibly due to dense breast tissue.[1]

A radiological technologist must be specially trained in order to perform a high quality mammogram that possesses clarity and image contrast. The mammography technique used currently in the United States is film screen mammography via x-ray exposure. With this technique, microcalicifications as small as 40 μm can be detected and characterized.[1]

Features observed in mammography x-rays can be used to divide breast cancer into two categories. The first category is a spiculated, soft tissue mass which is the most specific feature of cancer. The second category which is seen in 60% of the

mammography detected breast cancers is microcalcifications. In addition, mammography is used as an accurate guide for the biopsy of a nonpalpable mass. This method that is commonly used in the United States is called the Mammography Guided Hookwire Needle Localization Technique.[1] After the specimen has been removed, specimen radiology is performed to confirm the removal of the abnormality.[1]

Aside from mammography, other methods of detecting breast cancer are available. For example, ultrasound is useful in distinguishing between cysts and solid masses. Another method is CT (computerized axial tomography) also sometimes referred to as a CAT scan. CT is helpful in locating a mass near the chest wall. Other methods that are still under investigation include magnetic resonance imaging and digital mammography.[1] All of the above screening programs are sometimes called "secondary prevention."[3]

Screening for the detection of breast cancer does pose some disadvantages. One drawback is the possibility of the induction of the radiation-induced cancer by mammogram. A delay of at least ten years is seen before the development of breast cancer due to exposure of radiation. For example, a series of five mammograms is equivalent to one rad which is enough radiation exposure to trigger the development of breast cancer in some patients. Another screening process that possesses its own disadvantages is the BSE (Breast Self Examination). Monthly BSE's are good in that cancerous masses can be detected earlier than the once a year clinical exam. However, the prize in discovering a mass is not a pleasant one and as a result many patients may delay seeking treatment. Therefore, the success of BSE is dependent on psych-social

factors. A third disadvantage is based on the distress a patient feels in the form of anger when screening processes did not detect cancer at a curable stage. As a result, doctors are inclined to perform biopsies as screening procedures to avoid missing cancer at a stage that can be cured. Biopsies are financially more costly, carry a small risk of postoperative mortality due to general anesthesia, possess a delay in healing, scar pain, and breast disfigurement.[3] In conclusion, even with the shortcomings of screening for breast cancer, screening is still justifiable when considering the number of deaths the cancer can cause.[3]

Once breast cancer has been diagnosed via mammogram, physical exams, SBE, or any method, the next step is treatment. The first step is to obtain a physical exam and complete medical history incorporating the patient's history along with their family history. This aids in assessing the type of surgical procedure and anesthesia to be employed. The next step is biopsy. The goal of the procedure is to determine how far along the cancer has progressed. There are different types of biopsies, each possessing individual advantages and disadvantages. One such type of biopsy is aspiration / needle biopsy. Aspiration via a needle is fast, efficient, and is performed in a doctor's office with or without a local anesthetic. Fine needle aspiration is used to obtain a cellular specimen from a solid mass. Depending upon the pathologist's experience, the sample may be inadequate or not accurate. Another form of biopsy that is fast is an incisional biopsy. In this form of biopsy, the in situ disease can be evaluated. However, the seriousness of the disease may be downplayed. Incisional biopsy is used in evaluating large masses that are possibly malignant. Excisional biopsy follows along the same lines

as an incisional biopsy, except that the mass is completely removed. Another form of biopsy used for a nonpalpable mass is needle directed excisional biopsy. However, this technique also shares the same drawback as incisional biopsy of possibly down playing the extent of the tumor. A final form of biopsy is stereotactic breast biopsy, which uses three dimensional coordinates to locate the site. Stereotactic breast biopsy offers three positive attributes. First of all, this form of biopsy utilizes accurate image guidance, meaning that the mass is not located based on mere chance. Secondly, this procedure can be performed on an out patient basis and not in surgery. And lastly, stereotactic breast biopsy is cost efficient.[7]

The final step, based on the results of the biopsy and size of the tumor, is to decide between conservative surgery with radiation or a mastectomy. If the tumor has an extensive intraductal component (EIC) around or within it, then the chance of recurrence is possible. The risk of recurrence is reduced if a wider excision or a mastectomy is performed. When should a mastectomy be performed? Certain circumstances dictate when a mastectomy should be performed. These circumstances are the following: patient preference, pregnancy, anticipated poor cosmetic results, and quality of radiation available.[1,2]

A patient who undergoes a mastectomy is generally encouraged to also have reconstructive surgery at the same time. There are two forms of reconstructive surgery. The first is an implant. A pocket is formed for the implant, however, if the pocket is too small, an expander is temporarily placed under the muscle. Once the pocket is large enough, the expander is removed and the implant takes its place. The second type of

reconstructive surgery is the utilization of a flap of the patients tissue, either from their back or abdomen called latissimus dorsi myocutaneous flap or transverse rectus abdominus muscle flap (TRAM), respectively. This tissue is used to reconstruct the breast.[1,2,7]

Breast deformity can be avoided when performing conservative surgery by maintaining a specific pattern of incisions called Cosmesis. Conservative therapy followed by radiation is limited to patients based on their age and preference, tumor size and location, and histologic factors.[1,7]

Both, mastectomy and conservative therapy possess risks. These drawbacks are the following: infection, edema of arm adjacent to breast that had node dissection, bleeding, deformity of breast, and scarring.

If the tumor has metastasized to other organs at the time of diagnosis, then in addition to local therapy, systemic therapy must also be exercised. There are two forms of systemic therapy. The first is chemotherapy. Chemotherapy consists of cytotoxic agents that attack the cell resulting in cell death. Some examples of chemotheraputic drugs include Methotrexate, 5-fluorouracil, cyclophosphamide, and doxorubicin. The second form of systemic therapy is hormone therapy. Polypeptides and steroids are two types of hormones that influence breast cancer cells.[1,2,7]

Chemotherapeutic agents are pleiotropic (having many different actions) and are therefore classified according to their primary action. For example, some agents interfere with DNA replication by forming cross linkages of DNA strands via alkylation of the base pairs of DNA. Another form of DNA interference deals intercalation of the drug

into the double helix structure. In addition, cytotoxins, also called antimetabolites, inhibit the synthesis of purine and pyrimidine. Normal cells are also affected by these cytotoxic agents. However, these agents tend to work on cells in more active stages of mitosis, these being the neoplastic cells. Other organs that have a high cellular growth rate also display associated side effects such as hair loss, bone marrow depression, and gastrointestional mucosal function. [1,2]

Estrogen receptors (ER) and progesterone receptors (PR) are found in some breast cancers. A high level of ER and PR in breast tumors is indicative of a better chance of recovery. A higher concentration of these receptors leads to an increased response to endocrine therapy. Hormone therapy either ceases the production of estrogen or hinders the uptake of estrogen by tissues dependent of the hormone.[1] Tamoxifen is an example of an agent used in hormone therapy. This agent binds to the ER with a greater affinity than estradiol. At this point in time, Tamoxifen is still undergoing further studies.[2]

Works Cited

1. Hayes, D. F. *Atlas of Breast Cancer*; Mosby Europe Limited, London, **1993**.

2. Fentiman, I. S. *Prevention of Breast Cancer*; R.G. Landes Company, Austin, **1993**. 2a page 2, 2b page 16.

3. Stoll, B. A. *Woman at High Risk to Breast Cancer*, Kluwer Academic Publishers, London, **1993**. 3a page 21, 3b page 95. .

4. Smith, T.A.D., Glagollm, J., Leach, M.O., Machin, L., McCready, V.R. "The Effect of Intra-tumour Heterogeneity on the Distribution of Phosphorous-containing Metabolites with in Human Breast Tumours: an In Vitro Study Using ^{31}P NMR Spectroscopy," *NMR in Biomedicine.* Vol. 4, 262-267, **1991**.

5. Gribbestad, I. S., Petersen, S. B., Fjosne, H. E., and colleges. "^{1}H NMR Spectroscopic Characterization of Perchloric Acid Extracts from Breast Carcinomas and Non-involved Breast Tissue", *NMR in Biomedicine.* Vol. 7, 181-194, **1994**.

6. Moran, L. A., Scrimgeour, K. G, Horton, H. R., Ochs, R. S., Rawn, J. D. *Biochemistry*, Neil Patterson Publishers, Englewood Clliffs NJ, **1994,** pp 10.5, 15.2, 20.30.

7. Wells, S. A.Jr., Young, L. V., and Andriole, D. A. *Atlas of Breast Surgery*, Mosby Europe Limited, London, **1993**.

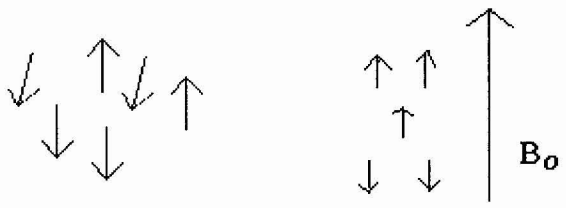
Chapter 2 NMR Basics

Section I: The Nucleus and Nuclear Magnetic Resonance Spectroscopy

The biochemical compounds that are increased in tumor cells are identified by NMR spectroscopy. There are many nuclei that can give NMR signals. Nuclei with a spin quantum number of I=1/2 are the most suitable nuclei for NMR analysis. Examples of such nuclei include hydrogen (^{1}H), phosphorus (^{31}P), fluorine (^{19}F), carbon (^{13}C), silicon (^{29}Si), and nitrogen (^{15}N). If the spin quantum number, I, equals zero, the nucleus does not possess a magnetic moment and is therefore not detectable by NMR. These nuclei do affect the chemical environment of neighboring nuclei, modifying their chemical shifts. A chemical shift is the change in frequency of a resonating nuclei. Examples of nuclei that are not NMR active include carbon (^{12}C), oxygen (^{16}O), sulfur (^{32}S), and silicon (^{28}Si). On the other hand, if the spin quantum number is equal to or greater than one, the nucleus exhibits both quadrupole and magnetic moments. The quadrupole moment aides in relaxing the nuclear spin. Due to the rapid relaxation, NMR signals of nuclei having $I \geq 1$ are broadened and are generally relatively difficult to observe (e.g. chloride (^{35}Cl and ^{37}Cl) (I = 3/2 for both), bromide (^{79}Br) (I = 3/2), boron (^{10}B and ^{11}B) (I = 3 and 3/2 respectively), nitrogen (^{14}N) (I = 1), and iodide (^{126}I)) (I = 5/2).[1]

What happens to these nuclei in an NMR spectrometer? First, the frequency of radiation used to determine NMR signals depends on the nucleus involved and the

applied magnetic field. With commercial Nuclear Magnetic Resonance spectrometers, the frequencies used are in the "FM" radio range between 800 MHz and 5 MHz. These values depend on the magnitude of the values for the magnetogyric ratio, γ. Gamma, γ, is a ratio of magnetic moment to angular momentum. When there is no applied field, the magnetic moment, μ is best described as many vectors randomly distributed in space. When a magnetic field is applied, the spins become oriented. (see Figure 2.1.1.a and b) Angular momentum is represented by the circular motion around the Z axis.



(a) (b)

Figure 2.1.1 Magnetic Vectors at Random Angles (a) in the absense of an applied field and (b) oriented by an external magnetic field. Each small arrow represents direction of a magnetic vector within a nucleus.

In most cases, γ is positive, indicating that the β states (i.e. spins are antiparallel to the magnetic field) are above the α states (i.e. paralled to the magnetic field). Alpha, α is represented by M_I when the spin quantum number, I is equal to +1/2 ($\uparrow$) where as β corresponds to M_I equal to -1/2 ($\downarrow$).[2-6] (see Figure 2.1.2)

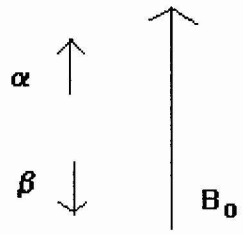


Figure 2.1.2 α and β States in a Magnetic Field.

As the external magnetic field strength is increased from zero, a couple of changes take place. First of all, the magnetic moment vectors, μ, come into phase and precess (sweep) around the Z-axis. (see Figure 2.1.3) This precession occurs at the so-called Larmor frequency. The greater the magnetic field, the greater the Larmor frequency.

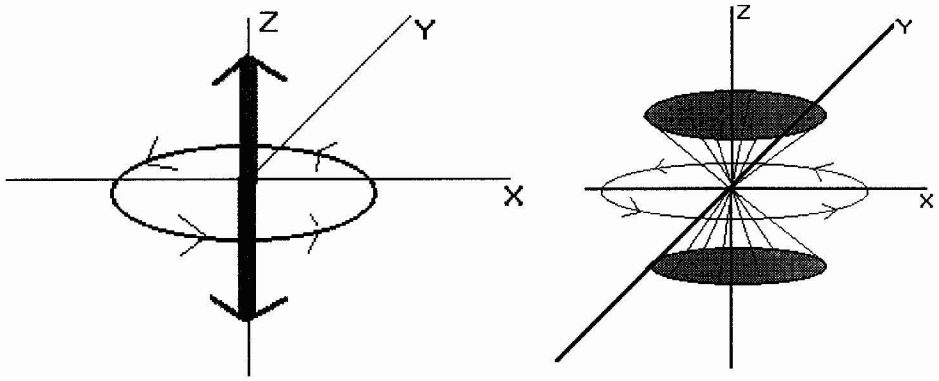


Figure 2.1.3 Magnetic Vectors Precessing Around Z-axis.

Secondly, the energies of the α and β spin states change states. If the magnetic moment is greater than zero, the α spins (i.e. parallel to the magnetic field) move to a lower energy level and the β spins move to a higher energy level. Before the magnetic field

was applied, there were equal populations of α and β spin states. However, after the magnetic field was applied, there were more α spins than β spins.[2,4,6]

Populations of the spin states are represented by the Boltzman distribution equation:

$$N_\beta / N_\alpha = e^{-h\omega/2\pi kT}$$

$$\text{Let: } \omega = \gamma B_o$$

$$\text{Then: } N_\beta / N_\alpha = e^{-h\gamma/2\pi kTBo}$$

ω_L is the larmor precession frequency, a function of gamma. Planck's constant is *h*. The Boltzman constant is *k*. B_o is the magnetic field. The imbalance of the spin states due to the applied field causes a net magnetization.[5,6] (see Figure 2.1.4)

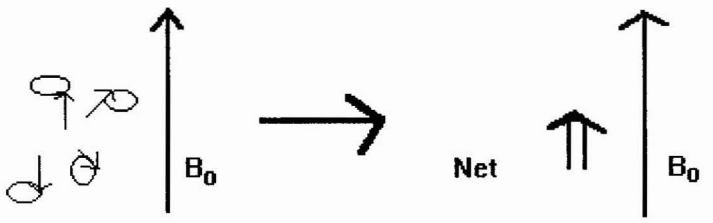


Figure 2.1.4 Imbalance of Spin States Resulting in Net Magnetization.

If a transient RF field at the resonance frequency of the nucleus is applied perpendicular to the magnetic field, B for a certain period of time, the magnetic moment vector will tilt toward the XY plane. The amount of time that the pulse is applied is in microseconds and is inversely related to the strength of the applied transient field. This pulse, if applied from the Y-axis, flips the net magnetization along the Y-axis. The

detector, which is also a radio frequency coil, is placed on the Y-axis and records the signal from the rotating magnetic vectors.[4,6] (see Figure 2.1.5)

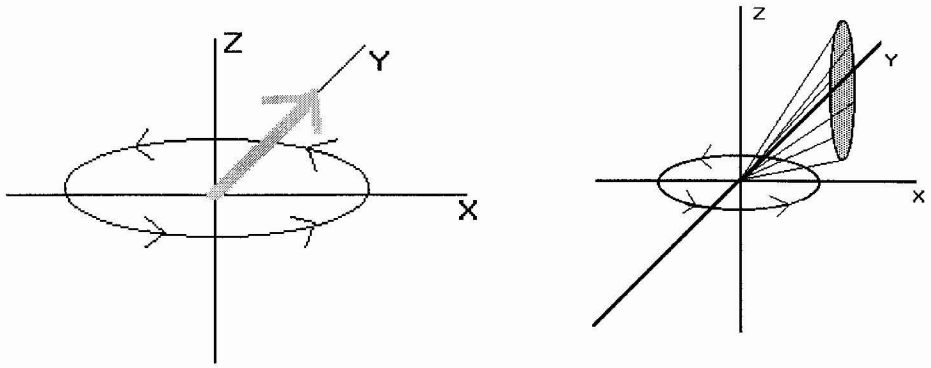


Figure 2.1.5 Magnetic Vector after a 90^{O} Pulse.

Resonance is reached when the larmor frequency of the nucleus is almost equivalent to the radio frequency of the applied field. At this point, energy is absorbed by the sample. The magnetic vectors of other nuclear types i.e. ^{13}C and ^{1}H precess at different rates (according to their environment) displaying exponential decays of the magnetic vectors as they relax to their original thermal alignments parallel or opposed to the permanent field. The signal and the exponential decay of the signal is displayed through what is called free induction decay, FID. The FID is the sum of the decay of different nuclei that precess at different frequencies and is represented by superimposed sine waves. (see Figure 2.1.6)

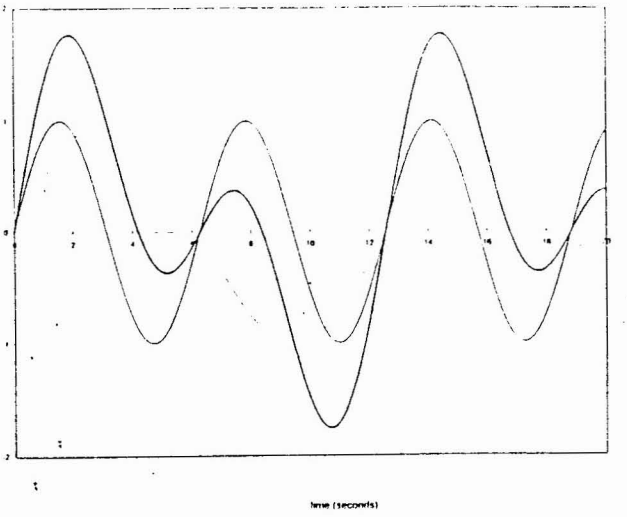


Figure 2.1.6 The sine of 1 Hz is represented by (- - -) line. The sine of 0.5 Hz is represented by the lighter (- - -) line. And, the sum of the two sines is represented by a dark, (—) solid line.

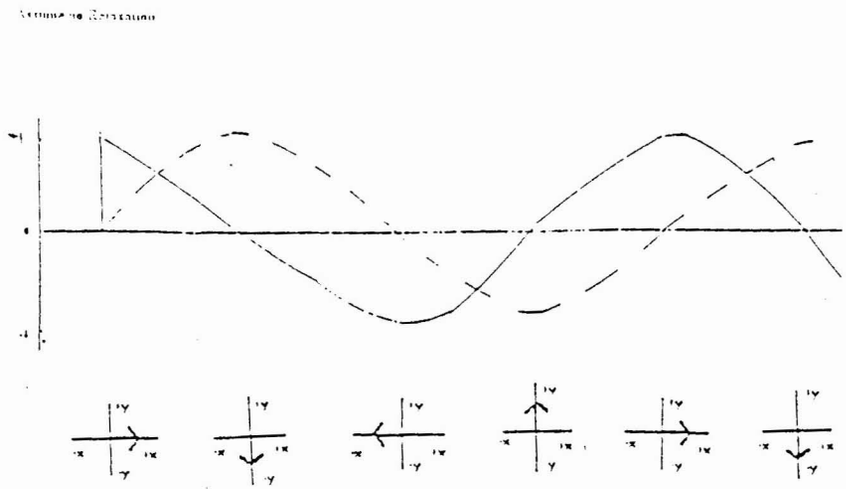
The FID signal is expressed by:

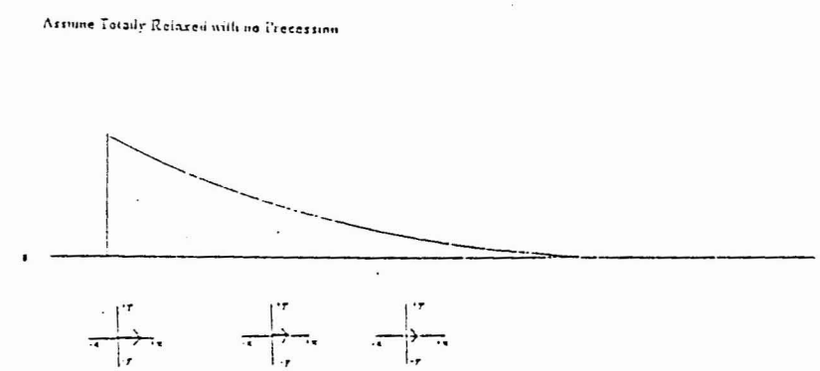
FID Intensity = Net Magnetization × Periodicity Term × Relaxation Term

$$FID = A \cos \omega_O t \, e^{-t/T}$$

A = amplitude, $T_{observed}$ = experimental transverse relaxation time

Through mathematical expressions, this FID undergoes fourier transformation, resulting in a spectra of possible singlets, doublets, triplets, etc. It can then be said that fourier transformation is the analysis of the FID's oscillating harmonic components. The exponential decay of the magnetic vectors deals with what is called relaxation time. (see Figure 2.1.7)





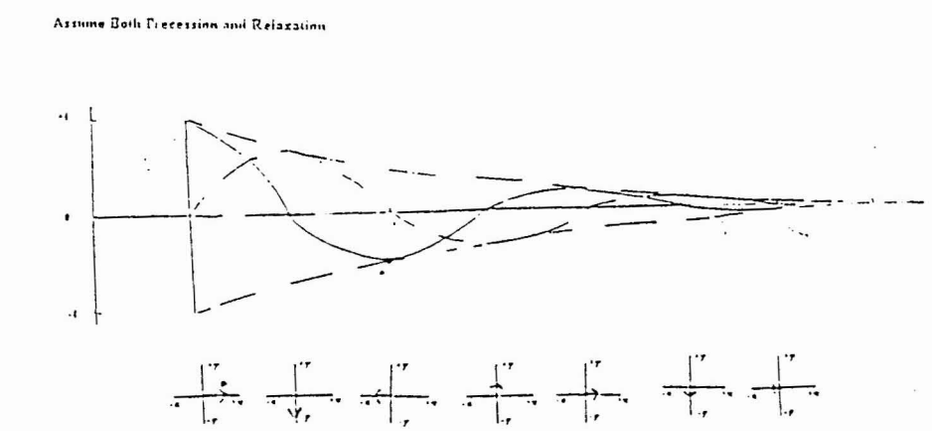


Figure 2.1.7 The top graph represents no relaxation with precesion, the middle graph represents total relaxation with no precession, and the bottom graph represents both relaxation and precession.

Since the magnetic spins are not in thermal equilibrium when a field is applied, they try to return to their equilibrium state. Once in thermal equilibrium, there are more α than β states.[4,6]

There are two mechanisms that explain why the net magnetic vector in the XY plane shrinks. The first explanation is called longitudinal relaxation time, T_1. As stated earlier, after a 90^O pulse, there are equal numbers of α and β spins. As these nuclei return back to their potential energy state, being more α than β spins, and energy is given up to the lattice. The lattice is considered the surrounding environment including neighboring nuclei in the same and surrounding molecules. Another name for this relaxation process is called spin-lattice relaxation. Spin-lattice relaxation is dependent upon the magnetic fields that are created due to the moving nuclei.[4,6]

A second form of relaxation is called transverse relaxation time, T_2. Transverse relaxation deals with how the spin of one nucleus interacts with the spin of neighboring nuclei. After a 90^O pulse, the spin's are all grouped together resulting in a large magnetic vector. However, this grouping of spins is not in thermal equilibrium and therefore tends to spread out in all angles around the Z-axis. This dephasing of the spins occurs exponentially. Since transverse relaxation time deals with the different larmor precession rates leading to the dephasing of the spins, another name for T_2 is spin-spin relaxation. (see Figure 2.1.8)

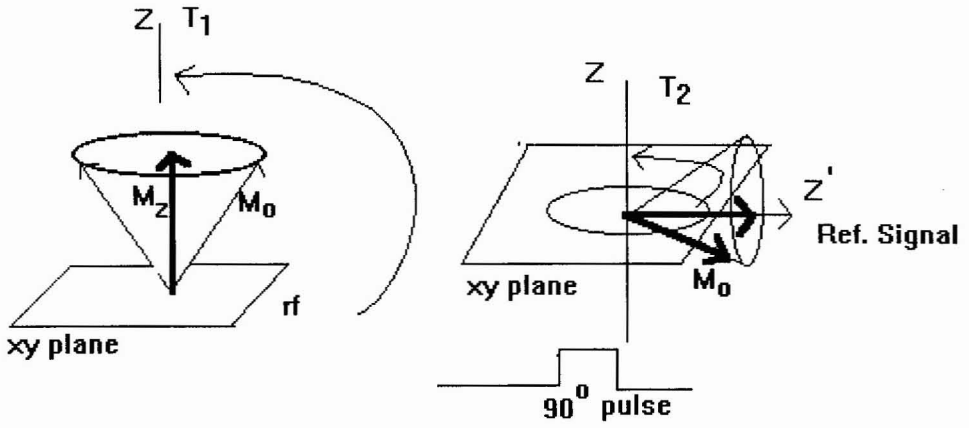


Figure 2.1.8 T_1 and T_2 Relaxation.

The exponential decay due to T_2 causes broadening of the spectral line. The expression for the spectral line width at half height is the following:

$$\Delta\nu_{1/2} = 1/\pi T_2$$

This is true if the field is homogenous. Unfortunately, the field is not completely homogenous and the spectral lines are broadened. This broadening is called inhomogenous broadening. T_2 is then represented as T_2^*, effective transverse relaxation time.[4-6]

Section 2: Components of the YSU NMR Spectrometer

The GEMINI 2000 Spectrometer located in Ward Beecher at Youngstown State University is a 400 MHz Fourier Transform NMR spectrometer operating at 9.34 Tesla and is composed of four major units. The first is the operator station which consists of a Sun computer, monitor, keyboard, optical mouse, plotter, and disk drives. This station allows the operator to select, monitor, process, save, and plot the data of NMR experiments.[8]

The second major unit is the NMR console. The console contains the bulk of the spectrometer's electronics including both analogue and analogue / digital converters. Visible in the rear of the NMR console is the digital cardcage, RF cardcage, blower assembly, the console power supply, the ACQ CPU mode switch, and the consoles ON/OFF switch.[8]

The third major unit of the NMR console is the preamplifier. There is a left leg and a right leg that collectively make-up what is called the preamplifier. More specifically, the left leg consists of three preamplifiers and control circuits. The control circuits are located on the outside of the leg and are the following:

1. meter for visually displaying the spinner rps
2. LED indicator light which is lighted when the spinner turns 10 rps or faster
3. speed control knob for adjusting the rps of the spinner
4. eject button for manual insertion and ejection of the sample

5. switch which allows selection of meter operation, either rps or amplitude of the tune signal
6. tune meter sensitivity knob

The inside of the left leg is composed of a Spinner Sense Tachometer board which transmits signals to the spinner LED and also to the acquisition system regarding the spin status. The Tachometer board also plays a role when displaying spinner speed by driving the meter. The air valves are also located inside the left leg. The air valves are controlled by the eject button when inserting or ejecting a sample from the magnet. The right magnet leg has two meters. One displays probe body cooling air flow rate and the other displays variable temperature air flow rates.[7-8]

The last major component of the NMR console is the magnet. The magnet assembly consists of a superconducting, gradient-compensated solenoid. (see Figure 2.2.1)

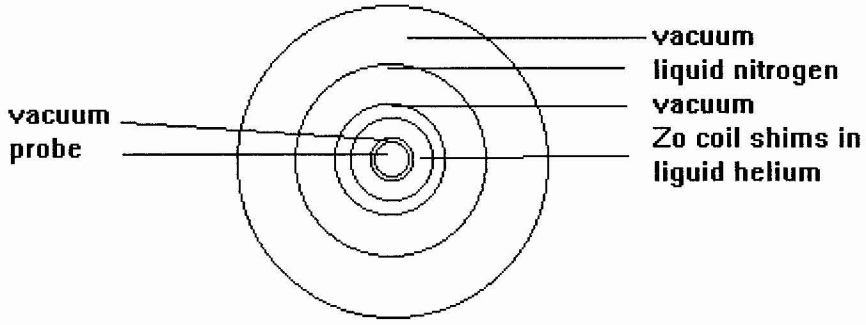


Figure 2.2.1 Cross Section Diagram of Solenoid.

The solenoid is immersed in liquid Helium which is contained within a cryostat and is maintained at a temperature 4.215 Kelvin. An outer jacket of liquid Nitrogen acts as a "radiation shield." As the liquid Nitrogen and Helium boil off as gases, they are vented to the atmosphere through vents at the top of the cryostat. The 'shims' are a set of field solenoid coils wrapped around the inside of the bore of the magnet. There are two sets of gradient coils. The first is a superconducting set that is adjusted only when installed which are in the liquid Helium cryostat. The second is the room temperature set that is adjusted with the insertion of each sample.[2] These shim coils surround the sample and counteract any field gradients to produce a homogeneous field. As a result, the spectrum displays peaks that are sharper and more defined and intense.[1]

The probe is inserted in the base of the cryostat in the active region of the magnet and holds the sample in the highest and most homogenous magnetic field region. This location is in the center of a coil called the Z_o gradient coil.[2] This superconducting N_b / T_a wire carries about 60 Amps. of current and is several miles long. These coils are used when shimming a sample. The probe is interchangeable depending on the experiment conducted. The probes currently at Youngstown State University are broadbanded, one for 5mm and one for 10 mm sample tubes. The probe assemble consists of an upper barrel that extends through the center at the top of the Dewar (cryostat) and the probe that inserts from the base of magnet. These two pieces are tightly joined together. The upper barrel contains the sample spinning mechanism, the tachometer pickup and the pneumatic sample insertion and ejection system. The sample which is in a 5 mm or 10 mm cylindrical NMR tube is placed into the upper barrel and transported to the upper part of

the probe via cushions of air. The sample is spun by an air turbine on its vertical axis.[7] The probe, gradient coils, and sample are all maintained at room temperature.[2] (see Figure 2.2.2)

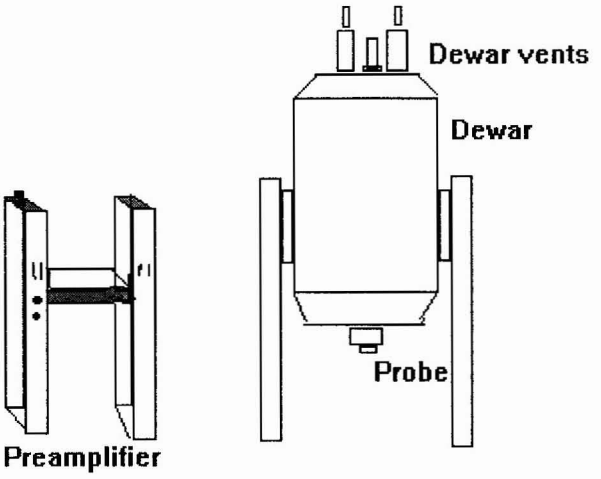


Figure 2.2.2 NMR Magnet Assembly.

Section 3 : Description of NMR Electronic Components

The Gemini 2000 is a broadband system that we currently use to observes ^{1}H, ^{13}C, ^{31}P, and ^{19}F nuclei with all other NMR active nuclei also observable. The Gemini 2000 can also decouple highfield (i.e. ^{1}H and ^{19}F) and lowfield (i.e. ^{13}C and ^{31}P) nuclei.[3,4] The electronic components of the Gemini 2000 spectrometer are the following:

I. host computer system

II. acquisition computer system

III. RF (radio frequency) system

I. The host computer system also called the data station, provides the menus for selecting, controlling, and viewing the results.

II. The second electronic unit, the acquisition computer system, contains four printed-circuit boards within the digital cardcage.[7,8] They are the following:

1. The Ethernet Interphase Controller board has 16k x 8-bit PROM plus 512 kB of DRAM.
2. The acquisition CPU board controls the acquisition system.
3. The Pulsed Controller board controls the RF system and manages pulse lengths, delays, frequency selections, and much more.
4. The Sum to Memory (STM) / Analog-to-Digital Conversion (ADC) board receives FID (free induction decay) signals from the rf (radio frequency), samples 0^{o} and 90^{o} NMR signals simultaneously, and digitizes the FID

signals for the Host computer. The STM/ADC board is a 12-bit 100 kHz ADC board.

III. Thirdly, the RF signals are generated by the RF boards. There are many RF boards and they are located on the lower cardcage. Some examples of RF boards include the following: RF control board, master clock board, and frequency transmitter. The following is a description of how the RF boards operate. First of all, the observe transmitter channel selects the frequency and power level of the RF signal that is used to irradiate the sample. Then, the RF signal is stabilized by the master clock board. The frequencies for irradiation are derived by ^{1}H and broadband transmitters and the correct power level is set by the dual-channel linear power amplifier. Finally, the receiver sets the gain, the signals are detected, and the FID is filtered for use by the acquisition system. The preamplifiers, which are located near the probe so as to avoid interference of unwanted, amplify NMR signals that are processed by the receiver. Sometimes the operator may wish to detach the ^{1}H nucleus from another nucleus such as ^{13}C, ^{15}N, or ^{31}P via the decoupled system.[7]

The Lock system is responsible for monitoring the intrinsic drift of the magnetic field by locking on a deuterium signal in the sample.[1] There are three parameters that need to be adjusted for optimal results. These parameters are the following: lock power, lock gain, and lock phase. The lock gain amplifies the signal received. Even though the gain does not directly affect the sample, if it is set too high, the spectra will have a lot of noise. On the other hand, if the lock power is too high the sample is directly affected resulting in broad bands and variations in amplitude. This is because the sample can not

relax due to the high level of energy going into it. The last parameter to be mentioned is the lock phase. The lock phase is adjusted for maximum lock signal. If the lock phase is off, the lock signal will be erratic and the shim will not adjust very well.[1]

Section 4: Basic NMR Procedures

Routine operation of the Gemini 2000 Spectrometer for this project included observations of ^{1}H, ^{13}C, and ^{31}P spectra. Variations of these experiments will be discussed in detail as performed in the experimental section.

The first step toward analyzing a sample is selection of a solvent. When choosing a NMR solvent from the many that are available, a few requirements must be taken into account. For example, the sample must be soluble in the solvent. Also, one must be aware that the solvent signals may cover sample peaks. Solvent viscosity is another factor that will affect the resolution. For example, less viscous solvents result in more rapid tumbling and more viscous solvents result in slower tumbling and increased line broadening.[2] The solvents classified as viscous include benzene, DMSO, dimethylformamide (DMF), pyridine, water, and toluene. Some non-viscous solvents are the following: acetone, chloroform, acetonitrile, dichloromethane, and methanol. Acetone often gives the highest resolution while more viscous solvents such as aromatics give poorer resolution.[2]

The most economical deuterated solvents include D_2O (heavy water) and $CDCl_3$ (deuterochloroform) of which the former is the solvent of choice for biomolecules and the latter is the solvent of choice for organic compounds. Other solvents such as deuterobenzene, deuterotoluene, deuteroacetone, deuterodimethylsulphoxide (DMSO), and deuteromethanol are more costly due to the many steps involved in the preparation of deuterated products.

Once the solvent has been chosen, the sample is placed in a 5 mm or 10 mm NMR tube that has already been cleaned. It should be noted that brand new NMR tubes are not very clean and need to be cleaned before use. When cleaning the NMR tube, acetone and distilled water are recommended while a small test tube brush should be avoided to prevent scoring of the glass. Oven drying a NMR tube allows dust to enter the tube and also alters the tube dimensions. A more preferable technique consists of blowing filtered, nitrogen gas into the NMR tube.[2]

Finally, to run a sample using the Varian NMR, the standard sample must first be removed by typing *e* for ejection or by manually pushing the ejection/insertion button located on the preamplifier. This standard sample helps maintain the lock system. When preparing to insert the sample to be tested, first align the sample properly in the NMR spinner turbine and make sure the NMR tube and turbine are free of fingerprints and other smudges. Then place the sample in the top of the Dewar where it is maintained by the eject air flow. Type *i* for insert or use the manual ejection/insertion button. Via decreased air flow, the sample will be lowered to the top of the probe. The green LED indicator light will be a solid green light when the spinner turns 10 rps or greater. At this point, using the mouse, *connect* on the acquisition menu. Once in the acquisition menu, click on *lock* and adjust the lock level to approximately 60-70%. If the lock level is too high, "saturation" will set in causing band broadening and variation in amplitude. The lock gain, for the most part is always set at 30. The lock gain is adjusted by increments of ten and provide the following adjustments: 10, 20, and 30. Thirty is used mainly for

chloroform while 10 and 20 are used for solvents having more than one deuterium molecule such as benzene, acetone, or toluene.

Next, the shims need to be adjusted. First, click on *shim* and notice Z1C, Z2C, Z1, Z2, Z3, and Z4 are present. Z1C and Z2C are course adjustments of Z1 and Z2, respectively and are the only adjustments that will be used in adjusting the shim on a routine basis. The Z1, Z2, Z3, and Z4 are fine adjustments and not routinely used by the operator. To begin shimming, using the Z1C and Z2C, choosing one at a time, adjust by increments of ± 1 until each component is fully optimized. Note that one component is not meaningful alone, each component interacts with the other for an optimum shim. When adjusting the Z1C and Z2C, if the lock level is maximized, return to *lock* and readjust the lock level to 60-70%. The bars for adjusting the shims will be all red when the lock level is maximized. While shimming, if the Z1C and Z2C bars are erratic, then adjust the lock phase by increments of ± 4 or ± 16 which is located by clicking on the *lock*.

When shimming has been fully optimized, *disconnect* and move the cursor to the upper left box, click the right mouse button, and type **SU** (set up). At this time, the console, preamplifier, and data station are communicating to one another and when they are ready, "*Set Up Complete*" will flash on the screen.

Using the mouse, click on *Main Menu*, *Set Up,* and select the type of nucleus desired (e.g. ^{1}H/chloroform). At this point, parameters for that nucleus will appear at the bottom of the screen and are in the following table:

sfrq	sweep frequency
tn	transmitter nucleus, type of nucleus selected
at	acquisition time, amount of time to collect the FID in seconds
np	number of points (np = 2 x at x sw)
sw	sweep width, the width of the window that the spectrometer will observe
fb	filter bandwidth
bs	block size, the number of collected transients sent to the data station, is of particular value in lengthy overnight acquisitions, bs preserves some data in the case of a crash.
tpwr	transmitter power level with linear amplifiers
pw	pulse width
d1	delay
tof	transmitter offset frequency, sets the center of the sweep width
nt	number of transients, sets number of transients to be collected
ct	collected transients, number of transients actually collected
alock	automatic lock
gain	sets amplifier gain
il	interleave arrayed and 2 D experiments
in	interlock
dp	double precision, gives maximum quality of data and word length
sp	start of plot, starting position of spectrum

wp	width of plot, width of spectrum in display window
vs	vertical scale, sets the height of the spectrum, routinely, vs = 160, sets the tallest peak on the screen
sc	start of chart in X - axis
wc	width of chart
hzmm	scaling factor for plots
is	integral scale
rfl	reference peak position
rfp	reference peak frequency
th	threshold
ins	integral normalization scale
dfrq	transmitter frequency of first decoupler
dn	decoupler nucleus
dpwr	decoupler power
dof	decoupler offset
dm	decoupler mode, turns decoupler on or off
dmm	decoupler modulation mode
dmf	decoupler modulation frequency

Referenced from **VNMR Command and Parameter Reference**, by Varian associates Inc. , 1995.[4]

To run the spectra, click on *Main Menu, acquire,* then *go/wft.* Go/wft (go, weighted fourier transform) tells the spectrometer to go and collect the data, then via various fudge factors, weighs the FID prior to fourier transforming the spectra. The FID is raw data represented by superimposed sine waves and can be displayed by typing **df** or **dfid**. The spectrum is obtained by foruier transforming the FID.

Once the spectrum has been collected, the next step is to process the spectra. First, click *Main Menu, process,* and then type **aph** (autophase) to phase the peaks so that the peaks are all above the baseline. Manual phasing may be required in some cases. To set the scale, first locate the internal standard. For example, in a chloroform solvent which contains 0.03%TMS (tetramethylsilane) , TMS could be used as the internal standard and is defined as being at 0 ppm. Once the internal standard has been located, expand around the standard, it may be necessary to increase the vertical scale temporarily, and place a cursor on the center of the peak. Next, type **nl** (nearest line) to align the line in the center of the peak, then type **rl(0p)** meaning the reference peak position is at zero ppm (parts per million), using TMS as the internal standard. Finally, type **dscale** to put a scale at the bottom of the spectra and type **dres** to determine the linewidth. A successful shim is determined by the linewidth. Linewidth is peak width at half the peak height. An acceptable line width is about 0.5 Hz or lower.

Now that the scale has been set and a good shim has been obtained, the vertical scale can be set at vs=160. As mentioned earlier, by using 160, the tallest peak will now be on the top of the screen. The width of plot (wp), how wide the plot is, and the start of plot (sp), where the plot begins, are determined by the operator and typed on the screen.

In addition, proton spectra need to be integrated. This will be a valuable aid in determining the structure of the spectra. To do this, first, click *Main Menu, process, integrate* (either full or part), *next, lvl/tlt,* and adjust the green line on the screen so that it looks like a series of level steps. When finished, the unnecessary integration lines can be clipped by clicking *resets* and using the cursor, clip around each peak.

Finally, to avoid any unnecessary peaks due to noise, the threshold can be set by clicking *th* representing threshold (while still in the same menu). A yellow line will appear, place the yellow line just below the shortest peak that is a part of the spectra.

The spectra is now ready to be printed. To do so, click *Main Menu, display, plot, plot, scale* (prints the scale), *all parameters* (prints the parameters that were listed prior along with any modifications), *peaks (*mainly for ^{13}C, prints ppm above each peak), and *page* (tells the printer to go ahead and print).

Section 4 up to this point has given a descriptive explanation of routine NMR procedures. A simplified version of the procedure that void the descriptive explanations are located on the following pages of this section. In addition, at this point, it should be noted that the following procedure with a few minor alternations was used for teaching sophomore organic students routine hands on operation of the NMR and in conjunction, was my first step in learning routine testing of the NMR.

BASIC VARIAN NMR PROCEDURES

1. Sign in Login Book and log into the computer.
2. ejection of sample: e

   insertion of sample: i (make sure green light is on and stays on)
3. Acquisition Menu

   * <CONNECT>
   * <LOCK>
   * adjust lock level to 60-70%
   * Lock gain is always at 30
   * <SHIM>
     - adjust Z1C and Z2C (course adjustment) using increments of ±1
     - if max out, will be all red, will then have to go to <LOCK> and lower lock power to 60-70% then return to shim Z1C and Z2C.
     - if shimming bounces around a lot, adjust lock phase using ± 4 or ± 16 increments by first going to <LOCK> then once adjusted return to <SHIM>
     - when shimming is good, <DISCONNECT> (ready to collect spectra)
4. Move cursor to upper left box, click, type SU and enter. Wait till says, "*Set up complete*".
5. <MAIN MENU>

* <SET UP>

* Select type of spectra you are going to run (e.g., proton, chloroform)

6. To run spectra:

* <MAIN MENU>

* <ACQUIRE>

  - 'show time' will tell you how long will take to run spectra

* <GO, WFT>

7. To process spectra:

* <MAIN MENU>

* <PROCESS>

  - type aph (autophase) to realign peaks
  - to get a scale, expand around the internal standard, put a cursor on it, type nl, type rl(0p), type dscale, type dres. Dres shows the linewidth value, dres helps determine peak at 1/2 height. Good linewidth is 0.5 Hz or less.
  - type VS = 160 (for ^{1}H and ^{13}C)
  - type wp = 10p and sp = -.5p for ^{1}H
  - expand on double peaks (wp = 2 ppm) for ^{1}H
  - type wp = 200p and sp = -.5p for ^{13}C
  - integrate ^{1}H by doing the following: <MAIN MENU>, <PROCESS>, <INTEGRATE> (full or part), <NEXT>, <LVL/TLT>, and to clip around the peaks, <RESETS>.

8. To print:

   * <MAIN MENU>

   * <DISPLAY>

   * <PLOT>

9. <PLOT>, <SCALE>, <ALL PARAMETERS>, <PEAKS> (for ^{13}C only), <PAGE>

Section 5: Analysis of the NMR Spectra

To analyze NMR spectra, a brief review of Section I of this chapter may be helpful along with an explanation of the shielding effect. When an external magnetic field is applied, a smaller magnetic field is produced from the circulating electrons around the nucleus. This second magnetic field is proportional to, yet opposes the external magnetic field. This is expressed in the following equation:

$$\delta B = -\sigma B$$

Sigma, σ is called the shielding constant and is dimensionless. The shielding constant, σ may be due to paramagnetic or diamagnetic contributions.[4-6]

Diamagnetic contributions evolve when the applied field produces a circulation of electrons in the ground state. This circulation of electrons produces an opposing magnetic field which shields the nucleus. Diamagnetic contributions are positive ($\sigma_d > 0$) and they shield the nucleus. The density of the circulating electrons is proportional to the degree of shielding of a proton along with the role that other groups play that are attached to the proton. This is called the inductive effect.[4-6]

On the other hand, paramagnetic contributions, are negative ($\sigma_p < 0$) and they deshield the nucleus. Paramagnetic contributions arise when the applied field forces the electrons to circulate throughout the molecule and fill the unoccupied orbitals. The degree of paramagnetic contributions depends on how easily the electrons move to the

unoccupied orbitals. This is indirectly proportional to the distance between the highest filled and lowest empty orbitals.[4-6]

These diamagnetic and paramagnetic currents create what is called the neighboring group contribution. Depending on the nucleus's environment, these currents will shield or deshield the nucleus. The shielding constant depends on three factors.

1. Changes in magnetic susceptibilities parallel and orthogonal to a group.
2. The angle, θ, that the magnetic vector is to a group.
3. The distance the nucleus is from a group.

An example is acetylene, HC≡CH. This molecule is linear and has shielded protons. However, if the protons were perpendicular, then they would be deshielded. On the other hand, protons near a double bond are just the opposite. Since ethene is non-linear, a paramagnetic current is created parallel to the axis.[3,4,6]

The effect that shielding has on proton nuclei is very small, only a few parts per million (ppm). However, the NMR is so sensitive that it can easily detect the shielding effects. The change in the frequency the nucleus resonates is called the chemical shift. As previously discussed, chemical shifts vary according to the environment. If the environment is the same, the chemical shift will be the same.

In order to accurately set a scale each time a sample is analyzed, one must use a reference. A commonly used proton NMR reference is tetramethylsilane, TMS. TMS gives a single, sharp peak at 0 ppm. TMS is used as a standard because it is inexpersive, available in almost all solvents, and easy to view in spectra. The measured chemical shift is dimensionless and is calculated by the following expression:

$$\delta = 10^6 \bullet ((\nu_{sample} - \nu_{reference}) / \nu_{reference})$$

$\nu_{reference}$ for ^1H may be the observed frequency, TMS.[6] A common, internal reference for ^{13}C is chloroform which has a chemical shift of about 77 ppm and is used in my work as a defined value of 77.0 ppm. A more frequently used reference for ^{31}P is trimethylphosphate (TMP) which has a chemical shift of 61.0 ppm.

Some common chemical shifts for ^1H, ^{13}C, and ^{31}P are listed in the following diagrams. (see Figure 2.5.1)

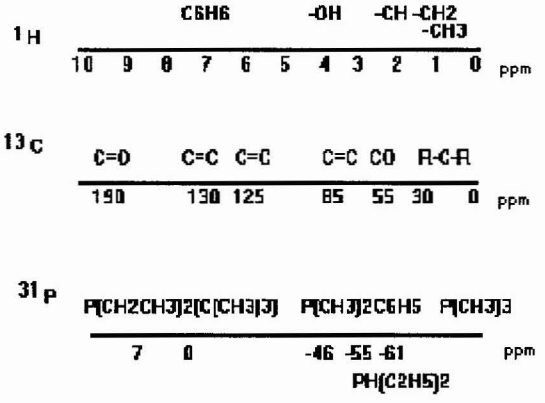


Figure 2.5.1 Common Chemical Shifts for ^1H, ^{13}C, and ^{31}P.

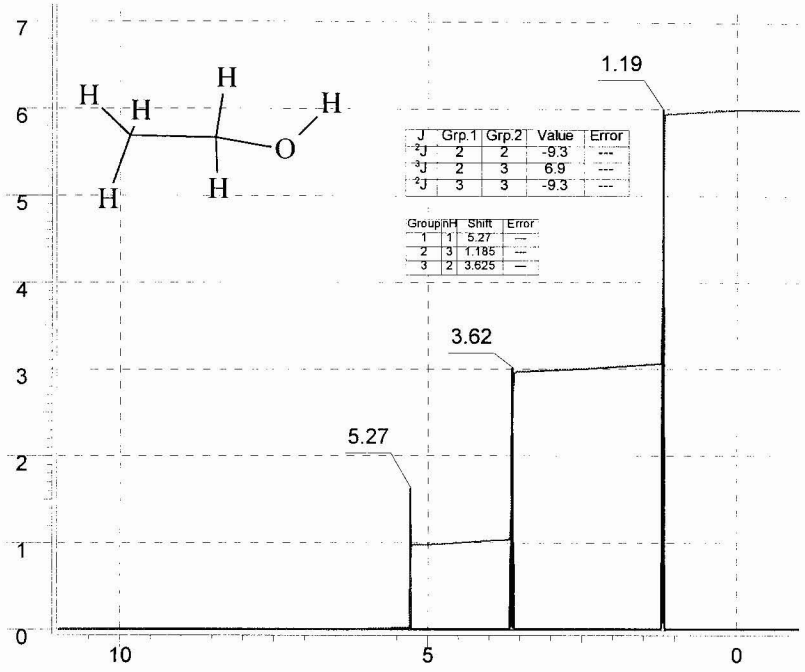
A common compound used to illustrate coupling and chemical shifts is ethanol, CH_3CH_2OH. CH_3 and CH_2 experience spin-spin splitting from one another. The CH_3 group interacts with the nucleus spins of the CH_2 group resulting in a triplet. This triplet is considered a splitting pattern and is called the CH_3 group's multiplicity. The

multiplicity is determined by the number of neighboring coupled protons plus one. This expression is represented by n+1.[9] The multiplicity of the CH_2 group is determined by the spin-spin splitting of the neighboring protons on the CH_3 group. Since there are three protons,

$$3+1=4.$$

Therefore, the multiplicity of the CH_2 group is a quartet.[3,4,6]

Unless the alcohol is dry and at a neutral pH, the hydroxyl proton (– OH) will be a singlet. This is due to the rapid exchange of –OH protons between molecules which removes the coupling between the -OH and -CH_2 groups.[5] Each absorption peak is represented by an integration line. Integration is the measurement of the area under a peak which is proportional to the number of hydrogens. (see Figure 2.5.2)



J	Grp.1	Grp.2	Value	Error
^{2}J	2	2	-9.3	---
^{3}J	2	3	6.9	---
^{2}J	3	3	-9.3	---

Group	nH	Shift	Error
1	1	5.27	---
2	3	1.185	---
3	2	3.625	---

Figure 2.5.2 A Proton NMR Spectra of Ethanol.

Another commonly analyzed nuclei is the ^{13}C nuclei. The shifts for ^{13}C nuclei are greater than that of ^{1}H nuclei. This is because ^{13}C nuclei have denser electron clouds resulting in greater shifts.[4,6] ^{13}C nuclei have a natural abundance of only about 1.1% and have only about 1/5700 the sensitivity of ^{1}H. Nondecoupled ^{13}C spectra is difficult to interpret due to the many overlapping multiplets. However, if the protons are decoupled, the result is a single sharp peak for ^{13}C.[9]

^{31}P nuclei have a natural abundance of 100%. ^{31}P nuclei follow the same rules for multiplicity as in ^{1}H nuclei. ^{31}P NMR is an easy spectra to observe. The protons are decoupled via broadband irradiation, yielding reduced line widths and an increased signal to noise ratio. The most commonly used external reference is phosphoric acid, H_3PO_4.[9,11]

Works Cited

1. Akitt, J.W. *NMR and Chemistry, an Introduction to Modern NMR Spectroscopy*; Chapman and Hall, New York, **1992**.

2. Derome, A. E. *Modern NMR Techniques for Chemistry Research*; Pergamon Press, New York, **1987**.

3. Pople, J. A.; Schneider, W. G.; and Bernstein, H. J. *High-Resolution Nuclear Magnetic Resonance*; McGraw-Hill Book Company, New York, **1959**; pp 4-11.

4. Moore, W. J. *Basic Physical Chemistry*; Englewood Cliffs, Prentice-Hall, New Jersey, **1983**.

5. Graybeal, J. D. *Molecular Spectroscopy*; McGraw-Hill Book Company, **1988**; pp 537-546, 200-250.

6. Atkins, P. *Physical Chemistry*; W. H. Freeman and Company, New York, **1994**; pp 625-652.

7. Kilman, C. *Introduction to Gemini 2000;* Varian Associates Inc., Califorina, **1994**.

8. *VNMR Command and Parameter Reference;* Varian Associates Inc., Califorina, **1995**.

9. Silverstein, R.M. et al. *Spectrophotometric Identification of Organic Compounds;* John Wiley & Sons, New York, **1991**.

10. Sanders, J. K.M. and Hunter, B. K. *Modern NMR Spectroscopy;* Oxford University Press, New York, **1993**.

11. Mason, J. *Multinuclear NMR*, Plenum Press, New York, **1987**, pp 369-370.

Chapter 3 NMR in the World of Oncology

Section 1: Advances of NMR into the Land of Medicine

"Researchers are now anxiously trying to discover whether we can apply MRS to grade and classify tumors, which could prove to be the most important medical contribution of the technique in the short term."[1] (Magnetic Resonance Spectroscopy, MRS, is spectroscopy based on the chemical shift.) The question is, what does nuclear magnetic resonance have to do with breast cancer? The use of NMR spectroscopy is being researched as a possible non-invasive tool in the world of oncology. Such roles that the NMR might partake in include diagnosis; monitoring of chemo- and radiation therapy, hormonal response, and metastatic aggressiveness; and tumor grading.[2,3]

A possible scenario may be the following sequence of events. To start with, a patient was diagnosed with breast cancer via Nuclear Magnetic Resonance or MRI even before a mass was visible by way of a mammogram. This speedy diagnosis was due to the discovery of certain tumor markers. As mentioned earlier, some tell-tale signs included an increase in concentration of PME, PDE, lactate, and certain nucleotides, along with a decrease in glucose concentration. Once the tumor was diagnosed, action was taken in battling the disease. In traditional methods, weeks may have passed before the detection of tumor response to chemo- or radiation therapy. However, with the advances made in nuclear magnetic resonance, increases and decreases in concentration of certain metabolites, such as PME and PDE, allow for speedier analysis of tumor

response to the treatment. For example, if the tumor is resistant to the drug used in chemotherapy, the mass will continue to grow, or at the very least, maintain its original size. However, if the cancer is susceptible to the drug, the mass will begin the decrease in size leading to a decrease in concentration of the metabolites mentioned earlier.

Section 2: What is Involved in Sample Preparation?

Tissue samples undergo specific extraction procedures. These extraction procedures must be implemented in order to analyze phospholipids and their metabolites, sugars such as glucose, and nucleotides. The two extraction procedures of interest are oerchloric acid (PCA) and chloroform methanol (CM) extraction's. Perchloric Acid extracts only water soluble metabolites. These metabolites are analyzed mainly by ^{1}H NMR. ^{1}H NMR allows the possible detection of metabolites along with sugars, amino acids, and lactate.[6] However, ^{31}P NMR is also useful in perchloric acid extraction's for the detection of lipid and phosphate metabolism. Chloroform methanol is employed mainly for the extraction of the tumors lipid components.[4] The key form of analysis for the chloroform methanol extraction's is ^{31}P NMR.

A point of interest in relation to the extraction protocol and NMR analysis of the extraction's is the stability of tissue samples. Sample stability was evaluated via a time study protocol. These studies were concerned with the changes in tissue metabolites once the blood supply was severed. At the point in which the tissue was removed from the blood supply, metabolite concentration either increased, such as lactate, or decreased depending on their nature. Consequently, an important quality control issue dealt with whether the increase or decrease in concentration was due to the cancer or the time factor once the tissue was removed from the blood supply. The time studies were employed starting at eight minutes after resection, then 28, 45, and 85 minutes. At these set time intervals, the tissue samples were placed in liquid nitrogen to halt any metabolic activity.

The results of the study indicated that PC, PE, nucleoside triphosphate, GPC, and GPE are stable at room temperature for about one and a half hours from the time of resection. However, it is still unknown as to the changes that may occur in the first few minutes that the tissue is removed from the blood supply.[5] The unknown changes within the first few minutes is mainly due to the difficulty in getting the tissue sample so soon after the resection.

Section 3: How are the Samples Analyzed?

Different nuclei are observed by NMR in biological studies. Proton (^{1}H) NMR has the greatest sensitivity and has been studied more extensively over the last few years.[6] In addition, ^{1}H NMR has recently gained the advantage of using shorter acquisition times and smaller sample volumes.[6] Phosphorous (^{31}P) NMR is also quite sensitive and has been used to determine the concentration of phosphorus containing metabolites. With ^{31}P NMR, studies have suggested that PME are increased in breast cancer and this increase is related to the rate of cell proliferation.[7] ^{31}P NMR is considered more valuable than ^{1}H NMR in breast cancer studies even though it has one fifteenth the sensitivity of proton. Why is ^{31}P NMR so valuable as opposed to ^{1}H NMR? ^{31}P NMR is helpful in providing information about the biological aspect of tissues. On the other hand, ^{1}H NMR is difficult to evaluate due to the extreme overlapping of the spectral peaks. This is due to the limited range of the chemical shifts and that almost every biochemical compound contains H (hydrogen) somewhere on the molecule. ^{31}P NMR is a valuable asset because ^{31}P studies biologically important phosphorus containing metabolites and also because the chemical shift ranges are greater than those of ^{1}H NMR. The following is a list of ^{31}P chemical shifts of biological metabolites.

PME 7.5 - 6.3 ppm

PE 6.87 ppm

PC 6.29 ppm

PDE 3.2 - 2.3 ppm

GPE 3.58 ppm

GPC 2.99 ppm

Pi 5.7 - 4.7 ppm

γ - NTP -2.0 - -2.8 ppm

α - NTP -7.5

β - NTP -15.4 - -16.3 ppm

PCr 0.5 - -0.6 ppm

These chemical shift assignments were taken from referenced literature.[4,5,7]

The main weakness of ^{31}P NMR is its lack of sensitivity in comparison to ^{1}H NMR. Nevertheless, to gain a more complete view, a combination of nuclei are analyzed in addition to extensive NMR analysis such as 2D NMR methods. Examples of nuclei analyzed include ^{1}H, ^{31}P, and ^{13}C. Examples of 2D NMR studies include ^{1}H COSEY (Correlation Spectroscopy) and HETCOR (Heteronuclear Chemical Shift Correlation). These studies aid in the identification of complex spectra. This is especially important because in some cases, certain nuclei are difficult to observe, such as ^{31}P in normal breast tissue.[8]

Works Cited

1. Knight, J.M., Shaw, I.C., and Jones, A.P. "Life begins at 50?", *Chemistry in Britain*; Vol. 32, No. 6, **June 1996**, p 38.

2. Moreno, A., Rey, M., Montane, J. M., Alonso, J., and Aru's, C. "^{1}H NMR Spectroscopy of Colon Tumors and Normal Mucosal Biopsies; Elevated Taurine Levels and Reduced Polyethyleneglycol Absorption in Tumors May Have Diagnostic Significance", *NMR in Biomedicine;* Vol. 6, **1993**, pp 111-118.

3. De Certaines, J.D., Larsen, V. A., Podo, F., Carpinelli, G., Briot, O., Henriksen, O. "In Vivo ^{31}P MRS of Experimental Tumours", *NMR in Biomedicine*; Vol. 6, **1993**, pp 345-365.

4. Lowry, M., Porter, D. A., Twelves, C. J., Heasley, P. E., Smith, M. A., and Richards, M. A. "Visibility of Phospholipids in ^{31}P NMR Spectra of Human Breast Tumours In Vivo," *NMR in Biomedicine;* Vol. 5, **1992**, pp 37-42.

5. Smith, T.A., Glagolm, J., Leach, M.O., Machin, L., and McCready, V.R. "The Effect of Intra-tumour Heterogeneity on the Distribution of Phosphorous-containing Metabolites within Human Breast Tumours: an In Vitro Study Using ^{31}P NMR Spectroscopy," *NMR in Biomedicine;* Vol. 4, **1991**, pp 262-267.

6. Gribbestad, I. S., Fjosne, H. E., Haugen, O. A., Nilsen, G., Krane, J., Petersen, S. B., and Kvinnsland, S. "In Vitro Proton NMR Spectroscopy of Extracts from Human Breast Tumours and Non-Involved Breast Tissue", *Anticancer Reaearch*; Vol. 13, **1993**, pp 1973-1980.

7. Smith, T.A.D., Bush, C., Jameson, C., Titley, J.C., Leach, M.O., Wilman, D.E.V., McCready, V.R. "Phospholipid Metabolites, Prognosis and Proliferation in Human Breast Carcinoma," *NMR in Biomedicine;* Vol. 6, **1993**, pp 318-323.

8. Ruiz-Cabello, J. and Cogen, J. S. "Phospholipid Metabolites as Indicators of Cancer Cell Function," *NMR in Biomedicine;* Vol. 5, **1992**, pp 220-233.

Part II

Experimental

Reagents and Extraction Conditions

All reagents were purchased from the Aldrich Chemical Company except for the following: potassium hydroxide was purchased from Spectrum Chemical Mfg. Corp., methanol was purchased from J. T. Baker, and chloroform was purchased from VWR Scientific. All reagents except for EDTA were prepared using deionized water. EDTA was prepared using deuterated water.

All pipettes, test tubes, and scalpels were properly disposed of after use due to the potential biohazard. The two stainless steel mortars and a porcelain pestle were washed with soap and water after extraction procedures. The tissue samples were weighed on weighing paper to the nearest 0.1 grams. NMR tubes (5 mm) were cleaned with acetone and deionized water and the caps were discarded between samples. The tissue homogenizer was a Tissue Tearor, model 985-370 from Biospec Products. The sonicator (ultrasonic cleaning bath) was from Fisher Scientific, model FS5. The point sonicator was a Branson Sonifier Cell Disrupter 185. The vortex was a Vortex Jr. Mixer from Scientific Industries Inc. All of the work areas were cleaned with 10 % bleach before and after extraction procedures. Gloves, goggles, and lab coats were always worn during extraction procedures due to the risk of potential biohazards and chemicals.

Tissue samples were obtained at Saint Elizabeth Medical Center in the frozen section room that was adjacent to the operating room at the time of surgery. The pathologist on call would cut out pieces of tissue from the mass that was removed from the patient. (Actual photographs of the tissue samples used in the extraction procedures can be found in appendix C.) I then began the first part of the extraction procedure in the frozen section room. Patient confidentiality was maintained at all times. The patients involved were those having breast reductions (giving non-cancerous tissue) and mastectomies (giving cancerous tissue if available and also, non-cancerous tissue).

Carbon, proton, and phosphorous NMR analysis was performed using a Varian Gemini 2000 400 MHz NMR spectrometer. The references used were the following: for phosphorous, MDPA (methylene diphosphonic acid) at 20.0 ppm with respect to 85% phosphoric acid; for carbon, $CDCl_3$ at 77.0 ppm, with respect to TMS at 0.0 ppm; for proton, TMS at 0.0 ppm.

Gas Chromatography was performed using the Shimadzu 9A GC. The column type was a Supelco SPE - 608. The solvent description was a Helium carrier / Nitrogen make-up. The detector was a FID detector.

Chloroform Methanol Extraction, Chloroform layer. Protocol A.

The tissue sample appeared to be mostly fatty tissue with a very small percentage of interspersed non-fatty tissue. The fatty tissue was yellow in color. The non-fatty tissue was white in color. The tissue was removed from the blood supply for approximately 6 min 49 sec before being submersed in liquid nitrogen. The sample

weighed 0.6 grams. The frozen sample was pulverized using a stainless steel mortar and pestle while submersed in liquid nitrogen. After the liquid nitrogen had boiled off, 1.2 ml of 10 mM EDTA was added, the sample was vortexed (The vortex is a rubber, vibrating surface that swirls the contents inside the test tube. It is a means of mixing the sample), and 2.25 ml of a chloroform and methanol mixture (2:1) was added to the sample. The suspension was vortexed and placed in an ice bath for 105 min. Chloroform (0.75 ml) was then added to the suspension. The mixture was again vortexed and 0.75 ml of Tris Buffer (in D_2O, pH 8) was added to the sample. The mixture was centrifuged at 3000 rpm for 15 min at 4°C. The aqueous layer (top) was separated from the chloroform layer (bottom) with a disposable glass pipettes. The sample was stored overnight at -30 ^{0}C. The chloroform layer separated from fatty layer which floated on top. Using a black marker, a line was drawn on the side of the test tube indicating the chloroform layer volume. The solvents were removed from the organic extract with a stream of nitrogen gas. The sample was then rediluted to the line with deuterated chloroform and 2.5 μl of 1M MDPA was added to the sample.

Spectroscopic data for chloroform layer of Chloroform Methanol Extraction. The following data was obtained: ^{1}H NMR ($CDCl_3$); 0.84 ppm (t, 18H, CH_2-C$\underline{H}_3$), 1.28 ppm (s, 138H, CH_2-(C$\underline{H}_2$)$_n$-CH_2), 1.6 ppm (m, 12H, C$\underline{H}_2$-CH_2.COO), 2.02 ppm (t, 12H, C$\underline{H}_2$-HC=C), 2.79 ppm (sextet, 6H, =CH-C$\underline{H}_2$-C=). 2.32 ppm (t, 8H, C$\underline{H}_2$-COO), 4.3 ppm (m, 4H, $\underline{H}_2$CO-C), 5.35 ppm (qt, 12H, -C$\underline{H}$=C$\underline{H}$-), 5.39 ppm (m, 2H, C-CH-O-CO). ^{13}C NMR ($CDCl_3$); 13.77 ppm (-CH_3), 22.53 ppm ($\underline{C}H_2$-CH_3), 24.74 - 27.03 ppm (-CH_2)$_n$, 29.56 ppm, (C=C-$\underline{C}H_2$-C=C), 34.00 ppm, (CH_2-$\underline{C}H_2$-COO), 61.97 ppm, (CH-$\underline{C}H_2$O),

68.82 ppm, ($(CH_2)_2\underline{C}HO$), 127.69-129.99 ppm ($\underline{C}H=\underline{C}H$), 172.94 ppm ($\underline{C}=O$). ^{31}P NMR - no signals observed.

Analysis of the organic layer of the chloroform methanol extraction on the Shimadzu 9A GC revealed linoleic acid, and arachidonic acid. The column was a SE30 and 30 meters in length. The injector and detector were both set at a temperature of 275 °C. The initial temperature was set at 100 °C for 5 min, then the temperature was increased to 190 °C (20 °C / min) for 13.5 min, and lastly, the temperature was increased to 250 °C (5 °C / min) for 25 min.

Fraction	Retention Time
linoleic acid	34.883
arachidonic acid	39.717

Variations of the Chloroform Methanol Extraction, Chloroform Layer. Protocols B, C, D, E.

Protocol	Variations of the original Protocol A.
B	Used a larger pestle for mincing of the tissue.
C	Sonicated the sample in a water bath for 10 min.
D	Sonicated the sample in a water bath for 20 min.
E	Varied the ration of Chloroform Methanol from (2:1) to (1:5).

Protocol B allowed the frozen tissue samples to be pulverized more easily and to a fine powder as opposed to small chunks of frozen tissue in Protocol A. In Protocols C and D the tissues were sonicated for 10 min and 20 min, respectively. Protocol E involved a change in the Chloroform Methanol ratio (1:5 instead of 2:1).

Chloroform Methanol Extraction, Chloroform Layer. Protocol F.

The tissue sample appeared to be mostly fatty tissue with a very small percentage of interspersed non-fatty tissue. The fatty tissue was yellow in color. The non-fatty tissue was white in color. The tissue was removed from the blood supply for approximately 33 min before being submerged in liquid nitrogen. The sample weighed 0.5 grams. The frozen sample was pulverized with a stainless steel mortar and porcelain pestle while submerged in liquid nitrogen. After liquid nitrogen had boiled off, 1.0 ml of 10 mM EDTA was added, the sample was vortexed, and 1.9 ml of a chloroform and methanol mixture (1:2) was added to the sample. The suspension was vortexed and placed in an ice bath for 60 min. Chloroform (0.6 ml) was then added to the suspension. The mixture was again vortexed and 0.6 ml of Tris Buffer (in D_2O, pH 8) was added to the sample. The mixture was sonicated in a water bath for 20 min. The mixture was centrifuged at 3000 rpm for 15 min at $4^{o}C$. The aqueous layer (top) was separated from the chloroform layer (bottom) with a disposable glass pipette. The sample was stored overnight at -30 ^{0}C. The chloroform layer separated from a fatty layer which floated on top. Using a black marker, a line was drawn on the side of the test tube indicating the chloroform layer volume. The solvents were removed from the organic extract with a

stream of nitrogen gas. The sample was then rediluted to the line with deuterated chloroform and 2.5 μl of 1M MDPA was added to the sample.

Spectroscopic data for chloroform layer of Chloroform Methanol Extraction. The following data was obtained: ^{1}H NMR ($CDCl_3$); 0.84 ppm (t, 18H, CH_2-C$\underline{H}_3$), 1.28 ppm (s, 138H, CH_2-(C$\underline{H}_2$)$_n$-CH_2), 1.6 ppm (m, 12H, C$\underline{H}_2$-CH_2.COO), 2.02 ppm (t, 12H, C$\underline{H}_2$-HC=C), 2.79 ppm (sextet, 6H, =CH-C$\underline{H}_2$-C=). 2.32 ppm (t, 8H, C$\underline{H}_2$-COO), 4.3 ppm (m, 4H, $\underline{H}_2$CO-C), 5.35 ppm (qt, 12H, -C$\underline{H}$=C$\underline{H}$-), 5.39 ppm (m, 2H, C-CH-O-CO). ^{13}C NMR ($CDCl_3$); 13.77 ppm (-CH_3), 22.53 ppm ($\underline{C}H_2$-CH_3), 24.74 - 27.03 ppm (-CH_2)$_n$, 29.56 ppm, (C=C-$\underline{C}H_2$-C=C), 34.00 ppm, (CH_2-$\underline{C}H_2$-COO), 61.97 ppm, (CH-$\underline{C}H_2$O), 68.82 ppm, ((CH_2)$_2$$\underline{C}$HO), 127.69-129.99 ppm ($\underline{C}$H=$\underline{C}$H), 172.94 ppm ($\underline{C}$=O). ^{31}P NMR - no signals observed.

Chloroform Methanol Extraction, Aqueous Layer. Protocol A

The tissue sample appeared to be a solid, white mass approximately 2.5 inches in diameter. The cancerous tissue was removed from the blood supply for approximately 16 min before being submersed in liquid nitrogen. The sample weighed 0.5 grams. The frozen sample was pulverized with a stainless steel mortar and porcelain pestle while submerged in liquid nitrogen. After the liquid nitrogen had boiled off, 1.0 ml of 10 mM EDTA was added, the sample was vortexed, and 1.9 ml of a chloroform and methanol mixture (2:1) was added to the sample. The suspension was vortexed and placed in an ice bath for 60 min. Chloroform (0.6 ml) was then added to the suspension. The mixture was again vortexed and 0.6 ml of Tris Buffer (in D_2O, pH 8) was added to the sample.

The mixture was sonicated in a water bath for 20 min. The mixture was centrifuged at 3000 rpm for 15 min at 4°C. The aqueous layer (top) was separated from the chloroform layer (bottom) with a disposable glass pipette. 25 µl of 1 M MDPA was added to the aqueous layer.

Spectroscopic data for aqueous layer of Chloroform Methanol Extraction. The following data was obtained: ^{31}P NMR (D_2O); 3.020 ppm, (Integration = 1.5), 3.171 ppm, (Integration = 1.2), 20.000 ppm, (Integration = 1), MDPA (internal standard).

Variations of the Chloroform Methanol Extraction, Aqueous Layer. Protocols B, C, D, E, F, G, H, I, J.

Protocol	**Variations of the Protocol**
B	varied ratio of chloroform methanol from (2:1) to (1:5)
C	varied ratio of chloroform methanol from (2:1) to (1:2)
D	used 0.4M EDTA verses 10 mM EDTA used Tris buffer pH 7 verses pH 8
E	used a point sonicator for 1 min and no sonication in a water bath
F	increased volume of EDTA to 4 ml / g
G	spiked tissue sample with PC and PE at beginning of extraction
H	no point sonication, 25 µl MDPA (18 mg/ml), tissue homogenizer, adjusted pH after addition of MDPA to pH 7.
I	the sample weight was obtained after being submersed in liquid nitrogen

	and then allowing the liquid nitrogen to completely evaporate.
J	the samples were homogenized at different time intervals; 10 s, 15 s, 30 s, 45 s, 60 s.

Protocol B used a change in the concentration of EDTA from 10 mM EDTA to 0.4 M EDTA. Protocol B also used a Tris buffer of pH of 7 as opposed to a pH of 8. In Protocol C, all of the sample was point sonicated for 1 min and no sonication in a water bath. (The point sonicator was suppose to provide a more, concentrated ultrasonic disruption of the cell membranes.) Protocol D used an increased volume of EDTA from 2 ml to 4 ml. In Protocol E, the tissue sample was spiked with 2.5 µl of 1M PC and 2.5 µl of 1M PE at the beginning of the extraction. In Protocol F, a tissue homogenizer was used for 1min, no point sonication was used, 25 µl of MDPA (18 mg / ml) was used and the pH was adjusted to 7. The sample weight in Protocol G was weighed after being submersed in liquid nitrogen and then allowing the liquid nitrogen to evaporate completely. In Protocol H, the samples were homogenized at different time intervals (10 s, 15 s, 30 s, 45 s, 60 s).

Chloroform Methanol Extraction, Aqueous Layer. Protocol K.

The tissue sample appeared to be mostly fatty tissue with a very small percentage of interspersed non-fatty tissue. The fatty tissue was yellow in color. The non-fatty tissue was white in color. The tissue was removed from the blood supply for approximately 12 min before being submersed in liquid nitrogen. The sample weighed

1.9 grams. The frozen sample was pulverized with a stainless steel mortar and porcelain pestle while submersed in liquid nitrogen. After the liquid nitrogen had boiled off, the sample was weighed in a preweighed test tube. The sample weighed 1.5 grams. (The decrease in weight was due to a loss of water.) Then 37.5 μl of 0.4 M EDTA was added, the sample was vortexed, and 5.6 ml of a chloroform and methanol mixture (1:2) was added to the sample. The mixture was homogenized with a tissue homogenizer for 10 sec while in an ice bath. The suspension was then left in the ice bath for 60 min. After 60 min, chloroform (1.9 ml) was added to the suspension. The mixture was then vortexed and 1.9 ml of Tris Buffer (in D_2O, pH 7) was added to the sample followed by vortexing again. The mixture was centrifuged at 3000 rpm for 15 min at 4°C. The aqueous layer (top) was separated from the chloroform layer (bottom) with a disposable glass pipette. 25 μl of 1 M MDPA was added to the aqueous layer and the pH was adjusted to 7.4.

Spectroscopic data for aqueous layer of Chloroform Methanol Extraction. The following data was obtained: ^{31}P NMR (D_2O); 6.496 ppm, (Integration = 0.2), 6.677 ppm, (Integration = 0.08), 20.000 ppm, (Integration = 1), MDPA (internal standard).

Chloroform Methanol Extraction, Chloroform Layer. Time Study.

The tissue sample appeared to be mostly fatty tissue with a very small percentage of interspersed non-fatty tissue. The fatty tissue was yellow in color. The non-fatty tissue was white in color. Tissue sample 1 was removed from the blood supply for approximately 6 min 49 sec before being submersed in liquid nitrogen. The sample

weighed 0.6 grams. Below is a table of the tissue sample, time the tissue was removed from the blood supply, and mass of the sample.

Tissue Sample	Time	Mass
1	6 min 49 sec	0.6 g
2	8 min 11 sec	0.5 g
3	14 min	0.4 g
4	22 min	0.6 g

The frozen sample was pulverized with a stainless steel mortar and pestle while submersed in liquid nitrogen. After the liquid nitrogen had boiled off, 1.2 ml of 10 mM EDTA was added, sample 1 was vortexed, and 2.25 ml of a chloroform and methanol mixture (2:1) was added to sample 1. The suspension was vortexed and placed in an ice bath for 105 min. Chloroform (0.75 ml) was then added to the suspension (sample 1). The mixture was again vortexed and 0.75 ml of Tris Buffer (in D_2O, pH 8) was added to sample 1. Below is a table indicating the sample and volume of reagents added to the sample. (CM is a chloroform methanol mixture.)

Sample	10 mM EDTA	CM (2:1)	Chloroform	Tris buffer pH8
1	1.2 ml	2.25 ml	0.75 ml	0.75 ml
2	1.0 ml	1.88 ml	0.63 ml	0.63 ml

3	0.8 ml	1.50 ml	0.50 ml	0.50 ml
4	1.2 ml	2.25 ml	0.75 ml	0.75 ml

The samples were centrifuged at 3000 rpm for 15 min at 4°C. The aqueous layer (top) was separated from the chloroform layer (bottom) with a disposable glass pipette. The sample was stored overnight at -30 ^{0}C. The chloroform layer separated from fatty layer which floated on top. Using a black marker, a line was drawn on the side of the test tube indicating the chloroform layer volume. The solvents were removed from the organic extract with a stream of nitrogen gas. The sample was then rediluted to the line with deuterated chloroform and 2.5 µl of 1M MDPA was added to the sample.

Spectroscopic data for chloroform layer of Chloroform Methanol Extraction. The following data was obtained: ^{1}H NMR ($CDCl_3$); 0.84 ppm (t, 18H, CH_2-C$\underline{H}_3$), 1.28 ppm (s, 138H, CH_2-(C$\underline{H}_2$)$_n$-CH_2), 1.6 ppm (m, 12H, C$\underline{H}_2$-CH_2.COO), 2.02 ppm (t, 12H, C$\underline{H}_2$-HC=C), 2.79 ppm (sextet, 6H, =CH-C$\underline{H}_2$-C=). 2.32 ppm (t, 8H, C$\underline{H}_2$-COO), 4.3 ppm (m, 4H, $\underline{H}_2$CO-C), 5.35 ppm (qt, 12H, -C$\underline{H}$=C$\underline{H}$-), 5.39 ppm (m, 2H, C-CH-O-CO). ^{13}C NMR ($CDCl_3$); 13.77 ppm (-CH_3), 22.53 ppm ($\underline{C}H_2$-CH_3), 24.74 - 27.03 ppm (-CH_2)$_n$, 29.56 ppm, (C=C-$\underline{C}H_2$-C=C), 34.00 ppm, (CH_2-$\underline{C}H_2$-COO), 61.97 ppm, (CH-$\underline{C}H_2$O), 68.82 ppm, ((CH_2)$_2$$\underline{C}$HO), 127.69-129.99 ppm ($\underline{C}$H=$\underline{C}$H), 172.94 ppm ($\underline{C}$=O). ^{31}P NMR - no signals observed.

Chloroform Methanol Extraction, Aqueous Layer. Time Study.

The tissue sample appeared to be mostly fatty tissue with a very small percentage of interspersed non-fatty tissue. The fatty tissue was yellow in color. The non-fatty tissue was white in color. The tissue was removed from the blood supply for approximately 7 min before being submersed in liquid nitrogen. Sample 8 weighed 2.0 grams. The frozen sample (8) was pulverized with a stainless steel mortar and porcelain pestle while submersed in liquid nitrogen. After the liquid nitrogen had boiled off, sample 8 was weighed in a preweighed test tube. The sample weighed 1.5 grams. (The decrease in weight was due to a loss of water.) Below is a table of the tissue sample, time the tissue was removed from the blood supply, mass of the sample before being submersed in liquid nitrogen, and after being submersed in liquid nitrogen.

Tissue Sample	Time	Mass before lq N_2	Mass after lq N_2
8	7 min 17 sec	2.0 g	1.5 g
9	12 min 12 sec	1.9 g	1.5 g
10	22 min 18 sec	1.9 g	1.2 g
12	37 min 19 sec	2.2 g	1.4 g

Then 37.5 µl of 0.4 M EDTA was added, the sample (8) was vortexed, and 5.6 ml of a chloroform and methanol mixture (1:2) was added to sample 8. The mixture (8) was homogenized with a tissue homogenizer for 10 sec while in an ice bath. The suspension was then left in the ice bath for 60 min. After 60 min, chloroform (1.9 ml) was added to

the suspension. The mixture (8) was then vortexed and 1.9 ml of Tris Buffer (in D_2O, pH 7) was added to the sample followed by vortexing again. Below is a table indicating the sample and volume of reagents added to the sample. (CM is a chloroform methanol mixture.)

Sample	**0.4 M EDTA**	**CM (1:2)**	**Chloroform**	**Tris buffer pH7**
8	37.5 µl	5.6 ml	1.9 ml	1.9 ml
9	37.5 µl	5.6 ml	1.9 ml	1.9 ml
10	30.0 µl	4.5 ml	1.5 ml	1.5 ml
12	35.0 µl	5.3 ml	1.8 ml	1.8 ml

The mixture was centrifuged at 3000 rpm for 15 min at 4°C. The aqueous layer (top) was separated from the chloroform layer (bottom) with a disposable glass pipette. 25 µl of 1 M MDPA was added to the aqueous layer and the pH was adjusted to 7.4.

Spectroscopic data for aqueous layer of Chloroform Methanol Extraction. The following data was obtained: ^{31}P NMR (D_2O); Sample 8, 20.000 ppm, MDPA (internal standard); Sample 9, 6.495 ppm, (Integration = 0.2), 6.677 ppm, (Integration = 0.08), 20.000 ppm, (Integration = 1), MDPA (internal standard); Sample 10, 20.000 ppm, MDPA (internal standard); Sampl 12, 20.000 ppm, MDPA (internal standard).

Part III

Results and Discussion

Chapter 1 Chloroform Layer of the Chloroform Methanol Extraction

^{31}P NMR spectra of extracts of human breast tissue have been previously used to identify phosphorous metabolites.[1] These papers only discussed the aqueous phase of the Chloroform Methanol extraction. In my project, I also analyzed the organic phase of the extraction to look for any non-polar marker compounds..

The tissue samples were obtained at Saint Elizabeth Medical Center in the frozen section room.[4] The frozen section room was set up for the pathologist to analyze tissue masses as they are removed from the patient during their surgery. This analysis provided the surgeon with a quick diagnosis concerning the nature of the mass removed, such as if the mass was cancerous. The frozen section room was where I waited for the breast tissue samples. The tissue was transported to the frozen section room in a plastic container. A pathologist usually inspected the tissue mass for any unexpected growths such as cancer, then gave me a portion of the tissue for my research. (Figure 1.1) The tissue mass was cauterized prior to removal from the patient to prevent excessive bleeding, but this usually resulted in a very, foul odor of burnt flesh. As a result, the tissue mass was only slightly bloody. The excised tissue was of irregular shape and typically about 100 cm^2 in total volume. The tissue was mainly fatty tissue which was

Figure 1.1 Photograph of a Tissue Sample

yellow in color. The non-fatty tissue was interspersed amongst the fatty tissue and was white in color. Cancerous tissue was also white in color, however the texture was different. The cancer mass was a firm solid as opposed to the non-cancerous tissue. Appendix C contains photographs of various tissue samples.

Once the tissue samples were obtained, they were immediately submerged in liquid nitrogen. The Chloroform Methanol Extraction was then performed. In Protocol A of the procedure, the tissue was pulverized with a mortar and pestle while submerged in liquid nitrogen. Then, 10 mM EDTA was added followed by the addition of a chloroform methanol mixture (2:1). The sample was then placed in an ice bath for 105 min. After the $4^{o}C$ incubation, chloroform was added to the mixture followed by Tris buffer, pH 8. The mixture was then centrifuged at 3000 rpm for 15 min at $4^{0}C$. As a result of centrifugation, the mixture separated into a top, aqueous layer; a middle layer of cellular debris; and a bottom, organic (chloroform) layer. The aqueous and organic layers were pipetted into individual test tubes. The solvents in the chloroform layer were removed via a stream of nitrogen gas and the sample was rediluted with deuterated chloroform. 1M MDPA was then added to the sample from the chloroform layer. The resulting liquid was yellow in color. To prepare the sample for spectroscopic analysis, about 30 % of the sample was put in a 5 mm NMR tube.

The ^{1}H and ^{13}C NMR analysis of the sample (Figure 1.2 and 1.3) produced spectra that qualitatively looked like those of triglycerides. The ^{31}P NMR spectra of these

Figure 1.2 Proton NMR Spectrum of the Organic Extract in Protocol A

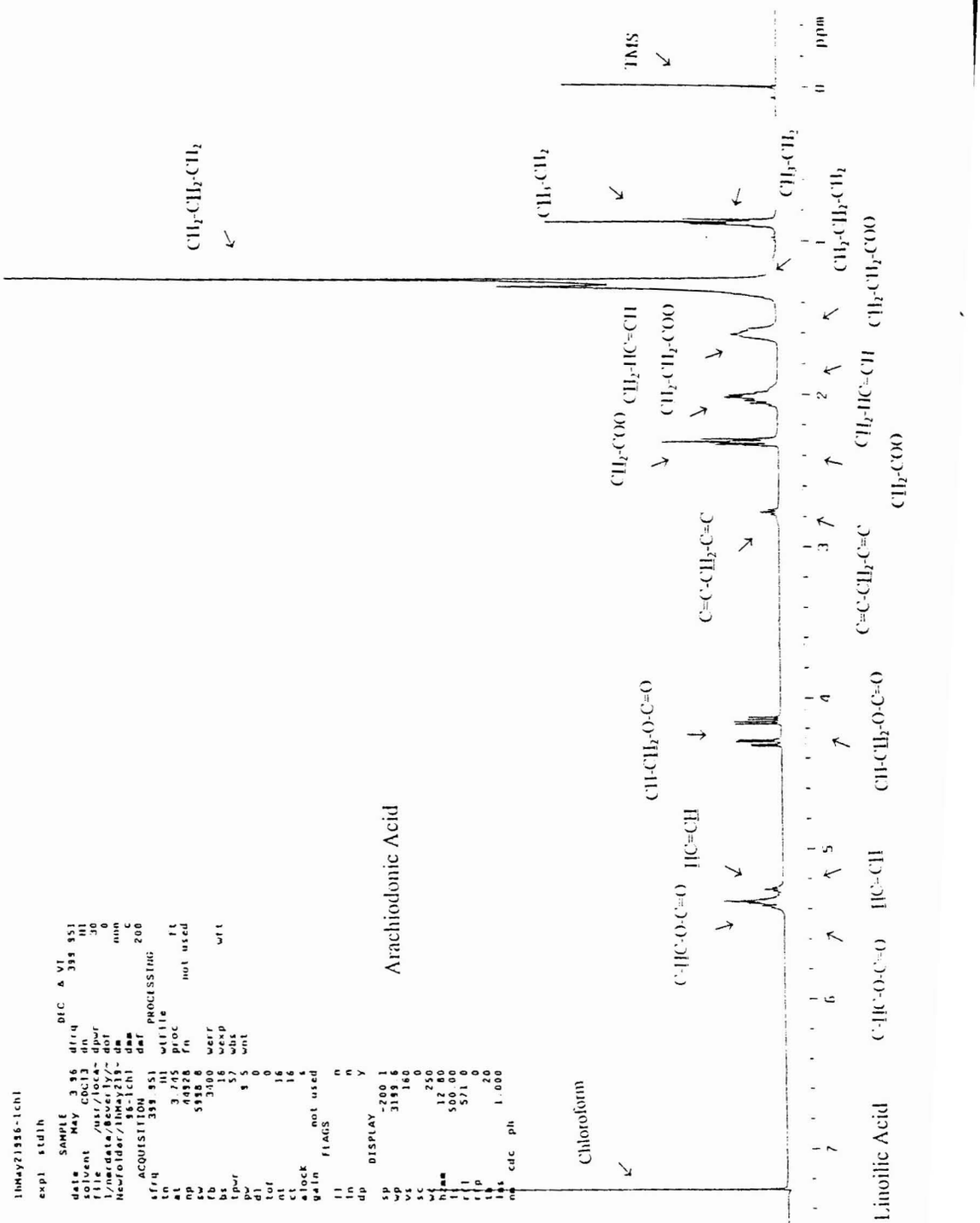
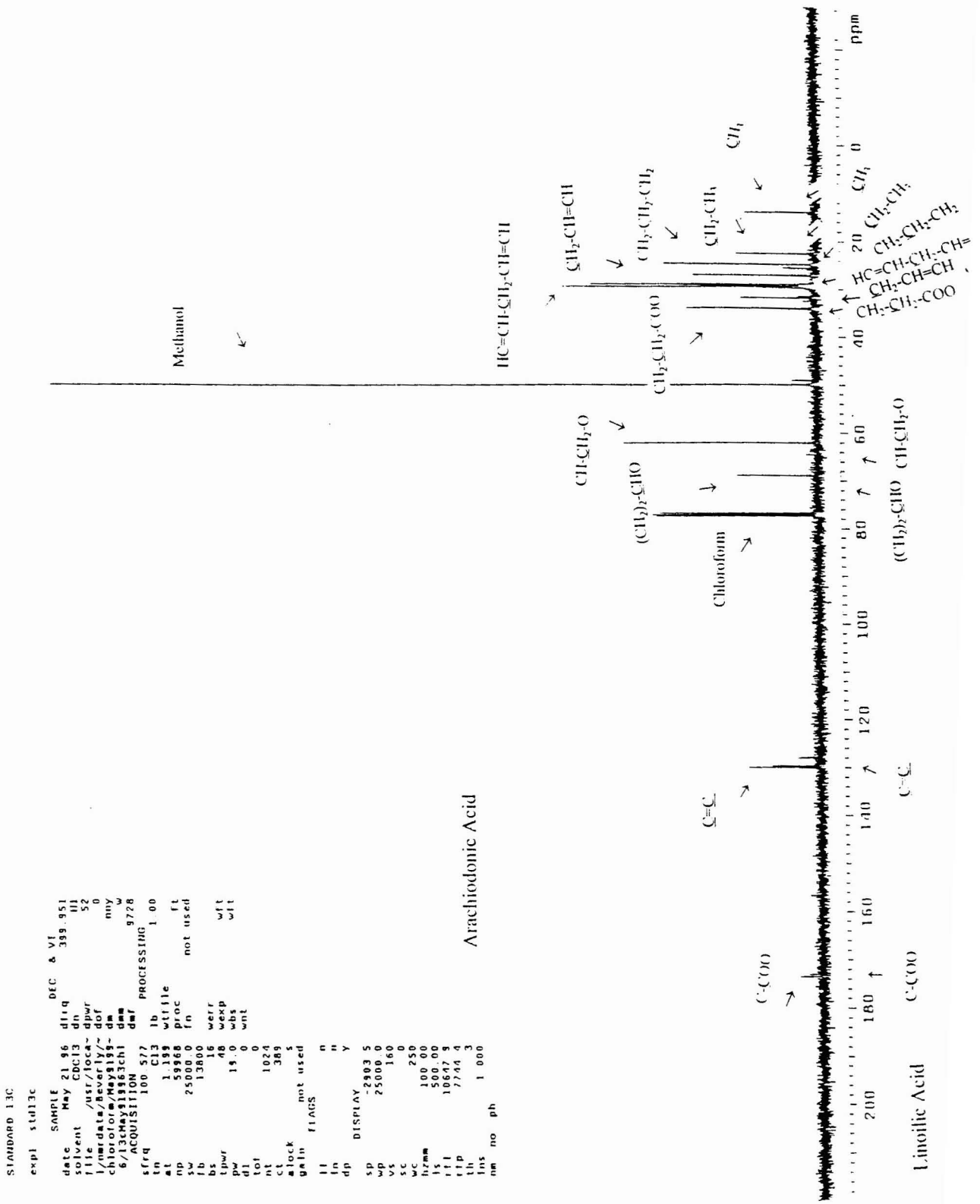


Figure 1.3 Carbon NMR Spectrum of the Organic Extract in Protocol A



samples did not contain any phosphorus peaks. Therefore, we can say that there were not detectable amounts of phosphorus containing metabolites in the organic extract. As a quick check, a sample composed of triglycerides, Land-o-Lakes butter, was analyzed via ^{1}H NMR. (Figure 1.4) The spectrum was very similar to that of the sample from Protocol A.

The organic extract of the breast tissue was analyzed by gas chromatography by Tamara Kerr. The triglycerides of arachidonic acid and linoleic acid were identified, amongst the components. The retention time of arachidonic acid was 39.7 sec. The retention time of linoleic acid was at 34.9 sec.

The ^{1}H and ^{13}C NMR ($CDCl_3$) spectra of the sample from Protocol A were shown in Figures. 1.3 and 1.4. The spectra was assigned with the aid of the ACD/CNMR and ACD/HNMR (Advanced Chemistry Development) programs. These programs are calculated based on the spectroscopic data bases. Figures 1.5, 1.6, 1.7, and 1.8 are the calculated spectra of the triglycerides of the arachidonic and linoleic acids, that were found in the sample from Protocol A

In the ^{1}H NMR ($CDCl_3$) spectrum, the triplet at 0.8 ppm was assigned to the methyl groups and the broad singlet at 1.3 ppm to the chain of methylene groups in the fatty acid side chains. The multiplet at 1.6 ppm was assigned to the methylene groups beta to the ester groups and the triplet at 2.3 ppm to the methylene groups alpha to the ester groups. The multiplet at 2.0 ppm corresponds to the allylic methylene groups (i.e. -CH=CH-C$\underline{H}_2$-CH_2), while the triplet at 2.8 ppm corresponds to the diallylic methylene

Group	nH	Shift	Error
1	1	5.507	0.02
2	4	4.255	0.08
3	2	2.336	0.22
4	2	2.336	0.22
5	2	2.322	0.23
6	2	1.615	0.35
7	2	2.094	0.19
8	1	5.338	0.16
9	1	4.95	0.81
10	2	2.734	0.17
11	1	5.071	0.69
12	1	5.071	0.69
13	2	2.759	0.17
14	1	5.071	0.69
15	1	5.071	0.69
16	2	2.734	0.17
17	1	4.963	0.8
18	1	5.268	0.12
19	2	2.009	0.2
20	2	1.25	0.13
21	2	1.25	0.02
22	2	1.25	0.09
23	2	1.615	0.35
24	2	2.094	0.19
25	1	5.338	0.16
26	1	4.95	0.81
27	2	2.734	0.17
28	1	5.071	0.69
29	2	2.759	0.17
30	1	5.071	0.69
31	1	5.071	0.69
32	1	5.071	0.69
33	2	2.734	0.17
34	1	4.963	0.8
35	1	5.268	0.12
36	2	2.009	0.2
37	2	1.25	0.13
38	2	1.25	0.02
39	2	1.25	0.09
40	2	1.615	0.35
41	2	2.094	0.19
42	1	5.338	0.16
43	1	4.95	0.81
44	2	2.734	0.17
45	1	5.071	0.69
46	1	5.071	0.69
47	2	2.759	0.17
48	1	5.071	0.69
49	1	5.071	0.69
50	2	2.734	0.17
51	1	5.268	0.12
52	1	4.963	0.8
53	2	2.009	0.2
54	2	1.25	0.13
55	2	1.25	0.02
56	2	1.25	0.09
57	3	1.035	0.37
58	3	1.035	0.37
59	3	1.035	0.37

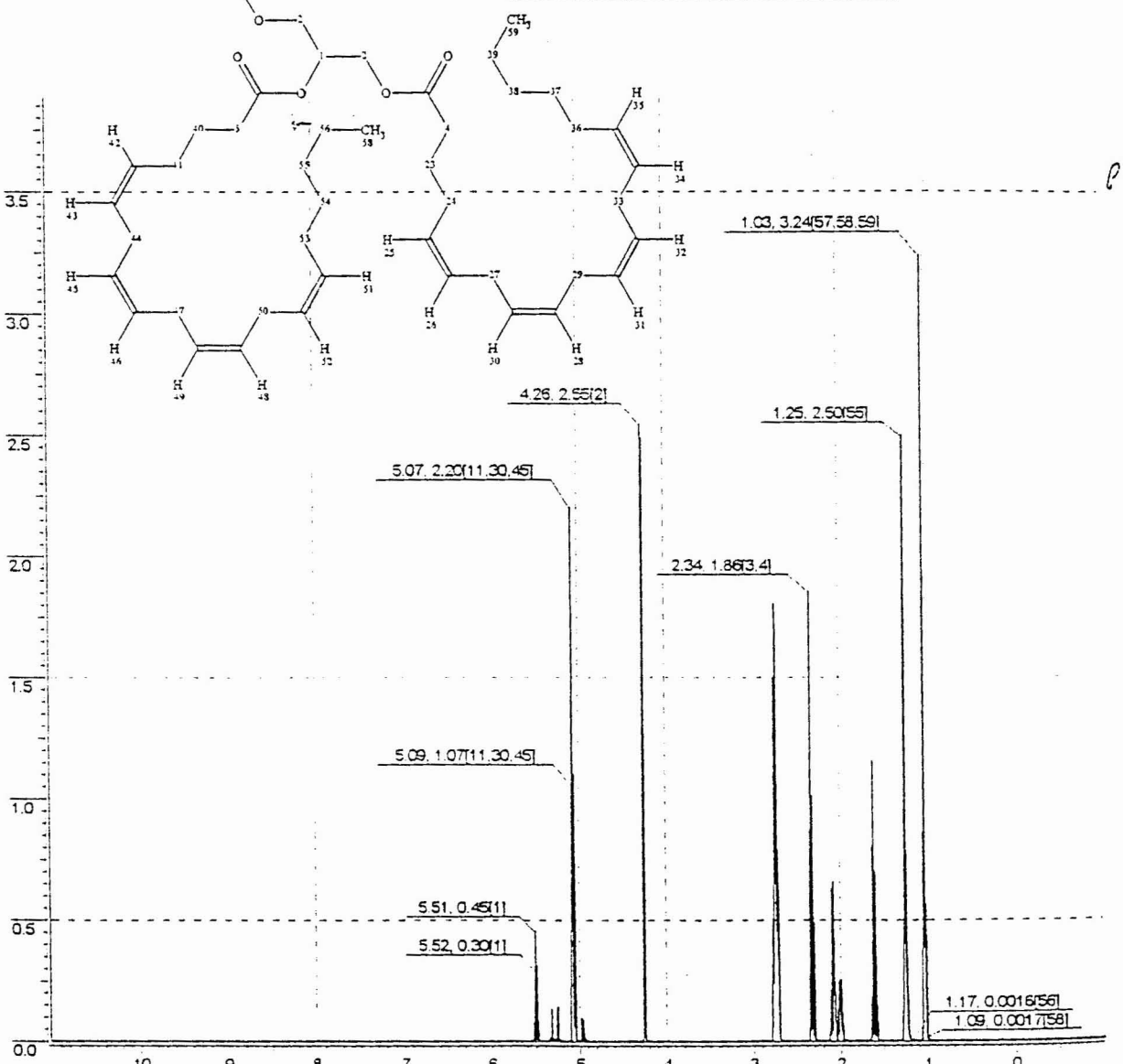


Figure 1.5 Proton NMR ACD Spectrum of the Triglyceride of Arachidonic Acid

Carbon No.	CHn	Chem. Shifts	Conf. Limits	Carbon No.	CHn	Chem. Shifts	Conf. Limits	Carbon No.	CHn	Chem. Shifts	Conf. Limits
1	CH	69.05	3.3	22	CH	131.4	3.8	43	CH_2	30.55	0.8
2	CH_2	62.24	4.5	23	CH_2	27.28	3.2	44	CH_2	23.1	0.8
3	CH_2	62.24	4.5	24	CH_2	31.06	1.8	45	CH_2	14.22	0.1
4	C	172.51	1.9	25	CH_2	30.55	0.8	46	CH_2	24.71	1
5	C	172.85	0.7	26	CH_2	23.1	0.8	47	CH_2	25.4	1.3
6	C	172.51	1.9	27	CH_2	14.22	0.1	48	CH	130.37	3.1
7	CH_2	33.37	1.6	28	CH_2	24.71	1	49	CH	129.56	0.5
8	CH_2	33.91	1.8	29	CH_2	25.4	1.3	50	CH_2	29.35	19.3
9	CH_2	33.37	1.6	30	CH	130.37	3.1	51	CH	129.17	4.8
10	CH_2	24.71	1	31	CH	129.56	0.5	52	CH	129.17	4.8
11	CH_2	25.4	1.3	32	CH_2	29.35	19.3	53	CH_2	29.13	19.5
12	CH	130.37	3.1	33	CH	129.17	4.8	54	CH	129.17	4.8
13	CH	129.56	0.5	34	CH	129.17	4.8	55	CH	129.17	4.8
14	CH_2	29.35	19.3	35	CH_2	29.13	19.5	56	CH_2	29.35	19.3
15	CH	129.17	4.8	36	CH	129.17	4.8	57	CH	128.96	1.1
16	CH	129.17	4.8	37	CH	129.17	4.8	58	CH	131.4	3.8
17	CH_2	29.13	19.5	38	CH_2	29.35	19.3	59	CH_2	27.28	3.2
18	CH	129.17	4.8	39	CH	128.96	1.1	60	CH_2	31.06	1.8
19	CH	129.17	4.8	40	CH	131.4	3.8	61	CH_2	30.55	0.8
20	CH_2	29.35	19.3	41	CH_2	27.28	3.2	62	CH_2	23.1	0.8
21	CH	128.96	1.1	42	CH_2	31.06	1.8	63	CH_3	14.22	0.1

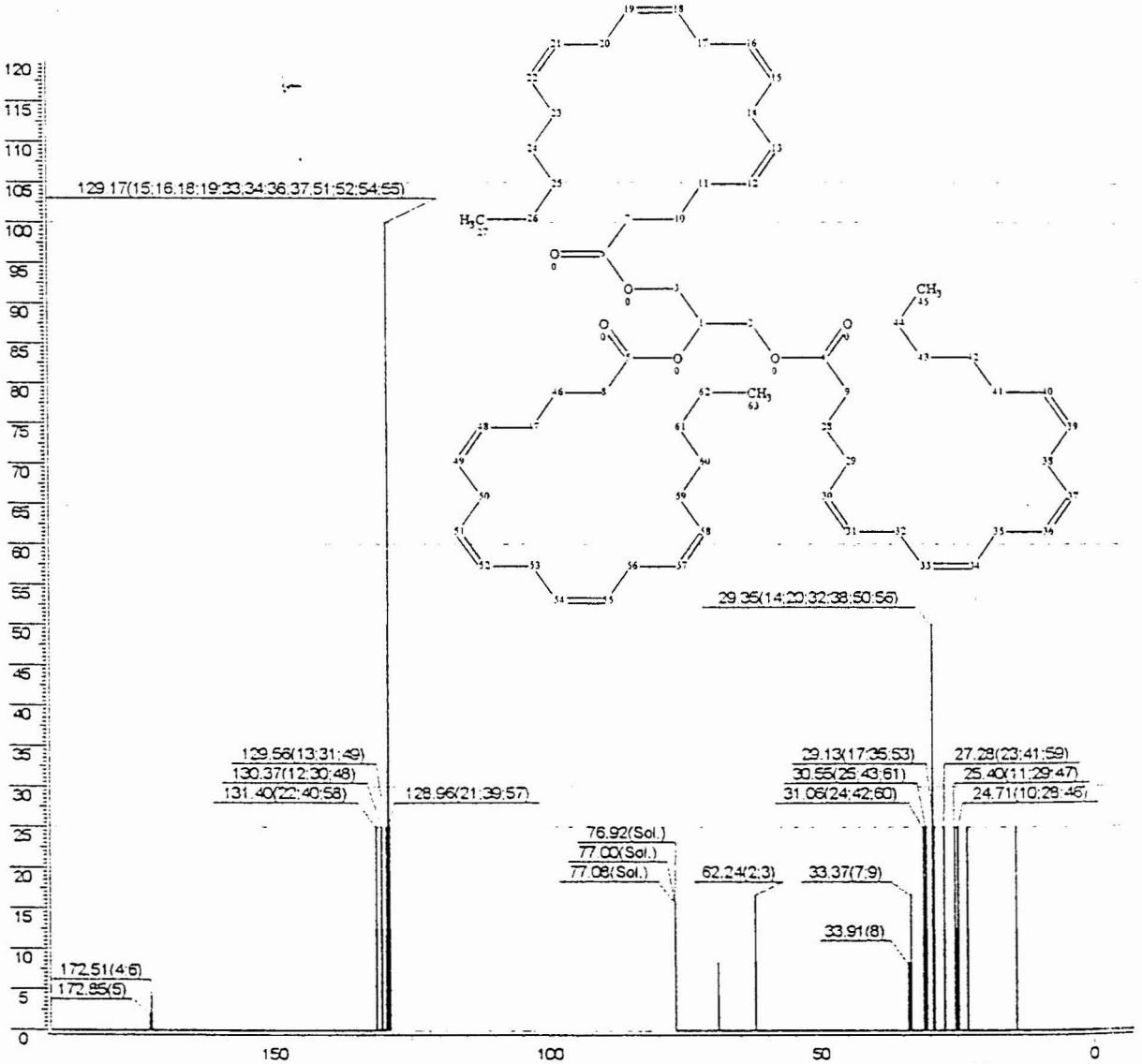


Figure 1.6 Carbon NMR ACD Spectrum of the Triglyceride of Arachidonic Acid

Carbon No.	CHn	Chem. Shifts	Conf. Limits	Carbon No.	CHn	Chem. Shifts	Conf. Limits	Carbon No.	CHn	Chem. Shifts	Conf. Limits
1	CH_2	62.24	4.5	20	CH	131.4	3.8	39	CH_2	30.55	0.8
2	CH	69.05	3.3	21	CH_2	27.28	3.2	40	CH_2	23.1	0.8
3	CH_2	62.24	4.5	22	CH_2	31.06	1.8	41	CH_2	14.22	0.1
4	C	172.36	1.1	23	CH_2	30.55	0.8	42	CH_2	27.22	4.4
5	C	173.27	0.7	24	CH_2	23.1	0.8	43	CH_2	29.35	1.2
6	C	172.36	1.1	25	CH_2	14.22	0.1	44	CH_2	29.01	0.5
7	CH_2	34.05	0.5	26	CH_2	25.17	0.7	45	CH_2	28.63	3.1
8	CH_2	34.32	0.2	27	CH_2	28.9	1.1	46	CH_2	28.8	2.8
9	CH_2	34.05	0.5	28	CH_2	29.01	0.5	47	CH_2	31.35	7.1
10	CH_2	25.17	0.7	29	CH_2	28.63	3.1	48	CH	130.5	3.2
11	CH_2	28.9	1.1	30	CH_2	28.8	2.8	49	CH	129.56	0.5
12	CH_2	29.01	0.5	31	CH_2	31.35	7.1	50	CH_2	29.58	19.1
13	CH_2	28.63	3.1	32	CH	130.5	3.2	51	CH	128.96	1.1
14	CH_2	28.8	2.8	33	CH	129.56	0.5	52	CH	131.4	3.8
15	CH_2	31.35	7.1	34	CH_2	29.58	19.1	53	CH_2	27.28	3.2
16	CH	130.5	3.2	35	CH	128.96	1.1	54	CH_2	31.06	1.8
17	CH	129.56	0.5	36	CH	131.4	3.8	55	CH_2	30.55	0.8
18	CH_2	29.58	19.1	37	CH_2	27.28	3.2	56	CH_2	23.1	0.8
19	CH	128.96	1.1	38	CH_2	31.06	1.8	57	CH_2	14.22	0.1

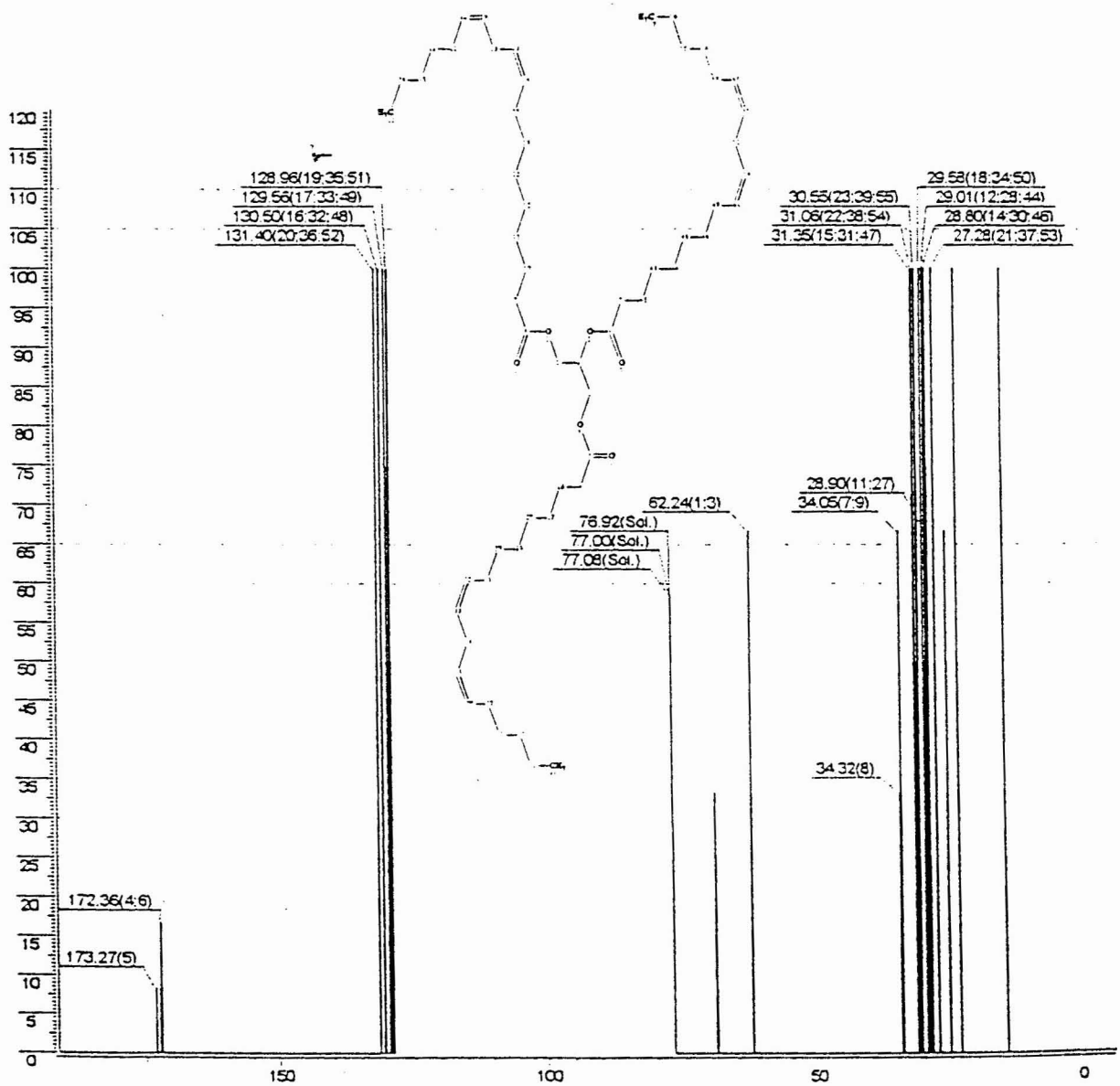


Figure 1.8 Carbon NMR ACD Spectrum of the Triglyceride of Linoleic Acid

groups (-CH=CH-C$\underline{H}_2$-CH=CH-). The multiplet at 4.3 ppm corresponds to glycerol methylene groups bonded to the oxygen of the ester groups. The multiplet at 5.35 ppm corresponds to the alkene groups and the multiplet at 5.39 ppm is the glycerol methine group.

In the ^{13}C NMR ($CDCl_3$) spectrum, the methyl groups were observed at 13.8 ppm, the methylene groups adjacent to the methyl groups were observed at 22.5 ppm and the chain of methylene groups in the fatty acid chain were in a range of 24.7 - 27.0 ppm. The diallylic groups linking the two alkene groups were observed at 29.6 ppm. and the allylic groups were observed at 29.5 The methylene groups alpha to the ester groups were observed at 34.0 ppm. The methylene groups which are part of the glycerol portion of the triglycerides were observed at 62.0 ppm, while the methine group of the glycerol was at 68.82 ppm. The alkene groups were between 127.7 - 130.0 ppm and the ester carbonyl groups were observed at 172.9 ppm.

Variations of Protocol A did not result in any significant changes in the NMR spectra aside from small changes in the relative peak height. Once the vertical scale was increased, the peaks appeared to be almost identical. In Protocol B, the size of the pestle was increased in order to better pulverize the tissue. With the larger pestle, the tissue was able to be pulverized to a fine powder. Protocols C and D used an ultrasonic sonicator (through water) for 10 min and 20 min, respectively. Protocols E and F used variations of the chloroform methanol mixture ratio (1:5 and 1:2, respectively) to see if changes in the polarity affected the sample. Again, none of these changes in protocol seemed to affect the sample according to the NMR spectra.

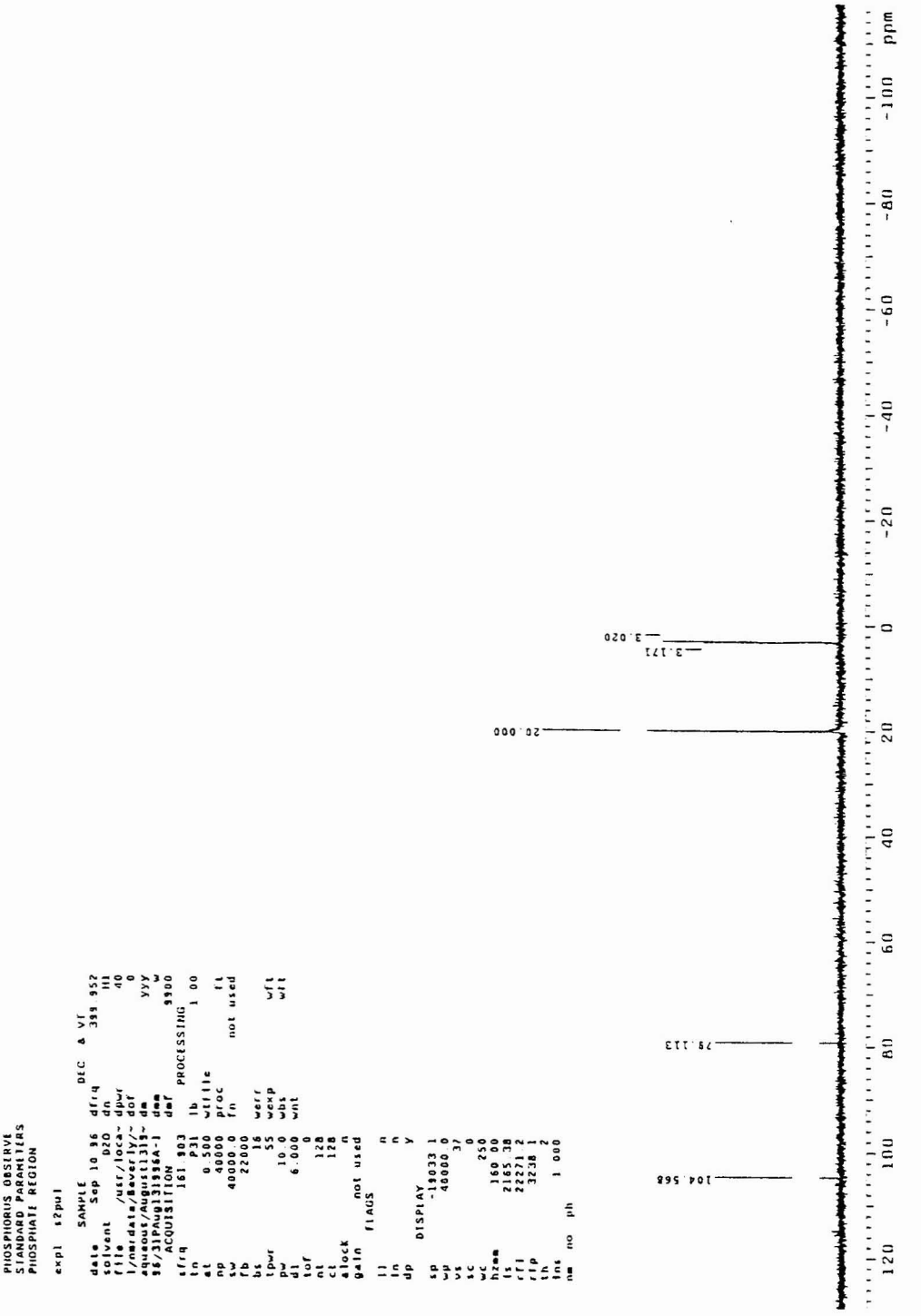
Chapter 2 Aqueous Layer of the Chloroform Methanol Extraction

The aqueous layer of the Chloroform Methanol Extraction is the focus of my work on the phosphorus containing metabolites found via ^{31}P NMR analysis. Observed metabolites include phosphocholine, phosphoethanolamine, and inorganic phosphate. A variety of protocols were performed in attempting to optimize the extraction of these metabolites.

In Protocol A, the tissue sample was pulverized with a mortar and pestle while submerged in liquid nitrogen, Then, 10 mM EDTA was added followed by the addition of a chloroform and methanol mixture (2:1). The sample was then placed in an ice bath for 60 min. After the 4°C incubation, chloroform was added to the mixture followed by Tris buffer, pH 8. The mixture was then centrifuged at 3000 rpm for 15 min at 4°C. As a result of centrifugation, the mixture separated into a top, aqueous layer; a middle layer of cellular debris; and a bottom, organic (chloroform) layer. The aqueous and organic layers were pipetted into individual test tubes. MDPA was added to the aqueous layer.

The ^{31} P NMR (D_2O) spectrum of the organic extract from Protocol A (Figure 2.1) displayed a peak at 3.0 ppm, a peak at 3.2 ppm, and a peak at 20.0 ppm due to the internal standard, MDPA (methylene diphosphonic acid), and two additional weak peaks that were not identified in this research project, one at 79.1 ppm and the other at 104.6 ppm. The peak at 3.0 ppm may be due to the inorganic phosphate and the peak at 3.2

Figure 2.1 Phosphorus NMR Spectrum of the Aqueous Extract in Protocol A



ppm may be due to phosphoethanolamine, (PE). However, the pH was not adjusted to a pH of 7.4 at this point in the project and therefore the chemical shift was not at the expected 6.7 +/- 0.3 ppm for inorganic phosphate and 7.7 +/- 0.3 ppm for PE. For this sample, the ^{31}P acquisition time was set to 0.5 sec with a delay of 6 sec.

The sample concentrations were based on the peak integrals with respect to the internal standard. Known concentrations of the phosphocholine (PC) and phosphoethanolamine (PE) were analyzed via ^{31}P NMR and integrated according to the internal standard, MDPA. (Figure 2.2 and 2.3) The PC concentration curve was not linear which may be due to PC adhering to the sides of the NMR tube. The peak concentrations were obtained according to the peak integral of the internal standard, MDPA, which was set at one.

The PC, PE, and inorganic phosphate peaks were identified, in general, by the ranges discussed in the literature.[1-3] More specifically, the peaks were identified according to the spiked concentrations of PC and PE prior to the extraction procedure and also spiked concentrations of PC, PE, and inorganic phosphate after the extraction procedure, prior to ^{31}P NMR analysis. The spiked PC chemical shift was in the range of 7.2 +/- 0.3 ppm and the spiked PE chemical shift was in the range of 7.7 +/- 0.3 ppm.. The spiked inorganic phosphate was in the range of 6.6 +/- 0.3 ppm. These chemical shifts were very pH and concentration dependent. It was not until Protocol D that the pH was adjusted to 7.4.

Numerous variations of Protocol A were performed in an attempt to extract phosphocholine and phosphoethanolamine. In addition, the NMR parameters for these protocols were also changed to 0.5 sec for the acquisition time and 6 sec for the delay.

Figure 2.2 PC Concentration

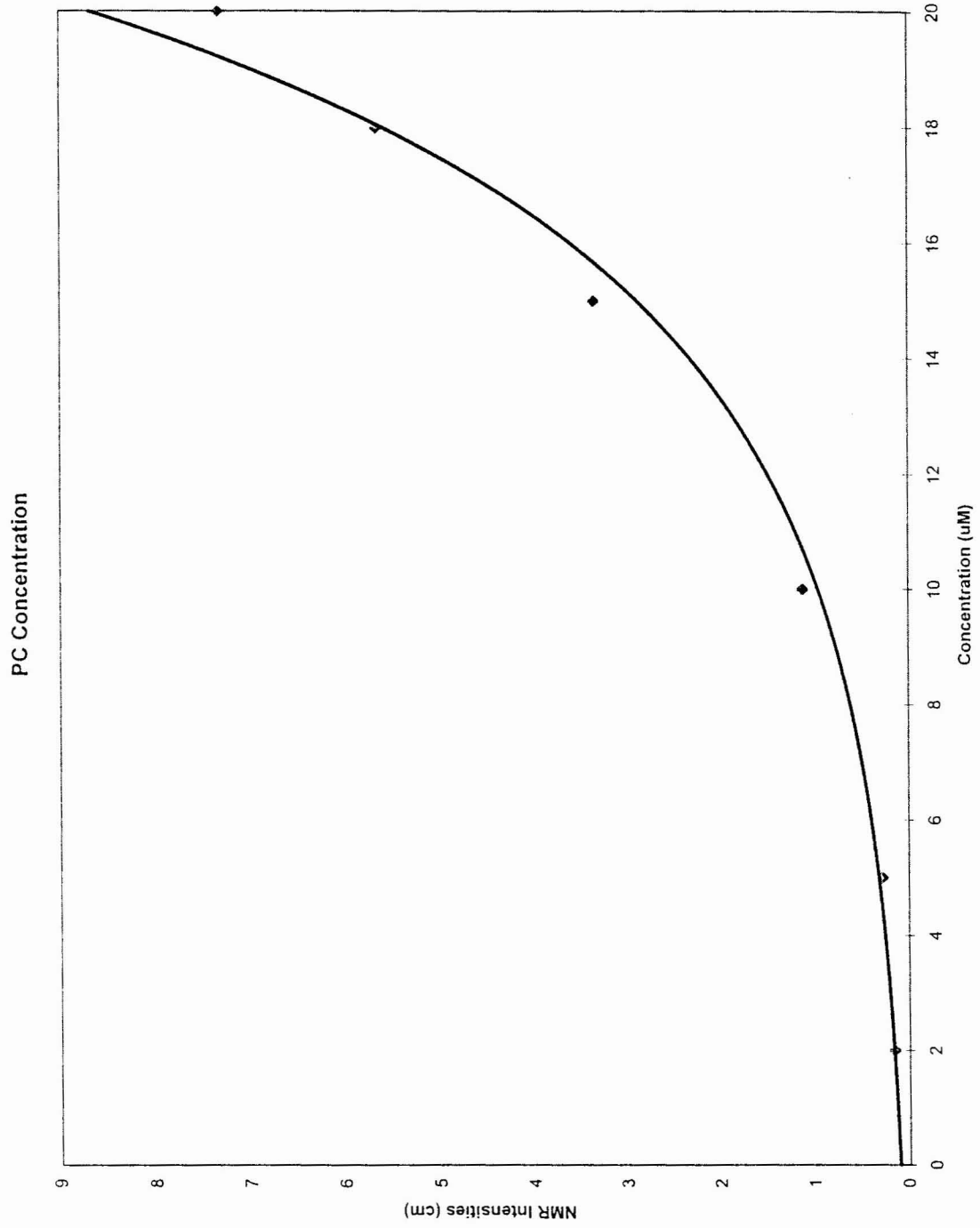
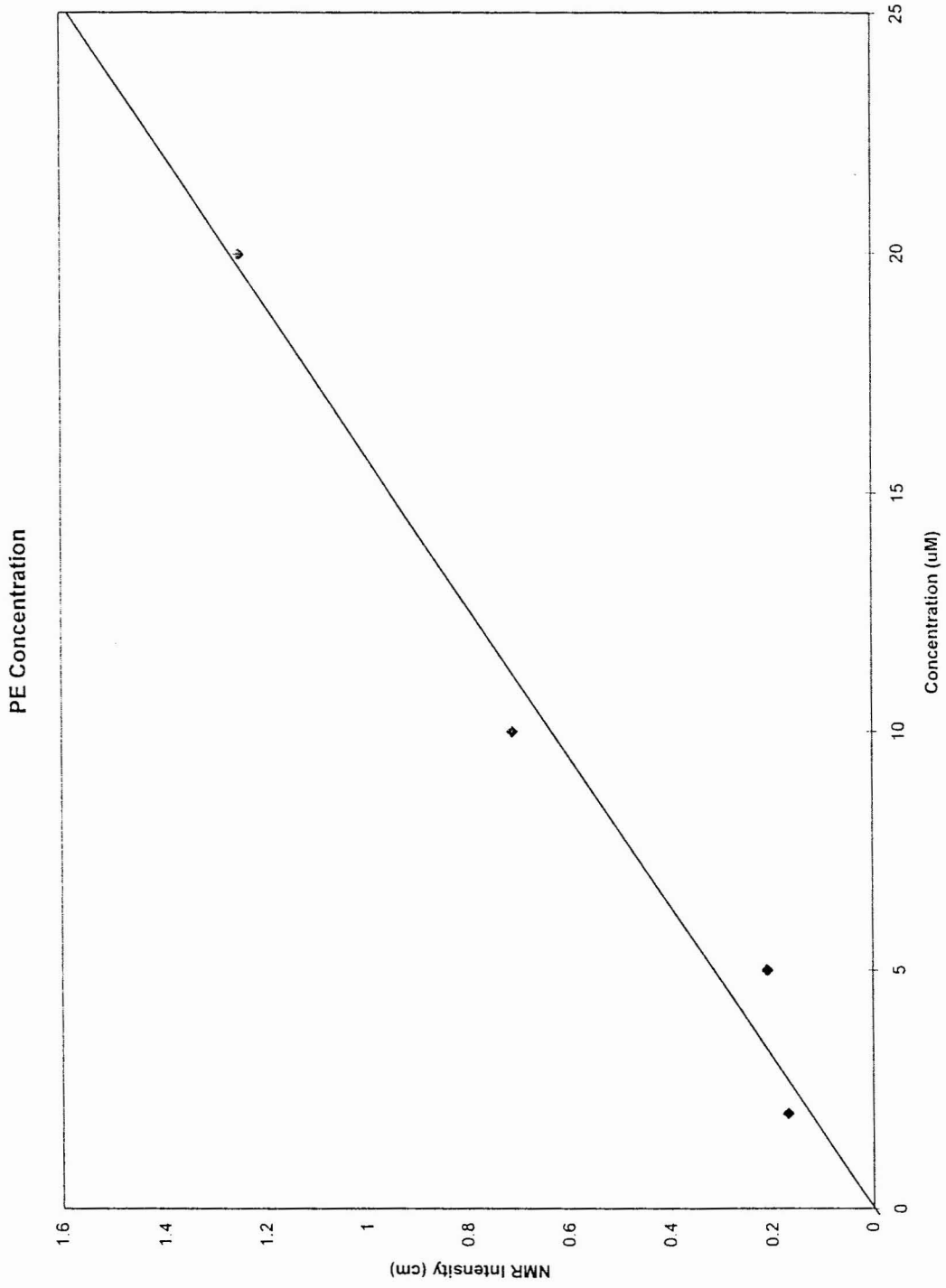


Figure 2.3 PE Concentration



Below is a table of the various protocols, their chemical shifts, and their concentrations relative to the standard.

Protocol	MDPA Peak	Peak A	Concentration Peak A	Peak B	Concentration Peak B
A	20.0 ppm	3.0 ppm	1.5	3.2 ppm	1.2
B	20.0 ppm	3.3 ppm	0.0006	none	none
C	20.0 ppm	5.5 ppm	0.1	none	none
D	20.0 ppm	6.4 ppm	0.05	6.7 ppm	0.01
E	20.0 ppm	6.4 ppm	0.05	none	none
F	20.0 ppm	6.7 ppm	0.05	none	none
G	20.0 ppm	7.2 ppm	0.1	7.7 ppm	0.2
H	20.0 ppm	6.4 ppm	4	7.7 ppm	2
I	20.0 ppm	6.7 ppm	0.2	none	none
J Sample 1	20.0 ppm	6.4 ppm	0.03	none	none
J Sample 2	20.0 ppm	none	none	none	none
J Sample 3	20.0 ppm	none	none	none	none
J Sample 4	20.0 ppm	none	none	none	none
J Sample 5	20.0 ppm	6.7 ppm	0.2	none	none
K	20.0 ppm	6.5 ppm	0.2	6.7 ppm	0.08

In Protocols B and C, the single peaks may be due to inorganic phosphate. In the literature, Dr. T. Smith and colleagues[2] reported the inorganic phosphate to be greater than that of any of the other metabolite peaks, such as phosphocholine (PC), phosphoethanolamine (PE), glycerol 3-phosphocholine (GPC), and glycerol 3-phosphoethanolamine (GPE). In addition, the chemical shifts were very dependent upon pH and metabolite concentration. In this part of the study, the pH of the extract was not adjusted to 7.4. Based on this information, the peaks may be due to inorganic phosphate.

In the remaining protocols, the pH was adjusted to 7.4. For Protocols D through K, the inorganic phosphate appeared around 6.4 +/- 0.3 ppm. Protocol H displayed a peak around 7.7 ppm that was due to PE. In Protocols D and K, the peak that was observed at 6.7 ppm may be due to PE. According to the literature by Dr. T. Smith and colleagues[3], PE represents the second greatest metabolite concentration, with inorganic phosphate being the highest in concentration. With this in mind, PE may be the metabolite that appeared at 6.7 ppm, but was not positively identified. Protocol G had two peaks, one at 7.2 ppm and the other at 7.7 ppm, that were due to the spiked PC and PE, respectively.

Through the variations in the protocols, 12 out of the 15 tissue extractions demonstrated a peak that was due to the inorganic phosphate and 4 out of the 15 tissue extractions demonstrated a second peak that was due to PE. Unfortunately, PC was never observed via ^{31}P NMR.

Again, these extraction samples were very pH and concentration dependent in relation to their chemical shift. In addition, these metabolites either appear to decompose

with time or were bound/cleaved by some cationic species such as Ca^{2+}. In the future, $\{^{13}C\}^{1}H$ analysis may yield better results due to its greater sensitivity.

Chapter 3 Time Studies of the Chloroform Methanol Extraction

In the literature, Dr. T. Smith[3] reported studies of the stability of metabolites in breast tissue in relation to the effect of time between removing the tissue from the blood supply and submersing the tissue in liquid nitrogen. In these studies, it was concluded that tissue samples remained stable for approximately one hour after being removed from the blood supply. However, observations were not made prior to 8 min after resection. The above mentioned literature's analysis was performed using ^{31}P NMR of the aqueous phase of the Chloroform Methanol Extraction. Consequently, I have tried to shorten this untexted period of time.

In the chloroform phase of the (Chloroform Methanol Extraction) Time Study, the tissue samples were pulverized with a mortar and pestle while submerged in liquid nitrogen. 10 mM EDTA was added to the sample(s), followed by the addition of a chloroform methanol mixture (2:1). The sample(s) was then placed in an ice bath for 60 min. After the 4°C incubation, chloroform was added to the mixture(s) followed by Tris buffer, pH 8. The mixture(s) was then centrifuged at 3000 rpm for 15 min at 4°C. As a result of centrifugation, the mixture(s) separated into a top, aqueous layer; a middle layer of cellular debris; and a bottom, organic (chloroform) layer. The aqueous and organic layers were pipetted into individual test tubes. The solvents in the chloroform layer were removed via a stream of nitrogen gas and the sample(s) was rediluted with deuterated chloroform. 1M MDPA was then added to the (chloroform layer) sample.

The ^{1}H NMR ($CDCl_3$) spectra of the Chloroform Layer Time Study was assigned using the ACD/CNMR program as described previously. The four samples (6 min, 8 min, 15 min, and 22 min) involved in the time study revealed identical spectra. Therefore, from approximately 6 min to 22 min, the triglycerides (arachidonic acid and linoleic acid), remained stable, as would have been expected.

In the Aqueous Phase of the (Chloroform Methanol Extraction) Time Study, the tissue sample(s) was pulverized with a mortar and pestle while submersed in liquid nitrogen. 0.4 M EDTA was added to the sample(s), followed by the addition of a chloroform and methanol mixture (2:1). The sample(s) was then placed in an ice bath for 60 min. After the 4°C incubation, chloroform was added to the mixture followed Tris buffer, pH 7. The mixture(s) was then centrifuged at 3000 rpm for 15 min at 4°C. As a result of centrifugation, the mixture(s) separated into a top, aqueous layer; a middle layer of cellular debris; and a bottom, organic (chloroform) layer. The aqueous and organic layers were pipetted into individual test tubes. MDPA was added to the aqueous layer and the pH was adjusted to 7.4.

The ^{31}P NMR (D_2O) spectra of the Aqueous Layer Time Study were assigned, as described previously, with the aid of spiked samples of known PC, PE, and inorganic phosphate, along with reported literature results. The measurements were performed using an acquisition time of 0.5 sec with a delay of 6 sec and the pulse width was set to 2.

^{31}P NMR of Sample 1 revealed only the internal standard, MDPA, at 20.0 ppm. Sample 2 displayed two peaks in addition to the internal standard, at 20.0 ppm. The first peak was observed at 6.5 ppm and the second peak was at 6.7 ppm. The peak at 6.5 ppm

should be performed and compared for similarities and differences. Another extraction procedure called the perchloric acid extraction should also be considered in attempt to extract the phosphorus metabolites. This extraction procedure focuses mainly on the ^{1}H NMR spectra. In addition, a more sensitive form of ^{13}C NMR, via indirect detection (i.e. $\{^{13}C\}^{1}H$ NMR), will soon be available in the department for analysis, which may enable better detection of the phosphorus metabolites.

Chloroform Methanol Extraction, Chloroform layer. Protocol A

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- stainless steel mortar and pestle
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 10 mM EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 8
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 10 mM EDTA (2 ml / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol / conc. hydrochloric acid (2:1:0.01), (3.75 ml / gram of tissue). Vortex.

7. Let mixture stand for 60 minutes at $4^{o}C$ via an ice bath.

8. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH ~8.0, (1.25 ml / gram tissue).

9. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

10. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

11. Express off the organic solvents via nitrogen gas.

3/18/97

Chloroform Methanol Extraction, Chloroform layer. Protocol B

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 10 mM EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 8
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 10 mM EDTA (2 ml / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol / conc. hydrochloric acid (2:1:0.01), (3.75 ml / gram of tissue). Vortex.

7. Let mixture stand for 60 minutes at $4^{o}C$ via an ice bath.

8. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH ~8.0, (1.25 ml / gram tissue).

9. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

10. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

11. Express off the organic solvents via nitrogen gas.

3/18/97

Chloroform Methanol Extraction, Chloroform layer. Protocol C

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 10 mM EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 8
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 10 mM EDTA (2 ml / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol / conc. hydrochloric acid (2:1:0.01), (3.75 ml / gram of tissue). Vortex.

7. Let mixture stand for 60 minutes at $4^{o}C$ via an ice bath.

8. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH ~8.0, (1.25 ml / gram tissue).

9. Sonicate in a water bath for 10 min.

10. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

11. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

12. Express off the organic solvents via nitrogen gas.

3/18/97

Chloroform Methanol Extraction, Chloroform layer. Protocol D

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 10 mM EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 8
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 10 mM EDTA (2 ml / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol / conc. hydrochloric acid (2:1:0.01), (3.75 ml / gram of tissue). Vortex.

7. Let mixture stand for 60 minutes at $4^{o}C$ via an ice bath.

8. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH ~8.0, (1.25 ml / gram tissue).

9. Sonicate in a water bath for 20 min.

10. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

11. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

12. Express off the organic solvents via nitrogen gas.

3/18/97

Chloroform Methanol Extraction, Chloroform layer. Protocol E

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle
- sonicator

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 10 mM EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 8
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.
2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.
3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.
4. Pulverize frozen tissue into a fine powder using a mortar and pestle.
5. Add 10 mM EDTA (2 ml / gram tissue).
6. Add an ice-cold mixture of chloroform / methanol, (1:5), (3.75 ml / gram of tissue). Vortex.
7. Let mixture stand for 60 minutes at $4^{o}C$ via an ice bath.

8. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH ~8.0, (1.25 ml / gram tissue).

9. Sonicate in a water bath for 20 min.

10. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

11. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

12. Express off the organic solvents via nitrogen gas.

3/18/97

Chloroform Methanol Extraction, Chloroform layer. Protocol F

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle
- sonicator

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 10 mM EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 8
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 10 mM EDTA (2 ml / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol, (1:2), (3.75 ml / gram of tissue). Vortex.

7. Let mixture stand for 60 minutes at $4^{o}C$ via an ice bath.

8. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH ~8.0, (1.25 ml / gram tissue).

9. Sonicate in a water bath for 15 min.

10. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

11. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

12. Express off the organic solvents via nitrogen gas.

3/18/97

Chloroform Methanol Extraction, Aqueous layer. Protocol A

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 10 mM EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 8
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 10 mM EDTA (2 ml / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol, (2:1), (3.75 ml / gram of tissue). Vortex.

7. Let mixture stand for 60 minutes at $4^{o}C$ via an ice bath.

8. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH ~8.0, (1.25 ml / gram tissue).

9. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

10. Remove the aqueous phase with a disposable pipette and add 2.5 µm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

3/18/97

Chloroform Methanol Extraction, Aqueous layer. Protocol B

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 0.4 M EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 10 mEDTA (2 ml / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol, (1:5) (3.75 ml / gram of tissue). Vortex.

7. Let mixture stand for 60 minutes at $4^{o}C$ via an ice bath.

8. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

9. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

10. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

3/18/97

Chloroform Methanol Extraction, Aqueous layer. Protocol C

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 0.4 M EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 10 mM EDTA (2 ml / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol, (1:2), (3.75 ml / gram of tissue). Vortex.

7. Let mixture stand for 60 minutes at $4^{o}C$ via an ice bath.

8. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

9. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

10. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4. Save chloroform and aqueous phase for analysis.

3/18/97

Chloroform Methanol Extraction, Aqueous layer. Protocol D

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 0.4 M EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 0.4 M EDTA (25 μl / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol, (1:2), (3.75 ml / gram of tissue). Vortex.

7. Let mixture stand for 60 minutes at 4^{o}C via an ice bath.

8. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

9. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

10. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

3/18/97

Chloroform Methanol Extraction, Aqueous layer. Protocol E

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle
- point sonicator

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 0.4 M EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.
2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.
3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.
4. Pulverize frozen tissue into a fine powder using a mortar and pestle.
5. Add 0.4 M EDTA (25 μl / gram tissue).
6. Add an ice-cold mixture of chloroform / methanol, (2:1), (3.75 ml / gram of tissue). Vortex.
7. Point sonicate for 1 min.
8. Let mixture stand for 60 minutes at $4^{o}C$ via an ice bath.

9. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

10. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

11. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

3/18/97

Chloroform Methanol Extraction, Aqueous layer. Protocol F

Cancerous and non-cancerous tissues are obtained and frozen va liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle
- point sonicator

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 0.4 M EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.
2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.
3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.
4. Pulverize frozen tissue into a fine powder using a mortar and pestle.
5. Add 0.4 M EDTA (4 ml / gram tissue).
6. Add an ice-cold mixture of chloroform / methanol, (2:1), (3.75 ml / gram of tissue). Vortex.
7. Point sonicate for 1 min.
8. Let mixture stand for 60 minutes at $4^{o}C$ via an ice bath.

9. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

10. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

11. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

3/18/97

Chloroform Methanol Extraction, Aqueous layer. Protocol G

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 0.4 M EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 2.5 μM methylene diphosphonic acid (MDPA)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform
- 1 M PC
- 1 M PE

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 2.5 μl of 1 M PC and 2.5 μl of 1 M PE.

6. Add 0.4 M EDTA (25 μl / gram tissue).

7. Add an ice-cold mixture of chloroform / methanol, (2:1), (3.75 ml / gram of tissue). Vortex.

8. Let mixture stand for 60 minutes at 4°C via an ice bath.

9. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

10. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

11. Remove the aqueous phase with a disposable pipette and add 2.5 μm of methylene diphosphonic acid (MDPA) to adjust the pH to 7.4.
Save chloroform and aqueous phase for analysis.

3/18/97

Chloroform Methanol Extraction, Aqueous layer. Protocol H

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle
- homogenizer

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 0.4 M EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 25 µl methylene diphosphonic acid (MDPA) (18 mg / ml)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 0.4 M EDTA (25 µl / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol, (1:2), (3.75 ml / gram of tissue). Vortex.

7. Homogenize tissue for 1 min.

8. Let mixture stand for 60 minutes at 4°C via an ice bath.

9. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

10. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

11. Remove the aqueous phase with a disposable pipette and add 25 µl of methylene diphosphonic acid (MDPA), adjust pH to 7.4.
Save chloroform and aqueous phase for analysis.

3/18/97

Chloroform Methanol Extraction, Aqueous layer. Protocol I

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle
- homogenizer

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 0.4 M EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 25 µl methylene diphosphonic acid (MDPA) (18 mg / ml)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample after being immersed in liquid nitrogen and allowing the liquid nitrogen to evaporate completely, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 0.4 M EDTA (25 µl / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol, (1:2), (3.75 ml / gram of tissue). Vortex.

7. Homogenize tissue for 1 min.

8. Let mixture stand for 60 minutes at 4°C via an ice bath.

9. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

10. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

11. Remove the aqueous phase with a disposable pipette and add 25 µl of methylene diphosphonic acid (MDPA), adjust pH to 7.4.
Save chloroform and aqueous phase for analysis.

3/18/97

Chloroform Methanol Extraction, Aqueous layer. Protocol J

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle
- homogenizer

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 0.4 M EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 25 µl methylene diphosphonic acid (MDPA) (18 mg / ml)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample after being immersed in liquid nitrogen and allowing the liquid nitrogen to evaporate completely, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 0.4 M EDTA (25 µl / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol, (1:2), (3.75 ml / gram of tissue). Vortex.

7. Homogenize tissue for 10 s, 15 s, 30 s, 45 s, and 60 s.

8. Let mixture stand for 60 minutes at 4°C via an ice bath.

9. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

10. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

11. Remove the aqueous phase with a disposable pipette and add 25 µl of methylene diphosphonic acid (MDPA), adjust pH to 7.4.
Save chloroform and aqueous phase for analysis.

3/18/97

Chloroform Methanol Extraction, Aqueous layer. Protocol K

Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000 rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle
- homogenizer

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 0.4 M EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 25 µl methylene diphosphonic acid (MDPA) (18 mg / ml)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample after being immersed in liquid nitrogen and allowing the liquid nitrogen to evaporate completely, weight range should be between 0.5 g and 2.0 g.

3. Immerse unfixed samples in liquid nitrogen within a measured time of excision. Record time immersed in liquid nitrogen.

4. Pulverize frozen tissue into a fine powder using a mortar and pestle.

5. Add 0.4 M EDTA (25 µl / gram tissue).

6. Add an ice-cold mixture of chloroform / methanol, (1:2), (3.75 ml / gram of tissue). Vortex.

7. Homogenize tissue for 10 sec.

8. Let mixture stand for 60 minutes at 4°C via an ice bath.

9. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

10. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

11. Remove the aqueous phase with a disposable pipette and add 25 µl of methylene diphosphonic acid (MDPA), adjust pH to 7.4.
Save chloroform and aqueous phase for analysis.

3/18/97

Chloroform Methanol Extraction, Chloroform layer. Time Study.

To Examine the stability of metabolites at room temperature within a designated time frame of severing the blood supply to the tumor via the CM extraction procedure. Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 10 mM EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 25 µl methylene diphosphonic acid (MDPA) (18 mg / ml)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Finely mince the sample using a scalpel.

4. Divide the sample into four equal parts and label (1, 2, 3, 4).

5. Immerse unfixed samples in liquid nitrogen at varing intervals of time. Record time immersed in liquid nitrogen.

6. Pulverize frozen tissue into a fine powder using a mortar and pestle.

7. Add 10 mM EDTA (2 ml / gram tissue).

8. Add an ice-cold mixture of chloroform / methanol, (2:1), (3.75 ml / gram of tissue). Vortex.

9. Let mixture stand for 60 minutes at 4°C via an ice bath.

10. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

11. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

12. Remove the aqueous phase with a disposable pipette and add 25 µl of methylene diphosphonic acid (MDPA), adjust pH to 7..
Save chloroform and aqueous phase for analysis.

13. Express off organic solvents via nitrogen gas.

2/22/97

Chloroform Methanol Extraction, Aqueous Layer. Time Study.

To Examine the stability of metabolites at room temperature within a designated time frame of severing the blood supply to the tumor via the CM extraction procedure. Cancerous and non-cancerous tissues are obtained and frozen via liquid nitrogen. Through a series of steps, the metabolites are extracted by chloroform-methanol (CM). CM extracts only lipid, soluble components of the tumor.

Equipment:

- scale (0.1 g - 3.0g)
- stop watch
- vortex
- cold centrifuge (3000rpm)
- Dewar flask(s)
- vortex
- Gilson adjustable pipette
- cooler
- pipette bulb
- scalpel
- stainless steel mortar
- porcelain pestle
- homogenizer

Consumables:

- pH paper (6-8)
- weighing paper
- test tubes
- liquid nitrogen
- 10 mM EDTA
- deuterated chloroform
- methanol
- 10 mM Tris buffer, pH 7
- 25 µl methylene diphosphonic acid (MDPA) (18 mg / ml)
- conc. HCl
- aluminum
- serological pipette (5ml)
- Gilson pipette tips
- chloroform

Procedure:

1. Observe and record visual nature of the sample, and record time tissue removed from patient.

2. Weigh the sample, weight range should be between 0.5 g and 2.0 g.

3. Finely mince the sample using a scalpel.

4. Divide the sample into four equal parts and label (1, 2, 3, 4).

5. Immerse unfixed samples in liquid nitrogen at varing intervals of time. Record time immersed in liquid nitrogen.

6. Pulverize frozen tissue into a fine powder using a mortar and pestle.

7. Reweigh samples in a preweighed test tube, once liquid nitrogen has evaporated off.

8. Add 0.4 M EDTA (25µl / gram tissue).

9. After ____ minutes, add an ice-cold mixture of chloroform / methanol, (2:1), (3.75 ml / gram of tissue). Vortex.

10. Homogenize tissue for 10 sec.

11. Let mixture stand for 60 minutes at 4°C via an ice bath.

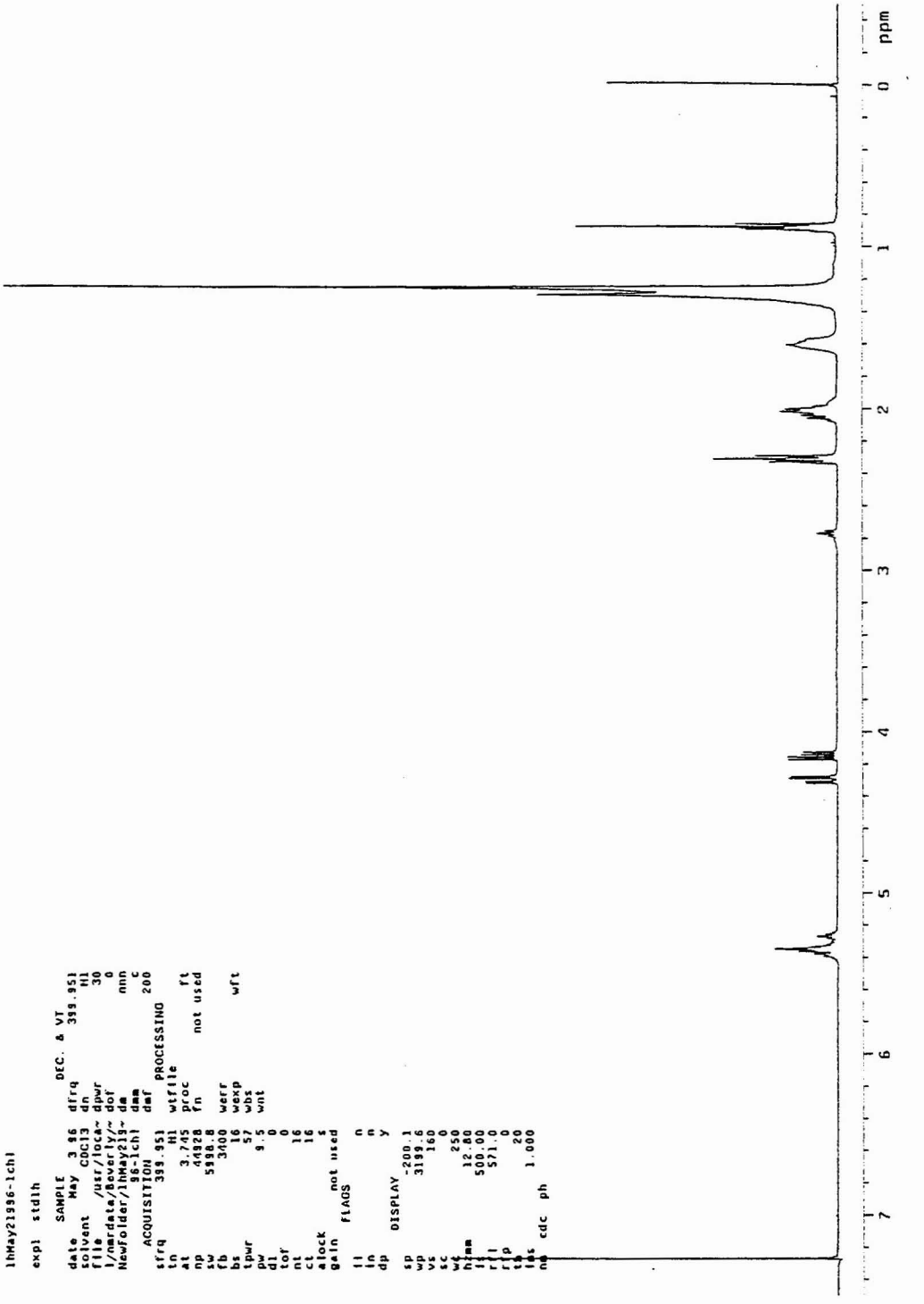
12. Add chloroform (1.25 ml / gram tissue), then add 10 mM Tris buffer, pH 7, (1.25 ml / gram tissue).

13. Using a cold centrifuge (4°C), centrifuge at 3000 rpm for 15 minutes.

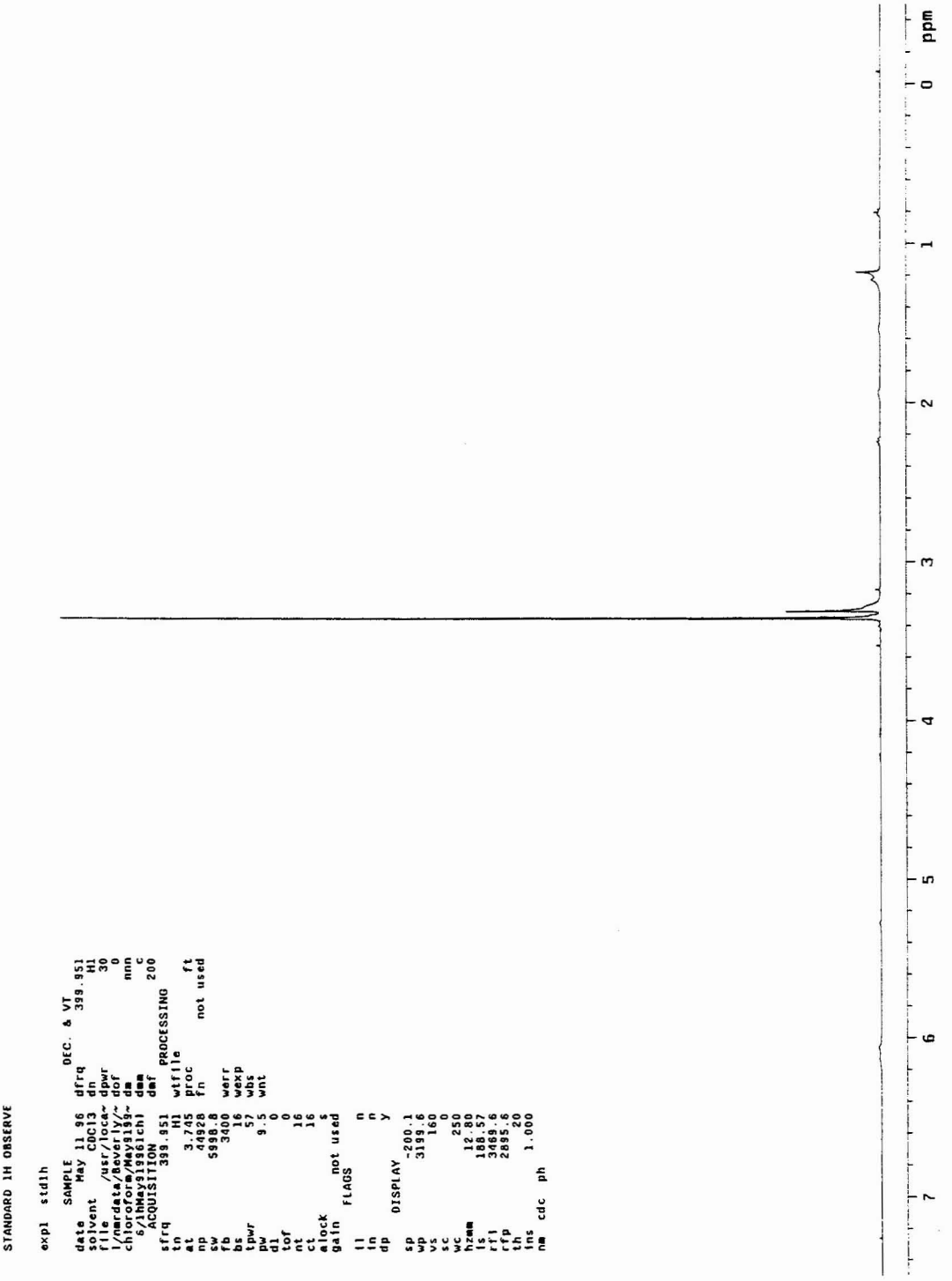
14. Remove the aqueous phase with a disposable pipette and add 25 µl of methylene diphosphonic acid (MDPA), adjust pH to 7..
Save chloroform and aqueous phase for analysis.

2/22/97

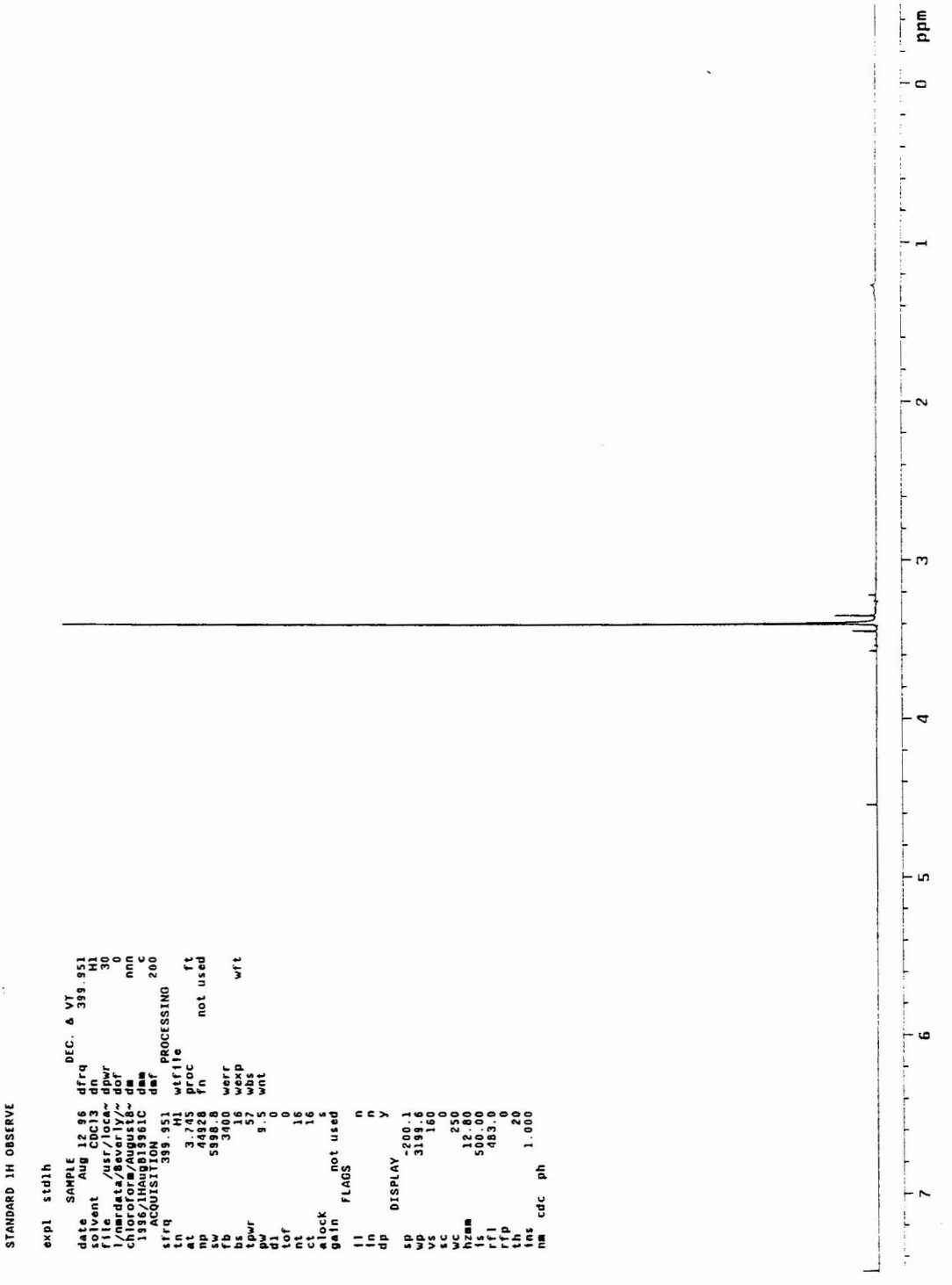
Chloroform Methanol Extraction, Chloroform Layer. Protocol A.



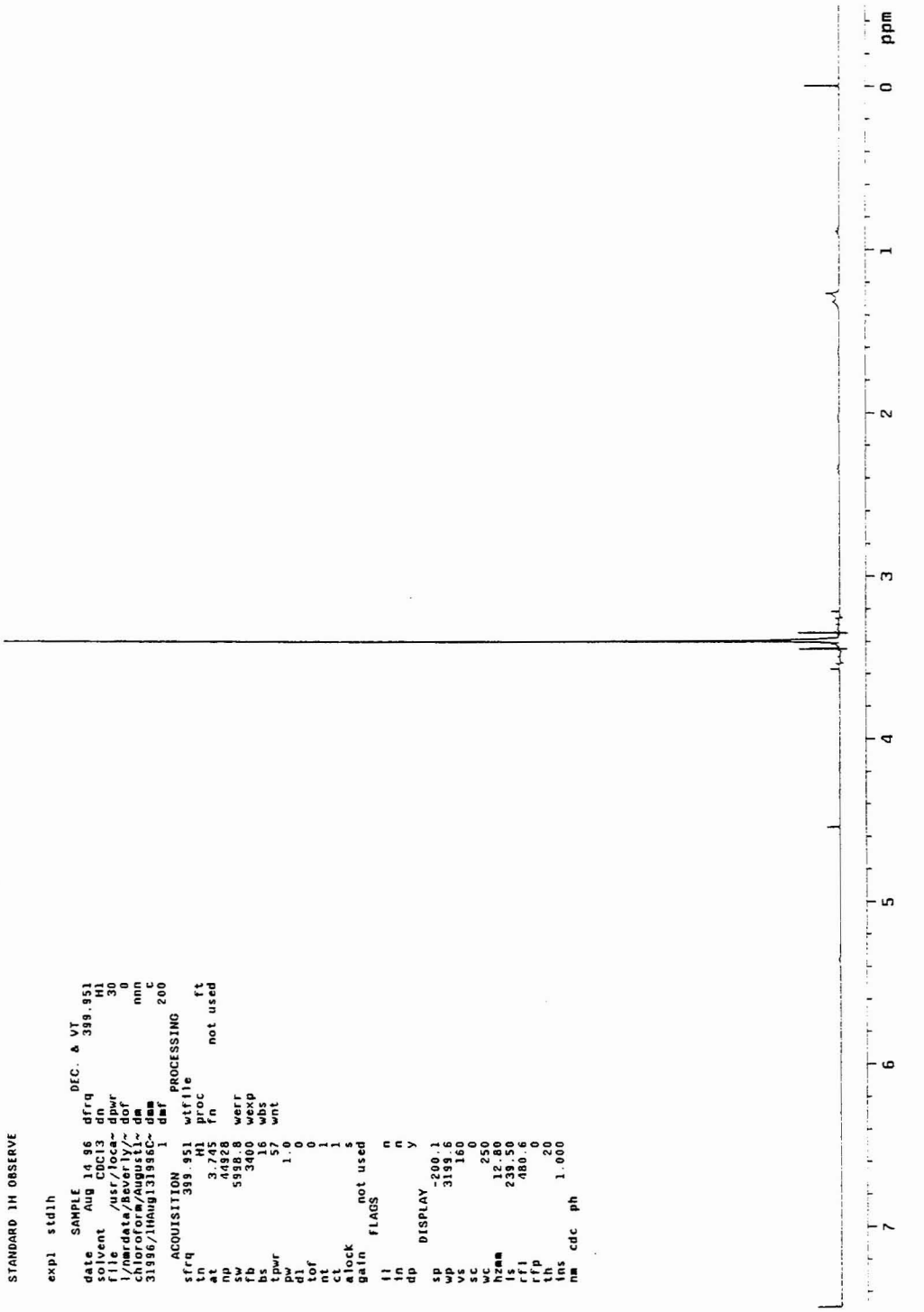
Chloroform Methanol Extraction, Chloroform Layer. Protocol B.



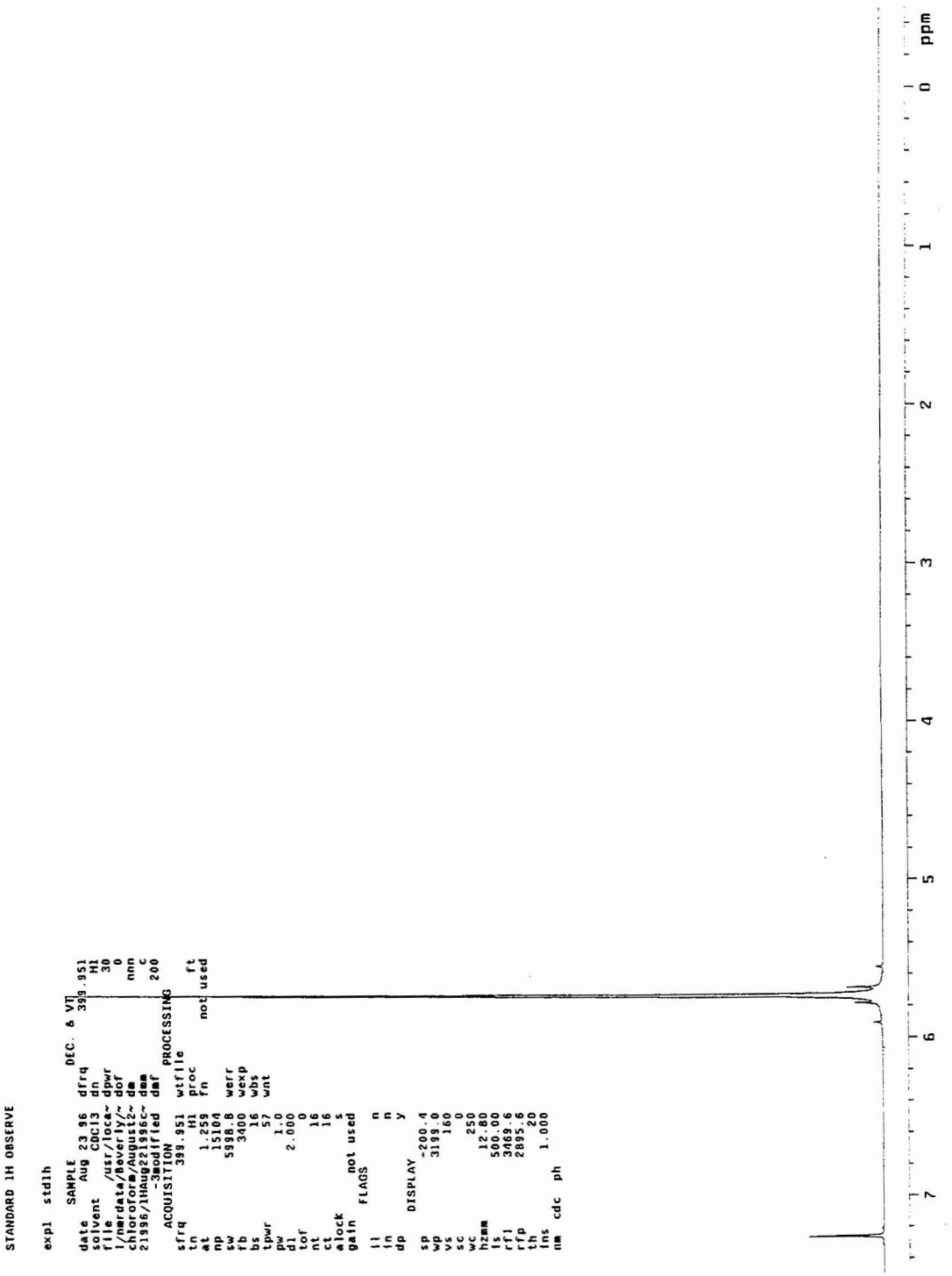
Chloroform Methanol Extraction, Chloroform Layer. Protocol C.



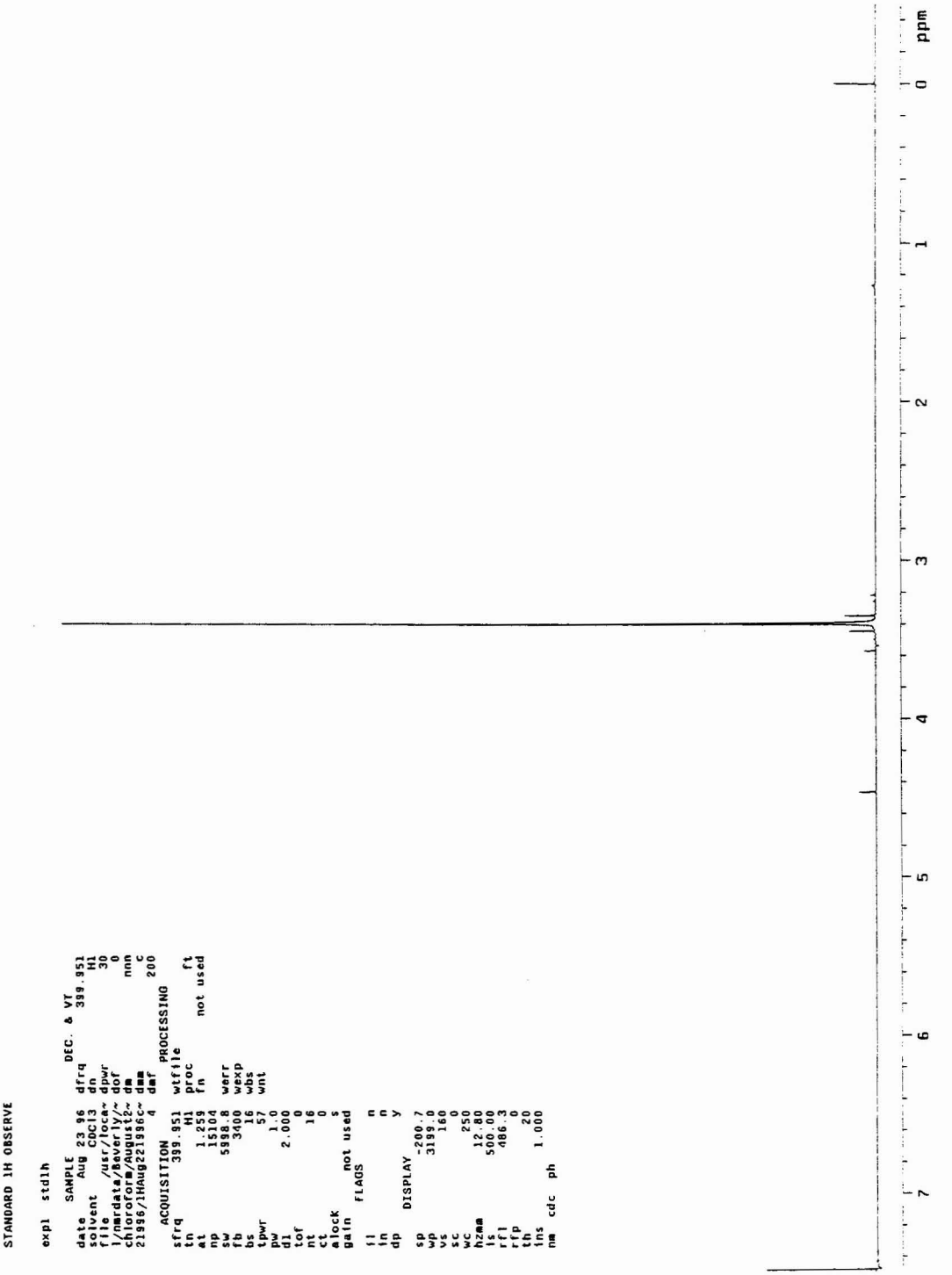
Chloroform Methanol Extracton, Chloroform Layer. Protocol D.



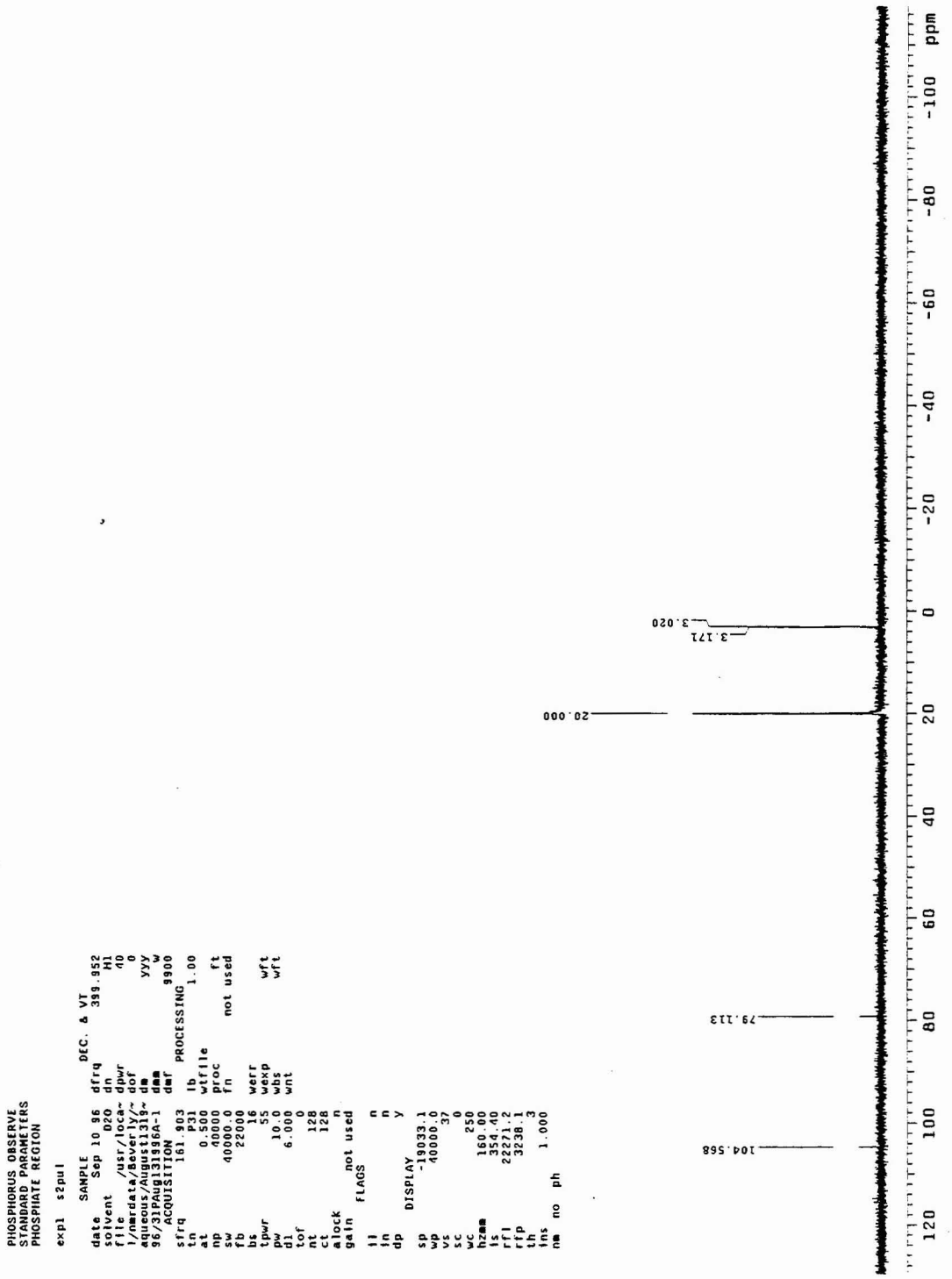
Chloroform Methanol Extraction, Chloroform Layer. Protocol E.



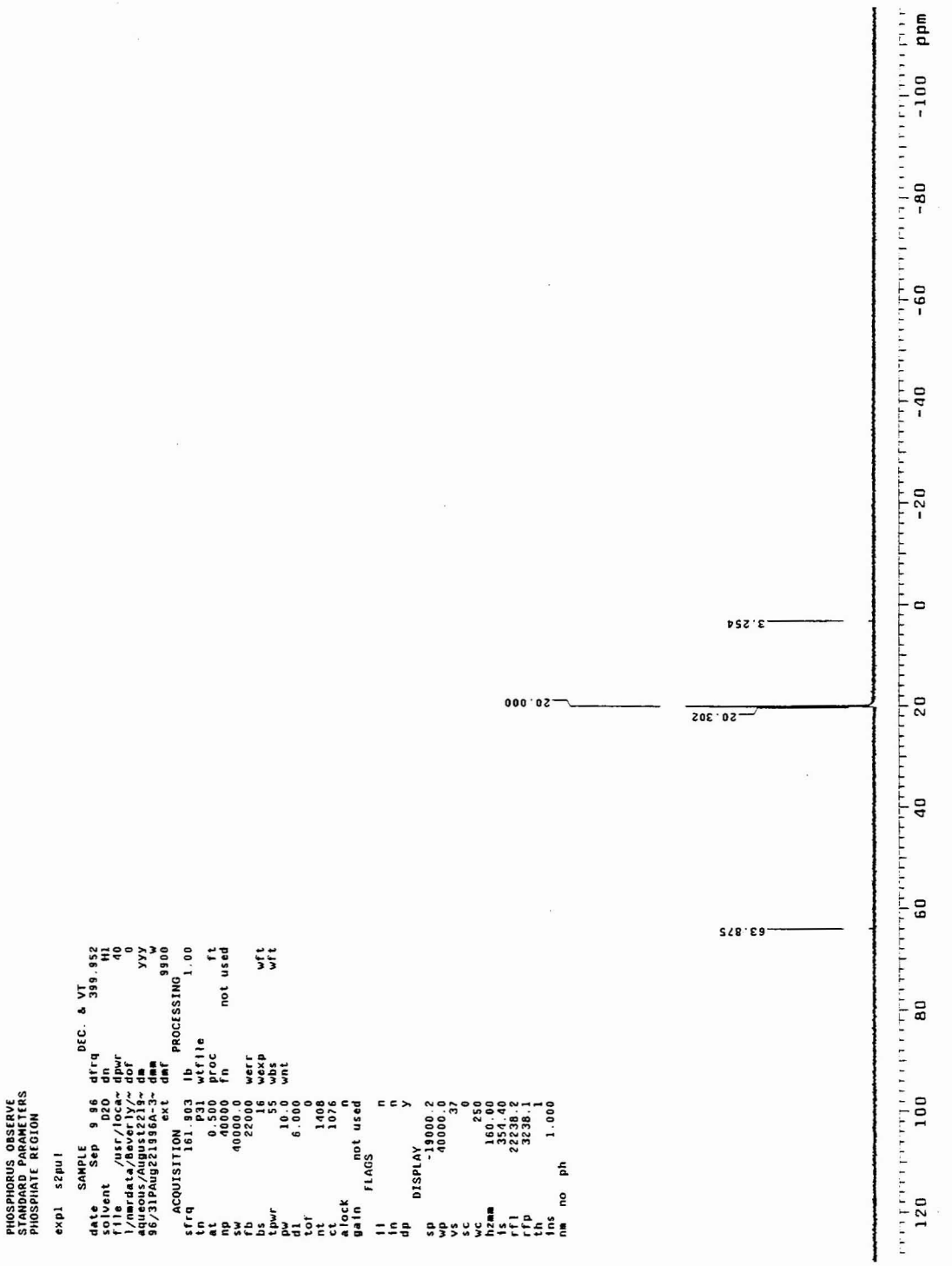
Chloroform Methanol Extraction, Chloroform Layer. Protocol F.



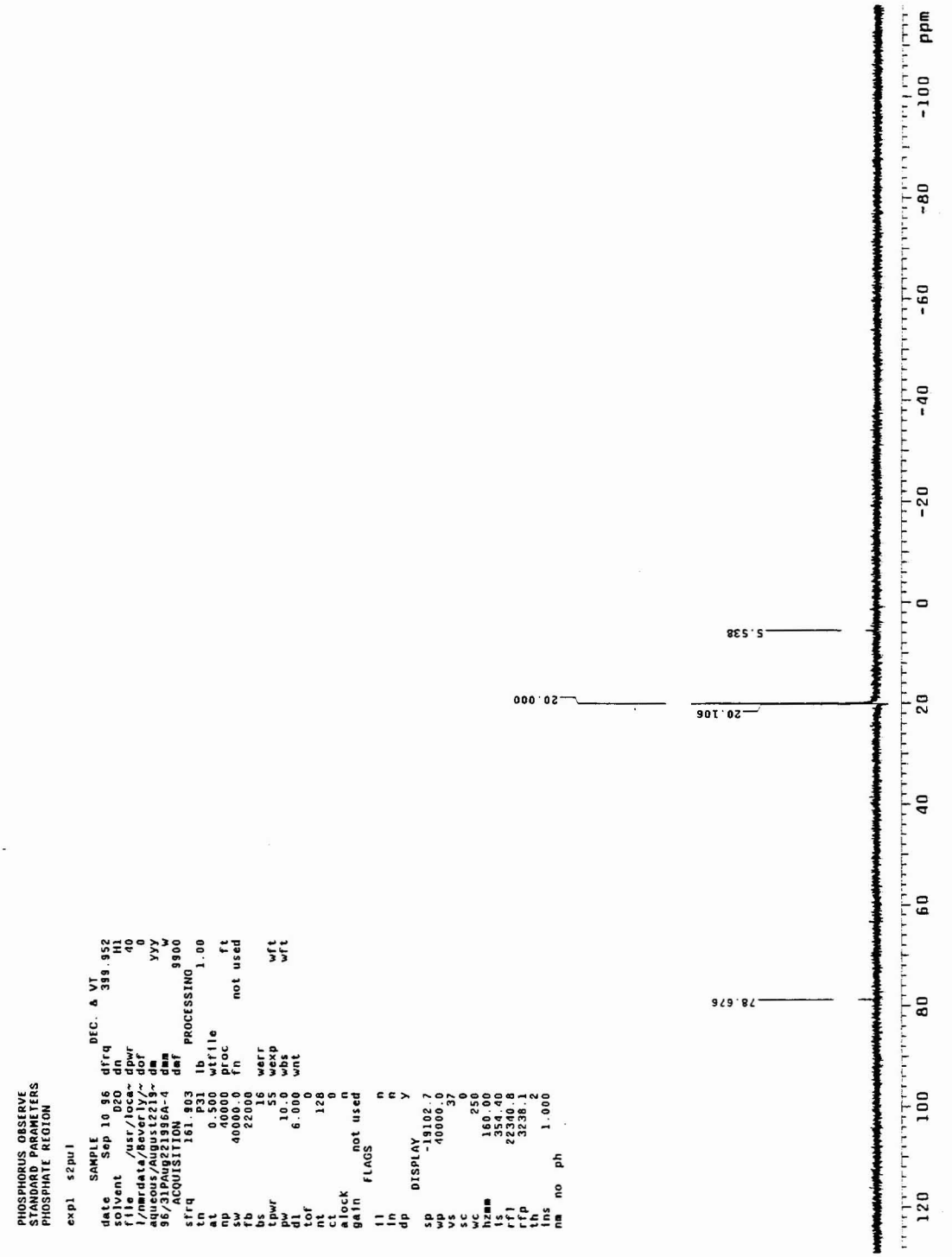
Chloroform Methanol Extraction, Aqueous Layer. Protocol A.



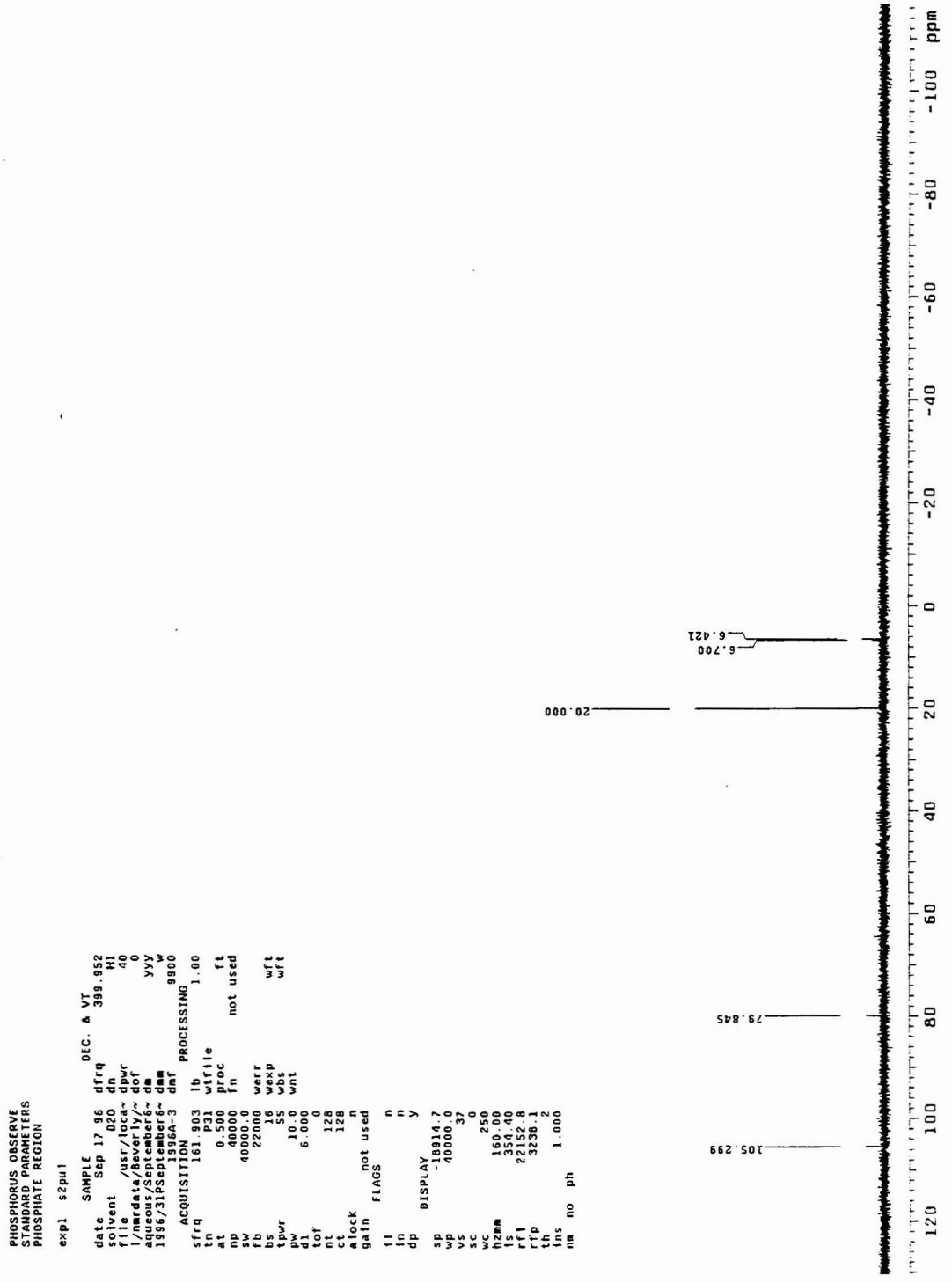
Chloroform Methanol Extraction, Aqueous Layer. Protocol B.



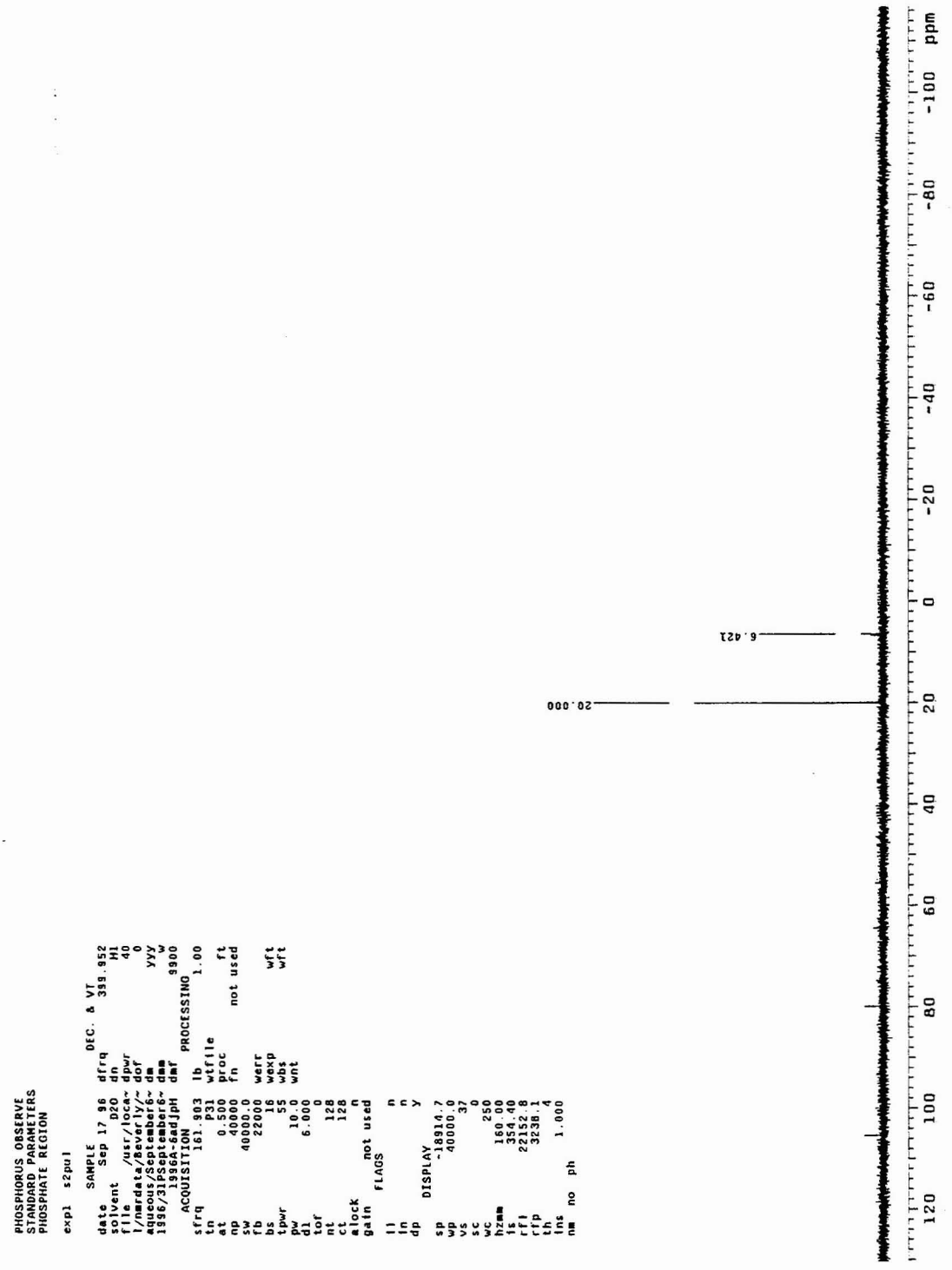
Chloroform Methanol Extraction, Aqueous Layer. Protocol C.



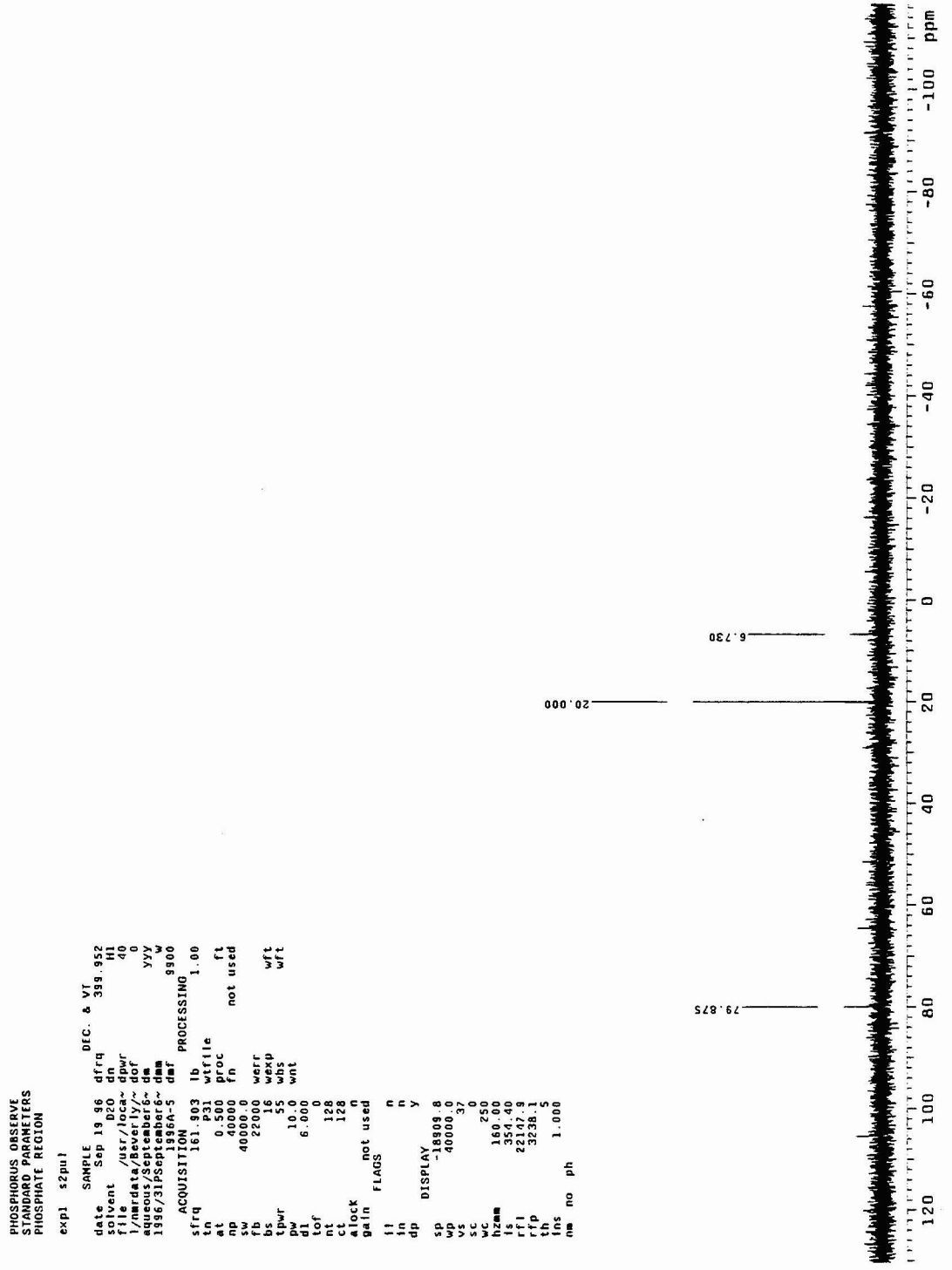
Chloroform Methanol Extraction, Aqueous Layer. Protocol D.



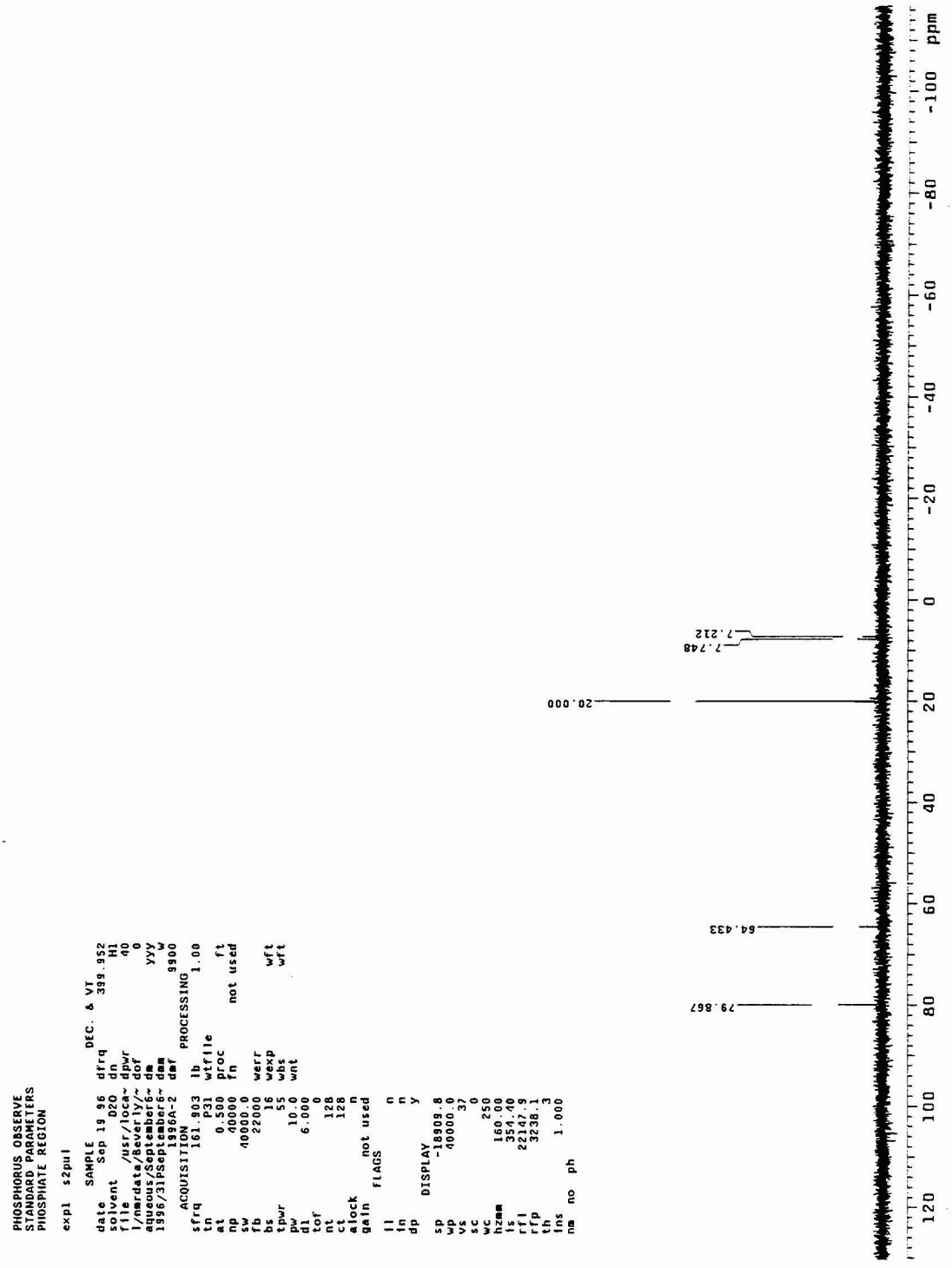
Chloroform Methanol Extraction, Aqueous Layer. Protocol E.



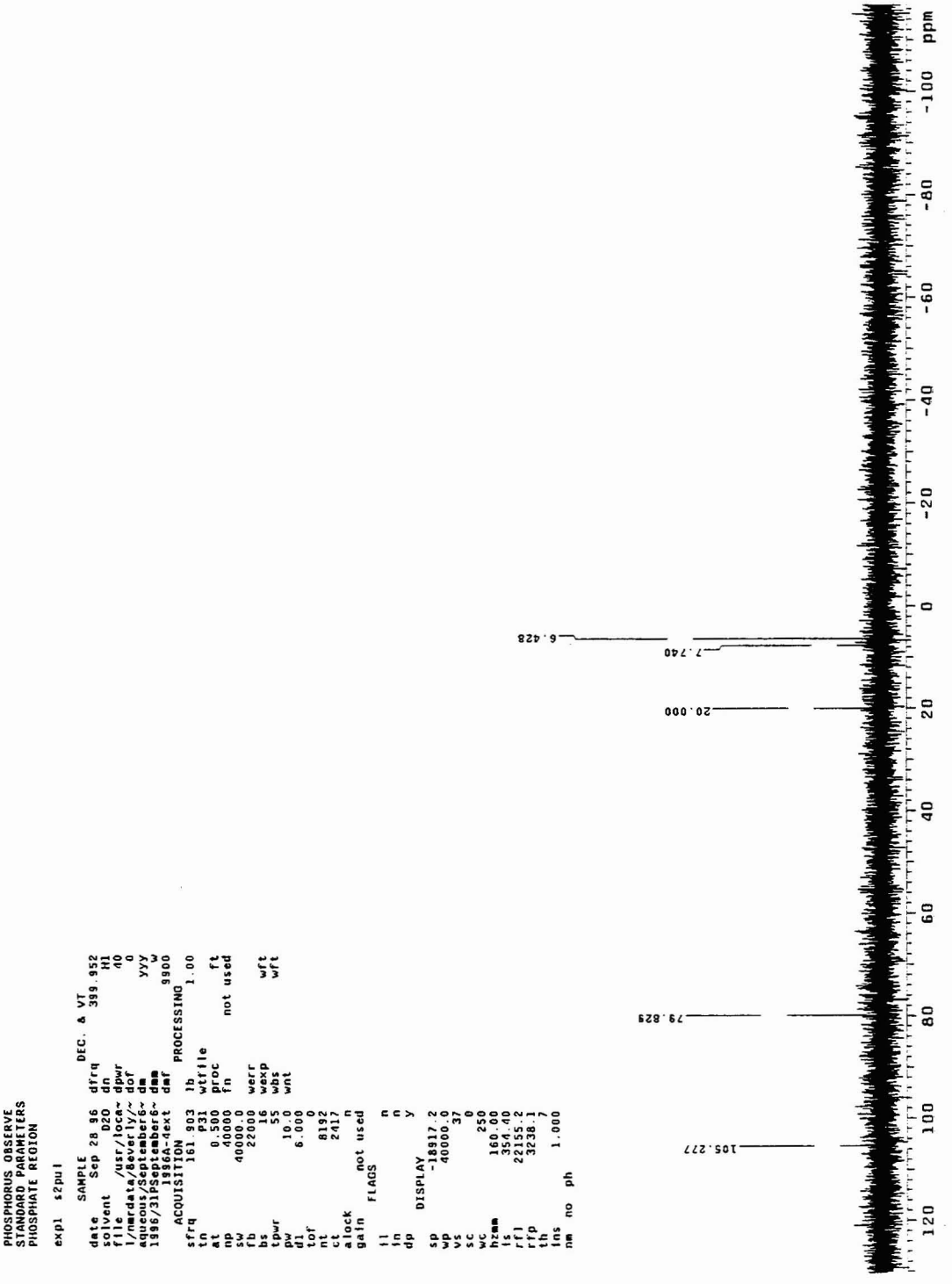
Chloroform Methanol Extraction, Aqueous Layer. Protocol F.



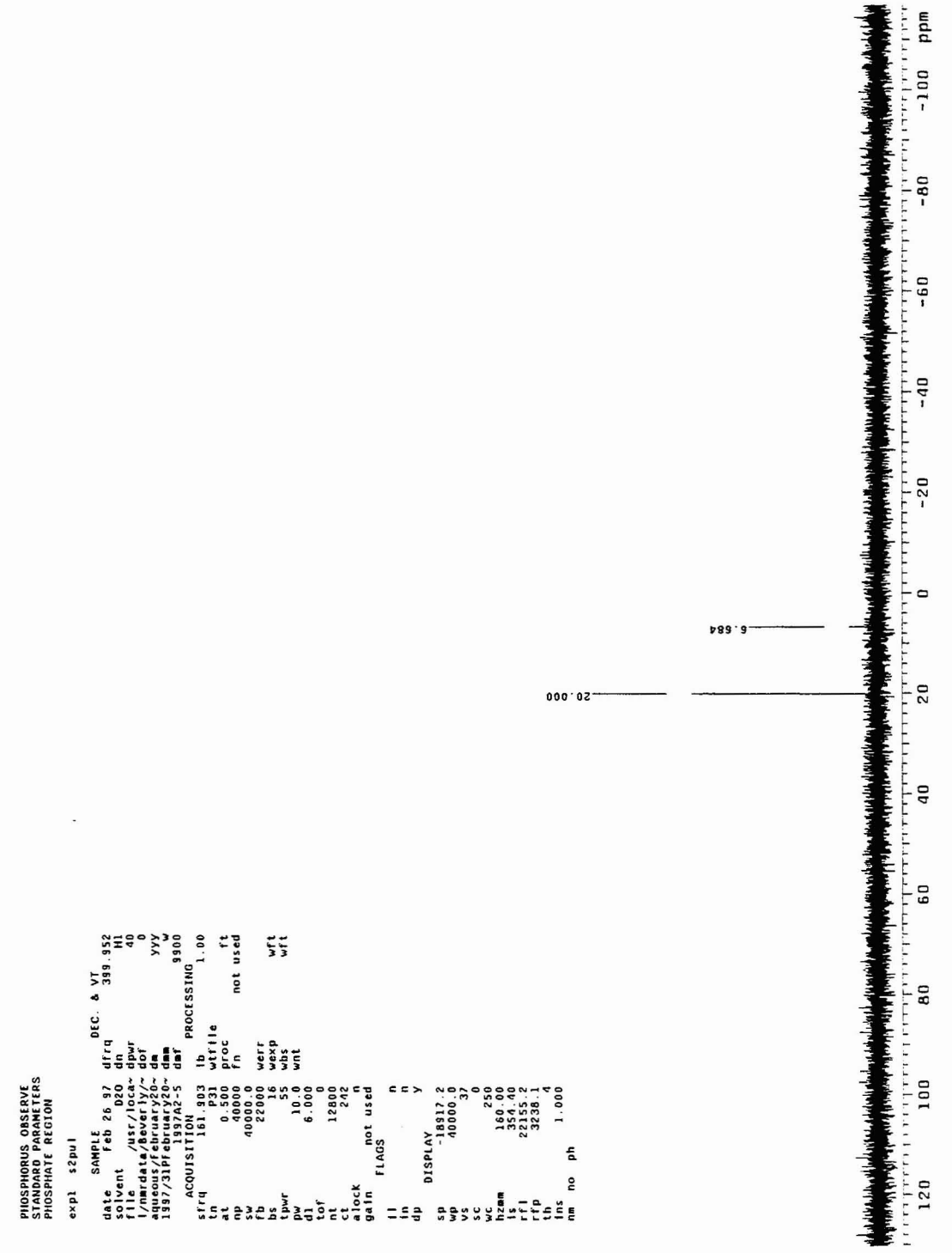
Chloroform Methanol Extraction, Aqueous Layer. Protocol G.



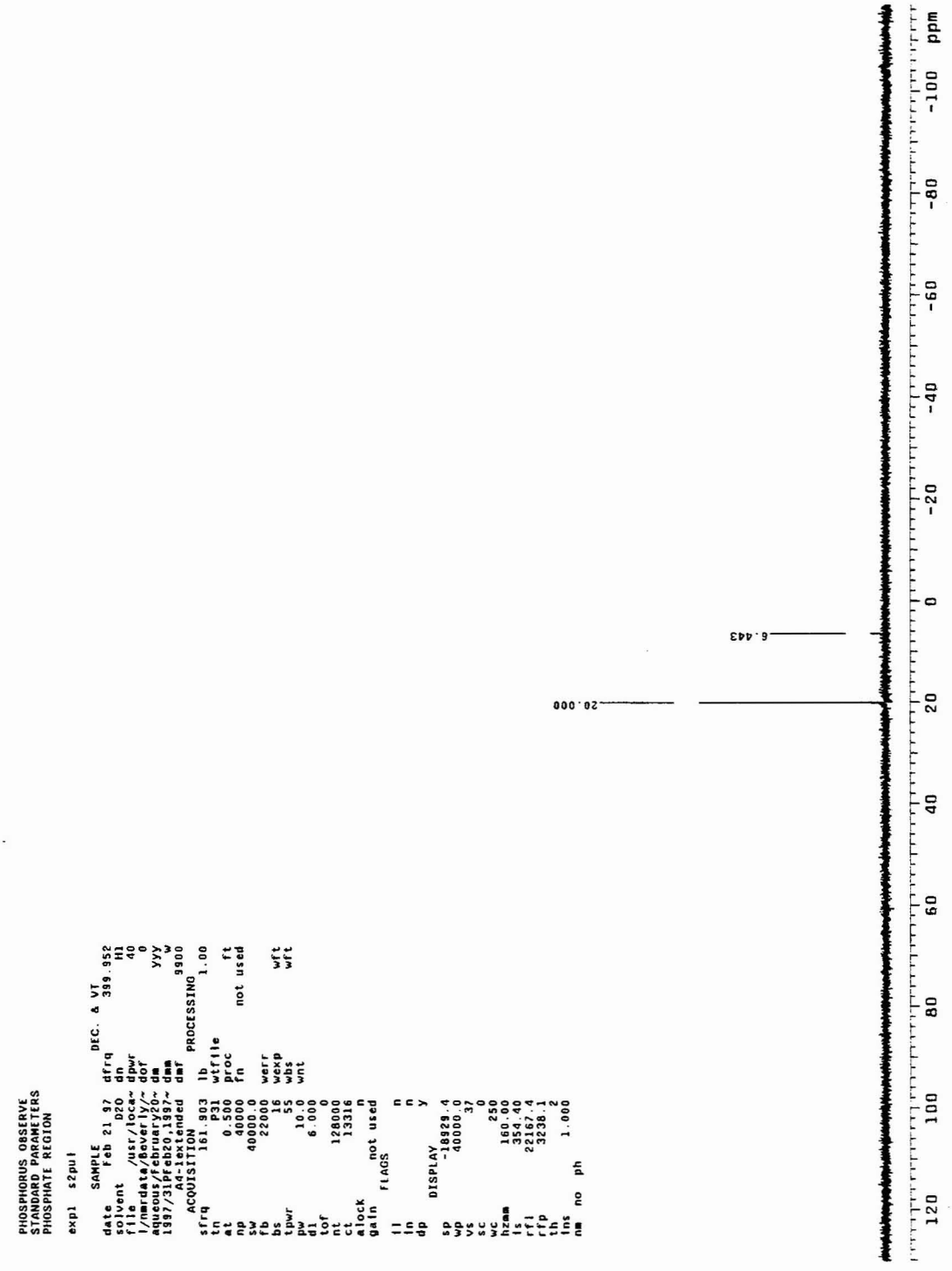
Chloroform Methanol Extraction, Aqueous Layer. Protocol H.



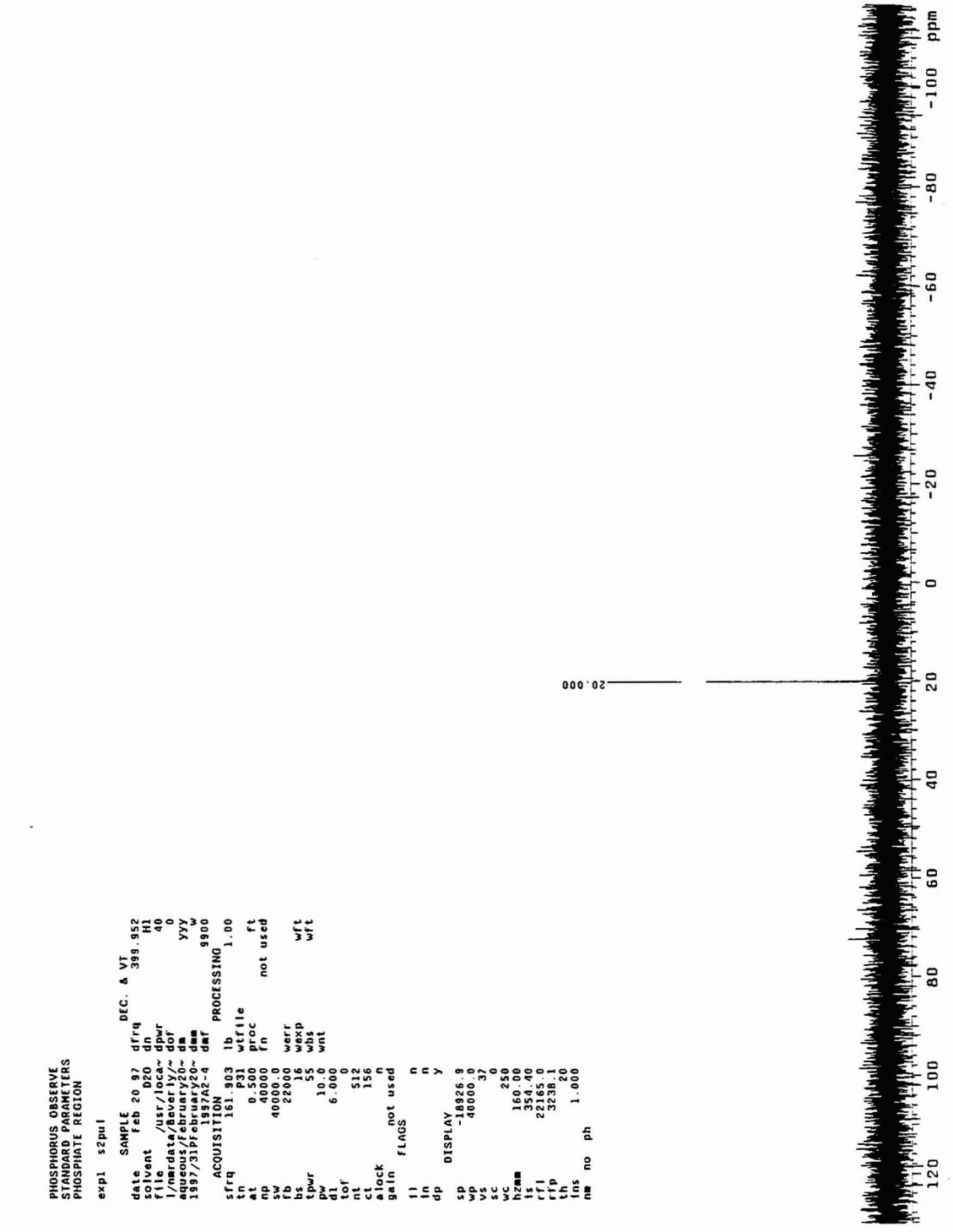
Chloroform Methanol Extraction, Aqueous Layer. Protocol I.



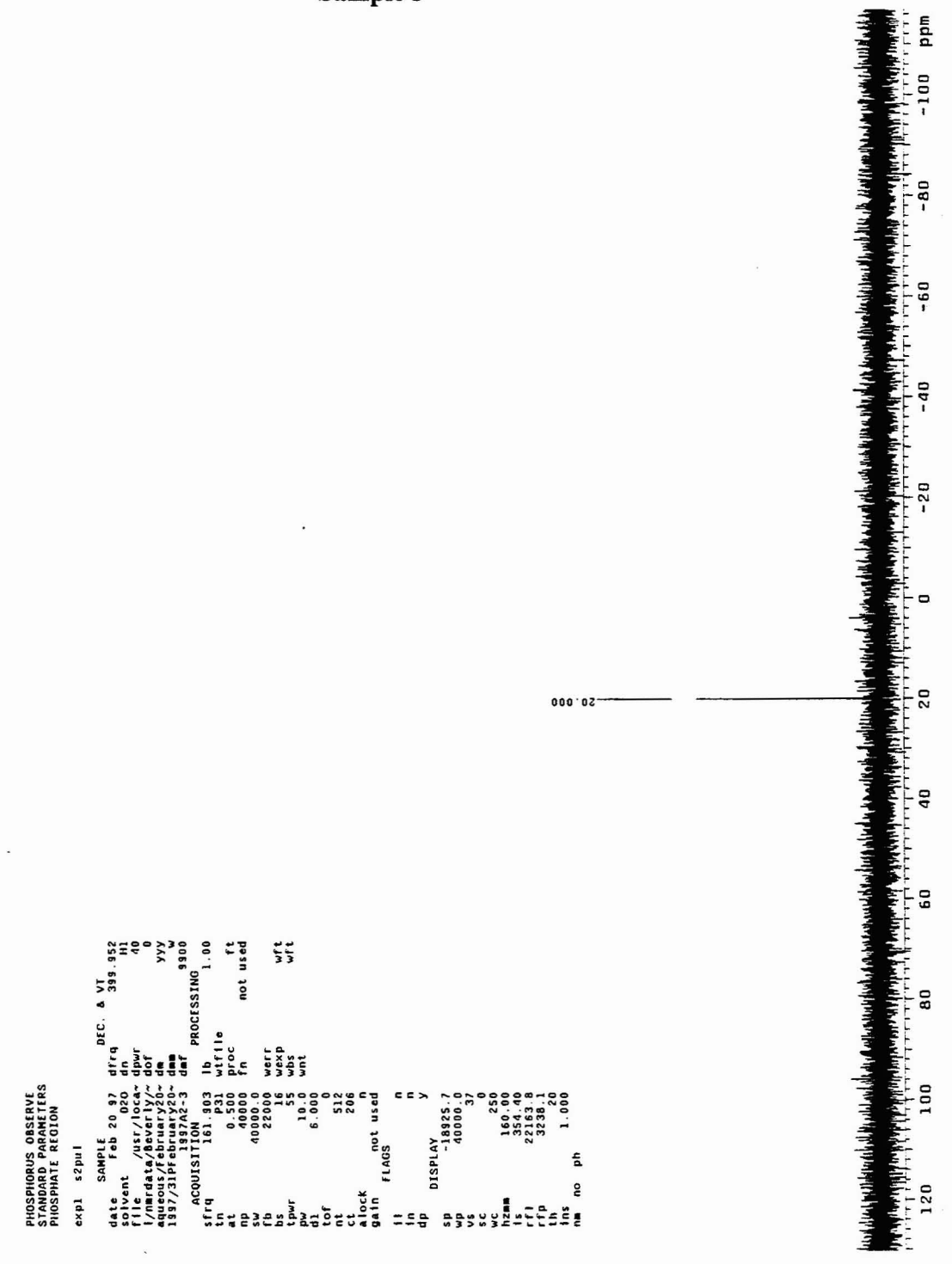
Chloroform Methanol Extraction, Aqueous Layer. Protocol J.
Sample 1



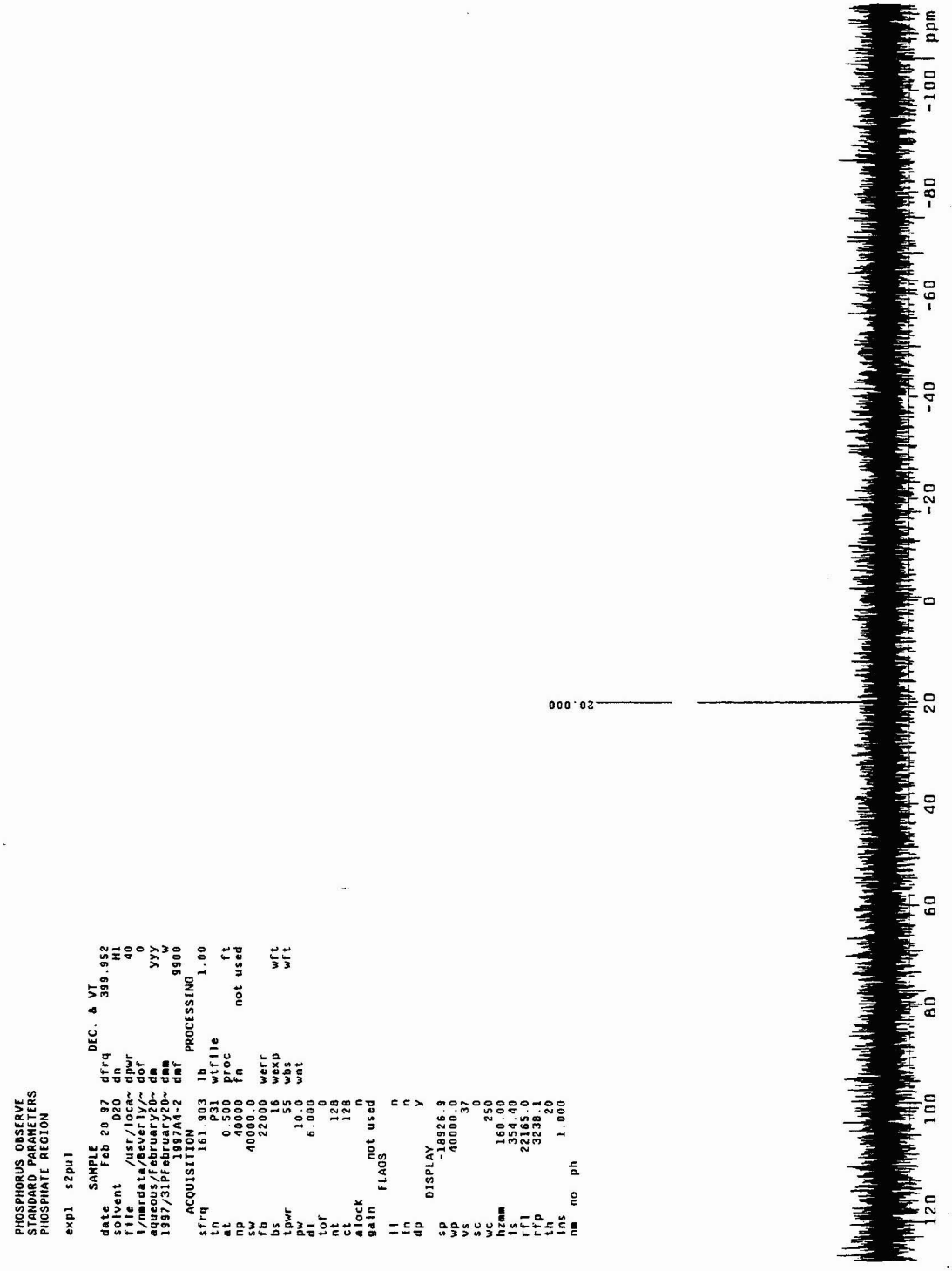
Chloroform Methanol Extraction, Aqueous Layer. Protocol J.
Sample 2



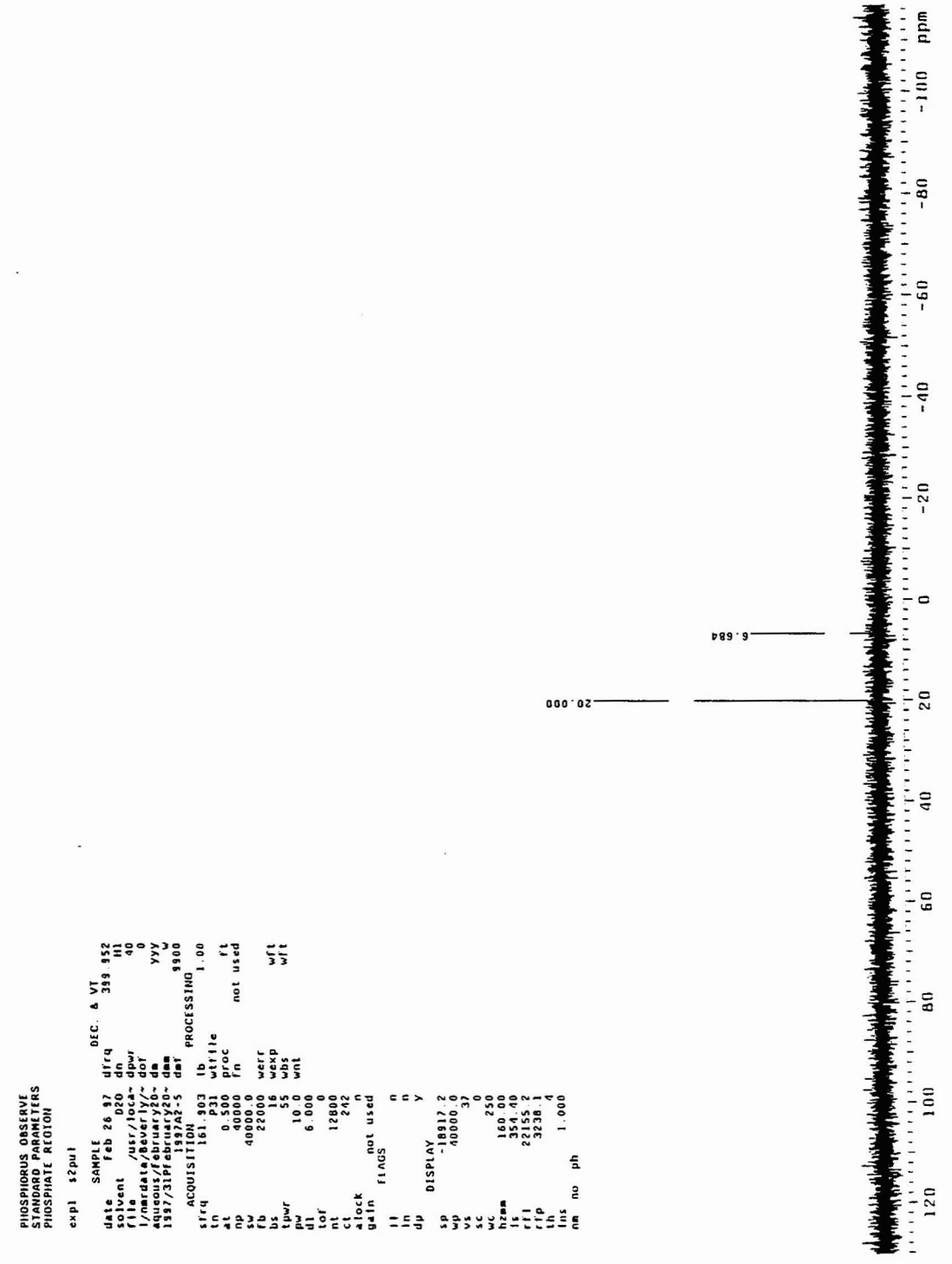
Chloroform Methanol Extraction, Aqueous Layer. Protocol J.
Sample 3



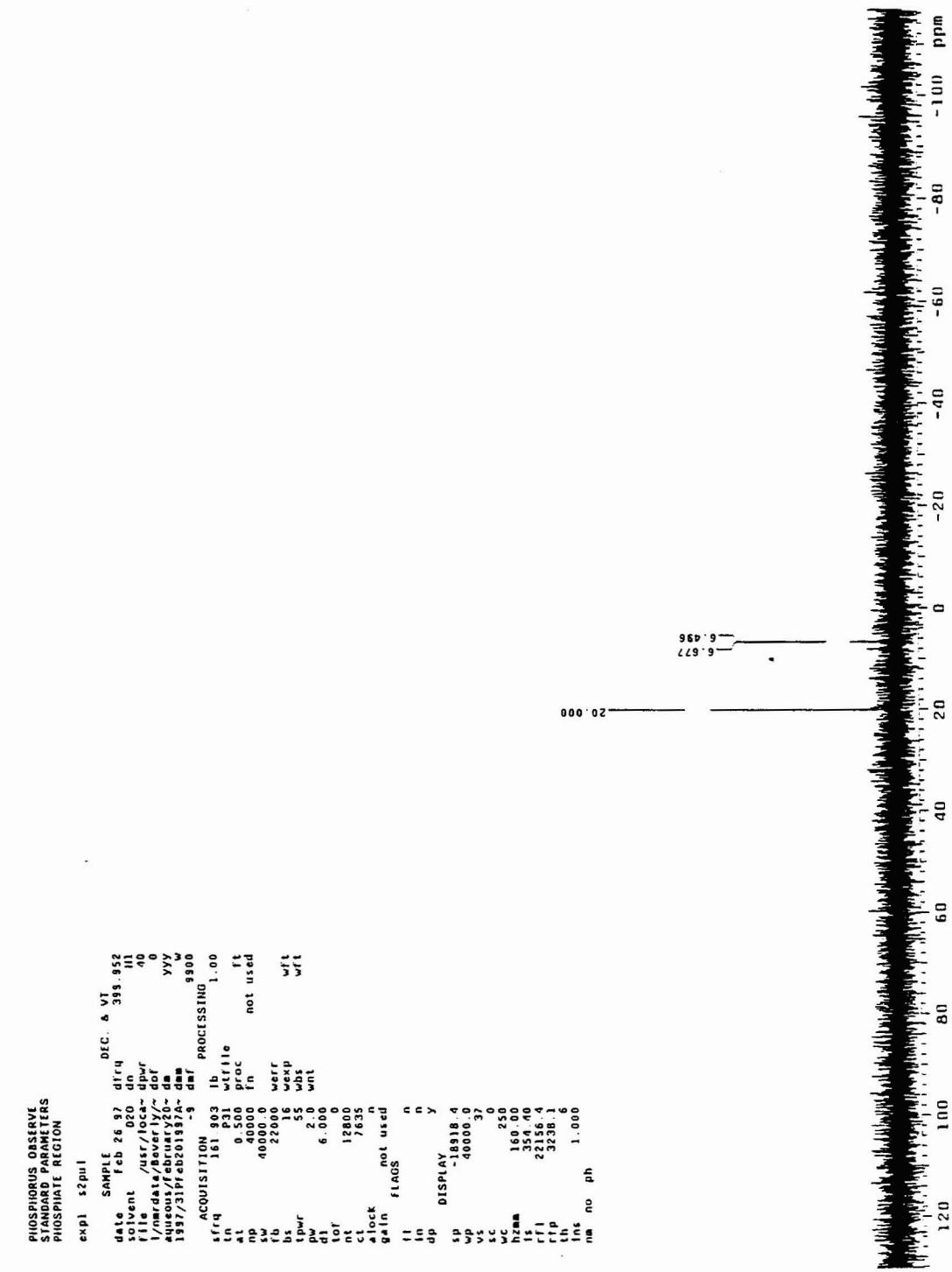
Chloroform Methanol Extraction, Aqueous Layer. Protocol J.
Sample 4



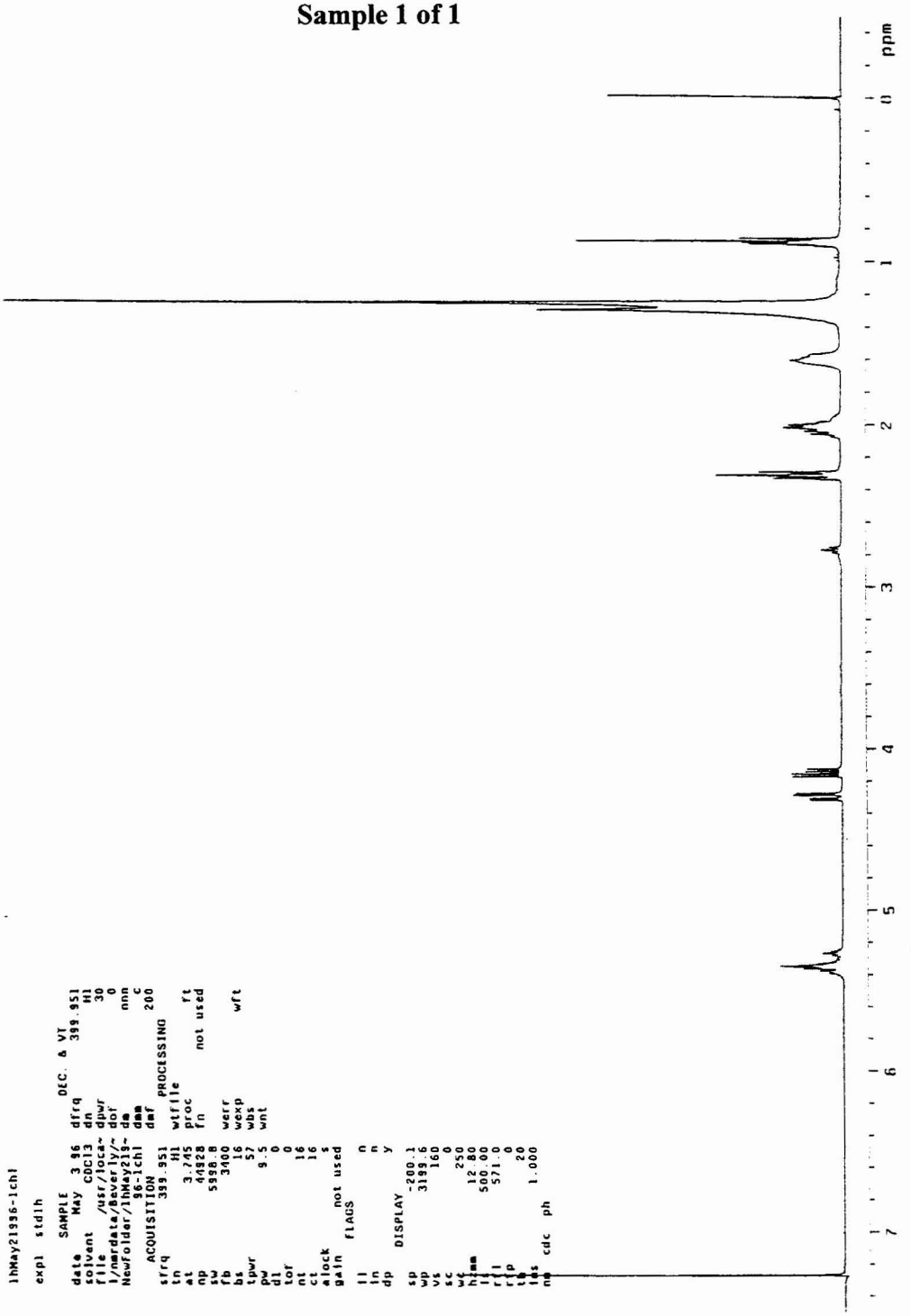
Chloroform Methanol Extraction, Aqueous Layer. Protocol J.
Sample 5



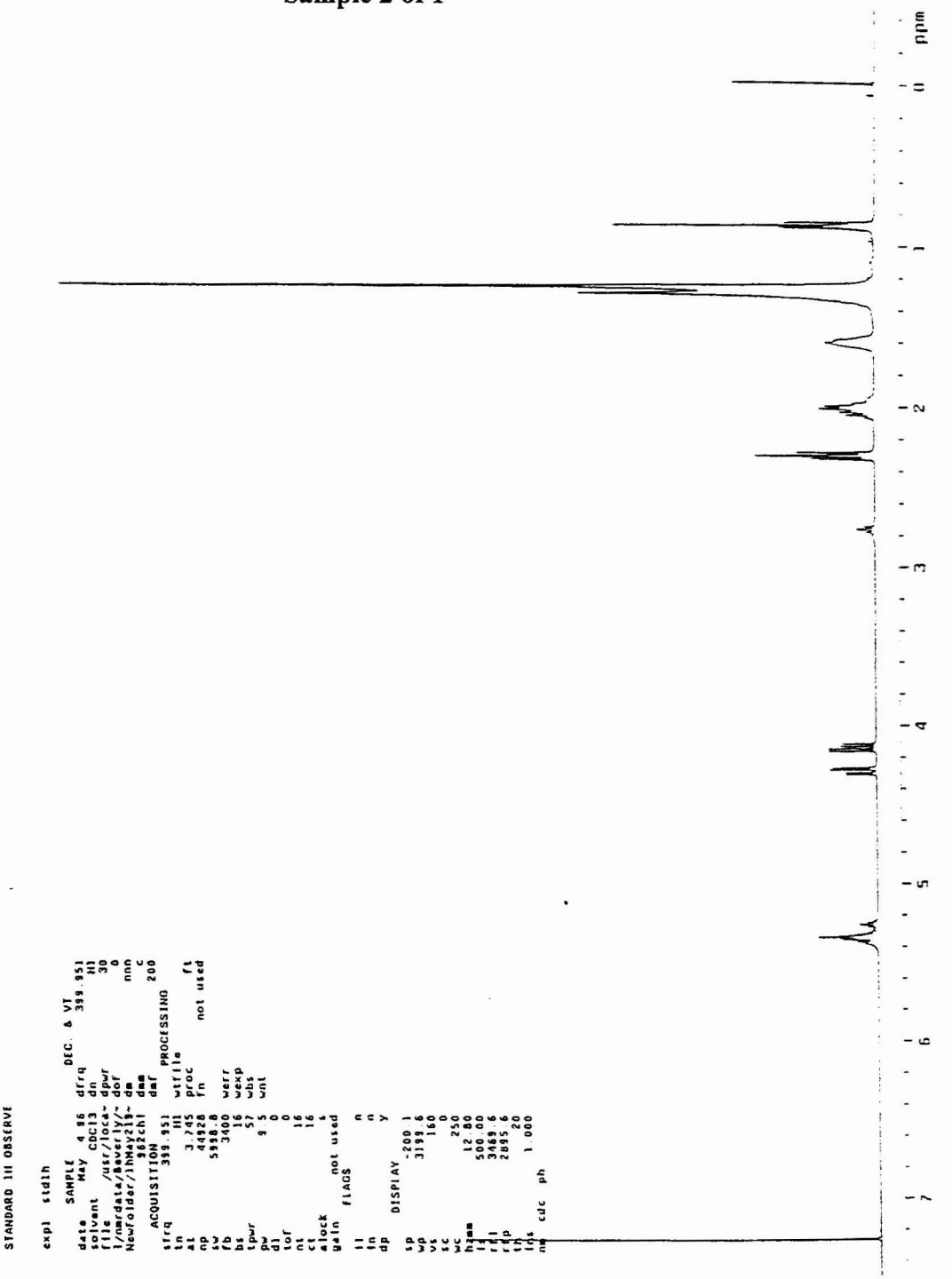
Chloroform Methanol Extraction, Aqueous Layer. Protocol K.



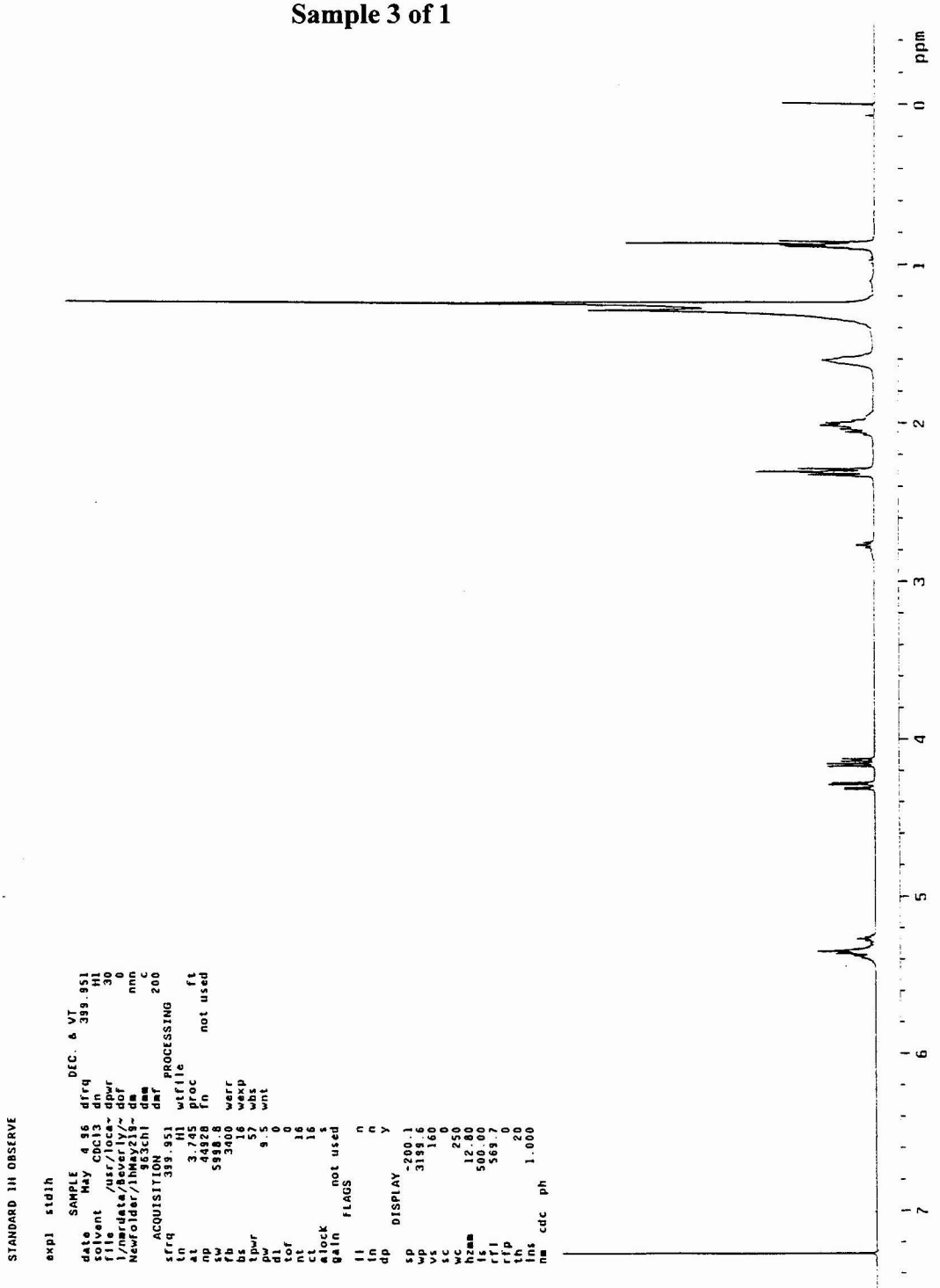
Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 1 of 1



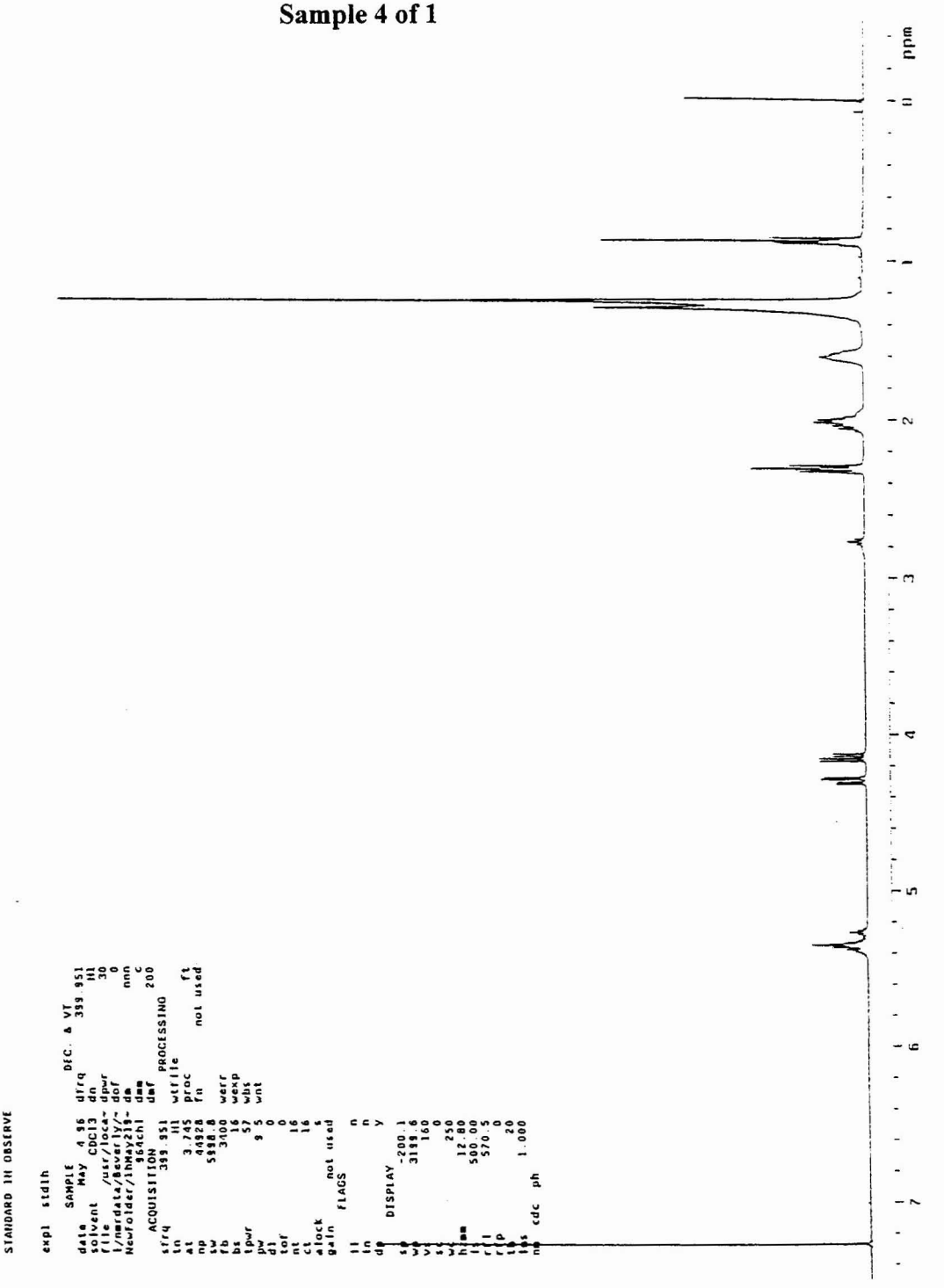
Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 2 of 1



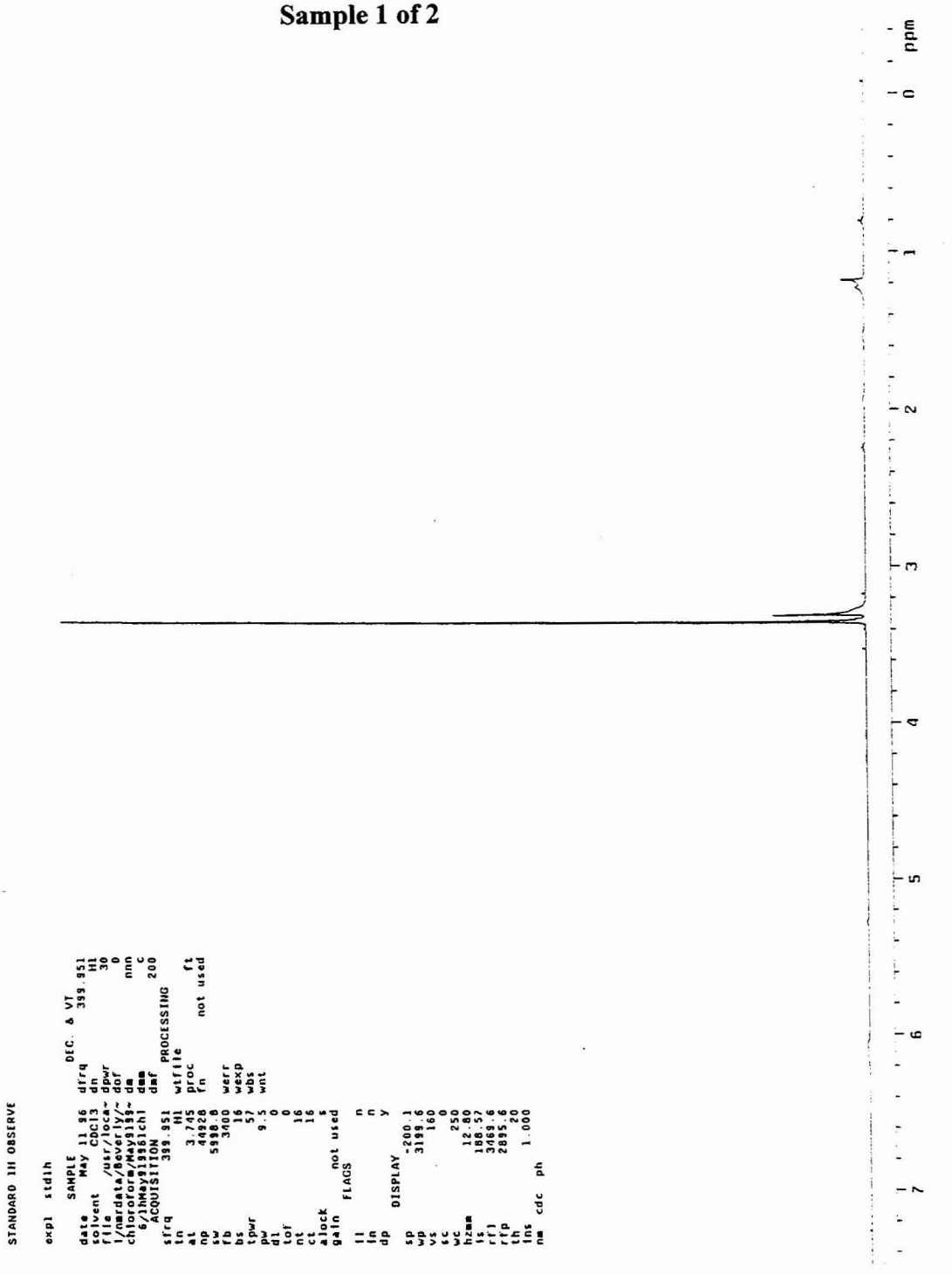
Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 3 of 1



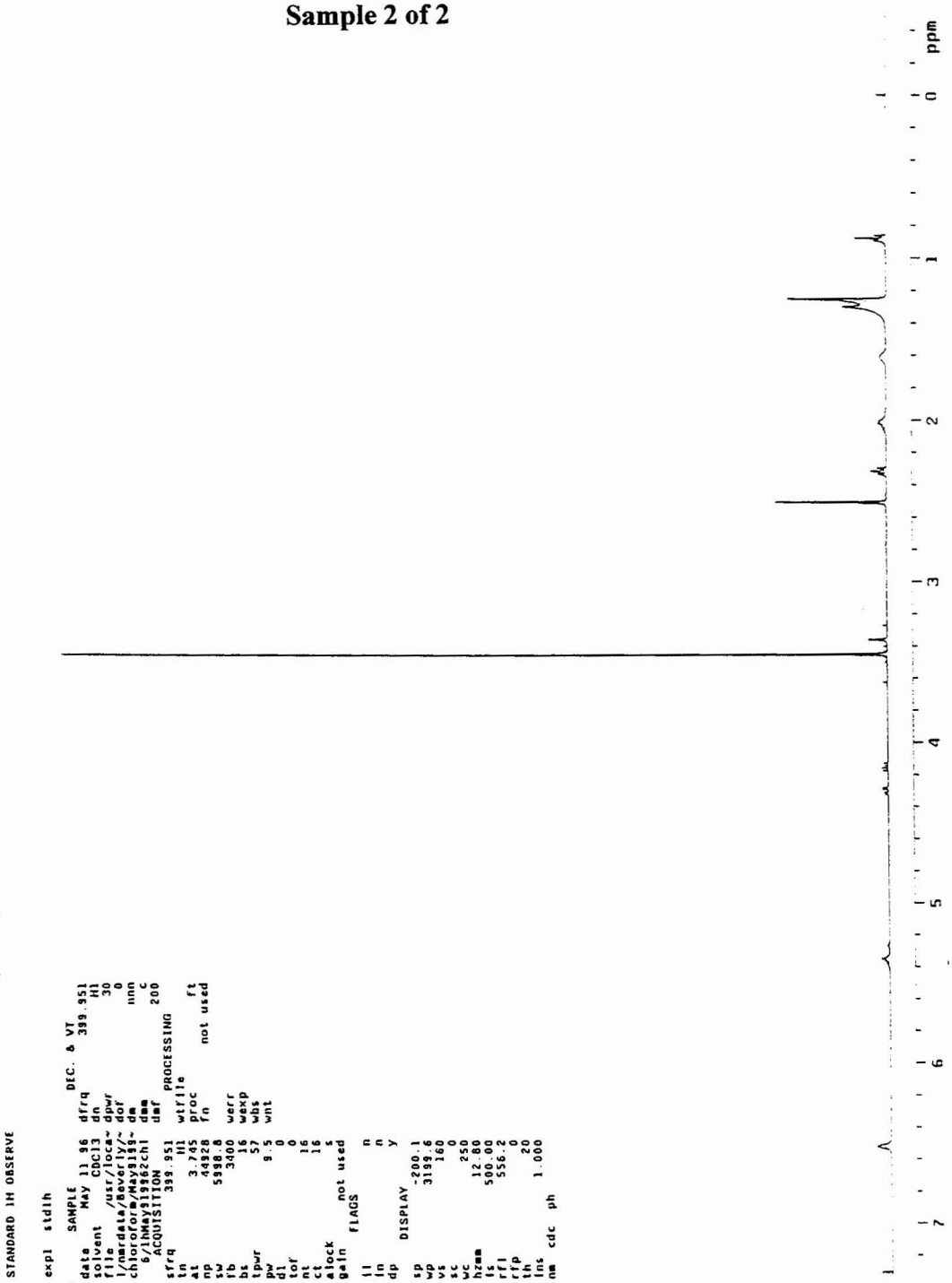
Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 4 of 1



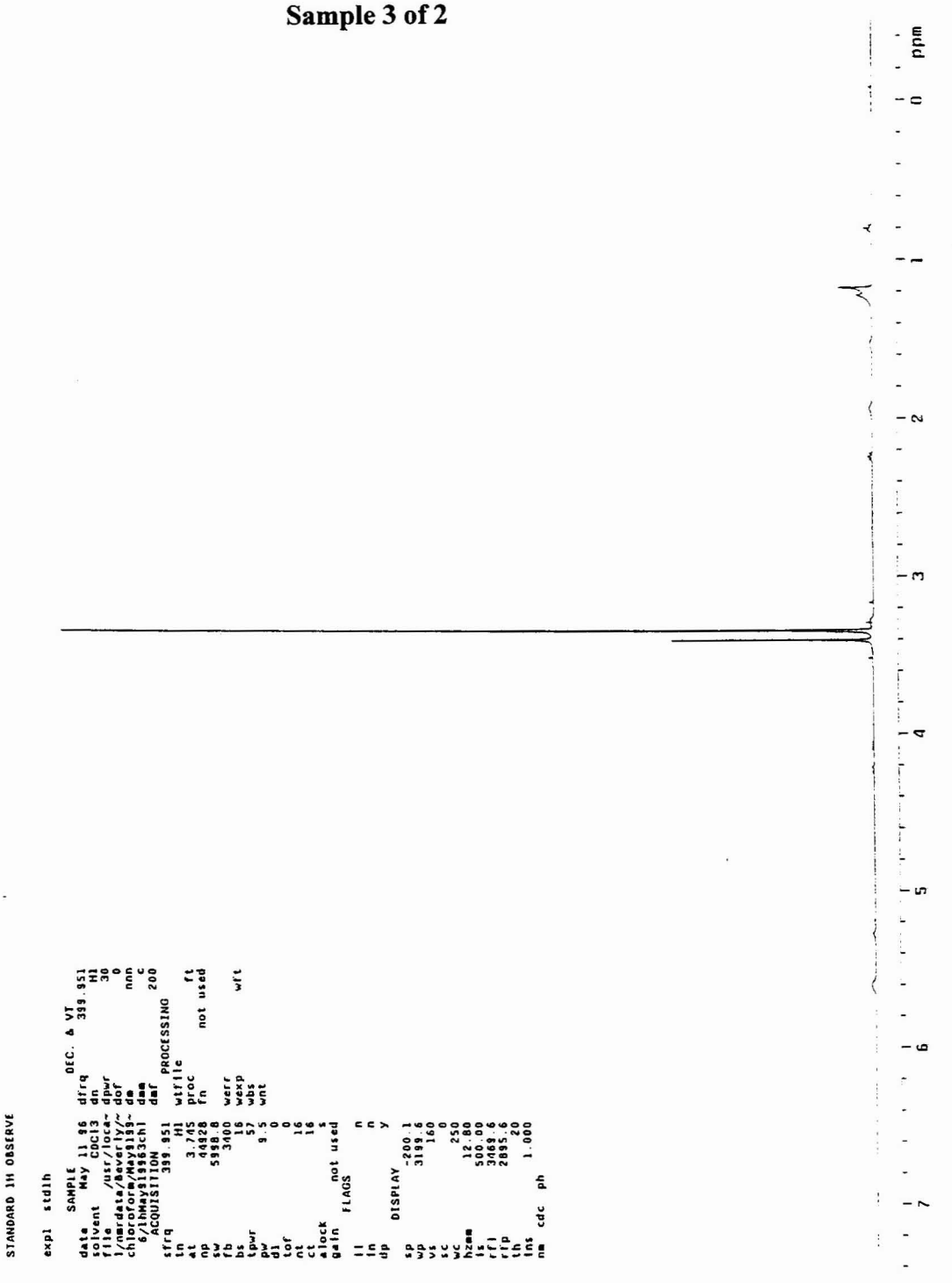
Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 1 of 2



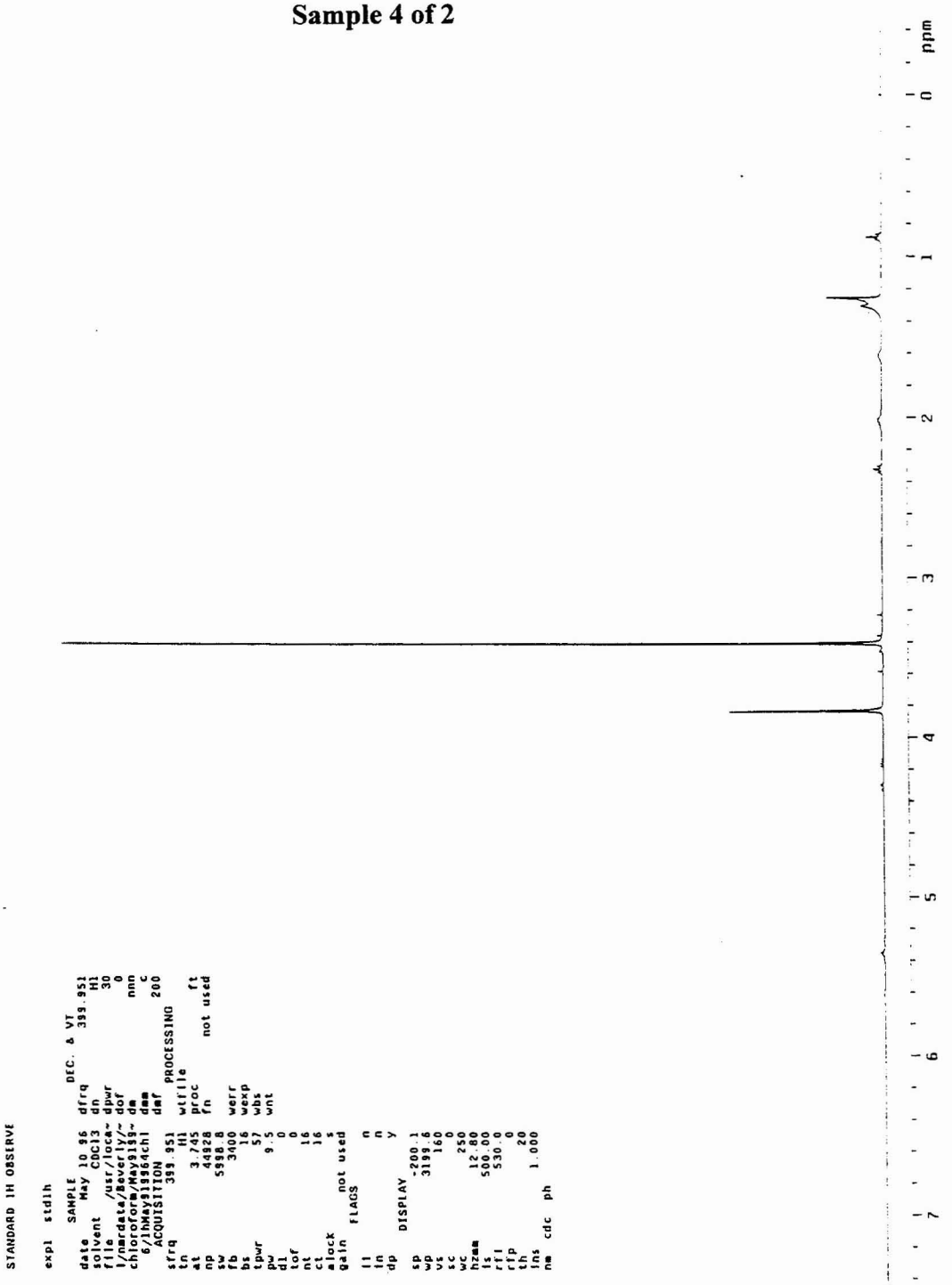
Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 2 of 2



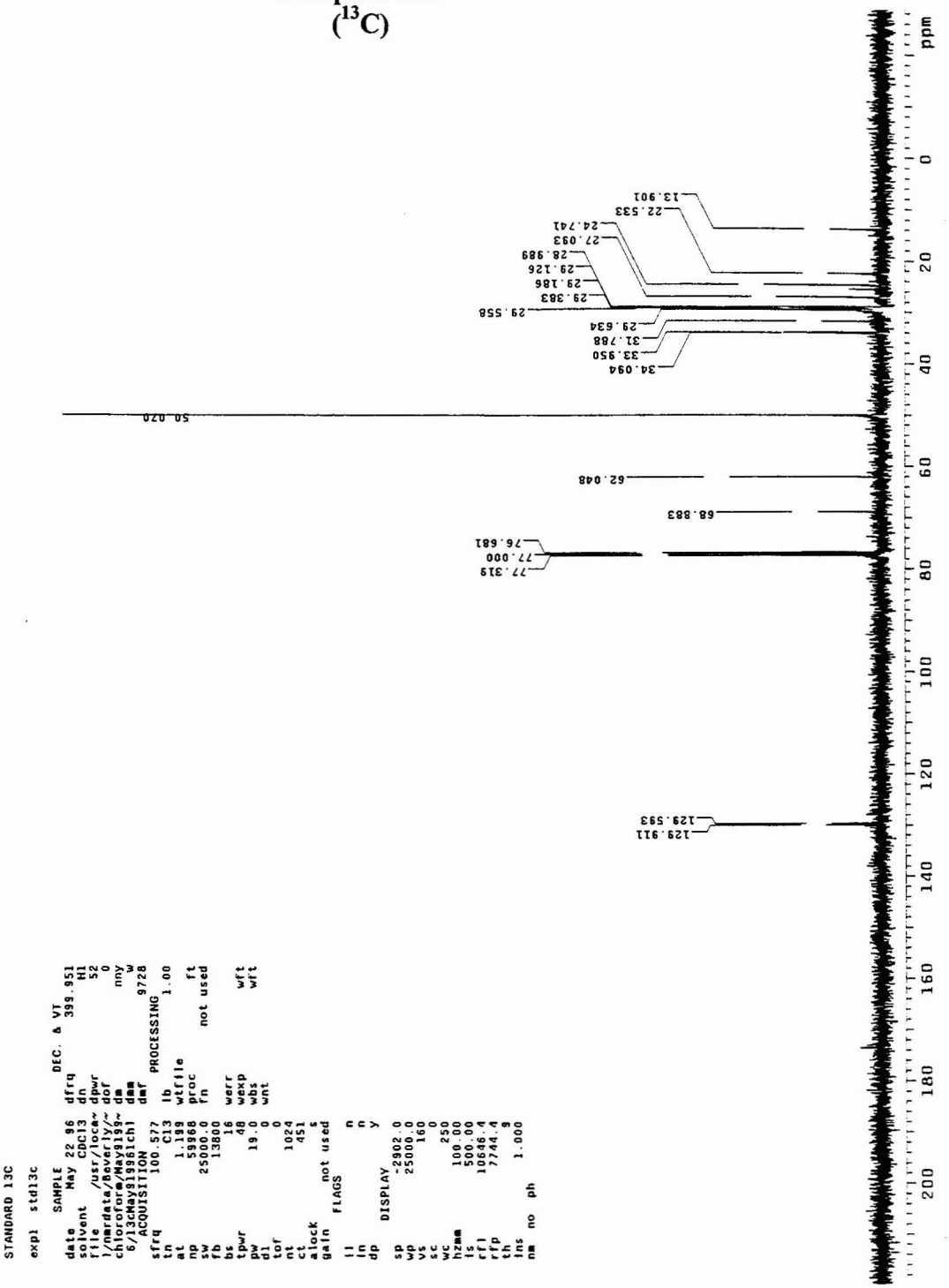
Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 3 of 2



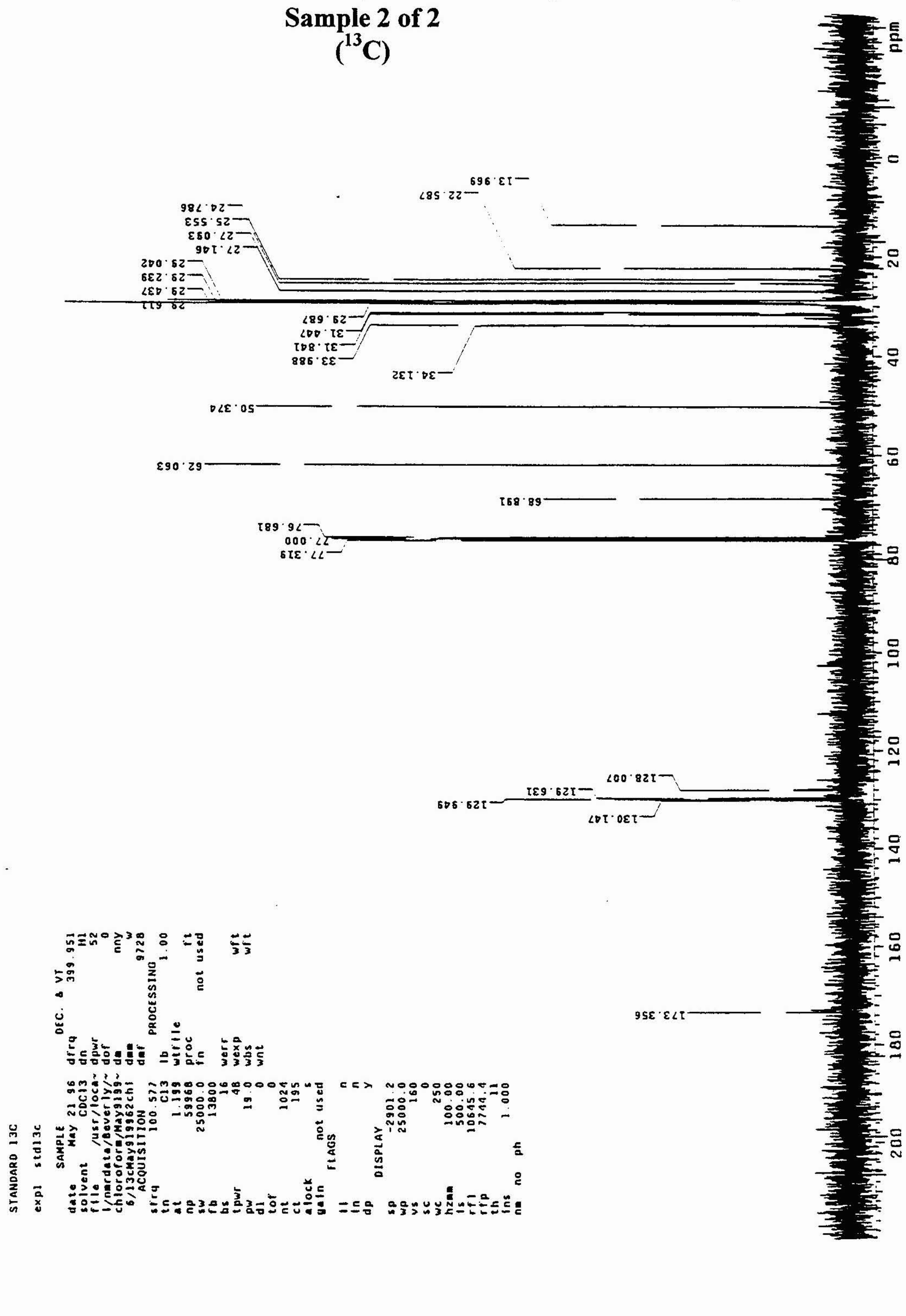
Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 4 of 2



Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 1 of 2
(^{13}C)



Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 2 of 2
(^{13}C)

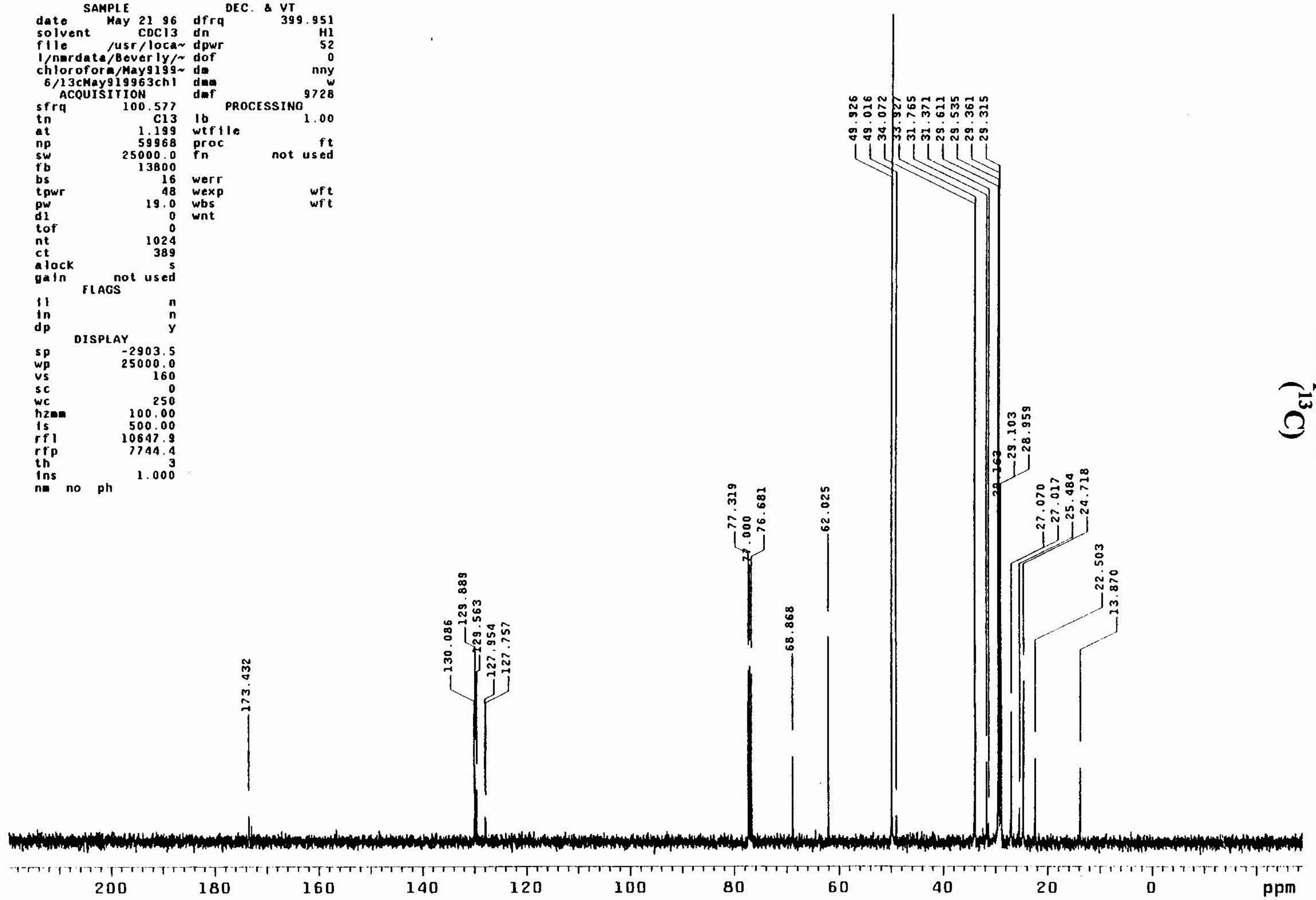


Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 3 of 2
(^{13}C)

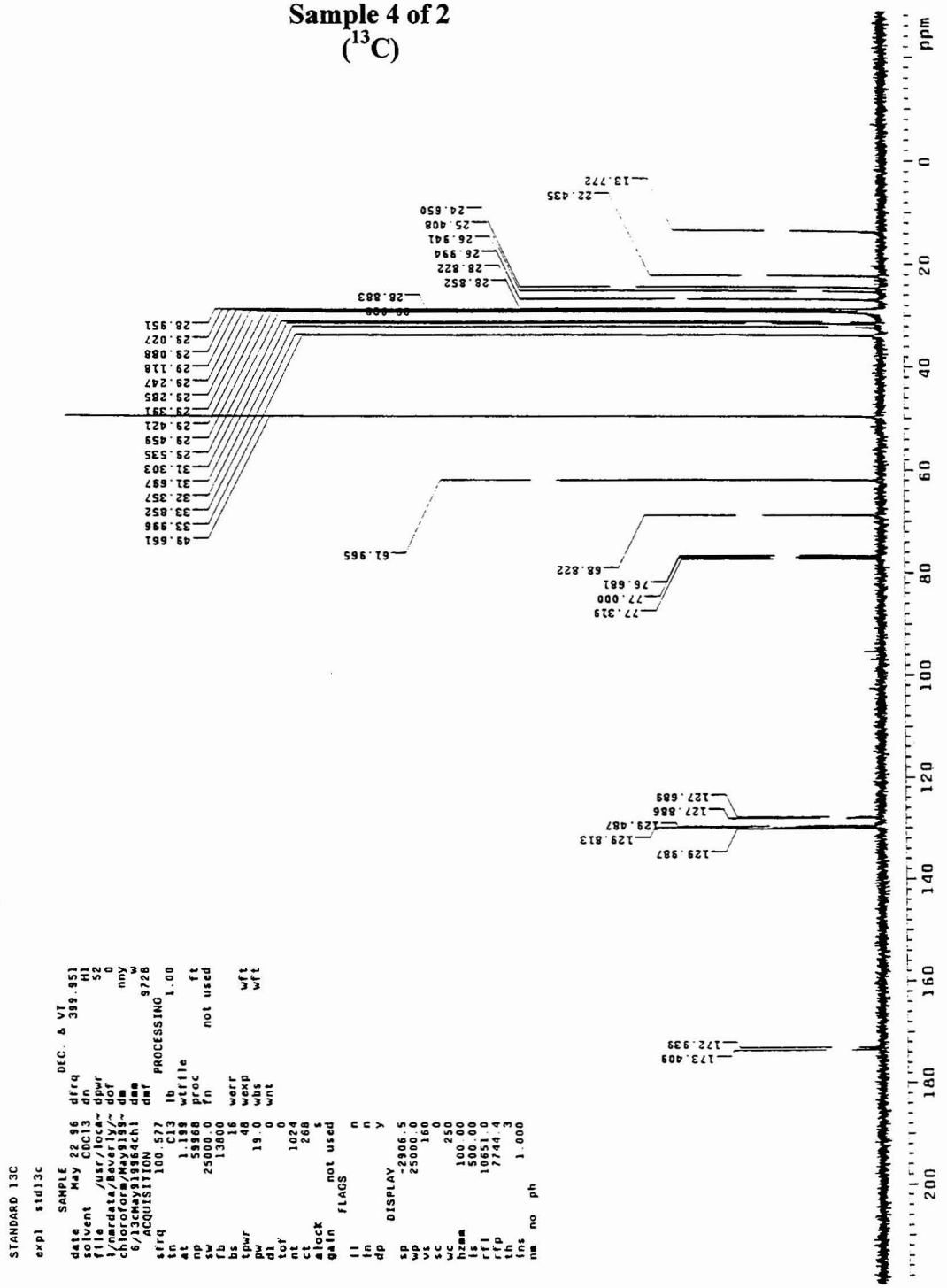
STANDARD 13C

expl stdl3c

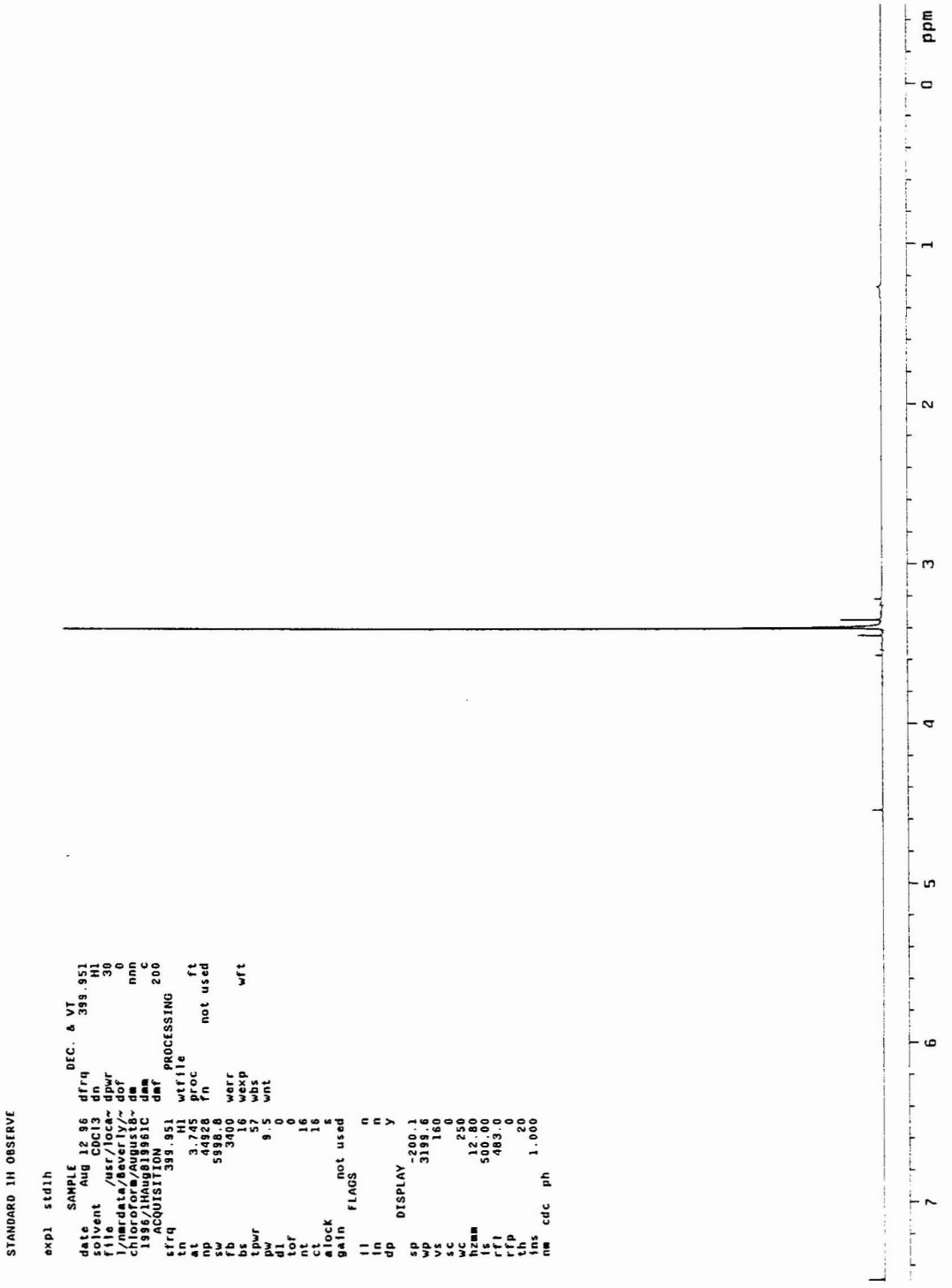
SAMPLE		DEC. & VT	
date	May 21 96	dfrq	399.951
solvent	CDCl3	dn	H1
file	/usr/loca~	dpwr	52
l/nmrdata/Beverly/~		dof	0
chloroform/May9199~		dm	nny
6/13cMay919963chl		dmm	w
ACQUISITION		dmf	9728
sfrq	100.577	PROCESSING	
tn	C13	lb	1.00
at	1.199	wtfile	
np	59968	proc	ft
sw	25000.0	fn	not used
fb	13800		
bs	16	werr	
tpwr	48	wexp	wft
pw	19.0	wbs	wft
d1	0	wnt	
tof	0		
nt	1024		
ct	389		
alock	s		
gain	not used		
FLAGS			
il	n		
in	n		
dp	y		
DISPLAY			
sp	-2903.5		
wp	25000.0		
vs	160		
sc	0		
wc	250		
hzmm	100.00		
is	500.00		
rfl	10647.9		
rfp	7744.4		
th	3		
ins	1.000		
nm no ph			



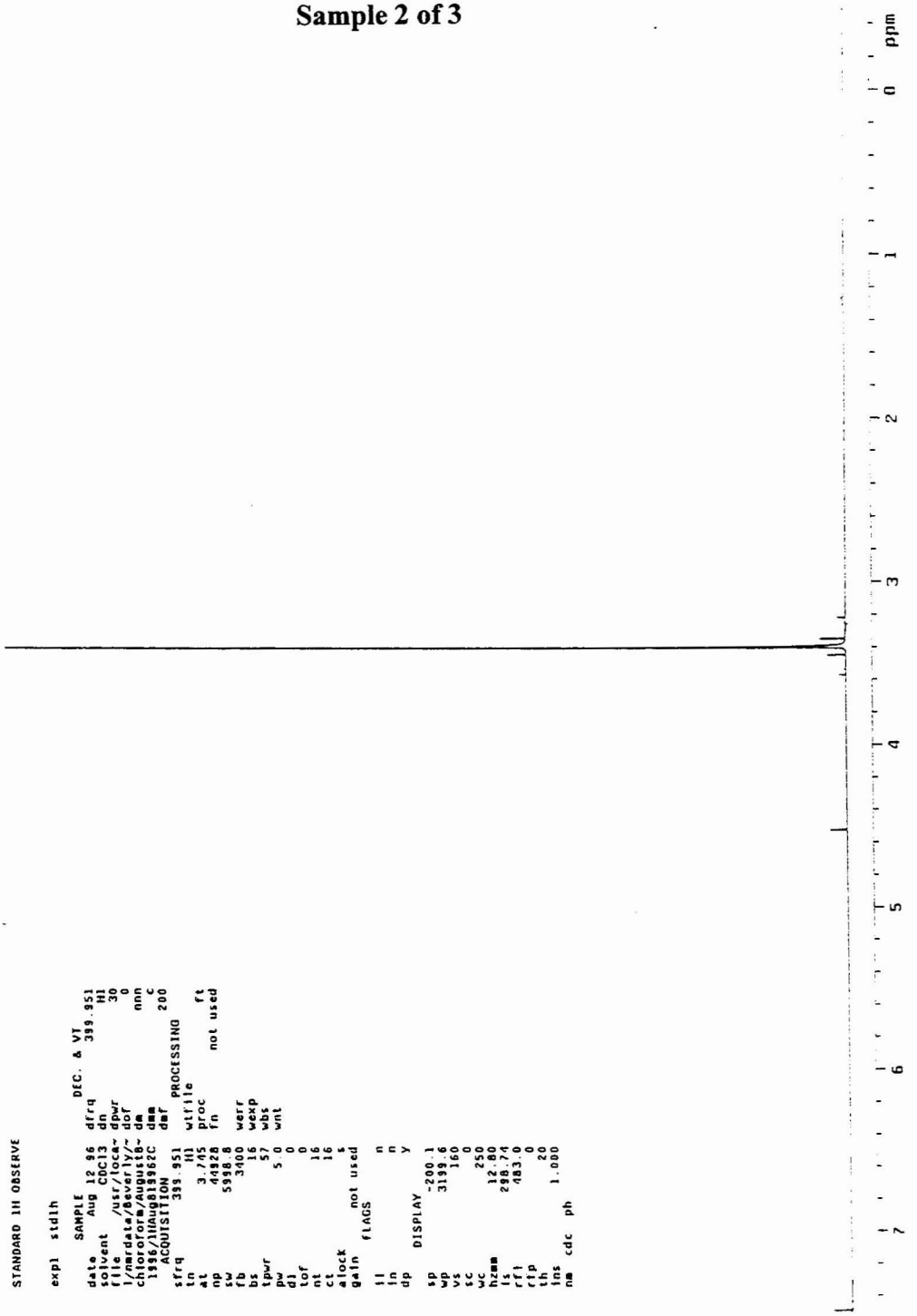
Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 4 of 2
(^{13}C)



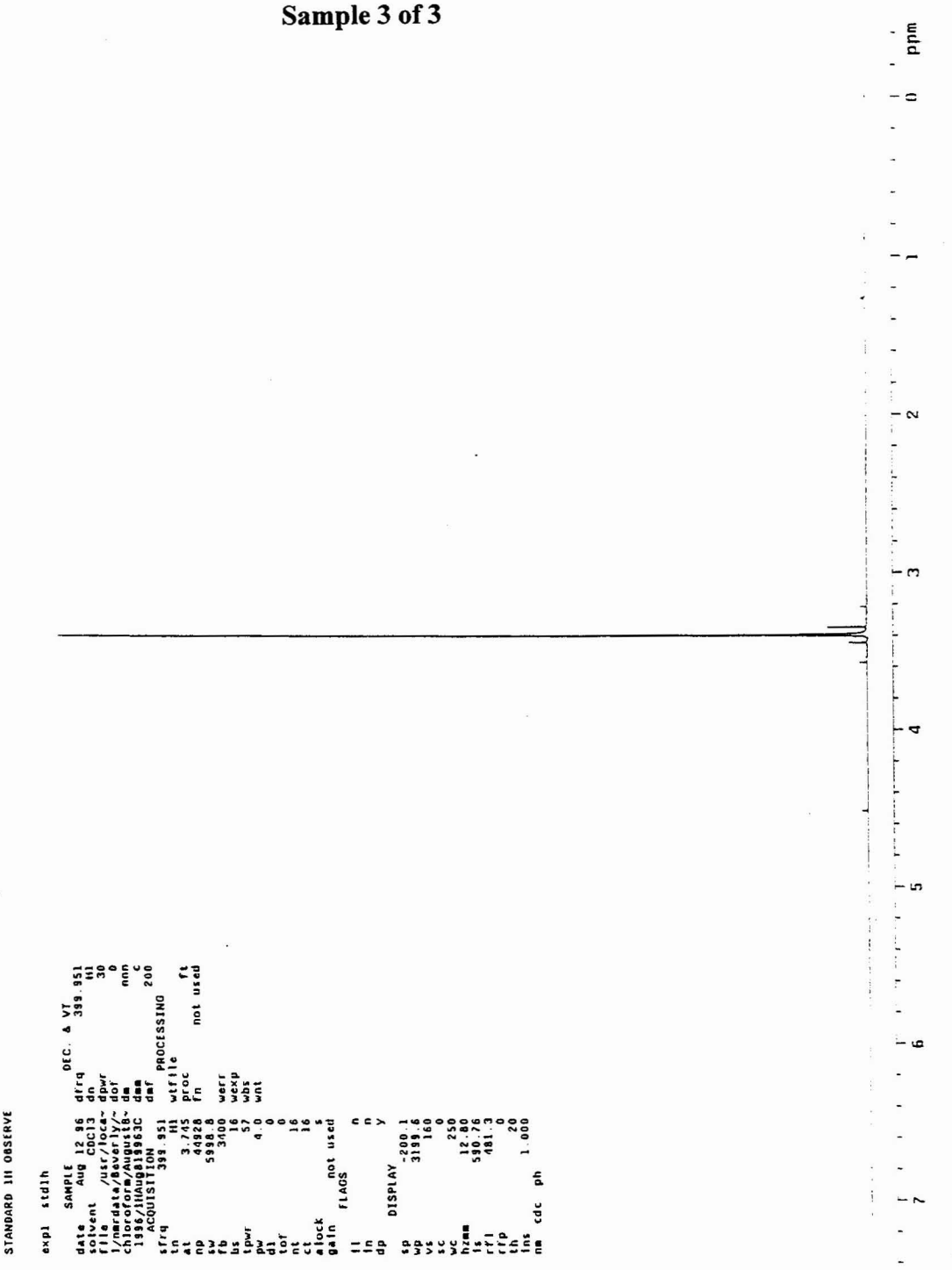
Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 1 of 3



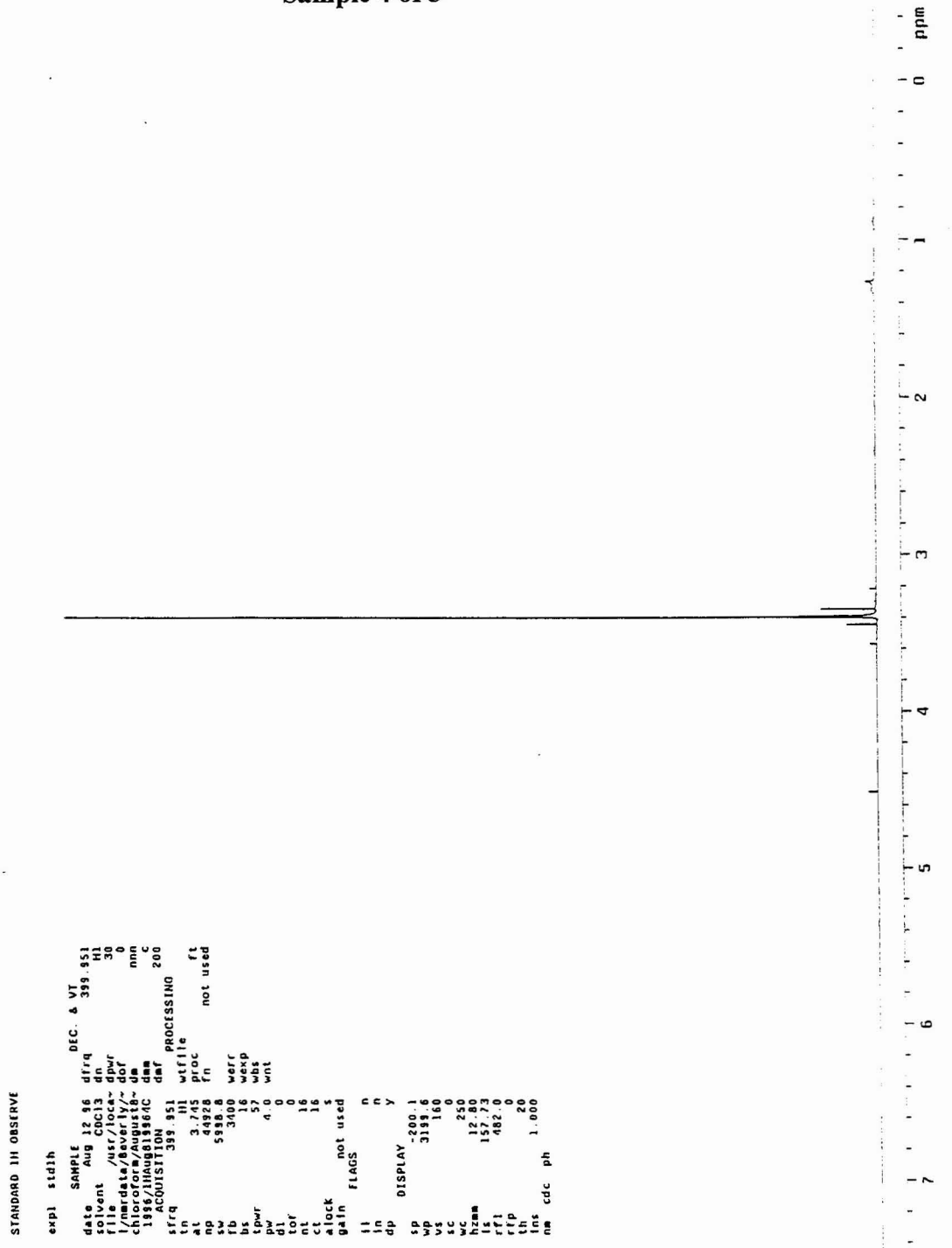
Chloroform Methanol Extraction, Chloroform Layer. Time Study. Sample 2 of 3



Chloroform Methanol Extraction, Chloroform Layer. Time Study. Sample 3 of 3

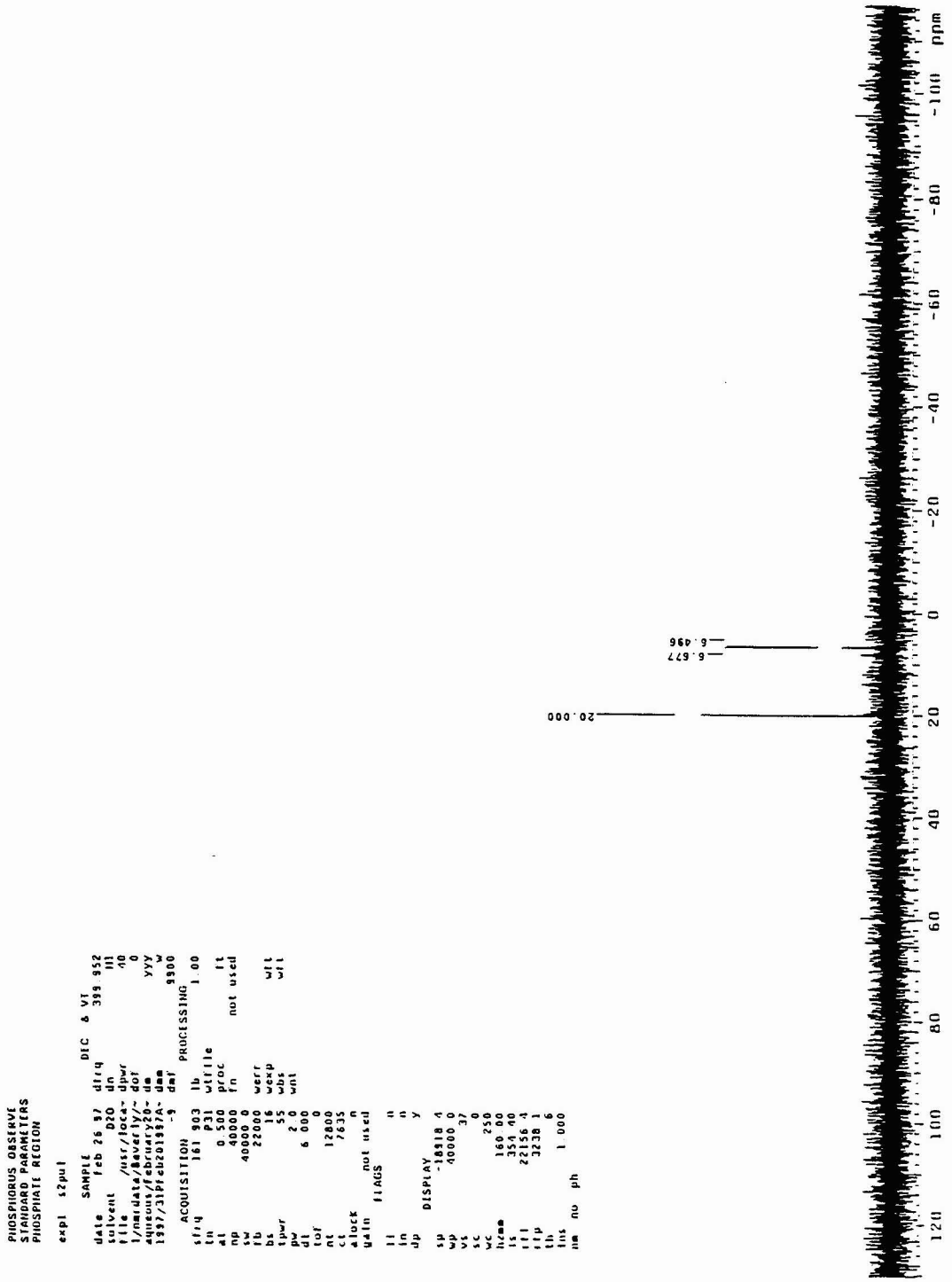


Chloroform Methanol Extraction, Chloroform Layer. Time Study.
Sample 4 of 3

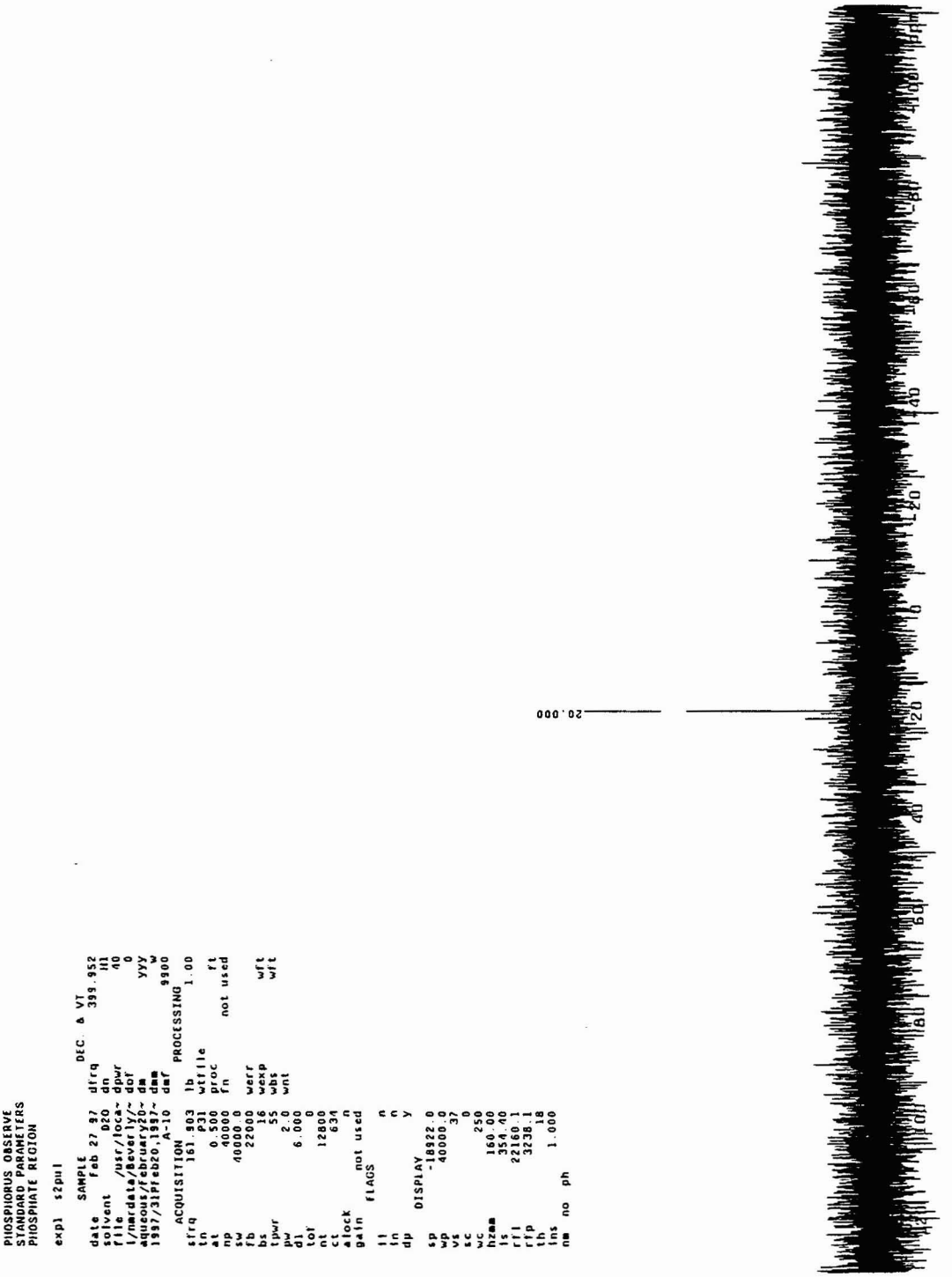


Chloroform Methanol Extraction, Aqueous Layer. Time Study.
Sample 1 of 1

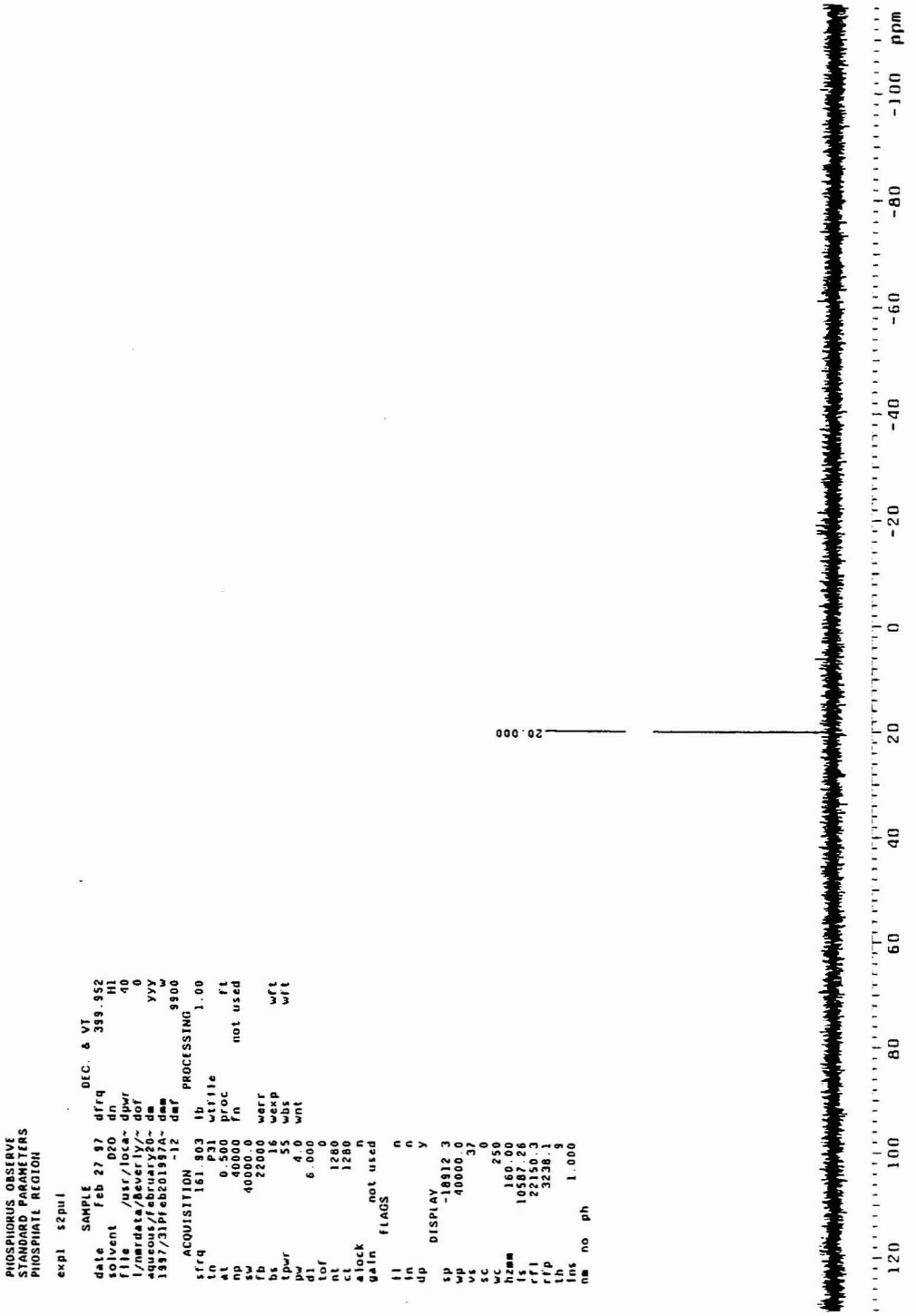
Chloroform Methanol Extraction, Aqueous Layer. Time Study.
Sample 2 of 1



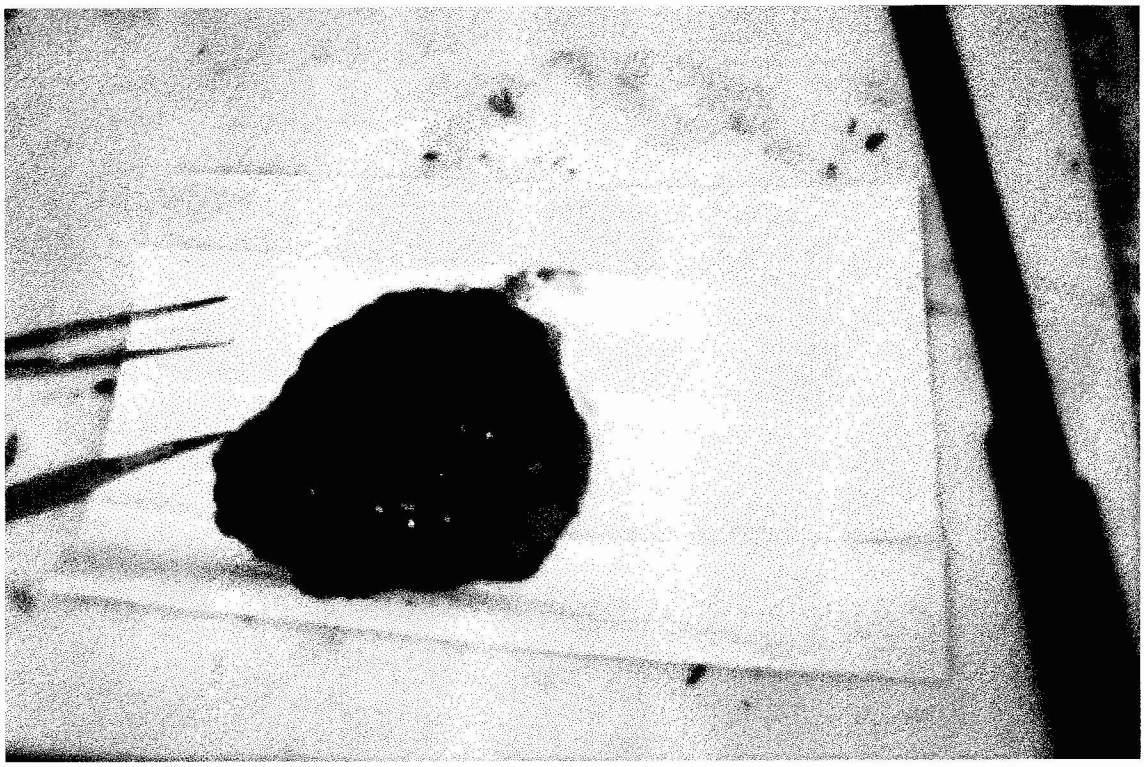
Chloroform Methanol Extraction, Aqueous Layer. Time Study.
Sample 3 of 1



Chloroform Methanol Extraction, Aqueous Layer. Time Study.
Sample 4 of 1



Tissue from a Breast Reduction



Breast Reduction Tissue that was cut into sections with a knife.

Breast Reduction Tissue that was minced into small pieces with a scalpel.