bcc: R. E. Keller - QUEENY.

February 27, 1967

Dr. M. J. Thomas
Research Division
Building No. 33
National Cash Register
Dayton 9, Ohio

Dear Dr. Thomas:

Attached is a photostat of the original paper of Dr. Jensen in Sweden, relating to polychlorinated biphenyls. I will be happy to have your ideas after you read it.

As far as the section on toxicology is concerned, it is true that chloracne and liver trouble can result from large doses. Whether or not this is at all relevant to small quantities existing in human fat is, of course, an entirely different question.

At any rate, I believe before we worry about the toxicological part of the problem, we should settle the analytical part.

Sincerely,

R. Emmet Kelly, M. D.
Medical Director

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att.

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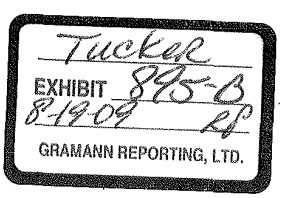

Mr Chairman, Ladies and gentlemen.
In honor to our Brittish guest I will try to hold this lecture in English.

As the title of this lecture states, I am today going to tell about the discovery of some hitherto unobserved chlorinated hydrocarbons having up to eight chlorine in the molecule and found in residue analysis. The chemical name of polychlorinated bifenyls (In the following called PCB). To get familiar with PCB I will start with the chemistry and toxicologi.

Chemistry

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The main characteristic of PCB is 1. Their very high stability. As an example they can be boiled with nitrin acid without being destroyed. 2. They are hardly metabolised in living organism. 3. if more than 4 chlorine are present they are non inflamable. It is clear that these three characteristics does it easy to understand that when they have entered the living organism the will have a low persistence . But it is difficult to explain how they find their way into the living organism. One thing seems to be clear, they don't come from agricultural use, but from a technical one and most probable it comes to the nature via wastes that are tried to be burnt up, because then we have them at once in the air, because of their non inflamability.

Toxicologi

The PCB were introduced in 1929 and as early as 1936 Jones and Aldon reported that 23 out of 24 men employed in manufacturing of PCB suffered from an acne form eruption of the skin. Acne did not appear until 6 to 8 months after the material was first used. In 1937 Drinker reported that rats exposed to chlorinated biphenyls in concentration of approximately 1 mg/m^3 for 16 hours a day for 6 weeks showed damage of the liver. After that time the allowed concentration of PCB in air is 0,5 mg/m^3. (For DDt the same value is 0.5 - 1 mg/m^3). The same authors finished their experiments in 1938, and related that these compounds have an injourious effect, manifested solely in the liver. Chlorinated bipohnyls appeared to be the most injourious chlorinated compounds of all tested.

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Greenburg, Mayer and Smith 1939 reported that PCB and polychlorineted naphtalenes are blamed for the death of three young workers, and that pregnant women and persons who have at any time had any liver diseases are particularly suspectible.

Wedel, Haller and Benton gave 1942 animals PCB including administration by inhalation, ingestion and skin absorbtion. Histological examination of the viscera showed important toxic effect only in the skin and liver, and the degeneration effects in the liver are essentially the same whatever was the method for the administration. Faribok (1955/ found as an occupational poison in the electrical industry, mixed tetra and penta chlorobiphenyl causes folliculitis, comedo, pyodermia and other skin affections, and that its principal toxic effect is fatty degeneration of the liver.

Miller (1944) injected 69 mg PCB (4 and 5 chlorine) subcontaneously in 32 guinea pigs. Eight to ten days after injection, fat droplets were noted in the liver cells, and after 16 days they were present in moderate or very large numbers. Rabbits and rats were also tested in this investigation, as well as the PCB was administated both continously, subcontinously or ingested in the food. In the feeding experiment 8 guinea pigs received 2 doses of 69 mg of the chlorinated biphenyl 1 week apart. Death occurred in 11 to 29 days.

Finally Mc Laughlin 1964 reported a method to test the chemical toxicity and teratogenic effect by injection into the yolk sac of fertile eggs prior to incubation. PCB was found between the eight compounds among 100 tested having the highest order of toxicity. No hatch was found at a level of 25 mg pr egg. At a level of 10 mg per egg, one chick hatched out of 20 injected eggs, but died 2 days later. Some embryos which were examined after they died, showed weak deformities (often a short upper brak) and growth retardation. Lead acetate resulted as an example in no hatch at a level of 1 mg per egg. Autopsy of the dead embyos have showed extensive brain damage. Mercuric chloride showed no hatch even at a level of 0,5 mg per egg.

As the analytical chemistry is a pronounced service science I have been in contact with many scientists from other fields during the work with residue analysis, and I have always found this contact very stimulating for my own work. This co-operation often demands that we are talking the same scientific language. Because of this need I will today try to give a lecture in low level analytical chemistry for biologists, illustrated by the residue analysis of polychlorinated biphenyls.

The lecture will be divided in the following three sub-divisions:

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1. Chemistry of PCB and their toxicology.
2. Analytical methods for residue analysis and proof of structures.
3. Behaviour of PCB in nature, differencies in metabolising rate of the PCB components, potensation in an ecological serie, concentration levels and examples of samples which have been proved to contain PCB.

A residue analysis can be divided in:

1. Extraction of the pesticides from the biological material, followed by a careful cleaning-up to take away interferring substances, most often fats.
2. Identification analysis by mean of gas chromatography. Thin-layer chromatography and mass spectrometry.
3. Quantitative analysis.

At an ecological laboratory in Riksmuséet in Stockholm 1-2 g of a sample is cut out of the biological material and transferred into a weighed and carefully cleaned test tube, and stored at -20° until analysis. Smaller samples have been used, min. 5 mg of body fat, and with dry materials such as hair, feathers, pine needles 100 mg are sufficient to reach the desired 10 ng/g level in residue analysis. In cases of water proofs 1 l. is used for reaching the 10 pg/g. level.

B.1(homog) In order to facilitate complete extraction of the fatty materials from the biological sample, the doulbe amount of finely powdered anhydrous magnesium sulphate is added to the sampling tube, and the whole is homogenised with an insertable homogenizer. The resulting powder is transferred into a special Soxhlet extractor. After 4 hours of extraction the solvent is evaporated, leaving the fat in a small weighed test tube at the bottom
Sox.-tube) of the extractor. This fat is dissolved in methylene chloride in such a way that 100 ul (0,1 ml) contain 20 mg of fat.

The 100 ul solution is now transferred to a little object glass, 3 x 7 cm, covered with a silicagel layer 1 mm thick, in order to form a line 0,7 cm from one end of the slide. Inserting this thin-layer plate into a vessel the bottom of which is covered by a few mm of methylene chloride, the solvent will be sucked up in the dry layer of silicagel, and at least reach the upper end of the plate. The fact is that the fat has a greater affinity to the powder on the plate than the chlorinated hydrocarbon have, — and we get a separation. The fat being more polar than the chlorinated hydrocarbons will never go longer than 2 cm before the

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solvent reaches the upper part of the glass.

7 ELUTION tube

The front of the fat appears quite visible against a lamp, and with the aid of a razor blade the zone above the fat is transferred to the elution tube and the chlorinated biocides absorbed on the powder can now be eluted by one ml of ether. The concentration is sufficient for detection of the chlorinated hydrocarbons down to the 10^{-12} g level.

5 go

The next step in the analytical procedure concerns the separation of the different chlorinated hydrocarbons that the sample may contain. As a matter of fact, this is a troublesome task. It is easy to estimate what is not present, but more difficult to say exactly one is present. We suffer from the negative demonstration, as will be shown later.

At first a few words about the separation of the components present in the sample and their visualization.

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The separation is accomplished by mean of a gas chromatograph fitted to a detector that transfers its impulse to a recorder.

The system is shortly described:

A spirally formed glass tube with an inner diameter of 2 mm and about 2 m in length is filled up by a support, covered with an thin layer of an oil. The tube is heated in the chromatograph to about 200°. Through the tube a stream of nitrogen continously follows. When about 10 ul (1/100 of 1 ml) of the purified sample is injected into the tube, the components of the sample will be evaporized and go forward through the column with the gas stream. As the constituents have different affinity to the column filling they will pass the column with different speed and it will take different time for them to reach the detector at the other end of the glass tube. If the temperature and the nitrogen flow are held constant this time, the retention time, has a specificvalue for a certain compound. This is true, but unfortunately it is also a fact that two components can have the same retention time. This is one of the bigger problems in gas chromatographic analysis of unknown samples, as will soon be obvious.

To make it possible to estimate the retention time it is necessary to visualise the chlorinated hydrocarbons. For that purpose more or less specific detectors are used. The detector most often used in posticide analysis is the so called electron capture detector, which can detect down to one picogram (= 10^{-12} g of lindan). Unfortunately this detector is not specific for chlorine, bu gives answer also for oxygencontaining compounds. The response here is much lower but can be counterbalanced if the concentration of the oxygen containing is much higher. 0281423

The principle for the electron capture detector is shortly:

At the end of the gas chromatographic tube is placed a little tube containing a foil made of titanium tritide. This is an radiant. The b-

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particles are reacting with the nitrogen molecules coming from the column. Then we get $+ N_2 \rightarrow e^- + N_2^+$. Over the detector we have a tension of 90 volt and by mean of the electrons we will get a constant electrical current over the detector. This standing current is transferred to a one-mV recorder as a constant baseline. When now a chlorinated hydrocarbon leaves the column this compound has a high affinity to the electrons and this means that the amount of electrons will diminish, and they will diminish proportionally to the amount of chlorine. The electrical current will also diminish and this is noted as a peak on the recorder. The area of the peak will be proportional to the amount of substance in the sample. By mean of a standard injection it is now possible to compare the retention time and the area of an unknown component with the retention time and area of the known standard. As said before this detector is not specific for chlrine but anyhow very useful, because of its high sensitivity. The system described has, as we have seen, two disadvantages:

1. Two different compounds can have the same retention time and be detected as one peak.

2. A registrated peak does not need to be chlorinated , because the detector is not specific.

If the sample is injected in two different columns with different chemical properties we have increased the chance for a good separation. If two compounds have the same retention time on one column they may not have it on another. When a result seems doubtful, - if the compound being responsible for a certain peak contains chlorine or not - it is possible to concentrate the sample and analyse it on a less sensitive detector such as the microcoumetric one, which is specific for chlorine. The compound is burned in a furnace and teh generated chlorine titrated directly.

As is seen from the two last mentioned possibilities it is anyhow possible to get a rather high degree of certainty in residue analysis, but it is a rather time-consumeing work.When using this method just described, we very often found that many chromatograms from residue analysis of most carefully purified samples still contain a large number of peaks. Many of these have retention times that do not agree with any known chlorinated pesticides, or their metabolites. This chromatogram can serve as an example. It was obtained by residue analysis of a sea-eagle found dead in the archipelago of Stockholm. In the rangs of the known peaks, there are so many unidentified that there also must be an obvious risk of the known peaks to be covered by unknown ones.

If this remark is found true, the reported results of many previous quan-

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titative anal[illegible]is must be brought into ques[illegible]n. In the present investigation it is shown that most of the unknown peak of chromatograms at residue analysis of chlorinated pesticides are due to polychlorinated biphenyls.

I will show a chromatogram of human fat analysed on a so called SP 96 column, the most often used type in pesticide analyses. Early retention times were in agreement with DDE, DDTop and DDTpp. Next slide shows the same sample analysed on a QF-1 column. Now the former 2 DDT peaks have divided into 4 peaks, and two of them are still in agreement with DDTpp and op., the two now were unknown.

Logically, these unknown components were at first thought to be metabolites of the insecticides. Against that spoke that neigther treatment nor concentrated sulfuric acid in ether. This treatment made it rather sure that the compounds did not contain oxygen. In Sweden residues of organic mercury have been investigated rather intensively in the Swedish fauna. As these compounds give very high responses to the electron capture detector it was also investigated if the unknown peaks could have a mercuric origin.

It was found that the water-ecological series had high residues of both mercury (Westermark, Johnels) and the unknown ones, when the same individuals were analysed. Anyhow, the pheasant suffering most from mercury poisoning only contained low levels of electron capturing compounds and these belonged to the normal insectisides. Therefore the unknown could hardly be mercurials or metabolites of them.

As the eagle sample giving the chromatogram shown in fig. 10. could be estimated to contain DDT and DDE up to 15 g/kg in extractable fat, the amount of unknown compounds also were suggested to be in the same range, and tehn sufficiently high to do a run on the combined gas chromatograph – mass spectrometer. If this could be done successfully it would be possible to get very important informations about the chemical nature of the unknown, for ex. the molekular weight numbers of chlorine etc. This method is up to now the method giving the highest degree of certainty in the low level analytical chemistry, amounts of 100 ng substance being enough.

As this method for identification of totally unknown residues surely will be very important in the future (when f.ex. a biologist has found that fishes in a river die) it may be possible by mean of this method to find out exactly what compounds are responsible for the death. For this reason, I will go into some details with this method. In the actual case we took the extract from 20 mg eagle and concentrated

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it as much as possible and made an injection on the gas chromatograph combined with the mass spectrometer. The result was the chromatogram shown on the next slide. Every time the recorder showed that a compound is leaving the column, the effluent is led to the mass spectrometer. Now just a few words about the mass spec.

The molecules leaving the column are bombed with electrons at E. We have now got the molecule positive charged, but with the same mass as before. This M^+ is accelerated in a vacuum and will then get a kinetic energi.

where is the speed. Next comes the magnetic field that tries to bend the direction of the molecule. This will be big for a small molecule and less for

If we have a sieve in the other end we can directly read the molecular weight. Added to this parent molecule M^+ we will also get addition informations, because of the fact that M^+ may not be stbble, a part of them will be broken down before they reach the sieve in the other end.

F.ex. M^+_{DDT} $M^+_{DDT} - CCl_3$

Mass spectrograms from the different unknown peaks in the eagle sample as shown. The mass numbers equal to the molecular weights of the unknowns could be read to 426,392, 358, 324. Astonishingly, the molecular differences were constantly 34 mass units. This difference shows a familarity in origin of the unknown. Now the fact is that chlorine exsists as a mixture of two isotopes with atom weights 35 and 37 in proportion 75:25. If the molecule has one chlorine, this will give two molecule peaks, one for Cl_{35} and one for Cl_{37}. If there are two chlorine we have the possibility of one with only Cl_{35}, one with both Cl_{35} and 37 and one with 2 Cl_{37} and therefore

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The relation of the peaks found on the different mass spec were:

Molecular weight	324	358	392	426
Chlorine content	5	6	7	8

An explanation of the familiarity of the compounds can be given if one substance is built from the former by substituting a hydrogen with chlorine

$$RH + Cl_2 \qquad RCl + HCl$$
$$M^+ + 70 \qquad M^+ + 34$$

Then it is possibl to calculate the molecular weight of the parent hydrocarbon PBC.

$M_{PBC} = M - x\,M_{Cl} + x\,M_H$, where M is the molecular weight of the component having x chlorine atoms. F.ex. for m = 426 and 8 Cl we will get M_{PBC} = 426 - 280 + 8 = 154 and equal with the other molekyls.

The most probable formula with carbon and hydrogen giving this molecular weight is $C_{12}H_{10}$ and this can only be satisfied when the parent-hydrocarbon is biphenyl, and the unknown being polychlorinated biphenyls.

This explanation was later fully verified by injection of a synthetic PBC on the mass spec.

Furthermore extensive gas chromatographic investigations proved that the PBC standard gave peaks with the same retention time as the unknown peaks from the sea eagle.

With the method just described I suppose that we have a new possibility to study the residues in the air because the pine needles can allways be . We have had great difficult; in quantifying the PCB, but when getting a little more time it will be possible. We have done a few calculations on a few species, and I suppose they are right within a factor 2. We have found the residue to be from

It has been my statement here to-day to present this method for studies of defiling of the nature, and with this method a new type of defiling agents has been found to be present in nature, and a few experiment have shown where they may be found.

Now this method is going to be used in the first hadn to estimate how the situation is in nature as a whole, and in the other hand to find the leaks throug which they find its way to nature. Some maybe are present here today to get news about the leaks, and to them I want to say come back in a year.

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So much I think I can say again that the PCB hardly can come from agriculture. As suppoft for this suggestion I can say that we have found PCB in eagle feathers from Rikamuseet from 1944, where hardly any chlorinated pesticides were used in agricultrue. One more thing that I find important to say is that in contrast to the mercury problem this does not seem to be a pure Swedish problem. I have just studied chromatograms taken from London air, and they clearly contain PCB, and dr. Holden has told me that he also find them in his fishsamples. But finally in waiting at more results I should like to point out one morething. It is proved that PCB comes to nature, we dont know now where they are used, but they are very persistant to chemicals and to fire. I think the poison jury should try to state that a content of PCB shall always be found in an open declaration.

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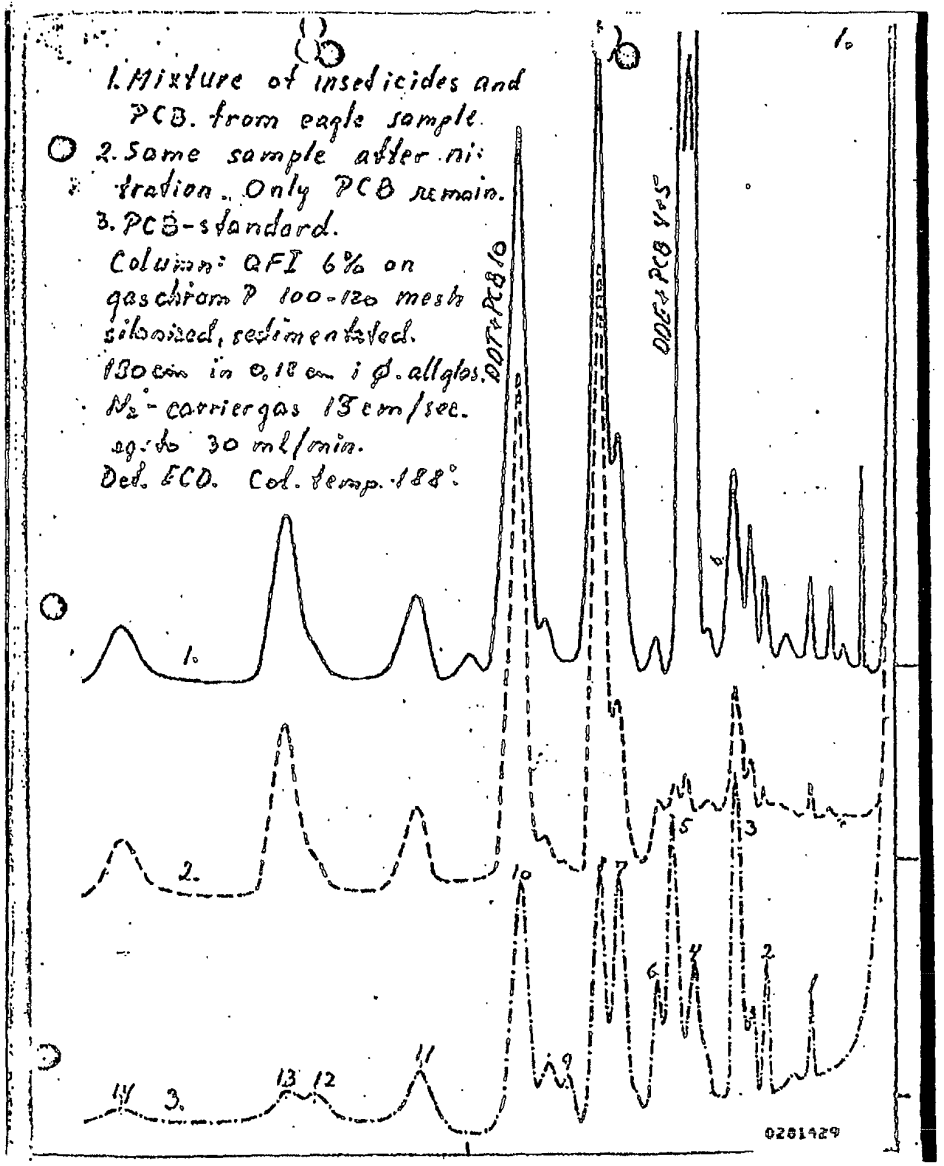

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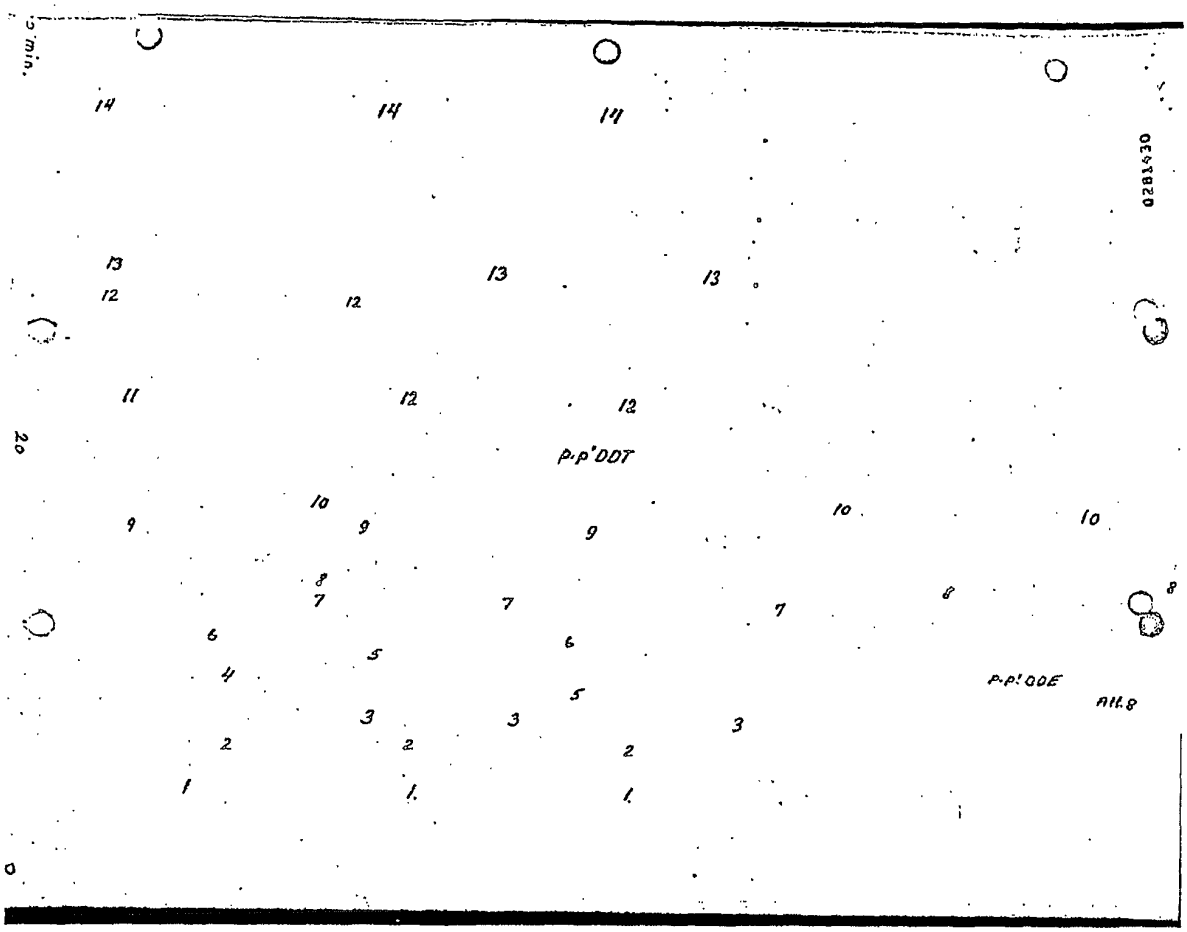

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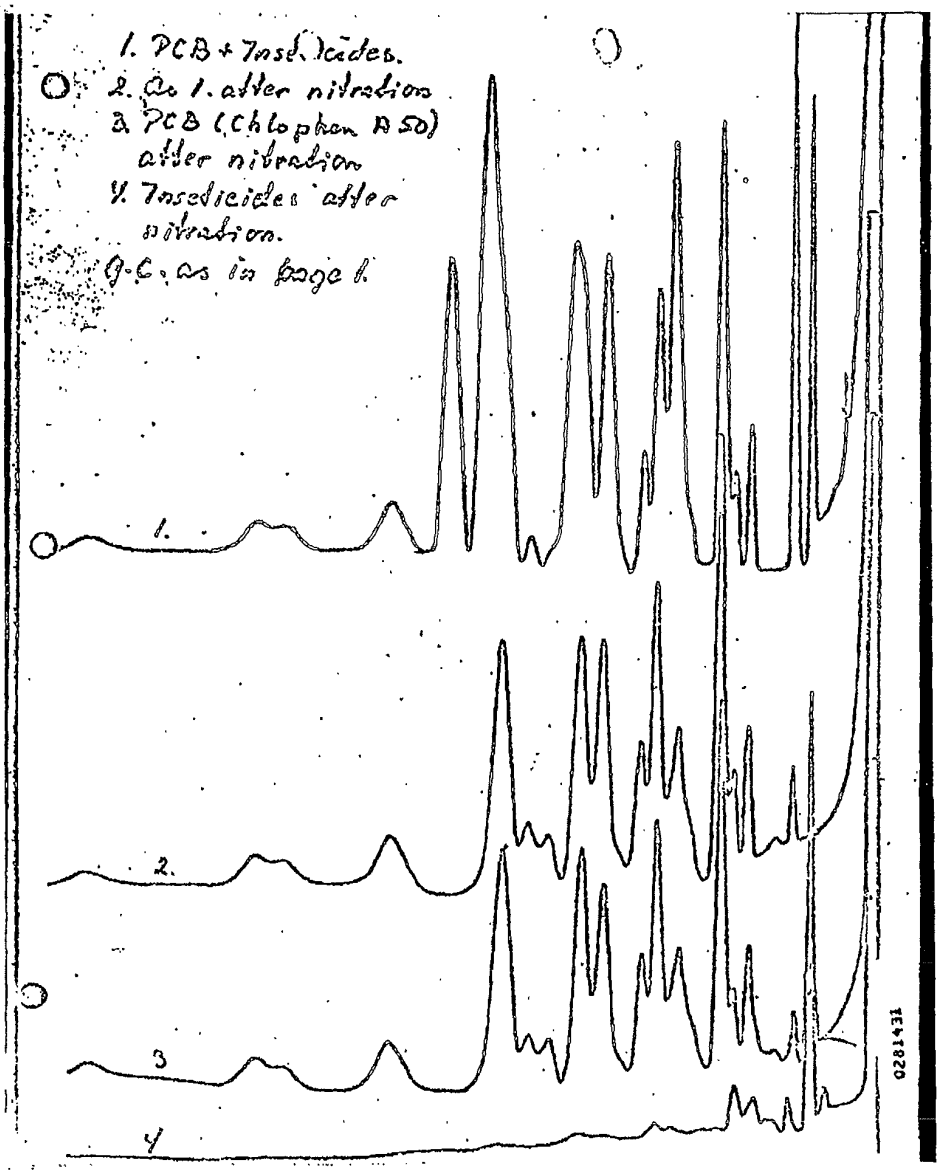

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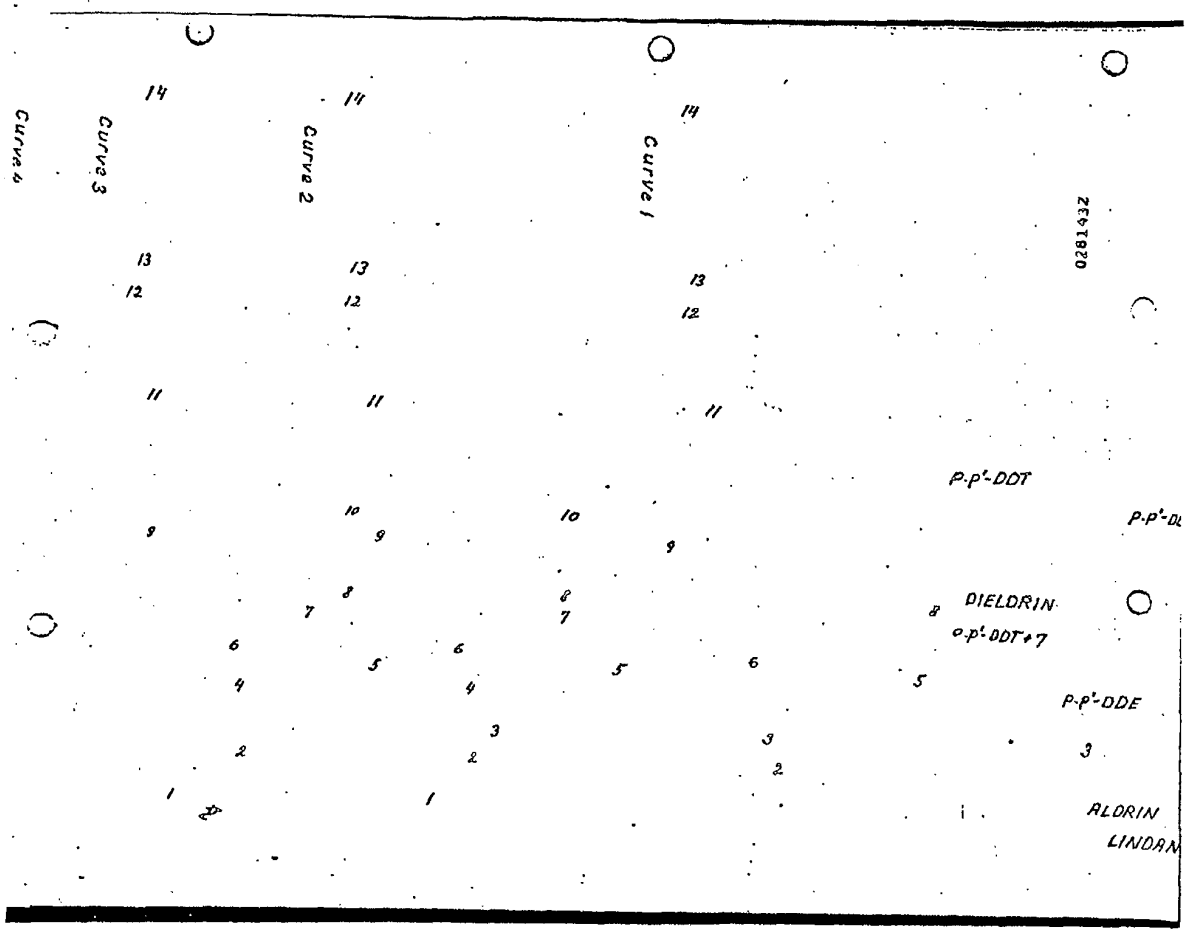

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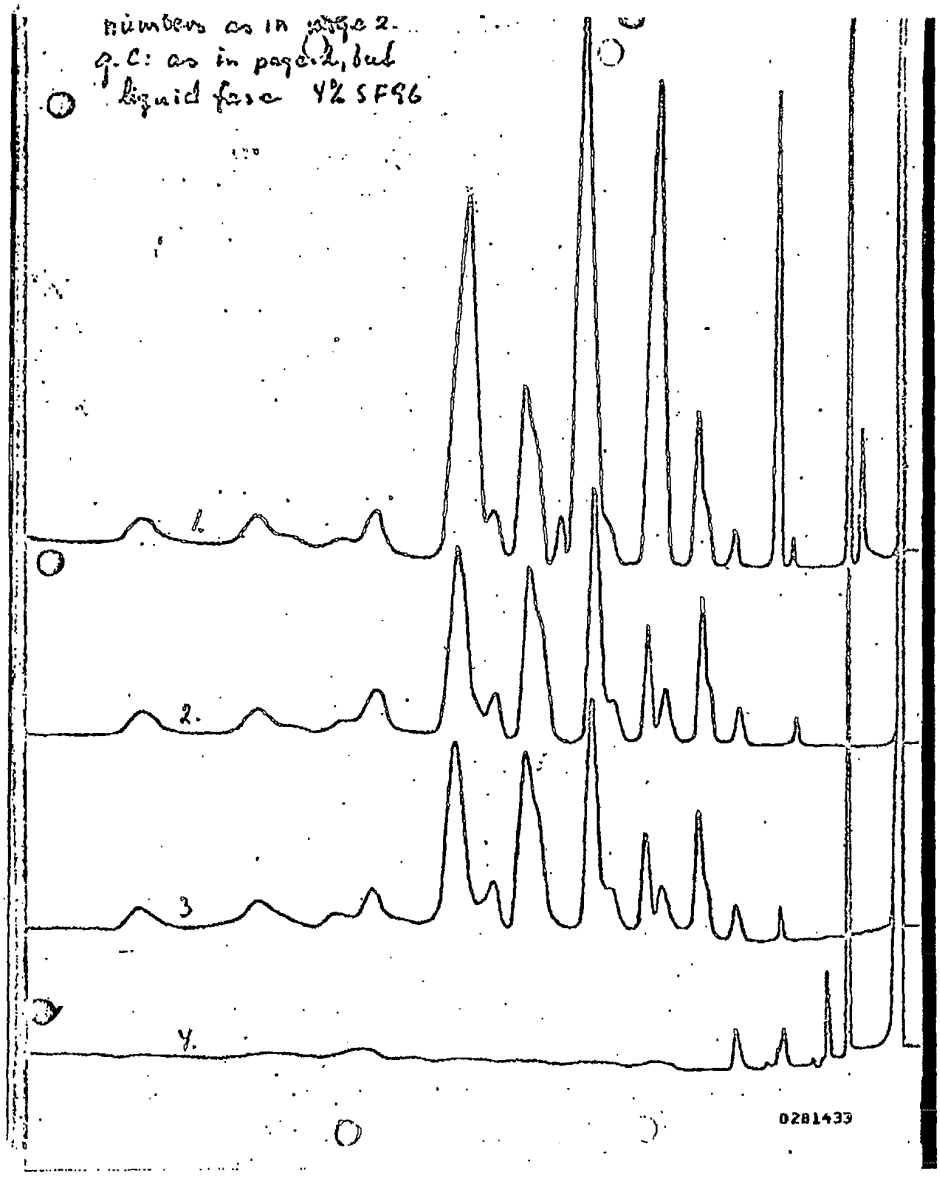

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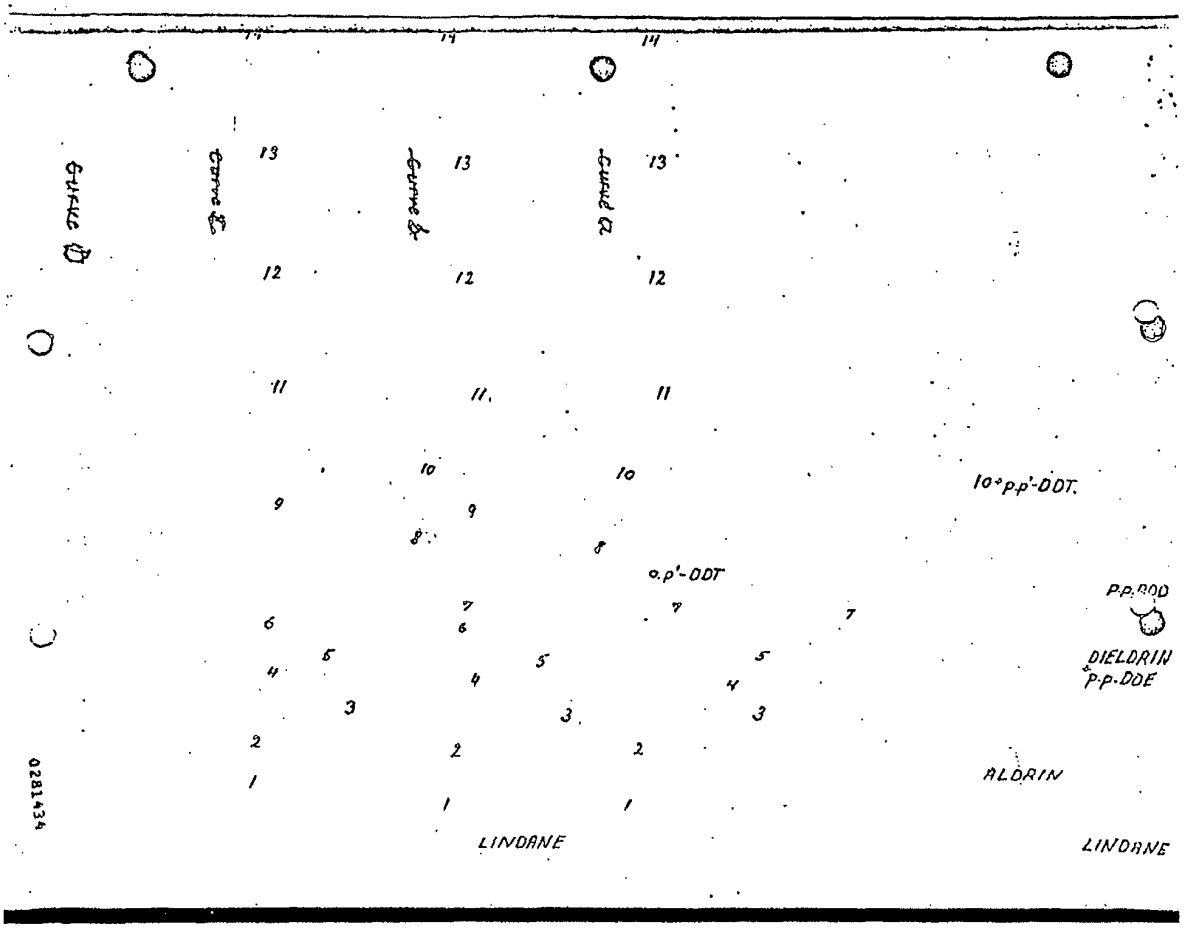

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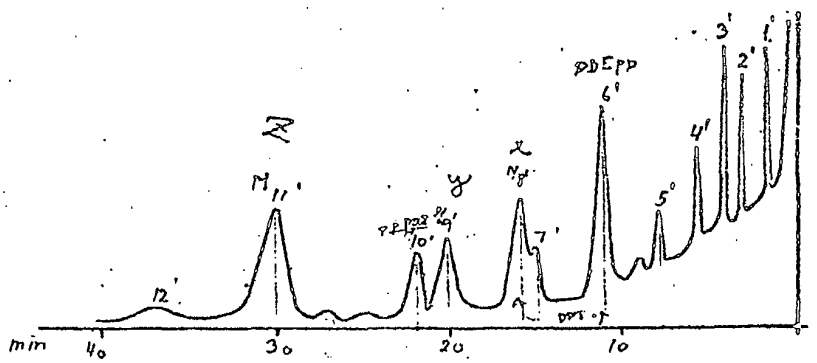

Fig 3. GC-MS chromatogram from eagle sample. Peak numbers represent the points of scanning.

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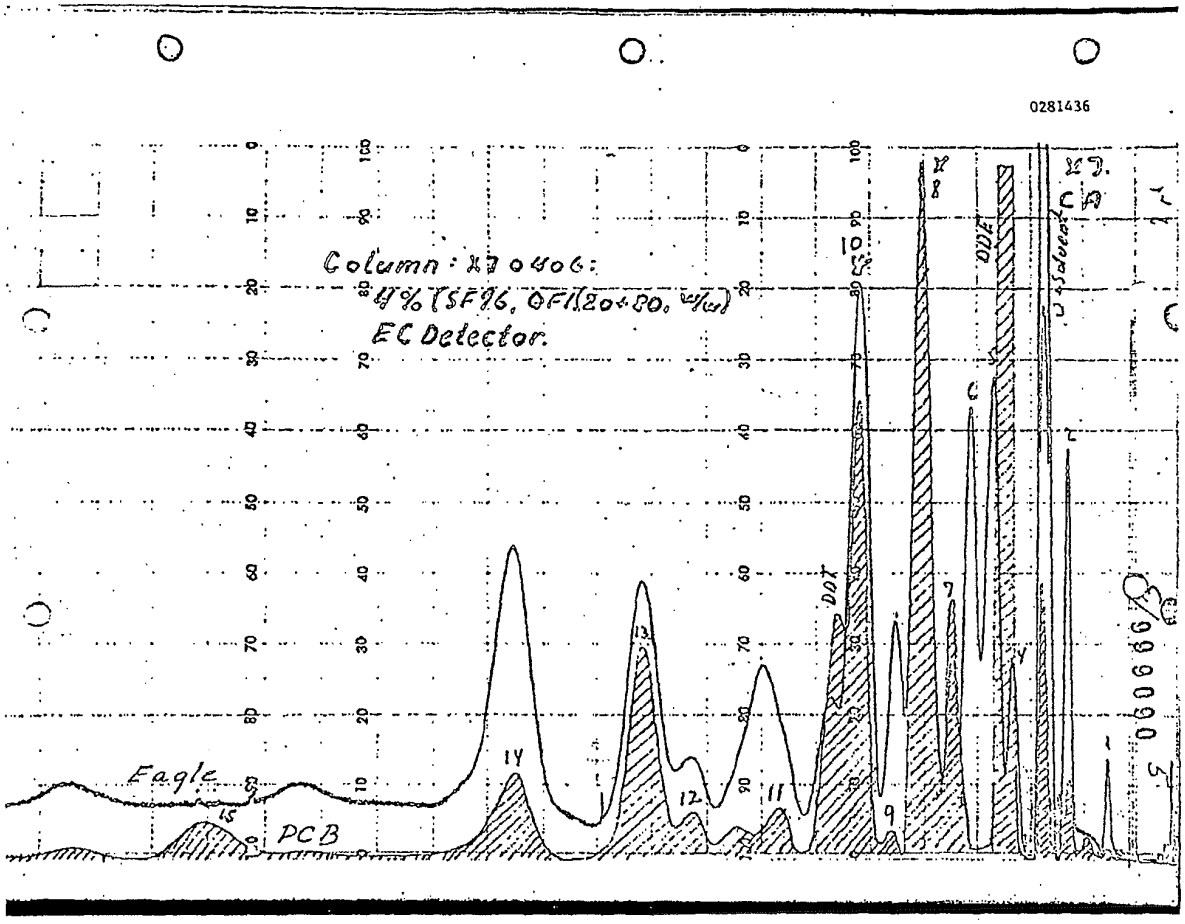

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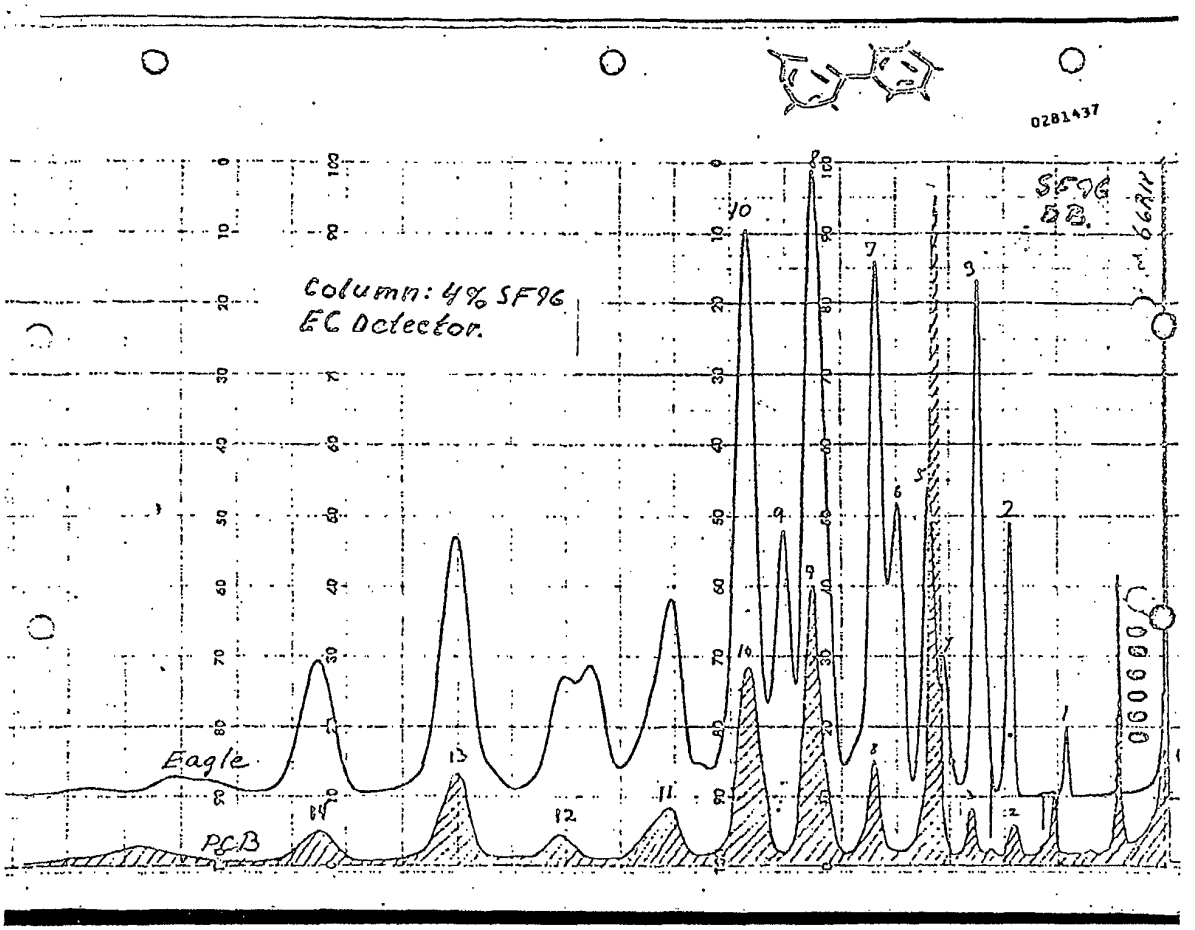

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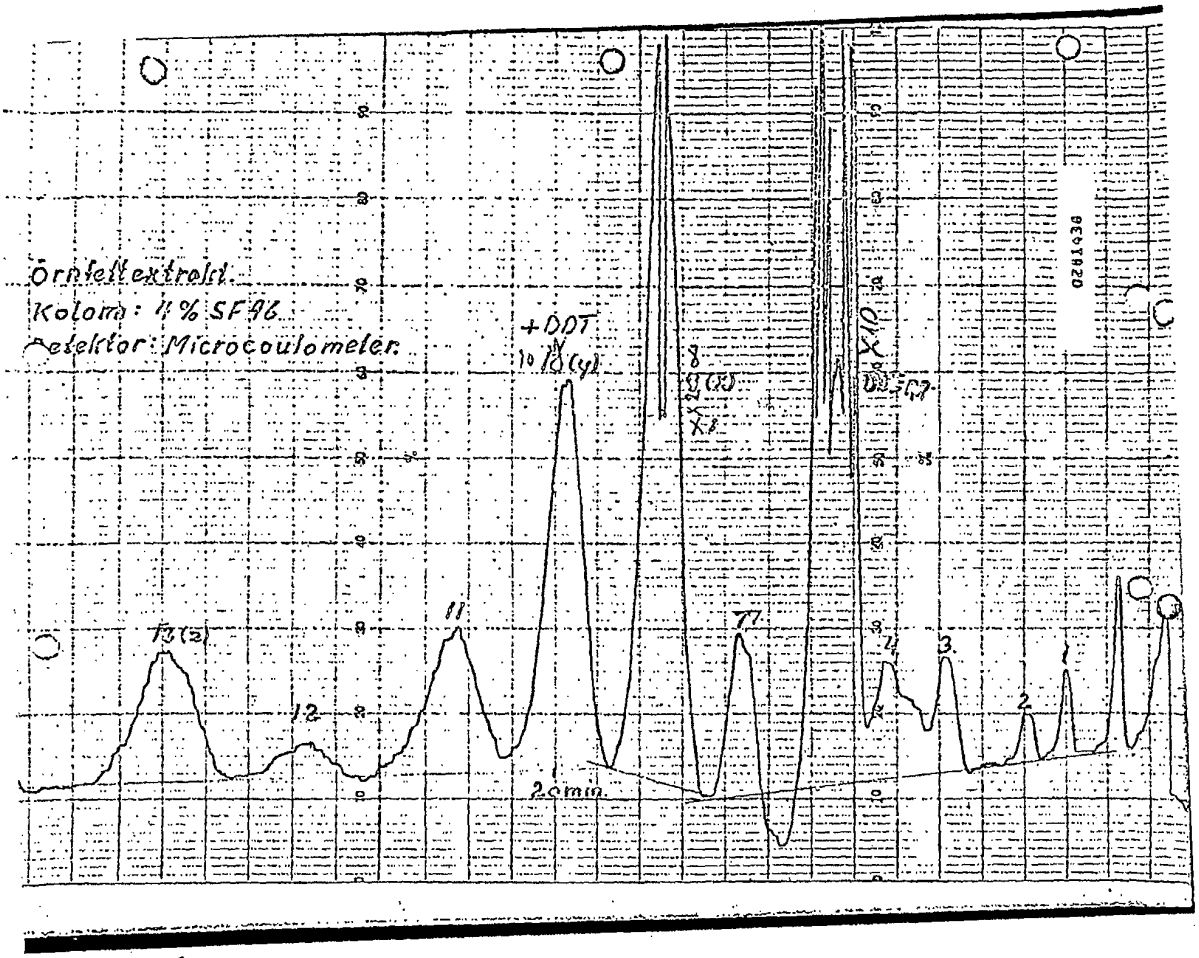

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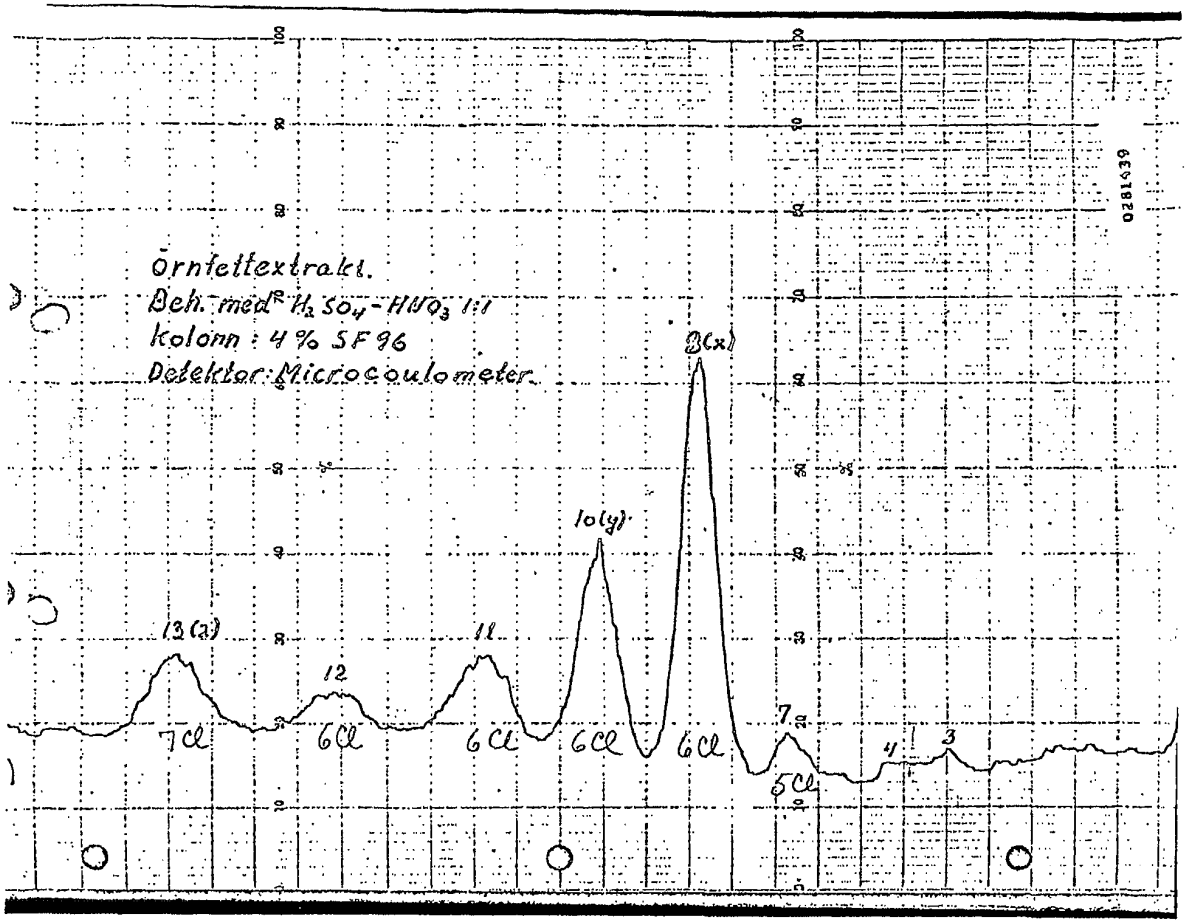

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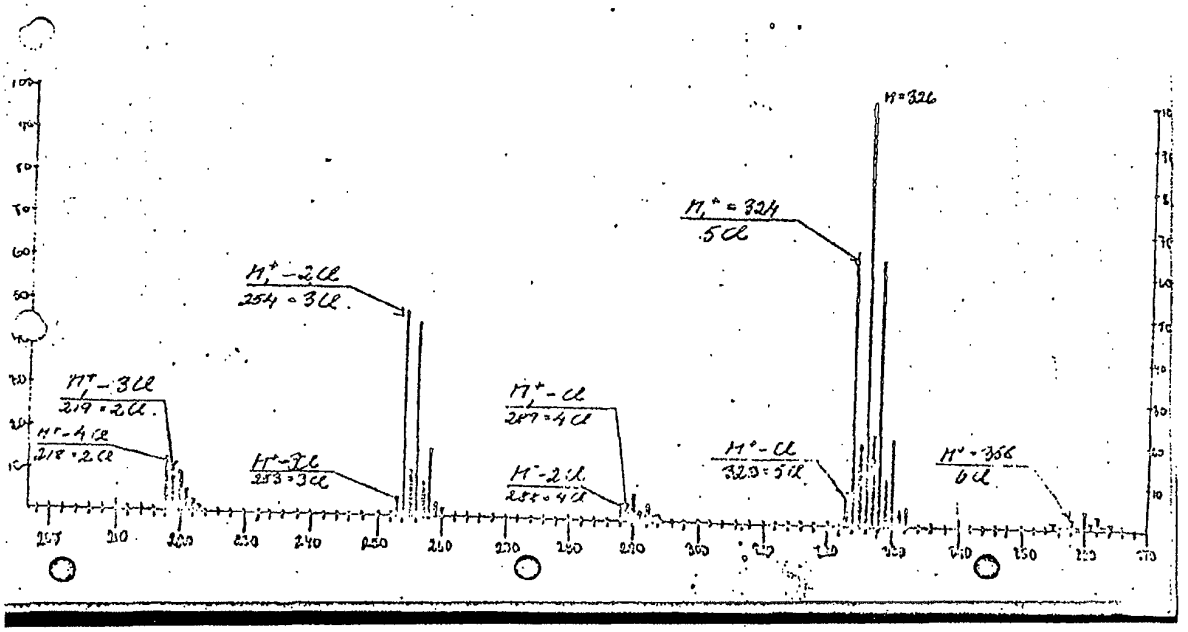

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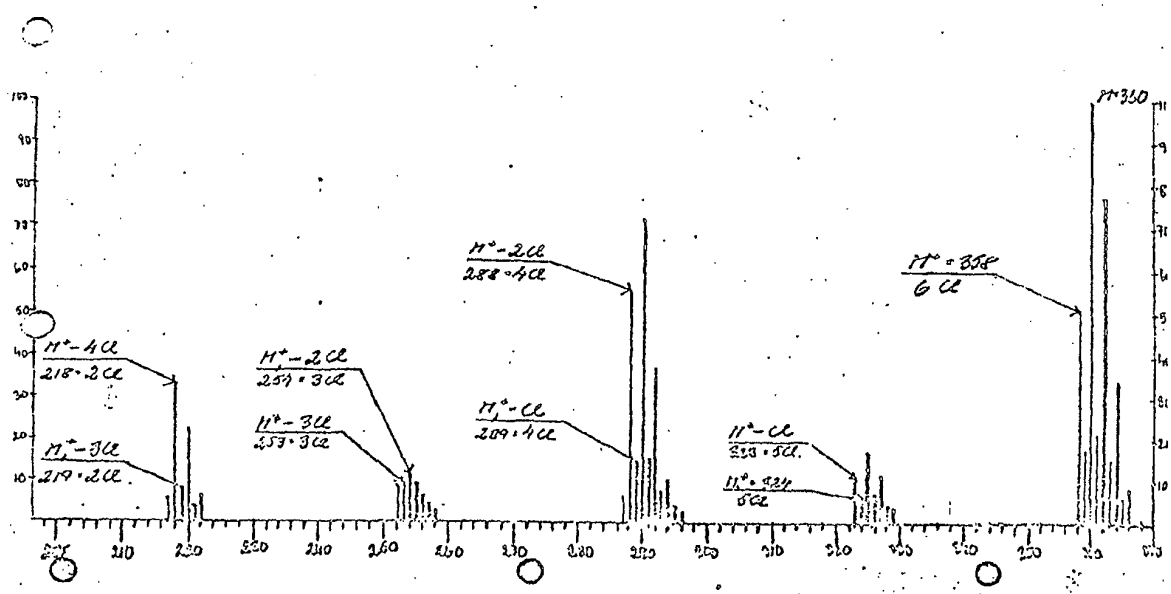

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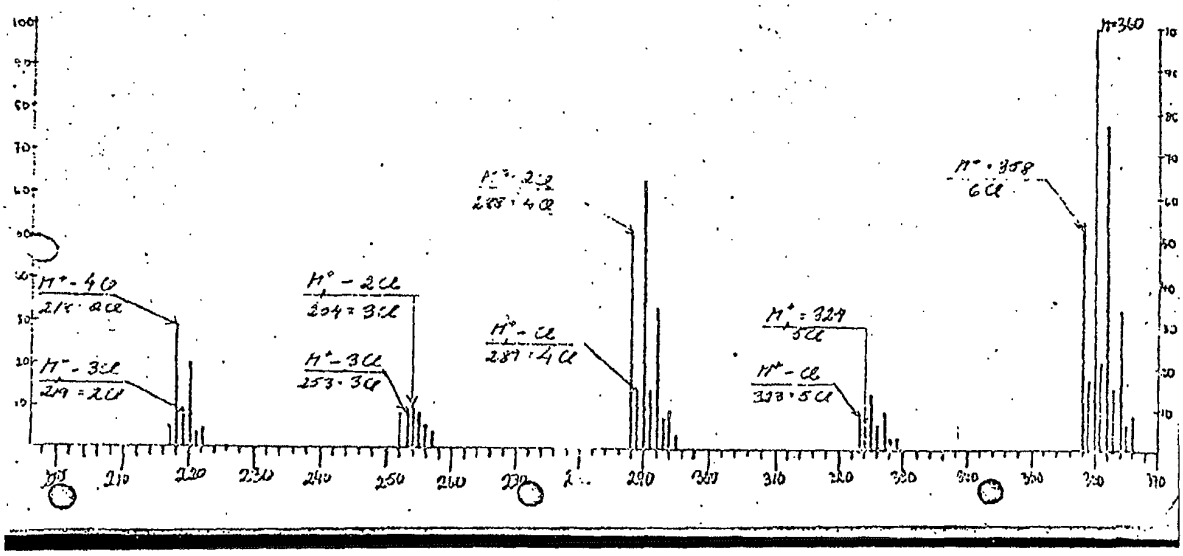

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Topp nr. 11.

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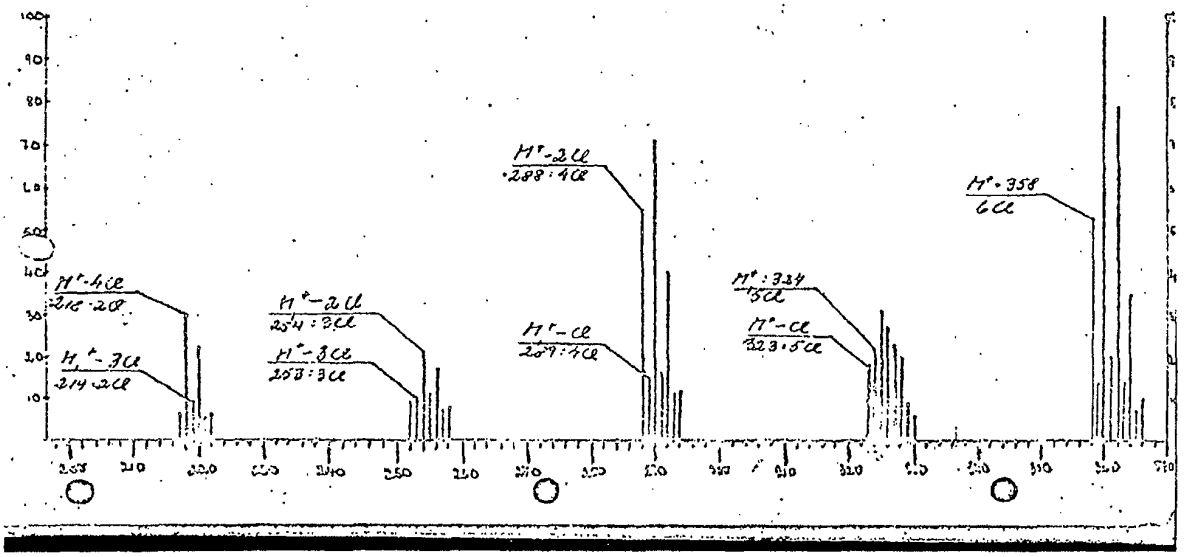

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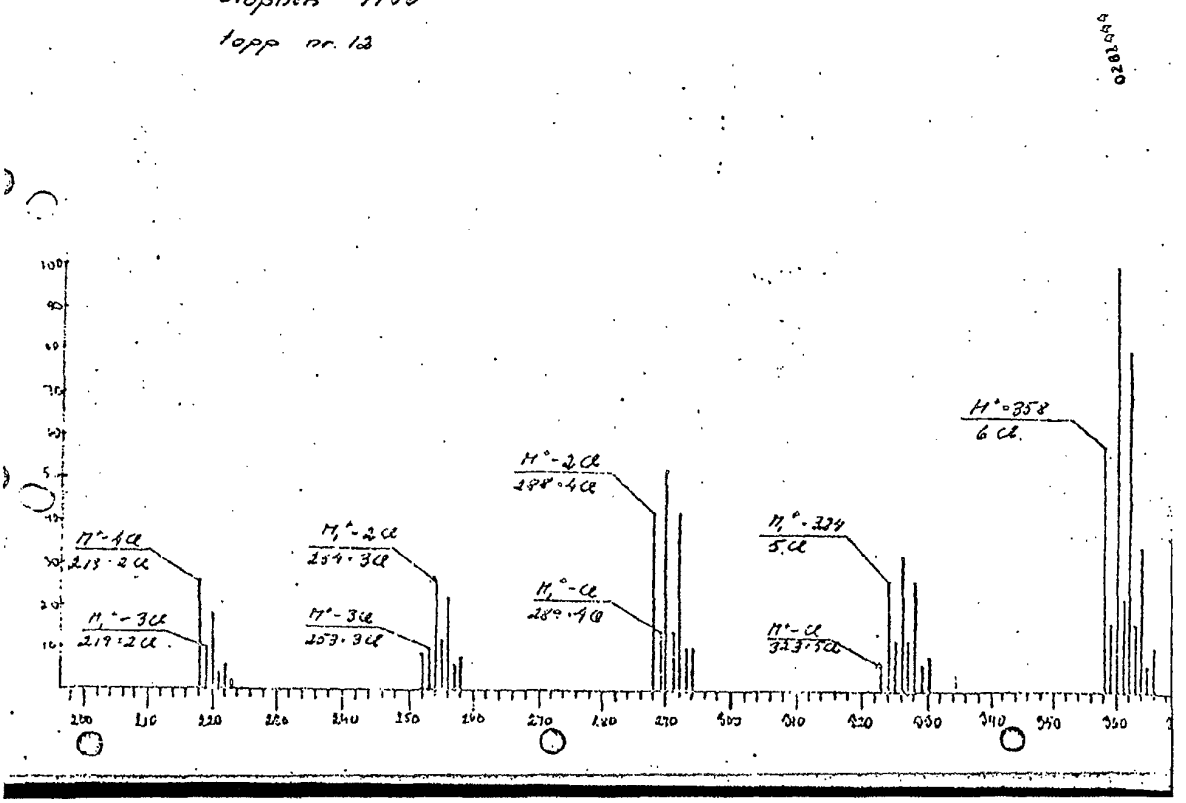
Clophen A50

lopp nr. 12



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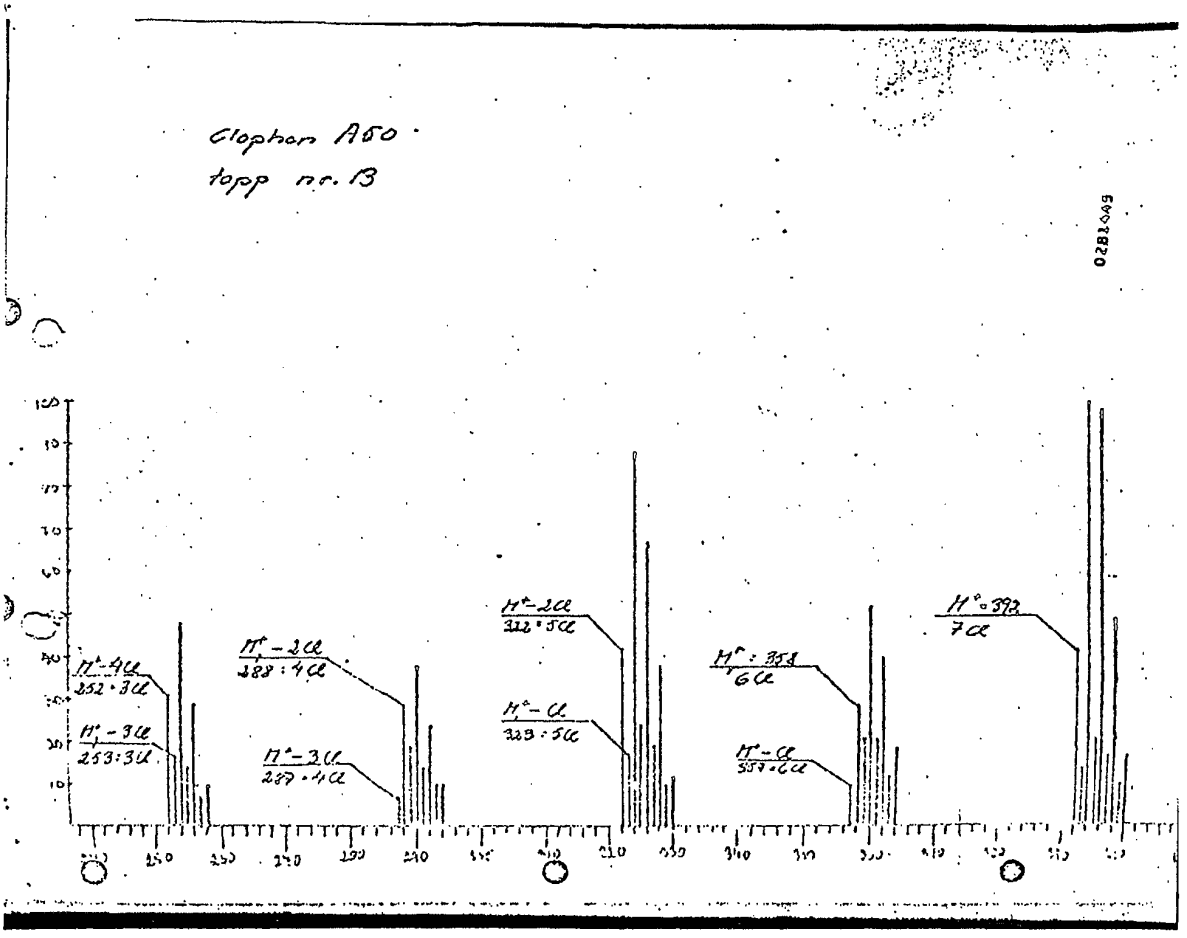

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