# Disease and Contaminants in Marine Mammals

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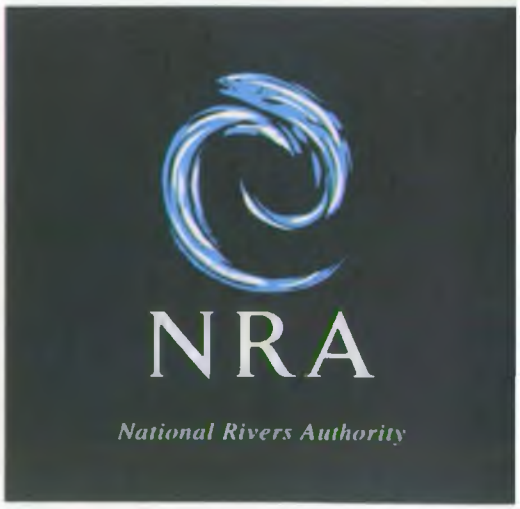

# DISEASE AND CONTAMINANTS IN MARINE MAMMALS - PROGRESS REPORT

PRS 2451-M

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Authors: L Taylor, C Watts, I Wilson, P Kendrick and
T ap Rheinallt

Contract Manager: T ap Rheinallt

Contract No: 4729

Client's Reference No: 3.2.3a



Any enquiries relating to this report should be referred to the author at the following address:

WRc Medmenham, Henley Road, Medmenham, PO Box 16, Marlow, Buckinghamshire SL7 2HD. Telephone: Henley (0491) 571531

# DISEASE AND CONTAMINANTS IN MARINE MAMMALS - PROGRESS REPORT

L Taylor, C Watts, I Wilson, P Kendrick and T ap Rheinallt

## SUMMARY

The objectives of the work were to determine the relationship, if any, between infections of phocine distemper virus (PDV) in seals and their body burdens of contaminants. To this end, broad spectrum survey mode GCMS was to be used to look for a wide range of compound types in extracts of seal blubber, with emphasis on PAHs and Red List compounds such as triazine herbicides, organophosphorus pesticides and trifluralin.

Some preliminary extractions and GCMS analyses were carried out. PCB congeners, p,p'-DDE and p,p'-DDT were identified in one of the extracts. However, no other compounds of potential interest were found. An open-ended broad spectrum approach to GCMS analysis of seal blubber is not feasible because of the high lipid content. It is recommended that future work should focus on using isotopically labelled internal standards to develop effective extraction and clean-up methods for some specific target compounds. Candidates for target compounds include triazine herbicides, organophosphorus pesticides, trifluralin, PAHs, nonyl and octylphenols and their mono and diethoxylates, and linear alkylbenzenes.

Since the analytical scheme could become extremely complex if a significant number of different compound types were to be targeted, it will be necessary to focus on a few types only.

To our knowledge, no attempt has yet been made to analyse seal blubber for any of these compounds and therefore the possibility remains that these and other non-chlorinated compounds are bioaccumulated in this mammalian tissue.

Report No PRS 2451-M, March 1990
14 Pages, 2 Tables, 11 Figures
Project Reference 3.2.3a

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## SECTION 1 - OBJECTIVES

To determine the relationship, if any, between infections of phocine distemper virus (PDV) in seals and their body burdens of contaminants.

## SECTION 2 - INTRODUCTION

There has been substantial concern over the past two years generated by the deaths of large numbers of common seals (Phoca vitulina) around the north west coast of Europe. These deaths have been attributed to a viral infection (phocine distemper virus: PDV) and the occurrence of this outbreak has been correlated, particularly in media reports, with chemical pollution of the North Sea.

There is no direct evidence indicating the extent to which contaminants may have rendered seals more susceptible to infection with the virus. There is, however, experimental and epidemiological evidence indicating that a variety of environmental contaminants may suppress the immune response system of mammals. Heavy metals, organotin compounds, certain pesticides, polychlorinated hydrocarbons and polycyclic aromatic hydrocarbons have been shown experimentally to be immunotoxic with the result, in some studies, that host resistance to infectious agents appeared to be depressed.

With specific regard to common seals, interest in the effects of pollution was kindled by the collapse of the population inhabiting the Dutch Wadden Sea area. This collapse was shown to be associated both with a decline in pup production and with high levels of PCBs in the tissues of seals from this area. An experimental study subsequently showed that feeding seals on fish from that area resulted in lowered reproductive success (Reijnders 1986).

A similar population decline has been observed in several seal species in the Baltic. Although initially this was linked to hunting, some populations have continued to decline despite the cessation of hunting.

As in the Wadden Sea, there was evidence of low reproductive success associated with high levels of PCBs and DDT. Also thought to be caused by PCBs (and possibly other chlorinated hydrocarbons) were a number of pathological conditions associated with hyperadrenocorticism in a high proportion of seals found dead or drowned in nets along the Baltic Coast of Sweden. The authors attributed the presence of these pathological conditions to the effects of PCBs on glucocorticoid levels and the immune system (Bergman and Olsson 1986).

Most recently, the levels of plasma retinol (vitamin A) and thyroid hormone in the seals from the above study of Reijnders (1986) have been reported. It was found that animals fed a diet of fish high in PCBs had significantly lowered levels of plasma retinol and thyroid hormone in association with the observed low reproductive success. As vitamin A is known to play an important role in resistance to microbial infections, it was suggested that the PCB-induced reduction in plasma retinol and thyroid hormone levels may be critically involved in the recently reported reproductive disorders and lethal viral infections in seals in the Baltic, the North Sea and the Wadden Sea (Brouwer et al 1989).

Concern about the possible effects of PCBs or other organic contaminants on marine mammals around UK and North Sea coasts is not limited to seals. There has been a decline in the populations of both common porpoises and bottlenose dolphins in the waters around the UK since 1940 (Evans and Scanlan 1988), and analysis of tissues from these animals has shown high levels of some compounds (Morris et al 1989).

PCBs have been reported to occur throughout the North Sea in suspended particles, sea water, sediments, zooplankton, fish and seabirds (Boon and Duinker 1986, Delbeke and Joris 1988).

Investigation of the possibility of a link between disease and body burdens of chemical contaminants in marine mammals has centred on the analysis of tissue samples from seals, porpoises, dolphins and whales for organochlorine compounds. PCB concentrations varying over a wide range (1.0-700 $\mu g.g^{-1}$ wet weight) have been reported in seal blubber

from the North Sea and elsewhere (Duinker et al 1988, and references therein; Law et al 1989, and references therein). The higher levels tend to have been reported by earlier studies and there are indications of reductions in concentration since the late 1970s. This reduction in concentration has been more rapid for DDT compounds than for PCBs, probably as a result of the earlier and more effective controls on its use (Helle et al 1985). The PCB congener profiles were almost always dominated by congeners 138 and 153 and among the DDT-type compounds the pattern of abundance was DDE>DDT>>TDE. PCB distributions in dolphins and porpoises from Cardigan Bay were also dominated by congeners 138 and 153, but 118 was also present at relatively high levels compared to seal blubber samples (Morris et al 1989). The ratio of DDT-type compounds was different to that in seals with TDE>DDE>DDT in blubber samples. This research has been carried out at a number of European research laboratories and is being co-ordinated in the UK by the NERC Sea Mammal Research Unit (SMRU) in Cambridge.

Most of the reported research has focused on the analysis of PCBs and organochlorine pesticides, with much less emphasis on other anthropogenic pollutants. This is because of the widespread distribution of these organochlorine compounds, their relative ease of detection and their physiochemical properties which make them both persistent and readily bioaccumulated. Other organochlorine compounds which have been analysed in seal tissues are the polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) which have similar physicochemical properties to the other organochlorines but have certain congeners with higher toxicity. Levels of these compounds in seal blubber were of the order of 20-80 $pg.g^{-1}$ lipid wt for the most abundant congeners (Oehme et al 1988, Olsson et al 1988). However, the levels found were species-dependent with grey seals (<25 $pg.g^{-1}$ lipid wt) and common seals ($\leq$10 $pg.g^{-1}$ lipid wt) showing lower levels.

To our knowledge there are no reports in the literature of the analysis of anthropogenic compounds other than organochlorines in seal tissue. Contact with other groups in the UK (MAFF, DAFS, SMRU etc) has shown that analysis here is still centred on PCBs, organochlorine pesticides

and PCDDs/PCDFs, although MAFF have done some organotin analysis of dolphin tissues. It seems sensible to attempt to look for other priority pollutants on the UK Red list (Table 1) which may have a propensity to accumulate in seal tissues. MAFF have been extending their methods to look for pentachlorophenol (a Red List compound), toxaphenes, chlordane and pyrethroids but have no plans to analyse for any other compounds at present. The objective of the work reported herein was to apply broad-spectrum survey mode GCMS to look for a wider range of compound types in extracts of seal blubber, with emphasis on Red List compounds such as triazine herbicides, organophosphorus pesticides and trifluralin as well as PAHs.

## SECTION 3 - EXPERIMENTAL

### 3.1 OVERALL APPROACH

A sample of seal blubber (sample code PV289/89D) was obtained from SMRU so that suitable extraction and clean-up techniques could be developed. These methods would then be applied to subsamples of seal blubber collected and stored by SMRU during 1988 and 1989.

### 3.2 OUTLINE OF METHOD DEVELOPMENT APPROACH

As a first approach to the analysis of the compounds of interest, the following basic steps were planned:

i. Use a general extraction technique which would be likely to be successful in extracting all compounds of potential interest.

ii. Carry out lipid clean-up to remove gross amounts of lipids and other co-extracted materials which would interfere with the GCMS analysis.

**Table 1 - UK Red List Compounds (April 89)**

Lindane*
DDT*
Pentachlorophenol*
Hexachlorobenzene*
Hexachlorobutadiene
Aldrin
Dieldrin*
Endrin
Polychlorinated Biphenyls*
Dichlorvos
1,2-Dichloroethane
Trichlorobenzene
Atrazine
Simazine
Tributyltin compounds*
Triphenyltin compounds*
Trifluralin
Fenitrothion
Azinphos-methyl
Malathion
Endosulfan

Mercury and Cadmium and compounds

*Compounds analysed for in UK seals

iii. Perform preliminary general survey capillary GCMS using full scan electron impact (EI) MS followed by interpretation of the data to identify any compounds present and to look specifically for triazine herbicides, organophosphorus pesticides, trifluralin and PAHs.

Seal blubber is composed almost entirely of fatty tissue, hence GCMS analysis of extracts cannot be attempted until the bulk of the fatty materials such as lipids have been removed. Compared to concentrations of compounds of interest, the amount of lipid is $10^3$-$10^6$ times greater. Removal of lipid is achieved using chromatographic clean-up methods. However, any clean-up may also remove compounds of analytical interest unless appropriate development work is done to optimise recoveries of specific target compounds. A single clean-up method will not be applicable to all potential determinands. If analysis is required for a range of chemical types then several separate clean-ups may be required and each clean-up may generate more than one separate fraction for analysis. This means that the analytical scheme could become extremely complex if a truly broad-spectrum approach to the analysis of seal blubber is to be attempted.

In view of this, following step (iii) above, it was planned to use the data obtained as a basis for determining which target compounds to pursue and then to assess what further extract clean-up and fractionation should be attempted in order to optimise the recoveries of the target compounds chosen. In addition advice would be sought from marine scientists and other experts, so that the analytical focus would still be consistent with the overall aims of the main study.

The methods described by Allchin et al (1989) have been used successfully for the analysis of PCBs and OCLs in difficult samples such as marine biota tissues, sludges and sediments. The initial stages of the method involving Soxhlet extraction and lipid clean-up are general approaches suitable for many types of organic determinands. Therefore, it was decided to use these techniques in our initial experiments. In addition to the Soxhlet extraction technique which Allchin et al (1989) described, we also decided to test a maceration technique in order to compare extraction efficiencies.

## 3.3 EXTRACTION OF SEAL BLUBBER SAMPLE PV 289/89D

### 3.3.1 Soxhlet Method

A portion (10 g) of seal blubber was very finely diced using a scalpel and then dehydrated by thoroughly mixing with anhydrous sodium sulphate (40 g). A clean Soxhlet apparatus was pre-extracted with hexane and the hexane discarded. The blubber/sodium sulphate mixture was put into the Soxhlet thimble and extracted with hexane (120 ml) for 24 hours. After extraction, the cooled extract was quantitatively transferred to a volumetric flask (100 ml) and the extract volume adjusted to 100 ml.

### 3.3.2 Maceration Method

A portion (5 g) of seal blubber was very finely diced using a scalpel and then extracted by maceration using an Ultra-Turrax macerator and separate portions of hexane (15 ml) and propan-2-ol (5 ml). After maceration the sample was centrifuged and the organic extract was removed. This sequence of extraction followed by centrifugation was carried out three times. The organic extracts were combined and then dried by passing through sodium sulphate. The dried extract was collected in a volumetric flask (100 ml) and the extract volume was made up to 100 ml with hexane.

## 3.4 LIPID DETERMINATION

As explained by Allchin et al (1989), lipid concentrations of the initial extract are determined for two reasons: "First to enable the final result to be expressed on a lipid weight basis if desired, and secondly because the alumina micro-columns used for the clean-up have a finite capacity for removal of lipids. If this capacity is exceeded, the resolution of the columns will be lost, with consequent inadequate clean-up of the samples."

Lipid determinations were carried out on both the Soxhlet and macerated extracts in duplicate, using aliquots equivalent to 1 g of the original blubber sample. The solvent was removed by evaporation and the residues were dried to constant weight in an oven at 105 °C. The mean weights of each pair of duplicate determinations were obtained and the lipid concentrations were calculated as percentages of the original samples.

## 3.5 LIPID CLEAN-UP

Crude blubber extracts were cleaned-up using glass chromatography columns (15 cm x 1 cm ID) packed with 5% deactivated alumina (10 g). A layer of sodium sulphate was added to the top of the alumina in order to maintain its activity.

An aliquot of a crude extract equivalent to 1 g of original blubber was concentrated to 1 ml using nitrogen blow-down and applied to a clean-up column. The extract was eluted with hexane (25 ml) and the total column eluate was collected. A lipid determination was performed on this eluate and the result was used to establish whether additional lipid clean-up was necessary. Further lipid removal was accomplished by redissolving the residue in hexane (5 ml) and repeating the clean-up step. The clean-up and lipid determination were carried out sequentially until most of the lipid was removed.

Separate lipid clean-ups were done simultaneously on a duplicate extract sample which would be submitted for GCMS analysis when sufficient lipid had been removed. The results from the lipid determinations carried out on the first extract were used to determine the number of clean-up steps required. As summarised in Figure 1, duplicate lipid clean-ups were performed in parallel for both Soxhlet and macerated extracts until extracts had been prepared that were suitable for GCMS analysis. The Soxhlet extract was cleaned up using three alumina columns and the maceration extract was cleaned up using four columns. The cleaned-up extracts were concentrated to 1 ml using nitrogen blow-down and then analysed by GCMS.

## 3.6 GCMS ANALYSIS

GCMS analysis was carried out using a Hewlett Packard 5890 GC equipped with a 60 m x 0.25 µm DB-1 fused silica capillary column and cool on-column injector. The GC was connected to a VG7070E double focusing magnetic sector MS via a heated direct interface. The MS was equipped with an electron impact (EI) source which was operated in the full scan positive EI mode at a temperature of 250 °C.

GCMS analysis used the following operating conditions:

GC Temperature Program: 30 °C for 4 minutes, then 8 °C $min^{-1}$ to 300 °C. Held for 20 minutes.

MS Mass Range: 700-20 amu was scanned at a scan speed of 0.5 sec/decade giving a scan cycle time of 0.97 seconds. Mass calibration was achieved using perfluorokerosene.

An injection (1 µl) of a mixed standard containing benzene, chlorobenzene, p-xylene, 1,1,1-trichlorethane, naphthalene, phenol, hexanoic acid, phenanthrene and hexadecane at a concentration of 10 µg $ml^{-1}$ per component was made to establish capillary column performance.

The cleaned-up extracts which were originally prepared using the Soxhlet and maceration techniques were analysed by GCMS. Total ion current (TIC) traces were obtained and peaks were identified by using the instrument data system to search the NBS/EPA library of standard compound mass spectra. In addition mass chromatography was used to search the data from each extract for specific target organic compounds including Red List compounds, chlorinated dibenzodioxins and furans, PAHs, and octyl and nonylphenols and their mono-and diethoxylates.

## SECTION 4 - RESULTS AND DISCUSSION

### 4.1 RESULTS OF LIPID DETERMINATIONS

Results of lipid determinations are summarised in Table 2. In terms of the initial crude extracts, GCMS analyses were carried out after 91% of lipid had been removed from the Soxhlet extract and 96% of lipid had been removed from the maceration extract.

### 4.2 RESULTS OF GCMS ANALYSIS

TIC traces for Soxhlet and maceration extracts are given in Figures 2 and 3 respectively. The tailing, broad and poorly defined peaks obtained in Figure 2 for the Soxhlet extract indicated that the extract required further alumina clean-up before any useful GCMS results could be obtained. No further interpretation was carried out on the data from this extract.

By comparison, the TIC trace obtained for the maceration extract showed peaks without gross tailing or broadening and therefore the GCMS data for this extract was interpreted as fully as possible.

Interpretation using library searching and mass chromatography techniques indicated that $Cl_3$, $Cl_4$, $Cl_5$, $Cl_6$, $Cl_7$ and $Cl_8$ PCB congeners were present as shown in Figures 4 - 9. Each figure shows the mass chromatograms for masses 90-400 as trace B at the top, with the mass chromatograms for the molecular ion M and M+2 below. In addition, peaks at scan 1896 and scan 2029 were identified as p,p'-DDE and p,p'-DDT respectively. Figures 10 and 11 show the background subtracted mass spectra for each peak together with the NBS/EPA library spectra which were used to make the assignments. No other compounds of interest could be found in this extract.

Table 2 - Results of Lipid Determinations

| Sample | Lipid Weight (mg) |
|---|---|
| Crude Soxhlet Extract | 772 |
| Soxhlet Extract after 2 Clean-ups | 170 |
| Soxhlet Extract after 3 Clean-ups | 70 |
| Crude Maceration Extract | 870 |
| Maceration Extract after 2 Clean-ups | 520 |
| Maceration Extract after 3 Clean-ups | 290 |
| Maceration Extract after 4 Clean-ups | 39 |

## 4.3 CONCLUSIONS

Compared to the Soxhlet method, the maceration technique using hexane and propan-2-ol was 10% more effective in extracting organic material from the original blubber. This technique also has the advantage that it can be carried out robotically. Extract preparation takes about one hour per sample and if large numbers of samples need to be extracted then this can be carried out automatically with only periodic supervision by a scientist or technician. This compares very favourably with the Soxhlet method which uses five times more solvent and takes 24 hours per sample.

Removal of at least 95% of the lipid is essential before a satisfactory GCMS analysis can be obtained; this involved the use of at least four alumina clean-up columns for 1 g equivalent of blubber extract.

Although only OCLs were identified in the preliminary analysis carried out so far, this does not rule out the possibility of other organic compounds of potential interest being present in the blubber sample. This is the case because the extraction and clean-up techniques used were general approaches and not optimised for any particular class of compound. Therefore, it is possible that some compounds of interest were removed from the extract with the lipid during the clean-up.

Clearly, an open-ended broad spectrum approach to GCMS analysis of seal blubber extracts is not feasible and target compounds need to be specified so that extraction and clean-up methods can be optimised.

## SECTION 5 - FUTURE WORK

It is recommended that future work should focus on using isotopically labelled internal standards to develop effective extraction and clean-up methods for some specific organic compounds.

Selection of target compounds should be based on criteria such as mammalian toxicity, environmental persistence, ability to bioaccumulate, amounts manufactured and whether commonly present in sewage sludge.

The compounds suggested by WRc include triazine herbicides, organophosphorus pesticides, trifluralin and PAHs. Compounds such as nonyl and octyl phenols and their mono and diethoxylates and linear alkylbenzenes which are known to be associated with sewage sludge could also be considered as candidate chemicals.

Since the analytical scheme could become extremely complex if a significant number of different compound types were to be targeted, it will be necessary to focus on a few types only, so that the analytical task remains manageable.

To our knowledge, no attempt has yet been made to analyse seal blubber for these compounds and therefore the possibility remains that these and other non-chlorinated compounds are bioaccumulated in this mammalian tissue.

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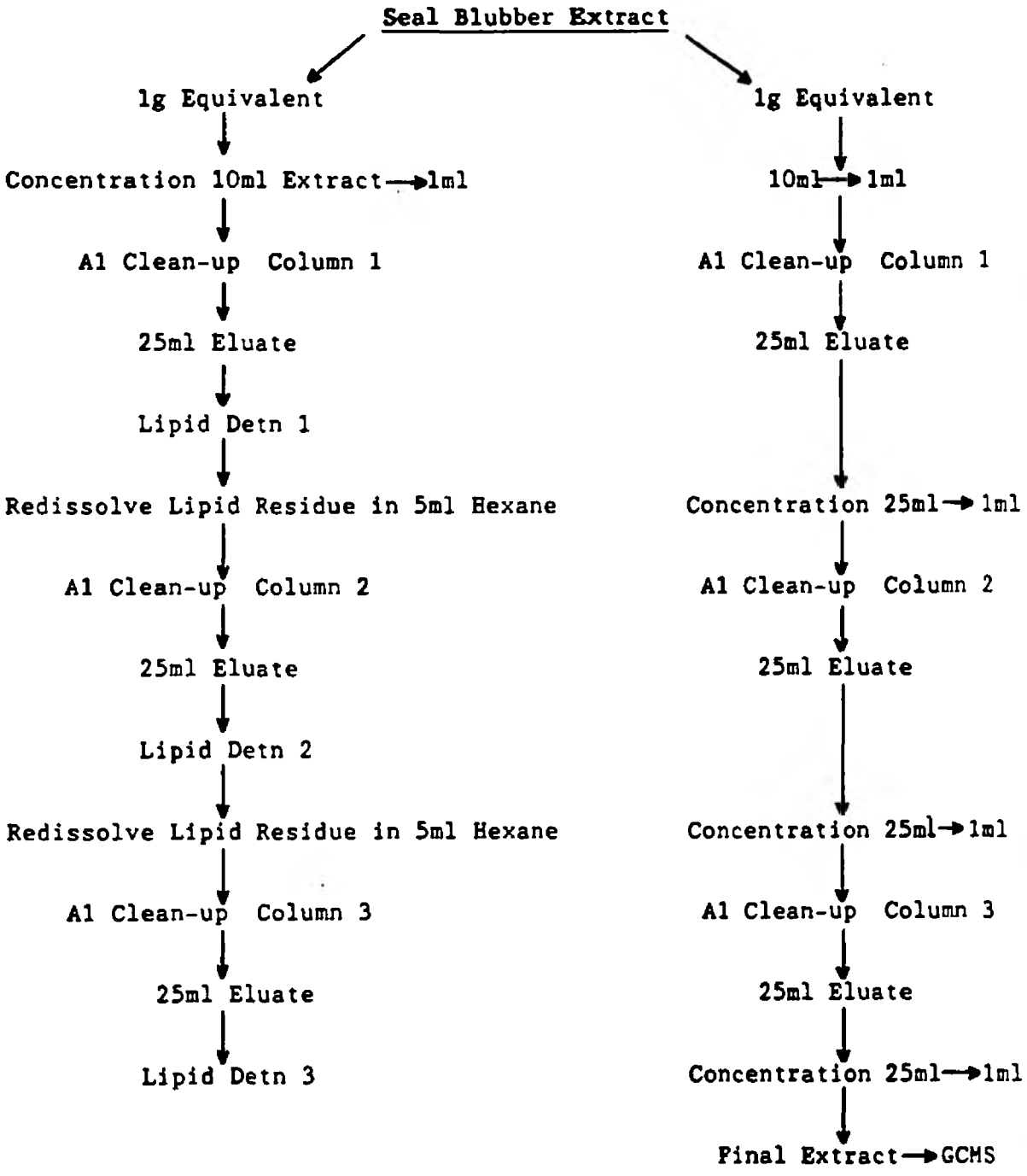
## LIST OF FIGURES

Figure 1 - Summary of Lipid Clean-up



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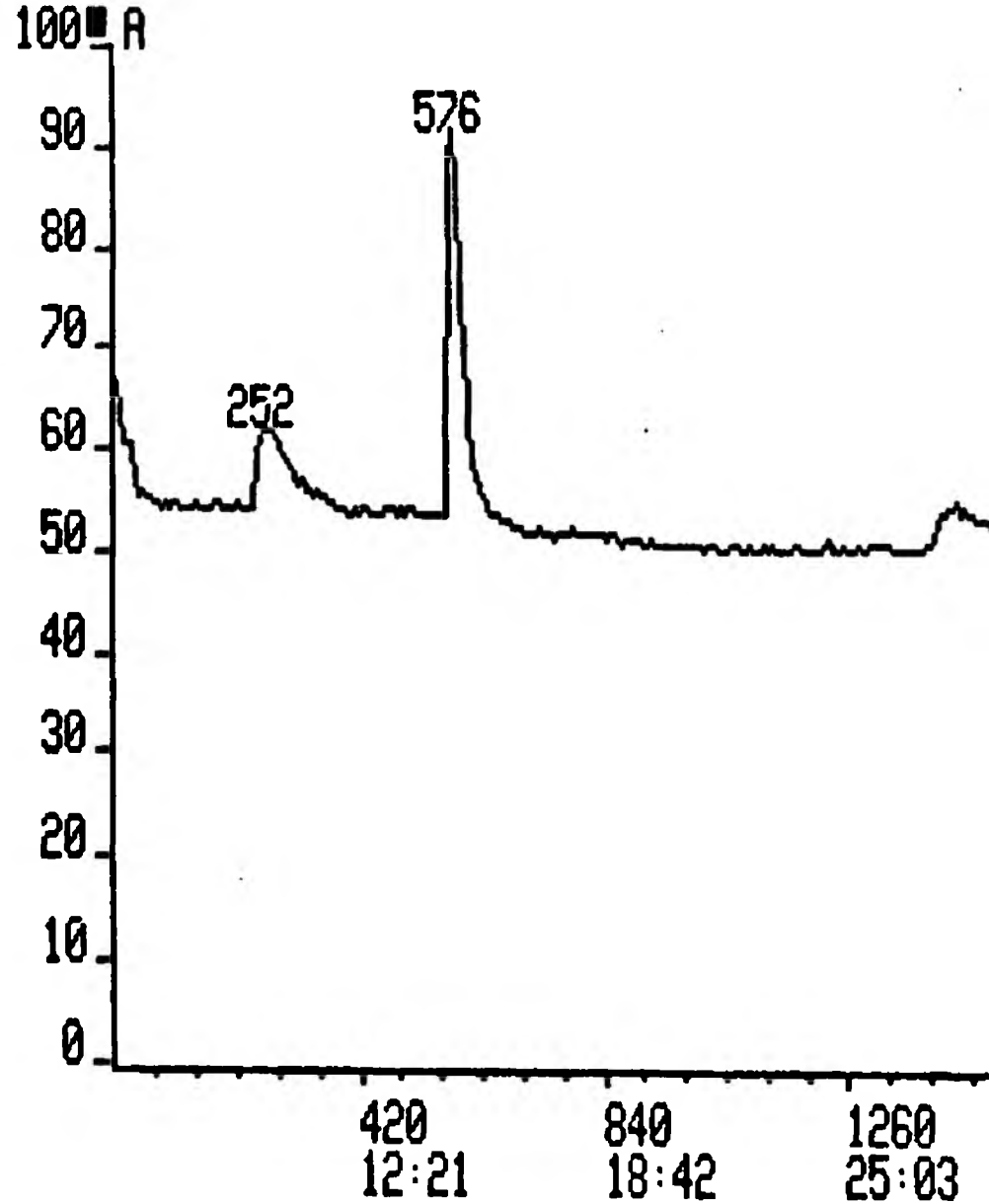

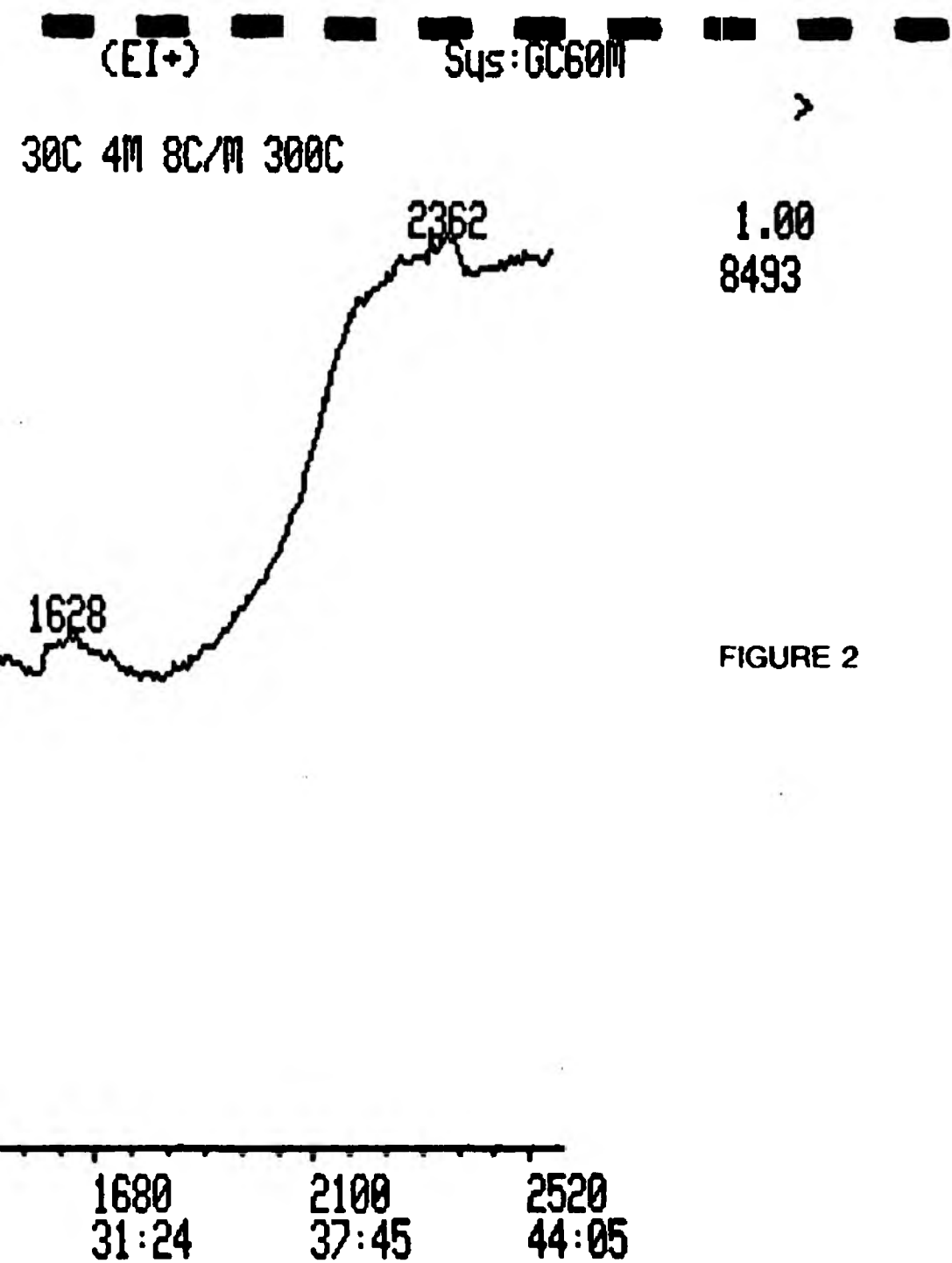
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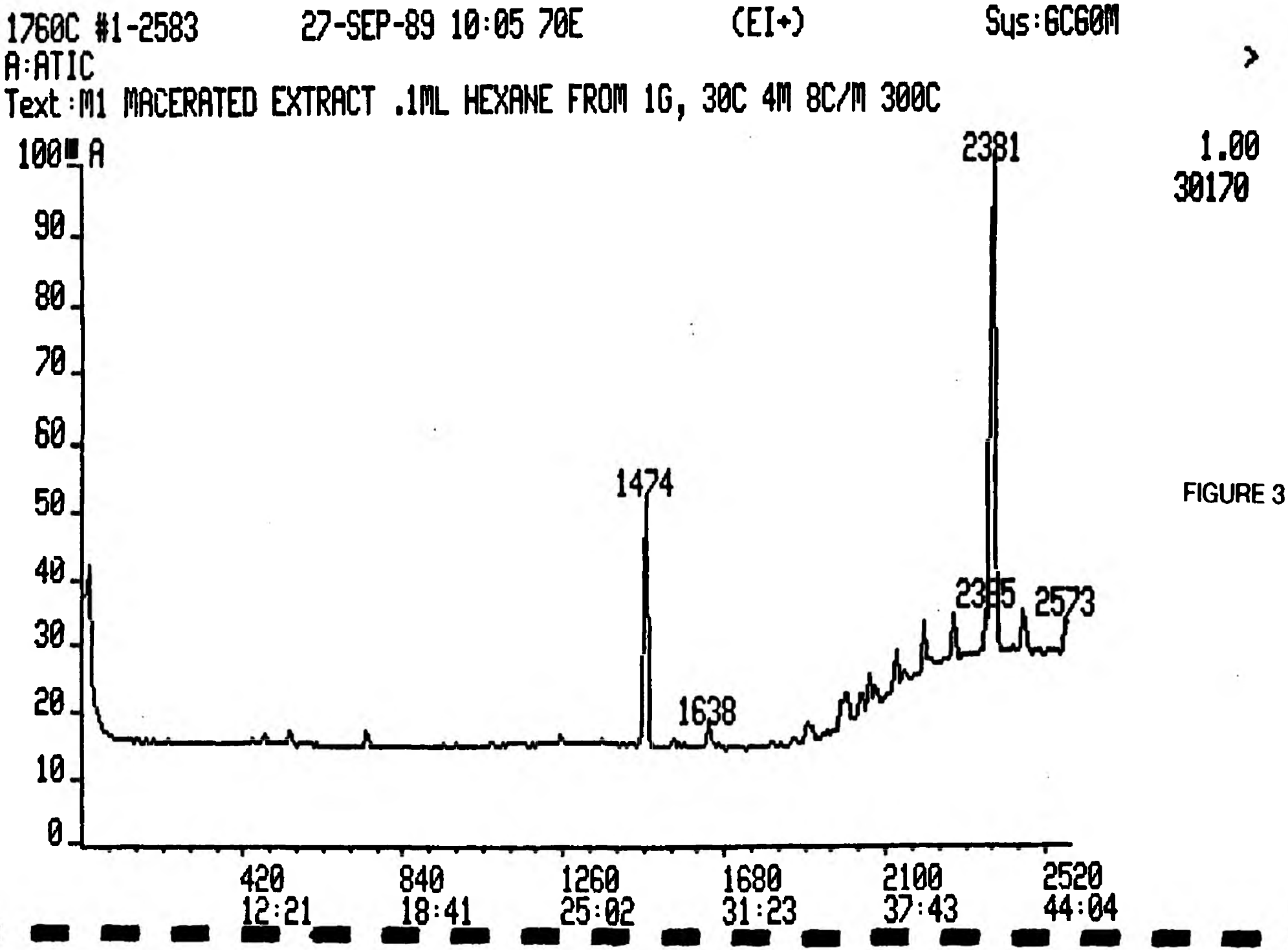
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90
80
70
60
50
40
30
20
10
0
1474
1638
2385
2573
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12:21
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18:41
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25:02
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FIGURE 3

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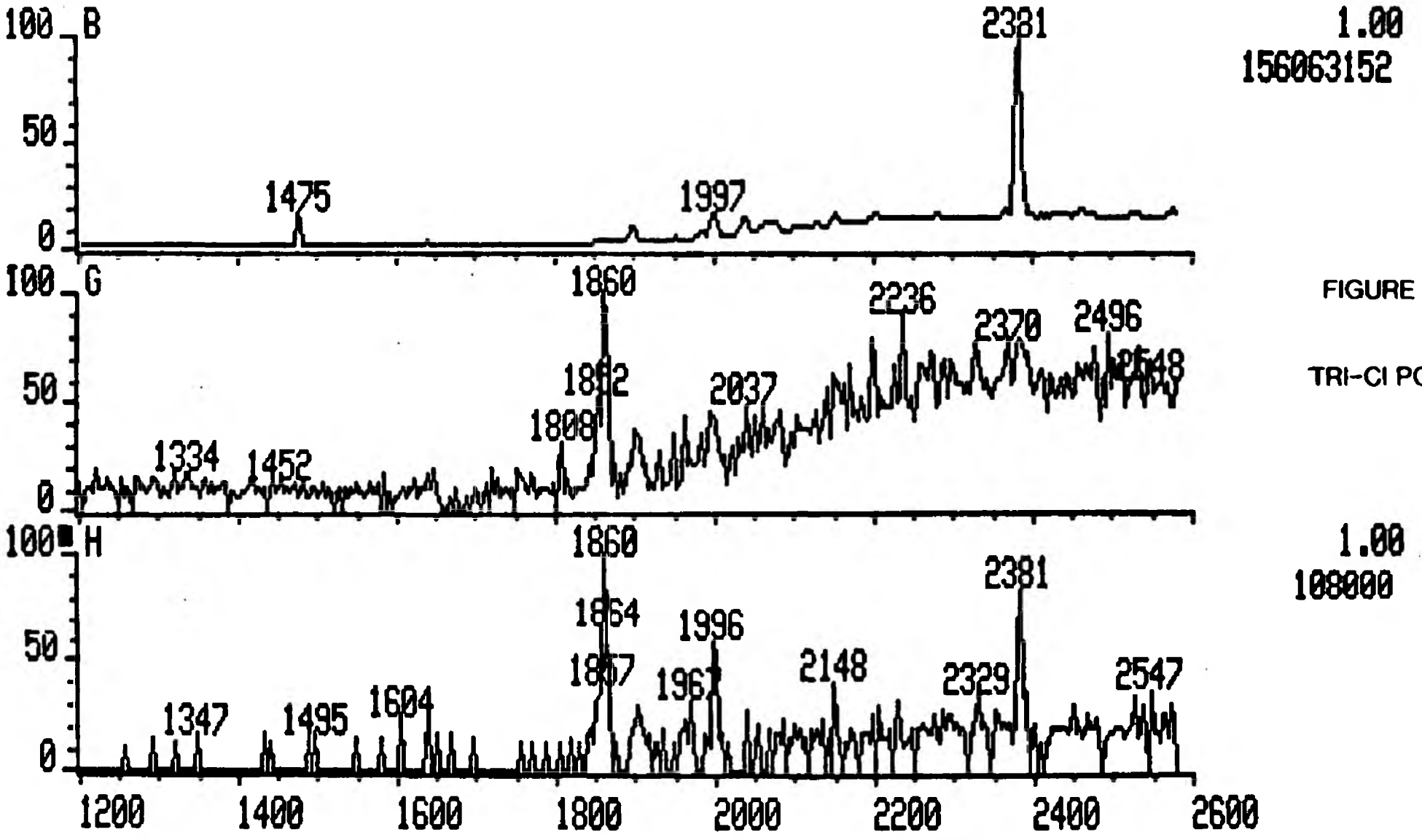


FIGURE 4

TRI-CI PCB

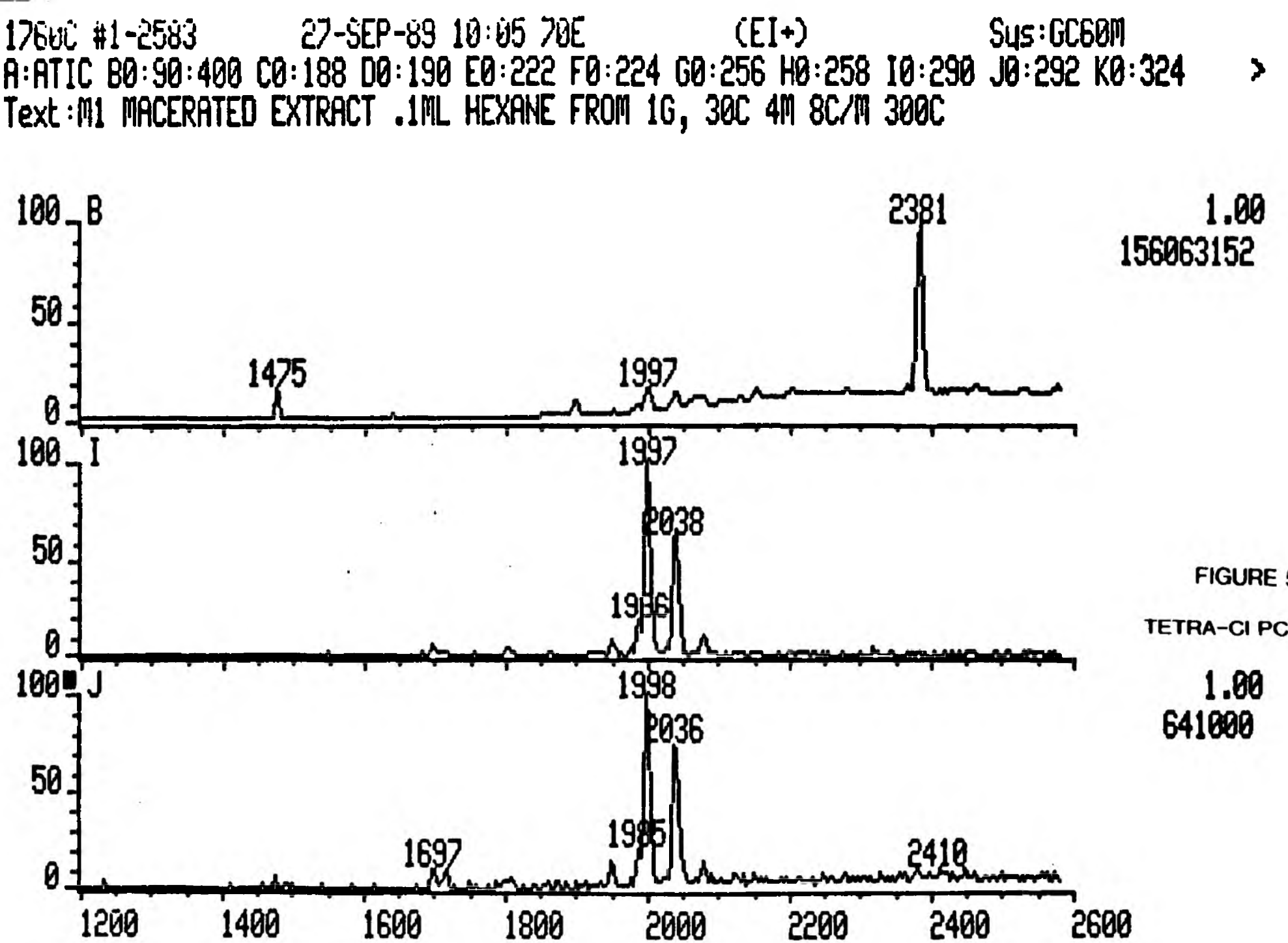


FIGURE 5

TETRA-CI PCBs

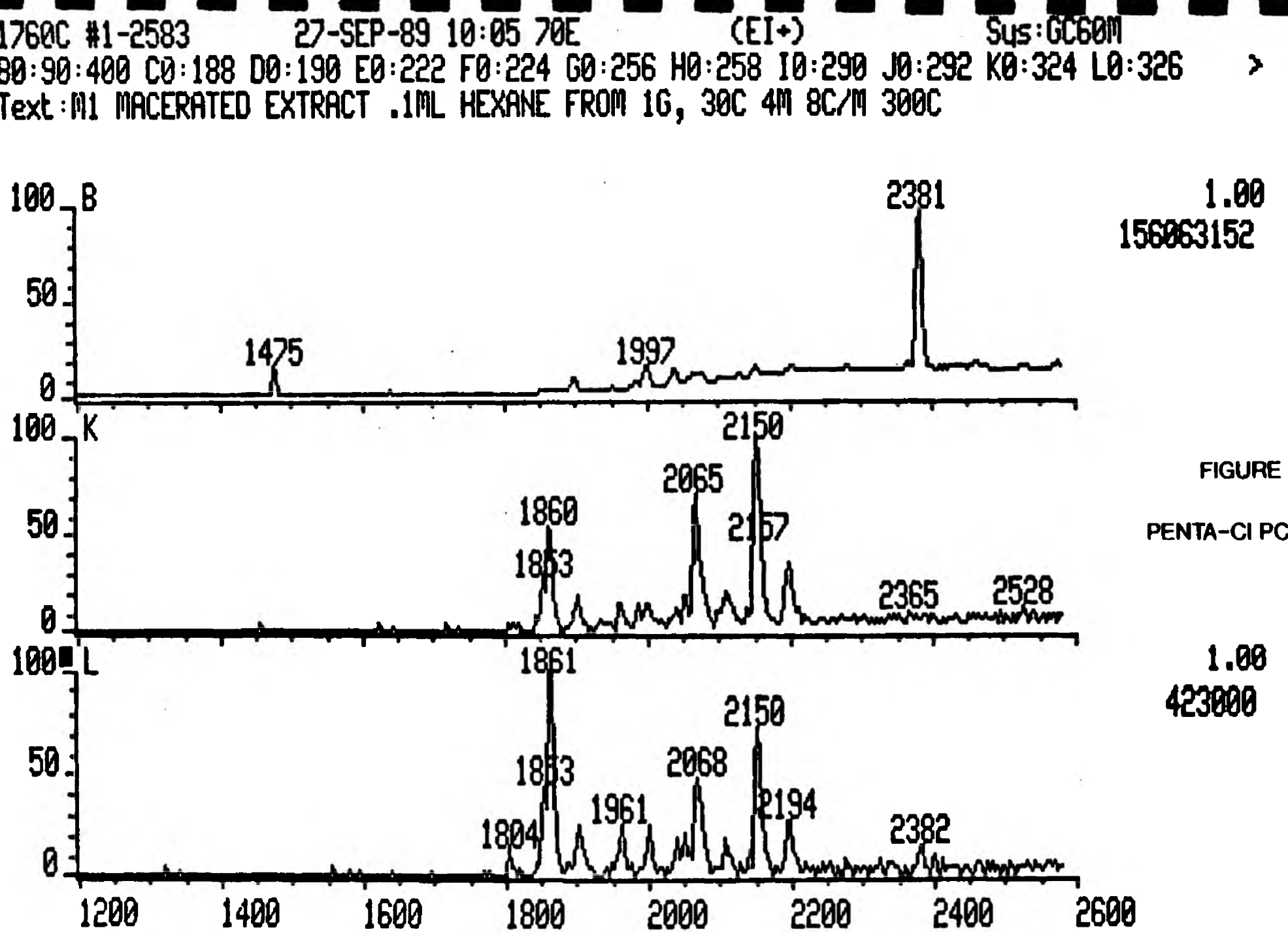


FIGURE 6

PENTA-Cl PCBs

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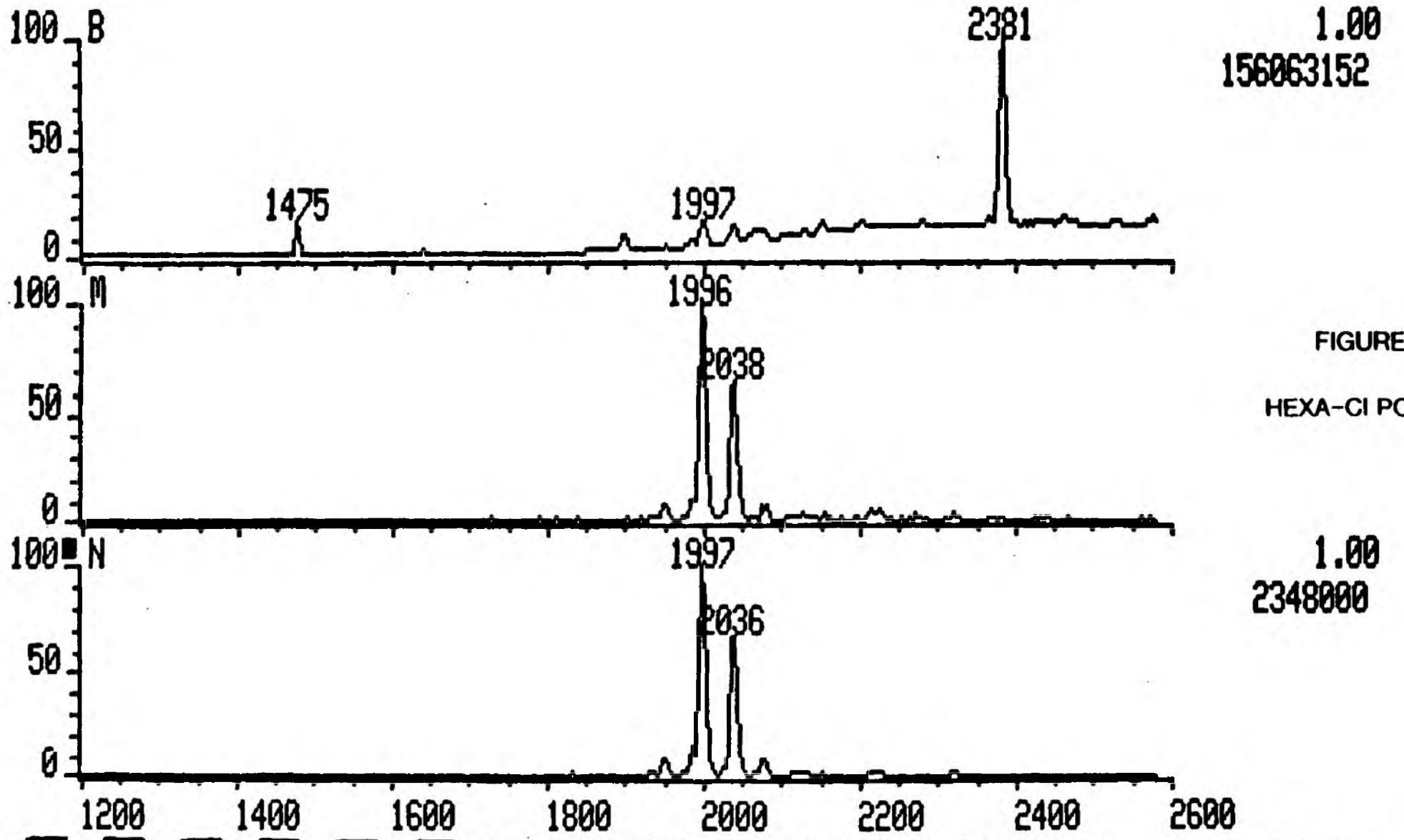



FIGURE 7

HEXA-Cl PCBs

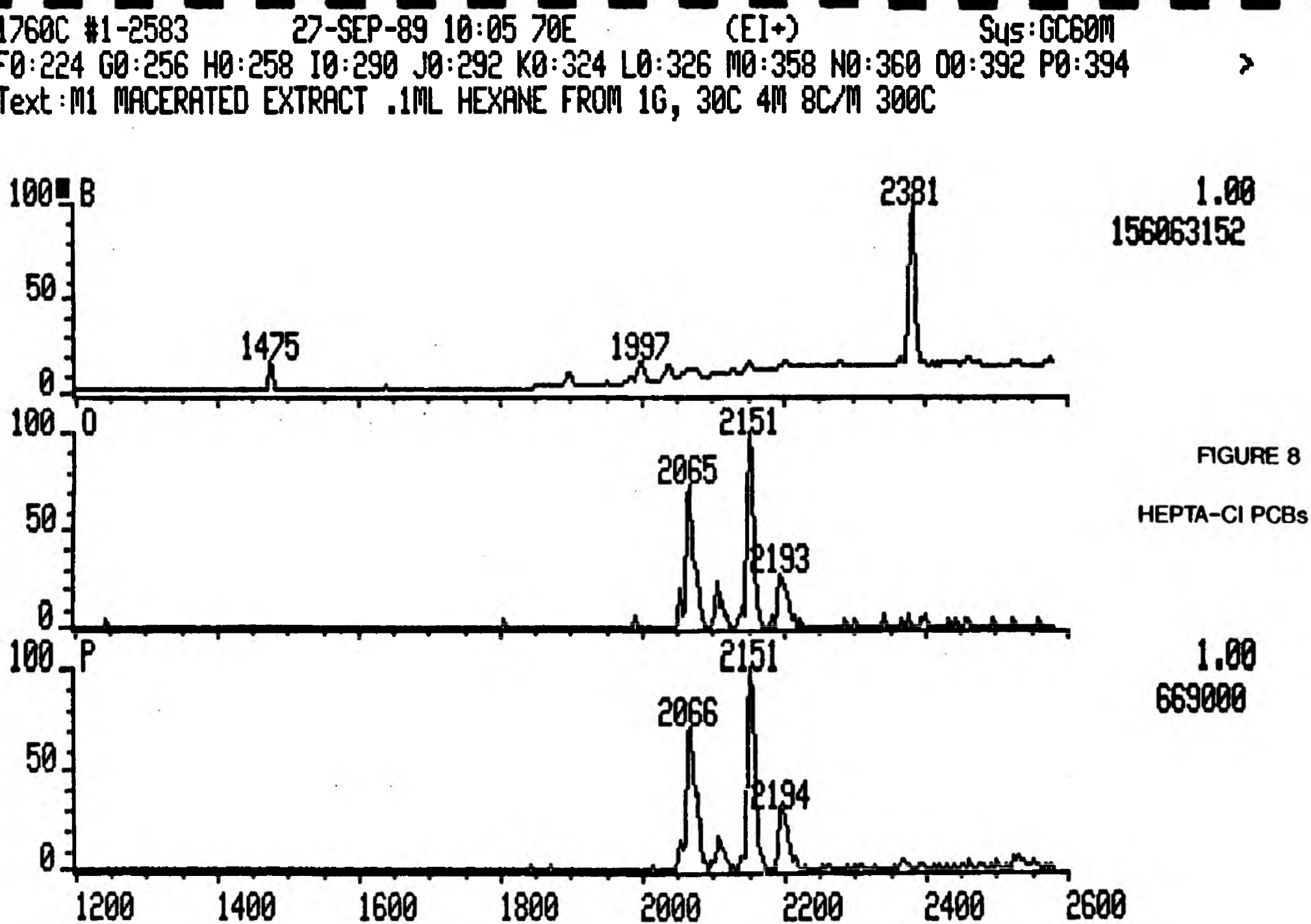



FIGURE 8

HEPTA-CI PCBs

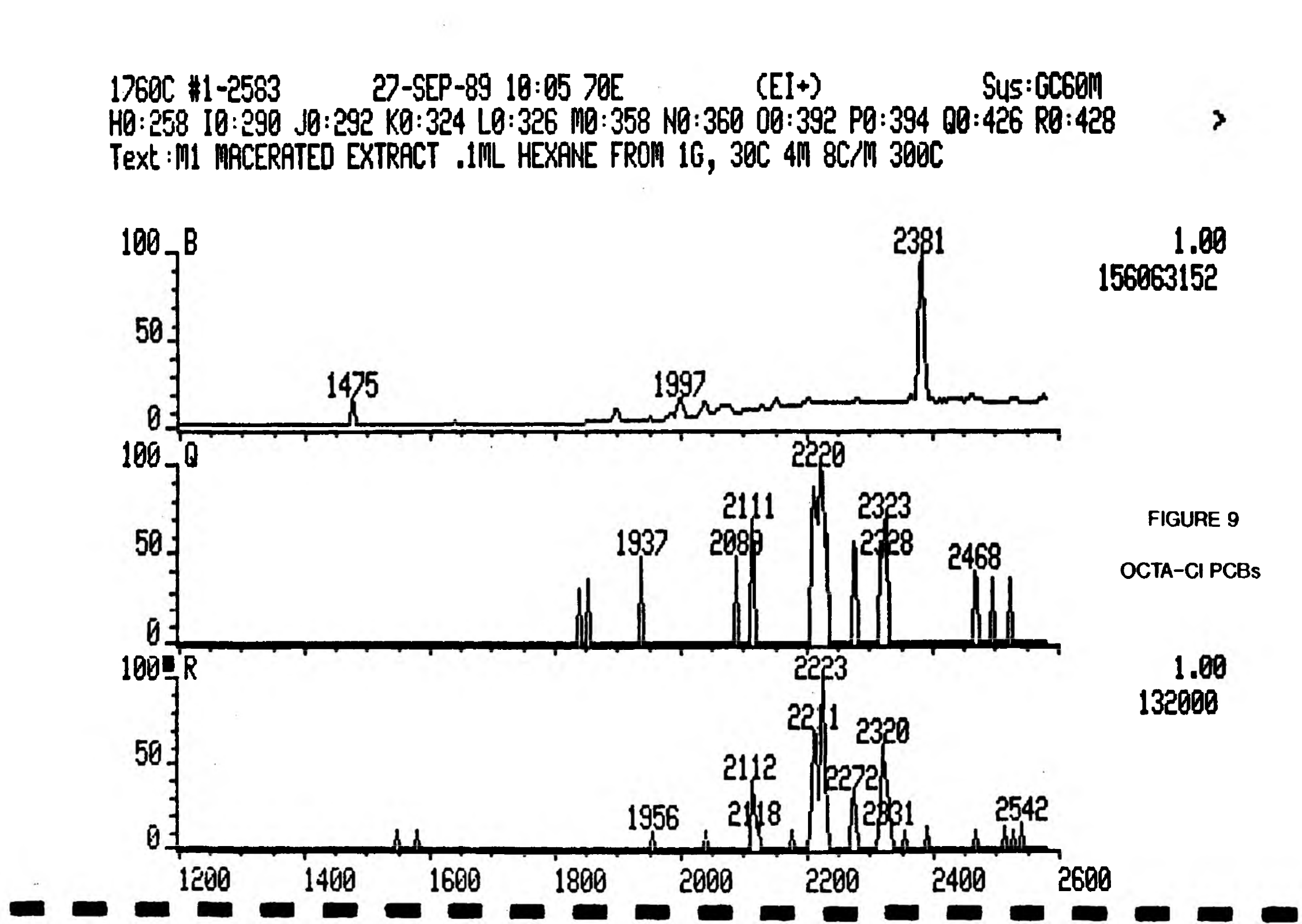
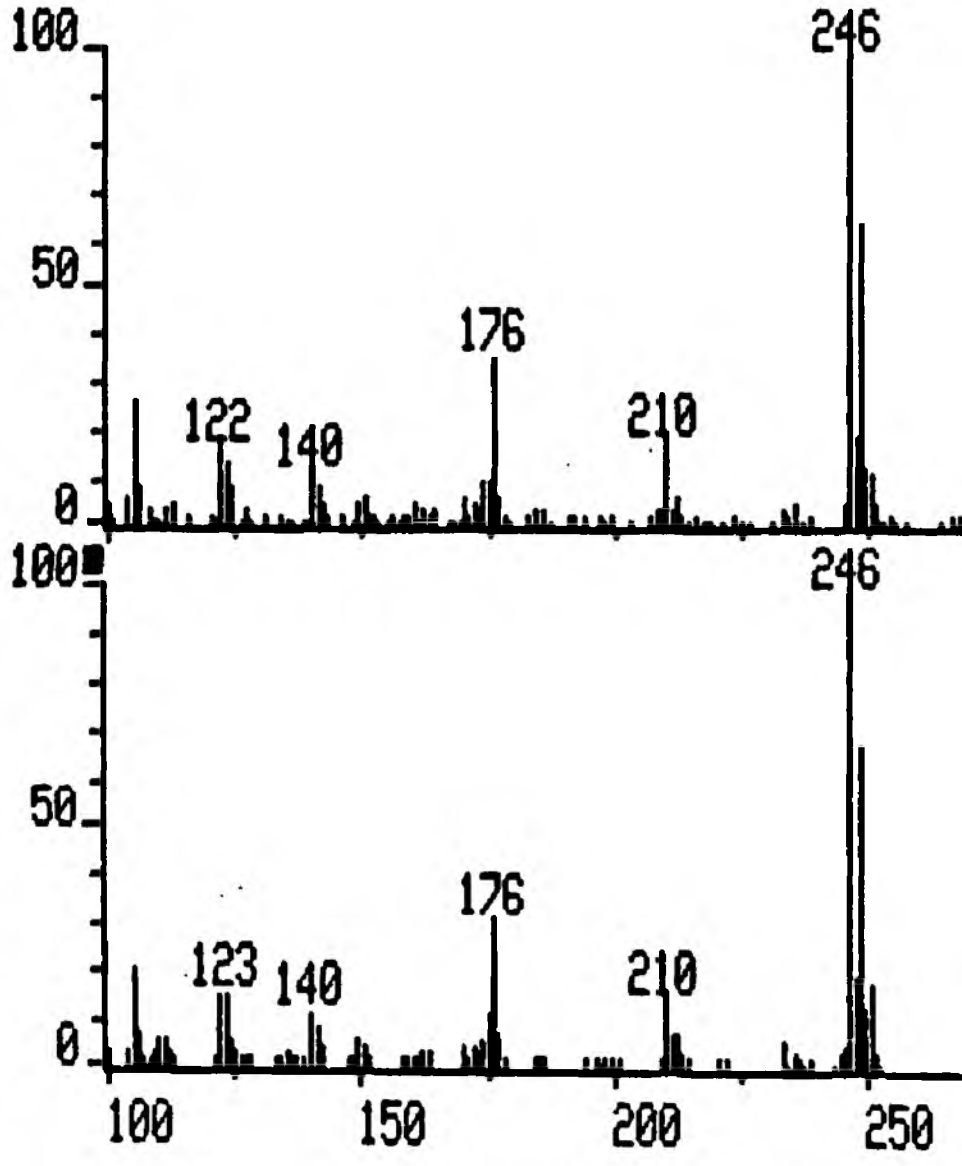



FIGURE 9

OCTA-Cl PCBs

P,P'-DDE
C14.H8.CL4.

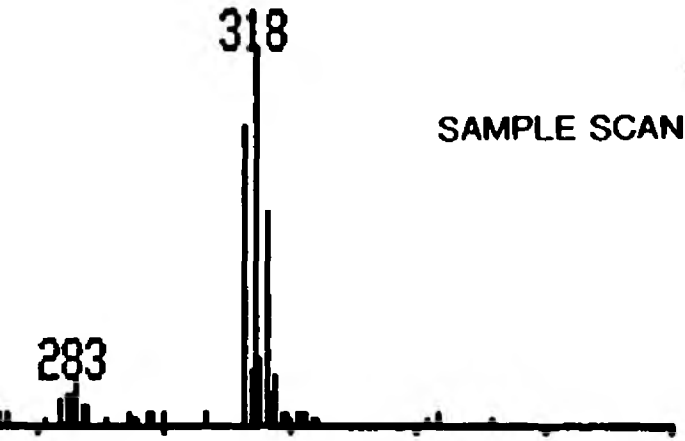


p891 m954 R914 RFN:72-55-9

Lib:NBS 30693 Bpk: 246 Mwt: 316

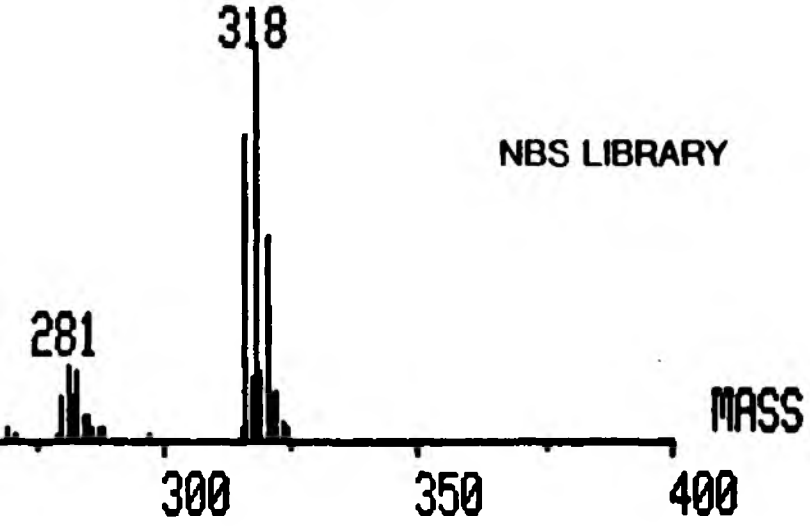
#1 1.0

1371000

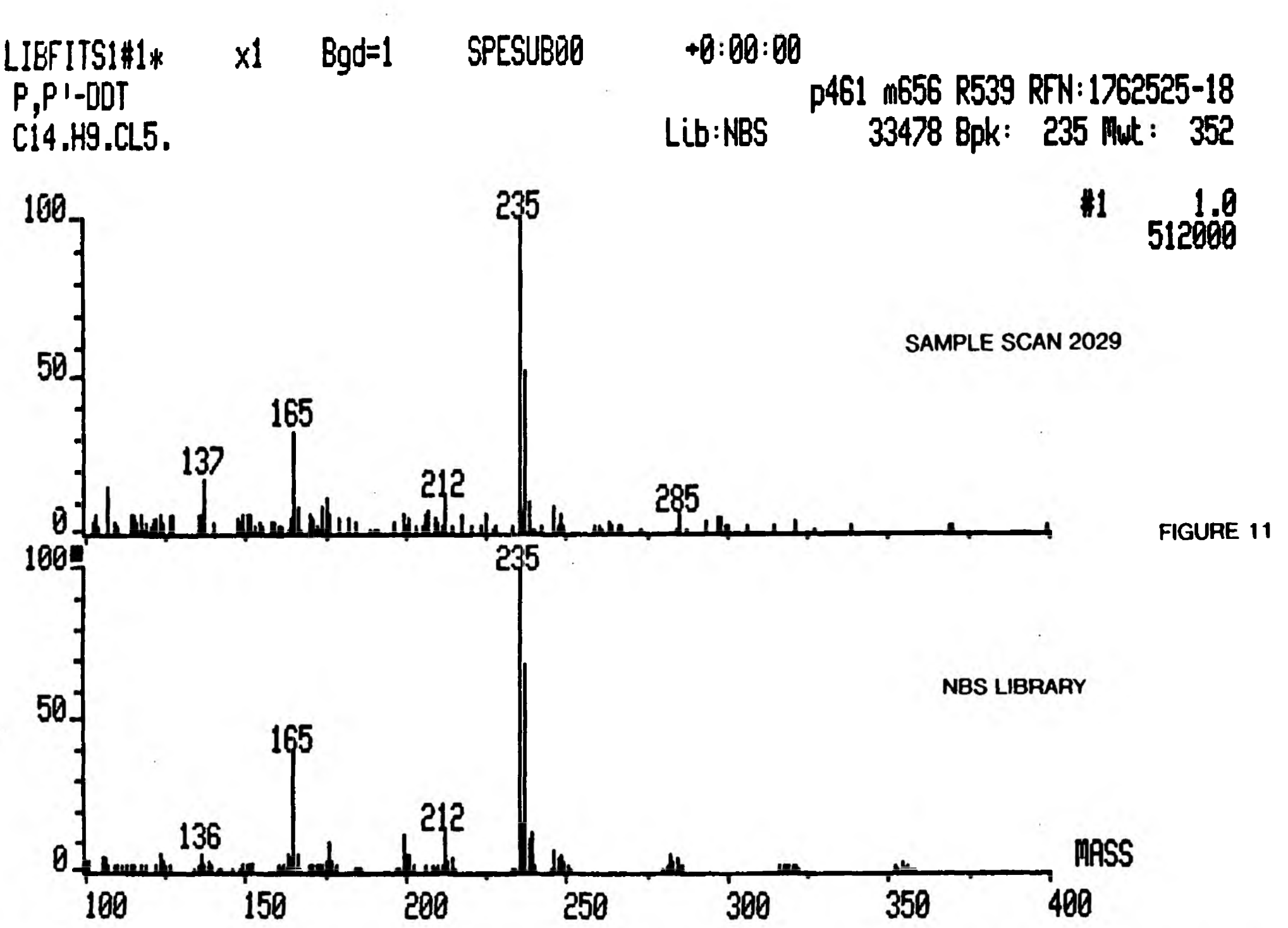



SAMPLE SCAN 1896

FIGURE 10




NBS LIBRARY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Bgd=1
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P,P'-DDT
C14.H9.CL5.
p461 m656 R539 RFN:1762525-18
Lib:NBS
33478 Bpk: 235 Mwt: 352
#1
1.0
512000
SAMPLE SCAN 2029
NBS LIBRARY
MASS
100
50
0
235
165
137
212
285
136
100
150
200
250
300
350
400

FIGURE 11