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083 Microbial Degradation of Hydrocarbon Mixtures in a Marine Sediment Under Different Temperature Regimes

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MICROBIAL DEGRADATION OF HYDROCARBON MIXTURES IN A MARINE SEDIMENT UNDER DIFFERENT TEMPERATURE REGIMES

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SUMMARY

Spills of petroleum hydrocarbons in the marine environment are a major concern because of their serious effects on marine life and on beaches. In Canada, most of the offshore oil and gas exploration takes place in the Arctic or in areas where the water temperatures are low during most of the year. Although hydrocarbons are degraded by microorganisms, the consequences of an oil spill may be particularly severe under low temperature conditions because biodegradation of the hydrocarbons is slowed.

To understand better the rate of biodegradation of various petroleum hydrocarbons in the marine environment we undertook a laboratory study in which we measured over 11 months the rate of degradation of three hydrocarbon mixtures under simulated conditions of a marine sediment at temperatures of 15°, 10°, and 5°C. The aim of this study was to predict from the results rates of degradation that may be expected at temperatures below 5°C. The three hydrocarbon mixtures used were a crude oil, a Bunker C oil, and a condensate. Low concentrations (1-3%) of the three hydrocarbons were mixed with sand and these samples were incorporated into sediment in which a flow of sea-water was maintained comparable to that of interstitial water in a sublittoral sediment influenced by moderate waves.

Total counts of aerobic bacteria in the hydrocarbon-containing sediment remained high throughout. Changes in the three hydrocarbon mixtures resulting from biodegradation were determined using solvent extraction and analysis by capillary gas chromatography. Under the experimental conditions chosen there was a significant leaching of the

more-volatile water-soluble hydrocarbons from the crude oil and particularly from the condensate. Daily degradation rates attributable to biological action were low and were estimated at 15°, 10°, and 5°C for the crude oil to be 40, 18, and 13. mg/m^2, respectively. For Bunker C the corresponding daily rates were 12, <2, and <2 mg/m^2, respectively, whereas for the condensate the estimated daily rate at 15°C was <5 mg/m^2 and considerably less at the lower temperatures. From these biodegradation rates the Q_{10} value (change in rate per 10°C change in temperature) for crude oil was estimated to be 3.1 which is in excellent agreement with other published data. Thus, theoretical, daily biodegradation rates for crude oil at 0° and -5°C are estimated to be 8.4 and 4.2 mg/m^2, respectively. In reality these rates are likely to be even lower.

RÉSUMÉ

Les déversements d'hydrocarbures en milieu marin constituent un grave problème vu les dangers qu'ils représentent pour les espèces marines et les plages. Au Canada, la prospection extracôtière de pétrole et de gaz se fait le plus souvent dans l'Arctique ou dans les régions où les températures de l'eau sont basses presque à longueur d'année. Bien que les micro-organismes puissent décomposer les hydrocarbures, les conséquences d'un déversement de pétrole peuvent être d'autant plus sérieuses dans des températures peu élevées puisque la biodégradation est ralentie.

Nous avons procédé à une étude en laboratoire afin de mieux constater le taux de décomposition des différents hydrocarbures en milieu marin. Pendant onze mois, nous avons mesuré le taux de décomposition de trois mélanges d'hydrocarbures (pétrole brut, soute de type C et condensats) en reconstituant les conditions des sédiments marins à des températures de 15° 10° et 5° C. L'étude avait pour but d'extrapoler, d'après les résultats, les taux de décomposition à des températures inférieures à 5° C. De faibles proportions (1-3%) des trois hydrocarbures ont d'abord été mélangées à du sable. Ces échantillons ont ensuite été incorporés dans des sédiments qu'on a soumis à un courant continu d'eau salée, comparable au courant caractérisé par des vagues modérées d'une eau interstitielle dans un sédiment sublittoral.

Le total des bactéries aérobies dans les sédiments renfermant des hydrocarbures est demeuré élevé tout au cours de l'expérience. Les changements attribuables à la biodégradation des trois mélanges d'hydrocarbures ont été identifiés au moyen de l'extraction par solvant et de l'analyse par chromatographie capillaire en phase gazeuse. Dans les conditions expérimentales, on a remarqué une plus grande lixiviation des hydrocarbures solubles à l'eau, donc plus volatils, dans le pétrole brut et surtout dans les condensats. Les vitesses quotidiennes de décomposition par action biologique étaient faibles. À 15°, 10° et 5° C, le pétrole brut s'est décomposé à un taux de 40, 18 et 13 mg/m^2 respectivement. Les soutes de type C se sont décomposées à des taux quotidiens de 12, < 2 et < 2 mg/m^2 respectivement. Enfin, à 15° C, les condensats se sont décomposés à un taux de < 5 mg/m , et beaucoup moins rapidement aux températures plus froides. La valeur Q_{10} (coefficient de multiplication pour chaque changement de température de 10 ° C) de ces taux de biodégradation a été estimée à 3,1 pour le pétrole brut, ce qui correspond de près aux données scientifiques déjà publiées. Ainsi, les taux quotidiens de biodégradation du pétrole brut sont théoriquement de 8,4 mg/m^2 à 0° C et de 4,2 mg/m^2 à -5° C. Les taux réels sont probablement inférieurs à ces chiffres.

INTRODUCTION

Petroleum hydrocarbons enter the marine environment from natural sources, offshore oil production, marine transportation, the atmosphere, wastes and runoff, and ocean dumping. Of the total hydrocarbons, estimated at 3.2 million tonnes per year, offshore oil production is estimated to account for 0.04 to 0.07 million tonnes (Steering Committee for the Petroleum in the Marine Environment. 1985). Although offshore oil production as yet accounts for only a small percentage of the total input, increased oil exploration in the North and offshore Newfoundland and Nova Scotia will make this source increasingly important to Canada. Of concern is the fate of petroleum hydrocarbons entering sea-water at temperatures below 15°C; indeed, in the North year-round and for much of the year offshore Atlantic Canada, water temperatures are below 5°C.

Various processes occur that affect the composition of petroleum hydrocarbons entering the marine environment, including physical and chemical processes such as evaporation, dissolution, vertical dispersion, emulsification, sedimentation, and photochemical oxidation. However, of equal importance are biological processes, which include degradation of the hydrocarbons by micro-organisms to intermediate organics and ultimately to carbon dioxide, uptake by larger organisms, and then metabolism, storage, or discharge (Steering Committee for the Petroleum in the Marine Environment. 1985). It has been estimated that biological processes can account for up to half of the total degradation and weathering loss of the petroleum hydrocarbon by these various processes.

Considerable work has been done on the degradation of petroleum hydrocarbons by biological processes (Watkinson 1978; Samson et al. 1980; Haines and Atlas 1982; Atlas 1984, 1985; Payne and McNabb 1984; Walker 1984). It has been shown that micro-organisms, particularly bacteria, are the major cause of biological degradation of hydrocarbons in the marine environment (Zajic and Supplisson 1972; Walker et al. 1976; Van de Linden 1978; Westlake et al. 1978; Steering Committee for the Petroleum in the Marine Environment. 1985). The rates of degradation of different classes of organic compounds in the petroleum mixture vary widely. The biodegradation of n-alkanes is most rapid (except for the most volatile fraction C_5-C_9), followed by simple aromatics, such as benzene, toluene, and the xylenes-isoalkanes, whereas cycloalkanes and condensed aromatics are degraded more slowly.

Some of the other factors influencing the rates of biodegradation of petroleum hydrocarbons are temperature, oxygen concentration, and mineral nutrient concentration. Although some work has been done on the rate of hydrocarbon biodegradation at low temperatures (ZoBell 1969; Mulkins-Philips and Stewart 1974; Atlas 1985; Steering Committee for the Petroleum in the Marine Environment. 1985; Strain 1985), a definite paucity of information still exists on degradation rates below 15°C. If a petroleum hydrocarbon is spilled relatively close to shore, most of it will be washed ashore and will end up partly in the upper intertidal zone and partly incorporated into the intertidal or sublittoral sediments. In those environments the rate of degradation of different petroleum mixtures may differ.

In this study, it was decided to determine the rate of microbial degradation of three types of petroleum hydrocarbon mixtures incorporated into a marine sediment under a range of temperatures. The experimental conditions chosen attempted to simulate conditions that would be found in a part of the sublittoral zone where the sediment is submerged all the time but where constant water movement prevents anaerobic conditions in the sediment.

The objectives of the study were:

- to design an experimental set-up to simulate the natural conditions under which petroleum hydrocarbons are degraded in a marine sediment;
- to conduct the experiments under three different temperature regimes: 5°, 10°, and 15°C;
- to monitor at regular intervals the populations of bacteria in the hydrocarbon-containing sediments as compared to a hydrocarbon-free sediment;
- to measure the overall rate of degradation and chemical change of the hydrocarbons under the experimental conditions; and
- to use the experimental results to predict the rates of degradation of the three petroleum hydrocarbon mixtures studied at temperatures below 5°C.

METHODS

SELECTION OF HYDROCARBONS, WATER AND SEDIMENT

The three petroleum hydrocarbon mixtures chosen for the study were a Venezuelan crude oil, a Venezuelan Bunker C oil, and a condensate sample from the Scotian Shelf off Nova Scotia. The crude oil and the Bunker C samples were obtained from the Esso Petroleum Refinery in Dartmouth, N.S., whereas the condensate was obtained from Mobil Oil Canada Ltd. (Selected physical and chemical data for these three mixtures are given in Appendix I). All three samples were used as received; no attempt was made to sterilize them or to remove any volatile hydrocarbons.

These mixtures were chosen to represent those hydrocarbons which most likely would be input into the marine environment in the event of a spill, either during drilling (crude oil and condensate) or during ocean transport (Bunker C). Their chemical compositions differ somewhat with the crude oil covering a wide hydrocarbon range (C_6-C_{32}), the Bunker C being concentrated at the heavier (higher boiling point) end (C_{11}-C_{32}), and the condensate at the lighter end (C_7-C_{27}).

Batches of 1000 L of sea-water were received weekly from Dalhousie University. This water originates from the Northwest Arm, south of Halifax and is sand-filtered before it enters the Dalhousie Aquatron facilities. Analyses for phosphate and nitrate and a total bacterial count were conducted on each batch of water.

The marine sediment used in the experiment was freshly collected beach sand from the intertidal zone of Lawrencetown Beach, 20 km east of Dartmouth, Nova Scotia. The dry weight of 1 L sand was 1.4 kg and

its water-holding capacity was 40%. The size distribution of the particles in the beach sand was as follows:

0.4% >2 mm

1.1% >0.6 mm

76.2% >177 µm

22.2% >75 µm

0.1% <75 µm.

DESIGN FOR MARINE SEDIMENT REACTOR

The original design called for a sediment reactor in which sea-water was pumped through a series of filtering flasks containing coarse sand and hydrocarbon sandwiched between two layers of uncontaminated sediment. It was planned that 11 of these dual-flask set-ups would be used in series for each of the three petroleum hydrocarbon mixtures at each of the three temperatures. Unfortunately, it was discovered after testing several variations of this design that it was impossible to maintain constant and equal flow rates through the 11 sets of flasks. Thus, a new design was required.

After a number of methods had been tried to simulate marine sediment conditions in the laboratory, the experimental set-up was chosen as shown in Figure 1.

The system used consists of a 20-L polyethylene bucket containing 10 L beach sand. This bucket is placed into a large polyethylene container. A total of 40 L sea-water is added to the system in such a way that the bucket is filled to the top and the remainder is filled into the outside container. Sea-water from the outside container is pumped via an airlift into an elevated container from which it

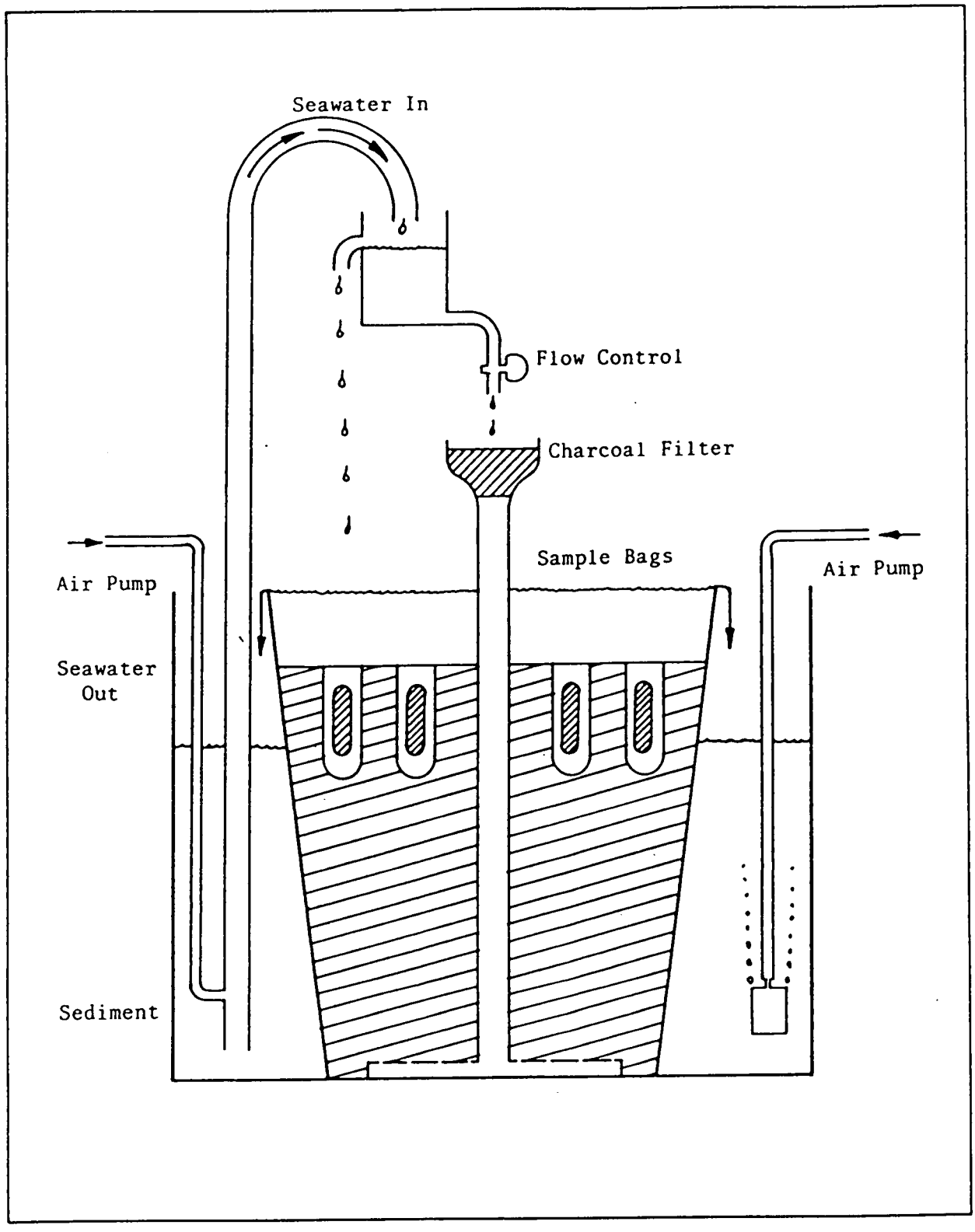

Figure 1. Marine sediment reactor.

overflows back into the outside container. Using the constant head of water in the elevated container, a controlled flow of sea-water of 300 mL/h is directed via a control valve through a charcoal filter into a plastic stand-pipe that is buried in the sediment in the bucket. At the bottom of the bucket the stand-pipe is connected to a perforated circular tube through which the water is distributed evenly and returns upwards through the sand to the top of the bucket where it overflows into the outside container completing the cycle.

In addition to being circulated, 50% of the sea-water in each system is replaced with fresh sea-water daily. At the same time 20 mL of a solution containing 0.5 g/L K_2HPO_4 and 2.0 g/L KNO_3 is added to each system to maintain a nutrient level throughout the experiment of not less than 10 µg atom/L nitrate and 1.5 µg atom/L phosphate. The water in the outside container is kept aerated using an aquarium air stone and an aquarium pump.

The hydrocarbon samples were mixed with beach sand and, with the help of a cardboard tube, were placed inside a clean layer of sand into bags made of fibreglass screen. These bags were buried in the sand of the bucket so that the upper sand surface of the bag was level with the sediment surface in the bucket. In the case of the crude oil and the Bunker C oil the sand core in the bag contained 1% by weight hydrocarbon. In the case of the condensate, the hydrocarbon content was 3% to compensate for anticipated greater loss by leaching. Each bucket held ten bags containing hydrocarbon samples and two control bags without hydrocarbons. Three systems each, one for each of the three hydrocarbons, were placed in controlled-temperature rooms at 5°,

10°, and 15°C, respectively. Bags containing the hydrocarbon samples were removed from the sediment at intervals and the content was analysed chemically and microbiolgically. At weekly intervals, total bacterial counts and measurements of dissolved oxygen were also taken of the fresh sea-water, the recirculating sea-water, and the sea-water in the test buckets.

MICROBIOLOGICAL ANALYSIS

Total bacterial counts were performed using Difco Marine Agar and the spread-plate technique. Dilutions of sea-water and sediment samples were carried out using a diluent containing 0.1% peptone and 2% NaCl. Plates were incubated at room temperature and were counted after 5 days.

CHEMICAL ANALYSIS PROCEDURES

Preparation of Standard Hydrocarbon Mixtures

Samples (∿20 mg) of each of the petroleum hydrocarbon mixtures already described were dissolved in 5 mL of dichloromethane (chromatographic quality) and were stored in a refrigerator at 1° to 2°C. These solutions were used as standards with which to compare the hydrocarbon mixtures extracted from the sediments in the marine sediment reactor.

Analysis of Hydrocarbon Mixtures

Solutions of petroleum hydrocarbons in dichloromethane were analysed on a Hewlett-Packard Model 5830A Gas Chromatograph using a silicone fused-silica capillary column (30 m x 0.25 mm id) to effect the component separation. Detection was by flame-ionization detector and quantitation was by comparing the area counts generated by the

microprocessor to those from hydrocarbon standards analysed in the same way.

Chromatographic conditions used were:

- injector temperature: 250°C
- detector temperature: 275°C
- carrier gas: He at 0.5 cm^3/min
- oven temp.: 35°C for 5 min, then programmed to 260°C @ 8° min^{-1}.

Extraction of Hydrocarbon Mixtures from Sediment Samples

To compare the composition of hydrocarbon mixtures extracted from the sediment in the marine sediment reactor to the standards, it was desirable to maximize the extraction efficiency and to minimize losses during concentration steps. The following procedures were evaluated:

- extraction of the wet oil in sand using methanol-benzene, followed by an aqueous acidic wash and pentane extraction of the aqueous layer;
- extraction with benzene only;
- extraction with dichloromethane only;
- drying the oil in sand mixture in air;
- drying the oil in sand mixture in an oven; and
- freeze-drying the oil in sand mixture.

It was found that the procedure yielding the best recovery of oil with the highest precision is extraction of the wet oil in sand mixture in a Soxhlet with dichloromethane, followed by removal of water using sodium sulphate and concentration to ∿10 mL using a rotary evaporator at room temperature and reduced pressure.

Comparison of Extracted Hydrocarbon Mixtures to Standards

Two separate factors were used to evaluate the extent of biodegradation of the hydrocarbon mixtures; first, loss of volatile hydrocarbons was assessed from the early part of the chromatogram because n-C_{17} and n-C_{18} are known to degrade at a faster rate than the isoprenoids pristane and phytane; second, the size of the UCM (unresolved complex mixture) was measured relative to the resolved hydrocarbons above them in the chromatogram. As degradation proceeds, the n-hydrocarbon peak heights decrease, whereas some of the products of degradation (which normally are not well resolved under these conditions) increase in concentration. The trend is thus a gradual increase in the UCM (the "hump") and a decrease in the size of the n-hydrocarbon peaks from about C_{13} to C_{25}.

OIL DEGRADATION EXPERIMENTS

Trial Run with Crude Oil

An initial experiment using a marine sediment reactor similar to that already described and a sample of crude oil was carried out at 10°C. This trial provided an opportunity to examine the suitability of the reactor set-up and also permitted verification of the reliability of the microbiological and chemical analytical techniques. During this 3-month pilot study, sample bags were pulled at various intervals. Analyses carried out on these samples included total aerobic bacterial counts using marine agar and 20°C incubation, and qualitative hydrocarbon analysis using gas chromatography. Although the air-lift, pump-driven, recirculating system could not be controlled very accurately, the system worked well, and aerobic conditions were maintained in the sediment.

Accelerated Degradation Experiment

Because the bacterial counts and chemical analyses of the preliminary sediment reactor experiment showed no clear evidence of bacterial hydrocarbon degradation after 11 weeks of incubation at 10°C, it was decided that an experiment should be set up with conditions more conducive to hydrocarbon degradation.

The experiment, which was carried out in rotating shake flasks (250 mL) at room temperature, was designed to establish whether hydrocarbon-degrading bacteria were actually being introduced into the system. Other factors examined in this study helped to confirm the design and protocol for the long-term project. The variables used in this set-up include: two types of sediments, two oil concentrations, two levels of nitrogen and phosphorus nutrients, and two levels of bacterial inoculum. For this experiment a bacterial inoculum was cultured starting with a mixture of bacteria from fresh sea-water, beach sand, and sea-water from the long-term experiment set-up after it had been in operation for about two months. This bacterial mixture was grown in a nutrient broth containing 2% NaCl and was used as inoculum.

Total content in each flask was 50 mL. In the shake-flask experiment, 40% by volume of either silica sand or beach sand was used. The beach sand was collected from the intertidal zone of Lawrencetown Beach (20 km east of Dartmouth) and was used immediately after collection. The washed silica sand was obtained commercially. The levels of crude oil used were 1% and 10% (oil:sediment) by weight. Flasks contained either sea-water only or a sea-water-mineral mixture (Table 1). Flasks remained either uninoculated or were inoculated with a culture of mixed bacteria, and the final bacterial concentration was

TABLE 1

Composition of mineral nutrient solution used in the accelerated degradation experiment

Mineral	Quality
K_2HPO_4	0.66 g
KH_2PO_4	0.41 g
$MgCl_2 \cdot 6H_2O$	0.10 g
$FeCl_2 \cdot 4H_2O$	0.05 g
$MnCl_2 \cdot 4H_2O$	0.002 g
$(NH_4)_2SO_4$	1.0 g
Seawater	1 L

adjusted to 10^9 cells/mL. Combinations used in this experiment are shown in Table 2. The contents of the flasks were analysed both microbiologically and chemically after incubation for 2 and 4 weeks. Analysis after 4 weeks showed no significant change.

Long-Term Experiments

Based on the results from the short-term and shake-flask experiments, two changes were made to the marine sediment reactor for the long-term experiments at 5°, 10°, and 15°C with each hydrocarbon mixture. First, freshly collected beach sand was used rather than silica sand, and a modification was made to the apparatus itself. A flow-control system was added to each reactor, and the flow rate through the sand was adjusted to approximately 300 mL/h. At this flow rate, the water in the sand columns containing the hydrocarbon mixtures is replaced about once daily. In addition, about one-half of the 40 L of sea-water contained in each system is replaced with fresh seawater daily.

Incubation of the samples of the three hydrocarbon mixtures at 5°, 10°, and 15°C was begun during December 1985 and January 1986. Each container held 10 bags of sample mixtures and two bags of control samples. Samples were pulled from the reactors at 1 to 2-month intervals and were split for microbiological and chemical analysis. The sample collection schedule for each oil at each temperature is given in Tables 3 to 5.

TABLE 2

Design of accelerated shake-flask degradation experiment

Level of oil:sediment	Crude	Crude + silica	Beach sand + crude	Sterile beach sand + crude	> nutrients + beach sand + crude
			Flask Number		
inoculated 1% crude after 2 weeks	1	3	5	7	33
inoculated 10% crude after 2 weeks	2	4	6	8	34
uninoculated 1% crude after 2 weeks	17	19	21	23	37
uninoculated 10% crude after 2 weeks	18	20	22	24	38

TABLE 3

Sampling schedule for crude oil

Time in system (mo)	Temperature, °C		
	5°	10°	15°
1	✓	✓	✓
2	✓	✓	✓
3			
4	✓	✓	✓
5			
6	✓	✓	✓
7			
8	✓	✓	✓
9			
10	✓	✓	✓

TABLE 4

Sampling schedule for Bunker C

Time in system (mo)	Temperature, °C		
	5°	10°	15°
1	✓	✓	✓
2			✓
3	✓	✓	✓
4			
5	✓	✓	
6			
7		✓	✓
8			
9		✓	✓
10	✓		✓
11		✓	

TABLE 5

Sampling schedule for condensate

Time in system (mo)	Temperature, °C		
	5°	10°	15°
3 days	✓	✓	✓
6 days		✓	
2			✓
3		✓	
4			
5	✓	✓	
6			
7		✓	
8			✓
9	✓	✓	

RESULTS

RECOVERY OF HYDROCARBONS

The maximum recovery of all three hydrocarbons from the marine sediment reactor was obtained by draining excess water from the sample mixtures and then by extracting with dichloromethane, removing the water with anhydrous sodium sulphate, and concentrating the solution to ∿10 mL using a rotary evaporator. An average recovery of 93% by weight was obtained for hydrocarbons with boiling points $>C_{12}$; the precision in peak areas from run to run on the gas chromatograph was ±5%.

Each hydrocarbon mixture gave different recoveries using this procedure. For example, after being mixed with beach sand and immersed in the marine sediment reactor at 15°C for one week, crude oil, Bunker C, and condensate gave average overall recoveries of 35, 72, and 10%, respectively. Testing each stage of the process-water leaching of the more soluble hydrocarbons, efficiency of extraction from the sample mixtures and concentration drying of the solutions gave the results shown in Table 6. These results reflect the different characteristics of the three mixtures. For example, with crude oil, about 30% of the total components that elute from the gas chromatograph are eluted before C_{10} normal hydrocarbon; only 5% of the total components in Bunker C which elute come off before C_{10}; in condensate the value is 45%. Figures 2 to 4 are sample gas chromatograms for each hydrocarbon mixture (the numbers of the chromatograms refer to the location of the n-hydrocarbon, i.e., 18 ≡ C_{18}).

With each hydrocarbon mixture, the major loss before biodegradation could occur resulted from leaching of the more water

TABLE 6

Mechanisms for loss of hydrocarbons in degradation experiments

Loss mechanism	Incremental loss, %		
	Crude oil	Bunker C	Condensate
In reactor 1 week	15	7	14
In reactor 16 h	25	15	35
Extraction from sand	7	4	6
Solvent concentration/drying	18	2	35
TOTAL LOSS	65	28	90
Average recovery	35	72	10

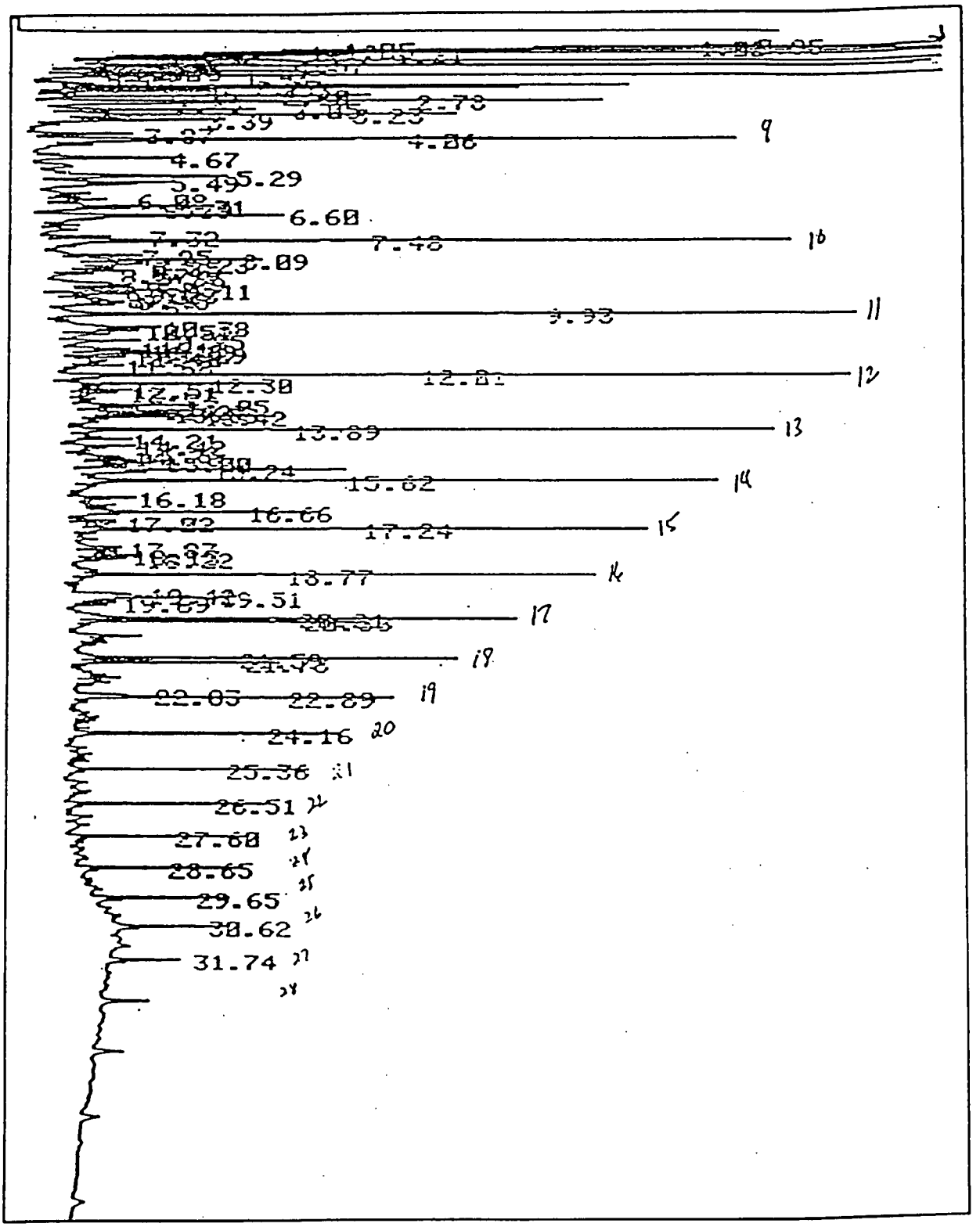

Figure 2. Sample gas chromatogram of crude oil standard.

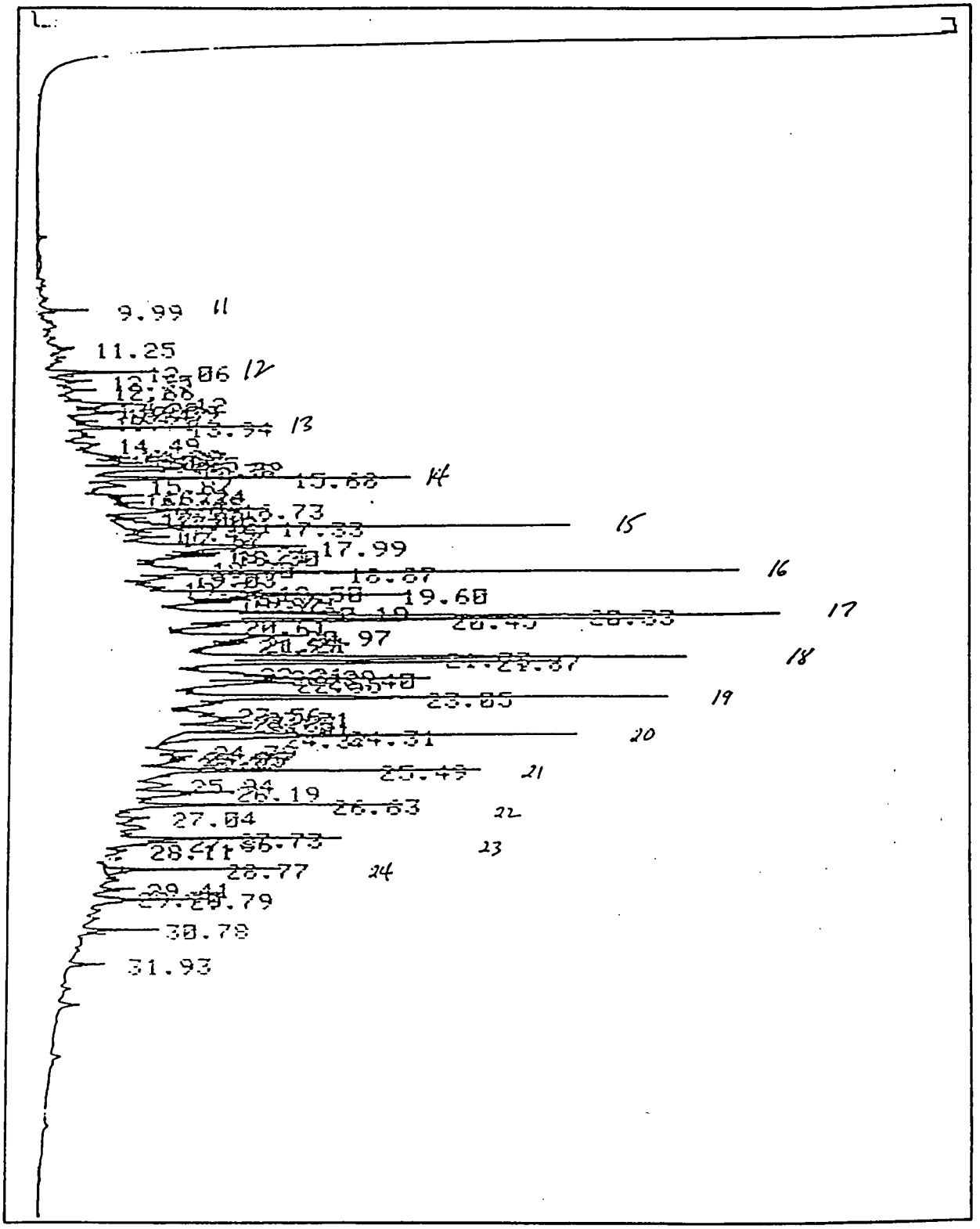

Figure 3. Sample gas chromatogram of Bunker C standard.

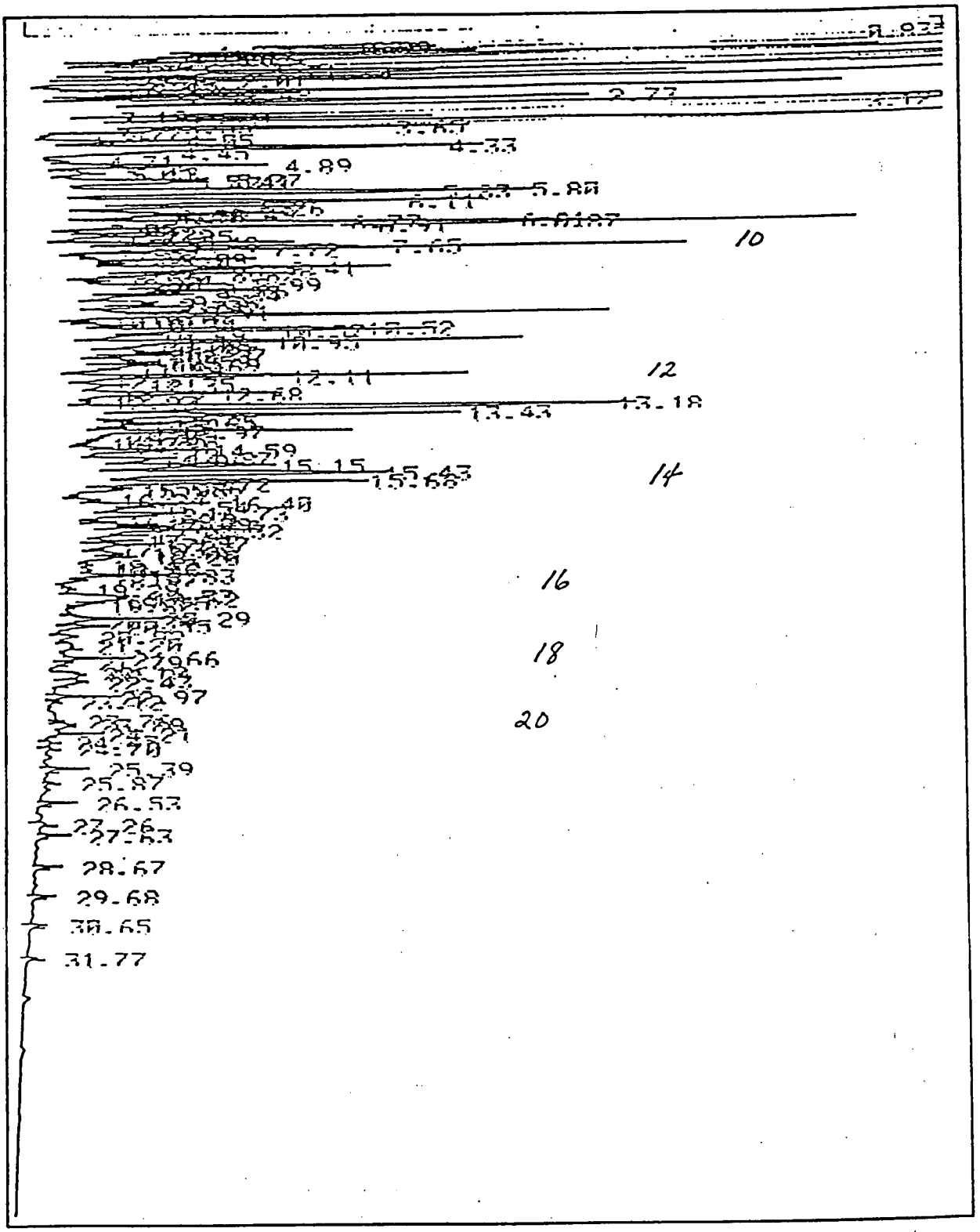

Figure 4. Sample gas chromatogram of condensate standard.

soluble organic compounds (short-chain, aliphatic hydrocarbons and aromatics). This loss varied between 22 and 49% and did not change significantly after the mixtures were in the reactor for 1 week (until biodegradation began to occur). Although only traces of hydrocarbons were found in the sea-water overflowing from the container holding the bags of oil in sediment, extraction of the charcoal filter after the system had been run for 1 week showed peaks in the chromatograms that matched those of the standard hydrocarbon mixtures.

THREE-MONTH TRIAL

Chemical Analysis

Five samples (including two sets of duplicates) of crude oil in silica sand, 1% by weight, were removed from the test system at 2, 5, and 11 weeks to check for biodegradation of the oil. Analysis of the extracted oil by capilliary gas chromatography showed that, except for the loss of volatiles ($<C_{10}$) discussed previously, there was little indication of biodegradation. The results are shown in Table 7.

The ratios of C_{17}/pristane and C_{18}/phytane, and those of C_{18}-C_{21}/UCM from the samples were compared to those of fresh crude oil. Although there appears to be a decrease in the C_{17}:Pr ratio from that in fresh crude oil in the samples, the change is probably not significant. The other two ratios are not indicative of any significant change in the oil, except for sample number 5, which was analysed after 11 weeks in the test system. Sample number 5 showed a decrease in the unresolved organic component relative to the n-hydrocarbons. These results were somewhat surprising, in that a measurable change in the parameters indicative of biodegradation was

TABLE 7

Extent of biodegradation in trial run

Sample	C_{17}/Pr	C_{18}/Ph	C_{18}/UCM*	C_{21}/UCM*
Fresh crude oil	1.43	1.33	3.8	3.0
Sample No. 1 **	1.33	1.47	4.0	3.5
Sample No. 2	1.18	1.43	-	-
Sample No. 3 **	1.33	1.33	3.1	2.9
Sample No. 4	1.33	1.33	3.8	2.9
Sample No. 5	1.35	1.39	5.5	3.9

UCM = Unresolved complex mixture ("hump").
** Duplicates.

anticipated, at least after 11 weeks at 10°C.

Microbiological Analysis

Total bacterial counts were carried out on the sea-water, the silica sand, and the crude oil. The results are listed in Table 8. Samples taken at intervals were also tested for total bacterial numbers, and, as shown in Table 9, an increase in bacterial counts was noted during the period of the test.

ACCELERATED DEGRADATION EXPERIMENT

Chemical Analysis

Oil from the shake flasks was extracted and analysed by gas chromatography, as described earlier. Results from the analysis after 2 weeks of incubation are given in Table 10. (Copies of the chromatograms are included in Appendix II.) The data presented are the C_{17}:Pristane and C_{18}:Phytane ratios and an estimation of the degradation as evidenced by the increase in the unresolved complex mixture (UCM).

These results indicate that, in all cases, the ratios have changed significantly from those of fresh crude oil. Comparing samples 1 and 3 (see Table 2), the addition of silica sand to the oil accelerates the degradation. A comparision between samples 3 and 5 (see Table 2) shows the effect of changing the substrate from silica sand to beach sand. Note, degradation of the C_{17} and C_{18} n-hydrocarbons is less with beach sand, although a similar loss of heavier hydrocarbons was observed. The use of sterile beach sand had little effect on the ratio of n-hydrocarbons to unresolved organics, but degradation of C_{17} and C_{18} increased with sterile sand. Increasing the level of nutrients with

TABLE 8

Bacterial counts of materials used in the sediment reactor

Materials	Colonies/mL (gm)
Silica sand	4.5×10^4
Crude oil	1.2×10^5
Sea-water (range)	9.6×10^3 - 5.6×10^4

TABLE 9

Plate counts of hydrocarbon sediment samples from reactor

Date	Colonies/gm of sample
2/7	6.8×10^4
24/7	7.9×10^4
3/9	1.1×10^6

TABLE 10

Measurement of oil degradation from shake-flask experiment after two weeks

Sample no.*	C_{17}/Pr	C_{18}/Ph	C_{18}/UCM	C_{21}/UCM
Fresh crude oil	1.43	1.33	3.8	3.0
1	0.91	0.53	1.3	0.58
2	1.0	0.48	0.74	0.60
3	0.12	0.10	0.17	0.11
4	0.50	0.35	0.30	0.16
5	0.59	0.42	0.34	0.13
6	0.70	0.62	0.65	0.50
7	0.20	0.21	0.30	0.11
8	0.80	0.75	0.70	0.45
17	0.59	0.37	0.69	0.33
18	1.2	0.91	1.1	0.92
19	1.5	1.0	2.2	1.3
21	0.83	0.43	0.30	0.18
23	0.20	0.17	0.34	0.12
33	0.77	0.23	0.13	0.10
34	0.45	0.28	0.20	0.16
37	0.23	0.10	0.12	0.10

* Sample nos. refer to those in Table 2.

oil and beach sand (sample no. 33) did not affect the C_{17}:Pr and C_{18}:Ph ratios, but more unresolved organics were present compared to the case in which no extra nutrients added.

The results from the uninoculated samples were a little surprising in that more degradation appeared to occur in the uninoculated samples than in those inoculated in the case either of the oil alone and or of the oil + beach sand with increased nutrients (sample nos. 1-17, and 33-37). However, in the others, somewhat less or similar degradation occurred in the uninoculated samples (sample nos. 3-19, 5-21, and 7-23). Analysis of samples after 4 weeks showed no change.

Microbiological Analysis

Bacterial counts remained high in the inoculated flasks; however, they also increased significantly in the flasks that contained only the microbial populations naturally present (Table 11).

In summary, the shake-flask experiment showed that extensive degradation can occur under appropriate conditions regardless of the type of sediment used and as the result of microbial action. In several of the samples, essentially no normal hydrocarbons remained. Additional inoculation with enriched bacterial cultures did not appear to be necessary for hydrocarbon degradation. We felt, therefore, that the test system, as used in the preliminary experiment, contained sufficient hydrocarbon-degrading bacteria that it could be used for the main long-term experiments at three different temperatures.

LONG-TERM EXPERIMENTS AT THREE TEMPERATURES

Chemical Analysis of Crude Oil Extracts

Samples of crude oil in sediment, which were removed from the marine sediment reactor according to the schedule in Table 3, were

TABLE 11

Accelerated shake-flask degradation experiment - total plate counts

Description of Experiment	Crude	Crude + silica	Beach sand + crude	Sterile beach sand + crude	>nutrients + beach sand + crude
1% crude (day 1)	1.2×10^3	1.0×10^4	7.3×10^4	1.2×10^3	7.3×10^4
10% crude (day 1)	1.2×10^4	2.2×10^4	8.4×10^4	1.2×10^4	8.4×10^4
1% crude after 2 weeks	2.0×10^4	1.3×10^6	1.4×10^9	2.0×10^9	1.2×10^7
10% crude after 2 weeks	2.3×10^4	1.6×10^6	8.0×10^8	1.1×10^9	3.3×10^7
inoculated 1% crude after 2 weeks	1.5×10^9	4.9×10^6	1.4×10^9	2.3×10^9	1.45×10^8
inoculated 10% crude after 2 weeks	6.5×10^4	2.2×10^7	2.1×10^8	1.1×10^9	3.0×10^8

analysed by gas chromatography to determine the extent of biodegradation as already described. The results obtained from samples that were immersed in the reactor for periods from 1 month to 10 months at 5°, 10°, and 15°C are given in Table 12. (Copies of the chromatograms are included in Appendix III.)

The results show that the most significant change in the crude oil removed from the reactor, even after 1 month, is the loss of the more volatile hydrocarbons ($<C_9$). For example in the standard crude oil sample, 21% of the components that elute under the chromatographic conditions elute before C_9 hydrocarbon. However, this percentage was reduced to about 3% after only 1 month in the reactor at 15°C, but remained relatively constant to the end of the experiment. This result reflects early loss of the more water-soluble hydrocarbons by a leaching process and is not the result of biodegradation.

Almost no change occurred in the C_{17}:Pr and C_{18}:Ph ratios until 10 months, but even then primarily at 15°C. Some change was observed in the ratio of the C_{21} peak to the unresolved complex mixture beneath it in the chromatogram; the largest change occurred with samples that were in the system from 6 months to 10 months, during which time the ratio decreased from 3.0 to 2.0. From these results, the extent of crude oil biodegradation is estimated at between 5% and 15% of the original (depending on the temperature), exclusive of the leaching losses of volatile hydrocarbons referred to already. These estimates are given in Table 13.

TABLE 12

Analysis of crude oil extracts from reactor at three temperatures

Time in system (mo)	5°C			10°C			15°C		
	C_{17}/Pr	C_{18}/Ph	C_{21}/UCM	C_{17}/Pr	C_{18}/Ph	C_{21}/UCM	C_{17}/Pr	C_{18}/Ph	C_{21}/UCM
0 (fresh crude oil)	1.3	1.4	3.5	1.3	1.4	3.5	1.3	1.4	3.5
1	1.2	1.4	3.3	1.3	1.4	3.1	1.2	1.5	3.2
2	1.2	1.4	3.3	1.2	1.4	3.2	1.2	1.4	3.0
4	1.2	1.4	2.8	1.3	1.5	3.0	1.3	1.5	2.8
6	1.4	1.6	2.8	1.3	1.5	3.4	1.4	1.5	3.0
8	1.3	1.5	2.9	1.2	1.5	2.9	1.3	1.5	2.6
10	1.4	1.5	3.0	1.3	1.5	3.0	1.1	1.0	2.0

TABLE 13

Estimation of the extent of biodegradation of crude oil in the reactor

Temperature, °C	Extent of loss of crude from biodegradation, %
5	5
10	7
15	15

Chemical Analysis of Bunker C Extracts

Samples of Bunker C in sediment, which were removed from the marine sediment reactor according to the schedule in Table 4, were analysed by gas chromatography to determine the extent of biodegradation. The results obtained from samples that were immersed in the reactor for periods from 1 month to 11 months are given in Table 14. (Copies of the chromatograms are included in Appendix IV.)

Unlike the results with crude oil, little change was observed with respect to loss of volatiles with the Bunker C samples. For example, although the sample contained less than 1% of hydrocarbons eluting before C_{11} after 1 month in the reactor, the standard Bunker C was very similar, with less than 5% of the total eluting before C_{11} hydrocarbon. Even after 11 months in the system little change was observed in the early part of the chromatograms. This result is not unexpected, because Bunker C has most of the volatile (and water-soluble) hydrocarbons stripped off in the refining process.

Examination of the ratios of n-hydrocarbon to isoprenoid and n-hydrocarbon to unresolved complex mixture in the Bunker C extracts showed that little biodegradation had occurred. Virtually no change was observed until 10 months at 15°C. Estimates of the extent of biodegradation of the Bunker C oil range from <1% at 5°C to 5% at 15°C (Table 15).

Chemical Analysis of Condensate Extracts

Samples of condensate in sediment, which were removed from the marine sediment reactor according to the schedule in Table 5, were analysed by gas chromatography to determine the extent of biodegradation. The results obtained from samples that were immersed

TABLE 14

Analysis of Bunker C extracts from reactor at three temperatures

Time in system (mo)	5°C			10°C			15°C		
	C_{17}/Pr	C_{18}/Ph	C_{21}/UCM	C_{17}/Pr	C_{18}/Ph	C_{21}/UCM	C_{17}/Pr	C_{18}/Ph	C_{21}/UCM
0 (fresh Bunker C)	1.6	1.5	2.4	1.6	1.5	2.4	1.6	1.5	2.4
1	1.4	1.5	2.4	1.5	1.6	2.2	1.4	1.5	2.4
2	not analysed			not analysed			1.4	1.5	2.8
3	1.3	1.4	2.7	1.4	1.6	2.8	1.2	1.4	2.8
5	1.3	1.4	2.4	1.4	1.5	2.7	1.3	1.4	2.7
7	not analysed			1.3	1.3	2.5	1.3	1.4	2.3
9	not analysed			1.3	1.6	2.4	1.3	1.4	2.4
10	1.5	1.5	2.7	not analysed			1.1	0.90	1.8
11	not analysed			1.5	1.6	2.8			

TABLE 15

Estimation of the extent of biodegradation of Bunker C in the reactor

Temperature, °C	Extent of loss of Bunker C from biodegradation, %
5	<1
10	<1
15	5

in the reactor for periods from 1 month to 9 months are given in Table 16. (Copies of the chromatograms are included in Appendix V.)

From the results, it appears that little biodegradation occurred in the condensate at any of the temperatures. Almost no change was detectable in the chromatograms over the test period aside from the loss of the more volatile (more water-soluble) hydrocarbons with time. We estimate that <1% biodegradation has occurred even at 15°C after 9 months (Table 17). Thus, the major effect in a spill of condensate is going to be weathering and leaching of the more water soluble hydrocarbons.

Microbiological Analyses

Table 18 shows a slight increase in bacterial numbers in the hydrocarbon containing sediment during the first month of the experiment. In most cases the bacterial populations decreased slowly thereafter and were at the level of the control sediment samples at the end of the 11 months. Exceptions were the sediments containing condensate at 10° and 15°C in which the bacterial numbers decreased slightly below the control values.

TABLE 16

Analysis of condensate extracts from reactor at three temperatures

Time in system	Temp., °C	% Eluted (GC) in the hydrocarbon range			
		$<C_{10}$	C_{10}-C_{14}	C_{14}-C_{17}	$>C_{17}$
Standard	-	50	26	19	5
3 days	5	43	30	21	6
3 days	15	44	32	18	6
6 days	10	41	34	20	5
6 days	15	43	33	19	5
3 weeks	15	40	35	20	5
2 months	15	36	38	21	5
3 months	15	26	44	26	4
5 months	5	28	40	22	10
5 months	10	41	35	19	5
7 months	10	32	38	18	12
8 months	15	28	39	22	11
9 months	5	34	39	19	8
9 months	10	34	38	19	9

TABLE 17

Estimation of the extent of biodegradation of condensate in the reactor

Temperature, °C	Extent of loss of condensate from biodegradation, %
5	<<1
10	<1
15	<1

TABLE 18

Microbiological analysis of sediment samples containing various hydrocarbons total plate counts at three temperatures (colonies/gm)

	5°C				10°C				15°C			
Mos	Crude	Bunker	Con-densate	Sediment only	Crude	Bunker	Con-densate	Sediment only	Crude	Bunker	Con-densate	Sediment only
0				$3.6x10^5$				$3.6x10^5$				$3.6x10^5$
1	$1.2x10^6$	$1.0x10^6$	$3.9x10^6$		$1.1x10^6$		$1.9x10^6$		$8.4x10^5$	$2.6x10^5$	$4.5x10^6$	
2						$3.9x10^5$	$4.2x10^6$		$4.3x10^5$	$5.1x10^5$	$4.9x10^5$	
3		$9.5x10^6$			$6.2x10^5$	$1.8x10^5$				$4.5x10^5$		
4	$1.1x10^6$					$6.4x10^5$	$1.0x10^6$		$1.1x10^5$		$1.5x10^5$	
5		$1.5x10^6$	$4.6x10^5$			$2.7x10^5$	$1.1x10^5$			$3.0x10^5$		
6	$1.4x10^6$	$5.4x10^5$			$5.1x10^5$				$2.6x10^5$		$4.7x10^4$	
7			$5.5x10^6$			$4.5x10^5$	$4.8x10^4$			$2.4x10^5$		
8	$9.9x10^5$	$3.0x10^5$			$2.2x10^5$				$2.0x10^5$		$2.4x10^4$	
9			$1.8x10^5$			$2.3x10^5$	$2.1x10^4$			$3.9x10^5$		
10					$1.7x10^5$				$5.1x10^4$		$7.8x10^4$	
11	$7.4x10^5$	$2.1x10^5$						$1.7x10^5$				$5.3x10^5$

DISCUSSION

DISCUSSION OF OBJECTIVES

Experimental Set-Up

In our experiment we attempted to simulate the natural conditions of a marine sediment in the sublittoral zone of a sandy beach. However, only one of many possible conditions could be simulated in our study. Riedl (1971) estimated that the interstitial flow in the intertidal zone of a high-energy beach is about 0.5 mm/s whereas the flow in a lentic low-energy, but still aerobic, beach can be as much as three orders of magnitude lower. In our experiment the interstitial flow was approximately 0.02 mm/s. Therefore, the conditions in our experiment would be comparable to a medium-energy beach or to sediment conditions some distance from shore, yet still under considerable influence of wave action. Under such conditions, the environment in the sediment would be still aerobic and would allow the growth of aerobic bacteria, which was borne out by our study. Aerobic conditions were maintained in the sediment at all temperatures, and populations of aerobic bacteria remained high both in the sediment and in the hydrocarbon sample throughout the experiment.

Temperatures

The experiments on each hydrocarbon mixture were conducted at three different temperatures (5°, 10°, and 15°C) and were held within 0.5°C of these temperatures throughout the course of the work.

Monitoring Bacterial Levels

During the course of the experiment, bacterial counts were performed of the hydrocarbon-containing sediment samples. Some increase in total bacterial numbers was noted in the samples as compared to the surrounding sediment, probably because of an increased carbon supply in the form of the hydrocarbon samples. McLachlan and Harty (1981) mentioned the possibility of inhibition of the interstitial water flow by the presence of oil in sediments. This would reduce the oxygen level in hydrocarbon-containing sediment and reduce the action of hydrocarbon degraders. In our experiments it was not possible to measure directly oxygen levels within the sediment-hydrocarbon sample. However, the presence of aerobic bacteria throughout the experiment suggests that aerobic conditions were maintained. A drop in bacterial numbers in the samples containing condensate at 10° and 15°C could indicate that the water flow was slightly more inhibited in these samples than in the samples containing the crude or Bunker C oils.

Monitoring Chemical Changes

The method chosen to monitor the changes due to biodegradation in the three hydrocarbon mixtures due to biodegradation (solvent extraction and analysis by capillary gas chromatography) was judged to be adequate. However, because the extent of biodegradation was in most cases very low, the use of gas chromatography-mass spectrometry might have provided a more detailed picture at the temperatures used. The major drawback in measuring the rate of biodegradation was the difficulty in determining the amount of hydrocarbon recovered from the

extractions. This problem arose because the oil in sediment mixtures were partitioned between biological and chemical tests immediately after they were removed from the reactor; it was desirable not to homogenize or dry them. However, use of the ratios of n-hydrocarbon to isoprenoid and n-hydrocarbon to unresolved complex mixture provided a clear indication of biodegradation, particularly with the crude oil.

BIODEGRADATION RATES

The results were used to estimate rates of biodegradation for each of the hydrocarbon mixtures at 5°, 10°, and 15°C. Whereas an estimate can be made at temperatures below 5°C for biodegradation of crude oil under these conditions, the low extent of biodegradation of the Bunker C and condensate did not permit estimates to be made.

Estimates of Rates at Three Temperatures

From the GC data, the loss of hydrocarbons can be used to estimate the rate of biodegradation at each temperature. In the case of crude oil, these daily rates are estimated to be 40 mg/m^2 at 15°C, 18 mg/m^2 at 10°C, and 13 mg/m^2 at 5°C in the type of beach sand used here. These daily rates are somewhat lower than those published by Gibbs and Davis (1976), 260 mg/m^2 at 12°C; and Johnston (1970), 40-90 mg/m^2 at 10°C.

For Bunker C, we estimate the daily biodegradation rates to be 12 mg/m^2 at 15°C and less than 2 mg/m^2 at both 10° and 5°C.

For the condensate, we estimate that the daily biodegradation rate at 15°C is $\leq$5 mg/m^2, whereas that at 10° and 5° is significantly less. As already discussed, the loss of hydrocarbons by leaching is very high initially (nearly 35% loss in 24 h), but levels off to a low rate of leaching after 1 month.

Estimate of Biodegradation Rates Below 5°C

From the biodegradation rates estimated for crude oil at 5° and 10°C, we can determine a Q_{10} value (change in rate per 10°C change in temperature). The Q_{10} value for crude oil is estimated to be 3.1, which is in excellent agreement with that reported by Gibbs and Davis (1976) from 6-26°C. Thus, theoretical daily biodegradation rates at 0° and -5°C are estimated to be 8.4 and 4.2 mg/m^2, respectively. In reality these rates are likely to be even lower.

IMPLICATIONS OF RESULTS

This work has shown that biodegradation of crude oil, Bunker C, and condensate in a simulated marine sediment proceeds slowly, even at 15°C. Although significant leaching of the more volatile, water-soluble hydrocarbons from the crude oil and particularly from the condensate occurred, very little leaching of the Bunker C occurred under these conditions. The low biodegradation rates are in agreement with the few values that exist in the literature and point out the importance of guarding against large-scale spills of petroleum mixtures, particularly of Bunker C and crude oil. A spill of condensate might not have the same effect because of its higher rate of leachability. However, its rate of biodegradation is very low and may be due in part to the higher concentration of volatile hydrocarbons which are reported to be toxic to some types of bacteria.

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APPENDICES

APPENDIX I

Physical and Chemical Properties of the Three Mixtures Used in the Experiments

Physical and Chemical Characteristics of the Crude Oil

Source:	Esso Petroleum Canada, Dartmouth Refinery
Type:	Venezuelan BCF 24
Density:	0.9029 kg/L
BS&W:	0.30%

Physical and Chemical Characteristics of the Bunker C Oil

Source:	Esso Petroleum Canada, Dartmouth Refinery
Type:	300
Density:	0.9828 kg/L
Flash Pt.:	101°C
Viscosity:	539 cSt @ 50°C
Pour Point:	+ 4.4°C
BS&W:	0.2%
Sulfur:	1.47%
V:	264 mg/L

Physical and Chemical Characteristics of the Condensate

Source: Mobil Oil Canada Ltd.

West Venture C-62, Scotian Shelf

Density: 0.800 kg/L

Viscosity: 2 m Pa·s

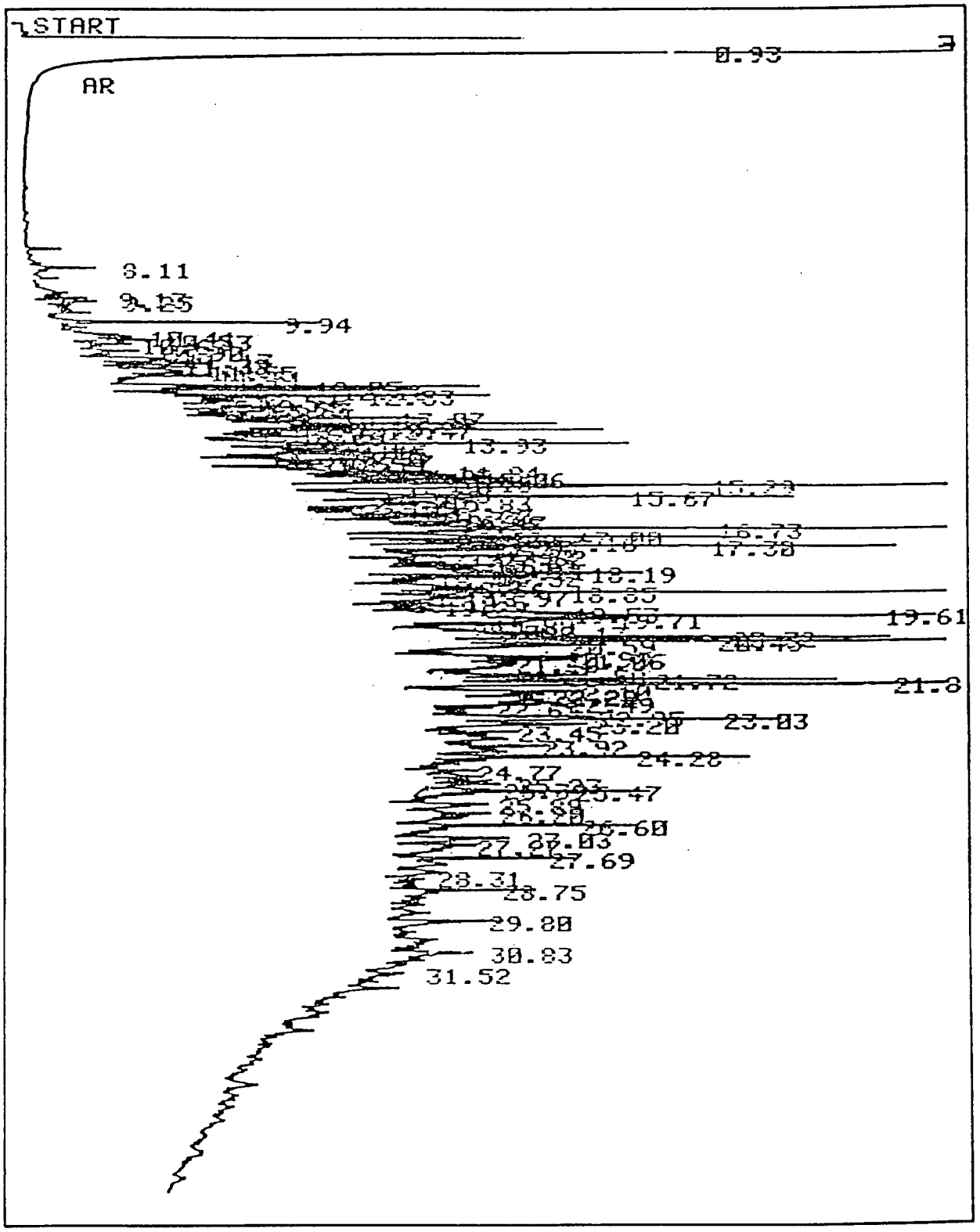
Approximate Component Concentrations

Component	Wt. %	Component	Wt. %
m- & p-xylenes	2.97	C_2-naphthalene	0.47
o-xylene	1.08	C_2-naphthalene	0.39
n-C_9	1.43	n-C_{14}	0.70
1,2,4-TMB	0.96	n-C_{15}	0.56
n-C_{10}	1.12	n-C_{16}	0.45
n-C_{11}	0.92	n-C_{17}	0.36
tetralin	0.31	pristane	0.09
naphthalene	0.40	phenanthrene	0.16
n-C_{12}	0.75	n-C_{18}	0.29
2-CH_3-naphthalene	0.98	phytane	0.03
1-CH_3-naphthalene	0.66	n-C_{19}	0.22
n-C_{13}	0.70	n-C_{20}	0.11
biphenyl	0.18	n-C_{21}	0.17

Source: Strain 1985.

APPENDIX II

Chromatograms of Hydrocarbons Analysed in Accelerated Shake-Flask Experiments



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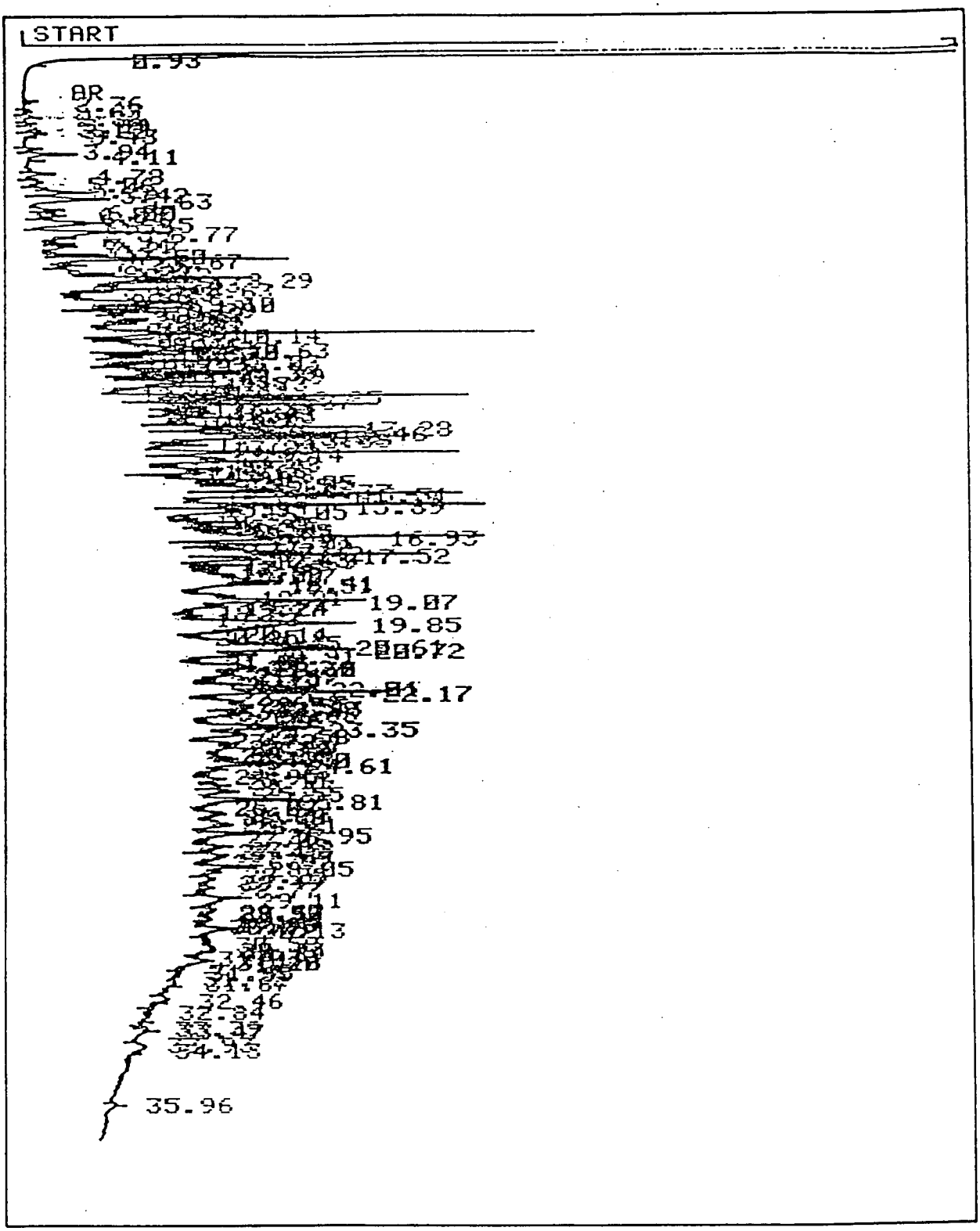

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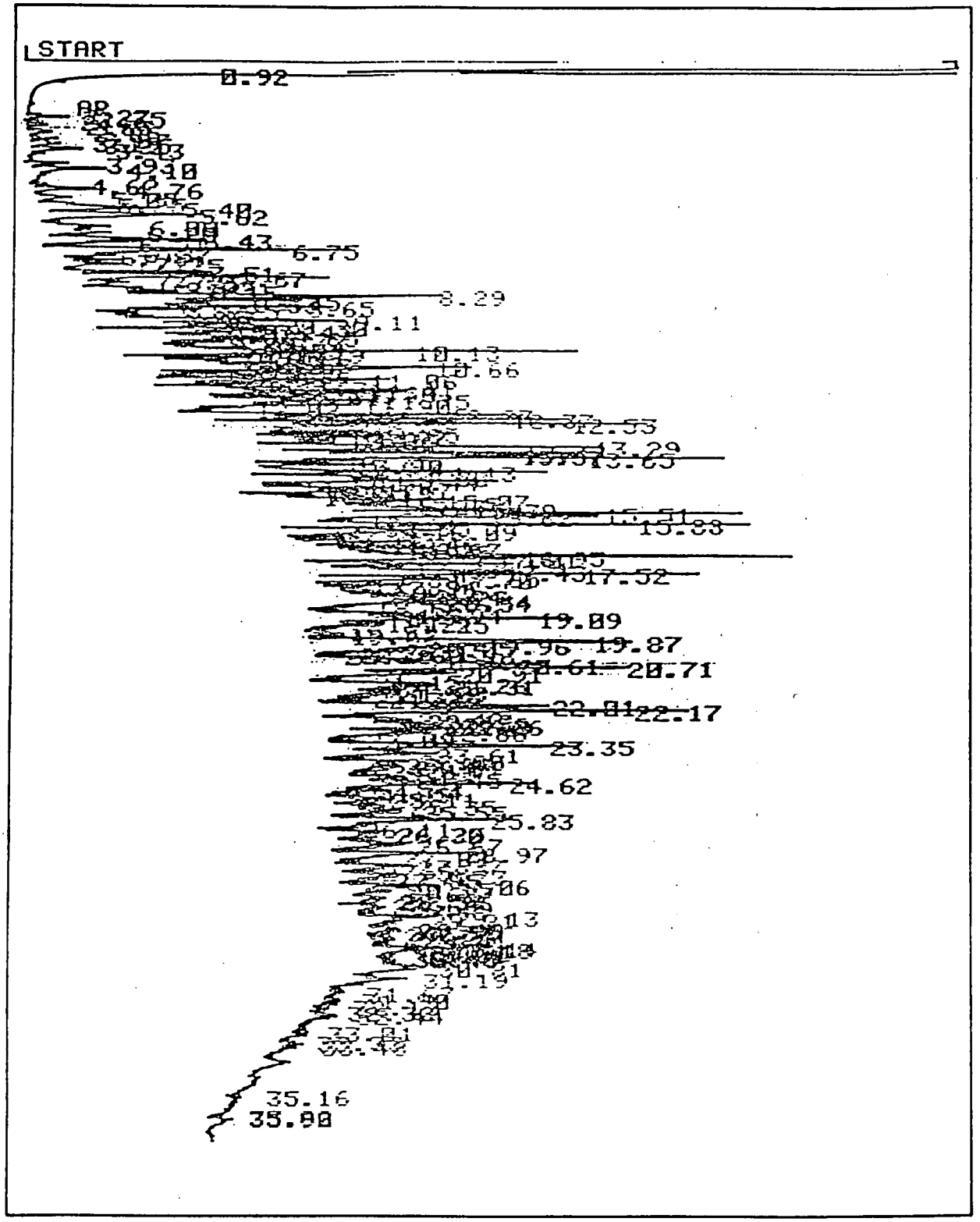

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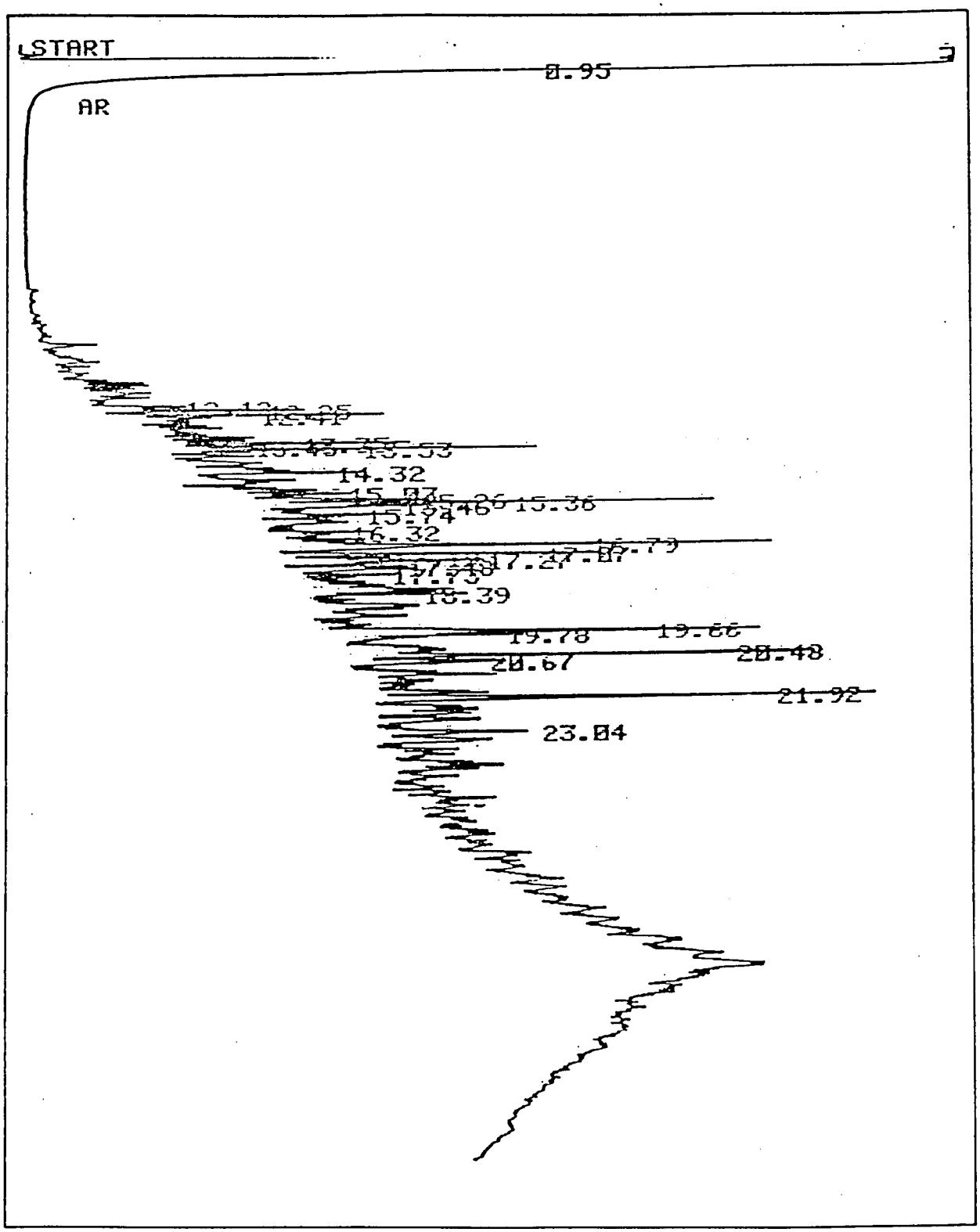
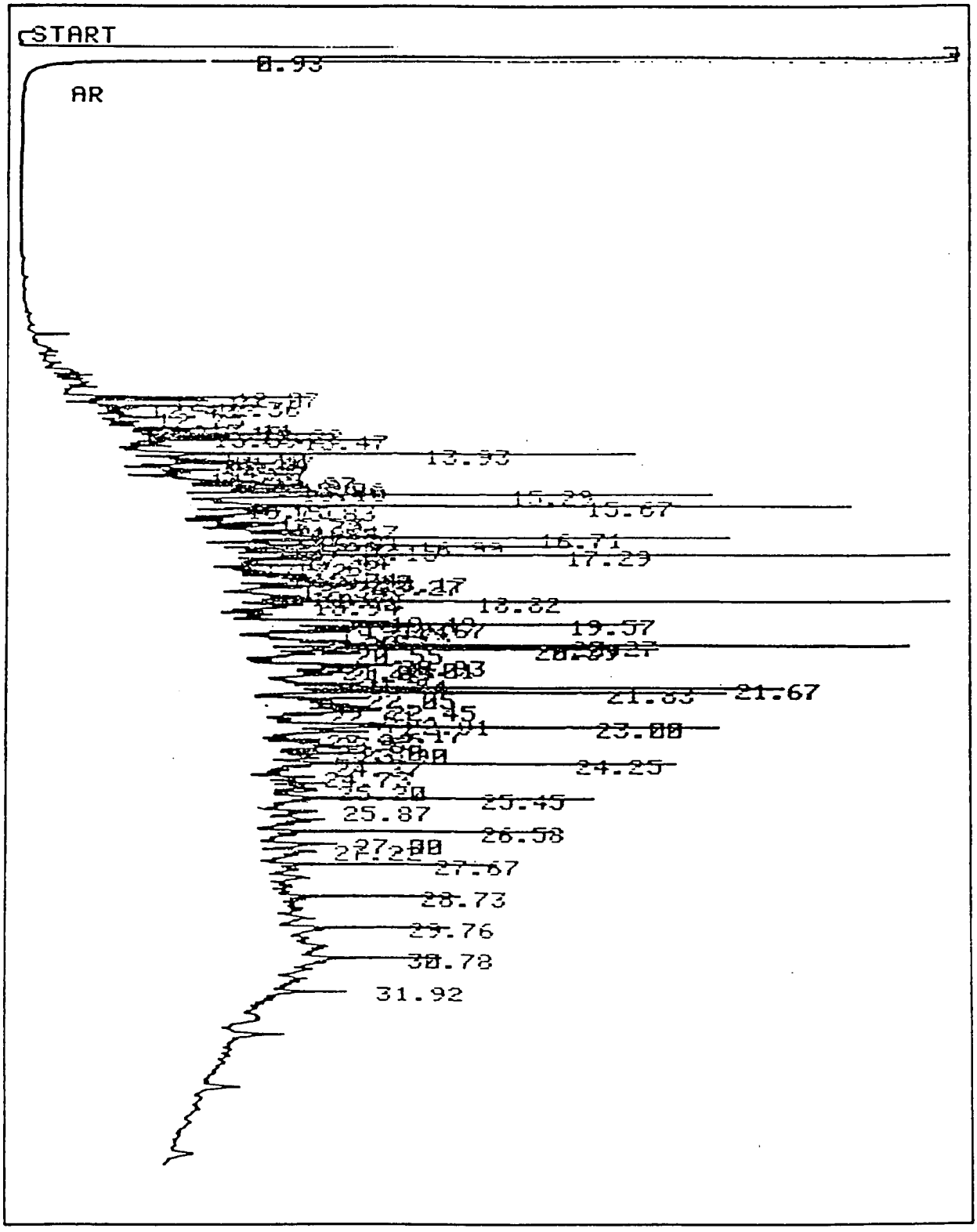
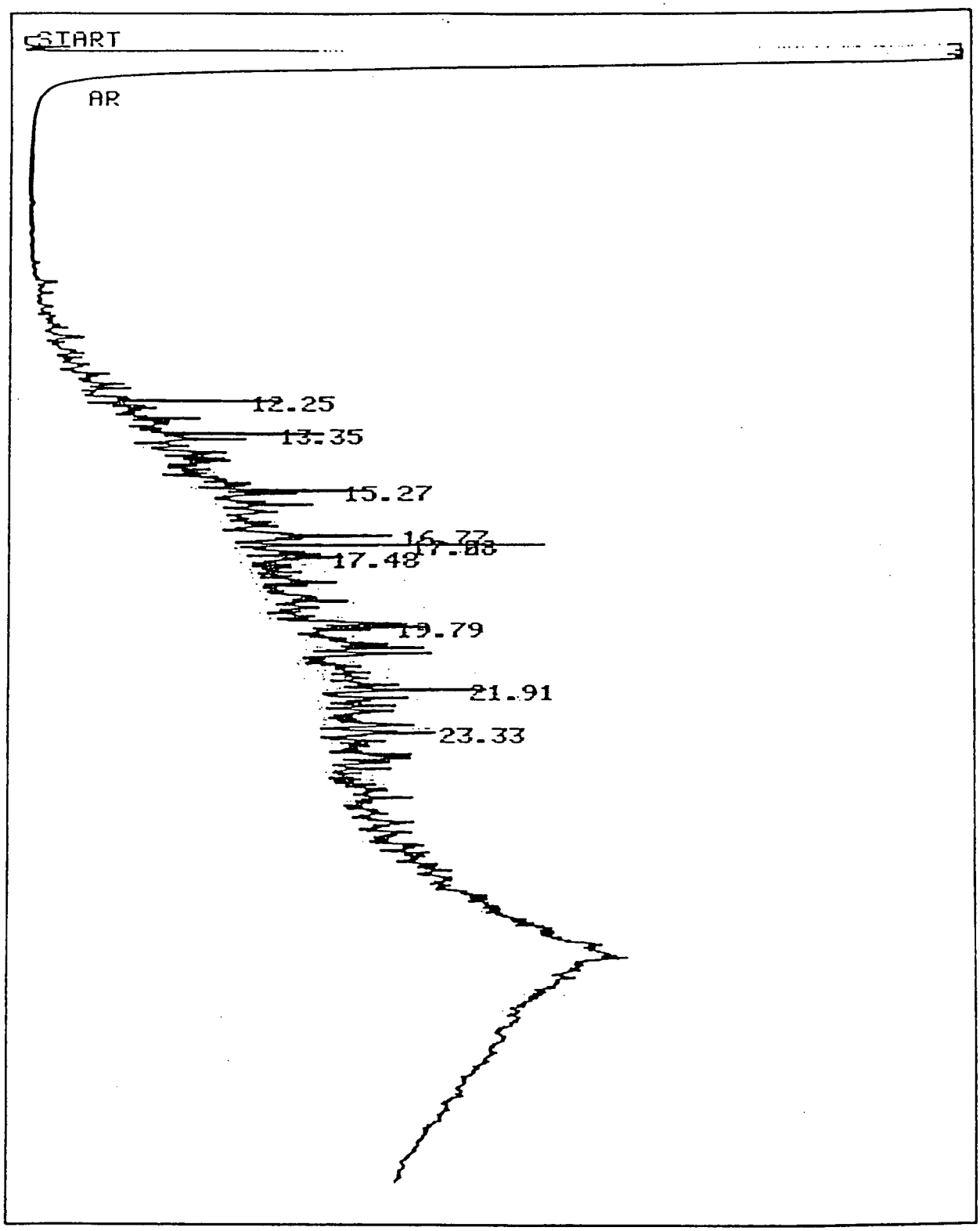


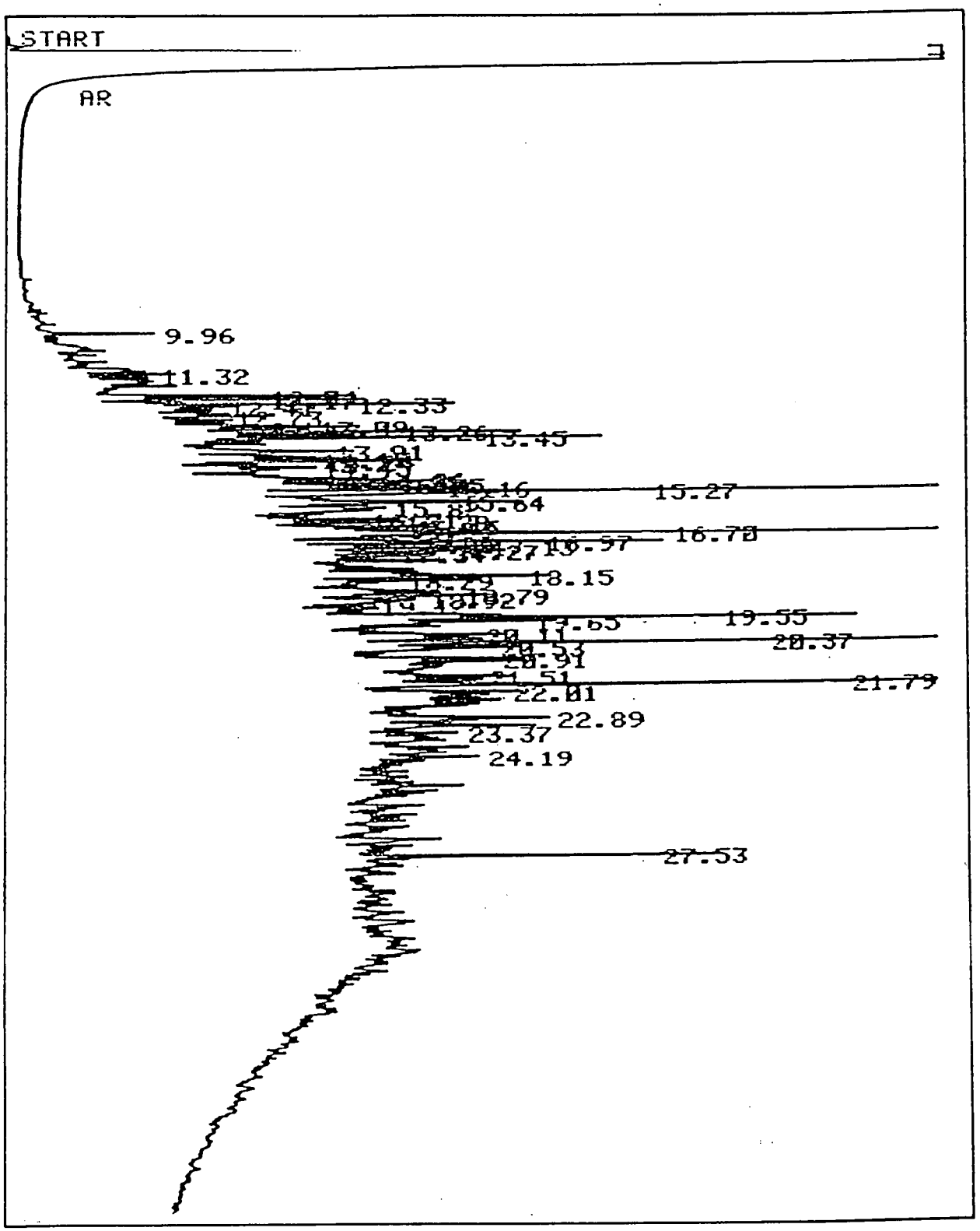
Extract of flask #7.



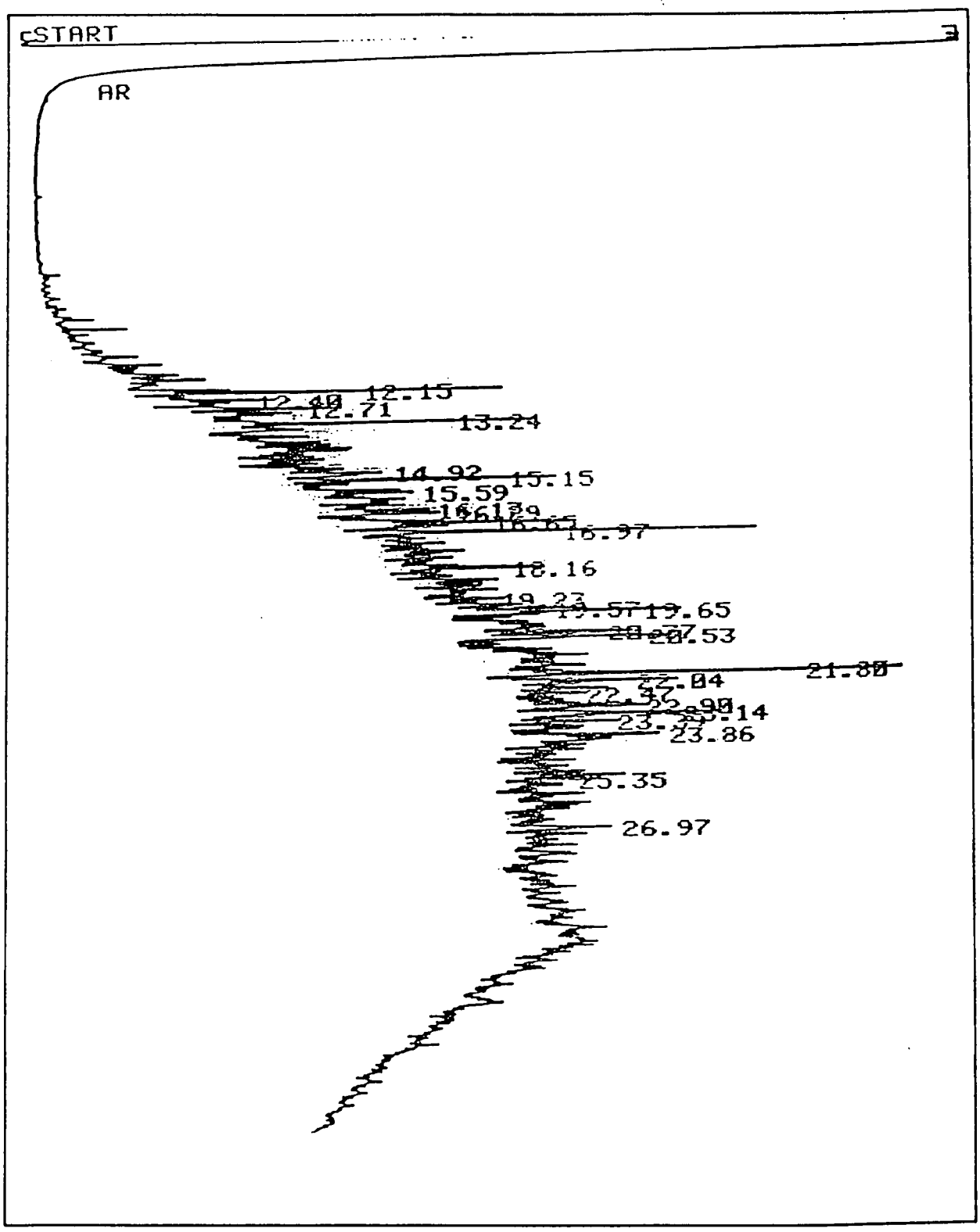
Extract of flask #19.



Extract of flask #21.



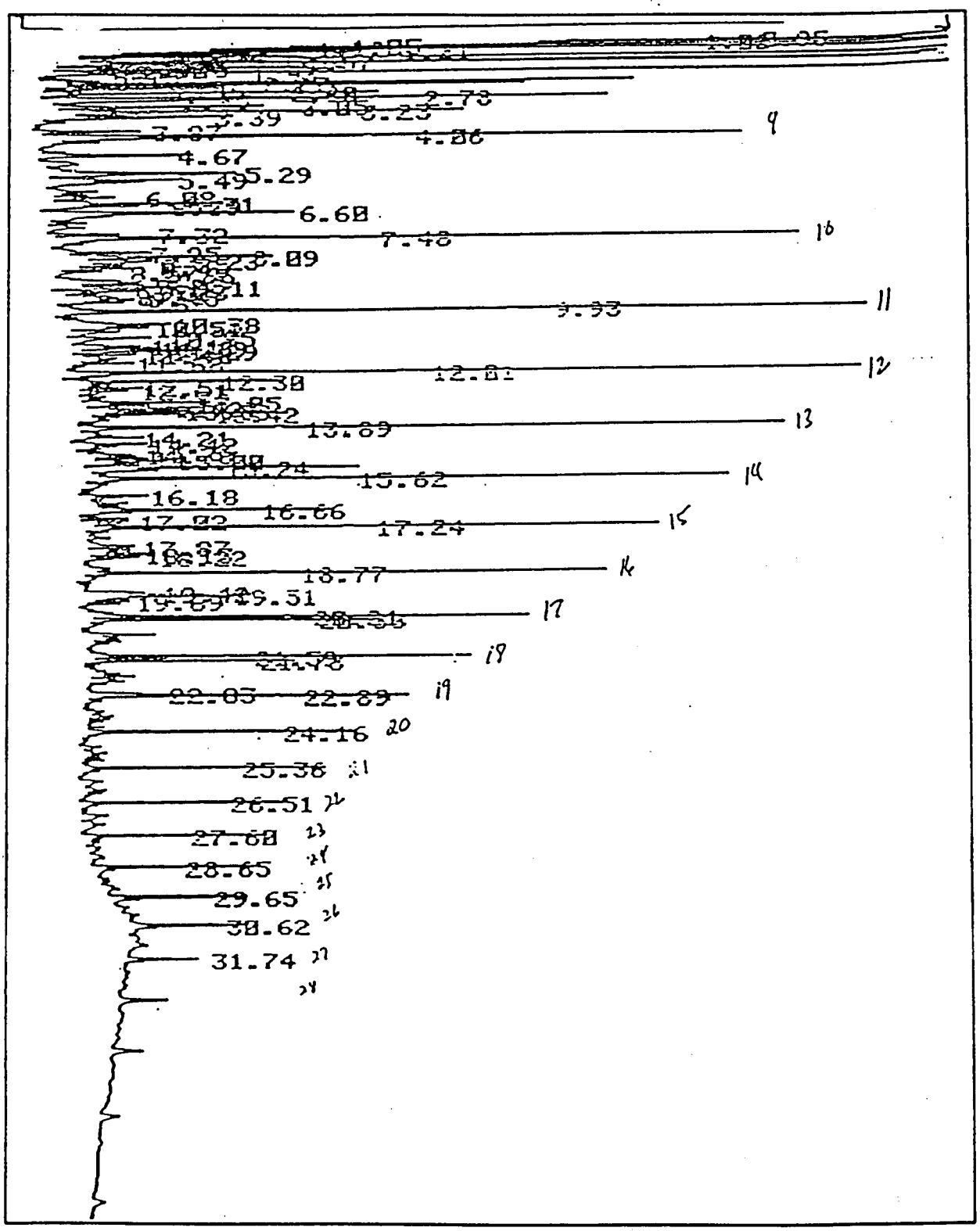
Extract of flask #23.



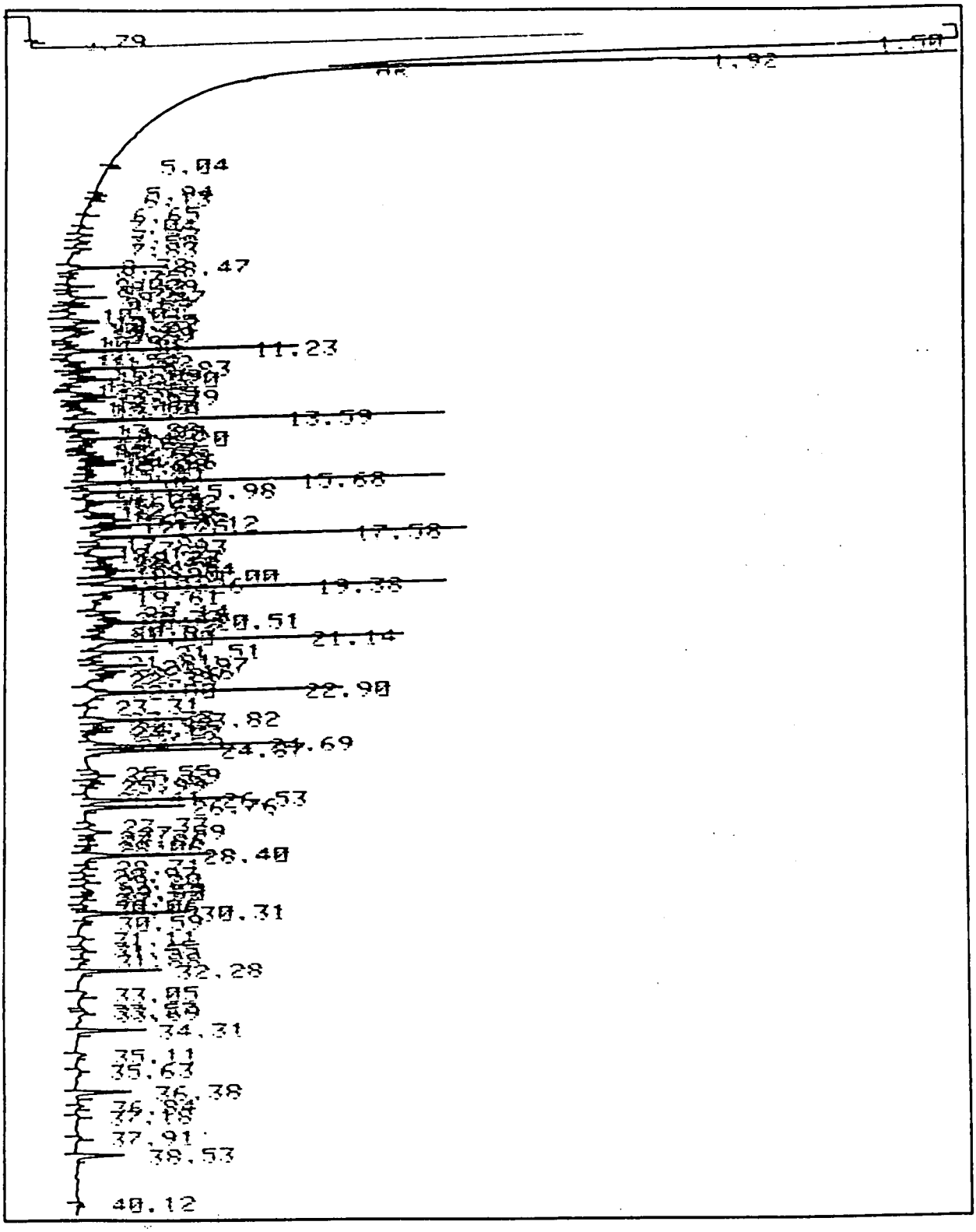
Extract of flask #33.

APPENDIX III

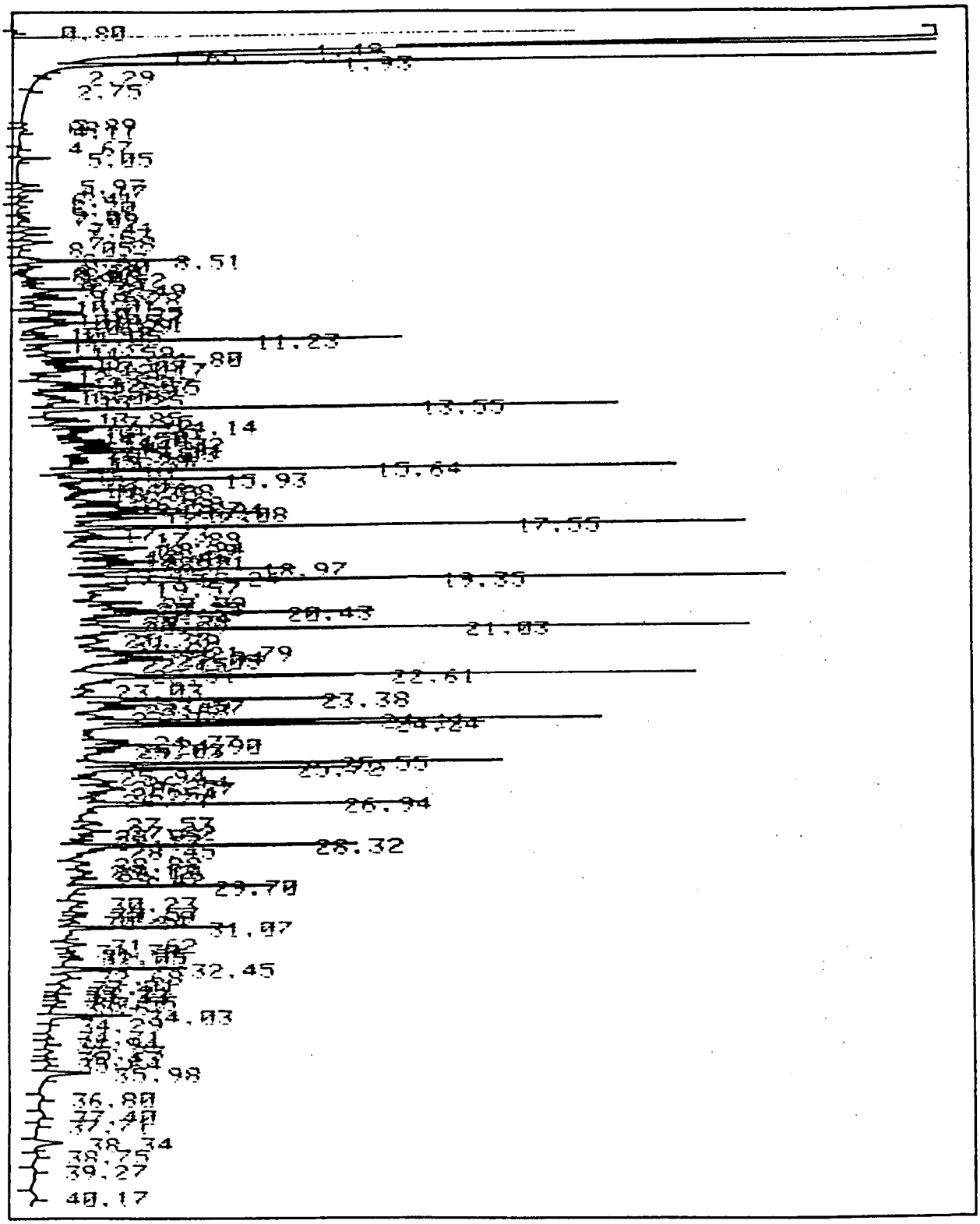
Chromatograms of Crude-Oil Extracts From the Reactor at Three Temperatures



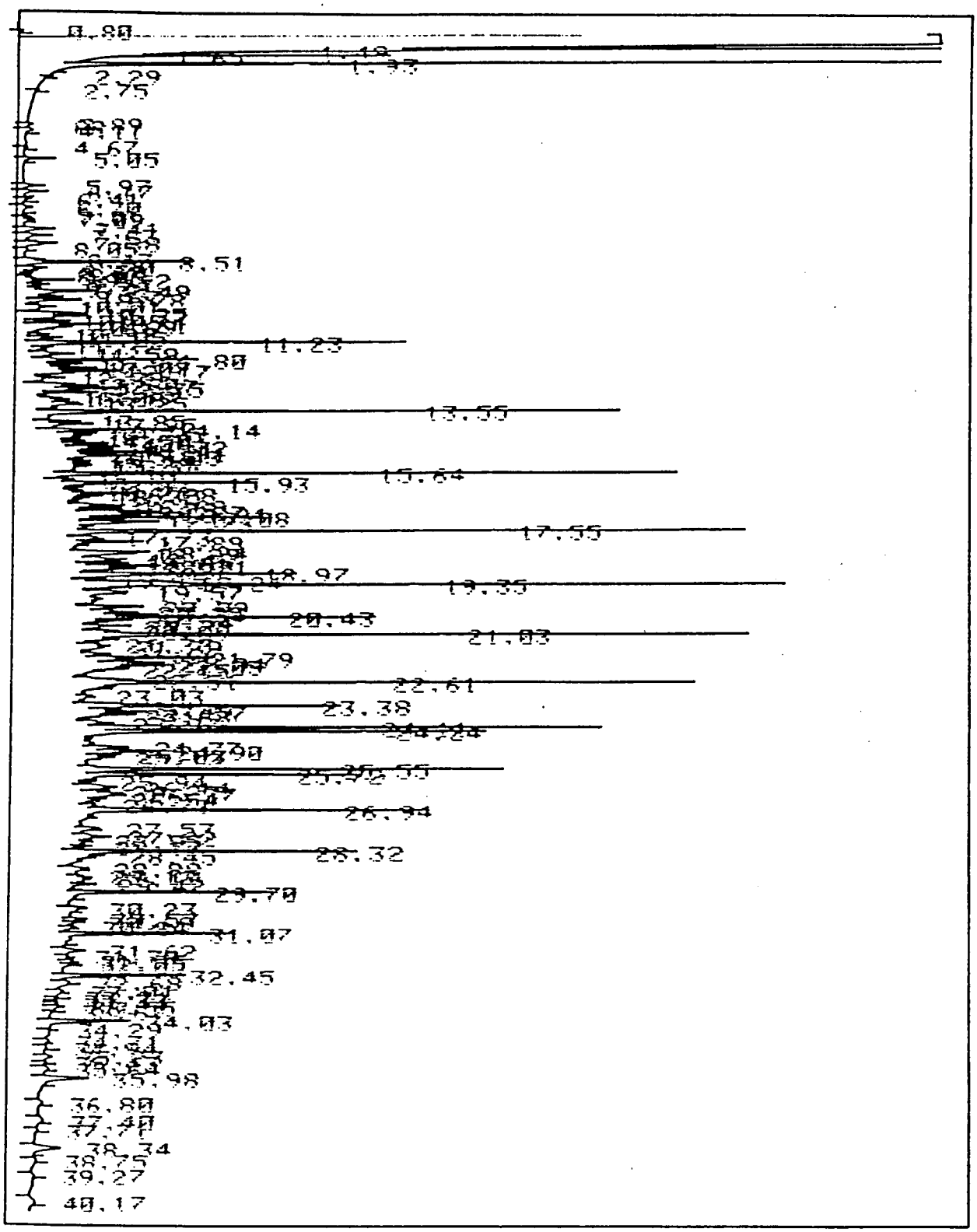
Sample gas chromatogram of crude oil standard.



Crude at 5°C for 10 months.



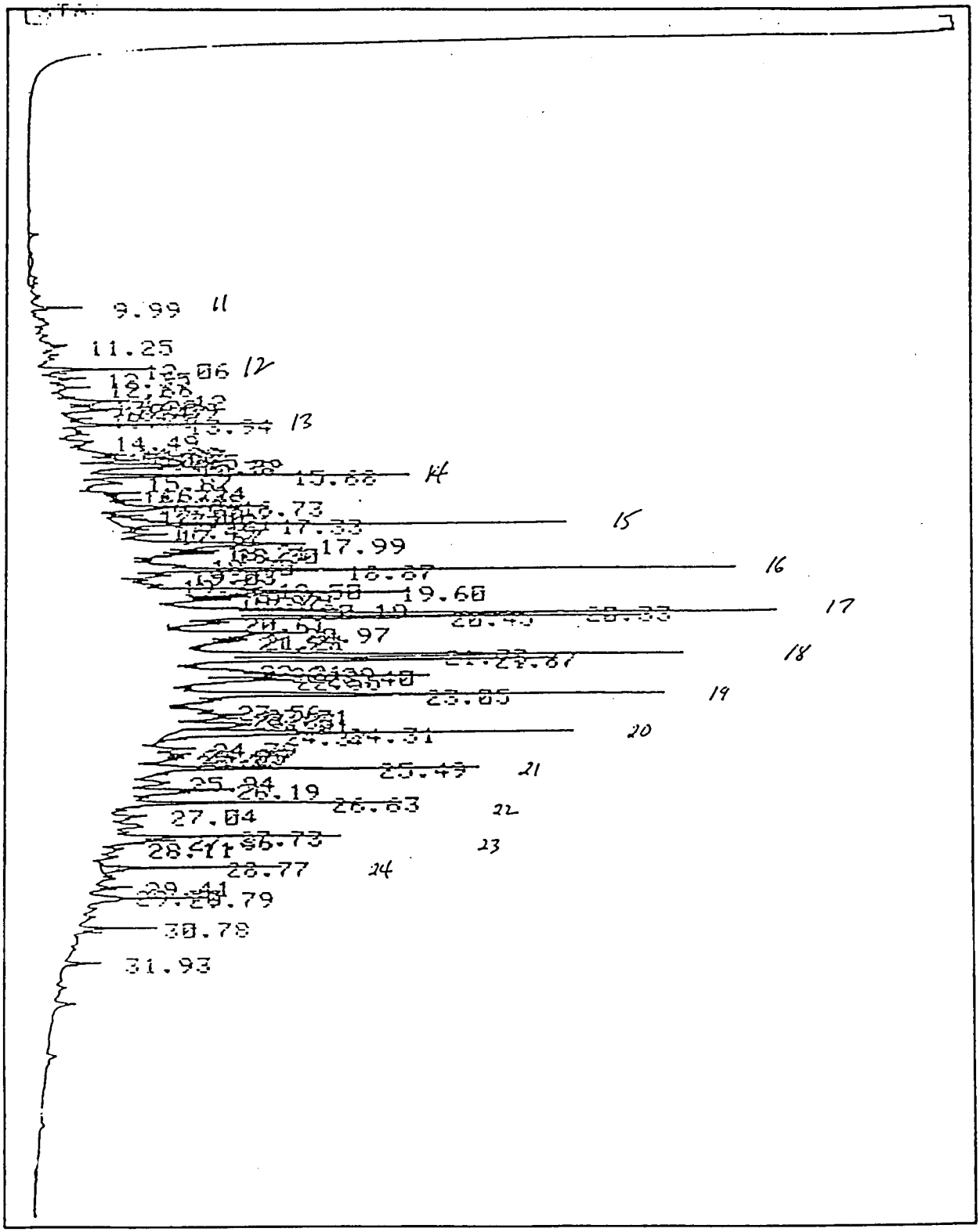
Crude at 10°C for 10 months.



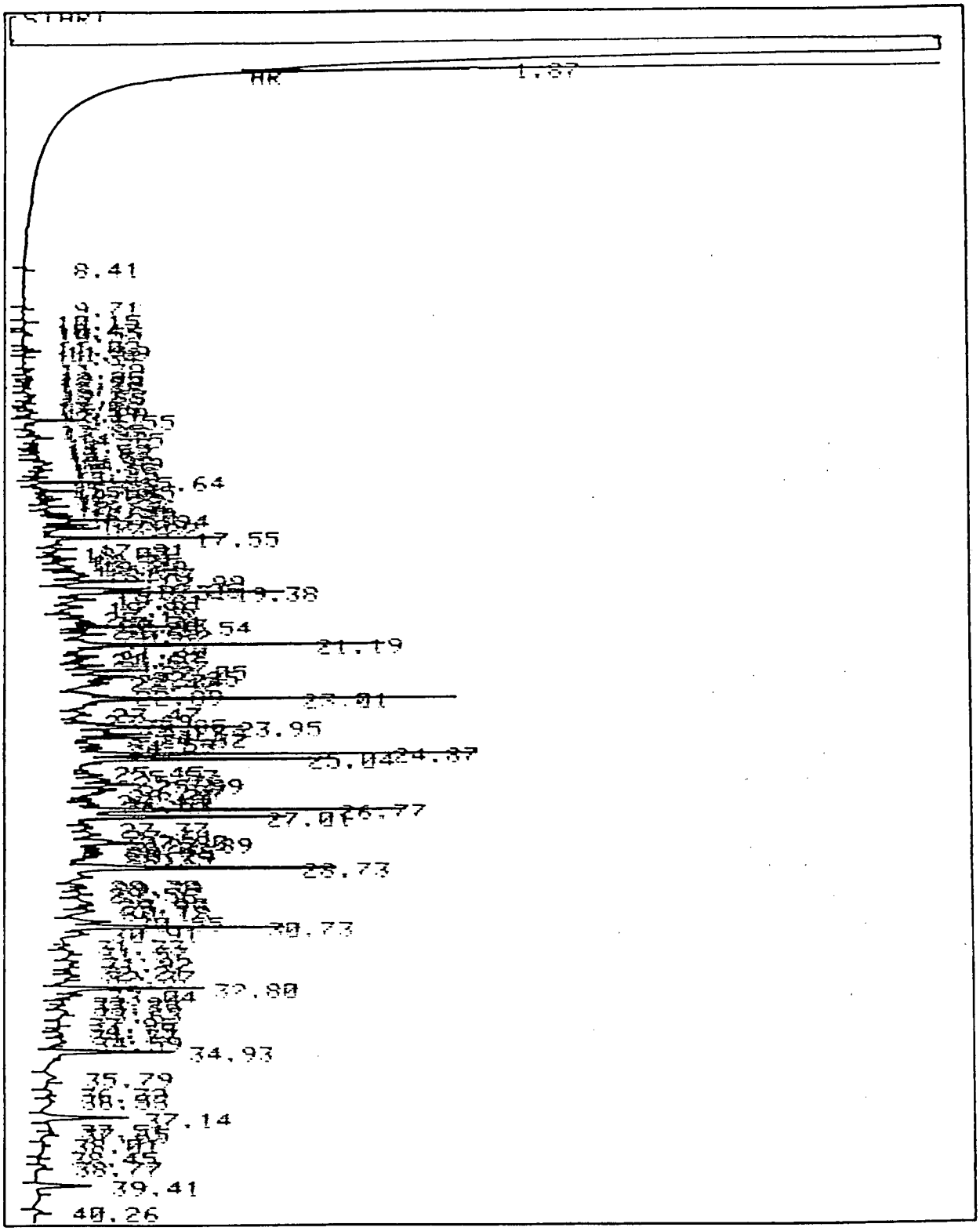
Crude at 15°C for 10 months.

APPENDIX IV

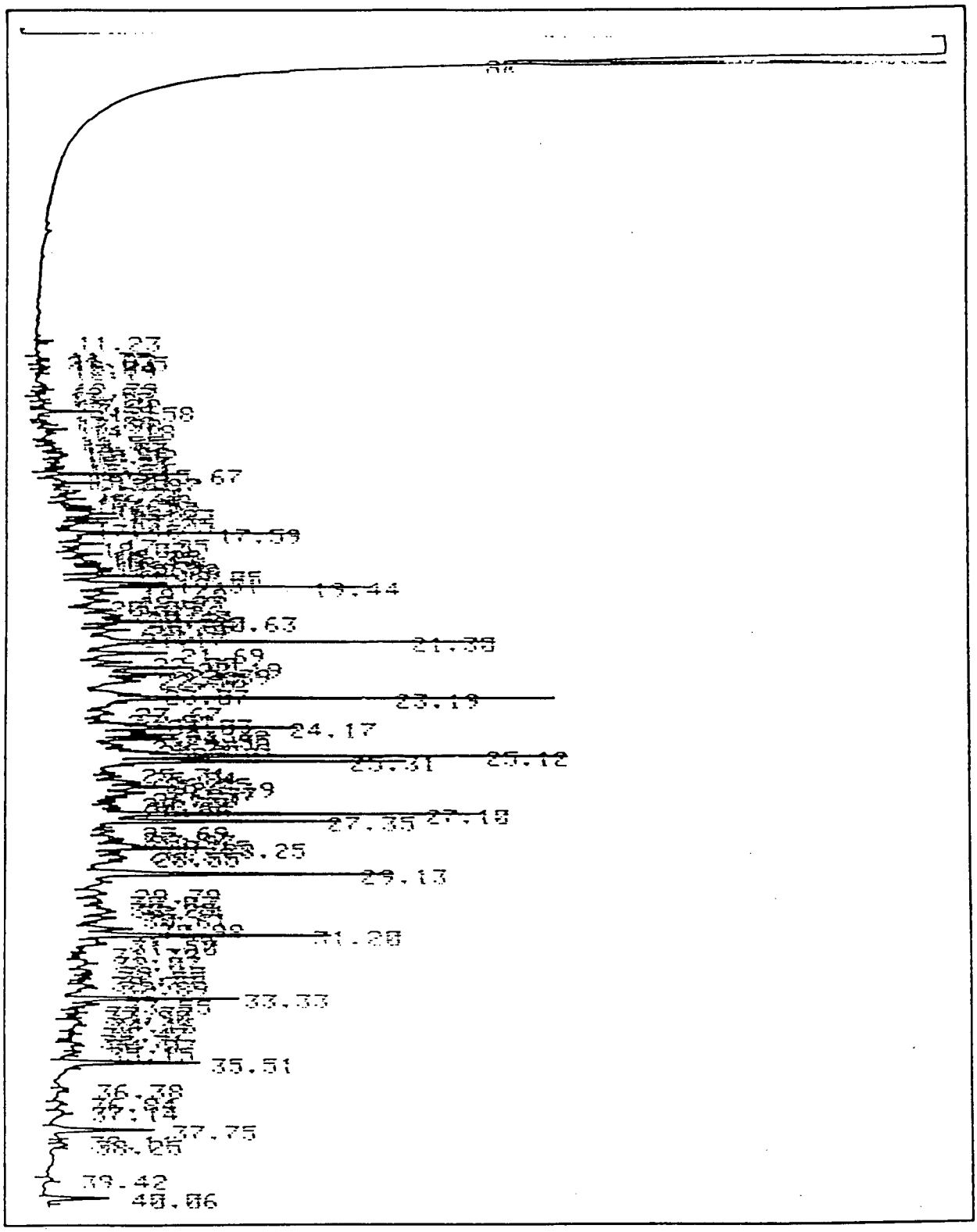
Chromatograms of Bunker C Extracts From the Reactor at Three Temperatures



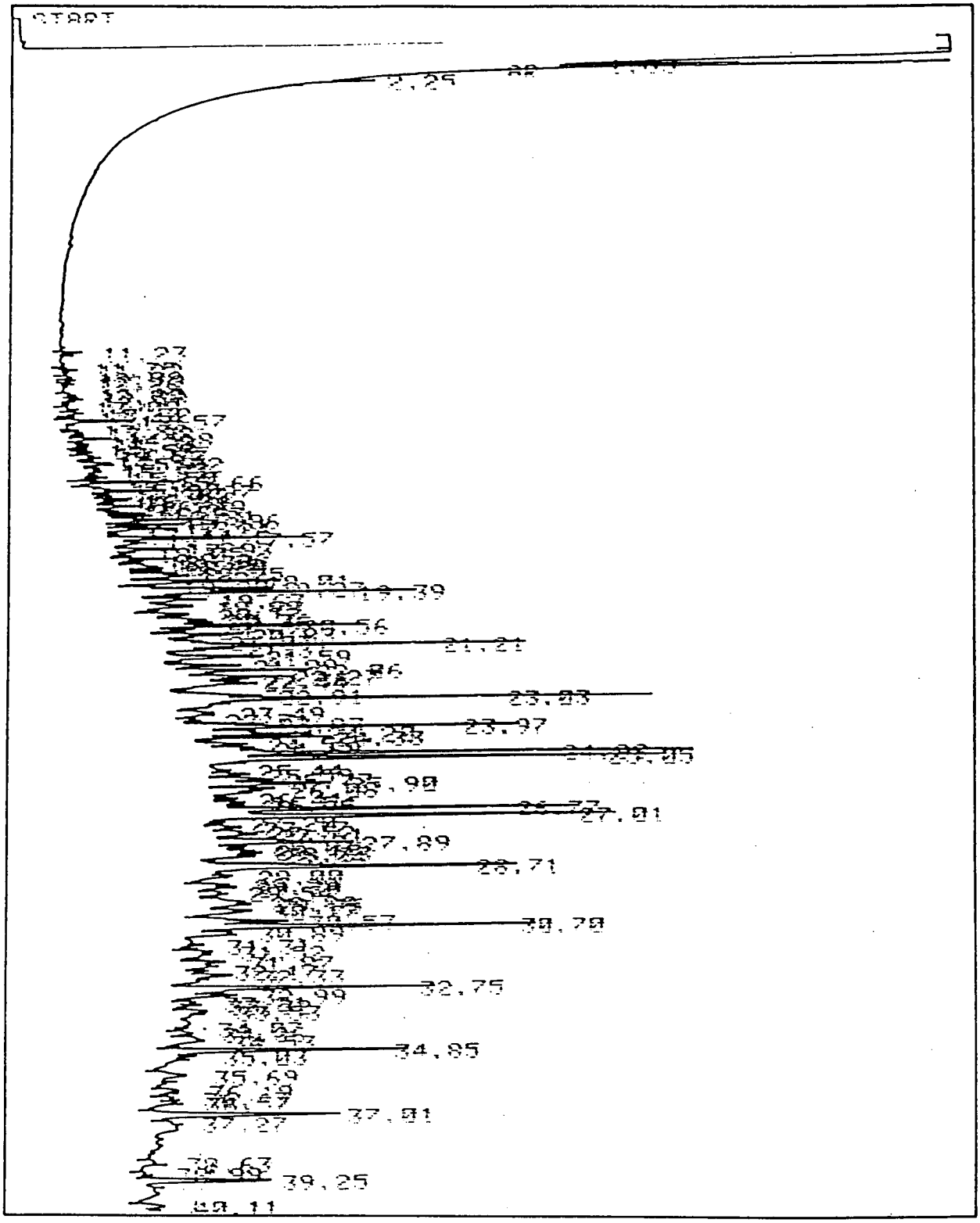
Sample gas chromatogram of Bunker C standard.



Bunker C at 5°C for 10 months.



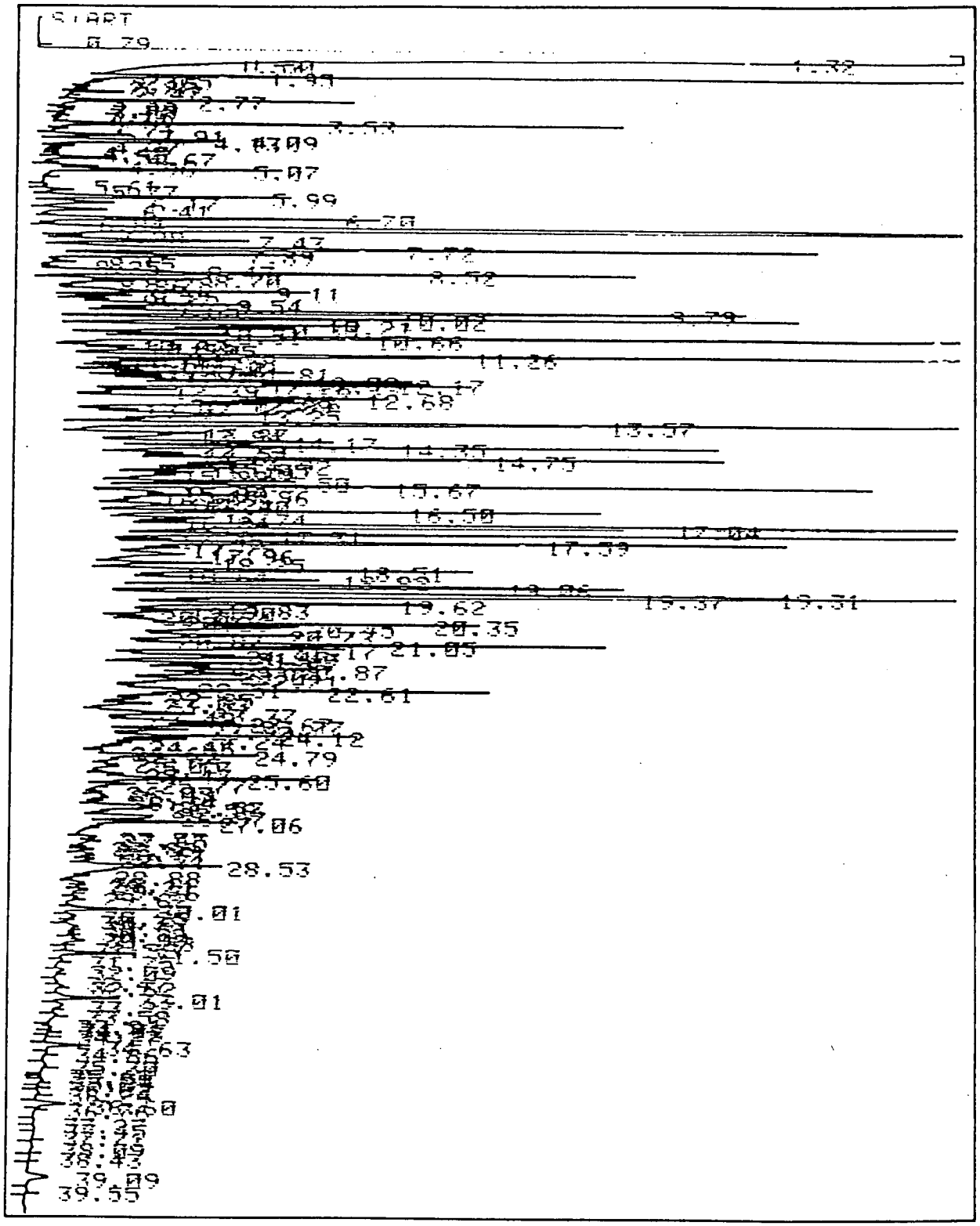
Bunker C at 10°C for 10 months.



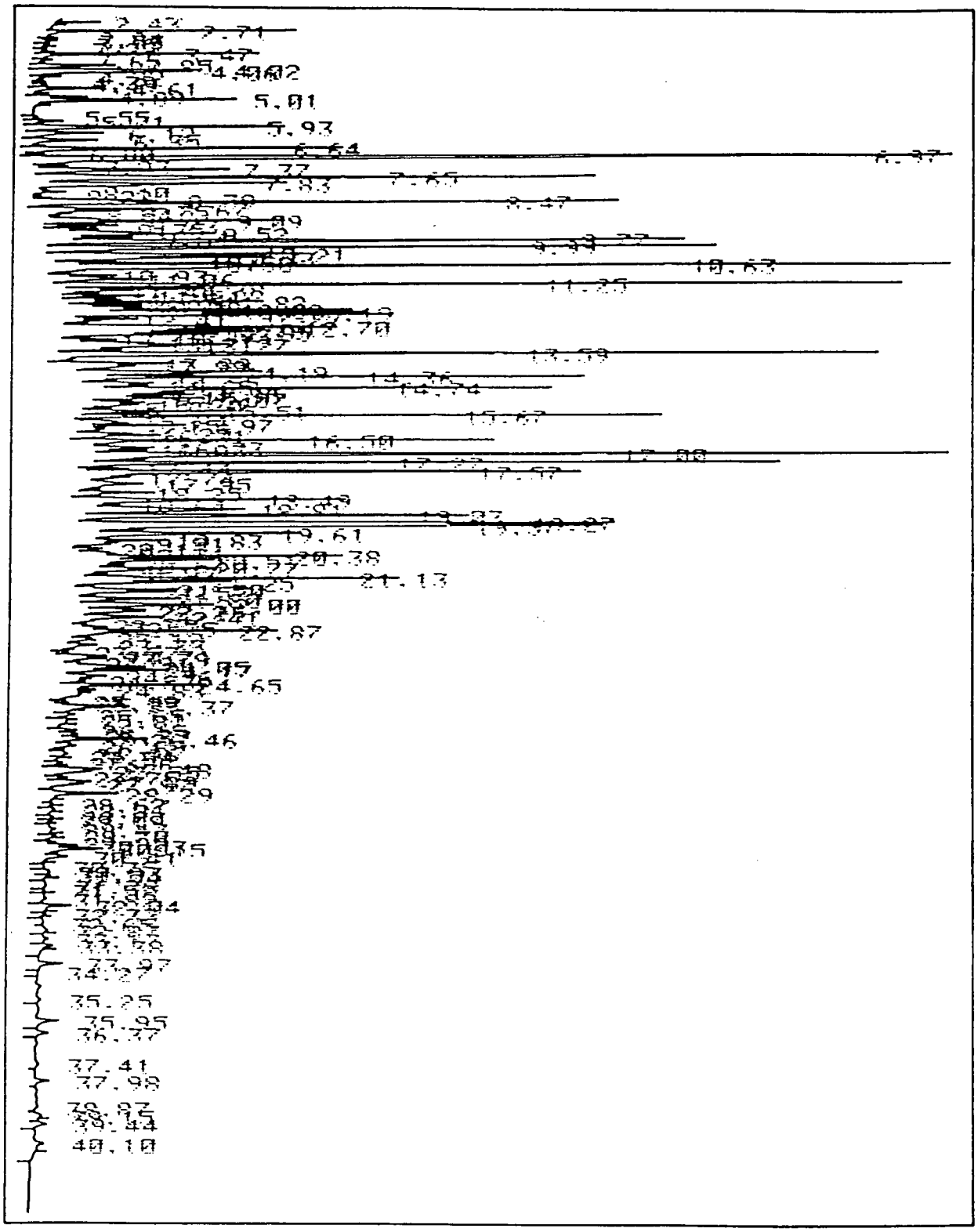
Bunker C at 15°C for 10 months.

APPENDIX V

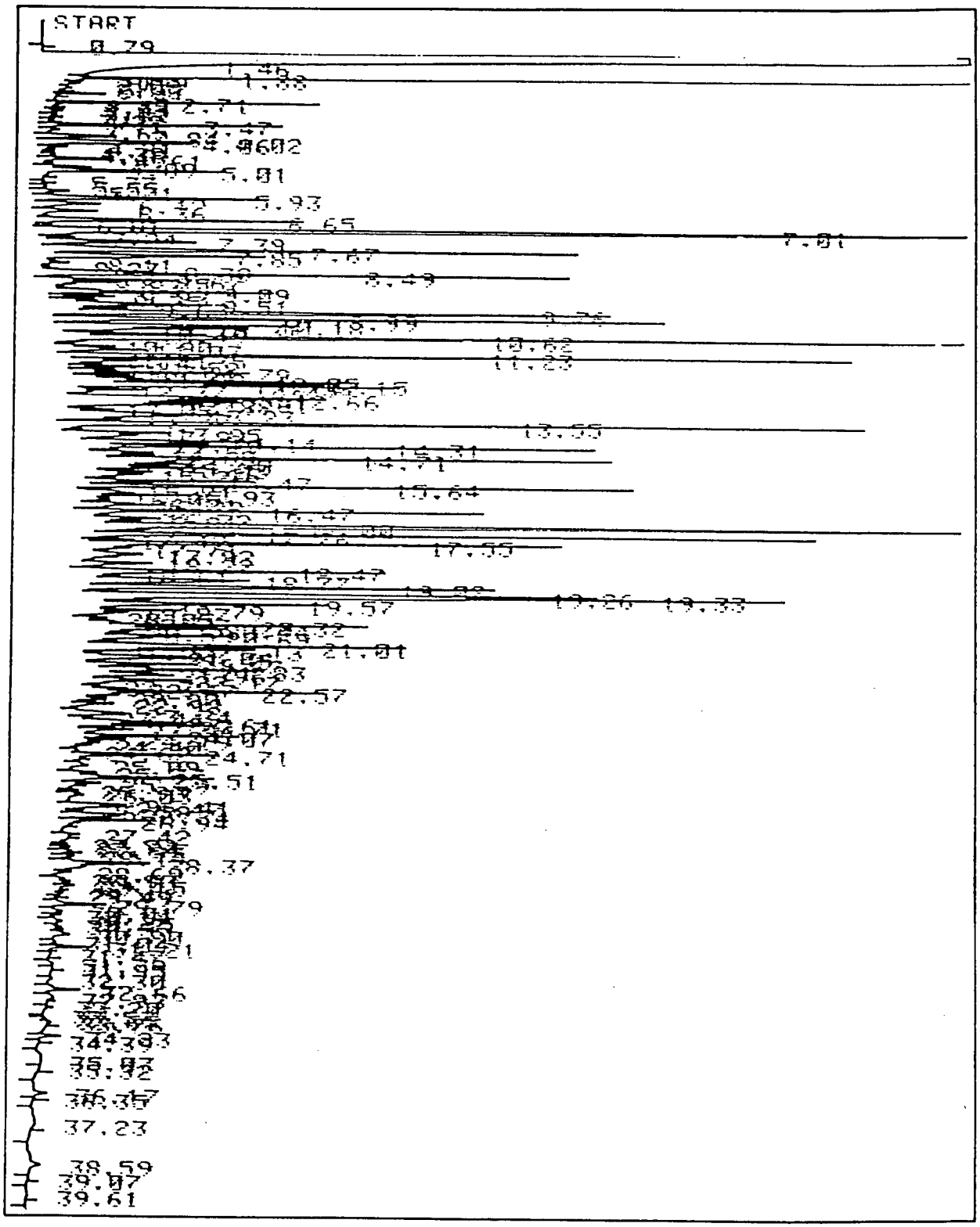
Chromatograms of Condensate Extracts From the Reactor at Three Temperatures



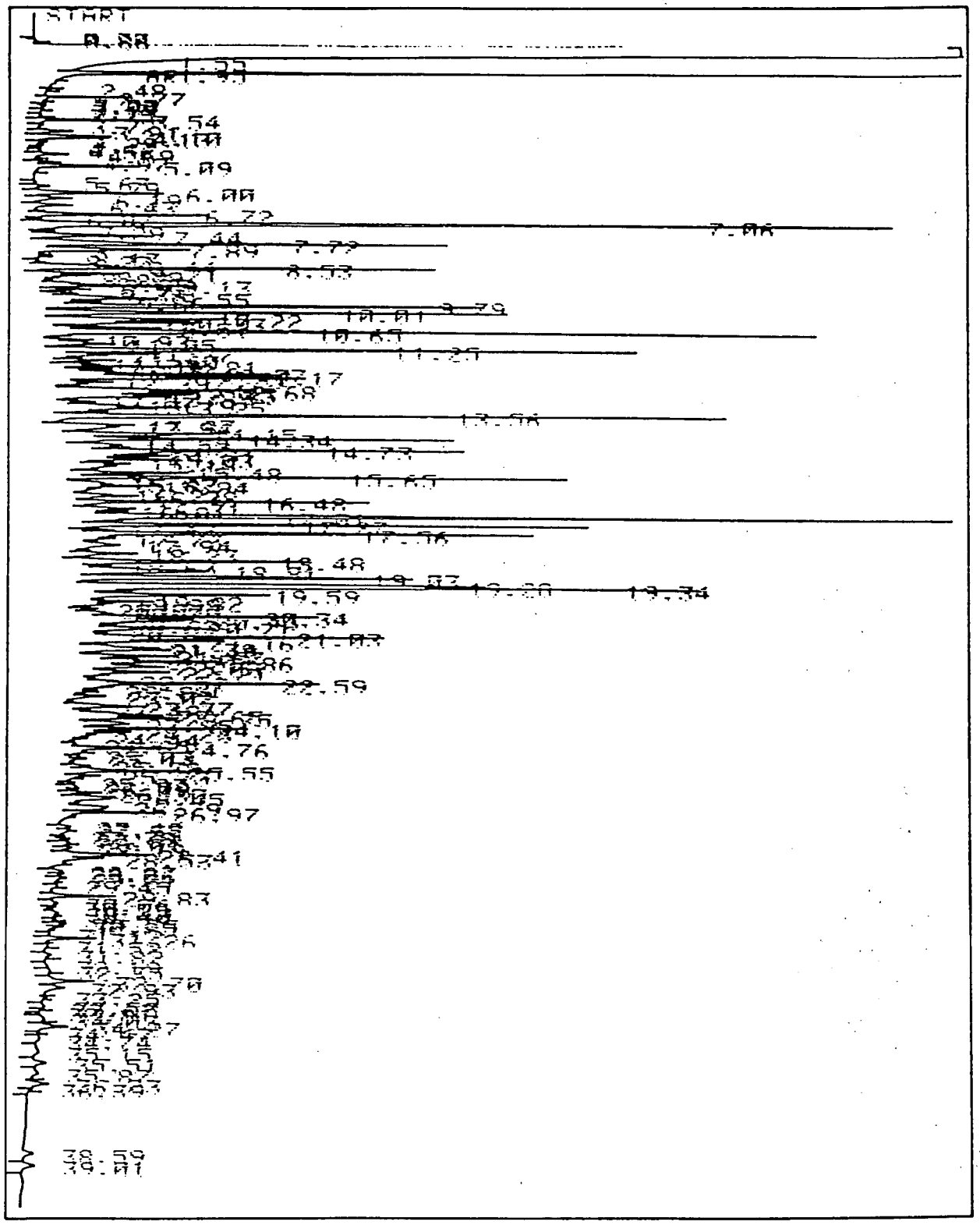
Sample gas chromatogram of condensate standard.



Condensate at 5°C for 9 months.



Condensate at 10°C for 9 months.



Condensate at 15°C for 8 months.