C H A P T E R - III

A COMPOSITE CHEMISTRY OF BIOLOGICALLY ACTIVE ROCK EXUDATE - SHILAJIT

S E C T I O N - I

COMPOSITE ANALYSIS OF SHILAJIT

ABSTRACT

Composite chemical analysis of Shilajit towards the origin has been described.

Multiple ion exchange separation furnished benzoic acid and protocatechuic acid together with a large quantity of acetic acid.

Light petroleum extract afforded a new sesquiterpene diol. However, biological markers could not be traced.

The bituminous rock exudate appears to be very complex in nature.

INTRODUCTION

'Shilajit' (SANSKRIT: Shilajatu)[1], is a blackish brown exudation from rock-surface found in lower Himalayan regions of India and Nepal. The ancient Hindu materia medica describes the aqueous solution of Shilajit to be efficacious against hypertension, chronic bronchitis, asthma, genito-urinary infections, dropsy and nervous disorders.[2] It is also used for hypoglycemic purpose, though some contradictions have been noted.[3]

Four varieties of this material are demarcated depending on the colour; namely gold, silver, copper and iron. However the last type is the most common and is considered biologically active.

The general appearance of Shilajit is that of a compact mass of dark gummy matrix interspersed with vegetable fibres, sand and earthy matter. The gummy substance dissolves in water and when washed leaves sand, fibres and a few black round button-like masses resembling peas. Water extract has decided urinous odour.

An interesting point about Shilajit exudations is the periodic secretions, which occur only in the months of May-June and September-October when the weather is very hot.[4] And that too from the rocks facing the sun. The above intermittent

phenomenon has naturally led to a scanty supply, so that many times it is adulterated with some other cheap materials.

It still remains a mystery that how Shilajit gets formed in the rock crevices at high altitudes and oozes out, though some hypotheses have been put forth.

From the physical characteristics and microscopic examination of the residue left after extraction with water, it would appear to be a substance of vegetable origin because of sand and vegetable fibres. A recent chemical analysis claims Shilajit be of plant origin. It has been shown that it owes organic constituents to the latex of Euphorbia royleana Boiss[5] - a cactus variety growing in the vicinity of shilajit exuding rocks. However, the point of contradition is that, the exudation is not found in every place where the plant grows commonly over similar rocky surfaces.[4] Another aspect which makes this supposition doubtful is the presence of hippuric acid and a high percentage of albuminoids.[2] Therefore the possibility that Shilajit may be of animal or microbial origin can not be ruled out. Some chemical investigations have been carried out.

Previous Work

Chemical analysis of crude and purified Shilajit by Hooper and R.N. Chopra et al.[2] shows presence of benzoic acid, hipouric acid, some fatty acids together with resin, waxy matter, guns and

albuminoids. S. Ghosal et al.[5] have isolated euphol, taraxerol, sitosterol, aromatic acids, benzocoumarins, triterpenes and some amino acids from Shilajit. No other report is available.

Based on the above organic constitution the medicinal value of Shilajit has been partially attributed to benzoic acid or benzoates[2].

Conclusion

At present it seems that the origin of Shilajit is inexplicable on the basis of available information. The option remains open for the possible animal or microbial interception, which may impart biological activity to this bituminous material.

Therefore subject needs further systematic chemical exploration. Present work has been an effort towards this direction.

RESULTS AND DISCUSSION

Authentic crude Shilajit obtained from Nepal (sample preserved in the laboratory) was a blackish brown mass with a very hard and slightly sticky surface. It was crushed into small pieces and exhaustively extracted with demineralized water at 60°C.

The dark brown aqueous portion thus obtained showed 29.6% of soluble matter while 65.2% remained insoluble in the form of stone pieces, sand and fibres. Aqueous extractive of Shilajit being biologically active was preferentially analysed as follows.

Water soluble portion

The above aqueous portion was concentrated on rotary evaporator to 1/6th of its volume. The concentrate was fractionated by successive solvent extractions into petroleum ether (.02%),* solvent ether (0.24%), ethyl acetate (.19%) and n-butanol (2.1%) fractions. Remaining highly hydrophilic solids on incineration left significant quantity of ash (10.69%) and atomic absorption spectra estimated cations of alkali-metals (Na^{+}- 179.5 ppm, K^{+}-600 ppm) as major and alkaline earth metals (Ca^{+2}-34 ppm, Mg^{+2}-125 ppm) as minor, together with the traces of copper (2.9 ppm).

Aqueous distillate obtained during concentration was basic

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* All percentages noted are by weight to crude Shilajit.

to pH paper (pH; 8-9). Therefore it was examined for volatile bases, volatile acids and volatile neutrals by usual sequential regeneration (Fig. 1).

Volatile acids and volatile neutrals showed no acid reaction and negative 2:4 DNP test respectively. While regeneration of volatile bases could provide solution of basic pH (8-9), which attained neutrality in 48 hr, even after preserving in cold room (0-2^{o}C); suggesting presence of highly volatile basic compounds.

A sequel to atomic absorption analysis was cation removal to disengage organic matter. Therefore the aqueous concentrate was sought for multiple ion exchange separation. Literature in this regard shows that the passage of some carbohydrates through strongly basic resin results in decomposition.[6] Such effect is not unlikely because, reducing sugars in contact with base in an atmosphere of oxygen are well-known to undergo enolisation with subsequent degradation of carbon chain.[7] Keeping this in mind a judicious combination of strong cation and weak anion exchangers was employed to demarcate aqueous extract into three basic types of portions - hydrochlorides and chlorides, neutrals and acids as shown in Fig. 2.

1. Hydrochlorides and chlorides. This brown coloured solid (pH - 7) was 7.4% by weight and turned slightly darker on keeping. According to the method of elution it should contain highly polar organic

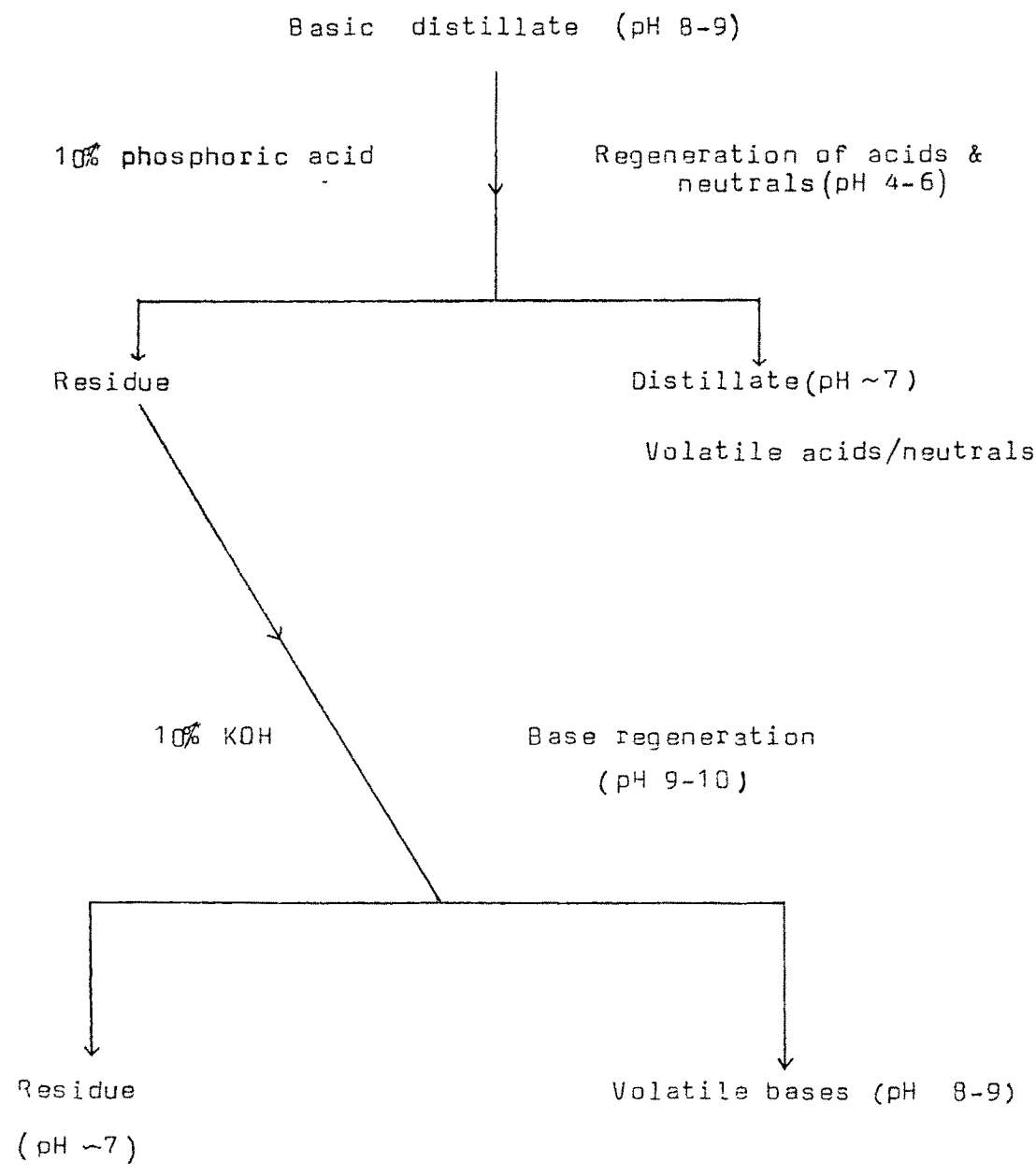


Fig. I : Separation of volatile bases, acids & neutrals by sequential regeneration

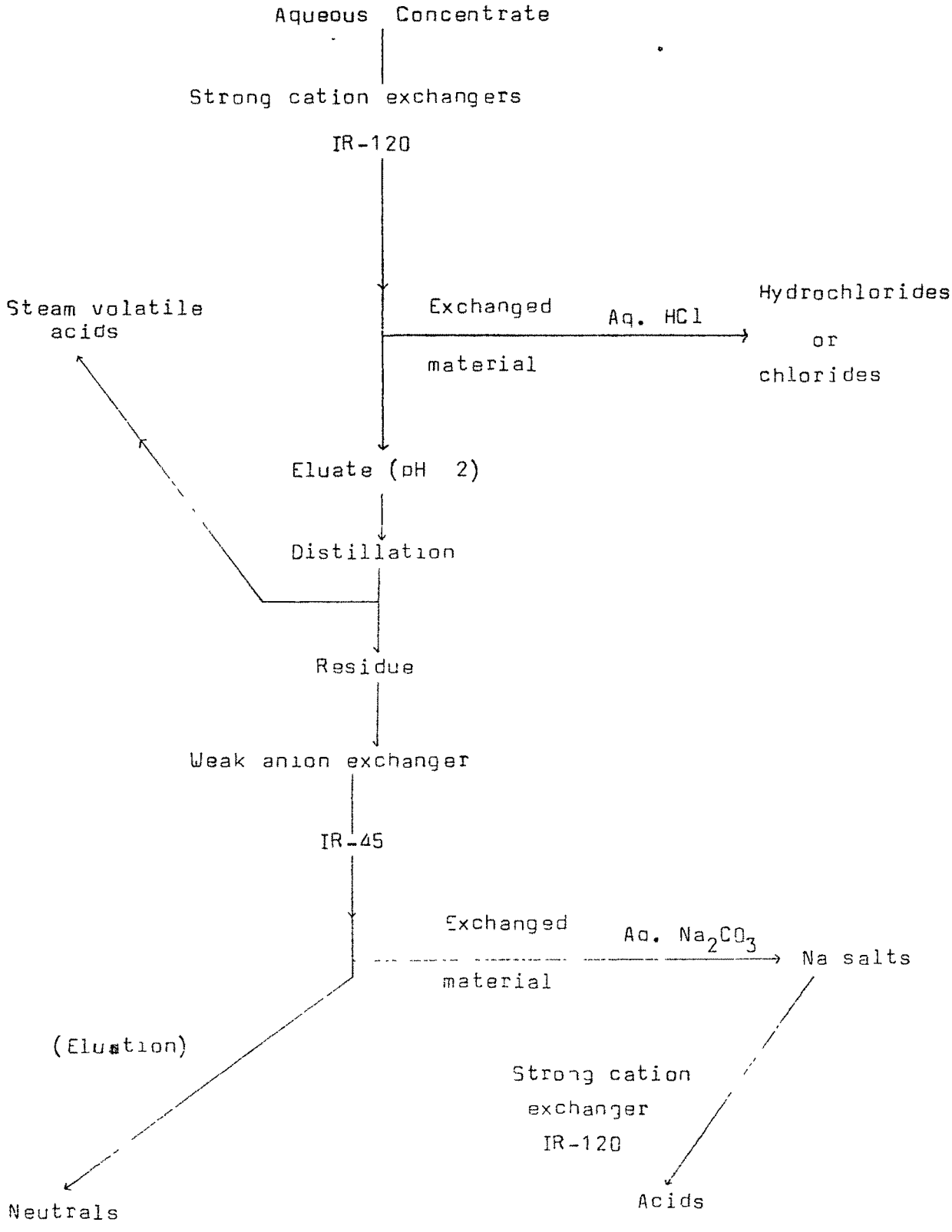


Fig. 2: Multiple ion exchange separation of aqueous concentrate

bases in the form of hydrochlorides and cations as chlorides. As cations were already estimated, the solid was examined for organic matter. It is possible that the fraction contains amino acids due to the Zwitter ion effect,[8] together with amines. However, ninhydrin indicator test on chromatoplate was found to be negative in acidic, basic and neutral solvent systems.[9a,b] This concluded major quantity of inorganic chlorides.

2. Neutrals. A highly hydrophilic neutral mass (2.34% by weight) would necessarily mean the possibility of carbobydrates or polyhydroxy compounds.

The fraction poorly responded to Tollen's reagent, Urea-HCl reagent and Fehlings solution (A and B).[10] As well, a systematic column chromatography on silica gel (grade: IIIB), with a slow elution and collection of milligram quantity of eluate in each fraction failed to give any pure isolate.

As it is preferable to deal with such a material in acetylated form, it was acetylated with dry pyridine and acetic anhydride. Acetylated product showed tailing on TLC, therefore it was readily esterified with the ethereal diazomethane solution. Organic portion thus obtained was distilled at reduced pressure (0.5-1.0 mm, 170-230^{o}C oilbath) to furnish 5 mg of straw coloured viscous liquid. As a preliminary test this distillate was compared with glucose penta-acetate on GLC (Fig. 3). The difference in retention times (glucose penta-acetate: 3.2-4 min; distillate: ·2-1.2 min, 10%

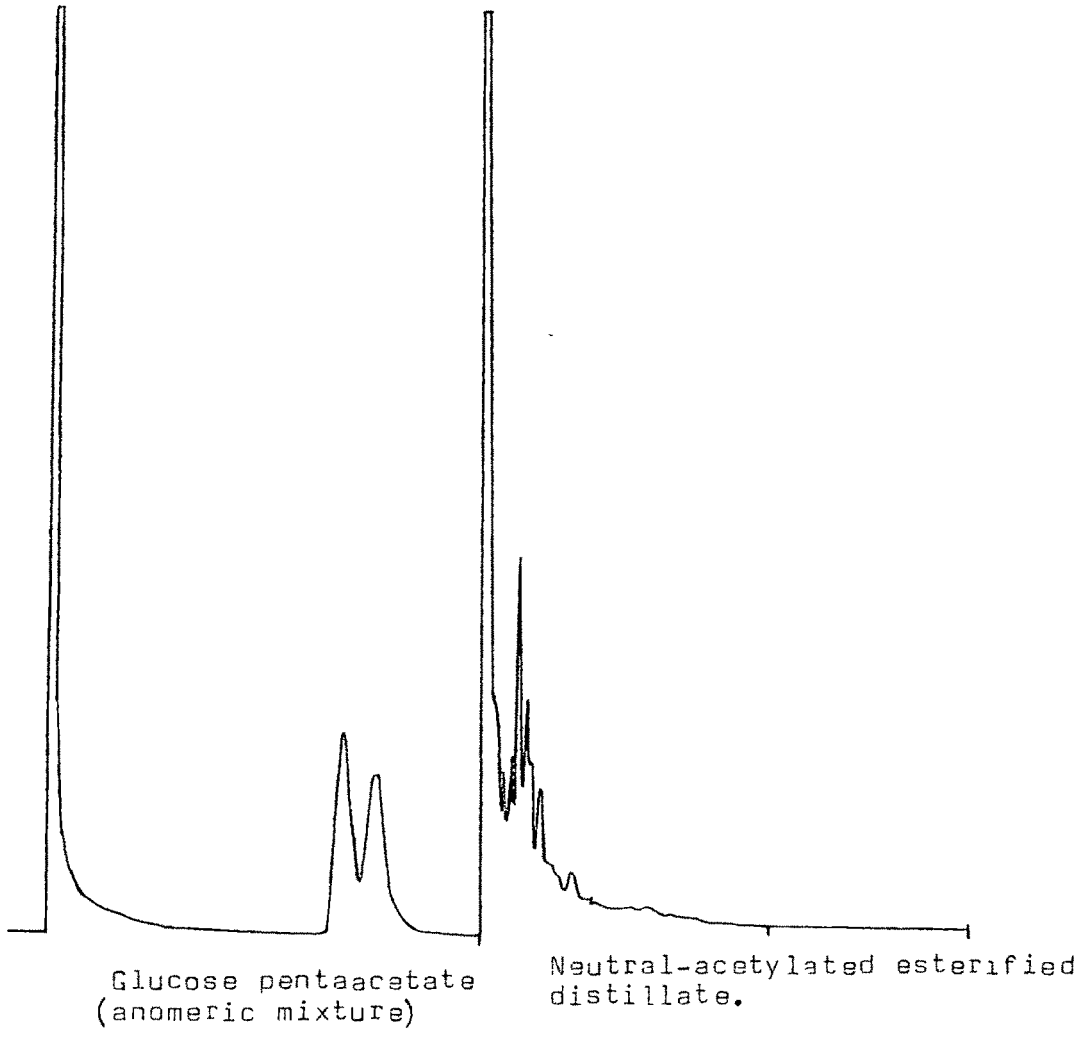


Fig. 3: Comparative GLC of glucose pentaacetate and neutral acetylated esterified distillate.

$OCQF_1$, col-200°, FID-270°) clearly indicated lower carbon number than glucose penta-acetate. When shooted for GC/MS analysis, minimum of 14 components could resolve. Efforts towards correlation of these mass spectra with the known acetates failed due to the difficult identity of molecular ion peaks. However, 3 compounds displayed specific base peaks at m/e 115 and m/e 103 corresponding to the loss of ketene from carbohydrate acetate fragments;[11] together with the respective parent ions as shown (Fig. 4).

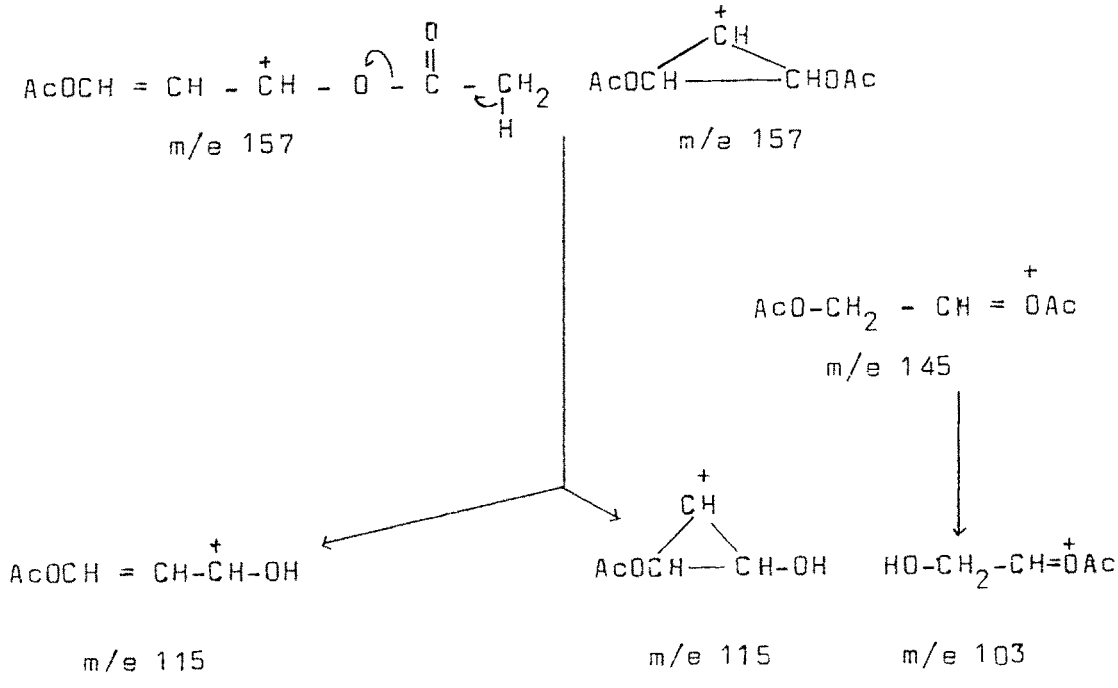


Fig. 4: Fragmentation showing loss of ketene for base peaks at m/e 115 and 103[11]

3. Acids. This brown sticky crust (3.77%) was found to be composed of only organic acids because of negative tests for SO_4^{--}, NO_3^- and PO_4^{---}. TLC pattern showed typically submerged elongated streaks. Fraction was comparatively soluble in methanol but still left a significant insoluble mass. This was attributed to some complex polymeric acids.

Methylation of total acid portion by i) CH_3-I, Ag_2O, DMF[12]; ii) ethereal diazomethane or iii) CH_3I, BaO, DMSO[13], often led to a trivial quantity of methylated product.

Finally methanol soluble portion free from solvent was triturated with hot water. Water soluble acids thus obtained chromatographically (SiO_2-gel, IIIB) eluted 7 mg of pure fraction (15% EtOAc in pet. ether) an amorphous powder (m.p.199-200°C).[14] By a direct comparison of ^{1}H-NMR spectra (DMSO-d^6) it could be characterized as protocatechuic acid[15] (3,4-dihydroxybenzoic acid). Result was supported with GLC where synthetically prepared methyl protocatechuate[16] was coinjected with the total acid ester at retention time 6.50 min (10% OV_4, 150°C). No other compound could be obtained.

This completed total analysis of non-volatile water soluble portion.

Volatile acids

The initial passage of neutral aqueous concentrate over strong cation exchanger (Fig. 2) liberated bound acids. Concentration of

this acidic eluate (pH=2) on rotatory evaporator at controlled temperature, co-distilled steam volatile acids in the distillate. Water was repeatedly added to the residue and distilled off till neutral. Qualitative and quantitative estimations were carried out as follows:

A negative chloride test depicted presence of only volatile organic acids. Normality of the acidic distillate, as determined by acid-base titration, was found to be 0.038. PTC esterification of aqueous distillate with n-cetyl-trimethylammonium bromide-CH_3I-KOH (25^oC)[17] afforded methyl benzoate (96% GLC purity, coinjection with the authentic ester). However, quantitative estimation showed normality contribution by benzoic acid to be only 0.0056. The difference in normality indicated loss of highly volatile ester at some stage. Attempts to control losses by operating at low temperatures and use of Perkin triangle for solvent removal etc. did not square with the desired acidity.

In this context literature describes some methods, especially to deal with volatile fatty acids.[18] Rarely some colour reactions with lanthanum and iodine have been used[19] but may interefere with other acids. Other methods like acidification, distillation, partition and titration have difficultly met success because of the problem in differential solubility or limitations in quantitative estimations. Improved results can be obtained when hydrophilic properties of the carboxylic acid group are progressively outweighed by the increasingly hydrophobic nature of the carbon chain.

In order to resolve this problem, conceptually different route was contemplated. A known volume of aqueous volatile acids was exactly neutralized by dilute KOH. Point of neutralization could be determined using phenolphthalein as an indicator with the aliquots. Thus acids were arrested in salt form. Water was distilled off (pH=7), which left fine colourless salt. A completely dried salt when subjected to ^{1}H-NMR analysis(D_2O) (Fig. 5) showed a sharp singlet at 2.0 δ apart from aromatic protons (multiplet, 8.1-7.6 δ and 7.7-7.38 δ) for K-benzoate moiety. There exists only one possibility for such a signal i.e. $\underline{CH_3}$-COOK. This was confirmed by individual spectra of authentic potassium benzoate and potassium acetate in D_2O. Indeed the spectrum was the exact addition of peaks of these two spectra. Proton intensities showed the ratio of 2.77:1 for potassium acetate to potassium benzoate respectively. This data is in perfect accordance with the yield of methyl bezoate and the total acidity of 0.038N in which benzoic acid and acetic acid contributed 0.0056 and 0.0319 respectively. Quantitative estimation showed a total of 0.45% of benzoic acid and 1.2% of acetic acid in 540 g of Shilajit.

It was surprising to note a large quantity of bound acetic acid present in Shilajit which was much more than benzoic acid. The former has not been detected in any of the previous works.

Having totally evaluated aqueous constituents, efforts were expended on the fractions of organic solvents, with a

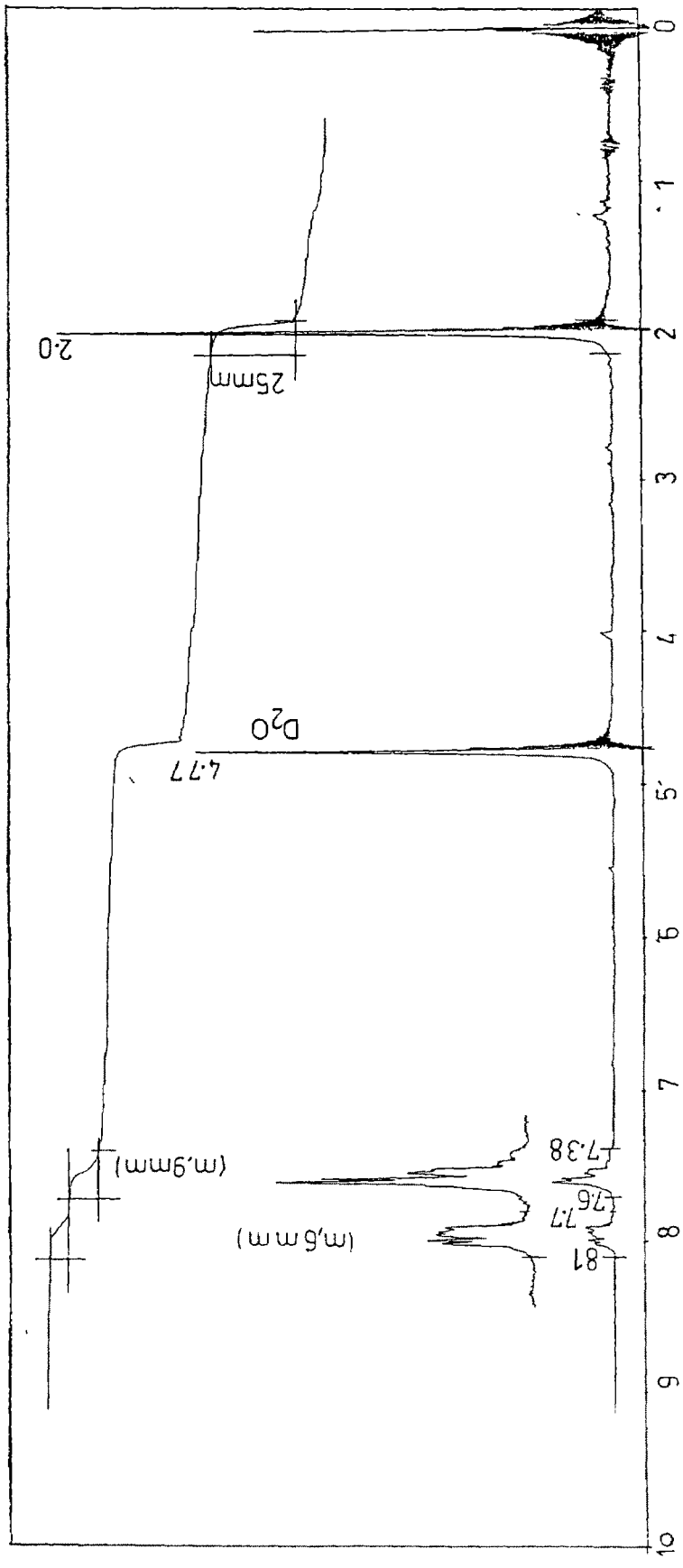


Fig. 5: ^{1}H-NMR spectrum of the mixture of potasium acetate and potasium benzoate

special stress on 'Biological markers'[20] (Biological markers are the compounds originating from microorganisms and enable chemists to investigate possible microbial interception).

Light petroleum fraction

This yellow coloured viscous fraction was 0.02% by weight to Shilajit. Development of overlapping coloured spots on chromatoplate, when sprayed with vanillin-phosphoric acid or anisaldehyde-sulphuric acid[10] suggested the possibility of terpenes. ^{1}H-NMR signals were in the region of 0.7 to 4.0 δ, with strong absorptions for methyl protons. GC/MS analysis displayed~10 resolved peaks (Fig. 6). A critical examination led to the following observations:

1. Petroleum ether fraction was a mixture of terpene hydrocarbons and terpene alcohols (comparatively hydrophilic), with retention times in the range of mono- and sesquiterpenes.

2. Two of the mass spectra showed molecular ion at m/e 154 and subsequent loss of H_2O to give m/e 136. Other peaks, especially at m/e 93 [M-(18+43)] and 121 [M-(18+15)] together with 136 (M-18) are typical of conjugated olefinic terpenes like β-ocimene, allo-ocimene or myrcene,[21] formed after dehydration on electron impact. Associated dignostic fragment at m/e 91 arises from stable tropolium ion (m/e 93) through a loss of two hydrogen atoms or a hydrogen molecule.[22]

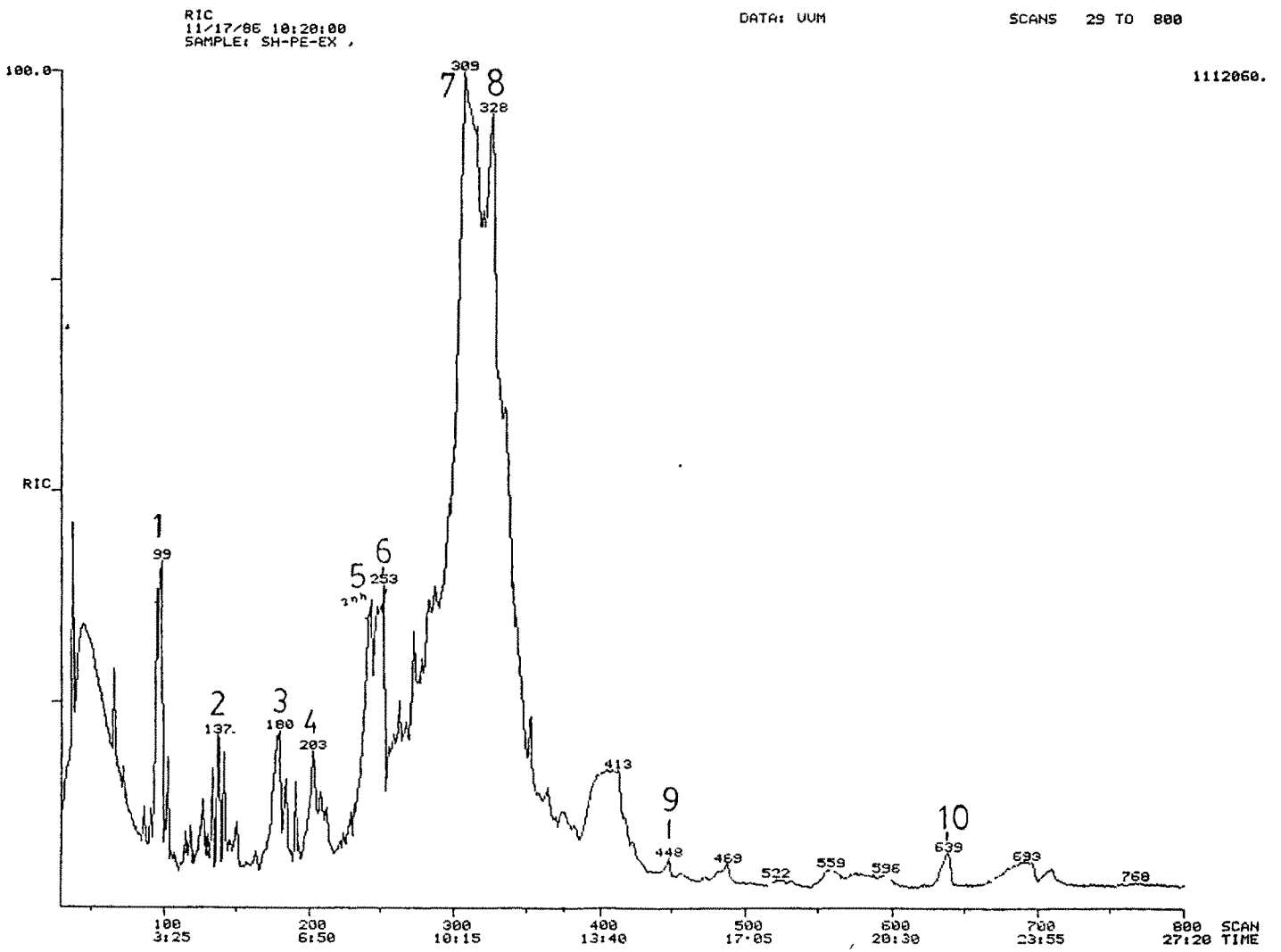


Fig. 6: GC of the GC/MS spectrum of light petroeum fraction

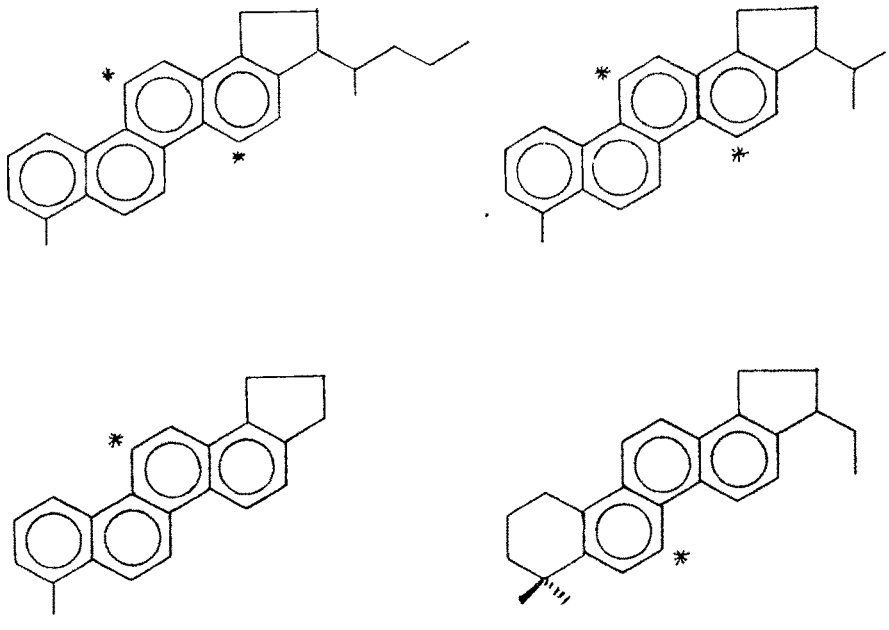
3. Frequently appearing base peaks in the mass spectra at m/e 41, 43 and 45 could be attributed to $CH_2=CH-\overset{+}{C}H_2$ (fragment derived from hydrocarbon chain), protonated cyclopropane[23] (favoured appearance potential in monoterpene hydrocarbons which decomposes by ethylene expulsion to methyl ion) and $CH_3-CH=\overset{+}{O}H$[24] (typical of branched alcohols, $CH_3-CHR-OH$) respectively. However, isoprenoid biological markers like phytane and pristane[25] (characteristic intense peaks m/e 113, 183); steranes and triterpanes[25] (base peaks m/e, 149, 191, 127); or other geohopanoids[26] (base m/e 191) could not be detected.

Therefore total light petroleum extract was subjected to separation on 'Chromatotron'. A systematic fractionation on this preparative rotatory disc was carried out with repeated solvent developments and step gradient elutions to furnish only 4 mg of crystals (Compound A), as a sole isolate. This compound was found to be a new sesquiterpene and its structure assignment forms Section-II of this Chapter. But it may be mentioned that the mass spectrum of this compound could not be superimposed on any of the spectra obtained by GC/MS analysis, though many important fragments were identical with the mass spectrum of component 8 (Fig. 6). Once again it became increasingly obvious that the complexity of the fraction had detrimental effect on resolution.

Solvent ether fraction

Assessment of this brown crust (0.24%) became necessarily important because of the presence of aromatic protons in the region 6.3 δ to 8.3 δ together with a singlet (2.52 δ) and a doublet (1.31 δ , J = 6 Hz).

The pool of biological markers also circumscribes polynuclear aromatic hydrocarbons (PAH) (Fig. 7).



* ^{1}H-NMR value for = 8.7 δ

Fig. 7: Some polynuclear aromatic hydrocarbons-chemical fossils.

It is generally believed that these chemical fossil fuels (PAH) are derived from biological remnants. Thus disproportionation reactions involved, help changing terpenoids and steroids into aromatic hydrocarbons and also yield cycloalkanes in energetically favourable manner.[27]

However, the possibility of PAH could be completely erased on the following spectral grounds.

1. ^{1}H-NMR (DMSO-d^6): General ^{1}H-NMR pattern of these polyaromatic systems give rise to a set of highly deshielded protons resonating at ~ 8.7δ,[28] which were absent.

2. UV (EtOH): Due to extended conjugation facility in electronic transitions is expected. This causes bathochromic shifts. In our case characteristic intense absorbance at longer wave length (e.g. phenanthrene[29]: 300-360 nm-multiple absorptions) could not be observed.

Ethyl acetate and n-butanol fraction

These fractions left significant residue on flame test. Solubility in organic solvents was also poor. Moreover weak signals in ^{1}H-NMR was a discouraging factor for significant organic contents and hence not investigated further.

Efforts were then directed to analyze insoluble stone which was left after exhaustive aqueous extraction of Shilajit

(vide supra). This solid part, trivial for pharmacology, was extracted in soxhlet sequentially with petroleum ether, dichloroethane and ethanol; and examined further.

Petroleum ether fraction

This waxy fraction (m.p. 76-80°C) was the most lipohilic part (0.11%) and partially retained the odour of Shilajit. From ^{1}H-NMR, IR and GC/MS (11 components resolved) of the distilate (170-300° oil bath/0.15-0.25 mm, GC-Fig. 8; SE 30, open tubular column, temp. 60-170°-programmed), it could be concluded that the fraction was rich in saturated aliphatic hydrocarbons.

Appearing potential of base peak at m/e 57 in eight mass spectra for CH_3-$(CH_2)_3$- fragment showed most likely branching at C_5, at more than one terminal. Though groups of peaks were spaced 14 mass units apart, an expected gradual decline of abundance with increasing fragment weight involved sharp breaks. From the above observation inference for branching of chain could be drawn[30] in which, better stabilization of secondary over primary carbonium ion is energetically favourable.

However, correlation with the known hydrocarbons proved difficult in view of the non-availability of computer data library.

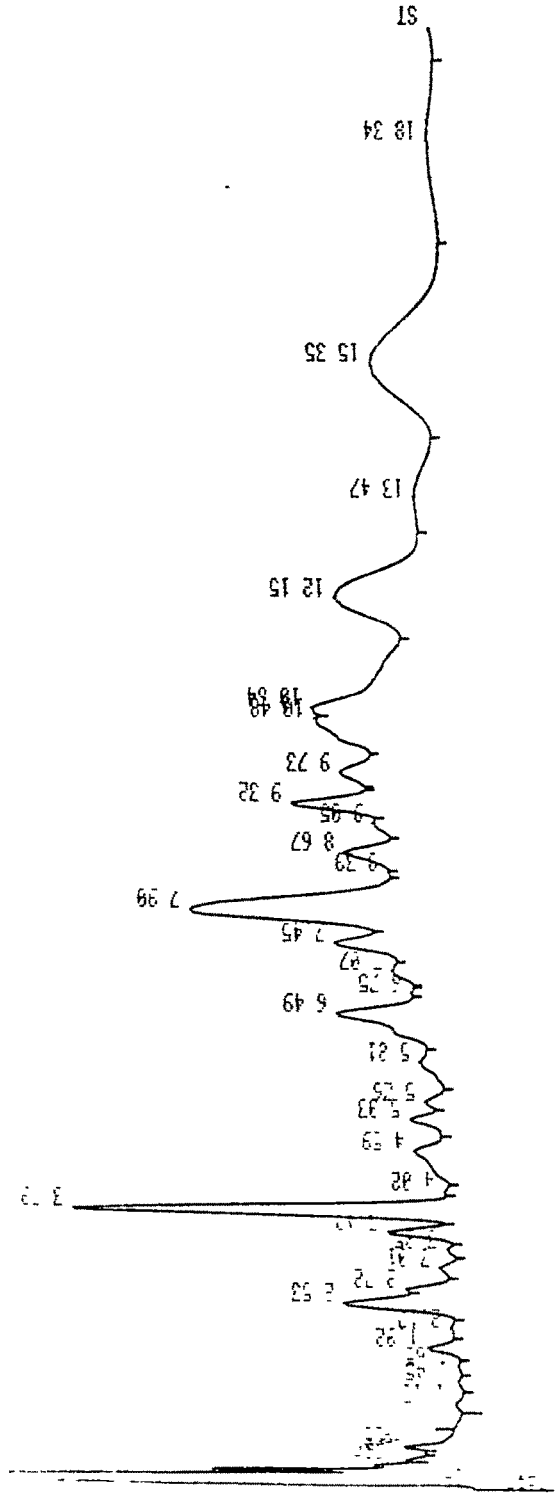


Fig. 8: GC of petroleum ether fraction of insoluble stone.

Dichloroethane and ethanol fraction

Fractions of dichloroethane (0.18%) and ethanol (0.37%) were found unimportant in the preliminary tests like incineration, solubility, ^{1}H-NMR and IR analysis.

As clarified by atomic absorption spectra this stone is calcium based with cations in ppm as follows- Na (4.73), K (14.0), Ca(182.7), Mg(33.8) and Cu(0.15).

Microbiological studies

Culture studies of crude Shilajit, till date, have shown the growth of thermophilic fungus and gram +ve bacillus, which multiply faster at 50^{o}C than at room temperature (30^{o}C).

Conclusions

A) Complexity: An overview of the above evaluation essentially shows complex mixtures at each stage. Rather it is difficult to answer such a complexity arising from vegetative origin, so that even a few milligrams of eluate either gives streak or a pattern of overlapping spots on chromatoplate.

B) Frequent exudations of Shilajit, especially in summer prompted us to look for the possibility of this material being a millenarian old sediment deposited under the rocks.

In this connection molecular paleontology has revealed a striking similarity in many such sediments[26] by way of

'biological markers'; which seem to be made up, principally, of microbial cell debris or derived from reworking of bacteria and fungi.

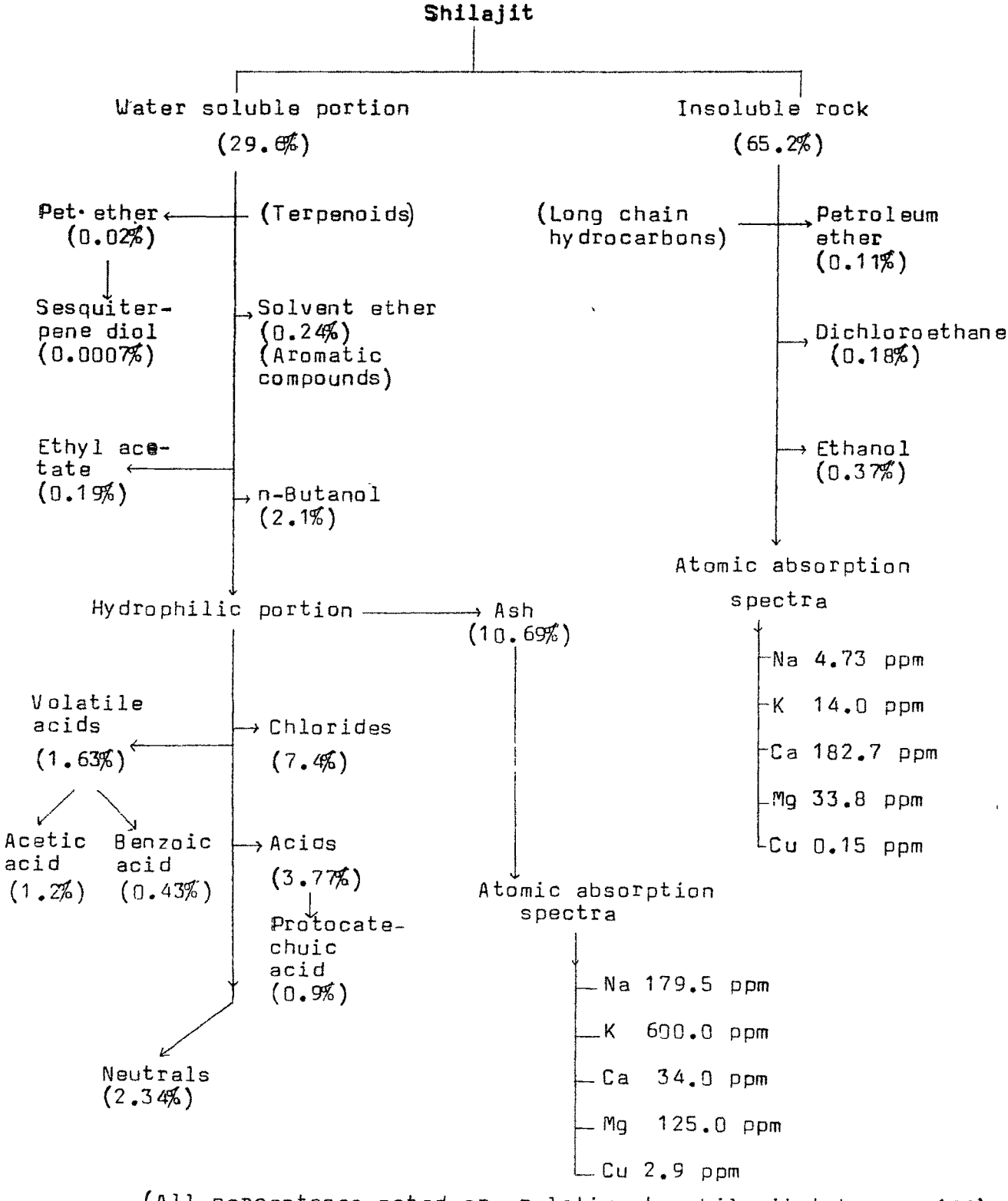
However, in the present study biological markers could not be traced. Rather it could be realized that screening for such infinitesimally small quantities from a complex material would require

i) initial molecular sieving[26] and

ii) direct comparison with the standard samples [25]

in order to preclude completely, the possibility of biological markers.

C) Composition: Percentage composition of Shilajit has been summarized in Fig. 9.



(All percentages noted are relative to shilajit taken as 100)

Fig. 9. Percentage composition of Shilajit

S E C T I O N - II

STRUCTURE ELUCIDATION OF COMPOUND A

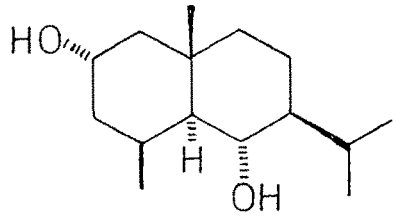
A B S T R A C T

Structure elucidation of (1α,2β,4aβ,6α,8β,8aα)-4a,8-dimethyl-2(1-methyl ethyl)-decahydronaphthalene-1,6-diol, has been described.

STRUCTURE ELUCIDATION

A solid compound (4.6 mg) was obtained, when light petroleum extract of the aqueous portion of Shilajit (Section-I) was fractionated on Chromatotron (see experimental). Slow crystallization from 30% ethyl acetate in petroleum ether furnished fine colourless needles (4 mg, 0.0007% by weight to Shilajit) which do not melt but sublime at ~160° (sealed tube).

Here we present relevant evidence under several headings which enabled us to assign structure [A] to the crystalline compound (Fig. 10).



A

Fig. 10: Structure of compound A.

Nature of the compound. Compound A on TLC gave bright violet colouration with anisaldehyde-sulphuric acid spray,[10] as expected for the terpenoids. R_f at 0.45 (solvent system: 80% EtOAc in pet. ether) was comparable to that of clovanediol. Negative tetranitromethane[31] and 2:4 DNP test indicated that the molecule was devoid of double bond or carbonyl function.

Also it can be seen from the following spectral analysis that an attempt was made to elicit maximum possible information with the perspective of material conservation; and without a single chemical transformation.

Compound A has the elemental constituion, $C_{15}H_{28}O_2$. (^{13}C-NMR: DEPT test; and m/e at 222 for $\overset{+}{M}-H_2O$).

FT-IR spectrum. Besides broad band at 3320 cm^{-1} for the O-H stretching, IR spectrum ($CHCl_3$) (Fig. 11) also exhibited strong O-H bending vibrations at 1135 cm^{-1} showing a strong possibility of secondary hydroxyl functions.[32] The characterstic absorption for other functional group was absent.

Number of hydroxyl functions: Resonance absorptions in ^{1}H-NMR (500 MHz FT ,$CDCl_3$) at 3.54 δ (triplet, 1H, J = 10 Hz) and 4.02 δ (triplet of triplet, 1H, J = 11Hz & 4 Hz) clearly depicted two H-C-OH groups. This conclusion could be further supported by ^{13}C-NMR (DEPT), where downfield signals at 64.2 and 69.1 ppm (methine carbons) were present. Also the loss of two water molecules in mass spectrum displayed an intense peak at m/e 204 (m/e 222-H_2O). With the above information in hand sequel called for the nature of carbon skeleton.

Carbon skeleton. ^{13}C-NMR spectrum of compound [A]($CDCl_3$) with proton broad band decoupling (Fig. 12) showed absorptions for 15 different carbons ranging from 15.4 ppm to 69.1 ppm (Table 1). Downfield signal for unsaturation or carbonyl function was absent which was in accordance with our earlier qualitative analysis.

Thus, a molecular formula of $C_{15}H_{28}O_2$ for saturated sesquiterpene diol enunciated that it should be bicyclic. The biogenesis of this group from precursor cis, trans-farnesyl pyrophosphate or nerolidyl pyrophosphate gives rise to a variety of bicyclic systems.[33] In order to pin-point one of these bicyclic systems, further elaboration of ^{13}C-NMR was necessary to distinguish carbons into methyl, methylene, methine and quaternary. An extensive study of the above spectrum towards this end, however, revealed some abnormal shifts. For example 5 signals appeared in the usual $-CH_3$ region instead of expected 4 (^1H-NMR). The same case could be observed for some methylene and methine carbons. Therefore the result had to be reconciled by the 'Distortionless Enhancement by Polarization Transfer' (DEPT experiment), conducted with the usual pulse sequence.[34]

The method led to distinguish differentially substituted carbons without having recourse to ^{13}C: 'Attached Proton Test (APT). Latter often causes severe overlap of multiplets with closely resonating carbons.

DEPT test (Figures 13 and 14) brought out the account of 15 carbons as 4 CH_3s, 4 CH_2s, 6 CHs and 1 quarternary* (Table 1). then it was noted that one CH_2 carbon was unusually shielded to

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* Low intensity of flipped signal at 51.2 ppm can be attributed to base line noise due to less sample. However, assignment of this signal to $-CH_2-$ becomes unequivocal, in view of the intensity in BB decoupled spectrum (Fig. 19). Quaternary carbons show weak resonance absorptions.

Table 1. ^{13}C-NMR shifts of Compound A .

Carbon number	DEPT assignments	Shift (ppm)
1	$-CH_2-$	51.2
2	-CH-OH	64.2
3	$-CH_2-$	43.7
4	$-\overset{\vert}{C}H-$	28.4
5	$-\overset{\vert}{C}H-$	(52.8)*
6	-CH-OH	69.1
7	$-\overset{\vert}{C}H-$	(51.8)*
8	$-CH_2-$	18.8
9	$-CH_2-$	42.9
10	$-\overset{\vert}{\underset{\vert}{C}}-$	36.5
11	$-CH_3$	(16.3)*
12	$-CH_3$	21.1
13	$-\overset{\vert}{C}H-$	26.4
14	$-CH_3$	21.5
15	$-CH_3$	(15.4)*

* Values in parentheses may be interchanged

resonate at 18.8 ppm together with two methine carbons at 26.4 and 28.4 ppm. While other CH_2 was downfield at 51.2 ppm along with two methine carbons at 52.8 and 51.8 ppm.

Keeping in mind the above values, different possibilities were considered to assign the type of carbon skeleton. However spiro (acorane, chamigrane), bicyclohexyl (laurane, cuprane) or bicyclic fused systems like eremophilane, himachalane, cyperane did suffer in many respects, especially for

(i) the required number of typical carbons
(ii) upfield value of one primary carbon (18.8 ppm) or
(iii) unsuitability to effect downfield shifts of two methine carbons with any placement of hydroxyl function.

An exhaustive but systematic exercise based on carbon shift arguments,[35] together with literature ^{13}C-NMR shifts[36] led to invoke eudesmane skeleton. The result could then be interpreted in favour of structure [A] (Fig. 15) by precise placement of hydroxyl functions as follows:

C-8 carbon. In the case of C-8 carbon two shielding effects are operating. One of it is a γ-effect of OH due to gauche arrangement with C-8 carbon, which necessarily is a shielding effect. Such effect is well studied in the cases of trans-decalols and 10-methyl-trans-decalols with shifts of 3.3 to 7.3 ppm upfield.[37] For example in (F), a comparable carbon appears at 20.4 ppm.

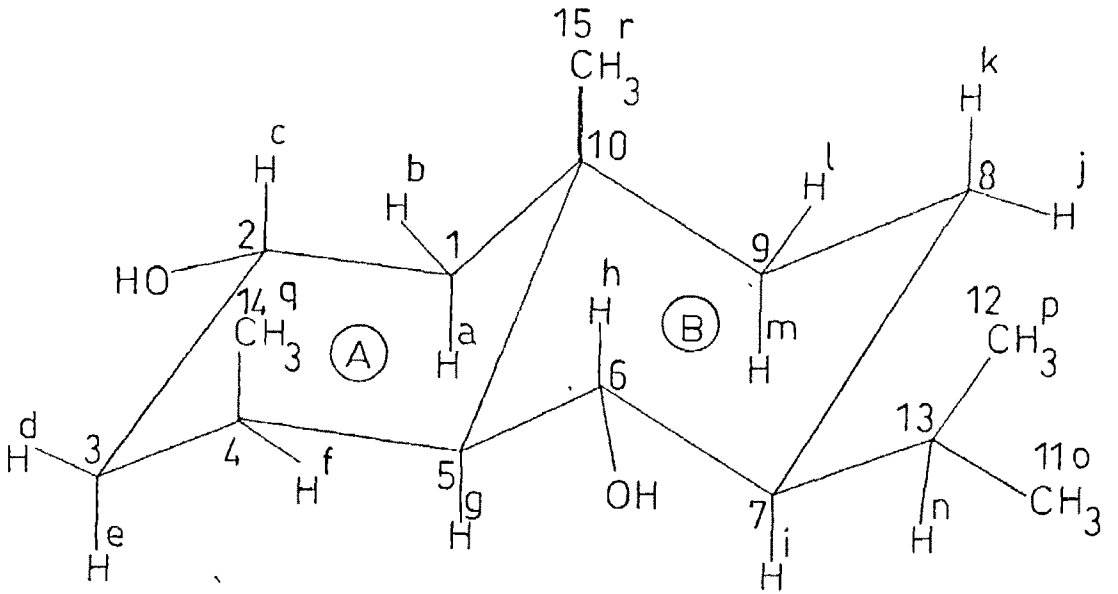
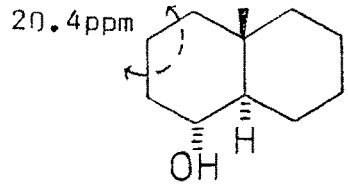


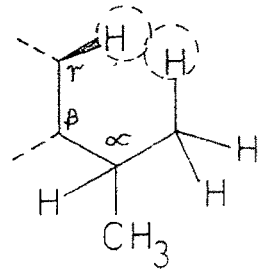
Fig. 15: Structure of Compound A *

* Connotations incorporated in Fig. 15 have been used in the discussion.



F

Another '4-bond removed' effect on C-8 can be envisioned. This is because of the fact that methyls of isopropyl group have also a γ placement with respect to C-8. Such spatial interactions are thought to arise by overlapping of Van der Walls radii of closely spaced hydrogens as shown in (G).[38] Thus a steric



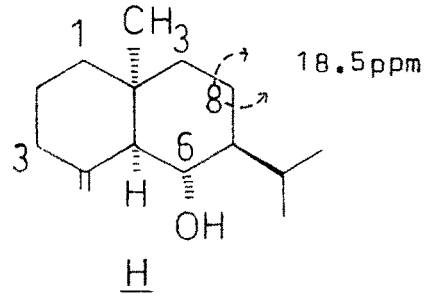
G

perturbation of C-H bond involved causes the charge to drift towards carbon with the expansion of bonding orbitals. Theoretically the effect can be calculated by the rule of additivity as under.

Substituent parameter of equatorial methyl, in cyclohexanes at γ-carbon is ca-0.6 ppm.[39] Therefore isopropyl group accounting for two such interactions should have shielding of 1.2 ppm. On

this ground with a base value of 20.4 ppm (vide supra), a shift at 19.2 ppm is expected, which is remarkably close to the value of 18.8 ppm.

Argument can be further supported by a comparable example of 10-epijuneol (H)[40] in which C-8 shift of 18.5 ppm is observed



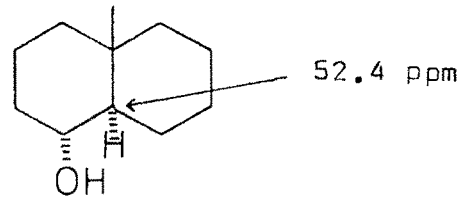
under the influence of β-isopropyl and α-OH functions. (A cis-fusion in 10-epijuneol would fractionally matter for C-8 carbon, as it is associated with the change of C-10 methyl to equatorial position).

Ring fusion. Geometry of ring fusion in decalin systems can be decisively concluded by ^{13}C-NMR. On the other hand ^{1}H-NMR values give only predictable picture, when two complementary isomers are available. Particularly notable value for cis-fusion is of structurally diagnostic methyl group at the ring junction which in case of trans-10-methyl decalols is less than 20 ppm.[41] In cis case a downfield shift at 27-28 ppm is observed. Furthermore, in 6,7-diequatorially substituted 10-methyldecalins trans-fusion exhibits downfield shifts for C-1 and C-9,[40] which has also been noted in our case.

C-5 and C-7-methines. Two downfield methine carbons at 52.8 and 51.8 ppm could be accounted by the placement of OH group at C-6. In such a case chemical shift of C-7 methine should be close to the carbon bearing isopropyl group in menthol (I).

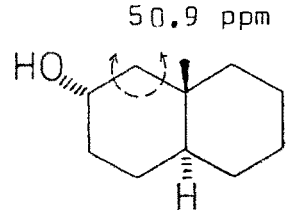
I

Indeed the value of 50.05 ppm[42] matches very closely to 51.8 ppm in compound [A] . Likewise C-5 snift of 52.8 ppm is in accordance with the observed value of 52.4 ppm for 4α-OH substituted 10-methyl-trans-decalin (J),[37] with minor increment from C-4 methyl.



J

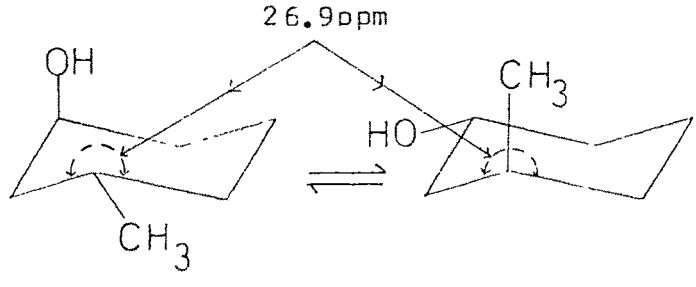
C-1, C-3 and C-9 methylene: It became imperative to place OH function at C-2 in order to accommodate only one downfield methylene carbon at 51.2 ppm. A reported practical model (K) is comparable with the ring (A) of compound A , which has been shown a similar shift of 50.9 ppm for C-1 carbon.[37] Also the



K

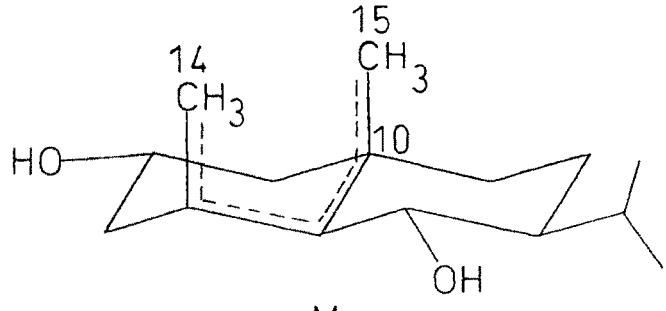
consequence of the above rationale led to assign ∝-configuration to C-2 hydroxyl. Downfield resonance for C-3 at 43.7 ppm and C-9 at 42.9 ppm have precedence of ref. 43 and 44 respectively.

C-4 Methine. Shift arguments as in the case of trans-3-methyl cyclohexanol[43] (C-3: 26.9 ppm, L) seemed reasonable for assigning the value of 28.4 ppm to C-4 methine carbon. Had hydroxyl been at C-3 then there would have been an increment at C-4 for further downfield shift in the range 35-40 ppm.



L

C-14 Methyl. Augmented value for C-14 methyl at 21.4 ppm seems more reasonable than other available values of ~15-16 ppm. Origin of such effect is attributable to syn-axial methyl-methyl-interactions (equivalent to gauche) which are accompanied by marked downfield shifts (M).



M

The fact has been noted earlier and violates the general premise which associates steric crowding with the upfield shifts.[37]

Other carbon values for methyls and methine of isopropyl are of general nature; hence not discussed. Thus it can be seen that all carbon shieldings given in Table 1, hold good with the assignments and lend strong support to the structure [A].

The above interpretations get reinforced by the high field ^{1}H-NMR spectrum.

^{1}H-NMR spectrum (500 MHz, FT). ^{1}H-NMR ($CDCl_3$) had to be recorded on Fourier Transform mode to obtain well-resolved spectrum with 1.5 mg of the sample (Figs. 16 and 17). Data as in Table-2, showed three doublets centred at 0.87 (3H, J = 7Hz), 0.95 (3H, J = 7 Hz) and 1.02 (3H, J = 8Hz) δ for the secondary methyls

$-CH_3^p$, CH_3^o (or vice versa) and CH_3^q respectively. A merged singlet at 0.94 δ (3H) was assignable only for the quaternary methyl CH_3^r. One interesting point to note was a significant coupling of CH_3^q with H^f (J = 7Hz) to give a doublet. This not only confirmed the trans-ring junction for decalin system but firmly established that the methyl at C-4 is β-configurated. Had it been α i.e. in trans-anti disposition. Then the splitting of methyl should have been immeasurable. Related studies as carried out by E.M. Banas et al.[45] also reveal the possibility of cis-anti-1 or cis-syn-1 conformations, which can give similar coupling constants (Fig. 18). The latter possibility of ring fusion has been ruled out on the basis of ^{13}C-NMR shifts.

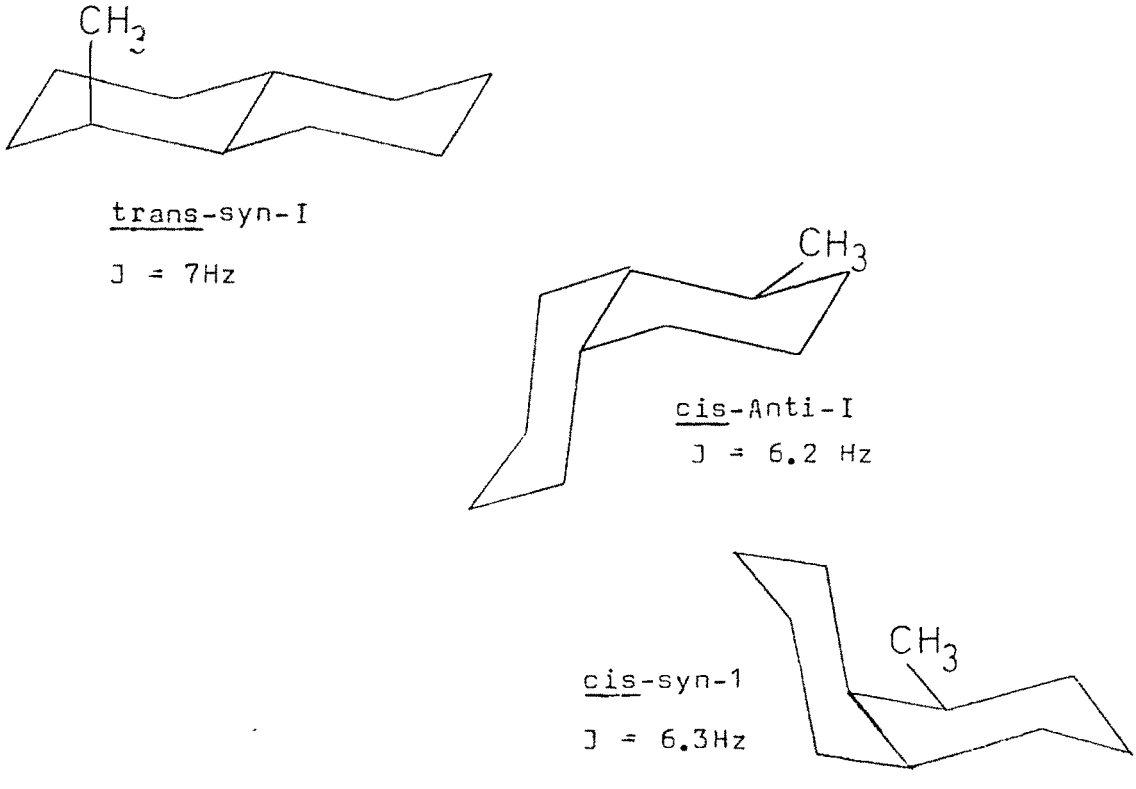


Fig. 18: Different ring fusions and coupling constant of methyl in 1-methyldecalins.

Resonance absorptions at 3.54 δ (triplet, 1H, J = 10Hz) and 4.02 δ (triplet of triplet, 1H, J = 11 & 4 Hz) for H^h and H^c respectively bear out two secondary hydroxyls and that they are not coupling with a common proton. Splitting of H^c by vicinal CH_2s is expected because of the bonding of latter with the chiral centres C-10 and C-4, leading to magnetic non-equivalence (MNE). Coupling constants of the order of 10 Hz place H^h and H^c axially.

Olefinic, acetylenic or protons α to carbonyl were absent, which was in accordance with our earlier conclusions. Other protons were seen to give either broad doublets or multiplets but precluded further unambiguous assignments. Therefore a more refined technique was called for.

2D NMR- ^1H-^1H correlation (500 MHz FT, homonuclear):

It has been shown very recently[46] that the complexity of structure elucidation can be, in large part, solved by 2D NMR spectroscopy. This technique involves two simultaneous pulse experiments, with two frequency scales at right angles to each other and records exchange of magnetisation between two nucleii in other dimension. In short, the experiment correlates different nucleii interacting may be through bond-J-coupling, through space dipole-dipole effects or chemical exchange.

Here as a first step, we chose ^1H-^1H homonuclear-shift-correlation programme

(500 MHz, FT) ($CDCl_3$) to scan contour map: COSY(AU), as shown in the Fig. 19. The experiment enabled us to confirm earlier assignments and establish some others for typical protons as follows.

It was confirmed by connectivities that CH_3^p and CH_3^o are methyls of isopropyl group due to the coupling with a common proton appearing at 2.15-2.24 δ (1H, multiplet). Sequel was the assignment of H^h to the above multiplet. Though value of H^n appears quite downfield, such deshielding of isopropyl tertiary proton has also been observed by T. Ohmato et al.[47] in a comparable structure. Proton H^f could be recognized by its connectivity with CH_3^q and hence multiplet at 2.4-2.49 δ was attributed to H^f.

Furthermore CH_3^q protons were observed to couple with one more proton centred at 1.76 δ (quartet of broad doublet, J = 10 and 2Hz). Such interactions can only be explained via 'W coupling'. Similar four bond extended W pathway of scalar couplings are commonly observed in saturated cyclic compounds with fixed configurations even when free rotating methyl groups are involved.[28] Though in our case two protons, namely H^e and H^g can exhibit such W conformation; choice for H^g being the above proton seemed more tenable in view of the observed J value of 10 Hz, which is equal to the one in a triplet split of H^h. Further multiplicity giving quartet (J = 2Hz) suffices our reasoning. However, inability of such a proton to couple with H^h in COSY could not be explained.

Table 2. ^{1}H-NMR shifts of Compound [A]

Proton	Shift (δ)	Multiplicity, J Hz)
CH_3^p	0.87	d, 3H, J = 7Hz
CH_3^r	0.94	s, 3H
CH_3^o	0.95	d, 3H, J = 7Hz
CH_3^q	1.02	d, 3H, J = 8Hz
CH_2s, CHs,-OH(2)	1.1-1.55	m, 10H
H^g	1.76	q bd, 1H, J = 10Hz & 2Hz.
H	1.94-2.01	m bd, 1H, J = 14Hz
H^n	2.15-2.24	m, 1H
H^f	2.4-2.49	m, 1H
H^h	3.54	t, 1H, J = 10Hz
H^c	4.02	tt, 1H, J = 11Hz & 4Hz.

Solvent: $CDCl_3$ with TMS as internal standard

Proton at 1.98 δ (broad doublet, J = 14Hz) appeared to show strong geminal coupling and connectivity at 1.34 δ. However, further conclusions could not be drawn in view of strong proton interactions in the region of 1.1 to 1.55 δ. Rigorous examination for defined splitting pattern in the area proved unwieldy mainly because of overlapping signals. Indeed, such complex multiplicities can arise in systems like decalins where apart from bonded interactions, secondary non-bonded effects like peri-effects[48] (between protons on C-1 and C-9), 1,3-diaxial interactions and W-couplings[28] are also pronounced.

2D NOESY (AU): In order to examine the possibility of molecular deformations and subsequent dipolar effects, 2D NOESY (AU) experiment was conducted with symmetrized matrix (Fig. 20). Contour display comparable to that of COSY, in all the probability stands for the restrained entropy of a rigid system. On the other hand in cis-decalins substituents like methyls are free to rock between the axial and equatorial conformations. While trans-decalins exhibit a better conformational locking.

GC/MS (Fig. 21): Mass spectrum albeit devoid of molecular ion peak showed fragment ion at m/e 222 corresponding to the loss of a water molecule. Such facile dehydrations are especially noticeable in cyclohexanol moieties.[30] Furthermore, it is reasonable to assume the highest abundance of fragment ion m/e 161 arising from complete dehydration followed by cleavage at the bulky isopropyl group. Structure [A] is likewise supported by some important electron impact-induced fragments as shown in Fig. 22.

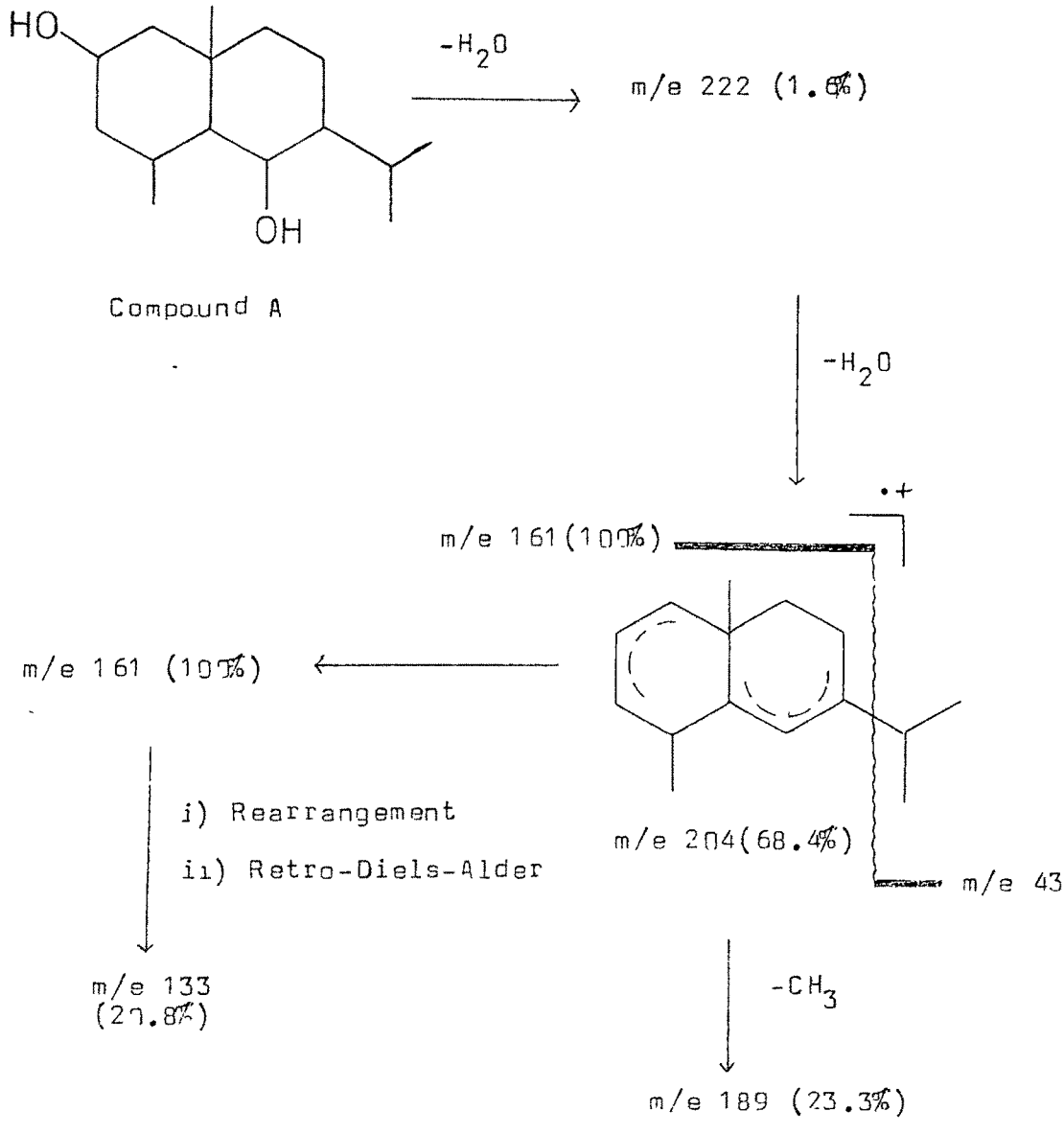
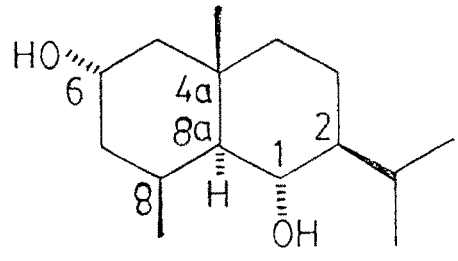


Fig. 22: Fragmentation of A in the mass spectrum

All the above data entwines the decisive evidence for the structure [A] - (1α, 2β, 4aβ, 6α, 8β, 8aα)-4a,8-dimethyl-2(1-methyl ethyl)-decahydronaphthalene-1,6-diol. (IUPAC Nomenclature)



A

IUPAC Numbering

However, it will be unrealistic not to consider some perturbations due to likely ring-compression effects and hence the interchangeability of ^{13}C assignments with close values. Further sequential rarefaction by ^{1}H-^{13}C heteronuclear correlation, carbon-carbon connectivity experiments (2D ^{13}C: COSY) and finally single crystal X-ray forms subject matter for our ongoing research.

EXPERIMENTAL

For General Methodology, refer Experimental of Chapter I.

The following instruments were used for the specific spectral/analytical data.

Brucker, AM-500, 500 MHz FT spectrometer (^{1}H-NMR); Homonuclear shift-correlated 2-D ^{1}H-^{1}H: NMR (COSY.AU), with unsymmetrized matrix and homonuclear dipolar-correlated 2-D ^{1}H-^{1}H: NMR (NOESY.AU), with symmetrized matrix were run on the same instrument (AM-500) using Aspect 3000 (DISB 861) software at Tata Institute of Fundamental Research, Bombay, India. FT-IR and ^{13}C-NMR (Broad band decoupling and DEPT) were recorded at the Department of Chemistry, Harvard University, Cambridge, Massachusetts, USA.

Hitachi-2-8000, Polarized Zeeman Spectrometer was used for obtaining atomic absorption spectra at National Chemical Laboratory, Pune, India. GC/MS spectra were recorded on Finnigan-Mat-1020 (70 eV, glass column, SE 54, carrier gas-helium) at National Chemical Laboratory Pune, and on Hevelett-Packard 39858 (70 eV, methyl silicone column, carrier gas- helium) at Indian Petrochemicals Corporation Limited, Vadodara, India. Chromatotron by Harrison Research 840, Moana Court, Palo Alto, California, USA was used for separation.

Microbiological studies were carried out at the Department of Microbiology, M.S. University, Baroda, India.

Aqueous extraction of crude Shilajit

Authentic crude Shilajit (Kora Shilajit) procured from 'Singh Darbar Vaidyakhana', Kathmandu, Nepal was crusned into small pieces (~1 cm x~1 cm)in pestle and mortar, 540 g of these pieces, were soaked in demineralized water (D.M. Water) at 60°C and extracted repeatedly with 250 ml of water each time to give ∿4.5 L of brown coloured aqueous extract. Insoluble rock weighed 352 g (65.2%).

Water soluble portion was carefully concentrated on rotary evaporator at 60°C/90 mm to 700ml. From the aliquot of this concentrate, soluble solids were found to be 160 g (29.6%). Distillate was of basic pH: 8-9.

Solvent extractions of aqueous extractive

The above concentrate was successively extracted by shaking vigorously in separatory funnel with the solvents of upgrading polarity as given in Table 3.

Table 3. Organic solvent extracts of the aqueous concentrate.

Entry No.	Solvent	No. of extractions x volume (ml)	Wt. of fraction (g)
1	Pet. ether	4 x 150	0.110
2	Solvent ether	6 x 200	1.27
3	Ethyl acetate	6 x 200	1.00
4	n-Butanol	7 x 200	11.3061

Highly hydrophilic portion left after solvent extraction was estimated for ash contents.

Quantitative and qualitative estimation of ash: Soluble solids from the above part (0.5105 g) were incinerated in silica crucible till constant weight (12 hr), to give 0.2457g of grey coloured ash (47.7%; i.e.10.69% by wt. to crude Shilajit).

This ash was dissolved in conc. HCl (2 ml; analytical grade) and diluted to 25 ml with D. M. water. Estimation of this solution was carried out by atomic absorption spectra to give cations in ppm as follows.

Na^{+} - 179.5 ppm, K^{+}-600 ppm, Ca^{+2} -34 ppm, Mg^{+2}-125 ppm and Cu^{+2} 2.9 ppm.

Multiple ion exchange separation (Fig. 2)

a) Treatment with strong cation exchanger. Aqueous concentrate (pH=7) was diluted to 1.5 l. 300 ml of this solution (solid contents = 30 g) was doubly diluted to 600 ml and then passed over regenerated strong cation exchange resin column (IR-120; wet volume- 480 cc; column - 35 cm x 3.6 cm). Eluate was acidic (pH=2). Complete elution was effected by passage of 300 ml D.M. water, 500 ml methanol followed by 250 ml ethyl acetate to give neutral eluate. Solvent was removed in rotary evaporation ($60^{o}C$/90 mm) to furnish acidic distillate (pH-2). Water was repeatedly (4 x 25 ml) added to the residual portion and distilled off to give neutral distillate and 15.5 g of brown residue.

Separation of hydrochlorides and chlorides: Above resin column was slowly eluted with 5% aqueous HCl (analytical grade, 1 L). Eluate was concentrated on rotary evaporator ($60^{o}C$) to give brown coloured solid which was made completely neutral by repeated addition and distillation of D.M. water. Thus adsorbed material was obtained in the form of hydrochlorides and chlorides (12.0 g).

b). Treatment with weak anion exchanger for the separation of neutrals: Decationized brown mass (15.5 g) was soluble in methanol. Therefore its solution in methanol (150 ml) was passed over a column of regenerated weak anion exchanger (IR-45; 46 cm x 2.8 cm) and eluted with methanol (1.5 l) to furnish, after solvent removal on rotary evaporator ($50^{o}C/90$ mm), 3.8 g of neutral material as a brown crust.

Acids adsorbed on IR-45 resin column were desorbed as follows:

Separation of acids: In order to avoid breaking of the column due to evolution of CO_2, above exhausted IR-45 resin was taken out in a beaker and treated in cold with 1 N Na_2CO_3 solution (400 ml). Acids were desorbed in the form of sodium salts with the evolution of CO_2. Supernatant solution was decanted after standing for 1 hr. Procedure was repeated with more portions (3 x 150 ml) of 1 N Na_2CO_3 solution for complete desorption.

Solution of sodium salts thus obtained was treated with regenerated strong cation exchanger (IR-120; 400 ml wet volume) in a beaker. CO_2 evolution could be seen due to excess of Na_2CO_3. Mixture was allowed to stand for 3 hr and decanted. Resin was repeatedly washed with D.M. water (Total ~ 1 L) till neutral. All washings were pooled (pH= 6) and water was removed on rotary evaporator (60^oC/90 mm) to furnish 6.1 g of acids.

Acetylation and esterification of neutral fraction

Neutral fraction (1.0 g) was acetylated by dissolution in dry pyridine (17 ml, refluxed and distilled over KOH) followed by addition of freshly distilled acetic anhydride (12 ml) in cold. Reaction mixture was then stirred at room temperature (30^oC) for 24 hr. Excess of acetic anhydride of pyridine were distilled off (100^o oil bath 60 mm). Residue was taken in ethyl acetate, washed with water (3 x 30 ml), 10% $CuSO_4$ solution (2 x 25 ml), water (2 x 25 ml), brine (2 x 20 ml) and finally dried over anhydrous Na_2SO_4 to afford 0.5053 g of acetylated product.

0.5 g of the above product was dissolved in chloroform (15 ml) and esterified with 30 ml of ethereal diazomethane solution (prepared by using 1.5 g of nitrosomethyl urea in usual way at $0-5^oC$). Mixture was tested for excess of diazomethane and left in cold for 2.5 hr. Solution was then filtered after attaining room temperature through Whatman No. 1. filter cone. Residue was repeatedly washed with chloroform (5 x 3 ml) and

pooled with the filtrate. Solvent removal furnished 100 mg of esterified product.

34 mg of the above acetylated-esterified product on bulb distillation furnished 5 mg of viscous straw coloured liquid.

Oil bath temperature $^{o}_{C}$	Pressure (mm)	Weight (mg)
150-230	0.5-1.0	5.0

GC of distillate (Fig. 3).

Product thus obtained was subjected to GC/MS analysis.

Isolation of protocatechuic acid from acid fraction

Acid fraction (5.5 g) was repeatedly treated with hot ($50^{o}C$) dry methanol (5 x 10 ml) and filtered through Whatman filter cone. Residue was again washed with (2 x 10 ml) of hot methanol to give brown coloured filtrate. Solvent removal furnished 4.5 g of methanol soluble fraction.

0.7 g of methanol soluble acid fraction was well-triturated with 5 ml of hot water ($70-80^{o}C$) and filtered over ordinary filter cone. Procedure was repeated and final wash (2 ml) left stick brown residue (0.4589 g). Filtrate was then taken on rotary evaporator and removal of water furnished 0.239 g of water soluble acids which were column chromatographed after adsorption on silica gel (0.5 g) as given in Chromatogram I.

Chromatogram-I

Flash Chromatography

Column: Silica gel, IIIB Wt. of silica gel: 14.5 g

Column length: 24 cm Column diameter: 1.0 cm

Flow rate: 30 ml/min.

Fr. No.	Solvent system	Vol. collected (ml)	Wt.(g)	Remarks
1	Pet. ether	3 x 100	0.007	
2	5% EtOAc in pet. ether	3 x 50		
3	10% -do-	4 x 20	0.0150	
4	15% -do-	11 x 20	0.007	Protocatechuic acid R_f: 0.43 50% EtOAc in pet. ether
5	25% -do-	4 x 100	0.0200	
	50% -do-	5 x 50		
6	75% EtOAc in pet ether	4 x 50	0.0740	
	100% ethyl acetate	4 x 50		
7	50% MeOh in EtOAc	2 x 50	0.0700	
	Methanol	2 x 50		
			0.2020	Recovery

TLC pure fraction 4 was characterized as protocatechuic acid by ^{1}H-NMR.[15]

^{1}H-NMR (DMSO-d^6 + $CDCl_3$) δ : 7.86 (s, 2H, -OH, D_2O exchangeable), 7.44 (s, submerged, 1H, ortho to -OH and -COOH), 7.38 (d, 1H, Ortho to -COOH, J = 8Hz), 6.81 (d, 1H, meta to -COOH, J = 8Hz).

Coinjection of total acidic fraction with the authentic protocatechuate ester: Protocatechuic acid for this purpose was prepared according to Ref. 16 from vanillin. Authentic acid

thus obtained (m.p. 200°C) was esterified together with the aliquot of methanol soluble acids using ethereal solution of diazomethane. Reaction mixtures were kept in cold for 24 hr and monitored by TLC. Products thus obtained could be coinjected on GLC as shown in the Fig. 23.

Steam volatile acids

Normality of the acidic distillate: Acidic aqueous distillate obtained during decationation (Fig. 2) was titrated against 0.05 N NaOH solution using phenolphthalein as an indicator.

Table 4. Normality of the aqueous distillate

Vol. of distillate - 25 ml Normality of NaOH- 0.05 N.

Reading No.	Burette reading (ml)	Mean burette reading(ml)
1	18.8	
2	19	19
3	19	

$N_1V_1 = N_2V_2$ $0.05 \times 19 = N_2 \times 25$, $N_2 = 0.038$ N

PTC esterification of aqueous distillate: Acidic distillate (80 ml) was neutralized with 10% KOH (2.1 ml) and concentrated on rotary evaporator to 3 ml. To this was added 2.1 g of n-cetyl-trimethylammonium bromide, 6 ml of dichloromethane and 6 ml of

methyl iodide (excess): and stirred magnetically for 14 hr at 27°C.

Organic phase was separated. Aqueous phase was repeatedly washed with dichloromethane (2 x 20 ml). Organic portions were pooled, washed thoroughly with water (5 x 25 ml), brine (20 ml) and dried over anhydrous Na_2SO_4. Careful solvent removal on Perkin triangle furnished 82.0 mg of crude product which on passage through silica-gel gave 90% pure methyl benzoate (60 mg, coinjection).

R_t: 9 min (10% SE 30, 100°C)

Preparation of K-salt of aqueous distillate for ^{1}H-NMR:- 80 ml of acidic distillate was carefully neutralized with 5% KOH (4.2 ml). Water was removed on rotary evaporator to give K-salts as white powder. It was further dried in drying piston and then ^{1}H-NMR (D_2O) was recorded.

^{1}H-NMR (D_2O) (Fig. 5) δ : 8.1-7.6 (m, 6mm), 7.7-7.38 (m, 9mm), 2.0 (s, 25 mm).

Ratio of K-acetate to K- benzoate is 2.77:1 from proton integration.

Therefore, 80 ml of acidic distillate contains 55 mg of benzoic acid and 153 mg of acetic acid. Normality contribution by acetic acid - 0.0319 N. Normality contribution by benzoic acid - 0.0056 N.

Organic solvent extracts of aqueous portion

Light petroleum extract:

GC/MS of this fraction is shown in Fig. 6.

Fractionation on 'Chromatotron: 110 mg of the above portion was loaded on rotary plate and separation was effected by multiple solvent development and step gradient elution under N_2 blanket as in Chromatogram II.

Chromatogram II

Plate thickness: 1 mm Adsorbent: SiO_2-TLC grade

Activation: 110°C/2 hr Flow rate: 4 ml/min

Frc. No.	solvent system	Volume (ml) each	Weight (g)	
1-5	7% EtOAc in pet. ether	10	0.0023	
6-18	-do-	10	0.0030	
19-24	-do-	10	0.0110	
	Disc completely dried under N_2 flow (2 hr).			
25-33	15% EtOAc in pet. ether	10	0.0120	
34-44	-do-	10	0.0120	
	Disc completely dried under N_2 flow (2 hr)			
45-50	30% EtOAc in pet. ether	12	0.0046	← Pure
51-55	-do-	12	0.0095	R_f: 0.45 in 80% EtOAc in pet. ether

Chromatogram II (contd.)

	Disc completely dried under N_2 flow (2 hr)		
56-59	50% EtOAc in pet.ether	12	0.0064
60-65	-do-	12	
	Disc completely dried under N_2 flow (2 hr)		0.0400
66-75	70% EtOAc in pet ether	12	
			0.1009 recovery

Fractions 45 to 50 were pooled and solid thus obtained was recrystalized from 30% EtOAc in pet.ether (0.45^{ml}) to furnish 4.0 mg of needles.

This crystalline material (R_f: 0.45, 80% EtOAc in pet.ether) gave violet spot on spraying with anisaldehyde sulphuric acid.

Analysis of insoluble stone

Residue left after water extraction (352 g) was packed in a filter paper thimble and exhaustively extracted in soxhlet, successively with hot petroleum ether, dichloroethane and ethanol as given below.

No.	Solvent	Time (hr)	Weight (g)
1	Pet. ether	64	0.60
2	Dichloro ethane	64	0.96
3	Ethanol (denatured)	24	2.00

Petroleum ether fraction: This waxy fraction melted in a range of 76-80°C. It was distilled in a bulb distillation unit at reduced pressure.

Distillation: Material taken for distillation: 160 mg

Oil bath temperature °C	Pressure	Wt of distillate
170-300	0.25-0.15	102 mg
	Residue	47 mg
	Loss	11 mg

Above distillate was analyzed for GC/MS.

Atomic absorption spectra of stone: Stone was incinerated in silica crucible to a constant weight (24 hr) and then standard solution was prepared as for earlier experiment.

Analysis in ppm - Na(4.73), K(14), Ca(182.7), Mg(33.8) and Cu (0.15).

SECTION II

Structure elucidation of compound A

FT-IR ($CHCl_3$) cm^{-1} (Fig. 11): 3320 (O-H stretch), 2957-2931 (C-H stretch), 2361, 2337, 1135 (O-H bend, secondary).

GC/MS : R_t : 4.7 (Column - Methyl silicone crosslinked, cap. col., 50M x 0.2 mmid, column temo 280°C)

Mass: m/e 222 (M^+-H_2O, 1.6); 161(100), 55 (85.9), 81(84.9), 204 (68.4), 69 (62.8), 57(53.9),95(53.9), 67 (51.0).

^{13}C-NMR data: Table 1

^{1}H-NMR data : Table 2

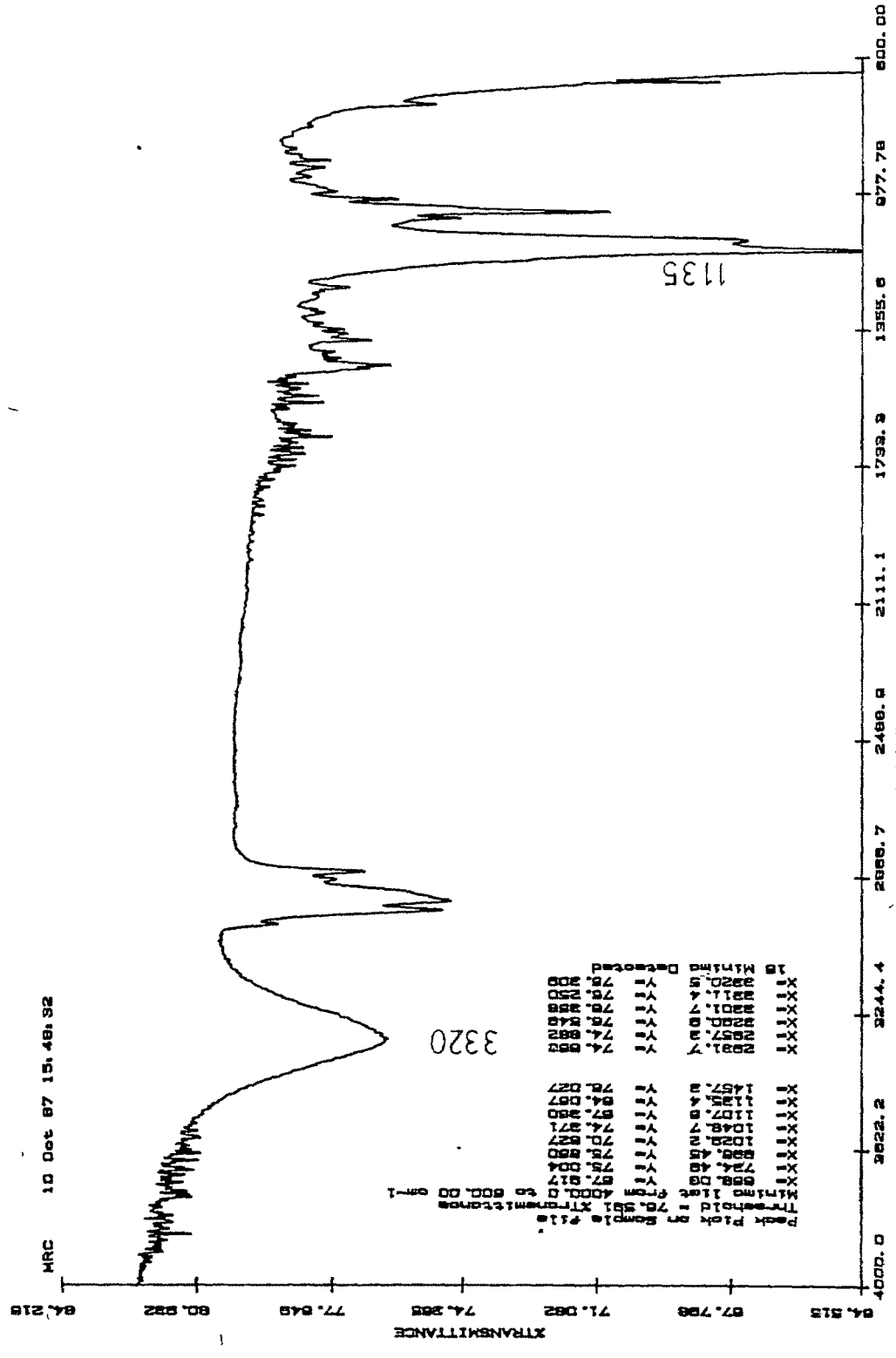


Fig. 11: FT-IR spectrum of Compound A

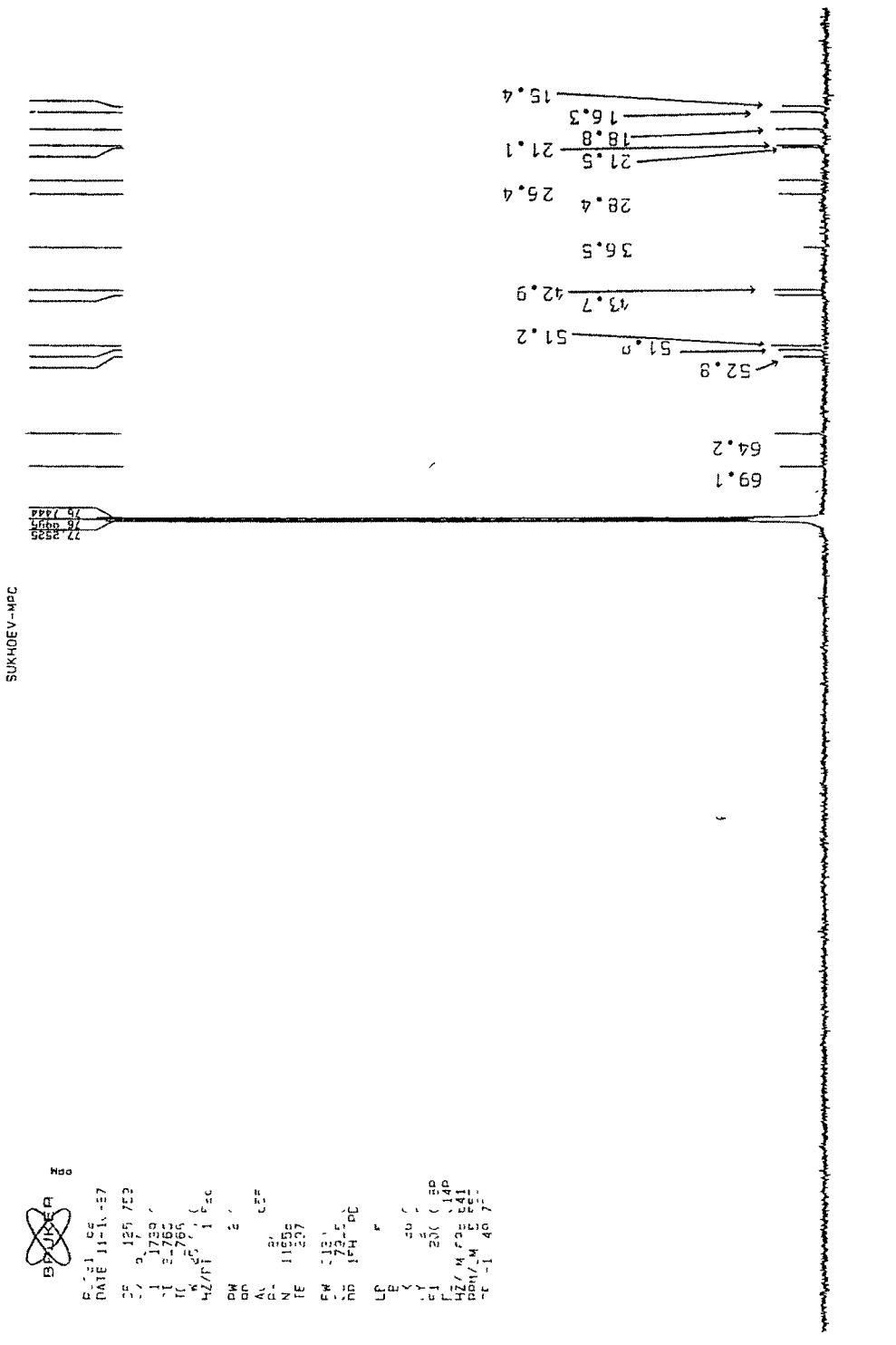


Fig. 12: ^{13}C-NMR (broad band decoupling) spectrum of compound A

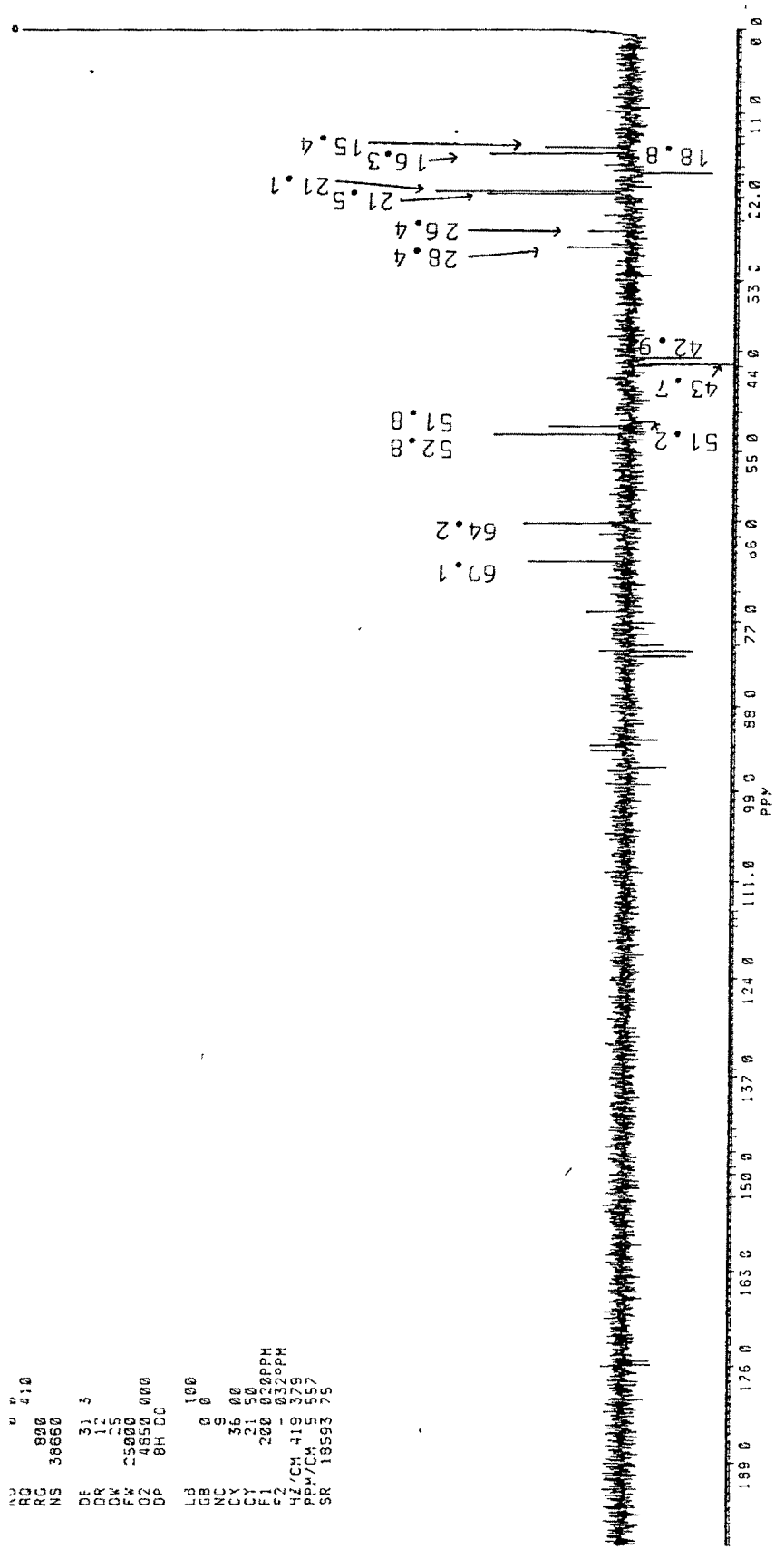


Fig. 13: ^{13}C-NMR DEPT spectrum (1-200 ppm) of compound A.

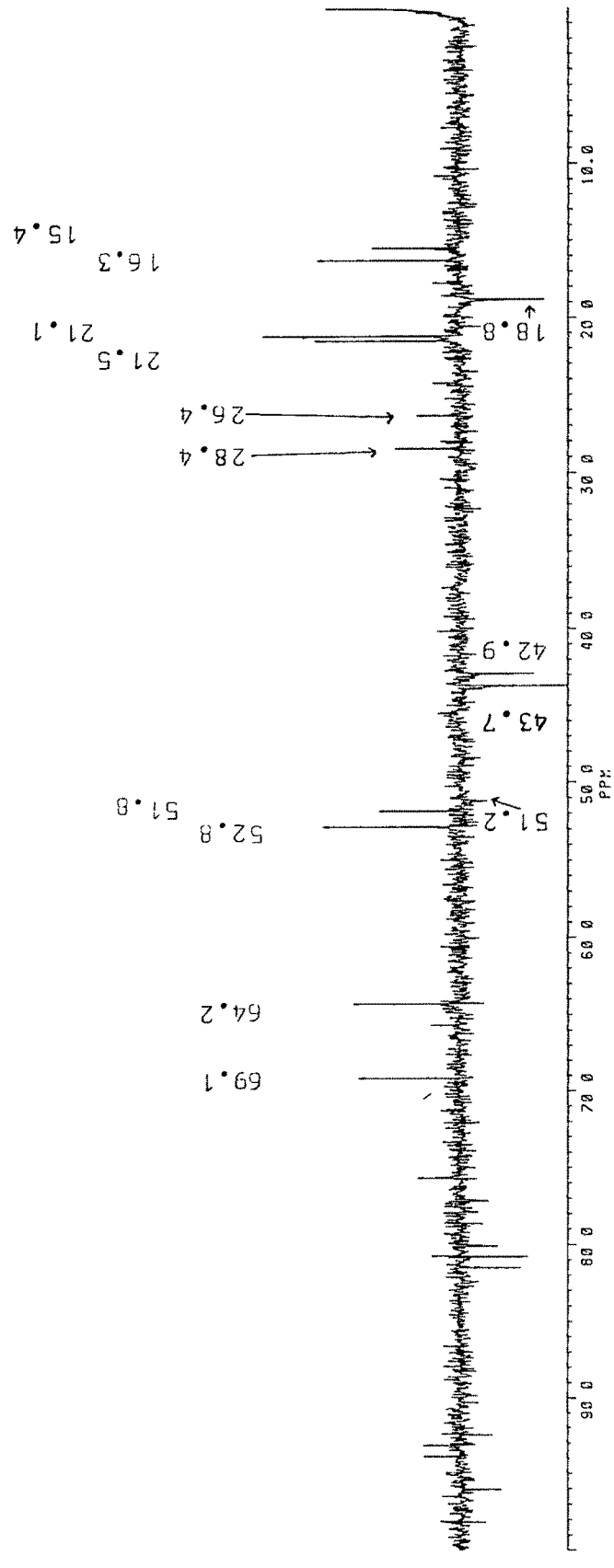


Fig. 14: ^{13}C-NMR: DEPT spectrum (1-100 ppm expanded) of compound A

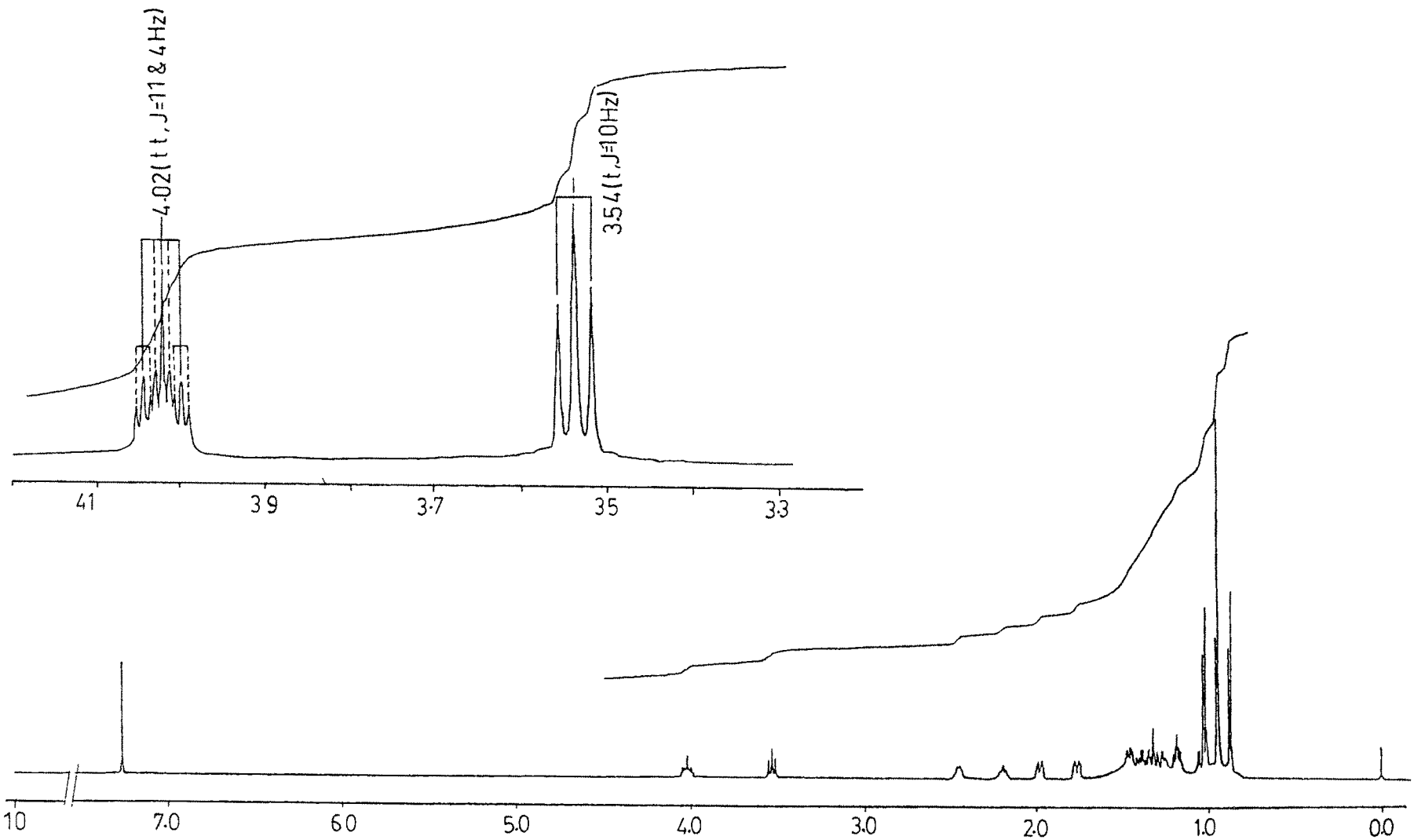


Fig. 16: ^{1}H-NMR spectrum (500 MHz, FT, 3.3-4.1 δ expanded) of compound A

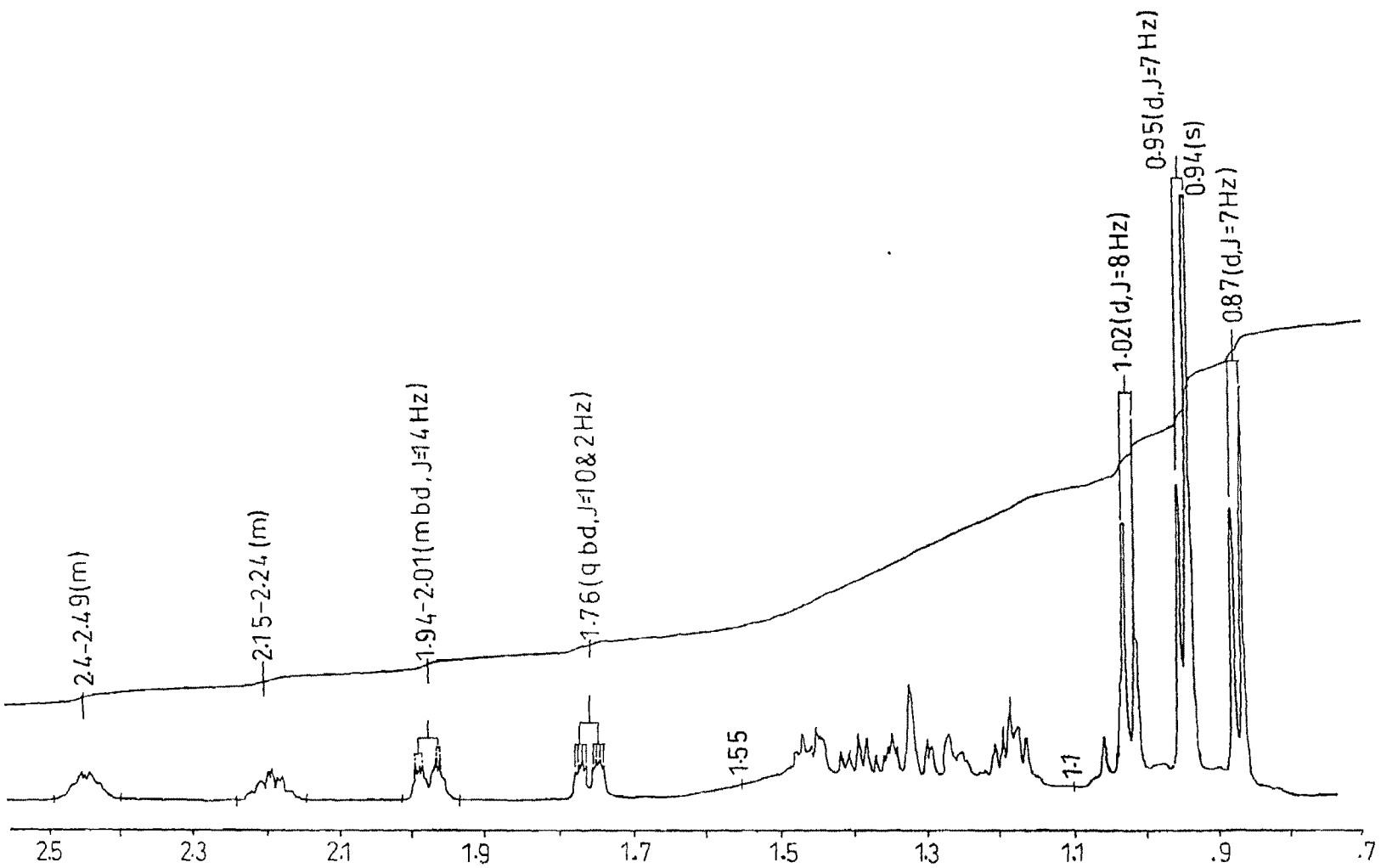


Fig. 17: ^{1}H-NMR spectrum (500 MHz, FT, 0.7-2.5 δ expanded) of compound A

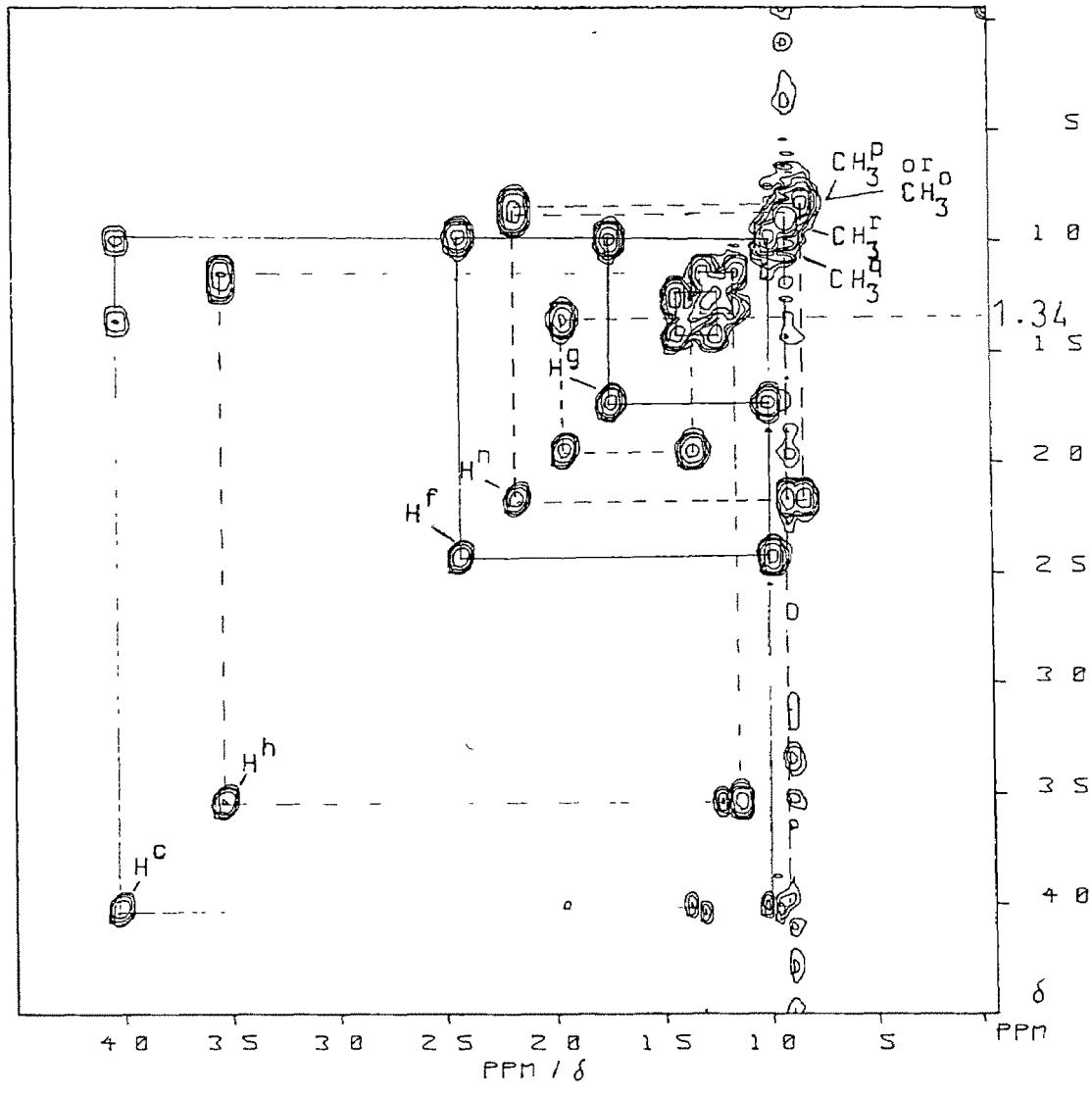


Fig. 19: 2D-NMR $^{1}_{H}$-$^{1}_{H}$- homonuclear correlation spectrum COSY (AU)

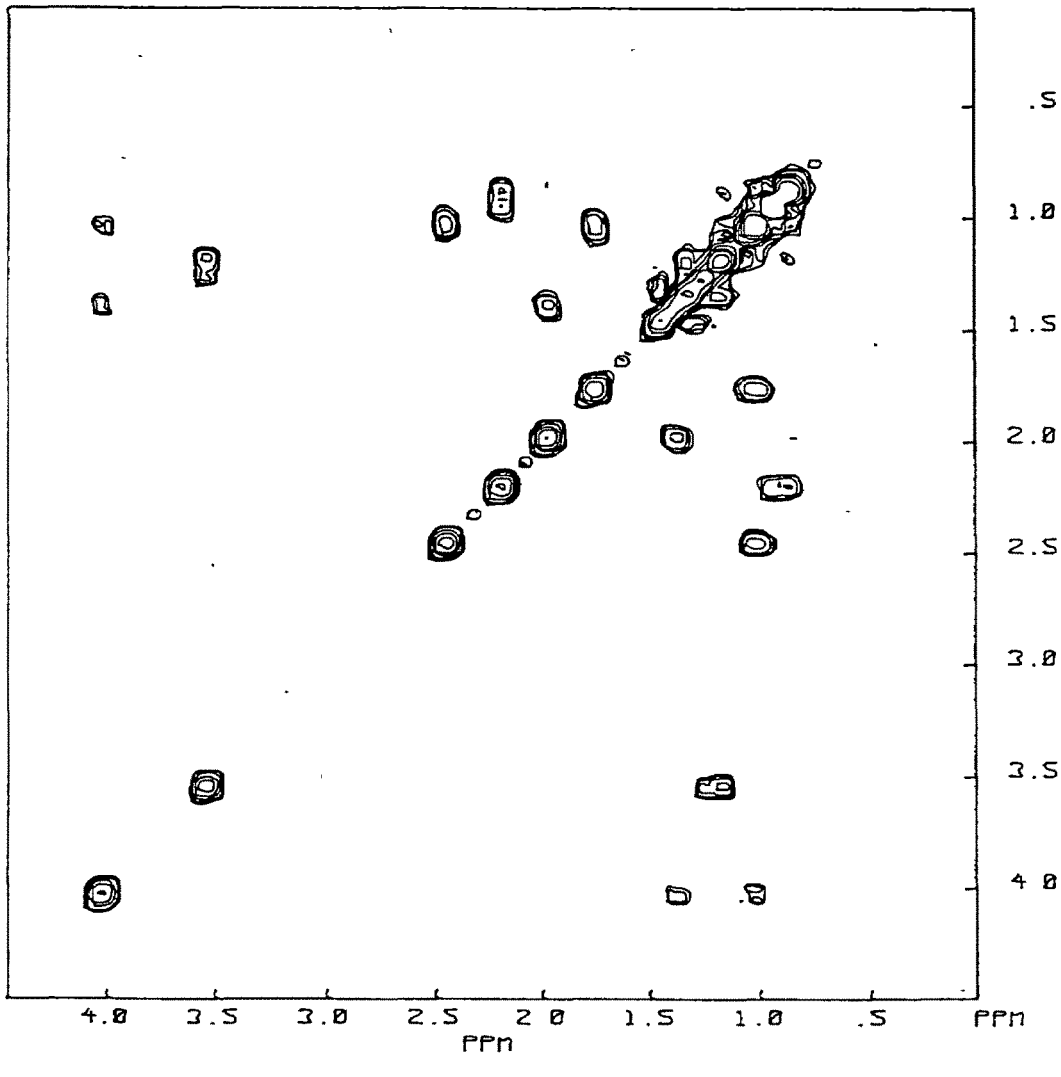
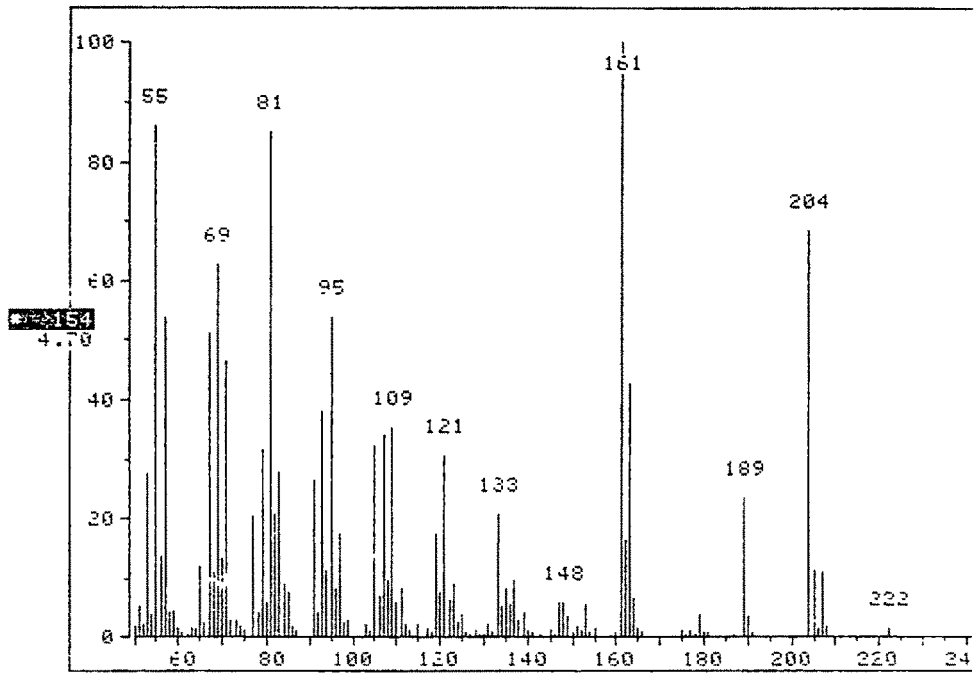


Fig. 20: 2D-NMR- NOESY (AU) spectrum



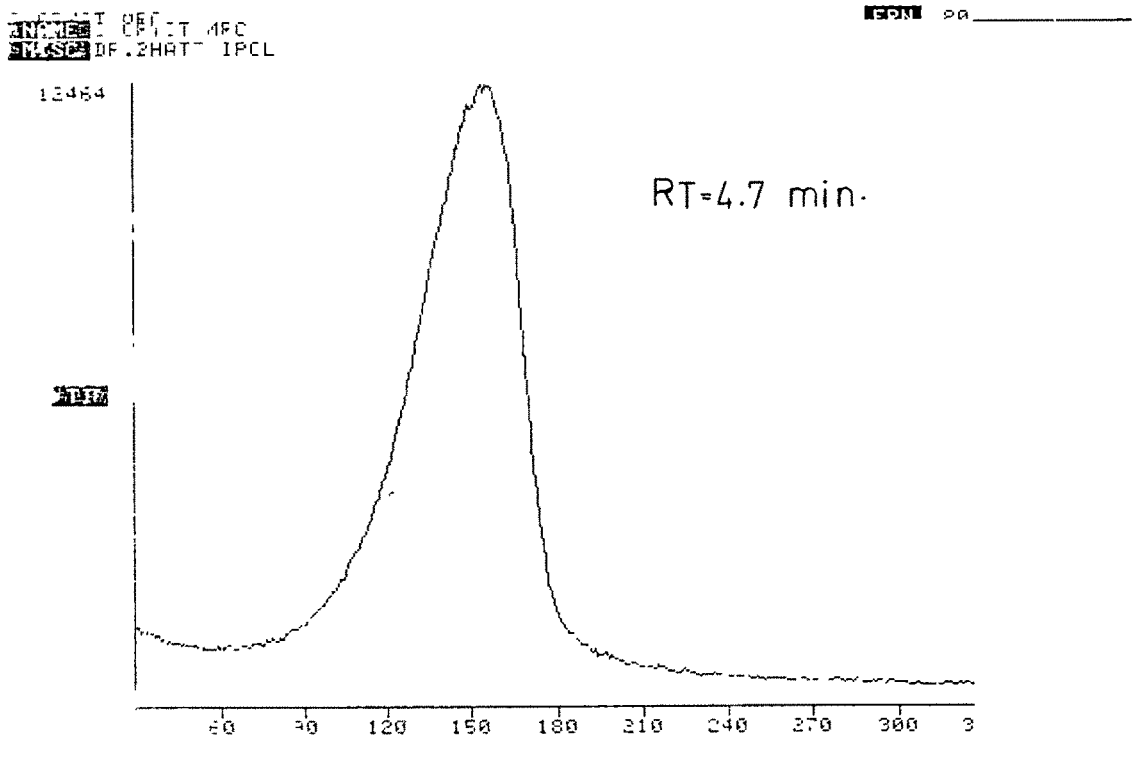


Fig. 21: GC/MS spectrum of Compound A

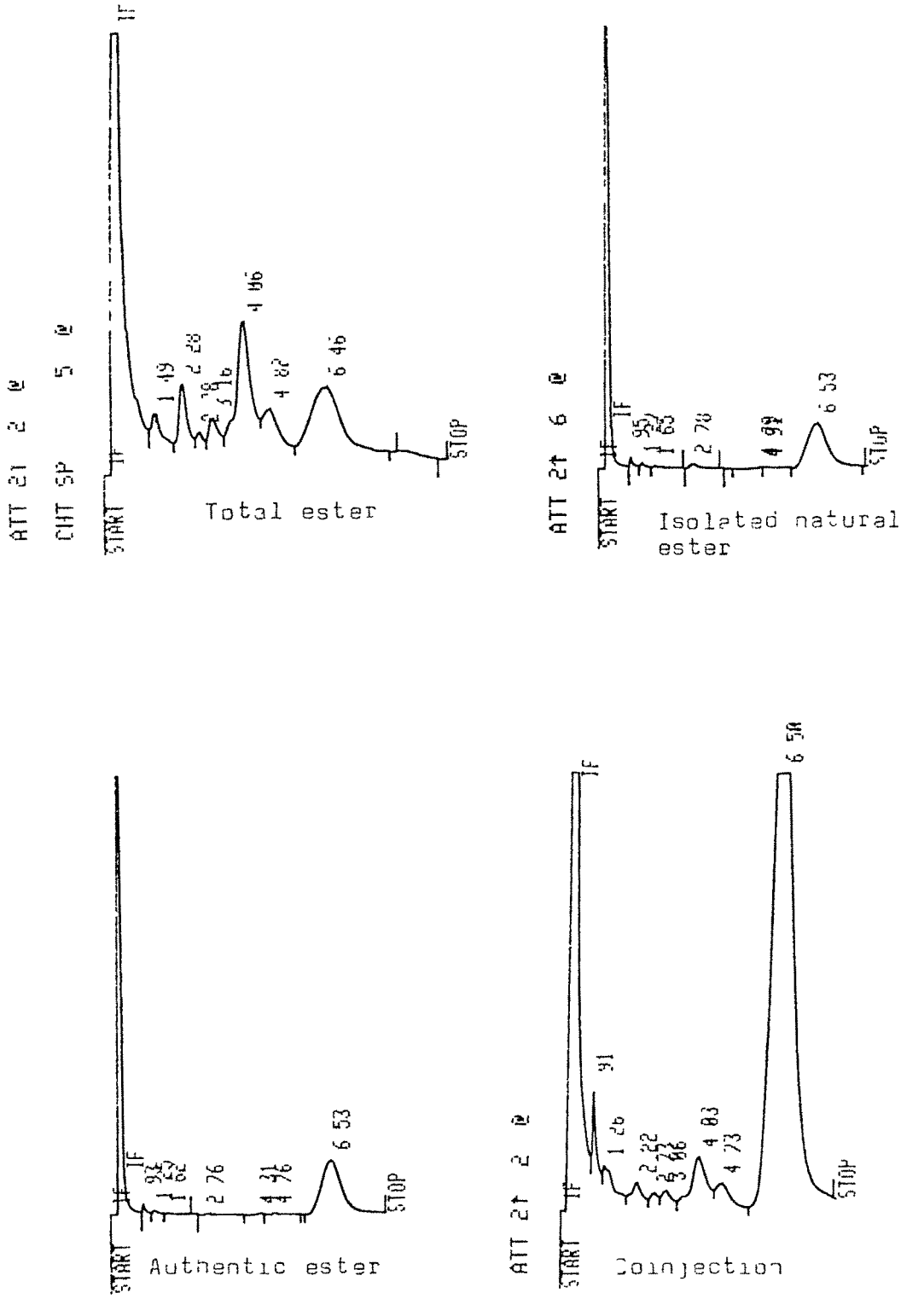


Fig. 23: GLC study by coinjection of methyl protocatachuate

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