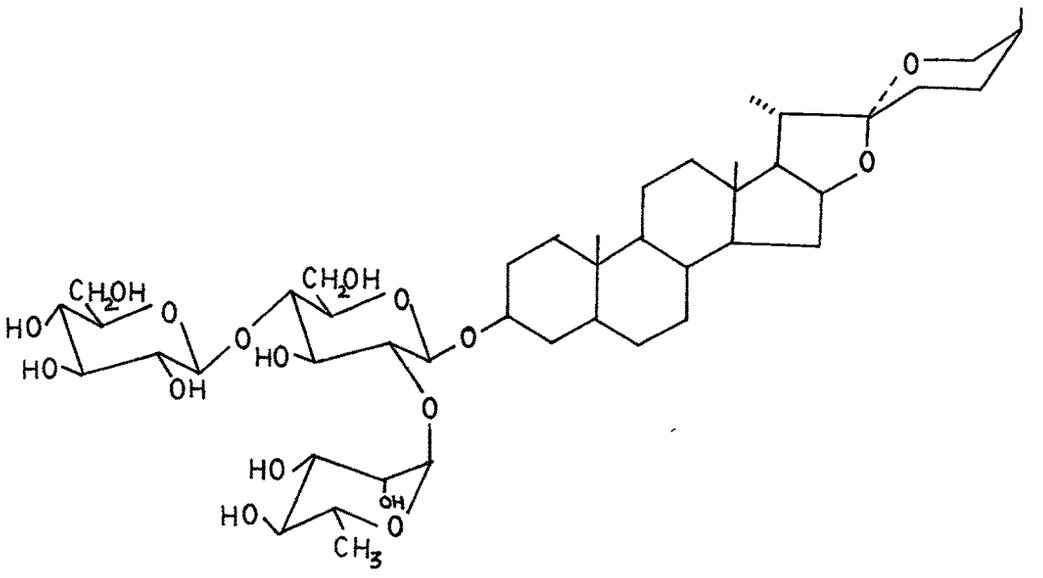
CHAPTER-3

APPLICATION OF FABMS AND 13C-NMR SPECTROSCOPY IN STRUCTURE DETERMINATION OF SAPONINS-STRUCTURE OF SHATAVARIN-IV

Isolation of shatavarin-IV is described in chapter-2. The present chapter deals with various spectral techniques and their applications in the structure determination of steroidal saponins. Fast-Atom-Bombardment-Mass-Spectrum (FABMS) of shatavarin-IV was studied. ^{1}H-NMR and ^{13}C-NMR spectra of peracetate of shatavarin-IV were recorded and examined carefully.

Shatavarin-IV was earlier isolated from <u>shatavari</u> by Ravikumar[1] and its structure determined by physical methods and chemical degradations. On acid hydrolysis it gave sarsasapogenin as its aglycone and glucose and rhamnose in the proportion of 2:1, as sugars. The sequence of monosaccharides was determined by Electron-Impact-Mass-Spectra of permethyl shatavarin-IV. Position of linkages were determined by comparison of products obtained by methanolysis of permethyl shatavarin-IV with samples prepared by standard methods. The stereochemistry of linkages of sugars were determined by ^{1}H NMR spectral study of permethyl shatavarin-IV and by molecular optical rotation differences using Klyne's rule. From the above evidences shatavarin-IV was formulated as sarsasapogenin-3-O-{α-L-rhamnopyranosyl-(1$\rightarrow$2)-[β-D-glucopyranosyl-(1$\rightarrow$4)]}-β-D-glucopyranoside (I).



I

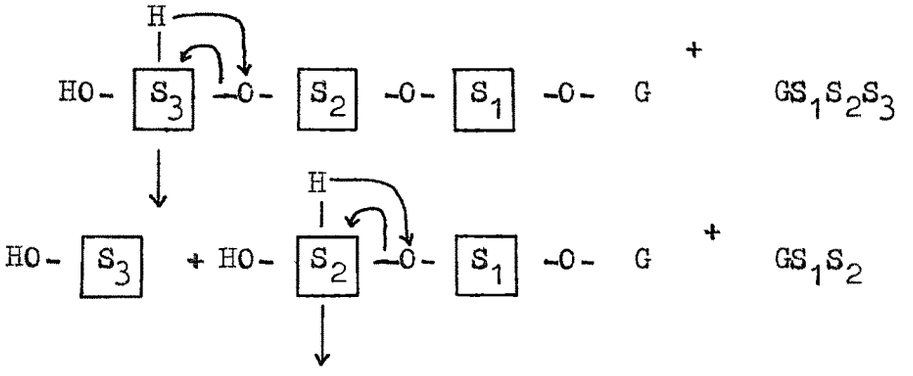
Since structure of shatavarin-IV was well established earlier, FABMS and ^{13}C-NMR techniques were applied to it to evaluate their scope. Shatavarin-IV proved to be a good model compound for structure elucidation by these techniques. These spectral methodswere then applied to other steroidal saponins isolated from shatavari.

APPLICATION OF FABMS

Mass spectrometry has proved to be an important technique in the field of natural products[2]. It has been found to be a powerful tool in the determination of sequence of oligosaccharides[2] and cardiac glycosides.

Various new MS techniques are used for the determination of sequence of monosaccharides in glycosides.

Due to low volatility and their thermal lability, much work could not be done on underivatized glycosides. Reichstein[3] reported the Electron Impact Mass Spectra (EI-MS) of cardiac glycosides with one sugar unit. Two other groups also reported EI-MS of cardiac glycosides[4,5]. in 1971. Brown et al.[5] applied the technique of Field-Ionization (FI) with Electron-Impact (EI-MS) to record the mass spectrum of a trisaccharide cardenolide. The formation of ions can be explained as shown in Fig.1. The cleavage of monosaccharides is comparable to partial hydrolysis of the glycoside. In a stepwise reaction, the monosaccharides are knocked out and the ions corresponding to the monosaccharide and the glycoside without it are recorded.



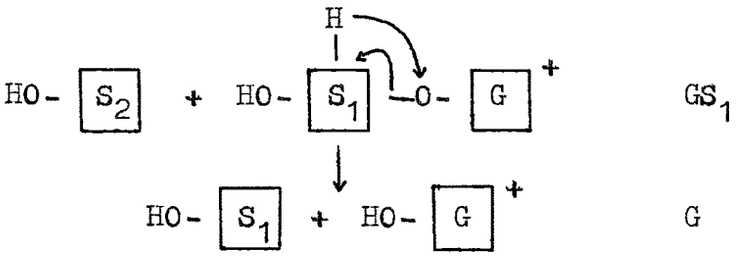


Fig. 1 Fragmentation of a sugar chain.

Before the development of newer techniques of mass spectrometry, it was not possible to record the full mass spectrum of underivatized saponins due to their low volatility. Since mass spectrometry can be beneficially used for the determination of sugar sequence, the saponins were converted to more volatile derivatives like peracetates and permethyl ethers.

Sugar sequence in shatavarin-IV was successfully determined by EI-MS[6]. Shatavarin-IV was converted into its permethyl derivative which was used for the mass spectral study. However, molecular weight could not be obtained. EI-MS was applied to ginsenoside acetate and the related dammarane type triterpene saponin acetates by Komori et al[7]. These volatile derivatives gave $[M^+-Ac]$ peak. Sugar sequence could be derived but molecular weight could not be recorded. Kawasaki et al.[8] reported the EI-MS of permethylated oleanan type triterpene saponins, they were able to get the molecular weight and sequence of monosaccharides.

It has been known that trimethylsilyl ethers of alcohols are prepared readily and have higher volatility than other derivatives. Kochetkov[9] and Kamerling et al.[10] reported the EI-MS of trimethylsilyl derivatives of oligosaccharides. Trimethylsilyl derivatives of dammarane type ginseng saponins were successfully tried by Tanaka et al.[11]

Kawasaki and his coworkers[12] reported the EI-MS of acetates and permethyl ethers of steroidal saponins. But they were not successful in determining the molecular weight. It was not until 1979 when underivatized steroidal saponins were tried. Sih et al.[13] reported the EI-MS of underivatized steroid saponins at an ionizing voltage of 70 eV. But due to decomposition, no molecular ion peak was observed making it impossible to determine the molecular weight accurately. However, chemical structure of sugars and their partial sequence could be obtained.

Techniques other than EI-MS are successfully applied to natural products. The Field-Ionization-Mass-Spectrometry (FI-MS) has been applied to structural analysis of permethylated oligosaccharides,[14,15] underivatized nucleosides[16] and cardiac glycosides[5,17].

For underivatized glycosides, there is limited scope in mass spectrometry because of low volatility and their thermal unstability. Field-Desorption-Mass-Spectrometry (FD-MS) is frequently used in case of underivatized glycosides. In FD-MS very little of thermal energy goes into internal excitation as only a small amount of energy which is equivalent to the energy of desorption is supplied.[18,19] This method is the best method for the determination of molecular weight. The ions corresponding to $[M^+ + H]$ and $[M^+ + Na]$ are observed distinctly. Sometimes $[M^+ + K]$ is also observed. FD-MS of underivatized steroid and triterpene saponins have been reported.[20-24] Schulten et al.[20] reported the FD-MS of dioscin. In the FD-MS, intense peaks appeared corresponding to $[M^+ + H]$, $[M^+ + Na]$ and $[M^+ + K]$. The Na and K ions are present as impurities either in the sample or in the emitter. The FD-MS not only gives molecular weight but also gives the information about the sequence of sugars and their individual chemical structure, from fragment ions derived by the direct bond cleavage in the oligosaccharide moiety in the saponin. The formation of fragment ions have been discussed in relation to the mechanism of glycosidic bond cleavage by acid hydrolysis.[25]

The new technique of Plasma-Desorption-Mass-Spectrometry which utilizes energetic fission fragments from the decay of

^{252}Cf to volatilize and ionize the solid sample, was successfully applied to non volatile and thermally labile biological molecules by Macfarlane and Torgerson.[26,27] Nakanishi[28] used this technique for steroidal saponins from Cornus florida. Strong peaks were observed corresponding to $[M^+ + Na]$, $[M^+ + K]$ and $[M^+ + 2Na - H]$.

However, for polar, non volatile and thermally unstable compounds, recently developed technique of Fast-Atom-Bombardment-Mass-Spectrometry (FABMS) has gained wide acceptance. This technique originally pioneered by Barber and his coworkers[29,30] is mainly used for solid organic samples which due to their low volatility become useless in EI-MS. So far it has been applied to a number of biologically active molecules,[31-33] antibiotics[34,35], carbohydrates[36] and cardiac glycosides[37]. In most cases, these applications have involved only determination of molecular weights. The more extensive structure assigning capabilities of FABMS have been mainly applied to peptides[38-41].

The technique of FABMS depends on the sputtering phenomenon which can be described simply as follows[30]. If a solid is bombarded with high velocity particles, say rare gas ions of 8 KeV energy, then material will be removed into the gas phase. This results from the momentum transfer from

the impinging particle to the target with setting up of collison chains, some of which will be in the form of positively or negatively charged ions. Instead of fast ion beams as the sputtering agents molecular beam of fast neutral species can be used.

$$A_{\alpha}^{+} + A_{\beta} \longrightarrow A_{\alpha} + A_{\beta}^{+}$$

Fast ion	Fast atom
KE = V	KE $\simeq$ V

The sputtered ion source produces ions from the solid at room temp.

It is observed that a true molecular ion is not observed, but $[M^{+} + H]$ or $[M^{+} - H]$ predominates, in which the molecule forms a stable ion by addition or loss of a proton. Molecular weight is thus determined from $[M + H]^{+}$ in the positive ion spectra or $[M - H]^{-}$ in the negative ion spectra, both the spectra are generally produced with equal sensitivity. Molecules that are alkali metal salts form stable species, in high abundance by addition or by loss of alkali metal cation. Cationized species can also be formed from mixtures of organic molecules with salt present as impurities in the sample. This facility can be used

beneficially by judicious doping of the sample with alkali metal salts or by protonating agent. e.g. addition of potassium chloride to a sample suspected to be a sodium salt will shift all the peaks containing sodium ions by 16 u for each sodium ion present in the various species observed in the spectrum.

The fragment ions present at reasonable abundance in most FABMS have been shown to arise by gas phase decomposition reaction steps originating with the molecular ionic species. The fragmentation is not dependent on thermally induced reactions, since the sample is usually maintained at ambient temp. throughout the analysis.

According to Williams and his coworkers[42] the $[M + H]^+$ or $[M - H]^-$ ions (i) are formed by proton transfer reactions which may occur, as the molecules are bombarded and pass into gas phase and/or (ii) are those already existing in the glycerol matrix; in such cases, the preexisting ions pass into the gas phase due to momentum transfer which they receive from an Ar atom which impacts near, but not upon the sample ion.

A remarkable and extremely useful feature of FABMS is the occurrence of low abundance ions at essentially every mass upto (and slightly beyond) $(MH)^+$ or $(MH)^-$. It is

proposed that these ions are arising from direct or nearly direct impact of Ar atoms on the sample and its supporting matrix. Glycerol and thioglycerol are the most commonly used solvents for dissolving the sample. The advantages of this technique are,

(1) Most polar non volatile compounds can be used, without their derivatisation into volatile derivative. Ionization occures from the solid which is at room temp.

(2) The method works well in both the polarities, i.e. +ve ion and -ve ion FABMS can be recorded with equal intensity. The method gives the same information about molecular weight by formation of $[M + H]^+$ in FAB^+ and $[M - H]^-$ in the FAB^-.

(3) The most important advantage is, that the mass spectra may be obtained from compounds of relatively high molecular weight, which led its use for the bigger molecules.

FABMS of shatavarin-IV : Due to its capability of recording high molecular weight, it was decided to use FABMS technique for the steroid saponins of shatavari. Since structure of shatavarin-IV is well-established and the sequence of sugars well-characterised using mass spectrometry and chemical methods, shatavarin-IV was chosen as a model compound for mass spectral study. As expected, even in underivatised saponin shatavarin-IV, molecular weight could be calculated precisely. More important information which was obtained

was regarding the sequence of monosaccharides. The FABMS was found to be the most suitable technique for steroidal saponins which are highly polar.

When this work was started in 1983, there was no report on application of FABMS to steroidal saponins. However, recently Schulten et al.[43] reported comparison of FD-MS and FABMS of steroidal saponins of Paris polyphylla and found FABMS as a confirmatory and supporting method. We found that FABMS is superior to other techniques in a way that it not only gives molecular weight and higher fragments, but also smaller fragment ions are observed which are characteristic and informative, which are usually observed in EI-MS. Unambigous molecular weight determination and elucidation of sugar sequence of saponins is thus feasible with the help of FABMS.

In the FAB^{+}MS pseudomolecular ions are observed at $[M^{+}+H]$ and $[M^{+}+Na]$ which confirm the molecular weight. As it is already known molecular weight of shatavarin-IV is 886 (calculated for $C_{45}H_{74}O_{17}$). In the FAB^{+}MS (Fig. 2) an intense peak appears at m/z 887 with 11.47% intensity which corresponds to $[M^{+}+H]$. All the fragments are derived from the pseudomolecular ion $M^{+}+H$. Shatavarin-IV shows a signal at m/z 741 with 4.35% intensity, due to loss of 146 mass units from $[M^{+}+H]$, which is a result of the loss of

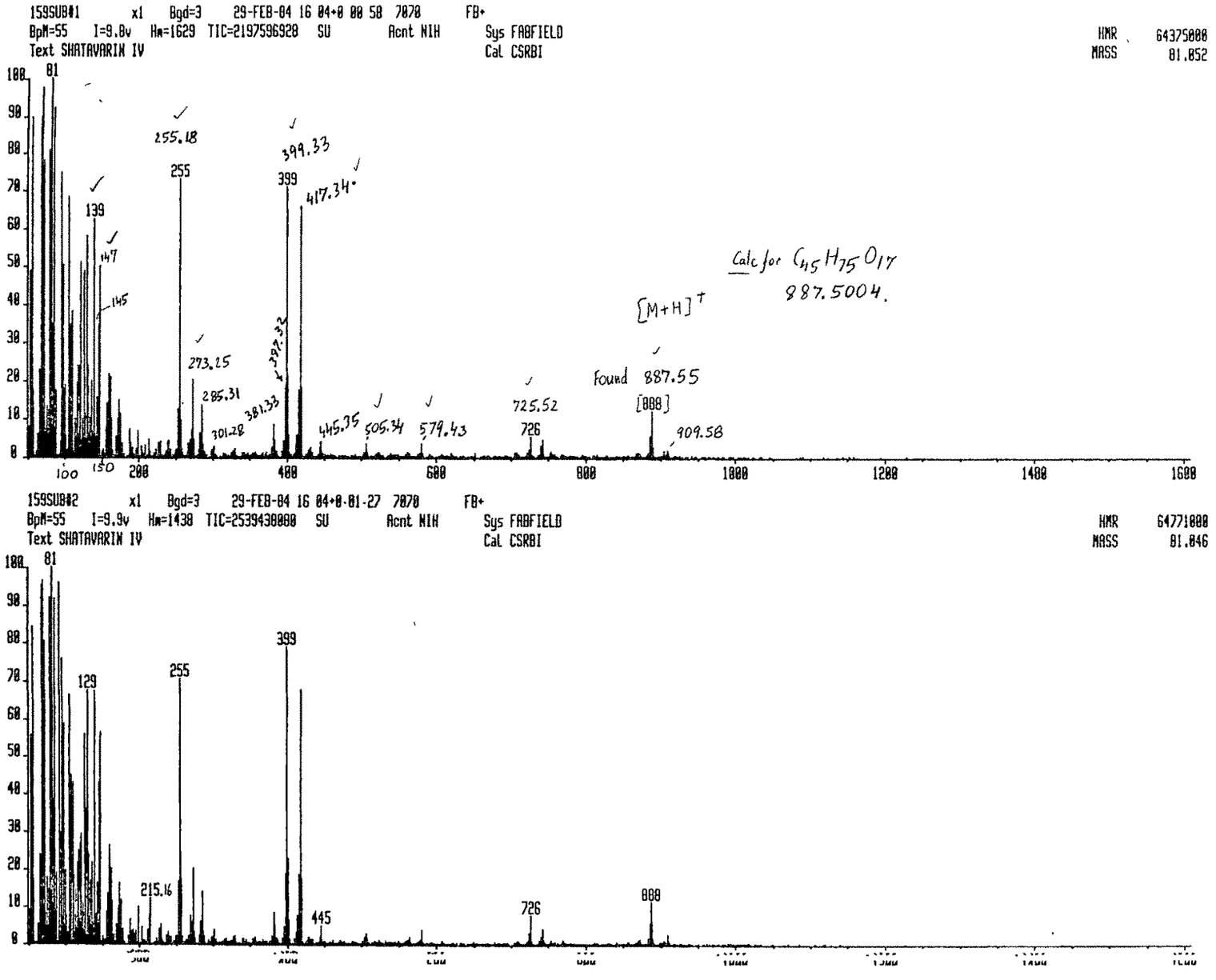


FIG. 2 : FABMS OF SHATAVARIN-IV

terminal rhamnose unit from the molecule by a protonation mechanism analogous to acidic solvolysis.[21] In this proton induced cleavage of the glycosidic oxygen and charge localization can induce a characteristic electron shift from the ring oxygen resulting in the formation of a glycosyl ion. This type of ion is almost always found in the FD-MS of sugars and their derivatives.[44-46]

The compound shows a signal at m/z 725.52 which arises by the loss of 162 mass units from the pseudomolecular ion $[M^+ + H]$, which corresponds to loss of terminal glucose unit. The loss of glucose and the loss of rhamnose from the molecular ion indicate that glucose and rhamnose form the two terminal sugars. Thus, $[M^+ + H - gluc]$ and $[M^+ + H - rham]$ are the two ions which are consistent with the branched sugars sequence. The signal at m/z 579 arises due to loss of both glucose and rhamnose from the molecule. i.e. $[M^+ + H - (gluc+rham)]$. The ion at m/z 417 is formed by loss of 162 mass units, which can be accounted for the loss of glucose unit. This ion at m/z 417 corresponds to $[sarsasapogenin + H]^+$, which implies that a glucose is connected to sarsasapogenin at C-3, which is connected to a glucose and a rhamnose in branched manner.

This ion at m/z 417 is observed due to loss of glucosyl-rhamnosyl-glucoside from $[M^+ + H]$ and this reveals

the aglycone mol. wt. 416 . For steroidal sapogenins, stabilization of the glycosidic bond in 3 position of the aglycone is a generally observed phenomenon, observed in mass spectrometry. Water elimination from the aglycone (m/z 417) produces a peak at m/z 399.

Signals observed at m/z 399, 397, 285, 255 and 139 are characteristic of the sapogenin.[47,48] The formation of the fragment ions can be explained as in Fig.3. Presence of glucose is confirmed by ions appearing at m/z 163, 145 and 127 and that of rhamnose is confirmed by the ions at m/z 147 and 129.

Ions	m/z
$[M^+ + H]$	887
$[M^+ + Na]$	909
$[M^+ + H - rham] = 887-146$	741
$[M^+ + H - gluc] = 887-162$	725
$[M^+ + H - (gluc+rham)] = 887-308$	579
$[M^+ + H - (gluc+rham+gluc)] = 887-470$	417

Fig. 3. Fragments formed in the FAB^+MS of shatavarin-IV.

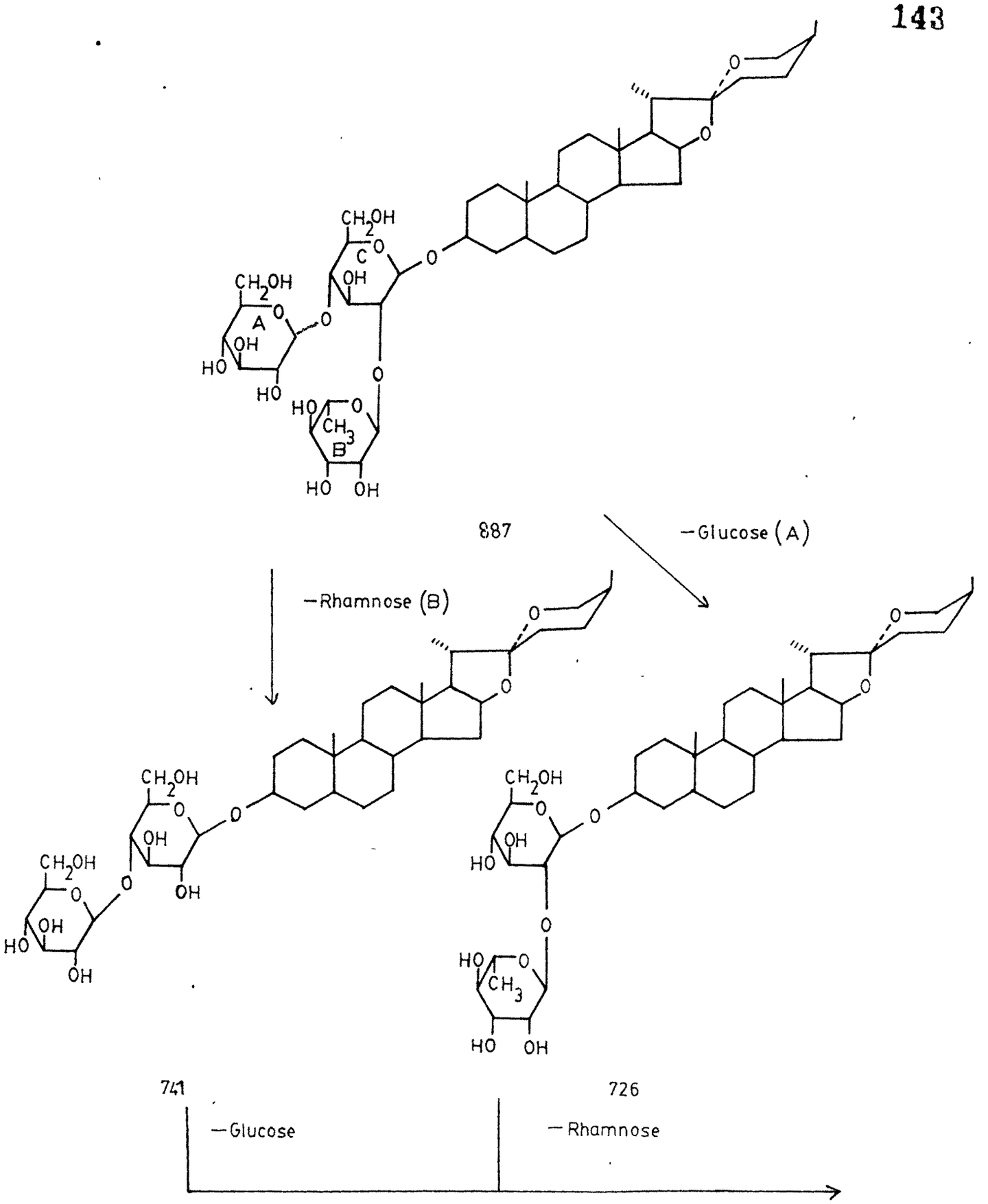


FIG.4 : FORMATION OF IONS IN THE FAB$^+$MS OF SHATAVARIN-IV.

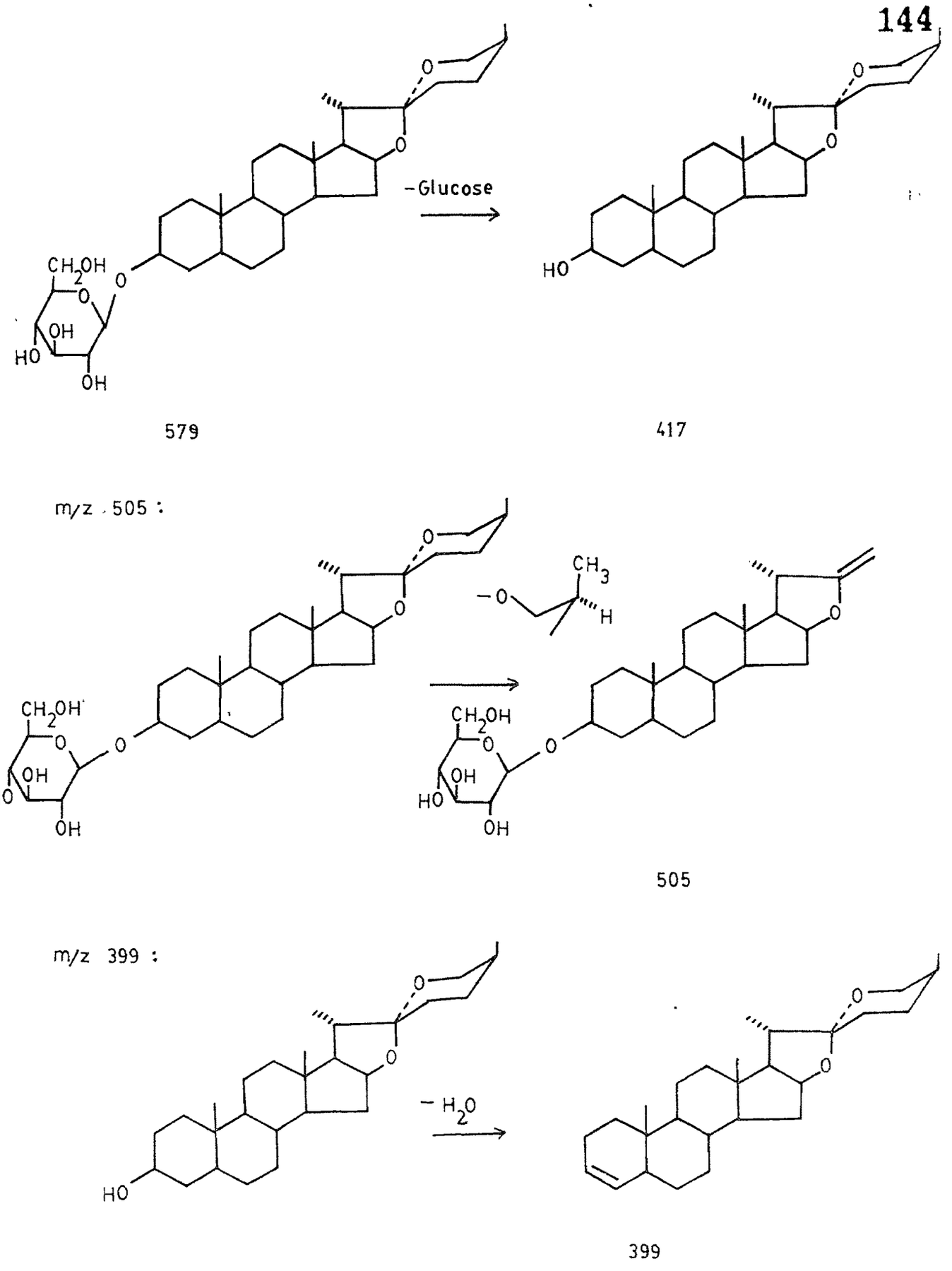
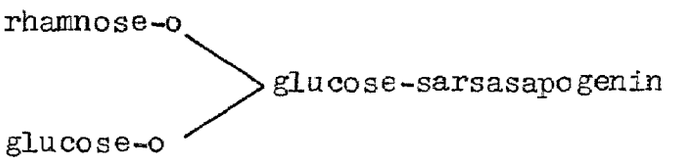


FIG.5 : FORMATION OF IONS IN THE FAB⁺MS OF SHATAVARIN-IV.

FIG.6 - FORMATION OF IONS IN THE FAB⁺

From FABMS structure of shatavarin-IV can be assigned as,



(II)

The FABMS of shatavarin-IV is shown in Fig.2. The signal at m/z 505 is obtained by the loss of sapogenin side chain. i.e. by opening of ring F. Formation of important fragment ions is shown in Figs 4-7.

It is evident from the above results that FABMS can help in partial assignment of the structure of saponin. However, no information can be collected regarding the position of linkages of sugars.

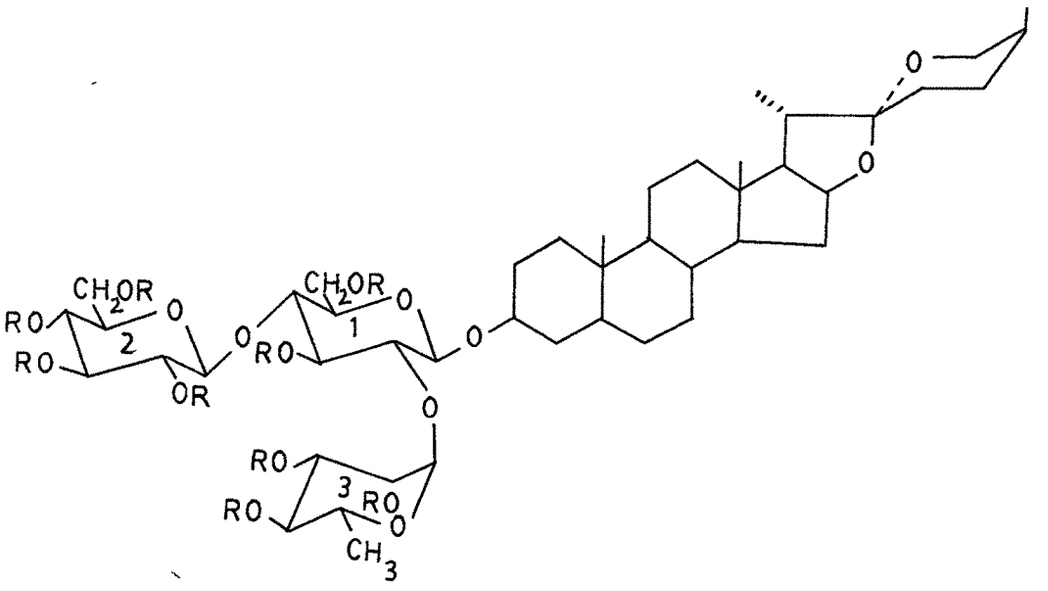
APPLICATION OF ^{13}C-NMR SPECTROSCOPY

^{13}C-NMR spectroscopy has proved to be an important tool in the structure elucidation of organic compounds. In glycosides[49-52] and polysaccharides[53-57] ^{13}C-NMR spectroscopy has proved useful in the determination of ring size, nature of anomeric linkages and position of substitution. ^{13}C-NMR spectroscopy may be used in natural products chemistry in a variety of ways and at various[58] stages of isolation; e.g. checking the identity of two compounds,

detection of stereoisomers or other closely related structures. For unknown compounds ^{13}C-NMR furnishes key information such as the total carbon number, the number of CH_3, CH_2, CH and quaternary carbons, the number of oxygen and nitrogen containing functions. However, the assignment of signals is sometimes very difficult due to overlapping of the signals in complex natural product molecules, ^{13}C-NMR of about all types of naturally occuring compounds have been reported.

^{13}C-NMR spectrum of shatavarin-IV : The ^{13}C-NMR spectrum of peracetyl shatavarin-IV (III) was recorded. Careful examination of the spectrum showed that ^{13}C-NMR spectroscopy can be conveniently used for the determination of position of linkages in case of steroidal saponins. Not only it gives an idea about the points of attachment of sugars, but also gives information about the attachment of a particular monosaccharide residue. e.g. glucosylation and rhamnosylation have different glycosylation shifts of α-carbon atoms, which can be easily differentiated.

Earlier Djerassi and Eggert[59] reported ^{13}C-NMR spectra of steroidal sapogenins, which were reassigned by Tori et al.[60] Mahato et al.[61] recorded the ^{13}C-NMR of steroidal sapogenin and saponins and reported fully assigned values of dioscin and gracillin.



R= $COCH_3$

II

The spectrum was recorded in deuterochloroform. ^{13}C chemical shifts of methyl-β-D-glucopyranoside[57], methyl-β-D-glucopyranoside tetraacetate[62,63] and glucose pentaacetate[64] are reported in the literature, ^{13}C-NMR of rhamnose tetraacetate was recorded in deuterochloroform and values were assigned to it. The signal assignment in glycoside was by comparison with those in its aglycone sarsasapogenin[59,60] and known chemical shift rules[65]. ^{13}C chemical shifts and glycosylation shifts i.e. chemical shift changes from aglycone and methyl glycoside to saponin are shown in Table-1, 2 and 3.

Glycosylation of S- and R-alcohols have been studied in detail.[66,67] In case of S-alcohol, characteristic signal shifts are observed at α- and β- positions of the -OH group in which glycosylation takes place. The carbinyl carbon (α-carbon) signals of aglycone alcohols are shifted downfield by about +8.0 ppm. In case of ^{13}C-NMR spectrum of peracetyl shatavarin-IV, the C-3 signal of the aglycone has shifted downfield by +8.3 ppm. The signals of the two methylene carbons (in case of alcohols) are shifted upfield by -2 ppm (in case of pro R-carbon atom, i.e. carbon syn to the pyranose ring oxygen atom) and -4 ppm (in case of pro S-carbon atom i.e. carbon anti to the pyranose ring oxygen atom).

The signals of sugars were assigned by comparison with those already reported earlier and from the following three assumptions[68].: 1) o-glycosylation of sec-OH except anomeric -OH in a sugar causes Ca +10 ppm downfield shifts of the α-carbon atom but has little effect on other carbon atoms. 2) glycosylation shifts for a sugar i.e. signal shift from methyl glucoside to sec-alcohol glucoside are Ca -3 ppm for the anomeric carbon but less than 1 ppm for the other carbons.[66,69] 3) o-acetylation of sec-OH causes Ca +2 ppm downfield shift of α-carbon atom and Ca -3 ppm downfield shift of other signals,[70] which can be seen from Table-2.

TABLE-1 : ^{13}C-NMR ASSIGNMENTS* OF AGLYCONE IN PERACETATE OF SHATAVARIN-IV

Carbon No.	Sarsasapogenin[59,60]	Aglycone in peracetate of shatavarin-IV
1.	29.9	31.9 (+2.0)
2.	27.8	26.3 (-1.5)
3.	67.0	75.351 (+8.3)
4.	33.6	31.9 (-1.7)
5.	36.5	35.6 (-0.9)
6.	26.6	26.7 (+0.1)
7.	26.6	26.7 (+0.1)
8.	35.3	35.2 (-0.1)
9.	40.3	40.5 (+0.2)
10.	35.3	35.2 (-0.1)
11.	20.9	20.62 (-0.3)
12.	39.9	40.5 (+0.6)
13.	40.6	40.8 (+0.2)
14.	56.4	56.7 (+0.3)
15.	31.7	31.9 (+0.2)
16.	80.9	81.0 (+0.1)
17.	62.1	62.5 (+0.4)
18.	16.5	16.5 (0.0)
19.	23.9	24.1 (+0.2)
20.	42.1	40.8 (-1.3)

(contd.)

TABLE-1 : (contd.)

Carbon No.	Sarsasapogenin[59,60]	Aglycone in peracetate of shatavarin-IV
21.	14.3	14.4 (+0.1)
22.	109.5	109.5 (0.0)
23.	27.1	27.3 (+0.2)
24.	25.8	26.3 (+0.5)
25.	26.0	26.3 (+0.3)
26.	65.0	65.0 (0.0)
27.	16.1	16.2 (+0.1)

* ppm from TMS.

TABLE-2 : ^{13}C- CHEMICAL SHIELDINGS* OF ALDOPYRANOSE, THEIR METHYL GLYCOSIDES AND THEIR ACETYLATED DERIVATIVES

Sr. No.	Compound	C-1	C-2	C-3	C-4	C-5	C-6
1.	Methyl-β-D-glucopyranoside[57]	104.7	74.2	76.9	70.8	76.9	61.9
2.	Methyl-β-D-glucopyranoside tetraacetate[62,63]	101.4	71.3	72.9	68.5	71.8	62.0
3.	β-D-glucopyranoside pentaacetate[64]	91.78	70.35	72.85	67.92	72.85	61.59
4.	Smilagenin-β-D-glucopyranoside pentaacetate[3]	99.3	72.2	73.4	69.5	72.2	62.6
5.	Methyl-α-L-rhamnopyranoside[57]	101.9	71.0	71.3	73.1	69.4	17.7
6.	α-L-rhamnopyranoside+ tetraacetate.	91.29	69.24	69.03	71.20	69.31	17.57

* ppm from TMS

+Data collected by the author.

TABLE-3 : ^{13}C CHEMICAL SHIELDINGS* OF SUGARS OF PERACETATE OF SHATAVARIN-IV

Carbon No.	Methyl-β-D-glucopyranoside tetra-acetate	∝-L-rhamnopyranoside tetra-acetate	SUGARS OF PERACETATE OF SHATAVARIN-IV.	
C-1'	101.4		99.4	
C-2'	70.358		77.1 (+6.8)	
C-3'	72.853		72.2 (-0.6)	
C-4'	67.923		75.3 (+7.4)	Glucose-1
C-5'	72.853		72.2 (-0.6)	
C-6'	61.593		62.5 (+1.0)	
C-1''			99.4	
C-2''			70.2 (-0.1)	
C-3''			72.2 (-0.6)	Glucose-2
C-4''			67.0 (-0.9)	
C-5''			72.2 (-0.6)	
C-6''			62.5 (+1.0)	
C-1'''		91.25	99.4	
C-2'''		69.24	67.0 (-2.2)	
C-3'''		69.03	67.0 (-2.03)	Rhamnose-3
C-4'''		71.20	70.2 (-1.0)	
C-5'''		69.31	68.7 (-0.6)	
C-6'''		17.57	17.4 (-0.1)	

* ppm from TMS

^{13}C-NMR helps in determining the interglycosidic linkages.[71] In case ^{13}C-NMR of peracetate of shatavarin-IV, the C-2' signal is observed at +6.8 ppm downfield and C-4' signal appears at +7.4 ppm downfield. It has been observed that glycosylation of sugar hydroxyls produce a sizable downfield shift in the resonance of hydroxylated carbon.[72] When glucose is the glycosylating sugar a downfield shift of the order of 7-8 ppm is evident in the resonance of hydroxylated carbon atom, this size shift is typical of β-D-glucosylation, generally in disaccharides.[73] All the remaining carbon atoms are shifted upfield by 3-4 ppm due to acetylation.

It becomes evident from the ^{13}C-NMR spectrum of peracetate of shatavarin-IV, that glucose-2 is connected at C-4 and rhamnose is connected at C-2 position of glucose-1.

^{13}C-NMR thus gives precise idea about the position of linkages of sugars, but does not give much information regarding the stereochemistry of linkages. i.e. whether the glucose/rhamnose are α-linked or β-linked.

The clarification of the stereochemistry at the point of linkages of the sugars can not be assignable on the basis of ^{13}C-NMR. However, this is easily done with the help of ^{1}H-NMR spectroscopy as already reported by Ravikumar[1] for

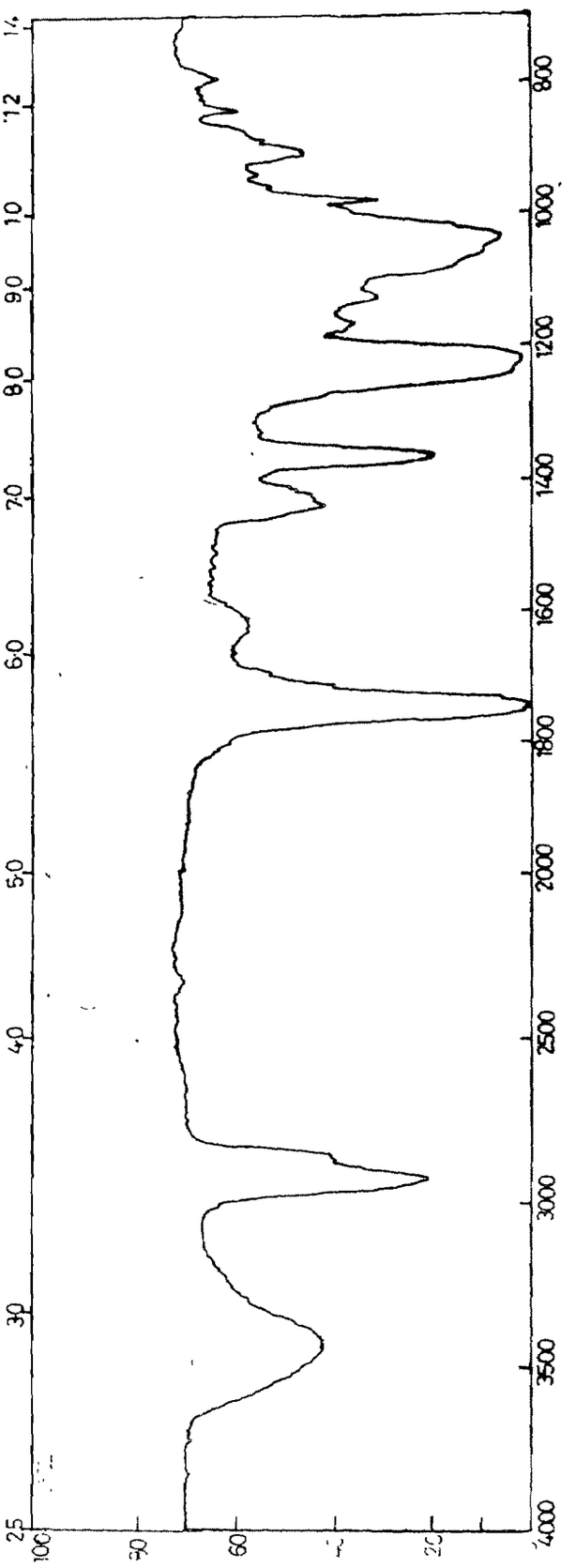


FIG.8 : IR SPECTRUM OF PHRACETATE OF SHATAVARIN-IV.

anomeric proton of ring B is equatorial. J value measured is 3 Hz. Hence, rhamnose is α-linked.

All the spectral data is consistent with the structure of shatavarin-IV formulated as (I).

EXPERIMENTAL

For general remarks please refer to page No. 100
IR spectra were recorded on Perkin-Elmer D-55 IR Spectrometer. ^{1}H-NMR spectra were recorded in deuterochloroform on Perkin-Elmer R-32 Spectrometer using Tetramethylsilane as internal standard. FABMS were recorded using VG Micromass 7070E-HF mass spectrometer. Xenon fast atom beam (8 KeV) was used to bombard the sample dissolved in glycerol on a stainless steel target. ^{13}C-NMR spectra were recorded on Jeol FX-100 Spectrometer in deuterochloroform using tetramethylsilane as internal standard.

PERACETYL SHATAVARIN-IV

Shatavarin-IV (100 mg) was dissolved in dry pyridine (6 ml) and mixed with acetic anhydride (6 ml) and kept at room temp. for 48 hours. Acetic anhydride and pyridine were removed under vacuum (30 mm, 75-85°), the residue was taken up in chloroform (100 ml), washed with water (25 ml), 5% aqueous sodium carbonate solution (25 ml x 3), water (25 ml), $\frac{N}{2}$ hydrochloric acid (25 ml x 2) and finally with water (25 ml x 4). The chloroform layer was dried with anhydrous sodium sulfate and the solvent was removed to get yellowish white foamy powder, which was purified by chromatography.

CHROMATOGRAM

Wt. of material : 0.1400 g.

Adsorbent : 10 g. silica gel, IIa.

Column dimension : 1.5 cm x 15 cm.

Fr. No.	Eluent	Vol.of Fr	Wt.of Fr. (gms)	Remarks
1.	Benzene+ethyl acetate :: 95+5	15 ml x 6	-	-
2.	Benzene+ethyl acetate :: 90+10	15 ml x 6	-	-
3.	Benzene+ethyl acetate :: 85+15	15 ml x 6	0.1084	Yellowish white powder.
4.	Benzéne+ethyl acetate :: 80+20	15 ml x 6	-	-
5.	Ethyl acetate	15 ml x 6	0.0250	rejected.
		Total...	0.1334 g (95%)	

FR. 3 showed a single spot on TLC. m.p. 128-31°, $[\alpha]_D^{26}$ - 45.4° ($CHCl_3$; C, 1.1), IR : ν_{max}^{KBr} cm^{-1} 1740 (-OAc). ^{1}H-NMR, singlets at 1.94, 2.0, 2.02, 2.08, 2.12, 2.16, 2.2 ppm accounting for 27 H. ^{13}C-NMR ($CDCl_3$) recorded.

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SUMMARY

General application of various techniques of spectrometry to steroidal saponins is discussed chosing shatavarin-IV as a model compound. Fast-Atom-Bombardment-Mass-Spectra successfully showed the sequence of sugars. ^{13}C-NMR spectroscopy was used to elucidate the position of linkages of the monosaccharides. ^{1}H NMR study of peracetate of shatavarin-IV revealed the nature of linkages of the sugars. From the above mentioned spectral techniques, structure of shatavarin-IV was confirmed as sarsasapogenin-3-o-{α-L-rhamnopyranosyl-(1 $\rightarrow$ 2)-[β-D-glucopyranosyl-(1 $\rightarrow$ 4)]}-β-D-glucopyranoside.