Chapter-II

Material and method

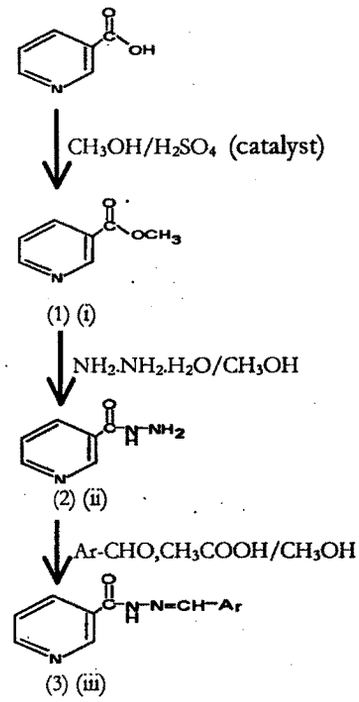
Material & method

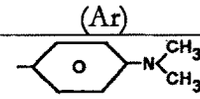
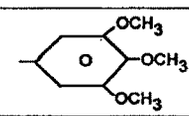
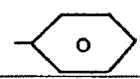
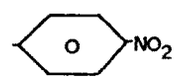
I have initialized my work on the esterification of nicotinic acid by using methanol and prepared its derivative and purified by using column chromatography. The structures of the compounds are confirmed by the spectroscopic data.

The purified and identified compounds were further used for the process of bioassay on insect pests and microbes.

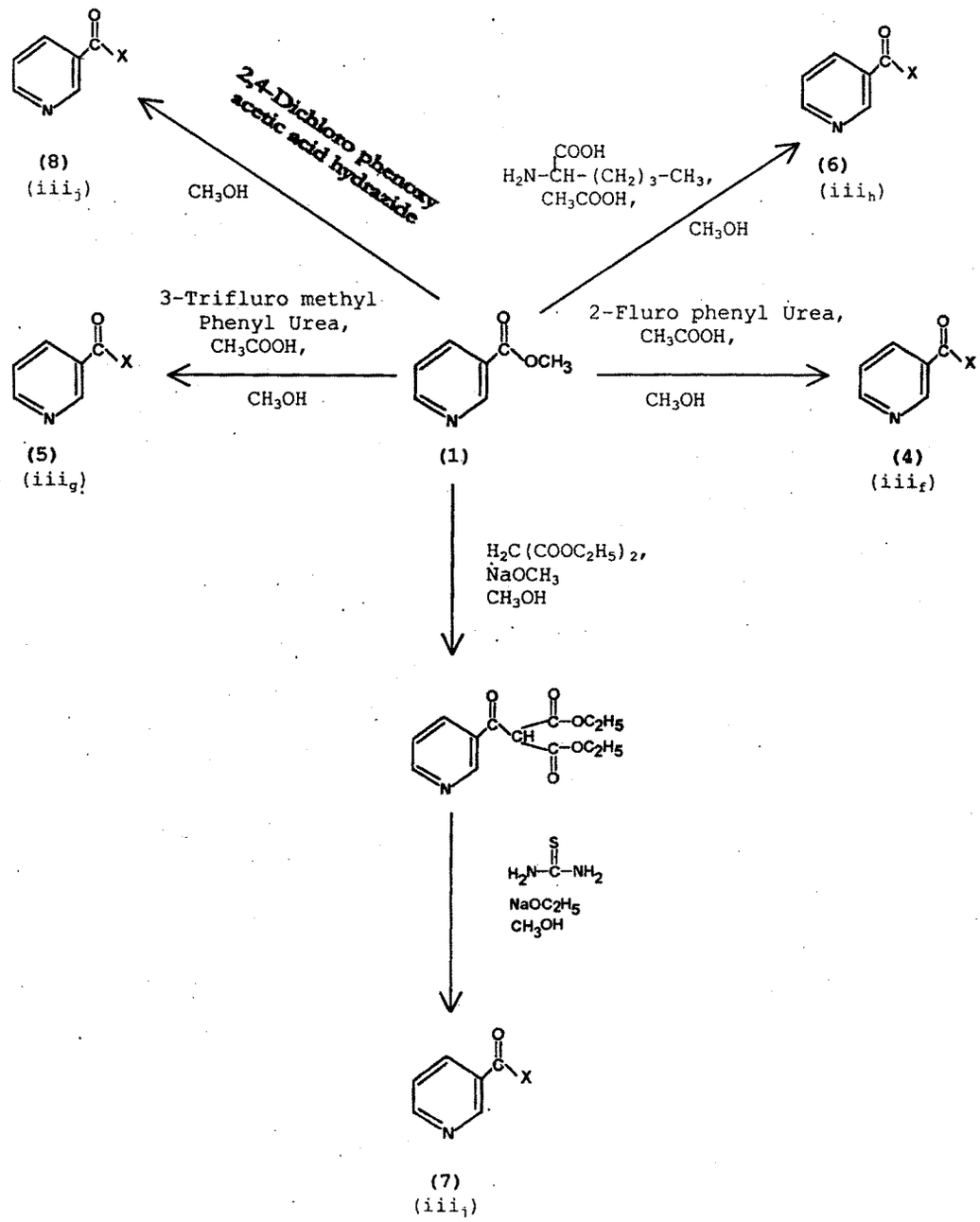
A) Synthesis of Nicotinic acid derivatives:

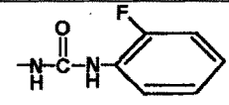
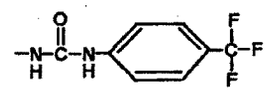
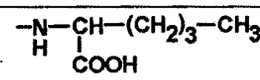
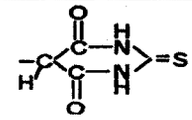
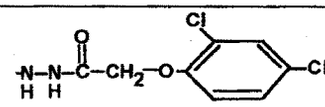
Scheme-1



Compd. No.	Substituents (Ar)
$3iii_a$	
$3iii_b$	
$3iii_c$	
$3iii_d$	
$3iii_e$	

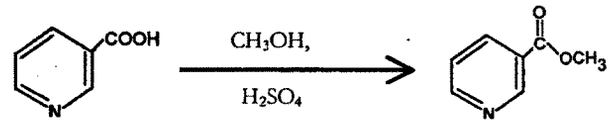
Scheme-2


(8)
(iii$_j$)
2,4-Dichloro phenoxy acetic acid hydrazide
CH_3OH
$H_2N-CH-(CH_2)_3-CH_3$,
COOH
CH_3COOH,
CH_3OH
(6)
(iii$_h$)
3-Trifluro methyl Phenyl Urea,
CH_3COOH,
CH_3OH
(5)
(iii$_g$)
(1)
2-Fluro phenyl Urea,
CH_3COOH,
CH_3OH
(4)
(iii$_f$)
$H_2C(COOC_2H_5)_2$,
$NaOCH_3$
CH_3OH
$H_2N-C(=S)-NH_2$
$NaOC_2H_5$
CH_3OH
(7)
(iii$_i$)

Compd. No	Substitutents
4iiif.	
5iiig.	
6iiih.	
7iii.	
8iiij.	

i) Synthesis of Methyl nicotinate (1):

In a round bottomed flask take a nicotinic acid 10 gm. (0.5 mole) to which 30 ml of methanol was added with constant stirring, followed by addtion of 4 ml. conc.H_2SO_4 drop wise and the reaction mixture refluxed on a water bath for about an 13 hrs., cooled and neutralized the reaction mixture by 10% sodium carbonate and extracted in 20 ml chloroform. The chloroform is removed by distillation and the residual mass was collected to get solid crystalline product (1)[23]. Yield: 70%, M.P. 38°C.

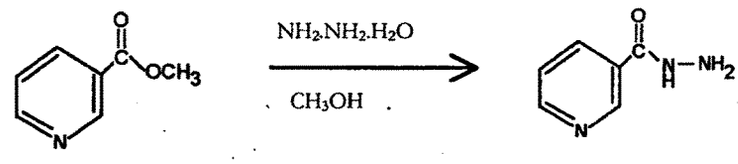


Nicotinic acid Methyl nicotinate

(1)

ii) Synthesis of Nicotinyl hydrazide (2):

The mixture of Methyl nicotinate (0.1 mole.) and hydrazine hydrate (0.1 mole.), in methanol (20 ml.) was heated on the water bath for 2 hrs., cooled and removed the solvent by rotary evaporation to get solid, which was purified by recristallization from ethanol, Yield 75%.

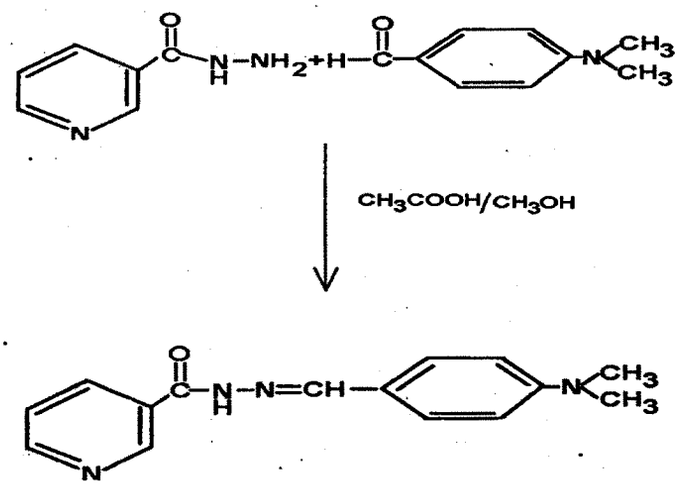


Methyl nicotinate Nicotinoyl hydrazide

(2)

iiia) Reaction of Nicotinyl hydrazide with p-dimethylamino benzaldehyde(3):

The mixture of nicotinoy hydrazide (0.05 mole) and amino benzaldehyde (0.05 mole) was heated in methanol (10 ml) in presence of catalytic amount of acetic acid. After completion of reaction the mixture was cooled, when crystalline product separated out which was filtered dried, recrystallized from ethanol to get hydrazone[24] (iiia), yield 80%, M.P.132^{0}C.

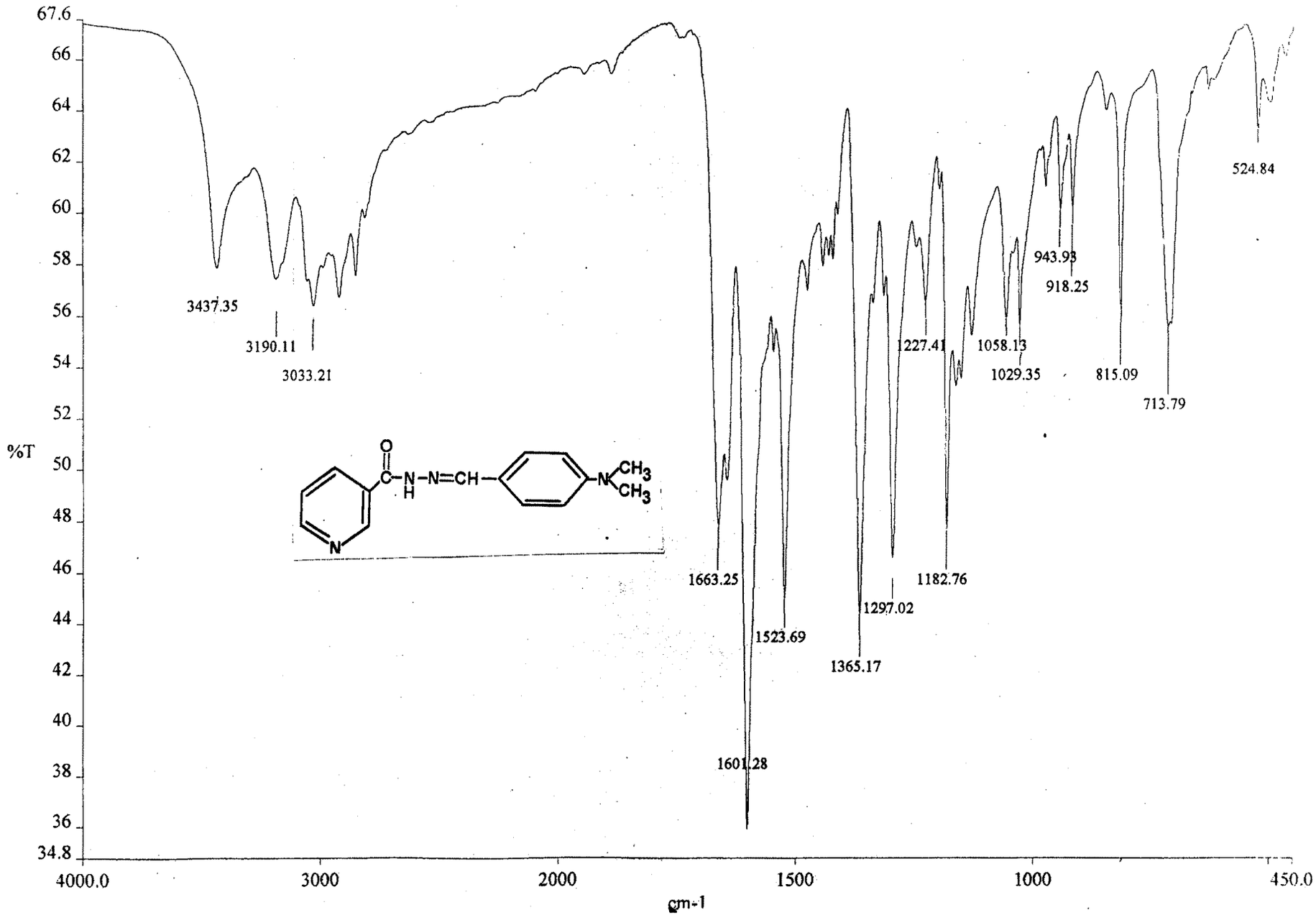


(iii_a)

IR (KBr): $\nu_{max,}$ 3437, 3190 (NH), 3033(CH), 1663, 1601(>=0), 1523(C=C), 1365(C-N), cm^{-1}. ---------- (Fig. No- 1)

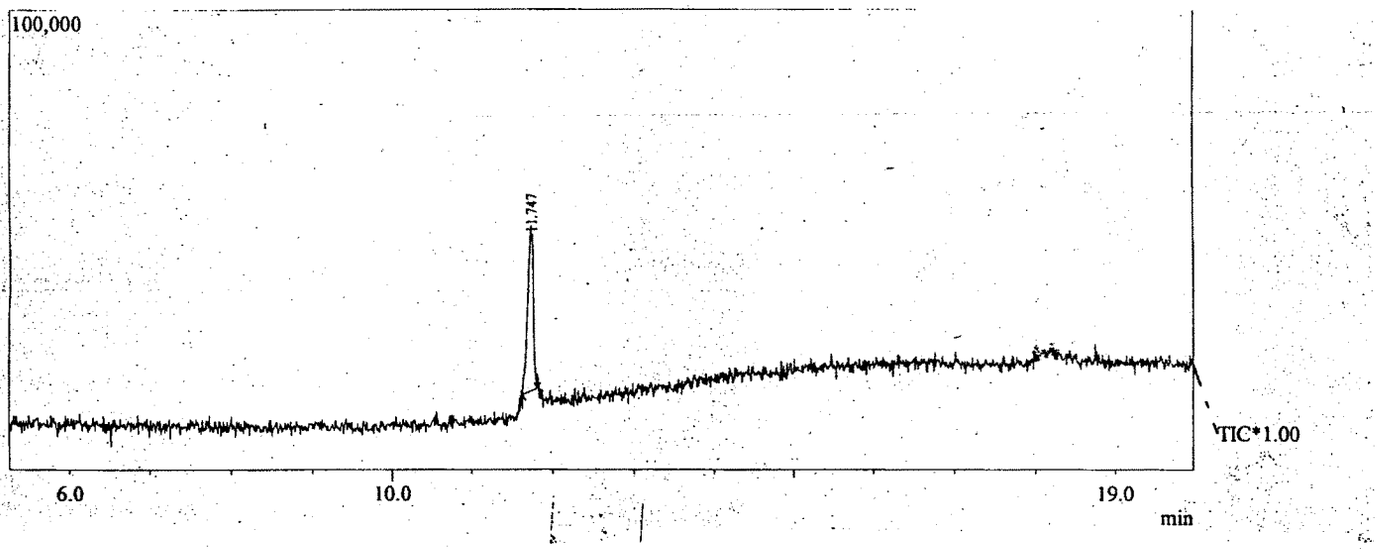
Mass (m/e): 268(M^+), 162,146,132,118,106,91,78,65,51---------- (Fig. No- 2)

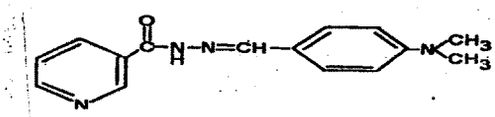
C.F.C. Shivaji Uni. Kolhapur Date: 1/14/05

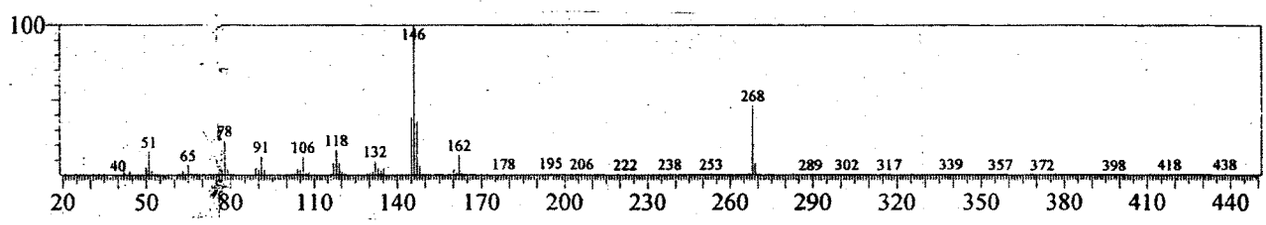


20

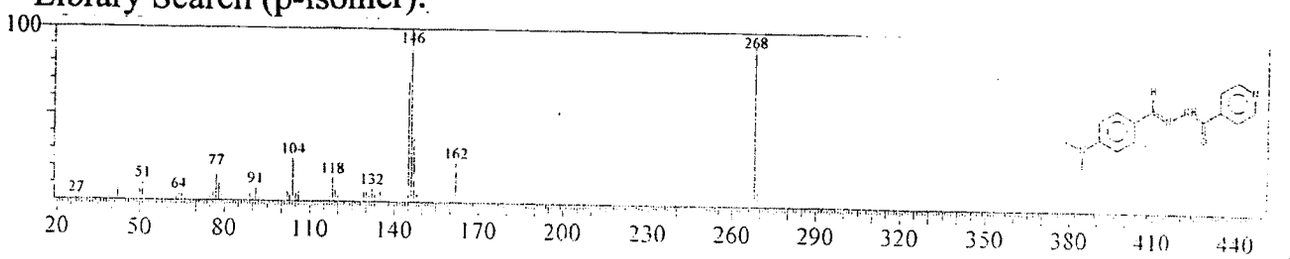
GCMS DATA (iiia)







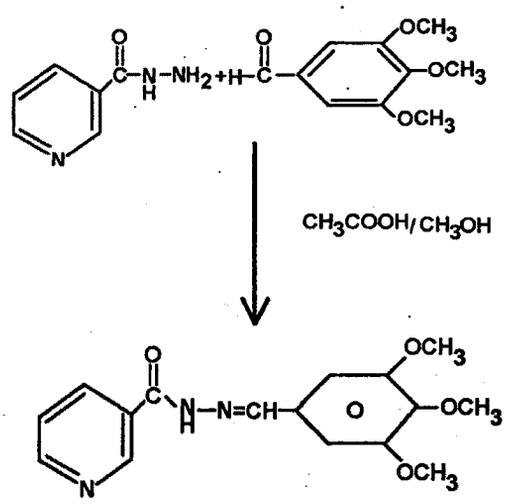
Library Search (p-isomer):



Fig, No-2

iiib] Reaction of nicotinyl hydrazide with trimethoxy benzaldehyde(3):

To the mixture of nicotinic acid hydrazide (0.05 mole) trimethoxy benzaldehyde (0.05 mole) in methanol (10 ml) to which 3 drops of glacial. acetic acid were added and refluxed on a water bath for five hrs: After the completion of reaction, the reaction mixture cooled when white product separated out was filtered and dried[24], M.P. 200^{0}C.



(iii_b)

IR (KBr). $\nu_{max,}$ 3434, 3184(NH), 2994, 2986, 2860,(C-H), 1641(>C=0), 1577(C=C&C=N), 1127 (Trisubstituted benzene) cm^{-1}

---------- (Fig. No-3)

Mass (m/e): 315(M^+), 193,178,163,150,135,118,106,78,66,51. -----(Fig. No-4)

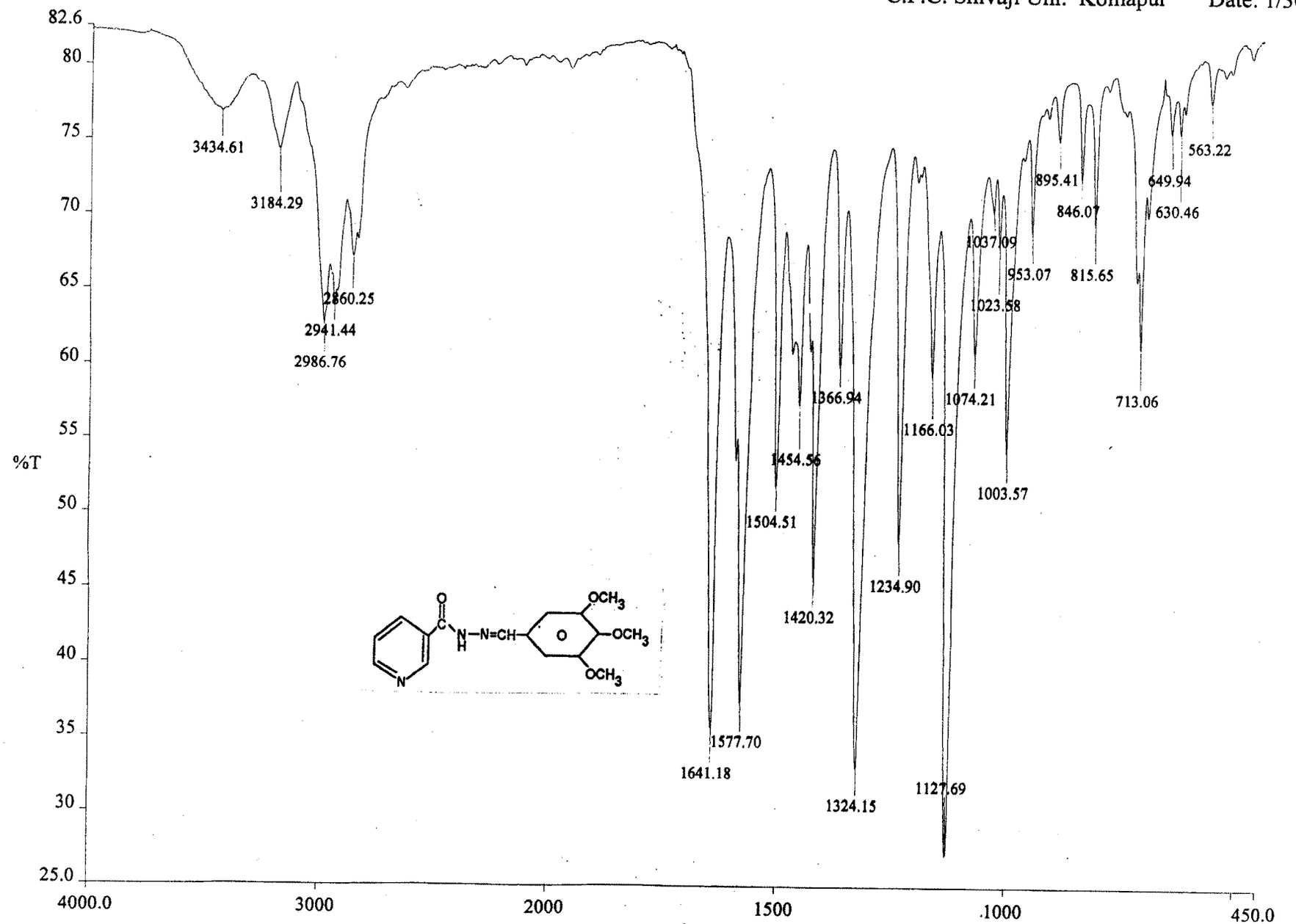
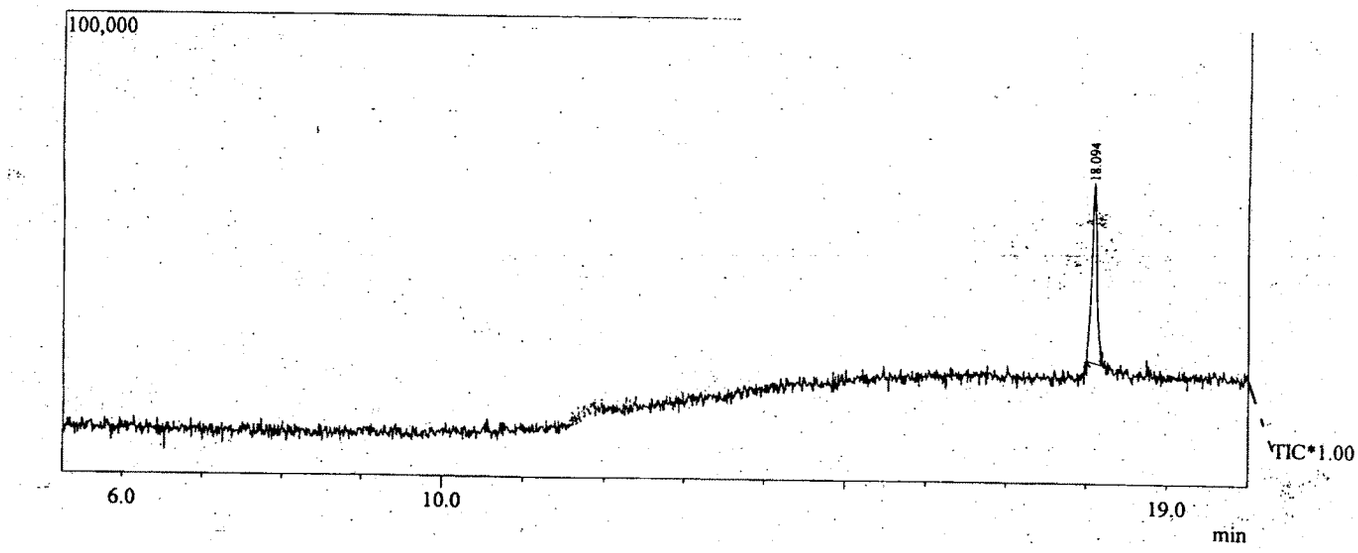
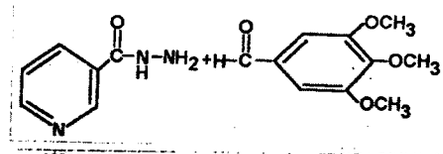


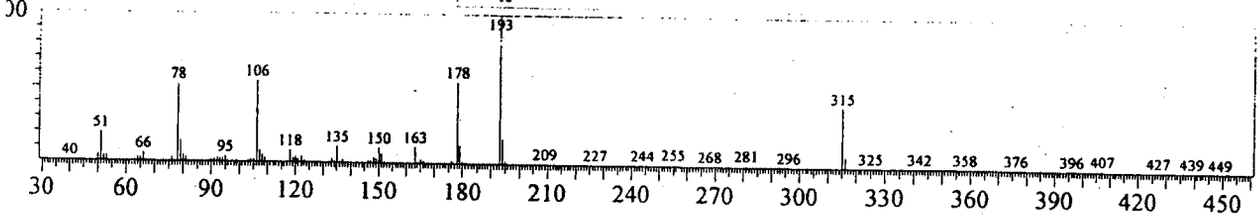
Fig. No-3

Description: Spectrum Name: CS - 2B.002 Date Created: wed jan 30 00:44:58 2002

GCMS DATA (iiib)







Library Search (o-isomer):

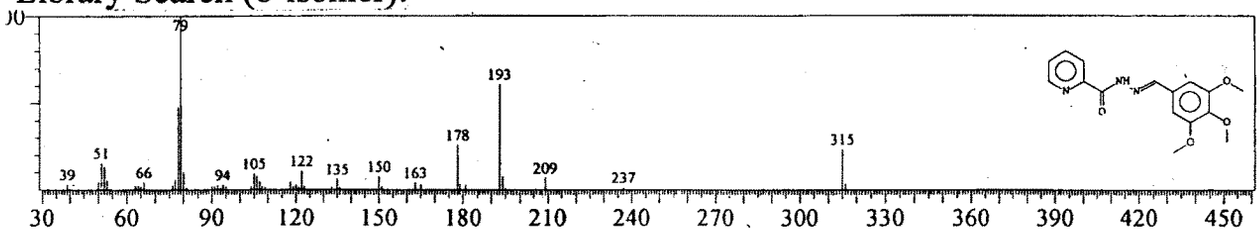
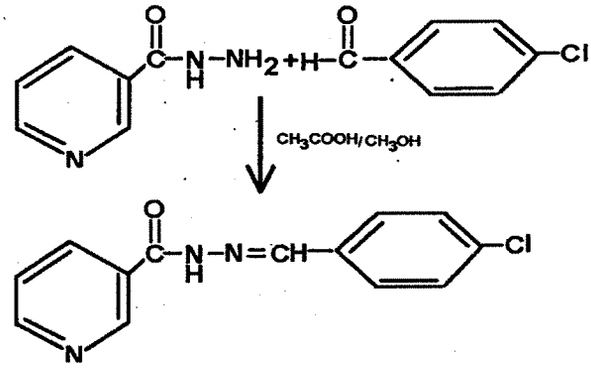


Fig. No-4

iiic] Reaction of Nicotinic acid hydrazide with p-Chloro benzaldehyde. (3):

The mixture of Nicotinyl hydrazide(0.05 mole) and p-Chloro benzaldehyde (0.05 mole) in methanol (15 ml) to which 3 drops of conc. acetic acid was added and the mixture get refluxed on a water bath for 5 hrs. After completion of the reaction the white crystalline product filtered dried and the purity checked by tlc-technicque[24], yield 70%, M.P.175^{0}C.



(iii$_c$)

IR (KBr): $\nu_{max,}$ 3258(NH), 2923(C-H), 1686, 1660(>C=0), 592(C=C&C=N), 1282(CH) cm^{-1}---------- (Fig. No-5)

Mass (m/e): 259(M^+), 224,148,137,122,106,89,78,65,51. ----------(Fig.No-6)

C.F.C. Shivaji Uni. Kolhapur Date: 1/30/02

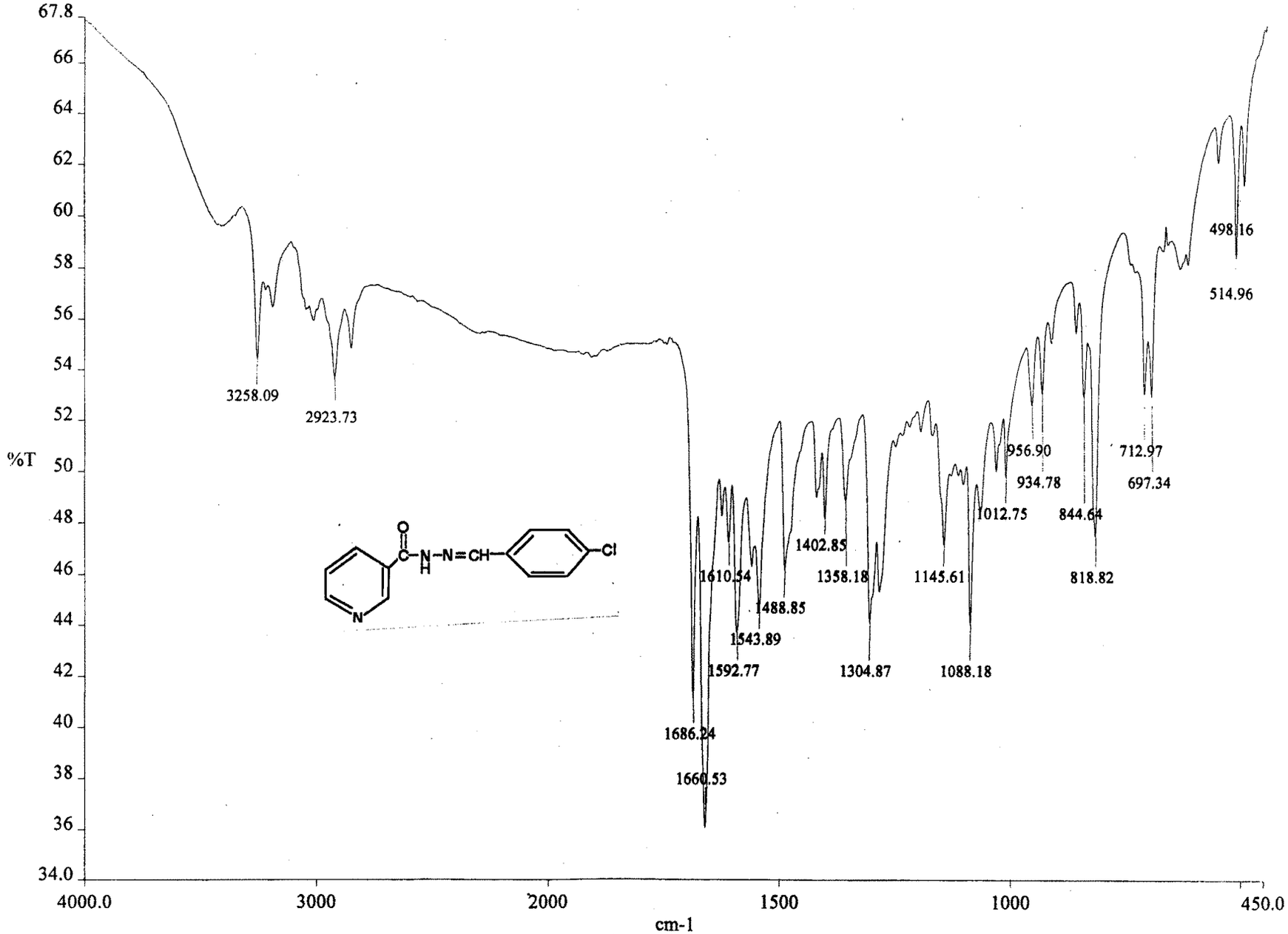
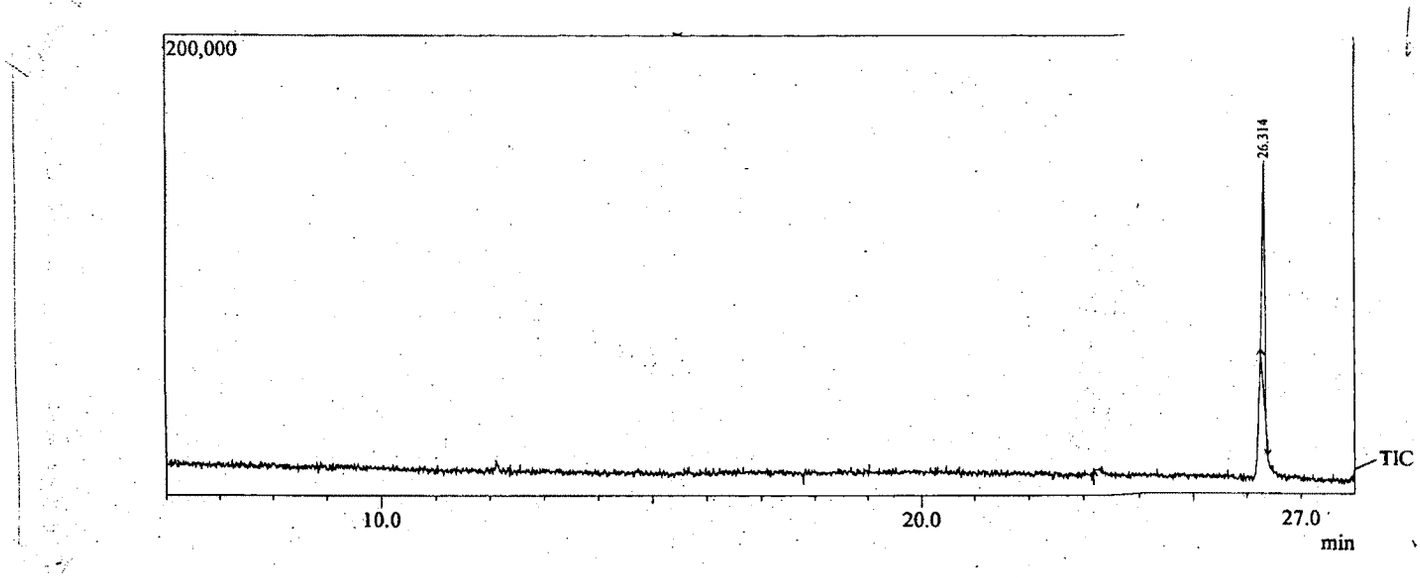
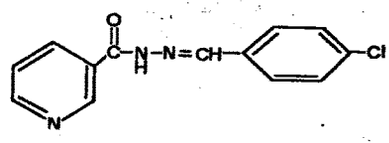


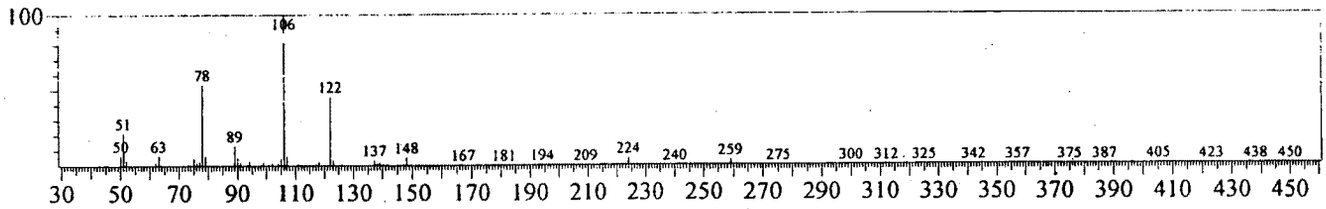
Fig. No-5

Description: Spectrum Name: CS - 3C.002 Date Created: wed jan 30 00:49:09 2002

GCMS DATA (iiic)



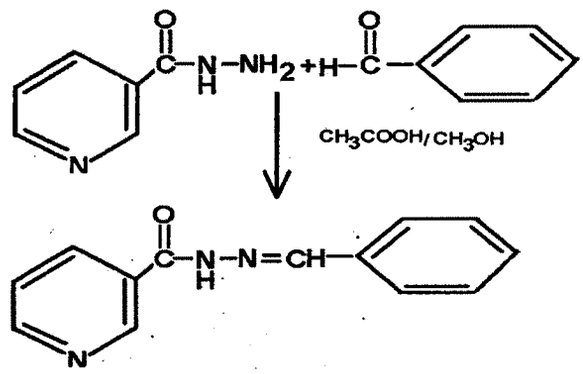




Fig; No-6

iii_d] Reaction of Nicotinyl hydrazide with benzaldehyde.(3):

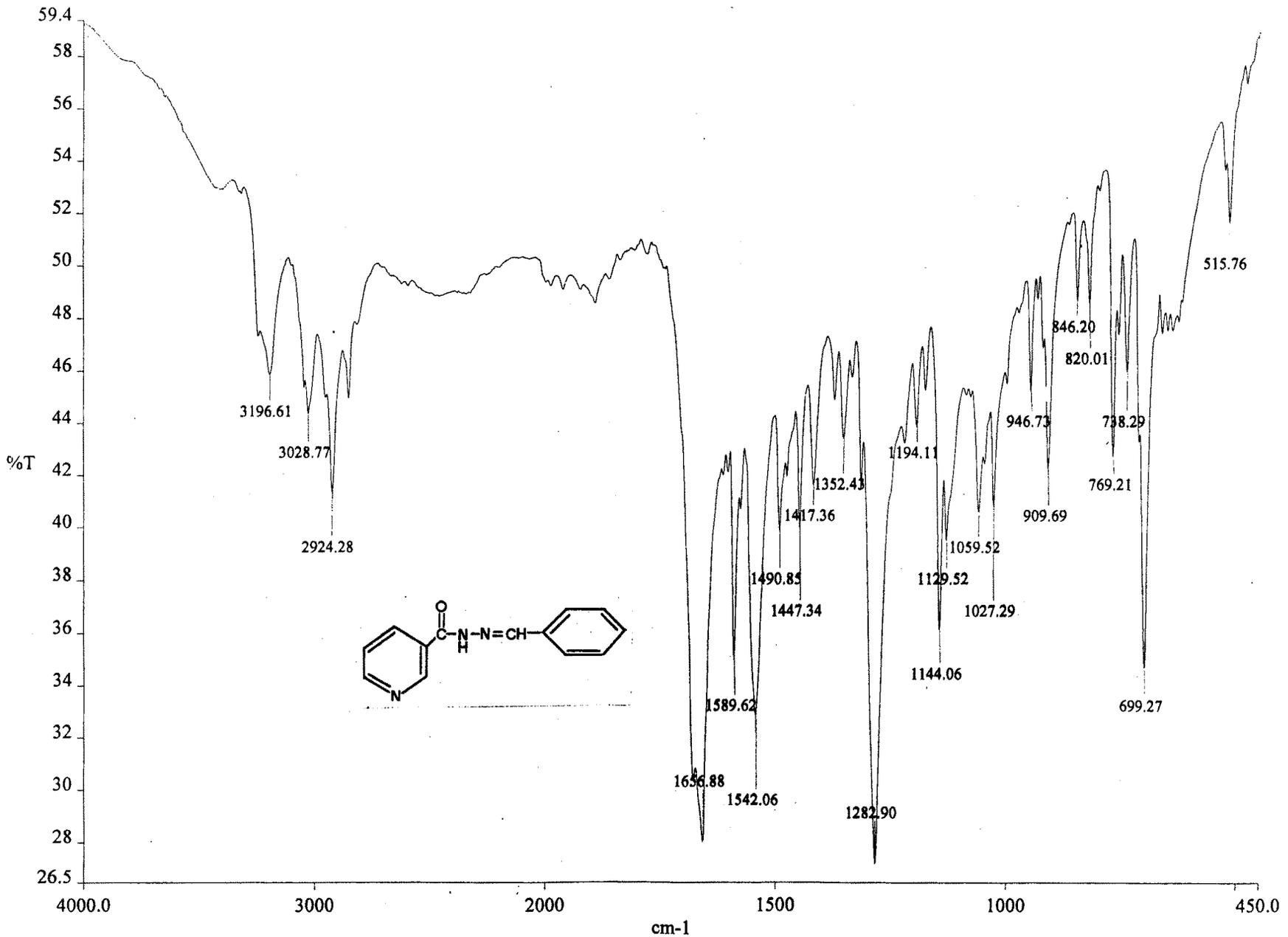
Nicotinic acid hydrazide (0.05 mole) and benzaldehyde (0.05 mole) in a methanol (15 ml) to with 3 drops of glacial acetic acid were added refluxed the mixture on water bath for 5 hrs., then cooled to get a solid, which was filtered and recrystallized form methanol as an solvent, yield 75%, M.P.130^{0}C.



(iii_d)

IR (KBr). ν_{max}, 3196(NH), 3028(CH), 1656(>C=0), 1589, 542(C=C&C=N), 1282(NH) cm^{-1}---------- (Fig. No-7)

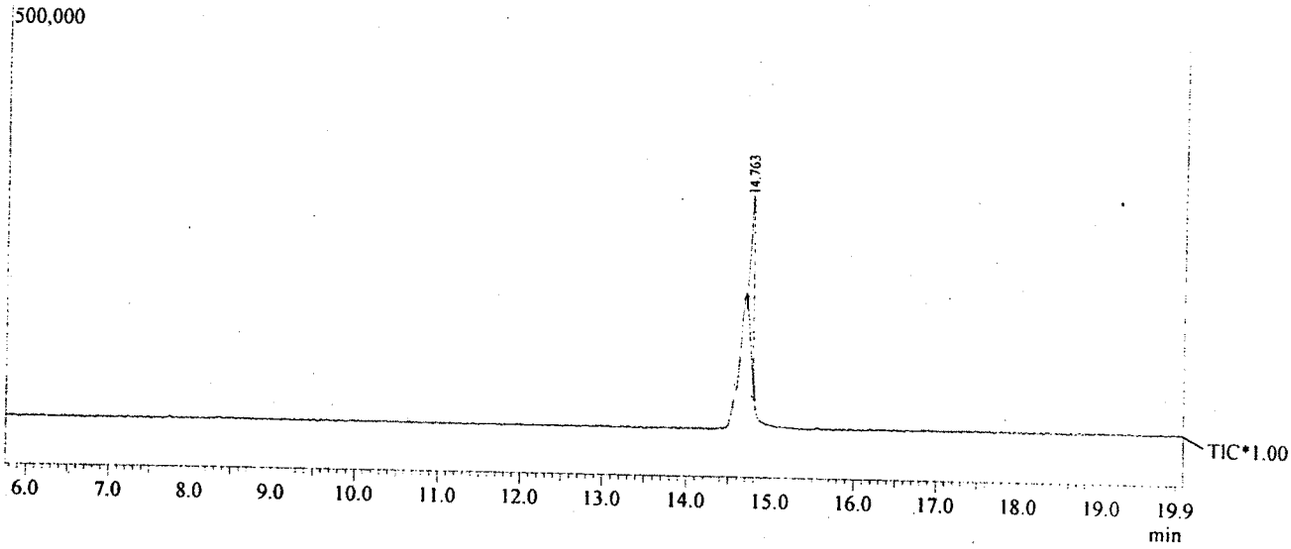
Mass (m/e): 225(M^+), 148,122,106,89,78,65,51. ----------(Fig. No- 8)

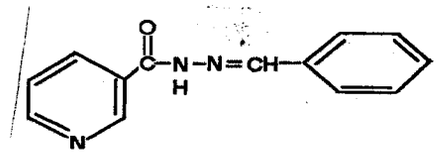


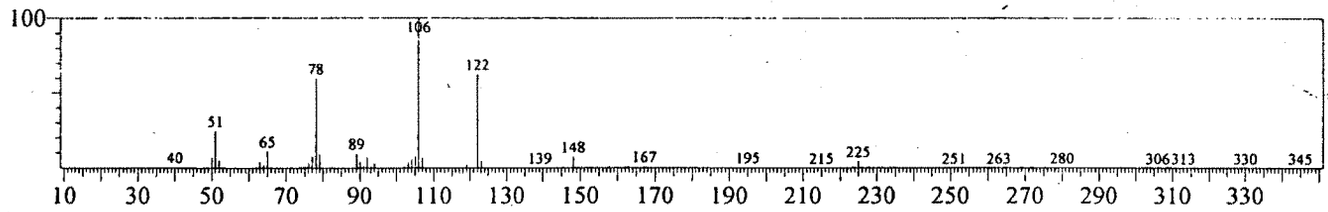
Fig, No-7

Description: Spectrum Name: CS - 4D.002 Date Created: wed jan 30 00:55:50 2002

GCMS DATA (iiid)







Library Search (p-isomer):

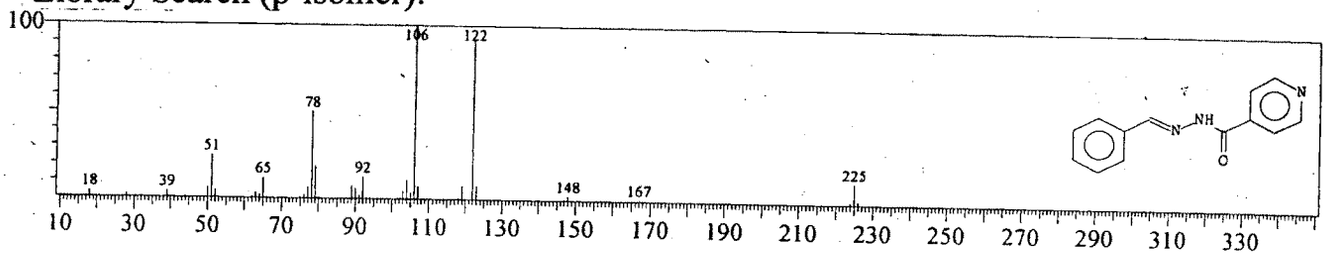
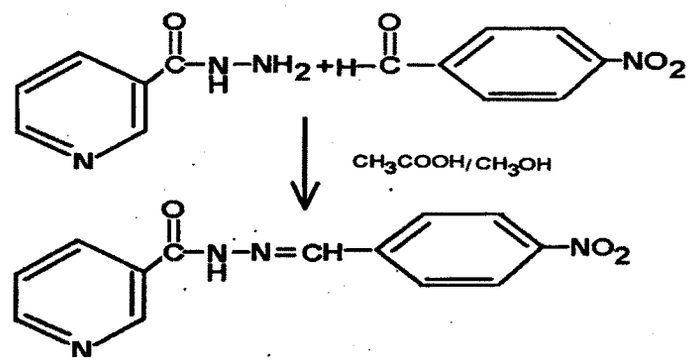


Fig. No-8

iiie] Reaction of Nicotinic acid hydrazide with p-nitrobenzaldehyde. (3):

The mixture of Nicotinic acid hydrazide (0.05 mole) and p-nitro benzaldehyde (0.05 mole) in methanol (15 ml) to which 3 drops of glacial acetic acid where added and the reaction mixture refluxed on the water bath for 5 hrs. After completion of the reaction the reaction mixture cooled to get crystalline product. The final product was recrystallized from methanol[24], yield 70%, M.P.250^{0}C.



(iii_e)

IR (KBr). ν_{max}, 2924,2853(CH), 1595,1518(C=C&C=N), 1103(1,3-disubstituted benzene)---------- (Fig. No-9)

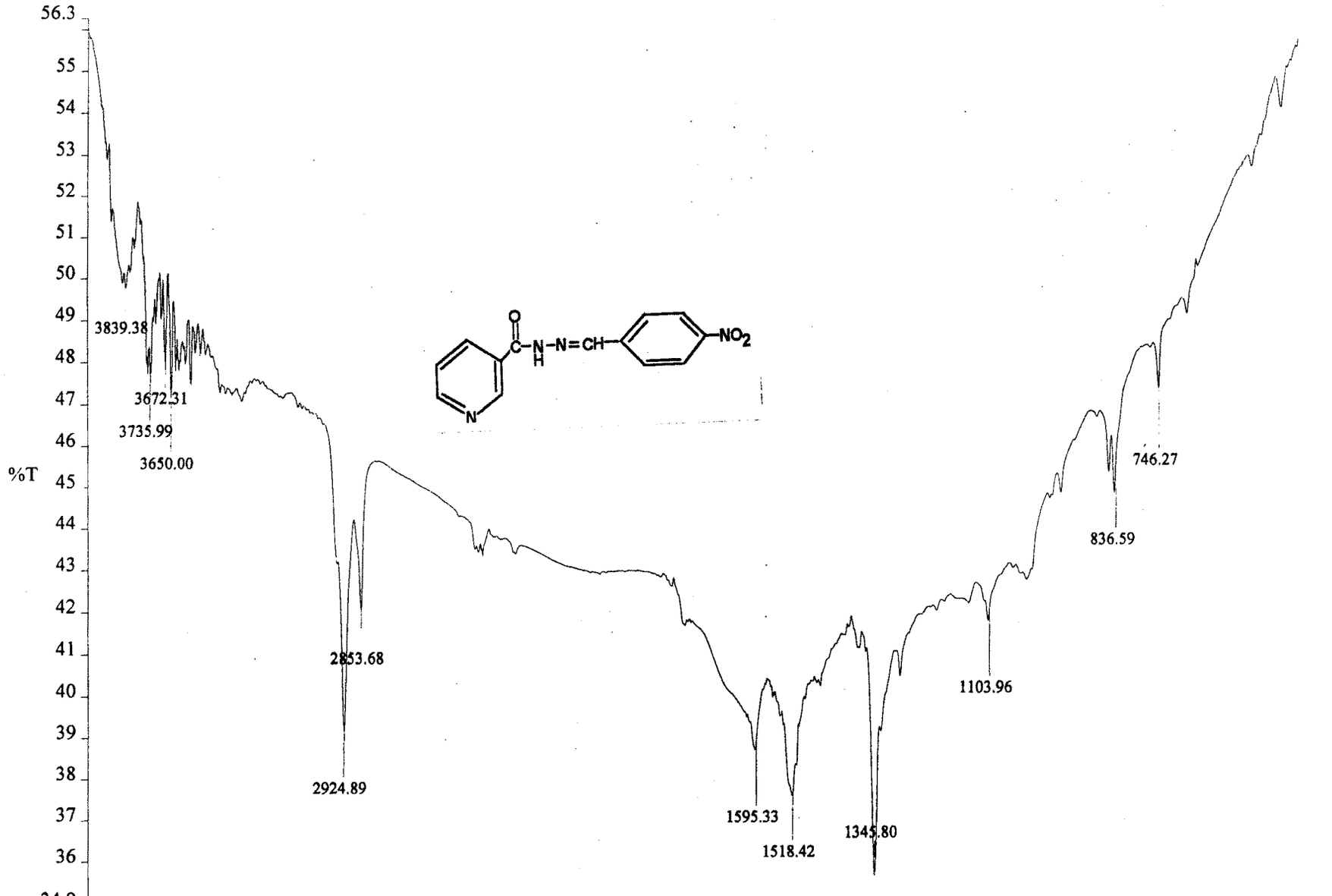
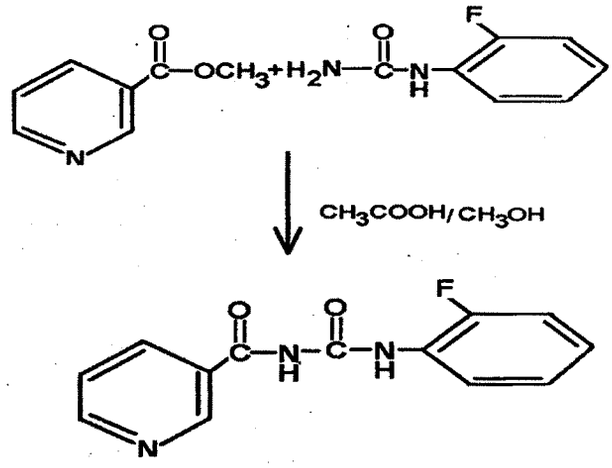


Fig. No-9

Date Created: wed jan 30 00:07:48 2002

iii$_f$] Reaction of Methyl Nicotinate with 2-Fluoro phenyl urea.(4) :

To the mixture of Methyl nicotinate (0.05 mole) and 2-Fluoro phenyl urea in a methanol (15 ml), 3 drops of glacial acetic acid were added and the reaction mixture refluxed on water bath for 5 hrs. Then cooled, to get solid which was recrystallized from methanol, yield 70%, M.P.177^{0}C.

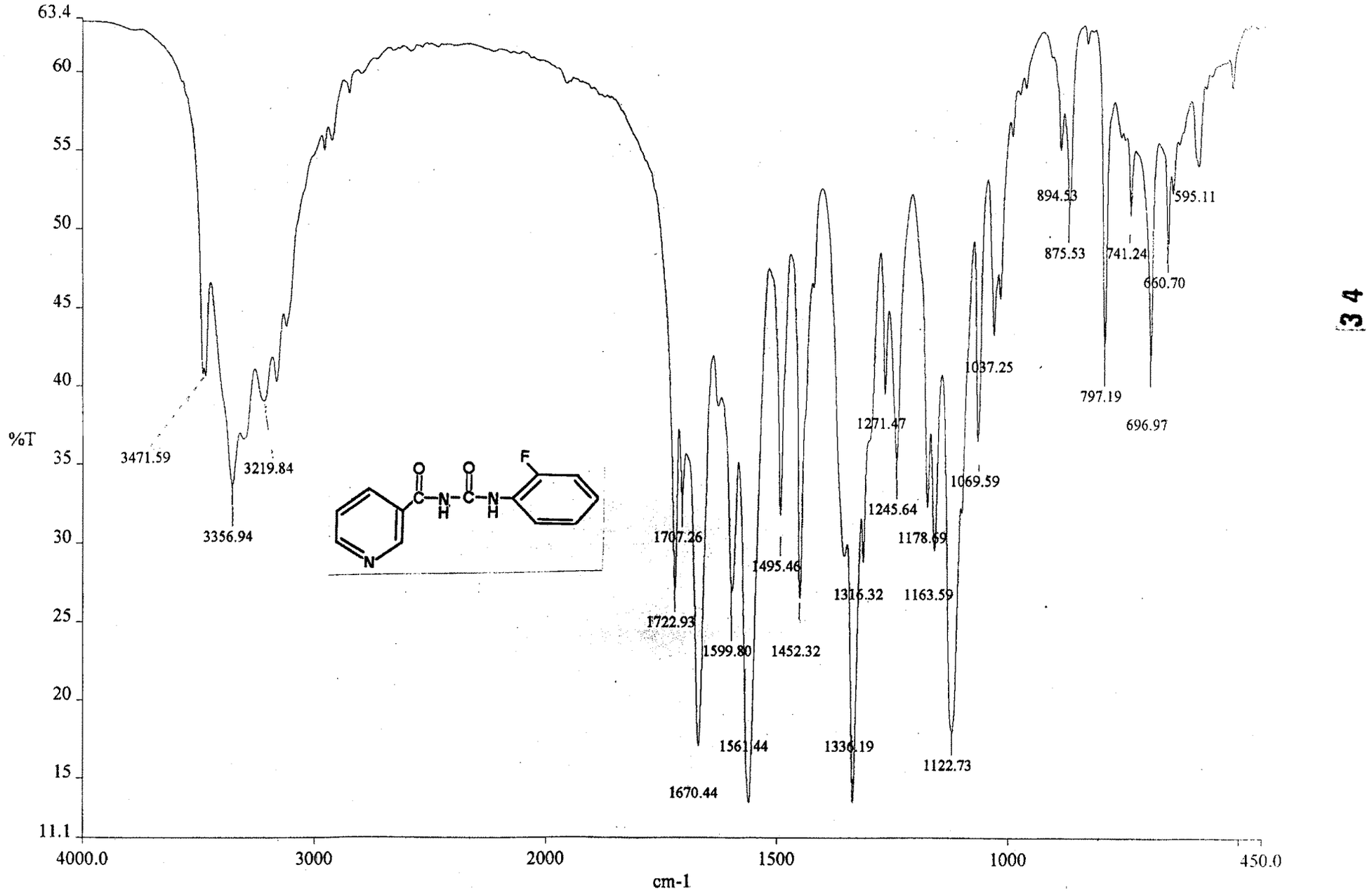


(iii$_f$)

IR (KBr). $\nu_{max,}$ 3471, 3356, 3219(NH), 1722, 1707, 1670(-Co-NH-Co-), 1599,1561(C=C&C=N), 1336(NH), 1122(1,3-disubstituted benzene) cm^{-1}---------- (Fig. No-10)

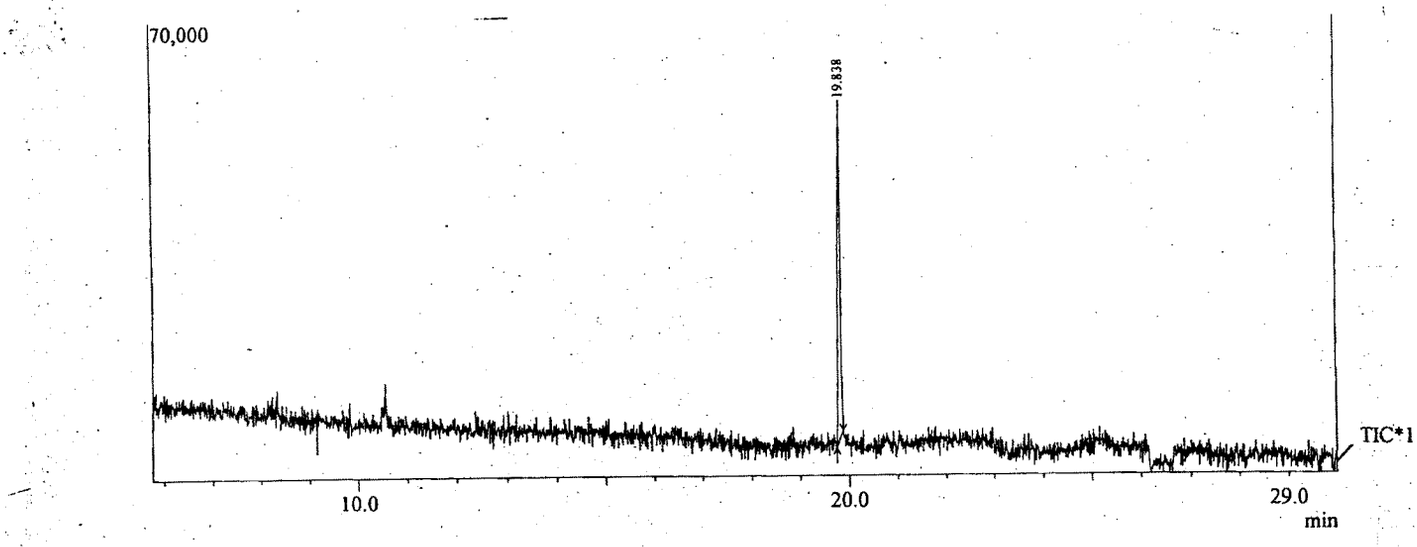
Mass (m/e): 253(M^+), 154,112,111,91,83,64,57,44. ----------(Fig. No-11)

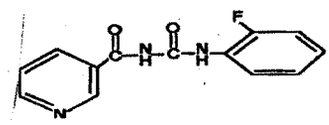
C.F.C. Shivaji Uni. Kolhapur Date: 1/14/05



34

GCMS DATA (iiif)





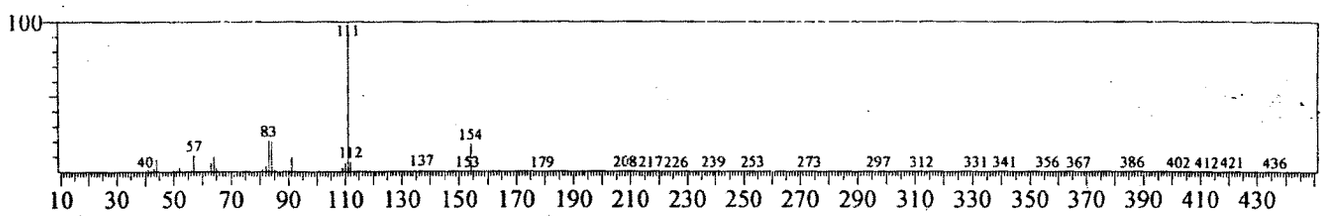
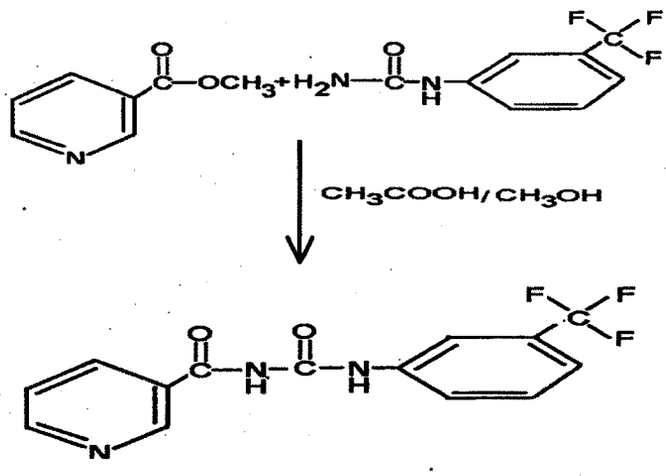


Fig. No-11

iii$_g$] Reaction of Methyl nicotinate with 3-trifluoro methyl phenyl urea.(5) :

To a mixture of Methyl nicotinate (0.05 mole) and 3-(trifluoro methyl) phenyl urea in a methanol(10 ml) to 3 drops of acetic acid were added and the mixture further heated on a steam bath for 5 hrs. After the completion of reaction the solvent was removed under vacuum. The solid obtained was recrystallized from methanol, yield 75%, M.P.177^{0}C.



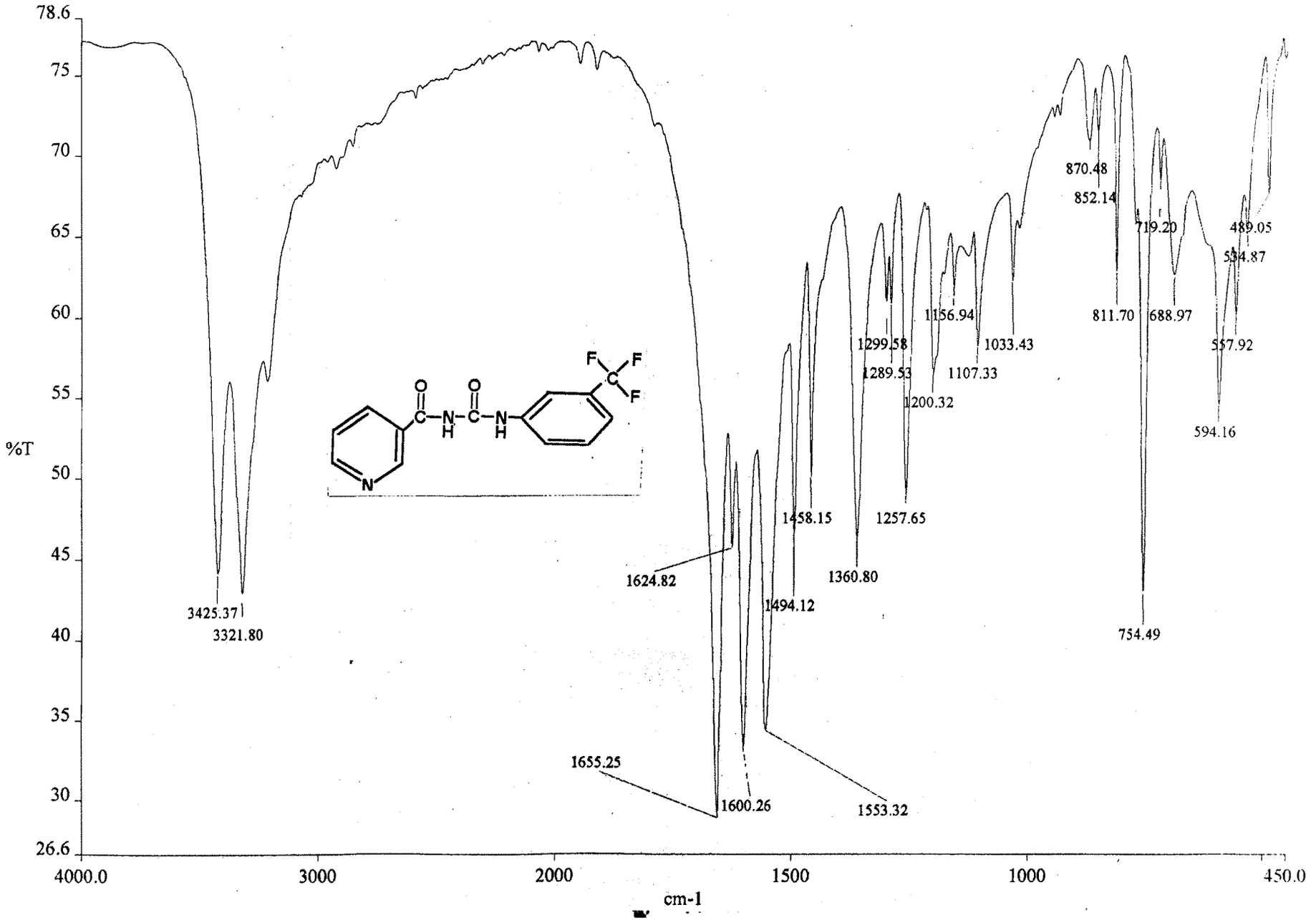
(iii$_g$)

IR (KBr). $\nu_{max,}$ 3425, 3321(NH), 1655(>C=0), 1625(C=C), 1600, 1553 (C=C&C=N), 1361(C-N), 754(benzenering with 4 adjecent H-atom) cm^{-1}---------- (Fig. No-12)

Mass (m/e): 306(M^+), 244,187,168,159,145,139,132,109,90,75,69,63.

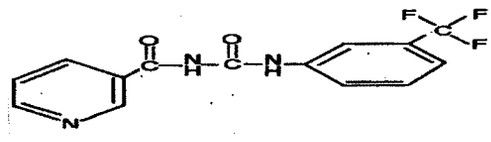
----------(Fig. No- 13)

C.F.C. Shivaji Uni. Kolhapur Date: 1/14/05



37

MS DATA (iiig)



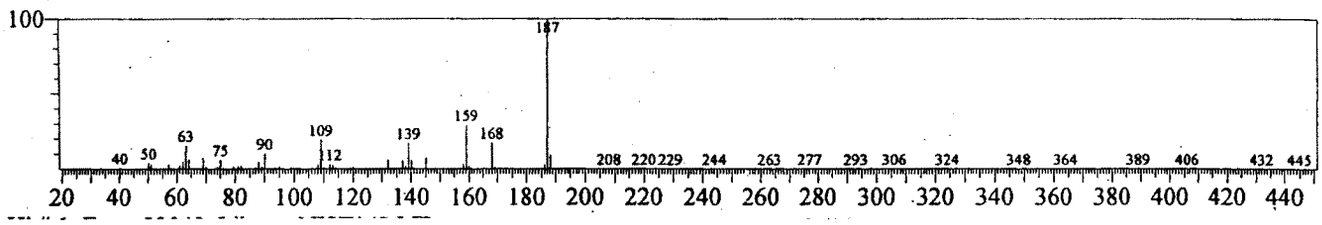
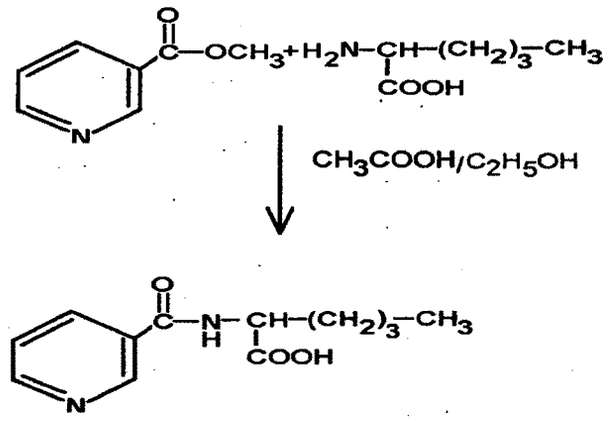


Fig. No-13

iiih] Reaction of Methyl nicotinate with DL-nor-leucine(6):

The mixture containing Methyl nicotinate (0.05 mole) and DL-nor leucine (0.05 mole) in an ethanol (10 ml), 3 drops of glacial acetic acid were added and the reaction mixture refluxed on a water bath for 5 hrs. After reaction completion of the reaction the mixture was cooled to get a solid, which were recrystallized from ethanol, yield 70%, M.P.220^{0}C.

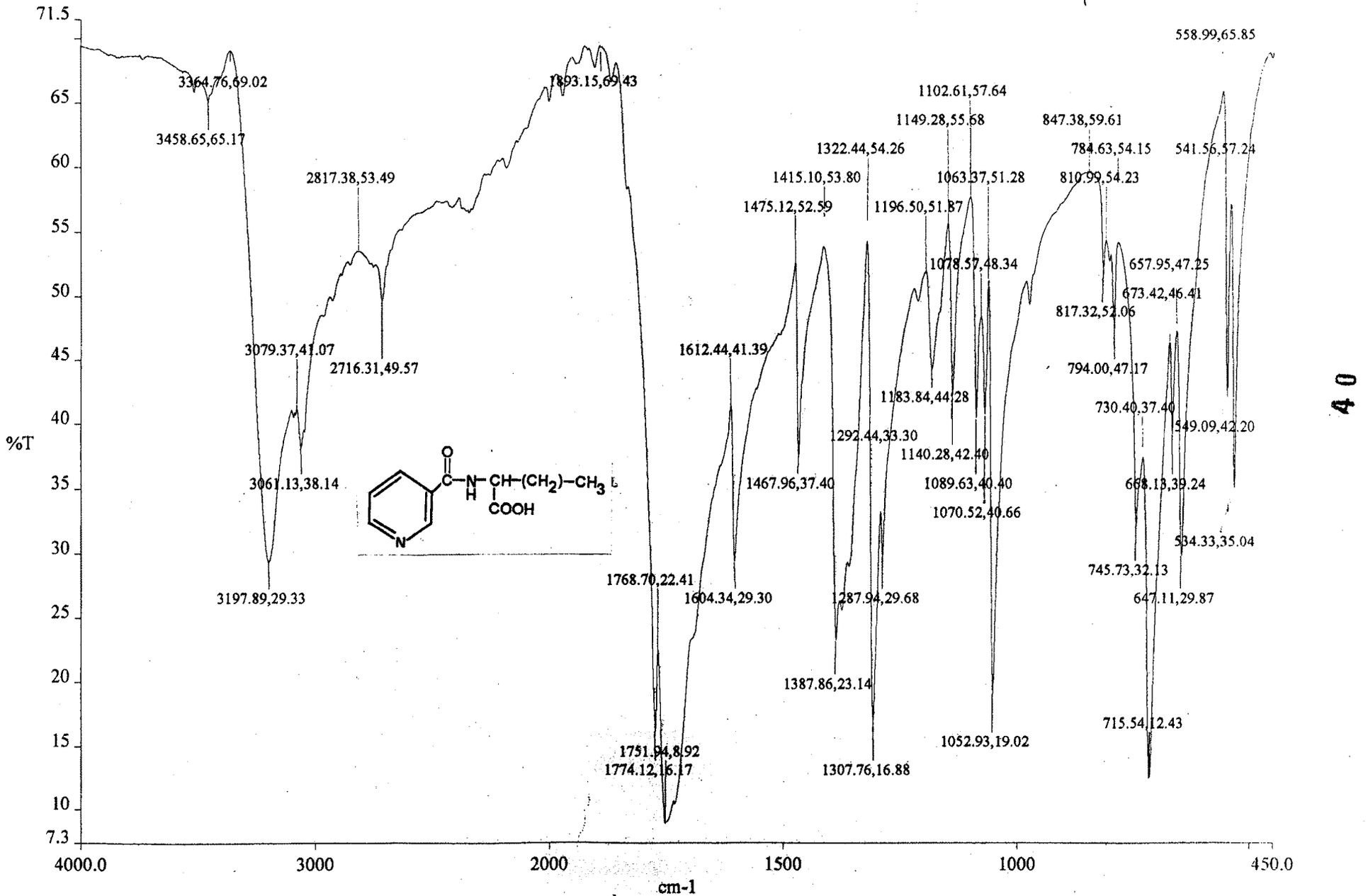


(iii_h)

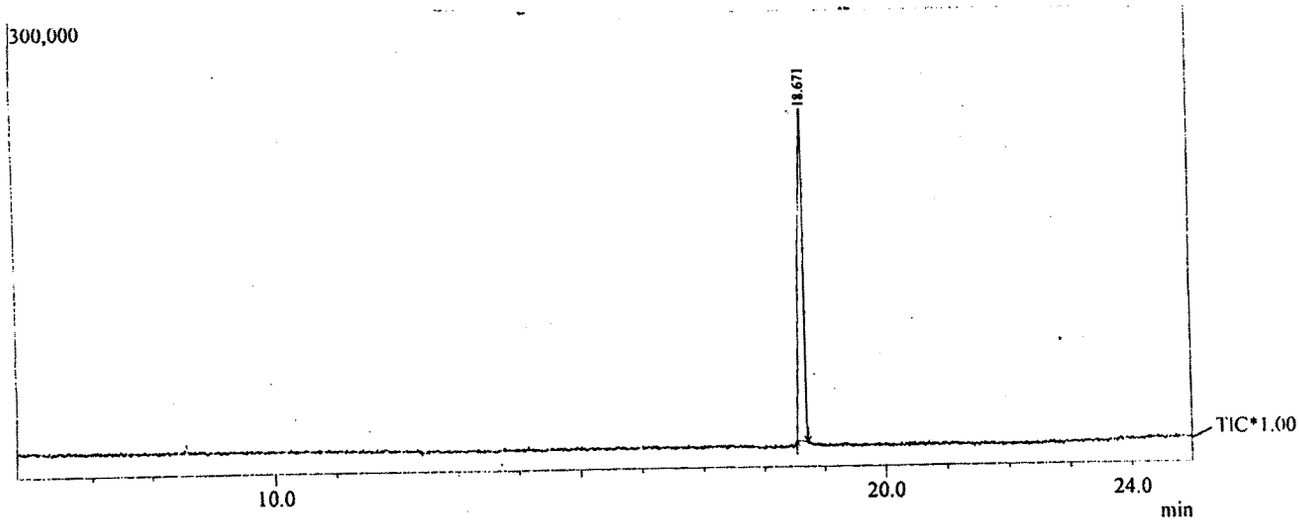
IR (KBr): $\nu_{max,}$ 3458, 3197(NH), 3061(C-H), 2716(NH), 1774, 751(>C=0), 1604(C=C&C=N), 1467(-CH_2-), 1387(-CH_3), 1307, 1052(C-H), 1307, 715(NH) cm^{-1} ----------(Fig. No-14)

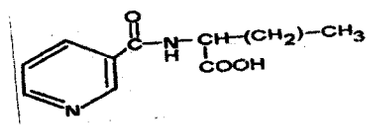
Mass (m/e): 147,104,90,70,66,50,38. ----------(Fig. No-15)

C.F.C. Shivaji Uni. Kolhapur Date: 8/2/04



GCMS DATA (iiih)





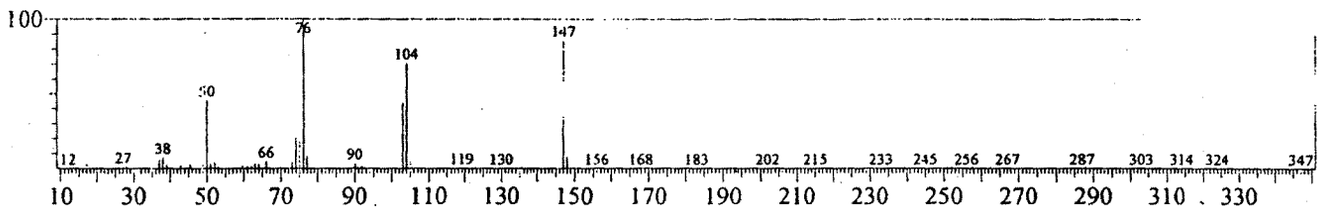


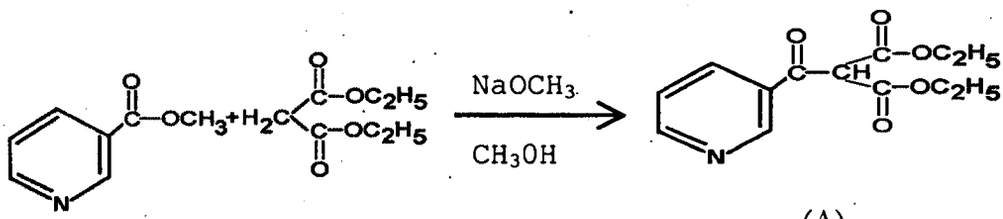
Fig. No-15

15110

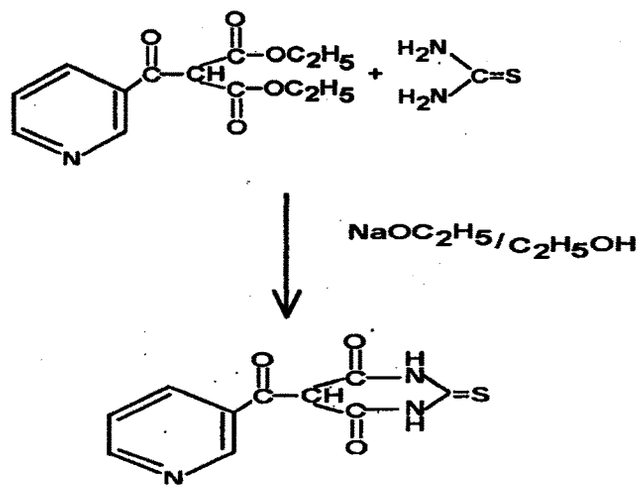
iii$_i$] Reaction of Methyl nicotinate with diethyl malonate and by thiourea.(7):

The mixture of Methyl nicotinate (0.05 mole) and diethyl malonate (0.05 mole) in methanol (10 ml) to which a pintch of $NaOCH_3$ was added and the reaction refluxed on a water bath for 5 hrs. The solvent was removed and dried it to get diester (A), which was used, as such for further reaction with thiourea[25].

The diester (A) (0.05 mole) and thiourea (0.05 mole) heated on a water bath for 5-6 hrs., cooled and concentrated to get solid product[23] purified from ethanol, yield 85%, M.P.168^{0}C.



(A)

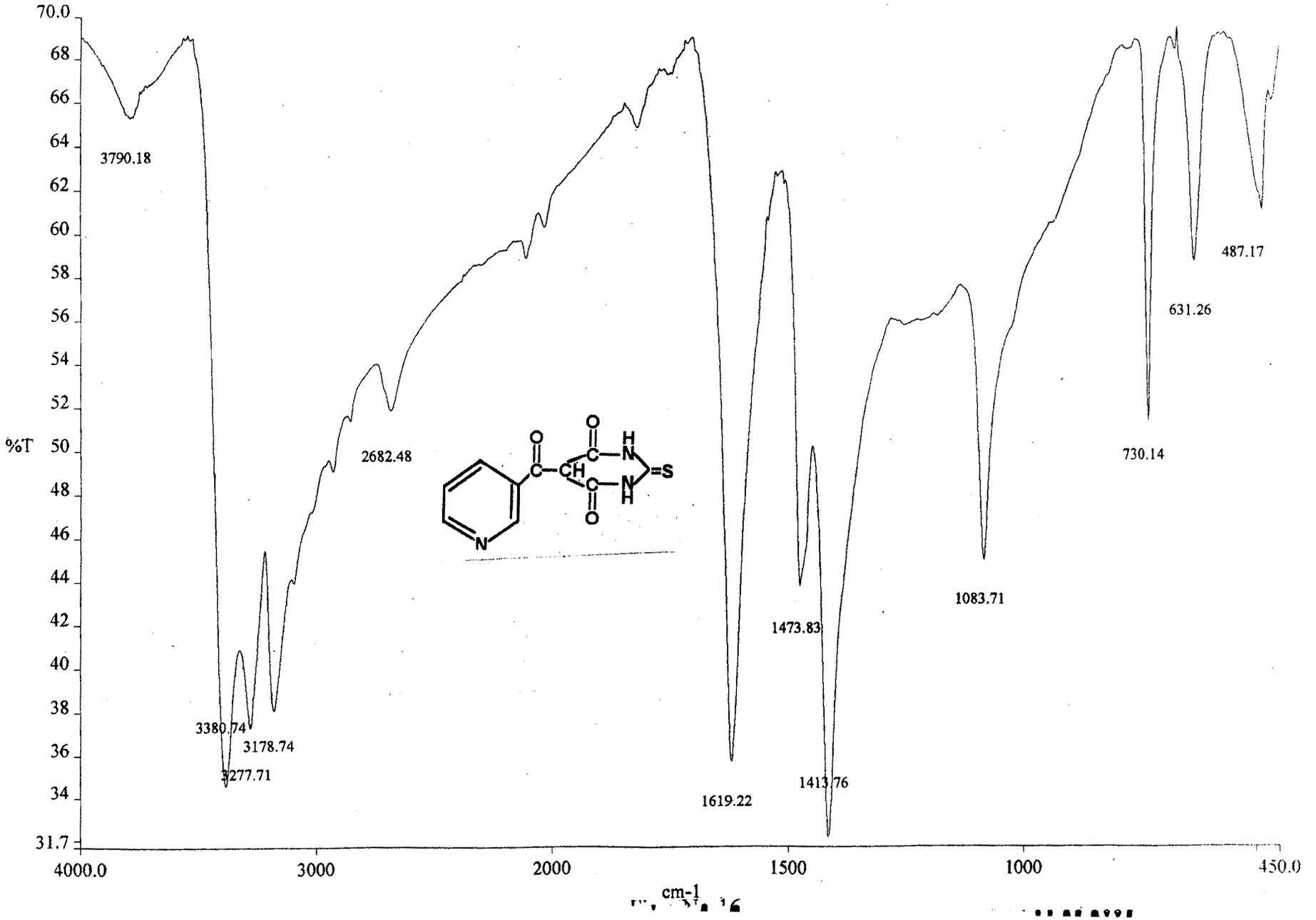


(iii$_i$)

IR (KBr): ν_{max}, 3380, 3277, 3178(NH), 1619(>C=0), 1473, 1413(C-H), 630(CH) cm^{-1} ---------- (Fig. No-16)

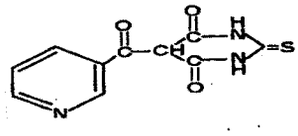
Mass (m/e): 249(M^+), 217,134,132,130,99,97,95,94,62,60,47.----(Fig. No- 17)

C.F.C. Shivaji Uni. Kolhapur Date: 3/5/05



43

MS DATA (iii$_i$)



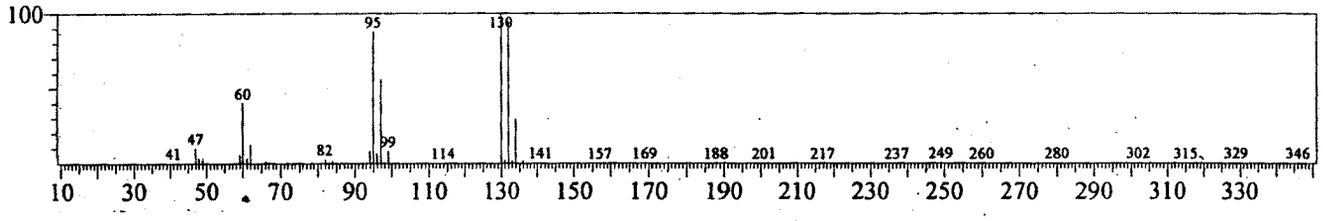
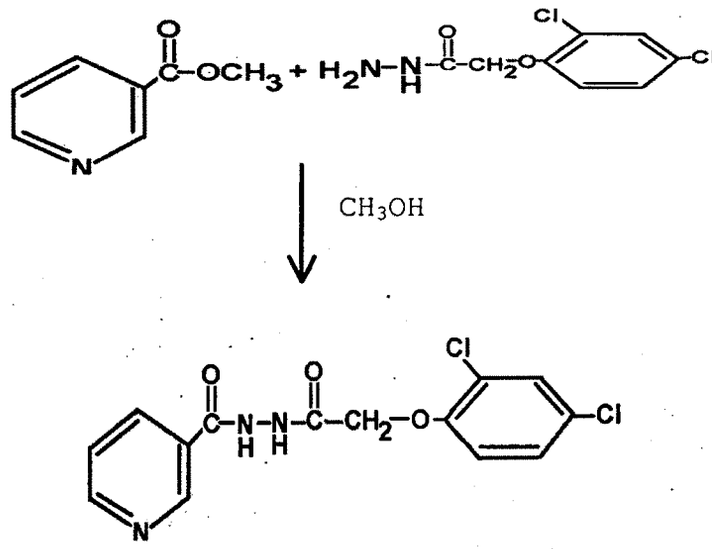


Fig. No-17

iii$_j$] Reaction of methyl nicotinate with 2,4-dichlorophenoxy acetic acid hydrazide.(8):

The mixture of Methyl nicotinate (0.05 mole) and 0.05 mole of 2,4-dichloro phenoxyacetic acid hydrazide in an methanol (15 ml), was refluxed on a steam bath for 6 hrs, cooled and the solid product that separated out, was filtered to get desired producted, yield 65%, M.P. 145^{0}C.

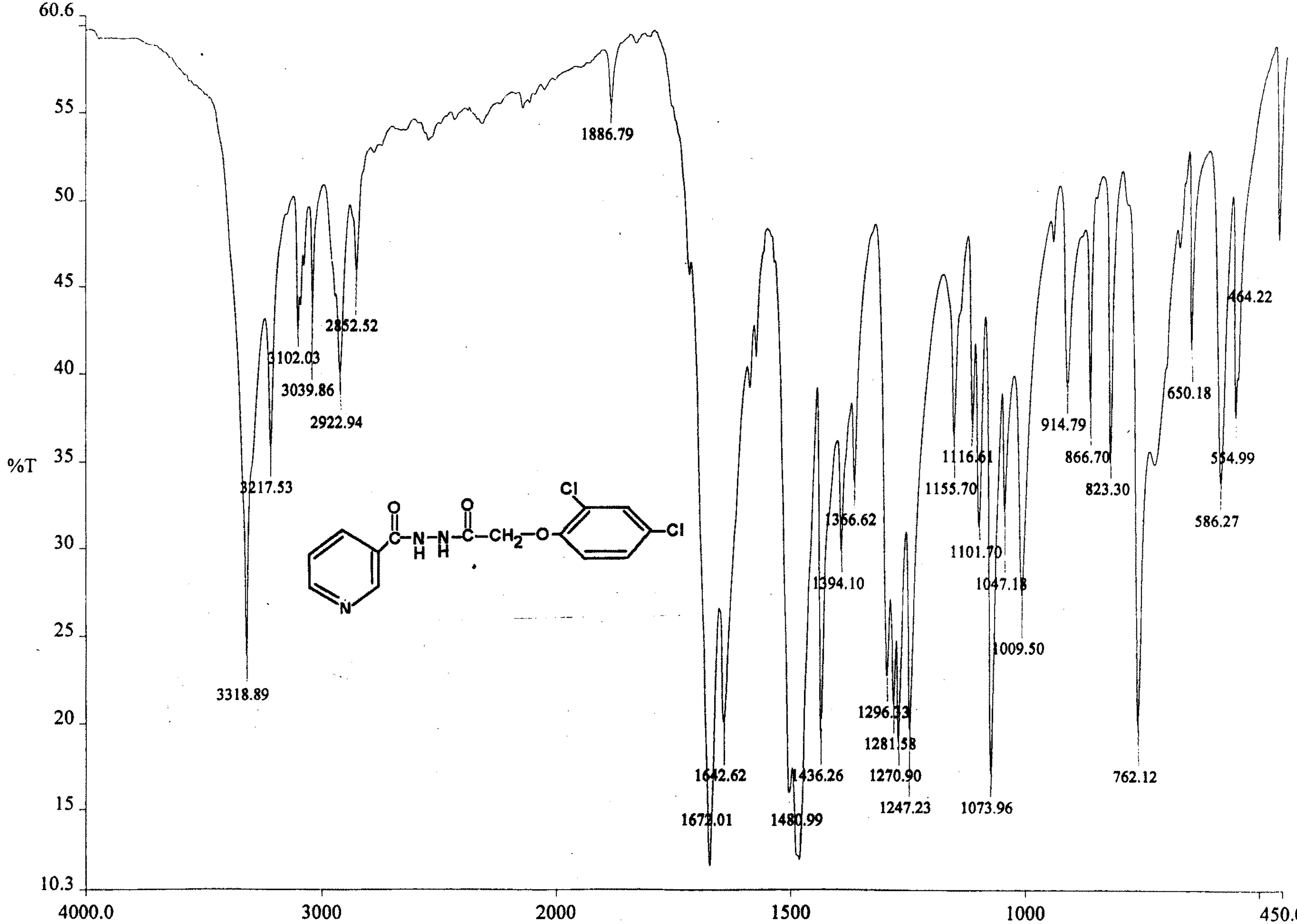


(jjj$_j$)

IR (KBr). $\nu_{max,}$ 3318, 3217, 3102(NH), 3039(CH), 2922, 2852(-CH_2-o-), 1672(>C=0), 1642(>C=0), 1500, 1480(C=C&C=N), 1436(C-H), 1270(R-O-Ar), 762(C-Cl) cm^{-1} ---------- (Fig. No- 18)

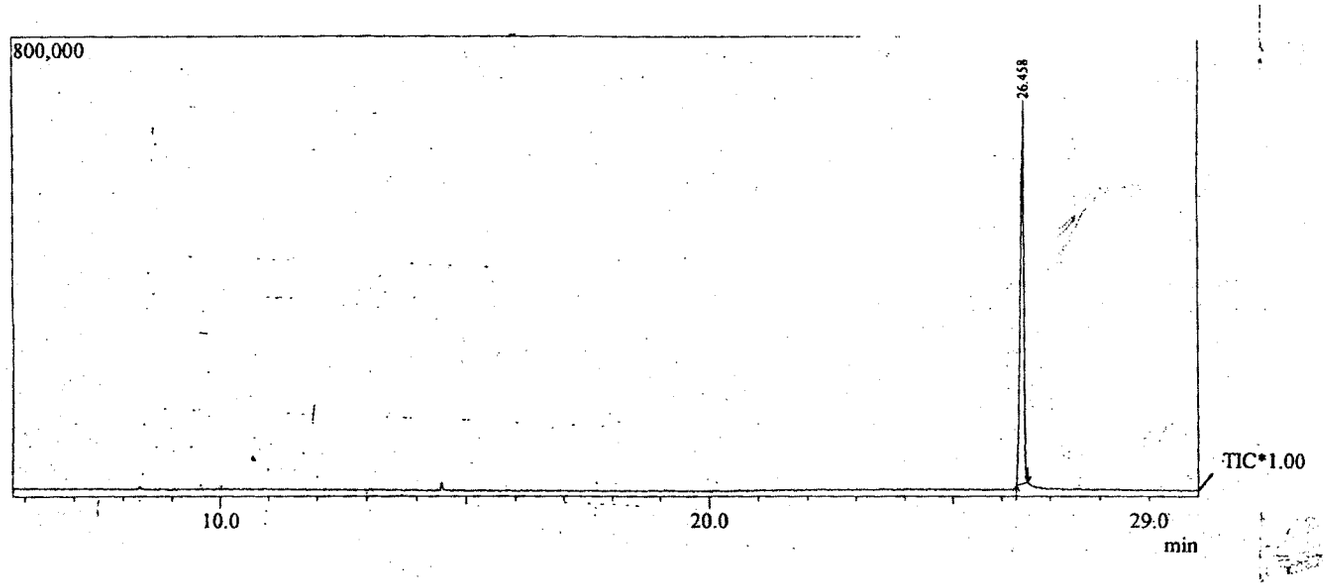
Mass (m/e): 336(M^+), 274,241,239,175,145,113,111,85,71,56,42.-(Fig.No-19)

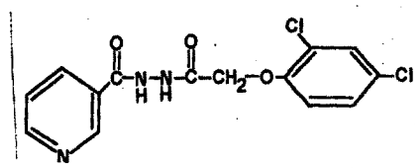
C.F.C. Shivaji Uni. Kolhapur Date: 1/30/02



Fig, No-18

GCMS DATA (iiij)





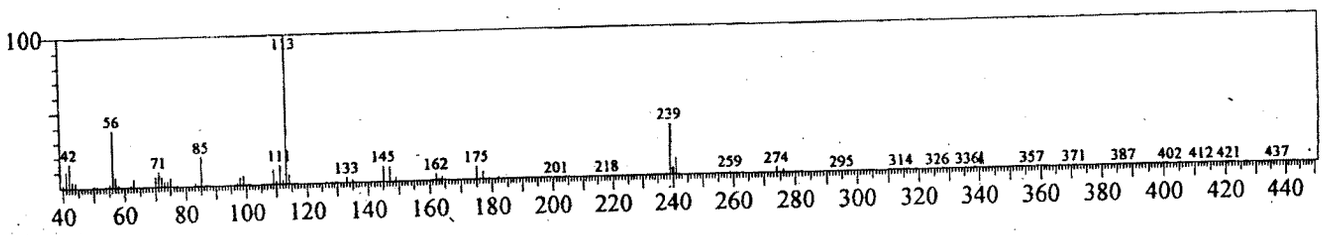


Fig. No-19.

B)Insecticidal bio-assay :

a] *Myzus persicae* (Tobacco aphid):

i) Performed on live plant:

The process of bioassay performed on the live plant i.e. on tobacco seedlings. The seedlings were collected from nursery bed in the month of July, and required 1 month for plantation to achieve 3 to 4 leaf stages. (Fig.1). These seedlings were planted in the plastic cups with a 3 part of soil and one part of well decomposed farmyard manure (Fig.3). These planted seedlings were kept in to a well-ventilated place with proper photo periodism. These seedlings were ready for testing within 3 to 4 days.

A standard solution of synthesized compound was prepared in an acetone at 100 ppm, 300 ppm and 500 ppm. These standard solution were applied on a leaves of the planted tobacco seedlings at the rate of 1ml by using a 1ml pipette. The second instars aphid collected from the sucker of tobacco plant, transferred at the rate of 30 young ones on the treated seedlings. After transplanting the young ones, immediately the seedlings were covered with a perforated plastic bags (Fig.4). Similarly, the experiment was performed on the control plant. After an interval of 24 hrs, 48 hrs and 72 hrs the mortality data have been collected and presented in a tabular form for further statistical analysis. The same procedure was repeated 3 times to achieve reproducibility of the results[21].

Fig-20: Tobacco seedlings grown on nursery bed.

Fig-21: Infected tobacco plants by aphid (*Myzus persicae*) in field.

Fig-22: Plantation of tobacco seedlings.

Fig-23: Application of chemicals and inoculation of the 'aphid on tobacco Plant.'

Fig-24: Dead aphids count (after certain intervals).

ii) Performed by residual thin film technique:

The process of bioassay mainly performed by residual thin film technique by preparing different concentrations of synthesized organic compounds at 100 ppm, 300 ppm and 500 ppm in acetone. These were then applied separately on petriplate at the rate of 0.5 ml each on the upper and lower plate and allowed to dry it to get thin film of residue on both the inner side of petriplates.

Aphids were previously collected from the sucker of tobacco plantation, and second instar aphids were transferred into each plate in a batch of 30 insects. After an interval of 24 hrs, 48 hrs and 72 hrs the data were collected and presented in a tabular form. Similar experiments on the control of the solvent acetone were performed and analyzed statistically[21].

b) ***Helicoverpa armigera*** :

The process of bioassay mainly performed on *Helicoverpa armigera.* The third instar larval stages of *Helicoverpa armigera* were collected from the field of chickpea in the month of November-December. Due to the canabolic nature of the individuals were kept in a separate container. The bioassay was done at 100 ppm, 300 ppm and 500 ppm concentrations of synthesized compounds.

The socked chickpea were treated at the different concentrations separately, dried and fed to collected larvae *Helicoverpa armigera* individually on a separate container (Fig.25). The same experiment was repeated with control in batch of 30 individuals. After an interval of 48 hrs, 96 hrs and 144 hrs; the number of individuals those going to shrink and immobilized due to test chemicals were counted. The whole data have been shown in tabular form and taken further for statistical analysis[21].

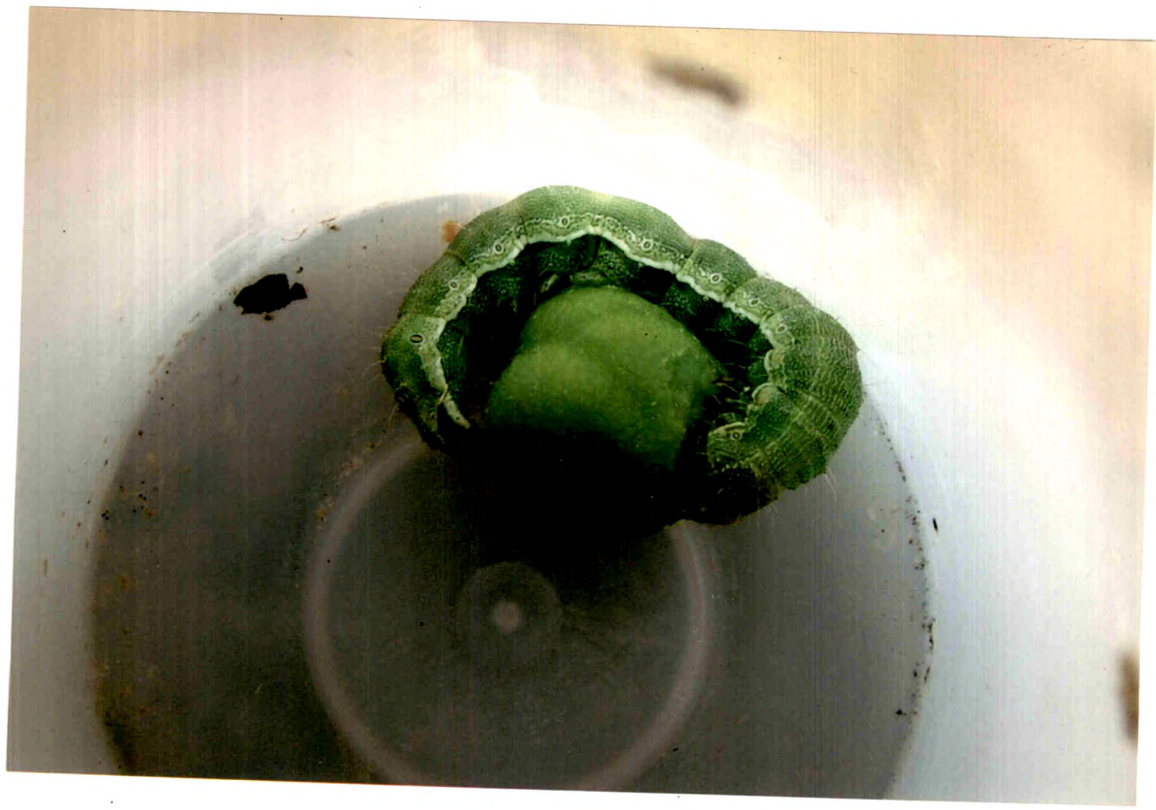


Fig-25:Feeding of treated food to *H. armigera* .

Fig-26: Process of bioassay (*H. armigera*).

c) Maize weevil (*Sitophilus zeamay*):

In the process of bioassay on maize weevil collected from the maize storage godown. The whole experiment was conducted to prepare different concentrations of synthesized organic compound in an acetone at 100 ppm, 300 ppm and 500 ppm. These solutions were applied at the rate of 1 ml on a moist sorghum grains in a petriplates separately. Similarly, the control experiment was performed using solvent acetone.

A Batch of 50 individuals of maize weevil was treated on a sorghum grains (Fig.27). After an interval of 8 and 16 days, (Fig.28) the numbers of dead maize weevils were counted and the data have been presented in tabular form for further statistical analysis[21].

Fig-27: A batch of maize weevil feeding on a treated sorghum grains.

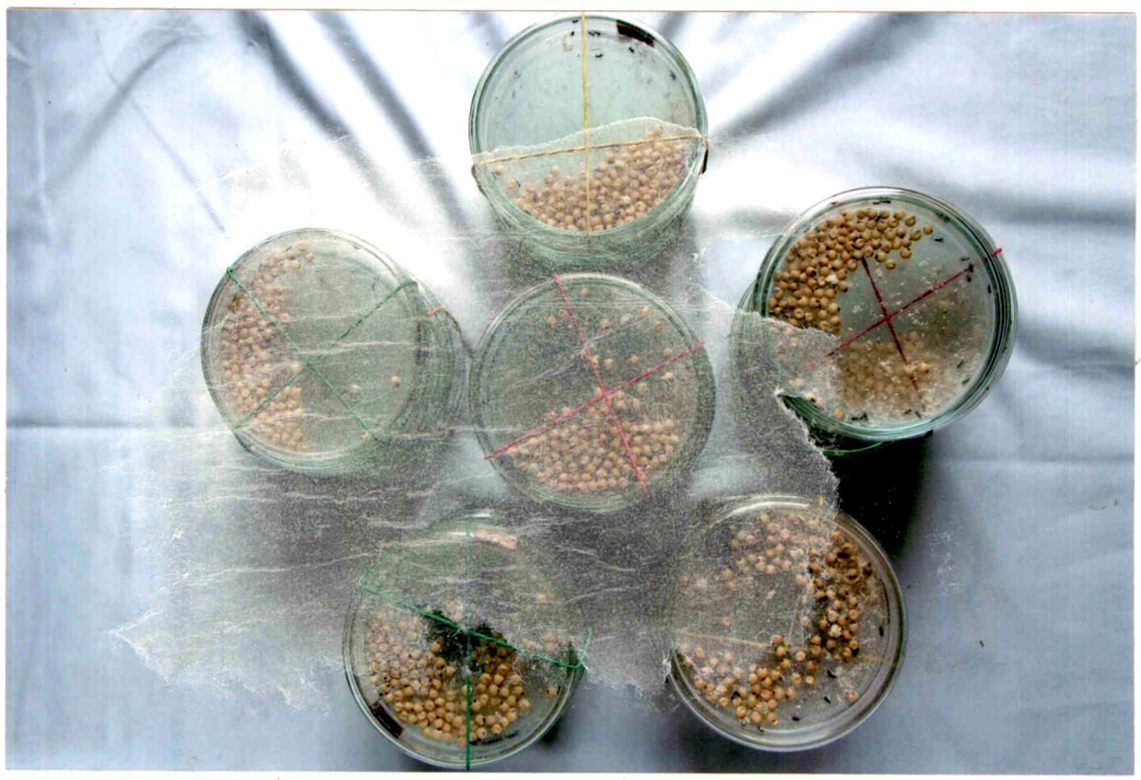


Fig-28: Bioassay on maize weevil.

C) Anti-microbial bioassay:

i] Antifungal bioassay on *Aspergills niger:*

The process of antifungal bioassay was achived on a potato dextrose agar medium (4gm.of PDA in 100 ml distilled water). The conical flask, Stoppard with cotton plug and kept in an autoclave, along with the paper wrapped petriplates to sterilize at 121^{0}C for 20min. Then neutralized and sterilized, the hot media was poured in a sterilized petriplates at the rate of 20 ml to solidify the PDA media. A pure *Aspergillus niger* culture was then inoculated and spread by using glass spreader and scooped up with metal cork borer with the 6 mm. in diameter.

The different concentrations of synthesized organic compound at 100 ppm, 300 ppm and 500 ppm in acetone were prepared and inoculated in a cup, at the rate (0.1 ml) using micropipette. (Fig.29).

The whole above procedure was performed under laminar flow with a spirit lamp. After the treatment the plates were kept for 48 hrs' and the zones of inhibition were measured by using vernier caliper, tabulated and used for further statistical analysis[22].

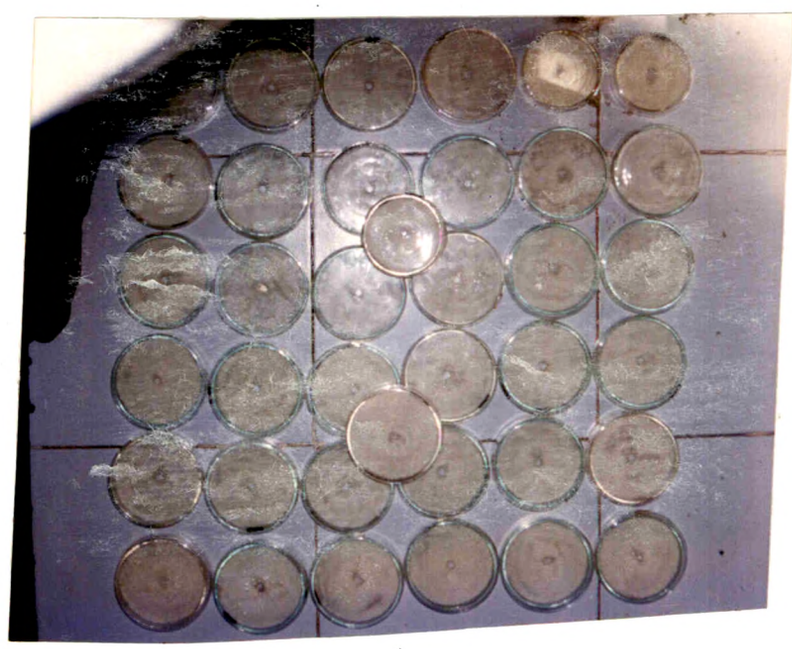


Fig-29: Zone of inhibition by *Aspergillus niger* in culture media.

ii) Antibacterial bioassay of *Pseudomonas flurous*:

The antibacterial activity of pyridine derivatives was studied comparatively by cup-plate method using gram negative bacteria *Pseudomonas flurous*.

Materials and methods:

1.Sterilized petridishes, pipettes and beakers.

2.Old growth culture in nutrient agar.

3. Sterilized test tubes.

4. Sterile 6 mm cork borer.

5. Sterile inoculation loop.

6. Sterilized fine pointed forceps.

Preparation of media:

Nutrient agar was prepared by dissolving bacteriological peptone (0.1%), sodium chloride (0.5%) in a distilled water and pH 7 kept neutral.

Preparation of sub-cultures:

The organisms used in the present study for the testing of antibacterial activity of the compounds. On the day of testing, the organisms were sub-cultured into sterile nutrient broth. After incubating the same for 3 hrs., the obtained growth was used as inoculums for the test.

Sterilization of media (glass wares):

The media used in the present study, nutrient agar and nutrient broth, were sterilized in a conical flask of suitable capacity by autoclaving the same at 15 lbs pressure for 20 min. The cork borer, glasswares, petridishes, test tubes and pipettes, were sterilized in hot air oven at 160^0 C for 1 hr.

Preparation of solutions:

Prepare stock solution in acetone at 100 ppm, 300 ppm and 500 ppm by test compounds and acetone is use as control.

Cup-plate method:

The method depends on the diffusion of an compounds through a cavity into through the solidified agar layer in a petridish. About 20 ml of molten nutrient agar was poured into each of the sterile plates. With the help of sterile cork borer, the cup were punched and scooped out of the solidified agar. The agar plates so prepared were divided into different sets and each set of the plates were inoculated with the suspension of organism by spread plate technique.

The cups of inoculated plates were then filled with 0.1 ml of the test solution, and the plates were allowed to stay in there, in their upright position for 2 hrs. Further, the plates were incubated at 37°C and kept overnight. The zone of on inhibition developed was measured for test organism for the particular compound.

Fig-30: Zone of inhibition on *Pseaudomonas flurous* in culture madia after 24 hrs.