CHAPTER-II

DYNAMIC PEPTIDE PROFILES OF GERMINATING MUNG BEAN: IN RELATION TO THEIR NATURE AND SEPARATION PATTERN

2.1 INTRODUCTION

As already discussed, in recent years, a vast array of bioactive peptides were isolated from different spectrum of life form and only some of these low molecular weight peptides have been characterized in details. Peptides in plant system possess definite role in amplifying signals (Lindsey *et al.*, 2002), nitrogen fixation (Mylona *et al.*, 1995), cell proliferation (Matsubayashi and Sakagami, 1996), generation of polarity (Souter and Lindsey, 2000), differentiation, self incompatibility and mediating biotic and abiotic stress elicitation with metabolic intermediates. Concept of bioactive peptides like Systemin, Phytosulphokine, ENOD 40, CLAVATA 3 and S-Locus Factors (Ryan *et al.* 2002) trigger challenges over the classical definition of narrow viewpoint of plant hormones. The genesis of numerous small peptides in different phases of plant system is not at all random but oriented through specific molecular programs that may not be associated with central dogma of protein synthesis (Lee *et al.*, 1996; Fletcher *et al.*, 1999).

Now, the basic aim of this work is to explore the separation profile of peptides isolated from germinating mungbean [*Vigna radiata* (L) Wilczek. cv. Sonali B1], which is one of the important cultivated variety of mungbean with excellent aroma, taste and flavour. Till now there are very few reports in plants solely related to peptide profile or fingerprint. This may be due to technical difficulty related to isolation, purification and characterization of peptides. More often peptides are characterized through Capillary zone electrophoresis (Heintz *et al.*, 2004; Wetterhall, 2004), Mass-spectrometry (Pu *et al.*, 1996), Liquid chromatography (Drykova *et al.*, 2003), Paper chromatography (Rydon and Smith, 1952), Paper electrophoresis (Bailey and Ramsaw, 1973), Capillary electro-chromatography (Kasicka, 2003) etc. based on the principles of the procedure and objectives of the work. The work presented in this chapter is an analysis of impending changing pattern of peptide profile present in different germinating hours of mung bean based on paper chromatography, capillary electrophoresis along with detection of amino acids present in these isolated peptides.

2.2 MATERIALS AND METHODS

2.2.1 Plant culture

Seeds of mungbean [*Vigna radiata* (L) Wilczek. cv sonali B1], were collected from Central Pulses Research Institute (C.P.R.I.), Berhampur, West Bengal, India; was weighed out (each set of 250 g.) and allowed to culture in sterile petriplates with absorbent cotton supplied with modified Hoagland solution with one-half strength of major nutrients and full-strength micronutrients. The nutrient solution was aerated continuously and changed weekly. Experiments were conducted inside a controlled environmental growth chamber with 14 hrs light period (350 μmol m^{-2} s^{-1}), 25°/20°C day/night temperature, and 80% relative humidity. The seedlings were grown for different germination time like 0h, 8h, 24h, 32h, 40h, 48h, 56h, 64h, 72h, 5days, 6days and 7days for fulfilling specific experimental objectives.

2.2.2 Isolation and purification of low molecular weight peptides

2.2.2a *Extraction:*

200 g. material of mungbean, of each set, were washed with distilled water and treated with 0.2% Sodium hypochloride solution to avoid pathogenic contaminations. Treated materials were rinsed with distilled water three to four times for removing the traces of Sodium hypochloride. The seedling pieces were frozen in liquid nitrogen, crushed (cryocrushed) and extracted with measured amount of chilled distilled water by blender at 4°C in cold room. After blending, the extracted material was passed through Cheese Cloth for initial sieving and then filtration was performed using Buchner funnel fitted with a layer of absorbent cotton and Whatman No. 1 Filter Paper through suction with aspirator for removing the unwanted tissues. The filtrate was Cold Centrifuged at 10,000 rpm for 30 minutes using Protease inhibitor Phenyl Methyl Sulfonyl Fluoride [PMSF] at 4°C to remove the Cell Wall Materials, Organelles and other cellular debris. The supernatant was collected and the whole extract [500 ml.] was lyophilized to reduce the volume. The lyophilized extract was subjected to ion-exchange chromatography.

2.2.2b *Ion exchange chromatography:*

The extracts were purified through cation exchange resin, Dowex-50 (Tsunasawa, 1982) and anion exchange resin, Dowex-1 (Watanabe, 2003) (Both purchased from Sigma Chemical Co., USA) filled in two separate glass column (60 cm x 2.9 cm).

The fresh cation exchanger resin H^+ form Dowex-50 (1.6 meq. /ml. charge) was taken in large volume of water and transferred carefully to suitable size column for avoiding trapped air bubbles. The resin was washed with 1 to $1^1/_2$ bed volume of 1 (N) HCl to convert all resin particles into H^+ form. The resin was washed with approx. 2-bed volume of distilled water until the effluent gave neutral pH [If the column is not used for long time then 50% ethanol should be added to displace the water. Excess 50% ethanol should be added at the top to prevent mould and bacterial growth. The column should be plugged with cork. This will prevent the resin to be putrefied and one can keep it for 4 to 5 months or longer period. Acetone should not be used otherwise it might dissolve the resin particles]. For charging extract, 900 meq. Cation Exchanger Dowex-50 in H^+ form was taken per kg. fresh weight equivalent plant material. The plant extract first passed through newly activated cation exchanger resin. The resin column was washed with distilled water, 50% ethanolic water and finally with water sequentially each of one bed volume. In this procedure, acidic compounds and hormones like IAA, GA and ABA were removed from the column. Only some basic or amphoteric compounds like cytokinin, amino acids, peptides and protein subunits were held up by the Cation Exchange Resin Dowex-50. After this the whole resin was poured to a big 1 L beaker and some quantity (approx. 50 ml.) of water was added. In cold it was then eluted and neutralized with approximate 50% liquid ammonia (Water: Ammonia :: 1:1) with constant agitation to avoid exothermic reaction [This part of work is an important task to be done very carefully otherwise exothermic system may damage the bioactive compounds of interest]. The cation resin (90 meq. was taken) was neutralized with 93 meq. Ammonia [If liquid ammonia was 15 (N), then 6.2 ml.], just to convert the resin H^+ form into NH_4^+ ammonium ion form. Then the liquid was poured to the column again carefully so that the air bubbles should not get entrapped. Now the column was again eluted with ½ bed volume of 3(N) NH_4OH and distilled water. The eluted material from the column was

taken for further processing. The column was washed and reactivated with 1(N) HCl as mentioned in the beginning, for further use.

The Ammonia was removed from elute with Lyophilization Instrument (Secfroid Lyolab B-II, Germany) fitted with Liquid Nitrogen Trap. The ammonia free extract was taken in approx. 30 to 40 ml. water and little dilute HCl was added with shaking and make the pH acidic 5 to 6. The liquid was then passed through the anion exchanger resin Dowex-2 in OH^- exchangeable ion form.

The Anion Exchanger Resin Dowex-1 was filled in a suitable sized column. Trapping of air bubbles was avoided by careful column packing. The Dowex-1 OH^- (1 meq. / ml.) was available to us. It is thermo-stable and workable in wide range of pH. After washing the resin (Total Charge 70 meq.) cation eluate was passed through the column. It was washed and eluted as like that of Cation Resin mentioned earlier. In this case Anion Resin was eluted with 1 (N) HCl instead of ammonia after taking out of resin from the column. The detailed procedure was mentioned at the beginning of Cation Resin. The elution of Anion Resin contained HCl. The Hydrochloric Acid was removed by Lyophilization in presence of Liquid Nitrogen Trap. The semidried sample, nearly free of HCl, was taken and dissolved in approx. 15 ml. water. The pH should be acidic and washed with peroxide free ether solvent to remove fats, lipids and traces of IAA, GA, ABA etc.

2.2.2c *Ether fractionation:*

50 ml. of Diethyl Ether was taken and made free from peroxide. Approximately 2.5 to 3.0 gm. of Ferrous Sulfate ($FeSO_4$) was dissolved, 0.6 to 0.8 ml. of conc. Sulphuric Acid was added and volume made upto 10 ml. Nearly 2 to 2.5 ml. of $FeSO_4$ solution was poured in the separating funnel containing 50 ml. of ether. It was shaken gently several times and then the aqueous Ferrous Sulphate layer was taken off and discarded. The ether was washed 3 to 4 times with equal volume of distilled water. This peroxide free ether was stored in ambered coloured bottle in deep freeze at -25°C.

The concentrated liquid of Anion elution (approx. 20 ml.) was washed four times with equal volume of peroxide free ether. This removes ether soluble lipid components

along with some phytohormones but highly water soluble peptides were recovered from aqueous fraction. The whole operation was done in cold at 4° to 8°C.

2.2.2d *Ultrafiltration:*

Ultra-filtration set up used was Millipore stirred cell fitted with Amicon Molecular Weight cut off Membrane Filter. It is a Nitrogen Gas Pressure (1 to 1.5 kg/cm^2) connection chamber with changeable permeable membrane to separate different species or groups by molecular sieving. The lyophilized ether washed material obtained from germinating seedlings of mungbean was undergone ultrafiltration through 10,000 Da (YM10), 3000 Da (YM3) and 500 Da (YCO5) cut off membrane filter consecutively. Precautions were taken for removal of Amino Acids and traces of IAA, GA, Cytokinin and ABA from plant extract by using 500 Da cut off ultrafiltration for three times in each case. The concentrated liquid of the stirred cell that contain mainly 3000 to 500 mol. wt. compounds were taken for peptide profiling. The molecules above 3000 and below 500 mol. wt. were eliminated. The filtered liquid left over in the stirred cell after 500 cut off was layered in the 500 ml. R.B. flask with B24 standard joint in a freezing bath (-40°C) and then lyophilized.

Now, as 200 g of fresh tissue were taken and the ultimate peptide (of desired molecular weight) extracted from sample, was dissolved in 2 ml distilled water; so, strength of peptide solution is 100 g/ml peptide present as fresh weight equivalent tissue.

2.2.3 One Dimensional Paper chromatography

Each isolated peptide solution of 100 µl (1 g fresh weight equivalent) was loaded on to Whatman No-1 chromatography paper (size-46 cm x 57 cm, thickness-0.16 mm), and separated by descending chromatography with two solvents separately [solvent1; isopropanol : ammonia : water :: 10 : 1 : 1 (v/v) and solvent 2; n-butanol : acetic acid : water :: 4 : 1 : 1 (v/v)]. The papers were stained with freshly prepared ninhydrin location reagent (Friedman, 2004). The retardation factor (R_f) values were determined.

2.2.4 Purification of semi purified extracts through sephadex LH-20 column

Semi purified extracts (2 ml) were loaded on Sephadex LH-20 column (80 cm x 3 cm; volume-566 ml), fitted with ISCO fraction collector, peristaltic pump and UV-recorder (Andrews 1965). Samples were eluted with 30% ethanol at a collection speed of 190 drops/tube (approximately 5ml/tube), drawn by pump set at a speed of 60 digit (ISCO WIZ pump) 30 ml/h. Recorder was set at 3 cm/h; 0.1 O.D full scale, with UV monitoring range at 280 nm. The semi-purified fractions were collected in 200 tubes. After removal of void volume (first 24 tubes), tubes were grouped into four major fractions (25 to 50 tubes for Fraction-1, 51 to 100 tubes for Fraction-2, 101 to 150 tubes for Fraction-3 and 151 to 200 tubes for Fraction-4) and lyophilized separately. Finally the lyophilized peptides were mixed with suitable volume of distilled water and used in chromatography or electrophoretic techniques for the purpose of detection. After LH-20 fractionation of peptide mixture of different germinating hrs, four fractions of peptides of 32 h and 6 days were again subjected to one dimension paper chromatography with two different solvents (solvent-1 and 2) separately.

2.2.5 Two dimensional paper chromatography

Two-dimension paper chromatography was performed with LH-20 purified peptides. At first, the last three fractions of LH-20 were mixed together in equal volume, then 100µl of that was spotted on to Whatman No-1 chromatography paper and separated by descending chromatography with two different solvent mixtures respectively in two arms [solvent-1 in short arm and solvent-2 in long arm]. Then papers were stained with freshly prepared ninhydrin location reagent (Friedman, 2004).

2.2.6 Capillary zone electrophoresis

After purifying through LH-20 gel exclusion chromatography, the peptides were subjected to capillary electrophoresis, Beckman P/ACE system 5010 (Beckman 1994). 60µl of sample was loaded in each case (with 50 sec injection time), detection wavelength 214 nm, used neutral gas nitrogen, capillary volume 50µm x 47cm (neutrally coated), voltage-18KV (8.4 µamps, temperature-20°C) detection time of 5 seconds was monitored. eCAP^{TM} citrate buffer pH-3 (20µM citrate) was used as running buffer

assuming the isoelectric points of peptides are greater than pH-4 using normal polarity. eCAP^{TM} Orange G (0.1% aqueous solution) is used as reference marker. Peak height (C_x =k.H_x), peak area (C_x=k.A_x) and area percentage were calibrated from electrophoregram using software System Gold Version 810. Molecular weight determination was performed by using standard peptides: Insulin (M_w - 5777.6), Insulin chain-A (M_w - 2531.6), Insulin chain-B (M_w -3495.9), Somatostatin (M_w -1637.9) [Sigma Chemical Co. USA] and CNBr treated lysozyme fraction A (M_w -1025.61) with the help of Smith's Statistical Package (Version-2.5 of Gary Smith) by $\log_{10} M_w$ vs. 1/RMT graph. Chromatograms of different peptides of germinating mung bean seedlings were presented graphically using Microsoft Excel.

2.3 RESULTS

2.3.1 Analysis of peptide spots of one-dimensional paper chromatography

The overall expression of one dimension paper chromatography is thoroughly documented in Figure 2.1.A. From that appearance, it is quite prominent, that in case of solvent-1, the separation pattern is little bit better than that of solvent-2, which indicates significantly towards the nature of peptides (better resolved in two dimensional paper chromatography). Though the same amount of 1 g fresh weight equivalent of purified plant extracts were spotted on chromatographic paper, it was observed that the amount of peptides present in 24h, 32h, 40h, 48h, 56h, 64h and 72h was drastically high than that of 8h, 5 days and 6 days (supported by apparent visibility and intensity of ninhydrin stained spots). Now, in both the solvents, some consistent characters were observed throughout all the germination hours – like, a definite gap zone (from, R_f-0.25 to 0.35, in solvent-1 and R_f -0.4 to 0.5, in solvent-2); a very good amount of peptides bear proline or hydroxy-proline in their amino terminal as seen by yellow colour of spot; in solvent-1, a very odd spot was present with highest R_f value (0.739) in case of 64h; in solvent-2, peptides of 8 h had a very weak separation. LH-20 purification was done with 32 h and 6 days germinating seedlings because these phases are crucial for developing mung bean. After LH-20 purification, different fractions showed better separation in one dimensional chromatography. The complete result is mainly documented in Figure 2.1.B. The overall expression shows that, the major amount of distinguishable peptides came within

fraction-1 and 2; though in fraction 1, some spots were overlapped. Lesser number of spots appeared in fraction 3 and 4 indicating that very low molecular weight peptides (taking higher elution time in LH20 column) were less abundant during these germination phases.

2.3.2 Two dimensional paper chromatographic profile

Two-dimensional paper chromatography was done with peptide mixtures of last three LH-20 fractions (just to make the peptide fingerprinting more dependable) of different germination hrs with solvent-1 and solvent-2, applying in two different arms. The peptides of 0, 8, 16, 24, 32, 40, 48, 56, 64, 72 hrs separated into 14, 10, 14, 14, 19, 16, 22, 14, 17 and 16 spots respectively (Table 2.1a-j and Figure 2.2-2.6) whereas the peptides of 4, 5, 6 and 7 days separated into 11, 13, 13 and 10 spots respectively (Table 2.1k-n and Figure 2.7-2.8). According to their positional clustering, peptides were grouped into ten different clusters (mentioned as A to J in the Table 2.1a-n and Figure 2.9). Consistent appearance of spots were observed in the region 'E', 'F' and 'G' throughout the germination period after water imbibition by dry seeds. High colour intensity of these spots indicates higher level of expression as germination proceeds. Spots were also fairly regular in region 'B' but their locations in paper were shifted with germination hours. In zone 'A' spots appeared and disappeared with germination phases and the intensity of the spots were also widely varied. Phase specific spots were emerged in 'H', 'I', and 'J' region. Only one spot was present in region 'H' before germination. In 32, 40 and 64 hrs some low intensity spots were appeared in region 'I' and "J'. Therefore dramatic changes of appearances and disappearances of spots were noticed in between 2 and 3 days. Like one dimensional chromatography, after 4 days, spots gradually faded out from many regions of two dimensional paper chromatogram.

Figure 2.1.A.i

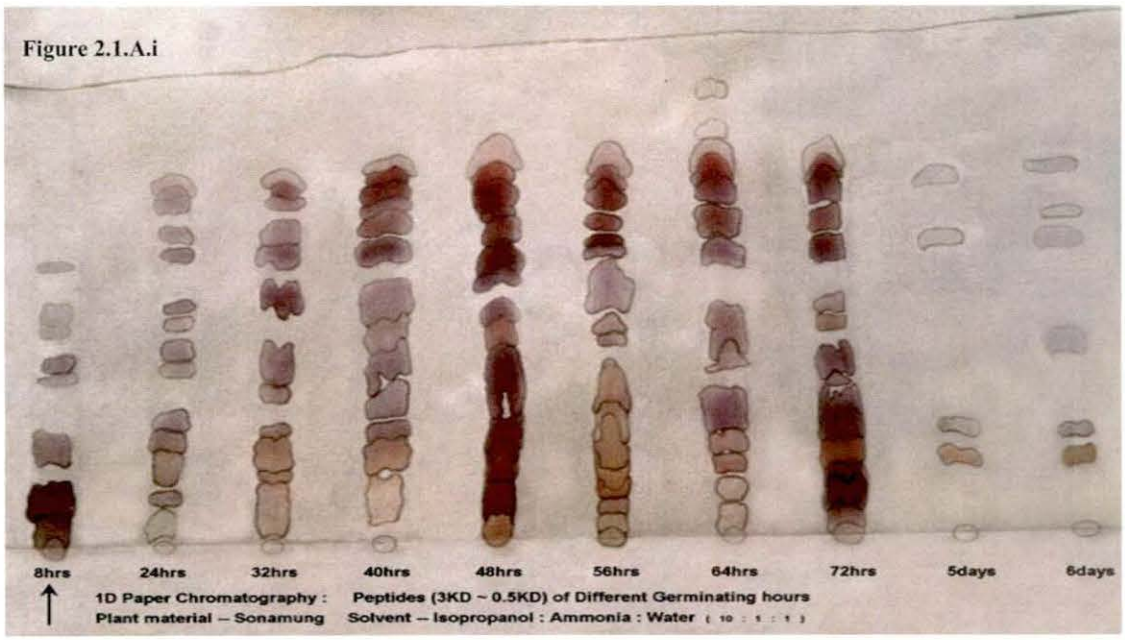

Figure 2.1.A.ii

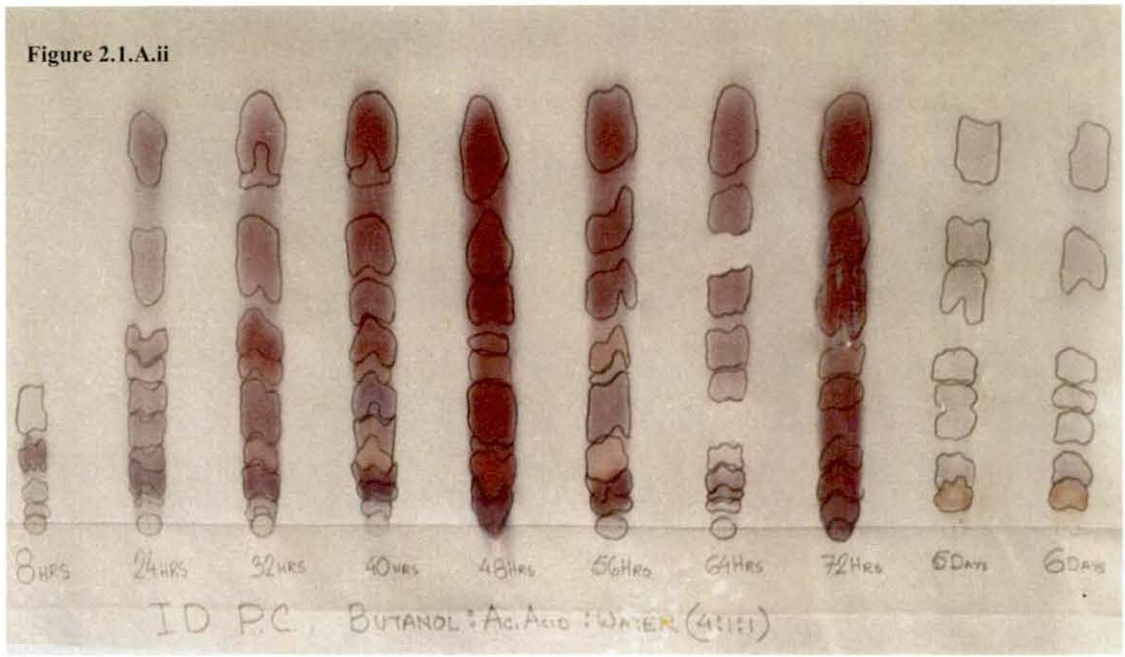

Figure 2.1.A Comparative One Dimension Paper Chromatography of isolated peptides from different hours of germination --

A.i – in Isopropanol : Ammonia : Water :: 9 :1 : 1 (v / v / v).

A.ii – in n-Butanol : Acetic Acid : Water :: 4 :1 : 1 (v / v / v).

Figure 2.1.B.i

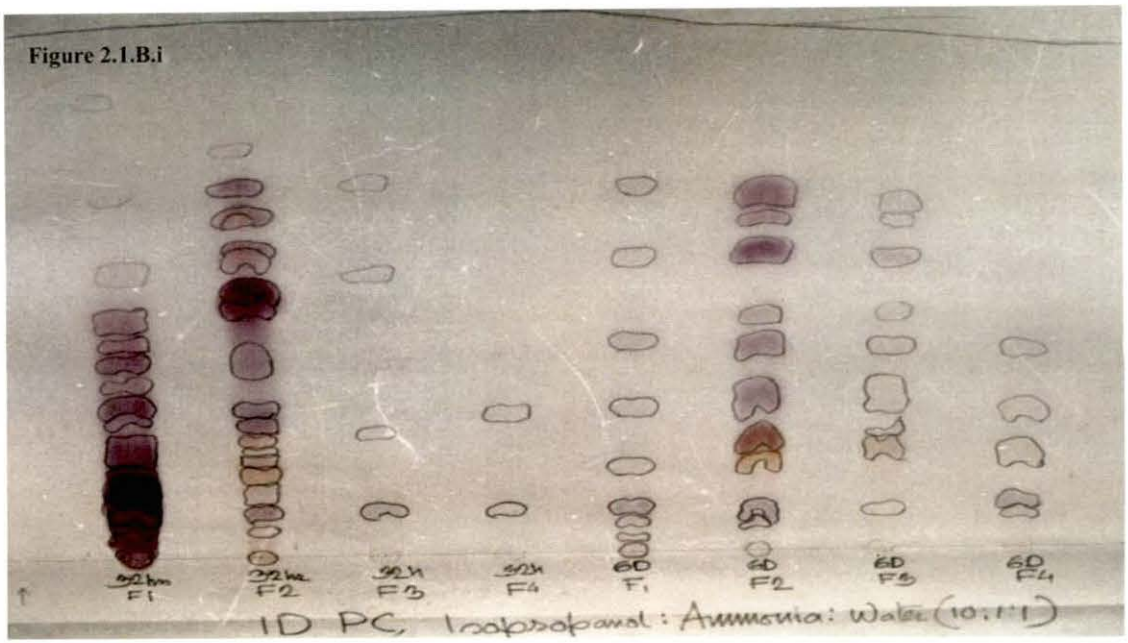

Figure 2.1.B.ii

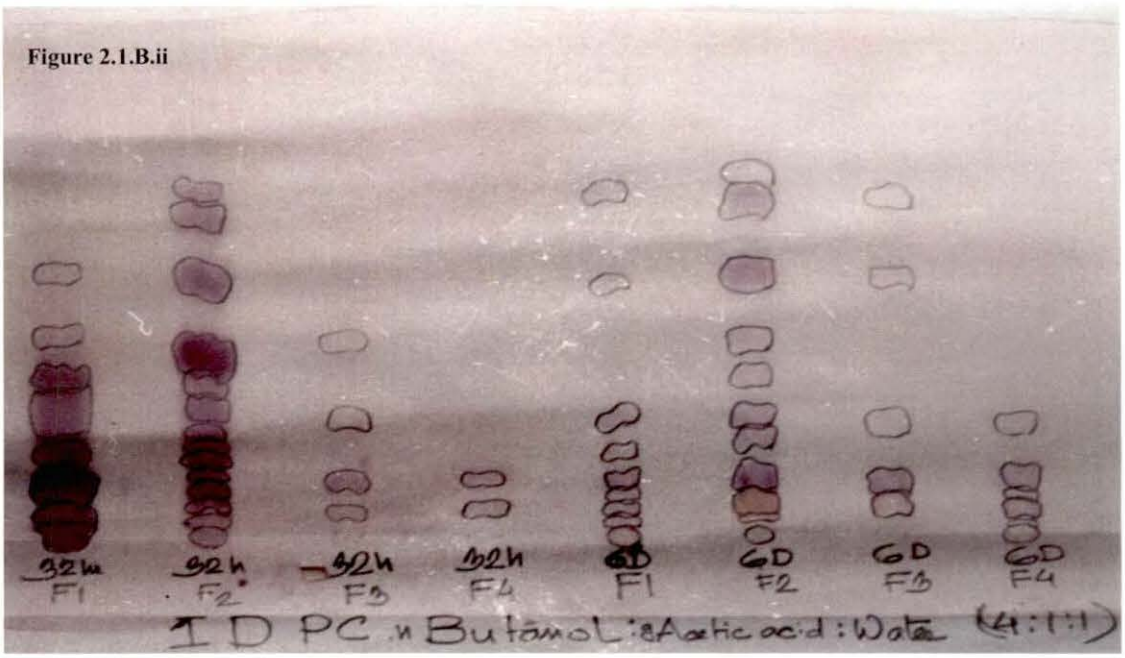

Figure 2.1.B Comparative One Dimension Paper Chromatography of different LH – 20 separated fractions of isolated peptides from 32 hrs. and 6 days of germination –

B.i – in Isopropanol : Ammonia : Water :: 9 :1 : 1 (v / v / v).

B. ii – in n-Butanol : Acetic Acid : Water :: 4 :1 : 1 (v / v / v).

Table 2.1 Categorical clustering of peptide spots appeared in 2D paper chromatography after ninhydrin development:

Table 2.1a Peptides isolated from 0h germination phase

Germination Phase: 0 h			Categorical Clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1	0.03	0.02	A										+++
2	0.08	0.05	A										+++
3	0.15	0.05	A										++
4A	0.23	0.08		B									+++
4B	0.34	0.06		B									++
5	0.05	0.10	A										++
6A	0.02	0.13	A										++
6B	0.10	0.13	A										+++
6C	0.16	0.14	A										++
6D	0.27	0.12		B									++
7A	0.20	0.20			C								++
7B	0.48	0.20								H			++
8	0.34	0.27					E						+
9	0.55	0.41							G				+

Table 2.1b Peptides isolated from 8h germination phase

Germination Phase: 8 h			Categorical Clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1A	0.03	0.09	A										+++
1B	0.06	0.08	A										+++
2A	0.11	0.14	A										+++
2B	0.25	0.15			C								++
3	0.15	0.20			C								++++
4	0.15	0.26				D							+++
5	0.20	0.37					E						+++
6	0.34	0.42						F					+++
7	0.42	0.48							G				+++
8	0.46	0.52							G				++++

Table 2.1c Peptides isolated from 16h germination phase

Germination Phase: 16 h			Categorical Clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1A	0.04	0.05	A										++
1B	0.07	0.05	A										+++
2	0.08	0.08	A										+++
3	0.12	0.13	A										++
4A	0.09	0.14	A										+++
4B	0.22	0.17			C								+
5A	0.13	0.20			C								+++
5B	0.15	0.20			C								++
6	0.16	0.26				D							++
7	0.21	0.31					E						++++
8	0.21	0.34					E						++++
9	0.29	0.37					E						++
10	0.34	0.41					E						++
11	0.38	0.46						F					++

Table 2.1d Peptides isolated from 24h germination phase

Germination Phase: 24 h			Categorical Clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1B	0.04	0.03	A										+
1B	0.06	0.03	A										+
1C	0.08	0.03	A										+
2A	0.07	0.09	A										++
2B	0.10	0.09	A										++
3A	0.03	0.13	A										+
3B	0.09	0.13	A										++
4	0.24	0.16			C								++
5	0.13	0.19			C								++
6	0.14	0.22				D							++
7	0.21	0.33					E						++
8	0.29	0.39					E						+++
9	0.37	0.45						F					++
10	0.42	0.49							G				++++

Table 2.1e Peptides isolated from 32h germination phase

Germination Phase: 32 h			Categorical Clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1A	0.08	0.05	A										++
1B	0.13	0.05	A										++
1C	0.17	0.06	A										+
2A	0.03	0.10	A										+
2B	0.18	0.10	A										++
3A	0.06	0.13	A										++
3B	0.11	0.13	A										+++
3C	0.23	0.16			C								+
3D	0.33	0.14		B									+++
4	0.16	0.20			C								+++
5	0.19	0.22				D							+++
6	0.07	0.30									I		+
7	0.26	0.32					E						++++
8	0.12	0.33									I		+
9	0.36	0.38						F					++++
10	0.42	0.42							G				++
11	0.47	0.46							G				+++
12	0.24	0.47										J	+
13	0.31	0.49										J	+

Table 2.1f Peptides isolated from 40h germination phase

Germination Phase: 40 h			Categorical Clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1A	0.07	0.05	A										+
1B	0.11	0.05	A										++
2A	0.08	0.11	A										++
2B	0.16	0.10	A										+
3A	0.05	0.15	A										++
3B	0.09	0.14	A										+++
3C	0.29	0.14		B									+++
4A	0.14	0.21			C								++
4B	0.18	0.23				D							++++
5	0.23	0.28				D	E						++
6	0.06	0.31									I		+
7	0.23	0.33					E						+++
8	0.10	0.35									I		+
9	0.33	0.38					E						++++
10	0.40	0.42						F					++
11	0.46	0.47							G				+++

Table 2.1g Peptides isolated from 48h germination phase

Germination Phase: 48 h			Categorical Clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1	0.09	0.03	A										++
2	0.01	0.08	A										+
3	0.08	0.09	A										+++
4A	0.14	0.10	A										++++
4B	0.18	0.11	A										++
5	0.04	0.13	A										++
6A	0.15	0.13	A										++++
6B	0.18	0.14	A										++
7	0.08	0.14	A										+++
8	0.11	0.15	A										+++
9A	0.09	0.15	A										+++
9B	0.13	0.15	A										++++
10	0.34	0.14		B									+++
11	0.17	0.25				D							+++
12	0.21	0.26				D							++++
13	0.24	0.31					E						++
14	0.26	0.34					E						++
15	0.28	0.36					E						++
16	0.37	0.40						F					+++
17A	0.43	0.44							G				++
17B	0.47	0.45							G				++
18	0.49	0.49							G				++

Table 2.1h Peptides isolated from 56h germination phase

Germination Phase: 56 h			Categorical Clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1A	0.07	0.03	A										++
1B	0.12	0.03	A										++
2	0.08	0.09	A										++
3	0.04	0.10	A										+++
4A	0.09	0.12	A										+++
4B	0.29	0.13		B									++++
5A	0.14	0.17			C								++
5B	0.17	0.17			C								++
6A	0.14	0.20			C								++
6B	0.17	0.22				D							++
7	0.22	0.30					E						+
8	0.33	0.35					E						++++
9	0.37	0.40						F					++
10	0.43	0.44							G				+++

Table 2.1i Peptides isolated from 64h germination phase

Germination phase: 64 h			Categorical clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1A	0.07	0.04	A										+
1B	0.12	0.04	A										+
2	0.05	0.10	A										++
3A	0.09	0.13	A										++
3B	0.32	0.14		B									+++
4	0.24	0.18			C								++
5	0.18	0.20			C								+
6	0.19	0.27				D							+
7	0.25	0.35					E						+++
8	0.09	0.38									I		+
9	0.35	0.39						F					+++
10	0.42	0.44							G				++++
11	0.22	0.47										J	+
12	0.47	0.48							G				++++
13	0.16	0.48										J	+
14	0.29	0.51										J	+
15	0.12	0.51										J	+

Table 2.1j Peptides isolated from 72h germination phase

Germination Phases: 72 h			Categorical clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1	0.07	0.06	A										++++
2A	0.12	0.08	A										++++
2B	0.17	0.08	A										++
3A	0.03	0.10	A										++
3B	0.07	0.10	A										+++
4	0.06	0.13	A										+++
5	0.09	0.15	A										++++
6	0.34	0.15		B									+++
7A	0.16	0.24				D							+++
7B	0.20	0.25				D							+++
8	0.26	0.29					E						+
9	0.25	0.33					E						+
10	0.37	0.40						F					++++
11A	0.43	0.45							G				++
11B	0.47	0.46							G				+++
12	0.49	0.50							G				+++

Table 2.1k Peptides isolated from 96h germination phase

Germination Phases: 96 h			Categorical clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1	0.05	0.01	A										+
2A	0.06	0.03	A										+
2B	0.09	0.05	A										+
3	0.03	0.07	A										++
4A	0.06	0.12	A										+++
4B	0.08	0.12	A										++
5A	0.03	0.15	A										+
5B	0.13	0.15			C								++
5C	0.16	0.17			C								+++
6	0.35	0.41						F					+++
7	0.41	0.50							G				++++
8	0.43	0.53							G				++++

Table 2.1l Peptides isolated from 120h germination phase

Germination Phases: 120 h			Categorical clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1A	0.07	0.03	A										++
1B	0.14	0.04	A										++
2A	0.05	0.12	A										++
2B	0.09	0.12	A										+++
3	0.05	0.14	A										++
4	0.14	0.17			C								+++
5	0.35	0.15		B									++
6A	0.17	0.26				D							++
6B	0.21	0.26				D							++
7	0.26	0.30					E						+
8	0.38	0.42						F					+++
9	0.44	0.49							G				++
10	0.49	0.52							G				++

Table 2.1m Peptides isolated from 144h germination phase

Germination Phases: 144 h			Categorical clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1	0.07	0.03	A										+
2	0.04	0.10	A										+++
3	0.08	0.12	A										+
4A	0.05	0.13	A										+
4B	0.09	0.15	A										++
5	0.34	0.14		B									++
6A	0.15	0.23				D							+++
6B	0.20	0.24				D							+++
7	0.24	0.29					E						++
8	0.37	0.39						F					++++
9	0.44	0.47							G				++
10	0.49	0.49							G				++
11	0.51	0.51							G				++

Table 2.1n Peptides isolated from 168h germination phase

Germination Phases: 168 h			Categorical clusters of spots										Intensity of spots
Spots	Rf_x	Rf_y	A	B	C	D	E	F	G	H	I	J	
1A	0.09	0.04	A										++
1B	0.15	0.04	A										+++
2	0.02	0.07	A										+++
3A	0.04	0.12	A										+++
3B	0.07	0.12	A										+++
3C	0.12	0.13	A										+++
4	0.35	0.15		B									+++
5	0.39	0.41						F					+
6	0.45	0.48							G				+
7	0.50	0.53							G				+
8	0.44	0.49							G				+

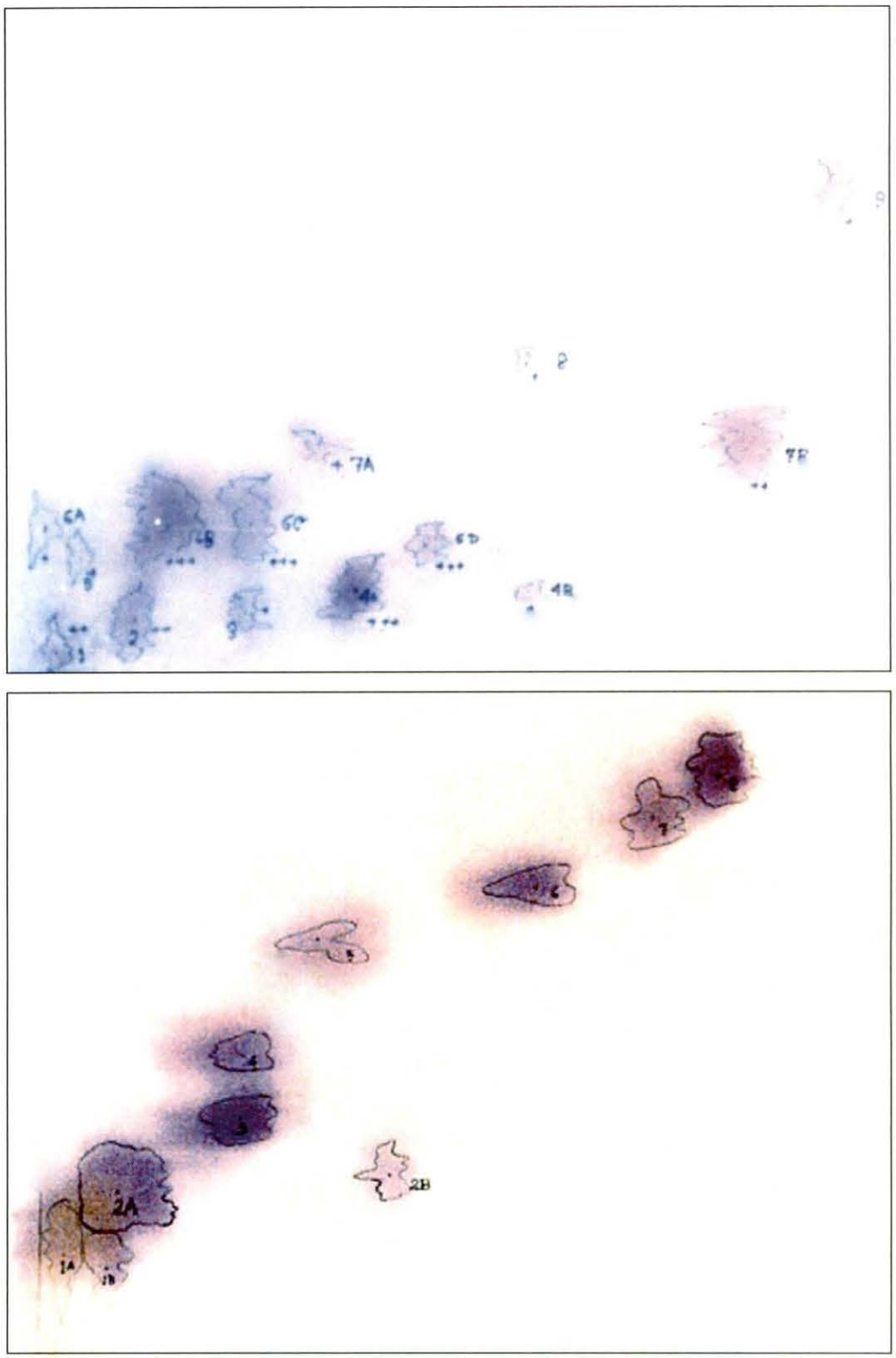

Figure 2.2 Two Dimension Paper Chromatography –

Above – Peptides of 0 h; Bottom- Peptides of 8 h

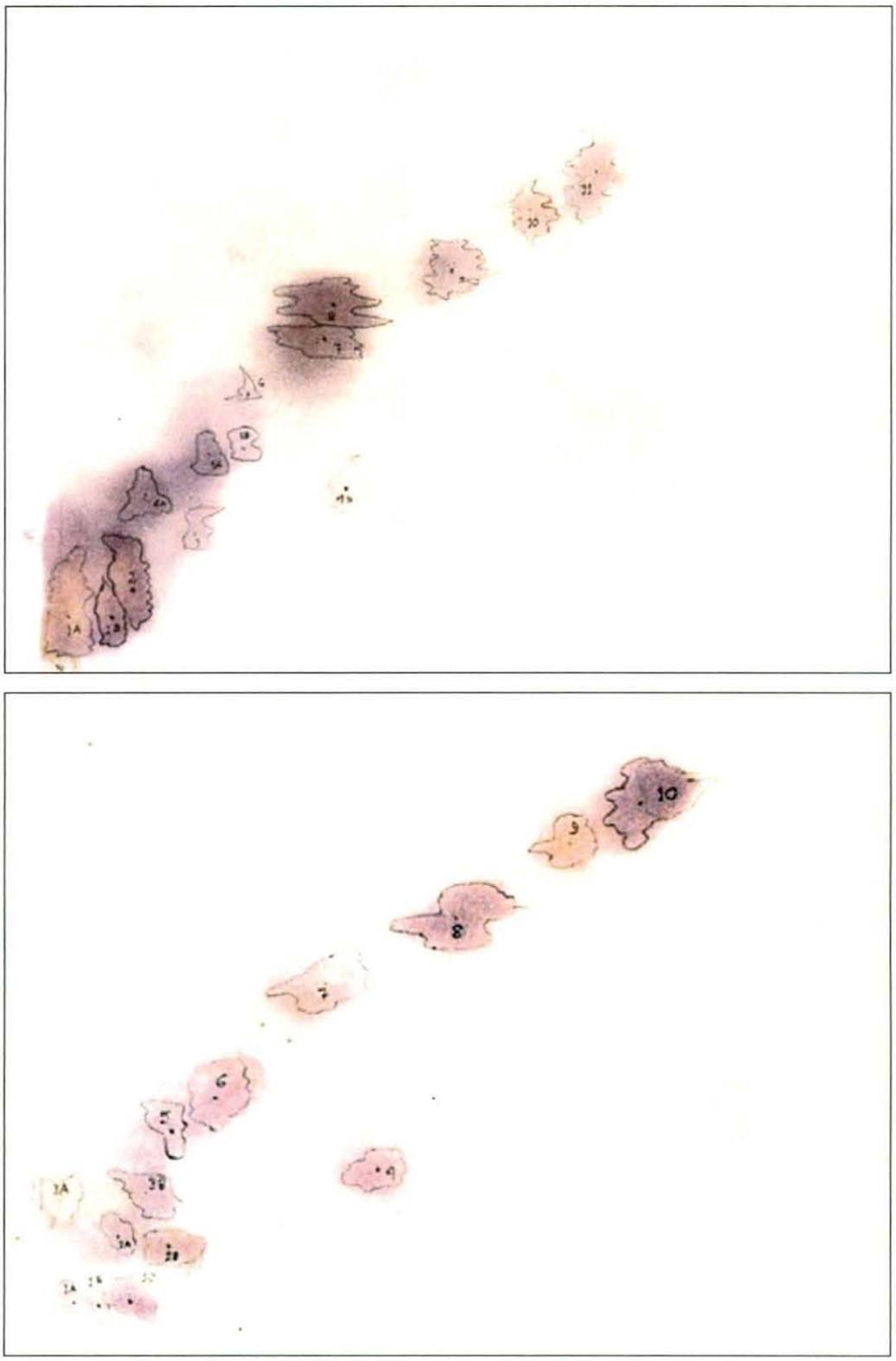

Figure 2.3 Two Dimension Paper Chromatography –
Above – Peptides of 16 h; Bottom- Peptides of 24 h

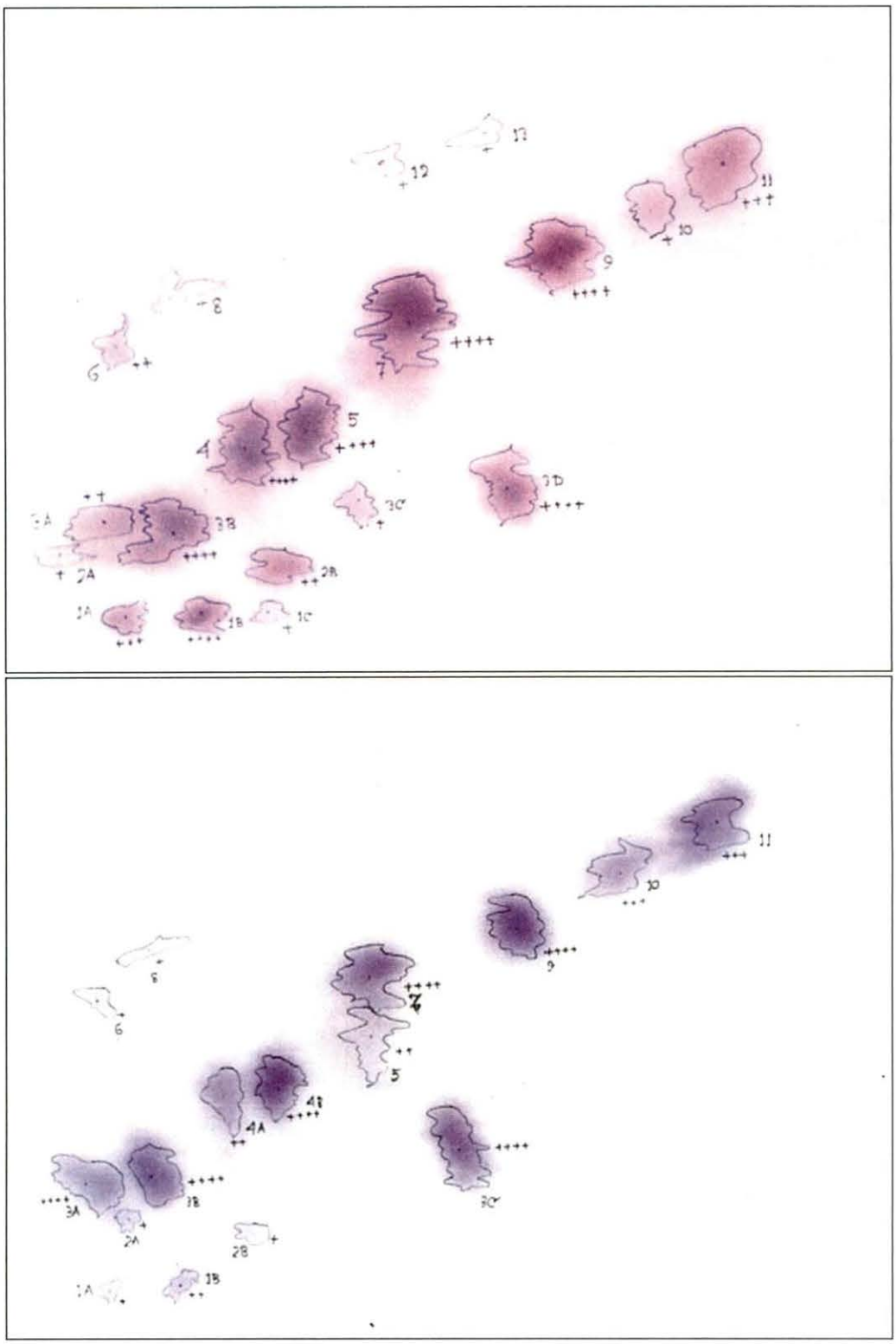

Figure 2.4 Two Dimension Paper Chromatography –

Above – Peptides of 32 h; Bottom- Peptides of 40 h

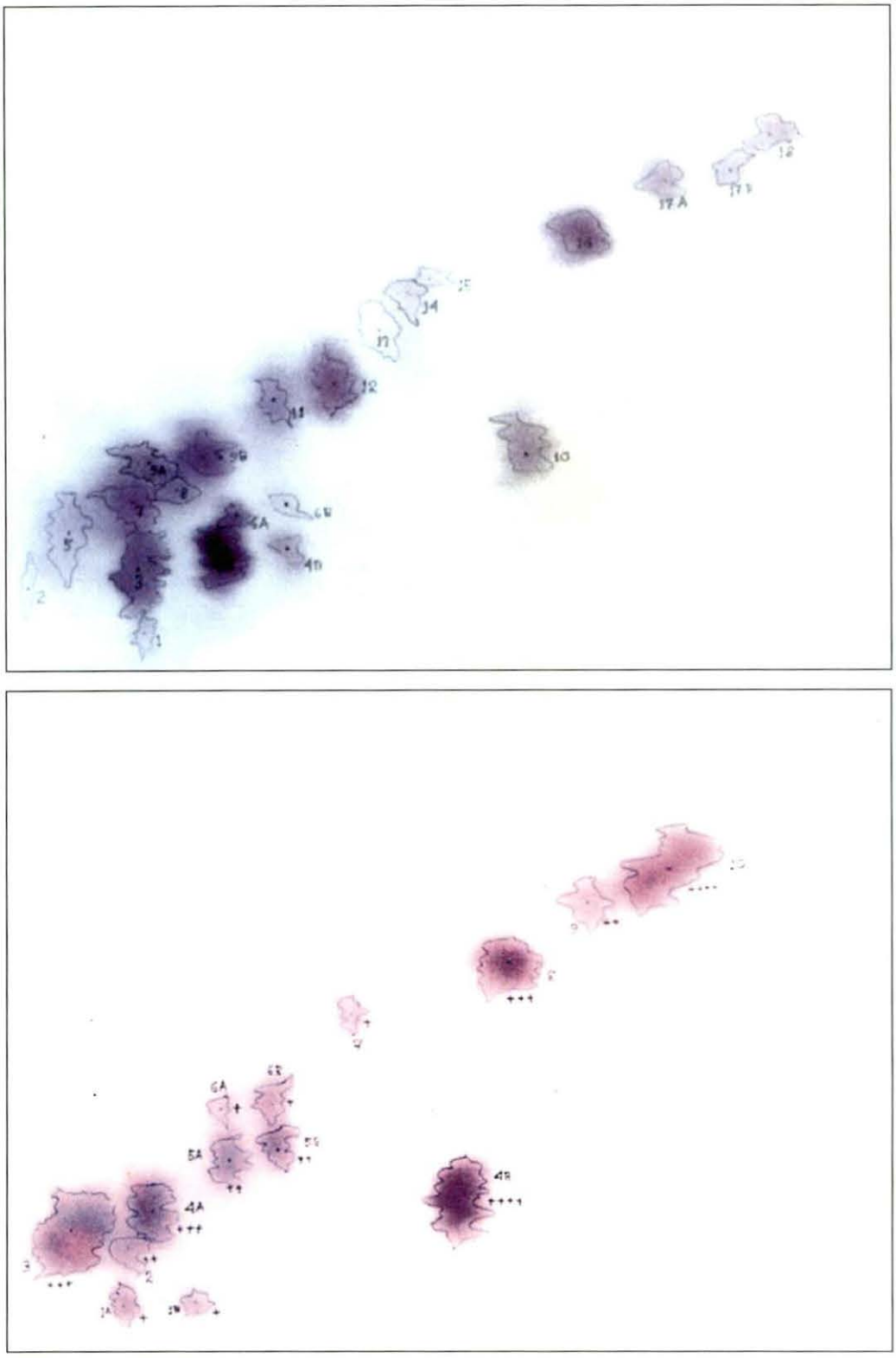

Figure 2.5 Two Dimension Paper Chromatography –

Above – Peptides of 48 h; Bottom- Peptides of 56 h

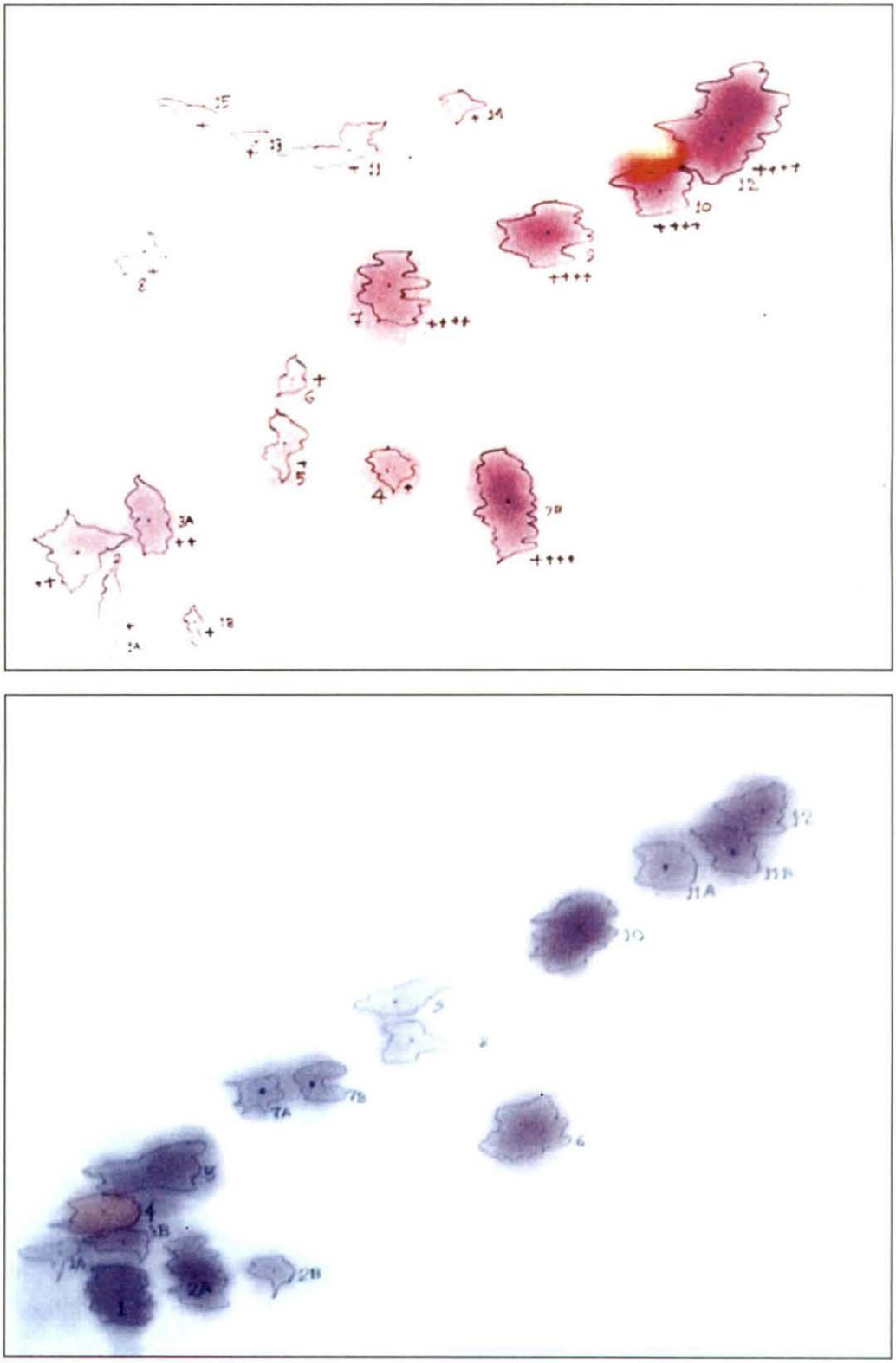

Figure 2.6 Two Dimension Paper Chromatography –

Above – Peptides of 64 h; Bottom- Peptides of 72 h

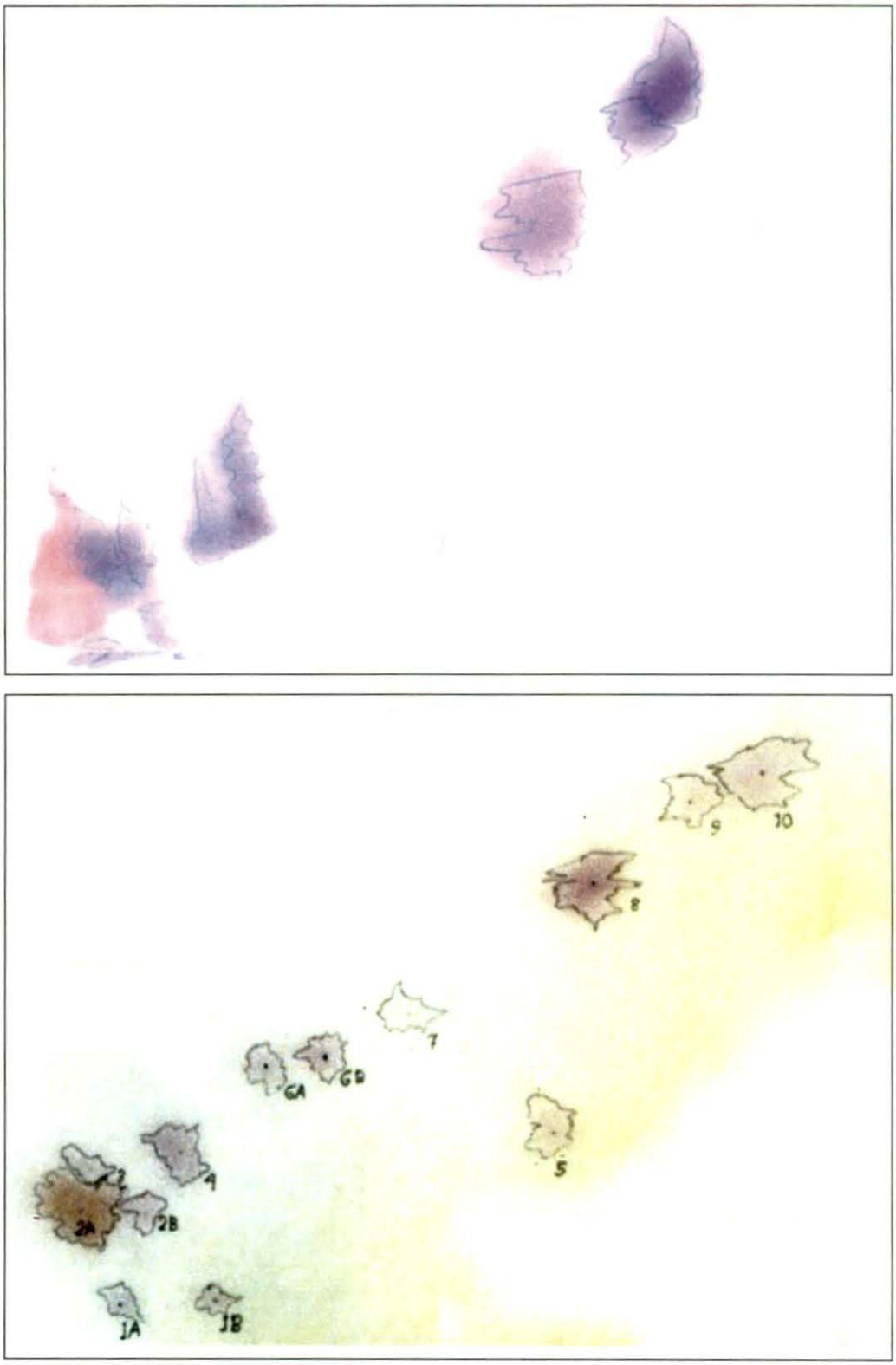

Figure 2.7 Two Dimension Paper Chromatography –

Above – Peptides of 4 Days; *Bottom- Peptides of 5 Days*

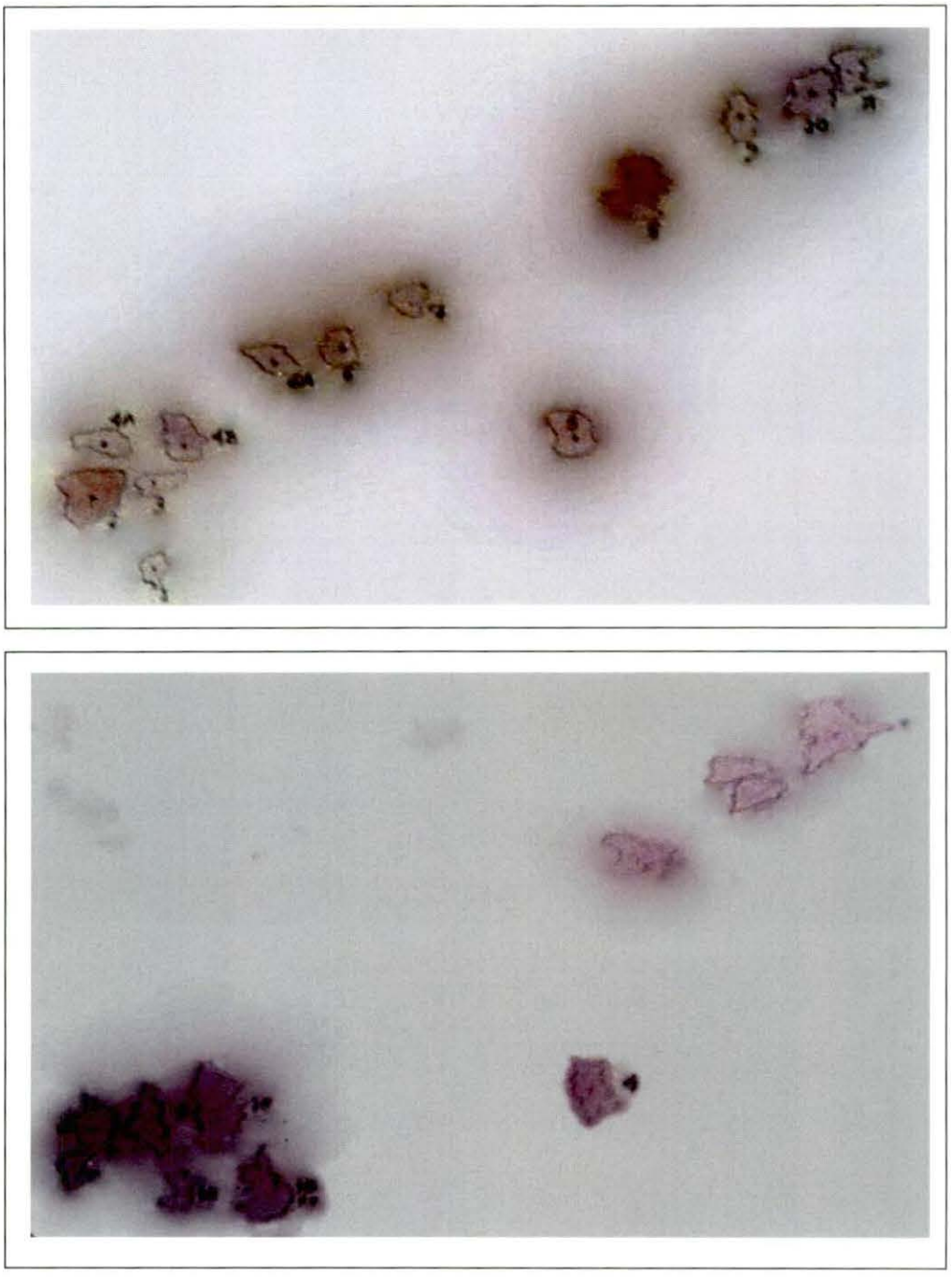

Figure 2.8 Two Dimension Paper Chromatography –

Above – Peptides of 6 Days; Bottom- Peptides of 7 Days

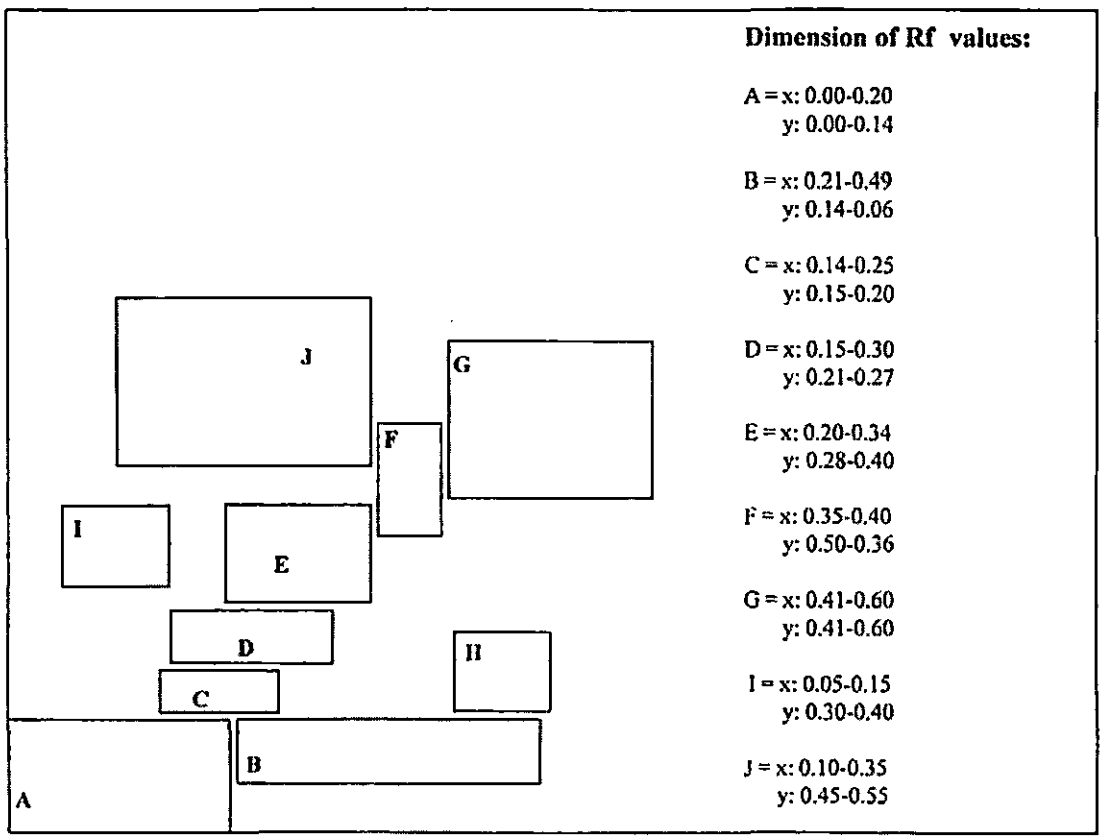

Figure 2.9 Domain of clustering of main peptide spots in 2-Dimensional Chromatogram was expressed by box diagram. Range of R_f values in each cluster was also incorporated

2.3.3 Capillary electrophoretic profile of peptides and electropherogram

Major peaks have been detected mainly in fraction-2 and fraction -3 by Capillary electrophoresis. Peptides of fraction–1, generally executed some overlapping peaks with trailing effect and fraction–4, contained lowest number of peaks. Major peptide peaks were distinguishable within the molecular weight range of 1000D to 3000D (Figure 2.10-2.21). Some consistent peaks near 2500 mol. wt. were observed in fraction F_2 after LH_{20} purification in between 24 h and 72 h germination period (Figure 2.12-2.18). Some peptides with mol. wt. higher than 4000 D in fraction F_1 were also present in minute quantity as detected by capillary electrophoresis. F_4 contains fewer amounts of peptides with widely distributed molecular range. At 0 h (before germination has started) and 8 h of germination period, very low molecular weight peptides below 2000 D were predominant in fraction F_4 (Figure 2.10-2.11). Probably these were the remnants of small degraded peptide fragments, generated during the desiccation phases of seed maturation. Comparatively high molecular weight peptides of nearly 3000 D were detected in major amount during the late phase of germination after 24 h when the radicle has already emerged (Figure 2.22A). After 3 days peptides were again fragmented as expressed from electrophoregram (Figure 2.22B). At the stage of 7 days of germinating seedlings, when they are almost independent, most of the peaks were clustered at fraction F_2 whereas F_1 contain only 1 peak and no peptides were eluted from LH_{20} column with F_3 or F_4 (Figure 2.21a-d).

Figure 2.10 ***Capillary Electropherogram***

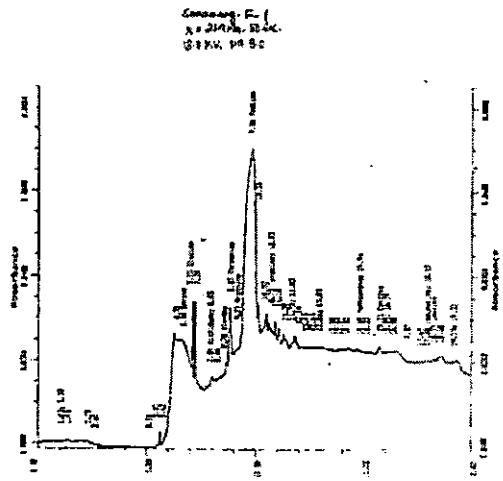

Figure 2.10a 0-Hour Fr-1

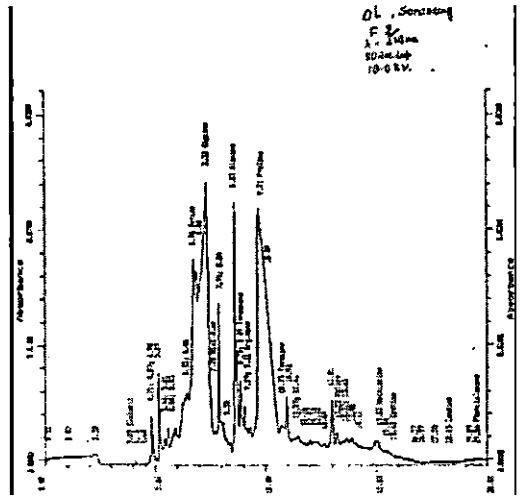

Figure 2.10b 0-Hour Fr-2

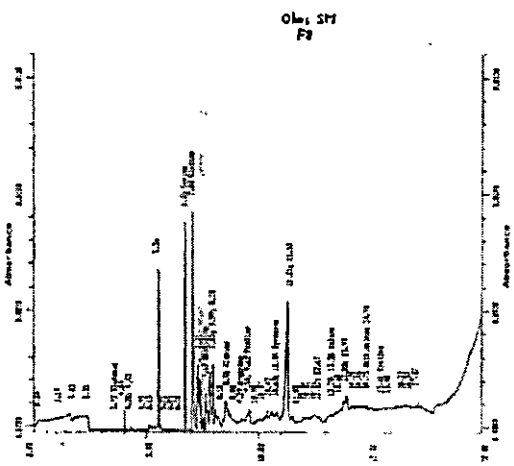

Figure 2.10c 0-Hour Fr-3

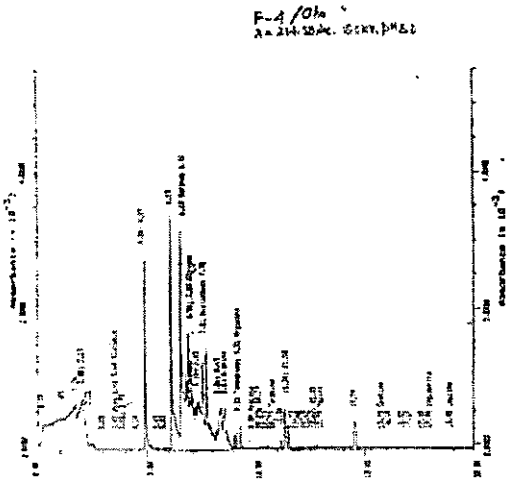

Figure 2.10d 0-Hour Fr-4

Figure 2.11 ***Capillary Electropherogram contd.***

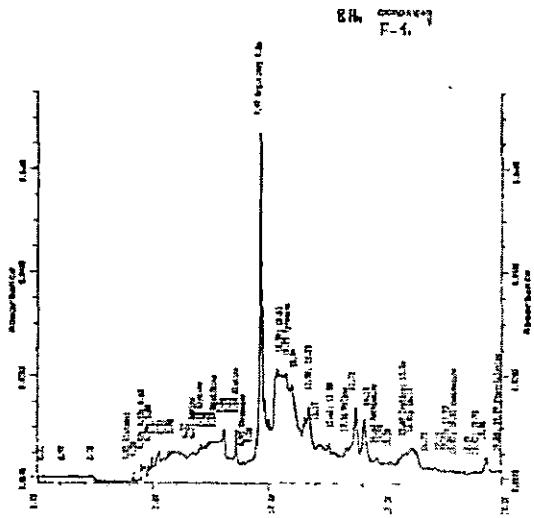

Figure 2.11a 8-Hour Fr-1

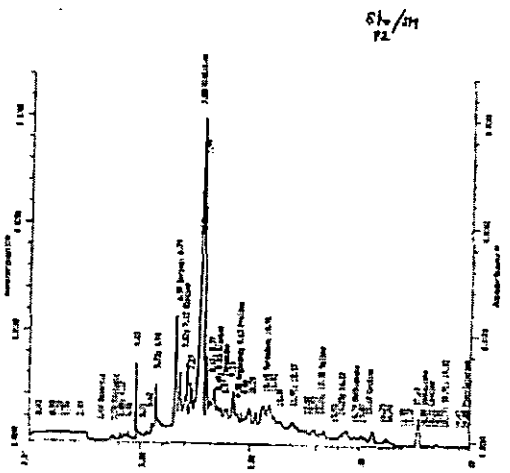

Figure 2.11b 8-Hour Fr-2

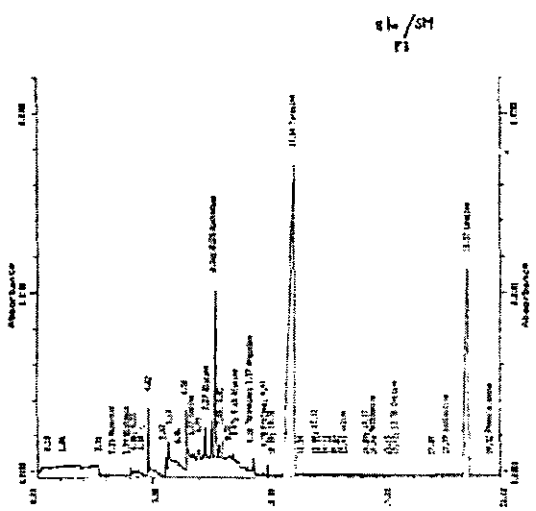

Figure 2.11c 8-Hour Fr-3

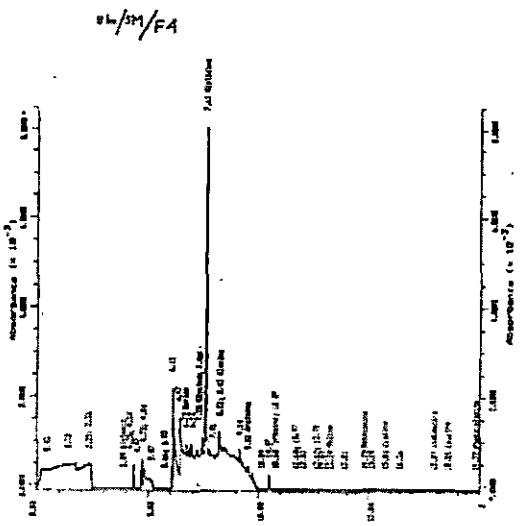

Figure 2.11d 8-Hour Fr-4

Figure 2.12 ***Capillary Electropherogram contd.***

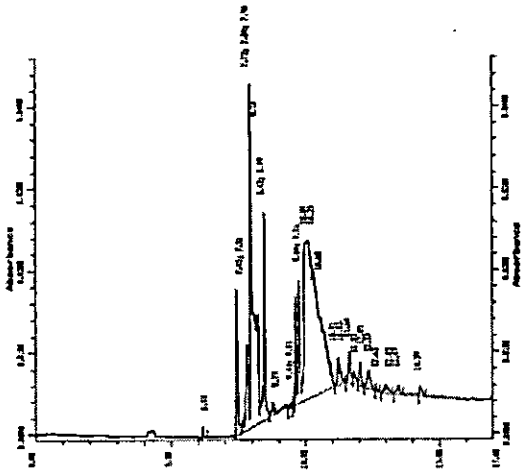

Figure 2.12a 24-Hour Fr-1

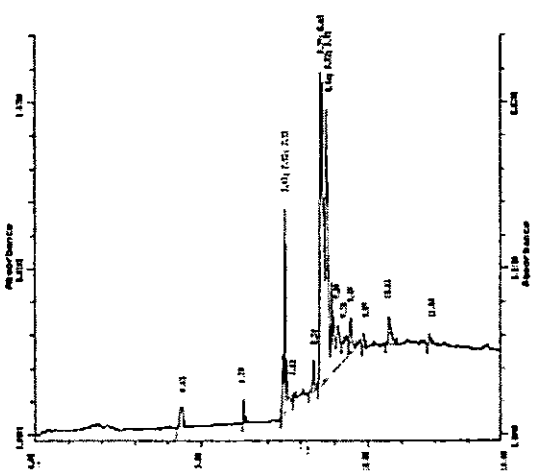

Figure 2.12b 24-Hour Fr-2

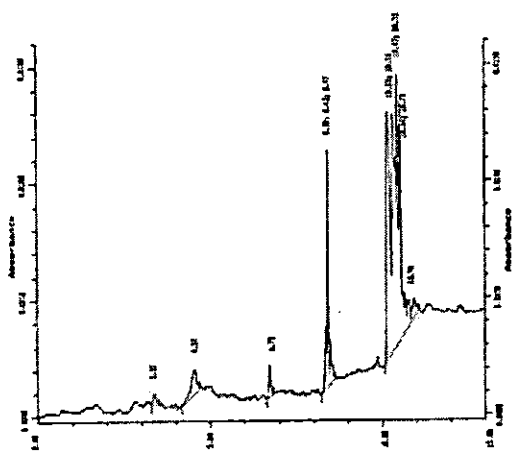

Figure 2.12c 24-Hour Fr-3

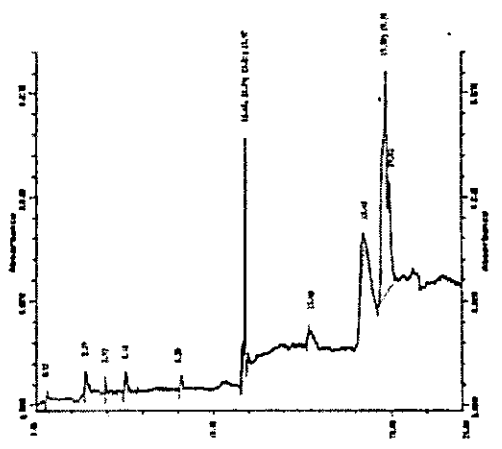

Figure 2.12d 24-Hour Fr-4

Figure 2.13 *Capillary Electropherogram contd.*

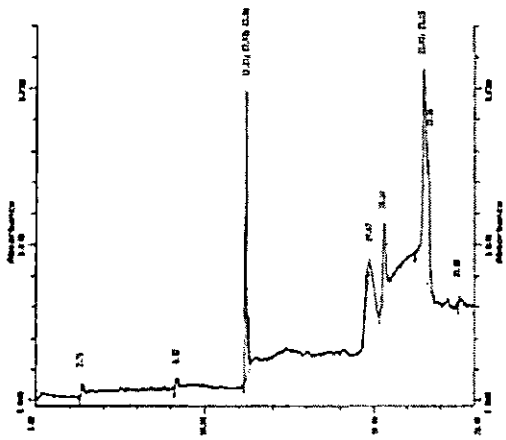

Figure 2.13a 32-Hour Fr-1

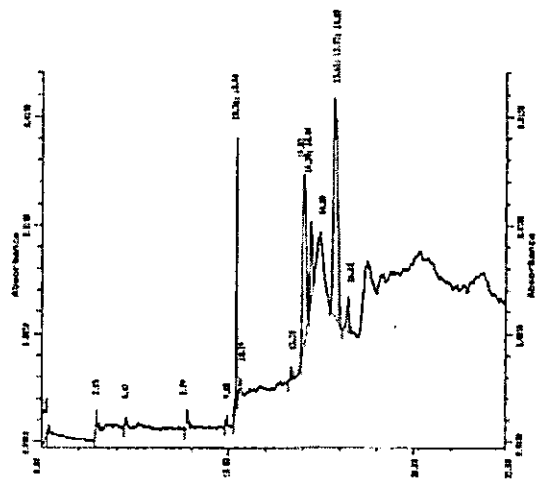

Figure 2.13b 32-Hour Fr-2

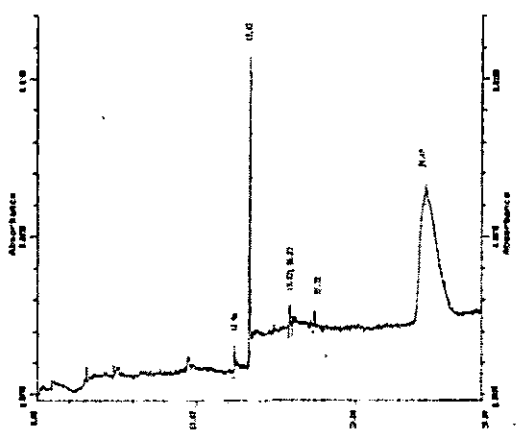

Figure 2.13c 32-Hour Fr-3

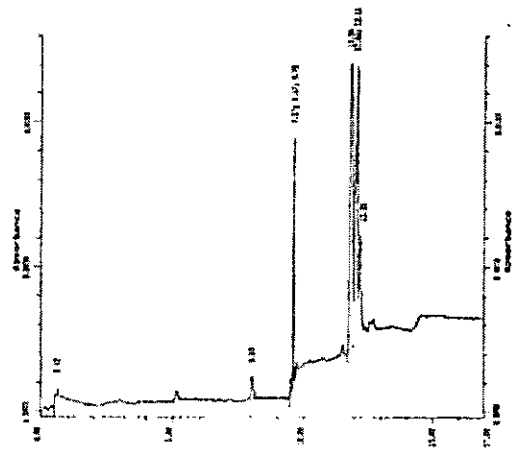

Figure 2.13d 32-Hour Fr-4

Figure 2.14 ***Capillary Electropherogram contd.***

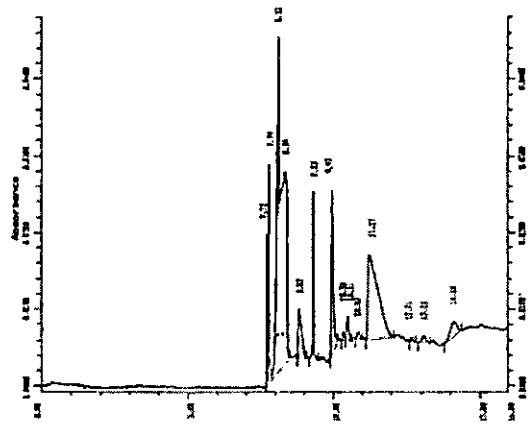

Figure 2.14a 40-Hour Fr-1

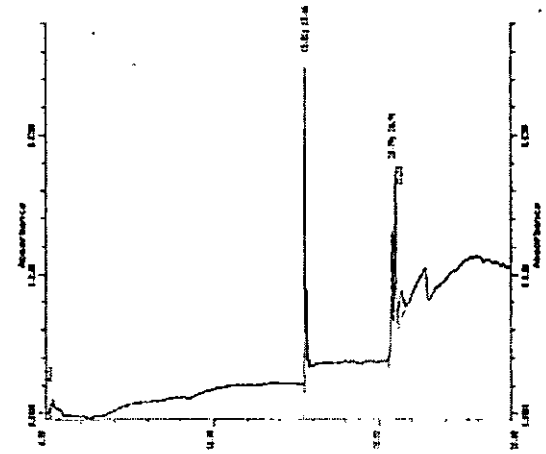

Figure 2.14b 40-Hour Fr-2

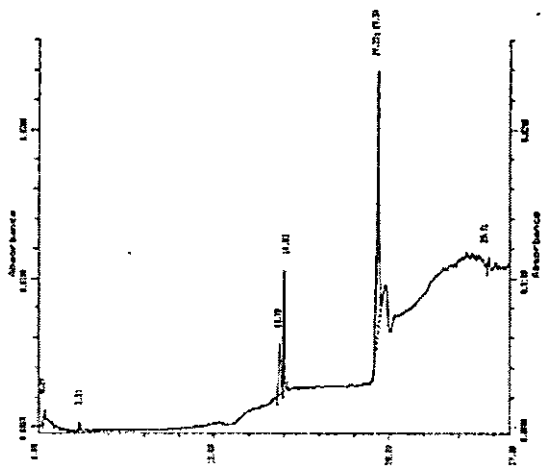

Figure 2.14c 40-Hour Fr-3

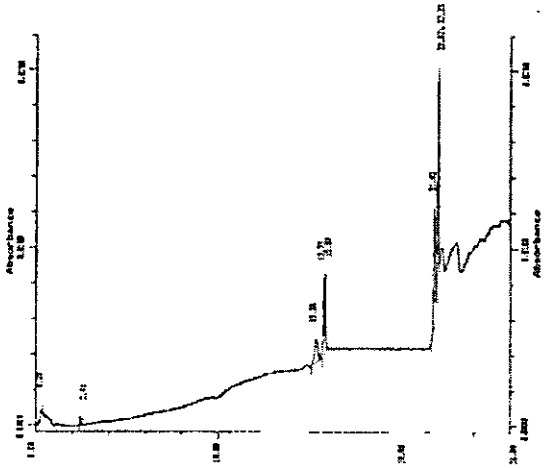

Figure 2.14d 40-Hour Fr-4

Figure 2.15 ***Capillary Electropherogram contd.***

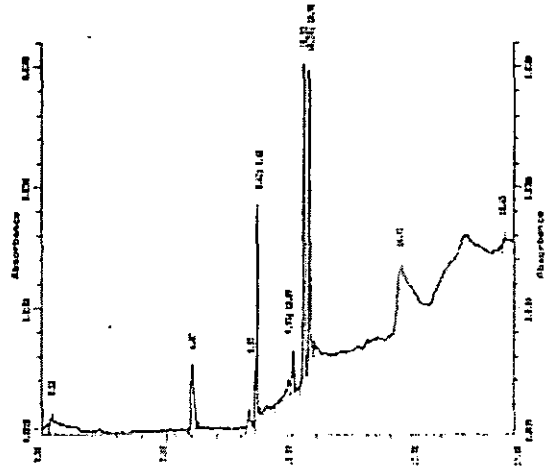

Figure 2.15a 48-Hour Fr-1

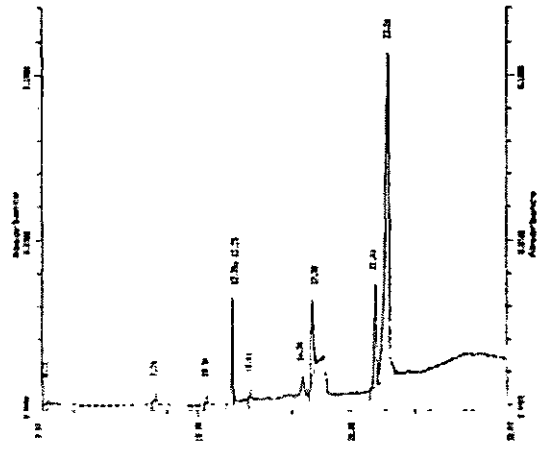

Figure 2.15b 48-Hour Fr-2

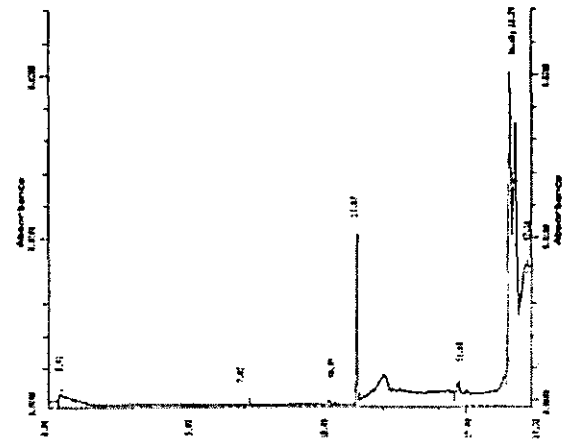

Figure 2.15c 48-Hour Fr-3

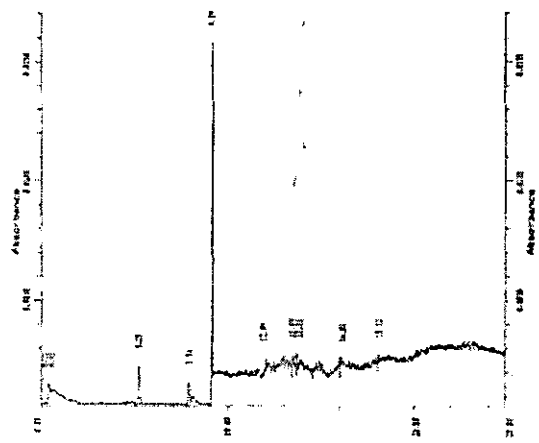

Figure 2.15d 48-Hour Fr-4

Figure 2.16 ***Capillary Electropherogram contd.***

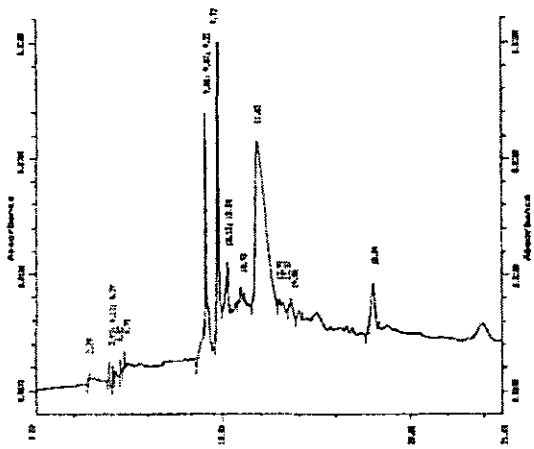

Figure 2.16a 56-Hour Fr-1

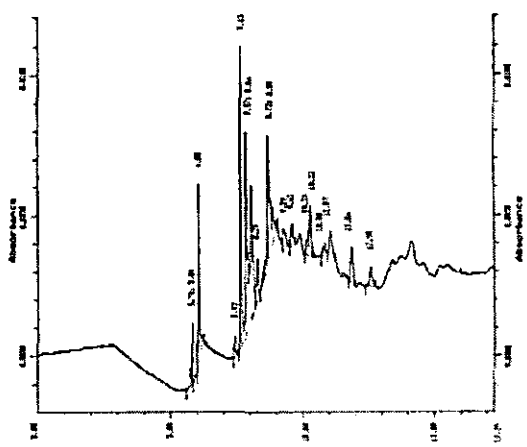

Figure 2.16b 56-Hour Fr-2

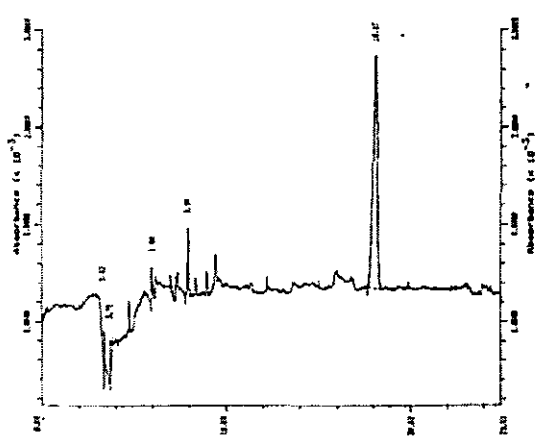

Figure 2.16c 56-Hour Fr-3

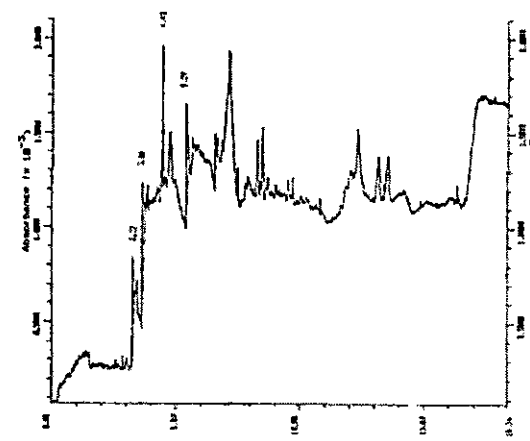

Figure 2.16d 56-Hour Fr-4

Figure 2.17 ***Capillary Electropherogram contd.***

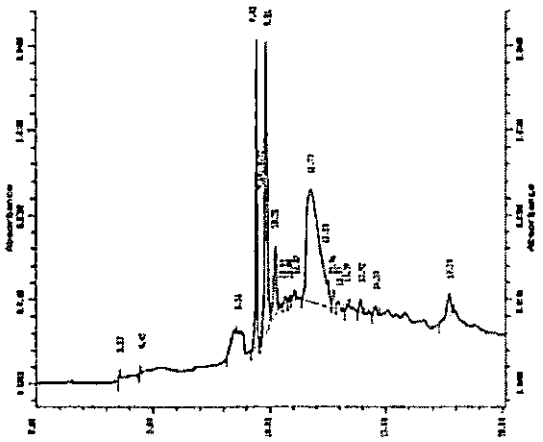

Figure 2.17a 64-Hour Fr-1

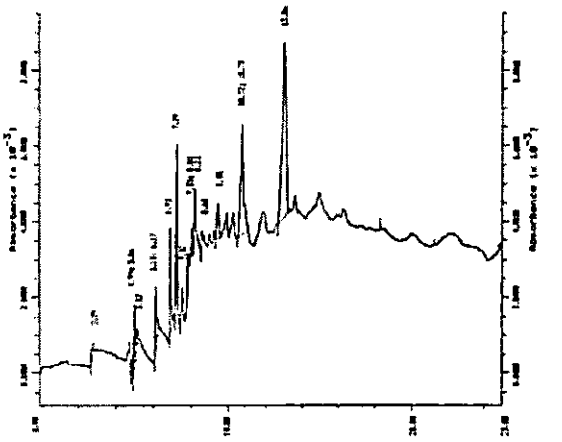

Figure 2.17b 64-Hour Fr-2

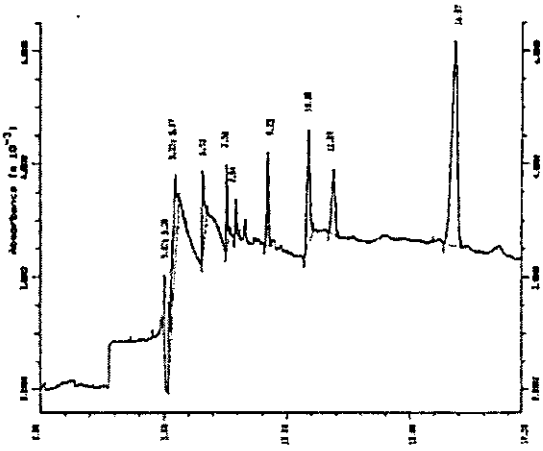

Figure 2.17c 64-Hour Fr-3

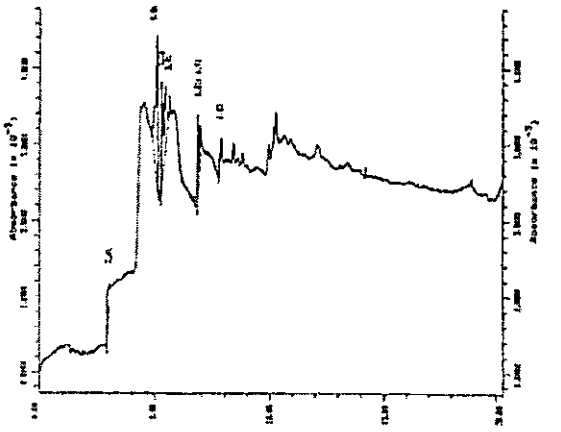

Figure 2.17d 64-Hour Fr-4

Figure 2.18 ***Capillary Electropherogram contd.***

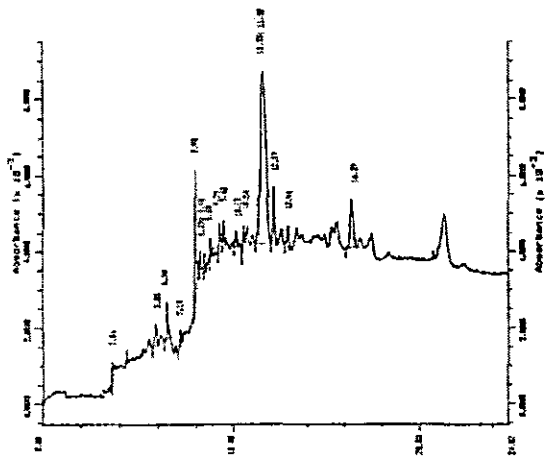

Figure 2.18a 72-Hour Fr-1

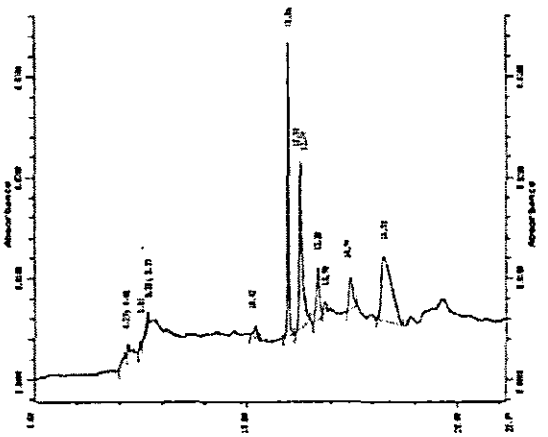

Figure 2.18b 72-Hour Fr-2

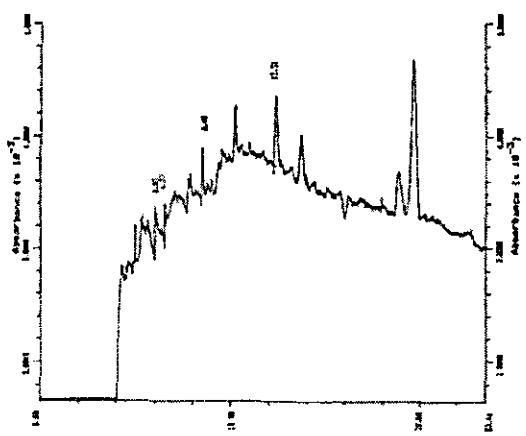

Figure 2.18c 72-Hour Fr-3

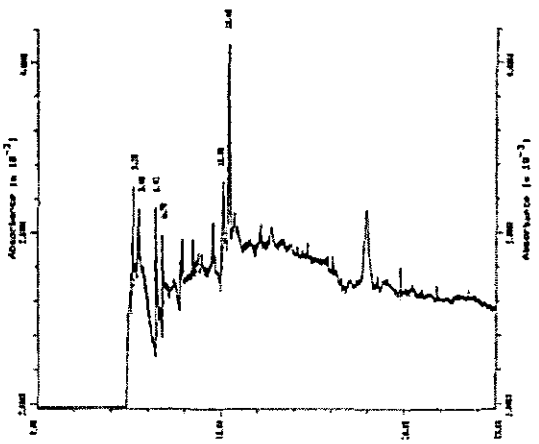

Figure 2.18d 72-Hour Fr-4

Figure 2.19 ***Capillary Electropherogram contd.***

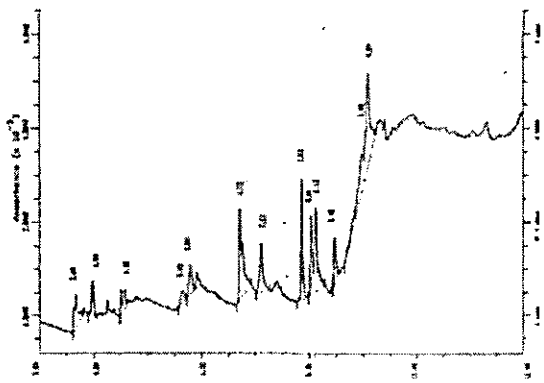

Figure 2.19a 5-Days Fr-1

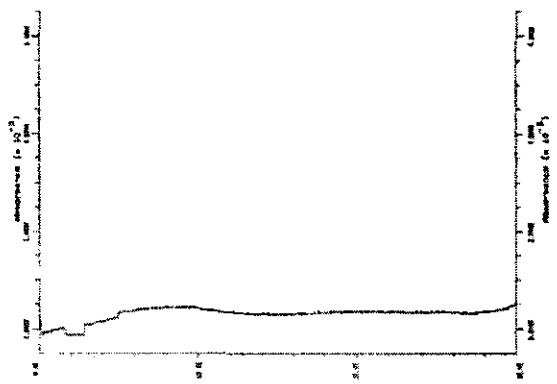

Figure 2.19b 5-Days Fr-2

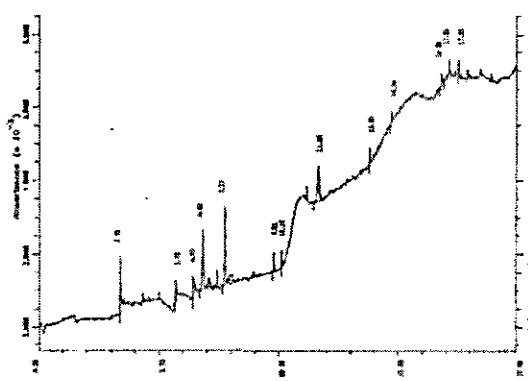

Figure 2.19c 5-Days Fr-3

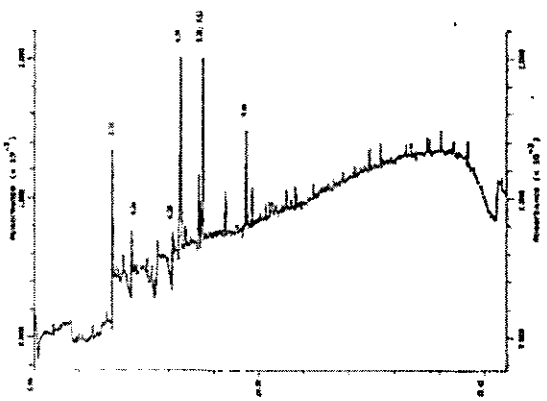

Figure 2.19d 5-Days Fr-4

Figure 2.20 ***Capillary Electropherogram contd.***

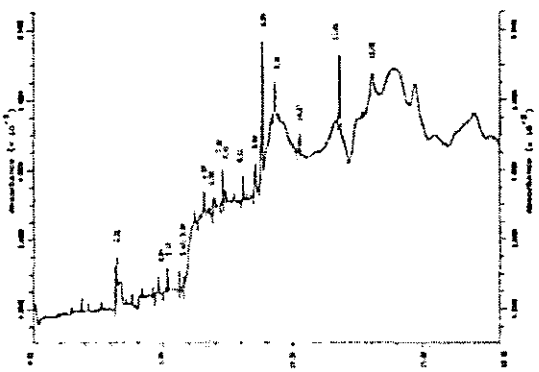

Figure 2.20a 6-Days Fr-1

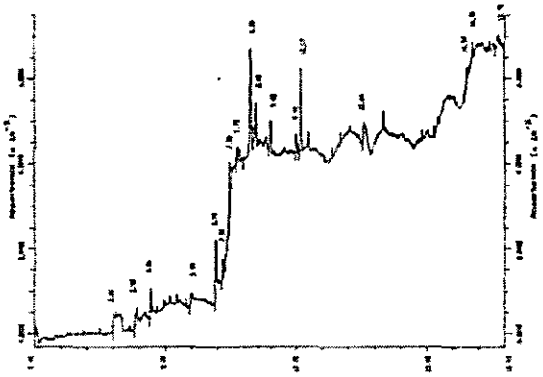

Figure 2.20b 6-Days Fr-2

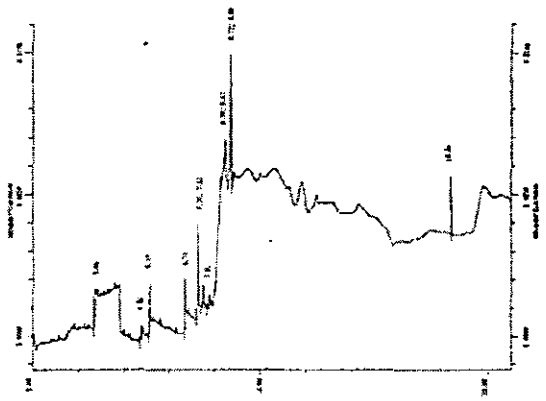

Figure 2.20c *6*-Days Fr-3

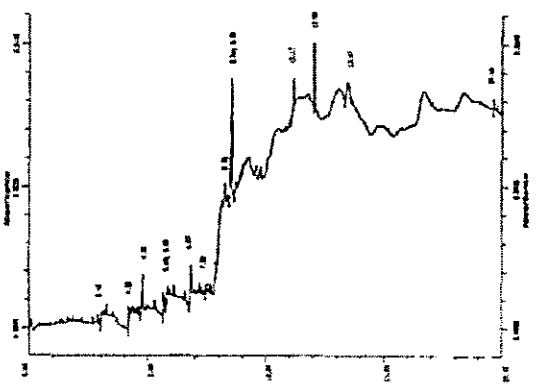

Figure 2.20d *6*-Days Fr-4

Figure 2.21 ***Capillary Electropherogram contd.***

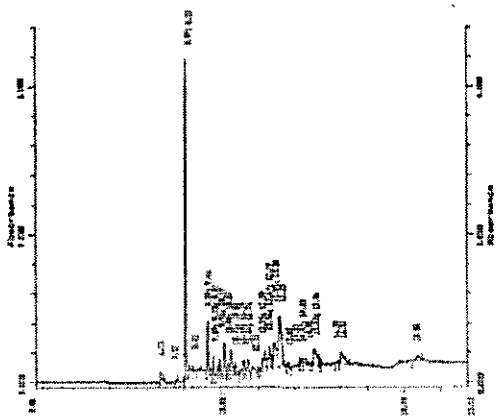

Figure 2.21a 7-Days Fr-1

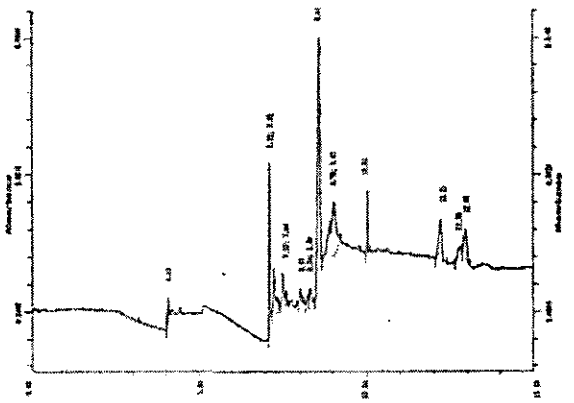

Figure 2.21b 7-Days Fr-2

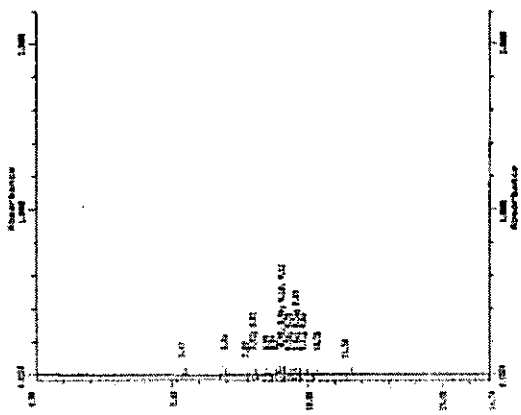

Figure 2.21c 7-Days Fr-3

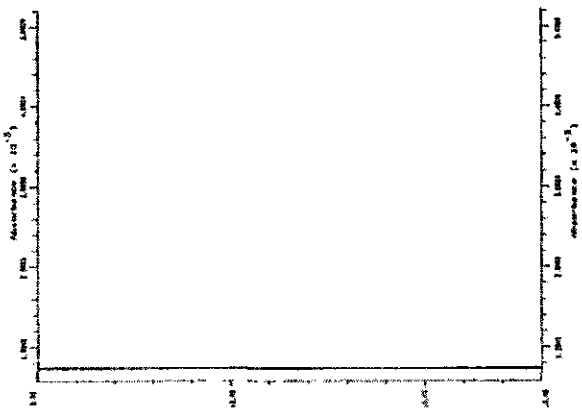

Figure 2.21d 7-Days Fr-4

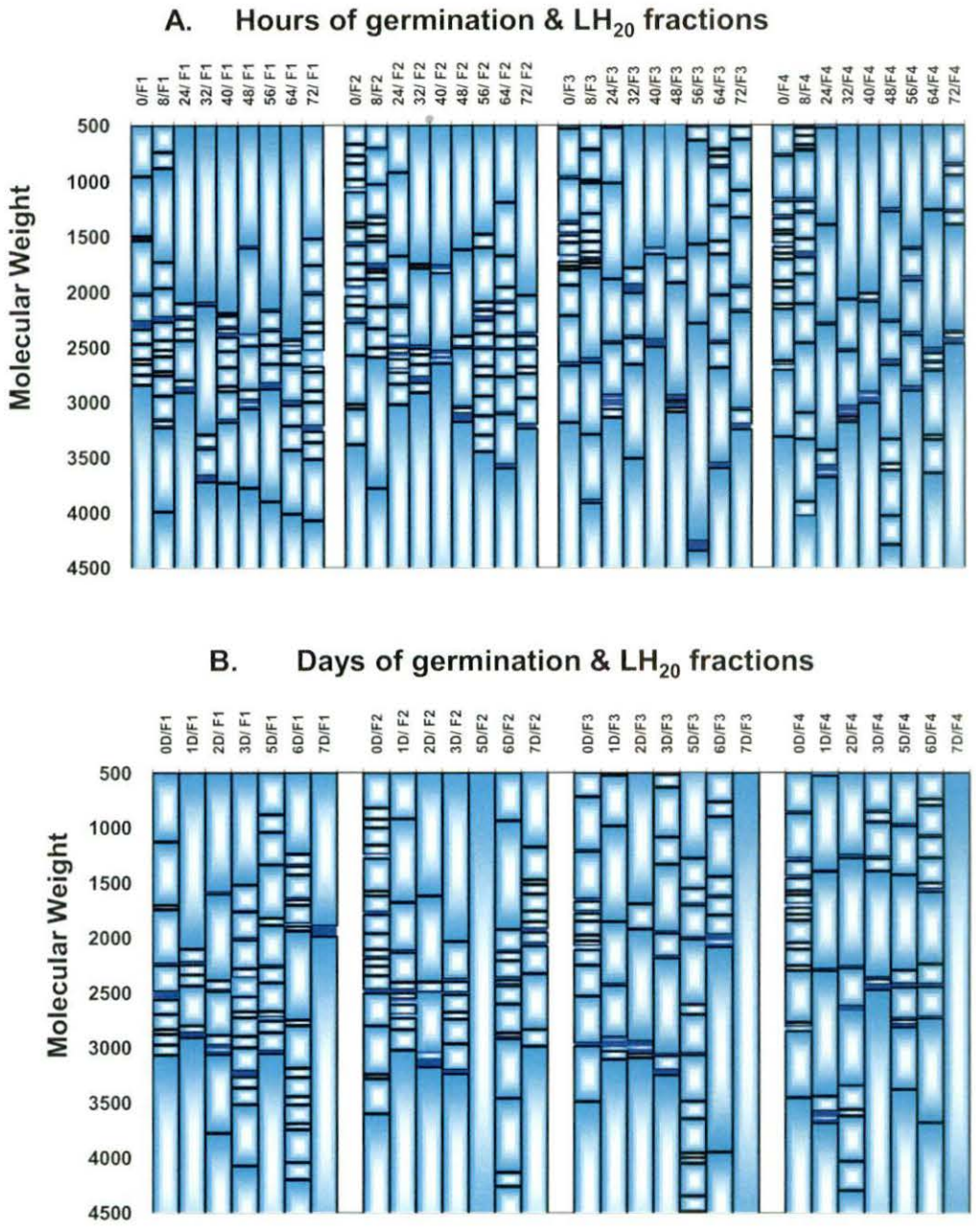

Figure 2.22 Two-dimensional Electropherogram of all the germination stages, prepared on the basis of Capillary electrophoretic representation. Thickness of bands indicates the area percentage of peaks occupying by one peptide in that fraction: **(A)** in different germination hours **(B)** in different germination days

2.4 DISCUSSION

Protein fingerprinting, in plants, has its wide application in different fields of study. This can be treated – to distinguish different legume cultivars or species (Ahmad and Slinkard 1992; Gardiner and Forde 1992; Gepts *et al.*, 1992; Takehisa *et al.*, 2001), as biological marker in biodiversity study (Tomooka *et al.*, 1992) or in functional aspect of spatial and temporal developmental context (Das *et al.*, 2006).

Peptides are also one of the most potent groups of bioactive molecules in the plant system (Lindsey *et al.*, 2002; Mandal *et al.*, 2006). They perform different biological activities with disciplined genesis (Lee *et al.*, 1996; Fletcher *et al.*, 1999). So, peptide fingerprinting is also a controlled and organized representation of its own kind with definite execution of internal metabolism.

In the present study, the primary focus was on the temporal difference in peptide pattern of germinating mung bean based on paper chromatography and capillary zone electrophoresis. The comparative analysis of peptide profile reveals mainly two groups of peptides. One of them is evident throughout the germination period. They might be called as "housekeeping peptides" with lower range of partition coefficient, as they showed a better separation in alkaline solvent [pH 12.11] (Lala, 1981) in all chromatograms. Actually they are more prominent in paper chromatograms with LH-20 separated fractions of 32 h & 6 days and also in electrophoregram (32 h and 6 days of germination periods are chosen because of their diverse nature; in 32h, the developing embryo is dependent on storage food reserve but in 6 days, it becomes nearly independent). These housekeeping peptides mainly ranges from 1500 Da to 3500 Da (consistency of peaks is evident throughout the germination in Capillary Zone Electrophoresis). From one dimension paper chromatography, it can also be predicted that a perceptible amount of these peptides contains proline or hydroxyproline in their amino terminal (Conn *et al.*, 1999).

Housekeeping proteins with a wide range of molecular weight have been already reported in mung bean (Das *et al.*, 2006). So, it is quite possible for that plant to carry such type of low molecular weight proteins, i.e. peptides, with same short of activity for some specific instances.

The other groups of peptides mainly appear from one day of germination and again nearly disappear at later stages. This shows that, they are mainly synthesized during the peak period of germination. These peptides are amphoteric in wide range of

pH (better resolved in two dimension paper chromatography) with moderate range of partition coefficient. The major part of these peptides came within first 150 tubes of sephadex Gel Exclusion Chromatography and their molecular weight ranges from 500 Da to 3500 Da. Depending on the nature and molecular weight, it may be predicted that these types of peptides are in most active form for transport, absorption and apoplastic migration. During germination, enzymatic hydrolysis of storage proteins forms a reservoir of small peptides which are translocated to the growing embryo for nutritional supply. Similar phenomenon is observed in pea seed germination (Liu *et al.*, 1996). Transport of these kinds of nutritional peptides has already been demonstrated in some monocots and *Arabidopsis* with specific peptide transporter (Waterworth *et al.*, 2000. Stacey *et al.*, 2002). Though, in Legumes, the picture is not so clear but similarities can be expected. Even, a large number of peptides have been recognized for their developmental phase related signaling behaviour (Ryan *et al.*, 2002). So, a number of those specific types of peptides affecting germination might be present in this group.

In conclusion, the present approach allowed us to recognize the abundance and dynamic shifting of low molecular weight peptides during various phases of seed germination and seedling establishment in *Vigna radiata*. Studying changes of individual peptide spots over the seedling growth period enable classification according to their existence and separation pattern. First group of peptides present throughout the germination periods were basically housekeeping in nature. Second group which increased gradually up to the stage of development of photosynthetic machinery and independence, included peptides may perhaps be involved in nutritional supply. Another group where spots appeared transiently just at the time of conversion from heterotrophic to autotrophic nature, included peptides possibly involved in developmental phase specific bioactivity. So the entire episode principally points on the fundamental budding picture of low molecular weight peptides in mung bean, during the awakening of its life. In the light this study, one can identify a wide bionetwork of different critically interdependent metabolic profile of peptides which in one theme, is just unexplainable.

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