CHAPTER NO. III.
STUDY OF AMINO ACID PATTERN OF THE PROTEIN HYDROLYSATES
BY CHROMATOGRAPHIC TECHNIQUE

Identification of amino acids:

The amino acids were identified by various standard techniques (i.e., by unidimensional ascending or descending, circular and two dimensional descending) paper chromatography and also by a technique worked out by the present authors. Whatman no. 1 filter paper was used throughout this work.

Paper chromatography essentially involved the following steps in succession.

1. Spotting of the sample solution: Using a fine capillary tube, small volume of protein hydrolysate or solution authentic amino acid was transferred to the filter paper and dried.

2. Development of the chromatogram: The loaded chromatograms were first equilibrated with the solvents vapour atmosphere, and developed by vertical or horizontal migration technique in proper chromatographic equipment using suitable solvents for development of the spot.

3. Drying and detection of spots: The developed chromatograms were dried between 60-70°C in a chromatographic drying oven for a few hours. The chromatograms were coloured either by dipping them in the staining reagent or by spraying
the staining reagent with an atomizer followed by air drying
or heating in an oven for colour development using the
following staining reagents.

Staining reagents used for detection of amino acids by the
authors:

1. Ninhydrin: 3.0-0.5% ninhydrin solution dissolved in
water saturated n-butanol.

2. Ninhydrin stained heated chromatogram was
after sprayed with 1.0% aqueous sodium
bicarbonate solution. Only phenylalanine
gave stable blue colour. When this
chromatogram was further sprayed with
1-N HCl solution, hydroxy proline turned red.

3. Sekaguchi reagent for arginine and other
guanidine derivatives. This reagent
consisted of two solutions described as
below:

Spray I: Solution of 0.1% alpha naphthol
dissolved in 1N sodium
hydroxide solution.

Spray II: Mixture of 100 ml, 5% aqueous
sodium hydroxide solution
containing 2ml bromine.
Procedure 1 - The chromatogram was first sprayed with reagent no. I and subsequently with reagent no. II. Arginine appeared as red spot.

4. Isatin reagent - A 0.2% solution of isatin was prepared in acetone containing 4% of acetic acid. Developed chromatogram was sprayed with isatin reagent and heated to 100°C for 10 minutes. Blue, lavender, or blue green colours were given by proline, hydroxy proline, phenylalanine, tyrosine, tryptophane, glutamic acid, arginine, lysine, methionine, histidine and cystine over a concentration range of, from less than 1 µg to 5 µg.

5. Folin reagent - 0.3 gm B - naphthoquinone-4-sodium sulphonate were dissolved in 10 ml of water. The solution was made up with acetone to 300 ml.

The developed chromatogram was dipped in Folin reagent and then heated in a drying chamber for 3-5 minutes at 100°C. The deep pink spots of amino acids undergo different colour changes, when the chromatogram was further dipped in for 1-1½ minutes into a solution, which has been freshly prepared by diluting 4 ml of aqueous 5 N-sodium hydroxide solution with 96% alcohol to 200 ml.

25. Saifer, A; and Oreskes, I. - Science; 119, 124-125, 1954.
6. Pauley's reagent: Solution I was prepared by dissolving 0.9 gm of sulphanilic acid in 9 ml of concentrated HCl and diluting the solution to 100 ml.

Solution II was 5.0% sodium nitrite solution, prepared in distilled water.

Solution III was 10% sodium carbonate solution prepared in distilled water.

Procedure: Solutions I & II in 1:1 ratio by volume, were mixed and allowed the mixture to stand for 4-5 minutes at temperature below 20°C. To this solution, carefully added 2 volume of aqueous sodium carbonate solution. Sprayed the oven dried chromatogram with this mixed solution. Histidine and tyrosine spots appeared red.

7. Ehrlich's reagent: 1% solution of p-dimethylaminobenzaldehyde was prepared in 96% ethanol and then the oven dried chromatogram was sprayed with this solution. After spraying the chromatogram was inserted for 5-10 minutes in a vessel saturated with HCl vapours. Tryptophane spot appeared purple.

Unidimensionally run paper chromatograms and segments of circular paper chromatograms were also subjected to multiple dipping technique of Jepson and Smith for better identification of amino acids.

EXPERIMENTAL

Preparation of unidimensional ascending paper chromatogram of authentic amino acids and the measurement of their \( R_f \) values in \( n\)-butanol : acetic acid : water (4:1:1.6) -

Two perfectly rectangular sheets 22½" x 11½" were taken. Origin line and positions of various starting points were drawn at 3 cm distance from each other and marked as 1, 2, 3, 4 --- etc. Solutions of authentic specimens of the amino acids of concentration 2mg/ml in 10% isopropanol were prepared. In case of sparingly soluble amino acids like cystine, tryptophane, tyrosine and methionine, one or two drops of 1 normal hydrochloric acids per 5 ml of the amino acid solution were added while making their solutions. At different positions solutions of different amino acid were spotted and dried as usual. The loaded sheets after equilibration with the solvent vapours, were irrigated with the solvent taken in a rectangular specimen jar. 48 hours total run period was given in two instalments. Developed chromatograms were dried and stained with ninhydrin solution. Photographs of the chromatograms are attached as figs. 4 & 5.

The \( R_f \) values of authentic specimens of the amino acids were determined by ascending paper chromatography using \( n\)-butanol : acetic acid : water (4:1:1.6) as solvent at 30°C and are given in table no.XIV.
Figs. 4 & 5: Ninhydrin stained, unidimensional paper chromatogram of authentic amino acids run in n- butanol : acetic acid : water (4:1:1.6). Spotting details are as under.

(1) Arginine, (2) Aspartic acid,
(3) Glutamic acid, (4) Threonine,
(5) Lysine, (6) Valine, (7) Phenylalanine,
(8) Leucine, (9) Histidine, (10) Glycine,
(11) Serine, (12) Tyrosine, (13) Tryptophane,
(14) Cystine, (15) Methionine, (16) Proline
and (17) Hydroxyproline.
TABLE NO. XIV

R<sub>f</sub> values of various amino acids in n-butanol : acetic acid : water (4:1:1:6) at 30<sup>0</sup>C.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Amino acid</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Arginine</td>
<td>0.06</td>
</tr>
<tr>
<td>2.</td>
<td>Aspartic acid</td>
<td>0.11</td>
</tr>
<tr>
<td>3.</td>
<td>Glutamic acid</td>
<td>0.18</td>
</tr>
<tr>
<td>4.</td>
<td>Threonine</td>
<td>0.20</td>
</tr>
<tr>
<td>5.</td>
<td>Lysine</td>
<td>0.03</td>
</tr>
<tr>
<td>6.</td>
<td>Valine</td>
<td>0.56</td>
</tr>
<tr>
<td>7.</td>
<td>Phenylalanine</td>
<td>0.75</td>
</tr>
<tr>
<td>8.</td>
<td>Leucine</td>
<td>0.78</td>
</tr>
<tr>
<td>9.</td>
<td>Histidine</td>
<td>0.05</td>
</tr>
<tr>
<td>10.</td>
<td>Glycine</td>
<td>0.13</td>
</tr>
<tr>
<td>11.</td>
<td>Serine</td>
<td>0.11</td>
</tr>
<tr>
<td>12.</td>
<td>Tyrosine</td>
<td>0.48</td>
</tr>
<tr>
<td>13.</td>
<td>Tryptophane</td>
<td>0.68</td>
</tr>
<tr>
<td>14.</td>
<td>Cystine</td>
<td>0.02</td>
</tr>
<tr>
<td>15.</td>
<td>Methionine</td>
<td>0.56</td>
</tr>
<tr>
<td>16.</td>
<td>Proline</td>
<td>0.27</td>
</tr>
<tr>
<td>17.</td>
<td>Hydroxyproline</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Mean values of 5 determinations.
Preparation of unidimensional paper chromatograms of various seed protein hydrolysates:

Similarly the various seed protein hydrolysates were chromatographed by the ascending technique using n-butanol : acetic acid : water (4:1:1.6) as solvent and the amino acids detected by comparison of their R_f values from those of the authentic specimens and also by specific staining techniques.

For confirming the presence of histidine and tryptophane, use of multiple reagents was made. The ninhydrin stained chromatogram was cut parallel to the line of origin of the spot and just above the position occupied by arginine. The lower portion of the ninhydrin stained chromatogram was further stained with Pauley's reagent to confirm the presence of histidine which appeared as red spot. The upper portion of the ninhydrin stained chromatogram was further sprayed with Ehrlich reagent to confirm the presence of tryptophane.

Photograph of the ninhydrin stained chromatogram of some seed protein hydrolysates is attached as fig. 6.

Fig.6 :- Ninhydrin stained unidimensional paper chromatogram of protein hydrolysates of seeds of Cucurbitaceae family. Spotting details are as under.
In a similar manner unidimensional ascending paper chromatograms with protein hydrolysates of seeds other than those of Cucurbitaceae family were prepared. Number of coloured spots with different staining reagents are given in the table No. XV.

**TABLE NO. XV**

Number of coloured spots with different staining reagents.

<table>
<thead>
<tr>
<th>No.</th>
<th>Seed/plant material</th>
<th>Ninhydrin</th>
<th>Cbrlich</th>
<th>Isatin</th>
<th>Pauluy</th>
<th>Polin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cucurbita maxima.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>2.</td>
<td>Cucurbita pepo.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>3.</td>
<td>Cucumis melo.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>4.</td>
<td>Cucumis sativus.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>5.</td>
<td>Momordica charantia.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>6.</td>
<td>Carthamus tinctorius.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>7.</td>
<td>Sterculia foetida.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>8.</td>
<td>Pongamia glabra.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>9.</td>
<td>Bassia latifolia.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>10.</td>
<td>Cassia tora.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>11.</td>
<td>Linum usitatissimum.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>12.</td>
<td>Cesalpinia bonduc.</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>13.</td>
<td>Oryza sativa kernel (dessi safari)</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>14.</td>
<td>Oryza sativa bran (dessi safari)</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>

**Note:** Ninhydrin stained chromatogram was heated and then sprayed with 1.0% aqueous sodium bicarbonate to ascertain the presence of phenylalanine, which gave stable blue colour.
From unidimensional ascending paper chromatograms using various specific staining reagents the presence of cystine, histidine, tyrosine, proline, phenylalanine, leucine and isoleucine, arginine and tryptophane could be confirmed in each seed and rice bran protein hydrolysate.

Preparation of the unidimensional descending paper chromatogram with authentic specimens of the amino acids and measurement of their Rf values in different solvent systems:

Line of origin of the spots parallel to the narrow edge of the filter paper sheet 22½" x 18½", at a distance of 3" from the edge was drawn with a pencil. Positions of various equidistant points on the line of origin were marked as 1, 2, 3, 4 --- etc. Different amino acid solutions were spotted at different positions on the line of origin and dried in usual manner. The loaded sheet was developed by unidimensional descending run technique in standard chromatography chamber with suitable solvent system till the solvent front reached almost, upto the other end of the filter paper. It took 18-22 hours with n-butanol : acetic acid : water (4:1:1.6), and isobutanol : acetic acid : water (4:1:1.6); 10-12 hours with pyridine : water (8:2); and 10-13 hours with n-butanol : pyridine : water (1:1:1) solvents. Developed chromatograms were dried and stained with ninhydrin solution.

Photographs of the chromatograms are attached as figs. 7 & 8.
Fig. 7: Ninhydrin stained unidimensional descending paper chromatogram run in n-butanol, acetic acid, water (4:1:1.6) solvent system. Spotting details are as under.


Fig. 8: Ninhydrin stained unidimensional descending paper chromatogram run in n-butanol:pyridine:water (1:1:1) as solvent. Spotting details are as under.


R_f values of different amino acids were measured at 30°C and recorded in the following table no. XVI.
**TABLE NO. XVI**

The \( R_f \) values of authentic specimens of amino acids in various solvent systems at 30\(^\circ\)C.

<table>
<thead>
<tr>
<th>No.</th>
<th>Amino acid</th>
<th>( R_f ) value in solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cystine.</td>
<td>0.03 0.04 - -</td>
</tr>
<tr>
<td>2.</td>
<td>Lysine.</td>
<td>0.06 0.09 0.12 0.14</td>
</tr>
<tr>
<td>3.</td>
<td>Histidine.</td>
<td>0.06 0.10 0.25 0.26</td>
</tr>
<tr>
<td>4.</td>
<td>Arginine.</td>
<td>0.10 0.10 0.19 0.17</td>
</tr>
<tr>
<td>5.</td>
<td>Aspartic acid.</td>
<td>0.14 0.16 0.13 0.14</td>
</tr>
<tr>
<td>6.</td>
<td>Glycine.</td>
<td>0.17 0.19 0.23 0.21</td>
</tr>
<tr>
<td>7.</td>
<td>Serine.</td>
<td>0.16 0.17 0.31 0.26</td>
</tr>
<tr>
<td>8.</td>
<td>Glutamic acid.</td>
<td>0.21 0.24 0.15 0.17</td>
</tr>
<tr>
<td>9.</td>
<td>Threonine.</td>
<td>0.25 0.27 0.33 0.34</td>
</tr>
<tr>
<td>10.</td>
<td>Alanine.</td>
<td>0.29 0.31 0.32 -</td>
</tr>
<tr>
<td>11.</td>
<td>Proline.</td>
<td>0.37 0.40 0.32 -</td>
</tr>
<tr>
<td>12.</td>
<td>Tyrosine.</td>
<td>0.39 0.45 0.55 0.61</td>
</tr>
<tr>
<td>13.</td>
<td>Valine.</td>
<td>0.56 0.62 0.51 0.54</td>
</tr>
<tr>
<td>14.</td>
<td>Methionine.</td>
<td>0.52 0.58 0.51 0.58</td>
</tr>
<tr>
<td>15.</td>
<td>Tryptophane.</td>
<td>0.50 0.59 0.51 0.65</td>
</tr>
<tr>
<td>16.</td>
<td>Phenylalanine.</td>
<td>0.60 0.68 0.53 0.69</td>
</tr>
<tr>
<td>17.</td>
<td>Leucine.</td>
<td>0.68 0.78 0.54 0.69</td>
</tr>
<tr>
<td>18.</td>
<td>Hydroxyproline.</td>
<td>0.17 0.20 - 0.33</td>
</tr>
</tbody>
</table>

Mean values of 5 determinations.
Preparation of unidimensional descending paper chromatogram of various seed protein hydrolysates in n-butanol : acetic acid : water (4:1:1.6)

Similarly the various seed protein hydrolysates were chromatographed by descending run technique and their amino acids detected by comparison of the Rf values from those of the authentic specimens and also by specific staining techniques. Protein hydrolysate solutions of different seeds were spotted and the chromatogram was developed with 40 ml of n-butanol : acetic acid : water (4:1:1.6) for 18 - 20 hours by descending technique. The sheet was then cut into three halves perpendicular to the line of origin of the spots and each was developed with a different staining reagent, eg, ninhydrin solution, isatin solution, diazotised sulphanilic acid solution etc. Photographs of the chromatograms was taken and attached as Fig. 9. The ninhydrin stained, isatin stained chromatograms were further subjected to multiple dipping technique of Jepson and Smith.

Fig 9 :- Unidimensional descending paper chromatogram of the protein hydrolysate of three different seeds of Cucurbitaceae family run in n-butanol, acetic acid, water (4:1:1.6) as solvent. Total run period 36 hours was given in two instalments. Each of the three portions of the developed chromatogram was stained with (a) 0.5% ninhydrin solution in water saturated n-butanol; (b) Isatin reagent;
(c) Diazotised sulphanilic acid.

Spotting details are as under.

(1) Cucurbita pepo, (2) Cucurbita maxima,
(3) Synthetic mixture of 18 amino acids, (4) Cucumis sativus.

In a similar manner unidimensional descending paper chromatograms were prepared with other seed protein hydrolysates. Staining with different reagents revealed the presence of cystine, histidine, tyrosine, proline, phenylalanine, leucine and isoleucine, tryptophane and arginine in hydrolysate of each seed and rice bran protein isolates.

CIRCULAR PAPER CHROMATOGRAPHY

In this technique, the development is carried out in a horizontal plan. By using different methods complete circles or circular arc chromatogram can be developed.

Technique for developing complete circles :-

A circular disc, diameter 10"-12", of Whatman filter paper No. 1 was taken. The protein hydrolysate solution was spotted at the centre of the disc and dried. The disc after equilibration with solvent vapours, was irrigated with the solvent by inserting one end of the filter paper wick through a small hole carefully made at its centre and dipping a portion of its other end in the solvent taken in a small petri dish.
The paper disc along with the petri dish of solvent, was housed in a bigger petri dish and the whole was covered with an inverted circular glass trough. When the solvent moved almost up to the periphery of the circular paper disc, it was removed and dried. Finally the chromatogram was stained with ninhydrin or other suitable staining reagent. The two main advantages of this technique are as follows:

(1) The disc may be cut into a number of segments and each segment in turn may be stained with a specific staining reagent for better identification of amino acids.

(2) It may be used as a preparative technique as well.

Chromatograms (Figs. 10 and 11) developed by this technique are attached.

Fig. 10: Ninhydrin stained circular paper chromatogram of protein hydrolysate of Momordica charantia (whole) seeds, developed in n-butanol: acetic acid: water (4:1:1.6). Total run period was 60 hours. Ninhydrin stained chromatogram was cut into two segments. One segment was after sprayed with 1% aqueous sodium bicarbonate solution, Phenylalanine band turned blue. The same segment was then sprayed with 1 normal HCl acid, hydroxyproline band turned pink. The other segment was after sprayed with Ehrlich reagent to ascertain the presence of tryptophane.

Fig. 11: Ninhydrin stained segment of a circular paper chromatogram of Pongamia glabra seed protein hydrolysate.
Development of circular arcs:

In this method, a circle of diameter 3-4 cm was drawn with a pencil at the centre of a circular disc of Whatman filter paper No. 1 of 12-13 inches in diameter. Positions of various equidistant starting points were marked as 1, 2, 3--- etc. on the circumference of the circle. The protein hydrolysate solutions of different seeds and solution of synthetic mixture of various amino acids, were spotted at different positions and dried. The chromatogram was irrigated using a filter paper wick, inserted in a small hole punched at the centre of the circular disc in a manner as described previously. Developed chromatogram was dried and stained as usual. Separation sequences of various amino acids in various solvents used were identical with the results of earlier workers.

Chromatogram (Fig. 12) developed by this technique is attached.

Fig. 12:-- Ninhydrin stained circular paper chromatogram of protein hydrolysates of four different seeds of Cucurbitaceae family, developed in n-butanol : acetic acid : water (4:1:1.6) solvent system. Total run period was 60 hours. Spotting details are as under.


Circular paper chromatography further supported the observations of unidimensional ascending and descending paper chromatography.
Fig. 12: Ninhydrin stained circular paper chromatogram of protein hydrolysates of five different seeds, run in n-butanol : acetic acid : water (4:1:1.6) as solvent. Spotting details are as under.
Two dimensional descending paper chromatographic technique:

The position of the starting points at a distance of 2½" from each of the two adjacent edges was marked on a Whatman filter paper sheet (22½" x 18½"). The protein hydrolysate solution was spotted and dried as usual. The spotted chromatogram was run in n-butanol: acetic acid: water (4:1:1.6) solvent system by descending technique in a chromatography chamber. When the solvent front moved up to the other edge of the sheet, it was removed from the cabinet and dried by heating in a chromatography drying oven at temperature below 70°C for an hour. Alternatively the sheet may also be dried at room temperature under fan in unpolluted, mosquito and insect free atmosphere for overnight. The chromatogram was then irrigated with another solvent pyridine: water (4:1) or n-butanol: pyridine: water (1:1:1) at right angle to the previous direction of the solvent, in another chromatography chamber. When the development was complete, it was removed from the chamber and dried and finally stained with ninhydrin solution for colour development.

Photographs of many of the fully developed, two dimensionally run paper chromatograms are attached. (Fig. 13 - 28).

Fig. 13: - Ninhydrin stained, two dimensionally developed paper chromatogram of the protein hydrolysate of Momordica charantia seeds. Run first with modified Partridge solution and then with n-butanol: pyridine: water (1:1:1).
The chromatogram revealed the presence of (1) Cystine, 
(2) Lysine, (3) Histidine, (4) Arginine, (5) Aspartic acid, 
(6) Glycine, (7) Serine, (8) Glutamic acid, (9) Hydroxyproline, 
(17) Phenylalanine, (18) Leucine and isoleucine, trace of methionine and one unidentified spot (19) of Rf value 0.11, 0.52 in butanol : acetic acid and butanol pyridine systems respectively.

Fig. 14: - Ninhydrin stained, two dimensional descending paper chromatogram of protein hydrolysate of Cucurbita pepo seeds. Run first with modified Partridge solution and then with n- butanol : pyridine : water (1:1:1) solvent systems.

The chromatogram revealed the presence of 
(1) Cystine, (2) Lysine, (3) Histidine, (4) Arginine, 
(13) Valine, (14) Methionine, (15) Tryptophane, 
(16) Phenylalanine, (17) Leucine & isoleucine and 
(18) Gamma amino butyric acid.
Fig. 15: Ninhydrin stained, two dimensional descending paper chromatogram of protein hydrolysate of Cucumis melo seeds. Run first with modified Partridge solution and then with n-butanol : pyridine : water (1:1:1) as solvent.

The chromatogram revealed the presence of
(1) Cystine, (2) Lysine, (3) Histidine, (4) Arginine,
(12) Tyrosine, (13) Valine, (14) Methionine, (15) Tryptophane,
(16) Phenylalanine, (17) Leucine & isoleucine and
(18) Gamma amino butyric acid.

Fig. 16: Ninhydrin stained, two dimensional descending paper chromatogram of protein hydrolysate of Cucurbita maxima seeds. Run first with modified Partridge solution and then with n-butanol : pyridine : water (1:1:1) solvent systems.

The chromatogram revealed the presence of
(1) Cystine, (2) Lysine, (3) Histidine, (4) Arginine,
(13) Valine, (14) Methionine, (15) Tryptophane,
(16) Phenylalanine, (17) Leucine & isoleucine and
(18) Gamma amino butyric acid.
Fig. 17: Minhydrin stained, two dimensional descending paper chromatogram of protein hydrolysate of Cucumis sativus seeds. Run first with modified Partridge solution and then with n- butanol : pyridine : water (1:1:1) as solvent.

The chromatogram revealed the presence of:
(1) Cystine, (2) Lysine, (3) Histidine, (4) Arginine,
(12) Tyrosine, (13) Valine, (14) Methionine,

Fig. 18: Minhydrin stained, two dimensionally developed paper chromatogram of the protein hydrolysate of Cesalpinia bonducella seeds. Run first with modified Partridge solution and then with n- butanol : pyridine : water (1:1:1) as solvents.

The chromatogram revealed the presence of:
(1) Cystine, (2) Lysine, (3) Histidine, (4) Arginine,
(18) Gamma amino butyric acid.
Fig. 19: Ninhydrin stained, two dimensionally developed paper chromatogram of the protein hydrolysate of Cassia tora seeds. Run first with modified partridge solution and then with n-butanol : pyridine : water (1:1:1) as solvents.

The chromatogram revealed the presence of
1. Cystine
2. Lysine
3. Histidine
4. Arginine
5. Aspartic acid
6. Glycine
7. Serine
8. Glutamic acid
9. Threonine
10. Alanine
11. Proline
12. Tyrosine
13. Valine
14. Methionine
15. Tryptophane
16. Phenylalanine
17. Leucine & isoleucine
18. Gamma amino butyric acid.

Fig. 20: Ninhydrin stained, two dimensionally developed paper chromatogram of the protein hydrolysate of Pongamia glabra seeds. Run first with modified Partridge solution and then with n-butanol : pyridine : water (1:1:1) as solvent.

The chromatogram revealed the presence of
1. Cystine
2. Lysine
3. Histidine
4. Arginine
5. Aspartic acid
6. Glycine
7. Serine
8. Glutamic acid
9. Threonine
10. Alanine
11. Proline
12. Tyrosine
13. Valine
14. Methionine
15. Tryptophane
16. Phenylalanine
17. Leucine & isoleucine
18. Gamma amino butyric acid.
Fig. 21: Ninhydrin stained, two dimensionally developed paper chromatogram of the protein hydrolysate of Sterculia foetida - seeds. Run first with modified partridge solution and then with n-butanol : pyridine : water (1:1:1) as solvents.

The chromatogram revealed the presence of
(1) Cystine, (2) Lysine, (3) Histidine, (4) Arginine,
(5) Aspartic acid, (6) Glycine, (7) Serine, (8) Glutamic acid,
(9) Threonine, (10) Alanine, (11) Proline,
(12) Tyrosine, (13) Valine, (14) Methionine, (15) Tryptophane,
(16) Phenylalanine, (17) Leucine and isoleucine, and
(18) Gamma amino butyric acid.

Fig. 22: Ninhydrin stained, two dimensionally developed paper chromatogram of the protein hydrolysate of Bassia latifolia seeds. Run first with modified Partridge solution and then with n-butanol : pyridine : water (1:1:1) as solvents.

The chromatogram revealed the presence of
(1) Cystine, (2) Lysine, (3) Histidine, (4) Arginine,
(5) Aspartic acid, (6) Glycine, (7) Serine, (8) Glutamic acid,
(9) Threonine, (10) Alanine, (11) Proline,
(12) Tyrosine, (13) Valine, (14) Methionine, (15) Tryptophane,
(16) Phenylalanine, (17) Leucine & isoleucine,
(18) Gamma amino butyric acid, and (19) Alpha amino butyric acid.
Fig. 23:-- Ninhydrin stained, two dimensionally developed paper chromatogram of the protein hydrolysate of Oryza sativa bran. Run first with modified partridge solution and then with n-butanol : pyridine : water (1:1:1) as solvents.

The chromatogram revealed the presence of
(1) Cystine, (2) Lysine, (3) Histidine, (4) Arginine,
(5) Aspartic acid, (6) Glycine, (7) Serine, (8) Glutamic acid,
(9) Threonine, (10) Alanine, (11) Proline, (12) Tyrosine,
(13) Valine, (14) Methionine, (15) Tryptophane,
(16) Phenylalanine, (17) Leucine & isoleucine, and
(18) Gamma amino butyric acid.

Fig. 24:-- Ninhydrin stained, two dimensionally developed paper chromatogram of the protein hydrolysate of Linum usitatissiumum seeds. Run first with modified Partridge solution and then with n-butanol : pyridine : water (1:1:1) as solvents.

The chromatogram revealed the presence of
(1) Cystine, (2) Lysine, (3) Histidine, (4) Arginine,
(5) Aspartic acid, (6) Glycine, (7) Serine, (8) Glutamic acid,
(9) Threonine, (10) Alanine, (11) Proline, (12) Tyrosine,
(13) Valine, (14) Methionine, (15) Tryptophane,
(16) Phenylalanine, (17) Leucine & isoleucine, (18) Gamma amino butyric acid, and (19) Hydroxy proline.
Fig. 25: Ninhydrin stained, two dimensionally developed descending paper chromatogram of the protein hydrolysate of Cucurbita pepo seeds. Run first with modified Partridge solution and then with pyridine : water (4:1) as solvents.

The chromatogram revealed the presence of cystine, lysine, histidine, arginine, aspartic acid, glycine, serine, glutamic acid, threonine, alanine, proline, tyrosine, (valine, methionine & tryptophane), phenylalanine, leucine and isoleucine and gamma amino butyric acid.

Fig. 26: Ninhydrin stained, two dimensionally developed descending paper chromatogram of the protein hydrolysate of Cesalpinia bonducella seeds. Run first with modified Partridge solution and then with pyridine : water (4:1) as solvent.

The chromatogram revealed the presence of (1) cystine, (2) lysine, (3) histidine, (4) arginine, (5) aspartic acid, (6) glycine, (7) serine, (8) glutamic acid, (9) threonine, (10) alanine, (11) proline, (12) tyrosine, (13) (valine, methionine, tryptophane), (14) phenylalanine, (15) leucine and isoleucine, and (16) gamma amino butyric acid.

* This solvent combination do not resolve valine, methionine, and tryptophane. They appear as a single spot.
Fig. 27: Ninhydrin stained, two dimensionally developed descending paper chromatogram of the protein hydrolysate of Cassia tora seeds. Run first with modified Partridge solution and then with pyridine : water (4:1) as solvents.

The chromatogram revealed the presence of (1) cystine, (2) lysine, (3) histidine, (4) arginine, (5) aspartic acid, (6) glycine, (7) serine, (8) glutamic acid, (9) threonine, (10) alanine, (11) proline, (12) tyrosine, (13) (valine, methionine, tryptophane)*, (14) phenylalanine, (15) leucine and isoleucine, and (16) gamma amino butyric acid.

Fig. 28: Ninhydrine stained, two dimensionally developed descending paper chromatogram of the protein hydrolysate of Carthamus tinctorius seeds. Run first with modified Partridge solution and then with pyridine : water (4:1) as solvent.

The chromatogram revealed the presence of cystine, lysine, histidine, arginine, aspartic acid, glycine, serine, glutamic acid, threonine, alanine, proline, tyrosine, (valine, methionine, tryptophane)*, phenylalanine, leucine and isoleucine and gamma amino butyric acid.

* This solvent combination do not resolve valine, methionine, and tryptophane. They appear as a single spot.
FIG. 27

FIG. 28
TWO DIMENSIONAL PAPER CHROMATOGRAPHIC TECHNIQUE WORKED OUT BY THE PRESENT AUTHORS

This technique is essentially a combination of unidimensional ascending or descending and circular paper chromatography for first and second development respectively using different solvent systems in each case. Spotting of the seed protein hydrolysate solution was done in duplicate on each of the two perfectly rectangular strips of Whatman filter paper No. 1 (14½" x 3½"), at starting points 1 & 2. Each strip was separately developed twice by ascending / descending technique in separate vessels using n-butanol : acetic acid : water (4:1:1.6) as solvent and dried. Each developed strip was cut into two halves perpendicular to the line of origin of the spot. One half of the strip in each case was stained with ninhydrin, followed by Pauley's reagent. The positions of various overlapping amino acid bands were located and marked on both the stained and unstained halves of the strip, (Fig.29). Separate bands were cut out from the unstained portion of the strip. Each cut out paper strip, rolled into a wick, was developed twice on a circular paper disc (diameter 25 cm) using pyridine : water (4:1) or n-butanol : acetic acid : water (1:1:1) as solvent.
Histidine, lysine; glutamic acid and threonine band gave good resolution in both pyridine : water (4:1) and n- butanol : pyridine : water (1:1:1) systems.

Good separation of aspartic acid : glycine and serine was achieved in pyridine, water system. The band containing gamma amino butyric acid, valine, methionine and tryptophane was resolved with n- butanol : pyridine : water (1:1:1) system. Chromatograms (Figs. 29, 30 & 31) developed by this technique, are attached.

Fig. 29 : - Ninhydrin stained unidimensional ascending paper chromatogram of Sterculia foetida seed protein hydrolysate developed in n- butanol : acetic acid : water (4:1:1.6) as solvent.

Fig. 30 : - Horizontally developed chromatogram of overlapping amino acid bands No. 1 & 3, using pyridine : water (4:1) as solvent. Band No. 1 separated into lysine and histidine; and band No. 3 into aspartic acid, glycine and serine.

Fig. 31 : - Horizontally developed chromatogram of overlapping amino acid band No. 4 using n- butanol : pyridine : water (1:1:1) as solvent. The band separated into glutamic acid and threonine.

Rf values of resolved and unresolved amino acid bands in various solvent systems were measured at 30°C and are given in Table No. XVII.
### Table XVII

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Tyrosine</th>
<th>Phenylalanine</th>
<th>Lysine</th>
<th>Histidine</th>
<th>Threonine</th>
<th>Alanine</th>
<th>Seroine</th>
<th>Aspartic Acid</th>
<th>Asparagine</th>
<th>Serine</th>
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**Mean values of 5 determinations**
The protein hydrolysates were qualitatively analysed both by two dimensional descending paper chromatography and the new technique worked out by the authors. The results of qualitative investigation by these two techniques of paper chromatography were identical and are given in table No. 18.
<table>
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<th>Plant</th>
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Note: The table indicates positive results for the specified plants and their products. The symbols (+) and (-) represent positive and negative results, respectively.
SUMMARY

The protein hydrolysates have been analysed qualitatively for the presence of amino acids by various techniques (viz. unidimensional ascending/descending, circular, two dimensional descending) paper chromatography and also by a technique worked out by the author, using suitable solvents. Differential and multiple staining methods have been adopted for detection of amino acid spots. The hydrolysates have thus been found to contain 18 amino acids viz, Cystine, lysine, histidine, arginine, aspartic acid, glycine, serine, glutamic acid, threonine, alanine, proline, tyrosine, valine, methionine, tryptophane, phenylalanine, leucine and isoleucine. Besides these, isatin positive spot for hydroxyproline has been observed in the hydrolysates of Momordica charantia and Linum usitatissimum. Presence of alpha amino butyric acid has been observed in the hydrolysates of Momordica charantia and Bassia latifolia seeds. Gamma amino butyric acid spot has been observed in the hydrolysate of all the seeds (except Momordica charantia) and rice bran. Asparagine and glutamine have been found absent in all the seeds and rice bran protein hydrolysates.