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# THE IDENTIFICATION OF THE FREE AMINO ACIDS PRESENT IN SOME GRASS POLLENS BY THIN-LAYER CHROMATOGRAPHY*

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SUMMARY

The free amino acid content of aqueous extracts (preserved in 50% glycerol) of eleven grass pollens (*Festuca pratensis* Huds., *Lolium perenne* L., *Poa trivialis* L., *Dactylis glomerata* L., *Cynosurus cristatus* L., *Anthoxanthum odoratum* L., *Arrhenatherum elatius* (L.) J. & C. Presl, *Holcus lanatus* L., *Agrostis tenuis* Sibth., *Phleum pratense* L., *Alopecurus pratensis* L.) has been investigated using a thin-layer chromatographic technique. α-Alanine, γ-amino-*n*-butyric acid, glycine, DL-leucine, L-lysine, L-proline and DL-serine were identified. No species differences were detected and there was no difference between the viable and non-viable pollen extracts examined.

## INTRODUCTION

All the common amino acids have been reported in pollen either bound to the protein or free[1]. While there are numerous references to the amino acid content of dicotyledonous[1-5], gymnospermous[6] and monocotyledonous[7-10] pollens there are few specific references to grass pollens.

AUGUSTIN[11] examined the amino acid pattern of whole extracts of *Phleum pratense* L. and *Dactylis glomerata* L. pollens and ultra-filtrates (using collodion membranes); both were reported to give identical amino acid patterns but it is difficult to interpret the chromatograms presented. The eluted base line fraction (allergenically active) on hydrolysis yielded cystine, lysine or arginine, histidine, aspartic acid, glycine or serine, threonine or glutamic acid, alanine, proline, tyrosine, valine, methionine, tryptophan, phenylalanine and the leucine group.

Four grass pollens (*Festuca rubra* L. subsp. *commutata* Gaud., *Dactylis glomerata* L., *Lolium perenne* L. and *Phleum pratense* L.) assayed microbiologically for their free or bound amino acids showed wide variation[12], the free amino acids identified being proline, alanine, glycine, serine and valine. However, SYNGE[13] reports that it is

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unwise to use a microbiological assay with unhydrolysed plant extracts since the bound amino acids can influence the behaviour of the micro-organism used.

## EXPERIMENTAL

Pollens from the following tribes of the family *Gramineae* were investigated:

| | | |
|---|---|---|
| *Festuceae*: | *Festuca pratensis* Huds. | (Meadow fescue) |
| | *Lolium perenne* L. | (Perennial rye-grass) |
| | *Poa trivialis* L. | (Rough meadow-grass) |
| | *Dactylis glomerata* L. | (Cocksfoot) |
| | *Cynosurus cristatus* L. | (Crested dog's-tail) |
| *Aveneae*: | *Arrhenatherum elatius* (L.) J. & C. Presl | (Tall or false oat grass) |
| | *Holcus lanatus* L. | (Yorkshire fog) |
| | *Anthoxanthum odoratum* L. | (Sweet vernal-grass) |
| *Agrostideae*: | *Agrostis tenuis* Sibth. | (Common bent or brown top) |
| | *Phleum pratense* L. | (Timothy) |
| | *Alopecurus pratensis* L. | (Meadow or common fox-tail) |

The maturing culms were collected and, as soon as possible, the cut ends were placed in long metal troughs containing water. The troughs had sloping sides to allow the flowering heads to hang beyond the edge of the trough and drop their pollen on strips of black, glazed paper. The pollens were shed about 8.30–9.30 a.m. with the exception of *H. lanatus*, which shed its pollen about 4 p.m.

### *Extraction procedure*

The pollen sample (2.0 g) was extracted with successive quantities of petroleum ether (60/80) until the solvent was colourless, filtered, the residue extracted with 50 % glycerol (25 ml) by shaking, at room temperature, on a mechanical shaker for 4 h and filtered.

*Viable pollen.* The pollens were extracted within 2 h[14] of being shed from the rips anthers.

*Non-viable pollen.* The pollens were stored (two–four days) at room temperature over silica gel and extracted when no tube growth was observed in the germination test[14].

### *Thin-layer chromatography*

Essential details are given in Table I.

## RESULTS AND DISCUSSION

The difficulties encountered in the resolution of amino acids in pollen extracts preserved in 50 % glycerol and methods for the identification of amino acids in the presence of glycerol based on the patterns obtained by plotting $hR_F$ values as a function of the pH of the layer or as a function of the sequence of the solvent system used have been reported[16,17]. The latter method has been used in the identification of the free amino acids present in grass pollen extracts. $hR_F$ values of the ninhydrin-

TABLE I

SUMMARY OF EXPERIMENTAL PROCEDURE FOR THIN-LAYER CHROMATOGRAPHY

| | |
|---|---|
| Adsorbent: | Silica Gel G (Merck), 250 $\mu$, air dried overnight. |
| Solvent systems: | (I) 96% Ethanol–water (70:30, v/v).<br>(II) Phenol–water (75:25, w/w; 20 mg NaCN added per 100 g mixture).<br>(III) *n*-Butanol–glacial acetic acid–water (80:20:20, v/v).<br>(IV) 96% Ethanol–water–diethylamine (70:29:1, v/v). |
| Method: | Ascending, in saturated chamber; 20–22°; 15 cm. |
| Load: | Pollen extracts (preserved in 50% glycerol), 2 $\mu$l. Reference amino acids, 2 $\mu$l (1% solutions of the reference compounds dissolved in 50% glycerol). |
| Detection: | The plates were dried at 110° for 10 min and sprayed with modified ninhydrin reagent[16]. After spraying, the plates were further heated to give optimum colour development of the amino acid spots. The position of the glycerol was readily distinguished as a whitish zone on a pinkish-buff background on prolonged heating of the sprayed plate at 110°. |

reacting components resolved in solvent systems I–IV are given in Table II and specimen chromatograms are illustrated in Fig. 1.

Examination of the chromatograms shown in Fig. 1 shows that there are seven ninhydrin-reacting spots resolved in solvent systems I and IV while six are apparently resolved in solvent systems II and III. However, the pink spot with an $hR_F$ value of 23 and 22 in solvent systems II and III respectively, in some instances showed a decided "waist" and these spots were subsequently each resolved into two. Since the free amino acids were similar in all the species examined, both in the viable and non-viable samples, the extracts from *Dactylis glomerata* L. and *Phleum pratense* L. pollens were selected for further investigation in order to identify the amino acids present.

Using the mean $hR_F$ values (shown in Table II) obtained in the four solvent systems the patterns for the unknown amino acids were built up (Fig. 2). After plotting the $hR_F$ values against the solvent system sequence there was no difficulty in joining up the appropriate points for the orange, yellow and pinkish-purple spots. The diagrams so obtained were then traced on tracing paper, using the same scale as for the known amino acids (patterns for 24 amino acids previously published[17]) and then fitted into these patterns. The orange spot corresponded with the shape for glycine, the yellow spot with L-proline and the slow moving pinkish-purple spot with L-lysine monohydrochloride (Fig. 3). Some difficulty, however, was experienced in joining up the points obtained for the four pink coloured spots. By plotting these points on tracing paper and superimposing on the patterns obtained for the known amino acids[17] the following was deduced:

$\alpha$-Alanine corresponded with points (2) or (3), (2) or (3), (1) or (2) and (2) or (3) in solvent systems I, II, III and IV, respectively.

$\gamma$-Amino-*n*-butyric acid corresponded with points (1), (2) or (3), (2) or (3) and (1) in solvent systems I, II, III and IV, respectively.

DL-Serine corresponded with points (2) or (3), (1), (1) or (2) and (2) or (3) in solvent systems I, II, III and IV, respectively.

TABLE II

MEAN $hR_F$ VALUES OF THE NINHYDRIN-REACTING COMPONENTS RESOLVED IN AQUEOUS EXTRACTS OF SOME GRASS POLLENS PRESERVED IN 50% GLYCEROL

V = Extract from viable pollen; N = extract from non-viable pollen.

| *Pollen extract* | | *Mean*[a] *$hR_F$ values in solvent system I* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Yellow*[b] | *Orange*[b] | *Pinkish-purple*[b] | *Pink*[b]*—in order of increasing $hR_F$ value* | | | |
| | | | | | *1* | *2* | *3* | *4* |
| *Anthoxanthum odoratum* L. | V | 30 | 37 | 3 | 25 | 41 | 48 | 53 |
| | N | 27 | 34 | 3 | 25 | 40 | 46 | 52 |
| *Poa trivialis* L. | V | 29 | 35 | 3 | 27 | 40 | 45 | 50 |
| | N | 30 | 34 | 2 | 26 | 41 | 45 | 49 |
| *Dactylis glomerata* L. | V | 30 | 35 | 2 | 26 | 39 | 46 | 49 |
| | N | 29 | 35 | 3 | 26 | 39 | 44 | 54 |
| *Lolium perenne* L. | V | 29 | 34 | 3 | 25 | 40 | 47 | 52 |
| | N | 28 | 35 | 3 | 25 | 41 | 45 | 50 |
| *Alopecurus pratensis* L. | V | 30 | 36 | 2 | 29 | 40 | 44 | 51 |
| | N | 31 | 34 | 3 | 29 | 42 | 44 | 50 |
| *Festuca pratensis* Huds. | V | 31 | 37 | 2 | 29 | 43 | 44 | 51 |
| | N | 30 | 39 | 2 | 28 | 40 | 45 | 54 |
| *Cynosurus cristatus* L. | V | 29 | 36 | 2 | 29 | 42 | 44 | 50 |
| | N | 30 | 36 | 2 | 28 | 39 | 47 | 49 |
| *Arrhenatherum elatius* (L.) J. & C. Presl | V | 32 | 34 | 3 | 28 | 40 | 47 | 49 |
| | N | 30 | 35 | 2 | 27 | 42 | 44 | 52 |
| *Holcus lanatus* L. | V | 31 | 37 | 2 | 28 | 43 | 43 | 49 |
| | N | 30 | 37 | 2 | 29 | 42 | 43 | 50 |
| *Agrostis tenuis* Sibth. | V | 32 | 36 | 2 | 29 | 42 | 43 | 50 |
| | N | 32 | 35 | 2 | 29 | 43 | 44 | 51 |
| *Phleum pratense* L. | V | 37 | 34 | 2 | 27 | 42 | 45 | 54 |
| | N | 35 | 36 | 2 | 29 | 42 | 45 | 52 |
| Mean $hR_F$ values | | 31 | 36 | 2 | 27 | 41 | 45 | 51 |

| *Pollen extract* | | *Mean*[a] *$hR_F$ values in solvent system II* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Yellow*[b] | *Orange*[b] | *Pinkish-purple*[b] | *Pink*[b]*—in order of increasing $hR_F$ value* | | | |
| | | | | | *1* | *2* | *3* | *4* |
| *Anthoxanthum odoratum* L. | V | 42 | 21 | 7 | 16 | 25 | 27 | 52 |
| | N | 42 | 21 | 7 | 16 | 25 | 27 | 51 |
| *Poa trivialis* L. | V | 40 | 20 | 7 | 16 | 24 | 26 | 51 |
| | N | 43 | 21 | 7 | 16 | 25 | 26 | 52 |
| *Dactylis glomerata* L. | V | 42 | 21 | 8 | 15 | 25 | 26 | 50 |
| | N | 42 | 20 | 8 | 16 | 26 | 26 | 51 |
| *Lolium perenne* L. | V | 42 | 20 | 7 | 15 | 24 | 26 | 52 |
| | N | 42 | 20 | 7 | 15 | 25 | 26 | 52 |
| *Alopecurus pratensis* L. | V | 44 | 21 | 7 | 15 | 24 | 26 | 54 |
| | N | 42 | 21 | 7 | 15 | 25 | 25 | 54 |
| *Festuca pratensis* Huds. | V | 43 | 21 | 7 | 16 | 25 | 25 | 53 |
| | N | 42 | 20 | 7 | 16 | 25 | 25 | 52 |
| *Cynosurus cristatus* L. | V | 42 | 20 | 8 | 17 | 25 | 25 | 53 |
| | N | 42 | 20 | 8 | 15 | 26 | 26 | 53 |
| *Arrhenatherum elatius* (L.) J. & C. Presl | V | 42 | 20 | 7 | 17 | 24 | 25 | 52 |
| | N | 42 | 20 | 7 | 16 | 24 | 25 | 52 |
| *Holcus lanatus* L. | V | 40 | 20 | 7 | 16 | 25 | 26 | 51 |
| | N | 40 | 21 | 7 | 16 | 25 | 27 | 50 |
| *Agrostis tenuis* Sibth. | V | 42 | 20 | 7 | 16 | 25 | 27 | 52 |
| | N | 42 | 20 | 7 | 16 | 25 | 27 | 53 |
| *Phleum pratense* L. | V | 43 | 20 | 8 | 15 | 25 | 27 | 54 |
| | N | 43 | 20 | 8 | 15 | 26 | 27 | 52 |
| Mean $hR_F$ values | | 42 | 20 | 7 | 16 | 25 | 26 | 52 |

*(continued on p. 261)*

TABLE II (*continued*)

| *Pollen extract* | | *Mean[a] $hR_F$ values in solvent system III* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Yellow[b]* | *Orange[b]* | *Pinkish-purple[b]* | *Pink[b]—in order of increasing $hR_F$ value* | | | |
| | | | | | *1* | *2* | *3* | *4* |
| *Anthoxanthum odoratum* L. | V | 12 | 17 | 3 | 18 | 20 | 24 | 40 |
| | N | 13 | 17 | 3 | 19 | 22 | 23 | 40 |
| *Poa trivialis* L. | V | 13 | 17 | 3 | 19 | 22 | 24 | 41 |
| | N | 13 | 18 | 4 | 19 | 23 | 24 | 40 |
| *Dactylis glomerata* L. | V | 14 | 18 | 4 | 19 | 22 | 24 | 42 |
| | N | 14 | 17 | 3 | 18 | 22 | 24 | 40 |
| *Lolium perenne* L. | V | 13 | 18 | 3 | 18 | 21 | 25 | 42 |
| | N | 13 | 18 | 3 | 18 | 21 | 24 | 42 |
| *Alopecurus pratensis* L. | V | 12 | 17 | 3 | 18 | 21 | 23 | 42 |
| | N | 13 | 19 | 3 | 18 | 21 | 24 | 41 |
| *Festuca pratensis* Huds. | V | 14 | 19 | 4 | 19 | 21 | 24 | 42 |
| | N | 14 | 18 | 4 | 19 | 21 | 24 | 41 |
| *Cynosurus cristatus* L. | V | 14 | 19 | 4 | 19 | 22 | 24 | 41 |
| | N | 13 | 19 | 4 | 19 | 21 | 24 | 40 |
| *Arrhenatherum elatius* (L.) J. & C. Presl | V | 12 | 17 | 4 | 18 | 22 | 24 | 42 |
| | N | 13 | 18 | 4 | 18 | 22 | 25 | 42 |
| *Holcus lanatus* L. | V | 13 | 18 | 4 | 17 | 22 | 25 | 41 |
| | N | 13 | 18 | 4 | 18 | 21 | 23 | 40 |
| *Agrostis tenuis* Sibth. | V | 12 | 18 | 4 | 18 | 21 | 24 | 40 |
| | N | 13 | 17 | 4 | 17 | 21 | 24 | 41 |
| *Phleum pratense* L. | V | 13 | 18 | 4 | 18 | 21 | 24 | 40 |
| | N | 14 | 18 | 4 | 18 | 21 | 24 | 40 |
| Mean $hR_F$ values | | 13 | 18 | 4 | 18 | 21 | 24 | 41 |

| *Pollen extract* | | *Mean[a] $hR_F$ values in solvent system IV* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Yellow[b]* | *Orange[b]* | *Pinkish-purple[b]* | *Pink[b]—in order of increasing $hR_F$ value* | | | |
| | | | | | *1* | *2* | *3* | *4* |
| *Anthoxanthum odoratum* L. | V | 25 | 30 | 6 | 23 | 38 | 42 | 68 |
| | N | 25 | 30 | 7 | 24 | 28 | 42 | 70 |
| *Poa trivialis* L. | V | 24 | 30 | 7 | 24 | 39 | 41 | 69 |
| | N | 23 | 30 | 7 | 23 | 38 | 42 | 68 |
| *Dactylis glomerata* L. | V | 25 | 32 | 7 | 24 | 40 | 43 | 68 |
| | N | 25 | 33 | 6 | 24 | 40 | 44 | 68 |
| *Lolium perenne* L. | V | 24 | 32 | 7 | 24 | 38 | 44 | 68 |
| | N | 24 | 32 | 7 | 23 | 38 | 44 | 69 |
| *Alopecurus pratensis* L. | V | 24 | 31 | 7 | 24 | 39 | 44 | 69 |
| | N | 24 | 30 | 7 | 24 | 39 | 43 | 69 |
| *Festuca pratensis* Huds. | V | 23 | 31 | 7 | 24 | 39 | 44 | 68 |
| | N | 24 | 31 | 6 | 23 | 39 | 43 | 70 |
| *Cynosurus cristatus* L. | V | 24 | 30 | 7 | 23 | 39 | 43 | 70 |
| | N | 24 | 30 | 7 | 24 | 38 | 43 | 68 |
| *Arrhenatherum elatius* (L.) J. & C. Presl | V | 25 | 30 | 7 | 23 | 39 | 43 | 68 |
| | N | 24 | 31 | 7 | 22 | 38 | 42 | 69 |
| *Holcus lanatus* L. | V | 25 | 30 | 6 | 23 | 38 | 42 | 69 |
| | N | 25 | 30 | 6 | 23 | 38 | 41 | 69 |
| *Agrostis tenuis* Sibth. | V | 23 | 31 | 7 | 22 | 38 | 41 | 68 |
| | N | 23 | 31 | 7 | 22 | 38 | 41 | 68 |
| *Phleum pratense* L. | V | 24 | 32 | 7 | 21 | 39 | 42 | 70 |
| | N | 24 | 31 | 7 | 22 | 38 | 44 | 70 |
| Mean $hR_F$ values | | 24 | 31 | 7 | 23 | 39 | 43 | 69 |

[a] These figures represent the mean of four replicates with the exception of *Dactylis glomerata* L. (viable and non-viable) and *Phleum pratense* L. (viable and non-viable) extracts on which twenty-five replicates were performed.

[b] Colour of spot after heating.

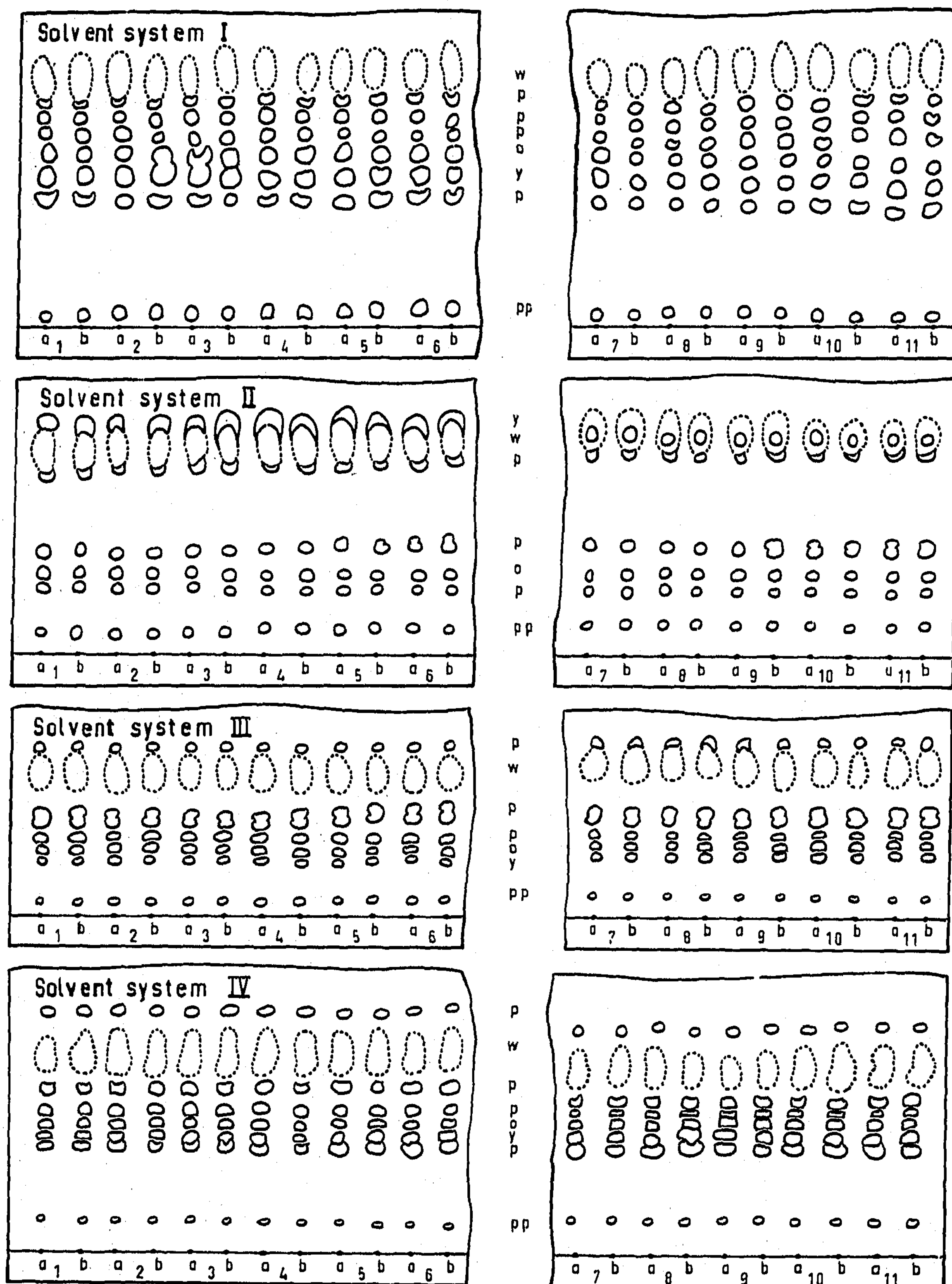


Fig. 1. Tracings of chromatograms of pollen extracts (preserved in 50% glycerol). Layer: silica gel, air dried. Distance: 15 cm. (– – – –) Glycerol; (——) amino acid. The colours obtained on heating the plate, after spraying with ninhydrin reagent[15] are the following: o = orange; p = pink; pp = pinkish-purple; w = white (glycerol); y = yellow. 1 = *Anthoxanthum odoratum* L.; 2 = *Poa trivialis* L.; 3 = *Dactylis glomerata* L.; 4 = *Lolium perenne* L.; 5 = *Alopecurus pratensis* L.; 6 = *Festuca pratensis* Huds.; 7 = *Cynosurus cristatus* L.; 8 = *Arrhenatherum elatus* (L.) J. & C. Presl; 9 = *Holcus lanatus* L.; 10 = *Agrostis tenuis* Sibth.; 11 = *Phleum pratense* L. a = Extract from viable pollen; b = extract from non-viable pollen. Solvent systems: (I) 96% ethanol–water (70:30, v/v); (II) phenol–water (75:25, w/w; 20 mg NaCN added per 100 g mixture); (III) *n*-butanol–glacial acetic acid–water (80:20:20, v/v); (IV) 96% ethanol–water–diethylamine

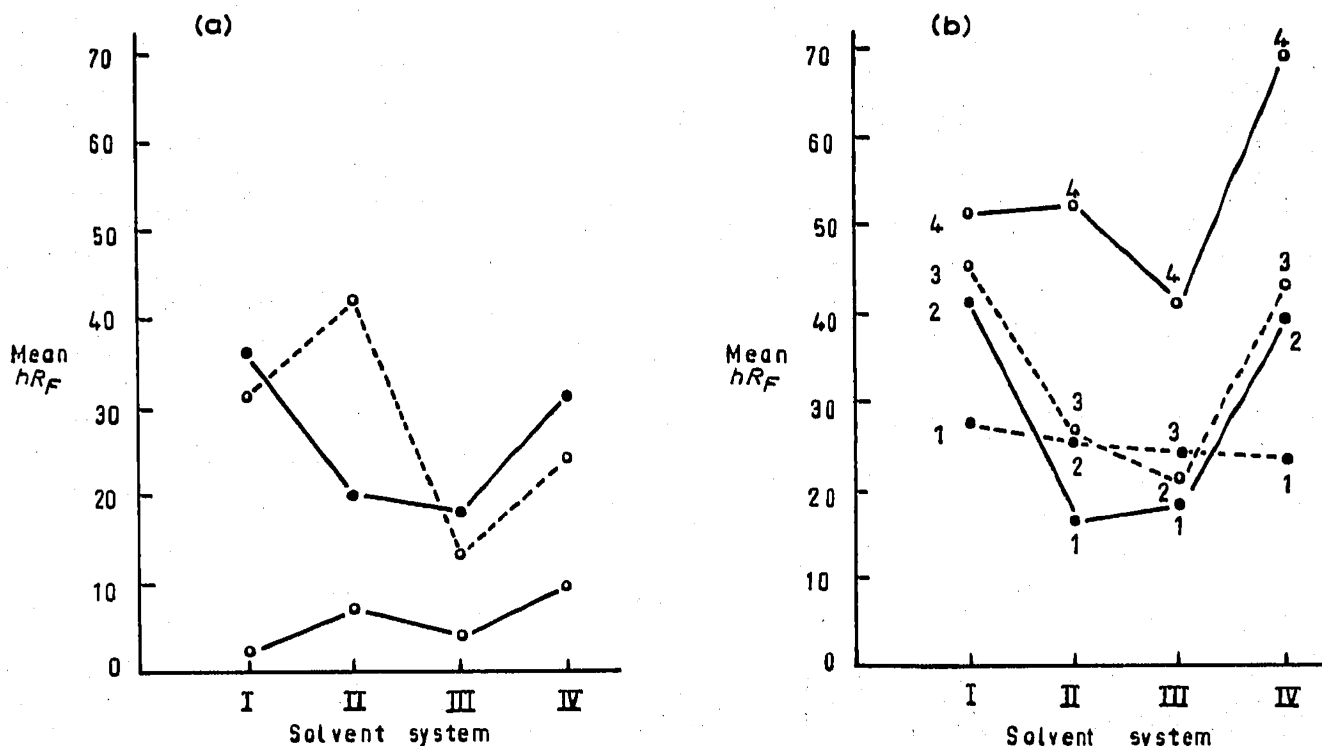


Fig. 2. Mean $hR_F$ values, plotted as a function of the solvent system sequence, for the ninhydrin-reacting components resolved in aqueous extracts of some grass pollens preserved in 50% glycerol. Layer: silica gel, air dried. Distance: 15 cm. (a) Components yielding orange (●——●), pinkish-purple (○——○), and yellow (○----○) spots. (b) Components yeilding pink spots: (○——○) pattern for DL-leucine, (○----○) possible pattern for α-alanine, (●——●) possible pattern for DL-serine, (●----●) possible pattern for γ-amino-*n*-butyric acid. Solvent systems: (I) 96% ethanol–water (70:30, v/v); (II) phenol–water (75:25, w/w; 20 mg NaCN added per 100 g mixture; (III) *n*-butanol–glacial acetic acid–water (80:20:20, v/v); (IV) 96% ethanol–water–diethylamine (70:29:1, v/v).

DL-Leucine corresponded with points (4), (4), (4) and (4) in solvent systems I, II, III and IV, respectively.

(The numbers in parentheses appear against the appropriate point in Fig. 2.)

From this information it was concluded that DL-leucine was almost certainly one of the components in the grass pollen extracts and that α-alanine, γ-amino-*n*-butyric acid and DL-serine accounted for the remaining three (Fig. 3), although with the latter it was not possible to decide precisely which points had to be joined up to get the true pattern. However, by running the *Dactylis* and *Phleum* viable and non-viable pollen extracts, a known mixture (prepared in 50% glycerol) containing glycine, L-proline, L-lysine monohydrochloride, α-alanine, γ-amino-*n*-butyric acid, DL-serine and DL-leucine and the individual amino acids in the mixture, also dissolved in 50% glycerol, on the same plate in the four solvent systems a clear picture was obtained. Fig. 4 shows that the position of the various spots in the grass pollen extracts corresponded both in rate of movement and colour on heating, after spraying with the ninhydrin reagent, with those of the single amino acids mentioned and when they were in admixture. It was concluded, therefore, that the free amino acids present in both viable and non-viable extracts of the pollen of all the species examined were α-alanine, γ-amino-*n*-butyric acid, glycine, DL-leucine, L-lysine, L-proline and DL-serine.

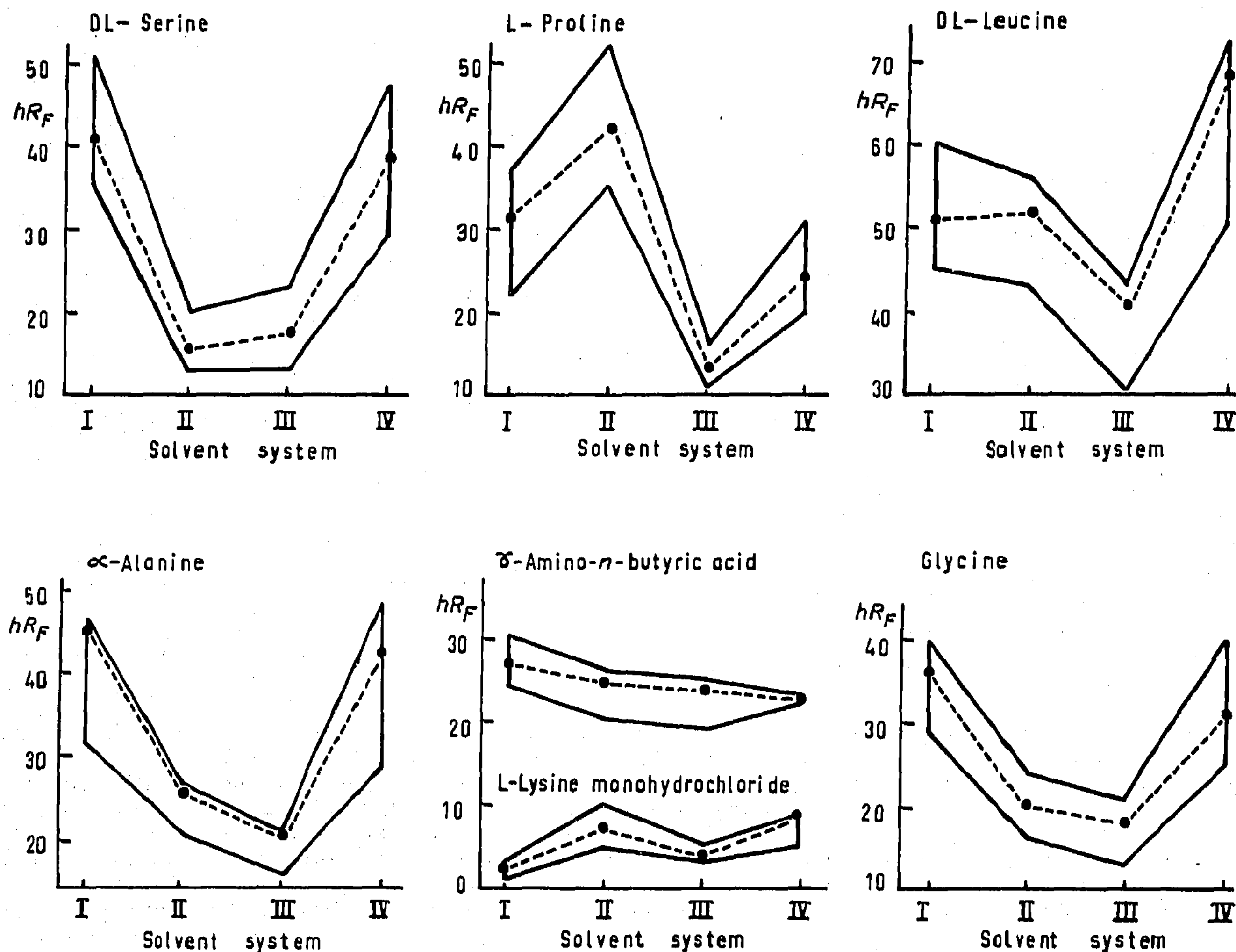


Fig. 3. $hR_F$ patterns for the amino acids resolved in aqueous extracts of some grass pollens. Solvent systems: (I) 96% ethanol–water (70:30, v/v); (II) phenol–water (75:25, w/w; 20 mg NaCN added per 100 g mixture); (III) *n*-butanol–glacial acetic acid–water (80:20:20, v/v); (IV) 96% ethanol–water–diethylamine (70:29:1, v/v).

The presence of γ-amino-*n*-butyric acid is of great interest since it has not previously been reported in any pollen. However, since SYNGE[13] observed that γ-amino-*n*-butyric acid had been found chromatographically in nearly all plant tissues and often represented a substantial fraction of the non-protein nitrogen, the presence of this amino acid in grass pollen is not surprising. Although BATHURST[12], using a microbiological assay technique, reported free valine in four grass pollens, no trace of valine was found in the extracts examined in the course of this work.

With respect to the free amino acid content of the grass pollens examined no species differences were detected and there was no difference between viable and non-viable pollen extracts.

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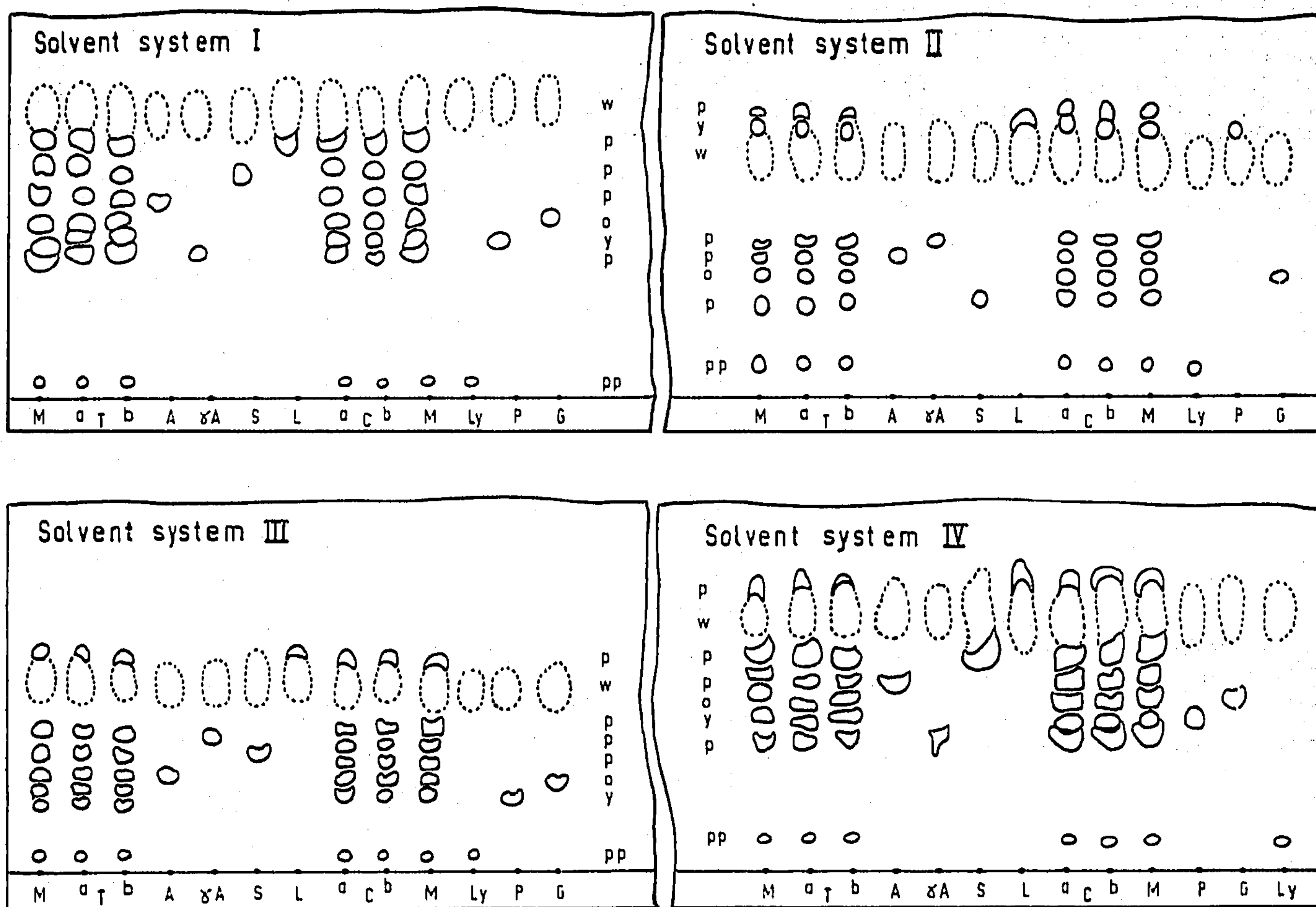


Fig. 4. Tracings of chromatograms of some amino acids and grass pollen extracts preserved in 50% glycerol. Layer: silica gel, air dried. Distance: 15 cm. (– – – –) Glycerol; (——) amino acid. The colours obtained on heating the plate, after spraying with ninhydrin reagent[15] are the following: o = orange; p = pink; pp = pinkish-purple; w = white (glycerol); y = yellow. A = α-Alanine; γA = γ-amino-*n*-butyric acid; S = DL-serine; L = DL-leucine; Ly = L-lysine monohydrochloride; P = L-proline; G = glycine; M = mixture of the reference amino acids. All the amino acids were dissolved in 50% glycerol. T = *Phleum pratense* L.; C = *Dactylis glomerata* L. a = Extract from viable pollen; b = extract from non-viable pollen. Solvent systems: (I) 96% ethanol–water (70:30, v/v); (II) phenol–water (75:25, w/w, 20 mg NaCN added per 100 g mixture); (III) *n*-butanol–glacial acetic acid–water (80:20:20, v/v); (IV) 96% ethanol–water–diethylamine (70:29:1, v/v).

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