CHROM. 5855

AN IMPROVED TECHNIQUE FOR THE ANALYSIS OF AMINO ACIDS AND RELATED COMPOUNDS ON THIN LAYERS OF CELLULOSE

VI. THE CHARACTERIZATION OF SMALL PEPTIDES BY THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

J. G. HEATHCOTE, R. J. WASHINGTON, B. J. KEOGH AND R. W. GLANVILLE
The University, Salford (Great Britain)
(Received October 25th, 1971)

SUMMARY

The autoanalyzer technique for the detection of amino acids by ion-exchange chromatography relies entirely on the time of elution of the respective peaks. This may result in confusion between amino acids and peptides in biological fluids. Such ambiguities may be removed by the simultaneous use of thin-layer chromatography. Relative chromatographic mobilities of some small peptides have been studied in both systems and some advances in the detection and characterization of these compounds have been made.

INTRODUCTION

The presence of small peptides in urine and other biological fluids presents many problems of identification in the analysis of amino acids and related compounds by the automatic ion-exchange method[1–6]. Thus many peptides give a low colour yield with ninhydrin and could be present in such fluids in relatively high concentration without detection at the wavelengths normally used (570 and 440 nm). Furthermore, because of the nature of the automatic method which relies entirely on the time of elution of the peak for characterization, compounds which are eluted from the column after the same time interval are likely to be identified incorrectly. HAMILTON[2] has reported several cases of confusion which have been caused by such coincidence of elution times between amino acids and peptides.

One way of overcoming these problems is to use a second separation technique at the same time which is based on a different physico-chemical principle, such as thin-layer chromatography (TLC). Cellulose thin layers have proved to be very useful for the separation of numerous simple nitrogenous compounds[7–9]. Furthermore the incorporation of a transition metal salt such as cadmium acetate into the ninhydrin reagent, which enhances sensitivity on TLC, also gives a different set of colour responses from those used in the automatic procedure. In this way, errors of identification may be prevented which could readily occur if either method were used alone[10].

We have examined a number of small peptides, chiefly dipeptides, simultaneously by both the automatic ion-exchange (Technicon) method and by TLC on cellulose in order to characterize such compounds unambiguously.

EXPERIMENTAL

Materials and equipment

Chromatographic equipment. The TLC equipment, glass tanks and 1 μl "microcaps" capillary pipettes were supplied by Shandon*. The automatic ion-exchange chromatographic system for the analysis of amino acids (Technicon** Autoanalyzer Model NC-1) was used throughout the work and the column was filled with "Chromobeads" (8 % cross linked ion-exchange resin "Type B").

Densitometer. The instrument used was the Joyce Loebl "Chromoscan"***, a double beam densitometer, with thin-layer attachment.

Cellulose powder. The cellulose powder used in this investigation was MN300 (without binder)§. Before use, it was washed by the technique described previously in Part I of this series[7].

Solvent for chromatographic development§§. The 2-methylbutanol-2 was of G.P.R. grade and the butanone and propanone were of M.F.C. grade. All other solvents were of "Analar" grade.

Detection reagents. Ninhydrin cadmium acetate (0.2 % w/v) reagent was used for the detection and estimation of amino acids and peptides on TLC. The reagent was prepared as previously described[8]. The ninhydrin reagent used in the Technicon Autoanalyzer was formulated in accordance with the operating manual.

Peptides and amino acids. Stock solutions (0.025 *M*) of the common amino acids and of the peptides for TLC were prepared in aqueous 2-propanol (10 % v/v) and these were kept refrigerated when not in use.

The dipeptides and tripeptides were obtained commercially§§§. Except for the optically inactive glycyl peptides, and also DL-alanyl–DL-alanine and DL-alanyl-DL-serine, all the peptides listed in Table I contained amino acids of the L-configuration.

The majority of the peptides were found to consist of a single component when each was examined by TLC and by ion-exchange analysis. However, the following five peptides: ser–gly, gly–ser, glu–ala, leu–ala, and gly–gly were contaminated with small amounts (up to 5 % w/w) of their constituent amino acids. One peptide, ala–ser, was found to contain 30 % (w/w) of alanine and serine. Control experiments showed that no hydrolysis of alanylserine occurred under the experimental conditions employed. The amounts of peptide applied to the Autoanalyzer had to be determined individually by control experiments.

Preparation of the cellulose layers. The purified cellulose powder (15 g) was spread as a slurry over five plates (20 × 20 cm) at an initial thickness of 400 μ. The coated plates were allowed to dry overnight in a horizontal position before use[7,8].

---

* Shandon Scientific Co. Ltd., 65 Pound Lane, London N.W.10, Great Britain.

** Technicon Instruments Co. Ltd., Hamilton Close, Houndmills, Basingstoke, Hants., Great Britain.

*** Joyce Loebl & Co. Ltd., Gateshead-on-Tyne, Great Britain.

§ Macherey, Nagel & Co. Ltd., Agents Camlab (Glass) Ltd., Cambridge, Great Britain.

§§ Hopkins and Williams Ltd., Freshwater Road, Chadwell Heath, Essex, Great Britain.

§§§ Sigma (London) Chemical Co. Ltd., 12 Lettice St., London S.W.6, Great Britain.

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF PEPTIDES AND AMINO ACIDS ON THIN LAYERS OF CELLULOSE

Peptide		*Colour of complex*		$R_F \times 100$		*Maximum colour yield*		
		Initial	*Final*	*Solvent No. 1*	*Solvent No. 2*	*Area* ($mm^2/\mu mole$) $\times 10^{-4}$		*Area at 490 / Area at 405*
						405 nm	*490 nm*	
Ala–ala	P1	Orange	Red	65	26	3.2	5.0	1.6
Ala–asp	P2	Orange	Red	56	1	2.2	4.4	2.0
Ala–glu	P3	Orange	Red	64	5	3.1	4.4	1.4
Ala–gly	P4	Orange	Red	30	17	3.4	5.2	1.5
Ala–gly–gly	P5	Orange	Red	47	13	3.4	4.2	1.2
Ala–phe	P6	Orange	Red	94	52	3.3	6.1	1.8
Ala–pro	P7	Orange	Orange-red	61	24	0.9	2.6	2.9
Ala–ser	P8	Orange	Red	52	17	2.9	3.0	1.0
Glu–ala	P9	Red	Red	68	1	3.2	4.7	1.5
Gly–ala	P10	Yellow	Orange	52	18	2.7	1.6	0.6
Gly–asp	P11	Yellow	Orange	43	0	2.9	1.5	0.5
Gly–gly	P12	Yellow	Orange	34	13	3.6	1.6	0.4
Gly–gly–gly	P13	Yellow	Orange	32	8	3.2	1.5	0.5
Gly–ile	P14	Yellow	Orange	81	49	1.8	0.4	0.2
Gly–leu	P15	Yellow	Orange	82	51	3.9	1.4	0.4
Gly–lys	P16	Yellow	Orange	14	11	3.5	2.2	0.6
Gly–pro	P17	Bright yellow	Yellow	47	17	3.4	0.8	0.2
Gly–ser	P18	Yellow	Orange	32	13	3.4	1.4	0.4
Gly–tyr	P19	Yellow	Orange	68	28	2.8	1.2	0.4
Gly–val	P20	Yellow	Orange	72	36	2.8	1.2	0.4
Leu–ala	P21	Red	Red	97	58	2.9	5.1	1.8
Leu–gly	P22	Red	Red	82	52	2.9	5.6	1.9
Leu–val	P23	Red	Red	100	77	3.1	5.0	1.6
Pro–gly	P24	Yellow	Mauve	58	24	0.2	0.3	1.5
Ser–gly	P25	Orange	Orange	43	16	2.5	1.7	0.7
Val–ala	P26	Pink	Pink	80	49	0.4	1.7	0.4
Val–gly	P27	Pink	Pink	67	38	0.2	0.9	0.4
Val–gly–gly	P28	Pink	Pink	65	28	0.4	1.3	3.2
Val–his	P29	Pink	Pink	23	19	1.3	3.8	2.9
Val–leu	P30	Pink	Pink	100	80	1.5	3.9	2.6
Val–met	P31	Pink	Pink	90	61	1.0	2.4	2.4
Val–phe	P32	Pink	Pink	96	67	1.0	3.3	3.3
Val–pro	P33	Pink	Pink	69	42	0.2	0.7	3.5
Val–ser	P34	Pink	Pink	68	34	0.5	1.6	3.2
Val–tyr	P35	Pink	Pink	90	57	1.0	3.7	3.7
Val–tyr–val	P36	Pink	Pink	96	73	0.3	2.5	8.3
Val–trp	P37	Pink	Pink	95	67	0.8	3.0	3.8
Val–val	P38	Pink	Pink	95	60	3.2	7.7	2.4
Alanine		Red	Red	57	18	3.6	7.4	2.1
Glutamic acid		Red	Red	56	2	3.1	7.8	2.5
Glycine		Orange-red	Orange-red	37	14	1.8	4.2	2.3
Histidine		Red	Red	11	21	1.6	4.0	2.5
Leucine		Red	Red	90	55	4.1	8.4	2.0
Lysine		Red	Red	16	13	4.2	8.5	2.0
Proline		Yellow	Yellow	58	24	1.8	1.1	0.6
Valine		Red	Red	79	35	3.4	7.4	2.2

Chromatographic solvent systems. The solvent systems of HEATHCOTE AND HAWORTH[8] were used for the separation of the peptides on TLC. These were, for the first dimension (Solvent No. 1): 2-propanol–butanone–1 *N* hydrochloric acid (60:15:25, v/v) and, for development in the second dimension (Solvent No. 2): 2-methylbutanol-2–butanone–propanone–methanol–water–(0.88) ammonia (50:20:10:5:15:5, v/v).

Ion-exchange chromatography. Sodium citrate buffers of pH 2.875, 3.80, 5.00 were prepared as described in the Technicon manual[3] for use at a flow rate of 30 ml/h over a total elution time of about 19 h.

Methods

Application of samples and development of plates. The conditions given previously[7–9] were followed. After application of the sample solution (1 μl) (equivalent to 2.5×10^{-2} μmoles), development with Solvent No. 1 (100 ml) was allowed to proceed until the solvent front had travelled 13 cm from the origin. The plate was removed, dried in a stream of cool air for at least 15 min and then heated in a convection oven at 60° for 15 min to remove final traces of hydrogen chloride. The plate was then cooled before development in Solvent No. 2 (170 ml) in a direction at right angles to the first dimension. After development, the solvent was removed by heating in a convection oven at 60° for 15 min. The plate was finally cooled in a current of cold air before spraying.

Detection of the peptides. The plates were sprayed with the cadmium acetate–ninhydrin reagent until they appeared translucent. After heating at 60° for 30 min and cooling, the initial colour of each peptide complex and the R_F value were noted. Estimations of the final colour of the complex were made after allowing the sprayed plates to stand overnight in an ammonia-free atmosphere away from light. This step was necessary because the final colour and maximum colour yield of the ninhydrin–peptide complexes were not attained until after some 18 h.

Densitometry. The instrument was used with slit No. 1005 (10 × 0.5 mm) and a gear ratio of 1:2 to scan each coloured complex after maximum colour intensity had been attained. The filters which had nominal wavelengths of 490 and 405 nm were chosen to give maximum response to the red and yellow-orange peptide complexes, respectively. The area (mm^2) under the densitometric curve was obtained for each wavelength from the relationship area = peak height × width at half height. The colour yield of each peptide was expressed as the calculated area (mm^2) per μmole of peptide. The ratio of the colour yield at 490 nm to that at 405 nm was also recorded for each peptide.

Ion-exchange chromatography. The standard Technicon Autoanalyzer was used with interference filters of wavelengths 570 and 440 nm. After preliminary trials the optimum loading of the column for each peptide (about 0.5 μmole per ml) was determined and an appropriate amount of the peptide was dissolved in 0.1 *N* hydrochloric acid; 1 ml of this solution was applied to the column. Norleucine could not be used as an internal standard because its peak obscured those of several peptides. Accordingly the amino acids aspartic acid (0.1 μmole) and histidine (0.1 μmole) were used instead.

The area under each eluted peak was calculated in arbitrary units as described in the Technicon manual. In order to correct for the slight variations in the areas

of the internal standards from experiment to experiment, the following procedure was adopted: For each experiment, the standard area was taken as half the sum of the aspartic acid and histidine peak areas. The median value of the standard area from all experiments was then taken as being the True Standard Area. All area values were then corrected against this True Standard Area.

In order to avoid the difficulties produced by variations in the peak elution times of the standards, the time interval between the elution peaks of aspartic acid (zero time) and histidine was designated as unity. The time of emergence of each peptide or amino acid peak was given a value on this scale which was known as the retention factor (R_{AH}) defined as follows:

$$R_{AH} = \frac{\text{elution time (min) of peak}}{\text{elution time (min) between standards}}.$$

A change in R_{AH} of 0.03 on the standard Technicon System for the analysis of amino acids corresponds to a time interval of about 20 min.

RESULTS AND DISCUSSION

Thin-layer chromatography

The R_F values and maximum colour yields at 405 and 490 nm are given in Table I for all peptides examined. The corresponding values for several representative amino acids are also included in this table for comparison. A two-dimensional TLC map of the peptide spots is shown in Fig. 1.

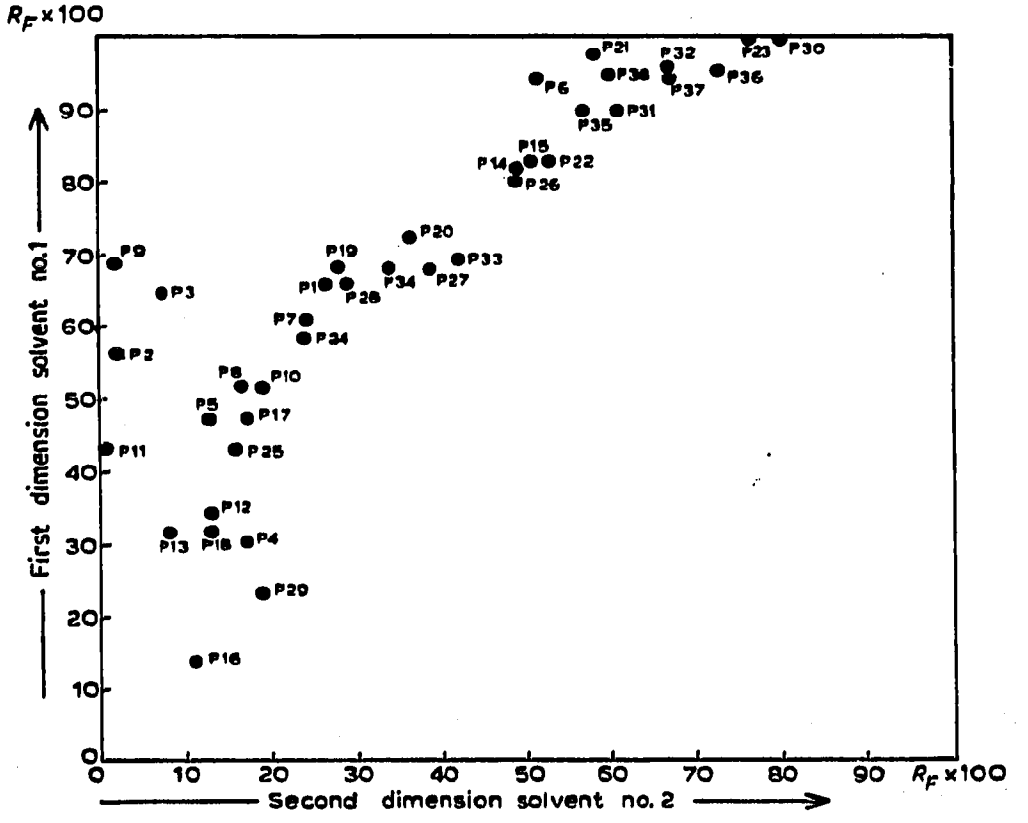

Fig. 1. Map of model peptides after chromatography on thin layers of cellulose.

The R_F values for any series of depeptides of constant N-terminal residue were influenced by the nature of the C-terminal (amino acid) residue. For a constant C-terminal residue, the R_F values tended to increase as the N-terminal amino acid increased in molecular size. This effect which is observed also with simple amino acids is probably due to the increase in solubility in the mobile organic phase which is brought about by the increasingly covalent character of the N-terminal amino acid residue.

The N-terminal amino acid appears to exert an appreciable influence over the observed colour of the cadmium ninhydrin complex formed by a peptide. For example, all the peptides with a glycyl N-terminal residue were found to give a yellow colour similar to proline with the detection reagent. Again, when the positions of the N-terminal and C-terminal residues are reversed in a dipeptide, the observed colour is determined by the nature of the new N-terminal amino acid residue. (The peptides containing proline seem to be an exception here (see Table II).)

The magnitude of the colour yield also seems to be influenced by the N-terminal residue. In the case of the N-terminal valyl peptides the colour yields are so low that such peptides would not be easily detected in routine TLC analysis of biological fluids.

TABLE II

INFLUENCE OF N-TERMINAL AMINO ACID RESIDUE ON THE COLOUR OF THE CADMIUM NINHYDRIN COMPLEX

Peptide	*Colour*	*Peptide*	*Colour*
Gly–ala (P10)	Yellow	Ala–gly (P4)	Red
Gly–val (P20)	Yellow	Val–gly (P27)	Red
Gly–leu (P15)	Yellow	Leu–gly (P22)	Red
Gly–ser (P18)	Yellow	Ser–gly (P25)	Orange
Gly–pro (P17)	Yellow	Pro–gly (P24)	Mauve

Ion-exchange analysis

Table III shows the retention factor R_{AH} and colour yield for each peptide at the standard (Technicon) wavelengths of 440 and 570 nm. The corresponding values for many common amino acids are included for comparison and a diagram showing the relative positions of the eluted peaks is given in Fig. 2.

The order of elution of the dipeptides from the column was almost invariably governed by the C-terminal residue. Thus for the series of dipeptides with N-terminal glycine, the order of elution is the same as that for the C-terminal amino acids aspartic acid, glycine, alanine, valine, tyrosine and lysine. The peptides glycyl serine and glycylproline occupy unexpected positions.

All the peptides examined were retained on the column far longer than the amino acid corresponding to the C-terminal amino acid, and an increasing retention occurred with increasing complexity of the molecule and especially of the N-terminal residue.

The colour yields of the valyl peptides are very low and column loadings in excess of 0.5 μmole were needed to obtain satisfactory peaks. Such peptides would not be readily detected in the routine analysis of a biological fluid. The dipeptides prolylglycine and alanylproline do not have any detectable absorption at wavelengths of

TABLE III

ELUTION PATTERN OF AMINO ACIDS AND PEPTIDES ON ION-EXCHANGE RESIN (TECHNICON)

Peptide or amino acid		$R_{AH} \times 100$	*Column loading (μmoles)*	*Peak area*[b]		*Colour yield* (*Area per μmole*)		*Area at 570 / Area at 440*
				440 nm	*570 nm*	*440 nm*	*570 nm*	
Aspartic acid		0	0.10	1.2	7.4	12.0	74.0	6.2
Threonine		3.4	0.10	1.3	5.8	13.0	58.0	4.5
Serine		4.2	0.10	1.3	6.3	13.0	63.0	4.8
Glutamic acid		10.7	0.10	1.6	8.4	16.0	84.0	5.3
Proline		14.1	0.10	1.5	0.3	15.0	3.0	0.2
Glycine		20.8	0.10	1.4	9.2	14.0	92.0	6.6
Gly–ser	P18	22.2	0.68	2.0	18.4	2.9	27.1	9.3
Alanine		25.3	0.10	1.3	8.0	13.0	80.0	6.2
Ala–asp	P2	25.4	0.50	2.5	16.8	5.0	33.6	6.7
Gly–asp	P11	28.8	0.32	0.3	1.8	5.6	56.0	10.0
Ala–ser	P8	28.9	0.40	1.3	9.8	3.3	24.9	7.6
Gly–gly	P12	30.6	0.51	2.6	23.7	5.1	46.5	9.1
Ser–gly	P25	35.3	0.50	3.2	29.0	6.4	58.0	9.1
Glu–ala	P9	37.1	0.46	10.1	35.3	22.0	77.0	3.5
Ala–gly–gly	P5	37.7	0.50	1.5	9.4	3.0	18.8	6.3
Valine		38.8	0.10	1.4	7.9	14.0	79.0	5.6
Ala–glu	P3	40.7	0.50	2.6	16.5	5.2	33.0	6.4
Ala–gly	P4	44.0	0.50	3.7	25.2	7.4	50.4	6.8
Cystine		44.5	0.10	1.6	4.1	16.0	41.0	2.6
Val–ser	P34	45.5	0.50	0.2	1.2	0.4	2.4	6.0
Methionine		46.7	0.10	1.3	8.4	13.0	84.0	6.5
Ala–ala	P1	47.9	0.37	2.0	16.5	5.4	44.6	8.3
Gly–gly–gly	P13	48.2	0.50	2.6	18.4	5.2	36.8	7.1
Gly–ala	P10	48.5	0.40	2.3	29.5	5.8	74.0	12.8
Val–gly	P27	54.1	0.50	0.6	2.7	1.2	5.4	4.5
Isoleucine		54.6	0.10	0.9	7.9	9.0	79.0	8.8
Val–ala	P26	55.3	0.85	0.2	1.2	0.2	1.4	7.0
Val–gly–gly	P28	57.4	0.50	0.1	0.6	0.2	1.2	6.0
Leucine		57.5	0.10	1.3	8.3	13.0	83.0	6.4
Gly–val	P20	58.9	0.46	3.6	30.5	7.8	66.5	8.5
Leu–ala	P21	59.2	0.50	1.3	9.6	2.6	19.2	7.4
Gly–pro	P17	62.3	0.46	4.1	34.5	8.9	75.0	8.4
Pro–gly[a]	P24	—	2.00	—	—	—	—	—
Ala–pro[a]	P7	—	2.00	—	—	—	—	—
Tyrosine		63.5	0.10	1.8	8.3	18.0	83.0	4.6
Val–pro	P33	65.8	0.50	4.0	21.7	8.0	43.4	5.4
Phenylalanine		66.3	0.10	1.3	8.0	13.0	80.0	6.2
Leu–val	P23	66.5	0.50	2.5	8.0	5.0	16.0	3.2
Val–val	P38	67.0	0.50	0.3	1.3	0.6	2.6	4.3
Leu–gly	P22	70.2	0.53	4.3	31.2	8.1	59.0	7.3
Val–ala	P26	70.5	0.50	0.4	2.4	0.8	4.8	6.0
Gly–ile	P14	71.6	0.53	5.1	36.8	9.6	69.5	7.2
Gly–leu	P15	72.4	0.48	4.4	37.4	9.2	78.0	8.5
Val–met	P31	72.4	0.50	0.5	2.0	1.0	4.0	4.0
Val–leu	P30	73.8	1.00	1.2	6.5	1.2	6.5	5.4
Gly–tyr	P19	84.1	0.50	2.8	24.4	5.6	48.8	8.7
Val–tyr–val	P36	84.7	1.00	1.0	6.5	1.0	6.5	6.5
Val–tyr	P35	86.0	0.50	0.3	1.4	0.6	2.8	4.7
Ala–phe	P6	87.0	0.50	4.4	31.5	8.8	63.0	7.2
Val–phe	P32	90.0	0.50	0.4	2.1	0.8	4.2	5.3
Lysine		95.0	0.10	2.1	9.5	21.0	95.0	4.5
Histidine		100.0	0.10	1.6	8.7	16.0	87.0	5.4
Val–his	P29	112.5	0.50	0.4	2.4	0.8	4.8	6.0
Gly–lys	P16	114.0	0.50	4.1	34.6	8.2	69.2	8.4
Val–trp	P37	121.0	0.50	0.2	1.1	0.4	2.2	5.5
Arginine		122.0	0.10	1.3	7.7	13.0	77.0	5.9

[a] Not observed at a column loading of 2 μmoles.
[b] "True Standard Area" at 570 nm was 8.05.

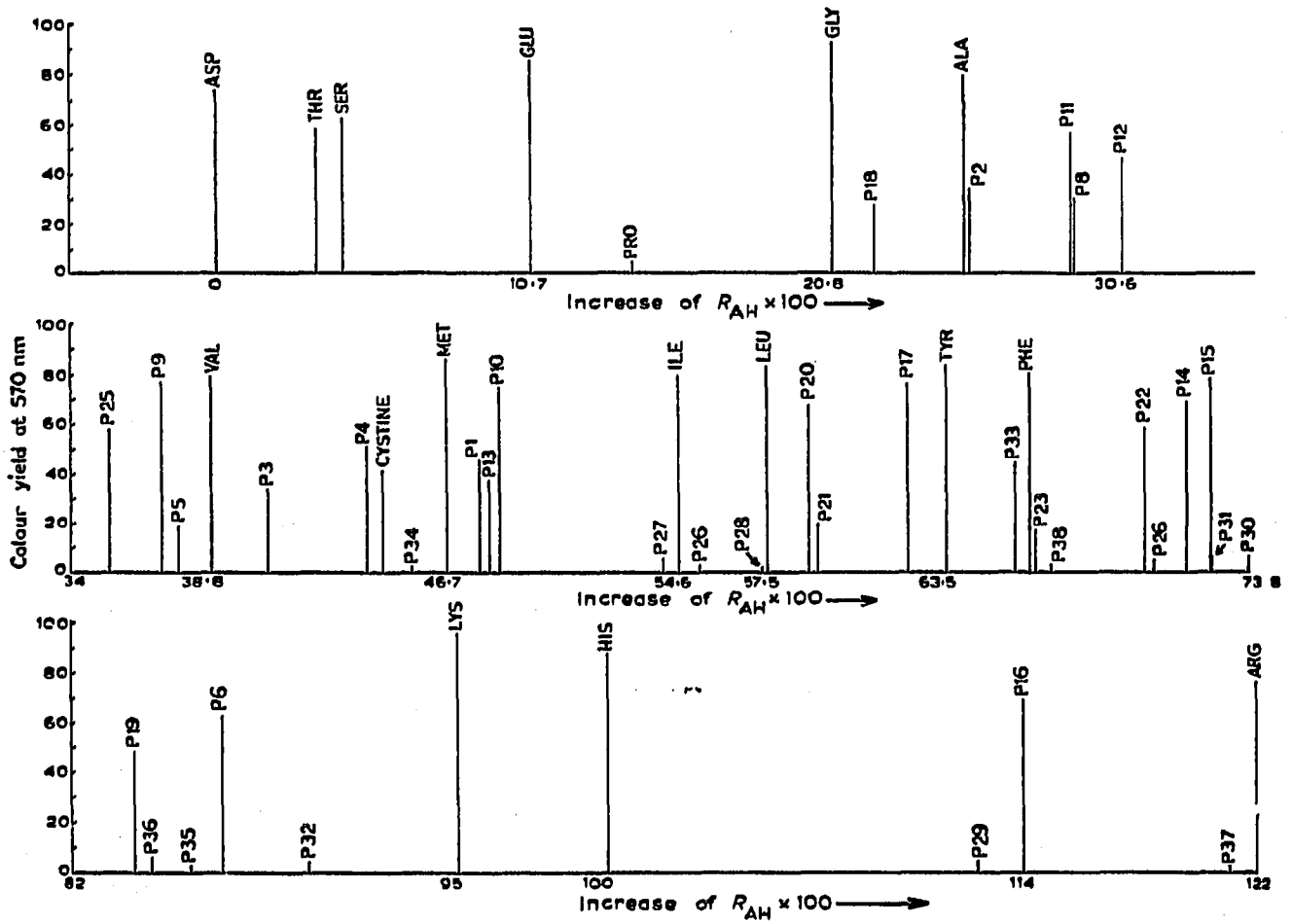

Fig. 2. Colour yield and position of elution of peptides on ion-exchange chromatography. Instrument, Technicon Autoanalyzer; the resin (Type B) and buffer conditions used were the same as for normal amino acid analysis.

440 and 570 nm and consequently no position of elution can be recorded. In contrast tc alanylproline, valylproline has an appreciable colour.

It will be seen from Table III and also Fig. 2 that many peptides have elution positions which coincide with the more common amino acids and these could readily be mistaken for the latter without the corrective adjunct of TLC examination.

The dominant role of the N-terminal amino acid in determining the colour, nature and intensity of the cadmium ninhydrin complex on TLC is not so evident in ion-exchange chromatography where ninhydrin alone is used. It is not known which factors other than the metal ion play a part in the peculiar colour formations observed on TLC but, undoubtedly, the variations in colour given by peptides could be useful pointers to sequence structure in simple peptides. R_F values alone are insufficient to characterize peptides and formulae designed to predict molecular structure by determining small incremental changes in these values are notoriously unreliable. However, R_F values could be valuable guides to structure if taken in conjunction with the colour of complex and the retention factor R_{AH} in a well-tried ion-exchange system. The resolution achieved by the amino acid autoanalyzer was probably better for the valyl and leucyl peptides than that given by TLC, where these peptides have high R_F values in both solvent systems. It is concluded that a combination of TLC

and of ion-exchange analysis is extremely useful for the detection and estimation of small unknown peptides.

REFERENCES

1 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
2 P. B. HAMILTON, *Anal. Chem.*, 35 (1963) 2055.
3 P. B. HAMILTON, *Techniques in Amino Acid Analysis*, Technicon International Division, S.A., Geneva, 1966, p. 17.
4 S. JACOBS, *Analyst (London)*, 95 (1970) 370.
5 *5th Colloquium in Amino Acid Analysis*, Technicon Monograph, No. 2, 1967.
6 G. ERTINGSHAUSEN, H. J. ADLER AND A. S. REICHLER, *J. Chromatogr.*, 42 (1969) 355.
7 C. HAWORTH AND J. G. HEATHCOTE, *J. Chromatogr.*, 41 (1969) 380.
8 J. G. HEATHCOTE AND C. HAWORTH, *J. Chromatogr.*, 43 (1969) 84.
9 J. G. HEATHCOTE, R. J. WASHINGTON, C. HAWORTH AND S. BELL, *J. Chromatogr.*, 51 (1970) 267.
10 J. KOWALSZYK, B. STASINSKA AND J. JANICKI, *Anal. Biochem.*, 41 (1971) 297.