A sensitive semi-quantitative method for amino-acid analysis

Methods have been developed for the analysis of hydrolysates of peptides, available in only 0.01 to 0.1 μmole amounts, using high-voltage electrophoresis (HVE) on filter paper followed by quantitative reaction of the separated amino acids with ninhydrin[1,2]. The technique has the disadvantage that certain groups of amino acids cannot be resolved, and although improved resolution may be obtained by HVE at two different pHs the procedure requires double the amount of peptide and ambiguities still occur. This difficulty has been overcome to some extent by the use of two-dimensional systems employing ionophoresis in one direction followed by either ionophoresis at a different pH[3-5] or by paper chromatography[6], in a direction at right angles. Such procedures suffer in comparison with the single-dimensional techniques in that they are less sensitive and more time-consuming. This paper describes a small-scale two-dimensional method that allows simultaneous fractionation of ten peptide hydrolysates in 3 h. Subsequent reaction with ninhydrin provides semi-quantitative analysis requiring only 2 to 20 mμmoles of peptide.

Experimental

A piece, 114 cm long, was cut from a roll of Whatman No. 3 MM filter paper 6 in. wide, and placed on a clean sheet of polythene 48 in. long by 8 in. wide. The paper was marked symmetrically with six pencilled lines at right angles to its length to delineate five rectangles 7 in. by 6 in. Five points were marked, 1.5 cm from one long edge of the paper and 4.5 cm from the right-hand (anode) edge of each rectangle, with the identifications of the hydrolysates to be applied alongside them. The paper was then impregnated, as described by ATFIELD AND MORRIS[2], with an aqueous buffer at pH 1.85 containing 74.25 ml of glacial acetic acid and 21.0 ml of 90% formic acid per litre. The five hydrolysates, prepared from between 2 and 20 mμmoles of peptide, were applied in volumes less than 1 μl to provide spots sufficiently compact for good resolution. HVE was carried out for 7 min at 20 kV (182 V/cm) and about 100 mA. The HVE apparatus was fitted with water-cooled plates 42 in. long and $6^1/_4$ in. wide, the emergent temperature of the cooling water being 11°. After ionophoresis the paper was dried in a current of air at 40° and cut along the six pencilled lines. The five rectangular pieces of paper so formed were each punched with two $^1/_4$ in. holes set 3 in. apart and $^1/_2$ in. from the edge of the long side opposite the point of application of the hydrolysate, and together with another five sheets previously prepared, assembled on two glass rods on the stainless-steel apparatus shown in Fig. 1.

This apparatus was mounted in a cubic glass tank of side 1 ft., with the two actuating rods passing through two holes in the lid, which were sealed with two corks when the lid was in position. The organic phase of one of the mixtures of solvents shown in Table I was contained in the rectangular glass dish that fitted onto the adjustable metal stands: a second dish containing the aqueous phase was placed beneath the stand on the bottom of the tank. After an appropriate interval for equilibration, during which the two actuating rods were raised to their fullest extent and the bottom edges of the papers about $^1/_2$ in. above the level of the solvent, the actuating rods were depressed until the papers just dipped into the solvent. Development was allowed to proceed until the solvent front reached the top edges of the papers,

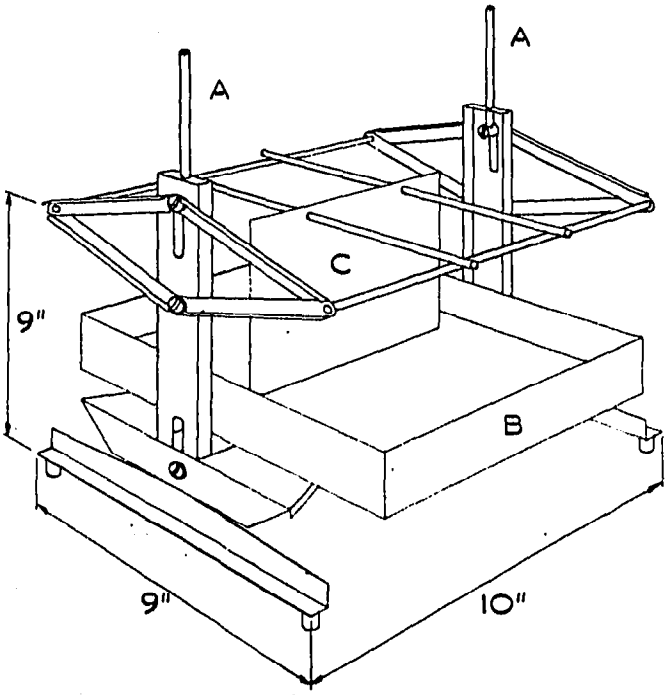


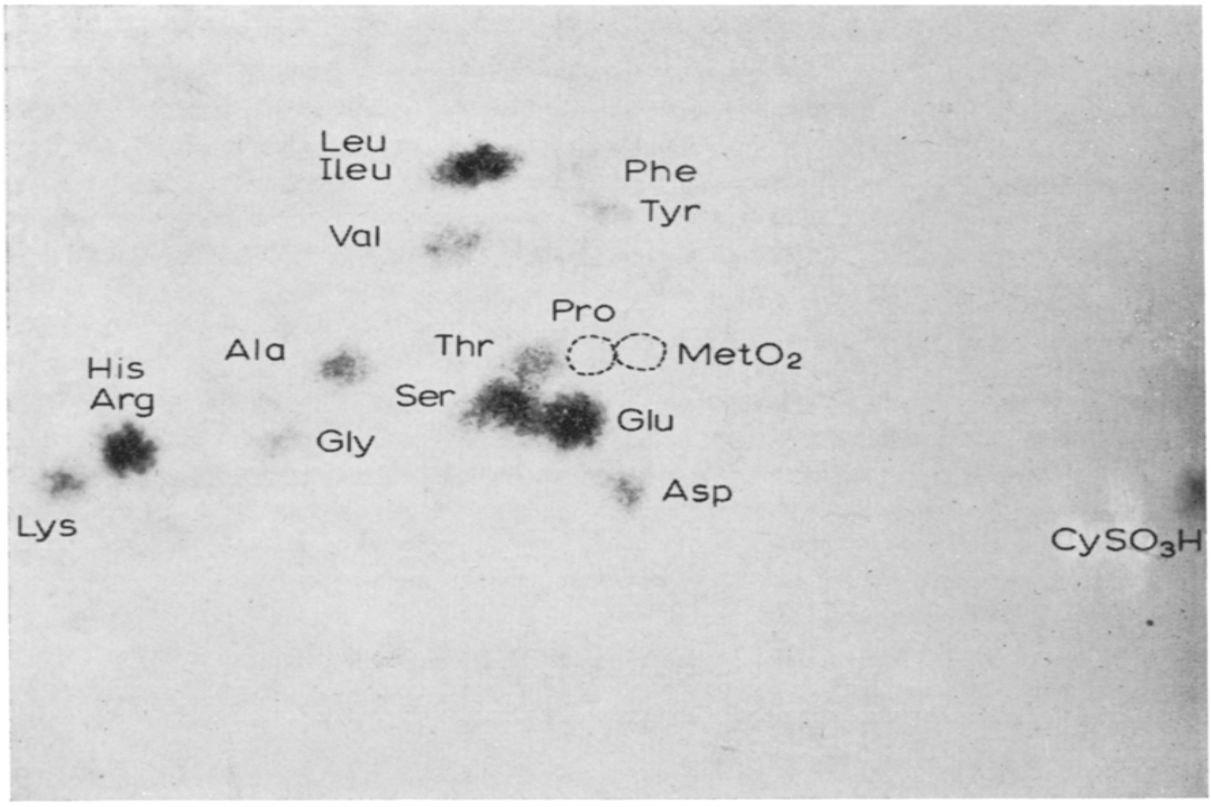
Fig. 1. Stainless-steel chromatogram support. (A) Actuating rods; (B) dish for mobile phase; (C) chromatogram.

usually about $2^1/_2$ h. The papers were then removed, dried in warm air, treated with cadmium–ninhydrin reagent[2,7] and stored overnight in an ammonia-free atmosphere for the colours to develop.

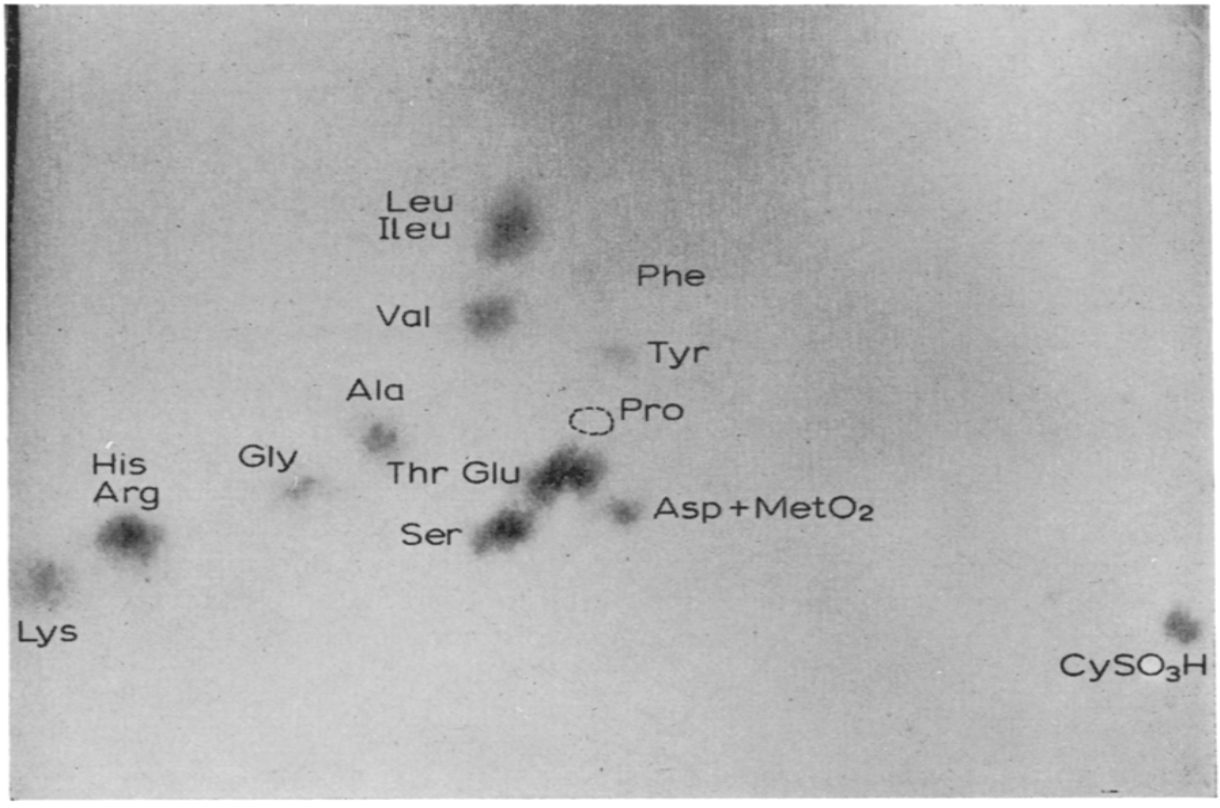
Quantitative measurements of the amounts of amino acids separated were made by cutting out the spots, allowing each of them to stand for 30 min with 1.0 ml of methanol in a small polythene-capped glass vial, and measuring the red colour extracted in micro-cells of 2 cm path-length at 500 mμ (260 mμ in the case of proline). After deduction of the reading given by an equal area of unstained paper, amounts of amino acids were obtained by reference to data obtained with known amounts of controls.

Results

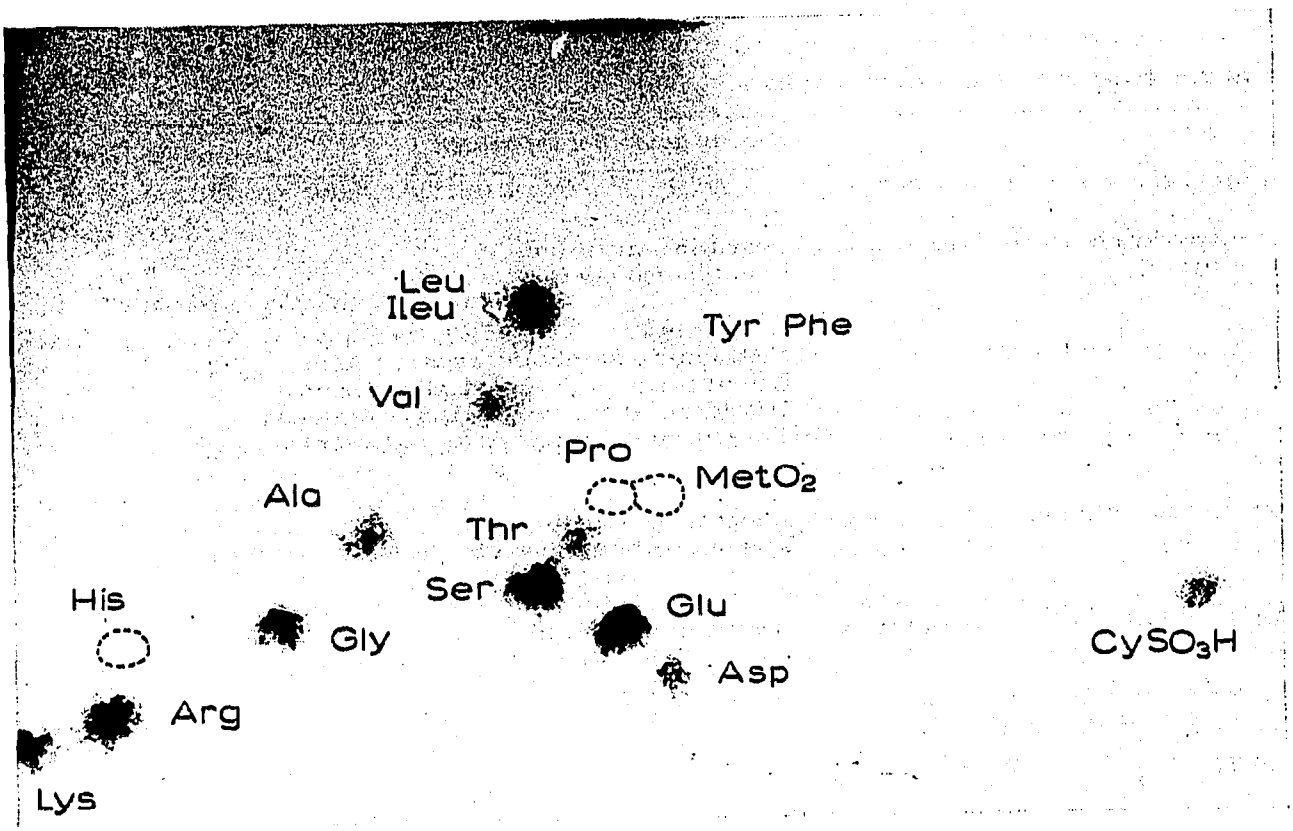
No developing solvent could be found that would fully resolve every amino acid normally found in hydrolysates of oxidised proteins. Table I lists the solvent systems that have been found useful and notes those pairs of amino acids that are not separated. The solvent system D requires a full day for chromatography. A double development with *sec.*-butanol–3 % aqueous NH_3 (5:2, v/v) will separate all amino acids, including isoleucine and leucine, in 7 h, but is unsuitable for quantitative measurements because of the background contamination with residual ammonia. Fig. 2 shows reproductions of actual separations obtained in representative experiments. The position of proline is marked with a dotted line as it is not clearly visible in the photographs. Cystine and methionine have not been included in the investigation because they do not occur as such in oxidised proteins, but according to their known electrophoretic mobilities and R_F values they should be completely resolved in most of the solvent systems quoted. Table II shows the results obtained in the


Leu
Ileu
Phe
Tyr
Val
Pro
Ala
Thr
MetO2
His
Arg
Ser
Glu
Gly
Asp
Lys
CySO3H

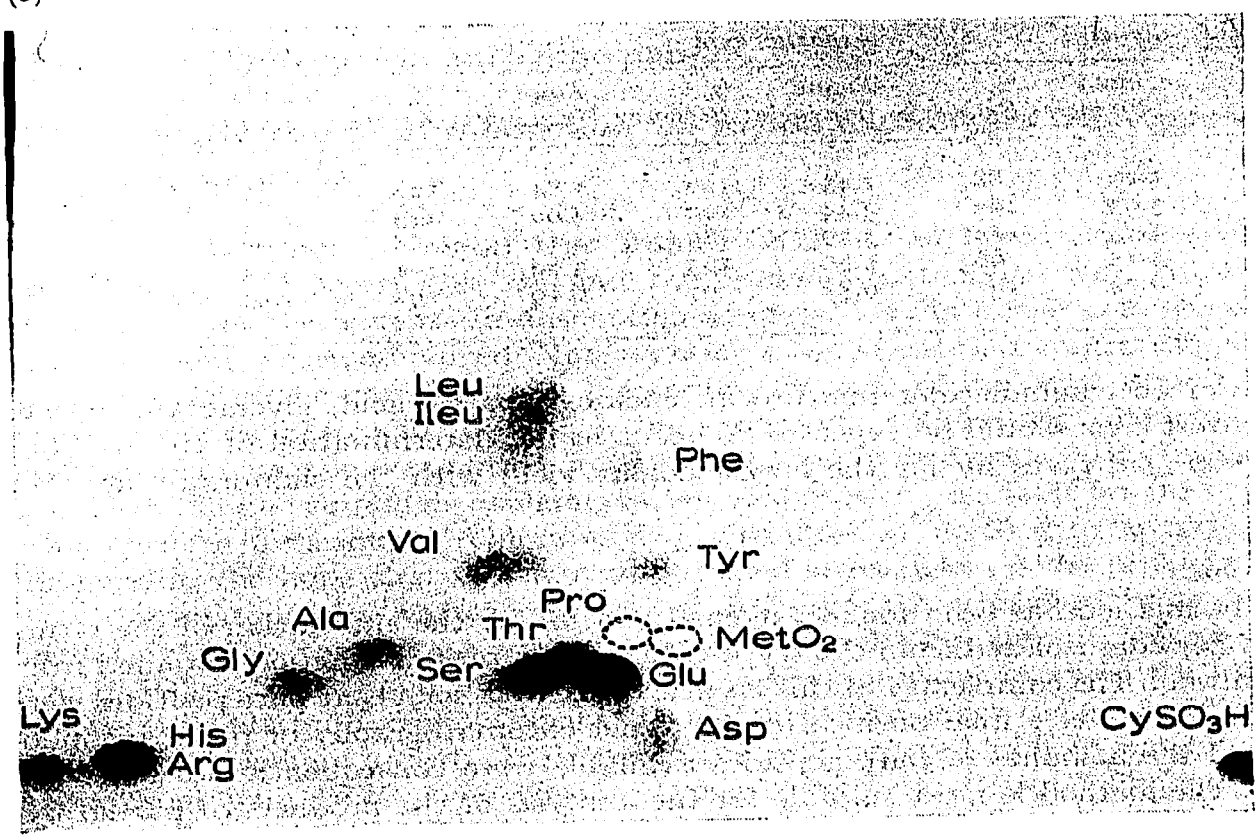
(a)


Leu
Ileu
Phe
Val
Tyr
Ala
Pro
His
Arg
Gly
Thr Glu
Asp+MetO2
Ser
Lys
CySO3H

(b)



(c)



(d)

Fig. 2. Chromatograms obtained after development with the solvents given in Table I. (a) Solvent A; (b) solvent B; (c) solvent C; (d) solvent D.

TABLE I

SOLVENTS FOR PARTITION CHROMATOGRAPHY

Solvent system	*Amino acids not separated*
(A)	
n-Butanol–pyridine–acetic acid–water (15:10:3:12)	Histidine–arginine Leucine–isoleucine
(B)	
n-Butanol–acetic acid–water (4:1:5)	Methionine sulphone–aspartic acid Leucine–isoleucine partially resolved Histidine–arginine partially resolved Threonine–glutamic acid partially resolved
(C)	
2,4/2,5-Lutidine–ethanol–water (11:4:5)	Leucine–isoleucine Tyrosine–phenylalanine partially resolved
(D)	
tert.-Amyl alcohol saturated with water. Double development	Histidine–arginine

TABLE II

SEPARATION AND ESTIMATION OF AN EQUIMOLECULAR MIXTURE OF LEUCINE, ASPARTIC ACID, GLYCINE AND LYSINE

Amino acid applied (*mμ mole*)	*Leucine*		*Aspartic acid*		*Glycine*		*Lysine*	
	Found (*mμ mole*)	% *Recovery*	*Found* (*mμ mole*)	% *Recovery*	*Found* (*mμ mole*)	% *Recovery*	*Found* (*mμ mole*)	% *Recovery*
1.44	1.26	88	1.43	100	1.84	128	1.36	95
2.84	3.03	107	3.44	122	4.06	143	2.69	95
4.23	4.73	112	3.79	89	4.12	98	4.07	96
5.70	5.45	96	5.89	103	5.79	101	5.85	103
6.70	6.74	101	6.51	97	6.19	92	7.53	112

analysis of an equimolecular mixture of leucine, aspartic acid, glycine and lysine. The greatest error would be expected to occur in the determination of glycine since this amino acid gives by far the lowest colour yield with the cadmium-ninhydrin reagent.

Discussion

The method has been found extremely useful for the elucidation of the structures of peptides isolated from the tryptic digest of a protein fraction obtained from oxidised wool. The complexity of this tryptic digest was such that pure peptides were only obtained after a final purification by paper chromatography or paper ionophoresis, and the amounts of pure peptides available were consequently small (< 0.3 μmole). The method is most suitable for the analysis of the products of partial hydrolysis of such a peptide, since a solvent system can usually be found that will separate all its constituent amino acids. The sensitivity and accuracy are good

enough to permit unequivocal determination of the amino-acid composition of small peptides.

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Wool Industries Research Association,
Torridon, Leeds (Great Britain)

M. C. CORFIELD
E. C. SIMPSON

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Chromatography on ion exchange papers

XVI. The adsorption of metal ions on cation exchangers from solutions of sodium perchlorate

The adsorption of metal ions from $HClO_4$ on sulphonic cation exchangers was discussed by NELSON *et al.*[1] and by LEDERER AND SARACINO[2]. No adequate explanation for the increase in adsorption of metal ions in higher concentrations of $HClO_4$ has so far been advanced. NELSON *et al.*[1] pointed out that accurate information for a number of the variables, *e.g.*, electrolyte invasion of the resin, activity coefficients of the metal ions in the supporting electrolyte, was lacking. We have shown[2] that this phenomenon was not confined to sulphonic polystyrene resins but occurred also with cellulose sulphonic exchangers. As no data were available for the behaviour of metal ions in various perchlorates as electrolytes we decided to investigate perchlorates in the hope that the data might shed some light on the problem.

Chromatography was carried out on Amberlite SA-2 resin paper as described previously[2]. The perchlorate solutions used as developing solvent had to contain 1 *N* $HClO_4$ so as to avoid hydrolysis of the metal ions and to avoid the formation of several fronts by demixion during development. Amongst the salts of perchloric acid only the sodium and the barium salt are sufficiently soluble in water to permit comparisons with $HClO_4$ over a wider range of concentrations. Preliminary results with barium perchlorate showed that it changed the equilibria owing to the divalent cation and hence only sodium perchlorate (containing 1 *N* $HClO_4$) could be compared