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The separation of acidic amino acids by an autoanalyser technique

Plants produce a large number of acidic amino acids, whose qualitative and quantitative distribution may have considerable relevance to phytochemical taxonomy of several families of plants. For this reason, we have studied chromatographic methods suitable for their adequate separation and identification. Previous publications have shown the usefulness, and occasionally the limitations, of paper chromatographic[1] and high-voltage paper electrophoretic[2] techniques in separating compounds of this type. Now, we report the separations achieved using a Technicon amino acid autoanalyser following the standard 21 h operating procedure utilizing a 140 × 0.6 cm column packed with type A chromobead ion-exchange resin[3]. The particular advantages of each of the three separation techniques are assessed.

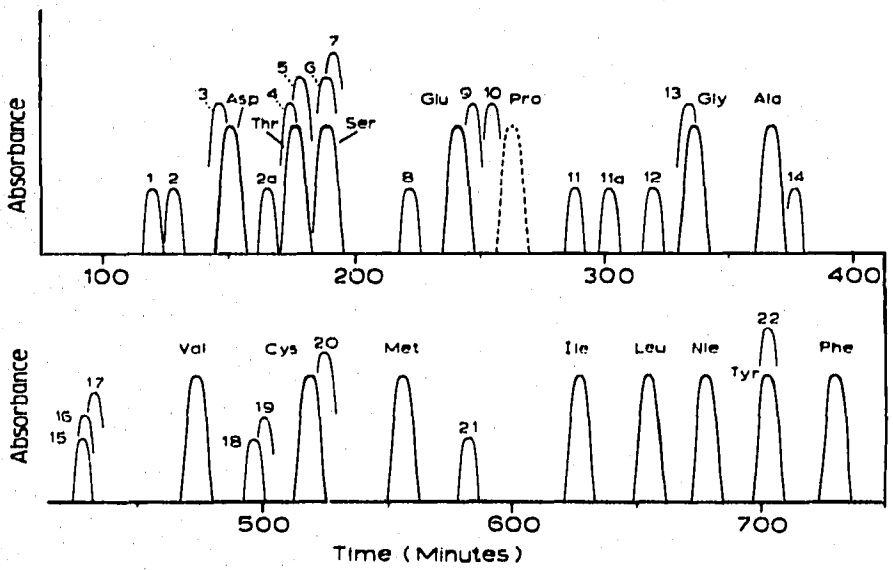

Fig. 1. Elution profile of amino acids obtained with a Technicon AutoAnalyser used under standard 21 h operating conditions. The common amino acids are shown in heavy lines, while the unusual amino acids have been drawn in light lines and elevated above the baseline in crowded areas. Table I provides a key to the numbered compounds.

Results and discussion

Fig. 1 illustrates the positions of the elution peaks of many unusual amino acids in comparison with those of fourteen protein amino acids and norleucine used as an internal standard. Table I provides a key to the compounds studied, and also lists the elution time determined for each compound.

Mixtures applied to the analyser contained selected protein amino acids, in 25 nmole amounts, and unusual amino acids (0.1–0.4 μmole). Certain generalizations can be made relating structural features of particular amino acids with their relative elution times. An increase in the length of an unbranched carbon chain leads to an increased elution time, *e.g.* compare C_4 aspartic acid (Asp) with C_7 α-aminopimelic acid (19). In a similar way, the elution time is increased when an alkyl group replaces a γ-proton of glutamic acid; *threo-* and *erythro*-γ-methylglutamic acids (11 and 11a)

TABLE I

KEY TO ABBREVIATIONS AND THE NUMERICAL CODE USED IN FIG. 1 AND THE ELUTION TIMES DETERMINED FOR EACH AMINO ACID

Code	*Compound*	*Elution time (min)*
1[a]	2(S),3(R),4(R)-β-Hydroxy-γ-methylglutamic acid[a]	120
2	*threo*-γ-Hydroxyglutamic acid	128
3	2(S),4(R)-γ-Hydroxy-γ-methylglutamic acid	140
Asp	Aspartic acid	151
2a	*erythro*-γ-Hydroxyglutamic acid	165
4	γ-Methyleneglutamic acid	175
Thr	Threonine	177
5	2(S),4(S)-γ-Hydroxy-γ-methylglutamic acid	179
Ser	Serine	189
6	*trans*-α-(Carboxycyclopropyl)glycine	189
7[a]	2(S),3(S),4(R)-β-Hydroxy-γ-methylglutamic acid[a]	192
8	S-Carboxyethylcysteine	222
Glu	Glutamic acid	241
9	*cis*-α-(Carboxycyclopropyl)glycine	247
10	S-Carboxyisopropylcysteine	255
Pro	Proline	263
11	*threo*-γ-Methylglutamic acid	287
11a	*erythro*-γ-Methylglutamic acid	302
12	α-Aminoadipic acid	319
13	*p*-Hydroxy-*m*-carboxyphenylglycine	333
Gly	Glycine	336
Ala	Alanine	367
14	*threo*- and *erythro*-γ-Ethylglutamic acid	374–378
15	α-Aminobutyric acid	428
16	γ-Ethyl glutamate (*i.e.* γ-ester)	440
17	α-(Methylenecyclopropyl)glycine	443
Val	Valine	474
18	γ-Ethylideneglutamic acid	497
19	α-Aminopimelic acid	501
Cys	Cystine	510
20	*m*-Carboxyphenylglycine	545
Met	Methionine	550
Ile	Isoleucine	627
Leu	Leucine	655
Nle	Norleucine	678
Tyr	Tyrosine	793
22	*m*-Carboxyphenylalanine	793
Phe	Phenylalanine	730

[a] Stereochemistry assigned as in private communication from Dr. E. A. Bell (Austin, Texas).

and *threo*- and *erythro*-γ-ethylglutamic acids (coincident at 14) are seen in Fig. 1 as peaks eluting after the parent compound. Increasing molecular size also gives rise to delayed elution of certain homologous sulphur-containing and aromatic amino acids: S-carboxyisopropylcysteine (10) appears later than S-carboxyethylcysteine (8), and *m*-carboxyphenylalanine (22) later than *m*-carboxyphenylglycine (20).

The unsaturated amino acids γ-methyleneglutamic acid(4) and γ-ethylideneglutamic acid (18) are eluted prior to the respective saturated compounds, *i.e.* the isomeric γ-methylglutamic acids (11 and 11a) and γ-ethylglutamic acid (14). In contrast, the C_7 amino acids, α-aminopimelic acid (19) and γ-ethylideneglutamic acid (18), although differing structurally in several important respects, are not resolved

as separate peaks by the analyser — the two compounds also behave very similarly during chromatography on paper with most commonly used solvent systems. High-voltage paper electrophoresis at pH 3.4 resolved these two amino acids more clearly, and their different colour responses with ninhydrin is a further aid in identification.

Substitution of a hydroxyl group either on glutamic acid (to give 2 or 2a) or on γ-methylglutamic acid (to give 1,3,5 or 7) markedly decreased elution time from the column; compare the peaks recorded for the parent amino acids, glutamic and γ-methylglutamic acids, respectively. (A similar observation was made by HAMILTON[4] in respect of β-hydroxyaspartic acids.) The diastereoisomeric forms of these hydroxyamino acids are well resolved by the analyser. Good separations also are achieved by electrophoresis at pH 3.4, but the isomers are not resolved by paper chromatography in most common solvent systems, although resolution is possible in selected solvents[5].

Substitution of hydroxyl groups onto aromatic rings again results in decreased elution times, *e.g.* compare phenylalanine (Phe) with tyrosine (Tyr), or *m*-carboxyphenylglycine (20) with its *p*-hydroxy derivative (13). The addition of a carboxyl group to a phenyl ring has a similar effect [*cf.* the positions of peaks of phenylalanine (Phe) and *m*-carboxyphenylalanine (22)].

The *cis*- and *trans*-isomers of α-(carboxycyclopropyl)glycine (6 and 9) were well separated from each other by the analyser and their positions were very distinct from that of α-(methylenecyclopropyl)glycine (17). These two isomeric dicarboxylic amino acids do not separate on paper chromatograms developed in 1-butanol–acetic acid–water, but a satisfactory separation can be achieved with aqueous phenol, in the presence of ammonia, or by paper electrophoresis using a buffer, pH 3.4 (ref. 2).

In summary, peaks attributable to acidic amino acids are seen to be spaced throughout the elution profile, occurring in regions typically associated not just with dicarboxylic amino acids but also with the neutral amino acids. Therefore, elution times, determined relative to other amino acids, can provide little insight into an "unknown" compound's identity, unless features of its basic carbon skeleton are known and the position of elution of the unsubstituted, saturated parent compound is recorded. When one recalls that at least another hundred neutral or basic amino acids are recognized as plant products, it is quite clear that the identification of an "unknown" amino acid in biological extracts requires the use of several independent chromatographic and electrophoretic techniques. In this respect, high-voltage paper electrophoresis can be used to quickly separate the acidic amino acid fraction from those of the neutral and basic amino acids — then the further resolution of these simpler mixtures using an amino acid analyser should permit the more certain identification of individual components, and also facilitate their quantitative assay.

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