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Note

Single-wavelength detection for amino acid analysis

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Since the first amino acid analyzer was developed by Spackman *et al.*[1], the determination of amino acids has been carried out by measuring ninhydrin colour development at two wavelengths, 570 nm for amino acids and 440 nm for the imino acids proline and hydroxyproline, followed by manual calculation.

Recently, computer systems have been introduced for the quantitative and qualitative analysis of amino acid chromatograms. Much work has been reported on the automatic off-line analysis of the amino acid chromatograms of protein hydrolyzates or naturally occurring compounds using a small computer[2,3]. Some modifications of the procedures are required in order to achieve a more accurate measurement of proline and hydroxyproline, because the determination of all amino and imino acids at 570 nm alone introduces problems owing to the low accuracy with these two imino acids, which give only small peaks at this wavelength.

This note describes a detection method using a shorter wavelength of 420 nm for the determination of all amino and imino acids with good reliability.

EXPERIMENTAL

A Hitachi Model KLA-3B amino acid analyzer (Hitachi, Tokyo, Japan) was employed, and a Hitachi Model 034 variable wavelength detector was connected with the analyzer in order to choose an appropriate wavelength. A Shimadzu Chromatopac Model 1A computer system with a logarithmic converter (Shimadzu, Kyoto, Japan) was connected with the amino acid analyzer.

Amino acid analyses were carried out on a two-column system: a 50 × 0.9 cm Hitachi custom resin 2612 column for acidic and neutral amino acids and a 10 × 0.9 cm Hitachi custom resin 2611 column for basic amino acids. Ninhydrin reagent was prepared using 2 ml of 20% titanium(III) chloride solution instead of tin(II) chloride solution per litre of the reagent[4].

RESULTS AND DISCUSSION

It is well known that the spectrum of the reaction products of ninhydrin with primary amino residues has two absorption maxima in the visible range, at about 570 and 400 nm, the absorbance of the ninhydrin colour developed, with proline and hydroxyproline decreases at longer wavelengths, and the background due to the nin-

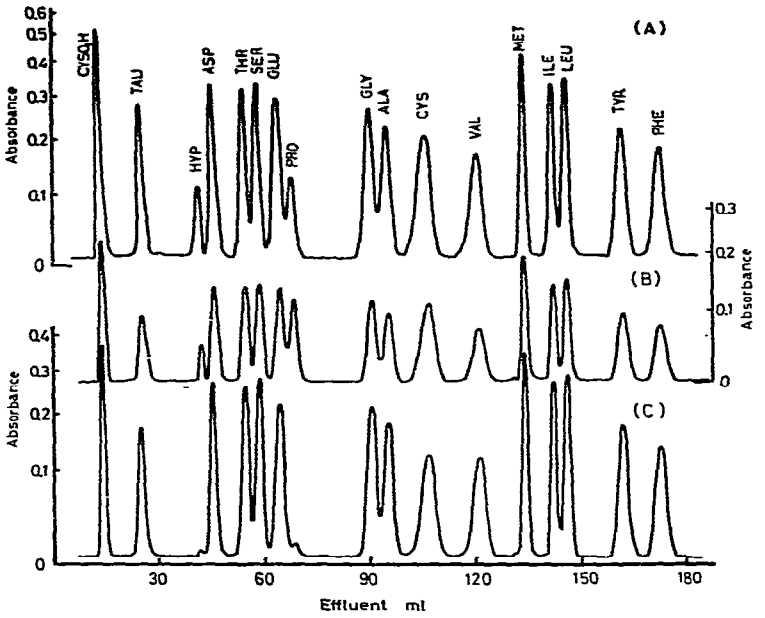

Fig. 1. Chromatograms of acidic and neutral amino acids with detection at various wavelengths: A, at 400 nm; B, at 420 nm; C, at 570 nm.

hydrin reagent increases at shorter wavelengths (below 440 nm). This high background is assumed to result in noise in the chromatograms and also to weaken the transmittance of the light source in the detection of amino acids at wavelengths below 440 nm. However, no problems due to the background of the reagent were found in our experiments.

Fig. 1 shows the chromatograms obtained at 400, 420 and 570 nm on a Hitachi Model 034 detector. The amount of each amino acid was 0.25 μmole, except for proline and hydroxyproline (0.50 μmole). When the absorbances of the colours developed with proline and hydroxyproline were measured at 570 nm rather than 440 nm, it was not expected that good results over a wide concentration range of the compounds would be obtained, for the reason given above. However, the base-lines for detection at 400 and 420 nm were as smooth as that at 570 nm and no problems occurred. The resolution between glutamic acid and proline was 0.9, which was sufficient for quantification when the ratio of the areas of these two peaks was not too large. However, when either peak was too large, the quantitative determination was inaccurate. While the ratio of the peak area for proline to that for glutamic acid at equivalent concentrations was about 1:36 measured at 570 nm, the ratio at 420 nm was about 1:3.

This single-wavelength detection method was applied to the amino acid analyzer–Chromatopac 1A computer system. The 640-nm interference filter in the detector of the KLA-3B analyzer was replaced with a 420-nm filter and the output of the detector was connected to the Chromatopac 1A computer system through a logarithmic converter.

Table I shows average retention times, average peak areas and their coefficients

TABLE I

COMPUTERIZED DATA FOR ANALYSIS OF A STANDARD AMINO ACID MIXTURE

Peak No.	*Amino acid*	*Average retention time, $\bar{t}$ (min)*	*Coefficient of variation of retention time, $\sigma/\bar{t} \cdot 100$*	*Average peak area*, $\bar{x}$*	*Coefficient of variation of peak area, $\sigma/\bar{x} \cdot 100$*
1	Cysteic acid	14.85	2.52	48890	1.87
2	Tau	25.98	0.80	28651	1.70
3	Asp	45.84	0.93	39071	1.01
4	Thr	54.42	0.77	39737	0.79
5	Ser	58.29	0.70	39491	1.31
6	Glu	63.66	0.71	47591	2.08
7	Pro	67.95	0.60	31934	1.19
8	Gly	89.10	0.29	44851	0.88
9	Ala	93.81	0.15	38565	1.23
10	Cys	103.20	0.46	62207	0.56
11	Val	119.10	0.23	39616	2.17
12	Met	133.98	0.44	43609	0.99
13	Ile	142.50	0.39	35952	1.98
14	Leu	146.40	0.38	42507	1.17
15	Tyr	162.51	0.39	44338	1.87
16	Phe	173.25	0.33	39606	1.34
17	Hyp	42.93	0.89	21684	1.49
1	Trp	32.13	2.14	38799	0.58
2	Lys	43.27	1.03	57237	0.78
3	His	52.65	0.92	49089	0.93
4	NH_3	68.79	0.63	19579	0.71
5	Arg	106.69	0.30	38636	1.21

* The concentration of each amino acid was 0.25 μmole, except for proline and hydroxyproline (0.50 μmole).

of variation for standard amino acids obtained in five replicate runs. The coefficients of variation of the retention time and peak area of each amino acid were satisfactory. Amino acids in amounts from 0.05 to 1.5 μmole were determined quantitatively by measurement of peak areas.

With the Chromatopac 1A computer system, determinations at wavelengths shorter than 420 nm were not examined, because the photo-intensity of the tungsten lamp was weak at shorter wavelengths, the background due to the ninhydrin reagent was high and sufficient power from the output of a selenium photo-cell could not be obtained.

The measurement of colours obtained with ninhydrin reagent using the 420-nm filter is an excellent method for computerized amino acid analysis with a simplified detection system, and it will be applicable to more sensitive detection system by using pulseless pumps and a stronger, stable light source.

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