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DETECTION WAVELENGTH AND INTEGRATION STRATEGY FOR AMINO ACID ANALYSIS*

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SUMMARY

Amino as well as imino acids were detected with ninhydrin at a single wavelength of 405 nm, one of the absorption maxima of Ruhemann's purple, instead of the conventional 570 and 440 nm. The advantages are: (1) no need for a wavelength switch before and after the imino acid peaks; (2) avoids summing of the signals of 440 and 570 nm; (3) improves the sensitivity for most of the amino acids, particularly glutamic, cystine, methionine and lysine. The increase in the wavelength bandwidth, in order to increase the signal-to-noise ratio, is somewhat limited compared with that at 570 nm.

INTRODUCTION

Though the available precolumn derivatization techniques for amino acid analysis are highly sensitive and rapid, they are suitable mainly for pure protein samples[1]. In 1948, Moore and Stein[2] published a method which permits the analysis of rather impure samples, which would present derivatization as well as separation difficulties to other techniques[1]. Since that time, detection has been carried out predominantly at 570 and 440 nm, the absorption maxima of the products of reaction of ninhydrin with amino and imino acids, respectively. Considered indisputable, this approach resulted in all commercial amino acid analysers being equipped with costly dual colorimeters. The introduction of electronic integrators raised the cost further. Various solutions have been devised to enable the use of single-channel integrators, such as a channel-summing circuit, added to a commercial amino acid analyser, which combined electronically the 570- and 440-nm outputs and transferred the resulting signal to the integrator. A similar situation exists in high-performance liquid chromatographic (HPLC) systems modified to perform the Moore and Stein reaction. Where sophisticated spectrophotometric detectors were employed, the wavelength was switched from 570 to 440 nm before the appearance of the imino acid peaks[3].

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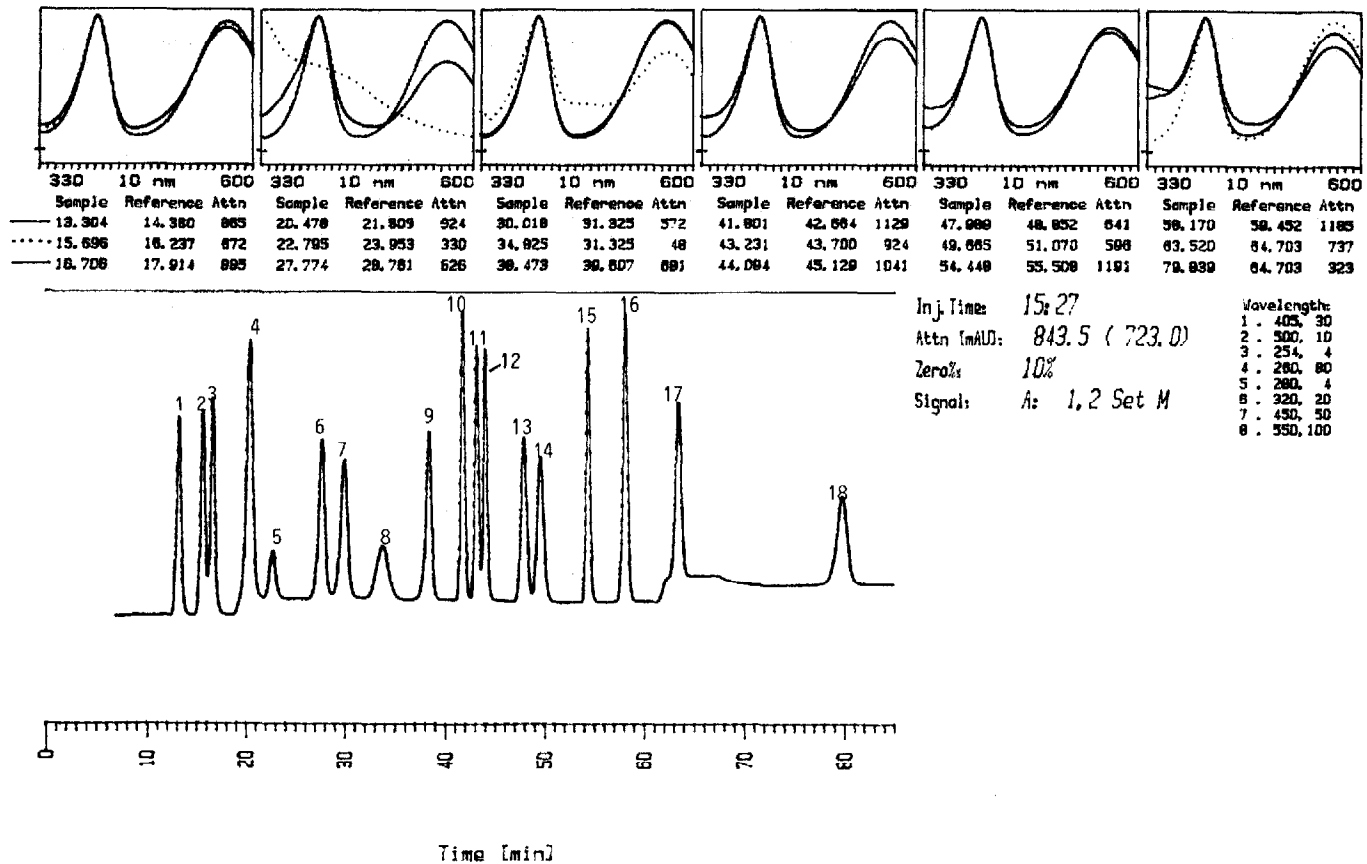

Fig. 1. Chromatogram and normalized spectra of a standard amino acid mixture. The concentration of the amino acids was 12.5 nmol, except for cystine, 6.25 nmol. Each frame contains the spectra of three amino acids, in their order of elution. The retention times of the relevant peaks are given in the "sample" column. Peaks: 1 = Asp; 2 = Thr; 3 = Ser; 4 = Glu; 5 = Pro; 6 = Gly; 7 = Ala; 8 = Cys; 9 = Val; 10 = Met; 11 = Ile; 12 = Leu; 13 = Tyr; 14 = Phe; 15 = His; 16 = Lys; 17 = ammonium; 18 = Arg. mAU = milli absorption units (full scale, 843.5; highest peak, 723.0).

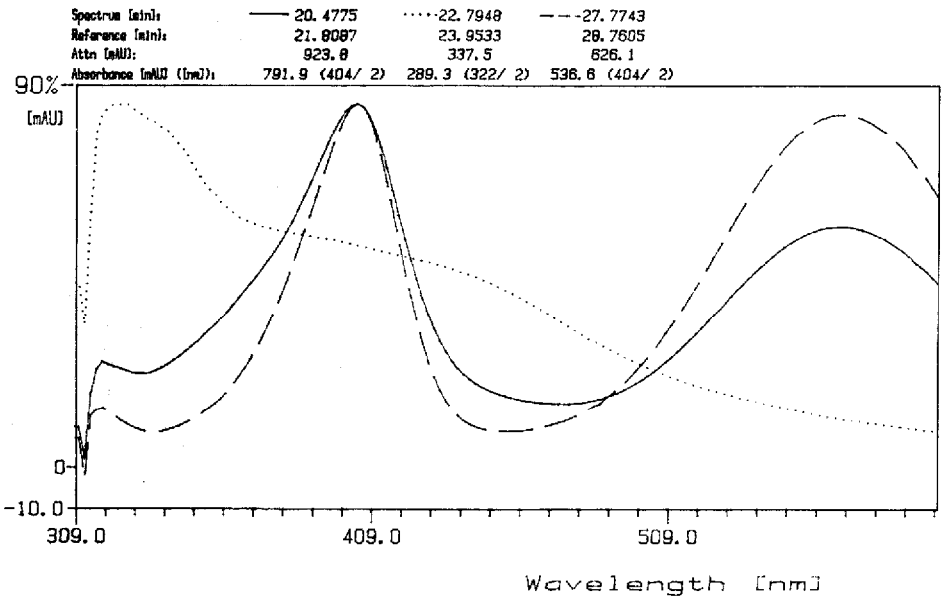

Fig. 2. Normalized spectra of glutamate (solid line), proline (dotted line) and glycine. Note the maxima at 440, 405 and 322 nm in the proline spectrum.

In many cases this means one has to decide whether (i) to sacrifice sensitivity, *e.g.*, glutamate in protein hydrolysates (Figs. 1 and 3) is included at 440 nm in order not to switch wavelengths between glutamate and proline, or (ii) to switch wavelengths between peaks and lose integration precision if peaks are not baseline resolved or a shift in retention time occurs. Ruhemann's purple shows an additional peak at 405 nm[4] which coincides with one of proline (Figs. 1 and 2). The latter is more pronounced than the one of 440 nm (both "peaks" are hardly more than shoulders on the ascending slope of the main peak, the maximum of which is at 322 nm, Fig. 2). The wavelength of 405 nm was suggested by Durham and Geren[5] as a means of improving the detection of proline.

EXPERIMENTAL

The chromatograph used was a binary version of the HP 1090A solvent-delivery system equipped with an HP 1040A diode-array detector, HP 9121 dual disc drive, HP 7470A printer-plotter and built-in DPU integrator (Hewlett-Packard, Waldbron, F.R.G.).

The post-column reaction system consisted of: (i) Milton-Roy minipump for delivery of ninhydrin and the wash solution; (ii) Rheodyne valves, to switch between ninhydrin and the wash solution; (iii) a post-column reaction bath (CRX 390, Pickering, Mountain-View, CA, U.S.A.), comprising an adjustable-temperature PTFE coil (0.25 mm I.D., volume about 0.5 ml). All components of the amino acid analyser were located outside the chromatograph. Parts i and ii are under the control of the chromatograph. Switching to any HPLC method necessitated only connection of the relevant column and solvent reservoirs.

The prepacked column, part No. 1193250 (250 × 3 mm), and the precolumn, part No. 119020 (20 × 2 mm), were purchased from Pickering. The stationary phase consisted of sulphonated divinylbenzene–polystyrene copolymer (10-μm diameter).

Materials

All chemicals were of analytical grade. Deionized water was acidified with sulphuric acid to a final concentration of 2 *N*, and distilled continuously, at constant volume, to remove the ammonia-like compounds. Fig. 1 illustrate an exceptional case where the buffers were prepared with deionized water.

No attempt should be made to use amino acid analyser solutions in the 1090A chromatograph unless the high-pressure pump check-valves are replaced with PTFE ones. Pickering sodium buffers, pH 3.28 (A) and 7.4 (B), or those made up in the laboratory were used. The pH of the laboratory buffer B was 7.45. This enabled elution of arginine at 75 min; the elution order of lysine and histidine is reversed.

The acetate buffer, 4 *N*, pH 5.2 (for ninhydrin solution, LKB formulation[6]) was prepared from 294.4 g potassium acetate, 136.0 g sodium acetate trihydrate, 4.0 g tripotassium citrate monohydrate, 100 ml glacial acetic acid made up to 1 l with water. When diluted by a factor of 3, the pH should be 5.2 ± 0.03.

The ninhydrin solution (modified LKB formulation[6]) was prepared (in order of addition) from 600 ml ethylene glycol, 10 g ninhydrin, 300 ml acetate buffer, 100 ml water and 800 mg $SnCl_2$. Nitrogen was bubbled through the solution for 30 min prior to the addition of $SnCl_2$ and for another 10 min there after. The solution was stored in a refrigerator, under 3 p.s.i. of nitrogen.

The wash solution (replaces ninhydrin solution between the elution of arginine and the next experiment; probably enables the use of $SnCl_2$) was prepared from 700 ml ethylene glycol, 100 ml isopropanol, 2 ml Triton X-100, made up to 1 l with water.

Chromatographic conditions

The column temperature was 45°C, the buffer and ninhydrin flow-rates 0.2 ml/min and the reaction bath temperature 130°C. The detection wavelength was 405 ± 5 nm and the reference wavelength was 500 ± 5 nm; 700 nm, which is out of the range of the detector, would have been the reference wavelength of choice, as neither the amino nor the imino acids absorb there[3].

RESULTS AND DISCUSSION

Fig. 3 depicts the chromatogram of a standard amino acid solution detected at 570 and 405 nm. The peak-height mode was used[7]. The ratios of the peak heights (405 nm/570 nm) are shown in Table I. Obviously the sensitivity for 10 of the 17 amino acids is greater at 405 nm. The increase for cystine was 47.6%; Friedman *et al.*[4] noted an even greater increase in sensitivity for cystine-related compounds at 405 nm compared with 570 nm.

It should be noted that an increase in the signal bandwidth, in order to improve the signal-to-noise ratio, has a greater effect on the 405-nm signal than that of the 570-nm one, as the peak at 405 nm is narrower (Figs. 1, 2).

The wavelength of 440 nm may be used if there is a need for channel-ratioing, to support peak identification, or in case of an extreme difference between the proline

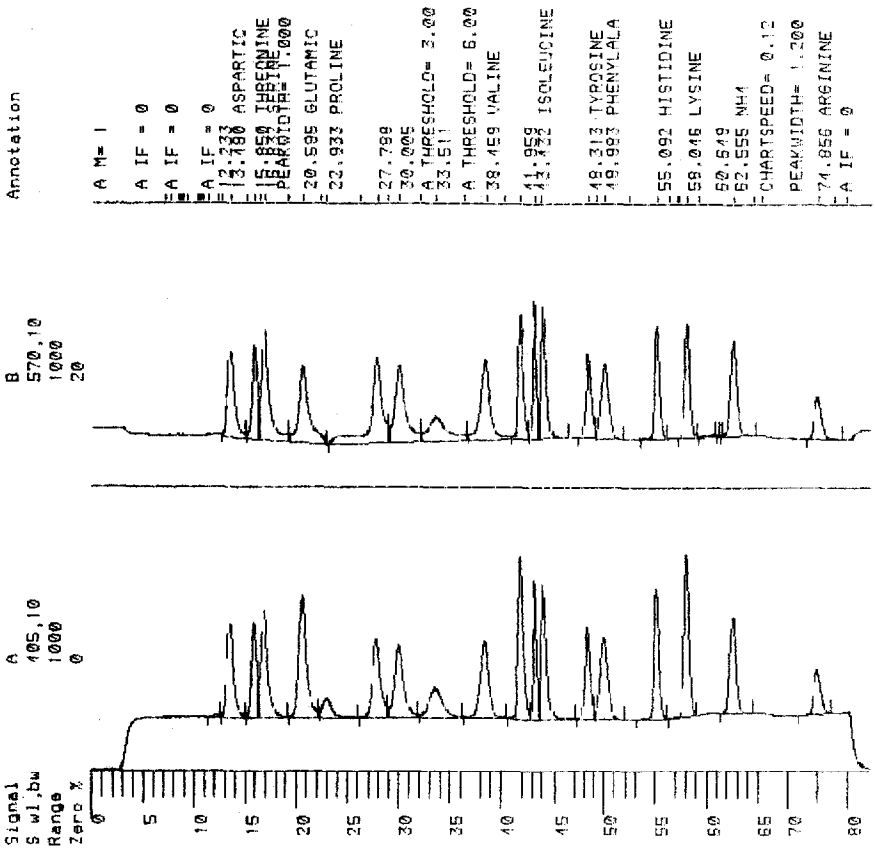

Fig. 3. Chromatogram of a standard amino acid mixture detected at 405 and 570 ± 5 nm. Proline appears at 570 nm as a negative peak. The order of the amino acids is as in Fig. 1. S wl = signal wavelength; bw = bandwidth.

peak and adjacent peaks. As the peaks of the primary amino acids are very weak in this region, and, as a result, narrower, an improved resolution may be achieved.

Hundreds of samples have been analyzed during the last 2 years, some of which were occasionally co-analyzed by prominent laboratories in Europe and Israel, with excellent agreement in the results.

TABLE I

CHANNEL-RATIO (%) OF THE SIGNALS AT 405 AND 570 nm, AT A BAND WIDTH OF 10 nm

Asp	*Thr*	*Ser*	*Glu*	*Pro*	*Gly*	*Ala*	*Cys*	*Val*
109.8	107.2	100.4	172.3	–	101.7	102.1	147.6	102.7
Met	*Ile*	*Leu*	*Tyr*	*Phe*	*His*	*Lys*	*Ammonium*	*Arg*
133.5	101.1	102.1	110.5	110.2	116.7	141.7	99.8	106.5

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