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MULTI-SAMPLE QUANTIFICATION OF AMINO ACIDS AND IMINO ACIDS WITH A SINGLE ANALYTICAL SYSTEM

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SUMMARY

An automated procedure is described for quantifying amino acids, imino acids, and other ninhydrin-positive compounds which is based on making absorbancy measurements at the single wavelength of 405 mμ. Experimental data indicate that the sensitivity of the modified capability is equal to or greater than that obtained using a conventional procedure. Additional evidence is presented that validates a further methodologic development which makes possible the analysis of three samples with the instrumentation designed for the analysis of only one sample.

INTRODUCTION

A voluminous literature concerning automated amino acid methods has accumulated since the first automatic recording system described by SPACKMAN *et al.*[1]. Notwithstanding the many noteworthy instrumental and procedural modifications, there is at least one aspect of the original method which has not been altered; namely the measurement of the ninhydrin chromogens of amino acids and imino acids at wavelengths of 570 mμ and 440 mμ, respectively. Accordingly, most commercial analyzers are equipped with at least two colorimeters and a dual recording system.

The present report describes what appears to be the first departure from the conventional use of two and three colorimetric recording systems. As described, the present procedure not only facilitates the use of a single recording system with optimum sensitivity for quantifying imino acids and amino acids, but as a consequence thereof, makes possible the simultaneous analysis of three samples with the instrumentation designed by its manufacturer to analyze only one sample at a time.

MATERIALS AND METHODS

A Technicon amino acid autoanalyzer of the type available in 1967 was used. Of particular note here is the fact that the analyzer was originally equipped with a two-coil heating bath, three colorimeters, and a three-point recorder. With respect to the colorimeters, one housed a 15 mm flowcell and a set of 440 mμ interference filters, one had a 15 mm flowcell and a set of 570 mμ filters, and the other one hand an

8 mm flowcell and a set of 750 mμ filters. Additionally, the manifold assembly consisted of the four tubes recommended by the manufacturer[2].

In accordance with the procedure described below, the following instrumentational modifications were made: (1) a third glass coil was placed within the heating bath used for color development; (2) two additional glass columns (140 × 0.636 cm) were packed to a height of 130 cm with Technicon Chromobeads, Type B; (3) the water jackets surrounding the chromatographic columns were connected in tandem so that all three columns were thermostatically controlled by a single water bath; (4) two additional sets of manifold tubing were placed along with the original set in the two-level end-blocks used with the proportioning pump; (5) 15 mm flowcells and sets of 405 mμ filters were placed within each of the three colorimeters; and (6) a four-arm metal connection (Swagelock "cross", Crawford Fitting Company, Solon, Ohio) was so placed on the outlet side of the high-pressure eluent pump that eluent buffer entered through one arm of the cross and was dispensed through the other three arms. Appropriate lengths of high-pressure Teflon tubing (1/16 × 0.03 in.) were used to connect the three outlets to the flanged Teflon fitting at the top of the three columns. In essence, the latter modification made possible the simultaneous elution of three columns using a single eluent pump and a single Technicon Autograd. The eluent pump rate was adjusted to deliver a total of 1.50 ml/min.

Two other departures from the suggested procedure[2] dealt with the production of the buffer gradient. One modification was the use of a previously reported gradient[3]. In brief, the gradient was produced by placing 60 ml of a citrate buffer (pH 2.88) in each of the first five chambers of the Autograd, and 60 ml of another citrate buffer (pH 5.00) in each of the four remaining chambers. The total volume of the resulting buffer gradient was sufficient for operating a single column system. With the novel three-column analyzer described herein, however, each of the nine chambers of the Autograd was filled to capacity; *i.e.*, each contained 160 ml of buffer.

Two operational procedures were used with the modified analyzer. In one procedure, all components were used for the separation and quantification of a known

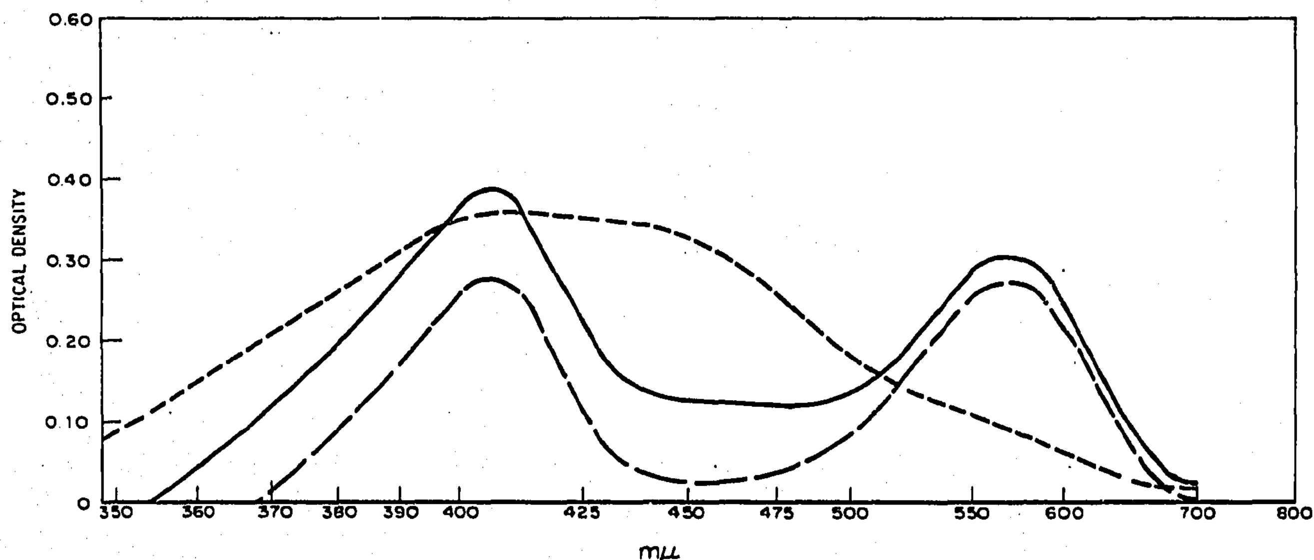

Fig. 1. Absorption spectra of the ninhydrin products of cystine (———), threonine (– – – –) and proline (------).

mixture of amino acids and imino acids. The other procedure was the aspiration of individually prepared solutions of amino acids and imino acids through the manifold tubing normally used for aspirating column effluent. With respect to the latter procedure, a set of calibration standards was prepared for each compound in 0.1 *N* HCl. Compounds examined in this study included the following: α-alanine, ammonium sulfate, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine hydroxyproline, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, and valine. Each set of standards was aspirated for a sufficient period of time to permit the recording of a plateau at maximum absorbance. Additionally, the repumped reactant fluid from each standard was not discarded; instead, each was collected and subjected to spectral analysis utilizing a Beckman Model DK-2 ratio-recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

The ninhydrin reagent and other solutions used with the analyzer were prepared in accordance with instructions furnished with the analyzer[2].

RESULTS

The rationale for quantifying ninhydrin chromogens of imino acids, amino acids, and closely related compounds with a single recording system is clearly demonstrated by the spectral data shown in Fig. 1. The spectral curves for the proline, cystine and threonine chromogens are shown to represent the three general types of spectra found among the 21 compounds examined. Spectra of the proline type were

TABLE I

RELATIVE ABSORBANCE OF NINHYDRIN CHROMOGENS

Compound	*Relative absorbance*[a]
Ammonium sulfate	1.00
Leucine	1.01
Isoleucine	1.02
Arginine	1.03
Threonine	1.03
Glycine	1.04
Alanine	1.04
Methionine	1.04
Tyrosine	1.04
Phenylalanine	1.04
Serine	1.05
Tryptophane	1.05
Aspartic acid	1.05
Valine	1.06
Histidine	1.11
Ornithine	1.15
Glutamic acid	1.21
Lysine	1.24
Cystine	1.36
Proline	1.06
Hydroxyproline	1.09

[a] Values are $A_{405}:A_{570}$ for all compounds except proline and hydroxyproline which are $A_{405}:A_{440}$.

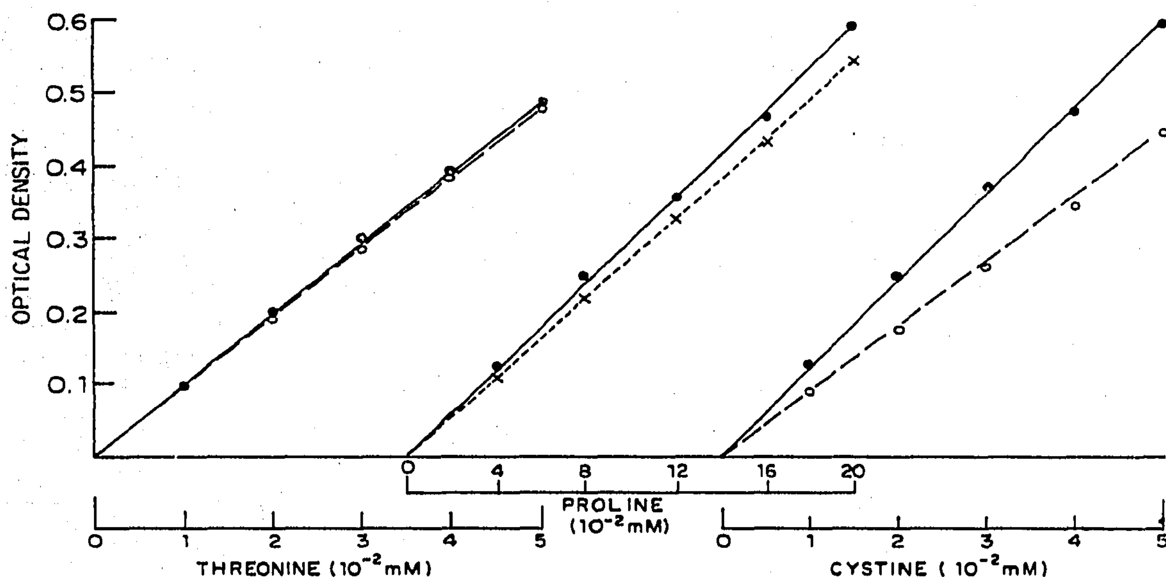

Fig. 2. Calibration curves for the ninhydrin products of threonine, proline and cystine (from left to right). Absorbance measurements for threonine and cystine products were made at 405 mμ (●—●) and 570 mμ (○---○); absorbancies for proline chromogen measured at 405 mμ (●—●) and 440 mμ (×---×).

clearly different from those of the cystine and threonine types, being characterized by the single, broad-absorption band. Differentiation between the other two spectral groups was less clear-cut as they were based on the relationship of absorbance at the two maxima, *i.e.*, at 405 mμ and 570 mμ. With respect to spectra of the threonine type, the absorbance values at the two wavelengths were virtually identical. This was not the case for spectra of the cystine type as the absorbance at 405 mμ was substantially higher than that at 570 mμ.

The data presented in Table I offer a summary of the spectra of the 21 ninhydrin chromogens. A noteworthy finding was that absorbancies at 440 mμ for imino acids and at 570 mμ for amino acids were less than absorbancies at 405 mμ.

The validity of making absorbance measurements at 405 mμ is demonstrated by the calibration data presented in Fig. 2. For brevity, the categorization employed above for absorption spectra is used for illustrating the linearity of absorbance measurements. These data not only substantiate the finding of equal or greater absorbance at 405 mμ, but, of even greater importance, indicate the quantitative soundness of using a single wavelength for quantifying the diverse ninhydrin chromogens.

As evidenced by the data shown in Table II, the separation and quantification of a mixture of amino acids further validated the use of the single recording system. Actually, these data were obtained from an operational procedure which differed from that suggested by the manufacturer[2] in one noteworthy respect; namely, a 15 mm flowcell and a set of 405 mμ filters were placed in the colorimeter which had originally been equipped with an 8 m flowcell and a set of 570 mμ filters. It is apparent from the data in Table II that not only was there less variation in area measurements at 405 mμ, but there was also a consistent gain in sensitivity. As might be expected from the data presented in Table I, the gain in sensitivity was especially marked for cystine, glutamic acid, lysine and histidine.

TABLE II

ANALYSIS OF CHROMATOGRAPHIC PEAKS BY DUAL AND SINGLE RECORDING SYSTEMS

Compound	*Area constants (H × W/μmole)*			
	Conventional recording system[a]		*Modified recording system*[b]	
	Mean (*n* = 3)	*Coefficient of variation*	*Mean* (*n* = 3)	*Coefficient of variation*
Aspartic acid	51.77	1.40	54.39	1.75
Threonine	52.93	1.94	55.10	1.96
Serine	53.87	1.67	55.85	1.10
Glutamic acid	51.80	2.51	63.76	2.17
Proline	13.63	7.35	14.98	4.38
Glycine	58.00	2.15	60.49	1.29
Alanine	51.07	2.16	53.34	2.64
Valine	48.63	2.22	51.71	1.51
1/2-Cystine	29.90	1.67	43.06	1.60
Methionine	53.90	2.60	57.12	2.28
Isoleucine	50.67	1.68	52.84	0.95
Leucine	57.87	1.93	59.04	0.87
Tyrosine	55.93	1.74	58.78	1.14
Phenylalanine	56.00	1.64	58.91	1.89
$(NH_4)_2SO_4$	26.17	3.98	28.77	2.68
Lysine	57.77	2.14	69.72	1.92
Histidine	59.07	2.13	65.21	1.04
Arginine	55.93	0.88	59.00	0.78
Mean		2.32		1.77

[a] Values based on absorbance at 570 mμ except for proline which was measured at 440 mμ.
[b] All values based on absorbance at 405 mμ.

The data presented in Table II were based on the analysis of 50 μl of a standard mixture in which each amino acid was present in a concentration of 2.5 m*M* except for the 1.25 m*M* cystine. Data obtained from replicate analyses of 10, 20, 30, and 40 μl of the same mixture revealed linear responses that were similar to those shown in Fig. 2.

After validating the use of the single colorimetric recording system for amino acids, imino acids and other ninhydrin-positive substances, efforts were made to utilize the triple colorimetric recording capability of the analyzer for the simultaneous analysis of three samples. In the latter endeavor, special efforts were made to utilize only the instrumental components which therefore had been used for the analysis of a single sample. It was recognized that certain expendable components such as manifold tubing, additional chromatographic columns and resin would be essential. It was desirable, however, that no additional instrumentation such as eluent and proportioning pumps, heating and circulating water baths, Autograds, colorimeters and recorders would be necessary.

The schematic drawing shown in Fig. 3 illustrates the basic features of the resulting three-sample analyzer. Although the use of the single colorimetric system described above was the most important factor leading to the desired operational procedure, a previously reported methodologic development proved to be a decisive factor. The latter factor was the modified buffer gradient[3] which, among other favorable features, made possible the elution of chromatograms with a substantially

reduced total volume of eluent buffer. The importance of the gradient volume is discussed below along with other aspects of the present procedure.

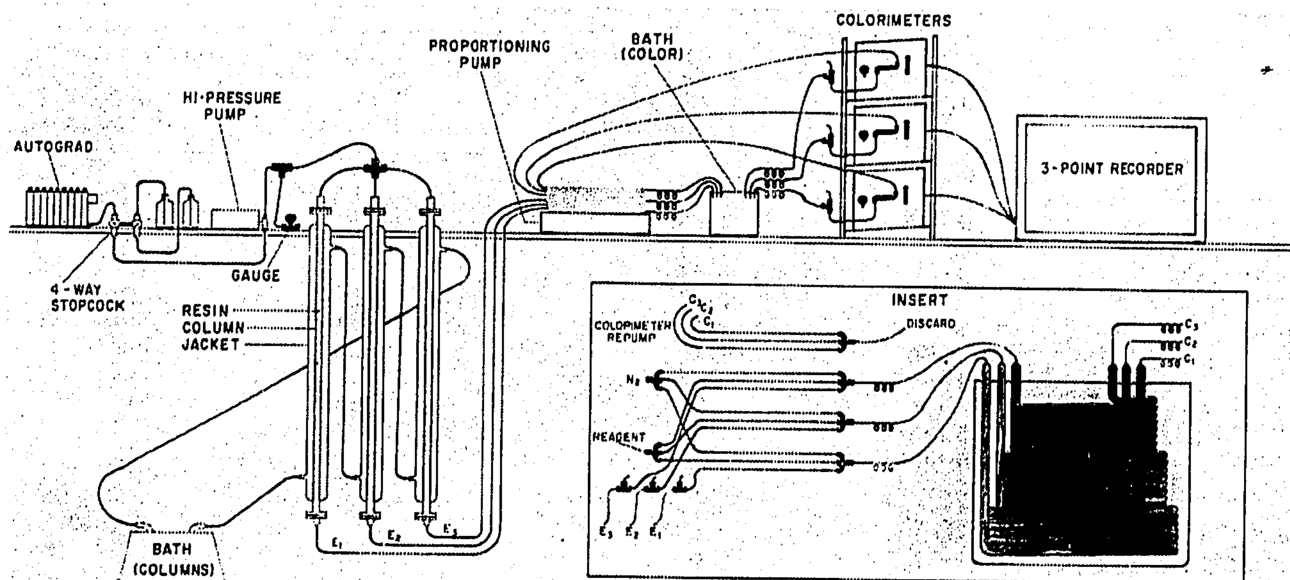

Fig. 3. Schematic drawing of 3-sample amino acid analyzer. Insert shows close-up view of manifold and glass coils contained within the heating bath used for color development. E_1, E_2 and E_3 denote effluents from the three columns. C_1, C_2 and C_3 denote the reactant fluids derived from effluents E_1, E_2 and E_3, respectively. See text for function and use of the individual components of the analyzer.

DISCUSSION

The present finding of two absorption maxima in the visible spectrum for ninhydrin products of amino acids is not unprecedented. Absorption maxima at 408 mμ and 578 mμ for the ninhydrin chromogen of alanine were reported by MEYER[4]. While those maxima are slightly higher than the 405 mμ and 570 mμ reported here, the slight shifts might be readily explained on the basis of procedural differences, particularly in the preparation of the ninhydrin reagent. As far as can be ascertained, however, advantage has not heretofore been taken of the 408 mμ (405 mμ) absorption maximum. The report of the first automatic amino acid analyzer[1] appeared a year after the report of MEYER[4]. The equipping of the three colorimeters in that original automated method necessitated two absorbancy measurements at 570 mμ and one measurement at 440 mμ. Moreover, it appears that all commercial automatic amino acid analyzers marketed since that time have retained that feature of the methodology.

In addition to the obvious advantage of using a single colorimetric recording system, the present data suggest that a more sensitive assay of imino acids as well as amino acids is obtained by absorbancy measurements at 405 mμ. The finding that the maximum absorbance of the ninhydrin products of proline and hydroxyproline was closer to 405 mμ than to 440 mμ was somewhat surprising as the latter wavelength has seemingly been universally accepted as the absorbance maximum.

The discovery that one colorimeter could be used to quantify ninhydrin-positive compounds was the most decisive factor in establishing the three-sample analyzer;

however, two other factors were important. One factor was to ensure that the eluent pump rate of 1.5 ml/min was uniformly distributed so that each column would be eluted at the rate of 0.50 ml/min. Two steps were necessary to achieve uniform elution rates. The first step was to pack the three columns in an identical manner. This was accomplished by the simultaneous filling of the columns with a single, homogeneous suspension of resin. The finding that uniformly packed columns did not consistently result in uniform eluent rates, however, led to the taking of a second step. In this connection, it was found that the porosity of the Teflon sintered disc inserted into the fitting at the base of the column differed substantially from one disc to another. This finding led to the careful screening and selection of discs prior to their use.

The other factor that had to be reckoned with dealt with the volume and/or character of the buffer gradient. According to the operational instructions furnished with the analyzer[2], a volume of 75 ml was needed in each of the nine chambers of the Autograd for a single chromatographic run. For the three-column capability, this would necessitate the placement of 225 ml of buffer in each chamber. As the capacity of each chamber was approximately 160 ml, it was apparent that either an Autograd having at least three additional chambers must be used or a modification of the buffer gradient was needed. Fortunately, the buffer gradient described in our previous work[3] was found to elute satisfactorily the three columns when all of the nine chambers were filled to capacity. It was essential, however, to tilt the Autograd forward during the final 30 min of each run in order to utilize the entire volume.

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