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HIGH-SPEED LIQUID CHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS BY POST-COLUMN SODIUM HYPOCHLORITE–*o*-PHTHALALDEHYDE REACTION

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SUMMARY

The performance and operating characteristics of a high-speed amino acid analysis system based on post-column reaction with *o*-phthalaldehyde and sodium hypochlorite are described. Thirty-minute analysis of all the 16 standard hydrolyzate amino acids is achieved on a 120-mm long column, packed with a 6-μm cation-exchange resin. With minor loss of resolution, a 22-min assay is possible. The column is useable with several commercial buffers, and analyses at the 10–100-pmole levels are demonstrated. Excellent precision (< 2% relative standard deviation), linearity (10–10 000 pmoles), and column lifetime are reported, together with quantitative correlation data with a ninhydrin system.

INTRODUCTION

Out of the myriad of methods in use today for amino acid determinations, most analysts requiring quantitative data still prefer the traditional post-column technique[1–3]. While the fundamentals of this technique have remained essentially unchanged since its inception in 1954[4], the performance, in terms of analysis speed and sensitivity, has undergone continual and dramatic improvements.

Whereas the analysis of protein hydrolyzates took 24–48 h in the 1950's[4–5], the same analysis can be performed in 20–30 min today. Responsible for this improvement are the advances in resin technology, in particular, the reduction of particle size from the 100–200 μm to the 6–7 μm range[1–2,5–9]. The smaller resin particles allow the packing of shorter columns with comparable efficiency. Coupled with low-dispersion instrumentation and compatible buffers, these columns facilitate faster analysis without sacrifice in resolution.

While faster analysis is desirable for increasing laboratory productivity, higher sensitivity is needed for structure determinations of proteins and peptides available in small quantities[1]. Here, the classical ninhydrin reagent has fundamental limitations. Characterized by slow reaction rates and high reaction temperatures, the predominance of ninhydrin is now challenged by the more sensitive fluorogenic *o*-phthalaldehyde (OPA).

The OPA method, developed by Roth and Hampaï[10–11] in the early 1970's, is

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five to ten times more sensitive than ninhydrin, as demonstrated by Benson and Hare in 1975[12]. However, secondary amino acids, are not detected unless these compounds are first oxidized with an oxidant, such as a dilute solution of of sodium hypochlorite[13], which is added either continuously[14], or in pulses during the elution of prolines[15]. The continuous addition method is preferred for routine analysis since no baseline disruption occurs during the analysis[14]. However, since primary amino acids are destroyed by the sodium hypochlorite reaction, very precise reaction conditions need to be maintained to yield reproducible analysis[13].

Although the sodium hypochlorite–OPA reaction for post-column amino acid analysis is well documented in the literature[2,9,14–16], no systematic analytical data on the precision, linearity, and sensitivity, are available. Particularly lacking is a quantitative comparison between the OPA and ninhydrin methods. This paper reports these data and describes the conditions for achieving high-speed, high-sensitivity analysis. In addition, column lifetime and compatability with several commercial buffer preparations are studied.

EXPERIMENTAL

Materials

Amino acid standards. Individual amino acids were obtained from Calbiochem (San Diego, CA, U.S.A.) and Sigma (St. Louis, MO, U.S.A.). A prepared mixture of protein hydrolyzate amino acids at a level of 2.5 μmoles/ml was obtained from Pierce (Rockford, IL, U.S.A.). Less concentrated standard solutions were prepared by serial dilution with a pH 2.2 buffer from Pickering Labs. (Mountain View, CA, U.S.A.).

Chromatographic buffers. Several commercial chromatographic buffers and a laboratory-prepared citrate formulation[17] were evaluated. The buffers were: Buffēlute (Pierce), Pico II (Pierce), sodium eluents (Pickering Labs.) and LKB buffers (LKB Biochem, Cambridge, U.K.).

Reagents. The reagents, OPA, 2-mercaptoethanol, sodium hypochlorite, phenol, boric acid, and sodium hydroxide, were obtained from various suppliers (Pierce; Fisher Scientific, Pittsburgh, PA, U.S.A. and Sigma). Ready-to-use reagents trademarked Fluoraldehyde (OPA reagent from Pierce) and Trione (ninhydrin reagent from Pickering Labs.) were used in the quantitative comparison study.

Columns. The column used in this study was a Perkin-Elmer high-speed amino acid analysis column (120 × 4.6 mm I.D.), packed with 6-μm sulfonated ion-exchange resin. Further details on this column will be published elsewhere[18].

Instrumentation. A schematic diagram of the liquid chromatographic (LC) system used is shown in Fig. 1. The system consisted of a Series 4 liquid chromatograph, equipped with a Model 7125S injector valve, an LC-10 filter fluorescence detector with a 10-μl flow cell (or an LS-1 fluorescence detector), and an LCI-100 computing integrator. For automated sample injection, an ISS-100 automatic sampler was used. Post-column reactions were performed with two Series 10 pumps in conjunction with a RTC-1 temperature control unit. The ninhydrin reaction was monitored with two LC-15B fixed-wavelength detectors (440 and 550 nm). All equipment was from Perkin-Elmer (Norwalk, CT, U.S.A.).

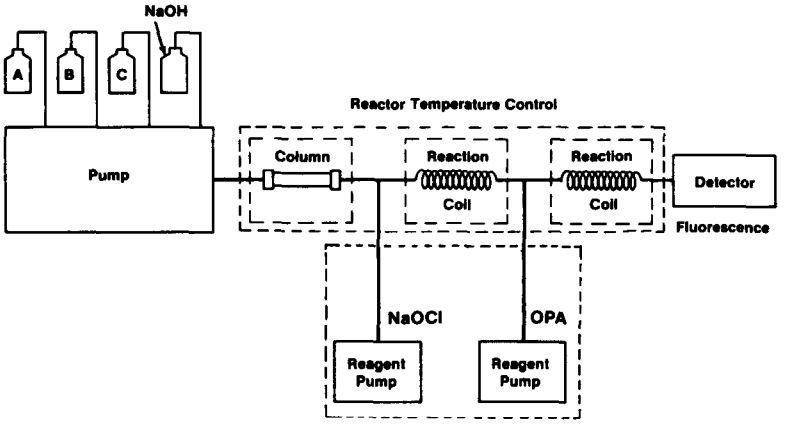

Fig. 1. Schematic diagram, showing the amino acid analysis system based on the sodium hypochlorite–OPA reactions.

Procedure

Reagent preparation. The OPA reagent was prepared by dissolving 800 mg of OPA in 20 ml of methanol and mixing the methanolic OPA solution into 1 l of degassed, filtered 0.3 *M* borate buffer at pH 10.4 in an amber bottle. A 2-ml volume of 2-mercaptoethanol (Pierce) and 2 ml of Brij-35 (30%, v/w, Pierce) were then added. The sodium hypochlorite reagent was prepared by adding 1 ml of sodium hypochlorite (4–6%) to 1 l of degassed, filtered borate buffer at pH 10.4.

Hydrolysis procedure. For the hydrolysis of proteins, the procedure of Jones and Gilligan[19] was followed.

LC Conditions. Standard chromatographic and reaction conditions are summarized below. Deviations from these conditions are listed in the figure legend. Column: Perkin-Elmer high-speed amino acid analysis column, 60°C; chromatographic buffers: Pierce Buffēlute: A (8 min), B (5 min), C (13 min), step gradient, flow-rate, 0.5 ml/min; OPA reagent: 800 mg/l OPA, 2 ml/l mercaptoethanol, 2 ml/l Brij-35 in 0.3 *M* borate buffer (pH 10.4), flow-rate, 0.5 ml/min, 0.5-ml reaction coil, 40°C; sodium hypochlorite reagent: 1 ml/l sodium hypochlorite (4–6%) in borate buffer (pH 10.4), flow-rate, 0.5 ml/min, 0.5-ml reaction coil, 40°C; detection: fluorescence, excitation filter at 360 nm, emission filter at 418–700 nm.

RESULTS AND DISCUSSION

System performance

Analysis speed and resolution. Fig. 2 shows a high-speed separation of hydrolyzate amino acids at the 2.5-nmole level under the standard conditions summarized in the Experimental section. The analysis time is 30 min. Typical cycle time between injections is 45 min. (with 15 min of column re-equilibration at 0.5 ml/min).

Resolution values, defined as: resolution = (peak height − valley/peak height) × 100%, between several critical pairs of amino acids, are often used to judge the performance of ion-exchange columns. These values are found to be as follows: Thr/Ser (97%), Ile/Leu (92%), Tyr/Phe (94%). The resolution of the Thr/Ser pair is

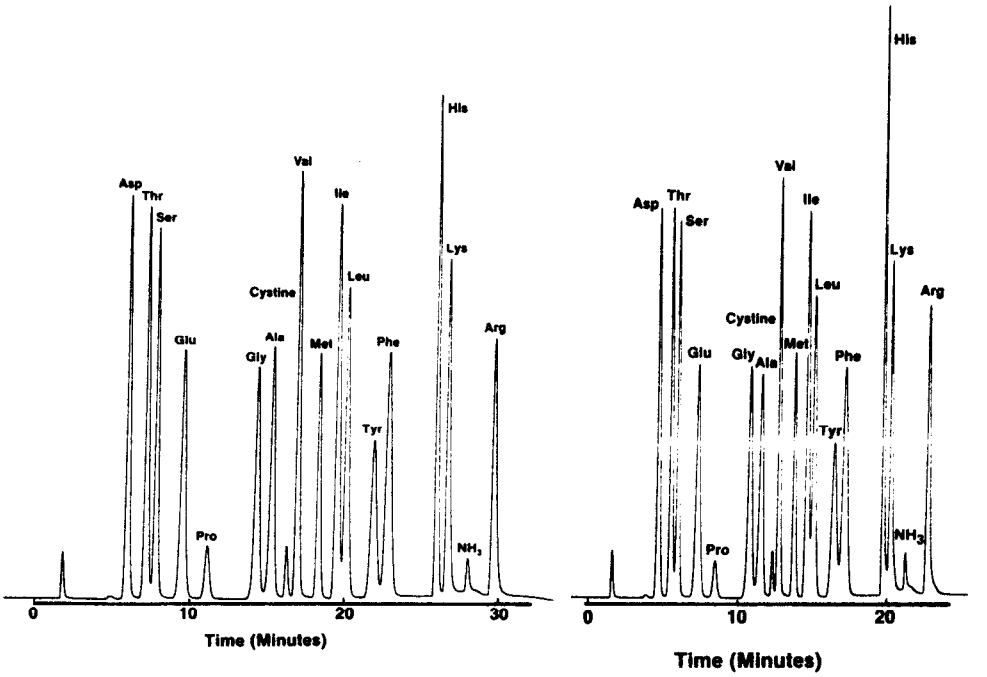

Fig. 2. Typical high-speed analysis of 16 standard hydrolyzate amino acids (2.5 nmoles of each). Conditions are summarized in the Experimental section.

Fig. 3. Analysis of 16 standard hydrolyzate amino acids in 22 min. Conditions as in Fig. 2, except flow-rate is at 0.7 ml/min; A (6 min), B (4 min), C (10 min).

further enhanced by the addition of 2.5% methanol in buffer A (from resolution of 90% to 97%). These resolution values are quite similar to those produced by a longer column (250 mm), packed with 9-μm resins with analysis times in the range of 60–80 min[2].

By increasing the eluent flow-rate from 0.5 ml/min to 0.7 ml/min with proportional changes in the gradient profile, a 22-min separation may be obtained (Fig. 3). Because operation is at a flow-rate higher than optimum, some resolution loss is apparent. However, a 30-min cycle time between injections is possible. These types of high-speed analysis can significantly increase laboratory productivity, and lower labor and buffer costs.

Precision. Table I shows the typical repeatability of retention times and peak heights under the conditions summarized in the experimental section. Based on 12 consecutive repetitive injections, retention time variability ranged from 2.5 to 5.4 seconds (relative standard deviation, R.S.D. = 0.8–0.3%). Average peak height precision was 1.38%, 1.75% and 3.5% R.S.D. for amino acid levels of 500, 100, and 12.5 pmoles respectively. Typical peak area repeatability was found to be 0.5–2% R.S.D. for amino acid levels above 100 pmoles. Peak height data were reported here since they are more accurate at levels below 100 pmoles. These precision values are similar to those of many amino acid analyzers[15].

Sensitivity. In post-column systems, the sensitivity is determined by a number

TABLE I

REPEATABILITY OF RETENTION TIMES AND PEAK HEIGHTS: HIGH-SPEED AMINO ACID ANALYSIS (SODIUM HYPOCHLORITE–OPA)

Peak	*Retention times*		*Peak heights (R.S.D., %)*		
	Min	*R.S.D. (%)*	*500 pmoles*	*100 pmoles*	*12.5 pmoles*
Asp	5.46	0.8	1.4	1.3	1.7
Thr	6.72	0.7	1.5	1.8	2.8
Ser	7.21	0.7	1.3	1.4	2.0
Glu	8.81	0.6	1.5	2.3	4.2
Pro	10.64	0.5	1.4	1.6	–
Gly	13.04	0.4	1.4	2.4	2.0
Ala	14.24	0.4	1.5	2.3	3.2
Val	16.93	0.3	1.6	2.3	4.1
Met	18.49	0.4	1.3	2.2	4.1
Ile	19.80	0.4	1.2	1.3	5.1
Leu	20.47	0.3	1.5	2.1	4.4
Tyr	22.41	0.3	1.8	1.5	5.2
Phe	23.29	0.3	1.5	1.8	3.6
His	25.55	0.5	0.7	1.1	5.0
Lys	26.86	0.3	1.5	1.9	2.8
Arg	30.23	0.3	1.1	0.8	3.2
	Average R.S.D.		1.38%	1.75%	3.5%

of factors, including column void volume, column efficiency, reagent chemistry, detector noise characteristics, pump pulsation, and buffer purity[1].

Fig. 4 shows a background chromatogram, and high-sensitivity analyses of amino acid standards at levels of 25 and 100 pmoles performed with the Buffēlute buffers. At 100 pmoles, all amino acids are clearly detectable. At 25 pmoles all the primary amino acids are detectable with an estimated detection limit of about 2–5 pmoles. To achieve these excellent levels of sensitivity, optimum reaction conditions and high-purity buffers and reagents must be used[1,20]. Additional care must be exercised to prevent buffer contamination by the laboratory atmosphere. Practical details and precautions for achieving high-sensitivity analysis will be published elsewhere[18]. The rising baselines in Fig. 4 are caused by the different fluorescent background in the changing eluents[9]. These effects can be minimized by using more concentrated borate buffers (*e.g.* 1.0 *M*), or can be eliminated completely by baseline subtraction techniques[22].

Linearity. Since primary amino acids are partially converted into non-fluorescent products by the hypochlorite oxidation, of special concern for quantitative analysis is the linearity of the sodium hypochlorite–OPA reaction system. Table II shows linearity data for all amino acids at two ranges (10–500 pmoles and 0.25–10 nmoles). The data show excellent linear correlation of all amino acids (linear correlation coefficients of >0.999) at these concentration ranges. Lys was linear only to 5 nmoles. The cause of the deviation from linearity of Lys above 5 nmoles is not known, but might be related to the concentration of surfactant (Brij-35) used to stabilize the Lys–OPA adduct[23].

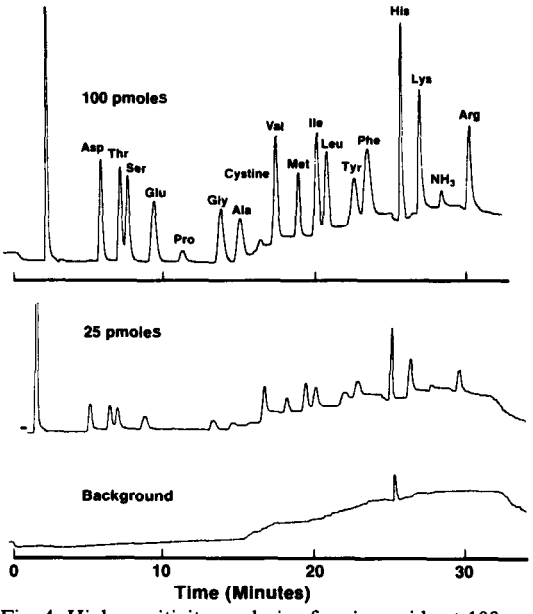

Fig. 4. High-sensitivity analysis of amino acids at 100 and 25 pmoles levels.

TABLE II

LINEARITY DATA: HIGH-SPEED AMINO ACID ANALYSIS (SODIUM HYPOCHLORITE–OPA)

Peak	*Linearity, correlation coefficient (r)*	
	0.25–10 nmoles	*10–500 pmoles*
Asp	0.9989	0.9992
Thr	0.9999	0.9992
Ser	0.9992	0.9991
Glu	0.9992	0.9992
Pro	0.9998	0.9997
Gly	0.9996	0.9994
Ala	0.9989	0.9992
Val	0.9997	0.9994
Met	0.9999	0.9994
Ile	0.9998	0.9995
Leu	0.9997	0.9996
Tyr	0.9999	0.9997
Phe	0.9999	0.9998
His	0.9999	0.9994
Lys	0.9990*	0.9999
Arg	0.9999	0.9999
Average	0.99958	0.99947

* From 0.25 to 5 nmoles only for Lys.

TABLE III

COMPARATIVE QUANTITATIVE DATA: NINHYDRIN *VS.* SODIUM HYPOCHLORITE–OPA

Sample: soy protein hydrolyzate.

Amino acid	*Ninhydrin (nmoles)*	*Sodium hypochlorite–OPA (nmoles)*	*Deviation (nmoles)*	*Deviation (%)*
Asp	13.0	13.3	0.3	2
Thr	4.4	4.35	0.05	1
Ser	7.4	7.3	0.1	1
Glu	17.2	17.9	0.7	4
Pro	7.05	6.9	0.15	2
Gly	7.8	8.0	0.2	2
Ala	7.15	7.3	0.15	2
Cys	0.2	0.2	0.0	0
Val	4.15	4.0	0.15	4
Met	0.35	0.35	0.0	0
Ile	2.0	1.95	0.05	2
Leu	2.45	2.3	0.15	6
Tyr	0.85	0.75	0.01	11
Phe	3.25	3.1	0.15	5
His	3.25	2.85	0.4	12
Lys	6.3	6.3	0.0	0
NH_3	14.85	14.4	0.45	3
Arg	5.25	5.15	0.1	2
			Average deviation	3.3%

Compatibility with commercial buffers and column lifetime. Several commercial buffers and a procedure for preparing citrate buffers[17] were evaluated in the course of the study. In our experience, the Pierce Buffēlutes were found to be the best buffers in terms of resolution, analysis time, and buffer background. The Pierce Pico II and Pickering buffers yielded good resolution and sensitivity at longer analysis time (35–50 min). The laboratory formulation yielded good resolution, though it is not recommended for analysis below 1 nmole, due to difficulties in avoiding contamination. The LKB buffers yield good separation of all amino acids except Glu/Pro.

Column lifetime is an important criterion for system stability and performance. In this study, the column was subjected to continuous testing for a 3-week period. Over 500 analyses were completed. No loss of column efficiency was found at the end of the experiment.

Comparative quantitative data: ninhydrin vs. sodium hypochlorite–OPA. The accuracy of the sodium hypochlorite–OPA method has previously been verified by Böhlen and Mellet[13] analyzing purified peptides. In this study, we demonstrate the correlation between the ninhydrin and the sodium hypochlorite–OPA post-column methods by analyzing the same sample using both techniques. Table III summarizes a comparison of quantitative data between ninhydrin and sodium hypochlorite–OPA. An identical sample of soy protein hydrolyzate was used. Excellent correlation was found between the two techniques with an average deviation of approximately 3%. Fig. 5 shows comparative chromatograms of the soy protein sample. These data, in

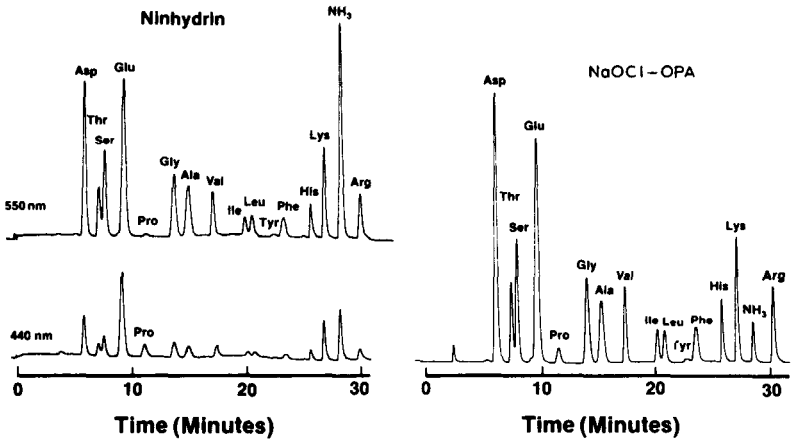

Fig. 5. Comparative chromatograms of a soy protein hydrolyzate sample by the ninhydrin and OPA methods. Chromatographic and OPA reaction conditions are summarized in the Experimental section. Ninhydrin reaction conditions are as follows: trione (ninhydrin): 0.5 ml/min; reaction coil 2.5 ml at 130°C; absorbance detector at 550 nm.

addition to the excellent precision and linearity results, support the accuracy of the sodium hypochlorite–OPA method.

CONCLUSION

This study demonstrates the analysis of the standard hydrolyzate amino acids in 22–30 min with good resolution, column stability, precision, and sensitivity. Using post-column sodium hypochlorite–OPA reactions and purified buffers, analysis in the range of 10–100 picomoles is possible. The system yields accurate quantitative data, as indicated by the excellent precision (< 2% R.S.D.), linearity, and correlation with the ninhydrin method.

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