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Amino acid analysis by liquid chromatography

An overview of five common methods

Today, more and more analysts realize that amino acids can be determined accurately using standard LC equipment. There are, however, questions — questions that plague even experienced chromatographers — which are certain to confuse newcomers, such as: which method to use; whether to use postcolumn or precolumn derivatization; or which derivatization reagents should be used — *o*-phthalaldehyde (OPA), ninhydrin, or dansyl chloride.

To complicate matters further, the best method of amino acid analysis is often dictated by specific application requirements. For example, a method that is useful for a protein chemist who requires accurate quantitation and baseline separation of all essential amino acids might not necessarily be the right method for a food chemist who performs quality control on food products; nor might the same method satisfy the needs of a clinical chemist analyzing picomole levels of amino acids in serum for disease diagnostic purposes. In practice, method selection is, more often than not, based on tradition, personal preference, and popular misconceptions.

The aim of this study, therefore, is to provide an overview of several methods commonly used for amino acid analysis:

Postcolumn:
- ion exchange, ninhydrin
- ion exchange, OPA (hypochlorite)
- reversed phase, OPA

Precolumn:
- dansylation, reversed phase
- OPA, reversed phase.

In the authors' view, the above five methods represent a good cross-section of LC techniques currently in use for routine amino acid assays. Other LC methods are not discussed here for various reasons. Fluorescamine postcolumn detection, as reported by Udenfriend (7), provides good sensitivity for primary amino acids, but it is less convenient and more expensive to run than the OPA method. Schuster (8) reported an easy method involving the separation of underivatized amino acids on an amino bonded-phase column with detection at 200 nm. However, because of poor column resolution and possible interferences at far UV wavelengths, the method might be severely limited in its applications. The analysis of pth-amino acids is extremely important for protein sequence determination (9), although it does not fall into the general category of amino acid analysis discussed in this paper. Finally, thin-layer chromatography (TLC), because of its semiquantitative nature and low resolution, is only useful for screening purposes.

In addition, gas chromatography (GC) of doubly derivatized amino acids, as developed by Gehrke et al. (11, 12), generated considerable interest in the mid-seventies because of its high resolution (13) and picomole sensitivity using either a flame ionization or a nitrogen selective detector. Several fundamental problems, including the extensive sample manipulation required, thermal degradation of some specific amino acid derivatives, and the possibility of multiderivatives formation, has prevented its effective implementation as a routine method.

The present study examines analytical parameters such as resolution, accuracy, sensitivity, and less well-documented factors such as convenience, cost, and limitations. With these goals in mind, the various methods of amino acid analysis were carried out and the procedures and performance of each method were documented. The pros and cons for all the methods are summarized in hopes that these comparative data might be helpful to analysts in their selection of the appropriate methods. Only the final analysis methods will be discussed here because methods for protein hydrolysis, sample cleanup, and extraction have been reported elsewhere (3, 4).

EXPERIMENTAL

The LC columns used were a PE Amino Acid column (250 mm × 4.6 mm), packed with 9-μm sulfonated polystyrene cation-exchange resin; a PE/HS-5 C18 column (125 mm × 4.6 mm), packed with a 5-μm C18 bonded phase support; a PE/HS-3 C18 column (100 mm × 4.6 mm) and a PE/3 × 3 column (33 mm × 4.6 mm), both packed with a 3-μm C18 bonded phase support (all Perkin-Elmer Corporation, Norwalk, Connecticut).

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The Perkin-Elmer Corporation

The LC system consisted of a Series 4 liquid chromatograph equipped with a Model 7125S injection valve, an LC-10 filter fluorescence detector, two LC-15B fixed-wavelength absorbance detectors, and a Sigma 15 chromatography data station. For automated sample injections, either a Model 420B autosampler or an ISS-100 intelligent sampling system were used. Postcolumn ninhydrin reactions were performed using a PCR-1 reagent pump in conjunction with a RTC-1 temperature control unit. Postcolumn OPA with hypochlorite oxidation used the PCR-2 dual reagent pump with the RTC-1 unit (all Perkin-Elmer).

Individual amino acids were obtained from Calbiochem (San Diego, California) and Sigma Chemical Company (St. Louis, Missouri). A prepared mixture of protein hydrolyzate amino acids at a level of 2.5 μmol/ml was obtained from Pierce Chemical Company (Rockford, Illinois).

For mobile phase preparation, HPLC-grade solvents such as CH_3CN, MeOH, and THF were purchased from MCB (Gibbstown, New Jersey). The local water supply was purified using a mixed-bed ion-exchange resin and an activated charcoal filter. Mobile phase additives such as H_3PO_4, CH_3COOH, NaH_2PO_4, l-hexane sulfonate, and sodium dodecyl sulfate were obtained from Fisher Scientific Company (Pittsburgh, Pennsylvania) and Eastman Kodak (Rochester, New York). For ion-exchange chromatography, the Sodium Eluent System from Pickering Laboratories (Mountain View, California) was used.

The reagents *o*-phthalaldehyde, 2-mercaptoethanol, ninhydrin, hydriandantin, and dansyl chloride were available from various companies, including Pierce, Fisher, and Sigma. Prepared reagents trademarked Fluoraldehyde (Pierce) and Trione (Pickering) were also used.

ION EXCHANGE, POSTCOLUMN NINHYDRIN

Figure 1a shows a schematic diagram of a postcolumn setup typically used in a dedicated amino acid system. Several buffers of various pH and salt content and a 0.2 *N* NaOH solution for column regeneration are often used in conjunction with a cation-exchange column packed with sulfonated polystyrene resin. The reagent pump feeds the derivatization reagent into the column effluent stream, which is then heated in a reactor coil; this is followed by on-line absorbance detection.

The equation describing the ninhydrin reaction is shown in Figure 1b. A molecule of amino acid reacts with two molecules of ninhydrin to form a molecule of Ruhemann's Purple with an absorbance maximum at 570 nm. Secondary amino acids such as proline and hydroxyproline form a different complex with an absorbance maximum at 440 nm. Because ninhydrin reacts slowly at room temperature, a reactor temperature of 100–135 °C for 1–2 min is generally used to develop the reaction. Higher reaction temperatures reduce reaction time and also lessen the extracolumn band-broadening effect.

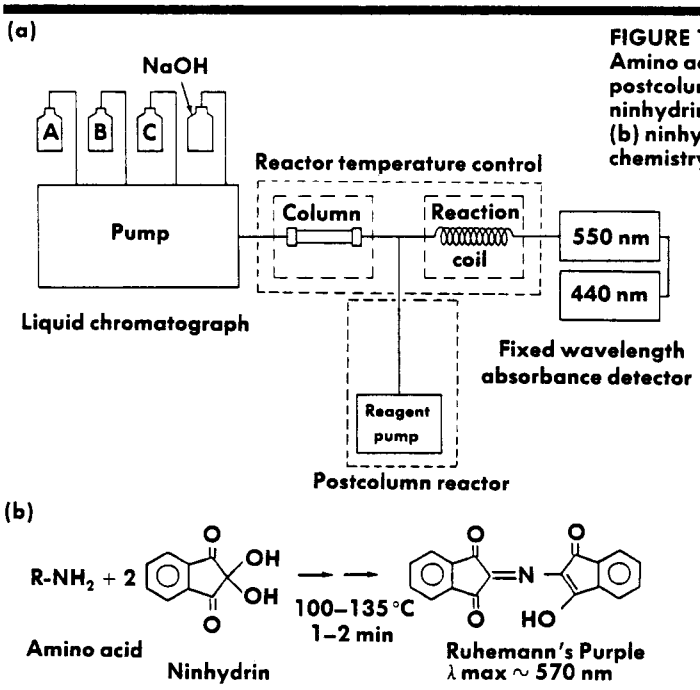

FIGURE 1: (a) Amino acid analyzer, postcolumn ninhydrin system; (b) ninhydrin chemistry.

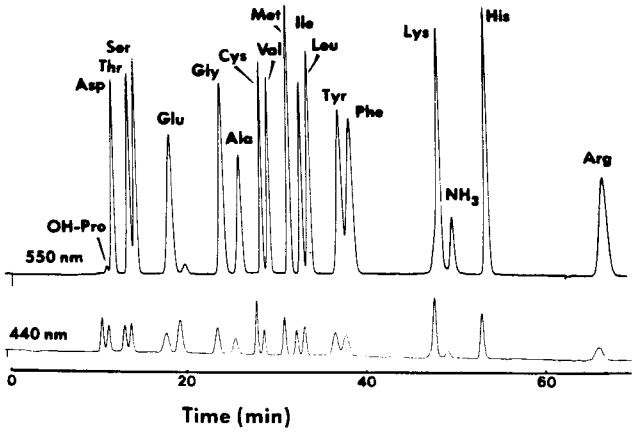

FIGURE 2: Amino acid analysis using postcolumn ninhydrin. Chromatographic conditions as described in text.

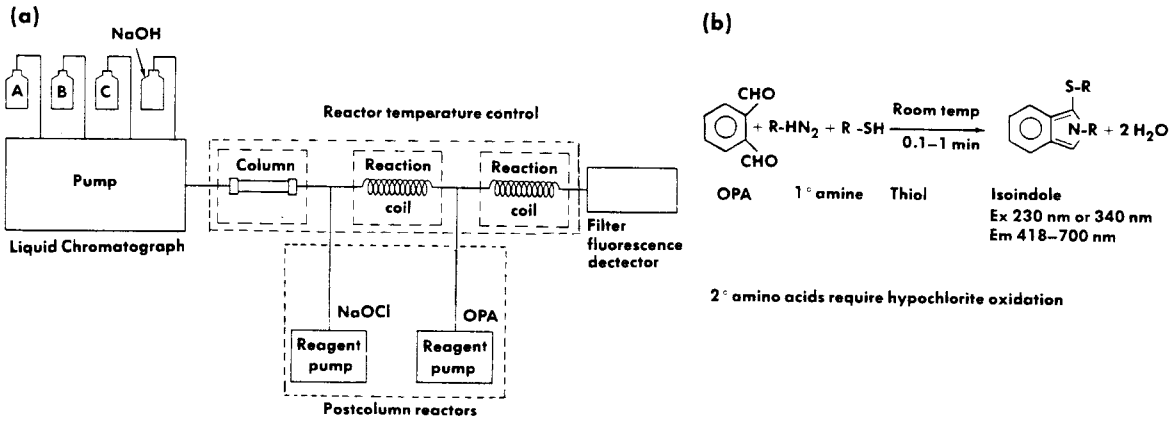

FIGURE 3: (a) Amino acid analyzer, postcolumn OPA (hypochlorite) system; (b) OPA chemistry.

Figure 2 shows a chromatogram of an amino acid standard mixture (10-nmol level). Column conditions were as follows — column: PE Amino Acid (250 mm × 4.6 mm) at 54 °C, packed with 9-μm sulfonated polystyrene cation-exchange resin; mobile phase: Pickering buffers Na 315 and Na 740, flow rate: 0.7 ml/min; Trione flow rate: 0.5 ml/min; reaction coil: 2.5 ml at 130 °C. An excellent resolution is obtainable in a time span of about 60 min. (Resolution >1.0 between Thr/Ser is a good column benchmark). Two monitoring wavelengths are generally used: 550 nm for primary and 440 nm for secondary amino acids.

The precision of the ninhydrin method was determined by repeating the analysis of an amino acid standard solution 12 times at a level of 12.5 nmol per injection. Coefficients of variation (CV) were found to be 0.2% and > 2% for retention times and peak areas, respectively. Excellent linearity for all hydrolyzate amino acids at levels ranging from 0.25–20 nmol were established for the ninhydrin system (coefficients of correlation $0.998 < r < 1.000$ for all amino acids with minimal intercepts). In conclusion, the postcolumn ninhydrin method is the classic method for amino acids, a proved technique that has been widely used for over 20 years. Although the method works well, its predominance is now being challenged by OPA.

ION EXCHANGE, POSTCOLUMN OPA

In the early seventies, Roth first described the fluorescence reaction of *o*-phthaladehyde (OPA) with amino acids (19–20). Since then, OPA has rapidly gained acceptance as an alternative to ninhydrin.

Figure 3b shows the equation in which OPA reacts with thiol and primary amine to form a highly fluorescent indole derivative. The reaction is complete in 0.1–1 min at room temperature under high pH conditions. The technique is ten times more sensitive than ninhydrin, as shown by Benson and Hare in 1975 (21). Secondary amino acids are not detected unless these compounds are first oxidized with an oxidant such as a dilute solution of NaOCl, fed by an additional reagent pump (Figure 3a). One can either add the NaOCl continuously throughout the whole run or pulse the NaOCl only during the elution of proline (22). The continuous addition method has the advantage of producing no baseline disruption (23) and should be the method of choice for routine analysis.

Figure 4 shows a chromatogram of a standard mixture at the 2.5-nmol level using continuous hypochlorite oxidation followed by OPA derivatization. Column conditions were as follows — column: PE Amino Acid (250 mm × 4.6 mm) at 54 °C, packed with 9-μm sulfonated polystyrene cation-exchange resin; mobile phase: Pickering buffers Na 315 and Na 740, flow rate: 0.7 ml/min; NaOCl reagent flow rate: 0.5 ml/min; OPA reagent flow rate: 0.5 ml/min; reaction coils: two 1.0-ml coils at 52 °C; detection: fluorescence. Note the flat baseline and the height of the proline peak. If detector noise were the only constraint, one could easily detect 1 pmol of amino acid; however, contaminants in the buffer limit the sensitivity of the postcolumn method.

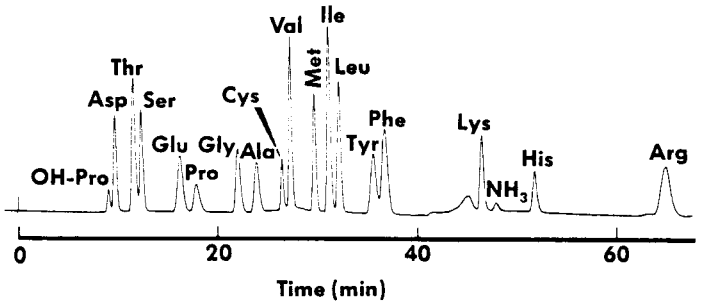

FIGURE 4: Amino acid analysis using postcolumn OPA (hypochlorite). Chromatographic conditions as described in text.

In this study, the precision of the method on retention times and peak areas was found to be less than 0.2% and 2% (CV), respectively (12 runs at 5-nmol level and 20-μl injections). Linearity is extremely important because hypochlorite also destroys primary amino acids and lowers their peak heights and areas. Therefore, in order to maintain good precision and linearity, the levels of NaOCl and thiol, the flow rates of chromatography buffers NaOCl and OPA, and the reaction coil temperature must be controlled precisely. Excellent linearity for all amino acids at levels from 0.1–10 nmol ($0.998 < r < 1.000$) has been established in this study.

REVERSED PHASE, POSTCOLUMN OPA

The third method involves the separation of amino acids on a reversed-phase column using ion-pair chromatography followed by on-line postcolumn detection. This OPA method was proposed by Radjai and Hatch in 1980 (25).

Figure 5 shows a chromatogram of several amino acids using 0.1% 1-hexane sulfonate in the mobile phase. Column conditions were as follows — column: PE/HS-5 C18 (125 mm × 4.6 mm), packed with 5-μm C18 bonded phase supports; mobile phase: solvent A = CH_3CN, solvent B = 0.5% acetic acid, pH 2.8, containing 0.1% 1-hexane sulfonate; flow rate: 1.5 ml/min, initially with 0% A for 2 min followed by 0–10% A in 10 min. Obviously, the polar acids are not adequately resolved. Better resolution can be effected by adding a longer chain ion-pairing reagent such as sodium dodecylsulfate (SDS), as is shown in the upper trace of Figure 5. When SDS is used in the mobile phase, however, column equilibration times become excessive (> 30 min). Because resolution between several amino acid pairs is poor, this method is not recommended at present.

No quantitative studies were undertaken at the time.

PRECOLUMN DANSYLATION

In contrast to postcolumn techniques in which individual amino acids are derivatized in the column eluent, precolumn reactions are performed in a vial with the entire sample mixture. These derivatives are then chromatographed.

One of the oldest precolumn techniques is dansylation, the chemistry of which is shown in Figure 6b. Dns-amino acids chromatograph well on a reversed-phase column at low picomole levels. The drawbacks are long reaction times, high reaction temperatures, and the formation of multiderivatives for basic amino acids.

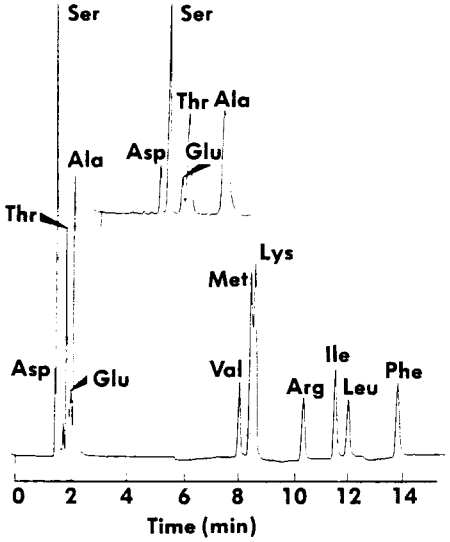

FIGURE 5: Amino acid analysis using reversed-phase OPA. Chromatographic conditions as described in text.

In this study, the procedure of Wiedmeier et al. was followed, using room temperature derivatization at pH 8.5 to minimize the formation of multiderivatives (26). Figure 6a shows the separation of several dns-amino acids. Column conditions were as follows — column: PE/HS-5 (125 mm × 4.6 mm), packed with 5-μm C18 bonded phase supports; mobile phase: CH_3CN/THF/NaOAc (pH 4.18), 5:5:90 to 45:5:50 in 20 min, then to 95:5:0 in 4 min; flow rate: 20 ml/min; detection: absorption at 298 nm.

The derivatization step is easy and can be set up in a short time. The chromatography is more difficult — problems were encountered in the separation of dns-Ile and dns-Leu in the columns used. This pair, however, has been baseline-resolved on different columns by others (27, 28). The quantitation aspects were not studied at length; but, as demonstrated by De Jong (27), dansylation under stringent reaction and chromatography conditions can yield good quantitative data for all amino acids (good correlation between dansylation and postcolumn ninhydrin — less than ±20% — was reported).

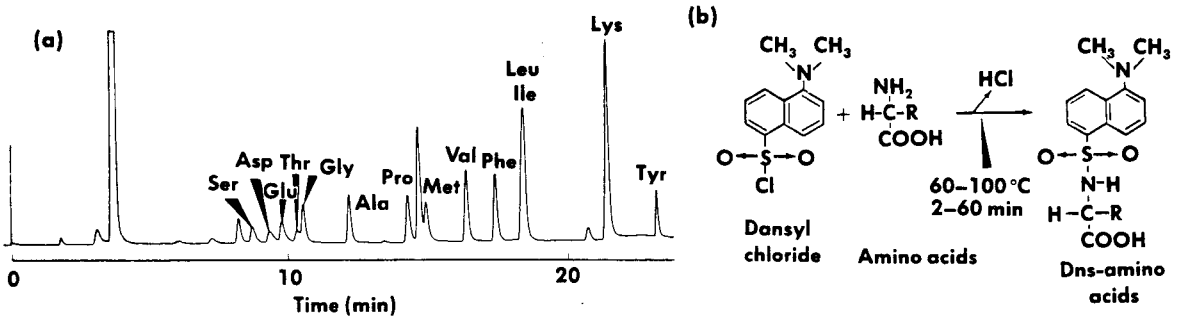

FIGURE 6: (a) Amino acid analysis using precolumn dansylation. Chromatographic conditions as described in text. (b) Precolumn dansylation chemistry.

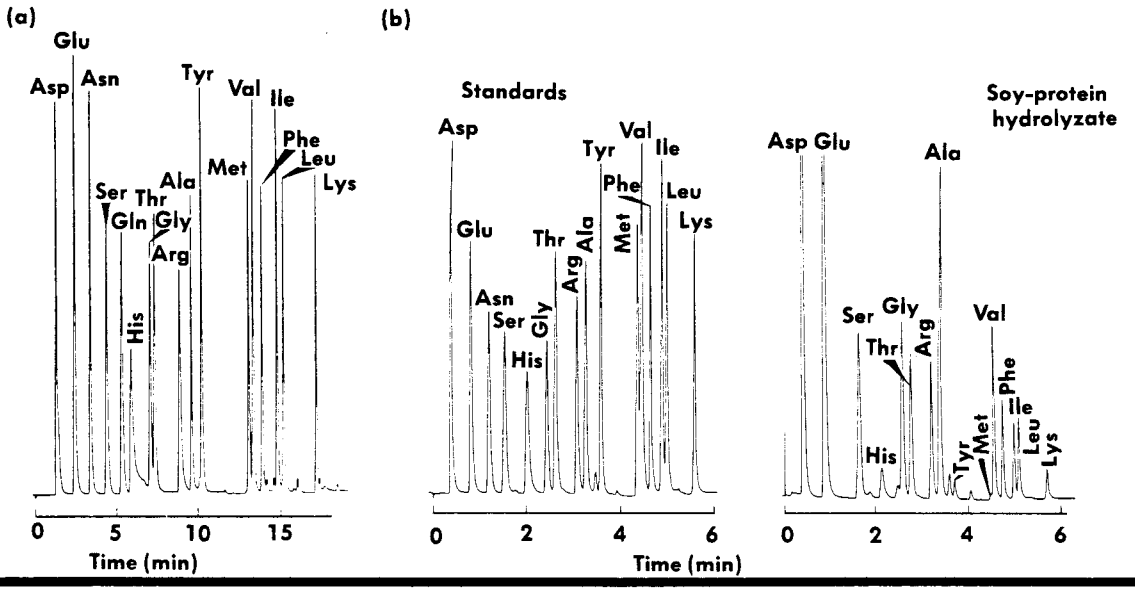

FIGURE 7: (a) Amino acid analysis using precolumn OPA on a 10-cm column. (b) Amino acid analysis using precolumn OPA on a 3-cm column.

PRECOLUMN OPA

In the last several years, considerable efforts have been put into the precolumn OPA technique. In this study, the procedure of Jones et al. was followed, using OPA derivatization with mercaptoethanol (MCE) at pH 9.5 (29).

Figure 7a shows a high resolution chromatogram of 17 amino acids using a 10-cm long, 3-μm column (PE/HS-3 C18). Other column conditions were — mobile phase: NaOAc buffer to MeOH, linear gradient in 16 min; flow rate: 1.3 ml/min; detection: fluorescence. Note the excellent resolution between Val/Phe and Gly/Thr pairs. Proline and cystine, however, are not detected.

Figure 7b shows a similar chromatogram on a 3.3-cm long, 3-μm (PE/3 × 3 C18) column with an analysis time of 5.5 min. Other column conditions were — mobile phase: NaOAc buffer to MeOH, linear gradient in 5 min; flow rate: 2.5 ml/min; detection: fluorescence. This very fast system was used to study the precision, kinetics, and stability of the derivatization. This method is unsurpassed for quick screening of amino acid profiles, as is shown in the chromatogram of soy-protein hydrolyzate (Figure 7b, right). It can be set up in minutes, reaction time is 1 min at room temperature, and >1.0-pmol level of sensitivity can be achieved routinely. Repeatability on retention times and peak areas of less than ±1% and ±5% (CV) were achieved routinely using a manual derivatization procedure (29). Umaget et al. (33) reported method precision of ±0.1% and ±3% on retention times and peak areas under different derivatization and chromatographic conditions. The instability of OPA-MCE derivatives, however, is a fundamental problem. Secondary amino acids and cystine are not derivatized by this procedure. Also, the quantitation of lysine was erratic (±16% CV). Good quantitative data have also been published by Stuart, Hill, Jones, Fernstrom, and Turnell; however, looking at the method as a routine quantitative tool, the consensus seems to be that this method holds promise but needs further refinements (29–32).

TABLE I: Comparison of common methods for amino acid analysis.

	Postcolumn		Precolumn	
	Ninhydrin	OPA/NaOCl	Dansyl	OPA
Accuracy/ Precision	Good	Good	*	*
Analysis Time (min)	30–80	30–80	20–60	5–60
Detection Limits (pmoles)	100†	2†	1–10	1–10
Resolution	Good	Good	**	**
Sec-Amino Acids	Yes	Yes	Yes	No
Limitations	–	–	Multiderivatives	Unstable Derivatives

* Dependent on derivatization procedure.
** Dependent on column efficiency and selectivity.
† Based on the detection limits of aspartic acid (OPA without NaOCl).

CONCLUSION

Table I summarizes comparative data on precision, speed, sensitivity, resolution, and limitations of all methods except the reversed-phase of free amino acids. The sensitivity data represent the best estimated values obtainable under routine conditions using the columns and instrumentation described previously. These data show that postcolumn systems remain the method of choice for accurate quantitation of amino acids. Ninhydrin and OPA-hypochlorite were found to be comparable, with OPA having a definite edge in high sensitivity assays. The data also show precolumn OPA to be extremely attractive in terms of resolution, speed, and sensitivity. This method might be used in food and clinical applications where extreme accuracy is not required.

Equipment cost of a typical modular postcolumn system described here ranges from $30K–40K, excluding autosampler and data-handling device. This compares very favorably to the cost of dedicated amino acid analyzers at $40K–80K. Commercial buffers and reagents are recommended for high sensitivity postcolumn analysis at a cost of $1–3 per assay. The equipment cost of a precolumn analysis system is considerably lower at $10K–20K (pump, injector, and detector) and reagent cost is substantially less. Because the accuracy of the precolumn methods are, in general, dependent upon the derivatization procedures, the user should nonetheless be prepared to expend time and effort in optimizing and validating reaction conditions.

Great strides in amino acid analysis have been made in recent years. The methods that previously were the property of experts and dedicated analysts have been successfully adapted into routine assays that can be performed on standard laboratory liquid chromatographs.

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