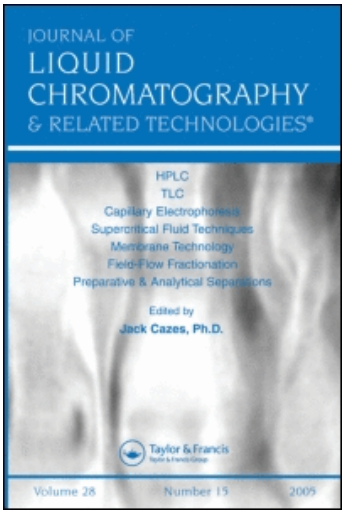

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AMINO ACID ANALYSIS OF PEPTIDES USING HPLC WITH EVAPORATIVE LIGHT SCATTERING DETECTION

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ABSTRACT

A new procedure for the amino acid analysis of peptides has been devised utilizing high performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD). This procedure eliminates the need for complex derivatization schemes inherent of previous amino acid analysis procedures since the ELSD detects the amino acids directly. This quantitative method detects and separates 18 of the common amino acids in a one hour run time using cation exchange chromatography coupled with ELSD. The procedure was tested by analyzing the hydrolysate of human parathyroid hormone 1-34 (PTH), a synthetic polypeptide. A standard digestion consisting of 24 hour hydrolysis at 110°C in 6 N hydrochloric acid with 3% phenol was used. Validation data reveal this is an accurate and precise procedure for the amino acid analysis of peptides.

The sensitivity of this technique is low compared to other state-of-the-art analytical techniques. Detection limits varied depending on the amino acid being analyzed and were as low as 200 picomoles for this new procedure. This approach yields a simple, universal method that is well suited for the amino acid analysis of peptides, of which sufficient quantity is available. Only basic HPLC instrumentation with an ELSD is necessary for this procedure.

INTRODUCTION

Amino acid analysis generally refers to a two-step procedure that quantitates amino acid molecules in proteins and peptides. The first step involves the digestion of a protein or polypeptide into free amino acids. The second step involves the separation of the free amino acids from each other and allows for their subsequent detection. This paper describes a new process of separating and detecting free amino acids from a hydrolysate solution of a peptide using HPLC with ELSD. No attempt was made to improve or to develop a new hydrolysis procedure.

The analysis of amino acids has historically been a complicated task owing to the absence of sufficient visible or ultraviolet chromophores in many of the naturally occurring amino acids. Derivatization has traditionally been performed in order to increase the detectability of the amino acids. In 1958, Spackman, Stein, and Moore described an amino acid analyzer that separated the free amino acids and then subsequently detected them by derivatizing the amino acids with ninhydrin.[1] The system was fully automated, and the derivatization was done on-line after the separation (post-column). Other post-column derivatizing agents have since been reported in the literature.[2-5] The high cost of automated amino acid analysis systems limits their availability in many laboratories.[6] Newer pre-column derivatization techniques avoid the use of costly automated equipment.[3,7-17] These techniques require only an HPLC, which is common in most analytical laboratories. The drawback is that the samples are prepared manually prior to separation, consuming more of the analysts time and introducing more potential error.

The evaporative light scattering detector is potentially useful in applications when ultraviolet (UV) detection is not possible. The theory of ELSD is based on the differences in volatility between the solvent and sample. ELSD operates by nebulizing the volatile effluent from the HPLC column into a fine mist. The mist is carried through a heated drift tube which evaporates the mobile phase and leaves behind non-volatile solute particles. The fine cloud of solute particles is carried at a high speed through a beam of light where the scattered light is detected by a photomultiplier. The amount of light scattered is

dependent upon the size and number of particles and therefore proportional to the concentration. The mobile phase used with ELSD must be free of non-volatiles which would be detected by the ELSD thus limiting its use for some traditional applications of ion-exchange/ion-pairing chromatography. An excellent reference introducing the basic principles and operation of the ELSD has been summarized by Dreux, Lafross and Morin-Allory.[18] In addition, numerous application papers have been published demonstrating the applicability of ELSD for detecting phospholipids,[19-25] triglycerides, fats and fatty acid esters,[26-31] carbohydrates,[32,33] synthetic polymers,[34] steroids,[35] inorganic ions,[36,37] surfactants,[38-40] and pharmaceutical compounds.[41-43]

Separation of underivatized amino acids has generally been accomplished using ion exchange chromatography with sodium or lithium counter ions. These ions are incompatible with ELSDs and cannot be used since they produce a large persistent background due to their low volatility. Recently, the successful separation of 20 underivatized amino acids was reported using a column switching system linking a reversed-phase column and an ion exchange column and subsequent detection with ELSD.[44] A method which separates the amino acids and avoids the use of multiple columns and column switching technology is desirable.

This paper presents a new separation method consisting of only ion exchange chromatography without the need for column switching technology. The system uses a weakly buffered aqueous mobile phase consisting of ammonium salts that are volatile and are compatible with ELSDs. Method development includes the selection of an appropriate analytical column, mobile phase composition, gradient profile, and HPLC parameters such as flow rate and injection volume. Validation studies were performed to thoroughly evaluate the new procedure with respect to linearity, precision, and recovery. Studies to determine the limit of detection (LOD) were also performed to evaluate the sensitivity of the method. PTH was used as the test substance to evaluate the ability of the method to analyze hydrolysates of peptides. The results of the amino acid analyses were compared to theoretical values and to the results from the certificate of analysis of PTH obtained from the manufacturer.

EXPERIMENTAL

Chemicals and Separation Media

All 18 amino acids and phenol (99.5%) were purchased from Sigma Chemical (St. Louis, MO). Ammonium acetate, (A.R.) and trifluoroacetic acid were purchased from Mallinckrodt (Paris, Kentucky). Hydrochloric acid, (ACS, 37.4%) used for the hydrolysis of the samples was purchased from Curtin

Table 1

Gradient Time Profile for the Ternary HPLC System

Time (Min.)	Mobile Phase A (%)	Mobile Phase B (%)	Mobile Phase C (%)
0	100	0	0
12	100	0	0
12.01	20	80	0
25	20	80	0
25.01	10	90	0
35	10	90	0
50	0	0	100
65	0	0	100

Mobile Phase A: 0.075% TFA pH 2.07 with ammonium acetate. Mobile Phase B: 0.1 % TFA pH 3.50 with ammonium acetate. Mobile Phase C: 0.1 M ammonium acetate.

Matheson Scientific (Houston, Texas). Water used was distilled and filtered through a deionizing Milli-Q™ water purification system from Millipore (New Bedford, MA). Human parathyroid hormone 1-34 acetate salt (lot FHPTH1349403), was purchased from Bachem California (Torrence, CA). USP/NF grade nitrogen (99%) was used for the ELSD. The amino acids were separated on a 25 cm x 4 mm I.D. Dionex IonPac® CS-10 cation-exchange column with a 10 cm x 4 mm I.D. IonPac® Dionex CS-10 guard column obtained from Dionex Corporation (Sunnydale, CA).

Analytical Equipment

The HPLC system consisted of a Shimadzu SCL-10A controller, three LC-10AS pumps, SIL-10A auto injector and a DGU-3A membrane degasser from Shimadzu (Kyoto, Japan). A Sedex 55 evaporative light scattering detector was used from Richard Scientific (Novato, CA). The data system consisted of a PE Nelson 900 series interface and a Hewlett Packard 1000 data system.

Chromatographic Conditions

The final operating conditions consist of a cation exchange ternary system with increasing pH gradient steps. The gradient time profile depicted in Table 1

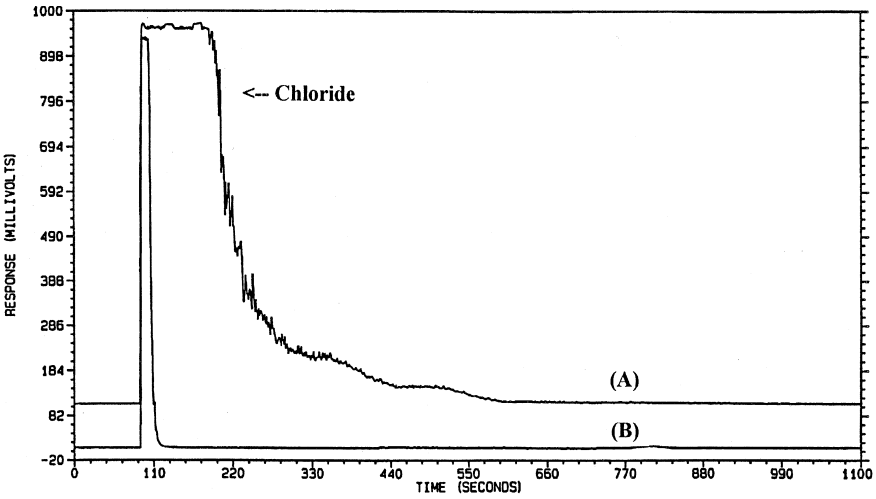

Figure 1. A chromatogram showing the effect of injecting the hydrolysate solution directly onto the HPLC column (A) versus drying the hydrolysate and then reconstituting with mobile phase (B). Baseline interferences are greatly reduced when the hydrolysate is dried.

was followed for the separation of the amino acids. Separation was performed on the column systems listed in the Chemicals and Separation Media section at ambient conditions (20-25°C) with a constant flow rate of 1.0 mL/minute. The injection volume was 10 microliters.

The ELSD was optimized for the greatest signal-to-noise ratio prior to sample analysis. The optimum drift tube temperature was 70°C and the nitrogen flow rate was optimized at 1.5 liters/minute, corresponding to an inlet pressure of 1 bar. A gain setting of 12 was used throughout the study for the ELSD.

Hydrolysis of Sample

PTH is the active fragment of the naturally occurring hormone that has been shown to increase bone density in humans.[45] PTH is a single chain peptide containing 34 amino acids with a molecular weight of 4118 Daltons. PTH is considered a medium sized polypeptide with no rigid three-dimensional structure since no disulfide bridges are present.[46] PTH is composed of 15 of the 20 common amino acids.

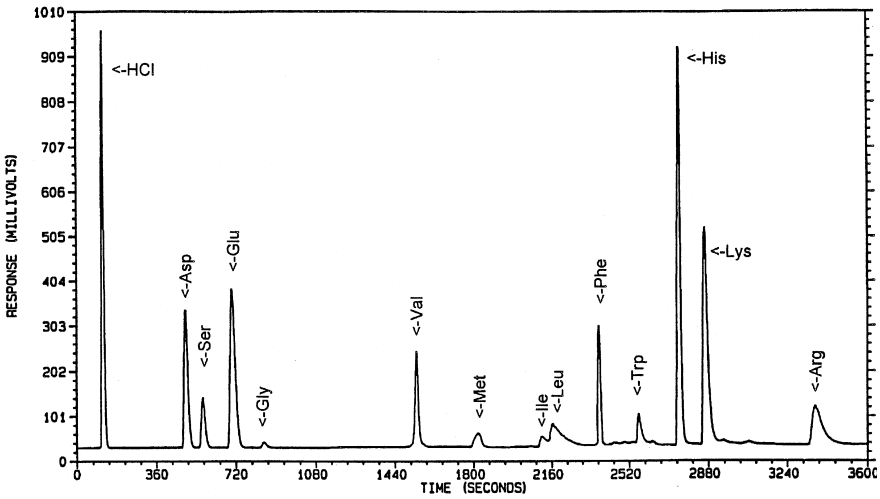

Figure 2. A chromatogram showing the amino acid profile of PTH 1-34 after digestion in 6 N HCl (3% phenol) for 24 hours at 110°C.

A standard, well studied digestion procedure was chosen to hydrolyze the sample into its free amino acids.[47] This digestion involves a typical acid hydrolysis with 6 M HCl heated at 110°C for 24 hours. A 3% concentration (w/v) of phenol was added to the hydrolysate solution to minimize the degradation of tryptophan. Phenol was chosen because it is an ideal additive when using ELSD. Phenol is a volatile additive and will not be detected by the ELSD. Thus it is unnecessary to separate phenol from the amino acids themselves.

The injection of concentrated HCl onto the column results in a massive chloride ion peak that interferes with the early eluting amino acids. It is desirable to have the chloride peak response return to baseline before the first amino acid elutes to make quantitation more reproducible.

Figure 1 shows the result of injecting the concentrated hydrolysate solution directly onto the chromatography system versus drying it prior to injection. The drying process effectively removes nearly all of the HCl from the sample.

A 5 mg sample of PTH was digested in 1.0 mL of the HCl/phenol solution. The samples were then completely dried and then reconstituted with 1.0 mL of mobile phase A (0.075% TFA pH 2.07 with ammonium acetate) prior to injection onto the HPLC system. A sample chromatogram of the reconstituted PTH hydrolysate using the described procedure is shown in Figure 2.

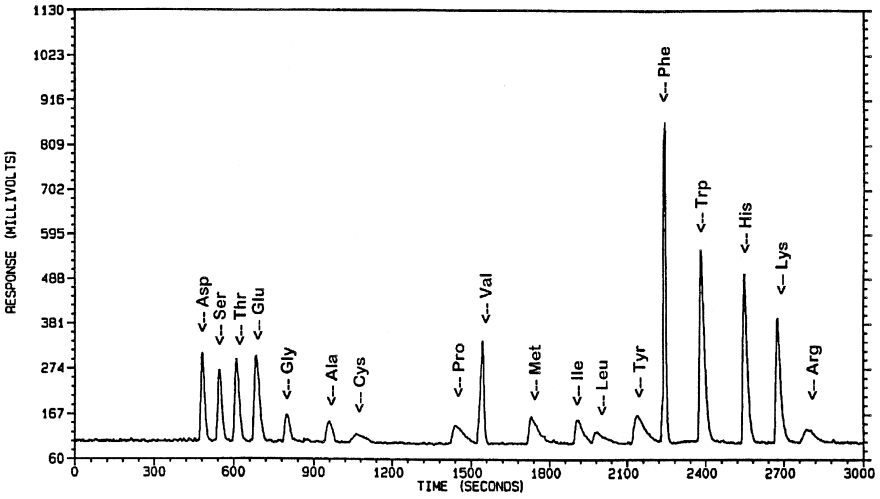

Figure 3. A chromatogram showing the separation of the 18 common amino acids using the final HPLC conditions.

RESULTS AND DISCUSSION

Method Development

Selection of an analytical column was the first step in developing the new method. The Dionex IonPac® CS-10 column produced the best separations, especially for the early eluting acidic amino acids. The column was designed for use with strong acids such as hydrochloric acid instead of sodium and lithium counter ions. The IonPac® CS-10 is a moderate capacity, high efficiency cation exchange column with sulfonic acid functional groups. It is suitable for use with solvents since the substrate is ethylvinylbenzene cross-linked with 55% divinylbenzene. This column is advertised for separation of alkaline earth cations, but works exceptionally well at separating amino acids. Prior to sample analysis the column was converted to the ammonium counter ion form by pumping through a 0.1M ammonium acetate solution for one hour.

The ternary gradient profile was optimized to achieve baseline separation of the 18 common amino acids in a single injection. Since an acid digestion is performed, no attempt was made to separate asparagine or glutamine. The digestion will convert these entities to their acid forms. The final gradient follows the general logic of the older post-column methods that use four step increasing pH gradients. This system consists of four changes in pH starting at

Table 2

Linearity Data for Each of the 18 Common Amino Acids

Amino Acid	Number of Points in Curve	Correlation Coefficient
Asp	11	0.9991
Ser	11	0.9975
Thr	10	0.0064
Glu	11	0.9976
Gly	9	0.9952
Ala	10	0.9862
Cys	6	0.9975
Pro	13	0.9973
Val	10	0.9990
Met	13	0.9916
Ile	9	0.9947
Leu	9	0.9812
Tyr	13	0.9903
Phe	13	0.9972
Trp	13	0.9940
His	13	0.9946
Lys	12	0.9968
Arg	9	0.9867

pH 2.07 and increasing to a final pH of approximately pH 6.8. The four step gradient is achieved with a ternary system by mixing portions of the different mobile phases in specific ratios to create the desired pH. It was necessary to have step gradients as opposed to gradual transition gradients to lock the amino acids into one charged state. The amino acids tail or produce split peaks if a gradual gradient is used during the first two pH changes. Figure 3 shows the separation of the 18 common amino acids using the newly developed method.

Method Validation

The validation examines the parameters of linearity, limit of detection (LOD), recovery and precision of the amino acids using the newly developed method.

The linearity of this method was evaluated by injecting 13 samples that were serial dilutions from a stock solution of the 18 amino acids to locate the working range. The samples represented a concentration range of

Table 3

Limits of Detection (LOD) for Each of the Common 18 Amino Acids

Amino Acid	LOD (Picomoles)
Asp	750
Ser	750
Thr	1000
Glu	750
Gly	2500
Ala	1000
Cys	7500
Pro	250
Val	1000
Met	200
Ile	2500
Leu	2500
Tyr	200
Phe	200
Trp	250
His	200
Lys	500
Arg	2500

approximately 25-7500 nmol/mL. The linearity was determined by plotting the log of peak area versus the log of concentration. A complete list of the data are tabulated in Table 2. Overall, the linearity was good with correlation coefficients between 0.9862 and 0.9991 over a wide concentration range. The values get consistently higher (0.999) when the concentration range is reduced by an order of magnitude.

The LOD is defined as the lowest concentration of sample that can be clearly detected above the baseline noise. Typically this value is estimated at 2-3 times the level of baseline noise. For this method, the limit of detection was determined experimentally for each of the 18 amino acids. The samples were purposely prepared at concentrations that would most likely not be detected. The LOD is determined by the first visible sign of the amino acid above and beyond baseline noise. The LOD is reported as the amount of amino acid injected onto the column and is reported in picomoles (pmol) to stay consistent when comparing other techniques that have been published in the literature. Table 3 lists the LODs for each amino acid. With the exception of cysteine, the overall the range of LODs was <250 to 2500 picomoles. A correlation between the shape of the peak and the detection limit was observed. In general, the

Table 4

Recovery Data for Each of the Amino Acids in PTH 1-34 Following Exposure to 6N HCl* for 24 Hours at 110°C

Amino Acid	RSD%	% Recovery
Arg	2.55	97.7
Asx	2.55	89.2
Glx	3.23	87.3
Gly	1.72	103.7
His	2.70	107.1
Ile	2.00	91.6
Leu	2.06	84.3
Lys	2.55	101.1
Met	16.22	69.1
Phe	1.51	105.3
Trp	61.37	46.3
Ser	1.55	78.2
Val	3.13	94.8

* with 3% Phenol. n = 5.

sharper the peak the lower the LOD. No explanation is presented as to why cysteine has a higher LOD than the other amino acids. Overall, the limits of detection for ELSD are higher than other reported techniques. Pre-column techniques have LODs of approximately 100 fmol to 20 pmol and post-column techniques have LODs of approximately 50-100 pmol.[48,49] It appears that ELSD is less sensitive than either pre-column or post-column derivatization methods. This may limit the use of ELSD systems when highly sensitive analyses are required.

Recovery of the amino acids that make up PTH was evaluated following their exposure to the acidic digestion solution. Five separate samples containing approximately 2000 nmol/mL of each amino acid were digested in the 6 N HCl solution with phenol at 110^{o}C. After 24 hours the samples were dried and then 1.0 mL of mobile phase was added to the vial. Upon reconstitution the samples were then injected onto the column. A four point standard curve was freshly prepared and used to calculate the recovery of the amino acids following the 24 hour acid digestion. The standard curve had an approximate concentration range of 400 to 4000 nmol/mL for each of the amino acids. The correlation coefficients for the amino acids range from 0.9879 to 1.0000. The data was plotted by taking the log of the peak areas versus the log of the standard concentrations. The recovery of each of the amino acids is listed in Table 4.

Table 5

Errors Associated with the Amino Acid Analysis of PTH 1-34 Using ELSD*

Amino Acid	Precision of Instrument (RSD%)	Precision of Method (RSD%)	Precision of Retention Time (RSD%)
Asx	2.65	4.06	0.42
Ser	4.90	6.97	0.29
Glx	2.93	4.02	0.08
Gly	3.59	2.20	0.17
Val	2.93	5.37	0.03
Met	2.56	14.99	0.50
Ile	3.80	3.54	0.39
Leu	2.31	1.21	0.21
Phe	2.84	5.25	0.47
Trp	1.99	84.12	1.45
His	2.41	6.97	0.13
Lys	1.66	7.85	0.12
Arg	3.22	5.70	0.11

* n = 5.

The precision was evaluated in three ways. First, five replicate injections of a single digested PTH sample were processed to determine the reproducibility of the peak areas of the method. This determines the error of the method that is attributed to the equipment and the HPLC gradient conditions apart from analyst error other than that associated with data integration. Second, five separate samples of PTH were individually digested, then processed to determine the overall precision of the method. Finally, the precision of the retention times was determined from the five individually digested samples. The retention times are defined at maximum peak height.

Table 5 summarizes the error associated with each of the amino acids in PTH. The error was reported as the relative standard deviation (%RSD) which is the standard deviation divided by the mean times 100. The error using ELSD for amino acid analysis appears to come equally from the digestion process and the instrument. The amount of instrument and gradient error associated with the five replicates of the single sample was approximately 2-5%. The precision of the five separately prepared samples ranged from 1.21% to 84.12% RSD. Disregarding the precision for tryptophan (84.12%) and methionine (14.99%) due to their instability in the acidic media, the rest of the amino acids ranged from approximately 4-7% RSD. Standard errors associated with post-column

derivatizations are approximately 4-9%.[50-52] Errors with ELSD are comparable to post-column derivatizations using commercial amino acid analyzers. The precision of the amino acid retention times ranged from 0.03% to 1.45 % for the five injections.

Comparison to Theory

Five separate samples of PTH were individually digested, then evaluated to determine the amino acid stoichiometry of the molecule. The standard curve consisted of four standards that bracketed the concentration range of the amino acids following the digestion. The standard curve represented a range of approximately 400-4000 nmol/mL of each amino acid. The samples were bracketed by a set of standards both before and after the sample injections. All data including peak areas and concentrations were converted to a log scale before data analysis. The samples were calculated from the standard curve and then converted back to nmol/mL. The sample results were then converted to moles by dividing by the molar concentration of sample which was diluted to 1.0 mL. Once the number of moles have been calculated, a normalization calculation is performed. Normalization involves dividing all of the amino acid results by a factor that is determined by the glycine result. The normalization factor is determined by the average percent recovery of glycine (n=5) of the digested samples based on theoretical stoichiometry. For this experiment the normalization factor was determined to be 0.8734 for PTH. This factor corrects for the known water and acetate in the sample. At this point recovery factors for all the amino acids are applied based on the recovery results in Table 4. The results are then reported as the average of the five samples.

Table 6 directly compares the amino acid analysis results obtained from ELSD with the manufacturer's (Bachem, California) certificate of analysis (COA) and to the theoretical amount of amino acid residues. The results from ELSD appear to compare favorably to those generated by Bachem California and to theory. Bachem's results were generated using a commercial amino acid analyzer which used ninhydrin as a post-column derivatizing agent. A statistical comparison of means was not performed because too few samples were run. Additionally, RSD data was not available from Bachem California. The overall recoveries for the amino acids using ELSD were approximately 90-110% of theory. Several of the tryptophan results were inconsistent possibly due to partial degradation during acid hydrolysis. This was observed in the validation of recovery samples as well as with Bachem's results.

The results generated with ELSD have shown that this technique offers a viable alternative to other traditional techniques that require derivatizations without compromising accuracy. The versatility of an ELSD lends itself to a variety of other uses.

Table 6

A Comparison of the Amino Acid Analysis Results of the Same Lot of PTH 1-34*

Amino Acid	Theory	ELSD	Bachem COA
Arg	2	2.02	1.95
Asx	4	3.91	4.16
Glx	5	4.83	5.22
Gly	1	1.00	0.97
His	3	3.10	2.77
Ile	1	1.04	0.89
Leu	5	5.24	5.44
Lys	3	2.85	3.02
Met	2	1.87	1.90
Phe	1	1.11	0.95
Trp	1	0.58	0.62
Ser	3	2.69	2.74
Val	3	2.97	2.74

* Results obtained with ELSD are compared with the manufacturer's (Bachem California) Certificate of Analysis (COA) and to theory.

ELSD can be used to determine levels of amino acids in matrices other than hydrolysates. Samples of free amino acids in solution such as urine or blood can be run assuming appropriate sample preparation steps are taken and adequate concentrations are present.

There are several obvious advantages for using ELSD. It is always desirable to detect analytes directly since it saves time and reduces the potential for error. The amount of time an analyst spends preparing samples and setting up systems is significantly reduced. An ELSD is considerably less expensive than a commercial amino acid analyzer. Also, perturbations from step gradients and reagent residues cause no interferences in the chromatography when using an ELSD. Another important advantage is that mobile phases used with ELSDs are completely compatible with LC/MS systems. Since only volatile buffers are used, no further method development is necessary. Scientists are often studying post modifications of amino acids in which hundreds of oxidized or methylated entities are possible. These modifications are often studied with mass spectrometry detection because the resulting entities can not be adequately separated from other common amino acids. The separation conditions described in this paper provide a starting point for the analysis of atypical amino acid modifications using MS detection.

CONCLUSIONS

A simple universal method which separates all of the 18 common amino acids from a hydrolysate solution has been developed and validated using HPLC with ELSD. This new method offers a greatly simplified alternative to the older amino acid analysis techniques which require derivatizations. Basic HPLC instrumentation and an ELSD are all the equipment required for this method. The accuracy and precision are comparable to other traditional methods. The use of this new procedure, however, may be limited when highly sensitive analyses are required. The HPLC conditions are also compatible with LC/MS systems.

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