AMINO ACID ANALYSIS: HYDROLYSIS, COLOR REAGENT, AND SENSITIVITY

by

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B. S., Kansas State University, 1977

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Grain Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1981

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ACKNOWLEDGEMENTS

I wish to thank my wife Patsy for her love, understanding, and tremendous help during this time period.

I also wish to thank Dr. Lynn S. Bates for his guidance.

INTRODUCTION

Automated ion-exchange chromatography is today the most widely used method for amino acid determinations. There have been improvements in resin technology and instrumentation, but research is still needed to improve current techniques.

One major detection problem is proline quantification. Proline, an imino acid, reacts with ninhydrin to yield a yellow colored compound instead of the purple complex that is formed by the reaction of the α-amino acids. The yellow color has an absorbance maximum that is different from the purple complex. One objective of this study was to develop a color reagent that will allow detection of both imino and α-amino acids at one wavelength.

There is increasing demand for more sensitive detection of amino acids. Analysis of very low levels of amino acids is of particular importance in specialized research where amino acids and proteins occur in low concentrations. A portion of this study was devoted to the development of methods to optimize color yield and thereby increase analyzer sensitivity.

Another important step in amino acid analysis is hydrolysis. Hydrolysis with HCl is the most common method used. Alternate hydrolysis methods need to be investigated for routine analysis of food and feed samples. The third portion of this study was timed hydrolysis of a cereal product to compare HCl and p-toluenesulfonic acid hydrolysates efficacy and amino acid destruction.

LITERATURE REVIEW

Color Reagents for Amino Acid Analysis

The detection of amino acids is most commonly done with ninhydrin (2,2-dihydroxy-1,3-indandione). Ninhydrin decarboxylates and deaminates the α-amino acids in stoichiometric amounts, to form carbon dioxide and an aldehyde with one less carbon than the α-amino acid. Ammonia, hydrindantin, and Rhuhemann's Purple (diketohydrindylidene-diketohydrindamine) are produced from this reaction in variable amounts. Rhuhemann in 1910 and 1911 discovered the reaction of ninhydrin with amino acids and proposed a possible reaction mechanism (McCaldin, 1960). Since that time many different mechanisms have been postulated. The currently most accepted mechanism is given in Fig. 1. (Bottom et al., 1978). Ninhydrin (1) is shown to tautomerize to 1,2,3-indantrione (2) which forms a Schiff's base with the amino acid. The ketimine formed (3) decarboxylates, yielding an aldehyde and the intermediate 2-amino-1,3-indandione (4). Condensation of this intermediate with another molecule of ninhydrin forms Rhuhemann's Purple. There is still controversy as to the exact role of hydrindantin (reduced ninhydrin) in the reaction mechanism (Lamothe and McCormick, 1973).

Moore and Stein (1948) were the first to adapt the ninhydrin reaction to quantitative determination of amino acids. In all previous work the color development had been carried out in tubes exposed to air. They felt that dissolved oxygen present in the reagents was responsible for the non-linear yields. By developing the color in tubes evacuated to 20 mm the relationship of color yield to amino acid concentration was found to be more linear. They experimented with adding either hydrindantin or a strong reducing agent to the reaction medium. Both were found to completely block the oxidative side reaction and give reproducible linear results. At that

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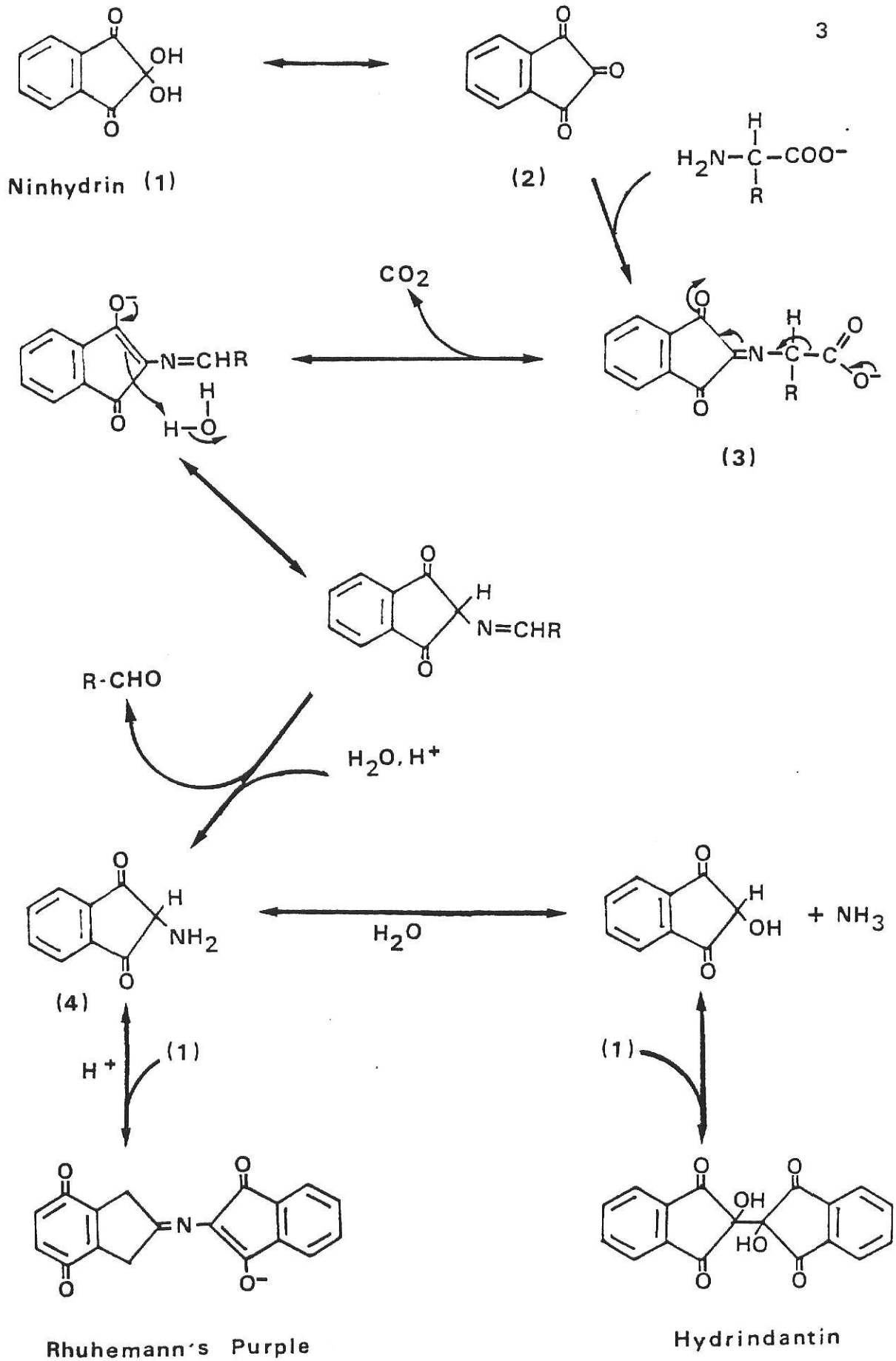


Fig. 1. Mechanism of the Ninhydrin Reaction

time there was not a source of crystalline hydrindantin so stannous chloride was added to the reaction medium to reduce ninhydrin to hydrindantin in situ.

Later in 1954, Moore and Stein returned to adding hydrindantin directly to eliminate the precipitation of tin salts. They also changed from a sodium citrate to a sodium acetate buffer to increase buffering capacity. The concentration of methyl cellosolve (the monomethyl ether of ethylene glycol) was increased to prevent hydrindantin precipitation. This reagent (2% ninhydrin, 0.3% hydrindantin in 3:1 methyl cellosolve and 0.4 M pH 5.5 sodium acetate buffer) when reacted with amino acids at 100°C yields a purple complex, absorbance maximum (Amax) 570 nm, with α-amino acids and a yellow complex, Amax 440 nm, with imino acids. Each amino acid produces a different amount of color per mole, therefore a separate color factor was used for each amino acid to obtain quantitative values.

Spackman, Stein and Moore (1958) developed the first automatic amino acid analyzer. Their 1954 fraction collector ninhydrin reagent was adapted for use in this instrument. Hydrindantin tended to precipitate at the 25% methyl cellosolve level that resulted when 2 parts effluent were mixed with 1 part ninhydrin reagent. The decreased level of oxygen in the automated system allowed hydrindantin to be used at 0.55 g per liter which eliminated precipitation, but the reagent was stable for only two weeks. A more stable reagent was formed by replacing hydrindantin with stannous chloride. At 0.4 g stannous chloride dihydrate per liter the reagent could be stored for one month without deterioration.

This original reagent is currently being used in many automatic amino acid analyzers. The precipitation problem with stannous chloride described by Moore and Stein (1954) is still present. Other reducing agents such as titanous chloride (James, 1971 and 1978) and sodium borohydride (Takahashi, 1978) have been used to replace stannous chloride. Both report equal or

better stability and color yield without the precipitation problem. Other methods have proposed adding the reductant to the eluent buffers (Knight, 1968, Niece, 1974, and Schwerdtfeger, 1962). An air stable ninhydrin reagent can then be used that is freshly reduced when mixed with column effluent. Kirschembaum (1965) substituted a portion of dimethyl sulfoxide (DMSO) for methyl cellosolve and reported very little precipitation with a 60% methyl cellosolve and 40% DMSO mixture. Moore (1968) completely replaced methyl cellosolve with DMSO and stannous chloride with hydrindantin. In order to dissolve all reagent constituents, he changed the sodium acetate buffer to a 4 M pH 5.2 lithium acetate buffer. Hydrindantin is 1.5 times more soluble in DMSO than in methyl cellosolve therefore precipitation is eliminated. DMSO does not contain peroxides which are present in methyl cellosolve. Lack of peroxides gives an increase in stability and color yield. Moore suggested the teflon tubing, which is permeable to oxygen be replaced with saran tubing.

Lamothe and McCormick (1972) studied the optimum pH value for the ninhydrin reaction. They found a pH of 5.0 to 5.2 produced optimal color yields in either methyl cellosolve or DMSO. Friedman and Williams (1974) looked at the stability of Rhuhemann's Purple (RP) under various conditions. They found RP to be stable in the pH range of 7.0 to 14.0 in methyl cellosolve, but RP is destroyed in the acid pH range by acid hydrolysis. When DMSO was substituted for methyl cellosolve, RP was found to be 10 times more stable in the acid pH range.

To further increase sensitivity in amino acid analysis the production of fluorescent compounds using fluorescamine and o-phthalaldehyde has been used for fluorescent amino acid quantification (Undenfriend et al., 1972 and Roth, 1971 and Roth and Hampai, 1973). Fluorescamine reacts with primary

amines at an alkaline pH and must be made and stored in acetone since it is unstable in water. o-Phthalaldehyde is stable in water but requires an alkaline pH and the presence of 2-mercaptoethanol for fluorescence development. Benson and Hare (1975) compared the sensitivity of ninhydrin, fluorescamine, and o-phthalaldehyde. They reported ninhydrin and fluorescamine to be equal in sensitivity, but o-phthalaldehyde to be 5 to 10 times more sensitive. However, these two compounds react only with primary amines and not imino acids such as proline.

Ninhydrin, fluorescamine, and o-phthalaldehyde are not good color reagents for proline detection. There is a need for a color reagent that will allow detection of proline at the same wavelength as the other amino acids. A possible color reagent is isatin. Isatin has been used in quantitative determination of proline by paper chromatography (Barrollier et. al., 1956 and Hrabětová and Tupý, 1960). The reagent used by Barrollier and his associates produced a color complex that had an Amax at 610 nm and followed Beer's law. In their study, proline was analyzed in a range of 5 to 20 ug with an accuracy of $\pm$ 2.5%. Isatin has promise because its Amax is very close to the 570 nm Amax of ninhydrin. This would allow a simple colorimeter to be used if a mixed reagent of ninhydrin and isatin were developed and their absorbance maxima peaks overlapped.

Sensitivity

Increased sensitivity in amino acid analysis has been achieved by reducing resin bed cross-section area and resin bead diameter, and by increasing the optical path length of the flowcell (Hare, 1977). There has also been experimentation with increasing the reaction bath temperature. The ninhydrin reaction is carried out in a post-column, teflon tube

reaction coil immersed in boiling water. The maximum color develops in 8 to 15 minutes at 95 to 100°C in open-ended coils (Spackman, Stein, and Moore, 1958). Anderson and his associates (1968) found that if the reaction coil is kept under high pressure the temperature can be raised to 135°C and maximum color development will occur in 1.3 minutes. A shorter color development time allows the use of a shorter reaction coil which will reduce post column mixing and peak broadening and in that way increase sensitivity.

Jonker and associates (1978) compared the color yield to reaction temperature with a reaction time of one minute (Fig. 2). The results show a maximum color yield at 140 to 150°C for the three amino acids used. This is consistant with findings by Anderson, et al. (1968). Conducting the reaction at 140°C will allow quantification at the maximum portion of the color yield curve instead of on the ascending portion. Jonker and his co-workers used a very elaborate system utilizing an air-thermostat of a liquid chromatograph (Perkin Elmer 1220) to control the temperature between 100° and 150°C.

Hydrolysis

With the increased precision in amino acid analysis the limiting factor in the determination of the composition of a protein is the extent to which the composition of the hydrolysate accurately represents the amino acid composition of the parent protein. A truly representative hydrolysate is difficult to obtain in practice due to the degradation of the labile amino acids while liberating the more slowly released amino acids.

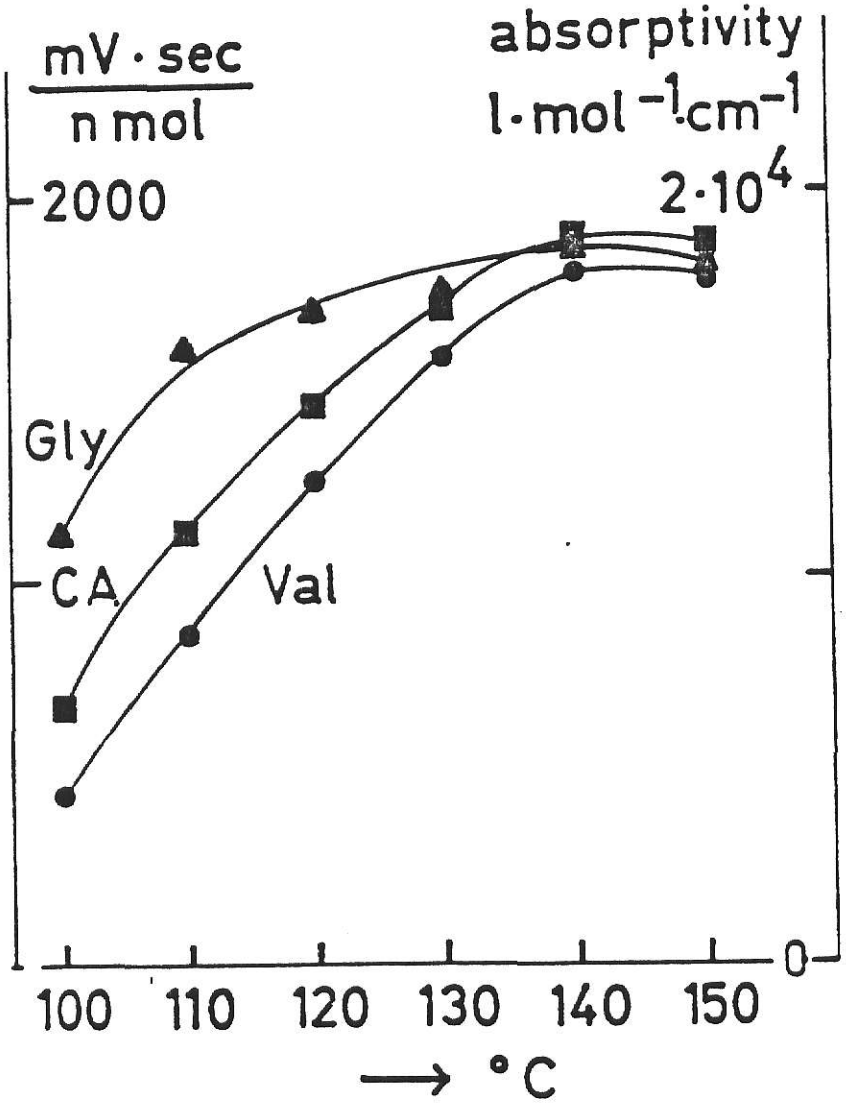


Fig. 2. Dependence of sensitivity on reactor temperature for reaction time of one minute (Jonker et al., 1978).

Hydrochloric acid is the most extensively used hydrolysis reagent. Moore and Stein (1963) describe its use with sealed pyrex tubes. A 5 mg sample of air-dried or lyophilized protein is weighed into a 16 x 125 mm heavy walled pyrex test tube. One mililiter of 6 N HCl is added to the tube. A section of the tube about 3 cm from the top is constricted with an oxygen flame to about a 1 mm bore. The lower half of the tube is inserted in a bath of solid carbon dioxide and ethanol to freeze the sample. When frozen, the tube is connected to a vacuum line and evacuated with an oil pump to 60 um. The tube is removed from the dry ice bath and the frozen solution is allowed to thaw slowly with the pump still on. As the bubbles form and rise in the tube, it is immersed momentarily in the dry ice bath to break the bubbles and allow the liquid to drain back. During this degassing process the pressure increases to about 80 um. When the pressure is back down to 60 um the tube is shaken to insure complete gas removal and then sealed. The hydrolysis is conducted at $110^{\circ}C \pm 1^{\circ}C$ for 20 or 70 hours. The temperature must be accurately controlled during hydrolysis. Following hydrolysis the tube is cooled to room temperature and centrifuged to remove any liquid from the walls. Then it is scored with a file at a point below the tapered end, cracked by the use of a hot glass rod and the cut end fire-polished. The hydrolysate is transfered to a round bottom flask and the HCl is removed under vacuum at $40^{\circ}C$ with a rotary evaporator. The sample residue is dissolved with water and taken to dryness again to insure complete removal of the HCl. The residue is transferred quantitatively to a 5 ml volumetric flask and brought to volume by the use of pH 2.2 buffer containing 5 ml per liter of thiodiglycol. The diluted sample is then filtered by vacuum filtration through Whatman No. 52 filter paper to remove insoluble humin. Aliquots of the filtrate

are then used for amino acid analysis (Thachuk and Irvine, 1969).

Another method of HCl hydrolysis refluxes the sample in excess HCl (Davies and Thomas, 1973). The sample (100 mg) is refluxed in a 150 ml flask with 100 ml 6 N HCl (distilled in an all glass apparatus). The flask is immersed in an oil bath to a depth where the liquid level is just above the oil level. Hydrolysis is conducted at 137°C ± 2°C and oxygen-free nitrogen is bubbled continuously through the liquid. Samples are hydrolyzed for 20 to 96 hours. After hydrolysis the samples are filtered through a G4 sintered glass filter and washed with 250 ml distilled water. The hydrolysate is made up to 500 ml. A rotary evaporator at 40°C is used to take a 25 ml aliquot of the hydrolysate to dryness. The residue is dissolved in 5 to 10 ml of 0.1 N HCl depending on the protein content of the sample.

During acid hydrolysis, threonine and serine are progressively destroyed (Rees, 1946). Their decomposition varies according to the protein being hydrolyzed and to unknown factors (Noltmann et al., 1962). As hydrolysis time is lengthened, aspartic acid, glutamic acid, proline, and arginine appear to be decomposed to a small but measurable degree and there is substantial decomposition of cystine and tyrosine (Hirs, Stein, and Moore, 1954). In the absence of air, tryptophan is relatively stable during acid hydrolysis except in the presence of serine, hydroxyproline, cystine, and carbohydrates (Gruen, 1973). Gruen believed the major factor in the extensive destruction of tryptophan is the degradative oxidation by cystine to cysteine. Oxidation during acid hydrolysis may lead to the formation of significant amounts of cysteic acid from cystine, methionine sulfoxide from methionine, and chlorotyrosine from tyrosine if the tubes are not evacuated below 100 um (Moore and Stein, 1963). Not all amino

acids undergo destruction. Smith et al., (1954) found alanine to be stable at both 20 and 70 hours of hydrolysis. Glycine, leucine, and phenylalanine appear to be stable for up to 140 hours of acid hydrolysis. Valine and isoleucine reach maximal values only after 70 hours of acid hydrolysis and remain constant up to 140 hours (Noltmann, et al., 1962).

Sulfonic acids are non-oxidizing strong acids and have been used to replace HCl in acid hydrolysis. Liu and Chang (1971) used p-toluenesulfonic acid (PTSA) as the catalyst for hydrolysis of proteins. Hydrolysis was carried out in heavy walled 18 x 150 mm ignition tubes. The protein (2 to 3 mg) was hydrolyzed under vacuum (20 to 30 um) at 110°C for 22, 48, and 72 hours with 1 ml of 3 N PTSA containing 0.2% 3-(2-amino ethyl)indole. After hydrolysis 2.0 ml of 1 N NaOH was added and the sample was transfered quantitatively to a 5.0 ml volumetric flask and brought to volume with water. After filtration through a 0.22 u millipore filter an aliquot was used for amino acid analysis. They noted that at 22 hours of hydrolysis 3 N PTSA had a lower yield of valine and isoleucine than 6 N HCl. After 48 and 72 hours the yields were quite similar for the two methods. Serine and threonine yields were somewhat higher with PTSA, but all the other amino acids, except tryptophan, gave comparable recoveries.

p-Toluenesulfonic acid can be used as a method of hydrolysis for tryptophan determination. Recovery of tryptophan is very high when purified proteins and PTSA are used. The results compare very well to those from methods of tryptophan determination that do not use acid hydrolysis. The addition of 0.2% 2-(2-amino ethyl)indole resulted in a higher and more reproducible recovery of tryptophan. However, an excess of carbohydrate in a sample causes extensive tryptophan destruction. The carbohydrate content is limited to 2.0 mg when 2 to 4 mg of protein are being hydrolyzed with

1.0 ml of acid.

A p-toluenesulfonic acid hydrolysis procedure is a more convenient method for routine amino acid determinations, because the hydrolysate can be placed directly on the column of the amino acid analyzer without prior removal of the solvent as required when HCl is used. A single hydrolysate can be used to determine all of the amino acids including tryptophan when the sample has a low carbohydrate content.

Davies and Thomas (1973) performed extensive hydrolysis studies on lysozyme, casein, and a microfungal protein comparing many hydrolysis reagents. They found lysozyme gave comparable values with HCl and PTSA, but casein and microfungal protein gave lower values for cystine and methionine when hydrolyzed with PTSA. Votisky and Gundel (1973) compared PTSA and HCl acid hydrolysates using fishmeal and grain maize. They reported PTSA to be a softer hydrolysis method giving lower yields than HCl. This study compares the recovery of amino acids from HCl and PTSA hydrolysates using a cereal product at twelve different time periods. From this a pattern of amino acid release and destruction can be followed for each amino acid and one can then compare equally PTSA and HCl hydrolysis.

MATERIALS AND METHODS

Comparison of HCl and PTSA Acid Hydrolysis

Approximately 100 mg of KSU flour were accurately weighed into each of 72 16 x 125 mm pyrex test tubes. Four ml of 6 N HCl were added to one half of the tubes and 4 ml of 3 N PTSA were added to the rest of the tubes. To all tubes 0.5 ml of 12.5 uM/ml norleucine, an internal standard, was added. Each tube was covered with a polypropylene culture tube cap (Bacti-Capalls, A. H. Thomas, Co.) and placed in a 100°C boiling water bath equipped with an air condenser to recover the water. The HCl and PTSA hydrolyses were conducted in separate reaction vessels. Time 0.0 hours began when the tubes were placed in the boiling baths. At 5, 10, 15, 20, 24, 30, 35, 40, 50, 60, 72, and 100 hours from time 0.0, three sample tubes were removed from both the PTSA and HCl hydrolysis baths to give three hydrolysates at each time for each method.

The PTSA samples were cooled and 1 ml of diluter buffer (Table 1) was added. The hydrolysate was transferred quantitatively to a 10 ml volumetric flask and brought to volume with deionized water. After cooling the HCl samples were transfered quantitatively to a 125 ml round-bottom flask with deionized water and dried with a rotary evaporator under vacuum at 40°C to remove the HCl. The samples were reconstituted with deionized water and taken to dryness a second time to insure complete removal of the HCl. One ml of diluter buffer was added to the dried sample before it was quantitatively transferred to a 10 ml volumetric flask and brought to volume with deionized water.

A 1 ml aliquot of each hydrolysate was placed in a 1.5 ml polypropylene microcentrifuge tube and centrifuged at 15500 rcf for 3 minutes to remove insoluble humin. The remaining hydrolysate was transferred to a 16 x 125 mm

Table 1

Buffers for Single Column Automatic Amino Acid Analysis

Buffer	pH	Ion	Sodium Conc. in M/L	# ml/L 88% Phenol
Diluter	2.20	Formate	2.0	0.0
A	3.25	Formate	0.2	1.0
B	4.25	Formate	0.2	1.0
C	6.80	Citrate	0.2	1.0
D	10.00	Borate	0.2	1.0
E	12.25	Borate	0.2	1.0

pyrex test tube, covered with parafilm and frozen. The clarified supernatant was loaded directly onto a Dionex D-300 kit or component analyzer (Sunnyvale, CA) by filling a 25 ul sample loop in a sixteen place automatic sampler. A standard containing 0.625 uM/ml of each amino acid and norleucine was analyzed after every seven samples. At injection the sample loop contents were pumped onto a single 0.4 x 15 cm stainless steel column of Durrum DC-5A spherical polysulfonated resin. Twenty minutes after injection the column temperature was changed from 45° to 65°C. The selection of buffers, column temperature, and sample injection were controlled by a Dionex CP-3 chromatographic programmer.

The buffer system (Table 1), a modification of Hare (1972), consisted of two sodium formate buffers (A and B), a sodium citrate buffer (C), a sodium borate buffer (D), and a sodium borate regeneration buffer (E). All buffers had a 0.2 M sodium concentration and were pumped at a flow rate of 18 ml/hr.

Detection of the amino acids was accomplished by pumping a ninhydrin reagent (2% ninhydrin, 0.400 g stannous chloride dihydrate in 875 ml of methyl cellosolve and 125 ml of 4 N pH 5.51 sodium acetate buffer) into the effluent stream at a rate of 18 ml/hr. The effluent-ninhydrin mixture was then carried through a boiling water bath in a coil of teflon tubing (0.3 mm i.d. and 18 feet long) to develop Rhuhemann's Purple. A photometer that alternately senses 570 nm, 440 nm, and a reference wavelength was used to detect the colored complex. Background interference was corrected by subtracting the absorbance at the reference wavelength from the absorbance at each analytical wavelength. An output for both the 570 nm and 440 nm wavelengths was charted on a chart recorder. Both channels were set at 1.0 on the photometer and the recorder full scale setting was 0.01 volts.

Peak areas were calculated by a Supergrator-3 programmable computing integrator (Columbia Scientific Industries). Integrator output was printed on paper tape and recorded by a Techtran 817A data cassette recorder. Recorded data were entered through a Perkin Elmer CRT terminal into an IBM 370 computer and calculated. Data from each hydrolysate was calculated in units of grams of amino acid per 100 grams of protein. The triplicate values for each hydrolysis time and each amino acid were averaged and a plot was made of time versus percent amino acid. The HCl and PTSA plots for each amino acid were graphed together for comparison. The data was also normalized by correcting to a 100% recovery protein basis.

Sensitivity

A standard containing 0.625 uM/ml of each amino acid and norleucine was analyzed by a Dionex automatic amino acid analyzer as described in the hydrolysis methods section. Flow rates of the effluent and the ninhydrin reagent were 18 ml/hr and 9 ml/hr respectively. The ninhydrin formulation consisted of 2% ninhydrin, 0.400 g stannous chloride dihydrate in 750 ml methyl cellosolve and 250 ml 4 N pH 5.51 sodium acetate buffer (Spackman et al., 1958). A reaction coil containing 18 feet of teflon tubing (0.3 mm i.d.) was used in a boiling water bath. Water in the reaction bath was then replaced by 85% acetoin (3-hydroxy 2-butanone). Acetoin has a boiling point of 148°C, is nontoxic and exhibits a pleasing aroma. The 85% acetoin boiled at 108°C and a standard was analyzed at this temperature. The areas of each amino acid at each temperature were compared.

After distillation to remove water the acetoin was placed in the reaction bath and brought to a boil (temperature = 138°C). A 20 ft length of teflon tubing (0.3 mm i.d.) was connected to the exit port of the flow

cell to increase back pressure to prevent boiling of the eluent-ninhydrin mixture in the coil. A standard was analyzed at this temperature but the run was not completed due to problems that developed from the 138°C temperature.

Water was added to the acetoin to lower the boiling point to 128°C. A standard was analyzed using an 18 ft coil in the 128°C acetoin bath. The coil was shortened repeatedly by 3 ft lengths and a standard was analyzed at each length. The peak areas of the 6, 9, 12, 15, and 18 ft coils were then compared to a standard analyzed using an 18 ft coil in a 100°C water bath. All analysies were done using a ninhydrin flow rate of 18 ml/hr and a eluent flow rate of 18 ml/hr. The photometer was set at 2.0 for both channels and the recorder full scale setting was 0.01 volts. The ninhydrin formulation contained 2% ninhydrin, 0.400 g stannous chloride dihydrate in 875 ml of methyl cellosolve and 125 ml 4.0 M pH 5.51 sodium acetate.

An 18 ft coil in a 100°C bath and a 6 ft coil in a 128°C bath were used to analyze 144 picomoles of each amino acid plus norleucine. All analyzer conditions were the same as described in the previous paragraph except the photometer setting was changed to 0.1 for both channels. The chromatograms and peak areas were compared.

Isatin

A set of eight 16 x 125 mm pyrex test tubes containing 0.1 ml of 12.5 uM/ml proline, 2.4 ml of deionized water, and 2.5 ml of 2% isatin were prepared to determine the optimum reaction time of isatin with proline. The tubes were placed in a boiling water bath and one tube was removed every 30 seconds. After cooling the absorbance of each tube was read at 570 nM

on a Beckman DB-G grating spectrophotometer. A comparison was made of reaction time versus absorbance.

The amount of isatin needed for optimum color development was found by pipetting 0.1 ml of 12.5 uM/ml proline into five 16 x 125 mm pyrex test tubes. To this 0.5, 1.0, 1.5, 2.0, or 2.5 ml of 2% isatin in methyl cellosolve was added. The mixture was brought to a 5 ml total volume with deionized water. The tubes were stirred with a vortex mixer and heated for 3 minutes in a boiling water bath. After cooling the absorbance was read at 570 nM and the number of ml of isatin was compared to absorbance.

The absorbance maximum and the pH optimum was then determined. Buffer solutions of 0.1 M were prepared for pH 2, 3, 4, and 5 using potassium acid phthalate (KHP), pH 6 and 7 using sodium dihydrogen phosphate, and pH 8 and 9 using trihydroxyaminomethane (TRIS). A trial was conducted at each pH by the following procedure:

Tube #1 - 3 ml buffer plus 3 ml 2% isatin in methyl cellosolve (Blank).

Tube #2 - 2.45 ml buffer plus 0.05 ml 12.5 uM/ml proline and 2.5 ml 2% isatin in methyl cellosolve.

Both tubes were mixed on a vortex stirrer and placed in a boiling water bath for three minutes, cooled, and scanned on a Beckman DB-G grating spectrophotometer from 750 to 320 nM. Both blank versus blank, and blank versus sample were scanned for each pH.

The exact pH optimum for the isatin-proline complex was found by utilizing a series of 0.1 M KHP buffers with a pH ranging from 2.5 to 4.0 with 0.3 gradations. Each tube contained 2.5 ml buffer, 2.5 ml 2% isatin in methyl cellosolve, and 0.05 ml of 12.5 uM/ml proline. Absorbance at 595 nM was read after mixing and heating for 3 minutes, and cooling. A plot of absorbance of absorbance versus pH was made.

The color yield of lysine and ammonia with isatin was examined. Three

16 x 125 mm pyrex test tubes were prepared with each containing 2.45 ml of 0.1 M pH 3.0 KHP buffer and 2.5 ml 2% isatin in methyl cellosolve. Either 0.05 ml of 12.5 uM/ml lysine, or ammonia, or proline were added. A fourth tube (blank) containing 2.50 ml KHP buffer and 2.5 ml 2% isatin was prepared. The tubes were mixed, heated in a boiling water bath for 3 minutes, cooled, and their absorbance read at 595 nM.

A combination isatin-ninhydrin reagent was prepared to detect amino acids in the column effluent on an automatic amino acid analyzer. The reagent was prepared as follows:

(I)
1. 875 ml methyl cellosolve
2. 125 ml 4 N pH 6.0 sodium acetate buffer
3. 20 g ninhydrin
4. 20 g isatin
5. 0.63 g hydrindantin

This reagent was used to analyze a standard containing 0.625 uM/ml of each amino acid plus an internal standard norleucine as described in the hydrolysis materials and methods section. The effluent and color reagent flow rates were 18 ml/hr. An 18 ft reaction coil was immersed in boiling water to develop the color. A ninhydrin reagent (II)-(2% ninhydrin, 0.400 g stannous chloride dihydrate in 875 ml methyl cellosolve and 125 ml pH 5.51 sodium acetate) was used for amino acid detection in a standard run at the same conditions. A comparison of color yield of each amino acid was made between color reagents.

Acetoin (128^{o}C) was used to replace the water in the reaction bath. An 8 ft coil was used to analyze a standard mixture of amino acids at the higher reaction temperature using the pH 6.0 isatin-ninhydrin reagent (I). A comparison of color yields was made between the two reaction temperatures.

A second isatin-ninhydrin reagent was prepared with the following formula:

(III) 1. 20 g ninhydrin
2. 20 g isatin
3. 0.63 g hydrindantin
4. 125 ml of 4 M pH 3.0 sodium formate
5. 525 ml dimethyl sulfoxide
6. 350 ml methyl cellosolve

Two standards were analyzed, one using the isatin-ninhydrin reagent (III) and the other using the ninhydrin reagent (II) from above. Flow rates were 18 ml/hr for both the effluent and ninhydrin, and an 18 ft coil was used in the boiling water bath for color development. Comparisons were made of the peak areas resulting from each color reagent.

An isatin-ninhydrin reagent (IV) was prepared that contained 2% isatin, 2% ninhydrin, and 0.63 g hydrindantin in 750 ml dimethyl sulfoxide and 250 ml 4 M pH 3.0 lithium formate buffer. Two standards were analyzed one using reagent (IV) and the other using ninhydrin reagent (II). Flow rates were 18 ml/hr for both the effluent and ninhydrin and an 18 ft coil was placed in boiling water. The peak areas from the two color reagents were compared.

Results and Discussion

Hydrolysis

Hydrochloric acid and PTSA hydrolysis methods were compared using triplicate flour samples at intervals from 5 to 100 hours of hydrolysis time. The hydrolysates were analyzed by an automatic amino acid analyzer and calculations were made in units of grams of amino acid per 100 grams of protein. These units were chosen in order to see the effects of hydrolysis on an amino acid independantly from the other amino acids.

The effect of PTSA and HCl hydrolysis on each amino acid is shown in figures 3 to 20. Average or extrapolated values were calculated from the timed hydrolysis data for both HCl and PTSA. Threonine, serine, and methionine are destroyed with time and their losses are corrected by extrapolation of the data back to time zero. The value at 100 hours of hydrolysis time was used for valine and isoleucine because these amino acids are released slowly. All the other amino acid values were the average from the most stable portion of the curve. The average or extrapolated values were also normalized to a 100% recovery basis (Table 2). These corrections provide the best amino acid values for each acid hydrolysis method. If extrapolated values are taken as 100% recovery, the percent released of each amino acid at each time can be calculated by dividing the amino acid value at each time by the 100% recovery value and multiplying by 100 (table 3 and 4). Percent recovery values of 97% and above are considered as totally released in the following discussion. A comparison of HCl and PTSA hydrolysis can be made by analyzing figures 3 to 20 and tables 2 to 4.

The PTSA and HCl hydrolysis curves of aspartic acid, glutamic acid, and leucine (Figures 3, 6, and 14) are parallel, with PTSA being the lower curve. Aspartic acid reached a maximum at 15 hours and leveled off with

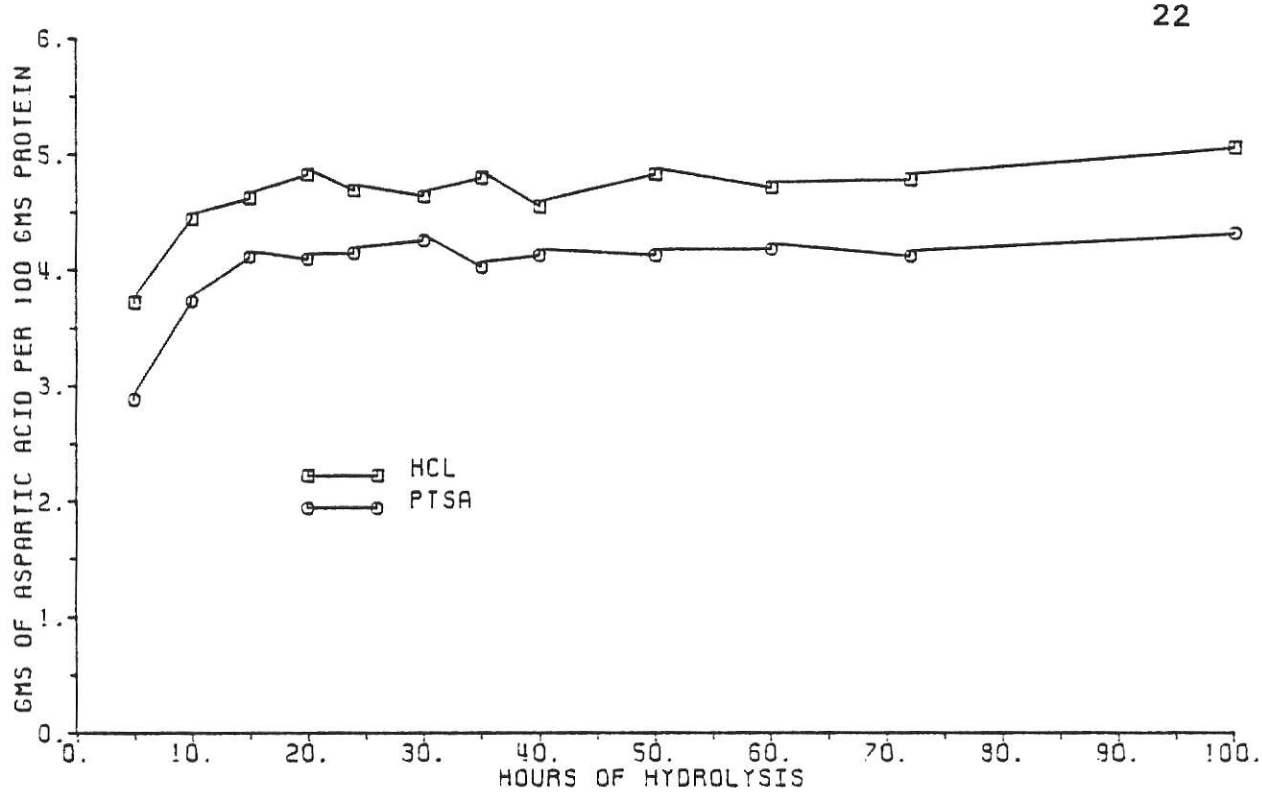


Fig. 3. The effect of HCl and PTSA hydrolysis with time.

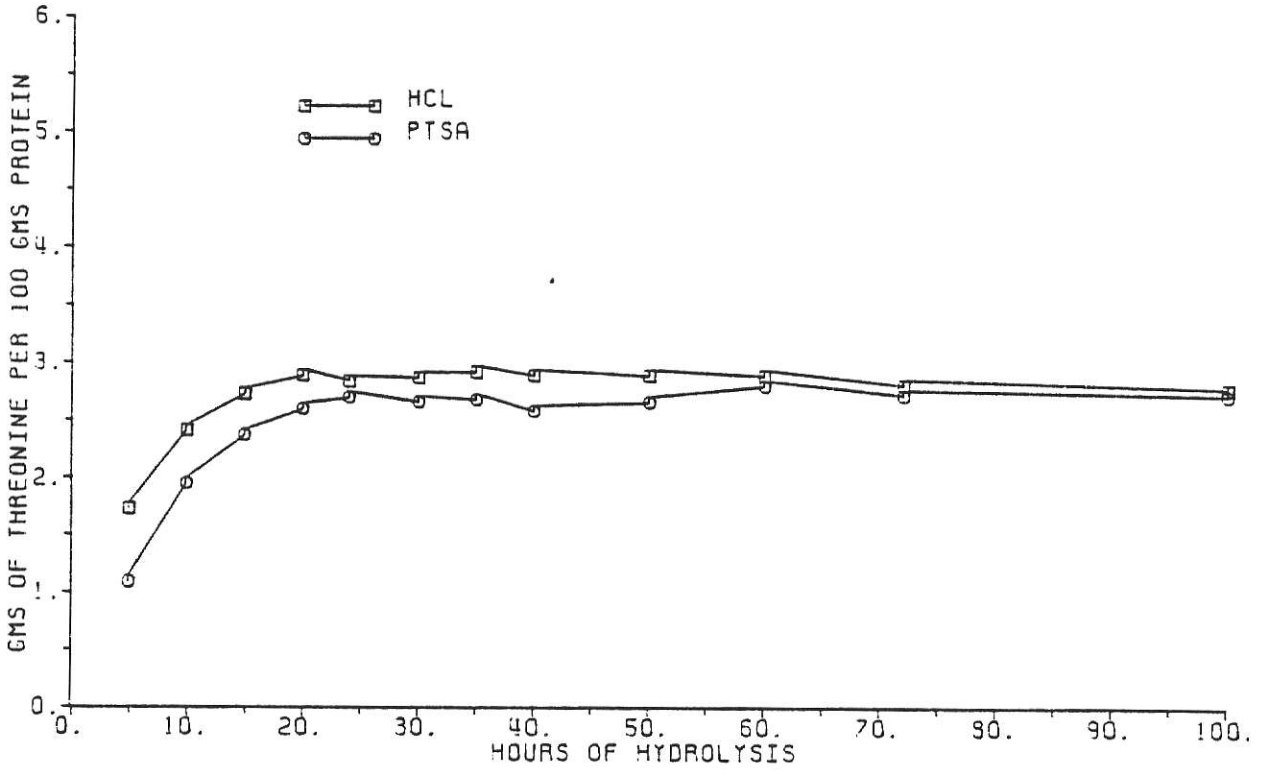


Fig. 4. The effect of HCl and PTSA hydrolysis with time.

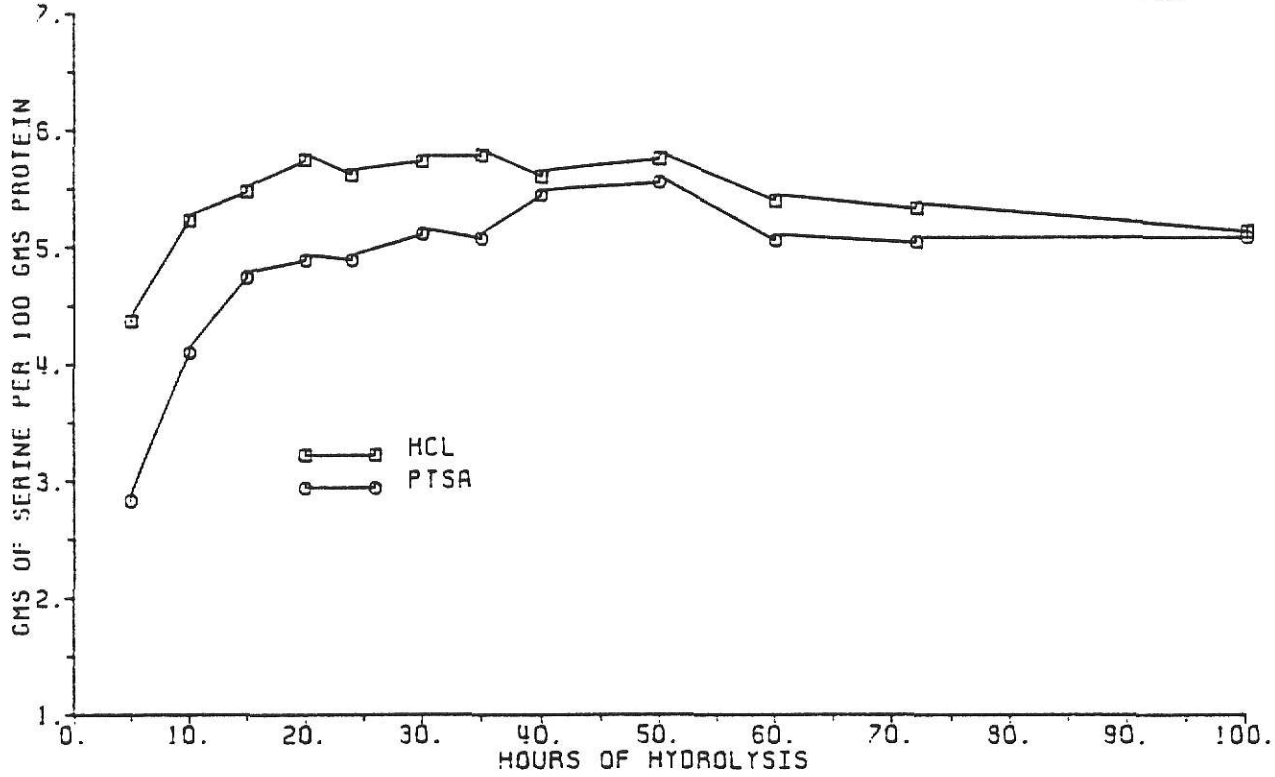


Fig. 5. The effect of HCl and PTSA hydrolysis with time.

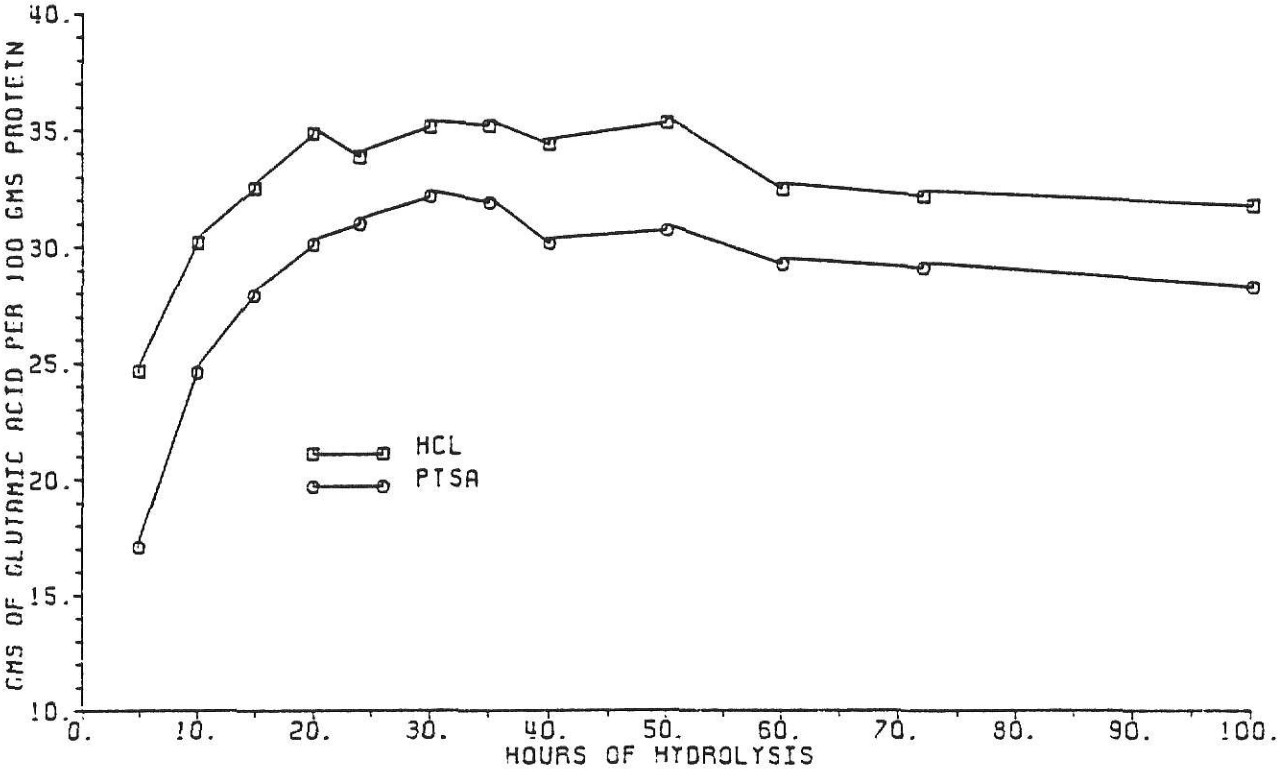


Fig. 6. The effect of HCl and PTSA hydrolysis with time.

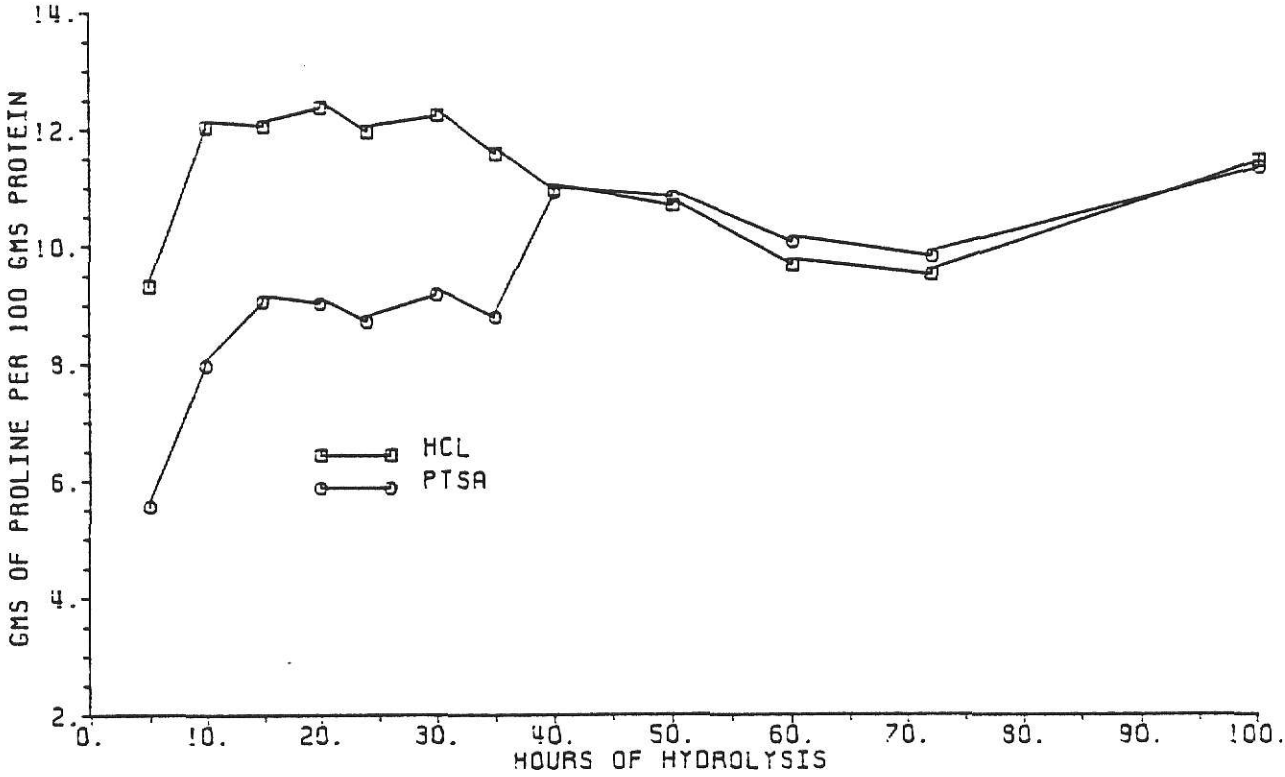


Fig. 7. The effect of HCl and PTSA hydrolysis with time.

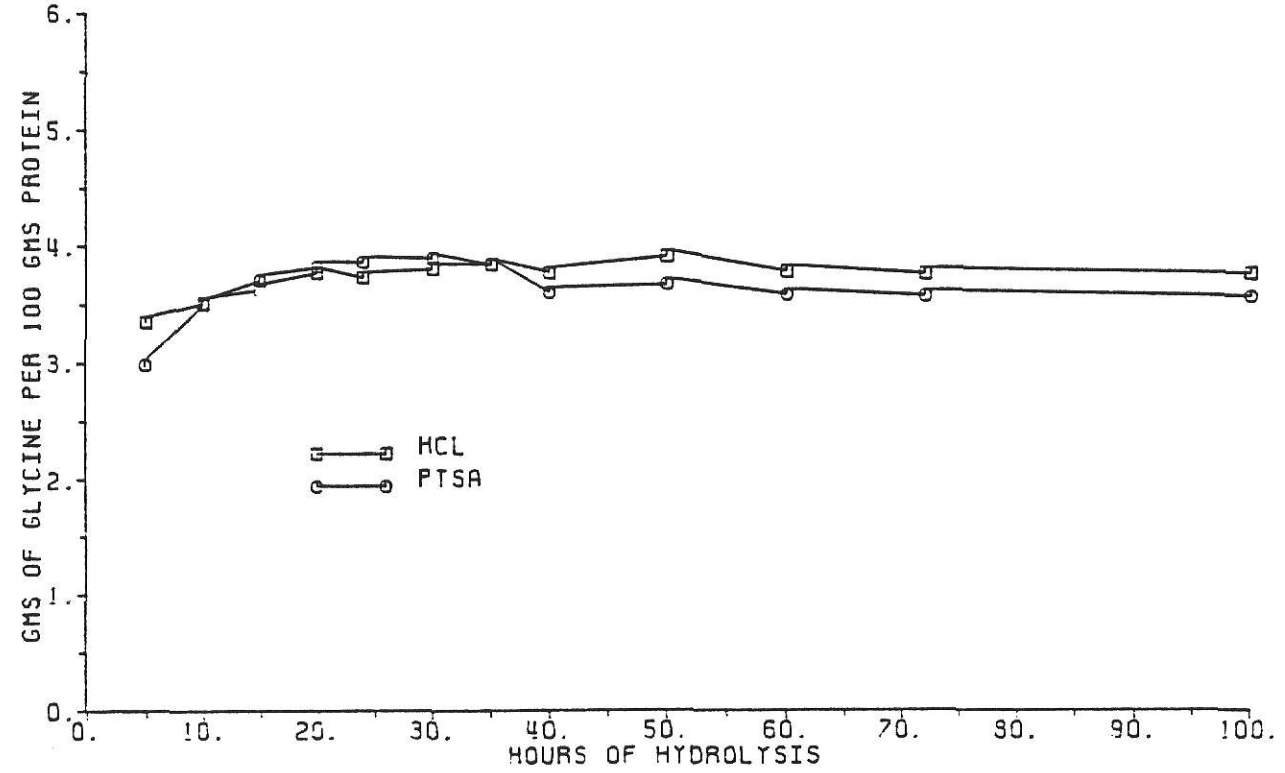


Fig. 8. The effect of HCl and PTSA hydrolysis with time.

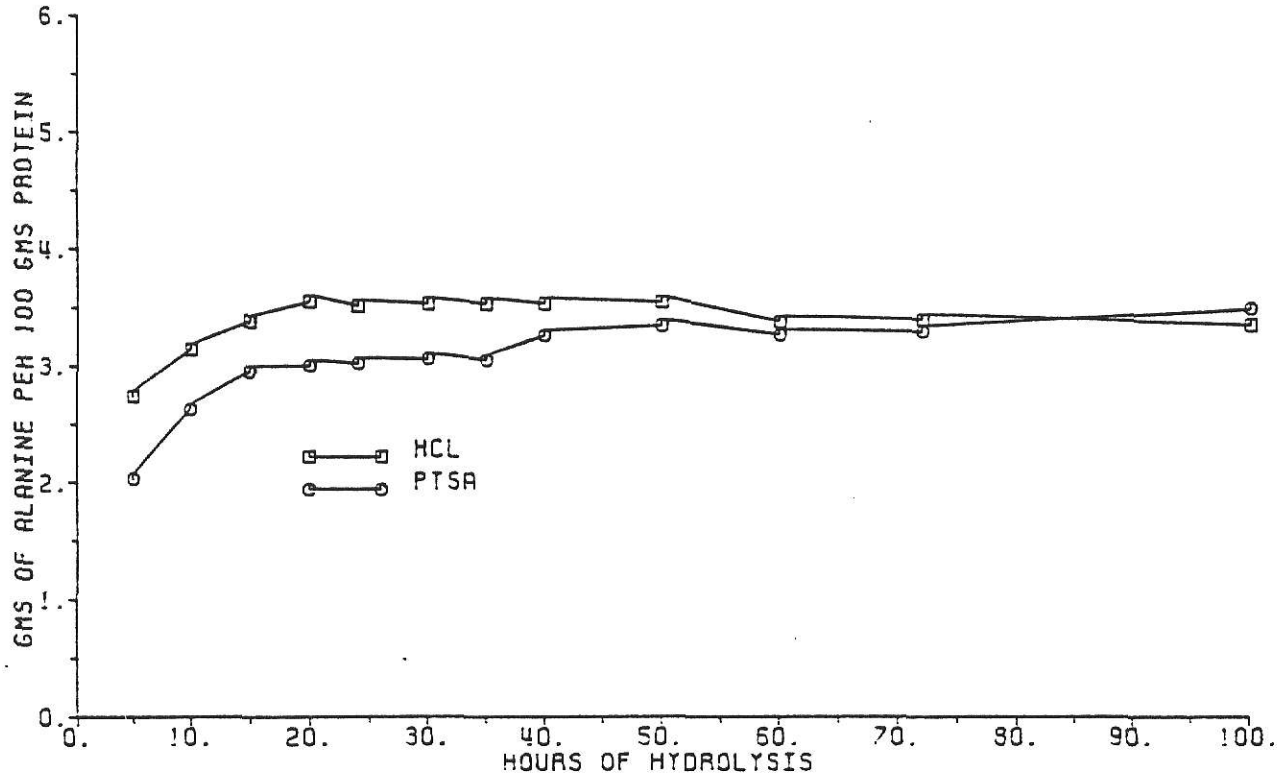


Fig. 9. The effect of HCl and PTSA hydrolysis with time.

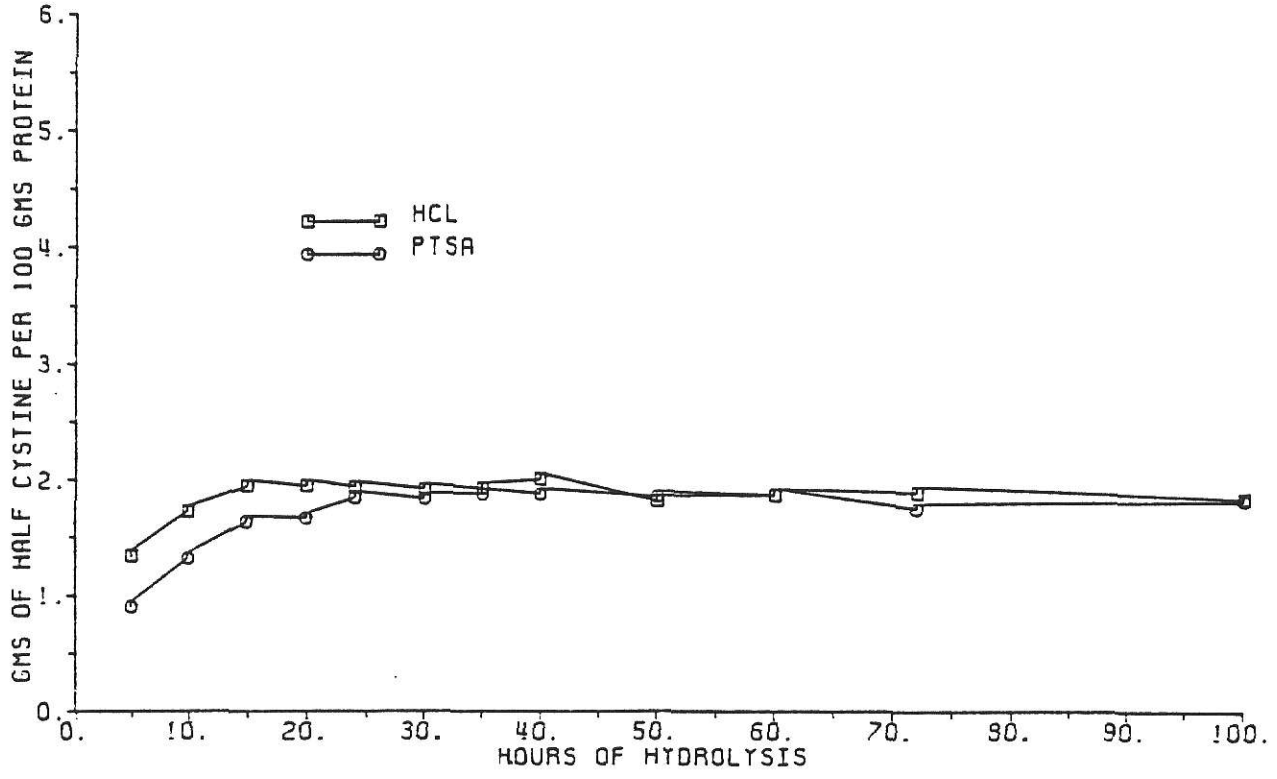


Fig. 10. The effect of HCl and PTSA hydrolysis with time.

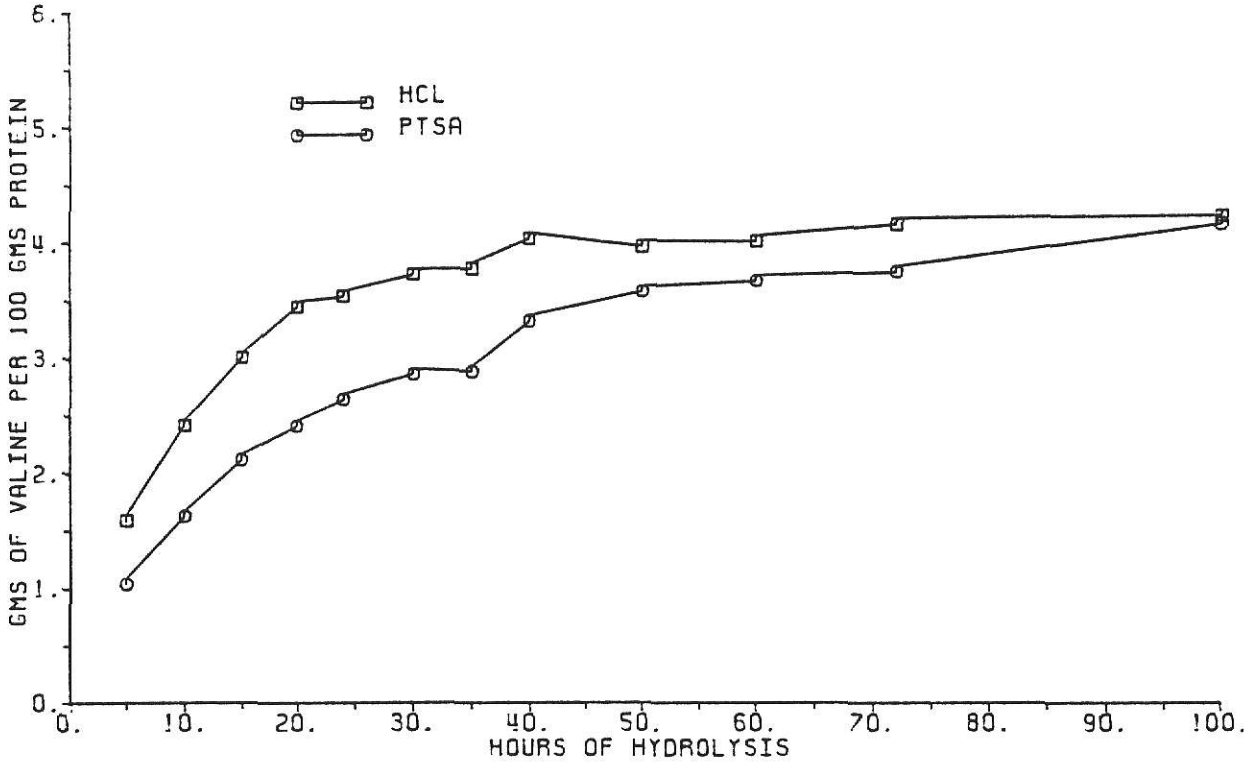


Fig. 11. The effect of HCl and PTSA hydrolysis with time.

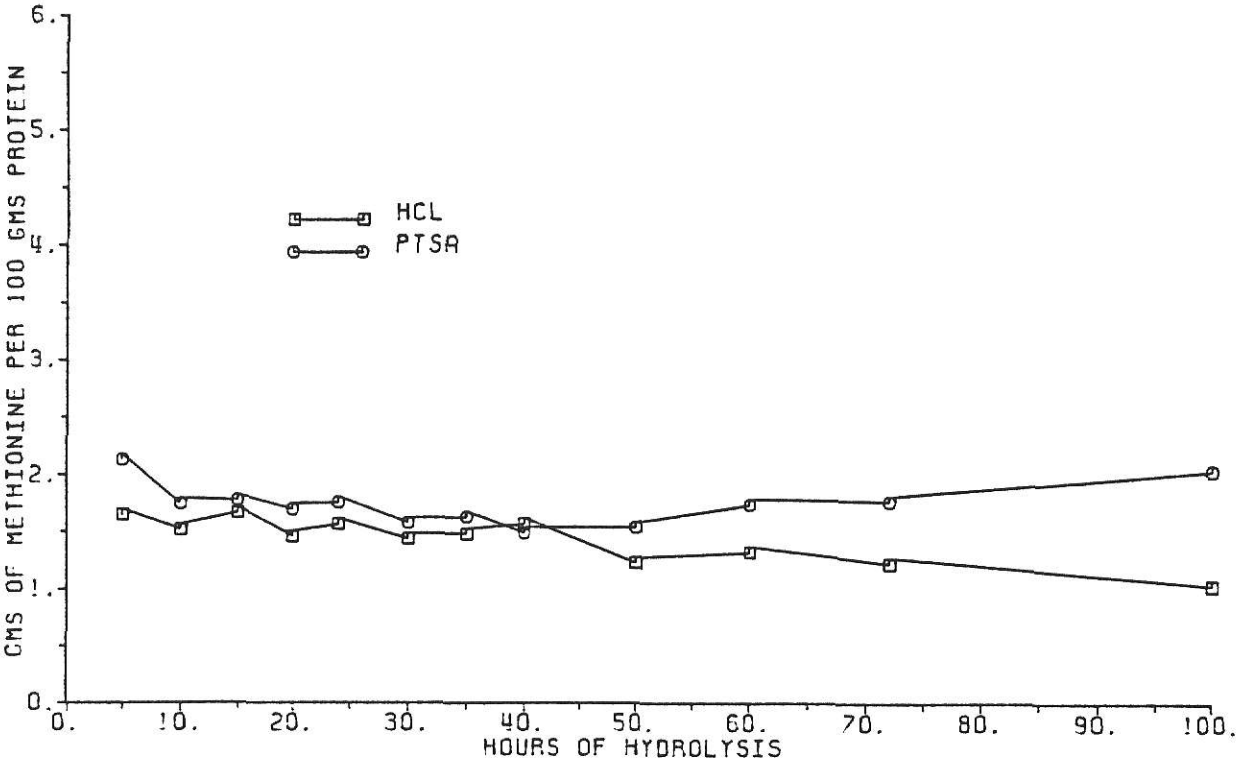


Fig. 12. The effect of HCl and PTSA hydrolysis with time.

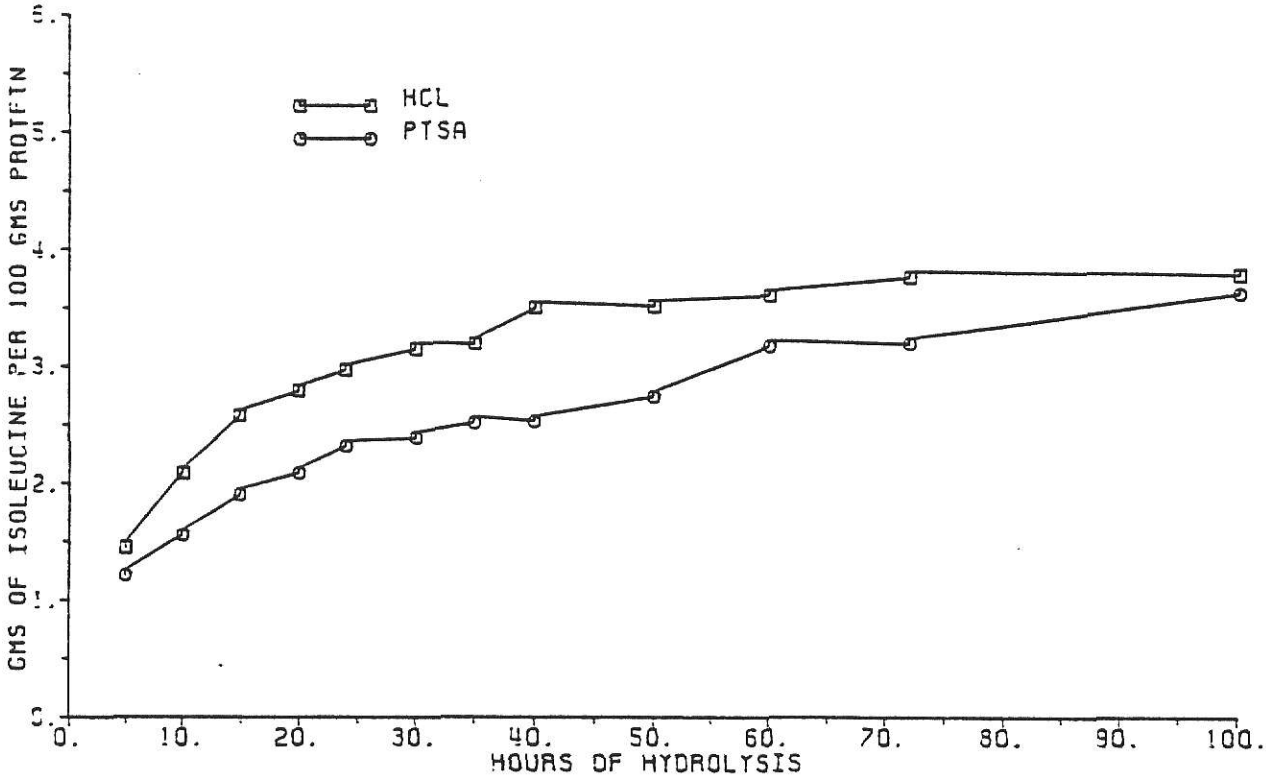


Fig. 13. The effect of HCl and PTSA hydrolysis with time.

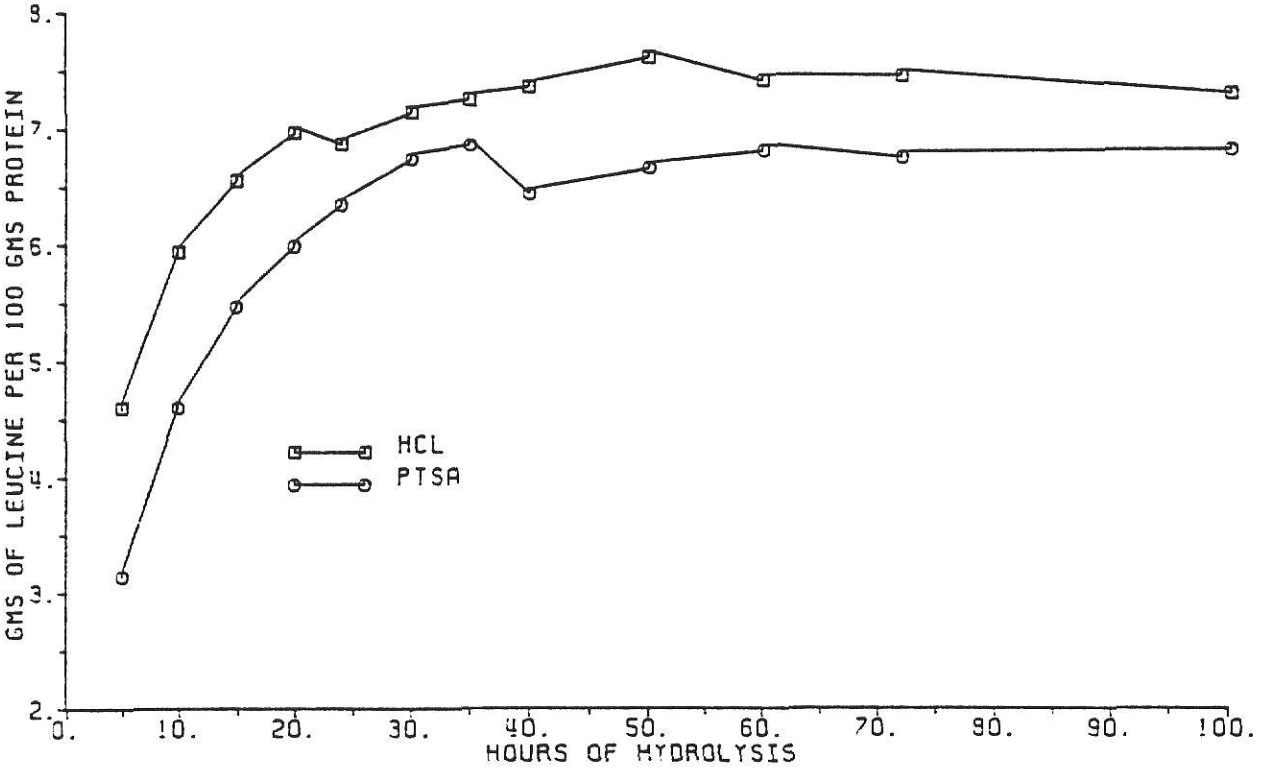


Fig. 14. The effect of HCl and PTSA hydrolysis with time.

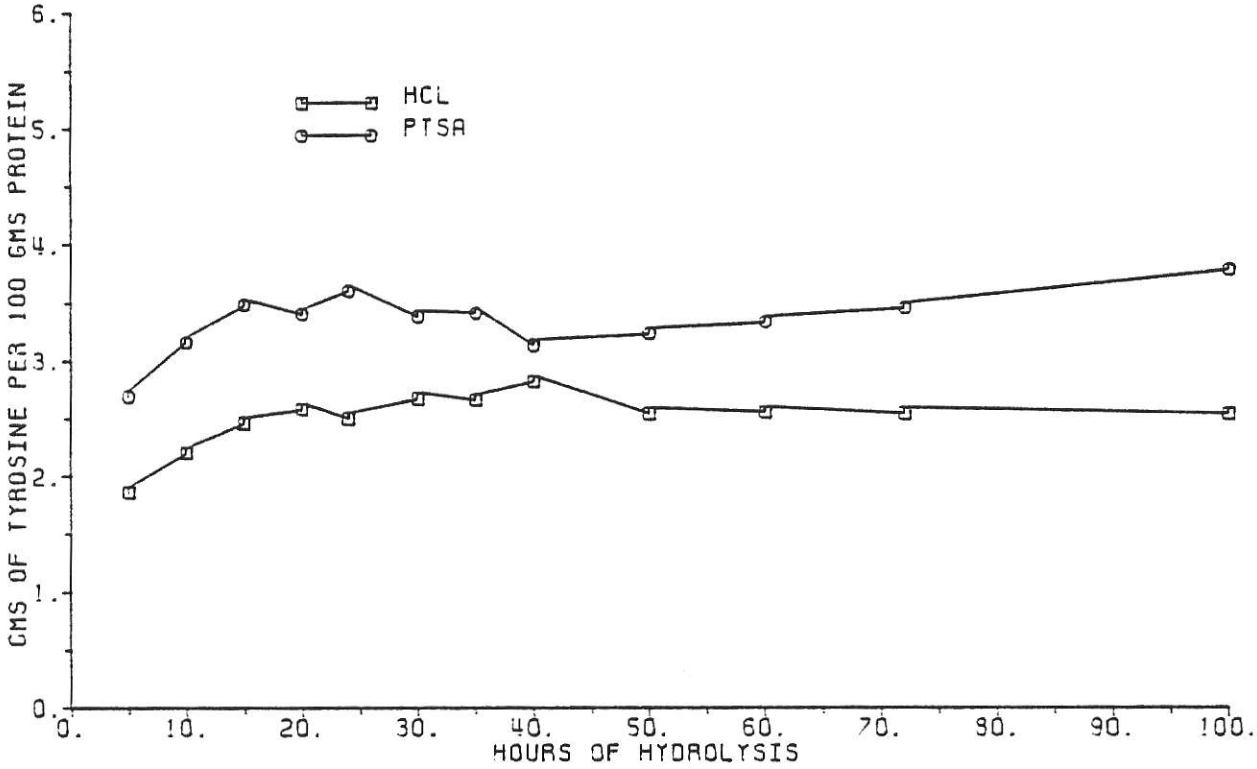


Fig. 15. The effect of HCl and PTSA hydrolysis with time.

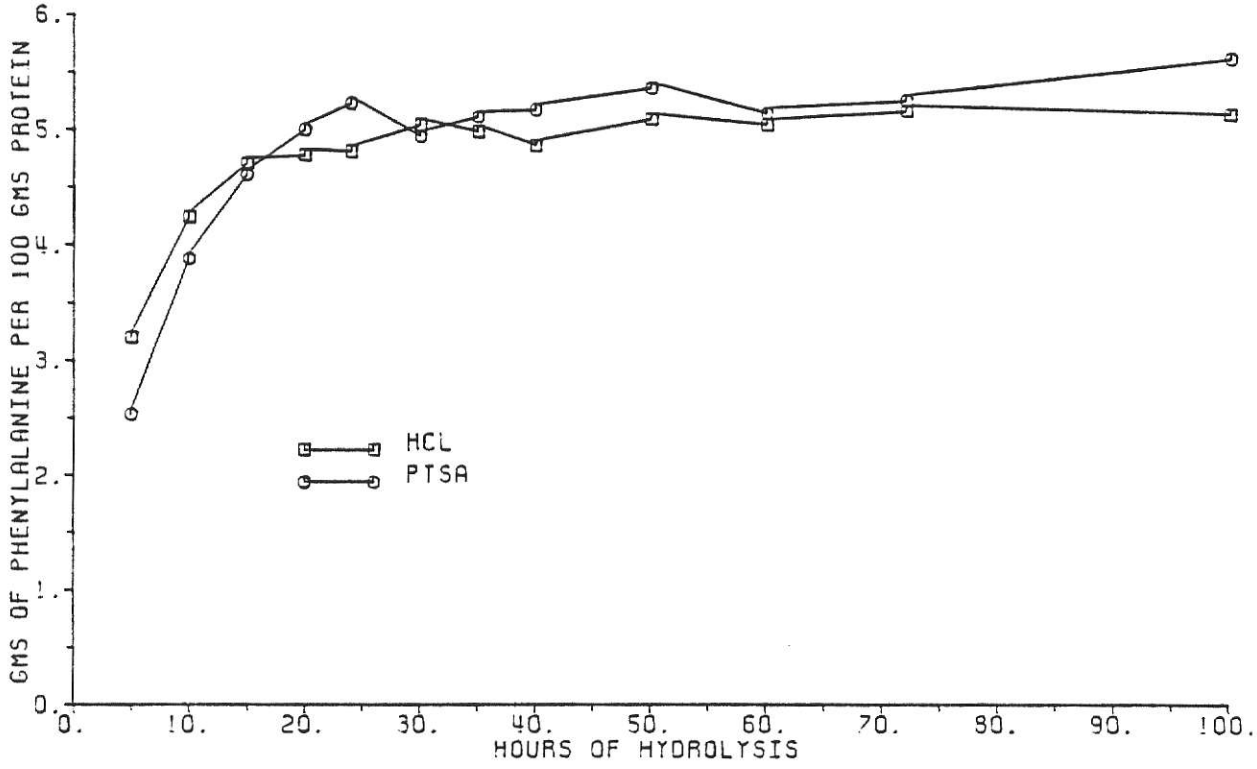


Fig. 16. The effect of HCl and PTSA hydrolysis with time.

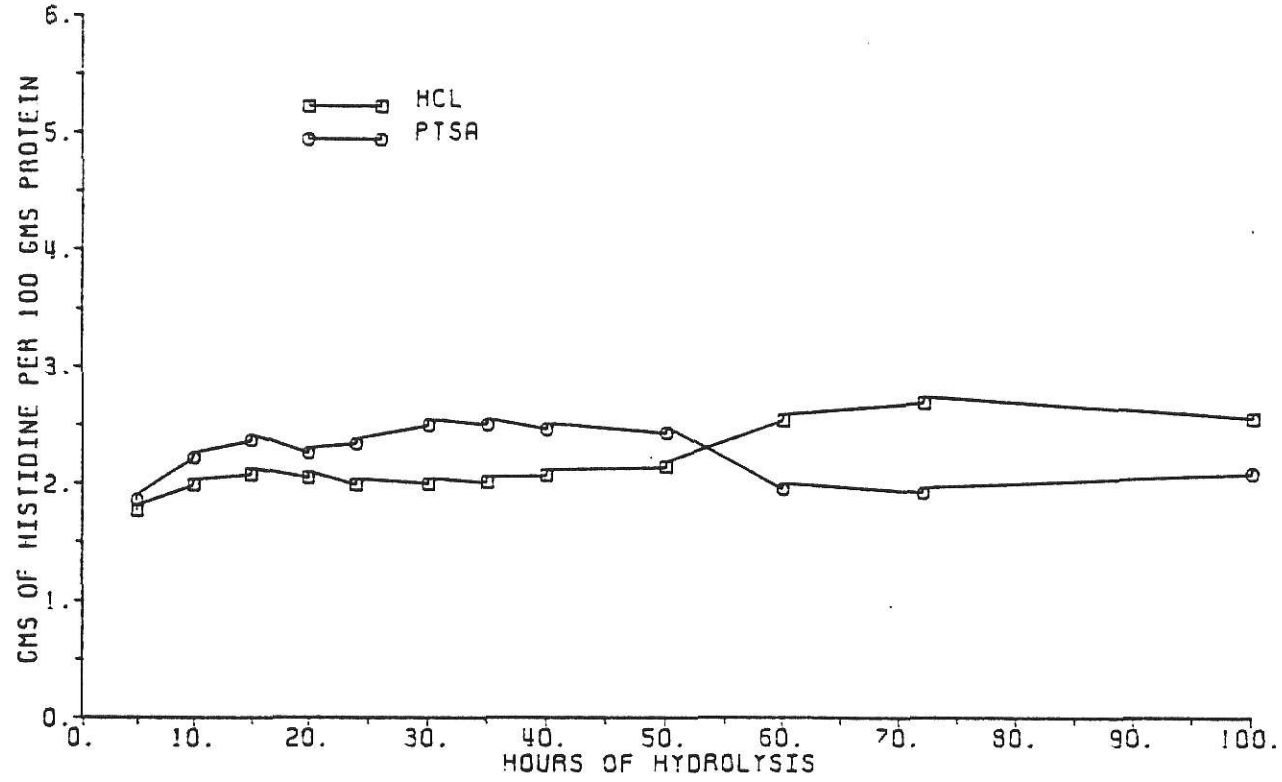


Fig. 17. The effect of HCl and PTSA hydrolysis with time.

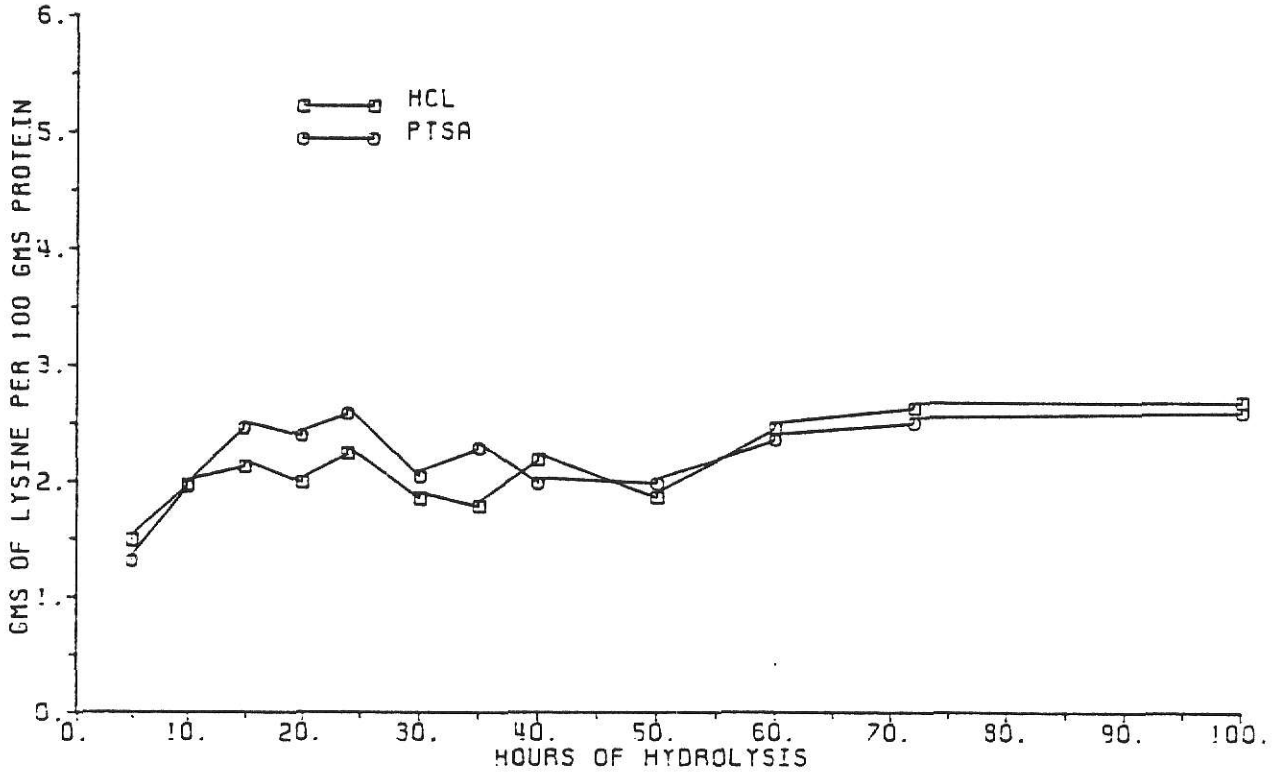


Fig. 18. The effect of HCl and PTSA hydrolysis with time.

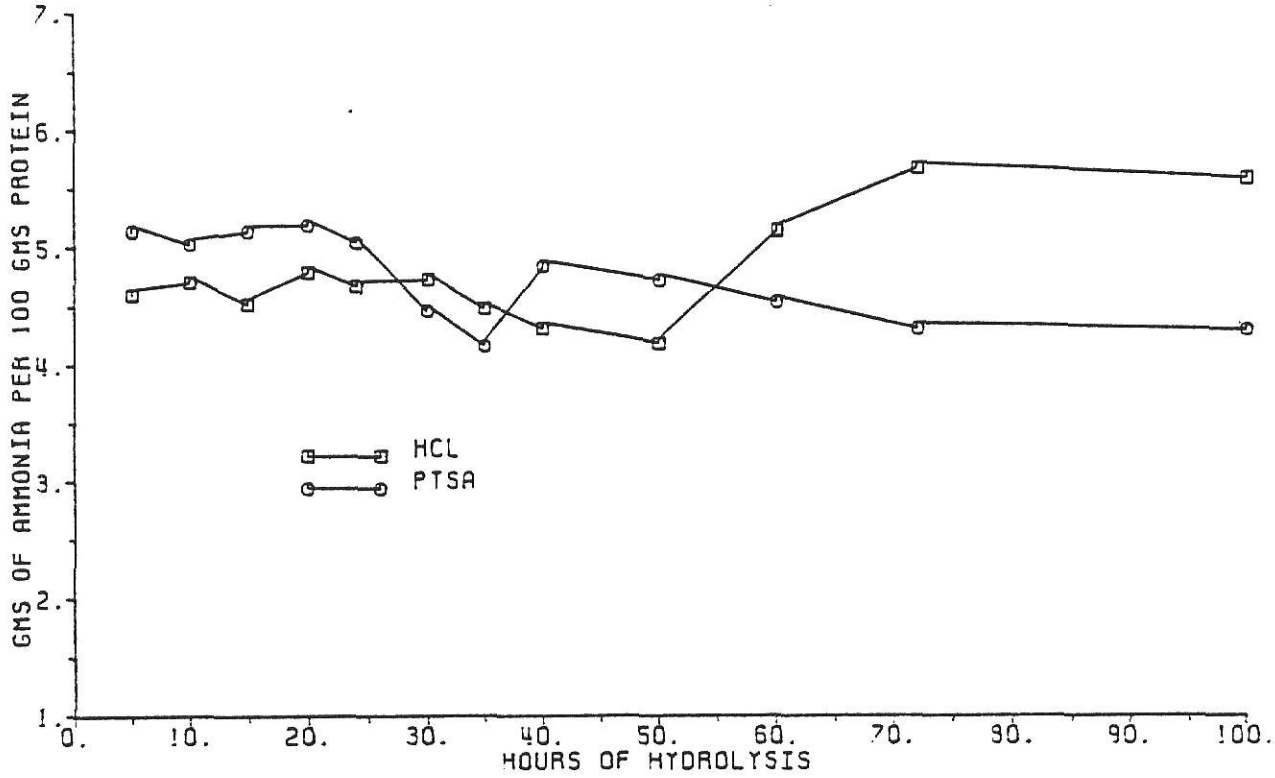


Fig. 19. The effect of HCl and PTSA hydrolysis with time.

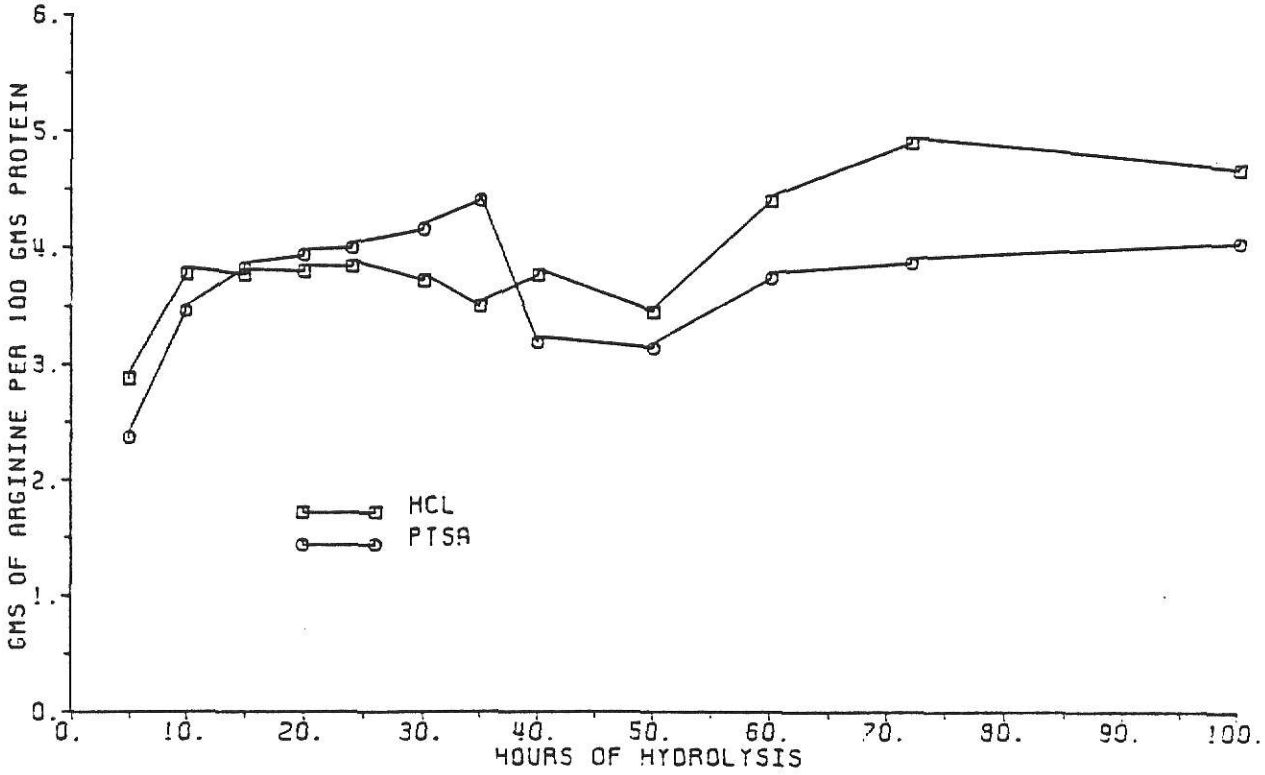


Fig. 20. The effect of HCl and PTSA hydrolysis with time.

Table 2

Average or Extrapolated HCl and PTSA Percent Protein Amino Acid Profiles

Amino Acids	HCl	Normalized HCl	PTSA	Normalized PTSA	Average[4]	5% Error[5]
Aspartic Acid	4.74	4.40	4.14	4.15	4.28	0.21
Threonine[1]	2.92	2.71	2.61	2.62	2.66	0.13
Serine[1]	5.94	5.51	5.09	5.10	5.30	0.26
Glutamic Acid[3]	35.16	32.61	32.04	32.13	32.37	1.62
Proline	12.16	11.28	9.00	9.02	10.15	0.51*
Glycine	3.79	3.52	3.71	3.72	3.62	0.18
Alanine	3.48	3.23	3.20	3.21	3.22	0.16
Half Cystine	1.90	1.76	1.84	1.84	1.80	0.09
Valine[2]	4.23	3.92	4.16	4.17	4.04	0.20
Methionine[1]	1.67	1.55	1.72	1.72	1.64	0.08
Isoleucine[2]	3.78	3.51	3.62	3.63	3.57	0.18
Leucine	7.39	6.85	6.79	6.81	6.83	0.34
Tyrosine	2.60	2.41	3.41	3.42	2.92	0.14*
Phenylalanine	4.99	4.63	5.20	5.21	4.92	0.25*
Histidine	2.21	2.05	2.28	2.29	2.17	0.11*
Lysine	2.18	2.02	2.35	2.36	2.19	0.11*
Ammonia	4.78	4.43	4.74	4.75	4.59	0.23
Arginine	3.89	3.61	3.83	3.84	3.72	0.19
Total	107.81	100.00	99.73	100.00		

1 - Values from extrapolation to time zero.
2 - Values at 100 hours of hydrolysis.
3 - Average of the 30 and 35 hour values.
4 - Average of HCl and PTSA Normalized values.
5 - 5% of the Average Column.

All other values are an average of the most stable portion of the curve.

* - Indicates the HCl and PTSA values are outside of the 5% error range of the average.

Table 3

Percent of Amino Acids Released with Time with HCl

Amino Acids	Hours of Hydrolysis											
	5	10	15	20	24	30	35	40	50	60	72	100
Aspartic Acid	78	94	97	102	99	98	101	96	102	99	101	106
Threonine	59	82	93	99	97	98	100	99	99	99	96	95
Serine	74	88	92	97	94	96	97	94	97	91	89	86
Glutamic Acid	70	86	92	99	96	100	100	98	100	92	91	90
Proline	76	99	99	102	98	101	95	90	88	80	78	94
Glycine	88	93	96	99	98	100	101	99	103	100	99	99
Alanine	79	90	97	102	101	101	101	101	102	97	97	96
Half Cystine	70	91	103	103	102	101	102	106	96	98	99	97
Valine	38	57	71	82	84	88	89	96	94	95	98	100
Methionine	99	91	100	87	94	86	87	94	74	79	73	62
Isoleucine	38	55	68	74	78	83	84	92	93	95	99	100
Leucine	62	80	89	94	93	97	98	100	103	100	101	99
Tyrosine	72	85	95	99	96	103	102	108	98	98	98	97
Phenylalanine	64	85	94	96	96	101	100	97	102	101	103	103
Histidine	80	90	94	93	90	90	91	94	97	115	122	115
Lysine	68	90	98	91	103	85	82	100	85	112	121	123
Ammonia	96	98	94	100	98	99	94	90	87	108	119	117
Arginine	74	97	97	98	99	95	90	97	89	113	126	120

Table 4

Percent of Amino Acids Released with Time with PTSA

Amino Acids	Hours of Hydrolysis											
	5	10	15	20	24	30	35	40	50	60	72	100
Aspartic Acid	70	90	99	99	100	103	97	100	100	101	99	104
Threonine	42	75	91	100	103	102	103	99	102	107	104	104
Serine	56	80	93	96	96	100	100	107	109	99	99	100
Glutamic Acid	53	77	87	94	97	100	100	94	96	91	91	88
Proline	62	88	101	100	97	102	98	121	120	112	109	126
Glycine	80	94	100	103	104	105	104	97	99	96	96	96
Alanine	63	82	92	94	94	95	95	102	104	102	103	109
Half Cystine	49	72	89	91	100	100	102	102	101	102	95	99
Valine	25	39	51	58	63	69	69	80	86	88	90	100
Methionine	124	102	103	99	102	92	95	87	90	101	103	119
Isoleucine	33	43	53	58	64	66	70	70	76	88	88	100
Leucine	46	68	80	88	94	99	101	95	98	100	99	100
Tyrosine	79	93	102	100	106	99	100	92	95	98	101	110
Phenylalanine	49	75	89	96	100	95	98	99	103	99	101	108
Histidine	82	97	104	99	103	111	110	108	106	86	84	91
Lysine	56	83	105	102	110	87	97	84	84	100	106	110
Ammonia	108	106	108	109	106	94	88	102	100	96	91	90
Arginine	62	90	100	103	104	109	115	83	82	98	101	105

virtually no destruction through 100 hours of hydrolysis with either method. Glutamic acid reached a peak at 25 to 30 hours and was slowly destroyed by both HCl and PTSA. Leucine reached a plateau with both methods in 30 hours and showed no destruction through 100 hours. If the lower yield of these three amino acids with PTSA was due simply to a slower release, the PTSA yields would slowly approach the HCl values as hydrolysis time increased. The higher HCl level could be due to the formation of ninhydrin positive destruction products by the harsher HCl environment. These destruction products when eluted with aspartic acid, glutamic acid, and leucine would produce an increase in the values of these three amino acids.

Threonine, serine, proline and alanine (Figures 4, 5, 7, and 9) showed lower yields initially with PTSA but their values approached the HCl levels as hydrolysis time increased. Threonine is totally released in 20 hours with HCl and PTSA, but HCl gradually destroyed threonine, while PTSA was stable. After 60 hours both methods gave essentially equal values. After 20 hours of hydrolysis serine is completely released by HCl and 96% released by PTSA. Proline is completely released in 10 hours with HCl and 15 hours with PTSA. The proline yield increased with time with PTSA and was gradually destroyed with HCl. The sharp increase in the proline values with both PTSA and HCl after 70 hours might be due to the formation of destruction products of other amino acids that co-elute with proline. After 15 hours alanine is completely released and is slightly destroyed with time by HCl. PTSA released 92% of alanine in 15 hours and continued to slowly release it through 100 hours of hydrolysis. At 100 hours the PTSA yield of alanine was equal to the maximum HCl values. These four amino acids are released more slowly by PTSA but as hydrolysis time increased the PTSA values become equal to the HCl values. All four amino acids were destroyed with HCl

while their yield was either stable or increased slowly with PTSA.

Half cystine (Fig. 10) was more slowly released by PTSA but after 15 hours PTSA and HCl values were equal and stable through 100 hours. Valine and isoleucine were slowly released (Figures 11 and 13) through 100 hours with both methods. The PTSA values were lower initially but equalled the HCl values at 100 hours. Both hydrolysis methods gave comparable yields of methionine (Fig. 12) at 35 to 40 hours. After 40 hours of HCl hydrolysis, methionine destruction began. The methionine yield with PTSA slowly increased through 100 hours.

The complete release of tyrosine (Fig. 15) was obtained with 20 hours of HCl hydrolysis and 15 hours of PTSA hydrolysis. The levels remained stable through 100 hours with both methods and PTSA gave a higher yield than HCl. The lower yield with HCl could be a result of the loss of tyrosine from the formation of bromo and chloro derivatives (Moore and Stein, 1963). These derivatives have not been found with PTSA hydrolysis.

Glycine, histidine, lysine, ammonia, and arginine have comparable recoveries with PTSA and HCl hydrolysis. The maximum yield of glycine (Fig. 8) was reached in 20 hours with HCl and 15 hours with PTSA and both were stable through 100 hours. Histidine (Fig. 17) was totally released in 10 hours by PTSA and 90% released in 10 hours by HCl hydrolysis. The maximum yield of lysine (Fig. 18) was obtained in 15 hours with both reagents. Both methods were stable through 50 hours of hydrolysis and increased from 60 through 100 hours. This could be due to the formation of ornithine, a destruction product of arginine (Murray et al., 1965), that co-elutes with lysine in the Spackman, Moore, and Stein accelerated automatic ion-exchange system (Bates, 1966). Ammonia (Fig. 19) levels were comparable with HCl and PTSA hydrolysies. Ammonia results from destruction of various amino acids and its actual presence in the sample (Blackburn,

1978). Arginine (Fig. 20) is totally released in 15 hours by PTSA and it is 97% released by HCl in 10 hours of hydrolysis and increases slightly as hydrolysis time progresses.

The results of other studies comparing HCl and PTSA hydrolysies were examined and compared to the results of this study. Liu and Chang (1971) compared acid hydrolysates of lysozyme, ribonuclease, and streptococcal proteinase and reported the PTSA yields to be comparable to those of HCl.

Egg white lysozyme, casein, and a fungal protein were used by Davies and Thomas (1973) to compare amino acid recoveries from HCl and PTSA. The recoveries were comparable from lysozyme, but the yields of half cystine and methionine with PTSA were lower from the casein and micro-fungal protein. All the other amino acid recoveries were comparable with the two methods.

Votisky and Gundel (1976) hydrolyzed fishmeal and grain maize and reported PTSA to be a softer hydrolysis reagent than HCl. They found PTSA gave lower yields of some amino acids and higher yields of the labile amino acids. In their study 22, 48, and 72 hour PTSA hydrolysates were compared to a 24 hour HCl hydrolysate. This did not allow comparison of the HCl and PTSA yields with time. Hydrolysis of grain maize with PTSA resulted in lower yields of threonine, proline, isoleucine, and valine, but higher yields of serine and tyrosine when compared with HCl. The PTSA hydrolysis of fishmeal gave lower aspartic acid, serine, glycine, and alanine levels but higher yields of half cystine, valine, methionine, and tyrosine when compared to HCl. All other amino acids showed comparable recoveries with both methods.

Hydrolysis is very complex and the yields of the various amino acids are dependant on the sample composition and the experimental conditions used (Blackburn, 1978). This makes it very hard to directly compare

results with past studies since they used different samples and hydrolysis conditions.

Liu and Chang (1971) suggested that PTSA would be a good hydrolysis reagent for routine analysis of amino acids. PTSA gave comparable recoveries of all amino acids and could be applied directly to the analyzer column without first taking the hydrolysate to dryness. Votisky and Gundel (1978) reported that with a single hydrolysate using PTSA one could analyze all of the amino acids including tryptophan, methionine, and cystine.

If the averaged or extrapolated normalized data is examined (Table 2) most amino acid values for PTSA and HCl are comparable. To determine if the normalized HCl and PTSA values are different an average was calculated of the HCl and PTSA normalized values. A 5% deviation was then calculated for this average value. A value of 5% was chosen because this is the precision error of the Dionex kit analyzer (Dionex Corporation, Sunnyvale, CA). The PTSA and HCl normalized values all fell within the 5% range from their mean except for proline, tyrosine, phenylalanine, histidine, and lysine. Proline, tyrosine, and lysine had a deviation of 11.1%, 17.5%, and 7.8% respectively. The deviation percentages of phenylalanine and histidine were not considered significant. These three amino acids show a difference between the two hydrolysis methods that is above instrument error.

When HCl is used for routine analysis the most accepted length of time for hydrolysis is 24 hours. The optimum hydrolysis time for PTSA can be determined by examining the percent amino acids released with time (Table 4). PTSA released the amino acids more slowly than HCl but it does not destroy the labile amino acids. A longer hydrolysis time is therefore possible since the labile amino acids are not as readily destroyed with PTSA. After examining Table 4, 30 hours was selected as the optimum length

of time for hydrolysis with PTSA. Thirty hours allows a more complete release of the amino acids without serious degradation of methionine.

Table 5 compares the 24 hour normalized HCl data to the 30 hour normalized PTSA data. The amino acid profiles are in units of grams of amino acid per 100 grams protein corrected to a 100% recovery protein basis. An average of the HCl and PTSA data and a 5% variation from this average were calculated. The proline, tyrosine, and histidine values varied 10.3, 17.6, and 14.2 percent from the average. Valine, isoleucine, and arginine varied 7.5, 7.9, and 7.0 percent respectively. The variations of valine, isoleucine, and arginine were not considered significant because the 5% error does not include any additional error from the preparation of triplicate hydrolysates. The 24 hour HCl and 30 hour PTSA values are comparable to the average or extrapolated values as both showed a difference in proline and tyrosine HCl and PTSA hydrolysis values.

Votisky and Gundel (1976) did not report a difference between HCl and PTSA proline and tyrosine values from the hydrolysis of fishmeal, but they did show a difference in the hydrolysis of grain maize. The same pattern was seen in this study with the hydrolysis of wheat flour and may be a characteristic of PTSA hydrolysis of cereals. PTSA and HCl hydrolysis of KSU flour gave comparable recoveries of all the amino acids except proline and tyrosine. This work showed that PTSA could be easily used for the routine hydrolysis of proteins for amino acid analysis.

Sensitivity

Low level concentrations of amino acids are detected by electronically amplifying the voltage output from the photometer on the amino acid analyzer. This amplifies both the signal from the photometer and the background noise.

Table 5

24 Hour HCl and 30 Hour PTSA Normalized Amino Acid Profiles

Amino Acids	24 hr HCl	30 hr PTSA	Average[1]	5%[2] Error
Aspartic Acid	4.45	4.37	4.46	0.22
Threonine	2.76	2.74	2.75	0.14
Serine	5.44	5.25	5.34	0.27
Glutamic Acid	32.83	33.08	32.96	1.65
Proline	11.59	9.43	10.51	0.52*
Glycine	3.61	4.00	3.80	0.19
Alanine	3.40	3.15	3.28	0.16
Half Cystine	1.88	1.89	1.88	0.09
Valine	3.43	2.94	3.18	0.16*
Methionine	1.52	1.62	1.57	0.08
Isoleucine	2.88	2.46	2.67	0.13*
Leucine	6.67	6.93	6.80	0.34
Tyrosine	2.43	3.47	2.95	0.15*
Phenylalanine	4.66	5.07	4.86	0.24
Histidine	1.93	2.57	2.25	0.11*
Lysine	2.17	2.10	2.14	0.11
Ammonia	4.53	4.60	4.56	0.23
Arginine	3.72	4.28	4.00	0.20*

1 - Average of 24 Hour HCl and 30 Hour PTSA.

2 - 5% of the Average.

* - Indicates the HCl and PTSA values are outside of the 5% error range from the average. Proline, Valine, Isoleucine, Tyrosine, Histidine, and Arginine deviate 10.3, 7.5, 7.9, 17.6, 14.2, and 7.0 percent respectively.

However, very low concentrations of amino acids cannot be detected because the background noise will exceed the signal.

One way to increase analyzer sensitivity is to enhance the color reaction yield so a higher signal is produced. This would allow lower concentrations of amino acids to be analyzed. The color yield of the ninhydrin reaction is not at a maximum at 100°C. Jonker et al. (1978) found that the maximum yield occurred at a reaction bath temperature of 140° to 150°C. At the higher temperature maximum color development occurs in 1.3 minutes, but the reaction coil must be kept under pressure to prevent boiling (Anderson et al., 1968). In their studies Jonker and Anderson and their associates used very elaborate devices to control the reaction bath temperature. By selecting a non-toxic liquid, with a boiling point at the desired temperature, to replace the water in the reaction bath the reaction temperature can be easily controlled.

Sensitivity is also limited by peak broadening which lowers resolution. Peak broadening is caused in part by post-column mixing in the reaction coil. The use of long reaction coils allows a greater degree of mixing to occur. If the color yield from the ninhydrin reaction is increased and the reaction time decreased a shorter reaction coil could be used which would decrease peak broadening and increase resolution and sensitivity.

Acetoin was used to replace water in the reaction bath because it has a boiling point of 148°C and it is non-toxic. It is available as an 85% solution that boils at 108°C. A standard amino acid mixture was analyzed at this reaction bath temperature and its peak areas were compared to the peak areas from a standard analyzed at 100°C. An 18 ft reaction coil was used with both analysies and the peak areas from each are given in Table 6.

Table 6

Change in Peak Area with an Increase in Reaction Temperature

Amino Acid	100°C 18' Coil	108°C 18' Coil	% Increase*
Aspartic Acid	66130	129454	196
Threonine	73498	139848	190
Serine	82416	151136	183
Glutamic Acid	89716	151836	169
Proline	19961	24336	122
Glycine	111410	178840	160
Alanine	64081	131316	205
Half Cystine	57889	78226	135
Valine	61779	138108	224
Methionine	99268	172780	174
Isoleucine	66796	143872	215
Leucine	89686	163216	182
Norleucine	82112	164220	200
Tyrosine	83358	165356	198
Phenylalanine	76384	167620	219
Histidine	175820	204248	116
Lysine	107916	205508	190
Ammonia	72130	143144	198
Arginine	89082	138892	156

*% Increase = (Area at 108°C/Area at 100°C) X 100

Raising the reaction temperature by 8°C increased the peak areas of all the amino acids. The average increase was 190% with a range of 116% to 224%. Proline and histidine gave the least response to the higher reaction temperature showing only 122% and 116% increase respectively. Ammonia gave a 200% increase and valine was increased the most with 224%.

The reaction bath temperature was raised to 138°C by distilling the acetoin to remove the water. At this temperature problems developed. The higher temperature increased the rate of tin salt precipitation in the coil and consequently plugged the teflon tubing. To help prevent this precipitation the ninhydrin formula was changed to 875 ml of methyl cellosolve and 125 ml of sodium acetate buffer and the flow rate was changed to 18 ml/hr for both the effluent and the ninhydrin reagent. The increased volume of methyl cellosolve helped reduce the precipitation problem. A second problem at the 138°C reaction bath temperature was coil leakage. At this temperature thin walled sections in the teflon tubing, a result of uneven extrusion, would burst under the higher pressure and temperature. A heavier walled teflon tubing was tried but leakage was still a problem. Therefore, the reaction bath temperature was lowered to 128°C by adding water to the acetoin. At this lower temperature tubing leakage was no longer a problem.

Standards were analyzed at 100°C with an 18 ft coil and at 128°C with coil lengths of 6 to 18 feet (Figures 21 to 26) to determine if sensitivity could be increased by using a shorter coil at the higher reaction temperature. The peak areas and the percent increase in area over water with increasing coil length is given in Table 7. Almost all of the amino acid areas showed a steady increase with increasing coil length. The maximum yields were found using the 18 ft coil at 128°C where peak area varied from proline which increased only 125% to ammonia which increased 2288%.

Fig. 21. Chromatogram of an Amino Acid Standard Analyzed with an 18 ft Coil and a Reaction Bath Temperature of 100°C.

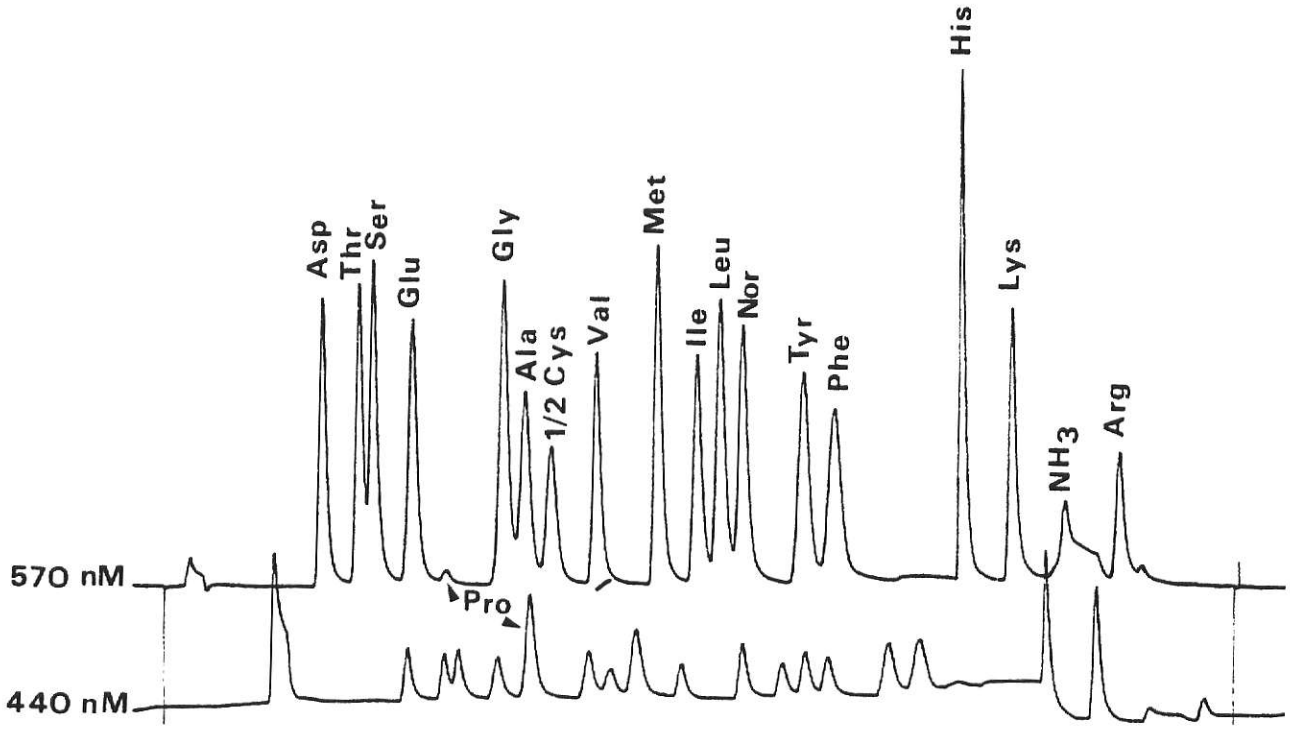

Asp
Thr
Ser
Glu
Gly
Ala
1/2 Cys
Val
Met
Ile
Leu
Nor
Tyr
Phe
His
Lys
NH3
Arg
570 nM
Pro
440 nM

Fig. 22. Chromatogram of an Amino Acid Standard Analyzed with an 18 ft Coil and a Reaction Bath Temperature of 128°C.

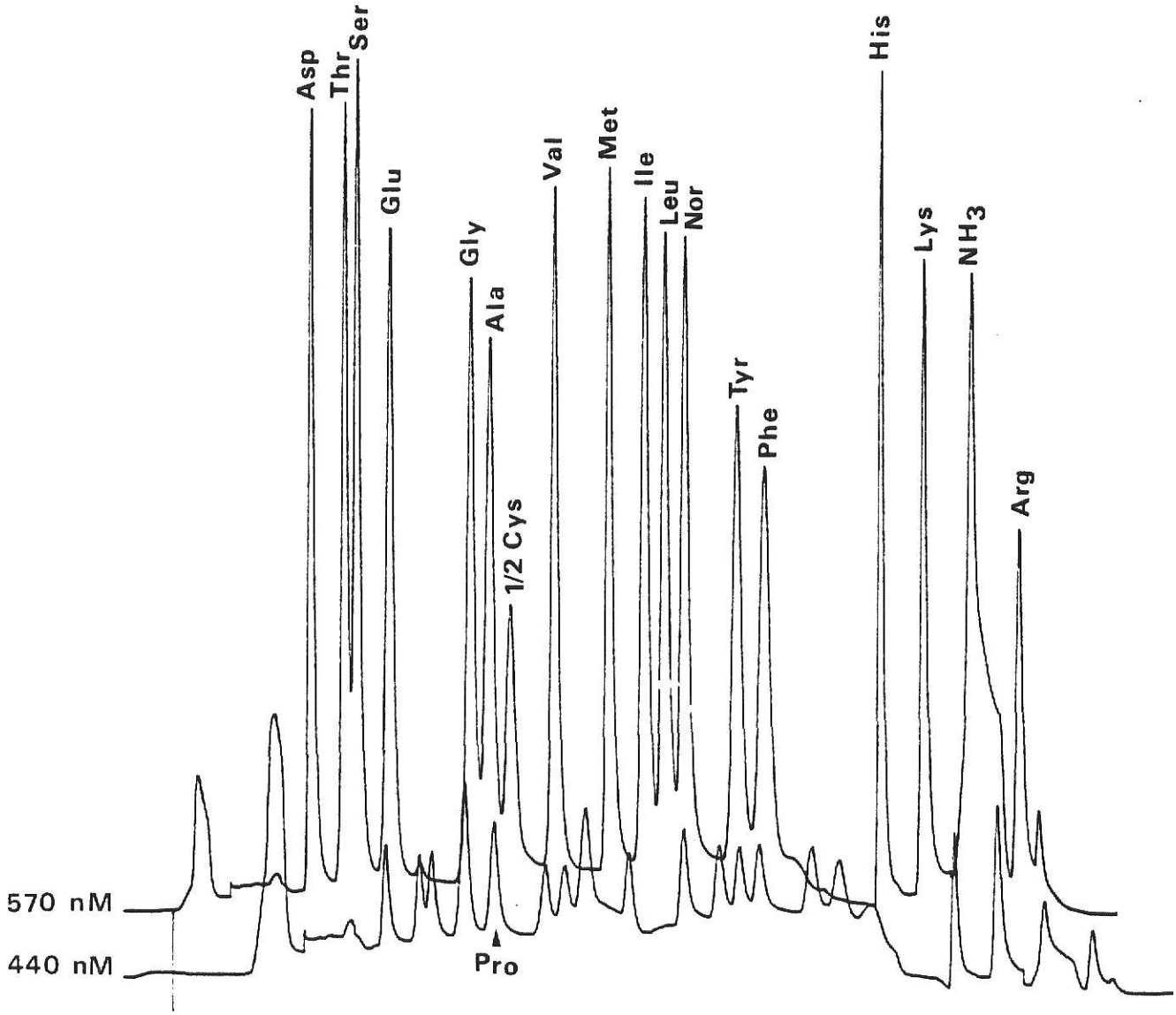

Asp
Thr
Ser
Glu
Gly
Ala
1/2 Cys
Val
Met
Ile
Leu
Nor
Tyr
Phe
His
Lys
NH3
Arg
570 nM
440 nM
Pro

Fig. 23. Chromatogram of an Amino Acid Standard Analyzed with a 15 ft Coil and a Reaction Bath Temperature of 128°C.

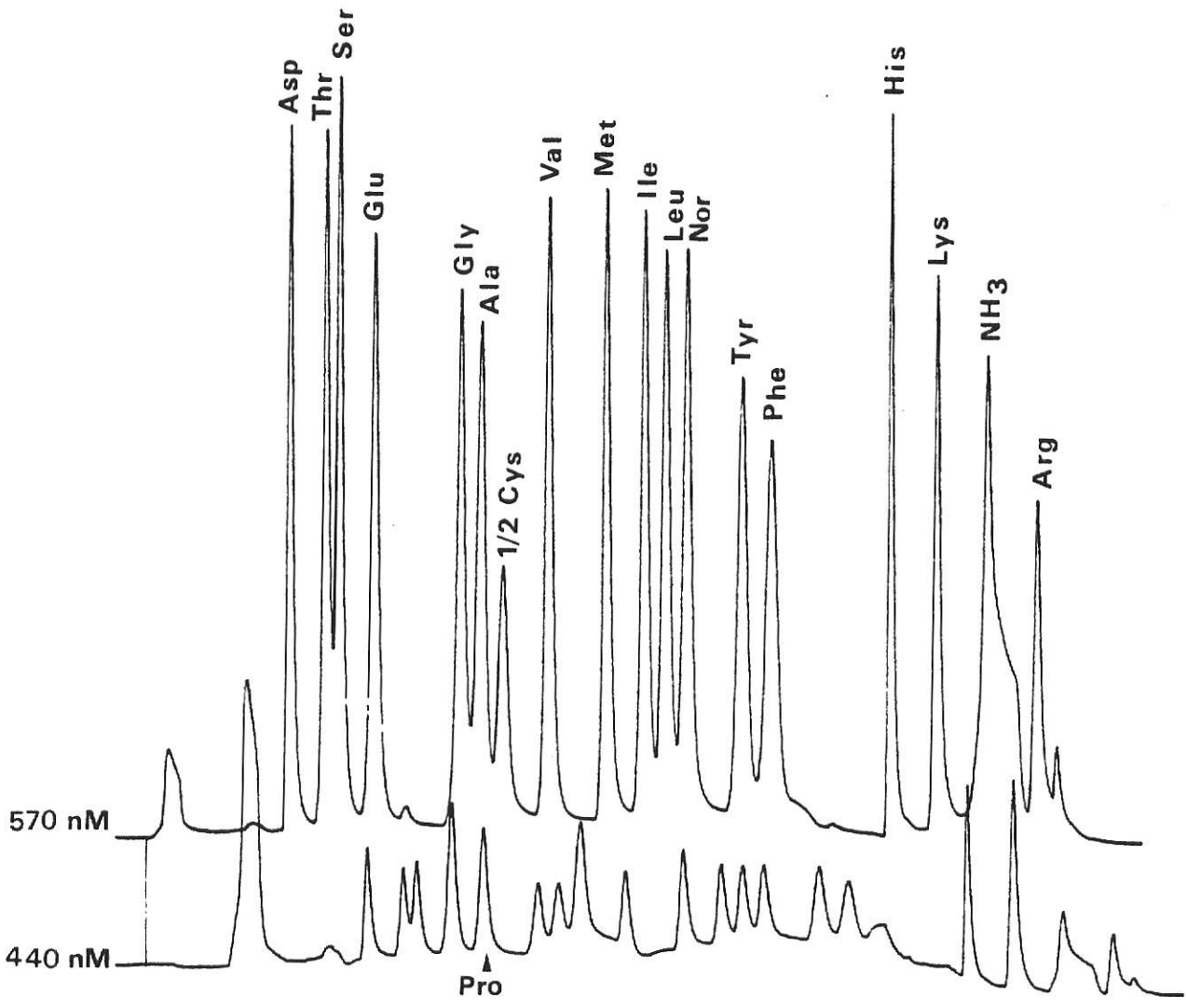

Asp
Thr
Ser
Glu
Gly
Ala
1/2 Cys
Val
Met
Ile
Leu
Nor
Tyr
Phe
His
Lys
NH3
Arg
570 nM
440 nM
Pro

Fig. 24. Chromatogram of an Amino Acid Standard Analyzed with a 12 ft Coil and a Reaction Bath Temperature of 128°C.

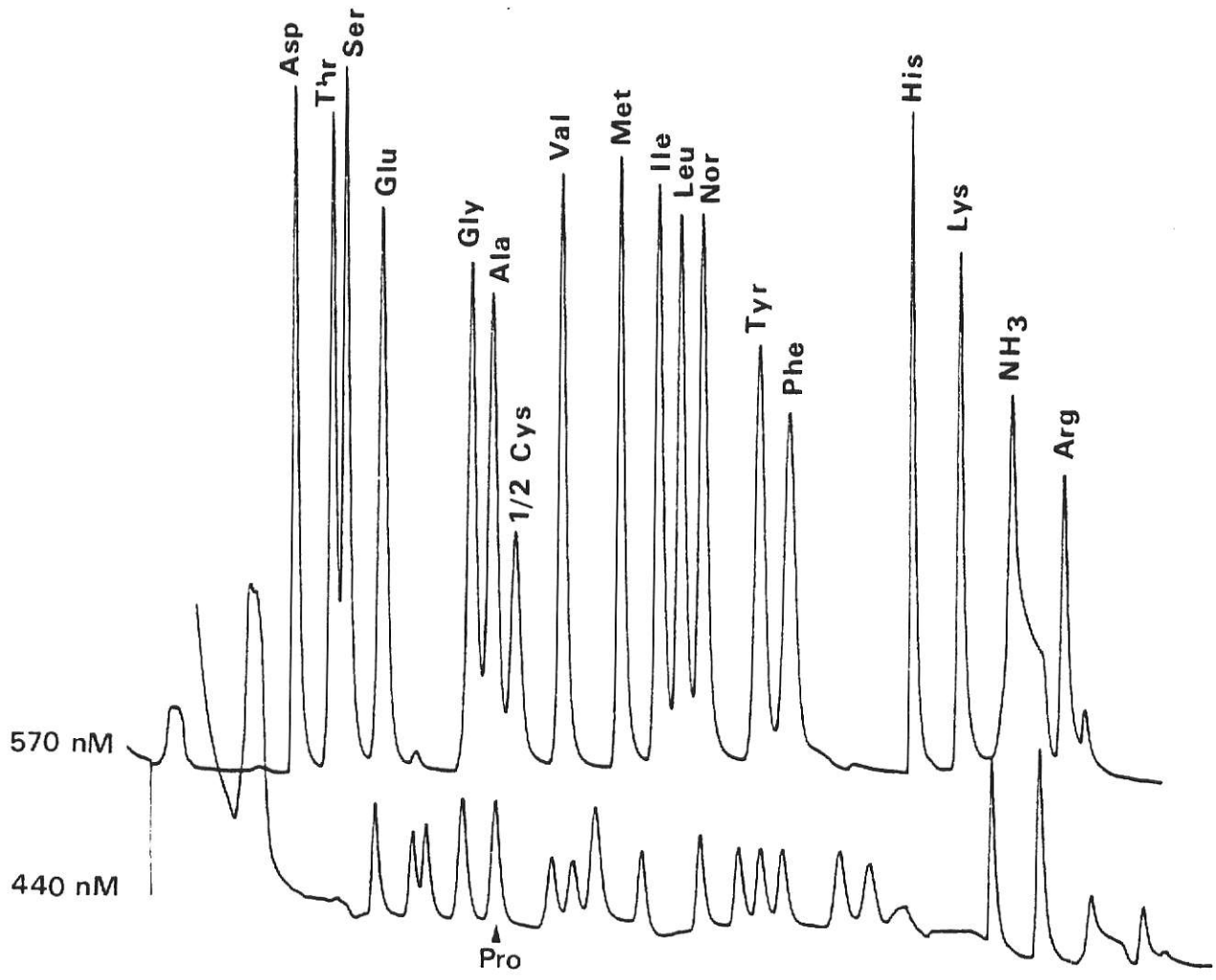

Asp
Thr
Ser
Glu
Gly
Ala
1/2 Cys
Val
Met
Ile
Leu
Nor
Tyr
Phe
His
Lys
NH3
Arg
570 nM
440 nM
Pro

Fig. 25. Chromatogram of an Amino Acid Standard Analyzed with a 9 ft Coil and a Reaction Bath Temperature of 128°C.

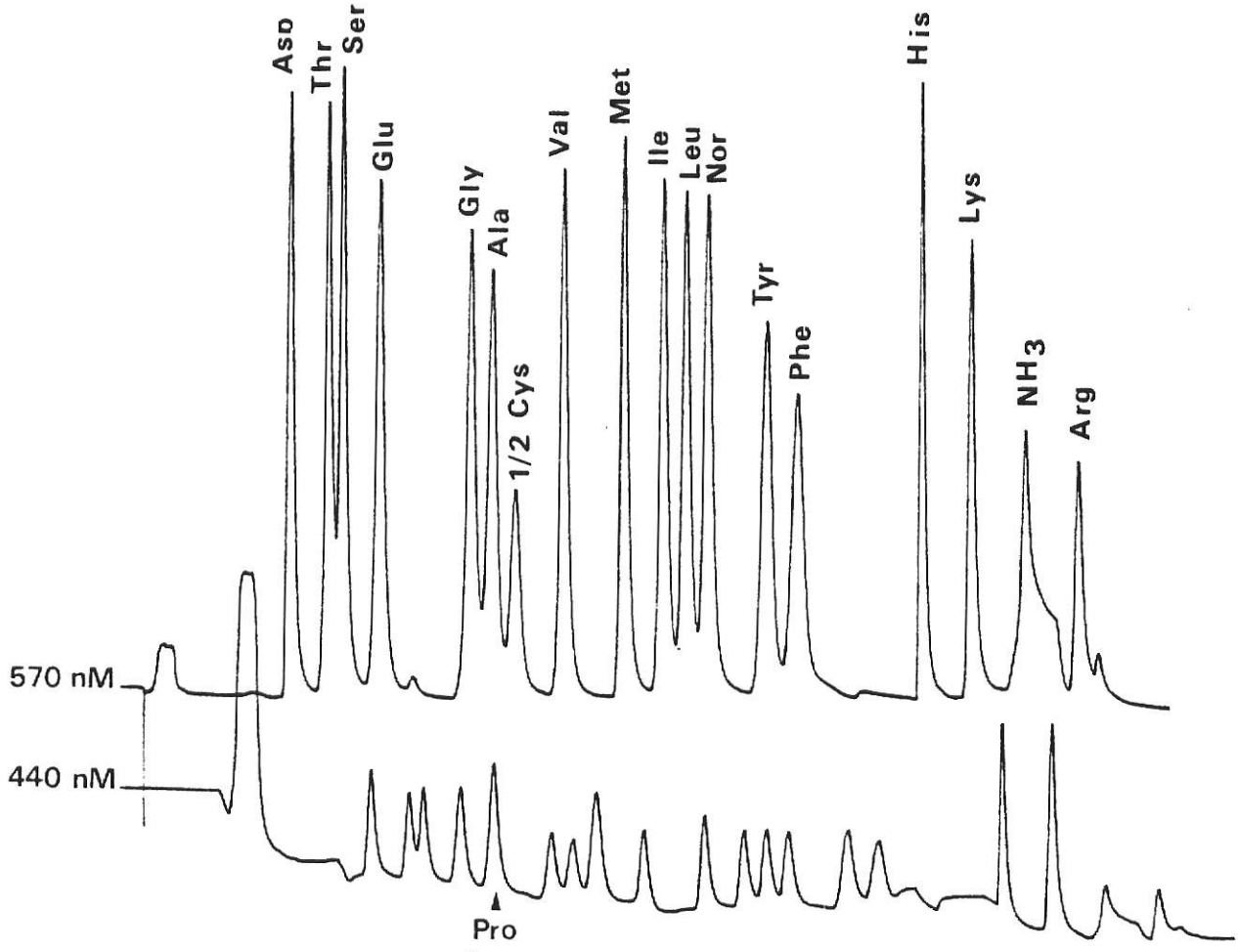

Asp
Thr
Ser
Glu
Gly
Ala
1/2 Cys
Val
Met
Ile
Leu
Nor
Tyr
Phe
His
Lys
NH3
Arg
570 nM
440 nM
Pro

Fig. 26. Chromatogram of an Amino Acid Standard Analyzed with a 6 ft Coil and a Reaction Bath Temperature of 128°C.

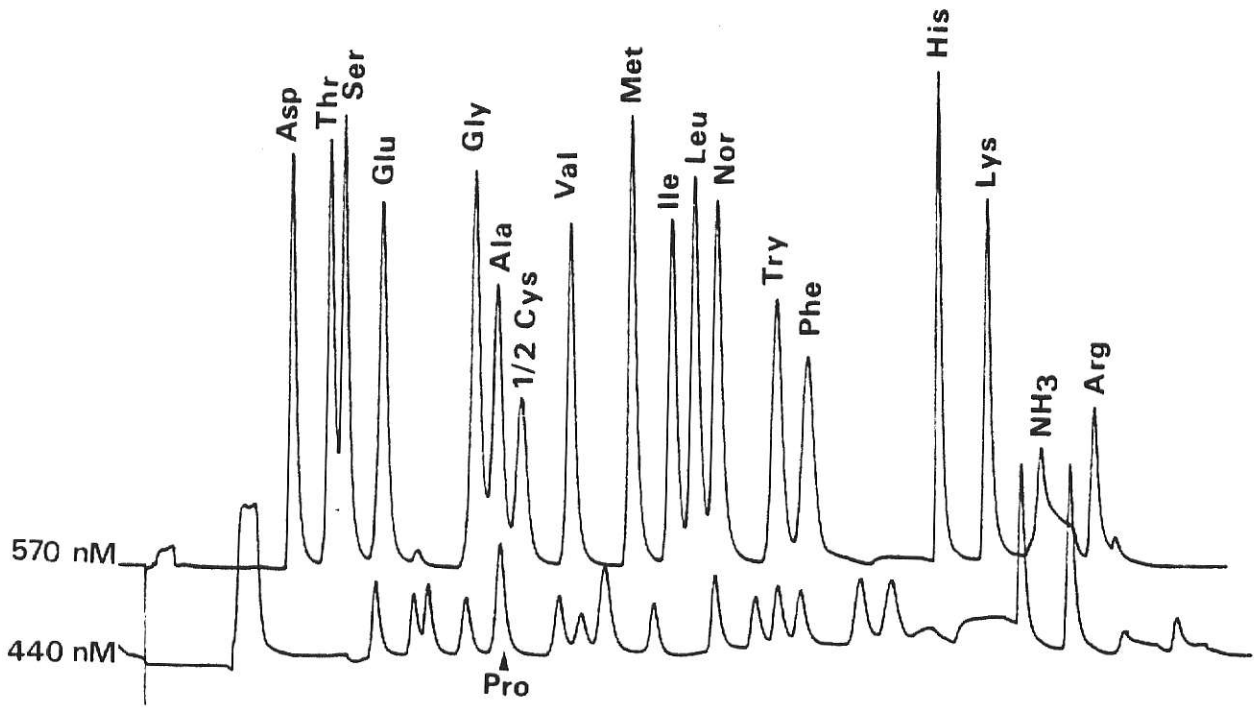

Asp
Thr
Ser
Glu
Gly
Ala
1/2 Cys
Val
Met
Ile
Leu
Nor
Try
Phe
His
Lys
NH3
Arg
570 nM
440 nM
Pro

Table 7

Effect of Temperature and Coil Length on Peak Area

Amino Acid	100°C 18'	128°C 18'	%*	128°C 15'	%	128°C 12'	%	128°C 9'	%	128°C 6'	%
Aspartic Acid	59255	164164	277	140520	237	130690	220	120372	203	80464	136
Threonine	60183	171964	286	147368	244	135048	224	121150	201	85062	141
Serine	70580	191024	271	166852	236	150644	213	135376	192	94572	134
Glutamic Acid	70200	190480	271	166384	237	152056	217	137872	196	95372	136
Proline	11250	14062	125	14531	129	13828	123	13828	123	11953	106
Glycine	78774	177360	225	153752	195	137496	174	125096	159	104382	132
Alanine	56123	175408	312	156900	280	141592	252	124584	222	81048	144
Half Cystine	43992	122090	278	98634	224	81018	184	69438	158	54620	124
Valine	54508	205704	377	169556	311	144616	265	127504	234	81784	150
Methionine	75970	195916	258	161000	212	139260	183	129574	170	99824	131
Isoleucine	55297	194492	352	164876	298	144200	261	128368	232	82840	150
Leucine	71360	188604	264	160632	225	141900	199	131392	184	97770	137
Norleucine	70434	225236	320	183696	261	153328	218	143292	203	100690	143
Tyrosine	65148	181148	278	156320	240	129122	198	122110	187	82532	127
Phenylalanine	65994	204132	309	170456	258	127042	192	123984	188	80524	122
Histidine	79304	151788	191	123906	156	107390	135	98766	124	76384	96
Lysine	54914	162992	297	132292	241	116004	211	100056	182	76782	140
Ammonia	17489	400072	2288	305096	1744	231184	1322	154384	883	65900	377
Arginine	30766	113184	368	95360	310	81044	263	64092	208	39587	129

* - % = (Area at 128°C/Area at 100°C) X 100

Proline color yield did not increase with coil lengths greater than 9 feet. Histidine color yield only increased 191% using the 18 ft coil at 128°C. These two amino acids react more completely with ninhydrin at 100°C than the other amino acids and therefore a very large increase in peak area is not seen at the higher reaction temperature. Ammonia reacts very slowly at 100°C and shows a very high peak area at 128°C. This increase in the color yield of ammonia causes an increase in background noise. The effluent buffers are contaminated with a low level of ammonia which is the major cause of background noise and baseline shifts in single column amino acid analysis (Hare, 1977). Under normal analyzer conditions the small amount of ammonia in the buffers does not react with ninhydrin rapidly enough to cause a great degree of background noise. However, the increase in the rate of the ammonia ninhydrin reaction at 128°C raises the noise level to an intolerable degree and amplifies the baseline. This will prevent use of coils longer than 6 feet in the 128°C reaction bath for low level amino acid detection.

Peak to valley ratios of the threonine-serine doublet were calculated to test for improved resolution resulting from the use of a shorter reaction coil. These ratios for each coil length are given in Table 8. No improvement in resolution was seen as the coil was shortened. This indicates that even in an 18 ft coil very little post column mixing occurs in the microbore teflon tubing used in this analyzer.

A 6 ft coil at 128°C and an 18 ft coil at 100°C were used for analysis of a 144 picomole standard. The 6 ft coil was used to minimize the background noise from ammonia which occurs at the higher temperature. A 144 picomole level was found to be the lowest amino acid concentration detectible with this analyzer and a 100°C reaction bath. Figures 27 and 28 are

Table 8

Peak to Valley Ratio of Threonine-Serine Doublet at Different Coil Lengths

	P/V*
18' Coil 100°C	75.30
6' Coil 128°C	75.70
9' " "	75.51
12' " "	76.00
15' " "	76.60
18' " "	77.00

* 100 - ((Height of Valley from Baseline/ Height of Serine Peak from Baseline) X 100).

Fig. 27. Chromatogram of 144 Picomoles of Each Amino Acid Analyzed with an 18 ft Coil and a Reaction Bath Temperature of 100°C.

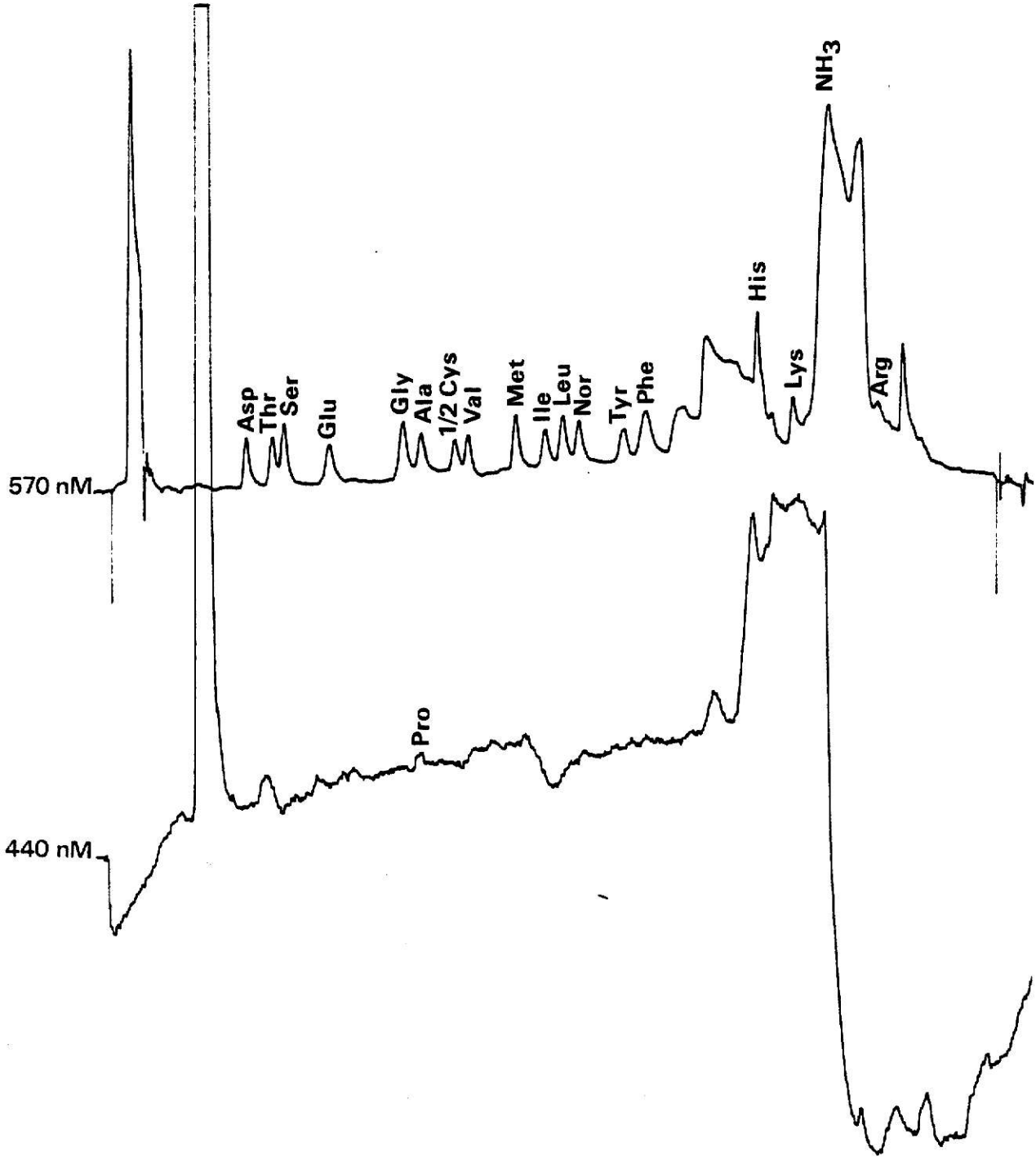
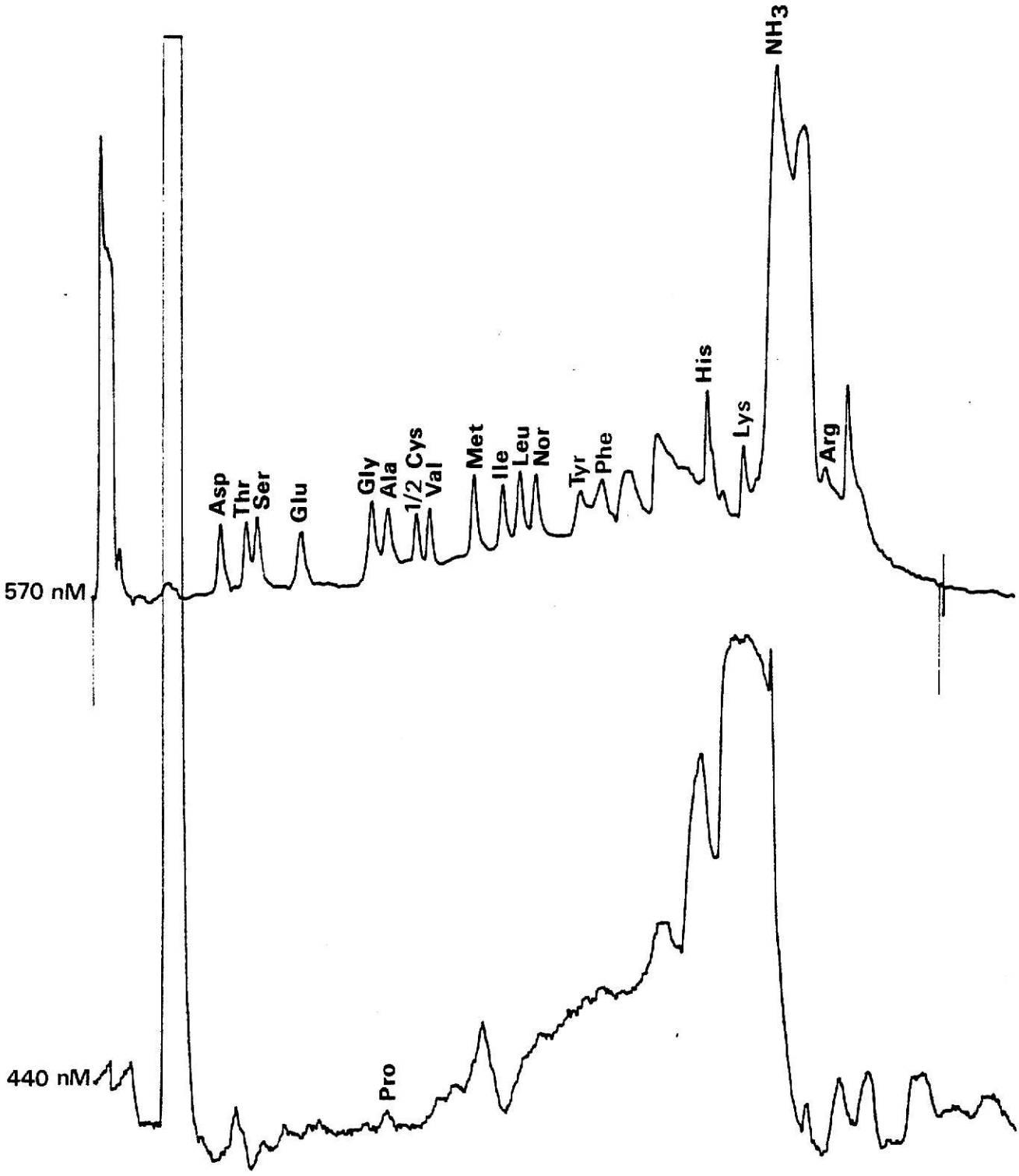

570 nM
440 nM
Asp
Thr
Ser
Glu
Gly
Ala
1/2 Cys
Val
Met
Ile
Leu
Nor
Tyr
Phe
Pro
His
Lys
NH3
Arg

Fig. 28. Chromatogram of 144 Picomoles of Each Amino Acid Analyzed with a 6 ft Coil and a Reaction Bath Temperature of 128°C.


570 nM
440 nM
Asp
Thr
Ser
Glu
Gly
Ala
1/2 Cys
Val
Met
Ile
Leu
Nor
Tyr
Phe
His
Lys
NH3
Arg
Pro

the chromatograms for both analysis, and peak areas are compared and given in Table 9.

The higher temperature bath increased peak areas by 67 to 200%. The peak area of proline at 128°C gave a 200% increase over the 100°C peak area. The 100°C proline peak was so small that it was very hard to distinguish it from the background noise. The 128°C bath increased the proline peak size so that a more accurate calculation of the area could be obtained. The peak areas of half cystine and histidine were increased very little at 128°C because of problems of peak integration with this low level amino acid concentration and high noise level. These peak area integration problems account for the discrimination between the 6' coil percent increase columns in Tables 7 and 9. The background noise and baseline shifts were higher in the high temperature chromatogram due to the increased ammonia reactivity. The high temperature reaction bath did increase peak areas of most of the amino acids but the higher temperature did not improve the signal to noise ratio and allow analysis of lower amino acid concentrations.

Isatin

Isatin has been used as a color reagent for proline detection in thin layer and paper chromatography. It reacts with proline to give a blue colored complex with an absorbance maximum of 610 nM. Proline forms a yellow complex with ninhydrin and is detected at 440 nM while the other amino acids form Rhuhemann's Purple and are detected at the 570 nM wavelength.

Preliminary tests were conducted to determine the optimum reaction conditions of isatin. The first experiment determined the optimum reaction

Table 9

Comparison of Peak Areas of 144 Picomoles of each Amino Acid

Amino Acid	100°C 18' Coil	128°C 6' Coil	% Increase*
Aspartic Acid	12732	19621	154
Threonine	13060	21326	163
Serine	16957	24875	147
Glutamic Acid	16154	27165	168
Proline	1875	3750	200
Glycine	20304	26006	128
Alanine	16962	20940	123
Half Cystine	14259	11367	80
Valine	11010	12260	111
Methionine	14607	27168	186
Isoleucine	10485	20763	199
Leucine	14513	24477	169
Norleucine	14084	23353	166
Tyrosine	9604	18818	196
Phenylalanine	19095	23739	124
Histidine	37583	25038	67
Lysine	13585	20180	148
Ammonia	340424	425848	125
Arginine	33298	50816	153

* % = (Area at 128°C/Area at 100°C) X 100

time at 100°C for best color development. Identically prepared standards were heated at variable time intervals of 0.5 to 4 minutes and their absorbances were read. The results indicated that the color was almost totally developed in 30 seconds (Table 10).

Next color development was studied as a function of isatin concentration. Tubes were prepared that contained variable concentrations of isatin and equal concentrations of proline. After a 3 minute reaction time absorbances were read (Table 11). The absorbance increased with increasing concentration of isatin, but a maximum was never reached. The highest concentration of isatin was therefore used for the following work.

Isatin color yield and absorbance characteristics as a function of pH was examined. Buffers were prepared with a pH range of 2 to 9. Tests were conducted at each pH by preparing a blank and a sample tube with the appropriate buffer. Scans from 750 to 320 nM of blank versus blank and blank versus sample were conducted at each pH. The highest color yield was seen at pH 3.0 and a secondary pH optima was found at pH 6.0. Color production was also seen at pH 2.0 and 4.0, but all other values produced no absorbance. The absorbance maxima was determined to be 595 nM from the pH 3.0 scan. However, no absorbance maxima shift was seen with changes in pH.

A set of potassium hydrogen phthalate buffers, pH 2.5 to 4.0 were used to accurately determine the pH optimum. Equal concentrations of proline and isatin were prepared with different pH buffers. They were allowed to react at 100°C for 3 minutes, were cooled and their absorbances were read at 595 nM. This was repeated three times and average absorbance versus pH (Fig. 29). A pH maximum of 3.0 was determined for the reaction of isatin and proline.

Table 10

Optimum Reaction Time of Isatin with Proline

Reaction Time	Absorbance
0.50 min	0.85
1.00	0.95
1.50	0.89
2.00	0.94
2.50	0.78
3.00	0.85
3.50	0.98
4.00	0.96

Table 11

Optimum Isatin Concentration for the Reaction with Proline

# ml of 2% Isatin	# ml of H_2O	# ul of Proline	Absorbance 570 nM
2.50	2.40	100	1.70
2.00	2.90	100	1.20
1.50	3.40	100	0.72
1.00	3.90	100	0.395
0.50	4.40	100	0.16
2.50	2.50	0	Blank

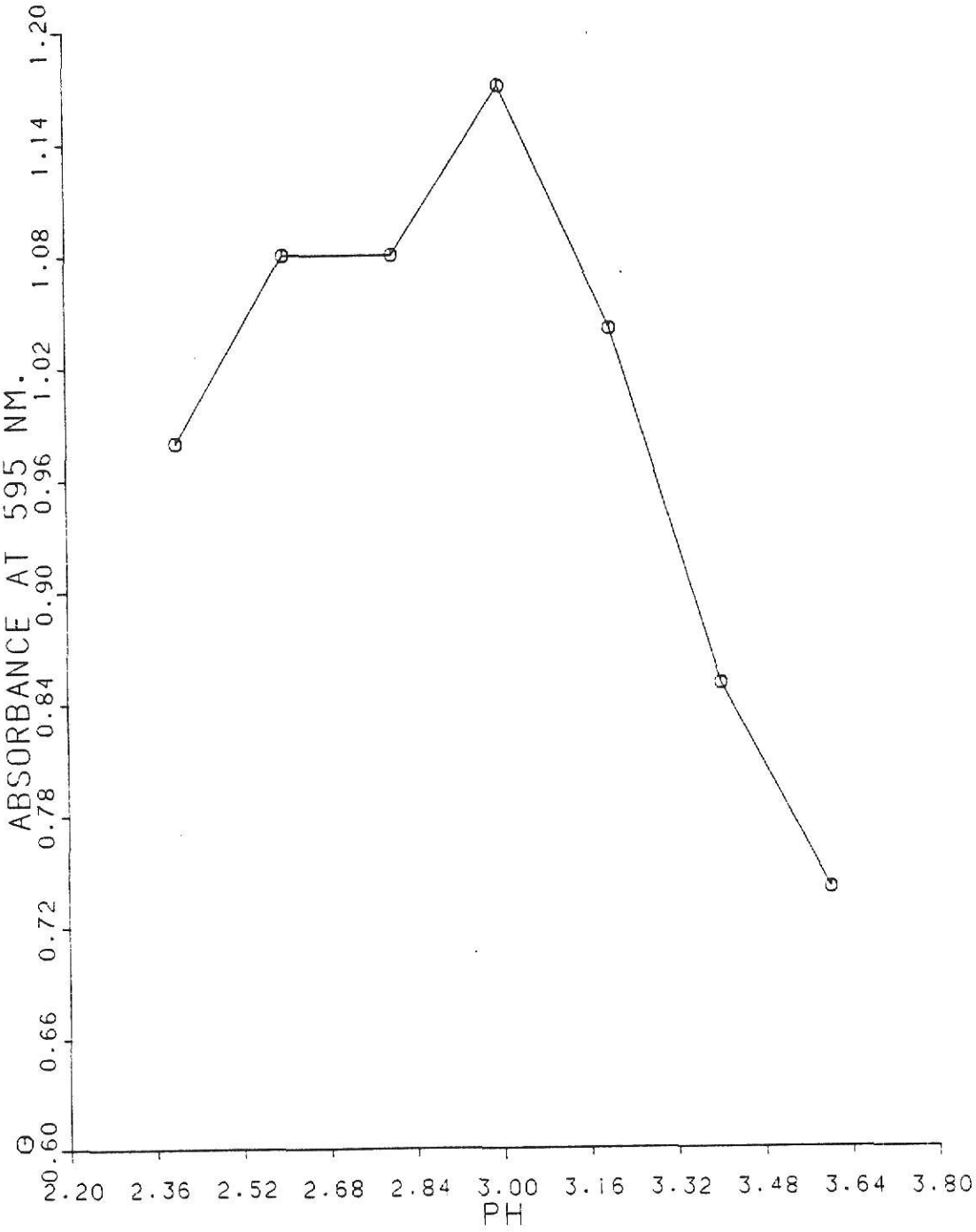


Fig. 29. pH versus Absorbance at 595 nM.

An experiment was conducted using varying concentrations of proline to determine if the isatin proline reaction follows Beer's law. A plot of absorbance at 595 nM versus the number of ul's of 12.25 uM/ml proline was made (Fig. 30). A straight line resulted which indicates the reaction does follow Beer's law in this concentration range.

The color yield of isatin with lysine and ammonia was examined. Standards of lysine, ammonia, and proline at the same molar concentration were mixed with isatin, heated, cooled, and their absorbances read at 595 nM. Proline had an absorbance of 0.9 and lysine and ammonia gave absorbances of 0.015 and 0.0 respectively. Lysine and ammonia gave a very low color yield with isatin.

An isatin ninhydrin color reagent was prepared using a 4 M pH 6.0 sodium acetate buffer. The pH optimum of 3.0 for isatin could not be used because acid hydrolysis of Rhuhemann's Purple occurs in the low pH range (Friedman and Williams, 1974). Hydrindantin was used because the effect of stannous chloride reduction of isatin was not known. Standard mixtures of amino acids were analyzed using the isatin-ninhydrin reagent and a ninhydrin reagent. The two color reagents gave equal proline areas but the isatin-ninhydrin reagent gave smaller peak areas of the other amino acids. The color yield of the isatin-ninhydrin was examined at 128^{o}C by replacing the water in the reaction bath with acetoin. The resulting chromatograph showed an increase in all amino acid peak areas except for the peak area of proline which was not increased. The isatin-ninhydrin reagent at pH 6.0 did not improve the color yield of proline in the 570 nM wavelength as was hoped.

A pH 3.0 isatin-ninhydrin reagent was prepared using a 4.0 M pH 3.0 sodium formate buffer. DMSO was used to replace 60% of the methyl cellosolve. DMSO was used because Friedman and Williams (1974) demonstrated

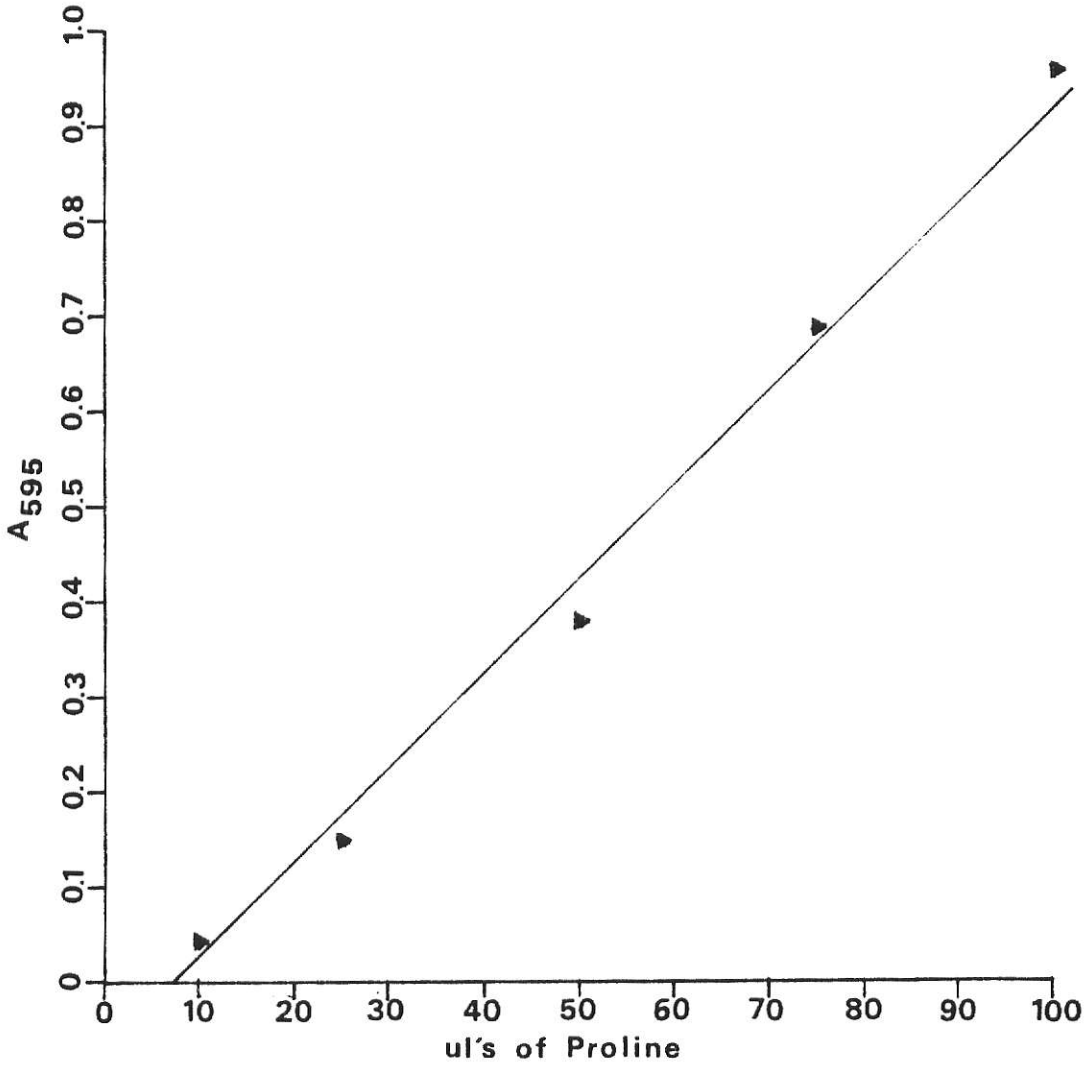


Fig. 30. # ul's of 12.5 uM/ml Proline Versus Absorbance at 595 nM.

the increased stability of Rhumann's purple under acid conditions in the presence of DMSO. While preparing this reagent, precipitation occurred when the DMSO was added. This was believed to be due to the low solubility of sodium formate in DMSO. A standard was analyzed with this reagent. The resulting chromatogram, when compared to a standard run with the normal ninhydrin reagent, showed better color yields of all the amino acids except proline. The pH was taken of the isatin-ninhydrin eluent mixture and was found to be 4.25. The precipitation of the sodium formate buffer decreased the buffering capacity of the color reagent and a pH of 3.0 could not be maintained when the color reagent was mixed with the effluent. A pH of 3.0 was needed for optimum reaction of isatin with proline.

Another isatin-ninhydrin reagent was prepared using a lithium formate buffer at pH 3.0. Lithium was used because Moore (1968) found that lithium acetate was more soluble in DMSO than the sodium salt. Comparisons were made between the lithium formate isatin-ninhydrin reagent and a ninhydrin reagent. The isatin-ninhydrin reagent was a very dark color, and this produced a very large background absorbance that was too high to allow peak detection.

The isatin-ninhydrin reagents did not give an increase in the absorbance of proline at 570 nM. This may be due to a competitive reaction of proline with isatin and ninhydrin. More work needs to be done in experimenting with other reaction conditions and reagents. However, isatin does not show promise as an added ingredient to the ninhydrin reagent. Isatin did not give an increase in proline absorbance at 570 nM. The narrow pH optima in the acid pH range is not conducive to use with ninhydrin. More research is needed to solve the problem of proline quantification.

SUMMARY

A comparison of HCl and PTSA acid hydrolysis of KSU flour showed comparable recoveries of all amino acids except proline and tyrosine. PTSA gave a lower recovery of proline and a higher recovery of tyrosine than HCl. A 30 hour PTSA hydrolysate was found to be comparable to 24 hours of hydrolysis with HCl.

The color yield of the ninhydrin reaction was increased by raising the reaction bath temperature. Ammonia showed a very large increase in color yield which greatly increased the background noise at the higher temperature. No gain in analyzer sensitivity was realized using the 128°C reaction temperature.

Isatin was investigated for the development of a color reagent that would allow the detection of proline at 570 nM. No increase in proline 570 nM absorbance was seen.

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AMINO ACID ANALYSIS: HYDROLYSIS, COLOR REAGENT, AND SENSITIVITY

by

MAX ALBERT JONES

B. S., Kansas State University, 1977

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Grain Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1981

The HCl and PTSA acid hydrolysates of KSU flour at 5, 10, 15, 20, 24, 30, 35, 40, 50, 60, 72, and 100 hours were compared. Both gave comparable recoveries except PTSA gave lower proline and higher tyrosine values than HCl.

The color yield of the ninhydrin reaction was increased by raising the reaction bath temperature from 100°C to 108°C and 128°C. An increase in peak areas and background noise resulted with no gain in sensitivity.

Isatin was investigated for use in an isatin-ninhydrin color reagent for proline detection at 570 nM. The proline 570 nM absorbance level was not increased.