Standard Hydrolysis Procedure:

The following is a vapor phase hydrolysis technique for multiple samples where samples are hydrolyzed in vial inserts that are placed inside a hydrolysis vessel. This is only one suggested method; there are numerous different methods in the literature.

1. Transfer samples for hydrolysis into Pyrolyzed autosampler vial inserts (250 µL) {optional step: pyrolyze inserts by heating in 400°C oven for 6 hours, cool overnight prior to using inserts for hydrolysis) (note: one may want to etch vials for later identification)
2. Dry samples in vial inserts with a Speed Vac concentrator
3. In a Hydrolysis vessel add the following reagents
    a. 989 µL of 6N HCl (Fluka, constant boiling HCl)
    b. 10 µL of 5% phenol (Sigma, make every 3 months and store 4°C under N_2)*
    c. 1 µL of betamercaptoethanol (Biorad, 6month lifetime and store 4°C)*

    *Note: Make sure you add acid into bottom of vessel and mix phenol and BME directly into acid solution at bottom of hydrolysis vessel
4. Add 6-8 vial inserts into one hydrolysis vial and seal vial
5. Put vial in ice bucket for 5 minutes than purge 3X with N_2 and Vacuum.
6. Pull vacuum from vial and close mininert valve
7. Hydrolyze 110°C for 24 hours
8. Open vial and remove inserts containing samples. Add 50 µL of H_2O to vial and then add 5-15 µL of reagent 2 to bring the pH of solution to between pH 2-3.
9. Add 100 µL of EZ:faast reagent 1 into vial insert and transfer all of the sample solution into a EZ:faast vial
10. Perform EZ:faast method as per manual

Reagent and Equipment sources:

Hydrolysis vials:	Kontes (P/N 896820-0000)
Vial inserts:	Fisher-(P/N 03-375-39C for 300 µL insert, 03-375-1C for 700 µL insert) VWR-(P/N 66009-992 for 250 µL insert, 66011-202 for 700 µL insert)*
6N HCl and 4N MSA	Fluka
Ultra pure Phenol	Sigma, Fisher, or Aldridge
Betamercaptoethanol	Biorad
3 Way glass valve for purge apparatus:	Kontes
Vacuum Pump	Many vendors
Speed Vac	Savant

*Note- for inserts with plastic springs, make sure you remove spring/seat before using insert for hydrolysis

Some Literature on the Subject that has similar methods:

How to Perform Vapor Phase HCl Hydrolysis*

1. Vacuum dry sample in clean 6 x 50 mm Pyrex tube.
2. Place sample tubes in a 40 mL screw cap bottle containing 200 µL 6N HCl plus a crystal of phenol. Cap with a modified mini-inert slide valve (Kontes).
3. Evacuate and flush with argon 3 times. Close slide valve on last vacuum step.
4. Heat 1 hr at 150°C. Following hydrolysis release pressure within a fume hood, pointing away from your face!
5. Transfer sample tubes to another container and vacuum dry. Store at -20°C under argon until submitted for AAA.

* from G.E. Tarr (1986) In: *Microcharacterization of Proteins* (J.E. Shively, ed.), Humana Press, pp. 155-194.

How to Perform Liquid Phase HCl Hydrolysis*

1. Vacuum dry sample in clean glass test tube (10 x 150 mm).
2. Add 100 µL 6N HCl containing 4% thioglycolic acid directly to the sample.
3. Evacuate the hydrolysis tube to <100 microns and flame seal.
4. Heat 22 hours at 110°C.
5. Open cooled tube, vacuum dry and analyze.

* M.C. Miedel, J.D. Hulmes and Y.-C.E. Pan (1989) J. Biochem. Biophys. Methods 18, 37-52.

Note: Under normal acid phase hydrolysis conditions TRP and CYS are both destroyed (MET also has low recoveries <50%). To analyze these and other specialty amino acids different methods must be used to obtain results for these amino acids. The major drawback with these techniques is that they often do not completely hydrolyze all amino acids and thus can give spurious results for other amino acids when using these methods.

Hydrolysis Method for Tryptophan Analysis

Hydrolysis with 4N Methane Sulfonic Acid (3)

1. Add 50 µL 4N methane sulfonic acid (Fluka) to dried sample in vial.
2. Seal tube in vacuo and heat 22 hours at 110°C.
3. Neutralize with 50 µL 4N NaOH.
4. Dilute with 90 µL water, 10 µL reagent (verify pH 2-3) and analyze via EZ:faast.

References:

1. D.J. Strydom *et al.* (1993) In: *Techniques in Protein Chemistry IV* (R.H. Angeletti, ed.) Academic Press, pp. 279-288.
2. Bozzini *et al.*, Applied Biosystems Research News, February 1991; K.A. West and J.W. Crabb (1992) In: *Techniques in Protein Chemistry III* (R.H. Angeletti, ed.) Academic Press, pp. 233-242.
3. B.N. Jones *et al.* (1981) J. Liquid Chromatography 4, 565-586.

Cysteine Analysis

To analyze CYS there are several choices including: carboxymethylation, pyradylethylation, carboxyamidomethylation. You perform these modifications BEFORE you hydrolyze using the standard techniques. I recommend using Reduction/ Alkylation with DTT and IAM. It is a simple method:

1. Dry sample down in an Eppendorf tube via speed vac concentrator.
2. Redissove sample 25-50 µL of 8M Urea/ 0.4M NH_4HCO_3 (no need to pH sample to 8.0)
3. Add 2.5 to 5 µL of 10mM dithiothreitol (DTT) and incubate at 50°C for 15 minutes.
4. Add 2.5 to 5 µL of 100mM iodoacetimide (IAM) and incubate at room temperature for 15 minutes in the dark.
5. Add 2.5 to 5 µL of 100mM DTT to quench the IAM
6. Transfer sample to hydrolysis vial and hydrolyze using the standard methods.

Nitro-tyrosine and Nitro-phenylalanine

Another option you have is to convert nitrotyrosine to aminotyrosine prior to hydrolysis or sequencing. Treatment of 3-nitroTyr with sodium hydrosulfite ($Na_2S_2O_4$) results in rapid, quantitative conversion into 3-aminoTyr (see Sokolovsky BBRC 27, p 20, 1967). AminoTyr is stable under conditions of hydrolysis and can be quantitated by AAA and by sequencing, though where it elutes I do not know. It also has nice absorbtion spectra, depending upon pH, which is nice for proof of existence (but not location). Conditions for conversion: treat the protein with 0.05M Tris, pH 8.0, 32-fold molar excess of sodium hydrosulfite, 10 minutes.

Some References regarding Hydroxyproline

One hydrolysis reference from ABRF (sounds similar to the standard method except less hydrolysis time):

Hydrolyze my sample in a snap-top epi-vial at 110°C for 16h in 0.25 mL constant boiling HCl on a heating block.
Dry down with nitrogen or on a Speedvac.

Other References:

Biochem 14 296-304 (1966)

Lamport and Northcote "Hydroxyprolin in primary cell walls of higher plants" Nature 118:665-66.

Annual Rev. Plant Physiol. Plant Mol. Biol. (1998) 49:281-309 Plant Cell Wall Proteins written by Gladys Cassab.

Analysis of Hydroxyproline and Hydroxyproline-Arabinosides of Plant Origin by High-Performance Anion-Exchange Chromatography-Pulsed Amperometric Detection" Campargues et.al. Anal. Biochem. 257:20-25 (1998).