COLLAGEN CONTENT FROM HYDROXYPROLINE

Sample Preparation

1. Place 1.6 – 3.5 g of powdered frozen sample in an aluminum weigh pan. Record weight. Place in a 110° C oven and dry sample for 30 min. Weigh and record dry sample weight. For uncooked samples, place 6 g of sample in weigh pan , record weights and heat in 110° C oven for 1.5 hr.

2. Using a mortar and pestle, grind sample to a fine powder.

3. Weigh 300 mg sample (record exact sample weight) and transfer to a 100 ml serum vial. Add 50 ml 6N HCl.

4. Seal vials with a rubber stopper and aluminum tear-away seal. Purge with dry nitrogen gas for 10 minutes. For exhaust, a disposable 20 gauge needle inserted off center in the stopper. For nitrogen purge, a 4" 20 gauge needle is inserted in the center of the stopper and pushed almost to the bottom of the vial. After 10 min nitrogen purge, first remove the nitrogen purge needle and then the exhaust needle.

5. Place vials in a pan and place the pan in a 110°C oven and heat for 20 hours.

6. Remove the pan of vials and allow the samples to cool 30 min. Because pressure can build up in the vial, hold thumb over stopper as you are peeling the seal and ease the stopper out of the vial, releasing the pressure gradually.

7. Using Whatman 54 paper, 12.5 cm, filter sample quantitatively into a 100 ml volumetric flask. Rinse the vial, lip of the vial, filter paper and then the funnel to obtain all the sample. Bring to volume with water and mix thoroughly.

8. Remove 50 ml and place into a 500 ml evaporating flask. Evaporate to dryness at 60°C in a vacuum rotary evaporator. After the sample dries down, rinse the flask with water to rehydrate the sample and then evaporate to dryness again.

9. Quantitatively transfer sample to a 50 ml volumetric flask using sodium diluent. To transfer the dried sample from the flask, use a Pasteur pipette and add diluent to flask. Swirl the flask to dissolve all the sample. Transfer to a 50 ml Nalgene beaker. Rinse flask several times. Transfer beaker contents to 50 ml volumetric, and then rinse beaker several times with diluent. Bring 50 ml volumetric flask to volume with diluent. Mix well.

10. Transfer sample to a 20 ml scintillation vial. Using a 3 ml Luer-lok syringe draw up sample. Filter sample through a 13 mm Swinney filter fitted with a 0.2 µm, Gelman Supor 200 membrane filter into a disposable glass tube. The remaining sample in the scintillation vial may be frozen for future re-runs.

HPLC Sample Prep

To a 2.0 ml HPLC vial add:

500 µl filtered sample

28 µl 2.5 µM Cysteic acid (Injected amount = 1 µg)

HPLC Analysis

Separation of amino acids is by ion-exchange chromatography and photometric detection of post-column derivatives of primary amines and ninhydrin.

1. Inject 50 µl of sample automatically into the Spectra Physics HPLC system, using SP8800 pump, and SP8880 autosampler.
   a. Column: 3mm x 250mm, 10 µm cation exchange resin, Na form, Pickering # 1193250
   b. Flow rate: 0.4 ml/min
   c. Run Time: 40 min
   d. Column Temperature: 54°C
   e. Gradient elution buffer: Sodium Eluent 2.70 (ChromTech #Na270) and Regenerant (Pickering PN RG011)
   f. Spectra FOCUS - Forward Optical Scanning Detector; set at 440 nm wavelength
   g. Used Trione (ninhydrin) color reagent (ChromTech #T200)

2. Calculations performed by SpectraSYSTEM software PC1000.
   The hydroxyproline content (ng) is calculated from the standard curve, the hydroxyproline area, and adjustment for cysteic acid area deviation from expectation. This value is plugged into an equation to account for both sample dilution during analysis and the amount of hydroxyproline in the collagen of muscle (Goll et al., 1963; Woessner, 1961) to give mg of collagen per g cooked muscle.

Reagents

1. ***Sodium Diluent, pH 2.20***
   Beckman P/N 239440; [Na^+] = 0.27 N

2. ***Sodium Eluent, pH 2.70***
   ChromTech P/N Na270

3. ***Trione/two part***
   ChromTech P/N T120C

Solutions

1. ***6 N Hydrochloric Acid***
   Add approximately 700 ml water to 2 liter volumetric flask. Slowly add 990 ml concentrated HCl. Bring to volume and store at room temperature.

2. ***2.5 µM Cysteic Acid in 0.1 N HCl***
   0.0189 g/50 ml 0.1 N HCl. Store refrigerated.

Standard Curve

Use trans-4-hydroxy-L-proline, Sigma Ultra.
Make a 1mg/ml solution with sodium diluent.
The cysteic acid is used to adjust for instrumentation variation.

	Hydroxyproline	2.5µM Cysteic Acid	Na Diluent
125 ng	2.5 µl	53 µl	944.5 µl
250 ng	5.0 µl	53 µl	942.0 µl
375 ng	7.5 µl	53 µl	939.5 µl
500 ng	10.0 µl	53 µl	937.0 µl
625 ng	12.5 µl	53 µl	934.5 µl
750 ng	15.0 µl	53 µl	932.0 µl
(injected)		(1 µg injected)	

References:

adapted from:

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