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Comparison between a Spectrophotometric and a High-Pressure Liquid Chromatography Method for Determining Tryptophan in Food Products

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A comparison is made between a spectrophotometric method and a high-pressure liquid chromatography (LC) method for tryptophan content of a variety of foods. The spectrophotometric method has an RSD of 2.53%. The LC method has an RSD of 2.03% with a recovery of 95.5 ± 2.4% for spiked samples. The mean tryptophan content of 18 samples by the spectrophotometric method was 0.38%, and that by the LC method was 0.35%.

Among the major challenges facing the food chemist today is the need for more accurate and cost-effective methods for nutrient analysis. Spectrophotometry and/or high-pressure liquid chromatography (LC) when coupled with appropriate sample preparation and workup procedures meet these criteria.

One of the nutritionally essential amino acids, tryptophan, has been analyzed by a variety of methods in the past. These methods have been reviewed by Friedman and Finley (1971). One of these methods, studied thoroughly by Spies (1967), which utilizes Pronase hydrolysis, derivatization with *p*-(dimethylamino)benzaldehyde, and spectrophotometric measurement appears to be well suited for food products. Tryptophan has also been measured by LC in biological samples by a variety of methods. These include separation on copolymer packings (Lefebvre et al., 1977, 1978; Kroeff and Pitrzyk, 1978), derivatization and colorimetric and/or fluorometric detection (LaPage et al., 1979; Hsu and Currie, 1978; Margolies and Brauer, 1978; Lammens and Verzele, 1978; Van Beeumen et al., 1978; Jornvall et al., 1978; Furukawa et al., 1977), direct fluorometric detection (Anderson and Purdy, 1977, 1979; Geeraerts et al., 1978; Krstulovic et al., 1977a,b; Meek and Neckers, 1977; Graffeo and Karger, 1976), and others (Rustun, 1978; Hancock et al., 1979; Riley et al., 1979; Krstulovic et al., 1978; Molnar and Horvath, 1978; Knudson et al., 1978; Grushka et al., 1977). Of these methods, reverse-phase chromatography coupled with direct fluorometric detection appeared to be the most viable method for food products.

Amino acids other than tryptophan are routinely analyzed by using ion-exchange chromatography following acid hydrolysis (Wall and Gehrke, 1976). Under these conditions tryptophan is labile and generally has to be analyzed separately. This is generally done by using basic hydrolysis or acid hydrolysis while protecting the tryptophan with an antioxidant followed by basic ion-exchange analysis on an amino acid analyzer, ultraviolet spectrophotometry, or fluorometry (Peters and Berridge, 1970; Berridge et al., 1971; Wapnir and Stevenson, 1969; Wilinson et al., 1976; Eftnik and Ghiron, 1976; Hassan, 1975; Lewis et al., 1976; Spackman et al., 1958). What was sought, therefore, was a method to complement the amino acid analyzer ion-exchange method and quantitate tryptophan accurately and efficiently. The spectrophotometric method was a modification of that of Spies (1967). The LC method developed utilized the hydrolysis developed by Spies and separation and quantitation similar to those used by Krstulovic et al. (1977a) for serum samples.

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EXPERIMENTAL SECTION

Apparatus. Spectrophotometer: Linear Absorbance, Model 6120A (Coleman, Norwalk, CT 06856). Pump: Model 110A, Constant Flow (Altex, Berkeley, CA 94710). Column: μ-Bondapak C_{18} (Waters Associates, Milford, MA 01757) or Lichrosorb RP-18 (Altex). Injector: Autosampler LC 420 equipped with a 20-μL loop (Perkin-Elmer, Norwalk, CT 06856). Detector: Spectrofluoromonitor LC650-10 (Perkin-Elmer); excitation at 295 nm with a 12-nm slit width and emission at 320 nm with a 12-nm slit width.

Materials. *Phosphate Buffer (pH 7.5).* Sodium phosphat dibasic (Na_2HPO_4) (4.40 g) and potassium phosphate monobasic (KH_2PO_4) (4.40 g) were dissolved in and diluted to 1 L with water. The pH was checked and adjusted to 7.5 if necessary.

Pronase Solution (4 mg/mL). Pronase (Calbiochem B grade, 45 × 10^3 PUK/g; Calbiochem, La Jolla, CA 92037) (100 mg) was placed in a 25-mL volumetric flask, and the flask was brought to volume with phosphate buffer. The mixture was prepared just prior to use. NOTE: Since the solution remains turbid, it was shaken before addition to each sample.

Sulfuric Acid (21.2 N). Concentrated H_2SO_4 (142 mL) was added in 25-mL portions to 85 mL of distilled water while the flask was swirled under cold tap water. The solution was cooled to room temperature before use.

p-(Dimethylamino)benzaldehyde (DAB). DAB (0.94 g) was dissolved and brought to 250-mL volume with 21.2 N sulfuric acid. The solution was prepared just prior to use.

Sodium Nitrite (0.048%). Sodium nitrite (24 mg) was dissolved and brought to 50-mL volume with distilled water.

Tryptophan Standards. For a stock solution (1 mg/mL), 100 mg of L(−)-tryptophan was dissolved and brought to 100-mL volume with phosphate buffer solution. An

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unheated ultransonic bath was used to aid in solution.

Standard Solutions (200, 100, 50, 25, 20, and 5 μg/mL). Various dilutions of stock solution were made by using appropriate pipets and volumetric flasks and diluting to volume with phosphate buffer.

Mobile Phase. One gram of sodium acetate was dissolved in approximately 100 mL of distilled water. Eighty milliliters of acetonitrile was added and the solution brought to 1000 mL with distilled water. The pH was adjusted to 4.0 with acetic acid.

Sample Preparation. Dry samples were ground and/or pulverized to pass through a 40-mesh screen.

Hydrolysis Procedure. A portion of sample containing approximately 25 mg of protein was weighed into a 10-mL volumetric flask. When the spectrophotometric method was used 2.0 mL of each of three standards (200, 100, and 20 μg/mL) was also pipetted into 10-mL volumetric flasks. A 1.0-mL amount of Pronase solution was pipetted into each of the flasks as well as an empty flask to be used as a Pronase blank (Pronase itself releases tryptophan during hydrolysis). The flasks were placed in an ultrasonic bath until the samples were wetted, and then 2 drops of toluene was added and the flasks were placed in a 40 ± 1 °C bath for 24 h. After being cooled to room temperature, the blank, samples, and standards were brought to volume with phosphate buffer.

Spectrophotometric Method. An 8.0-mL amount of acidic DAB solution was pipetted into a large test tube (15 mm i.d. × 150 mm). A 2.0-mL aliquot of the hydrolysate was pipetted into the test tube, and the contents were mixed on a vortex stirrer. The tubes were stoppered and placed in the dark for at least 6 h (usually overnight); 0.1 mL of sodium nitrite solution was then added to the test tube, and the tube was again mixed on a vortex mixer and then set aside for 30 min to allow color development. If necessary, solutions were filtered through Whatman GFA paper. The spectrophotometer was zeroed by using a solution of 8.0 mL of 21.2 N H_2SO_4, 2.0 mL of phosphate buffer, and 0.1 mL of sodium nitrite solution at 590 nm. The absorbance of the samples, standards, and blank was read at 590 nm. In cases where the hydrolysate was colored, a sample blank was prepared by mixing 2.0 mL of hydrolysate with 8.0 mL of 21.2 N H_2SO_4 and its absorbance was measured.

Calculations. (1) The sample blank absorbance was subtracted from the sample absorbance where applicable. (2) The Pronase blank was subtracted from the absorbance of the samples and standards. (3) The corrected absorbances of the standards were plotted against the microgram amount of tryptophan present in the volumetric flask (i.e., 400, 200, and 40 μg). (4) The microgram amounts of tryptophan in the sample were determined from the plot. (5) The percent tryptophan = $0.1T/S$, where T = micrograms of tryptophan present, S = milligrams of sample, and 0.1 = % conversion factor times conversion from micrograms to milligrams.

LC Method. The separation and quantitation were carried out with a flow of 1.5 mL/min. Tryptophan eluted at 2.5–4 min. Twenty microliters of each of the standards (50, 25, and 5 μg/mL) was injected, as was 20 μl of the Pronase blank and each of the sample hydrolysates. Tryptophan was quantitated as

$$\% \text{ Trp} = \frac{C}{W} \times \frac{P_X - P_B}{P_S} \times \frac{10 \text{ mL}}{1000 \text{ μg/mg}} \times 100\% = \frac{C}{W} \times \frac{P_X - P_B}{P_S}$$

where C = concentration of standard in micrograms per milliliter, W = weight of sample in milligrams, P_X = peak height of sample, P_B = peak height of blank, and P_S = peak height of standard.

Table I. Tryptophan Content by the Spectrophotometric Method

sample	Trp content, % run 1	run 2	av
(1) yeast	0.57	0.59	0.58 ± 0.014
(2) yeast	0.47	0.47	0.47 ± 0.00
(3) yeast	0.56	0.59	0.575 ± 0.021
(4) yeast	0.49	0.49	0.49 ± 0.00
(5) yeast	0.43	0.44	0.435 ± 0.00
(6) yeast	0.47	0.48	0.475 ± 0.007
(7) soy protein	0.89	0.88	0.885 ± 0.007
(8) yeast	0.52	0.54	0.53 ± 0.014
(9) meat and bone meal	0.16	0.17	0.165 ± 0.007
(10) lemon product	0.26	0.23	0.245 ± 0.021

Table II. Comparison of Tryptophan Content Obtained by Spectrophotometric and LC Methods

sample	Trp content, % spectrophotometric	LC
(1) protein powder	0.61	0.61
(2) protein powder	0.50	0.56
(3) protein powder	0.97	0.91
(4) protein powder	1.11	0.90
(5) protein powder	1.17	0.90
(6) liquid diet	0.05	0.06
(7) protein powder	0.18	0.18
(8) protein powder	0.20	0.21
(9) lemon product	0.26	0.28
(10) lemon product	0.23	0.26
(11) yeast	0.13	0.13
(12) dried food powder	0.10	0.13
(13) dried food powder	0.01	0.00
(14) yeast	0.47	0.39
(15) protein tablet	0.33	0.34
(16) protein tablet	0.26	0.27
(17) alfalfa	0.16	0.10
(18) alfalfa	0.14	0.10
mean	0.38	0.35
difference	0.03%	
correlation	0.983	

RESULTS AND DISCUSSION

The hydrolysis technique described above was very straight forward and was carried out with a minimal amount of manual effort. A variety of samples were analyzed by the spectrophotometric method in duplicate; the results are shown in Table I. As can be seen, the method is very reproducible. The pooled coefficient of variation was 2.53% relative for the 10 samples done in duplicate. This agrees with the results of Spies (1967).

A comparison was run between the spectrophotometric method and the LC method; the results are shown in Table II. As can be seen, the results obtained from the two methods are in good agreement; i.e., the difference between the means was only 0.03%. Samples no. 4 and 5 gave hydrolysates which were very highly colored. This color may have affected the ultimate value obtained spectrophotometrically, even though a sample blank was subtracted. If samples no. 4 and 5 are omitted from the data set, the mean by the spectrophotometric method is 0.287% and that by the LC method is 0.283% or a difference of 0.004% with a correlation of 0.988.

A number of samples were also analyzed in duplicate by LC. The results are shown in Table III. Also shown in Table III are the results of a recovery study. A quantity of tryptophan of the same magnitude as that found in the

Table III. Reproducibility and Recovery Obtained for Tryptophan Analysis by LC

sample	% Trp ± SD[a]	% recovery
(1) liquid protein	0.0349 ± 0.0008	95.9
(2) liquid protein	0.0371 ± 0.0002	100.0
(3) liquid protein	0.0345 ± 0.0003	98.0
(4) soy powder	0.515 ± 0.007	97.9
(5) soy powder	0.515 ± 0.007	91.1
(6) soy powder	0.560 ± 0.014	95.9
(7) yeast	0.394 ± 0.002	94.6
(8) yeast	0.026 ± 0.016	91.8
(9) yeast	0.438 ± 0.001	94.5
(10) yeast	0.422 ± 0.003	95.5
(11) yeast	0.445 ± 0.002	95.5
(12) yeast	0.438 ± 0.0007	95.1
(13) yeast	0.501 ± 0.006	96.0
av ± SD		95.5 ± 2.4

[a] Average of duplicate analysis.

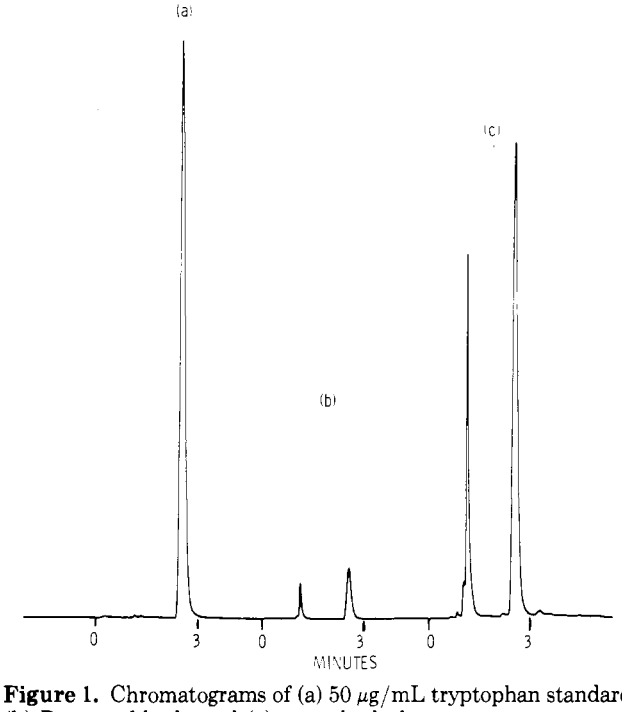

Figure 1. Chromatograms of (a) 50 μg/mL tryptophan standard, (b) Pronase blank, and (c) yeast hydrolysate.

sample was added to the sample before the Pronase hydrolysis step. As can be seen, reproducibility of the method is very good, giving a pooled COV of ±2.03% relative. The recovery results were also good, i.e., 95.5 ± 2.4% for the products analyzed.

The results of the study indicate that the LC method compares favorably with the published colorimetric method. The LC method is considerably easier and less time consuming to carry out. Quantitation is straight forward, since the chromatograms usually contain only one other peak in addition to the tryptophan peak (see Figure 1).

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