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A SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR ANALYZING RIBOFLAVIN IN FRESH FRUITS AND VEGETABLES

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ABSTRACT

Riboflavin (Vitamin B₂) in fresh fruits and vegetables, which is present in ng levels, was separated on a reverse-phase Altex-ultrasphere ODS column with water, methanol, and heptane sulfonic acid as the mobile phase. Ion-pairing reduced tailing of the peak. Quantity of riboflavin was based on fluorescence, excitation at 450 nm, and emission at 530 nm.

INTRODUCTION

Analysis of riboflavin has been improved with the use of high-performance liquid chromatography (HPLC), and several

methods have been developed for analyzing simultaneously riboflavin and thiamine or riboflavin and other water-soluble vitamins (1,2,3,4). These methods are applicable for, and generally limited to, sample material which contains about the same quantity of each analyzed vitamin, so long as the concentration of the analyzed vitamin is in sufficient quantity to give greater than a μg per 200 μl of the solution used for analysis. Unfortunately, these methods are not applicable for fresh fruits and vegetables which contain a ng range of riboflavin per 200 μl extract of dried sample, the amount used for injection. The above methods might be applicable if these plant samples could be simply and effectively concentrated 1×10^3 times.

A sensitive ng range method has been reported by Ang and Moseley (5) for analyzing riboflavin and thiamine in meat and meat products. In their method, riboflavin extracted from the sample was oxidized to lumiflavin, and the latter was separated on an HPLC silica column and quantified with a fluorescence detector. We tried this method for analyzing riboflavin in vegetables and found a component of unknown identity eluting about the same time as lumiflavin. The unknown component could not be separated by changing the concentration of methanol or by substituting propanol for methanol. The unknown component interfered in determining accurately the amount of riboflavin recovery. A best estimate of recovery of added riboflavin to various vegetable samples was 80 to 85%.

While we were developing a sensitive method applicable for fruits and vegetables, Fellman et al. (6) reported a method that might be useful for our needs. In their method, the extracted sample was passed through a Waters Sep-Pak cartridge to concentrate the sample and to remove substances that interfered in the chromatograph.

In our method described in this paper we found a way to analyze riboflavin in fresh fruits and vegetables which allows analysis of riboflavin without the need of concentrating the sample and the sample contains no substances that may interfere with the analysis.

MATERIALS AND METHODS

Riboflavin was separated on an Altex-ultrasphere ODS 5 μ m 4.6 mm x 25 cm column by using methanol, deionized water, and heptane sulfonic acid as the mobile phase. Various concentrations of methanol (Burdick and Jackson Laboratories, Inc., Muskegon, MI) HPLC grade were evaluated, and a 40% aqueous solution was found to be optimum for the best resolution. Heptane sulfonate was dissolved in deionized water and pH was adjusted to 2.8 with 20% phosphoric acid before being added to the mobile phase. The concentration of heptane sulfonic acid in the mobile phase was 0.005 M. The pH of the mobile phase was adjusted to pH 4.5 with 20% phosphoric acid, and the solution was pumped at a rate of 0.8 ml/min with a Waters 6000A solvent

delivery system (Waters Associates, Milford, MA). A 200 μ l portion of the sample was introduced into the mobile phase with the Waters WISP 710. A 4 mm x 25 mm guard column, containing Bondapak C_{18} /Corasil, was placed between the WISP and the analytical column, and was repacked after each 200 analyses. Quantity of riboflavin was based on fluorescence of a sample in the flow-thru cell as measured with an AMINCO fluoromonitor or a Perkin-Elmer fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT). The AMINCO fluoromonitor contained a PEN-Ray lamp with a tubular filter to convert 254 nm radiation to that peaking at 366 nm. The primary filter was a Corning No. 7-51 (Corning, NY), the secondary filter was a Wratten 2-A. The flow-thru cell of the fluoromonitor was kept at 20°C with water flowing through the jacket of the cell. With the Perkin-Elmer fluorescence spectrophotometer, model 650-10S, the excitation wavelength was set at 450 nm and the emission wavelength at 530 nm. The response of the spectrophotometer was 5 to 6 times greater than that of the fluoromonitor, probably because of the selected and minimal width of the excitation and emission wavelengths.

Sample Preparation. A 0.5-g ground dry vegetable sample was placed in 50 ml 0.1 N HCl and placed at 99°C for 30 minutes. Following acid hydrolysis and after the mixture cooled to room temperature, a 5 ml 2N NaOAc was added and incubated with 5 ml 5% mylase overnight at 38°. Preliminary study indicated that mylase

was as effective as takadiastase (the latter was no longer available), and that a 5% mylase was as effective as a 10% mylase mixture. The enzyme reaction was terminated with an addition of 2 ml 50% trichloroacetic acid and the mixture was placed in a 60°C water bath for 5 min. The mixture was then cooled, pH was adjusted to 4.00 with 4N NaOAc, and water was added to bring the volume up to 100 ml. The mixture was then filtered through a Whatman No. 40 filter, and the filtrate refiltered through a Durapore membrane disc filter with 0.22 μ m pores.

Destruction of Riboflavin. Purity of the riboflavin peak was determined in part by chromatographing sample extracts or rechromatographing eluants of the peak in which the riboflavin was destroyed. Destruction of riboflavin was accomplished by illuminating samples with a mercury lamp for 12 hours. Sample extracts were illuminated during the last 12 hours of enzyme hydrolysis. Combined eluants were illuminated when they were being concentrated under nitrogen for rechromatographing.

RESULTS AND DISCUSSION

Various concentrations of methanol were evaluated for their effective elution of riboflavin. The retention time decreased geometrically with increased concentration up to 50%. The retention times were 22.3, 10.4, 7.2, 5.6, and 5.5 minutes for a 20, 30, 40, 50, and 60 percent methanol solution, respectively,

at a flow rate of 1 ml/min. Retention time decreased slowly with continual use of a new column and eventually stabilized at about 9 minutes with the flow rate of 0.8 ml/min. Concentrations beyond 50% had no effect on the retention time. A 40% concentration was selected for separating riboflavin because this mixture allowed the elution of a large front prior to the riboflavin peak (Fig. 1-A). The front was due to substances in mylase as noted with chromatograms of standards processed with (Fig. 1-A) and without mylase (Fig. 1-B). The front was significantly smaller when the fluorescence was measured with the fluorescence spectrophotometer than with the AMINCO fluoromonitor. This probably was due to use of a selected and narrower wavelength with the fluorescence spectrophotometer than with the AMINCO fluoromonitor, which allowed fluorescence of fewer foreign components. The riboflavin peak had some tailing when eluted with only methanol and water solution. Addition of hexane sulfonate to the mobile phase rectified the tailing problem. Slight tailing of the peak continued with some samples when the concentration of hexane sulfonate was 0.0005 M, thus the concentration was increased to 0.005 M to assure clear resolution.

Purity of the riboflavin peak was determined by two tests. In the first test eluants from several runs were collected, reduced in volume, and reanalyzed with 20, 30, and 40% methanol mobile phase. Although only a single peak eluted, this does not preclude that no other component was present. In the second

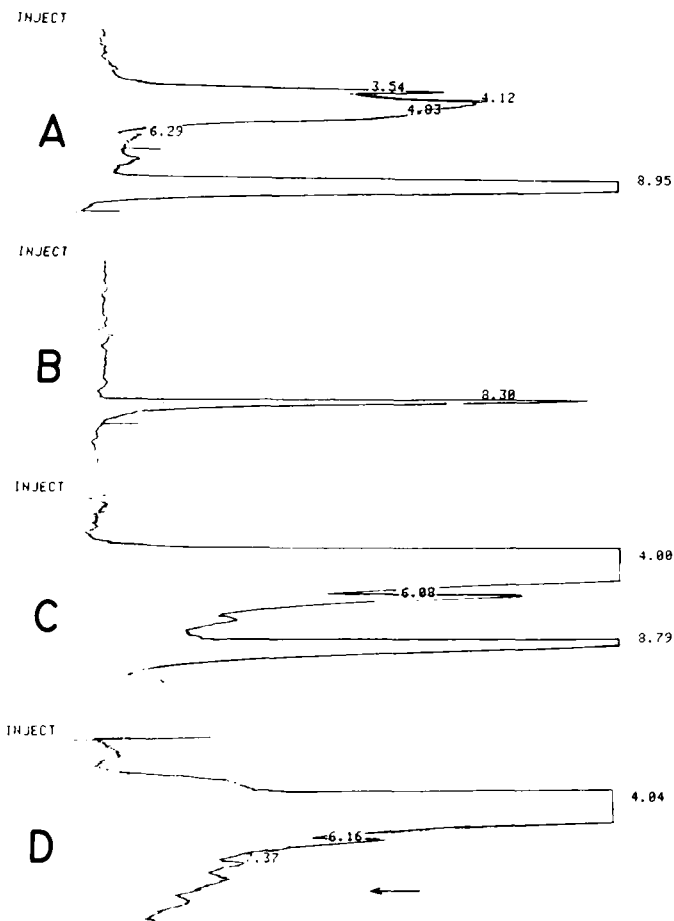


FIGURE 1. High performance liquid chromatograms of riboflavin separated through an Altex-ultrasphere ODS 5 μ m 4.6 mm x 25 cm column with methanol/water (40/60) mobile phase containing 0.005 M heptane sulfonic acid. (A) Riboflavin standard processed with mylase, which resulted in a large front before elution of the standard at 8.95 minutes. (B) Large front missing because riboflavin standard was processed without mylase. (C) Chromatogram of bell pepper samples in which the riboflavin is absent (arrow) because it was destroyed by illumination during the extraction process.

test, analyses were made with sample extracts and combined eluants in which riboflavin was destroyed by illumination. Chromatograms of the illuminated squash, sweetpotato, and bell pepper samples had a typical front but no peak at the time when riboflavin eluted as shown with chromatogram of bell pepper samples (Fig. 1C,1D). Chromatograms of illuminated eluants also had no riboflavin peak. On the basis of these analyses, assumption was made that no other fluorescing components eluted simultaneously with riboflavin. When a series of riboflavin standards ranging from 2 to 12 ng were analyzed, a linear response was obtained with the AMINCO fluoromonitor. The regression equation of the correlation was $y = 2.91 x + 0.342$ and the correlation coefficient was 0.988. A 0.2-ng standard could be measured with precision with the fluorescence spectrophotometer. The riboflavin content of vegetables is low, and standards ranging from 2 to 6 ng per injection were adequate for calculating the concentration. Consequently levels beyond 12 ng were not examined for linearity.

Recovery of added riboflavin standards to the sample extract ranged from 95 to 100% among the vegetable samples (Table 1). Repeated recovery studies gave similar results. In preliminary studies, the recovery for bell peppers and green beans was higher than the 95 and 96% obtained in these tests, thus the lower values probably are due to experimental error.

Table 1.

Riboflavin Content of a Few Vegetables as Determined by the High-performance Liquid Chromatography Method Described in Text, Percent Recovery, and the Content Indicated in the USDA Agriculture Handbook (1950) (7) and the Revised Edition, Agriculture Handbook 8-11 (1984) (8).

Vegetable sample	Riboflavin mg/100 g fresh weight	Percent recovery	USDA AH 8 mg/100 g fresh weight	USDA AH 8-11 mg/100 g fresh weight
Bell peppers	0.039 + .002	95	.08	.05
Green beans	0.163 + .010	96	.11	.105
Potatoes	0.048 + .001	98	.04	.04
Sweetpotatoes	0.060 + .003	99	.06	.147
Squash, summer	0.058 + .002	100	.09	.04

The riboflavin contents of bell peppers, potatoes, and summer squash were within the range of values reported in the revised USDA Agriculture Handbook 8-11 (8). The content of green beans was 50% higher than that reported in the revised handbook, which may be due to differences in cultivars. The value for sweetpotatoes was only 40% of that reported in the revised handbook; however, it is similar to that reported in the original handbook. In general, the riboflavin values reported in the revised handbook are lower than those reported in the original Handbook, but one of the exceptions is the values for sweetpotato. The increased value for sweetpotato is substantial and needs to be reexamined.

Sample Preparation. The AOAC procedure for extraction of riboflavin requires autoclaving the samples (9). We found that autoclaved standards and samples had approximately 16% lower instrument readings than nonautoclaved standards and samples; however, because the reduction was relative, the calculated concentration of riboflavin in vegetables was similar between the autoclaved and nonautoclaved samples. Acid hydrolysis of samples at 99°C did not affect the instrument readings, thus it was substituted for autoclaving. Acid hydrolysis at 99° was found to be necessary as indicated by the higher values with acid hydrolysis than without acid hydrolysis for green beans, potatoes, and sweetpotatoes (Table 2). The differences were not as great for bell peppers and squash.

TABLE 2

Riboflavin Content of Various Vegetables when Extracted with or without Acid Hydrolysis Followed by Enzyme Hydrolysis of Lyophilized and Ground Samples.

<u>Sample</u>	<u>With Acid Hydrolysis</u> mg/100 g fresh wt	<u>Without Acid Hydrolysis</u> mg/100 g fresh wt
Bell peppers	0.039	0.034
Green beans	0.163	0.130
Potatoes	0.048	0.036
Squash	0.058	0.052
Sweetpotatoes	0.060	0.033

The temperature of the water bath for acid hydrolysis must be near 99°C, because lower values resulted when the temperature was near 80°. The enzyme hydrolysis was found to be required for complete extraction of riboflavin, and the hot acid hydrolysis could not be substituted for it.

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Use of a company or product name by the U.S. Department of Agriculture does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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