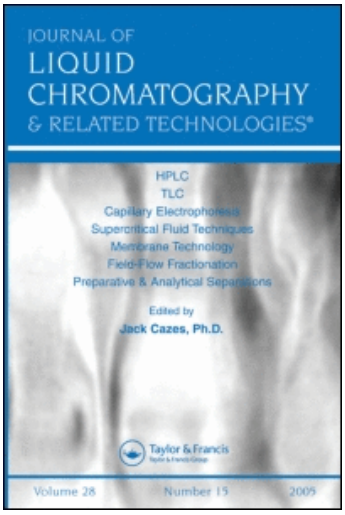

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Reliable System for the Analysis of Riboflavin in Foods by High Performance Liquid Chromatography and UV Detection

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RELIABLE SYSTEM FOR THE ANALYSIS OF RIBOFLAVIN IN FOODS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND UV DETECTION

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ABSTRACT

A high performance liquid chromatographic (HPLC) method has been developed to determine riboflavin in food samples. A reverse phase C_{18} column with ultraviolet detection was employed. Sample preparation included acid and enzimatic hydrolysis, followed by purification on Florisil and Sep-Pak cartridges. Recoveries of 98% were obtained. A detection limit of 0.4 ng/injection has been achieved.

INTRODUCTION

HPLC analysis of riboflavin in food samples has been carried out using UV (1-4) or fluorescence detection (5-14). The phosphated forms of the vitamin can only be hydrolyzed completely to free riboflavin by means of a phosphatase enzyme that ensures complete ex-

traction and faster filtration of the food samples by hydrolyzing any starch present. Fluorescence detection enjoys the advantage of lower detection limits. However, as pointed by Fernando et al. (14) the hydrolyses step with Takadiastase causes fluorescent interference of riboflavin in the sample extract and for most food products the use of disposable Sep-pak cartridges is necessary (3,7,11,13,19). The principal shortcoming of methods using UV detection derives from poor chromatograms, due to the presence of interfering compounds and lower sensitivity. We considered of interest to work out a procedure that could substantially overcome these drawbacks of the UV detection method.

In this work we describe an HPLC method to determine riboflavin in food samples using UV detection (254 nm). The detection limit was 0.4 nanogrames/injection.

MATERIAL AND METHODS

Apparatus and Liquid Chromatograph Conditions.

A Waters Associates liquid chromatograph equipped with a M-510 pump, a U6K injector and a 440 model UV absorbance detector, provided with a filter at 254 nm. The detector signal was recorded on a Houston Instrument Omniscribe recorder. A precolumn 3.2 mm i.d. x 4.0 cm packed with C_{18} Porasil B (Waters Associates) was also used. The chromatographic column was a 3.9 mm i.d. x 30 cm stainless steel column packed with μBondapack C_{18} (10μm) (Waters Associates). The mobile phase used were: a) methanol/acetic acid/ water (32/1/67) plus sodium hexanesulfonate 5mM, b) methanol/acetic acid/water (31/0.5/68.5) plus sodium heptanesulfonate 5mM/hexanesulfonate 5mM (25/75).

Extraction of Legume Samples.

All the procedure was carried out protecting the samples from sunlight. Sufficient ground materials (5-10g, which contain 40 μg of riboflavin) was hidrolyzed with 0.1 N HCl (30 mL) and 6N HCl (1mL) in an autoclave at 121° C for 15 min. The pH of the solution was then adjusted to 4.0-4.5 with sodium acetate (2N); 5 mL of freshly prepared aqueous enzyme solution (6% Taka-diastase) was added and the sample was incubated at 48° C for 3 hours. The sample solution, once cooled, was filtered through Whatman n° 40 filter paper and diluted to 100 ml with distilled water.

Purification of the Extract.

An aliquot of this extract (5 mL, equivalent to 2.0-2.5 microgrammes of riboflavin) was adjusted to pH 4.0-4.5 and was passed through a column (1.0 i.d.x 3.0 cm) packed with Florisil, (previously treated with 2% acetic acid (25 mL) and washed with 15 mL of water). The column was washed with 30 mL of distilled water, followed by 50 mL of a mixture of (triethylamine/ methanol/water, 7:30:13). This last eluent was collected and evaporated to dryness. The residue was disolved in a mixture of methanol/water (the same proportion as the mobile phase). An aliquot of this solution (1.0 mL) was passed through a C_{18} Sep Pak cartridge (Waters Associates) (previously conditioned passing 10 mL of a 5 mM solution of methanol-sodium hexanesulfonate and 10 ml of a 5 mM solution of sodium hexanesulfonate in distilled water). The interfering substances were removed by washing with 20 mL of aqueous 5 mM sodium hexanesulfonate. The vitamin was eluted from the cartridge using 2.0 mL of methanolic 5 mM sodium hexanesulfonate. This solution was filtered

through a Millipore filter (0.45 μm). At least 3 replicates for each sample were made.

Preparation of Standard Solutions and Recovery Test. Standard stock solutions of riboflavin (20 μg/ml) protected against light were prepared. 10-12 mg of riboflavin was dissolved in 500mL HCl 0.01N heating at 40°C and shaking during 20 minutes. Working standard solutions (0.2 μg/ml-0.04 μg/ml) were made on the day of use from the stock solutions by suitable dilutions. Aliquots of the standard stock solution (40-50 μg of riboflavin) were subjected to the extraction and purification procedures described above. Several replicates were carried out simultaneously.

RESULTS AND DISCUSSION

Different chromatographic conditions were assayed using standard riboflavin solutions. It was observed, with a μBondapack C_{18} column that an increase of the methanol concentration of the mobile phase caused a decrease of the k' value for riboflavine. The effect of the column temperature was also probed. The best conditions achieved were those indicated in Table 1.

A linear regression analysis of the relationship between peak height and the amounts of standard of five separate determinations, at several concentrations was carried out using a PC computer. The regression lines (y=a+bx) obtained with concentrations ranging 6-24 ng of standard riboflavin, gave correlation coefficients between 0.991-0.999. The detection limits (3s/b) (15), ranged between 1.7-0.4 ng/injection respectively, where

TABLE 1

Riboflavin Content and Chromatographic Conditions of Food Samples Analyzed by HPLC.

Foods	Riboflavin mg/100g dry matter*	Temperature Column (°C)	Mobile Phases
Chick peas:			
"Blanco lechoso"	0.06±0.00	t	a
"Sinaloa"	0.06±0.01	t	a
"Hostal"	0.05±0.01	t	a
Green beans[a]	0.18±0.01	14	b
Full Cream Milk Powder[b]	0.79±0.08	15	b

*) Means value ± standard deviation of at least 6 replicates.
[a]), [b]) Liofilized samples.
t) Room temperature

(b) is the slope and (s) the standard error of estimation, depending on the correlation coefficient obtained.

Table 1 shows the results obtained with chick peas (three varieties), green beans and powdered milk.

Riboflavin has been determined both alone and after separation from other water-soluble vitamins (20). We have chosen a common extraction for the determination of riboflavin, thiamin and niacin, but independent purification procedures and chromatographic conditions were carried out, for each vitamin (16,17).

The extraction method employed for the determination of riboflavin in foods usually begins with an acid

hydrolysis (8,14) or an acid and enzimatic hydrolysis (5,6,7,11,13), that permit to convert the protein-bound nucleotides [(riboflavin-5'-phosphate (FMN) and flavin adenine dinucleotide (FAD)] to free riboflavin in order to measure the total B_2 vitamin. The enzyme, in the case of starchy foods, such as legumes, has the added benefit of ensuring complete extraction of the vitamin and faster filtration of the sample by hydrolysing any starch present (10,18,19,23). On the other hand, the use of enzimatic hydrolisis produce fluorescent interference of riboflavin in the sample extract, that could cause an overestimation of the riboflavin when fluorimetric procedure are used, such as has been observed by different authors comparing their values with the AOAC procedure (3,19). Enzymes such as clarase and takadiastase impart fluorescing interferences to the analysis of riboflavin. Compounds coeluting and cofluorescing with riboflavin are reported to be present in these enzymes (14).

Consequently, HPLC analysis of riboflavin in food extract samples, without a previous purification step, is not advisable, due to the presence of interfering peaks. The low levels of vitamins and high amounts of other interfering material make the chromatographic determination of the direct extracts unfeasible. The use of filtration through membrane filters (0.22-0.45 μm) in order to obtain clear extracts for injection onto the analytical column or the use of Sep-Pak (Millipore Waters) disposible cartridges was inadequate for removing all interfering substances present in the hydrolyzed samples.

We turned our attention to adsorption methods, employing fuller's earth, used with some limitations by

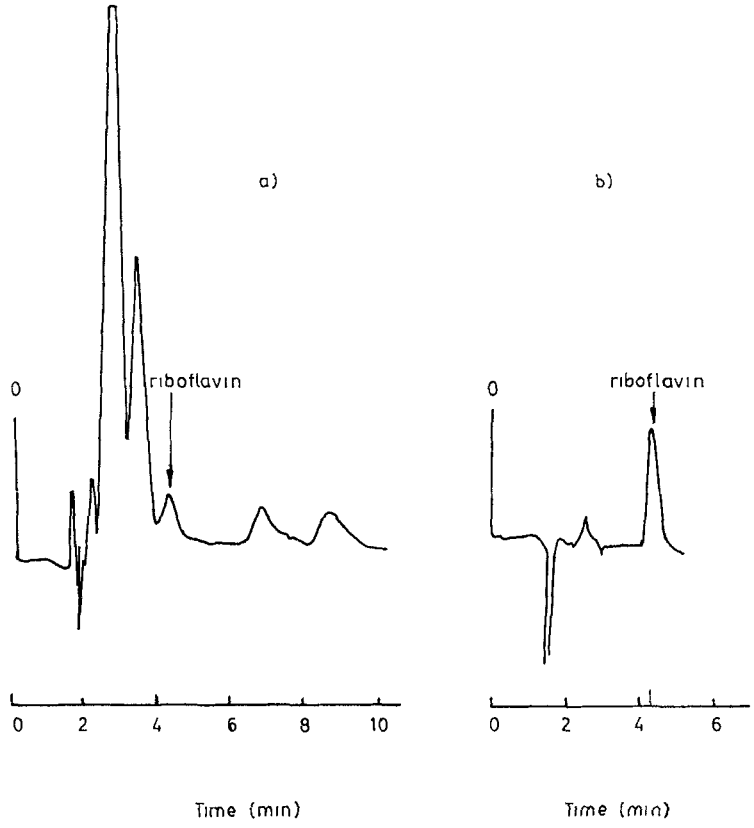

Figure 1.- Chromatogram obtained with: a) chick peas sample, b) standard riboflavin. Absorbance range 0.005. Flow rate 2 ml/min. Injection volume 50 μl. Detection at 254 nm.

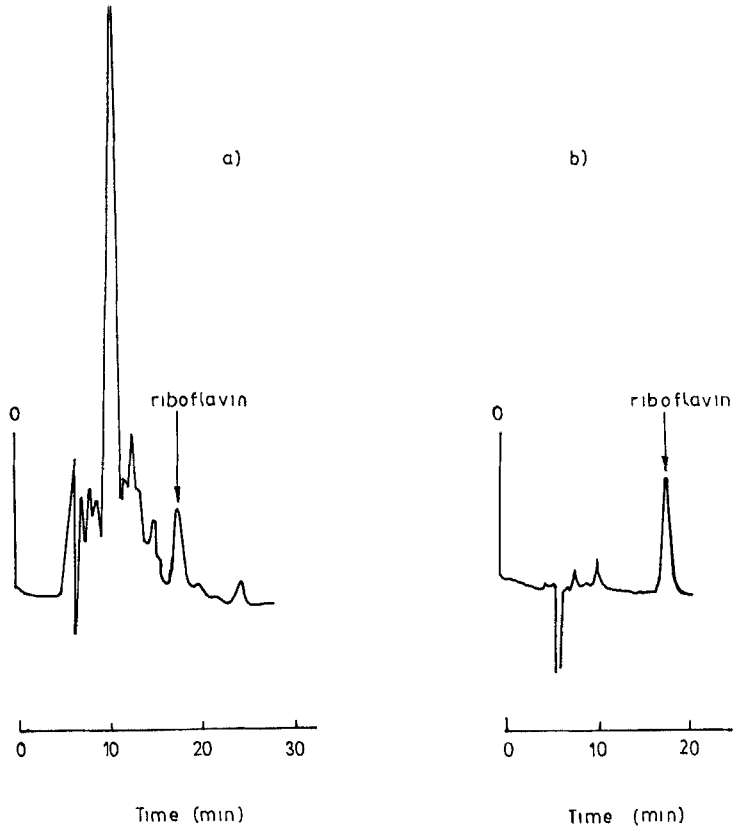

Figure 2.- Chromatogram obtained with: a) green beans sample, b) standard riboflavin. Absorbance range 0.01. Flow rate 0.5 ml/min. Injection volume 50 μl. Detection at 254 nm.

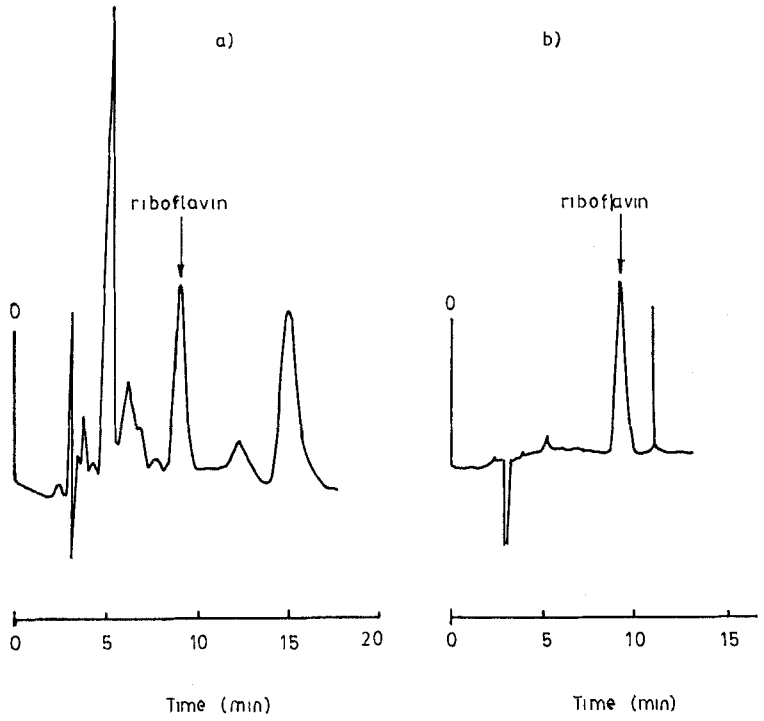

Figure 3.- Chromatogram obtained with: a) full cream milk powder sample, b) standard riboflavin. Absorbance range 0.01. Flow rate 1 ml/min. Injection volume 50 μl. Detection at 254 nm.

previous authors, to purifiy and enrich riboflavin extracts from natural sources (21-24). One of the main problems encountered in the use of Florisil for this purpose is the difficulty to elute the adsorbed vitamin quantitatively. An excellent recovery has been obtained when a mixture of triethylamine/ methanol/water (7/30/13) was used to elute the adsorbed riboflavin.

Table 2

Recovery of Standard Solutions of Riboflavin Subjected to Extraction and/or Purification Procedures.

	Recovery(*) %
a) Preparation of standard solution	100.2±2.6
b) Acid and enzimatic hydrolysis	99.7±2.1
c) Florisil column	98.7±3.1
d) Step b) followed by step c)	99.7±3.8
e) Step d) followed by Sep-pak Cartridge	97.9±3.9

(*) Mean value ± standard deviation of 6 different recoveries of thiamin estimated by peak height.

Once conditions have been optimized to obtain good recovery in Florisil columns with standard riboflavin solutions, we tried to purifiy our sample extracts. To obtain a good chromatographic peak of riboflavin with extracts that have been passed through a Florisil column, it was necessary to use also a C_{18} cartridge (Figures 1-3). Good recovery of standard riboflavin was obtained after going through the complete purification procedure (see Table 2). Riboflavin in solution degraded rapidly when exposed to ultraviolet or visible radiation, for this reason all the procedure was carried out protecting the samples from sunlight.

In conclusion we have applied this process to the determination of riboflavin in three varieties of chick peas, green beans and powdered milk. The clean-up procedure allows for the removal of the major interfering substances present in legumes and makes the method sufficiently sensitive for these food samples. The detection limit achieved (0.4 ng/injection) is reasonably low.

The determination of riboflavin by HPLC and UV detection has been previously applied to fortified and enriched food products (1,2) or to foods with a high amount of riboflavin (3,4). The present method extend this methodology to foods with lower riboflavin content.

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