

RESEARCH REPORT

Determination of Riboflavin Content in Goat and Cow Milk by High Performance Liquid Chromatography (HPLC)

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A high performance liquid chromatography method has been developed to determine riboflavin content in goat and cow whole milk. The present method involves acidification of the milk to precipitate bulk of the proteins and centrifugation affording a supernatant, which was diluted with 2% acetic acid to a constant volume. This solution was subjected to HPLC analysis using an ODS column. The method is simple, rapid and sensitive for riboflavin determination. The results obtained by the method compared well with the results of analysis by the "Official Methods of Analysis" of the Association of Official Analytical Chemists (AOAC).

Several analytical methods for the determination of riboflavin have been reported (Kamman, 1980). Most of the procedures involve a pretreatment to liberate the protein bound vitamin from flavoproteins. Fluorometric methods of detection are considered the most desirable because of their sensitivity, rapidity, and specificity. However, they require extra treatments to separate riboflavin from interfering fluorescent compounds. In the official methods of analysis of the Association of Official Analytical Chemists (AOAC, 1996, method 970.65), samples are treated with potassium permanganate (KMnO₄) and hydrogen peroxide (H₂O₂) to remove background fluorescence. Rashid and Potts (1980) treated milk samples with acidified lead acetate solution to separate extraneous protein and minimize background fluorescence. Fellman *et al.* (1982) have reported a C₁₈ reversed phase separation for the simultaneous determination of thiamin and riboflavin in selected foods including milk. This method required the removal of interfering substances using a 0.01M phosphate buffer.

The objective of this study was to develop a sensitive and specific HPLC method for the quantitative determination of riboflavin content in goat and cow milk in Kenya.

Although cow milk is the most popular in Kenya, various communities have consumed goat milk for many years. Recently, Kenya Government has been encouraging farmers to keep goats for milk to supplement the cow milk. The nutritional value of goat milk, has however, not been well studied. One of the important vitamins in milk is riboflavin (vitamin B₂). In the present study, the content of riboflavin in goat and cow whole milk was determined by a High Performance Liquid Chromatography (HPLC) method developed in our laboratory.

A Liquid Chromatograph (Shimadzu SPD - 10AV) equipped with Model SCL - 6A UV/Visible detector and a C-R6A chromatopac integrator was used for the analysis. A Kubota KR-20000T refrigerated centrifuge was used for centrifugation.

HPLC operating conditions were as follows:

Column : Inertsil ODS (C₁₈) 5 μ m 4.6 x 250mm
Solvent system : water:methanol:acetic acid (40:59.5:0.5 v/v)
Flow rate : 0.3ml/min.
Detector : 270 nm at 0.02 sensitivity.

Cow milk samples used in this study were obtained from the Jomo Kenyatta University of Agriculture and Technology experimental farm. The goat milk samples were obtained from Kakuzi farm in Maragua District. The samples were prepared for HPLC analysis on the same day of sampling as follows:

10ml of each milk sample was acidified to pH 3.0 with aqueous glacial acetic acid solution (1:1 v/v) added drop-wise with constant stirring. The acidified milk sample was stirred gently for an additional 5 minutes then centrifuged at 10000 rpm for 20 minutes. The supernatant was transferred to a 25ml volumetric flask and the sediment was washed twice with 5ml of 2% acetic acid solution. The washings were combined and centrifuged at 10000 rpm for 20 minutes. The second supernatant was added to the first supernatant in the volumetric flask and the volume made to the mark with 2% glacial acetic acid solution. This solution was filtered using cellulose acetate filter unit (0.45 μ m) for HPLC analysis.

Stock standard solution of 50 mg ml⁻¹ riboflavin (Sigma Chemical Company) was prepared in 2% aqueous glacial acetic acid. Working standard solutions in the range of 0.0 – 6 mg ml⁻¹ were prepared by serial dilution of aliquots of the stock solution in 2%

aqueous glacial acetic acid. A volume of 10 μ l of each of the standard solution was injected and the corresponding peak area units obtained with an integrator. The area (average of two runs) was plotted against the corresponding concentration of riboflavin injected.

A calibration curve was obtained by injecting 10 μ l of freshly prepared riboflavin aqueous standard solutions prepared as above. A volume of 10 μ l of each of the sample supernatants was injected and the riboflavin content was obtained indirectly from the calibration curve. An average of six determinations (two sample preparations, each injected three times) was used in calculating the riboflavin content of milk samples.

Each of the samples analyzed was spiked with known volume of a riboflavin standard solution containing an approximately equal amount of riboflavin to that of the sample and allowed to stand for two hours for equilibration to occur. The spiked samples were prepared for HPLC analysis and their riboflavin content was determined as mentioned previously. The average amount of riboflavin (average of two determinations) detected in the spiked samples was used in calculating percent recoveries. The riboflavin content of selected samples was also determined by the fluorometric AOAC method in order to confirm the reliability of the present method.

Solvent systems of varying compositions; water/acetonitrile, water/methanol and water/methanol/acetic acid were used as eluting/separating solvents in HPLC analysis of riboflavin. The best solvent system was obtained through trial and error. The best solvent system {water:methanol:acetic acid (40:59.5:0.5)} was used throughout this study. At acetic acid percentage lower or higher than 0.5%, the elution of riboflavin using the adopted mobile phase gave unpredictable peak areas due poor geometry. The detector response to riboflavin was also tested at different wavelengths ranging from 250-290 nm and the maximum response was obtained at 270 nm.

Calibration graph of riboflavin concentration versus detector response in the concentration range of 0.0-6.0mg/ml was linear ($r^2 = 0.9995$) and can be described by the equation, peak area = 516 \times concentration. As low as 10 ng of riboflavin (at a minimum area of 50units) could be detected reliably indicating the high sensitivity of the method.

Riboflavin was separated as a single peak with a retention time of 6.2 minutes in the

chromatograms of milk. The riboflavin peak purity was confirmed by two methods. When samples were co-injected with a riboflavin standard solution, the peak in the chromatogram increased in area. When the solvent aliquot of the riboflavin (retention time of 6.2 minutes) was collected and re-injected using solvents with different components (water-acetonitrile, 70:30 v/v, water-methanol, 60:40 v/v) only one peak with retention time matching that of the standard riboflavin was obtained. These results indicated that the riboflavin peak in all samples analyzed had no interfering compounds. This outcome indicated that the present HPLC method was specific for the quantification of riboflavin in all samples analyzed.

The mean percentage recoveries in duplicate analysis of riboflavin in cow milk were above 95% while for goat milk were between 87% and 93% (Table 1). This means that quantitation was possible for the cow milk and the milk matrix did not have any significant effect. The low recovery of riboflavin in the goat milk may be due to the high fat content of the milk. The contents of riboflavin in the milk samples as analyzed by AOAC method were comparable to those obtained by the HPLC method (Table 2).

Table 1. Recovery of riboflavin in cow and goat milks

Sample (milk)	Riboflavin content ($\mu\text{g/ml}$)		C. V %	Recovery %
	Determined	Spiked		
Cow 1	2.44 ± 0.01	1.2	8.11	97.2
2	2.00 ± 0.09	1.2	8.05	94.8
Goat 1	3.46 ± 0.07	2.0	8.20	87.4
2	3.42 ± 0.05	2.0	8.52	92.7

C.V = Coefficient of variation

Values given are means of duplicate determinations.

The values obtained from the AOAC method were not significantly ($p = 0.05$) different from those obtained by the HPLC method. The AOAC method consistently gave a slightly higher value than the HPLC method. Fernando and Murphy (1990) while determining thiamin and riboflavin content in soy products reported similarly higher values for riboflavin content when determined by AOAC method as compared to values obtained by HPLC method using a fluorescence detector. They attributed this to the

presence of other fluorescing compounds present in the sample. This probably accounts for the higher values observed for the AOAC method in the present study.

Table 2. Riboflavin analysis with HPLC and AOAC methods

Sample (milk)		Riboflavin content ($\mu\text{g/ml}$) [*] using	
		HPLC method	AOAC method
Cow	1	1.31 ± 0.01^b	1.42 ± 0.08^b
	2	1.11 ± 0.09^c	1.19 ± 0.07^c
	3	1.14 ± 0.05^c	1.20 ± 0.06^c
	4	1.03 ± 0.08^c	1.11 ± 0.06^c
	5	1.26 ± 0.04^b	1.32 ± 0.04^b
Goat	1	1.96 ± 0.07^d	2.12 ± 0.05^d
	2	1.69 ± 0.04^c	1.72 ± 0.06^c

^aMeans followed by the same letter are not significantly different ($p = 0.05$)

^{*}Values given are means of duplicate determinations.

Riboflavin content obtained by the HPLC method was slightly lower than that obtained by the AOAC method (ca $\pm 10\%$). This is probably because of the absence of interfering substances. In general, the content of riboflavin in the samples analyzed by the reported HPLC method was in agreement with that found in the literature (Arlin, 1977; Rashid and Potts, 1980).

The results obtained from this study indicated that the reported HPLC method has several advantages and can be used to quantify riboflavin in milk. The developed method is simple, rapid and sensitive to riboflavin. This method has a linearity of response over wide concentration range (0.0-6.0mg/ml).

So far as we know, there is scarce data on the riboflavin content of goat milk in Kenya. We therefore measured the content by the present method. As table 2 indicates, the goat milk samples afforded 1.69 ~ 1.96 $\mu\text{g/ml}$ of riboflavin which is significantly ($p = 0.05$) higher than that of cow milks (1.03 ~ 1.31 $\mu\text{g/ml}$).

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