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Analytical Methods Section

Determination by HPLC of changes in riboflavin levels in milk and nondairy imitation milk during refrigerated storage

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Riboflavin, a water-soluble light-sensitive vitamin, was determined in milks and nondairy imitation milks, by HPLC with ultraviolet-visible detection. The riboflavin content ranged from 1·16 to 1·31 $\mu g\ ml^{-1}$ in cows' milk, and from 1·33 to 1·44 $\mu g\ ml^{-1}$ in nondairy imitation milks.

When the opened containers were stored in a refrigerator at 8°C, in the dark, the loss of riboflavin in cows' milk samples ranged from 16·0 to 23·4%, and nondairy imitation milk samples ranged from 12·5 to 16·5% of the initial values.

INTRODUCTION

Milk and other dairy products supply significant amounts of riboflavin to the diet (Tanner *et al.*, 1988). Approximately 38% of the riboflavin in the American diet, for example, comes from milk and dairy products (USDA, 1987). However riboflavin is light labile (Toyosaki *et al.*, 1984), the intensity and the wavelength of the light (400–500 nm) determining the rate of riboflavin degradation in milk (Senyk & Shipe, 1981; Furuya *et al.*, 1984) and the development of off flavors (DeMann, 1978; Fanelli *et al.*, 1985). Hence, it is likely that a significant proportion of the vitamin is decomposed by the mechanism of photolysis, a process which is accelerated by the presence of active oxygen species (Toyosaki *et al.*, 1983). Furthermore it will suffer oxidative changes following heat treatments and storage (Lavigne *et al.*, 1987), due to the presence of oxygen (Lavigne *et al.*, 1989). After vacuum packaging, riboflavin retention is higher during storage in the absence of oxygen for all treatments (Lavigne *et al.*, 1989). The amount of riboflavin lost is dependent on the packaging material, so that milk stored in glass and plastic, in a refrigerator, suffers loss (Dimick, 1982) due to the penetration of light (Singh *et al.*, 1975; Gaylord *et al.*, 1986). From a nutritional point of view it is, therefore, extremely important to know if some riboflavin has been lost during storage.

Current dietary goals for improved nutritional status, written by the US Senate Select Committee on Nutrition and Human Needs (1977), suggest an increase in consumption of complex carbohydrates and a decrease in consumption of refined sugar, saturated fats and cholesterol. Since many of the richest sources of riboflavin, such as full milk, contain considerable quantities of saturated fats and cholesterol, the per capita consumption in the US has been gradually declining over the last few years. During this period, there has also been a pronounced increase in the consumption of beverage milks (Tanner *et al.*, 1988).

The purpose of this study was to determine the effect of refrigerated storage (+8°C) in opened containers, on water-soluble vitamin riboflavin in cows' milk and in imitation milk, to give an indication about what the consumer is actually taking, and to find out whether the concentration of riboflavin is similar in both kinds of milk.

Several methods have been used to analyze riboflavin, such as fluorimetry (AOAC, 1980; Rettenmaier & Vuilleumier, 1983); high-speed ion-exchange chromatography (Williams *et al.*, 1973) or microbiological (Bell, 1974), or HPLC (Rashid & Potts, 1980, Ashoor *et al.*, 1983; Finglas & Faulks, 1984; Stancher & Zonta, 1986). The HPLC technique described in the Materials and Methods Section was found to be the most convenient for the authors' samples.

MATERIALS AND METHODS

Reagents and samples

Riboflavin was purchased from Sigma Chemical Co., USA, and other reagents were obtained from E Merck, Darmstadt, Germany.

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The samples used in this work were purchased from local food stores: three UHT processed cows' milk liquids (A, B and C) and two UHT commercial nondairy imitation milk liquids a (D and E). All samples were full-fat. The nondairy imitation milks studied here were obtained by replacing the animal fat with vegetable fat. After the containers were opened (partially exposed to atmosphere), samples were kept in darkness in the refrigerator at 8°C ± 1·5°C for 6 days, in similar conditions to those which are usual in homes in Spain. The containers were in all cases 1-litre, non-returnable polyethylene-coated cartons.

Preparation of samples

Samples were prepared following the method of Rashid and Potts (1980). A 10% (w/v) lead acetate solution was adjusted to pH 3·2 using glacial acetic acid. A portion of this solution (2·5 ml) was added to milk (25 ml), or nondairy imitation milk; it was well mixed by stirring, and filtered through No. 42 Whatman paper; a fraction of the filtrate (10 ml) was collected. This filtrate was filtered again but now through Millipore HVLP 04700 filters (0·45 μm).

Samples were protected from light during preparation. Tubes and flasks were covered with aluminium foil and manipulations were carried out under subdued lighting conditions (Ashoor *et al.*, 1985).

Samples taken during the experiment were stored deep frozen and all of them were analyzed together in the same day.

Apparatus

A Waters Associates Liquid Chromatograph was used for HPLC analysis, equipped with a Model 730 data module, a Model 721 system controller, two Model 510 pumps and a Model 481 LC spectrophotometer and 10-μl loop.

HPLC operating conditions

The column used was a 15 × 0·39 cm 5μ Spherisorb ODS 2 with a C_{18} 5μ Bondapak guard column. The solvent system was water–acetic acid, methanol (70 : 30 v/v)/acetic acid (1·5 ml) in water (1 litre) with a flow rate of 0·6 ml min^{-1}. The detection was at 270 nm, and 0·02 a.u.f.s.

Quantification of riboflavin in samples

Quantitative data were obtained using the heights of the peaks of standards from chromatograms, and calibration curves were constructed by plotting peak heights corresponding to various concentrations. The same procedure was used to verify the linearity relationship. Two injections of 10 μl of freshly prepared riboflavin aqueous standard solution (4 mg $litre^{-1}$, and 2 mg $litre^{-1}$) (Ashoor *et al.*, 1983) were used to check the apparatus before any sample was injected. Six determinations of each sample were used to calculate the riboflavin content, corresponding to two different preparations of sample, each injected three times.

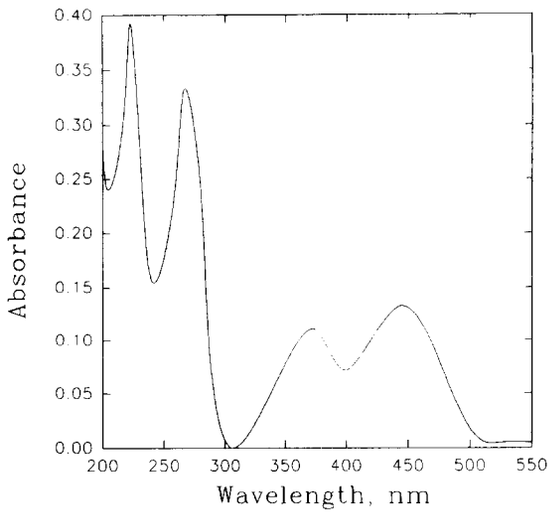

Fig. 1. Absorption spectrum of riboflavin standard of 4 mg $litre^{-1}$.

RESULTS AND DISCUSSION

Identification of riboflavin

The riboflavin absorption spectrum (Fig. 1) agrees very well with that described in the literature (Stancher & Zonta, 1986), with wavelength maxima at 223 and 270 nm. The peak of the chromatogram corresponding to riboflavin (Fig. 2) was characterized by comparing with authentic riboflavin, and the identity of the riboflavin peak in the samples was confirmed by spiking samples with known amounts of added authentic riboflavin standard solution (Lavigne *et al.*, 1987); small differences between the sample spectra and riboflavin standard may be observed (not shown in the figure), due to the background effect caused by the presence of trace impurities. To avoid interferences in the HPLC

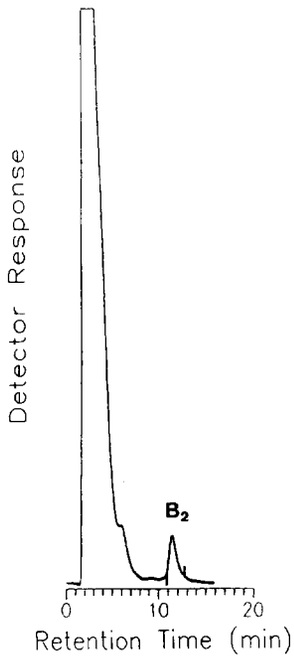

Fig. 2. Chromatogram of riboflavin extracted from nondairy imitation milk, 10 μl, detection wavelength 270 nm.

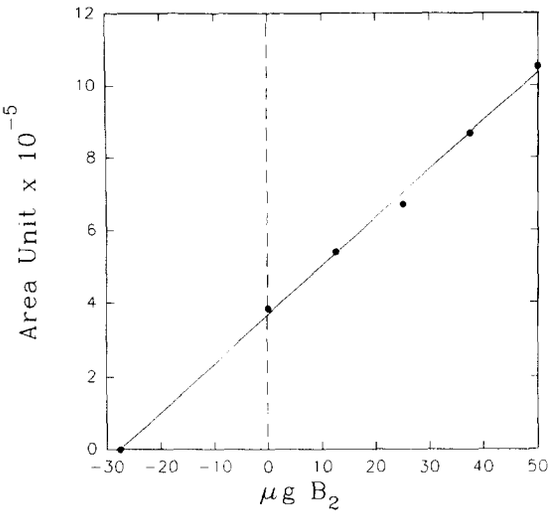

Fig. 3. Calibration curve for riboflavin extracted from sample 2 milk with the addition of amounts of standard (0·0, 12·5, 25·0, 37·5 and 50·0 μg).

system, the absorbance detection wavelength was set at the secondary maximum of riboflavin, 270 nm, so that the interfering peaks of the matrix did not overlap the riboflavin peak.

The LC conditions used in this study for the separation and quantitation of riboflavin were similar to those reported previously by Ashoor *et al.* (1983). Several different solvent mixtures were tried in order to shorten the analysis time; it has been previously shown that increasing the concentration of methanol in the mobile phase resulted in decreasing retention times for riboflavin (Lumley & Wiggins, 1981) and this is confirmed by the present results.

Linearity of response

Five samples of the same type of milk were spiked with various amounts of riboflavin (0·0, 12·5, 25·0, 37·5 and 50·0 μg). A linear relationship was noted between area and concentration with a correlation coefficient of 0·99732 (Fig. 3).

Recovery study

For the recovery studies, a sample of milk B was spiked with a known volume of a riboflavin standard (1·1 mg litre^{-1}). The spiked samples were prepared for HPLC analysis and their riboflavin content as determined as described previously in the Methods section. The average amount of riboflavin (the average of two determinations) detected in the spiked samples was used according to Ashoor *et al.* (1983) to calculate per cent recoveries. The recovery was 92·20 ± 1·2; similar to that obtained by Ashoor *et al.*, (1983), Toyosaki *et al.* (1986) and Lavigne et al. (1987) in milk samples.

Vitamin contents in cows' milk and imitation milk

The contents of a total of five different samples (A, B, C: cows' milk; and D and E: imitation milk) were analysed by HPLC. Figure 2 shows a typical chromatogram of separation of riboflavin in the nondairy imitation milk type D. Values of riboflavin are presented in Table 1 and ranged from 1·16 to 1·31 μg ml^{-1} in the cows' milk, and from 1·33 to 1·44 μg ml^{-1} in the two nondairy imitation milk samples.

It is interesting to note the wide variability in levels of riboflavin in the different containers of milk of equal freshness (Fanelli *et al.*, 1985), in different types of processed milks (from 1·13 to 1·31 μg ml^{-1}) (Toyosaki *et al.*, 1983), from 0·8 to 4·18 μg ml^{-1} for nondairy imitation milks and from 1·37 to 1·92 μg ml^{-1} for cows' milks with the same method (Kosikowski, 1971). It is perhaps not surprising that the vitamin content will vary from 0·3 to 1·5 μg ml^{-1} in a natural product such as milk, which is dependent, for example, on changes in the cow's diet (Fanelli *et al.*, 1985); the riboflavin concentrations are in agreement with values found in the literature for processed milk (Ashoor *et al.*, 1985; Lavigne *et al.*, 1987).

Riboflavin loss

Figure 4 depicts the riboflavin loss of these samples determined by HPLC. Riboflavin decomposition is usually reported as a percentage of the initial amount present (Fanelli *et al.*, 1985). Vitamin degradation in milk can occur slowly in the dark, during storage in the refrigerator (Fanelli *et al.*, 1985), and the level of riboflavin loss in a package would be a function of the packaging material, the amount of actual product exposed to light on the surface, and the penetration of light into the interior of the product (Woodcock *et al.*, 1982). Reported here are losses between 20·1 and

Table 1. Riboflavin analysis in milks and imitation milks

Samples	Riboflavin (μg ml^{-1})						
	Day						
	0	1	2	3	4	5	6
A	1·19 ± 0·03	1·19 ± 0·02	1·18 ± 0·05	1·18 ± 0·04	1·18 ± 0·03	1·10 ± 0·03	0·95 ± 0·03
B	1·31 ± 0·02	1·27 ± 0·03	1·23 ± 0·04	1·23 ± 0·02	1·23 ± 0·04	1·20 ± 0·02	1·10 ± 0·02
C	1·24 ± 0·02	1·15 ± 0·03	1·09 ± 0·02	1·08 ± 0·03	1·00 ± 0·03	1·00 ± 0·02	0·95 ± 0·04
D	1·44 ± 0·08	1·40 ± 0·03	1·35 ± 0·03	1·35 ± 0·03	1·36 ± 0·03	1·32 ± 0·02	1·26 ± 0·05
E	1·33 ± 0·03	1·27 ± 0·03	1·29 ± 0·07	1·21 ± 0·03	1·15 ± 0·06	1·14 ± 0·03	1·11 ± 0·05

Data are expressed as mean ± ID (n = 6).

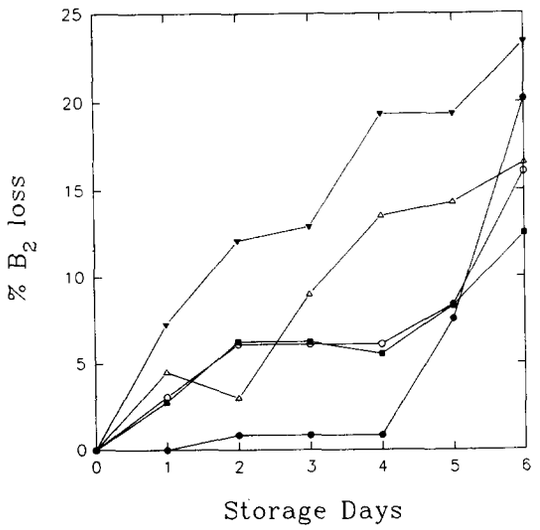

Fig. 4. Influence of refrigerated storage on riboflavin degradation in milk and imitation milk (●, A; ○, B; ▼, C; ■, D; △, E).

23·4% for A and C samples, losses of 16·0 and 16·5% for B and E samples, respectively, and one sample (D) with a lower loss of 12·5%. The expiry dates were similar in all these samples except in D, in which it was one month later, and perhaps due to that reason, it showed the lowest loss percentage. These values are similar to those reported by Dimick (1982) and Ashoor *et al.* (1985), who detected losses of 10–17 of riboflavin in milk stored in glass and plastic for 72 h, and by Fanelli *et al.* (1985), who observed a 23% loss for vitamin riboflavin stored in opened polyethylene containers. It is interesting to note that nondairy imitation milks are less prone to riboflavin losses than cows milk.

In summary, it can be concluded from this work, firstly that the nondairy imitation milk showed slightly higher riboflavin values and less losses than cows' milk, and secondly, that significant amounts of riboflavin are lost during storage in the refrigerator over a few days, and therefore it should be recommended that storage of long-life milk, after the container is opened, should be limited as much as possible.

REFERENCES

AOAC (1980). *Official Methods of Analysis*, 13th edn. Association of Official Analytical Chemists, Washington, DC.

Ashoor, S. H., Seperich, G. J., Monte, W. C. & Welty, J. (1983). HPLC determination of riboflavin in eggs and dairy products. *J. Food Sci.*, **48**, 92–4.

Ashoor, S. H., Knox M. J., Olsen, J. R. & Deger, D. A. (1985). Improved liquid chromatographic determination of riboflavin in milk and dairy products. *J. Assoc. Off. Anal. Chem.*, **68**(4), 693–6.

Bell, J. G. (1974). Microbiological assay of vitamins of the B-group in foodstuffs. *Lab. Pract.*, **23**, 235–42.

DeMann, J. M. (1978). Better protection for dairy foods needed with reduced lighting in dairy cases and light resistant packaging. *Modern Dairy*, Jan/Feb, 23.

Dimick, P. S. (1982). Photochemical effects on flavor and nutrients of fluid milk. *Can. Inst. Food Sci. Technol. J.*, **15**, 247–56.

Fanelli, A. J., Burlew, J. V. & Gabriel, M. K. (1985). Protection of milk packaged in high density polyethylene against photodegradation by fluorescent light. *J. Food Prot.*, **48**, 112–17.

Finglas, P. M. & Faulks, R. M. (1984). The HPLC analysis of thiamin and riboflavin in potatoes. *Food Chem.*, **15**, 37–44.

Furuya, E. M., Warthesen, J. J. & Labuza, T. P. (1984). Effects of water activity, light intensity and physical structure of food on the kinetics of riboflavin photodegradation. *J. Food Sci.*, **49**, 525–8.

Gaylord, A. M., Warthesen, J. J. & Smith, D. E. (1986). Influence of milk fat, milk solids and light intensity on the light stability of vitamin A and riboflavin in lowfat milk. *J. Dairy Sci.*, **69**, 2779–82.

Kosikowski, F. V. (1971). Nutritive and organoleptic characteristics of nondairy imitation milks. *J. Food Sci.*, **36**, 1021–5.

Lavigne, C., Zee, J. A., Simard, R. E. & Gosselin, C. (1987). High-performance liquid chromatographic-diode-array determination of ascorbic acid, thiamine and riboflavine in goats' milk. *J. Chromatogr.*, **410**, 201–5.

Lavigne, C., Zee, J. A., Simard, R. E. & Beliveau, B. (1989). Effect of processing and storage conditions on the fate of vitamins B_1, B_2, and C and on the shelf-life of goat's milk. *J. Food Sci.*, **54**(1), 30–34.

Lumley, I. D. & Wiggins, R. A. (1981). Determination of riboflavin and flavin mononucleotide in foodstuffs using high-performance liquid chromatography and a column-enrichment technique. *Analyst*, **106**, 1103–8.

Rashid, I. & Potts, D. (1980). Riboflavin determination in milk. *J. Food Sci.*, **45**, 744–5.

Rettenmaier, R. & Vuilleumier, J. P. (1983). A simple method for the determination of riboflavin in human milk. *Int. J. Vit. Nutr. Res.*, **53**, 32–5.

Senyk, G. F. & Shipe, W. F (1981). Protecting your milk from nutrient losses. *Dairy Field*, **3**, 81–4.

Singh, R. P., Heldman, D. R. & Kirk, J. R. (1975). Kinetic analysis of light-induced riboflavin loss in whole milk. *J. Food Sci.*, **40**, 164–7.

Stancher, B. & Zonta, F. (1986). High performance liquid chromatographic analysis of riboflavin (vitamin B_2) with visible absorbance detection in italian cheeses. *J. Food Sci.*, **51**(3), 857–8.

Tanner, J. T., Smith, J., Defibaugh, P., Angyal, G., Villalobos, M., Bueno, M. P. & McGarrahan. (1988). Survey of vitamin content of fortified milk. *J. Assoc Off. Anal. Chem.*, **71**(3), 607–10.

Toyosaki, T, Yamamoto, A. & Mineshita, T. (1983). Effect of superoxide anion on photolysis of riboflavin in serum of purchased bovine milk. *J. Jpn Soc. Nutr. Food Sci.*, **36**, 159–62.

Toyosaki, T., Yamamoto, A. & Mineshita, T. (1984). The photolysis mechanism of riboflavin in milk serum: The correlation between the superoxide and riboflavin decomposition. *Agric. Biol. Chem.*, **48**, 2919–22.

Toyosaki, T., Yamamoto, A. & Mineshita, T. (1986). Simultaneous analysis of riboflavin and its decomposition products in various milks by high-performance liquid chromatography. *J. Micronutr. Anal.*, **2**, 117–23.

USDA (1987). 1985 Nutrient Survey. *National Food Rev.*, Winter/Spring Issue, United States Department of Agriculture, Washington, DC.

US Senate Select Committee on Nutrition and Human Needs (1977). *Dietary Goals for United States*. Government Printing Office, Washington, DC, USA.

Williams, R. C., Baker, D. R. & Schmit, J. A. (1973). Analysis of water-soluble vitamins by high-speed ion exchange chromatography. *J. Chrom. Sci.*, **11**, 618–21.

Woodcock, E. A., Warthesen, J. J. & Labuza, T. P. (1982). Riboflavin photochemical degradation in pasta measured by high performance liquid chromatography. *J. Food Sci.*, **47**, 545–9.