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HPLC Determination of Riboflavin in Fortified Foods

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7.1
Introduction

Riboflavin, IUPAC systematic name 7,8-dimethyl-10-(1-D-ribityl)benzo[*g*]pteridin-2,4-dione, is a light-sensitive molecule. Earlier names derived from its discovery and first identification are lacto-, ovo-, and uroflavin. In alkaline solution it is decomposed to lumiflavin (7,8,10-trimethylisoalloxazine); in neutral or acidic medium, the photolysis proceeds to lumichrome (7,8-dimethylisoalloxazine) within hours (Figure 7.1). This also occurs in foods, when they are exposed to daylight for longer periods, for example, in milk and milk products.

Therefore, analytical manipulations and operations with riboflavin solutions should be performed in the dark or under red light. If protected from light, riboflavin is fairly stable against heat and oxygen. Riboflavin or vitamin B_2 is the active component (prosthetic group) of the flavin nucleotides FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide = riboflavin-5′-phosphate), which fullfill as coenzymes of oxidoreductases multiple functions involving catalytic hydrogen transfer reactions in the metabolism of carbohydrates, fats, and proteins [1]. Riboflavin is readily soluble in dilute mineral or organic acids but sparingly soluble in water (<10 mg per 100 ml at pH 7 and 27 °C) and very sparingly soluble in ethanol (<5 mg per 100 ml at 27 °C). It is also readily soluble but unstable in dilute alkali (Table 4.1). In contrast. FMN and FAD are water soluble (3 g per 100 ml at pH 7 and 25 °C). Absorbance spectra reveal absorption maxima in the visible and UV regions of the spectrum. Furthermore, in weakly acidic medium at pH 6–7 the molecule exhibits a characteristic yellow–green fluorescence with excitation maximum at 444 nm and emission maximum between 530 and 565 nm (Table 7.1). The degradation products after photolysis, lumichrome and lumiflavin, also fluoresce. After irradiation (λ_{ex} = 270 nm) in alkaline solution at pH 10–12, riboflavin is converted to lumiflavin, whereby the fluorescence is shifted to the green spectral range (λ_{em} = 418 nm). This conversion to lumiflavin followed by chloroform extraction allows a very sensitive quantification of riboflavin with a detection limit of 0.02 ng of vitamin B_2 [2].

In general, riboflavin is considered as a less “critical” vitamin, because the dietary reference intakes given by international boards, which vary between 1 and

Fortification of Foods with Vitamins, First Edition. Edited by Michael Rychlik.

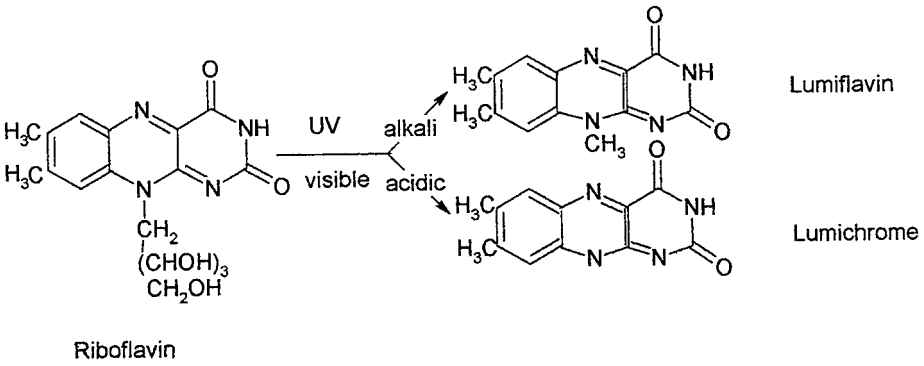

Figure 7.1 Conversion of riboflavin to lumiflavin and lumichrome.

Table 7.1 Physicochemical data for riboflavin according to [2].

Solubility	Sparingly in water (70–100 mg l^{-1}, 27 °C)
	Very sparingly in ethanol (45 mg l^{-1}, 27 °C)
	Readily in dilute alkali (decomposition) and in dilute acids
Absorbance (UV–Vis)	Maxima at 223, 267, 347, and 444 nm in HCl (0.1 mol l^{-1})
Fluorescence	Yellow–green in weakly acidic solution at pH 6–7 (λex 444 nm, λem 530–565 nm)

2 mg per day for adults, are met or even surpassed in population groups of Western countries [3]. Occasional fortifications of foods with this vitamin are restricted to multivitamin syrups, fruit juices, or breakfast cereals. Superior natural sources of this vitamin are milk and dairy products, but meat and whole meal products just as green vegetables are also relevant suppliers of this nutrient. A list of the riboflavin contents of foods is available in an online database [4]. The purpose of this chapter is to give an overview of high-performance liquid chromatography (HPLC) methods for vitamin B_2 quantitation.

7.2 Materials and Methods

7.2.1 Chemicals

Acetonitrile, ammonium chloride, chloroform, citrate–phosphate buffer (pH 5.5), 1,4-dioxane, formic acid, glacial acetic acid, hydrochloric acid, methanol, methy-

lene chloride, 4-nitrophenylphosphate, potassium dihydrogenphosphate, potassium perchlorate, sodium acetate, sulfuric acid, trichloroacetic acid, and urea can be obtained from common suppliers. Claradiastase is available from Fluka (Buchs, Switzerland) and takadiastase from Pfaltz & Bauer (Waterbury, CT, USA). The standards riboflavin, FAD, FMN, 7-ethyl-8-methylriboflavin and sorboflavin can be obtained from Sigma (Deisenhofen, Germany).

7.2.2 Extraction Procedure

Methods for the extraction of riboflavin from foods depend on whether the sum of free riboflavin plus its proportion in flavoproteins FAD and FMN is required or the simultaneous determination of FAD and FMN in addition to unbound vitamin. In fortified foods, riboflavin is commonly added in its free form. Equally in milk, dairy products, and eggs, loosely bound riboflavin is by far the predominant flavin derivative. In these cases, the procedure can be simplified by acidification to remove proteins following centrifugation or filtration through cellulose filters. For protein precipitation, methanol-acetic acid mixtures, dilute trichloroacetic acid, formic acid, or perchlorate are used [5–9]. Alternatively, liquid milk or an acetate buffer extract [0.02 mol l^{-1} acetate–methanol (1 : 1), pH 4] of the dairy product is passed through a conditioned Sep-Pak C_{18} cartridge to remove potential interferences followed by elution of riboflavin with the above acetate buffer–methanol mixture. This extraction procedure is also applicable to the analysis of foods fortified with riboflavin [10–12].

However, when milk proteins were solubilized by using formic acid–urea (6% formic acid containing urea, 2 mol l^{-1}), minor amounts of FMN and FAD can be quantified in addition to free riboflavin [13]. Obviously, these flavins were coprecipitated with milk proteins by simple acidification and escaped former analyses, thus providing an incomplete picture of the (total) riboflavin content in milk and dairy products. Older methods, based on the extraction of flavins from biological materials such as blood and urine by using acetonitrile at pH 7 or with 5% ammonium chloride solution at pH 5.5 and heating at 80 °C for 15 min to precipitate proteins, had the disadvantage that the protein precipitate may adsorb up to 20% of the riboflavin [14, 15].

Most food materials of animal and plant origin contain variable amounts of flavins in addition to free riboflavin. In a more sophisticated procedure, the individual flavins in raw and cooked meats and also in dairy products and cereals were extracted by using a methanol–methylene chloride mixture and citrate–phosphate buffer (0.1 mol l^{-1} at pH 5.5) [16].

If, on the other hand, the determination of total riboflavin is required, the extraction step should involve the release of riboflavin from the phosphorylated and protein-bound flavins by combined acid and enzymatic hydrolysis. A hydrolysis step with mineral acids followed by an effective enzyme treatment is the most important precondition for analytical accuracy, as incomplete hydrolysis is a relevant source of variations in riboflavin determination.

By autoclaving food samples at 121 °C for 15–30 min with dilute mineral acids, for example, 0.1 N HCl or H_2SO_4, protein-bound flavins are liberated and FAD is converted to FMN. Complete conversion to free riboflavin, particularly from starch- or protein-containing food matrices, requires subsequent enzymatic hydrolysis with takadiastase or clarase [17–24].

Both of these amylolytic enzymes also possess phosphatase activity and low protease activity. In view of the varying phosphatase activity in commercially available enzyme preparations, it may be necessary to determine their activity in preceding studies by using 4-nitrophenylphosphate as substrate. An estimation proved that 0.4–1.0 units of takadiastase were required in order to obtain a complete hydrolysis of up to 0.1 µmol of FAD and FMN, respectively. For practical purposes, this relation corresponds to 100–500 mg of enzyme preparation per gram of food sample. Irrespective of the enzyme preparations used, incubation periods should be at least or even exceed 18 h [19–21, 25].

7.2.3 Analytical Principles

HPLC analyses are mainly performed on C_{18} stationary reversed phases (RPs) with hydrophilic eluents based on water-methanol followed either by fluorimetric or rarely by ultraviolet (UV) detection. Less frequently used are C_{10}, amino, hydrophilic gels, and normal-phase chromatography based on silica materials.

Commonly, riboflavin, thiamin, and other B vitamins in foods and biological materials are analyzed simultaneously. In those cases, ion-pair chromatography with hexane- or heptanesulfonate or triethyl- or tetrabutylammonium phosphate is preferred to improve the resolution. Separation procedures include isocratic and gradient elution using mixtures of methanol or acetonitrile with water, acetic acid, or acetate or phosphate buffer, occasionally modified with 1,4-dioxane or formic acid. With gradient elution techniques using phosphate-buffered eluents based on methanol and/or acetonitrile, optimal resolution of FAD, FMN, and riboflavin and of simultaneously analyzed B-complex vitamins can be achieved [16, 26–30].

7.2.4 Detection Modes

Fluorescence detection as the preferred detection method avoids quantification problems due to interfering peaks that appear with UV detection. Because the fluorescence intensity is pH dependent, the mobile phases used for elution should contain buffers in the pH range 3.7–7.5 corresponding to the riboflavin fluorescence maxima (λ_{ex} = 440–450 nm, λ_{em} = 530–560 nm). The excitation maximum for fluorimetric detection of FAD and FMN is in the range 440–500 nm and the emission maximum is at 530 nm.

When employing a multistep gradient elution program based on citrate–phosphate buffer (pH 5.5) and acetonitrile on a polymer-based PLRP-S column

with subsequent fluorimetric monitoring at λ_{ex} 450/λ_{em} 520 nm, detection limits of flavins in various foods as low as 0.55 pmol (0.21 ng) of riboflavin, 1.96 pmol (0.89 ng) of FMN, and 14.19 pmol (11.15 ng) of FAD are obtained per injection [16].

In food samples with low riboflavin contents, as for example in meat products and vegetables, it could, however, be advisable to convert riboflavin to lumiflavin by UV irradiation in order to enhance the sensitivity of fluorescence detection. Accordingly, after acid extraction and enzymatic hydrolysis, an aliquot of the filtrate is adjusted to pH 10–12 (15% NaOH) and irradiated under a suitable UV lamp for 30 min. Thereafter, the sample is acidified (glacial acetic acid) and lumiflavin is extracted with chloroform. After drying over anhydrous sodium sulfate, the extract is injected on to an HPLC column and the lumiflavin fluorescence is measured at λ_{ex} = 270 nm and λ_{em} = 418 nm. The detection limit is given as 0.02 ng per injection [30]. Alternatively, filtered extracts of food samples with a low riboflavin content after autoclaving and enzymatic treatment can be further concentrated and purified on disposable Sep-Pak C_{18} cartridges [31]. UV detection is about 30 times less sensitive than fluorescence detection and, therefore, is only suitable for measurements of riboflavin in foods with higher natural contents (dairy products, eggs), fortified foods, or pharmaceuticals. Absorbance maxima of riboflavin are 270 and 446 nm [6, 8, 10, 11, 30, 32–34].

7.3 HPLC Intercomparisons

In intercomparison studies of methods that were organized by the EU Community Bureau of Reference (BCR), certified reference materials such as lyophilized meat products and vegetables and wholemeal flour were analyzed for water-soluble vitamins. Accordingly, autoclaving with HCl (0.1 mol l^{-1}) at 121 °C for 15–30 min followed by incubation with takadiastase (100–500 mg g^{-1} sample), or a mixture of takadiastase and claradiastase (10:1, m/m) at pH 4.0–4.5 and 37–45 °C for at least 3 h (with a tendency to longer incubation periods up to 18 h) evolved as the optimal extraction procedure for riboflavin in foods. The enzyme should in any case be in excess by using 60–500 mg of enzyme per gram of sample. The additional application of ultrasonication to the incubation mixture shortened the enzymatic hydrolysis to 1 h. The subsequent HPLC analysis involved separation on C_{18} material with water–methanol, water–methanol–acetic acid or phosphate buffer mixtures at pH 2.9–5 or with acetonitrile substituting methanol, and occasionally ion-pair chromatography with hexane- or heptanesulfonic acid. The type of HPLC column (normal phase or RP) did not affect the results. Fluorimetric detection was employed with excitation at 422–467 nm and emission at 510–525 nm. The detection limit was 20 pg of riboflavin absolute per injection.

The coefficient of variation of the total riboflavin content between or within laboratories (milk powder and pig liver) was 10–12%, showing an acceptable variability. Higher variability of up to 40% was found for the results for vegetables and wholemeal [19, 21, 23, 24, 35].

7.4 Conclusion

HPLC analysis is established as the method of choice for the determination of riboflavin in foods. The scope of the analysis determines the mode of procedure to be used. For the determination of the flavins FAD and FMN, including free riboflavin, a nondegradative extraction with dilute organic acids can be used. Determination of total riboflavin needs combined extraction by autoclaving with mineral acids followed by enzymatic hydrolysis. Chromatography is performed mainly on RP C_{18} columns by using isocratic or less frequently gradient elution with acidified or buffered methanol–water or acetonitrile mixtures followed by fluorimetric detection as the most sensitive quantification method. In fortified foods, riboflavin can also be determined using UV detection with adequate precision.

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