Journal of Chromatography, 511 (1990) 359–366
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 393

Note

Determination of flavins in dairy products by high-performance liquid chromatography using sorboflavin as internal standard

N. BILIC* and R. SIEBER
Federal Dairy Research Institute, Schwarzenburgstrasse 161, 3097 Liebefeld-Berne (Switzerland)
(First received July 21st, 1989; revised manuscript received February 12th, 1990)

Riboflavin, riboflavin-5-phosphate (flavin mononucleotide, FMN) and flavin adenine dinucleotide (FAD) are the principal forms of vitamin B_2. A variety of methods are available for their assays[1–3]. Microbiological methods and rat growth assay for riboflavin are not suitable for common laboratory applications[1]. Competetive protein binding assays are only specific for riboflavin[4]. Fluorimetric methods have been widely used because they are sensitive, rapid and applicable to the assay of individual flavins[3].

New developments in this area include assays of flavins by high-performance liquid chromatography (HPLC) with fluorescence detection. Applications of reversed-phase separation to the determination of riboflavin in various foods have been reviewed[5,6]. Assays for individual flavins in blood have been reported recently[7–9]. Important requirements are mild extraction procedures to prevent hydrolysis of both FAD and FMN.

HPLC assays for flavins or riboflavin have been carried out without use of internal standards and correction for losses in the extraction and in the chromatography was difficult or impossible. An internal standard must share structural and chromatographic properties with the flavins of interest, so as to co-purify them during the extraction and isolation procedures.

In this work we used sorboflavin as an internal standard for the assay of natural flavins by HPLC. It contains a glucityl side-chain on the isoalloxazine ring, in contrast to the ribityl chain in riboflavin. In our HPLC system, sorboflavin is well resolved and eluted between FMN and riboflavin. The substance is not commercially available, but can be prepared in the laboratory. Further a mild extraction procedure for flavins is presented, based on solubilization of the protein content followed by solid-phase extraction. Flavin losses due to coprecipitation, as observed in previous procedures, are largely avoided.

0021-9673/90/$03.50

EXPERIMENTAL

Apparatus

The HPLC system consisted of precision-flow metering pumps (Beckman Model 100A), a Supelco LC-18 7.5 cm × 4.6 mm I.D. column with a 3-μm packing, a 5μl loop, a Shimadzu C-R1A data processor and a Merck–Hitachi Model F1000 fluorescence spectrophotometer for HPLC applications equipped with a 12-μl flow cell. The excitation and emission wavelengths were set at 450 and 530 nm, respectively. The mobile phase was delivered at 1 ml/min. A Carl Zeiss PMQ II spectrophotometer was used to measure the absorption of flavin standard solutions.

Reagents and chemicals

Chemicals were of analytical reagent grade except acetonitrile (HPLC grade). Riboflavin, FMN monosodium salt and FAD disodium salt were from Merck. Water and deionized and doubly distilled.

Stock reagents and materials were 4 *M* urea, 1.5 *M* H_3PO_4, 10 and 12% aqueous formic acid, 1 *M* KH_2PO_4 (stored in a refrigerator) and silica gel C_{18}, 40–60 μm particle size, in ethanol (20 g in 80 ml).

The following reagent solutions were prepared prior to use: a solution for extraction of flavins consisting of 6% formic acid and 2 *M* urea (formic acid–urea), eluent for the extraction column [methanol–10% formic acid (1:4, v/v)] and mobile phase for HPLC, consisting of 14% acetonitrile in 100 m*M* KH_2PO_4, titrated to pH 2.9 with 1.5 *M* H_3PO_4.

Synthesis and purification of sorboflavin

The preparation of sorboflavin is based on the procedures described by Karrer and Benz[10] for riboflavin and by Berger and Lee[11] for arylamide-N-glycosides, but adapted for our laboratory without facilities for hydrogenation under both high pressure and temperature.

Methanol (200 ml) and a magnetic stirring bar were placed in a 250-ml boiling flask containing both 1 m*M* 4,5-dimethyl-*o*-phenylenediamine and 1 m*M* D-glucose. The mixture was refluxed for 8 h with stirring. The condenser was removed briefly in order to add 50–100 mg of allylpalladium (II) chloride as a hydrogenation catalyst. A stream of hydrogen, flowing through the fluid at 50 ml/min, was set up using PTFE tubing (1/8 in. O.D. × 2.1 mm I.D.) attached between the hydrogen bottle and the fluid (down the inside of the condenser). Refluxing under the hydrogen flow continued for 8–10 h. The fluid was cooled and filtered through a 0.45-μm PTFE membrane to remove the palladium. After evaporation of the solvent, 200 ml of glacial acetic acid and 10 m*M* alloxan were added to the residue and the mixture was stirred for 1 h at 50–60°C. The solvent was removed by evaporation. The residue containing sorboflavin was dissolved in 50–100 ml of water. Sorboflavin was purified on silica gel C_{18} (40–60 μm) packed in an Allihn filter tube to half the volume. The adsorbent was conditioned with ethanol and washed with water. The sorboflavin solution was loaded onto the adsorbent and passed through using suction. Non-flavin compounds and sorboflavin were eluted with 10 and 20% ethanol, respectively.

The sorboflavin obtained may be sufficiently pure for HPLC applications. If necessary, further purification may be carried out as described for flavin standard

solutions (see below). Using butanol–formic acid–water (77:10:13, v/v/v) as a solvent in paper chromatography, sorboflavin gave $R_F = 0.136$, in agreement with earlier observations[1,2].

Flavin standard solutions

Riboflavin (0.1 mg), FMN monosodium salt (0.15 mg) and FAD disodium salt (0.2 mg) were dissolved in 1 ml of water and separated on a 25 cm × 8 mm I.D. column filled with silica gel C_{18} (10 μm) using linear gradient elution at 2 ml/min [mobile phase A, 50 m*M* KH_2PO_4 (pH 5.5); mobile phase B, ethanol; gradient from 0 to 50% B in 30 min]. Measuring the absorbance at 450 nm, the major peak of each flavin was identified, collected and diluted with water to obtain an absorbance of about 0.3 (corresponding to concentrations of about 30 μM). The final concentration of each flavin was calculated using the molar absorptivity of $12.1 \cdot 10^3$ for both riboflavin and FMN and $11.3 \cdot 10^3$ for FAD[7]. Each standard solution was stored in 1-ml aliquots at −20°C. On the day of use, a 1 μM standard solution was prepared by transfering aliquots of purified flavin solutions into a 1-ml screw-capped volumetric flask (Pierce), followed by addition of 50 μl of sorboflavin solution (absorbance 0.2 at 450 nm) and dilution to volume with water.

Extraction of flavins

Solid-phase extraction columns were made on the day of use by filling filtration tubes (containing frits only) with 0.75 ml of silica gel C_{18} in ethanol, suspended by magnetic stirring. The ethanol was allowed to drain. A 1-cm glass microfibre circle (GF/D grade: Whatman) was placed on the adsorbent and gently pressed. The packing was rinsed with 2 ml of water.

A 1-g amount of cheese or 1 ml of milk was homogenized in or mixed with, respectively, 19-ml of formic acid–urea. Whole milk powder (0.2 g) was added to 10 ml of formic acid–urea and stirred for 30 min. The solutions were centrifuged to remove the fat. A 2-ml aliquot was mixed with 50 μl of sorboflavin solution (absorbance 0.2 at 450 nm) and passed through an extraction column, without application of pressure or vacuum. The packing was washed with 2 ml of 10% formic acid. The flavins were eluted by passing 1 ml of methanol–formic acid at a slow rate using a Vis-1 Single SPE Tube processor (Supelco) to provide gentle air pressure. The eluate was filtered and analysed by HPLC within 1 h.

RESULTS AND DISCUSSION

Reversed-phase HPLC with isocratic elution on a 7.5 cm × 4.6 mm I.D. column, filled wit a 3-μm packing, is effective for analysis of the flavins. Further advantages include short retention times, ranging from 1.2 to 2.9 min, in comparison with 8–20 min reported for riboflavin using other procedures[7–9]. Typical chromatograms of flavin standards and of flavins extracted from cheese are shown in Fig. 1. Because of both the short retention times and isocratic elution, the sample throughput can be fairly high. Also, clean-up of flavin extracts helps to prolong the column life considerably. The chromatograms in Fig. 1 were obtained on a column used for more than 500 separations.

The fluorescence intensity of flavins is pH dependent[3]. FAD exhibits a lower

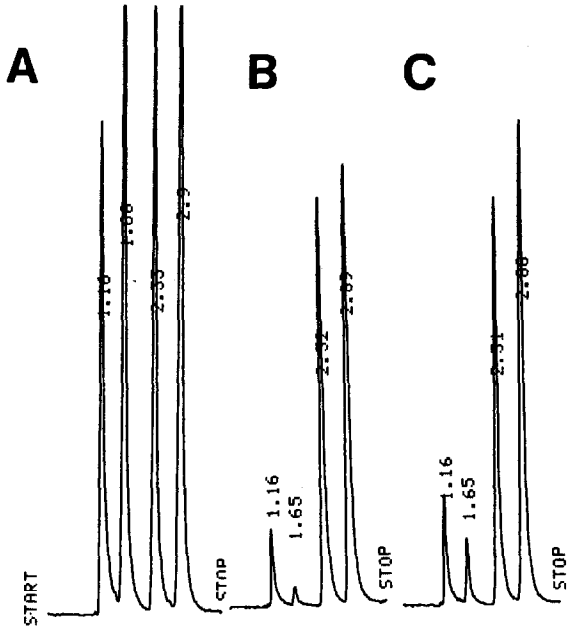

Fig. 1. Typical chromatograms of (A) flavin standards, each at a concentration of 1 μM, with retention times of 1.16, 1.66, 2.33 and 2.9 min for FAD, FMN, sorboflavin and riboflavin, respectively; (B) flavins extracted from 2 ml of a 5% Gruyère cheese homogenate; (C) flavins extracted from the same homogenate spiked with flavins, 100 pM each. Elution at 1 ml/min; injection volume, 5 μl; recording attenuation, 8 mV full-scale. For other conditions, see text. Numbers at peaks indicate retention times in min.

fluorescence efficiency than both FMN and riboflavin. A mobile phase of pH 2.9 was chosen to provide for the maximum fluorescence of FAD and for 85% of the maximum fluorescence of the two other flavins. We believe this to be a good compromise, allowing the analysis of all the flavins at low levels. The ionic strength of the mobile phase (100 mM KH_2PO_4) is important for separating FAD and FMN. At lower ionic strength, their peaks may overlap.

The determination of FAD by HPLC requires that the extraction procedure be sufficiently mild to prevent hydrolysis of the flavin to FMN. Exposure to both mineral acids and alkali should be avoided. Extraction with ice-cold trichloroacetic acid is possible if performed rapidly[1]. However, this procedure may destroy 4–5% of the FAD[13]. Other methods utilize extraction with acetonitrile at pH 7 or with 5% ammonium chloride at pH 5.5 and 80°C for 15 min[7,9]. Under such conditions, the protein precipitate formed may adsorb up to 17–20% of the riboflavin[8,9].

Our approach to the extraction of flavins includes a step to solubilize the protein content, which consists of poorly soluble casein. When this material is dissolved, the flavins are extracted by solid-phase extraction.

Formic acid was considered as a possible solvent for the present purposes, as it is known as a good solvent for peptides. Further, formic acid is perhaps the best solvent for riboflavin, dissolving 100 times as much riboflavin as water[14]. However, in our attempts to avoid hydrolysis of FAD, only dilute formic acid was used. It was soon shown that neither 5 nor 10% formic acid can dissolve casein or a piece of cheese

without the formation of a precipitate, unless supplemented by urea. A solution at pH 2 was adopted, containing both 6% formic acid and 2 *M* urea. It readily dissolves the required material and gives no precipitate on centrifugation at 4000 *g*.

A number of solvents were tried to desorb the flavins from an extraction column. It was observed by chance that methanol–formic acid is a very effective eluent for flavins. For instance, it is superior to methanol–0.15 *M* H_3PO_4 (Table I). By passing 1 ml of methanol–formic acid, more than 90% of the flavins were recovered in the eluate, in comparison with 20% or less using methanol–phosphoric acid. The reasons for these differences are not clear. Possibly higher protonation of the isoalloxazine ring at lower pH plays a role[14]. When the phosphoric acid had been replaced with 50 m*M* KH_2PO_4 (pH 5.5), the desorption rates of flavins remained almost unchanged (not shown). Eluents containing either phosphoric acid or phosphate buffer require a higher percentage of methanol (30% or more) for effective desorption of flavins. Consequently, eluates with 30% methanol may distort the FAD peak shape and shorten the column life.

Both FAD and FMN were examined for their stabilities in either methanol–formic acid or formic acid–urea. Each medium was spiked with either flavin and allowed to stand at ambient temperature in the dark for 24 h, then analysed by HPLC (Fig. 2). The fluids were filtered and analysed by HPLC without prior extraction. In methanol–formic acid, 8% of the FAD hydrolysed to FMN in 24 h. In another experiment (not shown), 9% of the FAD decomposed to FMN in the same period. These rates of FAD hydrolysis are very low, and may be neglected if flavin extracts are analysed within 1 h as proposed here. In formic acid–urea, the FAD was almost fully recovered, with trace amounts of FMN that could not be measured with the

TABLE I

DESORPTION RATES AND RECOVERIES OF FLAVINS FROM EXTRACTION TUBES.

A 2-ml volume of formic acid–urea was spiked with 1 n*M* each, of FAD, FMN and riboflavin, followed by addition of 50 µl of sorboflavin (absorbance 0.2 at 450 nm) and passed through an extraction tube. The adsorbent was washed with 2 ml of 10% formic acid. Volumes of 0.5 ml of either eluent were sequentially passed through the tube, collected and analysed by HPLC for flavin recovery.

Eluent	*Eluate No.*	*Recoveries of flavins in 0.5 ml of eluates (%)*			
		FAD	*FMN*	*Sorboflavin*	*Riboflavin*
Methanol–10% formic acid (1:4, v/v), pH 1.60	1	70	60	57.2	45.8
	2	22.9	33.3	37.1	46.1
	3	2.7	3.9	5.1	8
	Total:	95.8	97.2	99.4	99.9
Methanol–0.15 *M* H_3PO_4 (1:4, v/v), pH 1.85	1	4.7	3.3	1,3	0
	2	17.4	12.5	12.1	3.1
	3	21	18.5	22	9.2
	4	18	18.5	24	13.5
	5	12.8	16.6	19	17
	6	8	12	13	16.5
	7	4.3	7.3	7	13
	8	2.1	4.6	3.1	9.9
	Total:	88.3	93.3	101.5	82.2

TABLE II

DETERMINATION OF FLAVINS IN 2-ml ALIQUOTS OF CHEESE HOMOGENATES AND MILK DILUTIONS WITH AND WITHOUT ADDITION OF FLAVINS

The flavins (100 p*M* each) were added in a 100-μl volume.

No.	*Sample*	*Amounts of flavins measured (pM)*		
		FAD	*FMN*	*Riboflavin*
1	Aliquots of milk without addition of flavins ($n=8$)	32.9 ± 5	22.7 ± 4	427 ± 20
	Aliquots of milk with addition of flavins ($n=8$)	137 ± 11	128 ± 20	526 ± 18
	Recoveries of flavins added	104.1%	105.3%	99.0%
2	Aliquots of Sbrinz cheese homogenate without addition of flavins ($n=8$)	135 ± 19	19,6 ± 2	1300 ± 60
	Aliquots of Sbrinz cheese homogenate with addition of flavins ($n=8$)	237 ± 12	119 ± 11	1401 ± 28
	Recoveries of flavins added	102%	100.4%	101%
3	Aliquots of Gruyère cheese homogenate without addition of flavins ($n=8$)	163 ± 24	Not detected	1301 ± 30
	Aliquots of Gruyère cheese homogenate with addition of flavins ($n=8$)	255 ± 28	102 ± 10	1408 ± 36
	Recoveries of flavins added	92%	102%	98%

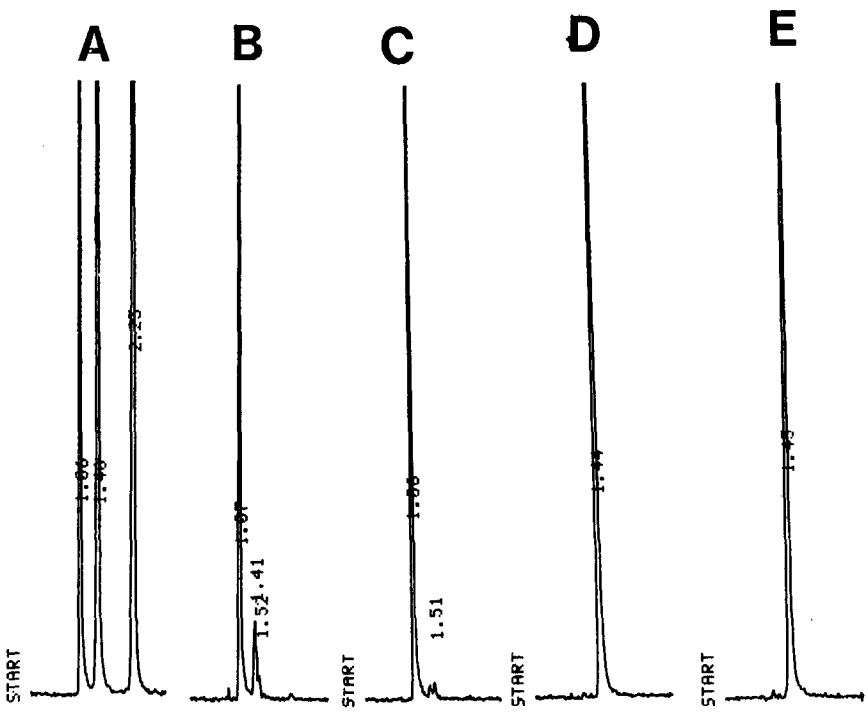

Fig. 2. Analysis of 1 μM flavin solutions. (A) FAD, FMN and riboflavin prepared in water and analysed within 2 h; (B) FAD in formic acid–methanol and (C) FAD in formic acid–urea analysed 24 h after preparation; (D) FMN in formic acid–methanol and (E) FMN in formic acid–urea analysed 24 h after preparation. Recoveries: (B) 94.4% as FAD and 8.5% as FMN, (C) 96.6% as FAD and (D) 98% and (E) 101% as FMN. The mobile phase contained 15% acetonitrile.

instrumental setup. In this case, hydrolysis rates of FAD may be guessed to be *ca.* 1% in 24 h or less, and may also be neglected. The FMN was shown to be stable in both media.

Recoveries of flavins for the whole procedure were checked in two ways. First, 2-ml aliquots of a 5% homogenate of Gruyère cheese were spiked with different amounts of each flavin to obtain 0.1, 0.2, 0.3, 0.4 and 0.5 μM concentrations. The results were evaluated using linear regression. The calculated slopes were multiplied by 100 to obtain percentage recoveries, which were 105.8, 99.7 and 100.2% for FAD, FMN and riboflavin, respectively. In the second approach, fixed amounts of flavins, 100 pM each, were added to 2-ml aliquots of either 5% cheese homogenates or 5% dilutions of milk in formic acid–urea. The flavin recoveries, given in Table II, are in the ranges 92–104% for FAD, 100–105% for FMN and 98–101% for riboflavin.

Both the within- and between-assay precisions of the method were tested on a cow milk containing all the flavins of interest. The milk was defatted by centrifugation and stored in aliquots at −20°C until the day of analysis. The results are given in Table III. The relative standard deviations are about 6% for both FAD and riboflavin and 12% for FMN; the low concentration of FMN might be a reason for the high value.

TABLE III

PRECISION OF THE ASSAY

Parameter	*pM in 2-ml aliquot of milk (mean ± S.D.)*		
	FAD	*FMN*	*Riboflavin*
Within-assay ($n=8$)	52.4 ± 3.4	17.8 ± 3	395 ± 25
Relative standard deviation	6.5%	11%	6,3%
Between-assay ($n=17$)	50.5 ± 3	15.1 ± 1.7	419 ± 28
Relative standard deviation	5.9%	12%	6.7%

At a signal-to-noise ratio of 3, the detection limits were calculated to be about 3 nmol/l for FAD and 2.5 nmol/l for both FMN and riboflavin.

The present procedure seems to be reliable for measuring the flavins in dairy material. The recovery and precision are within acceptable limits. The reliability is based on the use of sorboflavin as the internal standard, formic acid–urea as a good solvent for both the protein matrix and the flavins and methanol–formic acid as good solvent for desorption of the flavins.

Sorboflavin is stable in the procedure, readily soluble in aqueous solutions and exhibits spectral properties similar to those of natural flavins. The use of sorboflavin does not prolong the chromatography time, as its peak emerges between those of natural flavins. However, when stored at 4°C for 6 months or longer, sorboflavin may exhibit an new peak emerging shortly after riboflavin. This is of no consequence for the assay.

REFERENCES

1 J. Koziol, *Methods Enzymol.*, 43 (1971) 253.
2 W. N. Pearson, in P. György and W. N. Pearson (Editors), *The Vitamins*, Vol. 7, Academic Press, New York, 1967, p. 99.
3 S. Udenfriend, *Fluorescence Assay in Biology and Medicine*, Academic Press, New York, 1962.
4 S. E. Lotter, M. S. Miller, R. C. Bruch and H. B. White, *Anal. Biochem.*, 125 (1982) 110.
5 M. A. Marletta and D. R. Light, in A. P. De Leenheer, W. E. Lambert and M. G. M. De Ruyter (Editors), *Modern Chromatographic Analysis of the Vitamins*, Marcel Dekker, New York, Basle, 1985, p. 413.
6 P. M. Finglas and R. M. Faulks, *J. Micronutr. Anal.*, 3 (1987) 251.
7 H. Ohkawa, N. Ohishi and K. Yagi, *Biochem. Int.*, 4 (1982) 187.
8 A. J. Speek, F. van Schaik, J. Schrijver and W. H. P. Schreurs, *J. Chromatogr.*, 228 (1982) 311.
9 A. Lopez-Anaya and M. Mayersohn, *J. Chromatogr.*, 423 (1987) 105.
10 P. Karrer and F. Benz, *Helv. Chim. Acta*, 18 (1935) 426.
11 L. Berger and J. Lee, *J. Org. Chem.*, 11 (1946) 84.
12 W. Forter and P. Karrer, *Helv. Chim. Acta*, 36 (1953) 1530.
13 V. Massey and B. E. P. Swoboda, *Biochem. Z.*, 338 (1963) 474.
14 T. Wagner-Jauregg, in W. H. Sebrell, Jr. and R. S. Harris (Editors), *The Vitamins*, Vol. 5, Academic Press, New York, 1972, p. 3.