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Chromatographic determination of riboflavin and its derivatives in food

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Abstract

Three elution methods on two different reversed-phase C_{18} columns were developed to determine flavin[1] derivatives in raw egg white, raw egg yolk, egg powder, pasteurised milk, fermented milk products and liver (chicken, calf and pig). Additionally, 11 thin-layer chromatography solvent systems were used to confirm presence of flavins detected in assessed products. It was found that an Alphabond C_{18} column was not as effective as a Symmetry C_{18} column. Method A (mobile phase gradient of methanol–0.05 *M* ammonium acetate, pH 6.0 applied on an Alphabond C_{18} column) can be used for determination of flavin adenine dinucleotide, flavin mononucleotide, riboflavin 4′,5′-cyclic phosphate, riboflavin, 10-formylmethylflavin and 10-hydroxyethylflavin in products that do not contain 7α-hydroxyriboflavin. Method B (mobile phase gradient of methanol–demineralized water, on an Alphabond C_{18} column) can be useful to separate flavin coenzymes from other flavin compounds or to confirm the presence of 7α-hydroxyriboflavin and 10-hydroxyethylflavin in analysed samples. Method C (mobile phase gradient of methanol–0.05 *M* ammonium acetate, pH 6.0, on a Symmetry C_{18} column) allows separation of all flavins detected in tested products: flavin adenine dinucleotide, flavin mononucleotide, riboflavin 4′,5′-cyclic phosphate, riboflavin, 10-formylmethylflavin, 10-hydroxyethylflavin, 7α-hydroxyriboflavin, riboflavin-β-D-galactoside and riboflavin-α-D-glucoside.

Keywords: Food analysis; Riboflavins; Vitamins; Flavins; Nucleotides; Glycosides

1. Introduction

Several methods based on different principles are available for the determination of vitamin B_2 in food. The commonly estimated forms of this vitamin are riboflavin (7,8-dimethyl-10-ribityl-isoalloxazine, RF), flavin mononucleotide (riboflavin 5′-phosphate, FMN), and flavin adenine dinucleotide (FAD). The concentration of vitamin B_2 is usually determined as total riboflavin by converting FMN and FAD to RF prior to quantification. The standard method for determination of total riboflavin content in food is the AOAC fluorometric method [1]. In this method, samples are treated with $KMnO_4$ and H_2O_2 to remove background fluorescence. Such treatment, however, may destroy riboflavin [2]. It is also criticised for being tedious and time consuming. Additionally, the AOAC method has been reported as a procedure, which overestimates total flavin content due to the presence of interfering artefacts [3].

Besides RF, FAD and FMN there are also less known flavin derivatives present in nature. These are, e.g., 10-hydroxyethylflavin (10-HEF), 10-formylmethylflavin (10-FMF), 7α-hydroxyriboflavin (7-hydroxymethylriboflavin, nekoflavin [4], 7α-HRF), 8α - hydroxyriboflavin (8 - hydroxymethylriboflavin,

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[1]Derivatives of the 7,8-dimethyl isoalloxazine nucleus are defined also as ''flavins''.

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8α-HRF), 8α-hydroxy-FMN, riboflavin-α-D-glucoside (RFgluk), 8α-sulfonylriboflavin, 5′-peptydylriboflavin and some forms of isomeric alloxazinic structure such as lumichrome, 7- and 8-carboxylumichromes. These derivatives were found in human and animal milk [5–8], urine [5,6,9–18], blood plasma [19,20] and animal organs [21,22]. Presence of some flavin derivatives may also indicate photodegradation of flavins during food processing and storage. Riboflavin decomposition in food is usually reported as a percentage of the initial amount present (the rate of riboflavin loss) [23–30] or by the determination of lumichrome content, which is a photoproduct of riboflavin degradation in acid and neutral solutions [30,31]. Lumichrome, however, is not the only or final riboflavin degradation product so it can not be used as an exact measure of riboflavin deterioration [30,31]. It can be used to indicate qualitatively that riboflavin is degraded. Flavin derivatives may have vitamin character, be biologically inactive or antagonistic to riboflavin as in the case of 10-HEF and 10-FMF, which potentially inhibit the flavokinase-catalysed conversion of riboflavin to FMN [32]. Joseph and McCormick [33] revealed that the nutritional efficiency of RFgluk is similar to that of free riboflavin. Riboflavin and its coenzymes under alkaline conditions are photodegraded to biologically inactive lumiflavin [34]. The exact biological value of other flavin derivatives is not known. Significant concentration of derivatives, especially those that show antivitamin character should be taken under consideration in quantification of the vitamin B_2 value of food. From this point of view it seems to be important to know not only total riboflavin content but also flavin composition of food. Some of the above mentioned flavins such as 7α-HRF, 10-FMF, 10-HEF and riboflavin-β-galactoside (RFgal) have been already found in food products [8,35,36].

Recently, application of high-performance liquid chromatography (HPLC) methods with reversed-phase columns and UV–Vis or especially fluorometric detection allow determination of flavin derivatives, even those present in very small concentrations. HPLC methods with fluorometric detection are considered the most desirable because of their rapidity, specificity and sensitivity. HPLC determination of flavin derivatives is very often supported by thin-layer chromatography (TLC). Analytical TLC is rather used in qualitative analysis of flavins, although the quantification of the separated flavin compounds may be performed by the use of TLC–densitometry. Comparison of R_F values of the unknown flavin present in extract with that of flavin standards is a fast and useful tool for determination of riboflavin and its derivatives in food. An appropriate mobile phase may clearly differentiate riboflavin from their derivatives [6,8,35–39]. Preparative TLC is still used for isolation and purification of flavin compounds [35,36,39,40].

In this paper, HPLC and TLC methods of determination of riboflavin and its derivatives in selected food products are reported.

2. Experimental

2.1. Materials

The standards of RF (Reanal, Budapest, Hungary), FAD (Boehringer Mannheim, Germany), FMN (Merck, Darmstadt, Germany) were used without additional purification but for the quantitative analysis of flavins the FMN and FAD concentrations were corrected for the impurities. The purity of commercial FAD was 96.9% and FMN was 86.4%. 7α-HRF was a gift from K. Matsui (Osaka International University for Women, Osaka, Japan). 8α-HRF was prepared by acid hydrolysis [16] of 8α-bromo-2′,3′,4′,5′-tetraacetylriboflavin [41] obtained from 2′,3′,4′,5′-tetraacetylriboflavin [42]. 10-FMF and 10-HEF were synthesised according to the method of Fall and Petering [43] and riboflavin 4′,5′-cyclic phosphate (4′:5′-FMN) – according to the methods of Yagi and Okuda [44] and Huennekens and Kilgour [45]. RFgal and RFgluk were prepared according to the method of Tachibana [38] by incubation of riboflavin and lactose (RFgal) or maltose (RFgluk) with Taka-Diastase powder (Sigma, St. Louis, MO, USA) and purified using preparative TLC (solvent: *n*-butanol–glacial acetic acid–water, 15:3:7, v/v) and semipreparative HPLC. Yogurt bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* ST-36, *Streptococcus salivarius* subsp. *thermophilus* Lb-12) were from Chr. Hansen (Hørsholm, Denmark). Food products such as: eggs, egg powder, pasteurised milk, fermented milk products

(plain yogurt, kefir, sour milk, acidophilus milk, buttermilk) and liver (calf, chicken and pig) were bought at random in grocery stores.

2.2. Preparation and HPLC analysis of extracts

Flavins from 2 to 20 g of samples were extracted by method described previously [35] but instead of ammonium bicarbonate, pH 7.0, ammonium acetate, pH 6.0 was used. Milk and fermented milk products were sampled directly from the package at the time of analysis. Raw livers, prior to extraction, were ground in the food processor to prepare a homogenous sample. All extracts and standard solutions were filtered through a 0.45-μm filter (Sartorius, Göttingen, Germany) prior to injection into the HPLC column [35]. All samples and standard solutions were protected from light during the whole procedure. HPLC preparations were performed on a Waters Model 600E high-performance liquid chromatograph (Waters, Milford, MA, USA) equipped with an analytical Alphabond C_{18} column (300 mm×4.6 mm, 10 μm, Alltech, Carnforth, UK) or an analytical Symmetry C_{18} column (150 mm×3.9 mm, 5 μm, Waters) fitted with a μBondapak C_{18} or a Nova-Pak C_{18} precolumn, respectively. For identification of flavins three elution methods developed in our laboratory were used: (A) methanol–0.05 *M* ammonium acetate buffer, pH 6.0 and (B) methanol–demineralized water in the gradients described previously [35] (Alphabond C_{18} column); (C) methanol–0.05 *M* ammonium acetate buffer, pH 6.0 in the gradient described previously [36].

Semipreparative HPLC of flavins from food products was performed on a μBondapak C_{18} column (100 mm×25 mm, 10 μm) fitted with a μBondapak C_{18} precolumn (10 mm×25 mm, 10 μm) (Waters) using method A or B with a flow-rate 3 ml/min.

A Waters 474 scanning fluorescence detector with dual monochromator configuration was used at an emission wavelength of 530 nm with excitation at 450 nm for isoalloxazine derivatives, and emission wavelength of 430 nm with an excitation at 380 nm for alloxazine derivatives, with an emission slit width of 10 nm. Additionally, the Waters Model 991 or 996 photodiode-array detectors were used to differentiate flavins from other compounds on the basis of their absorption spectra.

The concentrations of FAD, FMN and RF were determined with use of their corresponding standard curves (method C) prepared under the same chromatographic conditions as used for determination of flavins in food products. A stock solution of RF (100 μg/ml) stored at 4°C and protected from light was stable for at least 4 weeks. Stock solutions of FAD and FMN (100 μg/ml) and working standard solutions of all vitamin B_2 forms were prepared freshly before every analysis. Quantification of 7α-HRF, 4′:5′-FMN, 10-FMF, 10-HEF and RFgal was performed using the standard curve prepared for RF. In the case of 7α-HRF, the concentration was corrected for the lower fluorescence intensity of this derivative [16].

Absorption spectra were obtained on the Waters Model 996 photodiode-array detector. Fluorescence emission spectra were measured using the MPF-44 A/E spectrofluorometer (Perkin-Elmer, Norwalk, CT, USA) in a mixture of methanol–ammonium acetate, pH 6.0 (25:75).

2.3. Thin-layer chromatography

For preparative TLC purposes, concentrated flavin extracts were passed through column packed with resorcinol-type resin R-15 synthesised and used according to the method of Koziołowa and Kozioł [46] to remove all interfering non-flavin compounds. Preparative TLC was performed on 2 mm silica gel plates (Kieselgel 60, Merck) using solvent I or IIa. Analytical TLC was performed with silica gel (Kieselgel 60, 0.2 mm, Merck) or cellulose plates (cellulose F_{254}, 0.1 mm, Merck). Solvent systems used for TLC were as follows: (I) *n*-butanol–glacial acetic acid–water (2:1:1, v/v), silica gel; (IIa) and (IIb) *n*-butanol–acetic acid–water (5:2:3, v/v), silica gel or cellulose, respectively; (III) chloroform–methanol–ethyl acetate (5:5:2, v/v), silica gel; (IV) *n*-butanol–benzyl alcohol–glacial acetic acid (8:4:3, v/v), silica gel; (V) collidine–water (3:1, v/v), cellulose; (VI) 5% $Na_2HPO_4 \cdot 12H_2O$, silica gel; (VII) *n*-butanol–formic acid–water–diethyl ether (77:10:13:15, v/v), silica gel; (VIII) *n*-butanol–ethanol–water (10:3:7, v/v), silica gel; (IX) isoamyl alcohol–ethyl methyl ketone–glacial acetic acid–water (40:40:7:13, v/v), silica gel; (X) *n*-butanol–isopropanol–water–glacial acetic acid (30:50:10:2,

v/v), silica gel; (XI) ethyl methyl ketone–acetic acid–methanol (3:1:1, v/v), silica gel.

2.4. Assay for conformation of microbiological origin of riboflavin-β-D-galactoside in plain yogurt

To confirm microbiological origin of RFgal in plain yogurt, pasteurised milk was inoculated under sterile conditions using lyophilised bacteria of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* (without measuring their amounts). The samples were incubated at 43°C to obtain yogurt. Flavins from ''produced'' yogurt were extracted using the method described in Section 2.2. and identified using HPLC methods. As a blank sample, extract of flavins from pasteurised milk were used.

To check whether RFgal can be obtained by incubation of riboflavin, lactose and yogurt bacteria, the mixture of 1 ml of riboflavin (600 μg/ml), 1 ml of lactose (16%) and 2 ml of activated yogurt bacteria was incubated at 43°C. The mixture was analysed by HPLC (method C) after at least 4 h and then every 12 h in the course of 96 h.

3. Results and discussion

3.1. Separation and chromatographic identification of flavins

Simultaneous determination of riboflavin and its derivatives, prior to their identification and quantification, requires an appropriate extraction procedure that does not cause any changes in the structure of flavins. The extraction procedure should be sufficiently mild to prevent hydrolysis of flavins, especially flavin coenzymes. The most frequently methods used are multiple hot water or buffer extractions (60–80°C) or extractions with use of organic solvents such as: methanol, phenol, acetonitrile, glacial acetic acid, formic acid or their mixtures [3,7,8,21,22,47,48]. Riboflavin and FMN in neutral aqueous solutions are stable even when heated to 100°C [49]. In diluted acids, however, the ester bond of flavin nucleotides is rapidly hydrolysed; FAD may be completely hydrolysed to FMN by allowing it to stand overnight at 38°C in 10% trichloroacetic acid. Extraction with ice-cold trichloroacetic acid is possible if performed over the course of maximal 30 min [49,50]. At pH values below 2, FMN considered as pure riboflavin 5′-phosphate undergoes isomerization to riboflavin 4′-, 3′- and 2′-phosphates. At pH values between 3 and 7, FMN hydrolyses with the maximal rate at pH 4. FMN is most stable in aqueous solutions at pH value about 7 [51]. Pure FAD in distilled water is not changed under heating below 60°C, but above 70°C is converted to riboflavin 4′,5′-cyclic phosphate (4′:5′-FMN) [44,52,53]. Crude FAD is rather stable in distilled water; the decomposition to 4′:5′-FMN occurs only above 90°C [49]. Thus the temperature for hot water or buffer extractions of flavins from tissue should be kept below 80°C. 4′:5′-FMN may be obtained by decomposition of FAD in alkaline solutions even in the cold [54]. It was also found that small amounts of 4′:5′-FMN are formed in solution of pure FAD with pH values between 4.5 and 7.5, left at room temperature for at least 24 h [35]. The compound of 4′:5′-FMN structure could had been earlier mistaken for FMN [39]. Taking into consideration that flavins are sensitive to light, alkaline or extremely acidic pH and phosphates enzymes, non-degradative extraction method developed by Russell and Vanderlice [48] and modified by Gliszczyńka and A. Koziołowa [35] was used. This method is a two-step extraction procedure using methanol, methylene chloride and ammonium acetate buffer, pH 6. This buffer was used because it does not cause any changes in composition of flavin extracts and it is good for their separation using HPLC methods. At pH 6, FAD has lower fluorescence intensity, thus its concentrations in assessed food products have to be determined using FAD standard curve prepared under the same chromatographic conditions as used for determination of flavins in food.

The HPLC methods described in this paper were developed in our laboratory. Some differences were found in the effectiveness of two reversed-phase HPLC columns used for analysis of flavin derivatives present in assessed products. Methods A and B applied on an Alphabond C_{18} column (mobile phase gradient of methanol–0.05 *M* ammonium acetate buffer, pH 6.0 and mobile phase gradient of methanol–demineralized water, respectively) have some disadvantages. Method A does not allow separation

of FAD and 7α-HRF, method B – FAD and FMN. However, method A can be used for determination of flavin compounds in such products as baker's yeast [35], raw egg white, raw egg yolk, egg powder and other products that do not contain 7α-HRF. In this method, FAD, FMN, 4′:5′-FMN, RF, 10-FMF and 10-HEF may be separated and quantified. Method B can be useful to confirm the presence of 7α-HRF and 10-HEF in analysed samples or to separate flavin coenzymes from other flavin compounds. Method C (mobile phase gradient of methanol–0.05 *M* ammonium acetate buffer, pH 6.0) applied on a Symmetry C_{18} column (Fig. 1, Table 1) is the best for separation of all flavins detected in the analysed products: FAD, FMN, 4′:5′-FMN, RF, 7α-HRF, 10-HEF, 10-FMF, RFgal, RFgluk.

TLC methods applied in this research were useful for confirmation of presence or additional identification of some flavin derivatives. The unknown flavin derivatives were separated from the other ones using

Table 1
HPLC retention times (t_r) of flavins in three elution methods

No.	Flavin	HPLC method (t_r in min[a])		
		A[b]	B[b]	C[c]
1.	FAD	6.51	4.50	5.83
2.	7α-HRF	6.51	8.80	4.23
3.	10-HEF	7.34	9.33	4.93
4.	4′:5′-FMN	8.35	–	8.82
5.	FMN	10.29	4.50	7.77
6.	RFgal	11.96	14.78	9.72
7.	RF	13.17	15.38	10.80
8.	10-FMF	15.40	16.55	13.09
9.	RFgluk	–	–	10.38

[a] Retention times are mean values obtained from few injections.
[b] HPLC separation was performed on a Alphabond C_{18} column using elution methods A with methanol–0.05 *M* ammonium acetate (pH 6.0) and B with methanol–demineralized water in appropriate gradients.
[c] HPLC separation was performed on a Symmetry C_{18} column using elution method C with methanol–0.05 *M* ammonium acetate (pH 6.0) in appropriate gradient.

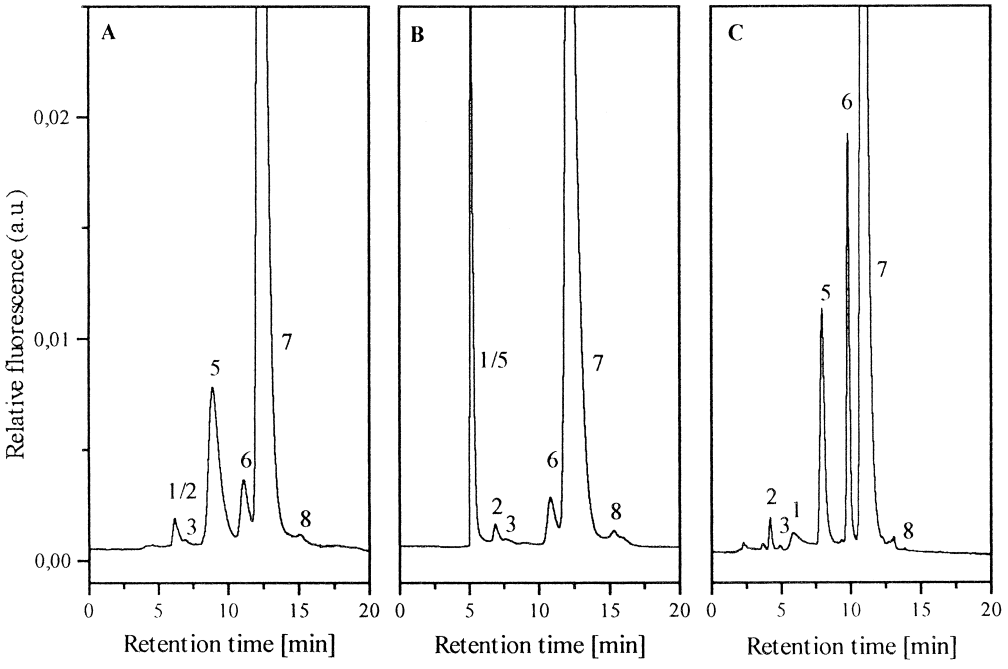

Fig. 1. HPLC chromatograms (methods A, B and C) of flavins extracted from plain yogurt: (1) FAD, (2) 7α-HRF, (3) 10-HEF, (5) FMN, (6) RFgal, (7) RF, (8) 10-FMF. The conditions were as follows: (A) mobile phase gradient of methanol–ammonium acetate, pH 6.0 on an Alphabond C_{18} column (300 mm×4.6 mm, 10 μm), (B) mobile phase gradient of methanol–demineralized water on an Alphabond C_{18} column, and (C) mobile phase gradient of methanol–ammonium acetate, pH 6.0 on a Symmetry C_{18} column (150 mm×3.9 mm, 5 μm); a Waters Model 474 scanning fluorescence detector with excitation and emission wavelengths of 450 nm and 530 nm, respectively. a.u.=Arbitrary units.

Table 2
R_f values of flavin standards and compounds 2 (7α-HRF) and 6 (RFgal) found in plain yogurt[a]

Flavin	R_f							
	I	IIa	III	IV	VIII	IX	X	XI
FAD	0.21	0.23	0	0	0.37	0	0	0
FMN	0.36	0.41	0	0.05	0.37	0.03	0.04	0.05
RFgal	0.56	–	0.14	0.10	–	0.06	0.21	0.21
7α-HRF	0.59	0.57	0.32	0.21	0.53	–	–	–
10-HEF	–	0.63	0.71	0.40	0.57	–	–	–
RF	0.68	0.64	0.55	0.32	0.64	0.38	0.50	0.52
10-FMF	0.80	–	0.86	0.76	–	–	–	–
Flavin 2	–	0.57	0.32	0.21	0.53	–	–	–
Flavin 6	0.56	–	0.14	0.10	–	0.06	0.21	0.21

[a] TLC on silica gel: (I) *n*-butanol–glacial acetic acid–water (2:1:1, v/v); (IIa) *n*-butanol–acetic acid–water (5:2:3, v/v); (III) chloroform–methanol–ethyl acetate (5:5:2, v/v), (IV) *n*-butanol–benzyl alcohol–glacial acetic acid (8:4:3, v/v); (VIII) *n*-butanol–ethanol–water (10:3:7, v/v), (IX) isoamyl alcohol–ethyl methyl ketone–glacial acetic acid–water (40:40:7:13, v/v); (X) *n*-butanol–isopropanol–water–glacial acetic acid (30:50:10:2, v/v); (XI) ethyl methyl ketone–acetic acid–methanol (3:1:1, v/v).

semipreparative HPLC and/or preparative TLC and then their retention times and/or R_F values were compared with that of synthetic flavins. Depending on the mobile phase, differentiation of RF and its coenzymes from the other flavin derivatives is possible (Tables 2 and 3).

3.2. Method validation

Reliability of methods was tested for recovery, linearity, precision and sensitivity. Both methods A and C, which allow separation at least RF and its nucleotides, were assessed. The methods proposed are fast and give satisfactory precision, specificity and accuracy but for quantitative determination of flavins method C was used because it allows simultaneous separation of all flavins found in food products.

The efficiency of the extraction method was determined based on the recoveries of FAD, FMN and RF from at least duplicate spiked samples. In all cases, the samples were spiked, at the beginning of an extraction procedure, with known concentration of flavin standards to approximate their levels in the food samples being analyzed. Spiked and unspiked samples were treated in the same way throughout the whole procedure. The results show (Table 4) that the recoveries of added RF and its nucleotides were no lesser than 95% in all analyzed samples.

Table 3
R_f and t_r values of flavin standards and flavin 4 (4′:5′-FMN) isolated from raw egg white and egg powder

Flavin standard	R_f[a]								HPLC[b]: t_r (min)
	I	IIa	IIb	III	IV	V	VI	VII	
FAD	0.14	–	0.16	0	0	–	0.62	0	5.27
FMN	0.30	–	0.25	0	0.03	0.13	0.53	0.06	7.05
4′:5′-FMN	0.46	0.41	0.32	0.23	0.11	0.45	0.57	0.08	8.22
RF	0.63	0.53	0.44	0.59	0.30	–	0.41	0.32	10.74
Flavin 4 from:									
Raw egg white	0.46	0.41	0.32	0.23	0.11	–	0.57	–	8.18
Egg powder	0.46	–	–	0.23	–	0.45	0.57	0.08	8.20

[a] TLC: (I) *n*-butanol–glacial acetic acid–water (2:1:1, v/v), silica gel; (IIa) and (IIb) *n*-butanol–acetic acid–water (5:2:3, v/v), silica gel and cellulose, respectively; (III) chloroform–methanol–ethyl acetate (5:5:2, v/v), (IV) *n*-butanol–benzyl alcohol–glacial acetic acid (8:4:3, v/v); (V) collidine–water (3:1, v/v), cellulose, (VI) 5% $Na_2HPO_4 \cdot 12H_2O$, silica gel, (VII) *n*-butanol–formic acid–water–diethyl ether (77:10:13:15, v/v), silica gel.

[b] HPLC: A Symmetry C_{18} column, mobile phase gradient of methanol–0.05 *M* ammonium acetate, pH 6.0.

Table 4
Recoveries of RF, FMN and FAD from spiked food samples

Product	Recovery (%)[a]		
	FAD	FMN	RF
Raw egg white	95.1	98.2	95.0
Raw egg yolk	–	–	95.9
Egg powder	98.3	98.5	98.0
Yogurt	96.4	97.2	95.5
Milk	97.0	97.2	96.2
Kefir	96.6	97.0	95.0

[a] Data obtained by elution method A.

The calibration curves were prepared with the standard solutions of FAD, FMN and RF at levels similar to those present in the assessed products but all curves were linear at least to 15 μg/ml for nucleotides and 20 μg/ml for RF. The repeatability of the standard curves (at least 3) was tested over several days and found to be good. The relative standard deviations (RSDs) for the slope of FAD standard curve obtained using methods A and C were 0.96% and 0.90%, respectively; for the FMN standard curve 1.71% and 1.66%, respectively and for RF standard curve 0.53% and 0.50%, respectively. A linear correlation coefficient (r) for the FAD standard curve was 0.9999 (r^2=99.98%) in elution method A and 0.9996 (r^2=99.92%) in elution method C. For FMN standard curve, an r value was 0.9987 (r^2=99.76%) and 0.9992 (r^2=99.86%) in elution methods A and C, respectively. For RF standard curve, an r value 0.9999 (r^2=99.98%) was obtained for both methods.

To study the precision of the methods, at least three to six determinations of flavins in the same sample were performed on the same day using the same reagents and apparatus. Standard deviations (SDs) and RSDs for total flavin content in tested food products were very similar in both elution methods, hence only data obtained in the method used for quantitative analysis (method C) are summarized in Table 5.

Table 5
Precision data for total flavin content in assessed food products

Product	Total flavin content (μg/100 g)	SD (μg/100 g)	RSD (%)
Raw egg white	326.6	4.0	1.22
Raw egg yolk	295.4	5.6	1.90
Egg powder	1273.7	20.7	1.63
Milk	163.1	4.6	2.82
Yogurt	205.9	3.5	1.70
Kefir	189.1	5.2	2.75
Sour milk	156.0	3.9	2.50
Buttermilk	158.1	4.3	2.72
Acidophilus milk	167.8	4.6	2.76

The detection limits of authentic samples under the working conditions proposed (emission slit width 10 nm, fluorometer gain 100, attenuation 1) were about 1 ng/ml for RF and FMN and about 6 ng/ml for FAD.

3.3. Flavin composition of food products

Typical flavins of milk and fermented milk products like plain yogurt, kefir, sour milk, acidophilus milk, buttermilk are: RF, FAD, FMN, 7α-HRF, 10-HEF and 10-FMF. Similar to the earlier studies [36], in the samples that were analysed we did not detect 8α-HRF, which has been found by Roughead and McCormick in cows' milk [8]. They reported that only trace quantities of this analogue were available, hence its identification was rather tentative. Plain yogurt and sour milk contain also another derivative identified as riboflavin-β-D-galactoside (RFgal, flavin No. 6 in Fig. 1) [36]. This flavin in plain yogurt is a product of the action of specific yogurt microorganisms: *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. It has been shown, by incubation of milk with these cultures and analysis of flavin extract obtained from ''produced'' yogurt, that enzymes of both of these microorganisms form this derivative during fermentation of milk (Fig. 2a). The identity of formed compound was confirmed by comparison of its t_R and R_F values (Table 2) with those of standard and additionally, by the tests described previously [36]. Treatment with hydrochloric acid, sodium hydroxide and sodium periodate yielded RF, LF and 10-FMF, respectively. It was susceptible to cleavage by β-galactosidase with formation of riboflavin and galactose. Glycosides of riboflavin may be formed by incubation of riboflavin and appropriate sugar donor with cultures (see Fig. 2b) or enzymes from different microorganisms [38,55–61] but up to now it was not reported that RFgal is present in some plant or animal tissues or in food. The only known glycosidic

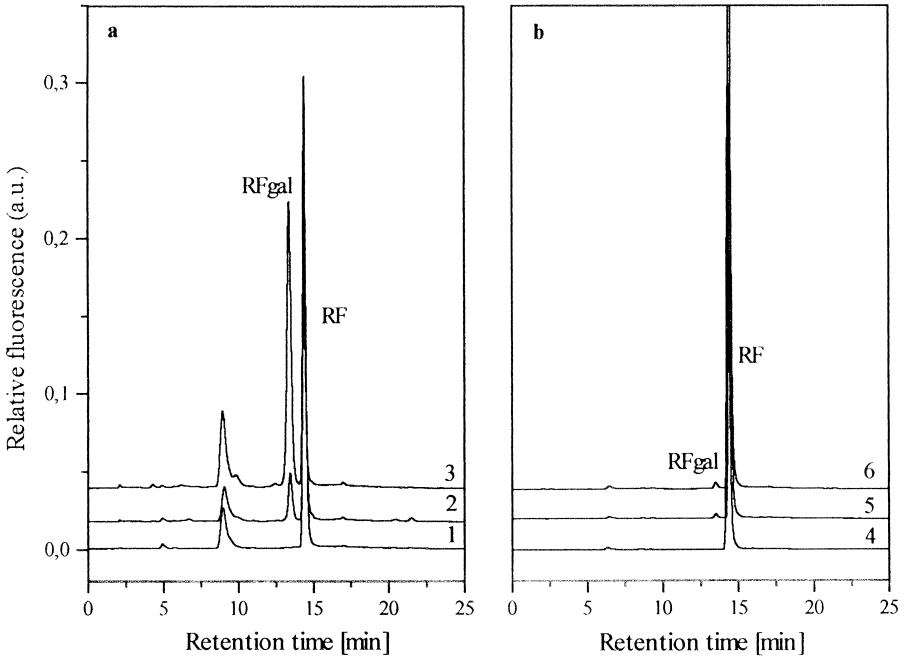

Fig. 2. HPLC chromatograms of (a) pasteurised milk incubated with yogurt bacteria and (b) mixture of riboflavin and lactose incubated with yogurt bacteria: (1) flavins of pasteurised milk, (2) flavins of pasteurised milk incubated with *Lactobacillus delbrueckii* subsp. *bulgaricus*, (3) flavins of pasteurised milk incubated with *Streptococcus salivarius* subsp. *thermophilus*, (4) mixture of riboflavin, lactose and yogurt bacteria at the beginning of incubation, (5) mixture of riboflavin, lactose and *Lactobacillus delbrueckii* subsp. *bulgaricus* after 48 h of incubation, (6) flavins of mixture of riboflavin, lactose and *Streptococcus salivarius* subsp. *thermophilus* after 48 h of incubation. The conditions were as follows: a mobile phase gradient C of methanol–0.05 *M* ammonium acetate (pH 6.0) on a Symmetry C_{18} column (150 mm×3.9 mm, 5 μm); a Waters Model 474 scanning fluorescence detector with excitation and emission wavelengths of 450 nm and 530 nm, respectively.

derivative of riboflavin occurring naturally was riboflavin-α-D-glucoside [14,21,22] first obtained by Whitby [62–64] by incubation of a rat liver enzyme with riboflavin.

On the basis of retention times of standards and flavins found in egg and egg powder (Fig. 3), it was found that RF and 10-FMF are the only flavins present in raw egg yolk, but raw egg white and egg powder contain also FAD, FMN and 4′:5′-FMN (traces of FAD and FMN found in egg yolk were rather coming from the residue of egg white remaining in yolk when they are separated). Occurrence of 4′:5′-FMN has not been reported in nature, yet. To ensure that 4′:5′-FMN is one of the egg flavin, this compound was isolated on semipreparative HPLC and/or preparative TLC and its retention times t_R and R_F values were compared with those of synthetic flavin applied as internal and external standard (Table 3). Additionally, the tests for identification of this derivative, described previously [35], were performed. This compound could not be hydrolysed by alkaline phosphatase. Treatment with hydrochloric acid yielded mainly FMN. It was susceptible to cleavage by sodium periodate with formation of 10-FMF; its electrophoretic mobility was smaller as compared to the FMN and FAD. These data and agreement of retention times in HPLC and R_F values on silica gel or cellulose with those of synthetic flavin used as internal and external standard suggested that the compound sign in Fig. 3 as 4 is 4′:5′-FMN. Studying flavin derivatives in baker's yeast we have found that degradation of FAD, especially under basic conditions is going through 4′:5′-FMN [35]. PH value of fresh egg white is about 7.9 and during few days after laying egg rapidly increases up to 9.2–9.3, maximally to 9.7. It

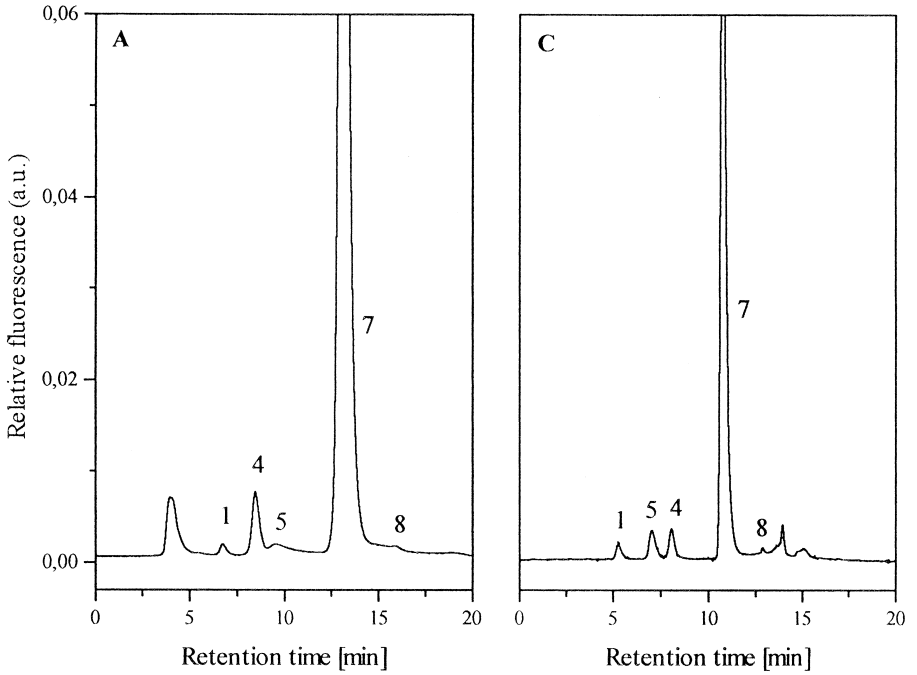

Fig. 3. HPLC chromatograms of flavins extracted from raw egg white: (1) FAD, (4) 4′:5′-FMN, (5) FMN, (7) RF, (8) 10-FMF. The conditions were as follows: mobile phase gradient of methanol–ammonium acetate, pH 6.0 on (A) an Alphabond C_{18} column (300 mm×4.6 mm, 10 μm), and (C) on a Symmetry C_{18} column (150 mm×3.9 mm, 5 μm); a Waters Model 474 scanning fluorescence detector with excitation and emission wavelengths of 450 nm and 530 nm, respectively.

can explain occurrence of this flavin in egg white and egg powder.

Flavin composition of meat products rich of vitamin B_2 such as liver or kidney are also being investigated in our laboratory. FAD, FMN and RF are the only vitamin B_2 forms found in chicken liver but pig and calf livers contain additionally RFgluk, and pig liver at least two other flavin derivatives (the third one indicated on chromatogram as Z (t_R=12.37 min) was found only in some analysed samples) (Fig. 4). Their absorption maxima (in mixture of methanol–ammonium acetate pH 6.0) are about 220, 267, 361 and 462 nm. The derivative Y (t_R=11.43 min) shows fluorescence maximum at about 553 nm, the derivative X (t_R=8.90 min) – 560 nm and riboflavin – 540 nm (Fig. 5). They display low fluorescence intensity, how it can be seen from comparison of HPLC chromatograms obtained by means of photodiode-array and fluorescence detectors (Fig. 4). These are riboflavin analogues; there is no doubt that they are not phosphate, peptide or sugar derivatives of riboflavin but their structures are still unknown. RFgluk has been already found in rat urine [14], liver [22] and in cat liver [21]. The enzyme responsible for the formation of this derivative has also been found in some plant grains [65] and in pig liver [66], what indicates possibility of formation of this flavin in livers. No clear physiological function or role in animal and microorganisms metabolism has been attributed to glycosidic derivatives of riboflavin. It has been speculated that formation of glycosides may be a way by which the organism stores vitamin in a stabilised form [33,67]. Because the flavin composition of animal livers is not yet completely recognised, quantitative analysis of these products was not performed.

We did not find derivatives with alloxazinic structure in any of the analysed samples.

3.4. Quantification of flavins

The mean content of individual flavins and total

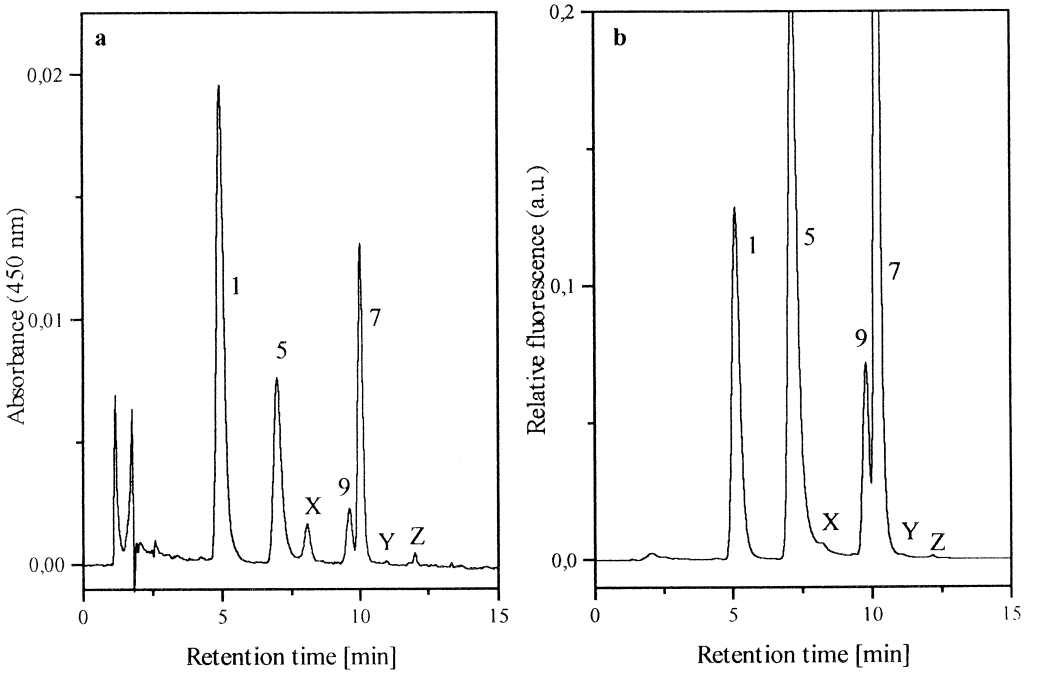

Fig. 4. HPLC chromatograms of flavins extracted from pig liver: (1) FAD, (5) FMN, (7) RF, (9) RFgluk, (X), (Y), (Z) unknown flavins. The conditions were as follows: a Symmetry C_{18} column (150 mm×3.9 mm, 5 μm); (a) a Waters Model 996 photodiode-array detector at 450 nm, and (b) a Waters Model 474 scanning fluorescence detector with excitation and emission wavelengths of 450 nm and 530 nm, respectively. The eluent for method C was a mobile phase gradient of methanol–0.05 *M* ammonium acetate (pH 6.0).

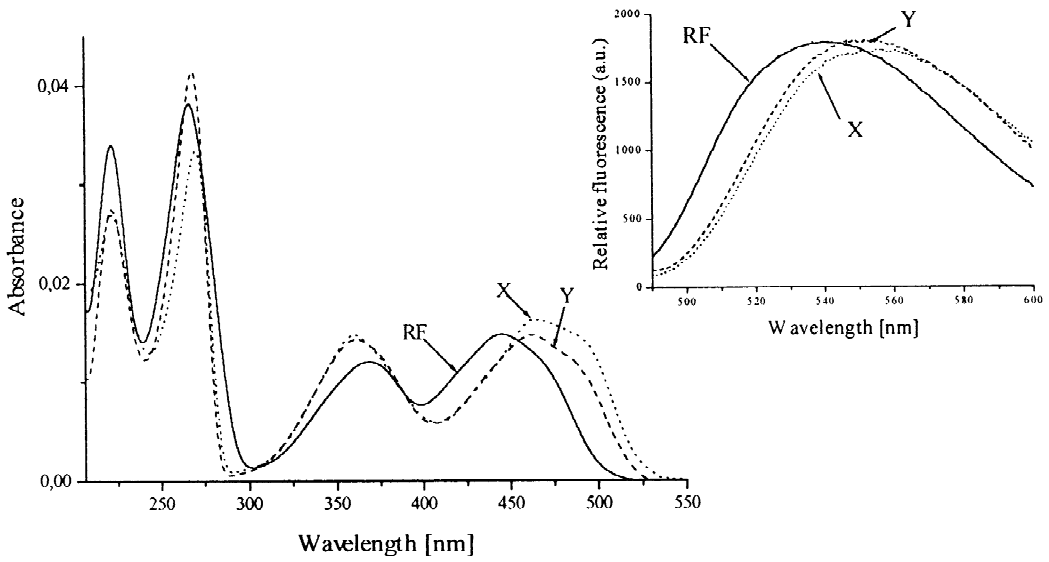

Fig. 5. Absorption and fluorescence emission spectra of riboflavin and unknown flavins extracted from pig liver.

Table 6
Distribution of flavins in assessed food products (in μg/100 g±SD)

Sample	RF	FAD	FMN	4′:5′-FMN	RFgal	7α-HRF	10-HEF	10-FMF	Total
Raw egg white	310.2±15.0 87.6%	12.5±7.6 3.5%	7.2±1.3 2.0%	13.8±2.2 3.9%	–	–	–	10.3±2.4 2.9%	342.4±21.6 100%
Raw egg yolk	352.3±68.9 98.2	–	–	–	–	–	–	6.3±1.8 1.8%	358.6±66.2 100%
Egg powder	1154.5±23.8 89.2	111.0±30.8 8.6%	Traces <0.05%	28.3±3.3 2.1%	–	–	–	Traces	1293.9±28.2 100%
Milk 2% fat	130.1±0.5 74.3%	31.1±1.0 17.8%	11.0±0.2 6.3%	–	–	2.5±0.6 1.4%	Traces	0.3±0.06 0.2%	174.7±0.8 100%
Milk 4.6% fat	127.6±0.8 78.2%	21.9±0.6 13.4%	11.2±0.2 6.9%	–	–	2.3±0.1 1.4%	0.2±0.05 0.1%	Traces	163.5±0.5 100%
Yogurt 1[a]	148.5±6.9 69.4%	16.0±1.8 7.5%	29.6±0.8 13.8%	–	14.2±0.4 6.6%	4.3±0.2 2.0%	Traces	0.5±0.1 0.2%	214.0±7.0 100%
Yogurt 2[a]	186.8±2.8 85.4%	2.8±0.6 1.3%	20.6±2.4 9.4%	–	5.3±0.1 2.4%	3.1±0.4 1.4%	Traces	Traces	218.8±3.5 100%
Bioyogurt 3[a]	160.3±3.4 78.6%	2.6±0.1 1.3%	32.3±3.9 15.8%	–	4.2±0.2 2.1%	3.5±0.9 1.7%	0.3±0.1 0.2%	0.2±0.0 0.1%	204.0±7.5 100%
Bioyogurt 4[a]	127.4±2.1 70.3%	37.6±4.9 20.7%	13.1±0.6 7.2%	–	0.5±0.3 0.3%	2.1±0.2 1.2%	Traces	0.6±0.1 0.3%	181.3±3.8 100%
Bioyogurt 5[a]	98.7±1.6 65.7%	18.3±1.3 12.4%	27.2±2.2 18.5%	–	2.0±0.8 1.4%	3.9±0.9 2.6%	Traces	Traces	150.0±2.1 100%
Kefir	142±1.4 72.2%	43.2±2.7 22.0%	8.3±0.1 4.2%	–	–	2.9±0.1 1.5%	Traces	0.2±0.03 0.1%	193.4±6.2 100%
Sour milk	110.6±1.7 70.1	34.3±2.3 21.7%	8.5±0.4 5.4%	–	1.9±1.1 1.2%	2.4±0.3 1.5%	Traces	Traces	158.2±5.3 100%
Buttermilk	117.8±3.1 77.2%	20.5±2.7 13.4%	11.1±0.5 7.3%	–	–	2.7±0.1 1.8%	Traces	0.3±0.04 0.2%	154.5±5.1 100%
Acidophilus milk	119.8±6.6 75.3%	30.4±2.2 19.1%	5.4±1.0 3.4%	–	–	3.1±0.2 1.9%	0.2±0.05 0.1%	0.2±0.05 0.1%	160.2±9.6 100%

[a] Reported in Ref. [36].

flavin content in assessed food products are reported in Table 6. All values are arithmetic mean of at least three measurements for three samples. In general, the contents of riboflavin in the samples analysed were in agreement with that reported in literature [3,68–70], although literature data for raw egg white and yolk are quite divergent. The content of vitamin B_2 in egg white may oscillate between 250 and 450 μg/100 g (in average 320 μg/100 g) and in egg yolk between 240 and 540 μg/100 g (in average 400 μg/100 g) [70]. According to Russel and Vanderslice [48] raw egg yolk contains even 690 μg of vitamin B_2/100 g. The data concerning the raw egg white and egg yolk (Table 6) are mean values of results obtained for eggs coming from different chicken farms. Total riboflavin content in raw egg white and yolk from the first chicken farm was 338.2±5.4 μg/100 g and 417.1±29.8 μg/100 g, respectively; for eggs from the second one (much bigger then the first one): 349.7±19.4 μg/100 g of egg white and 293.4±15.1 μg/100 g of egg yolk. There was no significant difference between total flavin content in raw egg white coming from different chicken farms, but total flavin content in egg yolk coming from small chicken farms was almost 1.5-times higher than in egg yolk coming from the large farm (417.1 and 293.4 μg/100 g, respectively). It indicates that the method of chicken nutrition has a higher influence on vitamin B_2 content in egg yolk than in egg white.

Observed flavin amounts in plain yogurt samples are variable probably because milk companies apply different types of raw materials, strains of microorganisms and technological parameters [36]. Total flavin content in plain yogurts and kefir is higher than in milk samples (with one exception, bioyogurt 5). Sour milk, buttermilk and acidophilus milk have total flavin content on the same level, similar to that of milk.

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