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Chromatographic determination of flavin derivatives in baker's yeast

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Abstract

The presence of flavin derivatives in baker's yeast was tested by high-performance liquid chromatography and thin-layer chromatography. In yeast samples, besides flavin adenine dinucleotide and flavin mononucleotide, small amounts of riboflavin and traces of 10-formylmethylflavin have been found. Total amount of flavins was calculated to be 17.9±2.9 μg/g of fresh yeast. The distribution of flavin adenine dinucleotide, flavin mononucleotide, riboflavin and 10-formylmethylflavin in total flavin content were estimated to be 71.5%, 25.8%, 1.7% and below 0.05%, respectively. In some samples we have additionally detected small amounts (0.8% of total flavins) of new flavin derivative which has been identified as 4′,5′-riboflavin cyclic phosphate by means of its chromatographic and chemical behaviour. This compound seems to be a product of flavin adenine dinucleotide degradation and probably has been earlier mistaken for flavin mononucleotide. Its formation is dependent on pH conditions.

Keywords: Yeast; Flavin nucleotides; Riboflavin; Formylmethylflavin; Vitamins

1. Introduction

Vitamin B_2, in principle, occurs naturally as riboflavin (RF), riboflavin 5′-phosphate (flavin mononucleotide, 5′-FMN) and flavin adenine dinucleotide (FAD), although there are also other flavin derivatives present in nature. 10-Hydroxyethylflavin (10-HEF), 10-formylmethylflavin (10-FMF), 7α-hydroxyriboflavin (7-hydroxymethylriboflavin, 7α-HRF, nekoflavin in cat's eyes [1]) and 8α-hydroxyriboflavin (8-hydroxymethylriboflavin, 8α-HRF) were found in cow's and human milk [2,3]. The last two derivatives were also detected in rat organs [4] and human urine [5,6]. 8α-Hydroxy-FMN was detected in rat kidney and its traces in other rat organs like brain, heart and liver [4]. A significant amount of riboflavin-α-glucoside has been found in rat urine [7], liver [4] and in cat liver [8]. The enzyme responsible for the formation of the last compound has been also found in pig liver [9]. Riboflavin-α-glucoside can be also a microbial product of the intestinal flora [8]. Among derivatives having an alloxazine structure, lumichrome (Lch) [2,3] and 7- and 8-carboxylumichrome [10] were found in body liquids.

These flavin analogues may 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 RF to 5′-FMN [11]. Presence of some flavin derivatives can be also an indicator of light-caused degradation of RF during food storage.

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Quantitative assessments of vitamin B_2 in food, however, mostly refer to the determination of total RF content or only RF. More recently [2–4,12], high-performance liquid chromatography (HPLC) methods with fluorescence detection have been used to qualify and quantify RF and all its derivatives in natural sources. These methods, supported by thin-layer chromatography (TLC) give a lot of possibilities for small-scale isolation and determination of all flavin compounds, even those present in very small quantities. We think that natural sources, like food, might contain not only common forms of RF like FAD and 5′-FMN but also its other derivatives.

In the present paper separation, identification and quantification of flavin derivatives found in baker's yeast 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 5′-FMN and FAD concentrations were corrected for the impurities. Purity of commercial FAD and 5′-FMN was 97.6% and 67%, respectively. 10-FMF was prepared by periodate oxidation of RF according to the method of Fall and Petering [13], 4′:5′-FMN was synthesised according to the methods of Yagi and Okuda [14] and Huennekens and Kilgour [15]. Sodium periodate (99.8%) was purchased from Aldrich (Steinheim, Germany), alkaline phosphatase from Sigma (St. Louis, MO, USA). Baker's yeasts were bought at random in grocery stores.

2.2. Extraction method

Determination of FAD and FMN requires an extraction method, which does not cause the hydrolysis of these flavins, therefore treatment with acid and alkali was avoided. 5′-FMN is stable in aqueous solution at pH=7.0 [16], FAD is relatively stable in distilled water and does not change below 60°C. Because of it flavins were extracted by modified for our purpose procedure described by Russell and Vanderslice [12]. The samples (12 g) were placed in extraction tubes, suspended in 19 ml of methanol–methylene chloride (9:10, v/v) and shaked for 60 s using a shaker. After addition of 9 ml of 0.1 *M* ammonium hydrogencarbonate of pH=7.0, the mixture was additionally shaked for 60 s and then centrifuged for 20 min at 27 000 *g* and 4°C. The decanted upper phase was filtered through 0.45 μm filter (Sartorius, Göttingen, Germany) and immediately injected into the HPLC. All samples and standard solutions were protected against light during the whole procedure.

2.3. HPLC separation of flavins

All HPLC analysis were performed on a Waters 600 E high-performance liquid chromatograph (Waters, Milford, MA, USA) equipped with analytical Alphabond C_{18} column (300×4.6 mm, Alltech, Carnforth, UK) fitted with a Waters Guard-Pak C_{18} precolumn insert. For separation and identification of flavins three elution methods developed in our laboratory were used:

(A) methanol–0.1 *M* ammonium hydrogencarbonate, pH=7.0, in the gradient methanol–ammonium hydrogencarbonate (30:70, v/v) at 0 min and linear gradient to 80:20 (v/v) within 20 min at the flow-rate 0.6 ml min/min;

(B) methanol–0.05 *M* ammonium acetate buffer, pH=6.0, in the gradient: isocratic at methanol–buffer (30:70, v/v) within 1 min and linear gradient to 70:30 (v/v) within 10 min at the flow-rate 1 ml min/min;

(C) methanol–demineralized water in the gradient: isocratic at methanol–water (30:70, v/v) within 1 min, linear gradient to 80:20 (v/v) to 4 min, isocratic at 80:20 (v/v) to 10 min, at the flow-rate 0.8 ml min/min.

Semipreparative HPLC of unknown flavin was performed on a αBondapak C_{18} column fitted with a αBondapak C_{18} Guard-Pak insert (Waters, Milford, MA, USA) using method A. The pooled fractions were concentrated and desalted using the same conditions but with water instead of buffer.

A Waters 474 scanning fluorescence detector was used at excitation/emission wavelengths of 450/530 nm for isoalloxazine derivatives and 380/430 nm for

alloxazine derivatives with a slit width of 10 nm. Additionally the Waters 991 photodiode array detector was used to differentiate flavins from other compounds on the basis of their absorption spectrum.

For quantification of flavins method A was used. The concentration of FAD, 5′-FMN and RF was determined from its corresponding standard curve obtained under the same chromatographic conditions (external standard method) because at pH=7.0 the fluorescence intensity of FAD is 15% of that of RF and 5′-FMN [17].

Fluorescence spectra were measured using a MPF-44 A/E spectrofluorometer (Perkin–Elmer, Norwalk, CT, USA).

2.4. Thin-layer chromatography (TLC)

To remove all non-flavin compounds, for analytical and 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 [18].

TLC was performed on silica gel plates (Kieselgel 60, 0.2 mm, Merck) and cellulose plates (cellulose F_{254}, 0.1 mm, Merck); for preparative TLC, 2 mm silica gel plates were used. Solvent systems used for chromatography were as follows:

(I) *n*-butanol–glacial acetic acid–water (2:1:1, v/v), on silica gel;

(IIa) and (IIb) *n*-butanol–acetic acid–water (5:2:3, v/v), on silica gel and cellulose, respectively;

(III) chloroform–methanol–ethyl acetate (5:5:2, v/v), on silica gel;

(IV) *n*-butanol–benzyl alcohol–glacial acetic acid (8:4:3, v/v), on silica gel;

(V) collidine–water (3:1, v/v), on cellulose;

(VI) 5% $Na_2HPO_4 \cdot 12H_2O$, on silica gel;

(VII) *n*-butanol–formic acid–water–dimethyl ether (77:10:13:15, v/v), on silica gel;

(VIII) *n*-butanol–ethanol–water (10:3:7, v/v), on silica gel.

Electrophoresis was run on Whatman No. 1 paper (23 cm in direction of migration) in 0.05 *M* phosphate buffer pH=8.0 at 300 V, 15 mA.

Identification methods of unknown flavin (4′:5′-FMN):

(i) For acid hydrolysis, equal volumes of unknown flavin and 1 *M* HCl were mixed, heated at 100°C during 1 h and analysed on analytical HPLC column.

(ii) For alkaline hydrolysis, equal volumes of unknown flavin and 1 *M* NaOH were mixed and after 4 h at room temperature analysed on analytical HPLC column.

(iii) Periodate oxidation was performed by adding double the volume of 0.05 *M* sodium periodate to the unknown flavin or flavin standard. The resulting solution was incubated in the dark for 4 h at room temperature and oxidation products were analysed using TLC and HPLC methods.

(iv) For enzymatic hydrolysis of samples with phosphatase, a reaction mixture containing equal volumes (0.1 ml) of an aqueous solution of the sample, 0.2 *M* buffer pH=9.8 and phosphatase (1.8 mg solid dissolved in 1 ml of distilled water) was incubated at 37°C for 4 h in the dark.

3. Results and discussion

3.1. Identification of flavins

Three elution methods were developed to identify and/or confirm the presence of flavin derivatives in baker's yeast. Proposed methods A (mobile phase gradient of methanol–0.1 *M* ammonium hydrogencarbonate pH=7.0) and B (mobile phase gradient of methanol–0.05 *M* ammonium acetate pH=6.0) allow complete resolution of FAD, 5′-FMN, RF, 10-FMF and unknown flavin. They may be used alternatively for separation of these flavin analogues. In method A, 10-HEF and 7α-HRF have retention times similar to FAD and 5′-FMN and in the case of samples rich with these coenzymes might be overlapped by them. To be sure that 10-HEF and 7α-HRF are present or not in yeast samples, elution method C (mobile phase gradient of methanol–demineralized water) was developed. This method, however, cannot be used for the separation of FAD and 5′-FMN (Table 1 and Table 3).

In Fig. 1 the HPLC elution profile of flavins in baker's yeast obtained from 50 μl injections is shown. Based on HPLC retention times and mobility on TLC plates (Table 1), compounds 1, 2, 3 and 4 were identified as FAD, 5′-FMN, RF and 10-FMF, respectively. The latter one was not found in all

Table 1
TLC values of R_F in methods I, IIa, VI and HPLC retention times (t_R) in A and C elution methods of flavins from baker's yeast and flavin standards

Flavin	TLC[a], R_F			HPLC[b], t_R (min):	
	I	IIa	VI	A	C
	0.19	0.21	0.37	11.08	4.47
Flavins	0.35	0.37	0.51	12.98	15.37
of baker's	0.52[c]	0.47[c]	0.54[c]	13.60	16.52[c]
yeast	0.67	0.58	0.61	21.35	
				25.86[c]	
Standards:					
FAD	0.19	0.21	0.61	11.08	4.52
5′-FMN	0.35	0.37	0.51	13.05	4.50
RF	0.67	0.58	0.37	21.45	15.38
10-FMF	0.76	–	–	25.93	16.55
10-HEF	0.72	–	–	11.85	9.33
LF	0.70	–	–	26.32	–
Lch	0.86	–	–	28.28	17.58
7α-HRF	0.54	–	–	11.15	8.80
8α-HRF	0.60	–		28.08	–

[a] TLC was performed on silica gel and solvents were: (I) *n*-butanol–glacial acetic acid–water (2:1:1, v/v); (IIa) *n*-butanol–acetic acid–water (5:2:3, v/v); (VI) 5% $Na_2HPO_4 \cdot 12\ H_2O$.
[b] HPLC separation was performed on Alphabond C_{18} column using fluorescence detector and elution methods: (A) methanol–0.1 *M* ammonium hydrogencarbonate pH=7.0 and (C) methanol–demineralized water in appropriate gradients described in Section 2.
[c] Traces.

samples. The identity of the appropriate flavins was additionally confirmed by spiking and co-elution the flavins in samples with standard flavins.

10-FMF is a photodecomposition product of riboflavin and it has got potential antivitamin character [11]. Small amounts of this derivative is of little nutritional importance but when present in high concentrations it should be taken into consideration in quantification of riboflavin value of food. It can be also an indicator of photochemical degradation of riboflavin during food storage.

Compound X has an isoalloxazine structure (Fig. 1) with a fluorescence excitation maxima at wavelengths of 372 nm and 445 nm, and emission maximum at 530 nm in water. Its chromatographic behaviour on TLC plates and HPLC column (Table 1) is different from standard compounds used. It was not present in each sample tested. We have noticed that this compound is a degradation product of FAD but we did not find any dependence between the source and amount of baker's yeast taken for extraction and appearance or final concentration of this unknown flavin. To confirm that degradation of FAD leads to this analogue we have partially hydrolysed commercial FAD in aqueous solution and separated unknown flavin from hydrolysed FAD and from the yeast sample using preparative TLC plates (solvent I)

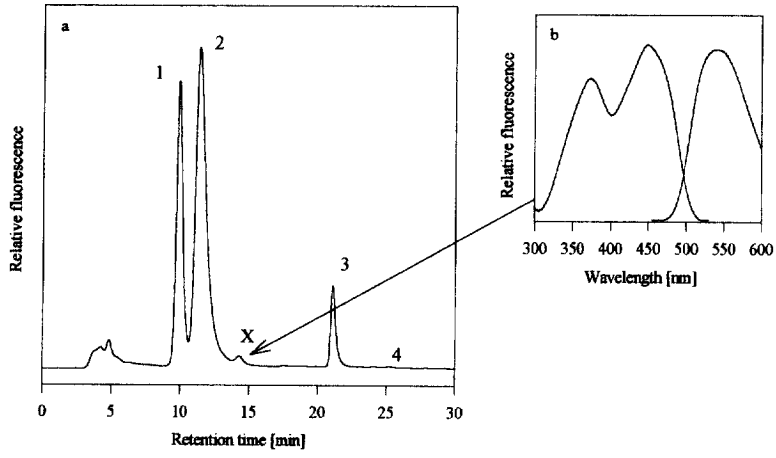

Fig. 1. (a) HPLC chromatogram of flavins extracted from baker's yeast. Mobile phase gradient of methanol–0.1 *M* ammonium hydrogencarbonate pH=7.0 (method A) on Alphabond C_{18} analytical column, fluorescence detection (λ_{ex}=450 nm, λ_{em}=530 nm); (1) FAD, (2) 5′-FMN, (3) RF, (4) 10-FMF, (X) unknown flavin. (b) Fluorescence excitation and emission spectra of unknown flavin X.

and a semipreparative HPLC column. The structure of both compounds, obtained from FAD and yeast, were established using a number of different tests (Table 2). Photolysis in aqueous and alkaline (1 *M* NaOH) solutions led to RF and lumiflavin (LF), respectively, which indicates that the unknown flavin is probably an ester of RF. To exclude the structure of mono- or diphosphoric acid ester of RF we have performed an acid hydrolysis in 1 *M* HCl and got 5′-FMN as a main product. Incubation with alkaline phosphatase did not change synthetic and natural unknown flavin which excludes the structure of mono- and diphosphate. Both were oxidised with sodium periodate to give 10-FMF. These results indicate that cleavage of the ribityl side chain of X takes place between C_2' and C_3' which excludes a structure of riboflavin 2′,5′-cyclic phosphate which was found in *Rhizopus oryzae* [19]. The pH–fluorescence curve of unknown flavin X is similar to RF and 5′-FMN (Fig. 2). The electrophoretic mobility is lower (41 mm from the start) in comparison to FAD (48 mm), 5′-FMN (61 mm) and higher than RF (23 mm). This data suggests that the unknown flavin is 4′:5′-FMN, which was further confirmed with a synthetic standard (Table 3). The R_F values of standards and electrophoretic mobility correspond to those reported by Nielsen et al. [20] and Yagi and Matsuoka [21,22], respectively.

Table 2
TLC values of R_F in methods IIa, IV, VI and HPLC retention times (t_R) in A elution method of unknown flavin X and standards after various treatments

Treatment	Compound	TLC[a], R_F			HPLC[b], t_R (min):
		IIa	IV	VI	A
Flavins	X from yeast	0.47	0.11	0.56	13.98
	X from FAD	0.47	0.11	0.56	14.02
	5′-FMN	0.38	0.03	0.52	12.88
	RF	0.57	0.28	0.38	21.65
	10-FMF	0.78	0.74	0.27	25.30
	LF	0.64	0.41	0.15	–
	Lch	0.84	0.85	–	28.62
Photolysis in aqueous solution	Product of photolysis:				
	X from yeast	0.57	0.28	0.38	21.70
	X from FAD	0.57	0.28	0.38	21.68
Photolysis in alkaline solution	Product of photolysis:				
	X from yeast	0.64	0.41	0.15	–
	X from FAD	0.64	0.41	0.15	–
	RF	0.64	0.41	0.15	–
Hydrolysis in 1 *M* HCl	Product of hydrolysis:				
	X from yeast	0.38	–	0.52	12.83
		0.57[c]	–	0.38[c]	21.61
	X from FAD	0.38	–	0.52	12.86
		0.57	–	0.38[c]	21.58
Oxidation with sodium periodate	Product of oxidation:				
	X from yeast	0.78	0.74	0.27	25.35
	X from FAD	0.78	0.74	0.27	25.25
	RF	0.78	0.74	0.27	25.36
Hydrolysis with phosphatase	Product of hydrolysis:				
	X from yeast	0.47	0.11	–	13.89
	X from FAD	0.47	0.11	–	14.03
	5′-FMN	0.57	0.28	–	21.57

[a] TLC was performed on silica gel and solvents were: (IIa) *n*-butanol–acetic acid–water (5:2:3, v/v); (IV) *n*-butanol–benzyl alcohol–glacial acetic acid (8:4:3, v/v); (VI) 5% $Na_2HPO_4 \cdot 12H_2O$.

[b] HPLC analysis was performed on Alphabond C_{18} column using fluorescence detector and elution method (A) methanol–0.1 *M* ammonium hydrogencarbonate pH=7.0 in appropriate gradient described in Section 2.

[c] Traces.

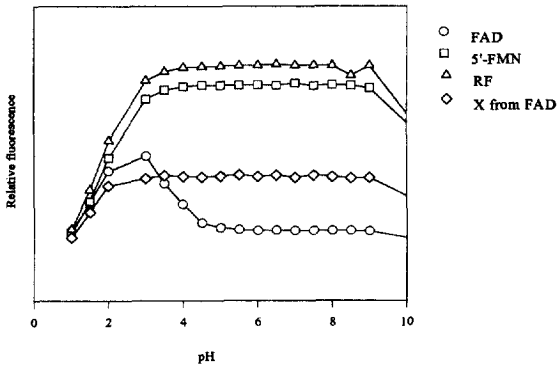

Fig. 2. Fluorescence versus pH profile for X from FAD and flavin standards (λ_{ex}=450 nm, λ_{em}=530 nm, 0.15 *M* phosphate buffer).

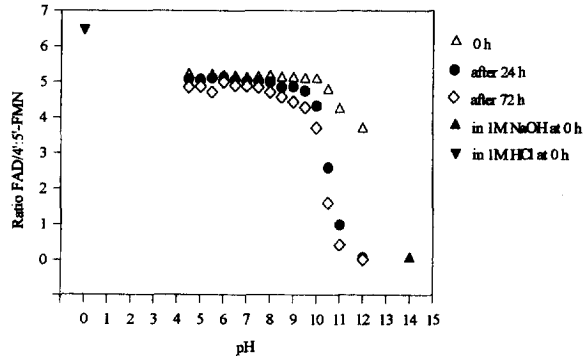

Fig. 3. Effect of pH and time on formation of 4':5'-FMN from FAD (0.15 *M* phosphate buffer, 1 *M* NaOH, 1 *M* HCl).

4':5'-FMN was obtained by Forrest and Todd [23] by treatment of RF with phosphoryl chloride in pyridine and treatment of FAD with concentrated ammonia. This compound was also produced by heating of FAD in aqueous solution [13]. The appearance of 4':5'-FMN in reaction of 5'-FMN with several carbodiimides [14] and with trifluoroacetic anhydride followed by treatment with ammonia [24] was also published. Carbodiimides have been shown to promote cyclization of 5'-FMN, so 4':5'-FMN may also appear as an impurity in the chemical synthesis of FAD using 5'-FMN, AMP and carbodiimides.

Our observations indicate that degradation of FAD, especially in alkaline solution is occurring through 4':5'-FMN; we have found that the appearance of 4':5'-FMN depends on the pH conditions and time. The formation of 4':5'-FMN in phosphate buffer was studied in the pH range 4.5–12.0, in 1 *M* HCl and 1 *M* NaOH, at ambient temperature (Fig. 3). In 1M HCl, small amounts of 4':5'-FMN and FAD are hydrolysed to 5'-FMN and then to RF. In 1 *M* NaOH, FAD is hydrolysed to 4':5'-FMN almost completely (Fig. 4). In the pH range 4.5–7.5, FAD and 4':5'-FMN are relatively stable, but after 24 h more 4':5'-FMN appeared. FAD is rather unstable at pH values above 10, and hydrolyses to 4':5'-FMN; after 24 h, at pH=12 it is almost completely converted to this compound (Fig. 4). A small amount of 5'-FMN was detected after 24 h which may

Table 3
Comparison of R_F values on TLC and retention time (t_R) on HPLC (methods A and B) of standards and unknown flavin X

Flavins	TLC[a], R_F									HPLC[b], t_R (min)	
	I	IIa	IIb	III	IV	V	VI	VII	VIII	A	B
FAD	0.20	0.23	0.24	0	0	0.37	0.62	0	0.32	10.63	6.85
5'-FMN	0.35	0.39	0.33	0	0.03	0.14	0.52	0.06	0.32	12.30	10.58
4':5'-FMN	0.51	0.48	0.40	0.24	0.12	0.46	0.57	0.08	0.44	13.35	8.63
Rfl	0.67	0.58	0.56	0.58	0.29	0.81	0.39	0.32	0.60	21.72	13.42
X from yeast	0.51	0.48	0.56	0.24	0.12	0.46	0.57	0.08	0.44	13.37	8.65
X from FAD	0.51	0.48	0.56	0.24	0.12	0.46	0.57	0.08	0.44	13.43	8.60

[a] TLC solvents were: (I) *n*-butanol–glacial acetic acid–water (2:1:1 v/v), on silica gel; (IIa) and (IIb) *n*-butanol–acetic acid–water (5:2:3, v/v), on silica gel and cellulose, respectively; (III) chloroform–methanol–ethyl acetate (5:5:2, v/v), on silica gel; (IV) *n*-butanol–benzyl alcohol–glacial acetic acid (8:4:3, v/v), on silica gel; (V) collidine–water (3:1, v/v), on cellulose; (VI) 5% $Na_2HPO_4 \cdot 12H_2O$, on silica gel; (VII) *n*-butanol–formic acid–water–dimethyl ether (77:10:13:15, v/v), on silica gel; (VIII) *n*-butanol–ethanol–water (10:3:7, v/v), on silica gel.

[b] HPLC analysis was performed on Alphabond C_{18} column using fluorescence detector and elution methods: (A) methanol–0.1 *M* ammonium hydrogencarbonate pH=7.0 and (B) methanol–0.05 *M* ammonium acetate pH=6.0 in appropriate gradients described in Section 2.

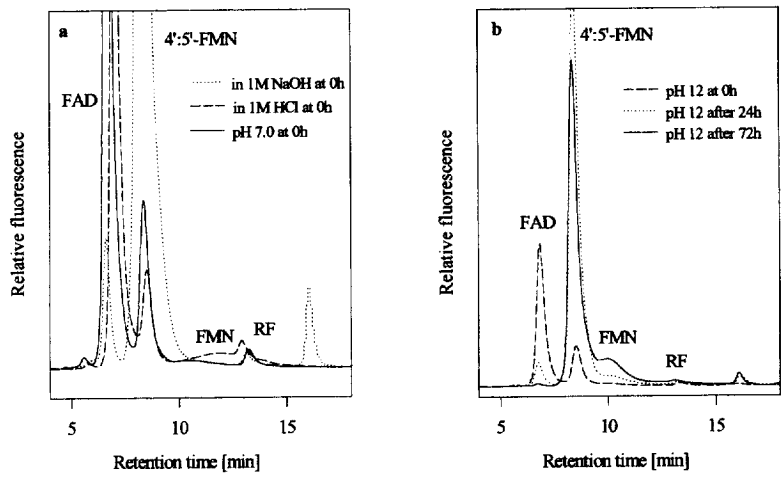

Fig. 4. HPLC elution profile of FAD and its degradation products at different pH conditions (a) and pH=12 (b). Mobile phase gradient of methanol–0.1 *M* ammonium acetate pH=6.0 (method B) on Alphabond C_{18} analytical column, fluorescence detection (λ_{ex}=450 nm, λ_{em}=530 nm).

indicate partial hydrolysis of 4′:5′-FMN. From Figs. 3 and 4 it is rather clear that the formation of 4′:5′-FMN even under moderate conditions is time dependent, but more detailed study is required.

3.2. Quantification of flavins

Seven samples in at least four replicates have been analysed and found to contain 17.9±2.9 μg total flavins/g of fresh yeast (55.4±8.7 μg/g of dry mass). FAD constitutes 71.5% of total flavins (12.8±1.9 μg/g of fresh yeast), 5′-FMN=25.7% (4.6±0.4 μg/g of fresh yeast), RF-1.7% (0.3±0.04 μg/g of fresh yeast), 4′:5′-FMN=0.8% (0.15±0.01 μg/g of fresh yeast). 10-FMF exists only in trace quantities (below 0.05% of total flavins). The recovery of individual flavins was more then 92%.

The published data on the vitamin B_2 contents in baker's yeast oscillates between 7.5 and 85 μg/g of dry mass [25]. This gap is due to the conditions of growth and the composition of the medium, which are not strictly constant in the technological process and may have a considerable influence on the vitamin content. Our results indicate that tested baker's yeast represents batches with average amounts of vitamin B_2.

This report constitutes a starting point for a more detailed study on the presence of different flavin analogues in food.

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