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Optimizing separation conditions for riboflavin, flavin mononucleotide and flavin adenine dinucleotide in capillary zone electrophoresis with laser-induced fluorescence detection

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Abstract

A method was developed for the quantitative determination of riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), using free solution capillary zone electrophoresis in uncoated fused-silica capillaries with laser-induced fluorescence (LIF) detection. Various factors influencing the separation and detection of flavin vitamers were investigated, including pH (5.5–10.5), concentration and nature of the run buffer (phosphate, borate and carbonate), applied voltage (15–30 kV), temperature (15–30 °C) and injection time. Optimal resolution and detection were obtained with a pH 9.8, 30 m*M* aqueous phosphate buffer at 15 °C and 30 kV of applied voltage. LIF detection was obtained with a He–Cd laser source using an excitation wavelength at 442 nm and $\lambda_{em} \geq 515$ nm. Riboflavin could be determined in the concentration ranges 0.5–350 μg/l with a rather low detection limit (LOD) down to 50 amol. The LODs of FAD and FMN were slightly higher, 300 and 350 amol, respectively. Combined with a simple clean-up procedure, the practical utility of this method is illustrated by the measurements of flavin derivates in foods and beverages, such as wines, milk, yoghurt and raw eggs.

Keywords: Wine; Food analysis; Vitamins; Riboflavin; Flavin mononucleotide; Flavin–adenine dinucleotide; Flavins

1. Introduction

Flavins are naturally occurring fluorescent compounds [1] that are involved as cofactor or/and substrate in many enzymatic redox processes [2]. Among flavins the most known is riboflavin (RF) or vitamin B_2 which is a micronutrient phosphorylated in cells to flavin mononucleotide (FMN) or further to flavin adenine dinucleotide (FAD). The chemical structures of RF, FAD and FMN are characterized by an isoalloxazine group substituted in the nitrogen at position 10 by a ribityl moiety (Fig. 1). These flavin nucleotides are prosthetic groups in the large family of proteins named flavoproteins including dehydrogenases, transferases, oxidases, and monooxygenases, which activate molecular oxygen [3]. RF is synthesized by microorganisms and higher plants, but not by animals, which must obtain their requirements of it from food.

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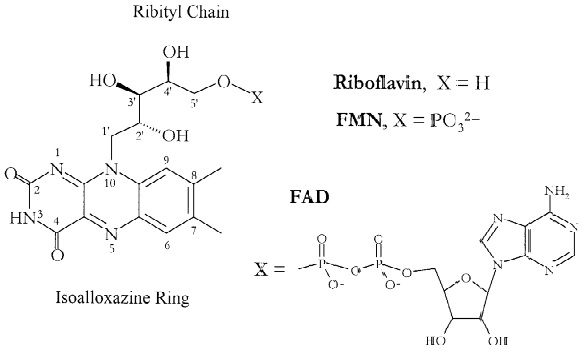

Fig. 1. Molecular structures of flavin chromophores.

The determination of the water-soluble riboflavin and related compounds in biological, food and beverage samples is important in biochemistry, nutritional and clinical chemistry. Analytical methods for these compounds rely on high-performance liquid chromatography (HPLC) with spectrophotometric detection taking advantage either of the fluorescence [4–7] or UV–visible absorbance [8–11] properties of the isoalloxazine chromophore. Moreover, electrochemical detection in liquid chromatography has also received some attention [12]. At present, the method most commonly used is fluorometric detection, with an excitation wavelength of 422 nm [13] or 450 nm [14]. Although reasonable resolution is given, HPLC determination of RF and flavin derivates is time-consuming and labor-intensive. In complex matrices, flavin analogues with the same elution time may lead to misleading identification and quantification. Some flavins may be biologically inactive or antagonistic to RF as in the case of 10-hydroxyethylflavin (10-HEF) and 10-formylmethylflavin (10-FMF), which potentially inhibit the flavokinase-catalysed conversion of RF to FMN [15,16]. As significant concentration of these flavin derivates in foods and beverages may occur, a detailed knowledge of the role of these compounds can be successfully accomplished only if a sensitive, highly efficient and rapid analytical method is available. Regardless of the application, optimal experimental conditions are essential for performing highly sensitive quantitative determinations. Our effort was to combine the advantages of highly sensitive laser-induced fluorescence (LIF) detection for flavin derivates, with the advantage of high resolving power and speed of capillary zone electrophoresis. LIF detector can provide a significant improvement in mass detectability compared to standard detectors for CE, as well as wide linear dynamic ranges [17,18]. Sensitivity at the level of 10^{-11} *M* can be reached with minimum detectable quantities in the zeptomole range [19].

The separation of riboflavin vitamers in human plasma by micellar electrokinetic chromatography (MEKC) has been successfully illustrated by Hustad et al. [20] using micelles of sodium lauryl sulphate in sodium borate at pH 7.9. Yet, for the determination of RF, FMN and FAD in biological tissues the main improvement in capillary zone electrophoresis was reported by Pérez-Ruiz et al. [21] while the present work was in progress. LIF detection was employed, but details about the CE experimental conditions were not described. The use of phosphate buffer at pH 8.5 was proposed, where RF is neutral and its determination in real samples may be a problem when native fluorescent and neutral compounds are present. However, according to its pK_a value of 10.2 [22] riboflavin is an ionisable compound, which renders it amenable to capillary zone electrophoresis (CZE) separation using basic or middle basic running electrolytes. Additionally, both these previous works [20,21] employed the argon-ion spectral line at 488 nm as an excitation source; such emitting line is higher than the maximum absorption at ~450 nm reported for riboflavins [23,24]. The use of a stable and intense laser line (e.g. He–Cd laser emitting at 442 nm), which is closer to the excitation maximum of these compounds, is most favourable in terms of signal-to-noise ratio for two reasons: (i) lower background signal as there is a greater difference between fluorescence emission and Rayleigh-scattered excitation light, and (ii) the emission intensity being proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength, a sensitivity enhancement may be expected. Thus, despite the major progress in capillary separations of flavins, there is still room for improvement as far as the optimization of CZE separation and LIF detection are concerned. Accordingly, the aim of the present investigation was to optimize the capillary electrophoretic conditions in conjunction with LIF detection for the determination of riboflavin vitamers. Effects of pH, type of buffer and its con-

centration, capillary temperature and applied voltage on mobility, resolution, sensitivity, and speed were carefully evaluated.

2. Materials and methods

2.1. Chemicals

All the chemicals used in this study were of analytical grade. Riboflavin 98%, flavin adenine dinucleotide 97%, and flavin mononucleotide 95%, α- and β-cyclodextrins, 99% and 98%, respectively, were purchased from Sigma–Aldrich (Steinheim, Germany); sodium hydroxide, disodium phosphate, sodium borate, sodium hydrogencarbonate and ammonium acetate were also obtained from Sigma–Aldrich. Methanol and methylene chloride of HPLC grade were purchased from Fluka (Buchs, Switzerland). Buffer solutions were prepared with ultrapure water supplied by a Milli-Q RG unit from Millipore (Bedford, MA, USA).

2.2. Samples and sample preparation

Commercial wines from different brands were kindly offered by local producers. Champagne Pommery as well as eggs, milk, and yoghurts were bought in grocery stores. Standard solutions of RF, FAD and FMN were prepared by dissolving in double-distilled Milli-Q water to give concentrations in the range 0.5–500 μg/l. All solutions were stored in amber glass bottles in a refrigerator at 4 °C. All samples were stored in the dark and each one was opened immediately prior to analysis. The wine samples were injected after dilution up to three times with water, and then passed through 0.22 μm membrane filters (Schleicher & Schuell, Dassel, Germany). The flavin compounds were extracted from food products by using the same method described by Gliszczyńska-Świgło and Koziołowa [25], in which an ammonium acetate solution at pH 6 was employed. The buffer solution used for electrophoretical runs were sonicated and filtered through a 0.45 μm membrane filter (Whatman); the pH of the running buffer was adjusted by addition of appropriate amounts of hydrochloric acid or sodium hydroxide.

2.3. Apparatus and method

CZE separation was performed on a Spectraphoresis Ultra Instrument (Thermo Separation Products, Fremont, CA, USA) equipped with an LIF detector ZETALIF (Picometrics, Ramonville, France) connected to a 20 mW He–Cd laser source. Uncoated fused-silica capillary (Thermo Separation Products), used throughout the analysis, had an internal diameter of 75 μm and an effective length of 84 cm to the LIF detector. Prior to use, the capillary was rinsed with 1 *M* NaOH and water for an hour and subsequently with the separation buffer for 30 min. Every morning at the beginning of a work day, the capillary was washed with 0.1 *M* NaOH (5 min), water (5 min) and run buffer (15 min). Between analyses, the capillary was rinsed with the electrophoretic buffer for 5 min. Samples were introduced into the anodic end of the capillary by pressure injection for 10 s at 0.8 p.s.i. (54 mbar) using aqueous buffer solutions at the concentration and pH determined by the experimental design. All experiments were conducted in normal polarity mode applying voltages ranging from 15 to 30 kV; the temperature of the capillary was maintained at values ranging from 15 to 30 °C by the instrument thermostating system. The LIF detector was operating at 442 nm as an excitation wavelength and the intensity of fluorescence was measured over the integration range above 515 nm, using a high-pass filter. Data processing was performed using Spectacle and PC1000 CE software version 3.5.

2.4. Procedure with standard solutions

Stock solutions of 500 μg/l RF, FMN and FAD in water were prepared and stored in darkness at 4 °C. Working standard solutions were prepared on the day of use by suitable dilutions. Aliquots of these solutions were treated as the samples. This concentration range was used for the systematic studies with CZE–LIF that involved the separation of flavin vitamers. The resulting peak areas were plotted against concentration for the calibration curve. The

flavin contents of the sample extracts were obtained by interpolation on the standard curves.

3. Results and discussion

3.1. Effect of experimental variables in CZE

3.1.1. Buffer nature, mobility of riboflavins and LIF detection

The influence of several parameters was investigated to identify key variables that affect signal intensity and separation efficiency of RF, FAD and FMN using standard solutions at the concentration of 100 μg/l. These parameters included the nature, pH and concentration of the run buffer, applied voltage, and capillary temperature. Using the conditions found to be suitable for the determinations of riboflavins as described by Pérez-Ruiz et al. [21], the separation of such compounds at pH 8.5 was examined. Three different aqueous buffers were tested: phosphate, carbonate and borate buffers. In agreement with the above cited work, the phosphate buffer solution (PBS) exhibited a higher electroosmotic flow (μ_{EOF}) and the most favourable signal-to-noise ratios for all flavins compared to the use of both borate and carbonate buffer solutions, BBS and CBS, respectively. However, to better assess the impact of run buffer on the CE separation of flavins, the effective electrophoretic mobilities were estimated. The evaluation of mobility rather than migration time has two main advantages in capillary electrophoresis [26]. Firstly, mobility is a physical constant of each species, which depends on charge and size under the experimental conditions employed (i.e. buffer composition, pH, and temperature), but is independent of capillary dimensions and applied voltage. This allows peak identification and simplifies inter-instrument method transfer. Secondly, it improves repeatability of migration times by reducing the dependence of electroosmotic flow.

The measured (i.e. apparent) electrophoretic mobility (μ_{mes}) of an analyte is defined by Eq. (1):

$$\mu_{mes} = \frac{\ell \cdot L}{V \cdot t_m} \quad (1)$$

where ℓ and L are the capillary length to the detector window and the total capillary length in centimetres, V is the applied voltage in volts and t_m is the measured migration time in seconds. The effective electrophoretic mobility (μ_{eff}) was evaluated by subtracting the μ_{EOF} from the measured electrophoretic mobility (see Eq. (2)):

$$\mu_{eff} = \mu_{mes} - \mu_{EOF} = \frac{\ell \cdot L}{V}\left(\frac{1}{t_m} - \frac{1}{t_{EOF}}\right) \quad (2)$$

The apparent mobilities of flavins at pH 8.5 using PBS, BBS and CBS are listed in Table 1. Such values are in good agreement with previous findings [21] estimated from plotted data in PBS at pH 8–9. In Fig. 2 are shown the electropherograms obtained after the time scale of raw data was transformed into a scale of effective electrophoretic mobility. Two major features may be recognized in these plots. First of all, the effective mobility of riboflavin compounds in BBS (middle plot in Fig. 2) was quite different from that observed in carbonate and phosphate buffers. Indeed, the migration order of FAD and FMN was reversed, which is probably due to borate complexation of FAD through the –OH groups of the ribosyl moiety. Such a complex migrates slower toward the cathode than FMN. Secondly, the appearance of riboflavin peak in the borate buffer was consistently found, exhibiting an effective mobility of $\sim -0.8\times10^{-4}$ cm^2 V^{-1} s^{-1}. Indeed, while riboflavin is neutral at pH 8.5 and should move with the electroosmotic flow, under the above experimental conditions the negative value of μ_{eff} may possibly be explained by borate complexation of the ribityl moiety [27]. Note that the same

Table 1
Apparent electrophoretic mobilities (μ_{mes}) of RF, FAD and FMN in phosphate, borate and carbonate buffers (30 m*M*) at pH 8.5

	Mobility ($\times10^{-4}$ cm^2 V^{-1} s^{-1})					
	PBS[a]				BBS[b]	CBS[c]
	15 °C	20 °C	25 °C	30 °C	15 °C	15 °C
RF	5.6	6.2	6.7	6.5	4.9	5.4
FAD	4.0	4.4	4.7	4.6	3.4	3.8
FMN	3.7	4.1	4.4	4.3	3.6	3.5

[a] Phosphate buffer.
[b] Borate buffer.
[c] Carbonate buffer.

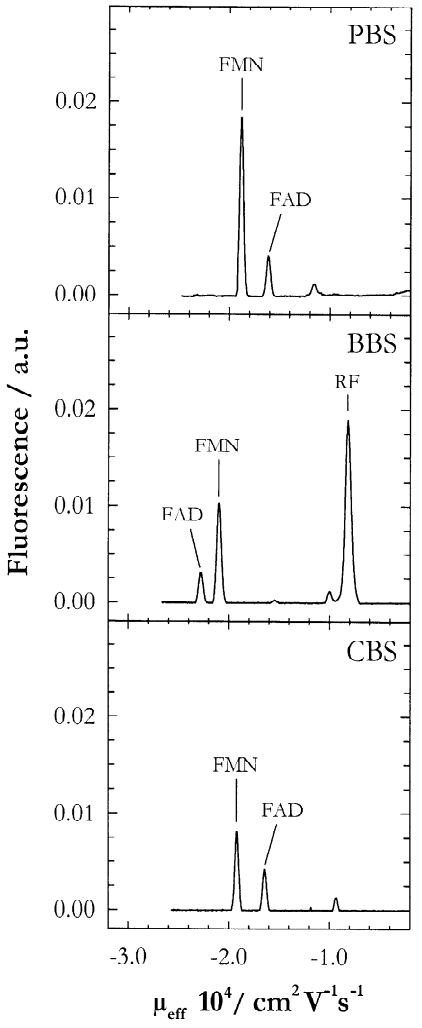

Fig. 2. Effect of buffer nature on the effective mobility of RF, FAD and FMN at the concentration of 100 μg/l each. Aqueous running electrolytes (30 m*M*) at pH 8.5 prepared with carbonate (CBS), borate (BBS), and phosphate buffer (PBS). Separation conditions: 92 cm capillary (84 cm effective length)×75 μm I.D. unmodified fused-silica capillary, 10 s and 54 mbar injection, 30 kV separation, capillary temperature 15 °C. LIF detection using a He–Cd laser source at 442 nm was employed. The identity of the small peak at low migration values in the electropherograms is not known, but this peak always appears with RF, so probably it is due to some contamination of the sample.

migration order found here RF<FMN<FAD, was detailed in another report [20], where borate buffer at pH 7.9 in MEKC was employed. A similar behaviour was not observed when PB and CB solutions were employed. The only drawback observed with CBS was related to greater current values compared to PBS. For these reasons and also because the signal-to-noise ratio of flavins was higher when PBS was used (see Fig. 2), all subsequent work was accomplished using aqueous phosphate solutions as electrophoretic buffers.

An important feature of the LIF detection used here was the replacement of the argon laser source at 488 nm, previously employed by other authors [20,21], by the He–Cd laser line at 442 nm as an excitation wavelength. In fact, the excitation maximum of flavins in aqueous solutions matches very well with the intense and stable emission line of the He–Cd laser source; the absorption peak of flavins is at ~450 nm, and the emission has a maximum at ~530 nm [23,28–31]. This renders the analytes amenable to be detected with a higher signal-to-noise ratio as the emission intensity is proportional to the intensity of the fluorescence excitation spectrum. In addition, the excitation source at 442 nm is most advantageous because the fluorescence emission is at a much different wavelength than the excitation, yielding a low background signal.

3.1.2. Effect of pH

In an attempt to find a suitable pH value for the run buffer and considering that the fluorescence intensity of flavins is pH-dependent [21,32,33], the effect of pH on migration times, baseline resolution and fluorescence intensity was investigated. pH values in the range between 5.5 and 10.5 were examined using 30 m*M* PBS. As can be seen from the electropherograms of Fig. 3, on increasing the pH there was a slight increase in the LIF intensity for all compounds up to a pH value of 9.8. However, in neutral or acidic buffers of pH 7 and 5.5, the observed peak shape of RF is not well-formed, probably due to quenching or changes of its fluorescence yield. In an attempt to clarify the reason of such a phenomenon other buffer solutions at pH 5–6 were examined. It was interestingly found that acetate and formate buffers exhibited the same behaviour (not shown), thus suggesting that the quenching effect is related to the pH of solution. This explanation was further supported by the fact that the well-shaped peak of RF was restored when

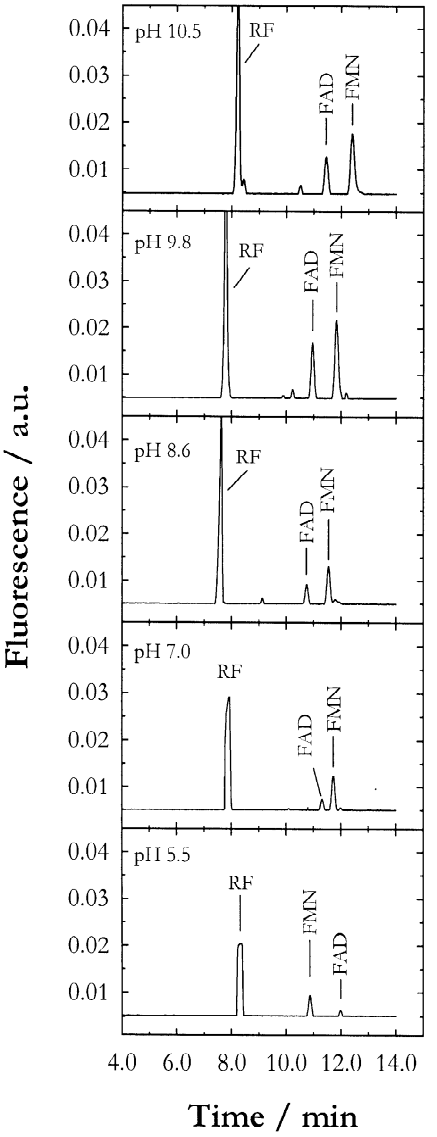

Fig. 3. Direct comparison of phosphate buffer pH on the free CZE separation of RF, FAD and FMN. Other conditions as in Fig. 2.

the same standard solution was introduced using the run buffer at basic pHs.

To ensure that the pH 9.8 was optimal for the separation of RF as an ionic compound in CZE, in Fig. 4 are plotted the effective mobilities of riboflavin vitamers in PBSs as functions of pH. While FAD and FMN are negatively charged ($\mu_{eff}<0$) at all examined pHs (pK_a for their phosphate groups are

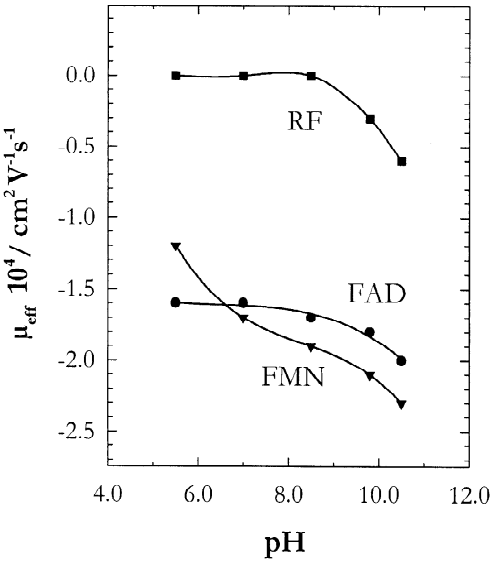

Fig. 4. Effect of pH on the effective mobility of RF, FAD and FMN. Separation conditions: electrophoretic buffer, 30 m*M* phosphate solutions running at +30 kV, capillary temperature 15 °C. LIF detection using a He–Cd laser source at 442 nm. Note that while the effective mobility of RF is null at pH values <8.5, at acidic pH values the mobility of FAD and FMN is reversed.

1.3 and 6.5 [34]), RF is neutral ($\mu_{eff}\cong 0$) from pH 5.5 to ca. 9, according to its pK_a value of 10.2 [22]. As demonstrated above, this applies in the absence of a reacting electrophoretic buffer (i.e. borate). Therefore, the optimal pH to separate and determine the riboflavin vitamers with symmetrical peaks was ~9.8, given that higher values do not improve resolution and/or sensitivity. Notably, at pH 5.5 the peak intensity of FAD and FMN was very low and their migration order was reversed compared to that observed at higher pH values. This last effect is probably due to changes of their effective mobilities (see Fig. 4) as a result of differences in their charges induced by acidic pH values.

3.1.3. Effect of ionic strength and buffer additives

The ionic strength of the PBS was tested at a constant pH of 9.8. As the concentration was changed from 10 m*M* to 50 m*M*, an increase in migration times was obtained (see Fig. 5). It is suggested that such an effect is related to a lower electroosmotic flow, resulting from a decrease of the zeta potential at the capillary wall–solution interface.

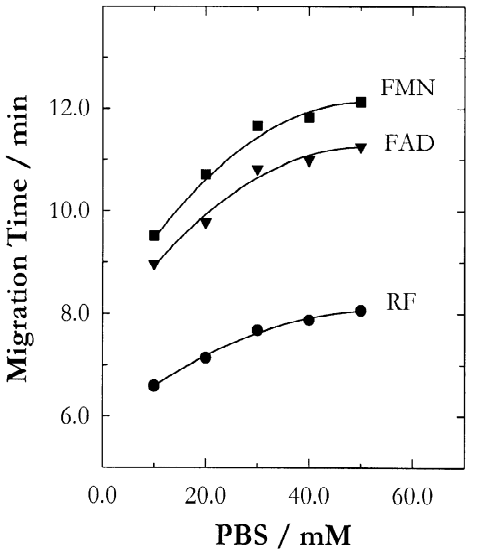

Fig. 5. Influence of phosphate buffer concentration at pH 9.8 on the migration time of RF, FAD and FMN. Other conditions as in Fig. 4.

On increasing the phosphate concentration, the analysis time was slightly longer, without significantly improving the separation of FAD and FMN. The difference of migration times between these two compounds was 33 s when 10 m*M* phosphate was used and became 52 s with 50 m*M* phosphate buffer. A good compromise between separation, run time and Joule heat generated inside the capillary was obtained with 30 m*M* PBS.

While cyclodextrins (CDs) are widely used as buffer additives to obtain chiral separations in CE, they can be also used to adjust the selectivity in non-chiral applications [35]. Indeed, several studies have shown that adding CDs can enhance the selectivity of CE because these compounds form inclusion complexes with a wide variety of guest organic molecules or ions [36]. Selectivity is taken to be a function of whether the guest molecule fits into the CD hydrophobic cavity. In the present study, the use of α- and β-cyclodextrin at the concentration 2–10 m*M* does not provide either any significant change of flavin migration or increased separation between FAD and FMN.

3.1.4. Effect of temperature and applied voltage

Temperature studies were performed to determine optimal capillary temperatures for the separation of flavins. The data (not shown) indicate that as the temperature of the capillary increased, migration time decreased. For example, the separation of FAD and FMN was 50 s at 15 °C and 41 s when capillary temperature was controlled at 30 °C. Such temperature-dependence of the migration time is thought to reflect alterations in the buffer viscosity. The optimal temperature in terms of separation and analysis time was chosen at 15 °C since higher values caused increased Joule heating.

The effect of applied voltages on peak efficiency of the three analytes was studied over the range 15 to 30 kV. At a constant temperature of 15 °C and using 30 m*M* PBS at pH 9.8, the increase of applied voltage led both to shorter migration times and sharper peaks for all flavins. The number of theoretical plates per metre calculated for FAD and FMN was 36,000 at 15 kV and increased to 56,000 at 30 kV. As expected, on increasing the applied voltage there is an increase in electroosmotic flow, leading to shorter analyses times and higher efficiencies. However, higher applied voltages exhibit also higher currents and increased Joule heating. The Ohm's plot was found to be linear with a zero intercept and a slope of $1/R$ up to the maximum voltage applied (not shown). The current associated with this separation plateaued at 60.4 μA corresponding to an applied power of 1.9 W/m. Such a value is very close to that recommended by Sepaniak and Cole (<2 W/m) [37] for effective electrophoretic separations without excessive Joule heating.

3.1.5. Injection time

Among other effects, duration of sample injection time was also examined in an effort to get a low detection limit. Sample solutions were hydrodynamically injected at a pressure of 54 mbar while the injection time was varied from 2 to 40 s. Peak areas of RF, FAD and FMN increased linearly on increasing the injection time, but the relationship of FAD was only linear up to 30 s (not shown). Generally, the volume of the sample recommended (e.g. plug length) should not exceed 1% of the whole capillary length in order to control efficiency and resolution. Here, an injection time of 10 s was chosen, which corresponds to a normalized injection length of ca. 0.14% [38].

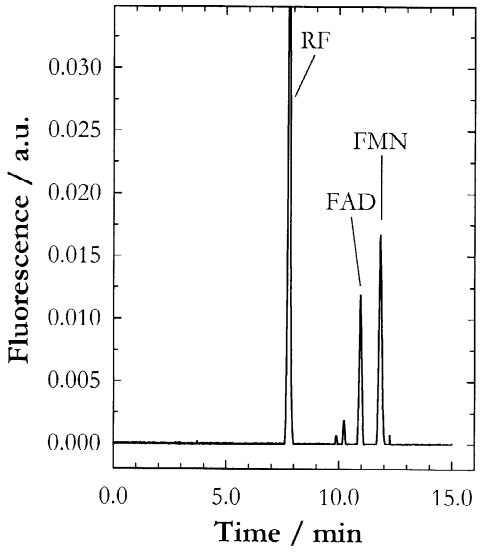

Fig. 6. Electropherogram in CZE–LIF of RF, FAD and FMN (each 100 μg/l) under optimized experimental conditions: electrophoretic buffer, 30 m*M* phosphate solution at pH 9.8; hydrodynamic injection at 54 mbar for 10 s; running at +30 kV; current, 60 μA. Effective capillary length, 84 cm; total length 92 cm. Relative fluorescence units (RFU) were recorded over the integration range above 515 nm.

3.2. Linear range and limit of detection

Choosing a PBS at pH 9.8 (30 m*M*), an applied voltage of 30 kV (corresponding to 326 V/cm), a temperature of 15 °C, and a hydrodynamic injection of 10 s at 54 mbar, resulted in a fast, sensitive and complete separation assay in less than 13 min. A typical CZE–LIF separation of riboflavin vitamers is illustrated in Fig. 6. Satisfactory separation of all three analytes was achieved with symmetrical peaks in the migration time window comprised between 7 and 12.5 min. The precision of migration times was evaluated by comparing the electropherograms of the same standard mixture at the beginning and the end of each day for weeks. The mean values of migration times (20 repetitions) and standard deviations (SD) of RF, FAD and FMN were 7.6±0.2 min, 10.7±0.2 min and 11.4±0.3 min, respectively.

For quantitative determinations the correlation between the peak area and concentration of each flavin was examined from the limit of detection up to 500 μg/l. As suggested by Baker [39], better correlations were obtained reporting the peak areas divided by the corresponding migration time as a function of concentration. Ten concentration levels of the standard solution and three replicate injections were used for calibration; the linear regression parameters are summarized in Table 2. The LODs were determined at a signal-to-noise ratio of 3 and found to be 0.5, 6 and 4 μg/l, for RF, FAD and FMN, respectively. The noise in the baseline was determined using the mean peak-to-peak noise. As the evaluated injection volume was ca. 40 nl (injection at 54 mbar and 10 s), the on-column detection limits of RF, FAD and FMN in standard solutions, corresponded to 50, 300 and 350 amol, respectively. Thus, detection of riboflavin in the 10^{-18} level (amol) is allowed, which is three orders of magnitude lower than conventional fluorescence detection in liquid chromatography [14]. At the concentration of 5 n*M*, the peak area of RF determined by the LIF detector had a coefficient of variation of 3.7% ($n=5$). Such an accurate and highly sensitive CZE–LIF method, combined with a simple extraction procedure, shows potential for quantitation of flavins in various samples in the picogram per

Table 2
Reproducibility, linearity and limit of detections of flavins evaluated by CZE–LIF detection[a]

Compound	RSD of migration time ($n=20$) (%)	Line equation, $Y=m$ ppb $+b$		r	Linear range (μg/l)	LOD[b]	
		b±SD	m±SD			μg/l	amol
RF	2.6	$(-17.2\pm0.1)\times10^3$	798.9±0.8	0.99946	0.5–350	0.5	50
FAD	1.9	$(-13.9\pm0.1)\times10^3$	578.3±0.9	0.99961	6–280	6	300
FMN	2.6	$(-12.5\pm0.1)\times10^3$	531.7±0.8	0.99949	4–350	4	350

[a] Concentration value expressed as μg/l.
[b] The limit of detection was calculated as being the concentration which gave a signal corresponding to a signal-to-noise value of 3; hydrodynamic injection of 10 s at 54 mbar.

millilitre range. The above optimized experimental conditions were thus used for the analysis and profiling of riboflavins present in selected food samples.

3.3. Determination of flavins in foodstuffs

While plants and microorganisms are able to synthesize vitamin B_2, external sources are needed to ensure the right intake for humans and animals. Thus, the applicability of this CZE–LIF method to analyze flavins in beverages and food samples was checked. Four different RF-containing matrices were chosen such as wine, milk, yoghurt and egg.

The analysis of wine samples were accomplished without extensive pretreatment [40,41], just filtration through 0.22 μm membranes and dilution up to three times with water. A typical electropherogram of a white wine sample (Pinot gris, vintage 1998) is shown in Fig. 7, panel A. In Table 3 are also listed the quantitative data of other samples, such as a second white wine (Locorotondo, 1997), a sample of champagne (Pommery, 2000) and that of spumante (Gancia, 2000). As expected, high levels of RF are present in all samples examined.

The CE–LIF method described in this paper was also used to evaluate the flavin-contents of milk, yoghurt and raw egg (both white and yolk). Extraction of flavin compounds from food samples has to be sufficiently mild to prevent their hydrolysis, especially when flavin coenzymes are involved. As described in Section 2, a solution of ammonium acetate at pH 6 was used in the present work. The extraction efficiency was evaluated from the recovery of flavins, using spiked and unspiked samples treated in the same way throughout the whole procedure. Recoveries of RF vitamers were higher than 90% in all investigated samples.

Fig. 7B shows a CZE–LIF separation of a sample of cow's milk. Although milk and dairy products are complex sample matrices, it was not necessary to precipitate proteins before introducing the sample. The mean contents of RF, FAD and FMN were evaluated as the average of three measurements performed on different samples. In general, the content of riboflavin in the samples analyzed was in agreement with that reported in the literature [25]. The amount of vitamin B_2 was 112 μg/100 ml,

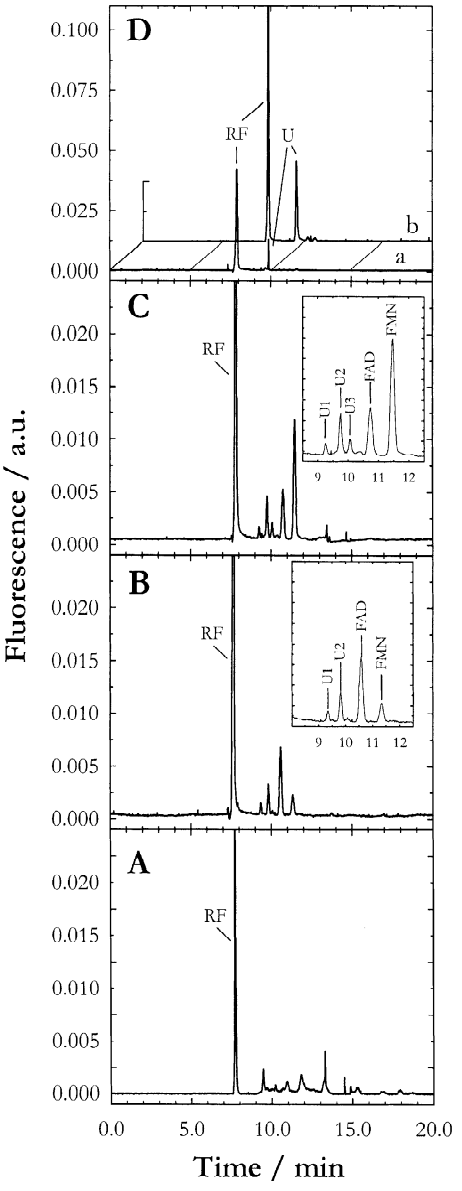

Fig. 7. (A) A sample of white wine (Pinot 1998) separated by CZE and LIF detection containing 130 μg/l of RF. (B) A sample of cow's milk: RF (112 μg/100 ml), FAD (28 μg/100 ml), and FMN (25 μg/100 ml). Inset shows expanded view of FAD and FMN peaks. U1 and U2 are unknown fluorescent compounds. (C) A sample of milk yoghurt: RF (138 μg/100 g), FAD (36 μg/100 g), and FMN (47 μg/100 g). Inset shows expanded view of unknown (U1, U2 and U3), FAD and FMN peaks. (D) Comparison made by CZE–LIF of raw egg yolk (a) and raw egg white (b) samples extracted from the same egg; U, unknown. The RF content was, respectively, 310 and 380 μg/100 g. Separation conditions as in Fig. 6.

Table 3
Flavin composition in beverages and foodstuffs (mean±SD, $n=3$)

Sample	RF	FAD	FMN
White wine[a]	120±3	ND[e]	ND
White wine[b]	130±3	ND	ND
Spumante Gancia	145±5	ND	ND
Champagne Pommery	183±5	ND	ND
UHT milk[c]	133±5	25±2	28±2
Cow milk[c]	112±2	28±2	25±2
Buffalo milk[c]	101±2	29±2	26±2
Sheep milk[c]	175±5	46±3	ND
Goat milk[c]	112±2	46±3	38±3
Yoghurt[d]	138±4	36±3	47±3
Raw egg white[d]	380±60	ND	ND
Raw egg yolk[d]	310±20	ND	ND

[a] White wine Locorotondo, vintage 1997.
[b] White wine Pinot 1998: concentration value expressed as μg/l.
[c] Concentration value expressed as μg/100 ml of product.
[d] Concentration value expressed as μg/100 g of product.
[e] Not detected.

while the contents of FAD and FMN were 28 μg/100 ml and 25 μg/100 ml, respectively. Only small differences in flavin composition and content were observed between fresh and UHT cow's milks. Such findings are in agreement with data reported by Saidi and Warthesen [42] who found that homogenization and pasteurization of milk increased the photostability of riboflavin. While electrophoretic peaks were usually identified by standard additions, here an additional separation was accomplished using borate as a run buffer. As mentioned above, the change of migration order allowed us to clearly identify FAD and FMN peaks. Although RF, FAD and FMN are the major flavins found in milk and dairy products, we found some unidentified peaks, U1 and U2 (Fig. 7B, inset). Additional efforts for their identification are needed perhaps by employing a more valuable analytical system, which might be CE in conjunction with mass spectrometry. Work is in progress along this direction.

Flavins present in plain yoghurt samples were also measured and a typical electropherogram is shown in Fig. 7C. We found riboflavin concentrations similar to those reported elsewhere [25]; the amounts of RF, FAD and FMN were 138, 36 and 47 μg/100 g, respectively. It should be noted, though, that the amount of flavins in plain yoghurts spans a wide range of values as a function of starting raw milk used, microorganisms and technological processes applied during fermentation. Thus, the content of vitamin B_2 may vary between 99 and 187 μg/100 g, with an average value that is generally higher than in milk samples [25]. As illustrated in the inset of Fig. 7C also the yoghurt sample examined contains at least three other unknown fluorescent compounds, U1, U2 and U3, which probably derived from traces of endogenous flavin derivates.

In panel D of Fig. 7, are compared the CZE–LIF separations of flavins present in the samples of raw egg yolk (plot a) and raw egg white (plot b). Whereas FAD and FMN were not detectable, the major peak was identified as RF with a mean content of 310 and 380 μg/100 g in raw egg yolk and white samples, respectively. As a result of the sample dilution, the peak heights of RF exhibits dissimilar intensity. The reported values for these samples are comprised in the range of 250–450 μg/100 g and of 240–540 μg/100 g for raw egg white and yolk, respectively [43]. In our study no significant differences were observed between eggs coming from chicken farms or eggs bought in a local store. Notably, the egg white sample exhibits a significant amount of an additional fluorescent compound (t_m = 9.5 min), which is also present at a lower amount in the corresponding extract of egg yolk. Such unknown compound might be 4′,5′-cyclic FMN, 10-FMF or both [25]. Apparently, 10-FMF that is a photodecomposition product of riboflavin exhibits a potential antivitamin effect, because it inhibits the flavokinase-catalysed conversion of riboflavin to FMN [16].

4. Conclusions

Optimal conditions to separate and determine vitamin B_2, FAD and FMN by CZE using LIF detection have been established. Such an optimization offers very favourable conditions over previous methods in terms of sensitivity, simplicity and applicability to food analysis. The CZE–LIF method is well suited to satisfy the demands for reliable, accurate and sensitive detection with minimal sample preparation and clean-up. Indeed, it was applied to the analysis of flavin derivates in wine, milk, yoghurt and egg samples, where the benefit of testing two run

buffers exhibiting different selectivity was also verified. The fact that the sensitivity of the detector is two or three orders of magnitude higher than a conventional fluorescence detector allows for dilution of the sample, thus reducing matrix effects and the interference of other native fluorescent compounds.

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