Riboflavin in Dietary Sources: Separation and Detection by CE–LIF

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This article demonstrates the suitability of capillary electrophoresis coupled to laser-induced fluorescence detection for riboflavin determination in common foods and beverages.

Introduction
Riboflavin (RF, Figure 1), or vitamin B2, is a naturally occurring micronutrient found at high levels in various foods and beverages. Good sources of riboflavin include milk, cheese, vegetables, liver and yeast.1 Currently, the RF product used in several pharmaceutical preparations and to fortify some foods is obtained through genetically engineered microorganisms derived from the soil bacterium Bacillus subtilis.2 Accordingly, this biotechnological product can be found in such foods as breakfast cereals, sauces, processed cheese, fruit drinks, baby foods, vitamin-enriched milk products and vitamin supplements. This article describes the successful application of capillary electrophoresis (CE) coupled with laser-induced fluorescence (LIF) detection for riboflavin determination in common natural products.

Riboflavin Properties and Occurrence
Flavins are extremely important biological cofactors because of their ability to transfer either one or two electrons in a wide variety of biological processes.3 This results from the fact that flavins are stable in oxidized, one-electron reduced and two-electron reduced forms in proteins. While this redox chemistry normally takes place in the ground electronic state, it is becoming evident that the excited electronic states, accessible by absorption of a photon, play an important physiological role in the areas of DNA repair (photolyases) and signal transduction (cryptochromes). Among flavins, RF is a widely distributed vitamin, which was first isolated from egg albumen in 1933.4 It has a key role in maintaining human health. Like the other B vitamins, it supports energy production by aiding in the metabolism of fats, carbohydrates and proteins. Riboflavin is also required for red blood cell formation and respiration, antibody production, and for regulating human growth and reproduction. It is essential for healthy skin, nails, hair growth and general good health, including regulating thyroid activity. Some evidence exists that riboflavin in high doses (400 mg daily) has some effect in migraine prophylaxis.5 Because of its properties, RF, otherwise known as E101, is used as an additive in flour, in bread and pastries, in breakfast cereals and in dietary products. It is heat-stable, though some is wasted when foods are cooked in boiling water.6 It is sensitive to light, and depending on how it is stored, can be destroyed by sunlight.

Recent scientific advances in cell and molecular biology have culminated in the genetic engineering of crops and microorganisms. In processed foods, RF is very likely to be genetically modified as it can be produced by a selected strain of Bacillus subtilis, altered to both increase production of riboflavin and to introduce an antibiotic (ampicillin) resistance marker. Despite the potential benefits of this new technology for improving the reliability and quality of the world food supply, public concerns have been raised about the safety of food derived from genetically modified organisms. Debates have focused principally on the perceived risks associated with the accumulation of novel compounds that may contribute to hazards in the human diet. However, biotechnologically produced RF and its use is continuously growing. As it is difficult to incorporate RF into many liquid products because of its poor solubility, a more expensive but more soluble form of riboflavin, riboflavin-5’-phosphate or E101A, has been prepared.

Basic Considerations on CE
The term electrophoresis is employed to describe the migration of charged particles under the influence of an electric field. When such a process is performed through a capillary column (50–150 µm i.d., with a length of about 20–100 cm), the
em electromigration of ions is known as CE. A high-voltage power supply up to 30 kV connects the reservoirs via the buffer-filled capillary. The separation is based on the tendency of identical ions, when dissolved or suspended in an electrolyte through which an electric current is passed, to migrate into sharply defined zones as a result of their characteristic mobility. Reported values range from $10^5$ to $10^6$ theoretical plates per metre, with exceptional values in the millions. This efficiency is considerably higher than that usually observed with high performance liquid chromatography (HPLC). Analysis times are in the region of 1–30 min depending on the complexity of the separation. Analytes can be detected on-column as they pass a window at the far end of the capillary. CE is easily interfaced with optical detection modes, that is, UV/visible absorption, conventional fluorescence and LIF emission, also exploiting direct and indirect detection. One of the issues facing all capillary format separations is the achievable concentration detection limit. Capillary separations exhibit excellent mass sensitivity and on-column detection of low femtomole (fmol) to 100 zeptomole (zmol) can realistically be achieved. However, only very small volumes can be injected under normal conditions (10–50 nL) and this results in relatively high concentration detection limits, especially for UV detection. LIF detection overcomes the optical limitations of the capillary in two ways. Intense light is available from the laser and this light can be efficiently focused into the narrow channel of the detection window. As a result, CE with LIF detection represents a powerful combination for quantifying riboflavin and flavin derivatives in foods, beverages and biological samples.

**Riboflavin Assay by CE–LIF**

Riboflavin and flavin-related compounds are ideal substrates for CE–LIF techniques. This class of compounds exhibits large absorption coefficients ($\geq 10.000 \ M^{-1} \ cm^{-1}$) and high quantum efficiencies (fluorescent quantum yields near unity). These properties are highly valuable for the fluorescence detection of flavins, especially those samples that present a relevant number of UV-absorbing compounds. As a result the separation of flavins has been traditionally achieved by reversed-phase HPLC coupled with fluorescence detection, allowing for low concentration detection limits. Recently much effort has been devoted to the determination of riboflavin and flavin cofactors in biological samples by CE and micellar electrokinetic chromatography. More recently, the experimental conditions in CE with LIF detection were optimized and successfully applied to the analysis of food samples. Here we describe a rapid, sensitive and very selective one-step method for riboflavin assay in foods and beverages by CE–LIF. Benefiting from its intrinsic fluorescent nature, riboflavin can be directly detected at very low amounts using a slightly alkaline phosphate buffer at approximately pH 10. The CE–LIF system used for determinations is identical to that described previously. Table 1 specifies the experimental details of CE–LIF detection for RF and flavin vitamers.

** Experimental**

**Chemicals:** All chemicals used in this study were of analytical grade. Riboflavin 98%, flavin adenine dinucleotide (FAD) 97% and flavin mononucleotide (FMN) 95% were purchased from Sigma-Aldrich (Steinheim, Germany), as were sodium hydroxide, disodium phosphate and ammonium acetate. Methanol and methylene chloride of HPLC grade were purchased from Fluka (Buchs, Switzerland). Stock solutions of RF, FMN and FAD (500 µg/L) in water were prepared and

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**Figure 1:** Vitamin B2 or riboflavin is a weak base, $pK_a$ 5 10.2. The molecule is in two distinct parts: a ribose sugar unit and a three-ring flavin structure known as lumichrome.

[Diagram of riboflavin molecule]
stored in darkness at 4 °C. Working standard solutions were prepared daily by suitable dilutions. Phosphate buffer solutions were prepared with ultrapure water obtained from a Milli-Q RG unit from Millipore (Bedford, Massachusetts, USA), then sonicated and filtered through a 0.45 µm membrane filter (Whatman, Maidstone, UK); the pH of the running buffer was adjusted by the addition of appropriate amounts of sodium hydroxide.

**Sample preparation:** Commercial wines were kindly offered by local producers. Milk, baker’s yeast and green leafy vegetables were bought in local markets. 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 using the same method described by Gliszczynska-Swiglo and Koziolowa.18 4 g of sample were placed in extraction tubes, suspended in 19 mL of methanol-methylene chloride (9:10) and shaken for 60 s using a shaker. After the addition of 9 mL of 0.1 M ammonium acetate at pH 6.0 and successive shaking for another 60 s, the mixture was centrifuged at 4200 rpm and 4 °C. The upper phase was filtered through 0.22 µm membrane filters (Schleicher & Schuell, Dassel, Germany). The flavin compounds were extracted from food products using the same method described by Gliszczynska-Swiglo and Koziolowa.18 4 g of sample were placed in extraction tubes, suspended in 19 mL of methanol-methylene chloride (9:10) and shaken for 60 s using a shaker. After the addition of 9 mL of 0.1 M ammonium acetate at pH 6.0 and successive shaking for another 60 s, the mixture was centrifuged at 4200 rpm and 4 °C. The upper phase was filtered through 0.22 µm filters and then analysed by capillary electrophoresis–laser-induced fluorescence (CZE–LIF). The flavin contents of the sample extracts were obtained by interpolation of the standard curves.

**Apparatus and method:** CZE separations were performed on a Spectraphoresis Ultra Instrument (Thermo Separation Products, Fremont, California, USA) equipped with a ZetaLIF detector (Picometrics, Ramonville, France) connected to a 20 mW He-Cd laser source. The uncoated fused-silica capillary (Thermo Separation Products, Fremont, California, USA) was rinsed with 1 N NaOH and water for an hour followed by 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 phosphate 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 54 mbar. Data processing was performed using Spectacle and PC1000 CE software version 3.5 (Thermo Separations, California, USA).

**Food and Beverage Analysis**

Dietary RF is present and readily available in liver, cheese, milk, meat, eggs, peas, beans, whole-grain cereals and even wines.19 It has been suggested that RF acts as an important enhancing factor in the formation of sunlight flavour in light-exposed white wines, even though the off-flavour cannot be entirely explained with the presence of RF as a photosensitizer.10 The analysis of wine samples was accomplished without an extensive pretreatment, just filtration through 0.22 µm membranes and dilution up to three times with water. In Figure 2 the typical electropherograms of three samples of Italian wines, (white, rosé and red), is shown. As expected, relatively high amounts of RF were determined, while no presence of FAD or FMN were found in all samples examined.10,17 Nevertheless, it should be mentioned that in the electropherograms of rosé and red wines (see plots (b) and (c) in Figure 2) the time window during which the migration of FAD and FMN occurs is overcrowded by several minor fluorescent compounds. Thus, the identification and quantification of very small amounts of FAD and FMN became less certain. The average value for red, rosé and white wines, and the respective standard deviations are

### Table 1: Details of the CE–LIF experimental conditions.*

<table>
<thead>
<tr>
<th>Capillary dimensions</th>
<th>(92 cm total length) 84 cm–75 µm i.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Running solution</td>
<td>30 mM phosphate buffer at pH 9.8</td>
</tr>
<tr>
<td>Separation voltage and temperature</td>
<td>30 kV in normal polarity mode and thermostating the system at 15 °C</td>
</tr>
<tr>
<td>Average current</td>
<td>60 µA</td>
</tr>
<tr>
<td>Injection</td>
<td>Pressure injection for 10 s at 54 mbar</td>
</tr>
<tr>
<td>Laser source and line</td>
<td>He-Cd laser source at 442 nm</td>
</tr>
<tr>
<td>Fluorescence detection</td>
<td>Fluorescence wavelength collected over the integration range above 515 nm.</td>
</tr>
</tbody>
</table>

*Apparatus: Spectraphoresis Ultra Instrument (Thermo Separation Products-Fremont, California, USA) equipped with an LIF detector ZetaLIF (Picometrics, Ramonville, France) using a 20 mW He-Cd laser source.

### Table 2: Riboflavin levels in white (n = 18), rosé (n = 8) and red (n = 27) wines.*

<table>
<thead>
<tr>
<th>Wines</th>
<th>Minimum value (µg/L)</th>
<th>Maximum value (µg/L)</th>
<th>Mean value (µg/L)</th>
<th>±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
<td>69</td>
<td>151</td>
<td>112</td>
<td>25</td>
</tr>
<tr>
<td>Rosé</td>
<td>74</td>
<td>193</td>
<td>115</td>
<td>45</td>
</tr>
<tr>
<td>Red</td>
<td>156</td>
<td>292</td>
<td>226</td>
<td>40</td>
</tr>
</tbody>
</table>

summarized in Table 2. As can be seen, a significantly higher content of RF is present in red wines while there is a similar average value in white and rosé wines. Greater content variability of RF was found for rosé wines and this is consistent with the fact that their colour is not simply related to grape variety, but mainly a result of the winemaking process.

The CE–LIF method described in this article was also used to evaluate the flavin contents of samples such as milk, baker’s yeast, green leafy vegetables and pharmaceutical vitamin supplements. Figure 3 shows a representative CE–LIF separation of a sample of UHT cow’s milk. The mean contents of RF, FAD and FMN were evaluated as the average of three measurements performed on different samples. Recoveries of RF vitamers from milk samples were higher than 90% in all investigated samples. The amounts of RF, FAD and FMN evaluated in some common foods and beverages are given in Table 3.

Flavins present in baker’s yeast samples were also investigated by CE–LIF (Figure 4). Four samples in three replicates were analysed and found to contain 1110 ± 10 µg total flavins per 100 g of fresh yeast. This level compares favourably to that evaluated by HPLC used in conjunction with conventional fluorescence detection,20 the amounts of RF, FAD and FMN were 194, 567 and 348 µg/100 g, respectively. FAD and FMN amount to 51 and 32% of total flavins, respectively, while the RF content ranges between 70 and 200 µg/100 g of fresh mass. It is probable that such a discrepancy is a result of the different medium conditions in which the growth is performed and its composition as well. Indeed, during the production process such conditions are not strictly fixed, thus considerably affecting the vitamin formation and corresponding content.

The amount of riboflavin in various types of vegetables was determined and the average values are listed in Table 4. For each vegetable, three series of the same fresh sample were weighed exactly and extracted using a simple protocol as described in the experimental section.18 As LIF detector sensitivity is several orders of magnitude better than an absorbance detector, a large sample dilution is allowed, thus reducing matrix effects and the interference of other native fluorescent compounds. Complete triplicate analysis was performed on all these complex matrix foodstuffs to allow the evaluation of average value and standard deviation. A typical electropherogram of a sample of lettuce leaves is shown in Figure 5.

The recovery rates of RF, FAD and FMN were quite satisfactory with a mean value (±SD) of 84 ± 6%. Published tables of nutrient composition data generally list only the total riboflavin (TRF) content of foods (USDA, 1976–1988),21 much of which is of questionable accuracy.22,23 The amounts of vitamin B2, FAD, FMN and TRF content calculated on fresh products were compared with published data in Table 4. As numerous factors affect the vitamin content, including harvest time, varieties and geographic conditions of crop cultivation, it is not unexpected to obtain different TRF values when comparing the same vegetable.24 It is worth noting, however, that so many natural and processed foods contain large amounts of riboflavin, which could satisfy the demand for human nutrition, without biotechnologically derived riboflavin supplements.

Finally, for those not acquainted with the power of CE–LIF measurements, Figure 6 is an additional elegant illustration. This figure shows the excellent selectivity of the CE–LIF method when applied to a pharmaceutical formulation containing nine water-soluble vitamins (ascorbic acid, folic acid, nicotinamide, pantothenic acid, pyridoxine, riboflavin, thiamine, biotin and cyanocobalamin). Upon dissolution of a tablet sample (50 mg in 50 mL of 1% acetic acid solution), heating at 65 °C and simultaneous shaking for 10 min,25 the amount of RF was easily determined, which corresponded to ~95% of the labelled amount. This result confirms that CE–LIF can be considered as an interesting and cost-effective alternative to liquid chromatography for the quality control of multivitamin preparations.

Conclusions

We have presented here a powerful analytical method, which has proved to be a valuable tool for sensitive and accurate
measurements of riboflavin in routine analysis of foodstuffs, beverages and pharmaceutical formulations. CE–LIF promises to pave the way for sensitive and more accurate determinations of riboflavin and flavin derivatives in real matrices and to verify the amounts of RF which undertake photodegradation as well. Future plans are to use CE–LIF to study all flavin-related compounds, both with and without vitaminic properties. In such instances, the availability of standards often compromises (or negates) definite identification. So it is anticipated that LIF detection can be used in combination with mass spectrometry, which may provide unambiguous structural elucidation of very similar flavins.

Acknowledgement

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Table 4: Amount of RF, FAD, FMN and total riboflavin in fresh vegetables evaluated by CE–LIF.a

<table>
<thead>
<tr>
<th></th>
<th>RF</th>
<th>FAD</th>
<th>FMN</th>
<th>TRF</th>
<th>TRF reportedb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lettuce</td>
<td>60±3</td>
<td>173±9</td>
<td>74±4</td>
<td>310±10</td>
<td>180</td>
</tr>
<tr>
<td>Savoy cabbage</td>
<td>34±2</td>
<td>80±4</td>
<td>49±2</td>
<td>163±5</td>
<td>40</td>
</tr>
<tr>
<td>Spinach</td>
<td>61±3</td>
<td>230±10</td>
<td>92±5</td>
<td>390±10</td>
<td>370</td>
</tr>
<tr>
<td>Rocket salad</td>
<td>87±4</td>
<td>112±6</td>
<td>43±2</td>
<td>242±7</td>
<td>90</td>
</tr>
<tr>
<td>Basil</td>
<td>167±8</td>
<td>ND</td>
<td>ND</td>
<td>167±8</td>
<td>310</td>
</tr>
<tr>
<td>Endive</td>
<td>43±2</td>
<td>51±3</td>
<td>16±1</td>
<td>110±4</td>
<td>70</td>
</tr>
<tr>
<td>Cauliflower</td>
<td>47±2</td>
<td>60±3</td>
<td>33±2</td>
<td>140±4</td>
<td>160</td>
</tr>
<tr>
<td>Zucchini</td>
<td>48±2</td>
<td>104±5</td>
<td>42±2</td>
<td>194±6</td>
<td>120</td>
</tr>
</tbody>
</table>

aConcentration value expressed as µg/100 g of fresh product. ND: not detected. bCappelli & Vannucchi, “Chimica degli Alimenti”, Zanchelli Editor (1999) Bologna, Italy, pp 626-628.
References


Donatella Nardiello received her Chemistry degree in March 2000 from the University of Basilicata, Potenza, Italy. Currently, she is a PhD student in Analytical Chemistry under the supervision of Professor Cataldi at the University of Basilicata. Besides capillary electrophoresis, her research activity includes liquid chromatography coupled to amperometric detection. She is a junior fellow of the Italian Chemical Society, Analytical Division. Sabino A. Bufo has extensive experience in the chemistry applied to agriculture and the environment. Main research topics are pesticides retention and degradation in water, soil and food. Tommaso R.I. Cataldi is Professor and Head Director of the Department of Chemistry at the University of Basilicata, Potenza, Italy. His current research includes capillary electrophoresis, electrochemical detection in I.C and the development of novel analytical methods for applications in biotechnology, clinical, forensic, food and environmental samples.