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Assay of Riboflavin in Sample Wines by Capillary Zone Electrophoresis and Laser-Induced Fluorescence Detection

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To routinely assay the concentration of riboflavin (RF) in wines, a rapid and sensitive method was developed and evaluated. The method is based on a simple sample preparation, capillary zone electrophoretic separation and laser-induced fluorescence detection (CZE-LIF). Sample preparation required only dilution and filtration. Under optimized conditions, the limit of detection of riboflavin was 0.5 μg/L, using a hydrodynamic sample introduction of 10 s at 54 mbar. The method was fully validated: the recovery of RF in wines was >95%. The concentrations of RF within the three sample types of Italian wines investigated here ranged from 69 to 151 μg/L with a mean value (±SD) of 112 ± 25 μg/L, from 74 to 193 μg/L with a mean value of 115 ± 45 μg/L, and from 156 to 292 μg/L with a mean value of 226 ± 40 μg/L, for white, rosé, and red wines, respectively. Such an accurate and highly sensitive CZE-LIF method represents a powerful improvement over previous methods in terms of sensitivity, simplicity, and efficiency. It is well suited to satisfy the demands for accurate and sensitive detection with minimal sample preparation and cleanup.

KEYWORDS: Wine; capillary electrophoresis; riboflavin; vitamin B_2; laser-induced fluorescence detection; flavin cofactors

INTRODUCTION

Riboflavin (RF) is a water-soluble vitamin (vitamin B_2) that was named from the Latin word *flavius* (yellow) to denote the deep color of crystals formed from the pure compound. It is a micronutrient that was first isolated from egg albumen in 1933 (*1*−*3*). RF is very stable during thermal processing, storage, and food preparation but is susceptible to degradation on exposure to light. Dietary riboflavin is present especially in liver, cheese, milk, meat, eggs, peas, beans, whole grain cereals, and even wines. Apparently, the main agent involved in the off-flavor of wine, as well as other beverages, is riboflavin, which acts as a photochemical sensitizer, provoking the photodegradation of sulfur-containing compounds (*4*−*6*). These substances are formed in grapes during ripening. Moreover, during the wine-making process sulfite is deliberately added to most wines as an antioxidant and antimicrobial agent. Owing to their high volatility and reactivity, sulfur-containing compounds have a profound effect on the flavor of wine. The outcome is that the photodegradation process leads to adverse effects on the RF content, wine color, and flavor as well, mainly when white wine is bottled in clear or green containers.

It is supposed that sensitive analytical techniques for detecting riboflavin and flavin cofactors (FAD and FMN) should be of general interest with regard to the extensive number of foods and beverages as well as biological samples in which they occur. Detailed knowledge of the role of RF in wines can be successfully gained only if a sensitive, reliable, and rapid analytical method is available, which should be applicable to all varieties of wines. Although high-performance liquid chromatography (HPLC) is a popular analytical technique for the determination of RF in foodstuffs and biological samples (*7*−*21*), and some reference papers and a book have been published that describe the technology in detail (*22*−*24*), the technique suffers from a number of drawbacks. These include the need to use gradient elution to separate the compounds of interest in a reasonable time set, a significant consumption of mobile phase, and unsatisfactory peak resolution, especially when real samples with a relevant number of UV-absorbing constituents, such as red wines, are involved.

Over the past decade capillary zone electrophoresis (CZE) has gained much in popularity, mainly because of the high resolutions that are attainable in the separation of both ionogenic compounds and neutral molecules (*25*). Poor sensitivity of traditional absorbance-based detectors is the major problem commonly associated with CZE−UV−vis applications, but laser-induced fluorescence (LIF) detection is recognized to be an extremely sensitive detection method. Mass detection limits may easily reach the attomole range because of the small injection volume (i.e., nanoliter samples). Recently, much effort has been devoted tor the determination of riboflavin and flavin

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cofactors in biological samples by capillary electrophoresis (*26*) and micellar electrokinetic chromatography (*27*). More recently, the experimental conditions in CZE with LIF detection were optimized and successfully applied to the analysis of food samples (*28*). Here we describe a rapid, sensitive, and very selective one-step method for riboflavin assay in wines of different origin, vintage, and variety by CZE-LIF. Benefiting from its intrinsic fluorescent nature, RF can be directly detected at very low amounts using a slightly alkaline phosphate buffer. The high selectivity of detection (i.e., excitation wavelength at 442 nm and emission wavelength >515 nm) would be specific enough to differentiate RF from other fluorescing compounds present in wines. To verify the quality and usefulness of the method, the analytical parameters linearity, sensitivity, precision, and percentage of recovery were determined. 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.

MATERIALS AND METHODS

Chemicals. All of the 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); sodium hydroxide, disodium phosphate, and ammonium acetate were also obtained from Sigma-Aldrich. Buffer solutions were prepared with ultrapure water supplied by a Milli-Q RG unit from Millipore (Bedford, MA).

Samples and Sample Preparation. All commercial wines from different brands were purchased from local markets or kindly offered by local producers. Standard solutions of RF, FAD, and FMN were prepared by dissolving in double-distilled Milli-Q water to give concentrations in the range of 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 buffer solution used for electrophoretical runs was sonicated and filtered through 0.45-μm membrane filters (Whatman International Ltd., Kent, U.K.); the pH of the phosphate running buffer was adjusted by the addition of appropriate amounts of hydrochloric acid or sodium hydroxide.

Apparatus and Method. CZE separation was performed on a Spectraphoresis Ultra instrument (Thermo Separation Products, Fremont, CA) equipped with a multiwavelength UV−vis scanning detector (Spectrasystem UV 3000) and an LIF detector ZETALIF (Picometrics, Ramonville, France) connected to a 20-mW He−Cd laser source; an uncoated fused-silica capillary (Thermo Separation Products), used throughout the analysis, had an internal diameter of 75 μm and an effective length of 62 cm to the UV detector and 84 cm to the LIF detector. Prior to use, the capillary was rinsed with 1 N NaOH and water for 1 h and subsequently with the separation buffer for 30 min. Every morning at the beginning of the 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 (30 mM) for 5 min. Samples were introduced into the anodic end of the capillary by pressure injection for 10 s at 0.8 psi (54 mbar). All experiments were conducted in normal polarity mode at an applied voltage of 30 kV; the temperature of the capillary was maintained at 15 °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.

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. The resulting peak areas were plotted against concentration for the calibration curve. The RF content of sample wines was obtained by interpolation on the standard curve.

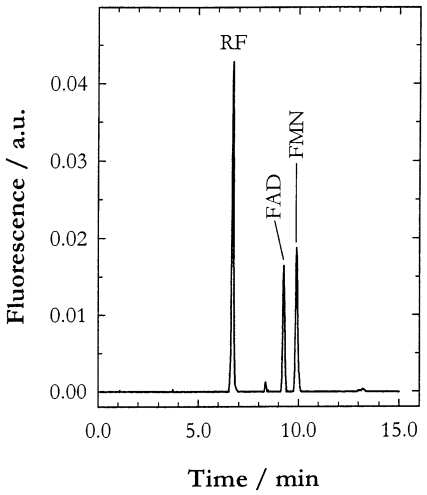

Figure 1. CZE-LIF separation of RF, FAD, and FMN at the equimolar concentration of 250 nmol/L. Experimental conditions: electrophoretic buffer, 30 mM phosphate solution at pH 9.8; hydrodynamic injection at 54 mbar for 10 s; running at +30 kV; current, 60 μA; effective capillary length, 84 cm; total length, 92 cm. LIF detection with an He–Cd laser source having an excitation wavelength of 442 nm was used. Relative fluorescence units (RFU) were recorded over the integration range above 515 nm.

Table 1. Reproducibility of Migration Times for Intra- and Interday Analysis of a Mixture of Flavins by CZE-LIF[a]

	intraday ($n = 5$)		interday[b] (7 days, $n = 21$)	
compound	t_m (min)	%RSD	t_m (min)	%RSD
riboflavin	7.1	2.5	7.6	3.4
FAD	9.4	1.8	10.2	3.9
FMN	10.8	2.6	11.4	4.3

[a] Separation conditions as in **Figure 1**. [b] The same capillary was employed during the experiments.

RESULTS AND DISCUSSION

Assay Performances of Riboflavin Vitamers. Figure 1 illustrates the CZE-LIF separation of an equimolar solution (250 nmol/L) of RF, FAD, and FMN. The following optimized experimental conditions were employed: 30 mM phosphate buffer at pH 9.8, an applied voltage of 30 kV, a temperature of 15 °C, and a hydrodynamic injection of 10 s at 54 mbar (*28*). Considering that FAD and FMN exhibit low pK_a values (*29*), the migration of RF, FMN, and FAD follows their charge/size ratio order (FMN > FAD > RF) with the RF position, in terms of migration time, being the first as its pK_a is 10.2 (*30*). Consistently, the use of a phosphate buffer at pH 9.8 ensures the separation of RF as an ionic compound, thus avoiding the electromigration with other neutral and native fluorescent compounds, which may occur in real matrices. Apparently, the fluorescence intensity of FAD and FMN is lower than that of RF, being approximately only 36 and 44%, respectively. Although intramolecular stacking of the adenine moiety on the isoalloxazine ring of the flavin has been invoked to explain the fluorescence of FAD when compared with RF (*31*, *32*), it is not clearly the reason for the lower intensity relative to FMN.

Table 1 summarizes the results for the reproducibility study carried out under the conditions of **Figure 1**. As can be seen, relative standard deviations (RSD) of migration times were

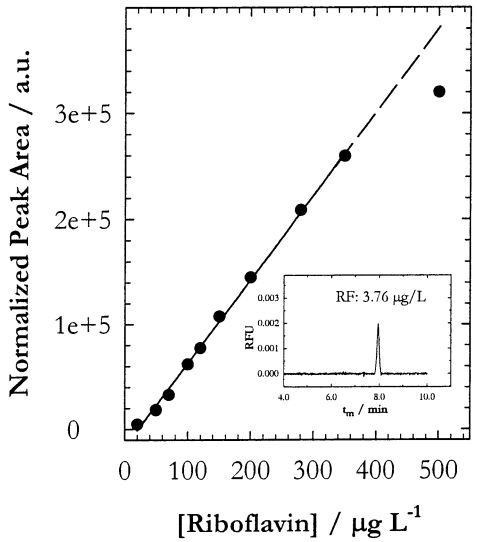

Figure 2. Calibration curve of RF obtained by CZE-LIF. (Inset) Electropherogram recorded at a riboflavin concentration of 10 nmol/L (3.76 μg/L). Conditions were the same as in **Figure 1**.

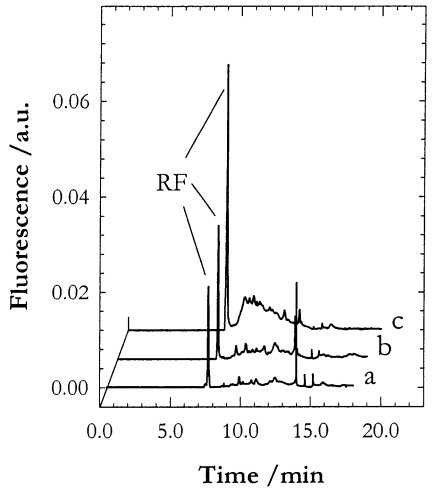

Figure 3. Typical separations by CZE-LIF of three Italian samples of white (a), rosé (b), and red (c) wines. The wine samples correspond to numbers 2, 1, and 11 in **Tables 2**, **3**, and **4**, respectively. Conditions were the same as in **Figure 1**.

<2.6% ($n = 5$) for the same day, whereas these values increase to 4.3% when the same experiment was repeated on seven different days ($n = 21$). The RSD of peak areas within-day and interday was between 2 and 5%, respectively.

Whereas FAD and sometimes FMN are present along with RF in fruit juices and beers, RF is the only form of flavin occurring in musts and wines (*6*). Therefore, our interest was mainly focused on the quantitative determination of riboflavin by CZE-LIF. The correlation between the normalized peak area (*33*), peak area divided by the corresponding migration time, and concentration was examined in the range of 0.5−500 μg/L. Ten concentration levels of the standard solution and three replicate injections were used for calibration; the linearity was guaranteed up to 350 μg/L. The concentration−response curve of RF, exhibiting a correlation coefficient $r = 0.9995$, is shown in **Figure 2**; the electropherogram in the inset was recorded using a very dilute solution of 10 nmol/L (3.76 μg/L). The baseline noise was determined using the mean peak-to-peak noise, and, at a signal-to-noise ratio of three, the limit of detection (LOD) was 0.5 μg/L. As the evaluated injection volume was ~40 nL (injection at 54 mbar and 10 s), the on-column LOD of RF corresponded to 50 amol. Clearly, it is possible to obtain a somewhat lower LOD by the use of longer injection times up to 30 s without exceeding 1% of the whole capillary length (*34*). As detector sensitivity is several orders of magnitude better than that of an absorbance detector, a large sample dilution is allowed, thus reducing matrix effects and the interference of other native fluorescent compounds (see next section).

Quantitative Determination of Riboflavin in Wines. We used the above-described assay to determine the RF content of several wine samples. Before the results obtained on white, rosè, and red wines are discussed, data relevant to the recoveries of riboflavin and flavin cofactors are presented. These data were evaluated by spiking the wine samples with pure compounds at the level of 50−100% of the measured content and performing triplicate assays before and after each addition. On the basis of their high water solubility, excellent recovery of flavin compounds was obtained, which on average ranged from 97.2 ± 1.0% for FAD and FMN to 104.5 ± 3.4% for RF, and coefficients of variation ranged between 0.9 and 3.3%. There were no consistent differences in recoveries from white, rosé, and red wines.

Table 2. Riboflavin Content (Micrograms per Liter) of Some Italian White Wines Determined by CZE-LIF[a]

white wine	vintage	mean ± SD ($n = 3$)
1. Guardiolo	1999	80 ± 2
2. Locorotondo	1996	151 ± 3
3. Locorotondo	1997	120 ± 2
4. Martina	2000	89 ± 2
5. Vulcanello	1998	84 ± 2
6. Greco	2000	126 ± 3
7. Tavernello	2000	94 ± 2
8. Vermentino di Sardegna	1999	135 ± 3
9. Cordicello	2000	80 ± 2
10. Amineo	1999	107 ± 2
11. Coda di Volpe	2000	127 ± 3
12. Fiano di Caserta	2000	146 ± 3
13. Fiano di Salerno	2000	119 ± 2
14. Fiano di Avellino	2000	139 ± 3
15. Pinot	1998	130 ± 3
16. Pinot	2000	69 ± 3
17. Leon de Tarapaca[b]	1999	103 ± 4
18. Spumante Gancia	2000	145 ± 5

[a] Three replicate analyses for each sample wine were performed. [b] White wine form Chile.

As the RF level seems to be a key factor in assessing the risk of sunlight-flavor appearance, routine analysis of musts and wines should be carried out, although it is not advisable to define a security level of RF below which the wine may be considered safe. Indeed, several compounds affect the light stability of wine, such as those playing a role in redox reactions, for example, transition metals and polyphenols, along with the actual precursors of the off-flavor, the sulfur-containing compounds. Thus, the rationale behind testing the RF content of wines as a function of cultivar, origin, wine-making techniques, etc., was twofold: (1) to determine the amount to RF and verify correlations between young wines and their aged counterparts and (2) to establish the possibility of analysis also in those samples that exhibit a significant presence of tannins and polyphenol compounds. In **Figure 3** the typical electropherograms of three sample wines, white, rosé, and red, are shown. As already stated, no presence of FAD and FMN was found in all samples examined. It should be mentioned, although, that in the electropherogram of red wines (see plot c in **Figure 3**), the time window where the migration of FAD and FMN occurs is

Table 3. Riboflavin Content (Micrograms per Liter) of Some Italian Rosé Wines Determined by CZE-LIF

rosé wine	vintage	mean ± SD ($n = 3$)
1. Brundisium	2000	129 ± 2
2. Pipoli	1999	139 ± 3
3. Pinot Rosa	2000	79 ± 4
4. Tavernello	2000	74 ± 2
5. Paternoster	1998	74 ± 2
6. San Severo	1999	75 ± 2
7. F.lli Vavallo	2000	156 ± 4
8. Don Giovanni	2000	193 ± 3

Table 4. Riboflavin Content (Micrograms per Liter) of Some Italian Red Wines Determined by CZE-LIF

red wine	vintage	mean ± SD ($n = 3$)
1. Mustilli	1995	227 ± 4
2. Mustilli	1996	214 ± 4
3. Mustilli	1997	240 ± 5
4. Mustilli	1998	224 ± 4
5. Mustilli	1999	239 ± 5
6. Tavernello	2000	196 ± 4
7. Lambrusco	2000	156 ± 3
8. Chianti	1999	231 ± 5
9. Barbera	2000	178 ± 4
10. Solopaca	2000	193 ± 4
11. Mionetto	2000	218 ± 4
12. Corvino	2000	256 ± 5
13. Napolitano	1997	292 ± 6
14. D'Angelo	1996	236 ± 5
15. Martino	1996	270 ± 5
16. Martino	1997	225 ± 4
17. Carato Venusio	1995	291 ± 6
18. Carato Venusio	1996	268 ± 5
19. I Portali	1996	203 ± 4
20. I Portali	1997	168 ± 3
21. Pipoli	1997	232 ± 5
22. Paternoster	1985	221 ± 4
23. Paternoster	1993	184 ± 4
24. Paternoster	1994	193 ± 4
25. Paternoster	1995	180 ± 4
26. Paternoster	1997	292 ± 6
27. Paternoster	1998	266 ± 5

overcrowded by several fluorescent compounds. This makes the identification and quantification of very small amounts of FAD and FMN less certain.

Numerous Italian wines were analyzed and, for each of them, the mean content of RF was evaluated as the average of three measurements performed on different aliquots of the same wine. The levels of RF in white, rosé, and red wines of different cultivars, origins, and vinifications are listed in **Tables 2**, **3**, and **4**, respectively. As expected, relatively high amounts of RF were present in all samples examined. Whereas the lowest value of RF in red wines was 156 μg/L (Lambrusco, 2000), the highest value of 292 μg/L was found in two different wines of the same vintage (Paternoster and Napolitano, 1997); the average value was 226 μg/L with a standard deviation of 40 μg/L. The mean value obtained for rosé wines was 115 μg/L with a relatively high standard deviation of 45 μg/L and outlier values of 74 and 193 μg/L for Tavernello and Don Giovanni wines, respectively. As far as white wines, the average value of RF was 112 μg/L, which is close to that reported by Mattivi et al. (*10*), namely, 98.6 μg/L, when wines produced in Spain, Slovenia, and Italy were analyzed. Note that no correlation was found between wine aging and flavin content, and this is probably due to the fact that RF concentration is influenced by several factors, such as grape variety and their ripeness grade, climatic and environmental conditions, cropping practices, and manufacturing procedures. In **Figure 4** are shown the average values (±SD) of RF determined in the present work. As can be

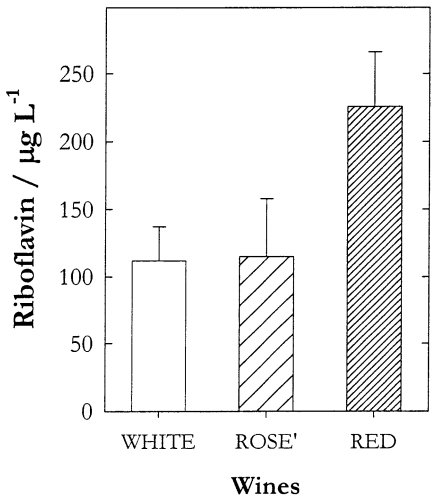

Figure 4. Plot of the RF mean levels (±SD) in white ($n = 18$), rosè ($n = 8$), and red ($n = 27$) wines.

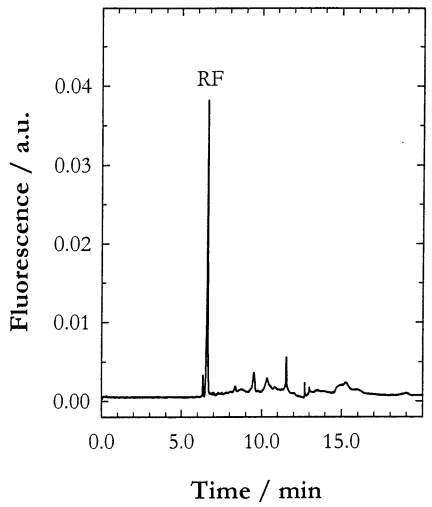

Figure 5. Sample of sparkling wine (Gancia, 2000) separated by CZE and LIF detection. Conditions were the same as in **Figure 1**.

seen, there is a slight increase in the average values of vitamin B_2 from white to rosé wines, whereas a significantly higher content in RF is present in red wines. However, the lowest value evaluated in red wines was higher than the highest value determined for white wines. Greater variability of the RF content was found for rosé wines, and this is consistent with the fact that their color is not simply related to the grape variety but is mainly due to the wine-making process.

Finally, we determined the amount of RF in a sparkling wine which is likely to develop the sunlight flavor. **Figure 5** shows the typical electropherogram of a spumante sample (Gancia, 2000); as expected, no presence of interfering substances was found. The amount of RF was relatively high (145 ± 5 μg/L) compared with the mean value obtained for white wines. This aspect is particularly important when white wines are bottled in clear glass containers that are more exposed to RF photodegradation. The proposed method enables simple, highly sensitive, and very rapid examination of a large number of samples.

Conclusions. The method proposed for the quantitative determination of riboflavin in wines and sparkling beverages by CZE-LIF offers very interesting features, such as simplicity, low cost, rapidity, and a very important improvement in sensitivity and selectivity as compared to conventional chromatographic techniques. A major benefit of the use of CZE with

LIF detection is that exceedingly high sensitivity is obtained. The method is well suited to satisfy the demands for accurate and sensitive detection of RF and flavin derivates with minimal sample preparation and cleanup. Although this method was developed and validated specifically for wines and beverages, it might be applied to several food samples. We believe that CZE-LIF is especially suitable for studies involving changes of concentration of soluble flavins biotechnologically produced by genetically modified microorganisms, so work is currently under way along this direction.

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