ELSEVIER

Food Chemistry 82 (2003) 309–314

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods Section

Assessment of riboflavin and flavin content in common food samples by capillary electrophoresis with laser-induced fluorescence detection

Tommaso R.I. Cataldi[a,*], Donatella Nardiello[a], Veronica Carrara[a], Rosanna Ciriello[a], Giuseppe E. De Benedetto[b]

[a]*Dipartimento di Chimica, Università degli Studi della Basilicata, Via N. Sauro, 85, 85100 Potenza, Italy*
[b]*Dipartimento dei Beni, delle Arti e della Storia, Monastero degli Olivetani, V.le San Nicola, 73100 Lecce, Italy*

Received 2 August 2002; received in revised form 8 November 2002; accepted 8 November 2002

Abstract

To routinely assay the flavin contents in foodstuffs, a rapid and sensitive method was developed, in which the powerful separation capabilities of high-performance capillary electrophoresis (CE) were exploited. The method is based on a simple sample preparation, electrophoretic separation and laser induced fluorescence (LIF) detection. The average content of water-soluble riboflavin vitamers in raw natural products (i.e. vegetables, wheat flours and tomatoes) and baker's yeasts was evaluated without interferences from the sample matrices. Such an accurate and highly sensitive CE-LIF technique represents a significant improvement over previous analytical methods in terms of sensitivity, simplicity and efficiency. Indeed, it is well suited to satisfy the demands for accurate and sensitive detection with minimal sample preparation and clean-up.

Keywords: Capillary electrophoresis; Vitamin B_2; Flavins; Vegetables; Wheat flour; Tomato juice; LIF

1. Introduction

Everyone is aware of the role of vitamins in the human diet. Riboflavin (RF) is a water-soluble vitamin (vitamin B_2) that occurs in several foods and beverages, such as liver, cheese, milk, meat, eggs, peas, beans, whole-grain cereals, and even wines (AMC, 2000; AOAC, 1995; Capo-chichi, Gueant, Feillet, Namour & Vidailhet, 2000; Gliszczyñska-Świgło & Koziołowa, 1998, 2000; Heelis, 1991; Web page, 2002). RF is very stable during thermal processing, storage, and food preparation, but is susceptible to degradation on exposure to light (Mattivi, Monetti, Vrhovšeket, Tonon, & Andrés-Lacueva, 2000). The analysis of riboflavin and flavin cofactors (flavin adenine dinucleotide, FAD, and flavin mononucleotides, FMN) is of considerable importance to the food and beverage industry during manufacturing and storage. It is supposed that sensitive analytical techniques for detecting flavin vitamers should be of general interest with regard to the relevant number of natural products as well as biological samples in which they occur (AOAC, 1995). A detailed knowledge of the riboflavin and flavin contents in vegetable products can be obtained only if a sensitive, reliable and rapid analytical method is available, which should be applicable to a large variety of samples. While HPLC is a popular analytical technique for the determination of riboflavin in foodstuffs and biological tissues, and some reference papers (Esteve, Farré, Frígola, & García-Cantabella, 2001; Ollilainem, Mattila, & Varo, 1990; Reyes, Norris, Taylor, & Potts, 1988; Ribarova, Shishkov, Obretenova, & Metchkneva 1987; Russell & Vanderslice, 1992; van den Berg, van Schaik, Finglas, & de Froidmont-Görtz 1996; Vidal-Valverde & Reche, 1990) and a book have been published that describes the technology in detail (Nielsen, 2000), 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 unsatisfac-

* Corresponding author. Tel.: +39-971-202-228; fax: +39-971-202-228.
E-mail address: cataldi@unibas.it (T.R.I. Cataldi).

0308-8146/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0308-8146(02)00567-8

tory peak resolution, especially when real samples with a relevant number of UV-absorbing constituents are under investigation.

Over the last decade capillary zone electrophoresis with laser induced detection (CE-LIF) has gained much in popularity. While high resolutions can be obtained in the separation of both ionogenic and neutral compounds (Khaledi, 1998), LIF detection is recognized to be an extremely sensitive detection method. Recently, much effort has been devoted for the determination of riboflavin and flavin cofactors in biological samples by capillary electrophoresis (Pérez-Ruiz, Martínez-Lozano, Sanz, & Bravo, 2001) and micellar electrokinetic chromatography (Hustad, Ueland, & Schneede, 1999). More recently, the experimental conditions in CE with LIF detection were optimized and successfully applied to the analysis of milk and wine samples (Cataldi, Nardiello, De Benedetto, & Bufo, 2002a; Cataldi, Nardiello, Scrano, & Scopa, 2000b).

Here we describe a routine, sensitive, and very selective one-step method for riboflavin assay in common natural products by CE-LIF. Benefiting from its intrinsic fluorescent nature, flavins can be selectivity detected at very low concentrations using a middle basic running electrolyte (i.e. phosphate buffer at pH 9.8). The proposed method enables simple, quantitative and very rapid examination of a large number of samples. It can be applied to monitoring the content of flavin vitamers in food and beverage production plants.

2. Materials and methods

2.1. Chemicals

All the chemicals used in this study were of analytical grade. Riboflavin 98%, flavin adenine dinucleotide 97%, flavin mononucleotide 95%, sodium hydroxide, disodium phosphate and ammonium acetate were purchased from Sigma-Aldrich (Steinheim, Germany). Buffer solutions were prepared with pure water supplied by Milli-Q RG unit from Millipore (Bedford, MA, USA). The buffer solution used for electrophoretical runs were sonicated and filtered through 0.45-μm membrane filters (Whatman International Ltd., Kent, UK); the pH of the phosphate running buffer was adjusted by addition of appropriate amounts of sodium hydroxide.

2.2. Samples and sample preparation

All samples were purchased from local markets or kindly offered by local producers. While samples of spinach leaves (*Spinacea oleracea* L.), lettuce leaves (Lactuca sativa var. capitata), zucchini (*Cucurbita pepo* conv. *giromontina*), savoy cabbage (*Brassica oleracea* L. var. *sabauda*), rocket salad (*Eruca sativa* L.), basil (*Ocimum basilicum* L.), endive (*Cichorium endivia* L.), cauliflower (*Brassica oleracea* L. var. *botrytis*) were lyophilized for moisture determination, baker's yeasts, tomato juice, and durum and soft wheat flours milled (*Triticum aestivum* L.) were used without any treatment. Spinach and lettuce leaves, zucchini, savoy cabbage, rocket salad, basil, endive and cauliflower were washed with deionized water, drained, and chopped to small pieces. Extraction of flavins from vegetables was performed as described elsewhere (Gliszczyñska-Świgło & Koziołowa, 2000). A 0.5–1.0 g sample was transferred in extraction tubes, suspended in 19 ml of a CH_3OH/CH_2Cl_2 mixture (9:10, v/v) and blended for ca. 1 minute. Upon addition of 9 ml of 0.1 M ammonium acetate at ca. pH 6, the mixture was additionally shacked and centrifuged for 20 min at 5000 rpm and 4 °C. The clear extract solution was completely transferred in a volumetric flask and diluted up to 50 ml using water; an appropriate amount was filtered through 0.22-μm membrane filters (Schleicher & Schuell, Dassel, Germany) and introduced into the CE system for quantification of the flavin content. All food samples were analyzed in triplicate and results were calculated as the average of the respective replicates.

2.3. Apparatus and method

CE separations were performed on a Spectraphoresis Ultra Instrument (Thermo Separation Products-Fremont, CA, USA) equipped with a multi-wavelength UV-VIS scanning detector (Spectrasystem UV 3000) and a LIF detector ZETALIF (Picometrics, Ramonville, France) connected to 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 one hour 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, then water and phosphate run buffer for approximately 5, 5 and 15 min, respectively. 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 capillary was maintained at a temperature of 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.

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. The resulting peak areas were plotted against concentration for the calibration curve. The content of riboflavin, FAD and FMN in the real samples was obtained by interpolation on the corresponding calibration curve.

3. Results and discussion

Before illustrating the results obtained on real samples, data relevant a standard mix separation and recoveries of riboflavin and flavin cofactors are presented. Details of the analytical method employed here are summarized in Table 1. A typical electropherogram in CE-LIF of riboflavin, FAD and FMN is illustrated in Fig. 1. As can be seen, using the optimized experimental conditions (Cataldi et al., 2002) excellent separation was achieved with symmetrical peaks in the migration time window comprised between 7 and 12.5 min. For quantitative determinations the correlation between the peak area and concentration of each flavin was examined in the range of 0.5–500 μg/l. As suggested by Baker (1995), 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. Considering an estimated injection volume of approximately 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 attomole (10^{-18} mole), respectively. At the concentration of 5 nmol/l, the peak area of RF determined by the LIF detector exhibited a coefficient of variation of 3.7% ($n=5$). Such an accurate and highly sensitive CE-LIF method, combined with a simple extraction procedure, shows potential for quantification of water-soluble flavins in most natural products.

Recovery data were evaluated by spiking the 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 riboflavin, and coefficients of variation comprised between 0.9 and 3.3%.

The usefulness of such well established experimental conditions in CE-LIF is illustrated by a comprehensive set of determinations. The method was applied to vegetable food samples with good results. Eight mixed vegetables (leaves or other plant portions) were analyzed, and all of them exhibited the presence of RF, FAD and FMN up to 167, 230, and 92 μg/100 g of fresh sample, respectively, suggesting that flavins are normal minor components in these raw natural products. Whereas Table 2 collects the flavin content determined by CE-LIF, in Fig. 2 the typical electropherogram of a sample extracted from zucchini purchased at a local store is shown. The mean composition was evaluated as the average of three measurements performed on different amounts of the same sample. Notably, comparison of the flavin content among common foods of vegetable origin (see Table 2) is only indicative of intervarietal differences. Indeed, additional work need to be performed

Table 1
Details of the CE-LIF experimental conditions

Capillary dimensions	(92 cm total length) 84 cm × 75 μm i.d.
Running solution	30 mM phosphate buffer at pH 9.8
Separation voltage and temperature	30 kV and 15 °C
Average current	60 μA
Injection	Pressure injection for 10 s at 54 mbar
Laser source and line	He-Cd laser source at 442 nm and 20 mW
Fluorescence detection	Fluorescence wavelength > 515 nm

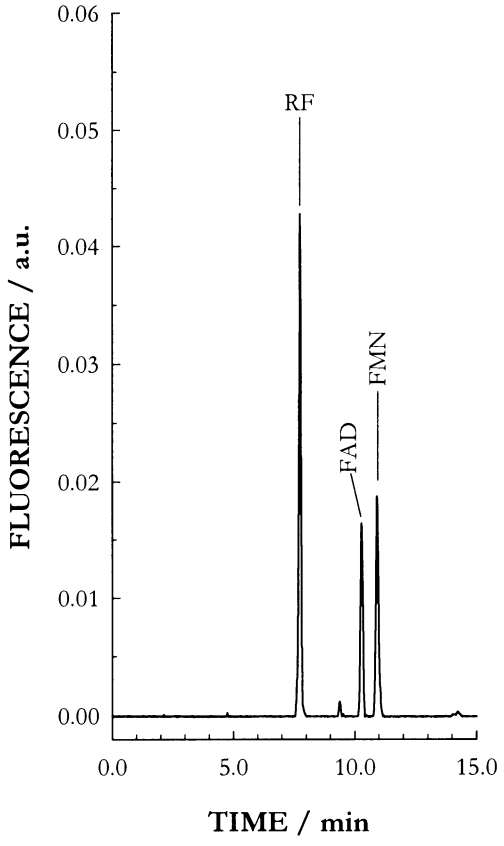

Fig. 1. CE-LIF separation of RF, FAD and FMN at the equimolar concentration of 250 nmol/l. The experimental conditions are collected in Table 1.

Table 2
Amount of RF, FAD, and FMN (μg/100 g of fresh product) in some vegetables samples ($n=3$) evaluated by CE-LIF[a]

	RF	FAD	FMN
Lettuce leaves	60±3	173±5	74±3
Savoy cabbage	34±2	80±4	49±2
Spinach	61±3	230±6	92±3
Rocket salad	87±4	112±4	43±2
Basil	167±5	ND[b]	ND
Endive	43±2	51±3	16±1
Cauliflower	47±2	60±3	33±2
Zucchini	48±2	104±3	42±2

[a] Separation conditions as in Table 1.
[b] Not detected.

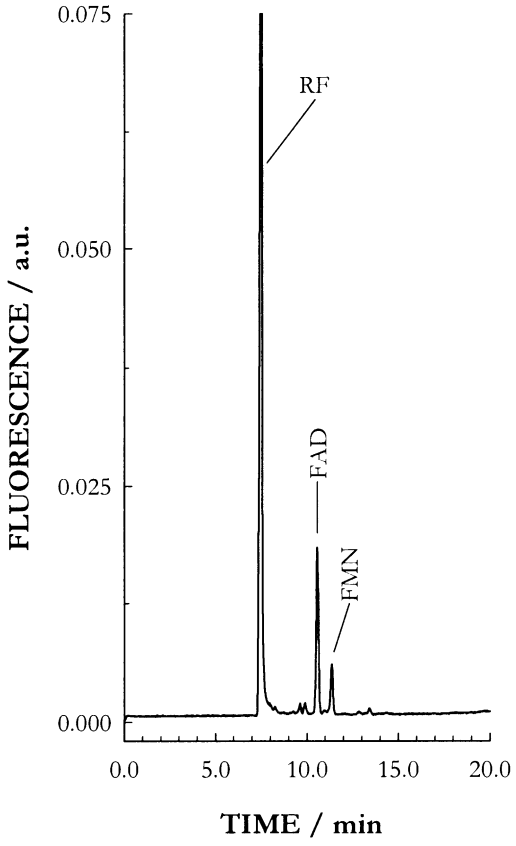

Fig. 2. Typical separation by CE-LIF of an extracted sample of zucchini.

with a representative number of samples relevant each product to validate the observed variability on the flavin level.

Analogous results were obtained for other common foodstuffs. Fig. 3 shows the electropherogram of flavins obtained for a sample of tomato juice. Although the dominant peak is that of RF, resolved peaks due to FAD and FMN, on a very flat baseline, were also clearly detectable. According to previous results (Cataldi et al., 2002b), concerning LIF sensitivity (e.g. fluorescence emission) of these compounds (i.e. RF > FAD≈FMN), the concentration of FMN (290.8 μg/l) was relatively high compared with the mean values of FAD (15 μg/l) and RF (137.3 μg/l). Whereas accurate identification of flavin vitamers was easily made by spiking each sample with small amount of RF, FAD and FMN, accurate quantification still needs to be checked by using certificate reference materials. Work is in progress along this direction. These preliminary results demonstrate, however, that the method is able to measure the very low concentrations of flavins in complex matrices by using a simple extraction procedure.

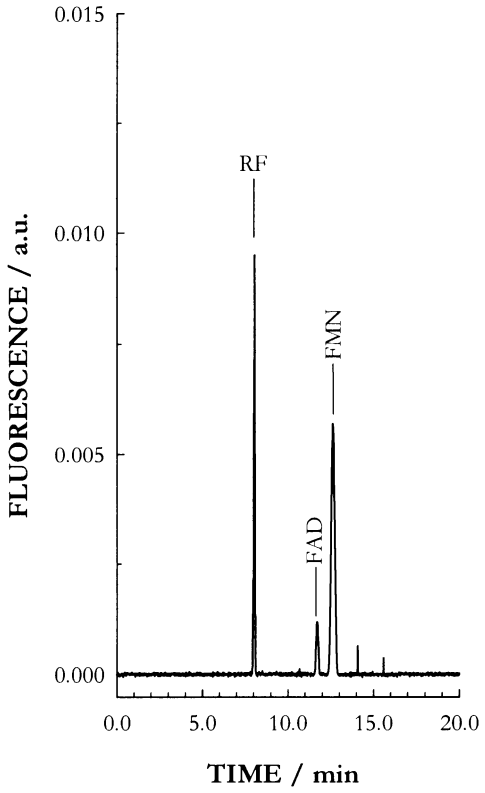

Fig. 3. CE-LIF electropherogram of a sample of tomato juice.

The method was also used to separate RF, FAD and FMN which occur in wheat flours and baker's yeasts. In Table 3 are summarized data of three commercially available wheat flours, durum, soft and maize on a 15% moisture basis, and Fig. 4 shows a typical electropherogram of a sample from durum wheat flour. All

Table 3
Flavins contents (μg/100 g) in commercially available flours evaluated by CE-LIF[a]

	RF	FAD	FMN
Soft-wheat flour	26.5±0.5	24±0.5	18±0.5
Maize flour	35.4±0.5	31±0.5	16±0.4
Durum-wheat flour	50.2±0.7	ND	ND

[a] Three replicate analyses for each sample were performed.

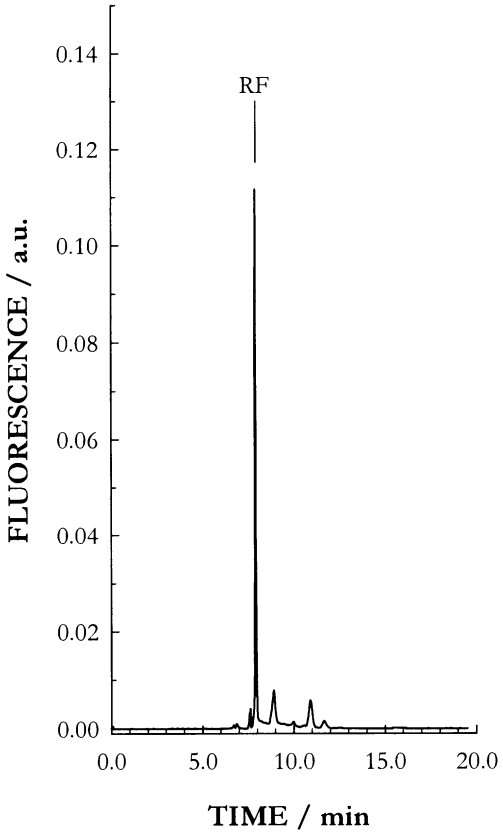

Fig. 4. CE-LIF electropherogram of a sample of durum wheat flour.

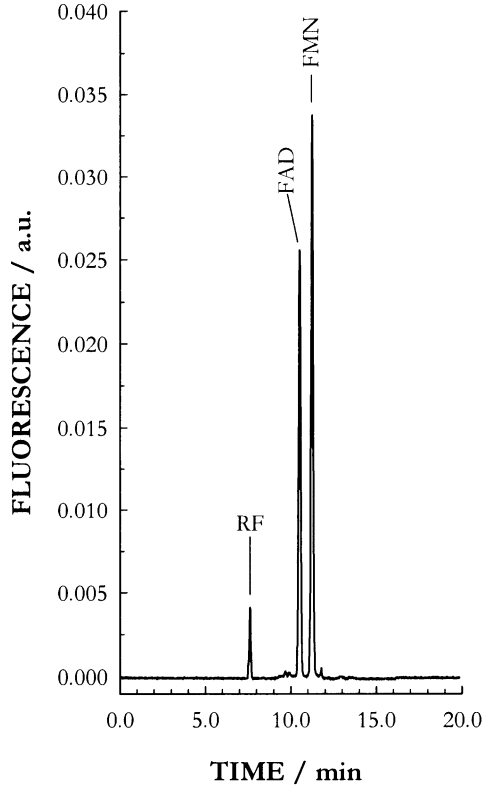

Fig. 5. CE-LIF electropherogram of a baker's yeast sample.

wheat flours assayed exhibited a very similar flavin pattern. Whether the generally lower variability observed for the flour samples is due to the relatively low contents, or is also related to specific problems by the cereal matrix, remains to be established. We wish to highlight that further studies are needed to investigate the role of flavin vitamers in these samples, and such quantifications may be accomplished by the developed CE-LIF method. In Fig. 5 the electrophoretic profile of flavins in a commercially available baker's yeast is shown. Resulting yeast flavin contents were 74 ± 2, 172 ± 3, and 205 ± 3 μg/100 g of product, for RF, FAD, and FMN, respectively. The amount of RF is within the range reported by Rose and Harrison (1970) on a dry mass basis.

The potential of CE-LIF has been illustrated by determining flavin vitamers in several food matrices with minimal sample preparation and clean-up. A major benefit of this analytical approach is that exceedingly high sensitivity can be obtained.

Acknowledgements

We gratefully acknowledge the financial support provided by a research grant "Assegno di Ricerca" to D. N. from University of Basilicata.

References

AMC, Analytical Methods Committee (2000). *Analyst*, *125*, 353–360.

AOAC, Association of Official Analytical Chemists. (1995). *Official methods of analysis* (16th ed.) [Methods 970.65 e 981.15 Vol. II Chapter 45, 9–12]..

Baker, D. R. (1995). *Capillary electrophoresis*. New York: John Wiley.

Capo-chichi, C. D., Gueant, J.-L., Feillet, F., Namour, F., & Vidailhet, M. (2000). Analysis of riboflavin and riboflavin cofactor levels in plasma by high-performance liquid chromatography. *Journal of Chromatography B*, *739*, 219–224.

Cataldi, T. R. I., Nardiello, D., De Benedetto, G. E., & Bufo, S. A. (2002a). Optimizing separation conditions for riboflavin, flavin mononucleotide and flavin adenine dinucleotide in capillary zone electrophoresis with laser-induced fluorescence detection. *Journal of Chromatography A*, *968*, 229–239.

Cataldi, T. R. I., Nardiello, D., Scrano, L., & Scopa, A. (2002b). Assay of riboflavin in sample wines by capillary zone electrophoresis and laser-induced fluorescence detection. *Journal of Agricultural and Food Chemistry*, *50*, 6643–6647.

Esteve, M.-J., Farré, R., Frígola, A., & García-Cantabella, J.-M. (2001). Simultaneous determination of thiamin and riboflavin in mushrooms by liquid chromatography. *Journal of Agricultural and Food Chemistry*, *49*, 1450–1454.

Gliszczyñska-Świgło, A., & Koziołowa, A. (1998). Chromatographic determination of flavin derivates in baker's yeast. *Journal of Chromatography A*, *822*, 59–66.

Gliszczyñska-Świgło, A., & Koziołowa, A. (2000). Chromatographic determination of riboflavin and its derivates in food. *Journal of Chromatography A*, *881*, 285–297.

Heelis, P. F.. In F. Muller (Ed.), *Flavins and flavoproteins* (pp. 171–193). Boca Raton: CRC Press.

Hustad, S., Ueland, P. M., & Schneede, J. (1999). Quantification of riboflavin, flavin mononucleotide, and flavin adenine dinucleotide in human plasma by capillary electrophoresis and laser-induced fluorescence detection. *Clinical Chemistry*, *45*, 862–868.

Khaledi, M. G. (1998). *High-performance capillary electrophoresis: theory, techniques, and applications*. New York: John Wiley.

Mattivi, F., Monetti, A., Vrhovšeket, U., Tonon, D., & Andrés-Lacueva, C. (2000). High-performance liquid chromatographic determination of the riboflavin concentration in white wines for predicting their resistance to light. *Journal of Chromatography A*, *888*, 121–127.

Nielsen, P. (2000). Flavins. In A. P. DeLeenheer, W. E. Lambert, & J. F. Van Bocxlaer (Eds.), *Modern chromatographic analysis of vitamins* (3rd ed.). New York: Marcel Dekker.

Ollilainem, V., Mattila, P., & Varo, P. (1990). The HPLC determination of total riboflavin in foods. *Journal of Micronutritional Analysis*, *8*, 199–207.

Pérez-Ruiz, T., Martínez-Lozano, C., Sanz, A., & Bravo, E. (2001). Determination of riboflavin, flavin mononucleotide and flavin adenine dinucleotide in biological tissues by capillary zone electrophoresis and laser-induced fluorescence detection. *Electrophoresis*, *22*, 1170–1174.

Reyes, E. S. P., Norris, K. M., Taylor, C., & Potts, D. (1988). Comparison of paired ion liquid chromatographic method with AOAC fluorimetric and microbiological methods for riboflavin determination in selected foods. *Journal of AOAC International*, *71*(1), 16–19.

Ribarova, F., Shishkov, S., Obretenova, N., & Metchkneva, L. (1987). Comparative determination of riboflavin in milk HPLC and lumiflavin methods. *Die Nahrung*, *31*(1), 77–79.

Rose, A. H., & Harrison, J. S. (1970). *The yeast (Vol. 3)*. London: Academic Press.

Russell, L. F., & Vanderslice, J. T. (1992). Non-degradative extraction and simultaneous quantitation of riboflavin, flavin mononucleotide, and flavin adenine dinucleotide in foods by HPLC. *Food Chemistry*, *43*(2), 151–162.

Vidal-Valverde, C., & Reche, A. (1990). Reliable system for the analysis of riboflavin in foods by high performance liquid chromatography and UV detection. *Journal of Liquid Chromatography*, *13*(10), 2089–2101.

van den Berg, H., van Schaik, F., Finglas, P. M., & de Froidmont-Görtz, I. (1996). Third EU MAT intercomparison on methods for the determination of vitamins B-1, B-2 and B-6 in food. *Food Chemistry*, *57*(1), 101–108.

WEB page available at http://www.roche-vitamins.com/home/what/what-gen/what-gen-vitamins/what-gen-vitamin-b2.htm [accessed October 2002].