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Laboratory Experiment

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Simultaneous Determination of Pyridoxine and Riboflavin in Energy Drinks by High-Performance Liquid Chromatography with Fluorescence Detection

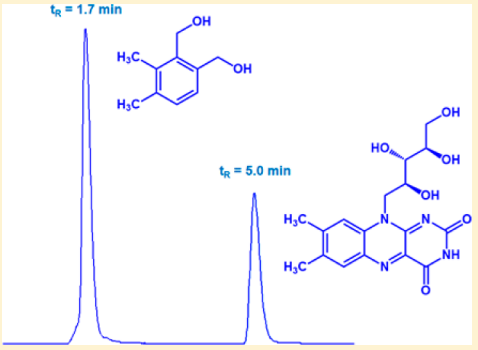
P. Martí-Andrés,[†] L. Escuder-Gilabert,[†] Y. Martín-Biosca,[†] S. Sagrado,[†,‡] and M.J. Medina-Hernández*,[†]

[†]Departamento de Química Analítica, Facultad de Farmacia, Universidad de Valencia, Avenida Vicent Andrés Estellés s/n, E-46100 Burjassot, Valencia, Spain

[‡]Centro Interuniversitario de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Unidad Mixta Universidad Politécnica de Valencia-Universidad de Valencia, Camino de Vera s/n, E-46022 Valencia, Valencia, Spain

Supporting Information

ABSTRACT: Energy drinks, as familiar consumer products, have been widely used in laboratory courses to help promote student interest, as well as to connect lecture concepts with laboratory work. Energy drinks contain B vitamins: pyridoxine (vitamin B6) and riboflavin (vitamin B2) of which amounts are high enough to be of concern. In this work, a fast and inexpensive high-performance liquid chromatography (HPLC) coupled with fluorescence detection method for determining pyridoxine and riboflavin simultaneously in energy drinks is developed. It takes advantage of the native fluorescence of B vitamins and provides high selectivity and sensitivity with low background noise. The method is suitable for undergraduate students, but it could be used for graduate level too including extra tasks such optimization and validation stages.



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The consumption of energy drinks has markedly increased in the past few years, becoming very popular among college students.[1] According to a recent study of the European Food Safety Authority (EFSA) on energy drinks consumption, 13.3% of young adults (18−29 years) consume energy drinks 4 or 5 times a week or more, yielding an estimated intake of 4.5 L per month per person.[2] The diversity of regulations on labeling, distribution, and sale of these drinks has allowed aggressive marketing campaigns, primarily targeted to young adults, which promote the stimulant effects of these beverages without warning about the possible negative consequences that some of their ingredients may have on their health.[1,3] The widespread and increasingly excessive consumption of these products has led to a marked increase in reported medical treatment. Chronic consumption of these products is becoming a focus of public health since the long-term health effects are ambiguous and inadequately studied.

Caffeine is the major active component of these drinks, and the risks derived from its consumption have been extensively studied.[3−5] Nevertheless, besides caffeine, energy drinks contain high amounts of B vitamins, particularly pyridoxine (vitamin B6) and riboflavin (vitamin B2).[1] Pyridoxine plays an essential role in the interaction of amino acid, carbohydrate, and fatty acid metabolism through the citric acid cycle by means of B6 coenzymes.[6] Riboflavin is widely distributed in plant and animal cells. Flavoproteins (proteins that contain a nucleic acid derivative of riboflavin) act as catalysts in biological redox systems, and are essential for carbohydrate, lipid and amino acid biosynthesis and metabolism, as well as for the activation of other vitamins.[6]

Riboflavin is absorbed in the upper jejunum by a fast and saturable transporter,[7] so an excessive intake does not pose a risk of toxic effects;[8] however, the dose per can in some of these beverages exceeds the recommended intake (1.6 mg/day).[9] The amounts of pyridoxine contained in these drinks are more concerning since the compound can induce a severe sensory neuropathy, even leaving patients unable to walk.[8,10] Photosensitivity and dermatitis have also been described due to pyridoxine toxicity.[8] The U.S. Recommended Dietary Allowance for a healthy adult is 1.3 mg/day of pyridoxyne.[11] Monster beverages contain declared values of 2 mg of pyridoxine per can, whereas Red Bull contains about 5 mg per can. Some other drinks contain amounts as high as 40 mg per can.[10]

Energy drinks, as familiar consumer products, have been widely used in laboratory courses to help promote student interest, as well as to connect lecture concepts with laboratory work.[12] These drinks contain caffeine, taurine, riboflavin, pyridoxine, other B vitamins, and various herbal derivatives.[1] Caffeine has been established as a target analyte in many undergraduate analytical chemistry courses, and several methods for its determination in different samples have been

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described.[12−16] Pyridoxine has also been determined by high-performance liquid chromatography (HPLC), using UV detection, in undergraduate analytical chemistry courses. In that case, the chromatogram exhibited relatively high noise around the pyridoxine peak and the standard addition method was used for the determination.[12] In a previous research paper, HPLC with fluorescence detection was proposed for determining riboflavin and pyridoxine together with other vitamins in various beverages, including energy drinks.[17] However, the method consisted in fact of two different methods since different columns and some other chromatographic conditions were used for each analyte; so riboflavin and pyridoxine had to be determined separately.[17] This is a practical inconvenient compared with a simultaneous multianalyte determination (an intrinsic advantage of HPLC).

In view of the public health situation, it could be interesting for students to analyze energy drinks in order to realize the high levels of substances other than caffeine these drinks contain and which may pose a risk for their health. For this purpose, in this paper a method for determining pyridoxine and riboflavin in several energy drinks, taking advantage of the native fluorescence of B vitamins is developed. HPLC coupled with fluorescence detection is proposed as analytical technique, which, in comparison with UV detection, provides higher selectivity and sensitivity, allowing the analysis of such complex samples with low background noise. Finally, the method is optimized to allow the simultaneous determination of both analytes under isocratic conditions, exploiting the potential of HPLC. This method could be used to demonstrate to students the differences between absorption and fluorescence used as HPLC detection methods, being specially suitable for an undergraduate instrumental analysis laboratory course,[18,19] but easily adapted to graduate courses if several advanced tasks are introduced.

■ EXPERIMENTAL SECTION

Apparatus and Materials

The chromatograms were obtained using a Jasco LC-2000 plus sytem (Jasco; Easton, MA) equipped with a fluorescence detector (FP-2020 Intelligent Fluorescence Detector), a Kromasil C_{18} column (5 μm, 150 × 4.6 mm) (Scharlab; Barcelona, Spain) and a manual injector. The injection port was equipped with a 20 μL loop for the manual injection of the samples. The excitation and emission wavelengths for each of the analytes were fixed after obtaining the fluorescence spectra from a PerkinElmer LS-45 fluorimeter (PerkinElmer; Waltham, MA).

Reagents

Pyridoxine hydrochloride, riboflavin (Guinama; Valencia, Spain), and methanol (Scharlab) were used for the preparation of the standard solutions. For the preparation of the mobile phases, HPLC-grade acetonitrile (Scharlab) and ultrapure water (Ultra Clear TWF UV, Siemens Water Technologies; Barsbüttel, Germany) were used. The buffer solution was prepared by dissolving an appropriate amount of sodium dihydrogen phosphate monohydrate (Scharlab) in ultrapure water and the pH was adjusted to 3.0 by adding 1 M hydrochloric acid solution prepared from concentrated hydrochloric acid (Scharlab). The different energy drinks were purchased from the university cafeteria and local supermarkets.

Mobile phases were filtered through disposable 0.45 μm Nylon filters (Micron separations; Westborough, MA) and sonicated in an ultrasonic bath (Elmasonic S60, Elma; Singen, Germany) prior to use, in order to eliminate any dissolved gas and avoid bubble formation in the chromatographic system.

Standard Solution Preparation

A 100 ppm stock solution for each of the analytes in methanol was prepared. Aliquots were taken from the stock solutions completing the volume with 50 mM phosphate buffer (pH 3.0) solution to prepare the different calibration standards.

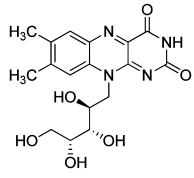
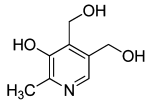
Sample Preparation

Samples were stored at 4 °C in their original can until use. A volume of 10 mL of each sample was sonicated in a 250 mL beaker until the bubbling stopped (approximately for 5 min) to minimize foam formation and avoid sample loss. After that, solutions were injected into the chromatographic system by triplicate.

HPLC Analysis

Acetonitrile−phosphate buffer (50 mM, pH 3.0) 20:80 (v/v) solution was used as mobile phase. The flow rate was set at 1 mL/min. Working solutions were injected into the chromatographic system using a 1 mL glass syringe (Agilent Technologies;

Table 1. Complementary Information about Riboflavin and Pyridoxine

	RIBOFLAVIN	PYRIDOXINE
Chemical structure		
pKa[a]	10.2 (experimental)	5.56, 9.40 (predicted)
logP[a]	-1.46	-0.77
Recommended Intake[b]	1.6 mg/day	1.3 mg/day
Maximum Recommended Intake[b]	-	60 mg/day
Minimum dose of reported effects	200 mg[b]	112 mg[c]
$t_{1/2}$[a]	66-84 min	15-20 days
Protein Binding[a]	60%	22%
Reported effects[c]	Urine color changes	Convulsions, dyspnea, hypermotility, diarrhea, ataxia, muscle weakness

[a]Drug Bank: see ref 20. [b]Food and Drug Administration: see ref 21. [c]See ref 8.

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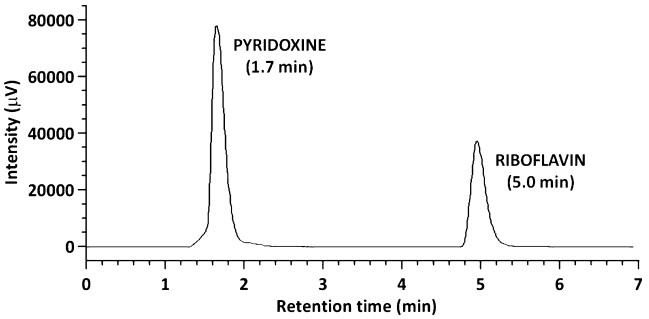

Figure 1. Chromatogram corresponding to the determination of pyridoxine and riboflavin in the sample *Monster Energy*. Excitation and emission wavelengths were 296 and 390 nm, respectively, until 3 min. Then, wavelengths were changed to 450 and 530 nm, respectively.

Table 2. Calibration Statistics for Riboflavin and Pyridoxine under the Proposed Separation Conditions

Analyte	Standard Solutions, N	Value for the Slope, b_1	Value for the Intercept, b_0	Coefficient of Determination, R^2
Riboflavin	5	30338	−8805.4	0.9992
Pyridoxine	5	18100	20127.0	0.9990

Waldbronn, Germany) coupled with a 0.45 μm filter (Micron separations). The initial excitation and emission wavelengths were set at 296 and 390 nm, respectively (optimum for pyridoxine). After 3 min, wavelengths were changed to 450 and 530 nm, respectively (optimum for riboflavin).

HAZARDS

Pyridoxine hydrochloride and riboflavin are not considered dangerous substances; however, unnecessary contact should be avoided. Methanol and acetonitrile are highly flammable and toxic when swallowed or inhaled and upon contact with skin. Vapor formation should be avoided. Sodium dihydrogen phosphate can cause irritation of the skin and mucous membranes upon contact. Concentrated hydrochloric acid is extremely corrosive. It must be handled under the hood. Solutions disposal should take into account the toxicity of the substances. Adequate personal protective equipment should always be worn in the laboratory.

RESULTS AND DISCUSSION

Table 1 shows the structure, the pK_a values, the logarithm of the *n*-octanol/water partition coefficient (logP) values, and several other characteristics of the analytes in the various samples. Especially noteworthy are the low recommended intakes of both compounds compared to the dose of reported effects.

Figure 1 shows the chromatogram corresponding to the determination of pyridoxine and riboflavin in the sample Monster Energy under the selected optimum conditions, including the change of excitation and emission wavelengths between the detection of both analytes. The optimization process is outlined in the Supporting Information as one of the possible tasks to be performed by graduate students (in a master's degree, for instance). In our opinion, it is important that students at advanced levels perform tasks such as optimization and method validation.

Calibration

The concentration ranges of calibration standards solutions prepared for each analyte were obtained taking into account their amounts declared by the manufacturers in the different samples. The range for pyridoxine was from 10 to 50 ppm, whereas that of riboflavin was from 2 to 10 ppm. Peak areas were used as dependent variables to obtain the calibration curves and their corresponding statistics are shown in Table 2. The original calibration data and the calibration graphs are included in the Supporting Information.

Recovery Study

To check the accuracy of the method, a recovery study was performed using three samples (Monster Energy, Monster Assault, and Red Bull). Spiked samples were prepared by adding 2 and 3 mL of 100 ppm standard solutions of riboflavine and pyridoxine, respectively, to 10 mL of samples. Recovery values were ranged between 90 and 105% for riboflavine and 96 and 102% for pyridoxine.

Sample Analysis

Figure 2A shows the experimental and declared concentration values of riboflavin in the analyzed samples. As can be observed, positive differences between experimental and declared values were obtained. These differences ranged between 17 (El Corte Inglés) and 49% (Hacendado). The precision of the experimental results (3 replicates) ranged between 0.3 (Hacendado) and 3.3% (Monster Energy). Figure 2B shows the results for

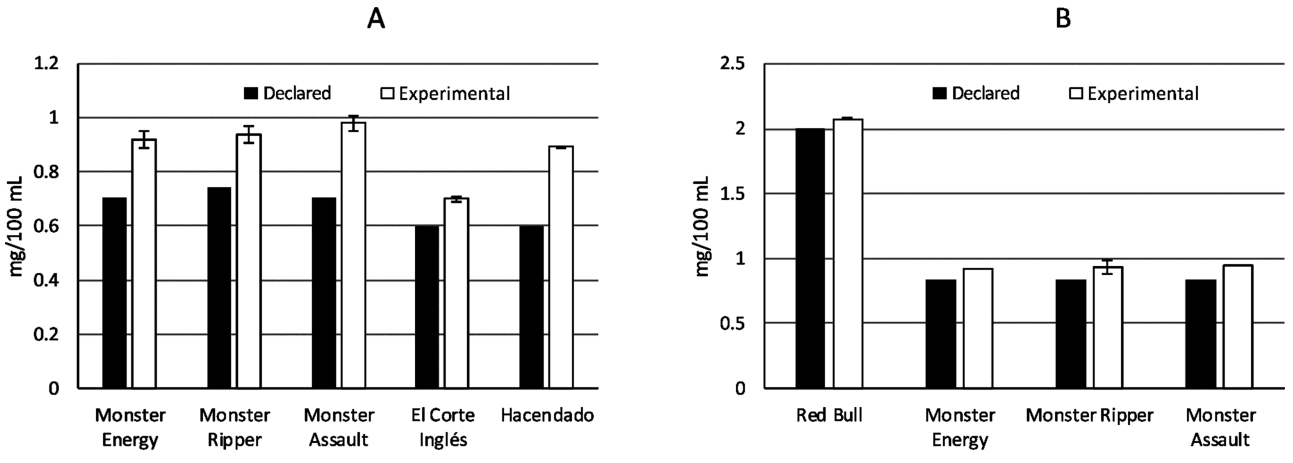

Figure 2. Declared and experimental values of riboflavin (A) and pyridoxine (B) in the different samples analyzed.

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pyridoxine. As before, positive differences were obtained; however, they were lower than those for riboflavin samples (from 4.0 for Monster Energy to 14.0% for Monster Assault). The precision of the results (3 replicates) ranged from 0.4 (Red Bull) to 5.4% (Monster Ripper). Since the recovery study suggests that maximum expected errors of the method are in the ±10% range, differences between experimental and declared values should be considered as reliable.

■ ASSOCIATED CONTENT

Supporting Information

Instructions for students and instructors. This material is available via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: maria.j.medina@uv.es.

Notes

The authors declare no competing financial interest.

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