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Development of a second-order standard addition fluorescence method for the direct determination of riboflavin in human urine samples without previous clean up and separation steps

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ABSTRACT

A new strategy for the determination of riboflavin (RF, or vitamin B_2) in human urine samples has been developed, combining excitation-emission fluorescence matrix (EEFM) data in standard addition mode and second-order chemometric analysis. The method is simple, fast and eco-friendly because it complies with the green analytical chemistry principles, avoiding the need of previous clean up and separation steps that consume high amounts of organic solvents. Successful results were obtained by different chemometric algorithms, namely parallel factor analysis (PARAFAC), unfolded partial least-squares/residual bilinearization (U-PLS/RBL) and multidimensional partial least-squares/residual bilinearization (N-PLS/RBL), all in the modified standard addition. These algorithms allowed us to achieve selectivity in a system, which requires standard addition and shows a significant background spectral overlapping with the studied vitamin. The quality of the proposed strategy was evidenced on the basis of the analytical recoveries from urine samples spiked with RF. The detection limits achieved in urine samples are encouraging compared to those obtained using chromatographic approaches. The relative prediction errors were lower than 5.6%.

1. Introduction

Riboflavin (RF) or vitamin B_2 is a water-soluble vitamin long established as a vital nutrient. It is an essential component of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) which are involved in the metabolism of many substances, e.g., glucose, fatty acids, amino acids, drugs, vitamins K and D [1,2]. It is found in a wide variety of foods: milk and cheese, meat and fish, eggs, wine and tea, these products provide about one-third of dietary requirements. Its deficiency may be due to insufficient intake, hormonal problems, drugs, alcohol or chelators reducing its bioavailability. Symptoms of RF deficiency include fatigue; digestive problems; cracks and sores around the corners of the mouth; swollen magenta tongue; soreness of the lips, mouth and tongue; red eyes and conjunctivitis [3,4]. Low intakes of RF can lead to poor growth and various functional abnormalities, including impaired iron handling and elevated plasma homocysteine concentration [5]. There is evidence that RF deficiency may increase both the risk of developing certain types of cancers [6] and the damage to proteins and DNA liver cells [7].

The amount in excess of RF in the body is rapidly excreted through urine because the vitamin is water-soluble and there is no capacity for storage. Free RF is the only flavin excreted by the body in significant amounts reflecting the short-term supply. When RF intake is low, excretion is proportional to the intake [3]. The most widely used analytical methods for the quantification of RF in human urine samples are liquid chromatography (LC)-fluorescence detection (FD) [8], LC-tandem mass spectrometry (MS/MS) [9,10], fluorescence with flow-injection analysis [11], capillary electrophoresis (CE) [12,13], voltammetry [14] and chemiluminescence [15]. These methods require rigorous extraction steps, the use of significant amounts of organic solvents and long analysis time.

Nowadays greener methodologies are very welcome [16], since they fulfill the principles of green analytical chemistry (GAC) [17] i.e. the absence of clean up and separation steps, and the minimization of the use of organic solvents and generation of toxic waste.

It is well known that the complexity of the biological matrices such as urine makes the determination of the target analyte difficult. The effect of a background on the analyte response leads to a change in the slope of the univariate signal-concentration relationship caused by inner filter effects or analyte-background interactions such as complex formation or protein binding. Nevertheless, this effect can be overcome by using the standard addition method [18]. Apart from that, the matrix

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constituents are potentially able to produce interference signal which does also affect the analyte response in a sample. In this case, the system requires both standard addition and second-order multivariate calibration achieving the second-order advantage for successful analyte quantification [19]. Second-order advantage refers the capacity of certain second-order algorithms to predict concentrations of sample components in the presence of any number of unsuspected constituents [20]. The algorithms that achieved the second-order advantage from standard addition data are parallel factor analysis (PARAFAC) [21], partial least-squares/residual bilinearization (U-PLS/RBL) [22,23] and multidimensional partial least-squares/residual bilinearization (N-PLS/RBL) [24,25].

In the present work, we have developed a GAC method to quantify RF in human urine samples. Second-order data were obtained by measuring excitation-emission fluorescence matrices (EEFMs) and processed by PARAFAC, U-PLS/RBL and N-PLS/RBL. The prediction capabilities of the employed algorithms were discussed.

It is relevant to highlight that it was the first time that the selectivity offered by the chemometric analysis was evaluated for the determination of RF using EEFMs and second-order standard addition calibration in a complex matrix. The feasibility of determining the target vitamin in human urine samples using sustainable resources was demonstrated. The new method represents other example of the power of coupling non-sophisticated analytical equipment with second-order data for the resolution of interfering samples.

2. Experimental

2.1. Reagents and solutions

RF and ofloxacin (OFL) were purchased from Sigma (St. Louis, MO, USA). The stock solution of RF (104 mg L^{-1}) was prepared in ultrapure Milli-Q water and OFL (204 mg L^{-1}), tested as a potential interferent, was prepared in 5×10^{-2} mol L^{-1} acetic acid. From these solutions, more diluted aqueous working solutions were daily obtained. Ultrapure Milli-Q water was used throughout the work.

2.2. Instrumentation

Fluorescence measurements were performed on an Aminco Bowman (Rochester, NY, USA) Series 2 luminescence spectrophotometer, equipped with a 150 W xenon lamp. EEFMs were measured from 320 to 460 nm (each 4 nm, excitation) and from 470 to 600 nm (each 1 nm, emission). In this way, the matrices were of size 36×131. Both the excitation and emission slit widths were of 8 nm using 1.00 cm quartz cells. The photomultiplier tube (PMT) sensitivity was fixed at 425 V and the temperature of the cell compartment was regulated at 21.0 ± 0.1 °C using a thermostatic bath (Cole–Parmer, IL, USA). The EEFMs were saved in ASCII format, and transferred to a PC for subsequent chemometric analysis.

2.3. Validation and test samples

RF is a light sensitive vitamin, so a pool of urine samples was placed in a clear glass bottle and exposed to white light of a lamp for 120 h [8,10]. This sample was analyzed to confirm that the concentration of RF was not detected by the proposed method. After this process, the pool sample was considered “blank urine” and used to prepare validation and test samples.

A validation set of 10 samples, by duplicate, was prepared. An aliquot of 40.00 μL diluted blank urine (1:125) and concentrations of RF at random numbers in the range 0.02–0.10 mg L^{-1} were placed in a 5.00 mL volumetric flask which was completed to the mark with ultrapure water. Afterwards, new solutions were prepared starting from the blank urine samples in order to carry out three successive additions of RF. Concentrations were increased by 0.02, 0.05 and 0.10 mg L^{-1} for RF on different aliquots of the original samples. We estimated the uncertainties in all these analyte concentrations to be of the order of ± 0.01 mg L^{-1}. Finally, the four solutions were cooled to 21.0 °C and their EEFMs were recorded in the conditions described in Section 2.2.

OFL is an antibiotic widely used and its excess is excreted by urine. As it will be demonstrated below, OFL has fluorescence signal that significantly overlaps with that of the studied compound. With the purpose of evaluating the method in the presence of this interferent drug, 8 test samples, by duplicate, were prepared containing 40.00 μL diluted blank urine (1:125), random RF concentrations in the range 0.02–0.10 mg L^{-1} and OFL in the concentration within the therapeutic range in human urine, in the order of 0.05–1.02 mg L^{-1} [26]. In a similar way than in validation samples, new solutions were prepared with three successive additions of RF to carry out the standard addition method.

2.4. Urine samples

The urine samples were collected from fasting healthy adult volunteers (female and male from 20 to 60 years). Fresh urine samples were immediately stored in a dark glass bottle at 4 °C after a preliminary centrifugation step at 10000 rpm for 15 min. The urine samples were diluted 1:25, an aliquot of 200.0 μL was placed in a 5.00 mL volumetric flask and completed to the mark with ultrapure water. The subsequent procedure was the same as described above for validation and test samples. A recovery study was carried out by spiking each urine sample with RF, by duplicate, at a final concentration level in the range 0.5–2.5 mg L^{-1}.

2.5. Chemometric algorithms and software

The theory of the second-order multivariate calibration algorithms applied in the present work is well established and can be found in the relevant references: PARAFAC [21], U-PLS [27], N-PLS [28] and PLS/RBL [22,23,29].

All routines of employed chemometrics algorithms were written in MATLAB 7.10 [30], and implemented using the graphical interface MVC2 [31], available on the Internet [32].

3. Results and discussion

3.1. Preliminary studies

In a first stage, the fluorescence properties of the studied analyte were evaluated. As previously reported [11,12], RF in aqueous medium presents two excitation maxima at approximately 368 and 440 nm, and a fluorescence emission maximum at 524 nm. The pH changes in the range 4–8 did not produce any significant modifications in the fluorescence signal [33]. Because the pH of the analyzed urine samples were between 5 and 7, it was not adjusted. The influence of the temperature of the cell was studied in the range 18.0–28.0 °C, recording the fluorescence intensity at 368 and 524 nm as excitation and emission wavelengths, respectively. It was found out that the optimum value obtained was at 21.0 °C.

3.2. Quantitative analysis

The need of standard addition method was corroborated through the different slopes of the univariate calibration curves for RF in water solution and in the presence of the urine matrix. The results for RF in water with five different concentrations in the range 0.0–0.25 mg L^{-1} were slope = 43.0(4), intercept = 0.16(8), $r^2 = 0.998$ (standard deviation in the last significant figures in parenthesis), while in a typical urine background, slope = 37.9(3), intercept = 4.74(4) and $r^2 = 0.998$. The results suggest a significant change in slope (a decrease of 5.1 units in slope, ca. 15 times larger than the average standard deviation (0.35)).