Detection and Assay of Riboflavin (Vitamin B2) Utilizing UV/VIS Spectrophotometer and Citric Acid Buffer

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Authors' contributions

This work was carried out in collaboration between both authors. Author RB designed the study, performed the statistical analysis, wrote the protocol and wrote all drafts of the manuscript. Author MLF managed the analyses of the study. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: Riboflavin is a B vitamin that is required for a wide variety of cellular processes. The absorbance spectrum of riboflavin was determined at different pH utilizing several buffers. The buffer at pH demonstrating stable absorbance peaks with high numerical values of molar absorptivity is followed by accurate and sensitive assay of riboflavin by spectrophotometer.
Study Design: The absorbance spectrum of riboflavin is determined in an aqueous buffer at various pH values. After identifying the absorbance peaks providing maximal molar absorptivity the assay of riboflavin in the identical buffer was undertaken.
Place and Duration of Study: Department of Chemistry, Durham Science Center, University of Nebraska, Omaha Nebraska between August 2013 to December 2013.
Methodology: An amount of riboflavin was dissolved in distilled water to make a stock solution of 0.0001100 molar concentrations. For determination of the absorbance spectrum one milliliter of the stock was diluted into various buffers of differing pH. The buffer having pH of 5.03 was selected for following assays with standard curve. Measurements of many aqueous solutions containing riboflavin were accomplished that included vitamin capsules/tablets and water vitamin mixtures. The data was evaluated

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meticulously utilizing several statistical numerical analysis methods.

Results: The riboflavin is highly soluble in a citric acid buffer. The standard curve extended from 6.60E-07 M to 1.100E-04 M (167x spread in concentration). The equation of the line was y = 11882x (intercept at origin) with Pearson r correlation of 0.9998 (R^2 = 0.9998). Concentration of riboflavin assayed can range from 0.000248 g per liter to 0.0414 g per liter. Accurate and reproducible results were obtained.

Conclusion: The B vitamin riboflavin can be assayed by UV/VIS spectrophotometer at 440 nm in aqueous media and using citric acid buffer at pH 5.03. The assay for riboflavin in aqueous mixtures showed high levels of accuracy and sensitivity.

Keywords: Riboflavin; vitamins; assay; spectrometer.

ABBREVIATIONS

M=molar; UV/VIS=ultraviolet/visible; g = gram; FMN=flavin mononucleotide; FAD= flavin adenine dinucleotide; IUPAC = International Union of Pure and Applied Chemistry; SMILES = Simplified Molecular-Input Line-Entry System; nm = nanometers; ε = molar absorptivity; cGMP = Current Good Manufacturing Practice; mg = milligram.

1. INTRODUCTION

The vitamin B group of water soluble vitamins differs substantially in chemical structure and biological activity [1]. In the body, the vitamin riboflavin (B2) carries out its action in the form of two coenzymes, a flavin mononucleotide (FMN) and a flavin adenine dinucleotide (FAD), which play a vital role in metabolism [1]. The vitamin riboflavin is distributed in all tissues, but at uniformly small concentrations [1]. Riboflavin is readily absorbed from the gastrointestinal tract with excretion directly correlated with the amounts ingested. This vitamin plays an important role in metabolism of ketone bodies, carbohydrates, proteins and fats, as well other aspects of energy metabolism.

When consumed orally this vitamin is not considered to be toxic partly due to its lower aqueous solubility [2] and because excess amounts of the vitamin is readily excreted into the urine [3]. Riboflavin has been popular as a supplement in the form of tablets or capsules and is often utilized in energy drinks or similar health-related beverages. There have been some studies conducted for applying riboflavin in clinical and therapeutic scenarios. For example, riboflavin in higher doses has been found to help prevent migraine headaches [4,5]. Simultaneous dosage of riboflavin and ultraviolet light with blood products has been found to reduce harmful activity of pathogens through reduction of replication [6]. Riboflavin is considered effective for protection against nuclear cataracts [7].

Various methodologies have been investigated for the determination of riboflavin vitamin. These include isocratic reversed-phase column high-performance liquid chromatography separation followed by fluorometric detection of the analyte [8]. Fluorescence detection following high-performance liquid chromatography [9,10]. Other works showed an assay approach with straight fluorescence following pre-treatment of samples [11]. Simultaneous detection of various vitamins, including riboflavin was found to be feasible utilizing planar chromatography followed by an application of fluorescence, ultraviolet-visible detection and confirmation with electro spray ionization mass spectrometry [12].

Considering the nutritional and clinical importance of riboflavin, it follows that methodology for detection and assay are needed. Further application of riboflavin in these areas is imminent so additional methods of detection are warranted. Presented here is an accurate and precise approach taking advantage of the aqueous solubility of riboflavin and its physical characteristics. A useful accurate methodology is a result that will be beneficial for application in industrial, pharmaceutical and clinical environments.

2. MATERIALS AND METHODS

2.1 Reagents and Instruments

Reagents utilized throughout this study were supplied by Sigma-Aldrich, PO Box 14508 St. Louis, MO 63178 USA. However, the vitamin riboflavin (FW = 376.37) was supplied by Eastman Chemical Company, 200 South Wilcox Drive, Kingsport, Tennessee 37660 USA. For spectrophotometric analysis, a Milton Roy Spectronic 21D instrument was utilized with one centimeter glass cuvettes.

2.2 Preparation of Standards and Test Samples

A stock solution of riboflavin was prepared by dissolving 0.04140 grams into one liter of distilled water making a concentration of 0.0001100 molar. This container was wrapped in aluminum foil to protect the riboflavin stock solution from light exposure. Stock solution of citric acid buffer of 0.025 molar was prepared in distilled water at pH values of 5.03, 6.03, and 3.02. A stock solution of 0.100 molar sodium borate buffers at pH 9.80 was prepared in distilled water.

For reading of an absorbance spectrum an aliquot of one milliliter of the stock solution riboflavin was placed in nine milliliters of desired buffer making a mixture of 1.100E-05 molar riboflavin and 0.0225 molar citric acid buffer (except in the case at pH 9.80, which was 0.090 molar sodium borate buffer). Absorbance values were obtained from 320 nm to 700 nm.

As many as fifteen mixtures were prepared for a standard curve was made with desired aliquot volumes from the stock solution of riboflavin combined with citric acid pH 5.03 buffer. Concentrations ranged from highest at 0.0001100 molar to 1.10E-06 molar with zero molar within the linear equation. Many specimens were prepared using known amounts of riboflavin solubilized in pH 5.03 citric acid buffer and concentrations determined by standard curve for comparisons. To ascertain feasibility for measuring industrial or beverage aqueous vitamin mixtures, a set of pH 5.03 citric acid buffered examples were prepared at a wide range of riboflavin concentration. In the case of tablets or capsules: 1) the tablet was weighed, 2) then ground in mortar and pestle, 3) the dry amount of solid to be dissolved in volumetric flasks was weighed again (a separate aliquot of ground powder can be captured for other assays and the amount of the vitamin pill preparation that was dissolved for riboflavin assay was thus known), 4) the desired amount of solid is carefully placed in volumetric container and dissolved in distilled water, 5) further use requires filtering out insoluble solid through Whatman #1 filter paper, 6) the filtered liquid is ready for assay or further dilution in desired buffer. In all cases of test specimens to be assayed in this study, the material was dissolved in a known volume of pH 5.03 citric acid buffer (all assays were carried out at pH 5.03).
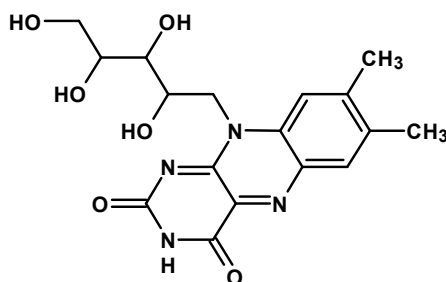
2.3 Computation and Statistical Analysis

Computation performed for identification of numerical outlier's using Grubbs' Test (extreme studentized deviate), means, and standard deviations were accomplished by Graphpad (www.graphpad.com/quickcalcs/). For statistical calculation of Pearson r, equation of a line from data that is graphed, and coefficient of determination (R^2) was taken from EXCEL (version 14.0.7106.5003, copyright 2010 Microsoft Corporation). Determination of statistical Pearson r, Spearman's rs, Kendall's tau, Mann-Whitney test, Kolmogorov-Smirnov test, Kruskal-Wallis test, Paired t-test were performed by PAST version 2.06 (copyright Hammer and Harper 1999-2011)[13].

3. RESULTS AND DISCUSSION

Riboflavin consists of a yellow to orange-yellow crystal-like powder and has a slight odor. The solid is not significantly affected by light but when in solution, the vitamin quickly degrades under light [1]. The sugar ribose comprises part of its structure but it is the ring-moiety or "flavin" that imparts the yellowish color to the molecule [1].

The riboflavin solid consisted of a fine powder and is sufficiently soluble in distilled water to produce a stock solution reaching 0.0001100 molar concentrations and should be protected from light while stored at room temperature. The molecular structure of the vitamin, some properties, IUPAC name and useful SMILES notation are indicated in Fig. 1. Note the ribose moiety and ringed-structure that provide the observed color of the vitamin. Interestingly the molecular structures of the B-vitamin group members differ substantially.

RIBOFLAVIN

7,8-dimethyl-10-(2,3,4,5-tetrahydroxypentyl)benzo[*g*]pteridine-2,4(3*H*,10*H*)-dione

OCC(O)C(O)C(O)CN2C3=NC(=O)NC(=O)C3=Nc1cc(C)c(C)cc12

Molecular Formula = $C_{17}H_{20}N_4O_6$

Formula Weight = 376.3639

Composition = C(54.25%) H(5.36%) N(14.89%) O(25.51%)

Fig. 1. Riboflavin chemical structure, properties, IUPAC name and SMILES notation

The absorbance spectrum of riboflavin was initially determined in a citric acid type buffer at three pH values 3.02, 5.03 and 6.03, with a fourth spectrum obtained in a sodium borate buffer at pH 9.80. All were measured from 320 nm to 700 nm. All spectra were obtained utilizing a riboflavin concentration of 1.100E-05 molar. The results obtained showed two very noticeable peaks with all pH values and all buffers, occurring at wavelengths 365 nm and 440 nm.

Altogether, the absorbance spectra at various pH and buffers are presented in Fig. 2 according to pH value. The absorbance values essentially decreased at approximately 500 nm to flatten out to the wavelength 700 nm. Two major absorbance peaks were discerned in all buffers utilized. Knowing the precise molar concentration (1.100E-04 molar) of riboflavin at each pH allows the computation of the relative molar absorptivity (ε). Values of molar absorptivity determined at pH of solutions at 3.02, 5.03, 6.03, and 9.80 (sodium borate buffer) was 12090.9 L $mol^{-1}cm^{-1}$, 11772.7 L $mol^{-1}cm^{-1}$, 12363.6 L $mol^{-1}cm^{-1}$ and 9363.6 L $mol^{-1}cm^{-1}$, respectively. The mean value for (ε) is then 11397.7 L $mol^{-1}cm^{-1}$ and standard deviation of 1377.4 L $mol^{-1}cm^{-1}$. Although the standard deviation is numerically appreciable there are no outlier's among values of (ε) by Grubbs' test (extreme studentized deviate) and applying two-sided significance level of 0.05. The pH value of 5.03 with citric acid was chosen for assay of riboflavin in various medium.
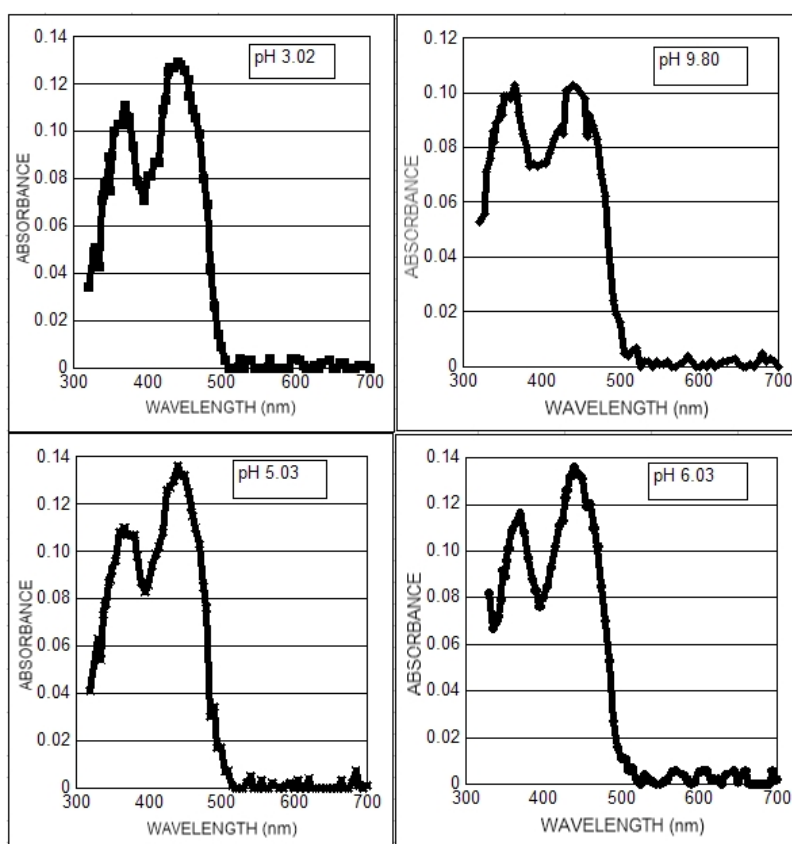

Fig. 2. Absorbance spectrum of riboflavin from 320 nm to 700 nm at different pH values (for pH 9.80 a 0.090 M sodium borate buffer was utilized). Spectrum acquired at pH values of 3.02, 5.03, and 6.03 were accomplished in a citric acid buffer. Riboflavin concentration for all spectra was 1.100E-05 molar.

A standard curve was formed by the use of 15 different values of riboflavin concentration which did result in a highly linear relationship (Fig. 3). The equation of the line determined by EXCEL showed $y = 11882x$ with $R^2 = 0.9998$ and Pearson r correlation value of $r = 0.9999$, indicating an extremely high strength of association and near perfect positive

linear relationship [14]. Having a coefficient of determination of R^2 = 0.9998 indicates that the model represents 99.98% of variance observed. For this relationship, the Kendall's Tau rank correlation coefficient of 1.000 indicated all pairs of data are concordant and in exactly the identical order (Spearman's rs = 1.000) [13]. In addition, all data fall within 95% ellipses, which indicate all members of the standard curve fall within 95% confidence interval [14]. The molar concentrations of this standard curve include the origin at (0,0) and begin at 1.100E-06 molar and extend to 1.100E-04 molar, which is a 100x range in concentration values.
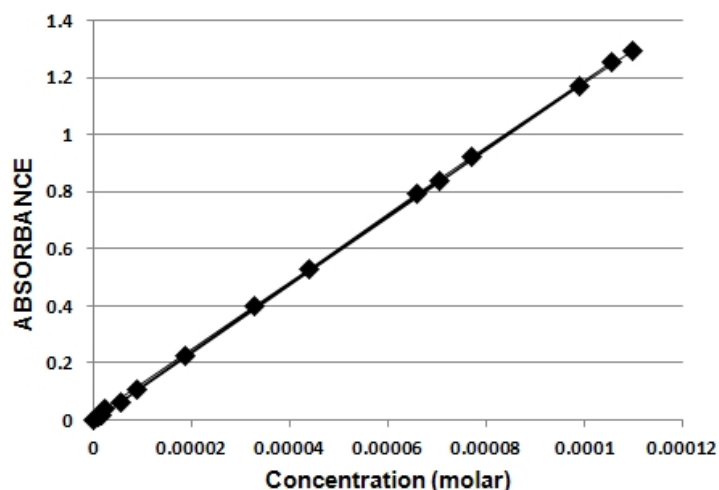

Fig. 3. Standard curve showing line equation y = 11882x with R^2 = 0.9998 and Pearson r correlation r = 0.9999.

The determination of riboflavin concentrations in prepared testing solutions of the vitamin via the standard curve was extremely successful and provided conclusive level accuracy with those mixtures. Representative outcomes are presented in Table 1 (comparison of actual molarity to calculated molarity determined by the standard curve).

The correlation coefficient Pearson r for actual and calculated molarities is 0.9997 (R^2 = 0.9994 or 99.94% of variance represented within the model). In addition, the Spearman's rs are 0.9993 and Kendall's tau of 0.9920. The Kolmogorov-Smirnov test of actual and calculated molarities produced a P = 0.99, indicating these are taken from two populations with equal distribution [14]. The Kurskal-Wallis test of actual and calculated molarities produced a P = 0.76, indicating these are from populations of equal medians. The Mann-Whitney test of the actual and calculated molarities showed P = 0.77, indicating these two populations have equal medians [14]. The t-test indicated the mean of the differences of these two populations is zero (P = 0.90) [14].

Further, the average percent recovery is 101.0% with standard deviation of 0.94% and mode of 100.4% (value that appears most often in a set of data [14]). The minimum percent recovery is 98.3% with a maximum of 102.9% (resulting in a range of percent recoveries of 4.60%). The skewness value of -0.148 indicates the percent recovery values are approximately symmetric [14].

Table 1. Comparison of actual and calculated Molar Concentration

Sample	Actual (molar)	Calculated (molar)	Percent recovery
1	0.00009900	0.00009889	99.9
2	0.00009680	0.00009721	100.4
3	0.00009460	0.00009510	100.5
4	0.00009240	0.00009341	101.1
5	0.00009020	0.00009131	101.2
6	0.00008800	0.00008837	100.4
7	0.00008580	0.00008601	100.2
8	0.00008360	0.00008483	101.5
9	0.00008140	0.00008265	101.5
10	0.00007920	0.00008012	101.2
11	0.00007700	0.00007743	100.6
12	0.00007480	0.00007574	101.3
13	0.00006600	0.00006733	102.0
14	0.00006160	0.00006295	102.2
15	0.00005280	0.00005386	102.0
16	0.00004840	0.00004974	102.8
17	0.00000880	0.00000884	100.4
18	0.00002200	0.00002213	100.6
19	0.00003300	0.00003358	101.8
20	0.00003850	0.00003964	102.9
21	0.00004950	0.00004865	98.3
22	0.00005280	0.00005428	102.9
23	0.00005720	0.00005807	101.5
24	0.00006160	0.00006245	101.4
25	0.00006820	0.00006868	100.7
26	0.00007040	0.00007070	100.4
27	0.00005280	0.00005386	102.0
28	0.00008580	0.00008601	100.2
29	0.00008800	0.00008837	100.4
30	0.00009900	0.00009889	99.9
31	0.00009680	0.00009721	100.4
32	0.00009460	0.00009510	100.5
33	0.00009240	0.00009342	101.5
34	0.00009020	0.00009132	101.2
35	0.00007700	0.00007743	100.6
36	0.00007480	0.00007575	101.3

To evaluate application of this methodology to aqueous preparations of vitamin B2 or similar (common reference as vitamin waters) a set of samples having riboflavin at known but a broad level of concentration was prepared. In each case, the amount of riboflavin is known and made to be pH 5.03 by citric acid buffer prior to assay. A broad range of buffer concentration is possible (ranging from 0.025 molar to 0.00063 molar).

Presented in Table 2 is the comparison of actual molarity to calculated molarity (derived from standard curve). Outcome was extremely good throughout the broad range of molarities that were tested and these results are typical. Concentration of riboflavin in molarity ranged from as low as 4.881E-06 molar 8.80E-05 molar (an 18x fold range in molarity concentration). The correlation coefficient Pearson r of actual versus calculated molarity values is 0.9999

with a coefficient of determination R^2 being 0.9998 (99.98% of variance explained). In addition, the Spearman's rs are 0.9991 with Kendall's tau of 0.9952. The two samples (actual and calculated molarity) are taken from populations having equal medians as shown by Mann-Whitney test (P = 0.87) and Kruskal-Wallis test having (P = 0.85). Results of paired t-test the mean of the difference between actual and calculated molarities are zero (P = 0.0620) [14]. In addition, the outcome of the Kolmogorov-Smirnov test indicated that actual and calculated molarities are populations of equal distribution (P = 0.99).

The percent recovery for vitamin waters evaluation showed a mean of 100.9 % recovery. The minimum and maximum recovery of riboflavin was 97.6% and 104%, respectively. The standard deviation of recovery was 1.6%. Having skewness of 0.115 indicates that the percent recovery is approximately symmetric for vitamin waters [14].

Table 2. Vitamin waters comparison of actual and calculated Molar Concentrations

Sample	Actual (molar)	Calculated (molar)	Percent recovery
1	0.00001571	0.00001616	102.9
2	0.000009167	0.000009258	101.1
3	0.00001222	0.00001271	104.0
4	0.00001000	0.000009763	97.6
5	0.00001467	0.00001456	99.3
6	0.00001257	0.00001296	103.1
7	0.00001100	0.00001111	101.0
8	0.000009778	0.000009763	99.8
9	0.00002197	0.00002200	100.1
10	0.00001372	0.00001375	100.2
11	0.00001212	0.00001222	100.8
12	0.000008462	0.000008584	101.4
13	0.000005789	0.000005807	100.3
14	0.000004783	0.000004881	102.0
15	0.00008800	0.00008800	100.0

The determination of riboflavin in a citric acid buffer by UV/VIS spectrometry was found to have high reproducibility. Example shown in Table 3 for representative concentration of 0.00002200 molar (2.200E-5 molar) compared to calculated molarity (determined by standard curve) which has a mean of 2.242E-05 molar. Calculated molarities are approximately symmetric having skewness of 0.173. The standard deviation of calculated molarity values is an extremely small 2.5E-07 molar (variance of 6.05E-14 molar).

Percent recovery of riboflavin was on the average of 101.9%, having standard deviation of 1.1%. The minimum percent recovery was 100.2 5 and maximum of 103.7%. Median percent recovery was 101.8% and values are approximately symmetric with skewness of 0.151. Coefficient of variation for percent recovery is 1.1%.

The numerical difference between the actual and calculated values is extremely slight with these values having an overall mean of 4.20E-07 molar and standard deviation of 2.5E-07 molar. A skewness of -0.173 indicates these differences are approximately symmetric.

Table 3. Comparison of actual versus calculated molarity for repetitive samples

Sample	Actual (molar)	Calculated (molar)	Percent recovery	Difference between actual and calculated (molar)
1	0.00002200	0.00002230	101.4	3.00E-07
2	0.00002200	0.00002239	101.8	3.90E-07
3	0.00002200	0.00002281	103.7	8.10E-07
4	0.00002200	0.00002205	100.2	5.00E-08
5	0.00002200	0.00002230	101.4	3.00E-07
6	0.00002200	0.00002247	102.1	4.70E-07
7	0.00002200	0.00002272	103.3	7.20E-07
8	0.00002200	0.00002264	102.9	6.40E-07
9	0.00002200	0.00002213	100.6	1.30E-07
10	0.00002200	0.00002239	101.8	3.90E-07

In June of 2007, the FDA established dietary supplement Current Good Manufacturing Practice (cGMP) regulations requiring that manufacturers evaluate their products through testing identity, purity, strength and composition. However, over the counter drugs may actually retain a wide variation in the amount of content of the targeted supplement. Dietary supplement use is widespread among U.S. adults aged 20 and over. The percentage of the U.S. population who used at least one dietary supplement increased from 42% in 1988–1994 to 53% in 2003–2006 [15]. A sum total of four over the counter products was evaluated for riboflavin content, two capsule forms and two tablet forms. In the case of capsules that contained already powder formed ingredients the use of mortar and pestle was not enhancing. Therefore, assert that capsule formed contained material may not require further grinding. Following solubilization by use of volumetric glassware, small aliquots of the dissolved material are analyzed in a citric acid buffer at pH 5.03 as described previously. The two commercial products could then be compared as to content and for these two cases, capsule supplement A and capsule supplement B, the (declared content/measured content) result became (25 mg/24.1 mg) and (7.5 mg/10 mg), respectively. For the case of tablets/pills the use of mortar and pestle was required and was accomplished carefully after recording the overall mass of the tablet. This was followed by grinding into powder-like consistency and dissolving a known measured amount of mass of the ground material. Similar use of volumetric containers and aliquot solubilizing in a citric acid buffer at pH 5.03 followed. The two commercial products could then be compared as to content and for these two cases, a tablet supplement C and a tablet supplement D, the (declared content/measured content) result became (20 mg/15.0 mg) and (100 mg/80.3 mg), respectively.

A dietary supplement is a product taken by mouth that contains a "dietary ingredient" intended to supplement the diet. The dietary ingredients in these products may include: vitamins, minerals, herbs (or other botanicals), amino acids and other substances that include enzymes, organ tissues, glandular and metabolites. Dietary supplements can also be extracts or concentrates and may be found in many forms such as tablets, capsules, soft gels, gel caps, liquids or powders. The production of vitamins, their use and application as personal supplements or commercial additives for enrichment of over the shelf products will continue to grow. The nutritional requirement for riboflavin (vitamin B2) and other of the B group of vitamins is well-established and widely published. It is reasonable to presume that additional other uses and clinical applications for these compounds will continue to grow. With these assertions in retrospect, clearly methodologies for the assay (or monitoring) the presence and quantity of these compounds will have growing necessity. The methodology

presented here, which applies ultraviolet-visible spectrometry in aqueous solution is shown to be accurate and precise. This approach should find application in industrial scenario (e.g. quality control, product quality, component security and screening), monitoring of adulteration (substitution of one material for an illicit material) of product, vitamin production and vitamin tablet/capsule assimilation, environmental monitoring and assessment of commercial vitamin authenticity.

4. CONCLUSION

Riboflavin is a member of the vitamin B group of nutriments essential for maintenance of normal metabolic functions. Riboflavin is assayed by ultraviolet/visible spectrometry in an aqueous citric acid buffer at pH 5.03. Absorbance spectrum determined at pH 6.03, 3.02, 9.8 and 5.03 showed absorbance peak at 440 nm with ε at 12363.6 L $mol^{-1}cm^{-1}$, 12090.9 L $mol^{-1}cm^{-1}$, 9363.6 L $mol^{-1}cm^{-1}$, 11772.7 L $mol^{-1}cm^{-1}$, respectively. An additional absorbance peak was discerned at 365 nm. A standard curve was developed ranging from concentration 1.10E-06 molar to 0.0001100 molar values, which is a 100x range in concentration. The assayed values of molarity were highly correlated with actual molarity at Pearson r = 0.9997. An aqueous citric acid buffer at pH 5.03 showed to be an effective solvent for determination of riboflavin from capsules, vitamin-type water and other aqueous based mixtures of this vitamin. Concentrations of molarity outcomes were shown to be reproducible. The percent recovery of assayed concentrations showed an average of 101.0% with standard deviation of 0.93%, minimum of 98.3% and maximum of 102.9%. The assay of riboflavin is of continued importance for industrial, quality control and clinical environments. The assay of riboflavin in aqueous citric acid mixture is effective and accurate.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=417&id=22&aid=3491