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Studies of Surfactants Effect on Riboflavin Fluorescence and Its Determination in Commercial Food Products and Vitamin Tablets.

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by

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ABSTRACT

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A simple and economical fluorometer using blue LEDs excitation sources and simple PMT detection had been built, assembled, optimized, and employed for measurement of fluorescence from riboflavin (vitamin B_2). Surfactants have been known to enhance the intensity of fluorescence of fluorescent compounds. Fluorescence analysis of riboflavin in the presence of various anionic, cationic, and nonionic surfactants was also conducted to determine if they could improve analysis. However, the surfactants employed did not seem to have any meaningful enhancement; in fact, some actually diminished the fluorescence intensity of riboflavin. The procedure was linear for riboflavin from 0.01 to 2.5 µg/mL. Reproducibility expressed as relative standard deviation was about 2%. The recoveries obtained range from 91.3 % to 100.21 % for the samples determined. The proposed method was successfully applied to the analysis of riboflavin in commercial vitamin tablets and cereal products. The results obtained were consistent with expected values as provided by the manufacturers. The method is simple, sensitive, economical, and rapid.

DEDICATION

When I survey all the mercies and graces showered on me by God my father through his Son Jesus Christ, my soul rises in worship and gets lost in wonder, love, and praise to him who sits on the throne and lives forever and ever.

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CHAPTER 1

INTRODUCTION

Vitamins

Vitamins are organic compounds required as a nutrient in small amounts by an organism. A vitamin cannot be synthesized in sufficient quantities by an organism, it must be obtained from the diet, and a lack of it results in obvious symptoms of deficiency. Vitamins are required for the normal functioning of our bodies. They are necessary for growth, health, vitality, general well being, and the prevention and cure of many health problems and diseases. There are 13 vitamins required in humans. They are vitamins A, C, D, E, K, and the B vitamins that include vitamin B_1 (thiamine), vitamin B_2 (riboflavin), vitamin B_3 (niacin), vitamin B_5 (pantothenic acid), biotin, vitamin B_6 (pyridoxine), vitamin $B_{12,}$ and folate (1, 2).

Vitamins are divided into two classes based on their solubility. Water soluble vitamins dissolve easily in water and are readily excreted from the body and hence do not get stored as much in the body. Water soluble vitamin need to be replaced in the body often. There are nine water-soluble vitamins; eight B vitamins and vitamin C. Vitamin B_{12}, although water-soluble, can be stored in the liver for many years. Fat-soluble vitamins, on the other hand dissolve in fat and are absorbed through the intestinal tract and stored in the body's fatty tissue. There are four fat-soluble vitamins: A, D, E, and K. Each vitamin is characteristically used in multiple reactions and, as a result, most have multiple functions (1, 2).

Vitamins have varied biochemical functions, including function as hormones (e.g. vitamin D), antioxidants (e.g. vitamin E), building and maintaining tissues and strengthening the immune system (e.g. vitamin C), and mediators of cell signaling and regulators of cell and tissue

growth and differentiation (e.g. vitamin A). The largest number of vitamins (e.g. B complex vitamins) functions as precursors for enzyme cofactor bio-molecules (coenzymes) that help act as catalysts and substrates in metabolism (2).

A substance is not considered a vitamin if its removal from the body does not cause a deficiency symptom. A deficiency interferes with many different body processes, including how the body uses other nutrients (1). Deficiencies of vitamins are classified as either primary or secondary. A primary deficiency occurs when an organism does not obtain enough of the vitamin in its diet. A secondary deficiency on the other hand may be due to an underlying disorder that prevents or limits the absorption or use of the vitamin due to a person's way of life such as smoking, excessive alcohol consumption, or the use of medications that interfere with the absorption or use of the vitamin (2).

Riboflavin

Riboflavin is an isoalloxazine derivative with a ribitol side chain and has the chemical name 7, 8-dimethyl-10 (1'-D-ribityl) isoalloxazine. Riboflavin forms of orange-yellow crystals and water solutions have intense greenish yellow fluorescence. Riboflavin exhibits fluorescence due to the pi conjugation and resonance of isoalloxazine and fluoresce in light of wavelength 440 to 500 nm. The intensity of fluorescence is proportional to the concentration of riboflavin in dilute solution (1, 3, 4). The molecular formula of riboflavin is $C_{17}H_{20}N_4O_6$ and has a molecular weight 376.36 g/mol (3, 4).The melting point is 290^{o}C. The molecular structure of riboflavin is shown in Figure 1.

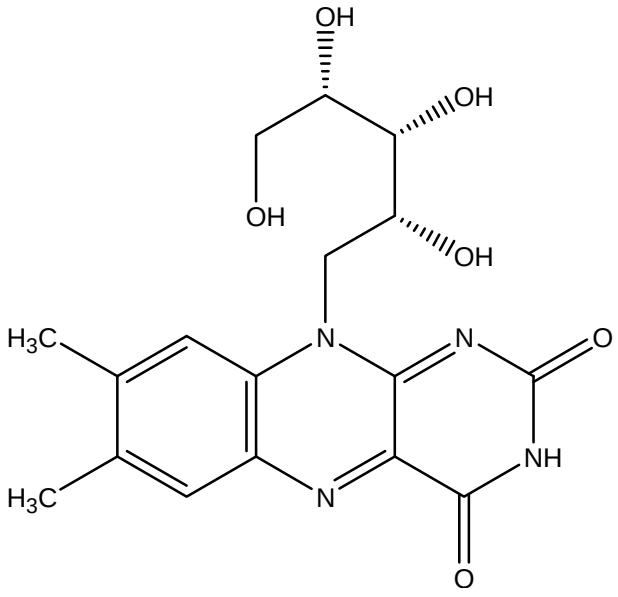

Figure 1: Molecular Structure of Riboflavin (Vitamin B_2)

Riboflavin, also known as vitamin $B_{2,}$ derives its name from its color. The root of this word is the Latin word "flavus" meaning "yellow" (5). It is worth noting that one of the naturally-occurring coloring agents in milk, called lactoflavin, is also a functional form of this vitamin (5). Riboflavin is a water- soluble vitamin, which means it is not stored in the body and has to be replenished on a daily basis. Excess riboflavin is excreted in urine and it is responsible for the bright yellow color of urine following high level supplementation with B-complex vitamins. There is no known toxicity to riboflavin. The highest concentrations of riboflavin in the body occur in the liver, kidneys, and heart (2, 5).

Riboflavin is a food coloring substance and it is also used to enrich or fortify some foods such as bread, cereals, baby foods, pastas, sauces, processed cheese, fruit drinks, vitamin-enriched milk products, some energy drinks, and supplements (1, 3). Milk, cheese, leafy green vegetables, liver, kidneys, legumes such as mature soybeans, yeast, almonds, and rock lobsters

are good sources of vitamin B_2 (1,3,5). Riboflavin is sensitive to light and is destroyed by exposure to light; consequently foods with riboflavin should not be stored in glass containers that are exposed to light. Approximately 50% of the riboflavin in milk contained in a clear glass bottle can be destroyed after two hours of exposure to bright sunlight (6). The most common forms of riboflavin available in supplements are riboflavin and riboflavin 5'-monophosphate. Riboflavin is most commonly found in multivitamin and vitamin B-complex preparations.

Functions of Riboflavin. Riboflavin is present in the body primarily as a basic constituent of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Coenzymes derived from riboflavin are termed flavocoenzymes, and enzymes that use a flavocoenzyme are called flavoproteins. The functions of riboflavin in the body include the production of energy, metabolism of the cofactor for homocysteine, recycling of glutathione, and maintaining the supply of vitamin B3 (6, 7)

Vitamin B_2 plays a significant role in the body's release of energy from carbohydrate, protein, and fat in food (1). When active in the body's energy production pathways, riboflavin typically takes the form of flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN). When riboflavin is converted into these FAD and FMN forms, it can attach to protein enzymes and allow oxygen-based energy production to occur. Flavoproteins are found throughout the body and particularly in locations like the heart and skeletal muscle where oxygen-based energy production is constantly required (5).

Studies indicate that people who have inadequate riboflavin status are more likely to have elevated homocysteine level than those whose riboflavin status is adequate. FAD among

other roles serves as a cofactor for an enzyme methylene tetrahydrofolate reductase (MTHFR) that is involved in the breakdown metabolism of homocysteine. High levels of homocysteine have been found to be associated with increased risk of cardiovascular disease (5, 6, 7).

Riboflavin also has antioxidant properties. It is a cofactor for the enzyme glutathione reductase that reduces the oxidized form of glutathione back to its reduced version. Glutathione is a small, protein-like molecule responsible for helping to prevent the inadvertent damage of structures in the body by highly reactive oxygen species such as hydroperoxides.(5)

Riboflavin plays a very important role in maintaining supplies of its fellow B vitamins. One of the pathways used in the body to synthesize vitamin B_3 (niacin) is by conversion of the amino acid tryptophan. This conversion process is accomplished with the help of an enzyme called kynurenine mono-oxygenase, and vitamin B_2 (in its FAD form) is required for this enzyme to function. (5, 6, 7)

Vitamin B_2 aids in the prevention as well as the treatment of migraine headaches, cataracts, rheumatoid arthritis, and a number of skin disorders such as acne (acne rosacea), dermatitis, and eczema (5). In the treatment of anemia, adding vitamin B_2 to iron supplements has shown to increase its effectiveness. Vital to maintaining a proper metabolism, riboflavin also helps to shore up the immune system by reinforcing antibody reserves, the body's first line of defense against infection. Along with iron, riboflavin is essential for producing the red blood cells that carry oxygen throughout the body. In addition, the body uses extra riboflavin to keep tissue in good repair and speed healing of wounds, burns, and other injuries (5).

Vitamin B_2 is capable of protecting the nervous system along with other B vitamin such as $B_{6,}$ folate, and niacin. It may therefore have a role in treating nervous system conditions

such as numbness and tingling, Alzheimer's disease, epilepsy, multiple sclerosis, and even anxiety, stress, and fatigue. Carpal tunnel syndrome may benefit from a treatment program including this vitamin when combined with vitamin B_6 (5). The body needs vitamin B_2 for reproduction and in the health of hair, nails, and skin (5). The ingestion of riboflavin with medication and its urinary measurement is an accepted method of compliance detection. Riboflavin is therefore frequently employed as a tracer of medication compliance in the treatment of patients with alcoholism, psychological disorders, and other conditions (8).

Recommended Daily Allowance (RDA). The RDA for riboflavin varies according to weight, metabolic rate, growth, and caloric intake of an individual. Riboflavin requirement must equal to the total energy needs and metabolism (9). The recommended daily allowance (RDA) for riboflavin is 1.7 mg/day for an adult man and 1.3 mg/day for an adult woman. The amounts found in many multivitamin supplements (20–25 mg) are more than adequate for most people. Women who are pregnant require an additional 0.3 mg per day and those who are lactating require an additional 0.5 mg per day. Riboflavin is best taken with B group vitamins and vitamin C. Extra amount might be needed when an individual is consuming alcohol, antibiotics, and birth control pills or doing strenuous exercise. If one is under a lot of stress or on a calorie-restricted diet, this vitamin could also be of use. Taking multivitamin or multimineral supplement containing 100% of the Daily Values (DV) will ensure an intake of at least 1.7 mg of riboflavin per day (6, 7, 9)

Deficiency of Riboflavin. Biochemical signs of depletion arise within only a few days of dietary deprivation. Deficiency of riboflavin is not common in the U.S. because the vitamin is

plentiful in the food supply. A shortage of this vitamin may manifest itself as cracks and sores at the corners of the mouth, eye disorders, inflammation of the mouth and tongue, and skin lesions. Dermatitis, dizziness, hair loss, insomnia, light sensitivity, poor digestion, retarded growth, and slow mental responses have also been reported. Burning feet can also be indicative of a shortage (5, 7, 9).

CHAPTER 2

ANALYSIS OF RIBOFLAVIN

Riboflavin is one of the water soluble B-group vitamins with a key role of preserving human health. The deficiency of riboflavin is associated with various diseases (10). The determination of riboflavin in foods, pharmaceutical preparations, and urine has been the subject of extensive research. Vitamin B_2 can be found in nature as the free riboflavin, but in most biological materials it occurs principally in the form of two coenzymes, flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) (6,11). Published tables of nutrient composition data commonly list only the total riboflavin (TRF) content of foods (12). Several methods for its analysis therefore typically involve a drastic acid treatment of the sample and conversion of these coenzymes into free riboflavin before quantification of total riboflavin (11, 13). A method for the analysis of riboflavin has to be sensitive, economical, reliable, and rapid. The analytical techniques that have been widely used and are recommended for the determination of riboflavin include Microbiological assay, High Performance Liquid Chromatography (HPLC), and Fluorimetric methods.

Microbiological Assay

Microbiological methods originated from the observation that certain microorganisms require specific vitamins for growth. Microbiological assay introduced by Snell and Strong (14) is usually applicable to the B vitamins. This method has been employed by many laboratories on a large variety of biological materials (15). The rate of growth of a species of microorganism that requires a vitamin is measured in a growth medium that contain various known quantities of a foodstuff preparation containing unknown amounts of the vitamin. The vitamin must first

be extracted from the sample in a water-soluble form, and in a state usable by the test organism (3). This method developed by Snell and Strong (14) is based upon the fact that the growth of *Lactobacillus casei* and its ability to produce lactic acid requires riboflavin in the medium (16). *Lactobacillus casei* grows readily, producing considerable quantities of the metabolite lactic acid which is determined by acid-base titration. The amount of acid produced is directly proportional to the riboflavin content (17). The riboflavin can be in the free form, but its combined forms, e.g. Flavoprotein, flavin adenine dinucleotide, and most probably riboflavin phosphate, are equally effective under the experimental conditions prescribed for the assay. Though the microbiological procedure postulated by Snell and Strong gives accurate results, it has the shortcoming of requiring several days to obtain results (18). Additionally, the microbiological method gives higher results than other methods on certain types of material, notably cereals and cereal products (15).

Barton–Wright and his co-workers (19) proposed a method that was a modification of the microbiological method proposed by Snell and Strong (14). Their new method produced better results with particular reference to cereals and cereal products. Barton-Wright and his co-workers used a number of different strains of *Lactobacillus helveticus* that were tested for quantitative response to riboflavin. Stock cultures of the organism were carried on a yeast-water glucose gar. The cultures were kept in a refrigerator at a temperature of approximately 4^{o} and removed at monthly intervals. The response of the organism to the added riboflavin was determined by turbidity produced as a result of growth of the bacteria. Response was also measured by direct titration of acid produced during the growth with 0.1 *N* NaOH, using bromothymol blue as indicator. Barton–Wright and his co-workers conducted florometric

analysis alongside the microbiological assay and found the agreement between the results obtained by the two methods to be good (19).

High Performance Liquid Chromatography (HPLC) Technique

High Performance Liquid Chromatography (HPLC) is a widely used analytical technique in many research and industrial laboratories. Many HPLC methods have been published in the past regarding the determination of water-soluble vitamins in foods or enriched foods (20). The HPLC methods all involved reversed-phase columns with mostly fluorescence detection and sometimes UV detection (21). The use of fluorescence detection reduces the number of interfering peaks on the chromatogram and therefore the riboflavin peak is well resolved. Ultraviolet detection on the other hand gives poor sensitivity, as UV-absorbing interfering substances often cannot be separated from the riboflavin peak (17). HPLC permit the analysis of riboflavin as well as the simultaneous assay of a number of B-group vitamins such as vitamin B_1, B_2 and B_6. Extraction of riboflavin is usually done by autoclaving or boiling with acid followed by an enzymatic digestion (21). The purpose of the acid treatment usually performed, with 0.1 M HCl in a water bath at 100 ^{o}C or in an autoclave at 121 ^{o}C, is basically to denature the proteins and to release the vitamins from their association with the proteins and, secondarily, if the foodstuff under study contains a lot of starch, to convert this polysaccharide into soluble sugars. With regards to enzymatic treatment, it allows the dephorsporylation of the vitamins (22).

Wehling and his co-workers (23) carried out the simultaneous determination of pyridoxine, riboflavin, and thiamin in Fortified Cereal Product by High Performance Liquid Chromatography. Following extraction, pyridoxine, riboflavin, and thiamin were separated

from other sample components by an ion–pairing mechanism using a reverse phase (ODS) column with an acidified methanol /water mobile phase containing sodium hexansulfonate. Pyridoxine and riboflavin were quantitated by fluorescence detection with 288 nm excitation. The fluorescent thiochrome derivative of thiamin was formed by postcolumn addition of an alkaline ferricyanide solution, and the thiochrome quantitated by a second fluorescence detector. A column-switching technique was used to minimize the required chromatographic time, allowing quantitation of all three vitamins in less than 25 min. Detection limits were 2 μg/g for pyridoxine and 1 μg/g for both riboflavin and thiamin. Relative standard deviations of the analytical procedure were reported as 1.66 %, 1.51 %, and 2.06 % for pyridoxine, riboflavin, and thiamin, respectively. Chromatographic specificity makes the high-performance liquid chromatographic method subject to less interference than the fluorometric method (23).

A sensitive method for the determination of thiamin and riboflavin in meat and meat products has been developed by Catharina Ang and Frederick Moseley. (24) They used high-pressure liquid chromatography and fluorometric detection. The most significant part of their work was the conversion of riboflavin to lumiflavin by UV irradiation and oxidation of thiamin to thiochrome prior to the chromatographic separation. These conversions enhanced the detectability of trace amounts of vitamins. The lowest detection limit for thiamin was 0.05 ng and for riboflavin 0.02 ng. Five samples of meats or meat products were analyzed and the recovery data for added vitamins were given. Mean recovery values ranged from 84.4 to 94.2% for thiamin and from 88.1 to 99.9% for riboflavin. (24)

Reyes and his co-worker (25) proposed an analytical technique for the simultaneous determination of riboflavin and thiamin in selected unprocessed and processed cereal products.

The samples used in the study were all purpose flour, wheat cracker, cheddar cheese cracker, vanilla wafer, chocolate chip cookie, shortbread cookie, and peanut butter cookie. The method involved a simple sample preparation followed by the oxidation of thiamin to thiochrome and then a Sep-pak clean-up and concentration. Finely ground samples containing about 10-40 µg of riboflavin and thiamin were weighed and to this 60 mL of 0.1N HCl was added. Samples and standards were autoclaved at 121^{o}C (15 psi) for 30 min. The samples were then cooled to room temperature and 2 N sodium acetate added to adjust pH to 4.0 -4.5. Five milliliters of 10% aqueous taka-diastase was added to this and incubated at 50^{o}C for at least 3 hours. The solution was then filtered and diluted for analysis. A stainless column packed with 10 µm particle size octadecylsilica was used to ensure a good separation of riboflavin and thiamin (thiochrome). Using single fluorescence detection, it was possible to measure low levels of the two vitamins from a single chromatogram. The detection limit of riboflavin was 0.01 mg and that of thiamin was 0.005 mg. Overall, the results obtained using their HPLC method was comparable with those obtained using the AOAC method (25).

Eva Barna and his co-workers (20) have reported the simultaneous determination of thiamine and riboflavin in meat and liver by high-performance liquid chromatography using UV detection. In preparing samples for analysis, homogenized spare rib, chop, ham, and liver samples were treated with acid at 121°C, for 30 minutes, followed by enzymatic digestion to release the vitamins. After a clean-up procedure, analysis was carried out with a Nuceosil ODS (3 µm) packed column at 45°C. The mobile phase used composed of phosphate buffer (pH 3.0) – acetonitrile (84:16, v/v) containing 5 mM sodium heptanesulphonate. They reported a

relative standard deviation of 5 % for thiamine, 12 % for riboflavin in meat, and 8 % for thiamine and 5 % for riboflavin in liver for four parallel determinations. (20)

Luiz Severo Silva and his co-workers (26) also employed HPLC in the determination of riboflavin in skimmed and full milk in the presence of tetracyclines. The proteins were first precipitated with trichloroacetic acid and separation carried out by reversed phase HPLC with fluorescence detection. A C_8 column was used with isocratic flow using a mobile phase consisting of 0.1 $molL^{-1}$ sodium acetate, 35 mmol L^{-1} calcium chloride, and 25 mmol L^{-1} EDTA :methanol (65:35v/v). The method was validated by means of the following parameters: linearity (0.9980), linear range (0.5 – 1.3 µg mL^{-1}), intra- and inter-assay precision (RSD ≤0.5 %), accuracy (recovery 92 %), and detection and quantification limits (0.75 µg mL^{-1} and 0.22 µg mL^{-1}, respectively). The method was applied for analysis of full cream and skimmed milk, showing robustness in the presence of tetracyclines, and when compared with a spectrofluorimetric method showed no significant difference ($p \leq 0.05$) between the results obtained (26).

Fluorometric Method

Analysis of riboflavin by fluorometric method is made possible for the reason that riboflavin fluoresces strongly when exposed to light of wavelength in the range of 440 to 500 nm, and the intensity of the fluorescence is proportional to the concentration of riboflavin in the solution examined (16, 27). The usefulness of florescence methods is being increasingly recognized for their excellent sensitivity, selectivity, non-invasiveness, and rapidity (28). This procedure first involves the extraction of riboflavin into solution followed by the assessment of its fluorescence. The application of this method is fairly straightforward in the absence of

interference from fluorescing impurities. In the presence of interfering substances, however, it is necessary to take elaborate precautions to eliminate their effects (16).

One of the methods used to cancel the effects of interfering substance involves the use of sodium hydrosulfite (dithionite), $Na_2S_2O_4$. This is made possible because riboflavin is not destroyed by mild oxidation or by reduction (27). Sodium hydrosulfite reduces riboflavin to a nonfluorescing form without altering the fluorescence of the interfering substances. A fluorometric reading is first taken of the riboflavin solution after which the riboflavin is destroyed and a second reading taken as a blank. The reading for the blank is subtracted from the total fluorescence of the unknown to determine the florescence due to the riboflavin alone (11, 27).

Another means of getting rid of the effect of interfering substances is to reduce with stannous chloride and sodium hydrosulfite all the fluorescent pigments including riboflavin. The riboflavin is afterward reoxidized by contact with air which brings it back to its original fluorescent form but leaves in reduced form some of the substances that would otherwise interfere with the analysis. (16, 27)

Another procedure entails the use of dilute permanganate under controlled conditions to oxidize interfering pigments without affecting the riboflavin. The excess permanganate is removed with hydrogen peroxide (16). The disadvantage in this procedure is the formation of minute bubbles of oxygen which tend to remain dispersed in the medium and gives a whitish tint to the riboflavin fluorescence. This interferes with the accuracy of the fluorescence measurement (30, 31).

There are other challenges that are encountered in fluorometric assay especially with regards to cereal and cereal products. Some of the difficulties include the presence of colloidal matter or fine suspended particles in the final riboflavin solution as prepared for visual or photoelectric assessment of its fluorescence. Such material cannot be removed by centrifuging or normal filtration and its effect in absorbing and scattering the blue exciting light can easily be so great as to mask completely any fluorescence. Another impediment to the analysis of riboflavin by fluorescence is the initial low concentration of riboflavin in some samples such as wheat that causes the fluorescence of final solutions derived from such materials to be too small to be measurable with any degree of accuracy (29).

Inayat Rashid and Donald Potts (30) developed a fluorometric method to determine riboflavin in milk. The method involved extraction with lead acetate and subsequent quantification of riboflavin using fluorescence. The milk products used in the analysis included skimmed, 2 % partially skimmed, homogenized, 2 % partially skimmed chocolate, and nonfat dry milk. The method was simpler, less expensive, and more rapid as compared with other methods for analyzing riboflavin. The method was sufficiently rapid to permit the assay of a large number of samples in one day. Recoveries in the range 90 % -110 % and standard deviations from 1.71 -3.16 were obtained depending on the product. Their method was an improvement of the AOAC (28) method that requires samples to be acidified and autoclaved. They also avoided the use of potassium permanganate and hydrogen required for the removal of background fluorescence of impurities as stipulated in the AOAC method.

An alternative approach to the analysis of riboflavin in complex food samples with a great variety of inherently fluorescent compounds has been described by Ewa Sikorska and his

co-workers (28). They conducted a simultaneous analysis of riboflavin and aromatic amino acids in beer using fluorescence and multivariate calibration methods. The major components of beer such as water, ethanol, and carbohydrates are non-fluorescent; however, beers contain a very wide range of minor constituents, including proteins, amino acids, vitamins, and phenolic compounds, some of which exhibit fluorescence. Riboflavin and aromatic amino acids were analyzed using their native fluorescence. They demonstrated the usage of intrinsic beer fluorescence combined with multivariate data analysis to simultaneously assay riboflavin and aromatic amino acid contents in beer (28).

Micellar Systems

The presence of hydrophilic and hydrophobic moieties in the structure of surface-active agents (surfactants) leads to several interesting consequences, the most important of which are adsorption at the water-air interface and self-association to form micelles, liquid crystals, or vesicles. In dilute aqueous solution, surfactants, also known as detergents, aggregate in the form of small colloidal particles or micelles in which the apolar groups (alkyl or alkylphenyl) form the micellar core, whereas the polar or ionic headgroups are localized at the aggregate surface. Upon reaching a characteristic concentration called the critical micelle concentration (CMC), surfactant molecules dynamically associate to form roughly spherical aggregates of colloidal dimensions commonly called micelles in equilibrium with the concentration of dissolved surfactant. At concentrations below the CMC in solution, the surfactant molecules exist as solvated monomers. The number of individual surfactant molecules present in a micelle aggregate is referred to as the aggregation number (*N*) (32, 33).

Florescence measurement can be enhanced by the judicious use of micelles in the analytical scheme. The use of micellar medium in analytical florescence measurements results in increased sensitivity, reduction in the number of potential interferences, and a greater experimental convenience (33). Depending on the nature of the head-group, a surfactant can be classified as cationic, non-ionic, zwitterionic, or anionic. Hexadecyltrimethylammonium bromide (CTAB) and Hexadecyltrimethylammonium chloride (CTAC) are examples of cationic surfactants. CTAB has a critical micelle concentration of 9.2×10^{-4} and an aggregation number of 61. CTAC conversely has a critical micelle concentration of 1.3×10^{-3} and an aggregation number of 78. The structures of CTAB and CTAC are shown in Figure 2

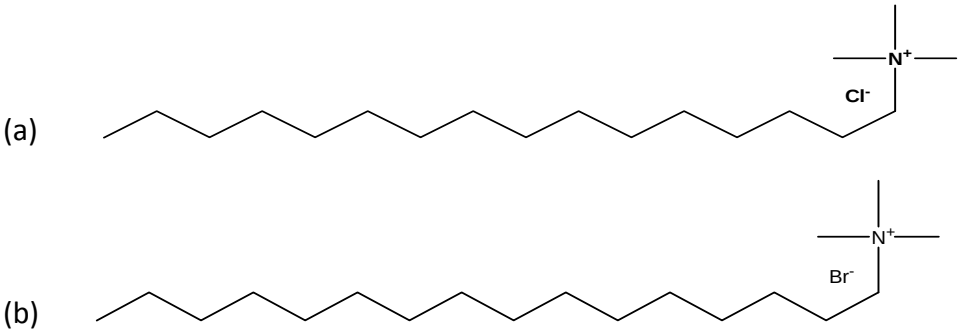

Figure 2: Molecular Structure of Hexadecyltrimethylammonium Chloride (CTAC), (a), and Hexadecyltrimethylammonium Bromide (CTAB), (b)

Examples of anionic surfactants are Sodium dodecylsulfate and 1-Ocatanesulfonic acid sodium salt. The structures of these two compounds are also shown on Figure 3 and Figure 4 respectively. Sodium dodecylsulfate has a critical micelle concentration of 8.1×10^{-3} and an aggregation number of 62.

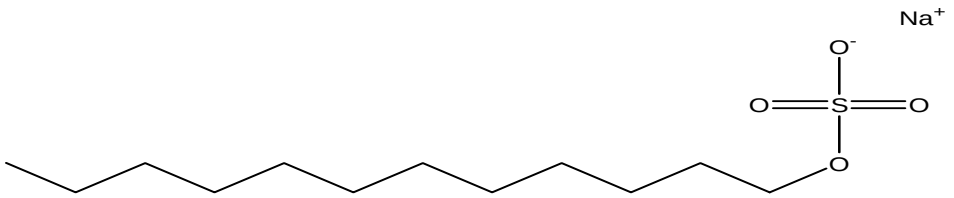

Figure 3: Molecular Structure of Sodium Dodecylsulfate (SDS)

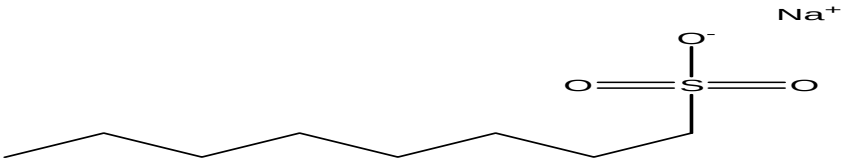

Figure 4: Molecular Structure of 1-Octanesulfonic acid Sodium Salt

Triton X-100 has a molecular formula of $C_{14}H_{22}O(C_2H_4O)_n$, The critical micelle concentration of Triton X-100 is 3×10^{-4} M and has an aggregation number of 143 (33).

Micelles are able to include or to organize solutes in their interior or in their colloidal surface. When a solute passes from the aqueous medium to the micellar medium, some changes are usually observed in several properties like reactivity, solubility, or spectroscopic characteristics; thus in fluorescence they have been important increases in the sensitivity, also the relative viscosity of these micellar microenvironments can inhibit quenching by molecular oxygen (34).

Garcia and co-workers (32) developed a method for the determination of thiamine, riboflavin, and pyridoxine in pharmaceuticals by synchronous fluorescence spectrometry in presence of micellar bis-2-ethylhexysulfosuccinate sodium salt (AOT) micelles. Pyridoxine (Vitamin B_6) and riboflavin (Vitamin B_2) both give a native fluorescence but for thiamine it was necessary for it to be oxidized completely to the one fluorescent derivative, thiochrome by alkaline ferricyanide treatment. Thiamine and pyridoxine were determined simultaneously,

while the riboflavin was analyzed separately due to its decomposition in presence of the thiochrome reagent. The concentration of AOT used was 3 X 10^{-4} M. Tablets were crushed, dissolved in water, and filtered. An aliquot was then dissolved in water with the corresponding quantity of AOT and analyzed by synchronous fluorescence using standard addition methods. The repeatability of the method as expressed in % R.S.D. was smaller than 1.4. The sensitivity was calculated from the slope of the straight line in the order of 10^{10} $Lmol^{-1}$ in all cases and detection limits were 12 μgL^{-1} for thiamine, 10 ugL^{-1} for pyridoxine and 9 ugL^{-1} for riboflavin (32).

Bhattar and his co-workers (34) had also carried out studies on fluorescence resonance energy transfer (FRET) between perylene and riboflavin in micellar solution of sodium dodecyl sulfate (SDS). This study was applied in the determination of vitamin B_2. FRET is a nonradiative process whereby excited state donor (D) transfers energy to a ground state acceptor (A). The rate of energy transfer is highly dependent on the extent of spectral overlap between the relative orientation of the transition dipoles and the distance between the donor and acceptor molecules. Bhattar and co-workers observed a strong overlap between the emission spectrum of perylene (donor) and riboflavin (acceptor). The water insoluble perylene was solubilized in non-fluorescent micellar solution. The micellar solution served as encapsulating system by providing a high viscosity microenvironment. Bhatter and co-workers noticed that the riboflavin fluorescence in solution of SDS is weak, but when placed in the SDS solution of perylene, the fluorescence intensity of riboflavin increased markedly and that of perylene decreased. Thus the process results in the quenching of perylene fluorescence and sensitization of riboflavin fluorescence. The fluorescence of perylene is quenched by riboflavin

and quenching is in accordance with Stern-Volmer relation. The efficiency of energy transfer was found to depend on the concentration of riboflavin. The value of critical energy transfer distance (R_o) was calculated by using Foster relation is 32.03 Å, and it was less than 50 Å, indicating efficient energy transfer in the system. The analytical relation was established between extent of sensitization and concentration of riboflavin, which helped to estimate vitamin B_2 directly from pharmaceutical tablets. There was a close agreement between observed values of vitamin B_2 and certified values in the samples (34).

A photophysical study on some dyes in aqueous solutions of triton X-100 had also been carried out by Bhowmik et al. (35). Triton X-100 is a neutral surfactant. The absorption and fluorescence spectral as well as photoelectrochemical studies of a few selective dyes, namely, anionic Erythrosin B, neutral riboflavin, and cationic Safranin O were carried out in aqueous solution of Triton X-100. The results showed that the ionic dyes Erythrosin B and Safranin O form 1:1 electron donor-acceptor (EDA) or charge-transfer (CT) complexes with Triton X-100 both in the ground and excited states, whereas neutral dye riboflavin in its excited state forms 1:1 complex with Triton X-100. In these complexes, the dyes act as electron acceptors whereas Triton X-100 acts as an electron donor. The Safranin O in presence of Triton X-100 show enhancement of fluorescence intensity with red and blue shifts respectively, while riboflavin shows normal quenching of fluorescence. A good correlation was found among photovoltage generation of the systems consisting of these dyes and Triton X-100, spectral shift due to complex formation and thermodynamic properties of these complexes (35).

Larguine and co workers (36) proposed a method for the determination of formaldehyde in aqueous samples by flow injection analysis in which the use of surfactant

enhanced the sensitivity of the method. In their procedure, formaldehyde reacts with acetylacetone, acetic acid, and ammonium acetate to form diacetylhydrolutidine, which was detectable by its fluorescence. The surfactants studied included Span 20, Span 85, Tween 85, Igepal CO-210, Igepal DM -970, Brij 30, Brij 70, and Triton X-405. Fluorescence enhancement for 15 % -70 % were observed in comparison with that without surfactant. The detection limit of 55 µg/L and a precision of 2.5 % at 1 µg/L level were obtained. A linear dynamic range OF 0.1-3000 µg/mL was achieved. Larguine and co workers applied their method in the determination of formaldehyde content in rain samples.

CHAPTER 3

METHODOLOGY

Fluorescence Spectroscopy

Fluorometric analysis is one of the most established analytical techniques used widely in many laboratories because of its extreme sensitivity, low detection limit, wide linear dynamic range, and selectivity in comparison with absorption methods. The first recorded observation of fluorescence was made by the Spanish physician and botanist Nicolas Monardes in 1565 from an extract of *Ligirium nephitciem*. The term fluorescence was however introduced by George Gabriel Stokes in 1852 after the fluorescent mineral fluorspar which exhibits a blue-white fluorescence. Stokes also introduced the concept of fluorescence as a light emission and established the technique of observing fluorescence with two different colored filters. In addition to this, he proclaimed the Stokes's Law that states emitted light is always of longer wavelength than the exciting light (37, 38, 39).

For a molecule to fluoresce it must first be able to absorb radiation. Only about 5 % to 10% of the molecules that absorb radiation eventually fluoresce. The benefit of this observable fact is obvious when considering possible interference in fluorescence. Molecules that do absorb high energy radiation such as UV radiation lose part of the absorbed energy by collision. The remainder of this energy is reemitted as radiation at longer wavelength when the molecules move from the excited state to the ground state. Fluorescence involves emission between states of the same multiplicity without change in electron spin, usually singlet to singlet. Consequently, florescence is short-lived, with a life-time of less than 10^{-5} seconds. The

fluorescence lifetime refers to the mean lifetime of the excited state. The general equation that illustrates the relationship between the fluorescence intensity I and the lifetime τ is:

$$I = I_o e^{-t/\tau} \qquad 3.1$$

Where I is the fluorescence intensity at time t, I_o is the maximum fluorescence intensity during the excitation, t is the time after removal of the exciting radiation, and τ is the average lifetime of the excited state. The fluorescence lifetime of most organic molecules is in the nanosecond region. Fluorescence is just one of the pathways by which a molecule can return to the ground state. An excited state has numerous potential routes available by which it can get to the ground state. The pathways from the excited state to the ground state are classified as either radiative or non-radiative (38, 39,)

Molecules posses a series of narrowly spaced energy levels and can go from a lower to a higher energy level by the absorption of a discrete quantum of light, equal in energy to the difference between the two energy states. Between each pair of main electronic states are the various vibrational levels of the molecule. The Jablonski diagram, also referred to as partial energy-level diagram as shown in Figure 5, gives an explicit representation of some of the processes involved in the electronic energy transitions of a molecule. The ground singlet state (S_o) the first (S_1) and second (S_2) excited singlet states as well as excited triplet state (T_1) are represented by a stack of horizontal lines. The ground-state energy of the molecule is represented by the lowest heavy horizontal line. The energy levels for the ground vibrational states of the three excited states are also represented by three upper heavy lines. At room temperature most molecules in solution exist in the ground state where all the electrons are paired (38, 40).

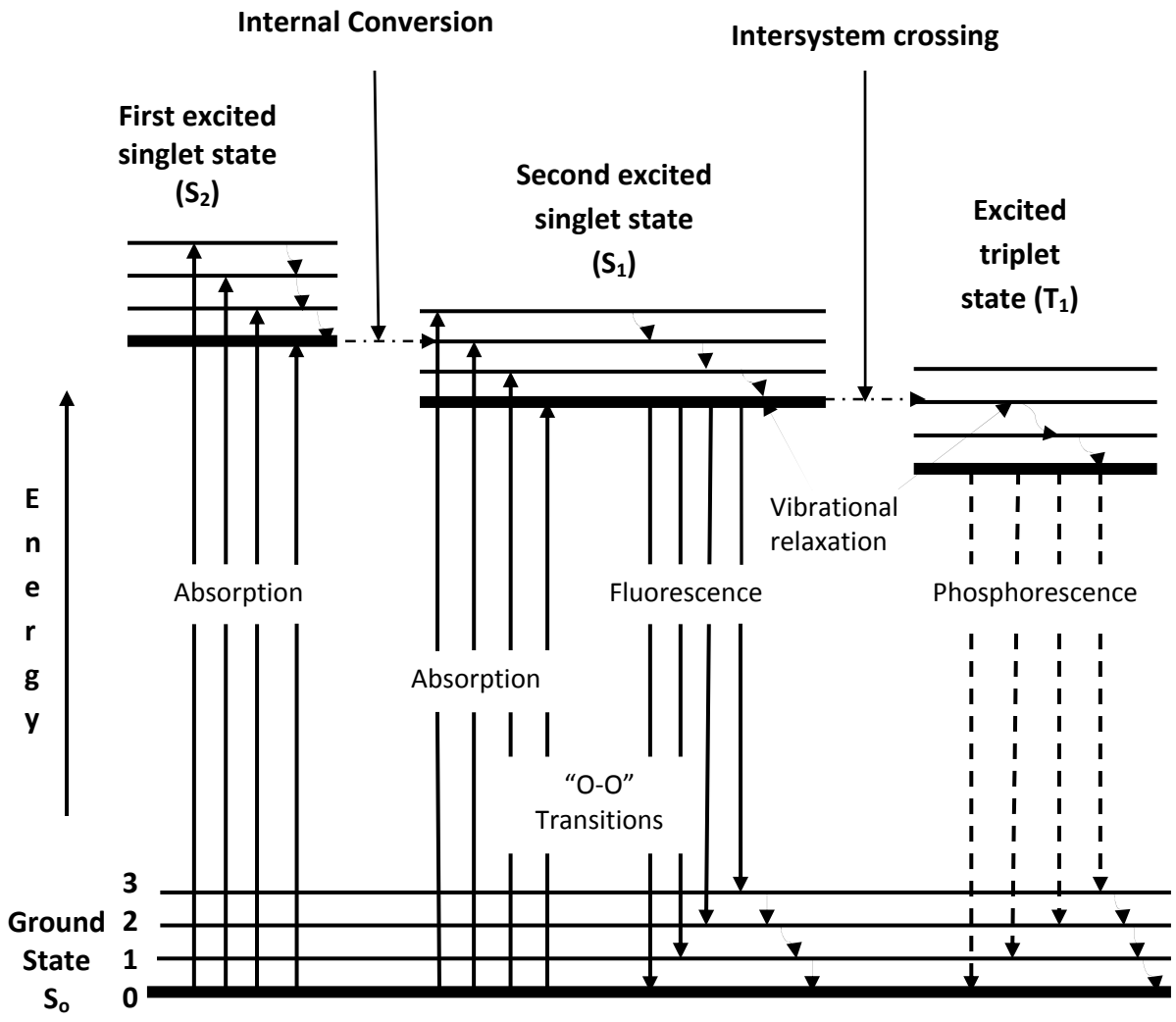

Figure 5: Jablonski Diagram

Note: Figure 5 is Jablonski diagram illustrating the processes involved in the formation of excited electronic states by photon absorption and subsequent radiative and non-radiative processes.

Paired electrons means electrons have opposite spins and are in the singlet state. On the other hand if electrons of a molecule have the same spin, they are unpaired and the molecule is in a triplet state. Singlet and triplet states are used in reference to the multiplicity of the molecule. (39, 40)

The process leading to fluorescence begins with absorption of a photon by a fluorophore within an average period of 10^{-15}seconds. This absorption results in an electron transition to an excited state of higher energy. A molecule may be promoted to any of several vibrational levels during the electronic excitation process. Collisions between molecules of the excited species and those solvent molecules at this point rapidly removes the excess energy from the higher vibrational level of S_1 in a process called vibrational relaxation (VR). Vibrational relaxation has an average lifetime of about 10^{-12} seconds. If the excited electronic state is higher than the S_1, internal conversion (IC), which is an isoenergetic transition of the excited molecule from a lower vibrational level of a higher excited electronic state to a higher vibrational level of a lower excited electronic state, occurs. Both vibrational relaxation and internal conversion are non-radiative processes. If all the excess energy is not further dissipated by collisions with other molecules, the electron returns to the ground, with the emission of energy. This phenomenon is what is referred to as fluorescence. Fluorescence from a solution, for that reason, always involves a transition from the lowest vibrational level of the lowest excited electronic state. Because some energy is lost in the short period before emission can take place, the emitted energy (fluorescence) is of longer wavelength than the energy that was absorbed (38, 39, 40).

The wavelengths of the emitted radiation are independent of the wavelength of excitation although the intensity of emitted radiation is proportional to the incident radiation. Another unique characteristic of excitation and emission transitions is that the longest wavelength of excitation corresponds to the shortest wavelength of emission. This is the “O-O”

transition that correspond to the transition between the O-vibrational level of S_o and the O-vibrational level of S_1 (38, 39).

In the excited state, a molecule can also undergo intersystem (ISC) crossing whereby the molecule reverses one of its electronic spin and transfer to a lower energy triplet state. In a triplet state, a molecule can undergo internal conversion and vibrational relaxation so as to move to the lowest vibrational level of the excited triplet (T_1). The transition of a molecule from this lowest vibrational level of the excited triplet state to the ground state is referred to as phosphorescence. Phosphorescence is much longer-lived than fluorescence because of transitions between states of different multiplicity is forbidden. The average lifetime of Phosphorescence ranges from10^{-4} to 10 seconds. Phosphorescence can thus persist for an appreciable time after the excitation source is turned off and this can be observed as an afterglow. Fluorescence emission, in contrast, ceases almost immediately after the excitation source is removed. The relatively long life of phosphorescence makes it prone to competition from radiationless processes hence only substances dissolved in a rigid medium phosphoresce. Phosphorescence, for that reason, is usually not observed in solutions where there can be collision with solvent or with oxygen. Phosphorescence measurements are made by cooling samples to liquid nitrogen temperature of about -196^{o}C to freeze them and minimize the other molecules (38, 39).

<u>Relationship Between Fluorescence Intensity and Concentration.</u>

G.G. Stokes established the relationship between concentration and fluorescence intensity and described the quenching of fluorescence at high concentrations and by the presence of foreign substances (37). Fluorescence intensity *F* is proportional to the radiant

power of the excitation beam that is absorbed by the system and can be derived from Beer's Law. That is,

$$F = \emptyset_f P_O (1 - 10^{-\varepsilon bc}) \quad [3.2]$$

Where Φ_f is the quantum yield and is a measure of the fraction of absorbed photons that are converted into fluorescent photons. P_o is the incident radiant power, ε is the molar absorptivity of the fluorescing molecules, b is the path length of the cell, c is the molar concentration, and εbc is the absorbance A. If the product εbc is large, the term $10^{-\varepsilon bc}$ becomes negligible compared to 1 and hence *F* becomes

$$F = \emptyset_f P_O \quad [3.3]$$

Alternatively, if εbc is small (≤0.05), the equation above could be approximated to be

$$F = 2.303 \emptyset_f P_O \varepsilon bc \quad [3.4]$$

Thus, for low concentrations, fluorescence intensity becomes directly proportional to the concentration and a plot of the fluorescence intensity of a solution versus concentration of the emitting species should be linear. This linear response is obtained until the concentration of the fluorescent species is large enough to absorb significant amounts of exciting light. The first part of the solution in the path absorbs more at higher concentration resulting in a decrease of fluorescence intensity with increasing concentrations. Thus at high concentrations, light scattering in addition to the inner filter effects become significant (37, 39, 40).

Apart from the concentration, the basic fluorescence intensity-concentration equation displayed as Equation 3.3 shows that there are three major factors that affect the fluorescence intensity. These factors comprise the quantum efficiency $\emptyset_f$, the intensity of the incident radiation P_o and the molar absorptivity of the compound ε. The quantum efficiency or yield is

the ratio of the number of molecules that fluoresce to the total number of excited molecules. The greater the value of $\emptyset_f$, the greater the observed fluorescence of the compound. For a highly fluorescent molecule, the quantum efficiency approaches unity under some conditions. A nonfluorescent molecule is one whose quantum efficiency is zero, or so close to zero that the fluorescence is not measurable With regards to the intensity of incident radiation P_o, it is observed that the more intense the source, the greater the fluorescence, yet a very intense source can cause photodecomposition of the sample. Laser, especially pulsed, have proven quite useful. Mercury and xenon sources with moderate intensity have often been used as well. Finally, the higher the molar absorptivity of a compound ε, the greater will be its fluorescence intensity. As a result saturated nonaromatic compounds are nonfluorescent (38, 39, 40).

Fluorescence and Chemical Structure

For a molecule to fluoresce, it must first absorb radiation. The greater the radiation absorbed by a molecule, the greater its fluorescence intensity. The absorption of radiation is highly specific, and radiation of a particular energy is absorbed only by a characteristic structure. Thus the intensity of fluorescence depends on the chemical structure of the fluorophore. Compounds or group of atoms which enhance fluorescence includes aromatic and heterocyclic compounds, electron donating groups, polycyclic compounds, and compounds containing multiple conjugated double bonds. These compounds have aromatic functional groups with low-energy $\pi \rightarrow \pi^*$ transitions. There are also compounds with certain functional groups which inhibit fluorescence. Compound with functional groups such as $–NO_2$, -COOH, -CH_2COOH, Br, I, and azo groups tend to hinder fluorescence. Compounds that are non-

fluorescent may be converted to fluorescent derivatives to facilitate analysis by florescence (39, 40, 41).

Fluorescence Instrumentation

The fluorometers and spectrofluorometers are composed of a light source, filters and monochromators, tranducers, cells and cell compartment, and a computer or electronic data system.

Sources. The excitation radiation for the sample is provided by the source. The magnitude of the output signal in fluorescence measurement and, for that matter, the sensitivity of the method is directly proportional to the radiant power of the source. There is therefore the need for a very intense source in fluorescence measurements.

A low-pressure mercury lamp equipped with a fused silica window is one of the most common sources in filter fluourometers. The output of a low-pressure mercury lamp includes a number of lines. Because the absorbance spectrum of most organic compounds is broad (20-30 nm), it is likely that a line will overlap the absorbance spectrum and excite the compound of interest. Some of the major merits of these lamps are that they are economical and have a long lifetime. Besides mercury lamps cadmium and zinc are frequently used lines sources (40, 42).

In spectroflurometers, a high-pressure xenon arc lamp with a continuum source of radiation from 200 nm well into the infrared is used. One important issue in the use of xenon lamps is the method of maximizing the amount of light that is focused on the entrance slit of the excitation monochromator because the more the light that impinges on the sample, the more the fluorescence and the better the sensitivity. Xenon lamps are available as either

continuous sources or pulsed sources. Continuous sources provide a significant amount of energy and hence are very useful for fluorescence. A pulsed source on the other hand makes it easy to measure phosphorescence because the compound is excited only when the source is on. Lamp sources are usually less costly and more applicable for the determination of multiple analytes with different excitation wavelengths (38, 40, 41).

Blue light-emitting diodes (LEDs) that emit radiation at 450-475 nm and appropriate for exciting some fluorophores are also used in certain fluorescence instruments. Such LEDs are used to make lighting products such as flashlights. An LED is a *pn*-junction device that when forward biased produces radiant energy. Mixtures of phosphors in some LEDs can provide wavelengths in the UV region to about 375 nm. LEDs have the advantage of long lifetimes and a smaller environmental impact. LEDs are readable at low ambient light levels as well as in fairly bright light, but they consume considerably more power and thus are not generally used in applications powered by batteries (40).

Besides the lamps used as source in fluorometers and spectroflurometers, lasers are also used as excitation sources in some fluorescence instruments. Laser provides isolated lines of a single wavelength for many applications. The brightness of a low-power laser at its output wavelength is 10^{13} times greater than that of the sun at its brightest wavelength. A laser light is typically plane polarized, coherent, and collimated. Laser source are very important when samples are very small, as in microbore chromatography and capillary electrophoresis where the amount of sample is a microliter or less. Other situations where the advantages of laser lamps are so obvious is in remote sensing, where the collimated nature of laser beam is needed as well as when highly monochromatic excitation is needed to minimize the effects of

fluorescing interferences. Tunable dye lasers pumped by a pulsed nitrogen laser or a Nd-YAG laser are some of the commonly used ones (40, 42, 43).

Detector. Due to its low emission intensities for very dilute samples, fluorescence instruments require very sensitive transducers. Photomultiplier tubes (PMT), which are extremely sensitive, are used to measure very low light level. They are made up of a cascade of similar metal surfaces. Electrons emitted from the photosensitive surface of a PMT due to incident photons strike a second surface, called a dynode, which is positive with respect to the photosensitive emitter. The function of the dynode chain of the photomultiplier is to amplify the signal and six to twelve dynodes are typically present. Electrons are accelerated and strike the dynode with more than their original kinetic energy. Each energetic electron knocks more than one electron from each of the dynode. These new electrons are accelerated towards a subsequent dynode, which is more positive than the preceding dynode. Upon striking the next dynode, even more electrons are knocked off and accelerated toward a following dynode. This process is repeated several times, so a gain of a million or more is achievable. Extremely low light intensities are translated into measurable electric signals. The potential across a photomultiplier may range up to 1000V. As the high voltage of the PMT is increased, the signal increases as does the noise. At high voltages the noise from the PMT can become very significant. The PMT are frequently operated in the photon-counting mode to give improved signal-to-noise ratios. Charge-transfer devices such as charge-coupled devices (CCDs) that permit the quick recording of both excitation and emission spectra are also used (38, 40, 42, 43)

In filter fluorometers, only filters are used for wavelength selection, whereas in spectrofluorometers two monochromators are employed for wavelength isolation. The wavelength selector transmits radiation that excites the sample molecules but excludes or limits radiation of the fluorescent emission wavelength. Fluorescence is most conveniently observed at right angles to the excitation beam as it is emitted from the sample in all directions. The radiation emitted passes through an emission wavelength selector that selects the fluorescence emission. The emission wavelength selector is placed between the sample and a photodetector located at a 90^{o} angle from the incident optical path. The isolated radiation then strikes the photodetector where it is converted to an electrical signal proportional to the intensity of the fluorescent energy. The output of the photodetector is amplified to give a reading on a meter or a recorder. The advantage of this right angle geometry is the minimization of contribution from scattering and from the intense source radiation. Thus the geometry and nature of the fluoremetric measurement accounts for its excellent sensitivity, which exceeds that of absorption methods by three to four orders of magnitude. While fluorometers are capable of measuring low concentrations of substances with good reliability, a spectrophotometer loses accuracy as the signal from the sample approaches that of the reference (40, 41).

Merits and Demerits of Fluorescence

There are a number of advantages associated with fluorescence. One of the chief advantages of fluorescence is its extreme sensitivity and good selectivity. Fluorometric methods can detect concentrations of substances as low as one part in ten billion, sensitivity 1000 times greater than that of most absorption spectrophotometric methods. The core

reason for this increase in sensitivity is that in fluorescence, the emitted radiation is measured directly and can be increased or decreased by altering the intensity of the exciting radiant energy. In absorption spectrometry, the difference between two finite signals is measured. The sensitivity is therefore determined by the ability to distinguish between the two signals, which is also dependent on the stability of the instrument and other factors. Additionally, in fluorescence, the signal depends linearly on concentration, and a much wider linear dynamic range of concentration of approximately three to four orders is possible. The selectivity of fluorescence is the result of two main factors. The first factor is based on the fact that there are fewer fluorescent compounds than absorbing compounds. All fluorescent compounds must necessarily absorb radiation, but not all compounds that absorb radiation fluoresce. The second reason is that two wavelengths are used in fluorometry, but only one in spectrophotomety. Two compounds that absorb radiation at the same wavelength will probably not emit at the same wavelength. The difference between the excitation and emission peaks ranges from 10 to 280 nm. Compounds that possess native fluorescence, those that can be converted to fluorescent compounds (fluorophors), and those that extinguish the fluorescence of other compounds can all be determined quantitatively by fluorometry (38,39)

There are a number of limitations in the use of fluorescence for analysis. The major drawback of florescence as an analytical tool is its serious dependence on environmental factors such as temperature, pH, and ionic strength. In addition to this, native fluorescence may be few compared to the number of molecules that go through absorption. There are however methods that are used to increase the number of molecules that fluoresce. These

methods include derivatization and complexation. Here the analyte is converted into a product with good fluorescence characteristics (38)

Photochemical decomposition is another major challenge in fluorescence analysis. The ultraviolet radiation for excitation may cause photochemical changes in, or decomposition of, the fluorescent compound, giving a gradual decrease in the fluorescence intensity reading. The fluorescence of a compound is also affected by the viscosity of the medium, increasing with increasing viscosity. Florescence measurements are again affected by quenching and temperature and these disadvantaged fluorescence.

Another disadvantage encountered with fluorescence technique is interference caused by highly fluorescent impurities present in the analyte solution. In addition to this, most fluorescent spectra tend to be broad and quite featureless. Above all, Beer's law can easily be violated when there are several components absorbing at approximately the same wavelength. To avoid interferences, florescence measurements are often combined with such separation techniques as chromatography and electrophoresis.

Another major concern with the use of fluorescence in making measurement is the need for a calibration curve for all analysis. Instrumental conditions may not always be identical between measurements. Contamination from reagents, laboratory glassware, and other interferents is more significant for florescence methods than for absorption methods. Precision and accuracy of florescence methods is usually poorer than spectrophotometric procedures by a factor of perhaps 2 to 5. The precision of florescence is often limited by source flicker noise and drift. The accuracy is often limited by concomitants, or particles, in the sample that cause additional fluorescence and scattering or that quenches the analyte fluorescence. One of the

major challenges of fluorescence is that of quenching. Quenching takes place as a result of substances that compete for the electronic excitation energy and decrease the quantum yield of fluorescence. Molecules that contain heavy atoms such as iodide and bromide substituent groups decrease the quantum yield. Although quenching of fluorescence is often a problem in quantitative measurement, the substances that causes these quenching may be determined indirectly by measuring the extent of fluorescence quenching. (38)

Proposed Research

A number of research works have been carried out in recent years to improve the sensitivity and selectivity of fluorescence measurement. Most of these methods however rely on instrumental means of improving fluorescence sensitivity and as a result require very expensive and sophisticated instrumentation. There is the need therefore for an economical non-instrumental means of achieving similar results. The rationale behind this project is to develop a method of analysis that makes use of micellar systems in enhancing the fluorescence of riboflavin. The objectives of the proposed method are as follows:

1. To develop a simple instrument based on LED excitation source.
2. To develop a sensitive, rapid, economical, and convenient method for the analysis of riboflavin in food products and vitamin tablets.
3. To establish optimal fluorescence conditions for the determination and quantitation of riboflavin in food products and vitamin tablets with special emphasis on ;
    a. The effects of different surfactants in enhancing florescence of riboflavin
    b. The effect of concentration of surfactant on riboflavin fluorescence
    c. The effects of combination of surfactants on the determination of riboflavin

d. The effect of combination of different concentration of surfactants on riboflavin determination

4. To establish the figures of merits for the fluorescence method: linear dynamic range, reproducibility, recovery, and accuracy.
5. To evaluate the applicability of our technique in the determination of riboflavin in commercial products.

CHAPTER 4

EXPERIMENTAL PROCEDURES, RESULTS, AND DISCUSSION

This chapter highlights the experimental procedures carried out to develop and establish the validity of the fluorescence method as well as the home built fluorometer used in the quantitative determination of riboflavin content in various brands of super B complex and cereals. The fluorescence method is assessed based upon its precision, accuracy, and applicability to the determination of riboflavin in super B complex and cereals. To validate the method, linearity, reproducibility, and recovery studies were performed.

Various surfactants have been used to enhance fluorescence signal (36, 44, 45). The effect of several surfactants on the florescence signal was examined in this study. Five different surfactants were examined. Two different cationic surfactants: hexadecylmethylammonium bromide (CTAB) and hexadecylmethylammonium chloride (CTAC), two anionic surfactants, sodium dodecyl sulfate (SDS) and 1- octanesulfonic acid, and one neutral surfactant: Triton X-100 were studied.

Experimental Procedures

Reagents Used

All the chemicals and reagents used were ACS certified and of the highest purity grade available from commercial sources and so were used without further purification.

1. Deionized Water, acquired from US Filter Company (Pittsburgh, PA).
2. Riboflavin, purchased from MP Biomedicals, (Inc. Solon, OH).
3. Glacial acetic acid, 99.8% purity, was obtained from Allied Chemical Company (Morristown, NJ).

4. Sodium acetate, obtained from MCB Manufacturing Chemist, Inc (Cincinnati, OH).
5. Sodium hydrosulfite, obtained from Fisher Scientific (Fair Lawn,NJ)
6. Sodium dodecyl sulfate (SDS), Hexadecyltrimethylammonium bromide (CTAB) and Hexadecyltrimethylammonium chloride (CTAC) from Aldrich Chemical Company (Milwaukee, WI).
7. 1-Octanesulfonic acid sodium salt, and Sodium dodecyl sulfate, obtained from Sigma Chemical Company (St. Louis, MO).
8. Triton-X-100 obtained from Eastman Kodak Company (Rochester, NY).
9. Enzyme Preparation, obtained from Fisher Science Education (Rochester, NY)

Preparation of Stock Solution

Riboflavin Stock Solution (20 µg/mL) – 20.0 mg of riboflavin was dissolved in 250 mL of deionized water in a 500 mL beaker. Then 10 mL of glacial acetic acid was added to the solution and transferred to a 1.0-L volumetric flask and diluted to the mark with deionized water. The stock solution was stored in a brown bottle at 4 ^{o}C and protected from undue exposure to light.

Preparation of Working Solution

Riboflavin working solution (5.0 µg/mL): 25 mL of riboflavin stock solution was pipetted into a 100-mL volumetric flask and diluted to the mark with acetate buffer solutin. This solution was prepared on a daily basis and was always protected from undue exposure to light.

Preparation of Sodium Acetate Buffer

Sodium acetate buffer – 4.1 g of sodium acetate was weighed out on an analytical balance and dissolved in 900 mL of deionized water. The pH was adjusted to 4.0 using glacial

acetic acid. It was transferred to 1.0-L volumetric flask and diluted to the mark with deionized water.

Preparation of Surfactant Solutions

All surfactants were prepared to approximately five times their critical micelle concentration to ensure the formation of micelles after fivefold dilution. The surfactant concentration must be greater than the critical micelle concentration (CMC)

CTAB - 0.168 g of CTAB was carefully weighed into a 50-mL beaker, and then 25 mL of deionized water was added. The solution was sonicated for 5 min and then transferred into a 100-mL volumetric flask and was diluted to the mark with deionized water.

CTAC - 0.208 g of CTAC was carefully weighed into a 50-mL beaker, and then 25 mL of deionized water was added. The solution was sonicated for 5 min and then transferred into a 100-mL volumetric flask and was diluted to the mark with deionized water.

SDS – 1.168 g of SDS was carefully weighed into a 50-mL beaker, and then 25 mL of deionized water was added. The solution was sonicated for 5 min and then transferred into a 100-mL volumetric flask and was diluted to the mark with deionized water.

1-Octanesulfonic acid sodium salt monohydrate, 98% - 1.172 g of 1-Octanesulfonic acid. Sodium salt monohydrate was carefully weighed into a 50-mL beaker, and then 25 mL of deionized water was added. The solution was sonicated for 5 min and then transferred into a 100-mL volumetric flask and was diluted to the mark with deionized water.

Triton X-100 – 61.08 µL of Triton X-100 was carefully measured into a 100-mL volumetric flask and diluted to the mark with deionized water.

Standard Solutions for Calibration Curve and Linearity Studies

To determine the linearity of the fluorescence signal with riboflavin concentration, the following procedure was used to generate a series of standard solutions for the calibration curve. Into seven different 10-mL volumetric flasks, 100, 200, 500, 1000, 2000, 4000, and 5000 µL of the riboflavin working solution was pipetted and then diluted to the mark with acetate buffer solution to make standard solutions of 0.05, 0.10, 0.25, 0.50, 1.0, 2.0, and 2.5 µg/mL, respectively. These samples were then ready for fluorescence measurement.

Preparation of Sample Solutions

Super B Complex. Each vitamin pill was crushed using a mortar and a pestle. Duplicate samples were weighed out from each of three tablets resulting in a total of six samples. 0.050 mg of Super B complex was accurately weighed into 50-mL beaker, and then 5 mL of acetate buffer solution was added. The sample was sonicated for 5 min and then stirred with a glass rod until dissolved. The dissolved solution was quantitatively transferred to a 100-mL volumetric flask and was diluted to the mark with acetate buffer solution. The solution was filtered through a No. 42 Whatman filter paper. A 1.00 mL aliquot of the filtrate was pipetted to a 10-mL volumetric flask and diluted to the mark with acetate buffer solution.

Cereal. The preparation of the cereal samples involves an extraction procedure that starts with an acid hydrolysis followed by an enzyme treatment for the complete breakdown of the phosphate ester. In the absence of an enzymic dephosphorylation step, flavin mononucleotide is expected to be present in the extracts and therefore low results might indicate insufficient dephosphorylation. One gram of cereal was carefully weighed out into a

125-mL conical flask. To this 35 mL of 0.1 M hydrochloric acid was added and placed in a water bath at 100 ^{o}C for 35 min. After allowing it to cool down, the solution was adjusted to pH of 4.5 with 2.5 M aqueous sodium acetate solution. Two mL of 5 % aqueous enzyme solution was then added and the mixture incubated in a 55 ^{o}C for 2.5 hours. The solution was diluted to 100 mL with acetate buffer solution in a volumetric flask and filtered through a filter paper. The filtrate was transferred into a 100-mL volumetric and diluted to the mark with acetate buffer solution. Fluorescence measurements were then carried out.

Preparation of Solutions for Reproducibility Studies

The solutions for these experiments were prepared in two ways. The first eight solutions were made using the standard working riboflavin solution (5.0 µg/mL). Into each 10-mL volumetric flask, 200 µL aliquots of the riboflavin working solution were pipetted and diluted to the mark with the acetate buffer solution. The samples were now ready for fluorescence measurement.

The second set of experiments was performed using super B complex sample to determine the reproducibility of the proposed procedure. Eight aliquots of 1.0 mL of the super B-complex sample solution prepared, according to the steps mentioned earlier, were pipetted into 10-mL volumetric flasks and then diluted to the mark with the acetate buffer solution.

Preparation of Solutions for the Recovery Studies

To determine the accuracy of the proposed method, recovery studies were carried out. A calibration curve and a series of spiked samples were prepared. Five standard riboflavin working solution ranging from 0.050 to 1.00 µg/mL were prepared as described for use in constructing a calibration curve. Nine aliquots of 1.00 mL super B complex solution prepared as

discussed previously were pipetted into nine 10-ml volumetric flasks. The solutions were then divided into three group triplicates. For the first triplicate, no standard riboflavin working solution was added, while 200 µL and 400 µL, of standard riboflavin working solution were added, respectively, to the second and third triplicates. The samples were then ready for fluorescence measurement.

Another set of experiments was conducted to determine the recovery of the proposed method using the cereal sample. The same procedure as described in the sample preparation section was used to prepare the cereal samples for the recovery studies except that the cereal samples were spiked with 750 µL and 1500 µL of standard riboflavin stock solution (20.0 µg/mL) for the second and third triplicates, respectively. The samples were then ready for fluorescence measurement.

Preparation of Commercial Samples

Cereal and Super B complex samples were used in the evaluation of the applicability of this method. About 1.0 g and 0.050 g of cereal and Super B complex were prepared following the procedures mentioned earlier in the chapter. Three 1000-µL aliquots of the prepared Super B complex sample solutions were pipetted into three separate 10-mL volumetric flasks and diluted to the mark with acetate buffer solution and fluorescence measurement carried out. The filtrates obtained after the sample preparation of the cereal products were transferred into a 100-mL volumetric and diluted to the mark with acetate buffer solution. Fluorescence measurements were then carried out.

Instrumentation

Fluorometric analysis is the method used in the analysis of riboflavin. Instrumentation used to measure fluorescence typically involves an excitation light source, an excitation wavelength selector, a sample holder to contain the sample solution, a means to select the fluorescence wavelength to be monitored, a detector capable of producing a signal proportional to the intensity of light striking it, and associated electronics and readout devices. A simple representation of such instrumental setup is displayed in Figure 6.

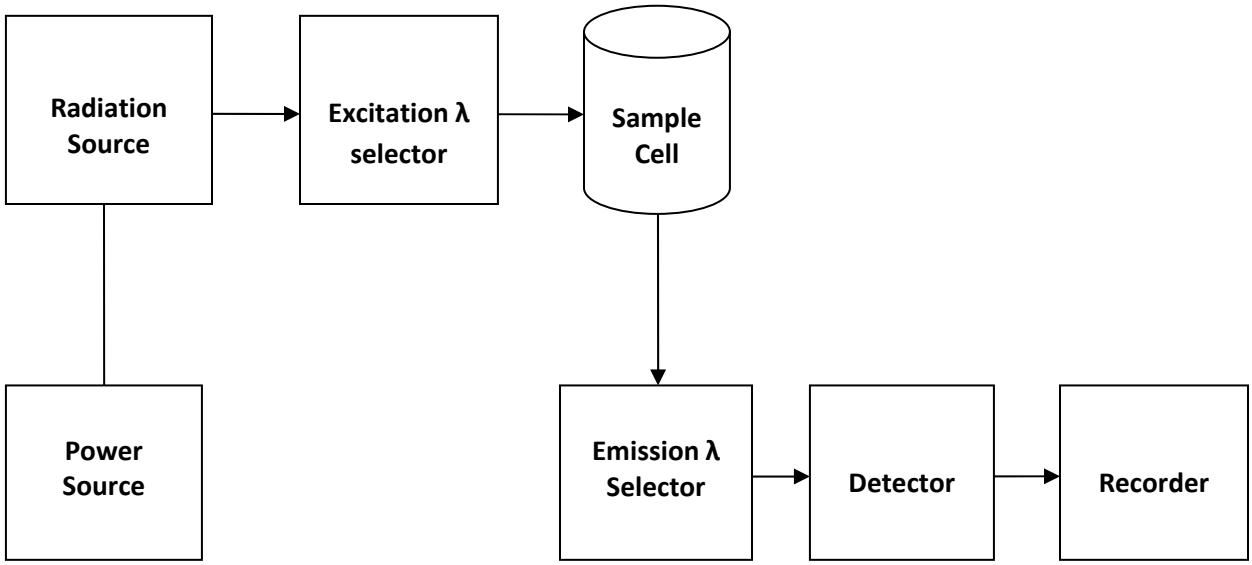

Figure 6: Schematic Diagram of the Home Built Fluorometer for Fluorescence Measurements.

For our analysis, we constructed a simple and economical fluorometer. Fluorescence signals were thus obtained with a homemade fluorometer built from economical commercial components. Blue excitation light source was provided by two light emitting diodes (LED) of wavelength between 450-500 nm. Two LEDs powered by two 9 V batteries were used to give

an intense light emission. The fluorescence emissions were analyzed at 90° from the excitation beam. There was no need for an emission wavelength selector (filter or monochromator) because the blue light emitting diode chosen emits light at around a wavelength of 490 nm. The light emitting diode is chosen to have a narrow bandwidth of emitted light that will bracket the excitation band of fluorescent molecules. A photomultiplier tube was used as detector and was housed in a chamber with slit on setting 2.0 mm or middle slit width. The photomultiplier tube provided a voltage signal proportional to the measured light intensity. A photomultiplier microphotometer is used to measure the signal. The fluorescence signal of each solution is first measured and then the solution is stirred with a little bit of sodium hydrosulfite, not more than 20 mg, just enough to destroy the greenish-yellow fluorescence. The fluorescence signal of the final solution is taken and then the difference calculated

Data Analysis

The homemade fluorometer was assembled to determine the intensity of fluorescence in the standard and sample solution. All experiments were conducted in triplicate and the data reported represent average values. Data were processed using Microsoft® Office Excel 2007 software for Windows® Vista. Means of the values of each sample were calculated. Standard deviations and relative standard deviations for them were all calculated using Excel. A plot of the calibration curves showing fluorescence signal and riboflavin concentration was obtained from the standard solutions prepared. The calculated mean values from the various samples were then substituted into the regression line equations obtained in order to get the riboflavin content.

Results and Discussions

In this section, the results of different experiments to develop, optimize, and determine the applicability of the proposed procedure are tabulated and discussed. A series of experiments were conducted to optimize the experimental conditions for the determination of riboflavin content in various brands of super B complex and cereal. The proposed method was evaluated by comparisons with values indicated by manufacturers.

Optimization of Instrumentation

Blue LED served as the excitation source for the home-built fluorometer. For efficiency, compatibility, and flexibility, an optical fiber was used for the collection of emitted fluorescence to the photomultiplier tube. The optical fiber restricted stray radiation. The use of two 9 V batteries resulted in inconsistent fluorescence reading after a time period and so they were replaced with constant power supply that provided consistent reading. Interference caused by stray radiations was a big challenge at the onset. To overcome this setback, an enclosure painted black on the inside was used and this protected the optical set up from external light.

Effect of Surfactants

The addition of surfactants to a solution containing a fluorescent compound has been shown to result in the enhancement of its fluorescence. (36, 44, 45) Surfactants therefore improve the sensitivity and selectivity of the analytical procedures. The factors responsible for the enhancement of fluorescence are however poorly understood. The micelle effect appears to be caused by the protection of the fluorescent compound from quenching in the bulk solvent (23, 45). The effects of various surfactants on fluorescence analysis of riboflavin were examined. The fluorescence characteristic of riboflavin is derived from the conjugate structure

it possesses. Anionic surfactants used were sodium dodecylsulfate (SDS) and sodium salt of 1-octanesulfonic acid, cationic surfactant used were hexadecylmethylammonium bromide (CTAB) and hexadecylmethylammonium chloride (CTAC), and the neutral surfactant used was Triton X-100.

The effect of these surfactants upon the fluorescence method was assessed by the addition of different volumes of surfactant to a 1.0 µg/mL riboflavin solution. Fluorescence measurement is enhanced in the presence of micelles and this occurs when the concentration of added surfactant is equal or greater than the critical micelle concentration. The measured fluorescence intensity for 1.0 µg/mL riboflavin solution with the different volumes of cationic surfactant hexadecylmethylammonium bromide (CTAB) and hexadecylmethylammonium chloride (CTAC) are shown in Table 1 and Table 2.

Table 1: Results of the Measured Fluorescence Intensity Signal of Riboflavin Solution with CTAB.

Volume of CTAB (mL)	0.0	0.5	1.0	2.0	4.0
Corrected Fluorescence Signal	12.20	11.10	11.70	11.65	11.75

Note: Different mL of CTAB solution (4.6×10^{-3} M) were added to 1 mL of 0.1 µg/mL riboflavin solution in a 10-mL volumetric flask and diluted to the mark with acetate buffer.

Table 2: Results of the Measured Fluorescence Intensity Signal of Riboflavin Standard Solution with CTAC.

Volume of CTAC (mL)	0.0	0.5	1.0	2.0	4.0
Corrected Fluorescence Signal	12.20	12.70	12.40	11.30	11.20

Note: Different mL of CTAC solution (6.5×10^{-3} M) were added to 1 mL of 0.1 µg/mL riboflavin solution in a 10-mL volumetric flask and diluted to the mark with acetate buffer.

The signal intensity of riboflavin generally decreased slightly with the addition of the cationic surfactants. Thus the cationic surfactants had some quenching effect on the fluorescence intensity signal of riboflavin. This might possibly be due to the nature of interaction between riboflavin and the cationic surfactants. The degree of fluorescence enhancement can be explained by the nature of the interaction between the fluorescent compound and the enhancer (46). The quenching effects of the cationic surfactants were not severe. Again when the cationic surfactants were applied to the commercial sample solutions, it resulted in an enhancement of the fluorescence of riboflavin by four times. The reason for the apparent quenching in fluorescence of the riboflavin standard solutions but significant enhancement in the case of the commercial sample solutions is not clearly understood. It is known that cationic micellar systems allow a smaller interhead group separation and thus might have a larger capacity to solubilize the riboflavin and as a result cationic micelles appear to show a larger enhancement effect than anionic micelles (46). A plot of fluorescence intensity signal versus mL of Raisin Bran cereal in the absence of CTAB surfactant and also in the presence of CTAB surfactants is displayed in Figure 7. The fluorescence signal of the of the sample solution measured in the presence of surfactants were about 70 % - 100 % higher than those measured in the absence of the surfactant. The difference in fluorescence signal might be attributed to the presence of other species present in the commercial samples. In the commercial samples, many other species for example, metal ions, are present some of which might quench the fluorescence of riboflavin, but when you add the cationic micelles, CTAB or CTAC, cationic species would be repelled from the micelle surface thus protecting the riboflavin if it is bound to those cationic micelles.

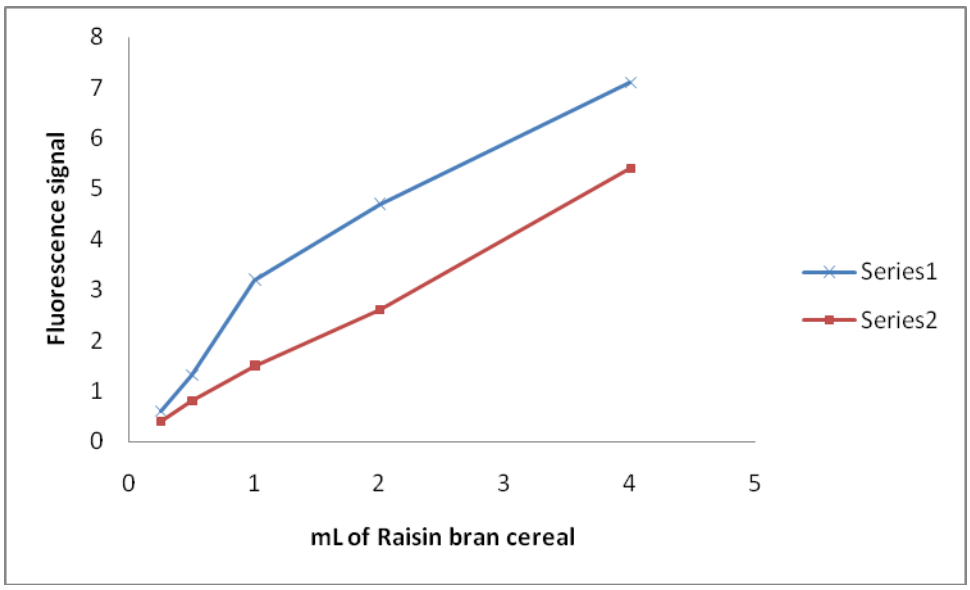

Figure 7: Plot of Fluorescence Intensity Signal versus mL Raisin Bran Cereal.

Note: Series 1 is sample solution in the presence of the cationic surfactant (CTAB)
Series 2 is sample solution in the absence of surfactant.

However, for the standard solutions prepared in just water, those quenchers are not present and no inherent fluorescence enhancement is observed. The greater signal observed for real samples in the presence of cationic micelles might be due to inhibition of fluorescence quenching when the micelles are present. Thus there is full quenching if micelle is absent, and less quenching, resulting in greater florescence signal in presence of micelle. (33)

The measured fluorescence intensity for 1.0 µg/mL riboflavin solution with the different volumes of anionic surfactants sodium dodecylsulfate (SDS) and 1-octanesulfonic acid sodium salt (OCT) are displayed in Table 3 and Table 4.

Table 3: Results of the Measured Fluorescence Intensity Signal of Standard Riboflavin Solution with SDS.

Volume of SDS (mL)	0.0	0.5	1.0	2.0	4.0
Corrected Fluorescence Signal	12.2	12.2	11.7	12.7	12.3

Note: Different mL of SDS solution (4.05×10^{-2} M) were added to 1 mL of 0.1 µg/mL riboflavin standard solution in a 10-mL volumetric flask and diluted to the mark with acetate buffer.

Table 4: Results of the Measured Fluorescence Intensity Signal of Standard Riboflavin Solution with OCT.

Volume of OCT (mL)	0.0	0.5	1.0	2.0	4.0
Corrected Fluorescence Signal	12.00	10.70	10.50	11.7	11.00

Note: Different mL of OCT solution (5×10^{-2} M) were added to 1 mL of 0.1 µg/mL riboflavin standard solution in a 10-mL volumetric flask and diluted to the mark with acetate buffer.

The values in Table 4 clearly shows there is no significant increase in the fluorescence signal of riboflavin standard solution with the addition of both sodium dodecylsulfate and 1-octanesulfonic acid. The fluorescence signal was however slightly higher in sodium dodecylsulfate than in 1-octanesulfonic acid sodium salt. Unlike the cationic surfactants, when the anionic surfactants were applied to commercial samples, there was no significant change in the fluorescence intensity signal of the riboflavin solution.

The result of the fluorescence intensity signal obtained for riboflavin standard solutions with the addition of different aliquots of the neutral surfactant Triton X-100 are displayed in Table 5 and Figure 8.

Table 5: Results of the Measured Fluorescence Intensity Signal of Riboflavin Standard Solution with Triton X-100.

Volume of Triton X-100 (mL)	0.0	0.5	1.0	2.0	4.0
Corrected Fluorescence Signal	12.20	12.70	12.90	13.2	13.15

Note: Different mL of Triton X-100 solution (1 X 10^{-3} M) were added to 1 mL of 0.1 µg/mL riboflavin standard solution in a 10-mL volumetric flask and diluted to the mark with acetate buffer

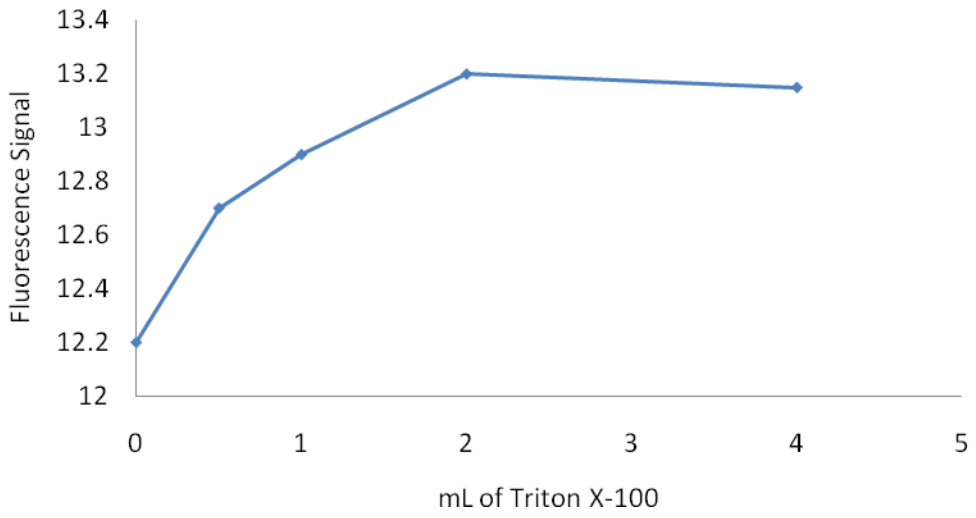

Figure 8: Plot of Fluorescence Intensity Signal versus mL of Triton X-100.
Note: The Fluorescence Signal is given in Arbitrary Units.

The addition of the neutral surfactant Triton X-l00 resulted in a slight increase in the fluorescence intensity signal of the riboflavin standard solutions and reached a maximum when 2 mL of the surfactant was added. This amount of surfactant in the riboflavin used was equivalent to the critical micelle concentration of the surfactant. Beyond that point, an increase in the amount of surfactant used did not result in any significant increase in the fluorescence signal. The result of the study on the effect of surfactant on the fluorescence of

riboflavin showed that most of the surfactants rather than improving the fluorescence intensity of riboflavin had a slight quenching effect on the proposed procedure. The effect of combination of different surfactants on the determination of riboflavin was also examined. Most of the combinations resulted in cloudy solutions and this reduction in solubility resulted in scattering of light. A combination of positive and negative surfactant, for example, hexadecyltrimethylammonium bromide (CTAB) and sodium dodecylsulfate showed opalescence problems and produced high background fluorescence thus they were not applied to the proposed method for the determination of riboflavin. Thus the effect of surfactants on pure riboflavin standard solutions is different from that of real samples because of other compounds present in the real samples.

Linear Dynamic Range

To show that the florescence signal produced was proportional to the concentration of riboflavin, a linearity study was carried out using the proposed fluorescence technique. Seven different concentrations of riboflavin within the range 0.05 -2.5 μg/mL were prepared for fluorescence measurement to establish a linear dynamic range. Each of the seven solutions was prepared in triplicate for the fluorescence measurement. The results for the three measurements were averaged and the relative standard deviations calculated. The results are tabulated in Table 6. The calibration curve is plotted and displayed in Figure 9. The regression line was observed to follow the equation $Y = 11.877 X + 0.0749$ with a correlation coefficient of 0.9998. The value of the correlation coefficient, $R = 0.9998$ indicates a very strong linear relationship between riboflavin concentration and fluorescence signal. These results were found to be consistent throughout the course of the project. Successive calibration curves for

reproducibility, recovery and application studies were constructed within this range of riboflavin concentration. The lowest detection limit was estimated to be 0.01 µg/mL. Below this riboflavin concentration, results obtained were not reproducible and did not satisfactorily fit into the linear relationship.

Table 6: Results of Linear Dynamic Studies

Standard Riboflavin Solution (µg/mL)	0.05	0.10	0.25	0.50	1.00	2.00	2.5
Average Fluorescence Signal	0.52	1.05	3.12	6.2	12.2	23.75	29.7
R.S.D. (%)	5.59	4.76	2.45	1.61	0.82	0.00	0.00

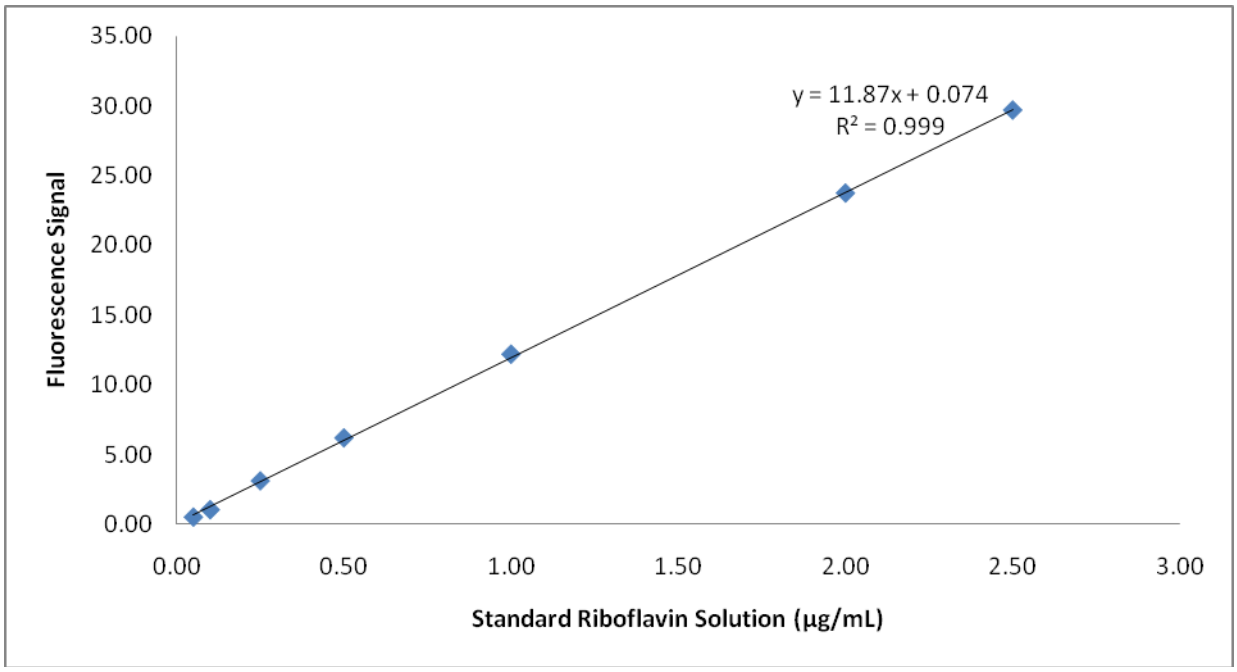

Figure 9: Calibration Curve of Riboflavin for Linear Dynamic Range Study.

Note: The fluorescence signal is given in arbitrary units.

Reproducibility Studies

A proposed analytical method has to be reproducible in order for it to be reliable and applicable for analysis. To evaluate the precision of the proposed fluorescence method, reproducibility studies were conducted. Eight samples each of two different concentration of riboflavin standard solution were used; one of a higher concentration and the other of a lower concentration of riboflavin. Additionally, eight aliquots of commercial samples each of Home Nature Brand and Spring Valley Brand of Super B complex were also used in order to determine the reproducibility of the measurement and the variability of these commercial items. The results of the first set of reproducibility studies are displayed in Table 7 and Table 8.

Table 7: Results of Reproducibility Studies using 0.25 µg/mL Standard Riboflavin Solutions

TRIAL	1	2	3	4	5	6	7	8
Fluorescence Signal	2.85	2.95	2.95	2.9	2.85	2.90	2.85	2.85
Average Fluorescence Signal			2.89					
Relative Standard Deviation			1.53%					

Table 8: Results of Reproducibility of Instrument Using 1.00 µg/mL Standard Riboflavin Solutions

TRIAL	1	2	3	4	5	6	7	8
Fluorescence signal	12.25	12.30	12.20	12.40	12.20	12.30	12.40	12.40
Average Fluorescence Signal			12.31					
Relative Standard Deviation			0.70%					

From the data obtained, the average signal was 2.89 for the 0.25 µg/mL riboflavin standard solutions and 12.31 for the 1.00 µg/mL riboflavin standard solutions. The relative standard deviations were 1.53 % and 0.70 % for the 0.25 µg/mL and 1.00 µg/mL respectively. Within the limit of experimental error, one can conclude that the precision of the proposed analytical method was very good. The second set of reproducibility studies carried out with commercial samples obtained from local stores in Johnson City, TN also yielded very good results as tabulated in Table 9 and Table 10.

Table 9: Results of Reproducibility Studies Using Spring Valley Brand Super B Complex

TRIAL	1	2	3	4	5	6	7	8
Fluorescence Signal	14.30	15.40	14.80	15.20	14.20	15.40	15.20	14.60
Average Fluorescence Signal			14.89					
Relative Standard Deviation			3.24%					

Table 10: Results of Reproducibility Studies Using Home Nature Brand of Super B Complex

TRIAL	1	2	3	4	5	6	7	8
Fluorescence Signal	20.7	21.00	20.8	20.20	20.60	19.80	19.60	20.90
Average Fluorescence Signal			20.45					
Relative Standard Deviation			2.56%					

Eight samples each of Spring Valley Brand and Home Nature Brand of Super B complex were prepared according to the procedures outlined earlier in this chapter. Fluorescence measurements of these samples were made after sample preparation. From the results

obtained from the experiments, the mean value of eight fluorescence measurements for Spring Valley brand of Super B complex was 14.89 and that of Home Nature brand of Super B complex was 20.45. The relative standards of deviations were 3.24 % and 2.56 %, for Spring Valley brand and Home Nature brand, respectively. The average of the relative standard deviation for all the reproducibility studies was 2.00%. The result thus demonstrated that the proposed procedure was reproducible for both pure riboflavin standard sample solutions as well as riboflavin present in commercial samples.

Recovery Studies

Recovery studies were carried out to determine the accuracy of the proposed method. Two sets of fresh riboflavin standard solution were prepared for use in constructing a calibration curve for two brands of Super B complex samples and two brands of cereal product samples as previously described in the sample preparation procedure section. The two sets of calibration were necessary because the experiments were carried out at different times. The linear regression equation Y = 12.253X – 0.0041 with a correlation coefficient of 0.9997 was used for all the calculations involving the super B complex tablets. The data obtained are tabulated in Table 11 and the resulting calibration curve is displayed in Figure 10.

Table 11: Average Fluorescence Signals of Standard Riboflavin Solutions for Recovery Studies.

Standard Riboflavin Solution (µg/mL)	0.05	0.25	0.50	1.00
Average Fluorescence Signal	0.52	3.12	6.2	12.2
R.S.D. (%)	5.59	2.45	1.61	0.82

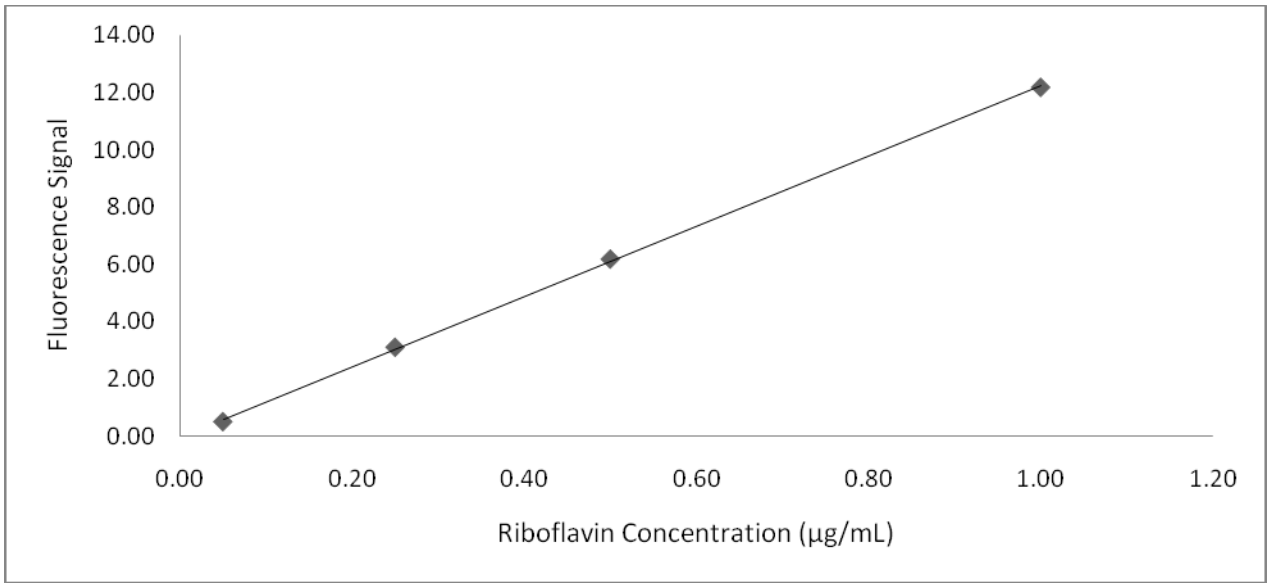

Figure 10: Plot of Florescence Signal versus Standard Riboflavin Concentration (µg/mL) for recovery studies. The fluorescence signal is given in arbitrary units.

The recovery studies were carried out on Spring Valley brand as well as Home Nature brand of Super B complex by spiking their sample solutions with two levels of riboflavin standard solutions. The commercial sample solutions used in the analysis were prepared as described previously. Home Nature Brand of Super B complex was used in the first study. Three triplicates of fixed riboflavin sample solutions were used. The first triplicates did not have any added riboflavin from the standard working solution. The second three aliquots had 500 µL of the 5.0 µg/mL of the riboflavin standard working solution put into each of them to give an added riboflavin concentration of 0.25 µg/mL. The last three had 1000 µL of 5.0 µg/mL of the riboflavin standard working solution put into each of them resulting in an added riboflavin concentration of 0.5 µg/mL. Fluorescence measurements were then carried out for the nine solutions. The results of the study are tabulated in Table 12. The recovery of the spiked amount of riboflavin was calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{Riboflavin (Real Sample + Added Standard)} - \text{Riboflavin (Real Sample)}}{\text{Riboflavin (Added Standard)}} x100\ \% \quad [4.1]$$

The difference between the average fluorescence signal of the analyte plus the amount of the riboflavin standard working solution added and the average fluorescence signal of the analyte alone is computed. Using the regression line equation, the difference in fluorescence signal is converted to concentration in µg/mL and then divided by the concentration of the added riboflavin standard solution.

The recovery range for the Home Nature brand for riboflavin is 97.19% to 102.97% with an average percent recovery of 100.21%.

Table 12: Results of Recovery Studies of Home Nature brand of Super B complex

Sample	Average Fluorescence Signal	Riboflavin Standard added (µg/mL)	Total Riboflavin found	Average Recovery (%)
1	13.73	0.000		
2	16.83	0.250	0.253	101.33
3	19.80	0.500	0.495	99.09
Mean Recovery		100.21 %		
Relative Standard Deviation		1.93 %		

Recovery studies for the Valley Spring brand of Super B complex were carried out in a similar fashion. The first three aliquots were prepared without added standard riboflavin working solution. Subsequently, 500 µL of the 5.0 µL of the riboflavin standard working solution was added to the second triplicates of Valley Spring band of Super B complex solutions in 10-mL volumetric flasks. The solutions were diluted to the mark with acetate buffer solution

to give an added riboflavin concentration of 0.25 µg/mL in each volumetric flask. The third triplicates were spiked with a riboflavin standard concentration of 0.75 µg/mL. Fluorescence measurements of the solutions were then made. The results of the measurement are tabulated in Table 13.

Table 13: Results of Recovery Studies of Valley Spring Brand of Super B complex

Sample	Fluorescence Signal	Riboflavin Standard added (µg/mL)	Total Riboflavin found	Recovery (%)
1	19.77	0.000		
2	22.85	0.250	0.252	100.79
3	28.70	0.750	0.729	97.25
Mean Recovery		99.02 %		
Relative Standard Deviation		2.41 %		

The difference in fluorescence signal to a large extent matched up with the concentration difference in the calibration curve. Percent recoveries were calculated based on stated formula. The percent recovery for the Valley Spring brand of Super B complex is 96.35 % to 102.97 % with 99.02 % as the average percent recovery. The recovery range for both brands of cereals is 97.16 % to 102.97 %. The percent recovery yielded values that are acceptable and within experimental errors, and the conclusion is that the proposed method is reliably accurate. Indeed, ideal values for the percent recovery should be around 100%, meaning that the matrix in which the analyte is present has an insignificant effect on its absorptive behavior thus allowing the analyte to be effectively determined without much interference. Moreover, the low relative standard deviation within each sample suggests a good deal of consistency and

precision of the method. A calibration shown in Figure 11 curve with riboflavin standard solutions of concentration 0.05, 0.1, 0.25, 0.5 was used in the recovery studies of the cereal product. The regression line equation given Y=7.949X + 0.139, with a correlation of coefficient of 0.9995 was described.

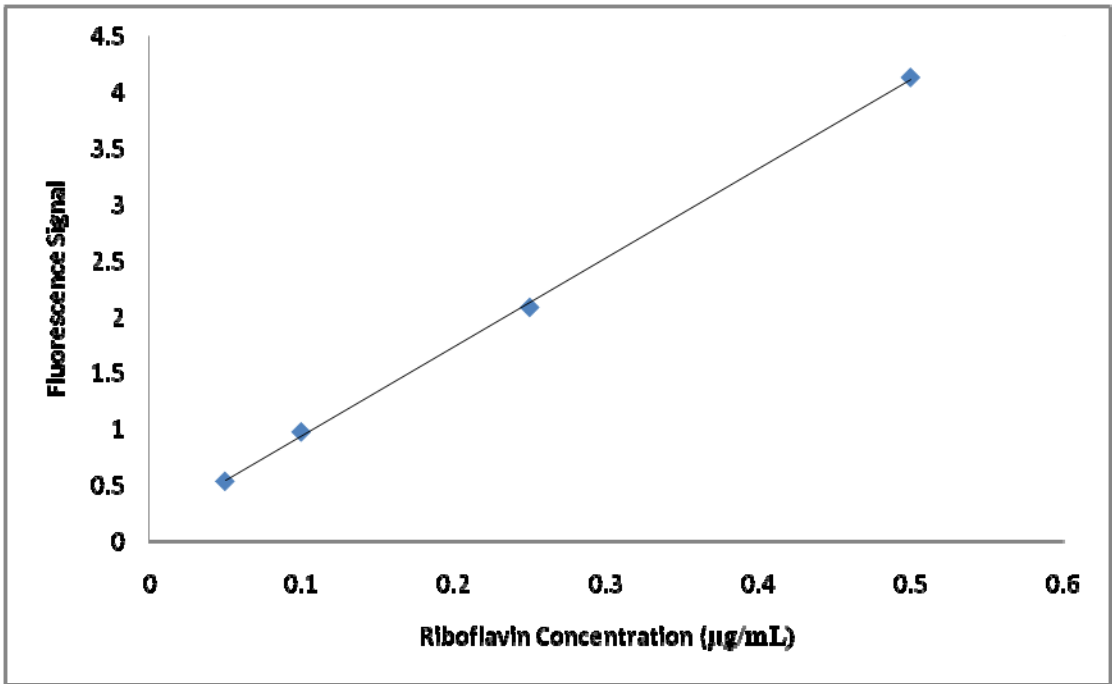

Figure 11: Plot of Florescence Signal versus Standard Riboflavin Concentration (µg/mL) for recovery studies on Cereal products. The fluorescence signal is given in arbitrary units.

Corn Flakes brand of cereal and Raisin Bran were also chosen for the recovery study. Three triplicates of sample solutions were used. The first triplicates did not have any added riboflavin from the standard working solution. The second three aliquots had 0.75 µL of the 20.0 µg/mL riboflavin standard stock solutions added to 1 g sample of the cereal product after which acid hydrolysis and enzymatic reaction were carried out as stipulated in the sample preparation section. The added riboflavin concentration of the final sample solution was 0.30

µg/mL. The last three had 1.5 µL of 20.0 µg/mL of the riboflavin standard stock solution added to 1 g sample of the cereal product after which acid hydrolysis and enzymatic reactions were carried out. The added riboflavin concentration of the final sample solution was as a result 0.60 µg/mL. Fluorescence measurements were then carried out for the nine solutions and the results tabulated in Table 14 and Table 15

Table 14: Results of Recovery Studies of Corn Flakes Cereal

Sample	Fluorescence Signal	Riboflavin Standard added (µg/mL)	Total Riboflavin found	Recovery (%)
1	2.60	0.000		
2	4.85	0.300	0.277	92.35
3	7.00	0.600	0.542	90.30
Mean Recovery		91.33 %		
Relative Standard Deviation		7.80 %		

Table 15: Results of Recovery Studies of Raisin Bran Cereal

Sample	Fluorescence Signal	Riboflavin Standard added (µg/mL)	Total Riboflavin found	Recovery (%)
1	1.43	0.000		
2	3.70	0.300	0.279	93.04
3	5.90	0.600	0.550	91.67
Mean Recovery		92.35 %		
Relative Standard Deviation		8.14 %		

Percent recoveries were calculated based on stated formula. The mean percent recovery for the Corn flakes is 91.33 % with a relative standard deviation of 7.80 %. The mean percent recovery of the raisin bran cereal on the other hand is 92.35 % with a relative standard deviation of 8.14 %. The percent recovery yielded values that are acceptable, and so we can infer that that the proposed method is consistently accurate. Indeed, ideal values for the percent recovery should be around 100 %, implying that the medium or matrix in which the analyte is present has an insignificant effect on the analyte. The values of the relative standard deviations obtained for the cereal products were higher than those of the vitamin tablets due to the elaborate extraction procedure involve in the analysis on the cereal products.

The overall average recovery for all the samples tested was 95.7 % and indicates that within limit of experimental error, the proposed method is reasonably accurate and suitable for the analysis of riboflavin in vitamin tablets and cereals. Recoveries for super B complex were higher than those of the cereal products. This can be explained by the elaborate sample treatment process required before fluorescence measurement in the case of the cereal products.

Application Studies

The goal of the next set of experiments was to apply the proposed method in the determination of riboflavin in commercial vitamin tablets and cereal products obtained from a local store. Two different brands of super B complex and four different brands of cereal products were chosen for this purpose. Super B complex and Cereal samples were prepared as mentioned in the experimental procedure section. Three samples of each type of super B

complex tablet and cereal were ready for the analysis, and triplicate measurements of fluorescence were made. Different calibration curves were required for the super B complex vitamin and the cereal products as these analyses differ from one another and were carried out separately. A plot of the data obtained for the analysis of the two brands of super B complex is shown in Figure 12. The regression line was found to follow the equation: Y =11.71X + 0.3975, and with a correlation coefficient of 1.00. The data for the plot are also displayed in Table 16.

Table 16: Average Fluorescence Signals of Standard Riboflavin Solutions for Application Studies

Standard Riboflavin Solution (µg/mL)	0.5	1.0	2.0	2.5
Average Fluorescence Signal	6.2	12.2	23.75	27
R.S.D. (%)	1.61	0.82	0.00	0.00

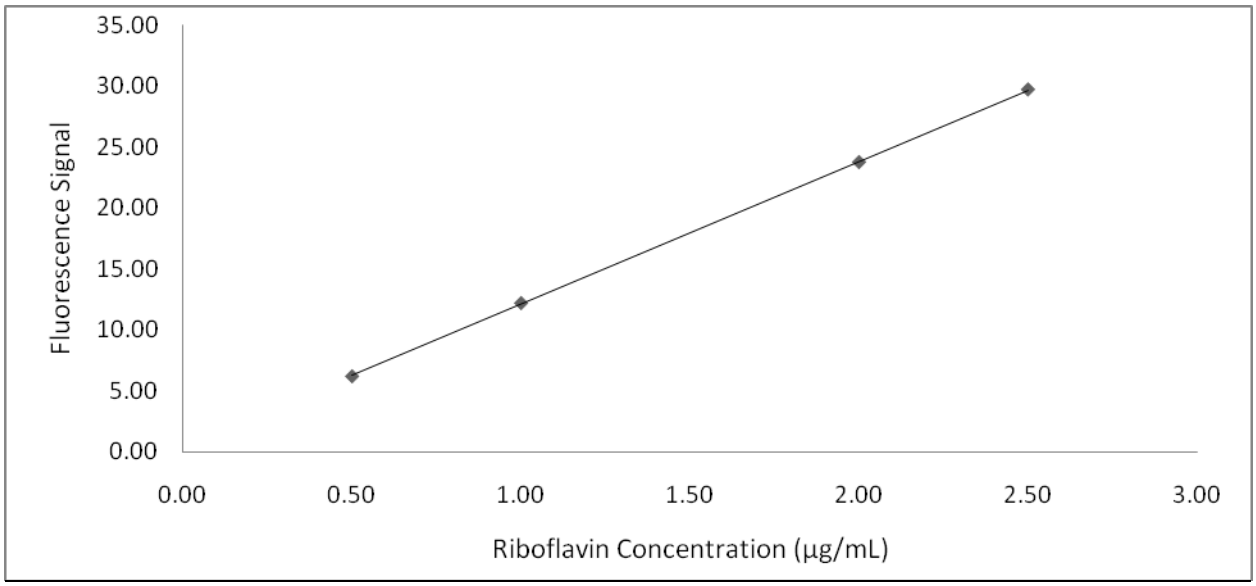

Figure 12: Plot of Fluorescence Signal versus Riboflavin Standard Concentration for Application Studies of Super B Complex.

Note: The fluorescence signal is given in arbitrary units.

Using the regression line equation, the fluorescence signals of the various sample solutions were converted to their corresponding riboflavin concentrations. The amount of riboflavin in each tablet were subsequently calculated and the result shown in Table 17.

Table 17: Results of the Determination of Riboflavin in Commercial Samples.

Sample	Label Claim (mg)	Found (mg)	R.S.D. (%)	Percentage of Label (%)
Home Nature Super B complex	20.00	20.54	3.01	102.69
Valley Spring Super B complex	20.00	20.69	2.80	103.45

The amount of Riboflavin in the super B complex as determined by our fluorescence method is 20.54 mg for Home Nature and 20.69 g for the Valley Spring super B complex. Comparing this with the amount indicated by the manufacturers, our values were a little bit higher.

A calibration curve displayed as Figure 13 with riboflavin standard solutions of concentration 0.05, 0.1, 0.25, 0.5 and 1.00 μg/mL was used in the determination of riboflavin in the commercial cereal products. The regression line is described by the equation: Y =7.949X + 0.139, and with a correlation coefficient of 0.9995.

Raisin Bran cereal and Corn Flakes cereal have a label content of 25 % Riboflavin per serving size, corresponding to serving of 59 g and 28 g, respectively. Kellogg's Special K cereal has a label content of 35 % riboflavin per serving size of 31 g. Smart Smart brand of cereal has a label content of 100 % riboflavin per serving size of 50 g. The exact amount of riboflavin per serving size was calculated based on the percent daily value (100%) of 1.7 mg.

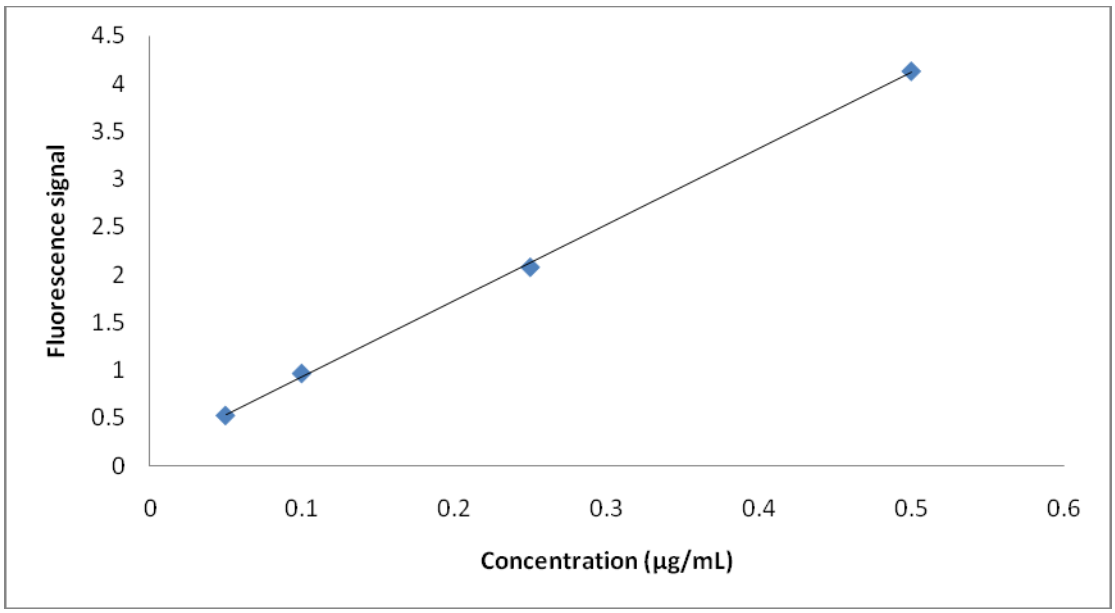

Figure 13: Plot of Fluorescence Signal versus Riboflavin Standard Concentration for Application Studies of Cereal Products. The fluorescence Signal is given in arbitrary units.

Table 18: Results of the Determination of Riboflavin in Commercial Samples.

Sample	Label Claim (mg)	Found (mg)	R.S.D. (%)	Percentage of Label (%)
Raisin Bran Cereal	0.425	0.466	18.40	109.62
Kellogg Special K Cereal	0.595	0.544	10.96	91.39
Corn Flakes Cereal	0.425	0.432	12.44	101.75
Smart Smart Cereal	1.70	1.63	8.02	95.99

According to the label claim as provided by the manufacturers, the amount of riboflavin in Raisin Bran cereal and Corn Flakes cereal as obtained from our method were slightly higher than the expected values; however, over-fortification is the norm in the food industry in order to ensure compliance with the label declaration (12). Conversely, Smart Smart and Kellogg's Special K cereal were a little lower than the anticipated values. On the whole, the percentage

of riboflavin in the cereals samples analyzed with our method to that provided by the manufacturers ranged from 91.39 % to 109.62 %. The results are also consistent as can be seen with an overall average relative standard deviation of 12.46 %. The results obtained are thus in agreement with expected values as provided by the manufacturers.

CHAPTER 5

CONCLUSION

A simple and economical fluorometer using blue LEDs excitation sources and simple PMT detection has been built, assembled, optimized, and employed for measurement of fluorescence from riboflavin (vitamin B_2). The fluorescence emissions were analyzed at 90° from the excitation beam. The photomultiplier tube used as a detector was housed in a chamber with slit on setting 2.0 mm or middle slit width. A photometer was used to measure the signal.

Surfactants have been known to enhance the intensity of fluorescence of fluorescent compounds. Fluorescence analysis of riboflavin in the presence of various anionic, cationic, and nonionic surfactants was also conducted to determine if they could improve analysis. The surfactants applied to riboflavin solutions, include Hexadecyltrimethylammonium bromide (CTAB), Hexadecyltrimethylammonium chloride (CTAC), Sodium dodecyl sulfate (SDS), 1-Octanesulfonic acid sodium salt, and Triton X-100. However, the surfactants employed did not seem to have any meaningful enhancement; in fact some actually diminished the fluorescence intensity of riboflavin. There is the possibility of efficient radiationless decay paths that return the riboflavin molecule directly to the ground state.

The proposed method was successfully applied to the analysis of riboflavin in commercial vitamin tablets and cereal products. The use of dilute hydrochloric acid in extraction of the riboflavin was to ensure the denaturation of protein and to release vitamin from its association with proteins. Enzymatic treatment of the cereals allowed the dephosphorylation of the vitamins. When surfactants were applied to commercial samples, an

increase in fluorescence signal was observed although there was no significant increase in fluorescence signal when the surfactants were applied to pure standard solutions. The difference in fluorescence signal could be due to the presence of other species present in the commercial samples such as metal ions that might quench the fluorescence of riboflavin. When surfactants like cationic micelles such as CTAB or CTAC are used, cationic species would be repelled from the micelle surface thus protecting the riboflavin if it is bound to those cationic micelles. The greater signal observed for commercial samples in the presence of cationic micelles might be due to inhibition of fluorescence quenching when the micelles are present.

The proposed fluorescence method for the analysis of riboflavin was evaluated in terms of its linearity, precision, and accuracy. A linear relationship was established between fluorescence signal and riboflavin concentration. Thus the procedure was linear for riboflavin from 0.01 to 2.5 μg/mL. Concentrations lower than this were less reproducible and did not fit in the calibration curve. Reproducibility throughout the project was very good. Reproducibility expressed as relative standard deviation was about 2 %. The recoveries obtained range from 91.3 % to 100.21 % for the samples determined.

In conclusion, the proposed fluorescence method can be used for the determination of riboflavin in vitamin tablets and cereals. Further study could be conducted to find out the effect other surfactants on riboflavin fluorescence and the discrepancy in fluorescence in commercial samples and that of pure standard solution.

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