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RESEARCH ARTICLE

Application of the Ratio Subtraction Ultraviolet Spectrophotometry Method for Simultaneous Quantification of B_1, B_2, B_3, B_6, and B_{12} Vitamins in Health Supplement Syrups

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Abstract

The present investigation aimed to simultaneously determine the concentrations of thiamine (B_1), riboflavin (B_2), nicotinamide (B_3), pyridoxine (B_6), and cobalamin (B_{12}) in health supplement syrups, commonly used as multivitamins. Precise quantification of these vitamins is essential to guarantee product efficacy, safety, and compliance with quality standards. The analytical approach employed was based on ratio subtraction ultraviolet (UV) spectrophotometry, utilizing methanol as a solvent to resolve the overlapping UV absorption spectra of the vitamin mixture. Carefully optimized divisor concentrations 12 µg.mL^{-1} for B_1, 22 µg.mL^{-1} for B_2, 18 µg.mL^{-1} for B_3, 11.5 µg.mL^{-1} for B_6, and 25 µg.mL^{-1} for B_{12} were selected to ensure accurate spectral differentiation of each analyte. The method was rigorously validated according to standard parameters, including linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ), all of which fulfilled established validation criteria. The quantified vitamin contents in health supplement syrup samples were 102.75% ± 2.20% for B_1, 101.03% ± 2.17% for B_2, 102.65% ± 2.21% for B_3, 102.11% ± 2.16% for B_6, and 101.26% ± 2.17% for B_{12}, all of which conformed to the specifications outlined in the United States Pharmacopeia 38 and National Formulary 33. The proposed ratio subtraction ultraviolet spectrophotometry method offers a straightforward, cost-effective, and selective solution for concurrent estimation of these vitamins in complex pharmaceutical matrices, without the need for sophisticated instrumentation or elaborate procedures.

Keywords: Ratio subtraction, UV spectrophotometry, Quantification, Health supplement syrup

Introduction

Health supplement syrup is a liquid form of dietary supplements or nutraceuticals that is intended to increase nutritional intake, such as multivitamins, which generally contains various essential vitamins that consistently function to meet the body's nutritional needs [1,2], including thiamine, riboflavin, nicotinamide, pyridoxine, and cobalamin. Thiamine (B_1) is a water-soluble micronutrient indispensable for maintaining optimal cellular activity. It serves as a critical coenzyme in the biochemical pathways responsible for the metabolism of carbohydrates, lipids, and amino acids [3]. The chemical structure of thiamine is made up of 2 rings are a pyrimidine ring and a thiazolium ring joined together by a methylene bridge. The pyrimidine ring is particularly 2,5-dimethyl-6-aminopyrimidine. The thiazolium ring contains 4-methyl-5-hydroxyethylthiazole [4]. Riboflavin (B_2), another essential water-soluble vitamin, significantly contributes to cellular energy production and metabolic regulation. It functions predominantly in redox reactions, acting as a precursor for the coenzymes flavin

mononucleotide (FMN) and flavin adenine dinucleotide (FAD), both of which are integral to various enzymatic processes. Chemically, riboflavin is known as 7,8-dimethyl-10-ribityl-isoalloxazine [5]. Nicotinamide (B_3), commonly referred to as niacinamide, is the amide derivative of niacin and serves as a precursor for the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). This compound is essential in numerous biochemical processes, such as energy metabolism and DNA repair. The systematic chemical designation for nicotinamide is pyridone-5-carboxamide or pyridone-3-carboxamide [6]. Pyridoxine (B_6) is a water-soluble vitamin integral to amino acid metabolism and functions as a coenzyme in more than 150 enzymatic reactions. It is available in multiple forms, including pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), along with their phosphorylated derivatives. The chemical designation for the active form of pyridoxine is pyridoxal 5'-phosphate [7]. Cobalamin (B_{12}) is a water-soluble vitamin characterized by a complex structure. It is essential for deoxyribonucleic acid (DNA) synthesis, the complex process of fatty acid metabolism, and the crucial development of healthy red blood cells. The chemical name for cobalamin is 5,6-dimethylbenzimidazole, and it is found in various forms, including its active forms, methylcobalamin and adenosylcobalamin [8].

Reliable determination of individual drug constituents is a critical requirement outlined in pharmacopeial monographs to maintain formulation stability. Ensuring accurate measurement of active substance concentration, as mandated by these quality standards, is fundamental to verify that each dosage form delivers the appropriate quantity of the therapeutic agent [9]. Various analytical techniques have been employed for the determination of health supplement components like thiamine, riboflavin, nicotinamide, pyridoxine, and cobalamin, both as individual analytes and in combination within complex formulations, often presenting significant analytical challenges due to matrix interference. A variety of analytical techniques have been employed for quantitative and qualitative evaluation, including high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) densitometry, reversed-phase liquid chromatography (RP-LC), hydrophilic interaction liquid chromatography (HILIC), voltammetric methods, pressurized planar electrochromatography (PPEC), high-performance thin-layer chromatography (HPTLC), mass spectrometry (MS), fourier-transform infrared spectroscopy (FTIR), raman spectroscopy, near infra-red (NIR), and ultraviolet-visible (UV-Vis) spectrophotometry [10-26].

Spectrophotometry offers a simple, rapid, and relatively straightforward analytical approach compared to many other methods. However, a primary challenge in the spectrophotometric analysis of binary, ternary, or more complex mixtures is the simultaneous determination of compounds without prior separation [27]. The ratio subtraction ultraviolet spectrophotometry method is particularly useful for the quantification of multiple components in a mixture, even when their UV absorption spectra exhibit significant overlap. Numerous investigations have focused on the concurrent spectrophotometric analysis of health supplement components like thiamine, riboflavin, nicotinamide, pyridoxine, and cobalamin have been published. These include methods such as the area under curve method in spectrophotometry, ultraviolet-visible spectrophotometric-assisted chemometric calibration models, spectrophotometric method based on continuous wavelet transform (CWT) and partial least squares (PLS) techniques, 1st-order derivative spectrophotometry, and multivariate regression spectrophotometry [22-26].

The ratio subtraction ultraviolet spectrophotometry method is a spectrophotometric technique capable of analyzing 2 or more compounds simultaneously without the need for prior separation. It's easily applicable to routine analysis, doesn't require derivatization, and avoids complex mathematical calculations, relying solely on constants within the ratio spectrum [27,28]. The utilization of response surface methodology ratio subtraction ultraviolet spectrophotometry method in mixture analysis has been extensively documented in the literature, including the quantification of isoniazid and pyridoxine hydrochloride using 0.1 N HCl as the solvent, and irbesartan and hydrochlorothiazide with 0.1 N NaOH as the solvent [9,27]. Accordingly, the present work seeks to validate the application of ratio subtraction ultraviolet spectrophotometry as a reliable approach for the concurrent determination of B_1, B_2, B_3, B_6, and B_{12} within a health supplement syrup matrix. While ratio

subtraction UV spectrophotometry has been previously applied to various binary and ternary pharmaceutical mixtures [27,28], with relatively few reports addressing its use this method for simultaneous quantification of 5 B-complex vitamins (B_1, B_2, B_3, B_6, B_{12}) especially in a liquid multivitamin syrup matrix. The integration of optimized spectral division order tailored to vitamin pharmacological relevance and spectral dominance further distinguishes this work from prior studies and offering a practical alternative to chromatographic methods.

Materials and methods

Materials

Analytical-grade standards of thiamine, riboflavin, pyridoxine, cobalamin, and nicotinamide were procured from the National Agency of Drug and Food Control of Indonesia to ensure compound identity and purity throughout the study. Methanol, supplied by E-Merck, was used as the solvent. Additionally, Sanvita-B® Vitamin B Complex Health Supplement syrup (Sanbe Farma) is obtained from pharmacies in Medan city, Indonesia, containing 5 mg of B_1, 2 mg of B_2, 20 mg of B_3, 2.5 mg of B_6, and 3 mcg of B_{12} per dose, was selected for analysis.

Equipments

The equipment used in this study included spectral data were acquired using a Shimadzu UV-Visible 1800 spectrophotometer interfaced with a personal computer running UV-Probe 2.42 software, a 1 cm cuvette, an analytical balance (Boeco), a sonicator (Branson), and various glassware (Oberoi).

Preparation of standard solution

Individual primary stock solutions of B_1, B_2, B_3, B_6, and B_{12} were individually prepared at a concentration of 1000 $\mu g.mL^{-1}$ by precisely measuring 25 mg of each vitamin and dissolving them in methanol using separate 25 mL volumetric flasks. From these, working standard solutions of 100 $\mu g.mL^{-1}$ were obtained by pipetting 2.5 mL of each stock solution into individual 25 mL volumetric flasks and making up to volume with methanol.

Preparation of absorption spectra

To facilitate the analysis of B_1, B_2, B_3, B_6, and B_{12}, standard solutions with different concentration levels were prepared from the working solutions, ranging from 4 to 20 $\mu g.mL^{-1}$ for vitamin B_1, 16 to 28 $\mu g.mL^{-1}$ for B_2, 6 to 30 $\mu g.mL^{-1}$ for B_3, 7.5 to 15.5 $\mu g.mL^{-1}$ for B_6, and 15 to 35 $\mu g.mL^{-1}$ for B_{12}. These calibration solutions were subsequently analyzed using a UV-Visible spectrophotometer, with spectral data recorded in the 200 - 400 nm wavelength range. This procedure enabled the identification of characteristic absorption maxima for each vitamin, forming the basis for subsequent quantitative evaluations.

Preparation of mixture spectra of B_1, B_2, B_3, B_6, and B_{12}

In this phase, mixed standard solutions containing B_1, B_2, B_3, B_6, and B_{12} were prepared to enable their simultaneous determination via spectrophotometric analysis. The absorption spectra of these multicomponent solutions were recorded to evaluate spectral overlap and to investigate the interaction among the components in the UV region, which is essential for accurate peak deconvolution in complex matrices.

Analysis of B_1 via ratio subtraction ultraviolet spectrophotometry method

The quantification of B_1 was conducted using the ratio subtraction ultraviolet spectrophotometry approach. This method was executed via the UV-Probe 2.42 software, which facilitated the analytical process through sequential mathematical manipulations involving spectral division followed by subtraction. The mixed spectrum was divided by the spectrum of B_{12} at 25 $\mu g.mL^{-1}$. Subtraction was then performed using a constant derived from the self-division of the B_{12} spectrum. This subtraction yielded a new spectrum, denoted as $B_1 + B_2 + B_3 + B_6/B_{12}°$. The resulting spectrum was then multiplied by the same divisor used for B_{12}, yielding the combined spectrum of $B_1 + B_2 + B_3 + B_6$. The process continued with the 2nd divisor. The $B_1 + B_2 + B_3 + B_6$ spectrum was divided by the spectrum of B_2 at 22 $\mu g.mL^{-1}$. The result was then subtracted by a constant, where the constant was obtained by dividing the B_2 spectrum by its divisor. This subtraction produced a new spectrum, $B_1 + B_3 + B_6/B_2°$. Multiplying this by the same B_2 divisor yielded the $B_1 + B_3 + B_6$ spectrum.

The 3rd divisor was then applied, the $B_1 + B_3 + B_6$ spectrum was divided by the spectrum of B_3 at 18 $\mu g.mL^{-1}$. After subtracting the constant from the self-division of B_3 to generated a new spectrum, $B_1 + B_6/B_3°$. Multiplication by the B_3 divisor provided the $B_1 + B_6$ spectrum. The last divisor used is the 4th divisor, the B1 + B6 spectrum divided by the B6 spectrum at 11.5 $\mu g.mL^{-1}$. A final subtraction was performed using a constant derived from the B_6 reference spectrum with results the $B_1/B_6°$ spectrum. Multiplying this final spectrum with a B_6 divider allows the isolation of a single B_1 spectrum from complex mixtures.

Analysis of B_2 via ratio subtraction ultraviolet spectrophotometry method

The determination of B_2 can be continued by taking the $B_1 + B_2 + B_3 + B_6$ spectrum, which was the result of the 1st divisor operation (B_{12}) from the B_1 determination. This $B_1 + B_2 + B_3 + B_6$ spectrum is then divided by the 2nd divisor, the spectrum of B_1 at 12 $\mu g.mL^{-1}$. The resulting ratio spectrum is then subtracted by a constant, where the constant is derived from the division of the B_1 spectrum by its own divisor. This subtraction yields a new spectrum, denoted as $B_2 + B_3 + B_6/B_1°$. Subsequently, this spectrum is multiplied by the same B_1 divisor to obtain the combined $B_2 + B_3 + B_6$ spectrum. The process continues with the 3rd divisor. The $B_2 + B_3 + B_6$ spectrum is divided by the spectrum of B_3 at 18 $\mu g.mL^{-1}$. The result is then subtracted by a constant, which is the B_3 spectrum divided by its divisor. This operation generates a new spectrum, $B_2 + B_6/B_3°$. Multiplying this by the B_3 divisor yields the $B_2 + B_6$ spectrum. The last divisor used is the 4th divisor, the $B_2 + B_6$ spectrum is divided by the spectrum of B_6 at 11.5 $\mu g.mL^{-1}$. The result is then subtracted by a constant, calculated by dividing the B_6 spectrum by its divisor. This produces a new spectrum, $B_2/B_6°$. Multiplying this final spectrum by the B_6 divisor allows for the isolation of the single B_2 spectrum from the mixture.

Analysis of B_3 via ratio subtraction ultraviolet spectrophotometry method

The determination of B_3 can be continued using the $B_2 + B_3 + B_6$ spectrum, which was the outcome of the 2nd divisor with B_1 during the B_2 determination after the initial division with B_{12} from the B_1 determination process. This $B_2 + B_3 + B_6$ spectrum is then divided by the 3rd divisor, the spectrum of B_2 at 22 $\mu g.mL^{-1}$. The resulting ratio spectrum is then subtracted by a constant, where the constant is derived from the division of the B_2 spectrum by its own divisor. This subtraction yields a new spectrum, denoted as $B_3 + B_6/B_2°$. Subsequently, this spectrum is multiplied by the same B_2 divisor to obtain the combined $B_3 + B_6$ spectrum. The process continues with the 4th divisor. The $B_3 + B_6$ spectrum is divided by the spectrum of B_6 at 11.5 $\mu g.mL^{-1}$. The result is then subtracted by a constant, calculated by dividing the B_6 spectrum by its divisor. This produces a new spectrum, $B_3/B_6°$. Multiplying this final spectrum by the B_6 divisor allows for the isolation of the single B_3 spectrum from the mixture.

Analysis of B_6 via ratio subtraction ultraviolet spectrophotometry method

The determination of B_6 can be continued using the $B_3 + B_6$ spectrum. This spectrum was obtained from the 3rd divisor with B_2 during the B_3 determination, following the 2nd division with B_1 in the B_2 determination and which in turn followed the initial division with B_{12} in the B_1 determination process. This $B_3 + B_6$ spectrum is then divided by the 4th divisor, the spectrum of B_3 at 18 $\mu g.mL^{-1}$. The resulting ratio spectrum is then subtracted by a constant, which is derived from the division of the B_3 spectrum by its own divisor. This subtraction yields a new spectrum, denoted as $B_6/B_3°$. Subsequently, multiplying this spectrum by the same B_3 divisor allows for the isolation of the single B_6 spectrum from the mixture.

Analysis of B_{12} via ratio subtraction ultraviolet spectrophotometry method

The determination of B_{12} can be achieved through a series of ratio subtraction operations. The mixed spectrum is divided by the spectrum of B_1 at 12 $\mu g.mL^{-1}$. The resulting ratio spectrum is then subtracted by a constant, which is derived from dividing the B_1 spectrum by its own divisor. This subtraction yields a new spectrum, denoted as $B_2 + B_3 + B_6 + B_{12}/B_1°$. Subsequently, multiplying this spectrum by the same B_1 divisor results in the combined $B_2 + B_3 + B_6 + B_{12}$ spectrum. The process then proceeds with the 2nd divisor. The $B_2 + B_3 + B_6 + B_{12}$ spectrum is divided by the spectrum of B_2 at 22 $\mu g.mL^{-1}$. The result is subtracted by a constant obtained from dividing the B_2

spectrum by its divisor, yielding a new spectrum: B_3 + B_6 + B_{12}/B_2°. Multiplying this by the B_2 divisor provides the B_3 + B_6 + B_{12} spectrum. The 3rd divisor is applied, the B_3 + B_6 + B_{12} spectrum is divided by the spectrum of B_3 at 18 µg.mL^{-1}. The outcome is subtracted by a constant, which is the B_3 spectrum divided by its divisor. This generates a new spectrum, B_6 + B_{12}/B_3°. Multiplying this by the B_3 divisor gives the B_6 + B_{12} spectrum. The last divisor used is the 4th divisor, the B_6 + B_{12} spectrum is divided by the spectrum of B_6 at 11.5 µg.mL^{-1}. The result is then subtracted by a constant, calculated by dividing the B_6 spectrum by its divisor. This produces a new spectrum, B_{12}/B_6°. Multiplying this final spectrum by the B_6 divisor isolates the single B_{12} spectrum from the mixture.

Analysis of health supplement syrup

To perform spectrophotometric analysis of the health supplement syrup containing 5 mg of B_1, 2 mg of B_2, 20 mg of B_3, 2.5 mg of B_6, and 3 µg of B_{12} per 5 mL, a volume equivalent to 20 mg of B_3 was precisely measured. The corresponding concentrations of the remaining vitamins within this volume were calculated proportionally to ensure accurate representation during analysis. The accurately measured syrup sample was quantitatively transferred into a 50 mL volumetric flask, diluted to the calibration mark with methanol, and subjected to sonication for 15 min to ensure complete dissolution. Filtration was carried out using Whatman No. 42 filter paper; the 1st 10 mL of filtrate was discarded to avoid potential matrix interferences, and the subsequent clear solution was collected. A 6.25 mL aliquot of this filtrate was then further diluted to 25 mL with methanol in a separate volumetric flask to obtain the final sample solution for analysis. An aliquot of 1.8 mL from the resulting filtrate was then transferred into a 10 mL volumetric flask. To this, 0.75 mL of B_1 working standard solution, 2.02 mL of B_2 working standard solution, 0.93 mL of B_6 working standard solution, and 2.5 mL of B_{12} working standard solution were added as an internal addition. The volume was then made up to the mark with methanol solvent. The absorbance of the final solution was measured in the 200 - 400 nm range. The prepared solution was subsequently subjected to analysis using the ratio subtraction ultraviolet spectrophotometry method for the quantification of B_1, B_2, B_3, B_6, and B_{12}. This analytical procedure is essential for confirming the actual concentrations of the active ingredients within the syrup formulation and for ensuring conformity with established pharmaceutical quality standards.

Method validation

Linearity

The linearity of the proposed analytical method was assessed for B_1, B_2, B_3, B_6, and B_{12} within their respective concentration ranges: 4 - 20 µg.mL^{-1} for B_1, 16 - 28 µg.mL^{-1} for B_2, 6 - 30 µg.mL^{-1} for B_3, 7.5 - 15.5 µg.mL^{-1} for B_6, and 15 - 35 µg.mL^{-1} for B_{12}. Absorbance values corresponding to each concentration level were recorded, and linear regression analyses were conducted to establish the relationship calibration equations and correlation coefficients. This evaluation was essential to confirm the method's ability to produce reliable and proportional responses over the tested concentration intervals with $r \leq 1$ [21,29].

Accuracy

The accuracy of the method was assessed by introducing known amounts of reference standards into the sample matrix. Recovery studies were performed at 3 concentration levels 80%, 100% and 120% where each level consisted of 70% analyte from the sample and 30% from the working standard solution. This procedure aimed to determine the method's capability to accurately recover and quantify the analyte content relative to its true concentration with recoveries between 90% - 110% [21,27,29].

Precision

Method precision, indicative of its reproducibility, was assessed by determining the relative standard deviation (RSD) of replicate measurements. An RSD value below 2% is generally accepted as a benchmark for adequate precision [21,27,29], reflecting the method's ability to produce consistent results under identical analytical conditions.

Limit of detection (LOD)

The limit of detection (LOD), which indicates the lowest concentration of an analyte that can be reliably identified, albeit not accurately quantified, was calculated using the formula: LOD = 3.3×(standard

deviation/slope) [9,21]. This parameter is essential in assessing the analytical method's sensitivity.

Limit of quantification (LOQ)

The limit of quantification (LOQ) denotes the minimum concentration of an analyte that can be quantified with satisfactory precision and accuracy. It was determined using the formula: LOQ = 10×(standard deviation/slope) [9,21]. This parameter is essential for defining the lowest concentration at which the analyte can be reliably measured.

Results and discussion

Absorption spectra of individual B_1, B_2, B_3, B_6, and B_{12}, as well as their mixtures, were obtained

Overlapping spectra were obtained from the maximum absorption spectra of B_1, B_2, B_3, B_6, and B_{12}, as well as their mixture. For the individual maximum absorption spectra, the following concentrations were used: 12 µg.mL^{-1} for B_1, 22 µg.mL^{-1} for B_2, 18 µg.mL^{-1} for B_3, 11.5 µg.mL^{-1} for B_6, and 25 µg.mL^{-1} for B_{12}. These spectra are presented in **Figure 1**.

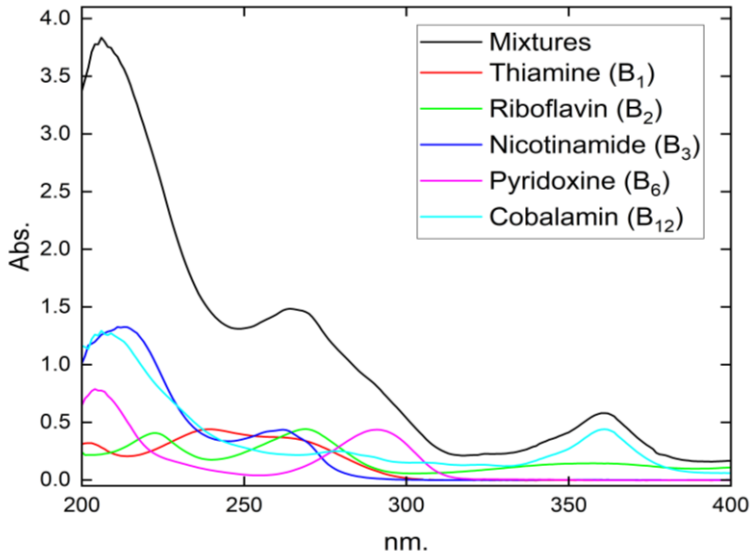

Figure 1 Overlapping spectra B_1, B_2, B_3, B_6, and B_{12}, as well as for their mixture.

Figure 1 illustrates that the spectral overlap of B_1 at 12 µg.mL^{-1}, B_2 at 22 µg.mL^{-1}, B_3 at 18 µg.mL^{-1}, B_6 at 11.5 µg.mL^{-1} and B_{12} at 25 µg.mL^{-1}, both individually and in their mixture, can be resolved using the ratio subtraction ultraviolet spectrophotometry method [28,30]. The ratio subtraction method is initiated by examining the overlapping spectral profiles of the components. Based on the spectral evaluation, a suitable initial divisor is selected, which serves as the foundation for subsequent ratio processing steps throughout the analysis [27,28,30].

Absorption spectra of B_1, B_2, B_3, B_6, and B_{12} using the ratio subtraction ultraviolet spectrophotometry method

The compound mixture contains 5 active ingredients, B_1, B_2, B_3, B_6, and B_{12}. To obtain the individual absorption spectrum of each compound from the mixture, the mixture undergoes a division step. Subsequently, a subtraction is performed using a constant, where this constant is derived from the compound divided by its own divisor. Finally, the 0-order absorption spectrum of each single compound is obtained by multiplying the resulting spectrum by the same divisor, all achieved using the ratio subtraction ultraviolet spectrophotometry method [27,28].

Thiamine (B_1)

To obtain the single absorption spectrum of B_1 from its mixture, the mixed spectrum undergoes a 4-step division process, as the mixture contains 5 active compounds. The sequence of these 4 divisors is determined by the preliminary orientation study of all 5 compounds. The 1st divisor used to determine the single absorption spectrum of B_1 from the mixture is B_{12} at a concentration of 25 µg.mL^{-1}. This is followed by a

subtraction with a constant, which is derived from the B_{12} spectrum divided by its divisor. Finally, multiplying this by the same divisor yields the absorption spectrum as seen in **Figure 2**.

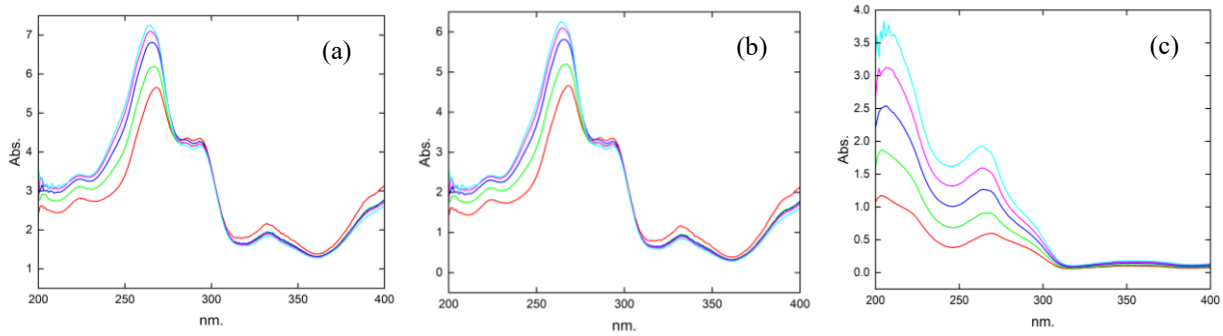

Figure 2 Spectra of the mixture with (a) B_{12} as the divisor 25 µg.mL^{-1}, (b) spectra subtracting the constant (B_{12}/B_{12}°), and (c) spectra after multiplying by the divisor.

The 2nd divisor used to determine the single absorption spectrum of B_1 from its mixture was B_2 at a concentration of 22 µg.mL^{-1}. This was followed by a subtraction with a constant, which was derived from the B_2 spectrum divided by its divisor. Finally, multiplying this by the same divisor yielded the absorption spectrum as seen in **Figure 3**.

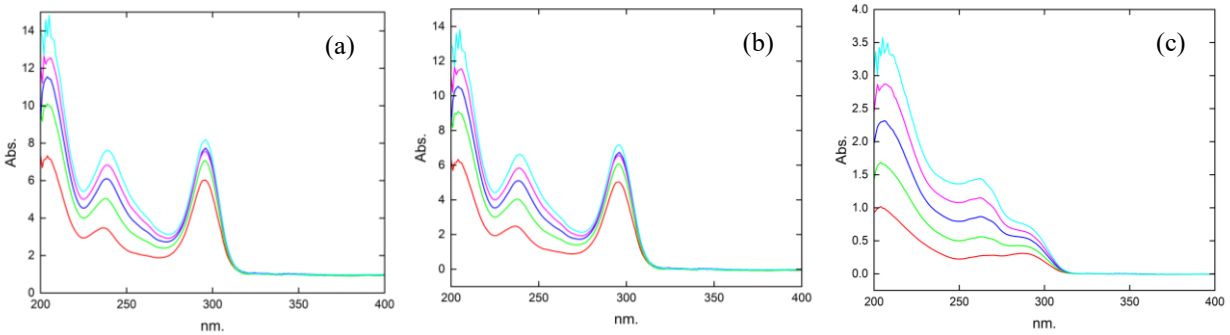

Figure 3 Spectra of the mixture with (a) B_2 as the divisor 22 µg.mL^{-1}, (b) spectra subtracting the constant (B_2/B_2°), and (c) spectra after multiplying by the divisor.

The 3rd divisor employed to determine the single absorption spectrum of B_1 from its mixture was B_3 at a concentration of 18 µg.mL^{-1}. This was followed by a subtraction with a constant, derived from the B_3 spectrum divided by its divisor. Finally, multiplying this by the same divisor yielded the absorption spectrum as seen in **Figure 4**.

The last divisor, the 4th, used to determine the single absorption spectrum of B_1 from its mixture was B_6 at a concentration of 11.5 µg.mL^{-1}. This was followed by a subtraction with a constant, derived from the B_6 spectrum divided by its divisor. Subsequently, the 0-order absorption spectrum of B_1, as obtained by the proposed ratio subtraction ultraviolet spectrophotometry method, resulted from multiplying this by the same divisor, as seen in **Figure 5**.

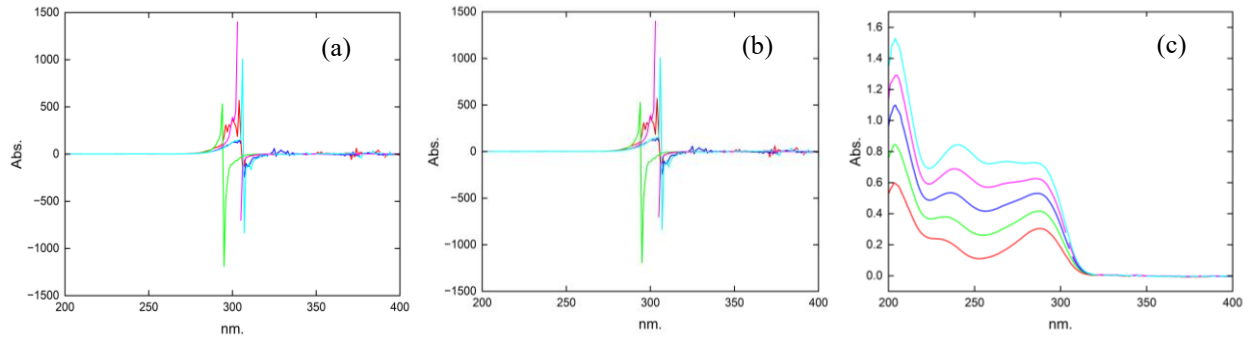

Figure 4 Spectra of the mixture with (a) B_3 as the divisor 18 µg.mL^{-1}, (b) spectra subtracting the constant ($B_3/B_3°$), and (c) spectra after multiplying by the divisor.

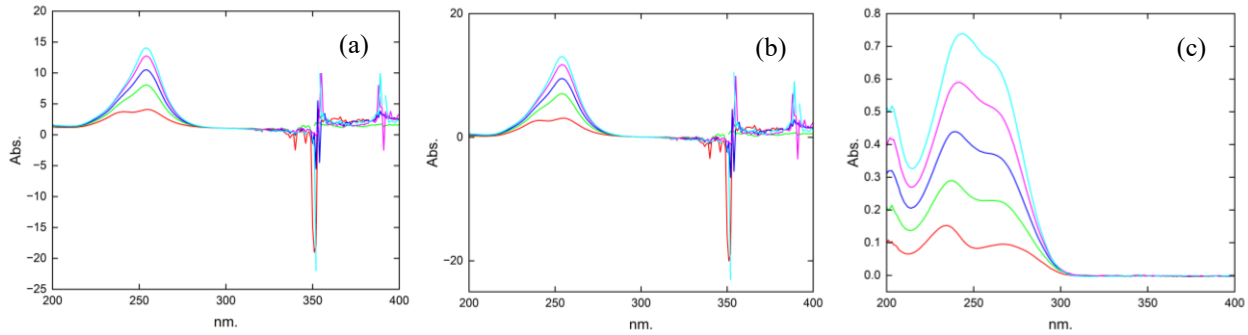

Figure 5 Spectra of the mixture with (a) B_6 as the divisor 11.5 µg.mL^{-1}, (b) spectra subtracting the constant ($B_6/B_6°$), and (c) 0-order absorption spectrum of B_1 obtained by the proposed ratio subtraction ultraviolet spectrophotometry method after multiplication by the divisor.

Riboflavin (B_2)

The determination of the single absorption spectrum of B_2 continues by taking the result from the 1st division with B_{12} during the B_1 determination, as shown in **Figure 2**. The 2nd divisor used to determine the single absorption spectrum of B_2 from its mixture is B_1 at a concentration of 12 µg.mL^{-1}. This is followed by a subtraction with a constant, which is derived from the B_1 spectrum divided by its divisor. Finally, multiplying this by the same divisor yields the absorption spectrum as seen in **Figure 6**.

The 3rd divisor used to determine the single absorption spectrum of B_2 from its mixture was B_3 at a concentration of 18 µg.mL^{-1}. This was followed by a subtraction with a constant, which was derived from the B_3 spectrum divided by its divisor. Finally, multiplying this by the same divisor yielded the absorption spectrum as seen in **Figure 7**.

The last divisor, the 4th, used to determine the single absorption spectrum of B_2 from its mixture was B_6 at a concentration of 11.5 µg.mL^{-1}. This was followed by a subtraction with a constant, derived from the B_6 spectrum divided by its divisor. Subsequently, the 0-order absorption spectrum of B_2, as obtained by the proposed ratio subtraction ultraviolet spectrophotometry method, resulted from multiplying this by the same divisor, as seen in **Figure 8**.

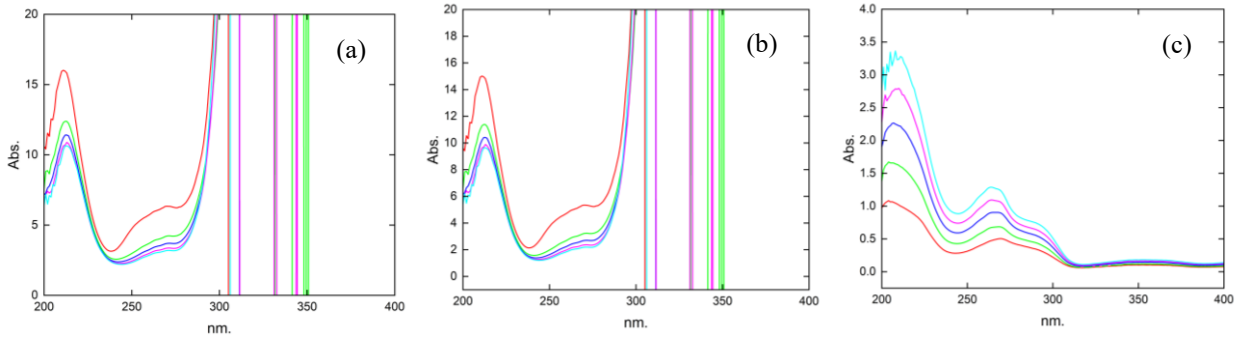

Figure 6 Spectra of the mixture with (a) B_1 as the divisor 12 µg.mL^{-1}, (b) spectra subtracting the constant (B_1/B_1°), and (c) spectra after multiplying by the divisor.

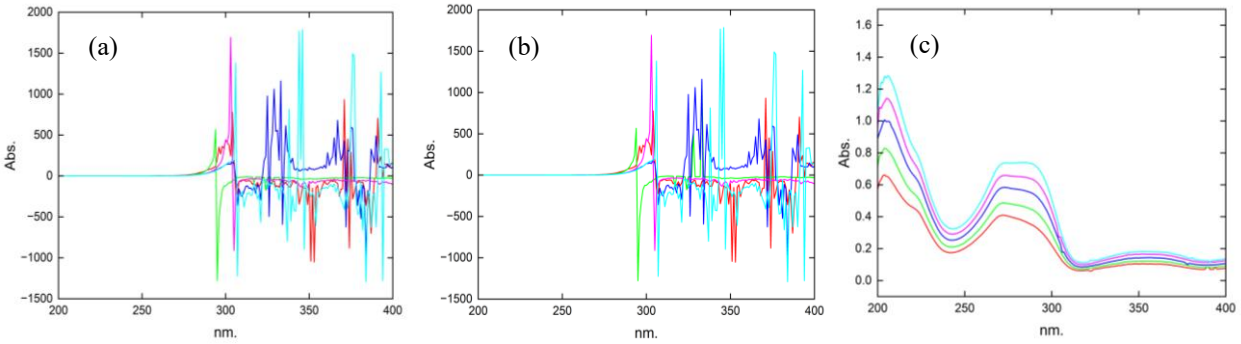

Figure 7 Spectra of the mixture with (a) B_3 as the divisor 18 µg.mL^{-1}, (b) spectra subtracting the constant (B_3/B_3°), and (c) spectra after multiplying by the divisor.

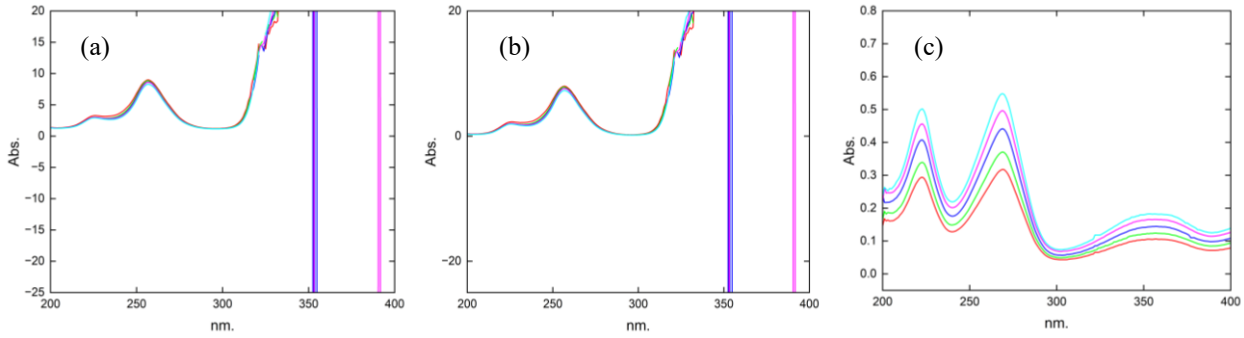

Figure 8 Spectra of the mixture with (a) B_6 as the divisor 11.5 µg.mL^{-1}, (b) spectra subtracting the constant (B_6/B_6°), and (c) 0-order absorption spectrum of B_2 obtained by the proposed ratio subtraction ultraviolet spectrophotometry method after multiplication by the divisor.

Nicotinamide (B3)

The determination of the single absorption spectrum of B_3 continued by taking the result from the 2nd division with B_1 during the B_2 determination (**Figure 6**), which itself followed the initial division with B_{12} from the B_1 determination (**Figure 2**). The 3rd divisor used to determine the single absorption spectrum of B_3 from its mixture was B_2 at a concentration of 22 µg.mL^{-1}. This was followed by a subtraction with a constant, derived from the B_2 spectrum divided by its divisor. Finally, multiplying this by the same divisor yielded the absorption spectrum as seen in **Figure 9**.

The final divisor, the 4th in the sequence, used to determine the single absorption spectrum of B_3 from its mixture was B_6 at a concentration of 11.5 µg.mL^{-1}. Following this, a subtraction was performed using a constant, which was derived from the B_6 spectrum divided by its divisor. Finally, the 0-order absorption spectrum of B_3, obtained via the proposed ratio subtraction ultraviolet spectrophotometry method, was

achieved by multiplying this by the same divisor, as shown in **Figure 10**.

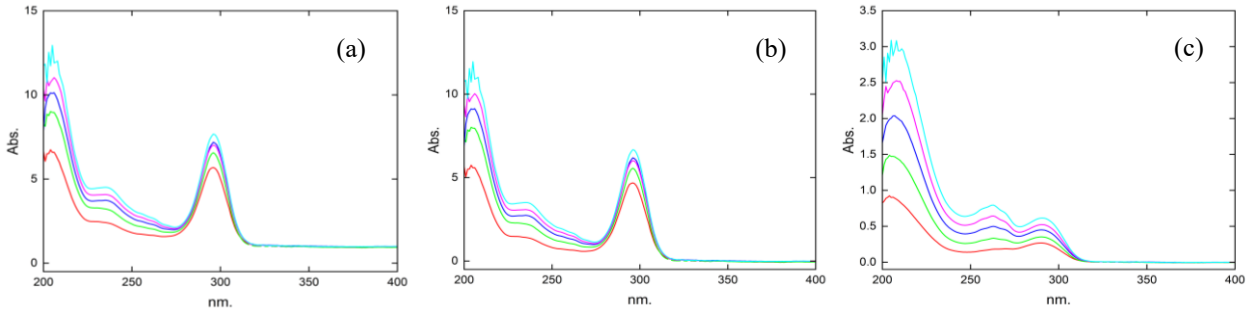

Figure 9 Spectra of the mixture with (a) B_2 as the divisor 22 µg.mL^{-1}, (b) spectra subtracting the constant ($B_2/B_2°$), and (c) spectra after multiplying by the divisor.

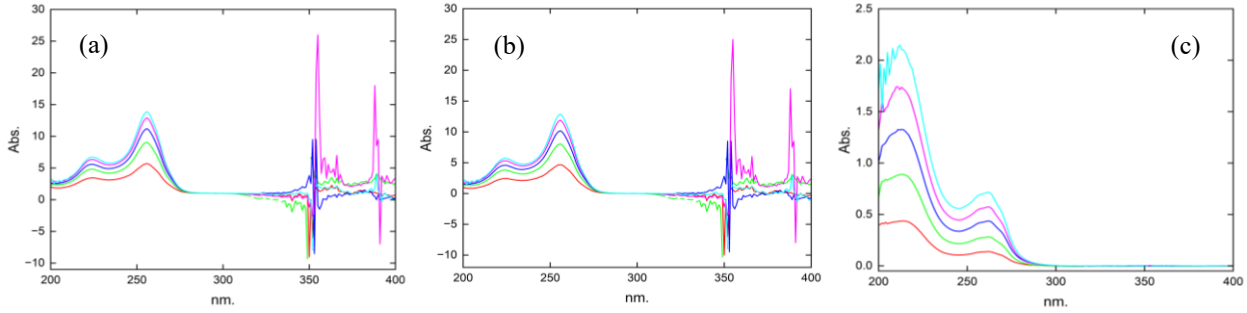

Figure 10 Spectra of the mixture with (a) B_6 as the divisor 11.5 µg.mL^{-1}, (b) spectra subtracting the constant ($B_6/B_6°$), and (c) 0-order absorption spectrum of B_3 obtained by the proposed ratio subtraction ultraviolet spectrophotometry method after multiplication by the divisor.

Pyridoxine (B_6)

The determination of the single absorption spectrum of B_6 proceeds by taking the result from the 3rd division with B_2 during the B_3 determination (**Figure 9**). This step followed the 2nd division with B_1 in the B_2 determination (**Figure 6**), which in turn followed the initial division with B_{12} in the B_1 determination (**Figure 2**). Subsequently, the final, 4th divisor used to isolate the single absorption spectrum of B_6 from its mixture was B_3 at a concentration of 18 µg.mL^{-1}. This was followed by a subtraction with a constant, derived from the B_3 spectrum divided by its divisor. Finally, the 0-order absorption spectrum of B_6, obtained by the proposed ratio subtraction ultraviolet spectrophotometry method, was achieved by multiplying this by the same divisor, as seen in **Figure 11**.

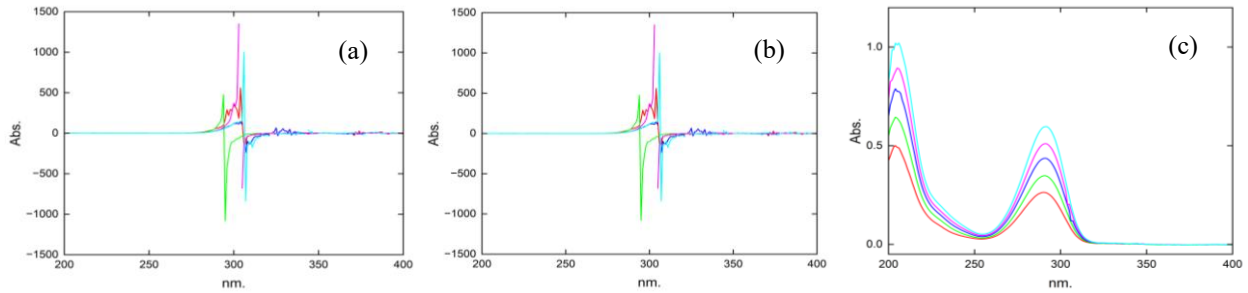

Figure 11 Spectra of the mixture with (a) B_3 as the divisor 18 µg.mL^{-1}, (b) spectra subtracting the constant ($B_3/B_3°$), and (c) 0-order absorption spectrum of B_6 obtained by the proposed ratio subtraction ultraviolet spectrophotometry method after multiplication by the divisor.

Cobalamin (B_{12})

The 1st divisor used to determine the single absorption spectrum of B_{12} from the mixture is B_1 at a concentration of 12 µg.mL^{-1}. This is followed by a subtraction with a constant, which is derived from the B_1 spectrum divided by its divisor. Finally, multiplying this by the same divisor yields the absorption spectrum as seen in **Figure 12**.

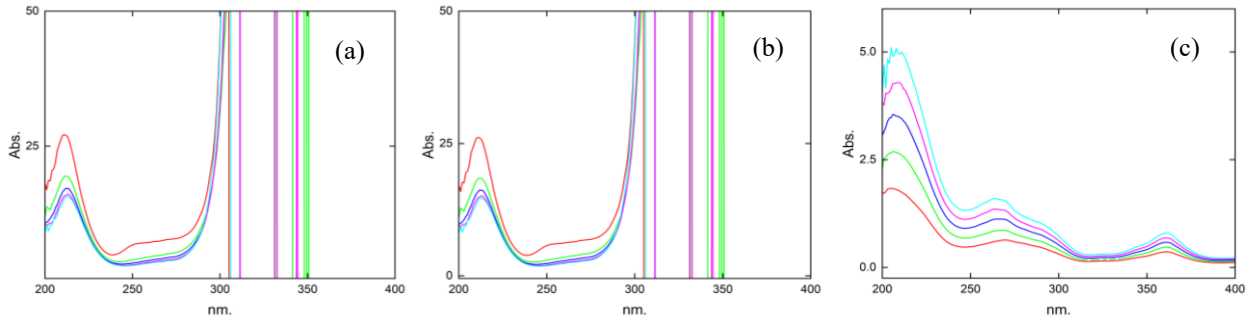

Figure 12 Spectra of the mixture with (a) B_1 as the divisor 12 µg.mL^{-1}, (b) spectra subtracting the constant ($B_1/B_1°$), and (c) spectra after multiplying by the divisor.

The 2nd divisor used to determine the single absorption spectrum of B_{12} from its mixture was B_2 at a concentration of 22 µg.mL^{-1}. This was followed by a subtraction with a constant, which was derived from the B_2 spectrum divided by its divisor. Finally, multiplying this by the same divisor yielded the absorption spectrum as seen in **Figure 13**.

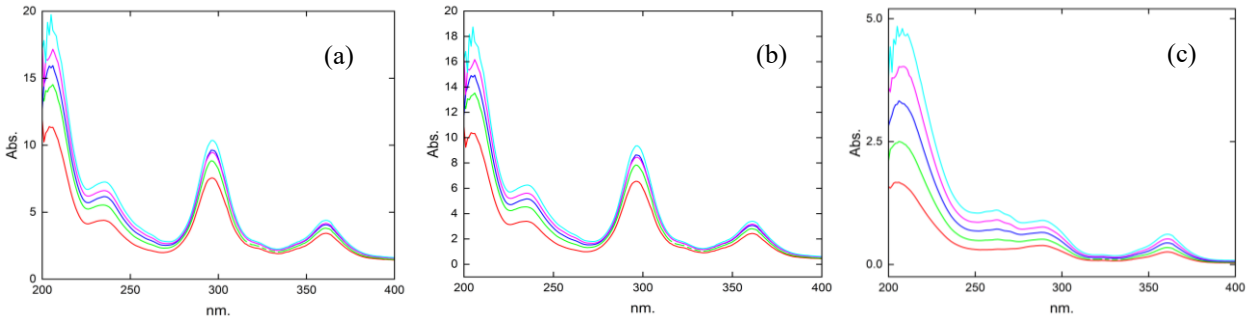

Figure 13 Spectra of the mixture of with (a) B_2 as the divisor 22 µg.mL^{-1}, (b) spectra subtracting the constant ($B_2/B_2°$), and (c) spectra after multiplying by the divisor.

The 3rd divisor employed to determine the single absorption spectrum of B_{12} from its mixture was B_3 at a concentration of 18 µg.mL^{-1}. This was followed by a subtraction with a constant, derived from the B_3 spectrum divided by its divisor. Finally, multiplying this by the same divisor yielded the absorption spectrum as seen in **Figure 14**.

The last divisor, the 4th, used to determine the single absorption spectrum of B_{12} from its mixture was B_6 at a concentration of 11.5 µg.mL^{-1}. This was followed by a subtraction with a constant, derived from the B6 spectrum divided by its divisor. Subsequently, the 0-order absorption spectrum of B_1, as obtained by the proposed ratio subtraction ultraviolet spectrophotometry method, resulted from multiplying this by the same divisor, as seen in **Figure 15**.

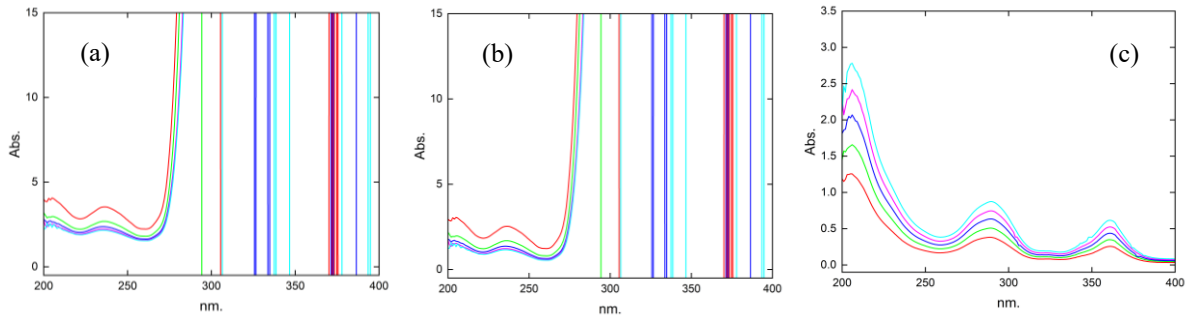

Figure 14 Spectra of the mixture with (a) B_3 as the divisor 18 µg.mL^{-1}, (b) spectra subtracting the constant ($B_3/B_3°$), and (c) spectra after multiplying by the divisor.

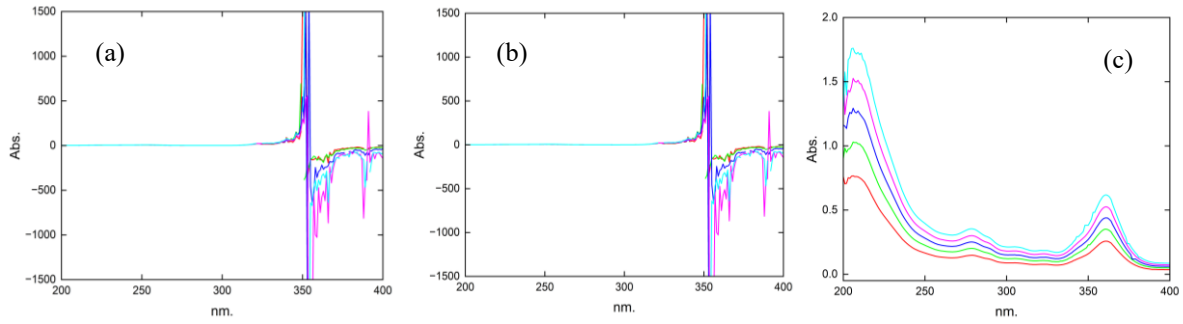

Figure 15 Spectra of the mixture with (a) B_6 as the divisor 11.5 µg.mL^{-1}, (b) spectra subtracting the constant ($B_6/B_6°$), and (c) 0-order absorption spectrum of B_{12} obtained by the proposed ratio subtraction ultraviolet spectrophotometry method after multiplication by the divisor.

As illustrated in **Figures 2** to **15**, the ratio subtraction ultraviolet spectrophotometry technique provides a structured and efficient strategy for the analysis of multicomponent mixtures, particularly in cases where spectral overlap occurs. This method is especially valuable for the quantification of pharmaceutical compounds, as exemplified by the simultaneous determination of B_1, B_2, B_3, B_6, and B_{12}. The analytical procedure begins by dividing the absorption spectrum of the mixture by that of a selected standard concentration, referred to as the divisor. The selection of appropriate divisor concentrations for each compound is essential, as it directly influences the resolution and accuracy of the subtraction process, thereby facilitating precise differentiation among the components. After the division step, the procedure proceeds with the subtraction of the divisor spectrum from the ratioed spectrum. This stage plays a crucial role in isolating the spectral contribution of each analyte by eliminating the influence of the divisor, allowing for clearer identification and quantification within complex mixtures. Subsequently, the subtracted spectrum is multiplied by the original divisor spectrum, a process that restores the spectral amplitude to its original scale while maintaining the resolution between overlapping signals. This sequence ultimately yields well-resolved, interference-free spectra for each compound present in the mixture. Notably, **Figures 5(c)**, **8(c)**, **10(c)**, **11(c)**, and **15(c)** depict the 0-order spectra of B_1, B_2, B_3, B_6, and B_{12}, respectively, as generated by this analytical approach. These figures also include the linear regression equations utilized to quantify the analytes based on their absorbance characteristics at specific wavelengths: 239 nm for B_1, 269 nm for B_2, 262 nm for B_3, 291 nm for B_6, and 361 nm for B_{12}. The respective concentrations were determined by referencing these spectra to their established calibration curves [9,27,28]. Compared to high-performance liquid chromatography (HPLC), which is highly selective but expensive and time-consuming, the proposed method offers a cost-effective and rapid alternative for multicomponent analysis. While chemometrics-based UV methods like PLS or CWT have shown success in resolving overlapping spectra, they require complex mathematical modeling and specialized software. In contrast, the ratio subtraction method achieves spectral deconvolution

using straightforward mathematical steps, making it more accessible for routine quality control laboratories.

The ratio subtraction UV spectrophotometry technique holds significant importance in pharmaceutical analysis, as it facilitates accurate quantification of individual components within complex mixtures, even when their absorption spectra exhibit substantial overlap. This level of precision is critical in verifying the correct dosage and therapeutic effectiveness of pharmaceutical formulations [27,30]. Despite its advantages, the ratio subtraction method has notable limitations. It becomes increasingly complex and less accurate when applied to mixtures with more than 5 analytes, especially when their spectra exhibit high degrees of overlap. Moreover, the accuracy of the method is highly sensitive to divisor selection, which requires careful optimization. As a purely spectrophotometric technique, it also lacks the selectivity of chromatographic or MS-based methods in highly variable matrices. Nevertheless, within the scope of this study, the method demonstrated sufficient accuracy, precision, and applicability for the simultaneous quantification of 5 B-complex vitamins in syrup formulation, as confirmed through method validation.

Method validation

Validation of the analytical method for determining B_1, B_2, B_3, B_6, and B_{12} encompasses several key parameters, including linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). These validation criteria are fundamental to evaluating the reliability and robustness of the method. A summary of the validation outcomes for all 5 compounds is presented in **Table 1**. The validation results confirm that the developed method aligns with the criteria outlined by International Council for Harmonisation (ICH) guidelines for the simultaneous quantification of B_1, B_2, B_3, B_6, and B_{12} in health supplement syrup. The adherence of all evaluated parameters underscores the reliability and suitability of this analytical approach. Previous studies have also reported that ultraviolet spectrophotometry utilizing the ratio subtraction ultraviolet spectrophotometry method yields robust and acceptable validation results [27,28,30].

Table 1 Method validation for the analysis of B_1, B_2, B_3, B_6, and B_{12} in ratio subtraction ultraviolet spectrophotometry method.

Parameter	B_1	B_2	B_3	B_6	B_{12}
Linearity	0.9998	0.9997	0.9999	0.9988	0.9999
Accuracy (%)	101.16 ± 0.63	100.40 ± 0.44	101.21 ± 0.56	101.09 ± 0.61	100.77 ± 0.72
Precision (%)	1.30	1.30	1.31	1.29	1.30
LOD (μg.mL^{-1})	0.49	0.85	0.62	0.98	0.57
LOQ (μg.mL^{-1})	1.47	2.58	1.88	2.98	1.73

Application of health supplement syrup quantification

The application results of the ratio subtraction UV spectrophotometry method for quantifying B_1, B_2, B_3, B_6, and B_{12} contained in health supplement syrup formulations presents in **Table 2**. The ratio subtraction ultraviolet spectrophotometry method was employed to determine the concentrations of B_1, B_2, B_3, B_6, and B_{12} for simultaneous quantification in health supplement syrups. This application aimed to ensure that these compounds met established pharmaceutical standards [31]. As shown in **Table 2**, the concentrations of B_1, B_2, B_3, B_6, and B_{12} in the health supplement syrup were found to be consistent with the required standards. These findings emphasize the critical role of rigorous quality control in the manufacturing of health supplement syrups and demonstrate the reliability of the ratio subtraction ultraviolet spectrophotometry method in ensuring that the formulation meets predefined compound specifications.

Table 2 Application of health supplement syrup quantification in ratio subtraction ultraviolet spectrophotometry method.

Component	Level (%)	Requirement (%)	The content (mg)	Claim on the label (mg)
Thiamine (B_1)	102.75 ± 2.20	90.00 - 110.00	5.14 ± 0.11	5
Riboflavin (B_2)	101.03 ± 2.17	95.00 - 115.00	2.02 ± 0.04	2
Nicotinamide (B_3)	102.65 ± 2.21	90.00 - 110.00	20.53 ± 0.44	20
Pyridoxine (B_6)	102.11 ± 2.16	95.00 - 115.00	2.55 ± 0.05	2.5
Cobalamin (B_{12})	101.26 ± 2.17	95.00 - 115.00	0.0030 ± 0.0001	0.003

Conclusions

The present study successfully developed and validated a ratio subtraction ultraviolet spectrophotometry method for the simultaneous quantification of 5 B-complex vitamins thiamine (B_1), riboflavin (B_2), nicotinamide (B_3), pyridoxine (B_6), and cobalamin (B_{12}) in a health supplement syrup formulation. The method employed a sequential ratio manipulation process involving spectral division, constant subtraction, and multiplication, allowing precise deconvolution of overlapping UV spectra. The analytical procedure was validated according to ICH guidelines, demonstrating excellent linearity ($r \leq 1$), high accuracy (recoveries between 90% - 110%), and precision (RSD ≤ 2%). Limits of detection and quantification for each vitamin confirmed the method's sensitivity. The quantified vitamin contents in real syrup samples were consistent with pharmacopeial specifications (United States Pharmacopeia 38 and National Formulary 33). Compared to conventional chromatographic and chemometric UV methods, the proposed technique is simpler, faster, and more cost-effective, requiring only basic instrumentation and minimal sample preparation. Therefore, this method is a promising alternative for routine quality control of multivitamin syrups, particularly in laboratories with limited access to advanced analytical technologies.

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Declaration of Generative AI in Scientific Writing

The authors affirm that generative AI tools, such as QuillBot and OpenAI's ChatGPT, were utilized solely for language refinement and grammar correction during the preparation of this manuscript. These tools were not involved in generating content or interpreting data. The authors bear full responsibility for the content and the conclusions presented in this work.

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