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# Fast and Efficient RP-HPLC Method for Simultaneous Determination of Water-soluble Vitamins in Some Nutraceutical Supplements

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THE purpose of this study is the development of analytical method for simultaneous determination of water-soluble vitamins ($B_1$, $B_2$, $B_3$, $B_6$, $B_9$, $B_{12}$ and vitamin C) in dietary supplement syrup, multivitamins and mineral dietary supplement syrup with iron, dietary supplement capsules and balanced supplemental nutrition for children and adult gum by using gradient RP-HPLC methods with PDA detector andcalculating the uncertainty for each matrix of analytical results based on the information from the validation process. The separation of water-soluble vitamins was performed on waters Spherisorb ODS2 (250 mm×4.6mm, 5µm) column. The wavelength was 272 nm at room temperature and the detection limits ranged from 2.4 to 8.3 ng/µl. The average recovery was 98%-102 % and correlation coefficient (r) was 0.9997 to 1.

**Keywords:** Water soluble vitamins, Nutraceuticals, Dietary supplements, Gradient RP-HPLC, Photodiode array detector (PDA)

## Introduction

Vitamins are organic nutrients required by the body to ensure normal growth and metabolism [1]. Vitamins are classified as micronutrients which exist in food in small quantities, and macronutrients as protein, carbohydrates and fats [2]. Although vitamins are essential for the prevention of several diseases and the maintenance of good health, the human body cannot make vitamins and it must get them from different foods and dietary supplement which are considered as vitamin supply [1-3]. The importance of vitamins in nutrition was understood in the 1920s and 1930s, where lack of them can cause various diseases in humans, and small concentrations are required to maintain good health [1,2]. Because of the critical role of vitamins in nutrition, qualitative and quantitative analyses are important issues and a challenging task for food manufacturers. Water soluble vitamins are thiamine ($B_1$), riboflavin ($B_2$), pyridoxine HCL ($B_6$), cyanocobalamin ($B_{12}$), nicotinic acid ($B_3$), folic acid ($B_9$), pantothenic acid ($B_5$), and vitamin C. Complex vitamins B and vitamin C cannot be stored in human tissues. Their excess is excreted with urine, but excess amounts of fat-soluble vitamins stored in adipose tissues and in the liver [1]**.** Nutraceuticals is any non-toxic food component or nutrient that provides medical or health benefits, including disease treatment or preventions. Nutraceutical mainly consists of Nutrients: substances have established nutritional functions e.g vitamins, minerals, amino acids, Herbals/ Phytochemicals as Herbs or Botanical Products (fatty Acid) and /or Dietary Supplements as Probiotics (helpful Bacteria), Prebiotics (digestive Enzymes), Antioxidants, Enzymes. Nutraceuticals are classified according to natural source, pharmacological condition and Chemical Constituent[4,5]. However, in case of real samples that may include vitamins and proteins, adsorption to the electrode surface is a serious problem [6]. On the other hand, the common Official analytical method is non-specific, tedious and time consuming as these methods involve

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pretreatment of the sample through physical, biological and complex chemical reactions to eliminate the interferences found [7]; it followed by individual methods for each different vitamin. The determinations of vitamins B and vitamin C in food were performed by spectrophotometry [8], electrochemical method [9,10], capillary electrophoresis [11]. HPLC method is a powerful tool for rapid analysis of pharmaceuticals and food staffs [12,13]. It was used for more complicated mixtures of two or more vitamins [14-20] with gradient elution program. The separations take place on normal-phase, ion-pairing, and reversed-phase chromatography columns.

As an extension of previous studieson fat soluble vitamins in milk powder, infant formula and edible oils [21,22], the present work was undertaken to develop a rapid and convenient method for simultaneous determination of water-soluble vitamins (B1, B3, B6 B9, B2, B12, and vitamin C) in some nutraceutical supplements. The developed method is simple, rapid, more sensitive with high accuracy and low RSD of retention time and peak area. The sensitivity and specificity of this method allowed a lower detection limits of the analytes. Therefore, the applied chromatographic system was successful for simultaneous separation and quantitative determination of the seven vitamins from their products in a single chromatographic run with high resolution and precision. Moreover, this method was developed to be convenient for the determination of high and low concentration vitamin in different matrices with different fat content.

## Materials and Method

*Apparatus and chemicals*

HPLC (Shimadzu LC-20, Japan), Centrifuge (Pro-Research, UK), pH-meter (Jenway3310, British), Mixer (Falk, Germany) and Ultra-sonic bath (Elma, Germany) were used in this study.

Dietary supplement milk, syrupy, capsules from Egypt companies.

*Preparation of vitamins solution*

The mixtures of water-soluble vitamins were prepared by dissolving 100 mg of Thiamine hydrochloride, nicotinic acid, folic acid, pyridoxine hydrochloride, vitamin C, cyanocobalamin, riboflavin in 500 ml of 4% ion pair agent (TCA). The standard solution was kept in the dark at 4 °C and was prepared fresh daily. This stock was used for preparation of more dilute solutions by appropriate dilutions.

*Extraction of samples*

Five grams or 5 ml of homogenized capsules, syrupy or powder were weighed, completed to the mark in 10 ml measured flask by using 4% TCA, mixed for 5 min, centrifugated for 15 min (6000 RPM), filtered by using 0.45 µm filter membrane and injected in HPLC instrument.

*Method*

High performance liquid chromatographic method was used for the determination of water-soluble vitamins. Separation was performed on a Waters Spherisorb ODS2 (250 mm × 4.6mm, 5µm) column. Gradient elution was performed with a mobile phase consisting of 0.2 g heptane-1-sulfonic acid sodium salt in deionized water: methanol (90:10, v/v) of pH 3 (solvent A), and 0.2g heptane-1-sulfonic acid sodium salt in deionized water: methanol (30:70, v/v) of pH 3 (solvent B) at the flow rate 0.6 ml min−1. Starting with 100 % solvent A then the composition was changed gradually during next 30 min to reach 100% of solvent B. The measurements were carried out at wavelength 272 nm, at room temperature with 0.6 ml $min^{-1}$ flow rate.

*Applied Hydrophobic-Subtraction Model*

In present study,the H-S model was applied to explain chromatographic behaviours of the analytes. For column selectivity comparison, besides the column information provided by the manufacturers, column parameters as H (Column hydrophobicity), *S*(Steric selectivity), B (column hydrogen bond basicity), A (Column hydrogen bond acidity) and *C* (cation exchange) obtained from H-S model are also conservatively used to interpret the stationary phase/analyte interactions when appropriate.

*Stability study of water-soluble vitamins*

Stability of seven water soluble vitamins was tested in three different pH conditions (acidic, basic and neutral) over a 24 hour at ambient temperature by using spectrophotometric.

- Thiamine was stable in acidic and neutral pH but steadily degraded over a one-day period and degradation was observed for the neutral condition.

-Riboflavin is degradation in basic solution but more stable in the acidic and neutral pH ranges.

- Nicotinic acid and pyridoxine were the most stable at different pH conditions.

- Folic acid is stable under neutral pH but decrease

under acidic and basic pH.

- Cyanocobalamin is degradation in basic pH but more stable in the acidic and neutral pH ranges.

- Ascorbic acid is the least stable of water-soluble vitamin of all with degradation being observed under acidic, neutral and basic pH conditions. It degraded extremely fast under basic pH after 5 hours.

**Results and Discussion**

Low cost and less consuming solvent HPLC method is the most suitable for the determination of multivitamin and mineral dietary supplement and in different matrices with different fat content because it has many advantages. It is highly precise, accurate, sensitive, and selective which give broad linear range with good correlation coefficient and good recovery. The chromatographic method was selected based on optimized gradient elution, modifier of mobile phase, types of column and other LC parameters.

The gradient elution is most frequently used in chemical separation. Table 3 and Fig. 2 showed that gradient-3 was preferred because it gives high resolution chromatogram. The low pH is suitable for analysis of ionic compounds by reversed phase column to avoid secondary interaction between ionized silanols on the silica surface and positive charge compounds [20]. In this method,pH 3the most suitable of mobile phase as shown in Fig. 3. The use of PDA detector, in the present study, gives higher signal to noise ratio than UV-VIS detector. Moreover, the slope of calibration curve of each standard in this method is higher than that previously reported methods [23-25]. Therefore, our developed method is more sensitive. Furthermore, in a previous study [26], ion-paired reagents, such as trifluoroacetic acid (TFA) and acetic acid were used for improving the separation of vitamins. However, TFA has UV cutoff below wavelength 240 nm, resulting in a shifted baseline and decreases the sensitivity. In the present study, heptane-1-sulfonic acid sodium salt was used instead of TFA to decrease the retention time and to give a sharp peak area hence increases the sensitivity. In addition, the use of suitable wavelength at 272 nm resulted in enhancing the signal and increases the sensitivity. Moreover, in this method, the relative standard deviation (RSD) of peak area is less than 5, therefore it is method more sensitive. Finally, the selection of Waters Spherisorb ODS2 column in the present study, gives sharper peaks with high sensitivity due to less band broadening.

Due to low concentrations of $B_9$ in nature, there is a critical need for preconcentration to facilitate their isolation and purification from a complex matrix like dietary supplement samples. Solid phase extraction (SPE) has been introduced for sample preparation. In this method,due the low limit of detection,there is no need for preconcentration for vitamin $B_9$.

New approach for calculating uncertainties of analytical results based on the information from the validation process is proposed. This complements the existing approaches proposed to date and can be applied to any validated analytical method. The total uncertainty calculated by

$$UC\% = \sqrt{precision^2 + bias^2}$$

*Selection of the appropriate column by chooses the suitable physical properties.*

Benefits of this approach are small initial time investment, time savings in the HPLC laboratory, more informed approach to column selection and more efficient than trial and error approach. There were five different types of chromatographic columns that have been used for separation of some water-soluble vitamins; intersil ODS and a waters spherisorb ODS2,Zorbax C18, Water Spherisorb ODS and Lichrospher 100 Rp18. The present study showed that Waters spherisorb ODS2 was give high resolution chromatogram. Physical parameters such as column dimensions are length and internal diameter of packing bed, particle Size (Spherical or irregular) is The average particle diameter, typically 3-20 μm, surface area ( m2/g ) is Sum of particle outer surface and interior pore surface, pore size is average size of pores or cavities in particles, ranging from 60-10,000Å,carbon load (% C) is amount of bonded phase attached to base material, end capping is Capping of exposed silanols with short hydrocarbon chains after the primary bonding step and embedded groups are provided by the column manufacturers. These parameters tell little about chromatographic performance of the bonded phases. The nature of which is important to the selectivity of the columns towards the analytes of interest. Choosing the appropriate HPLC column should be based both upon knowledge of the sample as molecular weight of seven water soluble vitamin less than 2000 so the pore size ≤ 150A° and in this case the goals for the separation method is separate multi components analysis(seven water soluble vitamins) so we need high large column

equal to 25-30cm and high surface areaso the column is high efficiency column and give high resolution chromatogram.

*Resolution measurement of waters spherisorb ODS2 column*

The results listed in Table 1 show the column parameters for an optimum separation of water-soluble vitamins. The capacity factor [K= ($RT_1$-$RT_0$) / $RT_0$], separation factor S=$k_2/k_1$ and the resolution [R=2($RT_2$-$RT_1$)/ ($W_2$+$W_1$)] were ranged in (1-9.9), (1.1-2) and (1.5-7), respectively, for water soluble vitamins separated on Waters spherisorb ODS2 column.

The column selectivity, originally called the separation factor (S), is defined as the ratio of the capacity factors of two adjacent peaks. When the value of S ≥ 1, this means that we obtained base lines separation for all the eluted mixtures [27]. A resolution value of 1.5 or greater between two peaks will ensure that the sample components are well separated [27].

*Optimization for separation of some water-soluble vitamins.*

The optimization work mainly depends on the choice of UV wavelength and the addressed program of the chromatographic elution, to maximize both resolution and sensitivity. So, the contents of mobile phases were 0.2 g heptane-1-sulfonic acid sodium salt in deionized water: methanol (90:10, v/v) adjusted to pH 3 (solvent A), and 0.2g heptane-1-sulfonic acid sodium salt in deionized water: methanol (30:70, v/v) adjusted to pH 3 (solvent B).

It was obvious from Table 2 and Fig. 2 that gradient-3 was preferred because a resolution value of 1.5 or greater between two adjacent peaks will ensurethat the sample components are

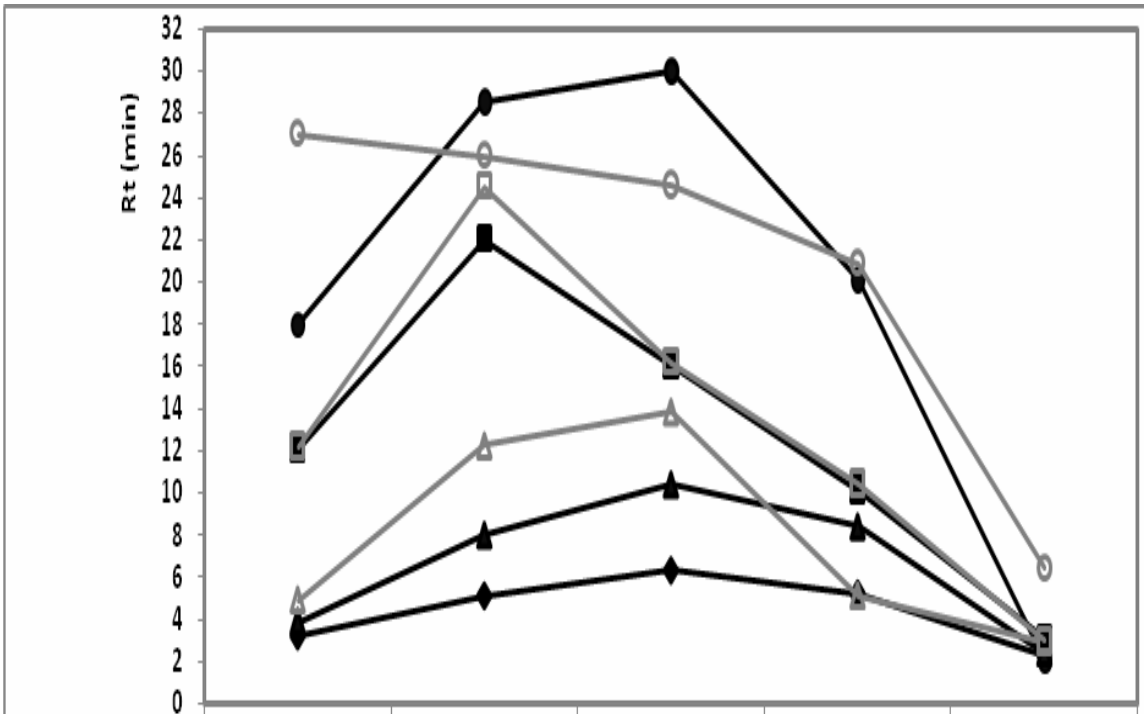
**TABLE 1. Physical parameters of five nonpolar columns for separation of water-soluble vitamins.**

| Column Name | Abbreviation | Column Size | Pore Size | Surface Area | Total Carbon |
|---|---|---|---|---|---|
| Water SpherisorbODS2 | Water ODS2 | (250 mm × 4.6 mm × 5 µm) | 150 A° | 320 $m^2/g$ | 18.5% |
| Intersil ODS2 | Intersil ODS2 | (250 mm × 4.6 mm × 5 µm) | 80 A° | 220 $m^2/g$ | 11.5% |
| Zorbax C18 | Zorbax C18 | (250 mm × 4.6 mm × 5 µm) | 80 A° | 220 $m^2/g$ | 6.2% |
| Water Spherisorb ODS | Water ODS | (150 mm × 4.6 mm × 5 µm) | 70 A° | 320 $m^2/g$ | 20% |
| Lichrospher 100 Rp18 | Lichrospher | (250 mm × 4.6 mm × 5 µm) | 100 A° | 320 $m^2/g$ | 21% |

**TABLE 2. Column parameters for an optimum separation of water-soluble vitamins.**

| Compound | RT min | k | S | W | N | R |
|---|---|---|---|---|---|---|
| Vit C | 5.1 | 1.0 | ---- | 0.6 | 1156 | ---- |
| Vit $B_3$ | 8.0 | 2.0 | 2.0 | 0.6 | 2844 | 4.8 |
| Vit $B_6$ | 12.2 | 3.7 | 1.8 | 2.0 | 595 | 3.2 |
| Vit $B_9$ | 22.0 | 7.46 | 2.0 | 0.8 | 12100 | 7.0 |
| Vit $B_{12}$ | 24.5 | 8.4 | 1.1 | 1.0 | 7744 | 2.7 |
| Vit $B_2$ | 26.0 | 9.0 | 1.1 | 1.0 | 10816 | 1.5 |
| Vit $B_1$ | 28.5 | 9.9 | 1.1 | 1.0 | 12996 | 2.5 |

Retention times (RT), capacity factors (K), separation factors(S), Column efficiency (N), Resolution (R) and peak width (W) show optimum condition for separation of water-soluble vitamins.




| | Intersil | water ods2 | zorbax | Lichrospher100 | water ods |
|---|---|---|---|---|---|
| vitamin C | 3.2 | 5.1 | 6.3 | 5.2 | 2.3 |
| vitamin B1 | 18 | 28.5 | 30 | 20.1 | 2.1 |
| vitamin B2 | 27 | 26 | 24.6 | 20.9 | 6.5 |
| vitamin B3 | 3.9 | 8 | 10.4 | 8.4 | 2.4 |
| vitamin B6 | 4.9 | 12.2 | 13.8 | 5.1 | 2.9 |
| vitamin B9 | 12.1 | 22 | 16 | 10.1 | 3.1 |
| vitamin B12 | 12.2 | 24.5 | 16.2 | 10.4 | 3 |

**Fig. 1. Effect of type of non-polar column on retention time (RT) of soluble vitamins; $B_1$, $B_2$, $B_{12}$, $B_9$, $B_6$, $B_3$ and vit. C.**

**TABLE 3. Three different gradient profiles for simultaneous vitamins separation by RP-HPLC.**

| Time | Gradient-1(A) | | | | Gradient-2(B) | | | Gradient-3(C) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Solvent | 0 | 15 | 30 | 50 | 0 | 15 | 37 | 0 | 15 | 30 |
| solvent A% | 90 | 40 | 10 | 10 | 92 | 47 | 3 | 100 | 50 | 0 |
| %Solvent B | 10 | 60 | 90 | 90 | 8 | 53 | 97 | 0 | 50 | 100 |

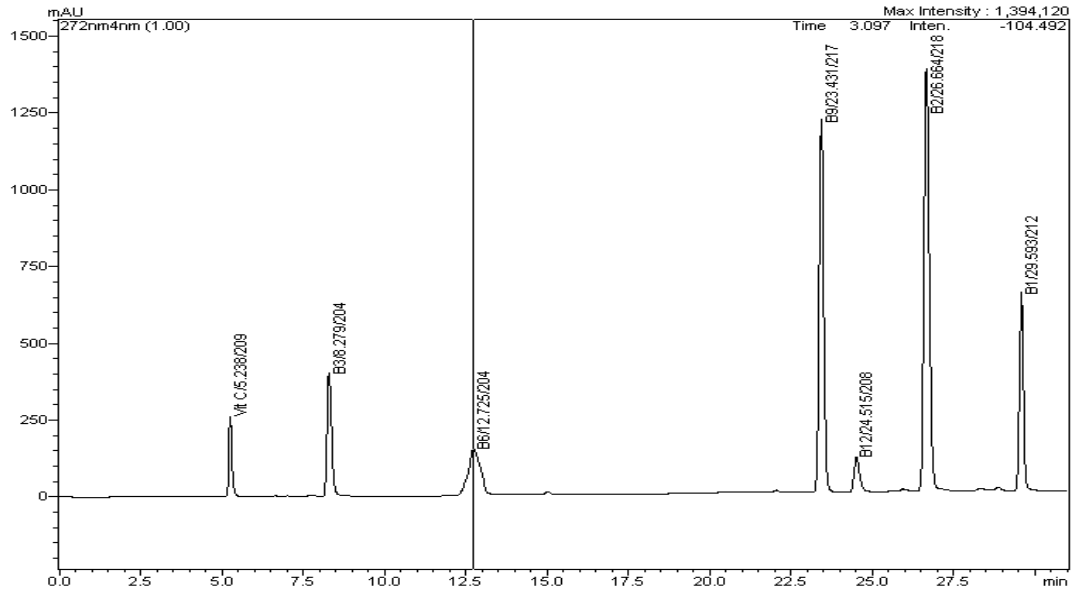



**Fig. 2. Chromatograms for RP-HPLC simultaneous vitamins separation gradiant-3**

well separated to a degree at which the area of each peak may be accurately measured and give vitamin C and six vitamins B with good resolution.

*The effect of pH on the retention time of some water-soluble vitamin's standards*

In the present study the effect of different pH values on the retention time of standards was studied by using two solvents (A and B); the pH of these solvents varied between ranges 2-7 by adding a few drops of sodium hydroxide or acetic acid. It was not possible to cover pH range less than 2 and more than 8 due to instability of the packing over the region since alkaline solution dissolves the silica support and at low pH breaks the Si-O linkage.

The curves show change of retention time of some water-soluble vitamins with the change of pH of mobile phase. The optimum pH obtained for best separation of water-soluble vitamins is at pH 3, because a resolution value of 1.5 or greater between two adjacent peaks will ensure that the sample components are well separated to a degree at which the area of each peak may be accurately measured (Fig. 3).

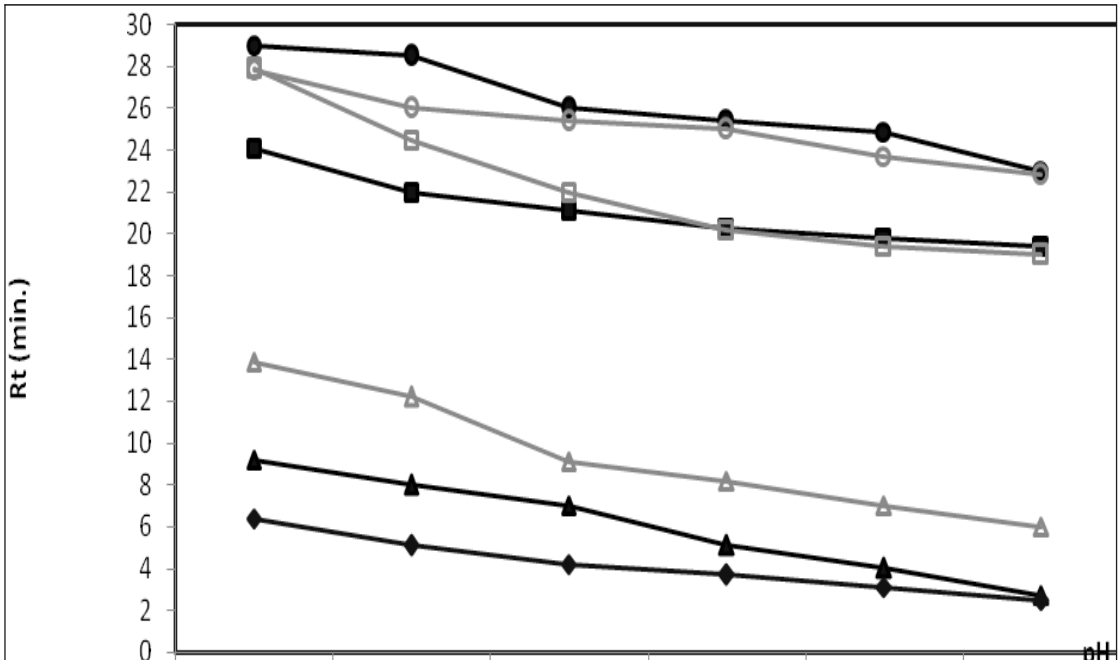
*The effect of flow rate on separation of water-soluble vitamins*

HPLC column is affected with change of flow rate because of changes of pressure and analysis time. A high flow rate may reduce the analysis time but may adversely affect the quality of chromatography, as it may not permit sufficient time for analyte separation and interact with stationary phase. Table 4 and Fig. 4 show that, the retention time of water soluble vitamins changes with the flow rate (0.6 ml/min to 1.2 ml/min); acceptable flow rate is (0.6 ml/min) which gave resolution value of 1.5 or greater between two adjacent peaks and this indicated that the sample components are well separated, but high flow rate not sufficient for separation of folic acid and cyanocoblamin, thiamin and riboflavin, and ascorbic acid and nicotinic acid, and give resolution value less than 1 between two adjacent peaks.

*Calibration curves and method validation*

Also, method validation studies were performed by measuring basic parameters such as precision, accuracy, linear region, limits of detection (LOD), and quantification (LOQ) [28], precision by using one-wayanova and recovery (Tables 3 and 4).

Detection limits of vitamins ($B_1$, $B_2$, $B_3$, $B_6$, $B_9$, $B_{12}$ and C) were 4.795, 2.412, 5.117, 4.921, 1.06,8.380 (ng/µl) and 3.230 (µg/µl), respectively, where the quantification limits were 14.532, 7.308, 15.505, 14.912, 3.225, 25.395 ng/µland 10.768 µg/µl, respectively, with recovery% in the

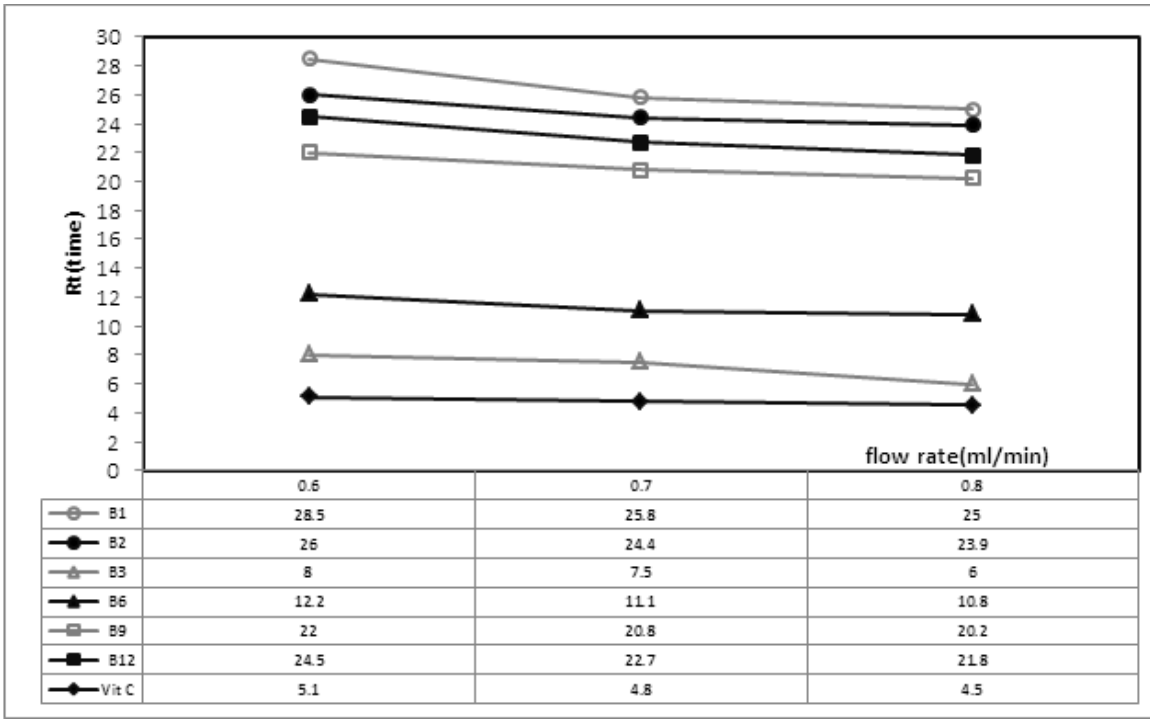



| | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
| Rt B1 | 29 | 28.5 | 26 | 25.4 | 24.9 | 23 |
| Rt B2 | 27.8 | 26 | 25.4 | 25 | 23.7 | 22.8 |
| Rt B3 | 9.2 | 8 | 7 | 5.1 | 4 | 2.7 |
| Rt B6 | 13.9 | 12.2 | 9.1 | 8.2 | 7 | 6 |
| Rt B9 | 24.1 | 22 | 21.1 | 20.3 | 19.8 | 19.4 |
| Rt B12 | 27.9 | 24.5 | 22 | 20.2 | 19.4 | 19 |
| Rt VIT C | 6.4 | 5.1 | 4.2 | 3.7 | 3.1 | 2.5 |

Fig. 3. Effect of pH on retention time (RT) of soluble vitamins; $B_1$, $B_2$, $B_{12}$, $B_9$, $B_6$, $B_3$ and vit. C.

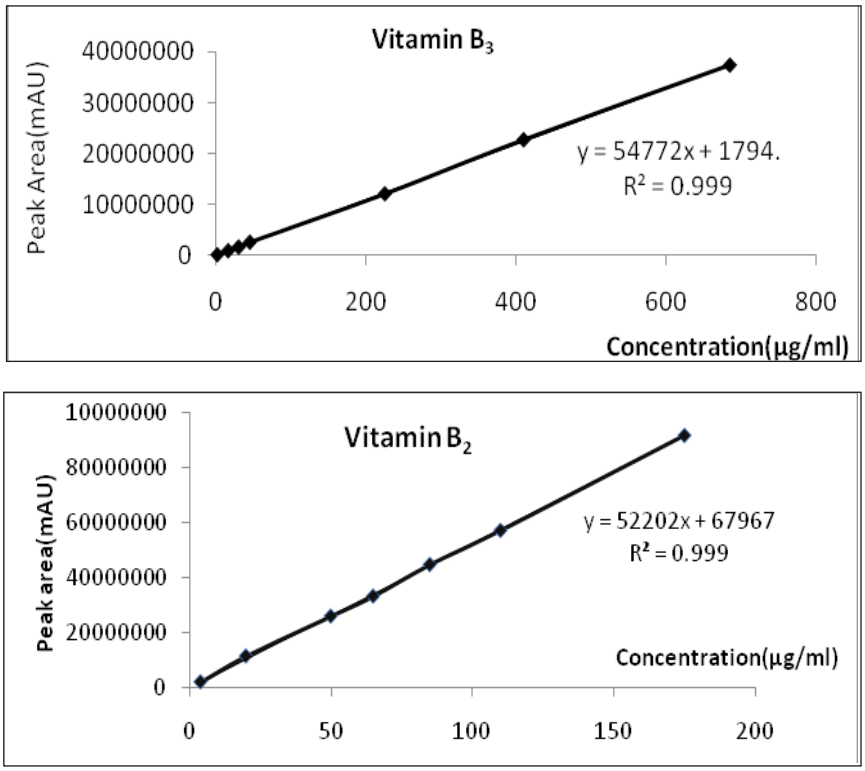
**TABLE 4. Method of validation for determination of different vitamins with the proposed method.**

| Vitamin | Rt (min) | LOD | LOQ | Accuracy | Linear range |
|---|---|---|---|---|---|
| $B_1$(ng/µl) | 28.5 | 4.795 | 14.532 | 101.62±3.60 | 12-1400 |
| $B_2$(ng/µl) | 26.0 | 2.412 | 7.3084 | 99.64±5.04 | 4-175 |
| $B_3$(ng/µl) | 8.0 | 5.117 | 15.505 | 99.78±2.18 | 1.6-685 |
| $B_6$(ng/µl) | 12.2 | 4.921 | 14.912 | 100.23±0.64 | 75-3150 |
| $B_9$(ng/µl) | 22.0 | 1.064 | 3.225 | 98.04±5.66 | 2-540 |
| $B_{12}$(ng/µl) | 24.5 | 8.380 | 25.395 | 100.30±1.98 | 20-350 |
| Vit. C (µg/µl) | 5.1 | 3.230 | 10.768 | 100.22±2.14 | 1.5-500 |




| | 0.6 | 0.7 | 0.8 |
|---|---|---|---|
| B1 | 28.5 | 25.8 | 25 |
| B2 | 26 | 24.4 | 23.9 |
| B3 | 8 | 7.5 | 6 |
| B6 | 12.2 | 11.1 | 10.8 |
| B9 | 22 | 20.8 | 20.2 |
| B12 | 24.5 | 22.7 | 21.8 |
| Vit C | 5.1 | 4.8 | 4.5 |

**Fig. 4. Retention time as a function of flow rate for some water-soluble vitamins; $B_1$, $B_2$, $B_{12}$, $B_9$, $B_6$, $B_3$ and) vit. C.**



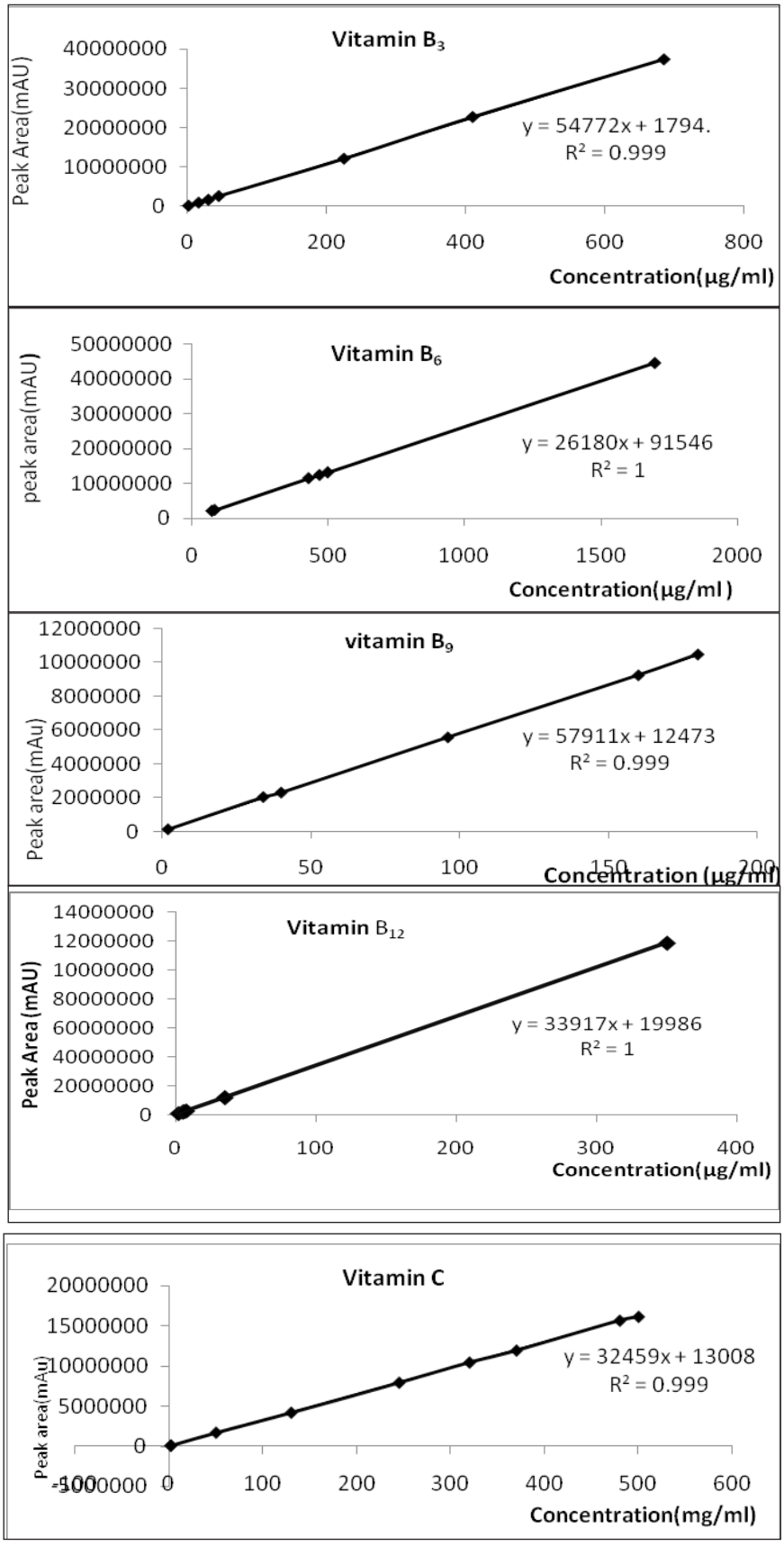



**Fig. 5. Calibration curve for water soluble vitamins.**

range of 98-102%.

*Quality control chart of water soluble vitamins*

In present work, we have applied the developed method on standard reference material® 1849 to calculate the bias and draw quality control chart for each standard that is found to be accepted according to West Gard rules.

*Quality control chart of vitamin $B_1$*

10 samples of standard reference material® 1849a detected by the same method of the food and nutraceuticals method

Mean =1.26 mg/100 g, SD=0.049

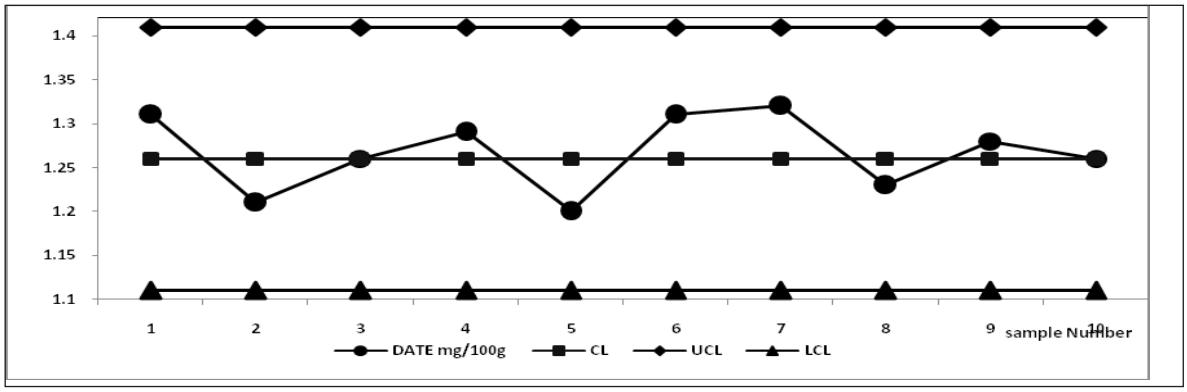



**Fig. 6. Quality control chart of vitamin $B_1$.**

CL= Control line = Mean, UCL = upper control line = Mean +3 SD and LCL = lower control line= Mean -3 SD.

*Quality control chart of vitamin $B_2$*

10 samples of standard reference material® 1849a detected by the same method of the food and nutraceutical method

Mean =2.037 mg/100 g, SD= 0.026

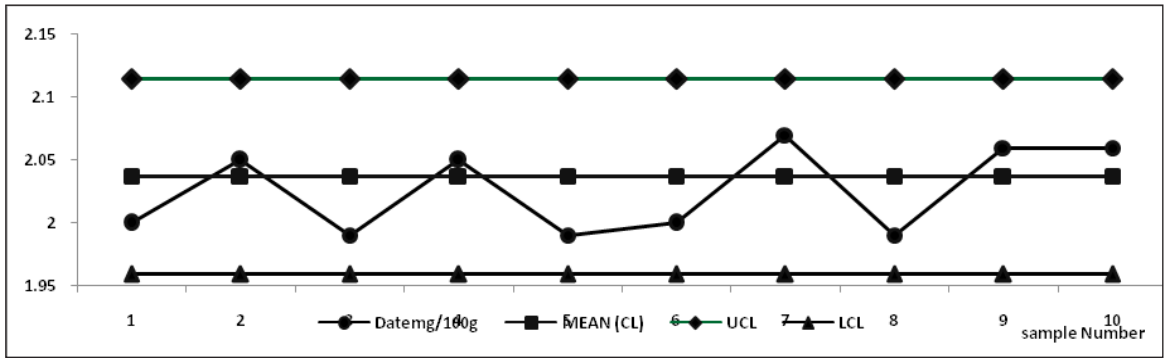



**Fig. 7. Quality control chart of vitamin $B_2$.**

*Quality control chart of vitamin $B_6$*

10 samples of standard reference material® 1849a detected by the same method of the food and nutraceutical method

Mean =1.34 mg/100g, SD= 0.0465

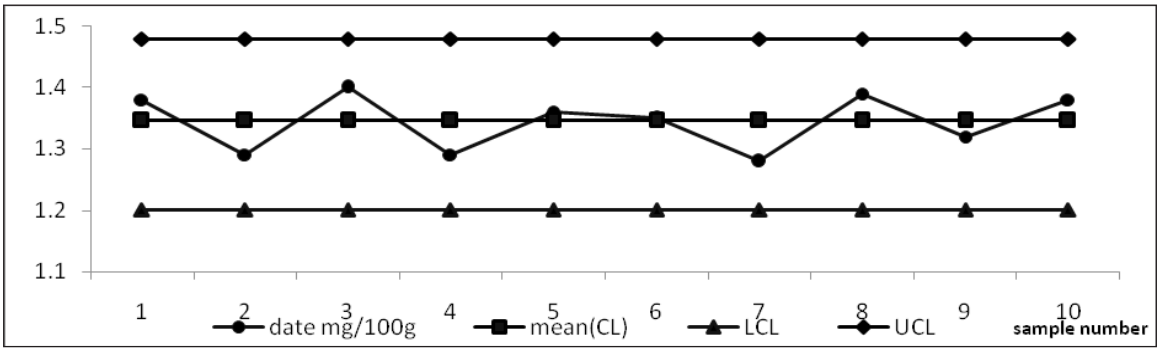



**Fig. 8. Quality control chart of vitamin $B_6$.**

*Quality control chart of vitamin $B_9$*

10 samples of standard reference material® 1849a detected by the same method of the food and nutraceuticals method
Mean =22.93 mg/100 g, SD= 0.31

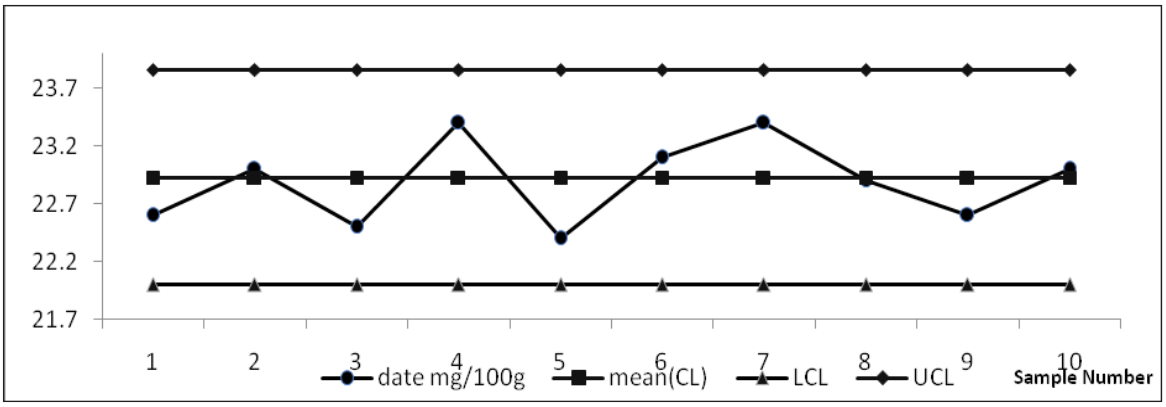



**Fig. 9. Quality control chart of vitamin $B_9$.**

*Quality control chart of vitamin $B_{12}$*

10 samples of standard reference material® 1849a detected by the same method of the food and nutraceuticals method
Mean= 0.482 mg/100 g, SD= 0.0425

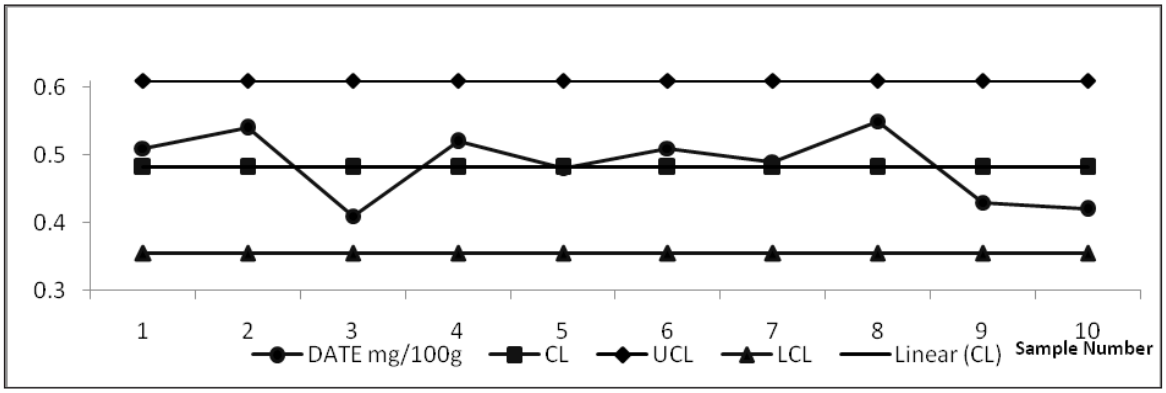



**Fig. 10. Quality control chart of vitamin $B_{12}$.**

*Quality control chart of vitamin C*

10 samples of standard reference material® 1849a detected by the same method of the food and nutraceuticals method
Mean =78.4 mg/100g, SD= 3.25

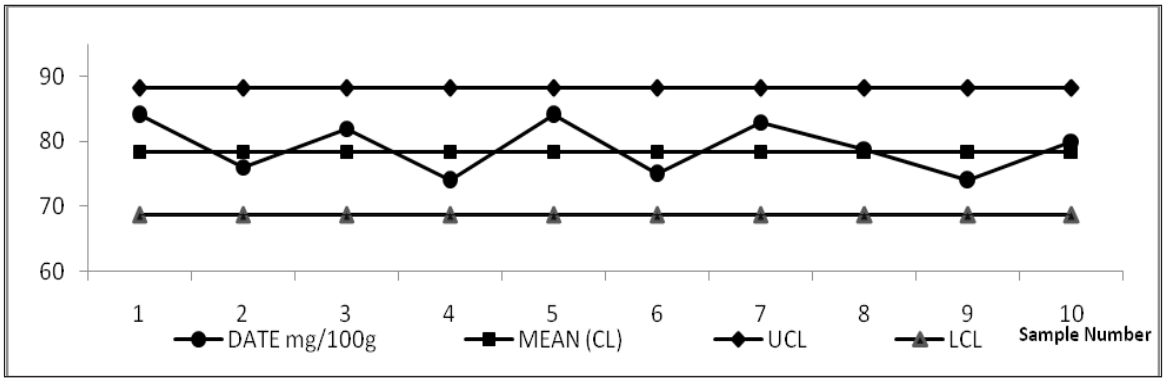



**Fig. 11. Quality control chart of vitamin C.**

*Uncertainty of water-soluble vitamins*

While verifying the accuracy of the measurement, the analyst generates information about different intermediate precision estimates. Since these estimates have been obtained by varying the factors that affect the measurement result representatively, they take into account the uncertainties of different steps in the analytical process. For instance, uncertainties due to the separation of the analyte of interest, matrix effects, environmental conditions, instruments or operators are considered as long as the factors that influence them are varied representatively. However, there may be other sources of variation which have not been considered in the assessment of accuracy. These sources may be related to global factors or local factors. The global factors are inherent to the measurement process. Therefore, these sources of uncertainty must always be considered in the overall uncertainty. They may be related to uncertainties not considered while assessing the accuracy or to the bias of the reference. Uncertainty of Bias% from CRM for balanced supplemental nutrition for children 1-10 years and spiking sample for other samples.

$$\text{bias} = \frac{\text{true value} - \text{labratory value}}{\text{true vale}} \times 100$$

True value from certification for CRM
Combined Uncertainty (Uc)

All the standard uncertainties for the individual sources of variation must be combined to produce an overall uncertainty for the measurement.
Combined Uncertainty

$$\text{UC\%} = \sqrt{\text{precision}^2 + \text{U bias}^2}$$

Precision by using one-way ANOVA to calculate reproducibility and repeatability.
Expanded Uncertainty (UE)

The combined standard uncertainty is then multiplied by a coverage factor, k, to provide an Expanded Uncertainty. For most purposes a value of 2 is used for k yielding a confidence level of approximately 95%.

Expanded uncertainty (UE)% = Uc × K

K: is a coverage factor

**TABLE 5. Concentration of seven water soluble vitamins in dietary supplement capsules .**

| Vitamin per capsule | Label concentration | Found concentration | Mean n=3 | SD | Recovery % | Uncertainty per capsule |
|---|---|---|---|---|---|---|
| | | 62 | | | | |
| Vitamin C mg | 60.0 | 61.17 | 62.00 | 0.83 | 103.3% | 2.7 |
| | | 62.83 | | | | |
| | | 1.61 | | | | |
| Vitamin $B_1$ mg | 1.4 | 1.4 | 1.50 | 0.11 | 107.1% | 0.13 |
| | | 1.5 | | | | |
| | | 1.71 | | | | |
| Vitamin $B_2$ mg | 1.6 | 1.63 | 1.66 | 0.13 | 103.8% | 0.08 |
| | | 1.46 | | | | |
| | | 19.2 | | | | |
| Vitamin $B_3$ mg | 18.0 | 16.4 | 17.20 | 1.72 | 95.6% | 0.62 |
| | | 16.08 | | | | |
| | | 2.05 | | | | |
| Vitamin $B_6$ mg | 2.0 | 1.99 | 1.97 | 0.09 | 98.6% | 0.06 |
| | | 1.878 | | | | |
| | | 106.1 | | | | |
| Vitamin $B_9$ µg | 100.0 | 105 | 105.00 | 1.1 | 105.0% | 5 |
| | | 103.9 | | | | |
| | | 1.03 | | | | |
| Vitamin $B_{12}$ µg | 1.0 | 0.87 | 0.95 | 0.08 | 95.0% | 0.06 |
| | | 0.95 | | | | |

Table 5 shows the found and label concentrations of vitamin C, $B_1$, $B_2$, $B_3$, $B_6$, $B_9$ and $B_{12}$ in nutrients for blood formation capsules with $B_{12}$, it was found to be 62.00±2.7, 60 mg/ capsule,, 1.50±0.13, 1.4 mg/ capsule, 1.66±0.08, 1.6 mg/ capsule, 17.20±0.62, 18.0 mg/ capsule, 1.97±0.06, 2.0 mg/ capsule, 105.00±5, 100 µg/ capsule and 0.95±0.06, 1.0 µg/ capsule, respectively. The recoveries were found to be 103.3%, 107.1%, 103.8%, 95.6%, 98.6%, 105% and 95%, respectively.

**TABLE 6. Concentration of five water soluble vitamins in dietary supplement syrup with iron**

| Vitamin per 5 ml | Label concentration | Found concentration | Mean n=3 | SD | Recovery % | Uncertainty per 5 ml |
|---|---|---|---|---|---|---|
| | | 49.91 | | | | |
| vitamin C mg | 50 | 54.07 | 53.00 | 2.75 | 106% | 3.30 |
| | | 55.10 | | | | |
| | | 1.1 | | | | |
| vitamin $B_1$ mg | 1 | 0.95 | 1.05 | 0.08 | 105% | 0.060 |
| | | 1.09 | | | | |
| | | 0.99 | | | | |
| vitamin $B_2$ mg | 1 | 0.95 | 0.99 | 0.04 | 99% | 0.011 |
| | | 1.02 | | | | |
| | | 4.51 | | | | |
| vitamin $B_3$ mg | 5 | 4.90 | 4.8 | 0.26 | 96% | 0.051 |
| | | 5.01 | | | | |
| | | 0.51 | | | | |
| vitamin $B_6$ mg | 0.5 | 0.46 | 0.48 | 0.03 | 96% | 0.024 |
| | | 0.48 | | | | |

Table 6 shows the found and label concentrations of vitamin C, $B_1$, $B_2$, $B_3$ and $B_6$ in multivitamins and mineral with iron syrup, it was found to be 53.00±3.30, 50, 1.05±0.06, 1, 0.99±0.01, 1, 4.80±0.05, 5 and 0.48±0.02, 0.5mg / 5 ml, respectively. The recoveries were found to be 106%, 105%, 99%, 96% and 96%, respectively.

**TABLE 7. Concentration of five water soluble vitamins in vitamins and mineral dietary Syrupy.**

| Vitamin per 5 ml | Label concentration | Found concentration | Mean n=3 | SD | Recovery % | Uncertainty per 5 ml |
|---|---|---|---|---|---|---|
| | | 1.15 | | | | |
| vitamin$B_1$ mg | 1.14 | 1.31 | 1.2 | 0.09 | 105.3% | 0.07 |
| | | 1.16 | | | | |
| | | 0.89 | | | | |
| vitamin$B_2$ mg | 0.9 | 0.77 | 0.89 | 0.12 | 98.9% | 0.01 |
| | | 1.01 | | | | |
| | | 10.95 | | | | |
| vitamin $B_3$ mg | 10 | 9.77 | 11 | 1.27 | 110% | 1.2 |
| | | 12.3 | | | | |
| | | 1.11 | | | | |
| vitamin$B_6$ mg | 1 | 0.85 | 1.05 | 0.17 | 105% | 0.06 |
| | | 1.18 | | | | |
| | | 53.26 | | | | |
| vitamin$B_9$ µg | 50 | 53.05 | 54 | 1.48 | 108% | 4.4 |
| | | 55.71 | | | | |
| | | 1.91 | | | | |
| Vitamin$B_{12}$ µg | 1.8 | 1.75 | 1.78 | 0.12 | 98.9% | 0.03 |
| | | 1.68 | | | | |

Table 7 shows the found and label concentrations of vitamin $B_1$, $B_2$, $B_3$, $B_6$, $B_9$ and $B_{12}$ in vitamins and mineral dietary supplement syrup, it was found to 1.20±0.07, 1.14 mg / 5 ml, 0.89±0.01, 0.9 mg / 5 ml, 11.00±1.2, 10 mg / 5 ml, 1.05±0.06, 1 mg / 5 ml, 54±4.4, 50 µg / 5 ml and 1.78±0.03, 1.8 µg / 5 ml, respectively. The recoveries were found to be 105.3, 98.9%, 110%, 105%, 108% and 98.9%, respectively.

**TABLE 8.Concentration of water-soluble vitamins in balanced supplemental nutrition for children 1-10 years .**

| Type of vitamin | LabelCon. per 100g | Mean Con. per 100g | SD n=3 | Recovery % | Uncertainty per 100 g |
|---|---|---|---|---|---|
| Vitamin C mg | 50.000 | 53.000 | 2.891 | 94.34 | 3.122 |
| Vitamin $B_1$mg | 1.350 | 1.32 | 0.320 | 97.78 | 0.04 |
| Vitamin $B_2$mg | 1.030 | 1.08 | 0.061 | 95.38 | 0.011 |
| Vitamin $B_3$ mg | 8.400 | 8.296 | 0.980 | 101.26 | 0. 99 |
| Vitamin $B_6$ mg | 1.300 | 1.279 | 0.040 | 101.65 | 0.03 |
| Vitamin $B_9$µg | 108.000 | 110.000 | 12.500 | 98.18 | 13 |
| Vitamin $B_{12}$ µg | 1.5000 | 1.487 | 0.043 | 100.87 | 0.015 |

Table 8 shows the found and label concentrations of vitamin C, $B_1$, $B_2$, $B_3$, $B_6$, $B_9$ and $B_{12}$ in balanced supplemental nutrition for children 1-10 years, it was found to be 53±3.112, 50, 1.32±0.04, 1.35, 1.08±0.011, 1.03, 8.3±0. 99, 8.4, 1.279±0.03, 1.3 mg / 100g,110±13, 108µg / 100g and 1.487±0.015, 105µg / 100g, respectively. The recoveries were found to be 94.34%, 97.78%, 95.38%, 101.26%, 101.65%, 98.18% and 100.87, respectively.

Table 9 shows the concentrations of vitamin C, $B_6$ and $B_9$in adult gum, it was found to be 28.9±1.5, 30 mg / gum, 0.96±0.05, 1 mg / gum and 198.9±5.51, 200 µg / gum, respectively. The recoveries were found to be 96.3, 96% and 99.45%, respectively.

**TABLE 9. Concentration of water-soluble vitamins in adult gum .**

| Type of vitamins | Label Con. per 100g | Mean Con. per 100g | SD n=3 | Recovery % | Uncertainty per 100 g |
|---|---|---|---|---|---|
| Vitamin C | 30 mg | 28.9 mg | 2.3 | 96.3 | 1.50 |
| Vitamin $B_6$ | 1 mg | 0.96 mg | 0.06 | 96 | 0.05 |
| Vitamin $B_9$ | 200 µg | 198.9 µg | 15 | 99.45 | 5.51 |

**TABLE 10. Compared with previous study [29], the present method is more sensitive and accuracy, precession method gives broad linear range with good correlation coefficients ($R^2$) .**

| Vit. | Regression Equation | | Intercept | | Slope | | Recovery | | $R^2$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pervious Work | Our Work | Pervious Work | Our Work | Pervious Work | Our Work | Pervious Work | Our Work | Pervious Work | Our Work |
| $B_1$ | Y =214.31 X-14.3 | Y =27133.61 X- 47379.71 | -14.3 | -47379.71 | 214.31 | 27133.61 | 98.5 ± 2.3 | 101.62±3.60 | 0.9992 | 1.0000 |
| $B_2$ | Y =282.51 X+2.9618 | Y=522028.84 X + 67966.82 | 2.9618 | 67966.82 | 282.51 | 522028.84 | 93.3± 3.8 | 99.64±5.04 | 0.9999 | 0.9997 |
| $B_3$ | Y =215.63 X-4.4514 | Y =54771.96 X+ 1794.07 | -4.4514 | 1794.07 | 215.63 | 54771.96 | 96.0 ± 4.3 | 99.78±2.18 | 0.9999 | 0.9999 |
| $B_6$ | Not Detected | Y =26280.89X+ 91546.91 | Not Detected | 91546.91 | Not Detected | 26180.89 | Not Detected | 100± 0.46 | Not Detected | 1.0000 |
| $B_9$ | Y =128.59 X-5.29 | Y =5779.75X+ 12473 | -5.29 | 12473 | 128.59 | 5779.75 | 86.7 ± 5.6 | 98.04±5.66 | 0.9999 | 0.9999 |
| $B_{12}$ | Y =339176.03X+ 199868.21 | Y =339176.03X+ 199868.21 | -0.3068 | 199868.21 | 58.378 | 339176.03 | 88.9 ± 5.7 | 100±3.50 | 0.9999 | 1.0000 |
| C | Y =18.163 X-0.35823 | Y =32459 X+ 13008 | 0.35823 | 13008 | 18.163 | 32459 | 88.3 ± 4.4 | 100.48±2.71 | 0.9999 | 0.9999 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| $B_1$ | 12996 | 2.5 | 0.14 % | 2.9 | 2.04 | | 12-1400 (µg/ml) |
| $B_2$ | 10816 | 1.5 | 0.17 % | 3.8 | 2.70 | | 4-175 (µg/ml) |
| $B_3$ | 2844 | 4.8 | 0.26 % | 3.5 | 1.85 | | 1.6-685 (µg/ml) |
| $B_6$ | Not Calculated 595 Not Calculated | 3.2 Not Calculated | 0.55 % | Not Detected | 3.38 | 10 – 500 µg/ l | 75-3150 |
| $B_9$ | 12100 | 7.0 | 0.11 % | 2.3 | 1.91 | | 20-5400 (µg/ml) |
| $B_{12}$ | 7744 | 2.7 | 0.15 % | 1.7 | 2.61 | | 2-350 (µg/ml) |
| C | 1156 | ------ | 0.11% | 0.9 | 1.01 | | 1.5-500 |

## Conclusion

A gradient RP-HPLC method was developed for rapid, simultaneous and simple determination of seven water-soluble vitamins ($B_1$, $B_2$, $B_3$, $B_6$, $B_9$, $B_{12}$ and C), in some nutraceutical supplements, in a single chromatographic run with high resolution and precision. This method was validated in terms of linearity, sensitivity and accuracy. Detection limits were 4.795, 2.412, 5.117, 4.921, 1.06,8.380 µg/L and 3.230 mg/L, respectively, and quantification limits of 14.532, 7.308, 15.505, 14.912, 3.225, 25.395 µg/Land 10.768, for vitamins $B_1$, $B_2$, $B_3$, $B_6$, $B_9$, $B_{12}$ and C, respectively, with good linearity and good correlation coefficients ($R^2$). Mean recoveries obtained were in the range of 98-102% which ensures the success of the method. The extraction and separation using high-performance liquid chromatography exhibited high response, sensitivity and fast separation.

## References

1. Semba, R. The discovery of the vitamins. – *NCBI. Int. J. Vitam. Nutr. Res.*, **82**(5),310-315 (2012). doi: 10.1024/0300-9831/a000124.

2. Rucker, R.; Suttie, J., McCormick, V. and Machlin, L. *Hand Book of Vitamins,* 4thed (2007). (Taylor and Francies Group, LLC).

3. Rucker, R.; Suttie, J.; McCormick, V. and Machlin, L. *Hand Book of Vitamins,* 3rd ed. (2004). (Marcel Dekker).

4. Singh, A. Chaturvedani, AK. Singh NP. and Baranawal A. Nutraceuticals: Meaning and regulatory scenario. *J. Pharma Innovation*, **7**(8), 448-451 (2018).

5. Bhowmik, D. Gopinath, H. Kumar, B. Duraivel, S. and Kumar S. Nutraceutical–A Bright Scope and Opportunity of Indian Healthcare Market. *J. Pharma Inn*, **1**(11), 29-41 (2013).

6. Buhlmann, P.; Pretsch, E. and Bakker, E. Carrier-Based Ion-Selective Electrodes and Bulk Optodes. *J. General Characteristics. Chem. Rev.*, **97**(8), 3083-3132 (1997).

7. Indyk, H., Persson, B., Caselunghe, M., Filouzi, E. and Wollard, D. Determination of vitamin $B_{12}$ in milk products and selected foods by optical biosensor protein-binding assay: method comparison, *J. AOAC Intl.*,**85**, **72**(2002).

8. Revanasiddappa, H. D., Veena, M.Facile and validated spectrophotometric methods for the determination of ascorbic acid. *Bulgarian Chemical Communications*, **40**(2), 104(2008).

9. Marszall, M.L.,Lebiedzinska, A., Czarnowski, W. and Szefer, P. High-performance liquid chromatography method for the simultaneous determination of thiamine hydrochloride, pyridoxine hydrochloride and cyanocobalamin in pharmaceutical formulations using coulometric electrochemical and ultraviolet detection. *J. Chrom. A*, **1094** (1-2), 91(2005).

10. Mimica, D., Bedioui, F. and Zagal, J.H. Reversibility of the L-cysteine/L-cystine redox process at physiological pH on graphite electrodes modified with coenzyme $B_{12}$ and vitamin $B_{12}$. *Electrochim. Acta*, **48**, 323(2002).

11. Fotsing, L.; Fillet, M. and Bechet, I. Determination of six water-soluble vitamins in a pharmaceutical formulation by capillary electrophoresis. *J. Pharm. Biomed. Anal.*, **15**, 1113 (1997).

12. Sebaiy, M. M., El-Adl S. M., Baraka M. M., Hassan A. A., Rapid RP-HPLC Method for Simultaneous Estimation of Some Antidiabetics; Metformin, Gliclazide and Glimepiride in Tablets. *Egypt. J. Chem*., **62** (3), 429-440 (2019).

13. Tahoun I. F. and Shehata A. B., Development and Validation of Simple Reversed Phase Method for Separation and Quantification of Tocopherols in Edible Oils. *Egypt. J. Chem*., **61** (3), 461-468 (2018).

14. Ramla, A. Determination of Water Soluble Vitamins [(B group: $B_1$, $B_2$, and $B_6$) by RP- HPLC. *J. Chem. Materials Res.*, **8**(10), 8 (2016).

15. Revanasiddappa, H. and Veena, M. Sensitive spectrophoto-metric method for the determination of ascorbic acid. *E. J. Chem.,* **5**, 10-15 (2008).

16. Rokayya, S., Yang, L.,Baokun, Q., Hengnan, W., Qiaozhi, Z.,Feifei, H., Ying, M., Jing, J. and Lianzhou, J. HPLC Analysis of Water-Soluble Vitamins ($B_2$, $B_3$, $B_6$, $B_{12}$, and C) and Fat-Soluble Vitamins (E, K, D, A, and β-Carotene) of Okra. *J.Chem.*, **2014**, 6 (2014). doi. org/10.1155/2014/831357.

17. Shafqat, U. Arshad, H. Javid, A. A simple and rapid HPLC method for analysis of vitamin-C in local packed juices of Pakistan. *J. Sci. Res*., **12** (8), 1085 (2012).

18. Philips, K.M. Terrago-Trani, M.T. Gebhardt, S.E. Exler, J. Patterson, K.Y. Haytowitz, D.B. Pehrsson, P.R. Holden, J.M. Stability of vitamin C in frozen raw fruit and vegetable homogenates. *J. Food Compos. Anal*., **23** (3),253(2010).

19. Chen, Z. Chen, B. Yao, S. High performance liquid chromatography / electrospray ionization

mass for simultaneous determination of taurine and ten water soluble vitamins in multivitamins tablets. *Anal. Chem. Acta*, **569**, 169 (2006).

20. Ekinci, R. and Kadakal, C. Determination of seven water-soluble vitamins in tarhana, a traditional Turkish cereal food, by high - performance liquid chromatography. *Acta Chrom*., **15**(15), 289 (2005).

21. Shehata, A. B., Rizk, M.S., Farag, A. M. and Tahoun I. F., Development of two reference materials for all trans-retinol, retinyl palmitate, α- and γ -tocopherol in milk powder and infant formula. *J. Food Drug Analysis,* **23**, 82-92 (2015).

22. Shehata, A. B., Rizk, M.S., Farag, A. M. andTahoun I. F., Certification of Three Reference Materials for α-and γ -Tocopherol in Edible Oils. MAPAN-*Journal of Metrology Society of India,* **29**(3),183–194 (2014).

23. Thomas, G. and Kristy, W. vitamin C quantification using reversed-phase ion-pairing HPLC. *Concordia College J. Analytical Chem.* **1**, 19-23 (2010).

24. Nishanta, S., Sudan, S. and Ajaya, B. Determination of Ascorbic Acid in Different Citrus Fruits of Kathmandu Valley. *J. Med. Biolog. Sci. Res.* **2** (1), 9-14 (2016).

25. Rahmatallah, R. and Neda, G. Determination of Folic Acid in Mint Vegetable, by High-Performance Liquid Chromatography. *Org. Chem.* **1**, 31-35 (2011).

26. Chan, M., Joung, H. and Won, J. Simultaneous determination of water-soluble vitamins excreted in human urine after eating an overdose of vitamin pills by a HPLC method coupled with a solid phase extraction. *Talanta*, **51**, 799-806 (2000).

27. Heudi, O. Kiline, T. Fontannaz, P. Separation of water-soluble vitamins by reversed-phase high performance liquid chromatography with ultra-violet detection: application to polyvitamined premixes. *J. Chrom.*, **1070**, 49 (2005).

28. ICH Harmonized. Tripartite Guide Line Validation analytical procedures: text and methodology Q2 (R1), *Parent Guidelines on Methodology* (2005).

29. Semahat, K., Onur, B., Filiz, A. and Huseyin, K. Simultaneous and accurate determination of water- and fat-soluble vitamins in multivitamin tablets by using an RP-HPLC method. *Quím. Nova,* **36** (7), 1044-1051 (2013).

## طريقة سريعة وفعالة لتقدير الفيتامينات الذائبة في الماء في بعض المكملات الغذائية باستخدام كروماتوجرافيا السائل عالي الأداء

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تم أعداد وتحسين الطرق التحليلية لتحديد كمية الفيتامينات القابلة للذوبان في الماء في بعض المكملات الغذائية. وقد توصلت هذة الدراسة إلىطريقهسريعة وفعالة لفصل وتقدير ستة انواع من فيتامين B بالاضافة إلى فيتامين C في المكملات الغذائية في وقت واحد باستخدام كروماتوجرافيا السائل عالي الاداء وباستخدام عمود فصل Spherisorb ODS2 (250 mm×4.6mm, 5µm)Waters عن طريق تحسين كل خطوة من خطوات طرق التحليل كالاستخلاص، وإعداد العينات، والفصل والكشف، والتحقق من صحة الطريقة المطورة وتطبيق الطريقة لتحديد إجمالي فيتامين B بالاضافة إلى فيتامين C.