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Determination of Water Soluble Vitamins [(B group: B1, B2, and B6) by RP- HPLC.

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Abstract
Vitamins are minor but essential constituents offload. They are required for the normal growth, maintenance and functioning of the human body.
Many different analytical methods has been done to determine Water Soluble Vitamins (B group: B1, B2, and B6) quantity in substances containing Water Soluble Vitamins, such as: (titration , spectrophotometer, electrochemical and chromatographic) methods .
Various analytical chromatographic conditions were tested in this search by using HPLC-RP (UV-Vis), we have reached to the following separation conditions:

1- Sorbent : C18 .
2- Moble phase : (MeOH+ H_2O + ACN + 0.1 %TFA)
(30 + 20 +50) v/ v
3- ø = 1 ml/ min .
4- λ_{max} = 254 nm

By the proposed method , we achieved a sharp symmetric peak during :

Vitamin	t_R(min)
Thiamine chloride hydrochloride, B1	3.9
Riboflavin , B2	9.3
Pyridoxine hydrochloride, B6	5.3

S =f (C) was applied in a various range depend on each vitamin, according to this concentration and liner equation we proceeded determining the quantity in each nutrition sample of the following :
Meat (fish, kidney,and liver) ,Yogurt , Beans(Kidney ,bean cowpea), Crackers (peanut, pistachio, and hazelnut), and RSD=(0.2159- 1.3600)% .
Keywords: Water Soluble Vitamins , vit B1 , vit B2, vit B6 , RP- HPLC , food sample.

1-Introduction

- This present paper describes a sensitive and simple RP-HPLC method with UV/VIS detection for determination of water soluble B-group vitamins

Vitamins are minor but essential constituents offload. They are required for the normal growth, maintenance and functioning of the human body.

1-1-Thiamine (Vitamin B1):

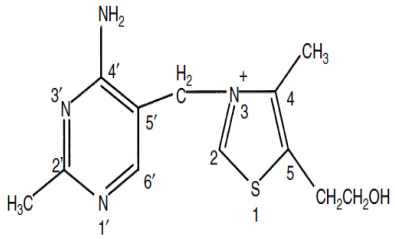


Figure (1): structure of vitamin B1

Thiamine is a key substance in carbohydrate metabolism, the requirement increases in a carbohydrate-enriched diet. The assay of trans ketolase activity in red blood cells or the extent of transketolase reactivation on addition of thiamine pyrophosphate can be used as indicators for sufficient vitamin intake in the diet [1] .Two commercially available forms of thiamine are thiamine hydrochloride and thiamine mono nitrate [2]. Thiamine hydrochloride is a colorless, crystalline powder with a yeasty odor and a salty nut-like taste. It melts at about 207°C, with decomposition. While the hydrochloride form is more soluble in water (1 g/ml) and therefore used in injectable and parenteral pharmaceuticals and food for food fortification, the mononitrate form is much less soluble (0.027 g/ml) and finds its use in dry blends, multivitamins, and dry products such as enriched flour [3]. As thiamine hydrochloride is often used as the standard reagent in vitamin B1 analysis, it is worth nothing, that this form is sparingly soluble in methanol, ethanol and glycerol and insoluble in fat solvents (ether, acetone, benzene, hexane and chloroform) [4]. Thiamine is generally stable in the dry state and can sustain high

temperatures up to 100°C [5]. Aqueous solutions of thiamine itself are acidic, and at pH below 5.0, they are even stable to autoclaving at 120oC-130oC and not susceptible to oxidation (2).

1-2-Riboflavin (Vitamin B2):

Riboflavin is the prosthetic group of Flavin enzymes, which are of great importance in general metabolism and particularly in metabolism of protein.[1]

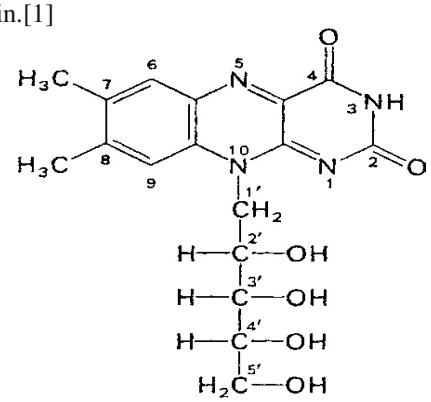


Figure (2): structure of vitamin B2

Riboflavin is an odorless orange crystalline powder with an unpleasant bitter taste and a melting point of about 280°C (14). In neutral aqueous solution, riboflavin exhibits a strong yellow-green fluorescence (15). Though classified as a water-soluble vitamin, riboflavin has a low solubility in water (10–13 mg/100 ml at 25–27.5oC; 19 mg/100 ml at40°C; 230 mg/100 ml at 100°C) [3,4]. It is sparingly soluble in absolute ethanol (4.5mg/100 ml at 27oC) and not at all in acetone, diethyl ether, or chloroform. Riboflavin solubility can be enhanced in dilute acid or alkali though it is not very stable in alkali, the presence of aromatic compounds is known to make riboflavin more soluble in aqueous solution, which is utilized in pharmaceutical preparations [7]. In contrast, FMN and FAD are much more soluble than riboflavin [4,7].

1-3-Pyridoxine (Pyridoxal, Vitamin B6):

Vitamin B6 activity is exhibited by pyridoxine or pyridoxal (R = CH2OH), pyridoxal (R = CHO) and pyridoxamine (R =CH2NH2). The metabolically active form, pyridoxal phosphate, functions as a coenzyme (cf. 2.3.2.3) of amino acid decarboxylases, aminoacid racemases, amino acid dehydrases, aminotransferases, serine palmitoyltransferase, lysyloxidase, δ-aminolevulinic acid synthase, and of enzymes of tryptophan metabolism. Furthermore, it stabilizes the conformation of phosphorylases.[1]

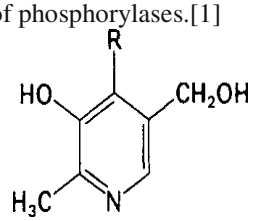


Figure (3): structure of vitamin B6

Free vitamers of B6 are commercially available as crystalline hydrochlorides is a white, odorless, crystalline powder with a slightly salty taste and a melting point of 204–206oC (with
Decomposition) [3]. It is readily soluble in water (22g/100ml), sparingly soluble in ethanol (1g/100ml) and practically insoluble in diethyl ether and chloroform. PN.HCl has pK values of 5.0 and 9.0 (25oC) and its 5% solution has the pH of 2.3–3.5 [7] .

- there are many analytical methods have been reported [8-10]for determination Water-Soluble Vitamins in its samples.

vit B1:

Spectroscopic, electrochemical, and capillary electrophoretic methodsSeveral recently published procedures using flow-injection analysis (FIA) coupled to spectrophotometric fluorescence or chemiluminescence detection are available [11, 12] Fluorescence based methods [13, 14]rely on conversion of thiamin to highly fluorescent derivativesincluding thiochrome or closely related fluorescent compounds.Electrochemical [15,16] and capillary electrophoretic [17 ,18] methods have not been extensively utilized for thiamin assay. LC has been commonly used for the analysis of thiamin. Reviews on LC methodology 80a historical perspective of method development and recent research approaches.[19 , 20] Because thiamin and riboflavin can be

Conveniently assayed concurrently or simultaneously, many methods are available to assaythe two vitamins from the same extract. These procedures are discussed [20 , 21]

vit B2 :

The spectral properties of riboflavin provide an ideal analyte for application of advanced spectroscopic methods based on fluorescence spectrometry. In addition, flow injection combined with chemiluminescence provides a good analytical approach. [22, 23] , Capillary electrophoretic methods have been effectively utilized for analysis of riboflavin, primarily from pharmaceutical products with high vitamin levels[24 , 25] LC methods for riboflavin have been studied , Specific methods for quantitation of riboflavin in foods include[26,27,28] , Various supports have been used for riboflavin chromatography C18 stationary phases are used for most commonly riboflavin analysis in foods and biological [29 ,30 ,31] .

vit B6 :

Spectral properties of vitamin B6 compounds and other hydroxypyridines were presented [32, 33, 34] recently summarized ultraviolet (UV) and fluorescence properties of the vitamin B6 group. For specifics of the spectral properties, the reader is referred to the excellent study provided [32], Spectroscopic, electrochemical, and capillary electrophoresis methods Excellent spectroscopic methods using spectrophotometric or fluorometric measurements are available for assay of vitamin B6. Usually, these procedures are applicable to pharmaceuticals.Liquid chromatography procedures are normally required for biologicals and foods owing to greater sensitivity requirements, Problems associated with the microbiological assay of vitamin B6 led to the early applicationOfLC procedures for food and other biological matrices. [35, 36] provide in-depth reviews of LC procedures for vitamin B6 analysis.

- This present paper describes a sensitive and simple RP-HPLC method with UV/VIS detection for determination of (B group: B1, B2,B6) vitamins .

2-Result and discussion:

2-1 Analytical conditions :

In this search we fix on chromatographic methods because it's our goal, (HPLC-UV) method was applied on a column C18 to analyses. RP-HPLC method was used for measurement of the concentration of (B group: B1, B2,B6) vitamins , Water-soluble vitamin standards of VB1, VB2, , and VB6 are prepared by accurately weighing 10 to 20 mg of the vitamin powder and adding 10 to 20 g of

DI water to make stock solutions of 1.0 mg/ml for each vitamin . Because of the limited solubility in water of VB2 (VB9), the concentration of stock solution of VB2 is decreased to 0.25 mg/ml in

DI water .

Table (1) : The optimum chromatographic conditions we have achieved were :

1- Sorbent : C18 .

2- Moble phase : (MeOH+ H_2O + ACN + 0.1 %TFA)
(30 + 20 +50) v/ v

3- ø = 1 ml/ min .

4- λ_{max} = 254 nm

MeOH:Methanol ,ACN : Acetonitrile , TFA : trimethylamine

By using above conditions , the peak of each vitamin (B1, B2,and B6) show in figures (4, 5,6).

From the figures, we found that the t_R of each studied vitamins are separate, table (2).

Table (2) : the amount of t_R for the studied vitamins in separate conditions as : C 18 ; Moble phase : (MeOH+ H_2O + ACN + 0.1 %TFA) (30 + 20 +50) v/ v ; ø = 1 ml/ min ; and λ_{max} = 254 nm .

Vitamin	t_R(min)
Thiamine chloride hydrochloride, B1	3.9
Riboflavin , B2	9.3
Pyridoxine hydrochloride, B6	5.3

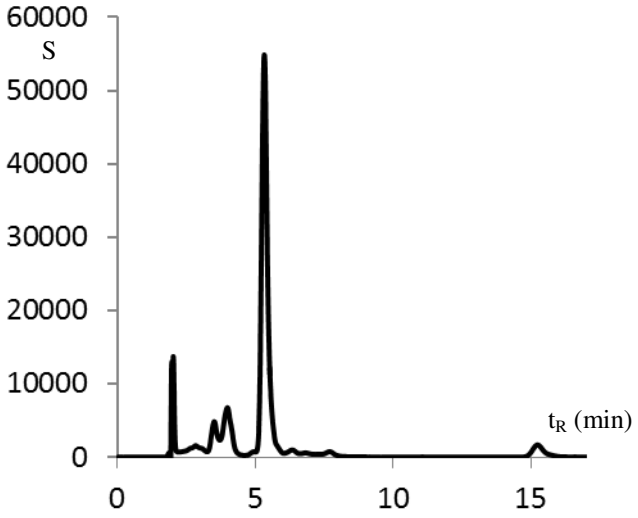


Figure (4) : Chromatographic peak of vitB6 , Sorbent : C18 , Moble phase : (MeOH+ H_2O + ACN + 0.1 %TFA) (30 + 20 +50) v/ v , ø = 1 ml/ min , λ_{max} = 254 nm .

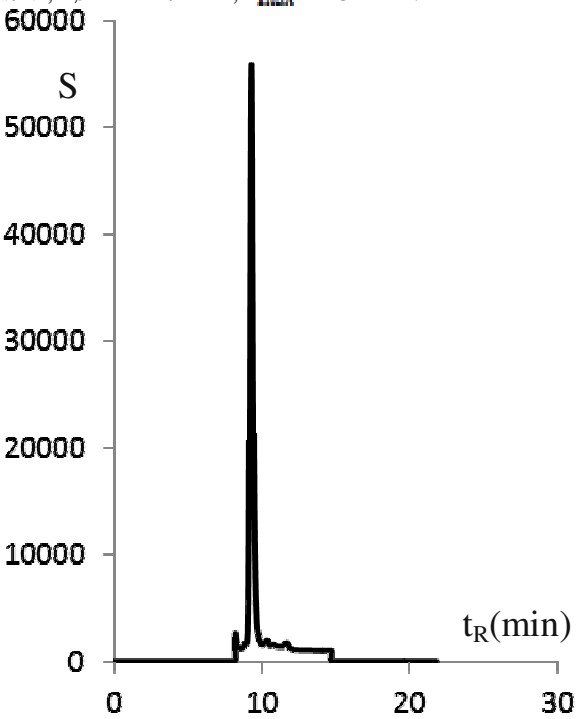


Figure (5): Chromatographic peak of vitB2 , Sorbent: C18 , Moble phase : (MeOH+ H_2O + ACN + 0.1 %TFA) (30 +20+50) v/ v , ø = 1 ml/ min ,λ_{max} = 254 nm .

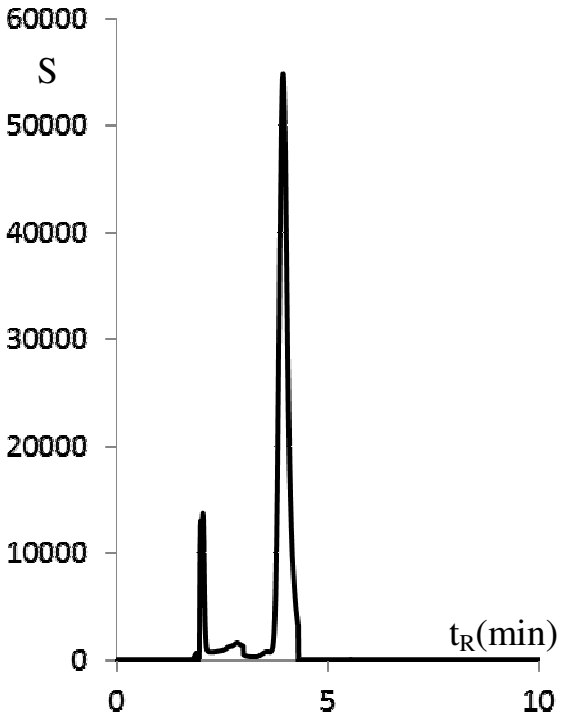
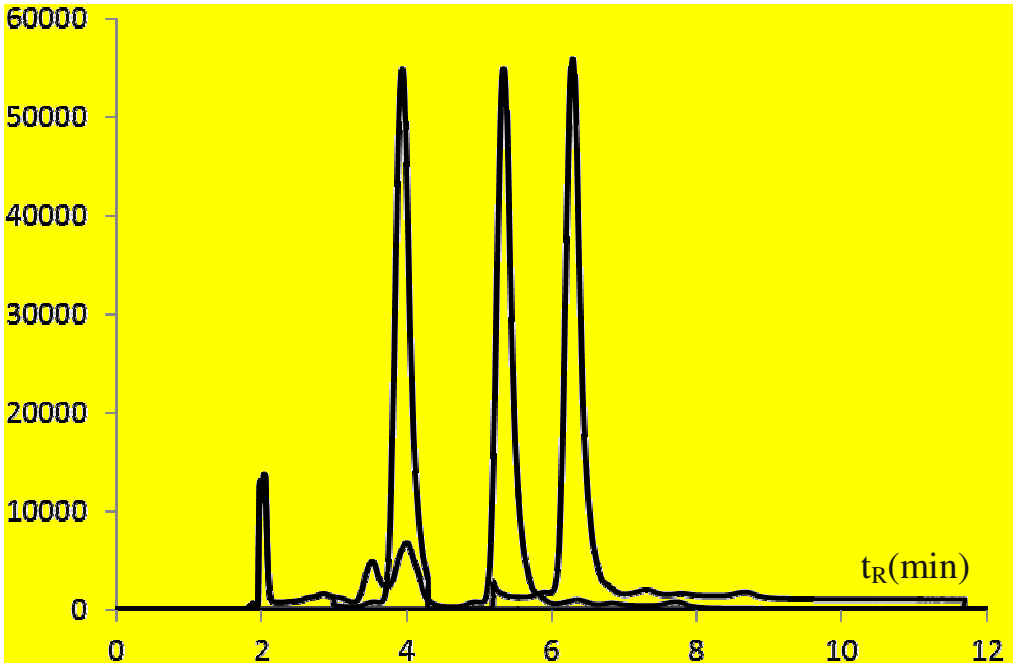


Figure (6): Chromatographic peak of vitB1 , in optimum conditions
Sorbent:C18, Moble phase: (MeOH+ H_2O + ACN + 0.1 %TFA)
(30 + 20 +50) v/ v, ø = 1 ml/ min, λ_{max} = 254 nm.



B1 B6 B2

Figure (7) : Chromatographic peaks of common solution (B group: B,B2,B6) , inseparate conditions as : C 18 ; Moble phase : (MeOH+ H_2O + ACN + 0.1 %TFA) (30 + 20 +50) v/ v ; ø = 1 ml/ min ; and λ_{max} = 254 nm.

We can see from fig. (7) That we can separate the common solution of (B group: B1, B2,B6) by using the optimum conditions , summarized in table (1) , that is the target of our search .

2-2 Study of slandered solutions, S= f(C):

Five common standers solution vit (B group: B1, B2,B6) were prepared and injected on C18 column and the analysis carried out by elution with (MeOH+ H_2O + ACN + 0.1 %TFA) , (30 + 20 +50) v/ v , λ_{max}= 254 nm , The liner relation between peak surface and the vit concentration , we achieved in the wide range : B1(1-50) ;B2(1-40) ;and B6(0.5-30) mg/ L. As show in figures (8, 9, and 10).

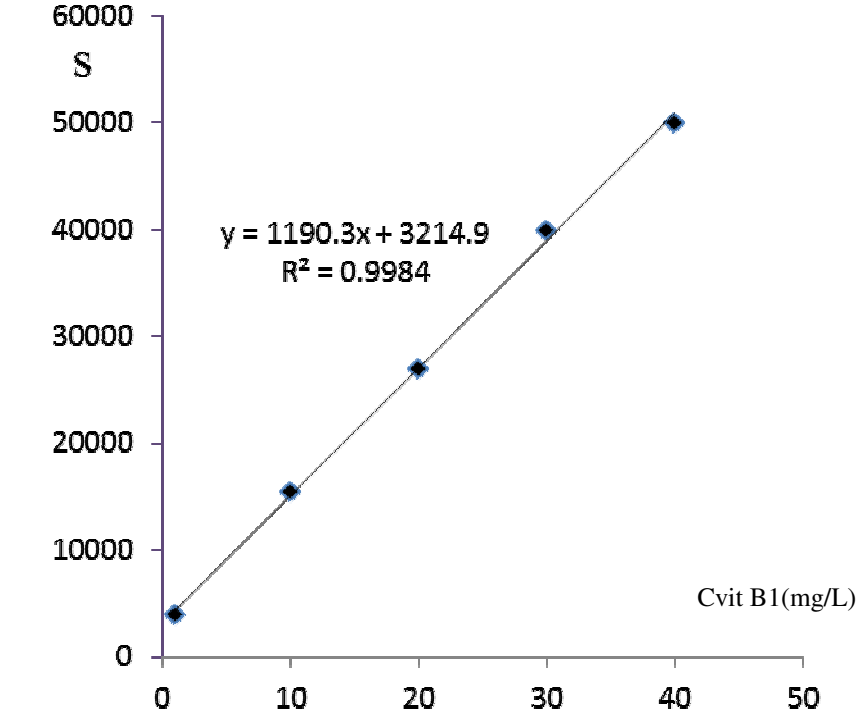


Figure (8) : the linear range between surface peaks and vit B1 concentration ,in separate conditions as : C 18 ; Moble phase : (MeOH+ H_2O + ACN + 0.1 %TFA) (30 + 20 +50) v/ v ; ø = 1 ml/ min ; andλ_{max} = 254 nm .

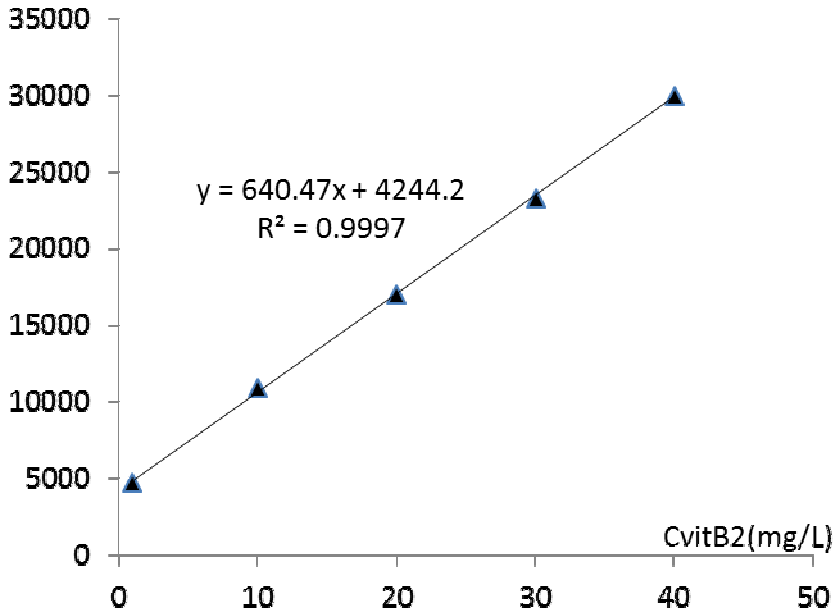


Figure (9) : the linear range between surface peaks and vit B2 concentration in separate conditions as : C 18 ; Moble phase : (MeOH+ H_2O + ACN + 0.1 %TFA) (30 + 20 +50) v/ v ; ø = 1 ml/ min ; andλ_{max} = 254 nm

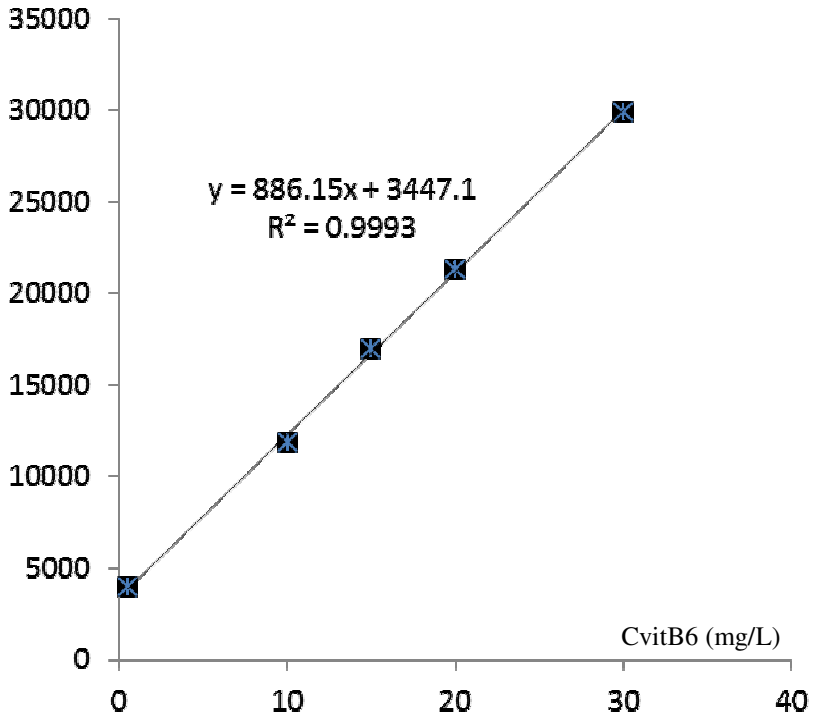


Figure (10) : the linear range between surface peaks and vit B6 concentration in separate conditions as : C 18 ; Moble phase : (MeOH+ H_2O + ACN + 0.1 %TFA) (30 + 20 +50) v/ v ; ø = 1 ml/ min ; and λ_{max} = 254 nm.

2-3-preparation of experimental (B group: B1, B2,B6)samples :

To be sure about the accuracy and precision of our proposed chromatographic method , the proposed method was applied on experimental common solution (B group: B1, B2,B6) samples, for that 3 slandered solutions were prepared, their concentrations include in the linear rang which we obtained above, and each concentration was repeated 3 times, then we have done some statistic study, table (3).

Table(3) : determination experimental vitamins (B group : B1,B2,B6) samples using HPLC-RP , stationary phase C18; Moble phase : (MeOH+ H_2O + ACN + 0.1 %TFA) (30 + 20 +50) v/ v ; ø = 1 ml/ min ; and λ_{max} = 254nm , (n=3 , α=0.95) .

Studied vitamin	Taken concentration mg /L	Found concentration $\bar{X} \mp \Delta X$ mg/ L	RSD%	Recovery %
B1	5.000	5.3082±0.028	0.7413	102.7
	25.000	25.4969± 0.016	1.3600	99.3
	35.000	35.7054±0.0167	0.9560	100.7
B2	5.000	5.3082±0.028	0.9613	102.7
	25.000	25.4969± 0.016	1.3600	99.3
	35.000	35.7054±0.0167	0.9560	100.7
B6	5.000	5.3082±0.028	1.0413	102.7
	20.000	20.4969± 0.016	1.3600	99.3
	25.000	25.7054±0.0167	0.9560	100.7

2-4- Natural samples :

The proposed method was applied in natural samples, Meat (fish, kidney,and liver) ,Yogurt , Beans(Kidney ,bean cowpea) , Crackers (peanut, pistachio, and hazelnut) as follows :

1- 50 gr from each sample mixed in mixer until a homogeneous crushed has been achieved
2- Added to homogeneous crushed sample: 0.1 N HCl for 30 min at 121°C , a transparent solution achieved, evaporated then added (MeOH+ H_2O + ACN) On ultra sound for 30 min until a homogeneous solution achieved , then centrifuged for 10 min .
3- isolated by two steps: first with ash less paper , second by special filter for HPLC .

4- Diluted to 100 ml by water for HPLC [these solution is : mother sample solution]

Each mother sample solution was diluted according to vitamin concentration by recording analytical signal (peak vitamin surface) at same time retention time for each vitamin (t_R), calculation the vitamin concentration in each sample was applied parallel by stander solution at same time, result in table(4) .

Table(4) : determination of (Bgroup : B1,B2,B6) samples using HPLC-RP, stationary phase C18;
Moble phase : (MeOH+H_2O + ACN + 0.1 %TFA) (30 + 20 +50) v/ v ;
ø = 1 ml/ min ; and λ_{max} = 254 nm , (n=3 , α=0.95)

Sample		Vitamin	Found concentration $\overline{X} \mp \Delta X$ mg/100 gr	RSD%
Meat	Fish	B1	0.2134± 0.016	0.7413
		B2	0.40± 0.117	0.5289
		B6	0.23±0.082	0.9560
	Kidney	B1	0.12±0.014	0.6613
		B2	0.38±0.124	1.3600
		B6	0.29±0.074	0.6560
	Liver	B1	0.15±0.036	1.0413
		B2	0.72±0.185	1.3600
		B6	0.76±0.098	0.9860
Yogurt		B1	0.10±0.014	0.2159
		B2	0.35±0.016	0.6987
		B6	0.32±0.183	0.5984
Beans	Kidney bean	B1	0.28±0.017	0.5989
		B2	0.21±0.013	0.6012
		B6	0.84±0.189	1.2126
	cowpea	B1	0.43±0.101	0.6412
		B2	0.18±0.075	0.6759
		B6	0.35±0.157	0.6021
Crackers	peanut	B1	0.23±0.021	0.5354
		B2	0.28±0.037	0.6211
		B6	0.30±0.112	0.6652
	pistachio	B1	0.27±0.015	0.5479
		B2	0.26±0.097	0.6912
		B6	0.29±0.078	0.7212
	hazelnut	B1	0.21±0.018	0.5221
		B2	0.24±0.014	0.6338
		B6	0.16±0.017	0.5032

From the table we concluded: a rapid and sensitive high performed liquid chromatography proposed method was carried out, accordingly we can determined (Bgroup : B1,B2,B6) in the wide range of samples , the method has a repeatedly and accuracy, we can observe from the lower RSD; and a high precision which can observed from the recovery, Accuracy is the degree of agreement between test results and true values. The precision of this method is the degree of agreement among individual test results when an analysis is applied repeatedly to multiple samplings. Precision is measured by injecting a series of standards and then calculating the relative standard deviation of retention times and areas or peak heights. Precision may be measured at three levels: repeatability, intermediate precision, and reproducibility.. Repeatability off low rates, gradient formation, and injection volumes can affect precision, as can response stability of the detector, aging of the column, and temperature stability of the column oven. The equipment should be inspected on a regular basis using the test methods recommended by the supplier to ensure reliability, high performance, and good analytical results.

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