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Preparation of New HPLC Stationary Phase and Study of Its Chromatographic Performance Toward the Separation of Some Water-Soluble Vitamins

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Abstract

A new stationary phase was prepared by the reaction of a crystal violet solution with silica gel. The capacity of the new prepared resin was calculated and the found average was 6.6meq./g. The resulted resin was highly rigid with high stability and used as stationary phase for HPLC column. Some water-soluble vitamin C, B_2, B_6, and B_{12} were examined by this column with isocratic eluention distilled water and methanol (2:98, (v/v)) as a mobile phase with flow rate of 0.5 ml/min and UV detection of 230 nm. The chromatographic performance of the packed column was characterized. The number of plate numbers (N), height equivalent of a theoretical plates (H), capacity factors (K′), selectivity factors (α), peaks asymmetry and Resolution (Rs) were measured by analyzing different analytes on the new columns using different mobile phase compositions and flow rates.

1. Introduction

Vitamins are essential for the normal growth and function of human and animal bodies. These compounds can be classified in two main groups: water-soluble and fat-soluble vitamins. Water-soluble vitamins include B_1, B_2, B_6, B_{12}, C, and B_5 and so on. They play different specific and vital functions in metabolism, and their lack or excess produces specific diseases. Food is the main resource of vitamins for human and animals. However, loss of vitamins, especially, water-soluble vitamins often occurs in the inappropriate processing and storage of food. Therefore, it is necessary to develop efficient analytical methods for the determination of vitamins for the quality control of food and relative products. In the past decades, traditional analytical methods including different physical, chemical and biological methods were used to analyze each vitamin, which were sometimes tedious and time-consuming. These methods are mainly microbiological procedures spectrophotometric, fluorimetric, electrochemical methods and thin-layer [1–3]. Recently a great progress has been achieved in rapid and specific methods for vitamin analysis. A lot of papers have been published concerning the separation and quantification of vitamins in a wide range of products, such as rice, milk, eggs, oral liquid tonics, and multi-vitamin formulation by more simple methodologies [4–7]. A choice of HPLC methods for water-soluble vitamins can be made: reversed-phase chromatography (RPC), ion-exchange chromatography (IEC), ion-pairing chromatography (IPC) [8-10], and normal-phase chromatography (NPC). Because of its simplicity and better column performance, RPC with RP-C18 as stationary phase is usually the best starting point. Among available RPC methods for water-soluble vitamins, individual vitamins, two or three vitamins can be chromatographed isocratically; the simultaneous chromatography of more complicate mixtures, in general, requires a gradient elution program involving complex buffer mobile phases or ion pairing reagents. Several detection methods an be applied, such as UV-Vis absorbance with variable wavelength, photodiode array, fluorimetric, or electrochemical. In contrast, the stationary phases used in the separation of water-soluble vitamins were almost uniform RP-C18 besides a few cyclodextrin packings. Though a lot of work has been done for quantitative analysis of the vitamins, the retention mechanism of these compounds still requires further to be understood, especially, on different stationary phases [11].

The packing materials have been developed in which the stationary phase is chemically bonded to an insoluble matrix (solid support). BPC involved a relatively non-polar stationary phase used in conjugation with polar mobile phase to separate a wide variety of less polar solutes [12]. Panfili et al. [13] carried out a similar study for the

determination of Tocopherols and Tocotrienols in Cereals. They monitored after every eight injections of Tocopherols and Tocotrienols into column of silica that the column was reactivated with a solution of 10% isopropyl alcohol in *n*-hexane as a mobile phase. Bhandare et al. [14] determination of arginine, lysine and histidine in drug substance and drug product by using normal phase column.

2. Experimental Part

2.1. Equipments:

High performance liquid chromatography used in this work was CECIL (Cambridge, England), UV-Visible detector model CE-1200, and injector equipped with 50µl sample loop. The HPLC system has been interfaced with Integrator model CE-1120, Sonicator Sonerex model Super PK 103H Mandolin (Germany), Shimadzu Fourier transforms infrared model FTIR 8300 (Kyoto, Japan) was used to measure the IR spectra for the prepared, Glass combination electrode was used to measure the pH of solutions (Germany) and Blank stainless steel columns of dimensions 15 cm ×0.4mm ID, was obtained locally.

2.2. Preparation of the Stationary Phase:

Four grams of Silica gel was first rinsed with 100ml deionized water and kept for about 24 hours so as to swell. A 10 ml of 1.5×10^{-3}M crystal violet was then added with stirring. The color of the silica was changed from white to deep violet. The content was led to settle for a period of the time approximately 24 hours. The solution was then decanted and discarded. The silica was washed many times with deionized water, then rinsed with ethanol and dried in the oven at 80 Cº over night and kept in a decicator for the future work. The FTIR spectrum for the resulting silica gel–crystal violet, crystal violet and silica gel is shown in Fig. (1).

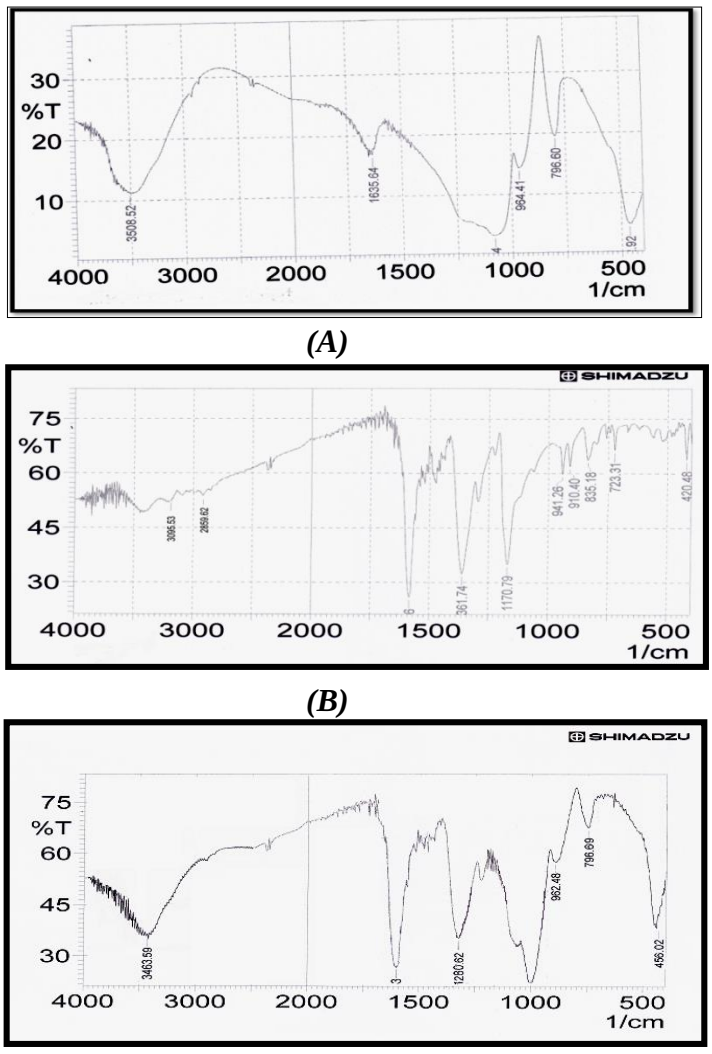


Fig. (1) FTIR Spectrum for (A) Silica Gel (B) Crystal Violet (C) Silica Gel-Crystal Violet.

2.3. Preparation of Standard:

A stock solution of 200 ppm of the standard vitamins was prepared by dissolving 5mg of each of vitamin C and diluted to 25 ml in methanol. The solvent used to prepare these solutions was usually the same as in the mobile phase employed for their separation.

Also vitamin B_2, B_6 and B_{12}were prepared by the same way by subsequent dilution of stock solution

Mixture of two or more of the above analytes was also prepared by mixing the appropriate volumes of the stock solutions. The solvent used to prepare these solutions before injection into HPLC was usually used as in case of the mobile phase employed for their separation.

3. Results and Discussion

Stationary phase in this work was done via adsorption of Crystal Violet on silica Gel resin which was hard and rigid. The resin was identified by FTIR in which the appearance of absorption band was different. The solubility has been examined using different solvents such as acetonitrile, benzene, chloroform, dioxane, DMF, DMSO, hexane, methanol and water. It was found that the polymer was insoluble and undecomposed in all of these solvents and was very stable. Column packing was done by using the slurry formed by mixing 4gm of resin powder with 15 ml of deionized water and homogenized in an ultrasonic bath and placed in the slurry reservoir, the column was packed using down-flow packing system. The study was carried out for the analysis of vitamins, Vitamin C, B_2, B_6 and B_{12} by using the silica gel-crystal violet column (15 x 0.4 cm). The retention times of vitamins gave a good sharp peak using change present of distilled water. Each vitamin injected alone as shown in Fig. (2) Then a mixture of these compounds was prepared and injected as shown in Fig. (3 and 4). The capacity factor **Ќ** for vitamins compounds chromatographed on the silica gel-crystal violet column were ranged from 1.58-1.91 in 2% and 2.67-6.31 at 8% as shown in Fig (5) and listed in Table (1). These indicated that there was a good competitive interaction between these compounds, stationary phase and mobile phase in both cases. The Retention time, number of plate numbers (N), height equivalent of a theoretical plates (H), capacity factors (K′), selectivity factors (α), peaks asymmetry and Resolution (Rs) were calculated and listed in Table (2).

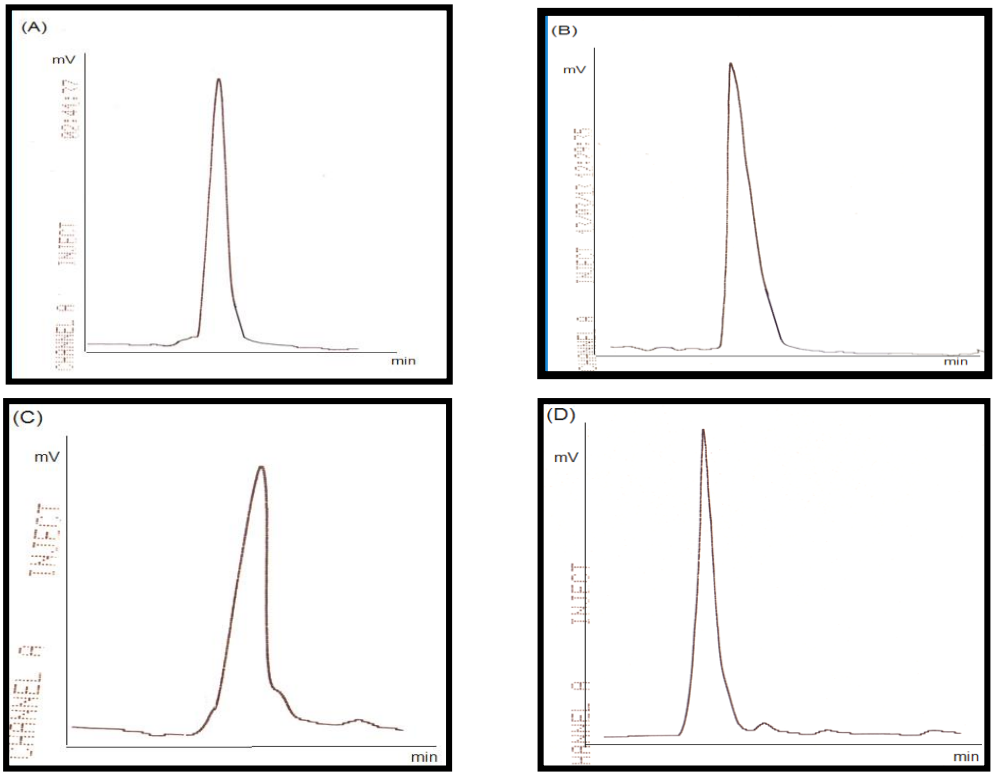


Fig. (2) Chromatograms of vitamins (A) vitamin B_6 25 ppm , (B) vitamin B_{12} 25 ppm, (C) vitamin B_2 25ppm ,(D) vitamin C 25 ppm . Using silica gel–crystal violet column (15 x 0.4 cm (i.d.)), detection wavelength 230nm, distilled water and methanol (2:98, (v/v)) as the mobile phase.

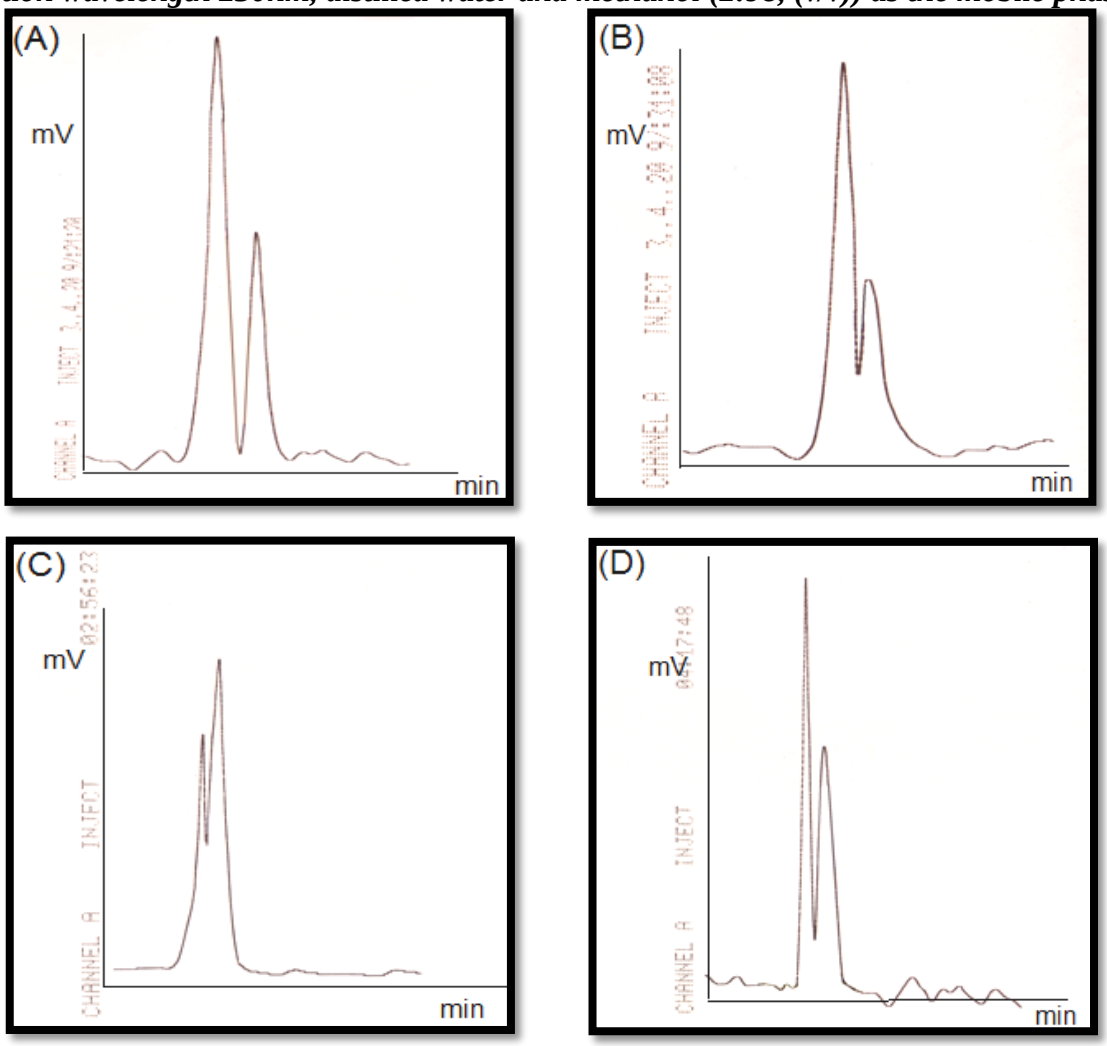


Fig. (3) Chromatograms of vitamins (A) vit.C 100ppm & vit.B_6 100ppm, (B) vit.C 5ppm & vit.B_2 50ppm, (C) vit.B_{12} 50ppm &vit. B_6 50ppm and (D) vit.B_2 50ppm &vit. B_6 50ppm. Using silica gel-crystal violet column (15 x 0.4 cm (i.d.)), detection wavelength 230nm, distilled water and methanol (2:98, (v/v)) as the mobile phase.

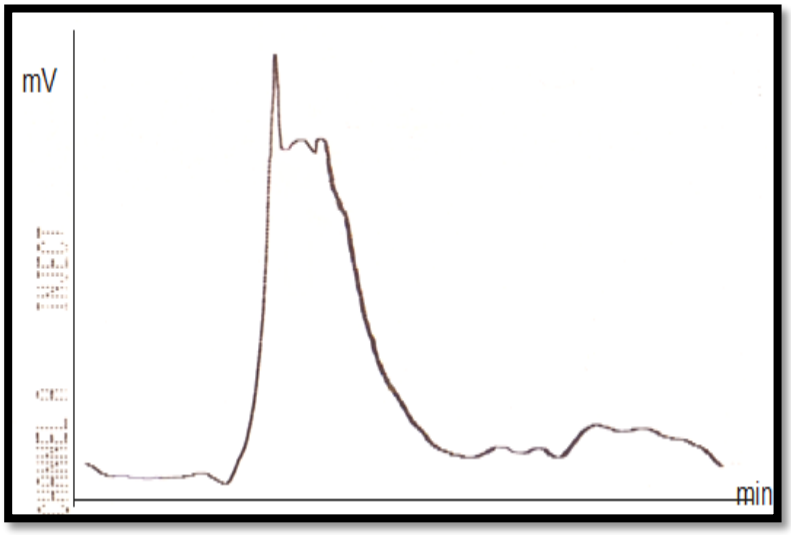


Fig. (4) Chromatograms of mixture vitamins (1) C 30 ppm, (2) B_2 30 ppm ,(C) B_{12} 30 ppm , (2) vitamin B_6 30 ppm. Using silica gel- crystal violet column (15 x 0.4 cm (i.d.)), detection wavelength 230nm, flow rate 0.5ml/min, distilled water and methanol (2:98, (v/v)) as mobile phase.

Table (2)

N, t_R , K′, α, H, Rs and Peaks Asymmetry of vitamins at 98% methanol -2% distilled water as a mobile phase, silica gel- crystal violet column (15× 0.4 cm (Id.)).

Name of compound	*Retention Time t_R*	*Plate number N*	*Capacity factor K′*	*Separation Factor (α)*	*H (cm)*	*Resolution factor Rs*	*Peaks Asymmetry*
Vit C	3.23	351.77	1.58	_	0.042	_	1.1
Vit B_{12}	3.58	277.77	1.86	1.17	0.054	2.1	1.02
Vit B_2	3.75	253.16	2.00	1.07	0.059	2.2	0.99
Vit B_6	4.22	206.08	2.37	1.18	0.072	1.4	1.18

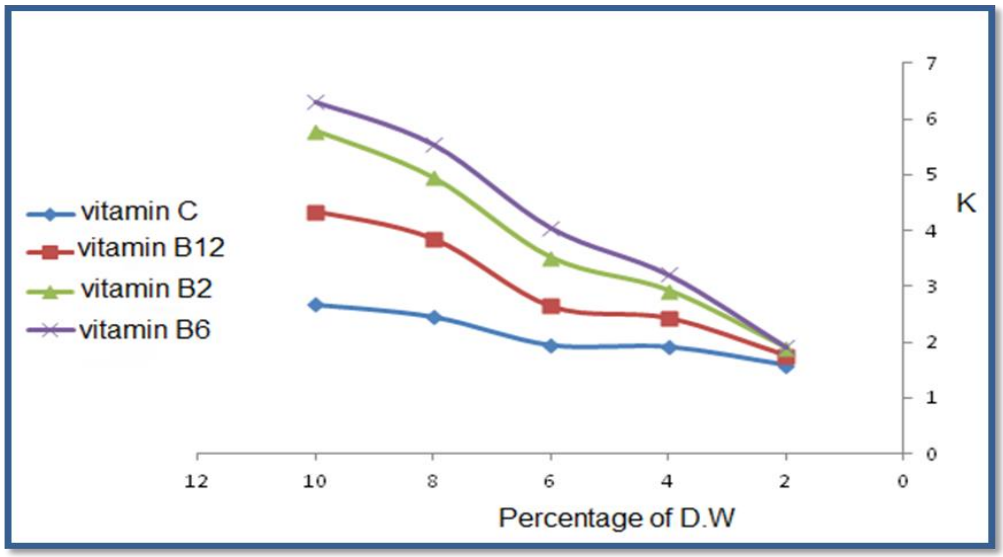


Fig. (5) Capacity factor, versus present of distilled water, using silica gel–crystal violet column (15×0.4 cm (id)), flow rate 0.5ml/min, detection wavelength 230nm and 10ppm of vitamin C, B_2, B_6 and B_{12}.

Table (1)

Capacity factor Ḱ and separation factors α variation with changing the composition of mobile phase for vitamins using silica gel–crystal violet column (15×0.4 cm (id)).

Vitamins	*Percentage of D.W. in mobile phase*									
	2%		*4%*		*6%*		*8%*		*10%*	
	Ḱ	**α**	**Ḱ**	**α**	**Ḱ**	**α**	**Ḱ**	**α**	**Ḱ**	**α**
C	1.58	--	1.91	--	1.94	--	2.45	--	2.55	--
B_2	1.76	1.11	2.43	1.27	2.64	1.36	3.85	1.57	3.91	1.59

B12	1.89	1.07	2.92	1.2	3.51	1.32	4.94	1.28	4.51	1.32
B6	1.91	1.01	3.21	1.09	4.05	1.15	5.55	1.12	5.56	1.22

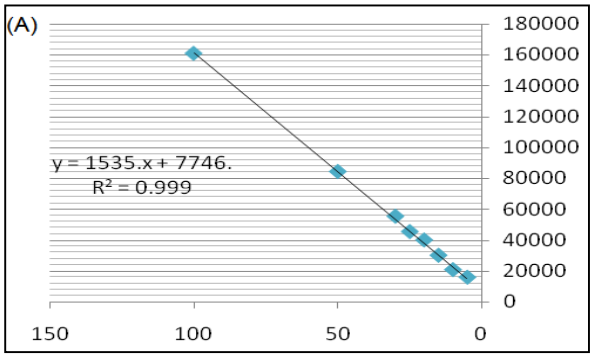
--Not detected.

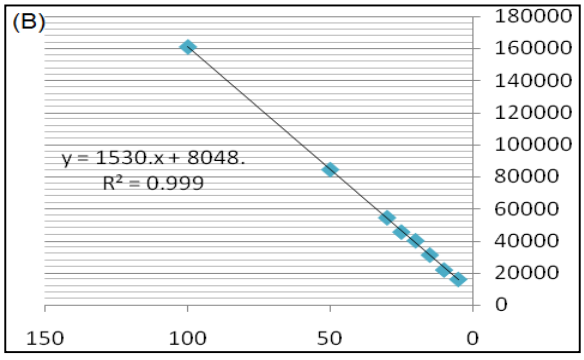
Separation factor **α** was ranged from 1.01-1.11, and 1.09-1.62 at 2% and 8% distilled water (the mobile phase) respectively.

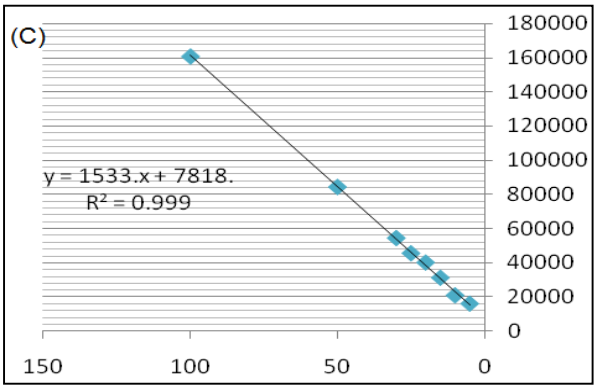
At 2% distilled water gave good separation factors, however, it may be possible to achieve a better separation as it is showed in Fig.(1). These results are summarized in Table (1).

The chromatograms of vitamin C, B_2, B_6 and B_{12} with the silica gel–crystal violet column, using 50 µl sample loop, with flow rate 0.5 ml/min are shown in Fig.(3-6). The mobile phase was a mixture of 2% distilled water and 98% methanol. These are analyzed in to vitamins and had given well shaped peaks as well as good detector responses. As it is shown in Fig.(3), the more polar vitamin B_6 has a retention time at 4.22 min and the non polar vitamin C has lower retention time at 3.23 min. This mean that the mechanism of interaction between vitamins and stationary phase depended on the polarity, and because of the appearance of non polar the stationary phase was relatively polar and the interaction depend on the hydrogen bonding of N-H and O-H groups of vitamins with O-H groups of silica gel–crystal violet of polymeric Column.

Calibration curves of vitamins compounds on silica gel–crystal violet resin column, in 2% distilled water-98% MeOH as mobile phase are shown in Fig.(4). A linear dependence of the peak areas on the amount injected is an evident for all compounds down to the detection limits. Vitamins showed a linear response range extending from at least 100 ppm to the detection limit which was as low as 1 ppm for most analytes. The slope values for the linear calibration curves were ranged from (1525.8- 1535.9) depending upon the kind of vitamin as shown as Fig. (6), the slope values are listed in Table (3). The correlation coefficients for all calibration lines were ranged from (0.9992- 0.9997) with an average value (0.9994). The detection limits of the vitamins are also listed in Table (3). It ranged from (1- 3) ppm for all vitamins in 2% distilled water-98% MeOH. These detection limits were calculated at S / N ratio of 3 or more.







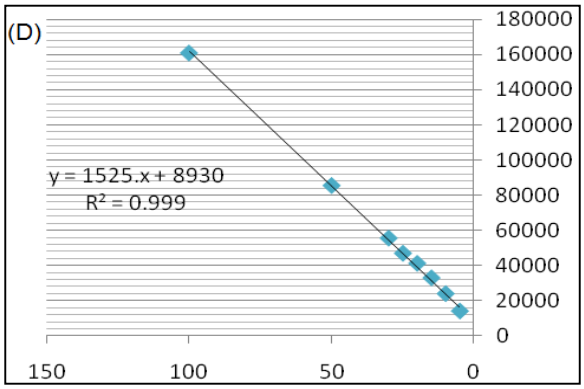


Fig. (6) Calibration curve for (A) vitamin C, (B) vitamin B_2, (C) vitamin B_{12}, (D) vitamin B_6. Samples were analyzed Using silica gel – crystal violet column (15 x 0.4 cm (i.d.)), detection wavelength 230nm, flow rate 0.5 ml/min, and using distilled water and methanol (2:98, (v/v)) as mobile phase.

Table (3)
linear equation , correlation coefficients R , and detection limits , samples were analyzed Using silica gel – crystal violet column (15 x 0.4 cm (i.d.)) ,detection wavelength 230nm , flow rate 0.5 ml/min , and using distilled water and methanol (2:98, (v/v)) as mobile phase.

Compounds	*Linear Equation*	*R*	*Detection Limit (ppm)*
Vitamin C	y = 1533.6x + 7818.4	0.9994	2
Vitamin B_2	y = 1535.9x + 7746.5	0.9993	3
Vitamin B_{12}	y = 1525.8x + 8930	0.9992	2
Vitamin B_6	y = 1530.3x + 8048.3	0.9997	1

The prepared standard mixtures solutions of the analyzed vitamins were injected for at least 3 times under the same conditions and their concentration were calculated by measuring the peak area of each vitamin and using their respective the linear equation. The recovery were ranged from 96.44% to 97.96% with an average of 97.17%, the relative errors were ranged from (-2.04_-3.56) % with an average of -2.83% as listed in Table (4).

Table (4)
% R.S.D, %Recovery, and % Relative Error for the Analyzed vitamins.

vitamins	*Conc. injected (ppm)*	*Average Conc. (calculated)* (ppm)*	*Absolute Error*	*Relative Error %*	*Recovery%*	*%R.S.D*
Vit. C	25	24.20	-0.8	-3.2	96.8	0.025
Vit. B_2	25	24.37	-0.62	-2.52	97.48	0.04
Vit. B_{12}	25	24.11	-0.89	-3.56	96.44	0.065
Vit. B_6	25	24.49	-0.51	-2.04	97.96	0.065

****using the linear equation for each vitamins and average of three concentration.***

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الخلاصة

تم تحضير طور ثابت جديد بطريقة كروموتوكرافيا السائل ذات الاداء العالي من تفاعل صبغة البنفسجي مع السليكا جيل. وقد تم حساب سعة الراتنج الجديد وقد وجدت 6.6 meq/g. درست استقرارية المجموعة الفعالة الجديدة المرتبطة مع مذيبات مختلفة مثل الميثانول, الايثانول, البنزين ,الكلوروفورم وغيرها من المذيبات وقد وجد أن الراتنج الجديد مستقر ولم يلاحظ أي استنفاذ أو إفراغ. تم تحليل بعض الفيتامينات بواسطة هذا العمود مثل (C,B_2,B_6 and B_{12}) باستخدام طورمتحرك يتكون من 2% ماء مقطر و98 % ميثانول وسرعة جريان 0.5 مل بالدقيقه عند طول موجي 230 nm باستخدام كاشف الاشعه فوق البنفسجيه.بعدها تم قياس كفاءة العمود المعبأ بحساب عدد

الصفيحات النظرية ,الارتفاع المكافئ للصفيحات النظرية , عوامل الاستيعاب وعوامل الانتقائية بواسطة تحليل محاليل مختلفة على العمود الجديد باستخدام أطوار متحركة مختلفة النسب وسرع جريان مختلفة.