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College of Science
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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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By
Saif Saad Abdull.Sattar
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نَرْفَعُ دَرَجَاتٍ مَّن نَّشَاء وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ

صدق الله العظيم

سورة يوسف (٧٦)

Supervisor certification

We certify that this thesis was prepared under our Supervision in the Department of Chemistry, College of Science, and Al-Nahrain University as partial requirements for the degree of master in chemistry.

Assistant Professor
Dr. Shahbaz A. Maki

Assistant Professor
Dr. Noor Mustafa Ali

In view of the available recommendation, I forward this thesis for debate by the Examining Committee.

Assistant Professor
Dr. Hilal S.Wahab
Head
Department of Chemistry
College of Science
Al-Nahrain University

Examining Committee's Certification

We the examining committee certifies that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted us a thesis for the degree of Master of Science in chemistry.

Signature:
Name: Dr. Sarmad B. Dikran
Title: Professor
(Chairman)
Date:

Signature:
Name: Dr. Khaleda H. Alsaidi
Title: Assistant Professor
(Member)
Date:

Signature:
Name: Dr.Fadhil M.Abid
Title: Professor
(Member)
Date:

Signature:
Name: Dr. Shahbaz A. Maki
Title: Assistant Professor
(Member/ Advisor)
Date:

Signature:
Name: Dr. Noor M. Ali
Title: Assistant Professor
(Member/ Advisor)
Date:

Approved for the College of Graduate Studies

Assistant Professor

Prof. Dr.Khulood Whyeb Abood

Dean of College of

Science Al-Nahrain University

الإهداء

إلى مثلي الأعلى ..... وقدوتي في الحياة .....إلى نبع المحبة الصافي .....إلى معنى العطاء والتضحية .....

والدي الحبيب

إلى رمز التفاني والإخلاص ...إلى من حملت المشاعل في درب الأجيال .....إلى من سعت وضحت في تعليمي وتربيتي .....إلى فيض الحنان الدافق .....إلى من أوقدت سنين عمرها لنكون.....

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إلى من شرفني الله بوجودهم .....إلى سندي ومشجعي بالشدائد ..... إلى مهد طفولتي ...ومرح شبابي....وأحباب قلبي.....

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سيف

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Praise is to God the Lord of the worlds and peace and blessings be upon the master of humankind Muhammad and his pure Progeny and his relatives and may God curse their enemies until the Day of Judgment.

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Summary

New stationary phase for high performance liquid chromatography has been prepared by the interaction of crystal violet solution with silica gel. The stability of the new attached functional group on the new stationary phase was studied against different solvents such as benzene, ethanol, acetonitrile, acetone, methanol, hexane, chloroform, and petroleum ether as well as NaOH and HCl solutions. The new prepared stationary phase was found stable and no depletion of the crystal violet moiety from the stationary phase was noticed with the above materials .The FTIR analysis has configured the attachment of crystal violet on the stationary phase as well as the physical appearance of the new stationary phase.

The new stationary phase has been packed into stainless steel column. The chromatographic performance of the packed column was characterized. The number of plate numbers N, height equivalent of a theoretical plates H, capacity factors Ḱ, selectivity factors α, and peaks asymmetry, and Resolution Rs were measured by analyzing different analytes on the new columns using different mobile phase compositions and flow rates.

These parameters were measured from analysis of some water-soluble vitamins such as vitamin C, vitamin B_2, vitamin B_6 and vitamin B_{12}. The analysis of vitamins using silica gel-crystal violet column has performed using dichloromethane and hexane (5:95, (v/v)) as the mobile phase.

The average value of plate numbers N was (356.4). The plate heights H was averaged (0.042) cm, capacity factors Ḱ were ranged from (1.83–4.96). Separation factors α were ranged from (1.27-1.63), and peak asymmetry value were range from (0.99-1.18). Resolution (Rs) were ranged from (0.86-1.56).

R.S.D were ranged from (0.025-0.065) %, recovery were ranged from (96.44-97.96) %, the relative errors were ranged from (-2.04_-3.56) %.

Calibration curves for all analyzed compound were linear from their detection limits to at least 50 ppm with correlation coefficient ranged from (0.9992- 0.9997).

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List of Abbreviations

Abbreviation	Translation
BPC	Bonded-phase column
CAFF	Caffeine
CPHX	Chlorophenoxamine
CTHE	8-Chlorotheophylline
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DCM	Dichloromethane
D.W	distilled water
FTIR	Fourier Transforms Infrared
GC	Gas chromatography
HPLC	High performance Liquid Chromatography
HETP	High Equivalent Theoretical Plate
i.d	inner diameter
K	Distribution coefficient
Ќ	Capacity factor
LC	Liquid Chromatography
meq.wt	Mille equivalent weight
N	Number of plate
NP-HPLC	Normal phase HPLC
PAHs	Polycyclic Aromatic Hydrocarbons
PS–DVB	poly (styrene–divinylbenzene)
RBC	Reversed Bonded phase Chromatography
RID	Refractive Index detecter
RPC	Reversed phase Chromatography
SGV	Silica gel-crystal violet stationary phase
SFC	Supercritical-fluid chromatography
TAG	Triacylglycerols
t_R	Retention time
UPLC	Ultra performance Liquid Chromatography
MPa	Mega Pascal

Chapter One

Introduction

1.1. Chromatography:

Chromatographic separations are accomplished by continuously passing one sample-free phase, called a mobile phase, over a second sample-free phase that remains fixed, or stationary. The sample is injected, or placed, into the mobile phase. As it moves with the mobile phase, the sample's components partition themselves between the mobile and stationary phases. Those components whose distribution ratio favors the stationary phase require a longer time to pass through the system. Given sufficient time, and sufficient stationary and mobile phase, solutes with similar distribution ratios can be separated. The history of modern chromatography can be traced to the turn of the century when the Russian botanist Mikhail Tswett (1872–1919) used a column packed with a stationary phase of calcium carbonate to separate colored pigments from plant extracts. The sample was placed at the top of the column and carried through the stationary phase using a mobile phase of petroleum ether. As the sample moved through the column, the pigments in the plant extract separated into individual colored bands. Once the pigments were adequately separated, the calcium carbonate was removed from the column, sectioned, and the pigments recovered by extraction. Tswett named the technique chromatography, combining the Greek words for "color" and "to write." There was little interest in Tswett's technique until 1931 when chromatography was reintroduced as an analytical technique for biochemical separations. Pioneering work by Martin and Synge in 1941 established the importance of liquid–liquid partition chromatography and led to the development of a theory for chromatographic separations; they were awarded the 1952 Nobel Prize in chemistry for this work. Since then, chromatography in its many forms has become the most important and widely used separation technique. [1] The chromatographic methods can be classified as shown in Table 1-1.

This classification is based on the typed stationary-phase and equilibrium involved in separation.

Table 1 -1 Classification of Chromatography Techniques [2]

Classification as mobile phase	*Stationary phase*	*Type of equilibrium*	*Specific method*
Liquid chromatography LC mobile phase liquid	Liquid adsorbed on a solid	Partition between immiscible liquids	Liquid-liquid or partition
	Organic species bonded to a solid surface	Partition between liquid and bonded surface	Liquid bonded phase
	Solid	Adsorption	Liquid-solid or adsorption
	Ion exchange resin	Ion-exchange	Ion-exchange
	Liquid in interstices of polymeric solid	Partition/sieving	Size-exclusion
Gas chromatography GC mobile phase: gas	Liquid adsorbed on a solid	Partition between gas and liquid	Gas-liquid
	Organic species bonded to a solid surface	Partition between liquid and bonded surface	Gas-bonded phase
	Solid	Adsorption	Gas-solid
Supercritical-Fluid Chromatography (SFC) mobile phase : supercritical fluid	Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface	

1.2. Liquid Chromatography:

Liquid chromatography began in the early 1900s, in the form known as classical column chromatography, a glass cylinder was packed with a finely powder such as chalk, a sample was applied to the top of the column and the solvent was poured onto the column, as the solvent flows down by gravity, the components of the sample begins to move through the column at different speeds and become separated.

Another form of liquid chromatography was introduced in 1940s, called paper chromatography, in which a strip of paper is, replaced the column, after the sample was spotted near the bottom of the paper strip; the paper was placed in container with solvent at the bottom. As the solvent migrated up the paper by capillary a paration took place, but in opposite direction, this open bed form of chromatography was modified by coating thin layer of powder silica onto a glass plate which is referred on thin layer chromatography.

High performance liquid chromatography represents the modern culmination of the development of liquid chromatography. It begins by placing samples in injection into the column and the solvent is continually pumped through the column and the separated compounds are continuously sensed by detector as they leave the column. [3]

1.3.High Performance Liquid Chromatography (HPLC):

HPLC is used in analytical chemistry and biochemistry to separate chemical compounds in mixtures for analysis or purification. Components in a mixture are separated on a column packed with silica-based particles (as stationary phase) by pumping a solvent (as mobile phase) through the column. Depending on the unique affinity of each

component (as the analyte) between the mobile phase and the stationary phase, each analyte migrates along the column at different speeds and emerges from the column at different times, thus establishing a separation of the mixture [(4)].

One of the primary drivers for the growth of this technique has been the evolution of packing materials used to effect the separation. The underlying principles of this evolution are governed by the Van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency). Since particle size is one of the variables, a Van Deemter curve can be used to investigate chromatographic performance [(5)].

According to the Van Deemter equation, as the particle size decreases to less than 2.5 μm, not only is there a significant gain in efficiency, but the efficiency does not reduce at increased flow rates or linear velocities. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time in gradient separations) can be extended to new limits, termed "Ultra Performance Liquid Chromatography", or UPLC. This can be result of using small particles size of the stationary phase as described by Van-Deemter equation.

The most important advantage of HPLC is that samples can separate much more quickly with very high efficiency. The efficiency of the chromatographic separation is usually described by the number of theoretical plates (N). The value of (N) is related to the height equivalent to theoretical plates (H) by: H= L / N Where (L) is the length of the column. The efficiency of HPLC is higher due to the large number of mass transfer equilibrium obtained with small values of (H).

The plate theory needs to assume that the solute during it passage through the columns, is always in equilibrium with the mobile phase and stationary phase. However this equilibrium never occurs. To take this non

equilibrium condition into account, the column is considered to be divided into a numbers of cells or plates; each plate is allotted a specific length. Thus the solute will spend a finite time in each plate; the size of the cell is chosen to provide sufficient residence time for the solute to establish equilibrium with the two phases; thus the shortest the plate, the faster will equilibrium and the more plates there will be in the column.

HPLC are widely used in pharmaceutical field by calculating the concentration of the active ingredients, drugs purification and separation of an enantiomers molecules, which allows the chemist to confirm which enantiomers is presents and calculate the ratio when both are present[6].

1.4. Modes of liquid Chromatography:

HPLC can be carried out in any of the classical modes such as normal and reversed phase as well as ion exchange (cationic or anionic) chromatography. Separation modes can be chosen for particular application depended on the properties of analytes to be separated, and can be optimized by choosing different combination of mobile phase and stationary phase materials [7].

1.4.1 Normal-phase chromatography (NPC):

Normal phase HPLC (NP-HPLC) was the first kind of HPLC chemist used. It separates analytes based on polarity. This method uses a polar stationary phase and a non-polar mobile phase, and is used when the analyte of interest is fairly polar in nature. The polar analyte associates with and is retained by the stationary phase. Adsorption strengths increase with increase in analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength not only depends on

the functional groups in the analyte molecule, but also on steric factors and structural isomers is often resolved from one another. Use of more polar solvents in the mobile phase will decrease the retention time of the analytes while more_hydrophobic solvents tend to increase retention times. Particularly polar solvents in a mixture tend to deactivate the column by occupying the stationary phase surface. This is somewhat particular to normal phase because it is most purely an adsorptive mechanism (the interactions are with a hard surface rather than a soft layer on a surface). NP-HPLC had fallen out of favor in the 1970's with the development of reversed-phase HPLC because of a lack of reproducibility of retention times as water or protic organic solvents changed the hydration state of the silica or alumina chromatographic media .Recently it has become useful again with the development of HILIC (hydrophilic interaction chromatography) bonded phases which utilize a partition mechanism which provides reproducibility. Typical stationary phases for normal phase chromatography are silica or alumina. The most non-polar compounds elute first and the most polar compounds elute last. The mobile phase consists of a very non-polar solvent like hexane or heptanes mixed with a slightly more polar solvent like isopropanol, ethyl acetate or chloroform. Retention increases as the amount of non-polar solvent in the mobile phase increases. [7]

Although the majority of recent LC studies on the characterization of polymers employs RP mode, normal-phase liquid chromatography (NPLC) has a longer history than RPLC and has been frequently applied to the analysis of various oligomers and polymers. [8]

Dessouky et al. [9] have determined of chlorphenoxamine hydrochloride (CPHX), caffeine (CAFF) and 8-chlorotheophylline (CTHE). The chromatographic separation was achieved using (150 x 6 mm) normal phase column operating with isocratic elution of ethyl acetate-heptanes

(50:50 v/v) at a constant flow rate of 1.0 ml min^{-1} .The effluent was monitored by an UV detector set as 254 nm.

Panfili et al. [10] 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. [11] determination of arginine, lysine and histidine in drug substance and drug product by using normal phase column. The above amino acids are eluted by n-hexane/chloroform (90:10, v/v) as a mobile phase. The authors noted that the increasing percentage of hexane was increased the retention time as well as tailing factor while decreasing the percentage of hexane was decreased the retention time as well as tailing factor.

Murahashi et al. [12] have used cyanopropyl column to determine of the 3-Nitrobenzanthrone in rain water. n-hexane /ethyl acetate (3 :1 , v/v) was used as mobile phase at flow rate of 1ml.min^{-1}. The effluent was monitored by a fluorescence detector. Detection excitation and emission wavelengths were 490nm and 560nm, respectively. Furthermore, silica gel columns have been employed for determination of simple lipids in human plasma and cyanopropyl column has been successfully used in the separation of short- and long-chain Triacylglycerols (TAG). [13]

1.4.2 Reversed-phase chromatography (RPC):

Reversed-phase chromatography is by far the most widely used technique in (HPLC) [14]. It is popular because it is applicable to most nonpolar analytes and to many ionizable and ionic compounds. Most of the stationary phases used in reversed phase chromatography are hydrophobic in nature. Therefore, analytes are separated by their degree of hydrophobic interaction with the stationary phase and matrices with hydrophobic components can also be retained in a similar manner [15].

Reversed-phase chromatography can be used to separate nonpolar, polar, and ionic compounds sometimes in the same separation. The technique also can achieve a larger range of separations than all other modes combined because it enables users to manipulate the mobile phase by changing organic solvent type, solvent composition, and pH; by adding modifiers such as surfactants, chiral reagents, competing bases, and ion-pair reagents; or by adjusting experimental conditions such as flow rate and temperature.

Reversed-phase chromatography generally is performed using octadecyldimethylsilane (C-18) or octyldimethylsilane (C-8) stationary phases bonded to high-purity, spherical silica gel. short-chain alkyl phases such as C-2 and C-4 and phenyl phases are used occasionally, but long-chain phases such as C-30 are used rarely and only for specialized applications. Polymeric materials such as poly (styrene–divinylbenzene) (PS–DVB) find some use as reversed-phase media (for example, with high-pH mobile phases) but generally provide lower column efficiencies than those of silica gel–based packing's. Water, usually buffered, mixed with a water-immiscible organic solvent (modifier), most commonly acetonitrile or methanol, is the preferred mobile phase. To retain most nonpolar and semi polar organic analyses, chromatographers generally begin method development by varying the water–organic solvent ratio in

the 20–80% range, often using gradient elution to find the optimum separation conditions more quickly[16].

Sudha and Venkata[17], described the role of reverse phase in determination of ziprasidone hydrochloride monohydrate in bulk and pharmaceutical dosage forms. For this, a binary mixture of methanol and phosphate buffer (55:45 v/v) as a mobile phase. The resulting chromatogram show the absence of additional peaks indicates non-interference of the common excipient used in the drug, it can be concluded that the proposed HPLC method is sensitive and reproducible for the analysis of ziprasidone HCl monohydrate in pharmaceutical dosage in a short analysis time.

Idress[18] found a procedure that use reveres phase high performance liquid chromatography to determine hydrochlorothiazidine; a thiazide diuretics, and enalpril maleate; an antihypertensive and a vasodilator in congestive heart failure, in pharmaceutical tables with free of interferences from excipient used in tablets formulation. The mobile phase was 3.0mM tetrabutylammoniumhydrogen sulfate in acetonitrile/water/triethylamine (14, 85.6, 6.4 V/V) adjusted to pH 4.1 by glacial acetic acid. The result found that the recovery of both components was 97.2-101.0 % and for enalpril maleate, the recovery was 97.9-101.5%.

1.4.3 Ion-exchange chromatography:

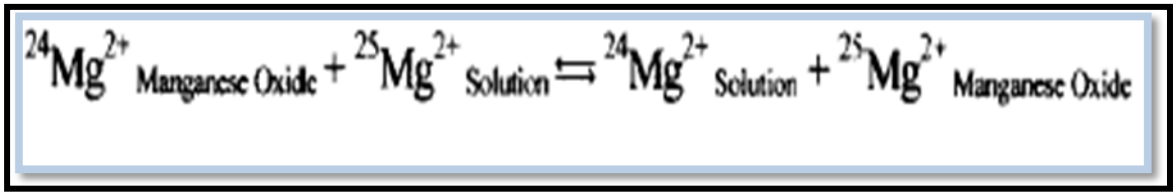
Ion exchange chromatography is a technique based on the difference in the strength of the interaction between a sample ion and an oppositely charged functional group [19].

Ion exchange is basically a process of nature occurring throughout the ages. The earliest of the reference were found in the bible, which says that “Moses” succeeded in preparing drinking water from brackish water by an ion exchange technique [20].

Ion exchange usually used for protein purification but may be used for purification of peptides or other charged molecules [21]. Proteins in the mobile phase will bind through electrostatic interactions to the charged group on the column. In a mixture of proteins, positively charged proteins will bind to a resin containing negatively groups; e.g. carboxymethyl group (-OCH2COO-) or sulfopropyl group (-OCH2CH2CH2SO3-); while the negatively charged proteins will pass through the column.

Yoshiteru et al. [22] developed a high performance anion-exchange chromatography with pulsed amperometric detection for simultaneous determination of relevant sugar alcohol, monosaccharide and monosacchride anhydrous (that comprise an important fraction of water soluble organic carbon in atmospheric aerosols).

Dong et al. [23], investigate the separation of magnesium isotopes by ion exchange using a manganese (IV) oxide with elution chromatography. The elute was ammonium lactate. The behavior isotopes of magnesium (II) were concentrated in manganese oxide (IV) phase, while the light magnesium (II) isotopes were concentrated in the solution phase. The separation by manganese (IV) oxide is primarily electrostatic based on



comparing the adsorption of cationic, anionic and uncharged organic compounds. Since at higher pH the manganese (IV) oxide has a net negative charged so it shows a high adsorption capacity for cations.

1.5. HPLC Detection system:

Besides the separation column, the analyte detector is considered the most important component of an HPLC system. The detector translates the changes in the chemical composition of the column effluent during the chromatographic run into electrical signals. The signals can then be recorded as chromatograms and processed to give the required information about the sample composition. The choice of the detector depends on several sample-related factors: the chemical properties of the analytes, the range of analyte concentration, and also the other chromatographic system components performances used for the separation. [24]

1.5.1. Refractive index detector (RID):

This type of detector relies on the Fresnel principle of light transmission through a transparent medium of refractive index [25]. It is designed to measure continuously the difference in the refractive index between the mobile phase ahead of and following the column. So a differential refractometer is used. Schematically, a beam of light travels through a cell that has two compartments: one is filled with the pure mobile phase while the other is filled with the mobile phase eluting the column (Figure.1-1). (RID), are used to detect a non-UV absorbing compounds, but they are less sensitive than UV-detectors [27].

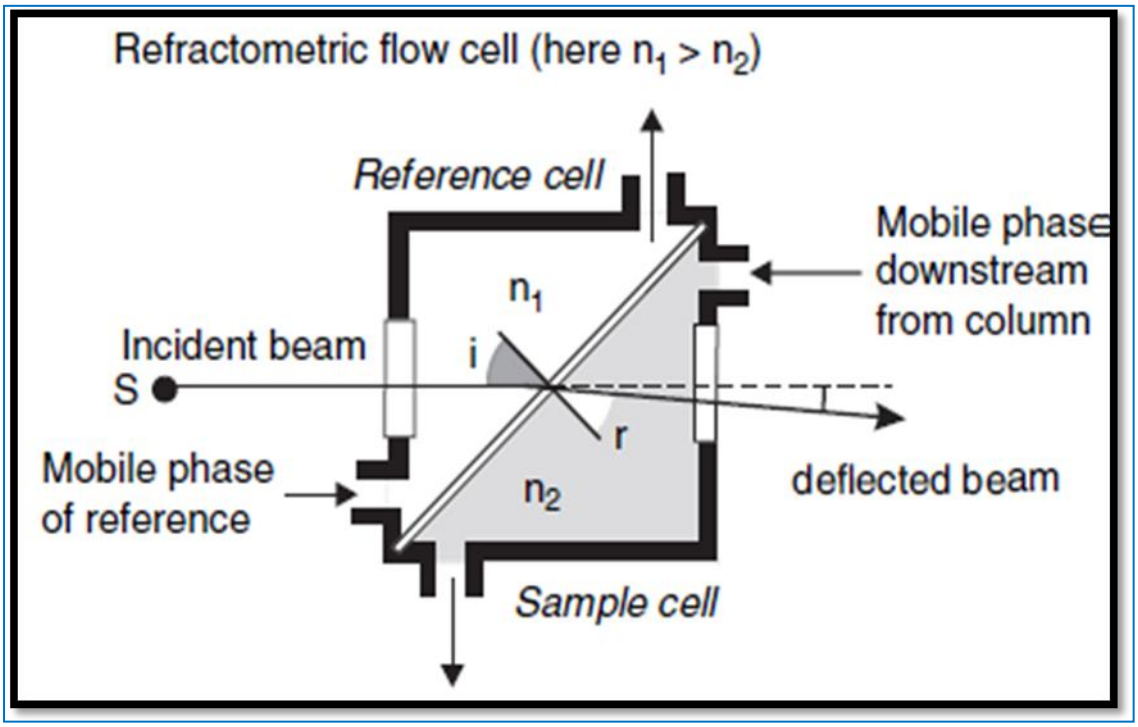


Fig. 1-1.Cell of Refractive index detector

1.5.2. UV-Visible absorption detector:

This detector is the most widely used in HPLC; it is relatively insensitive to temperature and flow variation. Detection limits at the nanogram level for certain types of compounds can be easily achieved. The UV-Vis detector has a linear response over a range of 5×10^{-3}M, with detection limits of 1×10^{-9}g $cm^{-1}$for highly absorbed compounds with large molar extinction coefficient.

Detection is based upon the Lambert–Beer Law $A = \epsilon\, b\, C$.

The absorbance A of the mobile phase is measured at the outlet of the column, at wavelength λ in the UV or visible spectrum. The intensity of the absorption depends upon the molar absorption coefficient ϵ of the species detected. It is essential that the mobile phase be transparent or possesses only a very little absorption (Figure. 1-2) [(26)].

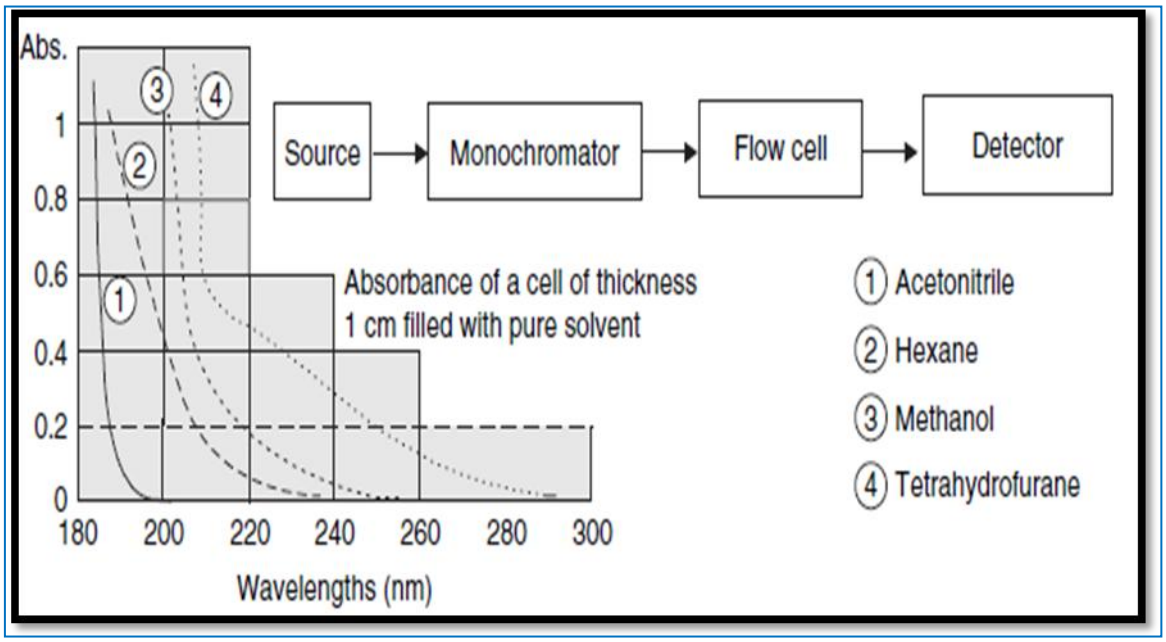


Fig. (1.2) Photometric detection at a single wavelength. Principle of a photometric detector along with the absorption spectra of several solvents used in liquid chromatography. Here the transparence limit of a solvent corresponds to an absorbance of 0.2 for 1 cm of optical path in the cell.

1.5.3. The Fluorescence Detector:

Compounds that fluoresce or which fluorescing derivatives can be obtained are picked up with high sensitivity and specificity by this detector. The sensitivity may be up to 1000 times greater than with UV detection. Light of a suitable wavelength is passed through the cell and the longer wavelength radiation emitted is detected in a right-angled direction. The light intensity and hence the sensitivity are increased by using a relatively large cell (20µl or greater) have a fixed excitation wavelength for which band width must not be too narrow and a fixed wavelength range for fluorescent light detection. [(28)]

1.5.4. The Conductivity Detector:

Conductivity detection is used to detect inorganic and organic ionic species in liquid chromatography. The principle of operation of a conductivity detector lies in differential measurement of mobile-phase conductivity prior to and during solute ion elution. The conductivity cell is either placed directly after an analytical column or after a suppression device required to reduce background conductivity, in order to increase the signal-to-noise ratio and, thus, sensitivity. The requirements for a typical conductivity detection cell are small volume (to eliminate dispersion effects), high sensitivity, wide linear range, rapid response, and acceptable stability. The cell generally consists of a small-volume chamber (5μL) fitted with two or more electrodes constructed of platinum, stainless steel, or gold. [29]

1.6. Column Packing:

The HPLC column is a heavy-walled stainless steel tube equipped with inlet and outlet fittings that is pressure packed with fine-diameter packing material suspended in a mobile phase. [30] Column packing supports for HPLC can be divided into two main classes, silica and polymer types. Silica has several desirable properties such as excellent pressure stability, high surface area, good control of size and shape, and ease of availability. Of the silica packings, pellicular and porous are the two major kinds. Pellicular packings developed first as an alternative to classical large particle LC supports, basically consist of a solid glass bead on which a 1–2μm porous layer of silica has been deposited. Interaction of the sample components occurs only in this thin layer. Originally 40-μm size particles were made but now smaller particles are available. Porous silica particles that are 10, 5, or 3 μm in size are more commonly packed in analytical

columns. Recently, columns packed with 2-μm particles have been reported in the literature. [31]

The pore size of the silica particles must be large enough to permit easy entrance and exit of the sample molecules. Porous zirconia particles coated with polybutadiene make a reversed-phase HPLC column packing that offers both excellent pressure stability and chemical inertness throughout the entire pH range. However, silica is not a perfect support for HPLC columns. An unfavorable characteristic of silica is its solubility at high pH. [32] Porous packing's have been in favor throughout the history of HPLC. The transition from large porous particles and pellicular materials to small porous particles occurred in the early 1970s, when micro particulate silica gel came on the scene and appropriate packing methods were developed. Irregularly shaped micro particulate packings were in vogue throughout the 1970s until spherical materials were developed and perfected. The spherical packings could be packed more homogeneously than their irregular predecessors, gave better efficiencies, and could be manufactured in higher purity. Indeed, the so-called Type B silica that was low in trace-metal content became the standard in the early 1990s and now most commercial silica-based analytical HPLC packing materials are of this higher level of purity. [33]

The "tap-fill" procedure was recommended for the dry packing of rigid particles with diameter dp >20 μm. The technique for dry-filling high efficiency LC columns is not very different from that used to prepare high efficiency GC columns [34]. The high-pressure "wet- fill" or slurry-packing techniques were, alternatively, used for packing particles with dp <20 μm. In this technique suitable liquids were used to wet the particles and eliminate particle aggregation during packing [35].

1.6.1. Down-Flow Method:

The down-flow method has been most widely used, and it permits the preparation of satisfactory columns of all types of micro particles packing. Rigid particles are required for the best results with this method. An apparatus such as that shown in figure (1-3) is used. The slurry mixture is rapidly forced downward into the column blank with a constant pressure pump [(34)]. Slurry flow rate is dependent on the pressure used and as the packed bed is formed, the flow rate decreases. Forcing the slurry mixture into the column blank at highest possible velocity generally produce the best column performance. This operation is carried out by pumping the slurry into the column blank at the highest pressure permitted by compression fittings connecting the column to the slurry-packing apparatus. The pump used for packing could be either reciprocating or pneumatic pump [(36)]. The very high initial velocity as the slurry enters the column blank may fracture weak particles, producing fine particles that tend to plug the column outlet and caused packing structure irregularities [(34)].

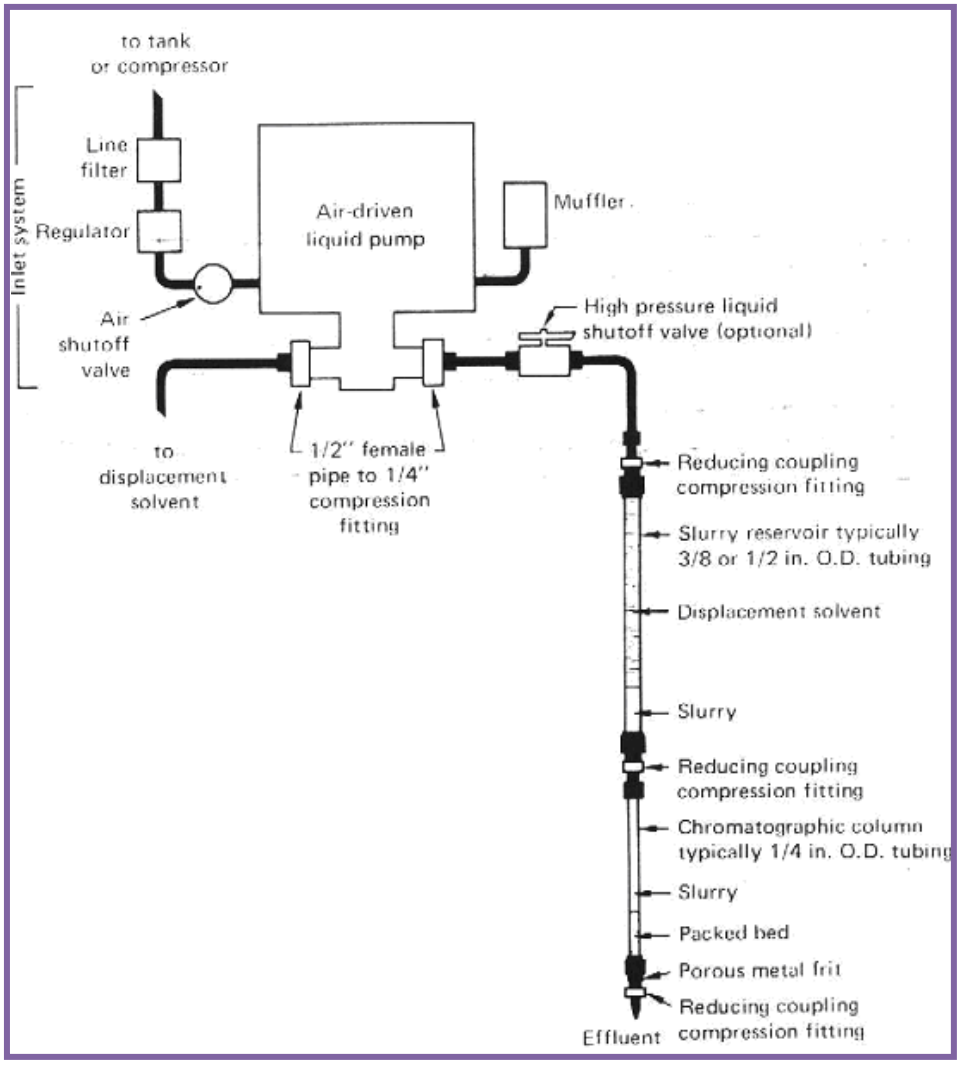


Figure (1-3) High-pressure slurry packing assembly [(34)].

1.6.2. Up-Flow Method:

An alternative technique for wet filling columns is the up-flow approach, which has been described by Bristow [(37)]. Experience with the up-flow packing method has not extensive, but column performance results have been about equivalent to those for the down-flow procedure. The equipment used for the up-flow packing is shown in figure (1-4). In this case the slurry is pumped up into the column blank from the reservoir whose contents are continuously diluted by incoming pressurized liquid [(38)].

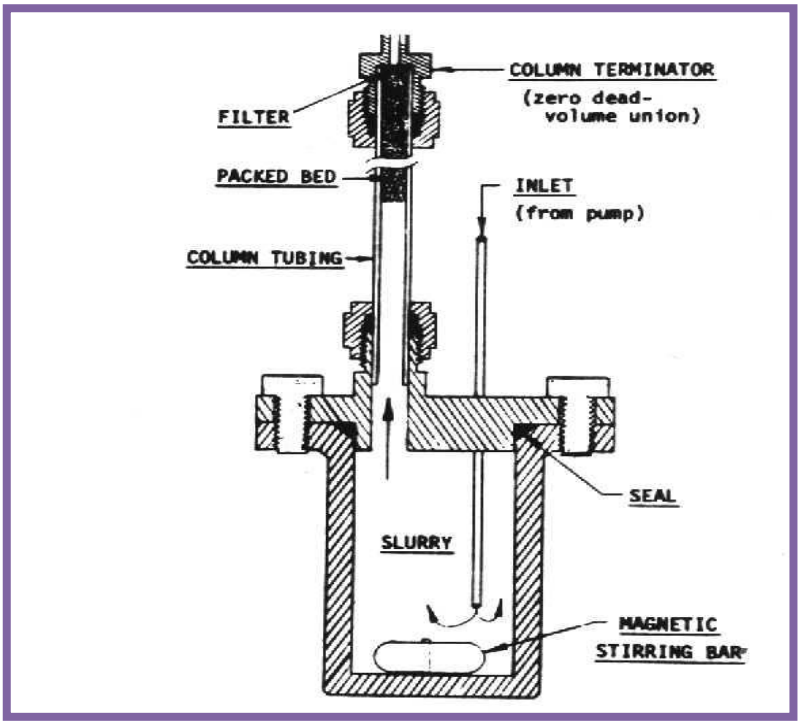


Figure (1-4) Up-Flow Packing System [38]

1.7. Water -Soluble Vitamins Characteristic:

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 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. In recent years, 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. A choice of HPLC methods for water-soluble vitamins can be made: reversed-phase chromatography (RPC), ion-exchange chromatography (IEC), ion-pairing chromatography (IPC), 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 can 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. [39]

1.7.1. Vitamin B_{12}:

Also called cobalamin, is a water-soluble vitamin with a key role in the normal functioning of the brain and nervous system, and for the formation of blood. It is one of the eight B vitamins. It is normally involved in the metabolism of every cell of the human body, especially

affecting DNA synthesis and regulation, but also fatty acid synthesis and energy production. It is the largest and most structurally complicated vitamin and can be produced industrially only through bacterial fermentation-synthesis. Vitamin B_{12} consists of a class of chemically-related compounds (vitamers), all of which have vitamin activity as shown as Fig (1-5). It contains the biochemically rare element cobalt. Biosynthesis of the basic structure of the vitamin is only accomplished by bacteria, but conversion between different forms of the vitamin can be accomplished in the human body. A common synthetic form of the vitamin, cyanocobalamin, does not occur in nature, but is used in many pharmaceuticals and supplements, and as a food additive, because of its stability and lower cost. In the body it is converted to the physiological forms, methylcobalamin and adenosylcobalamin, leaving behind the cyanide, albeit in minimal concentration. More recently, hydroxocobalamin, methylcobalamin, and adenosylcobalamin can also be found in more expensive pharmacological products and food supplements. The extra utility of these is currently debated. Vitamin B_{12} was discovered from its relationship to the disease pernicious anemia, which is an autoimmune disease in which parietal cells of the stomach responsible for secreting intrinsic factor are destroyed. Intrinsic factor is crucial for the normal absorption of B_{12}, so a lack of intrinsic factor, as seen in pernicious anemia, causes a vitamin B_{12} deficiency. Many other subtler kinds of vitamin B_{12} deficiency and their biochemical effects have since been elucidated.-(40)

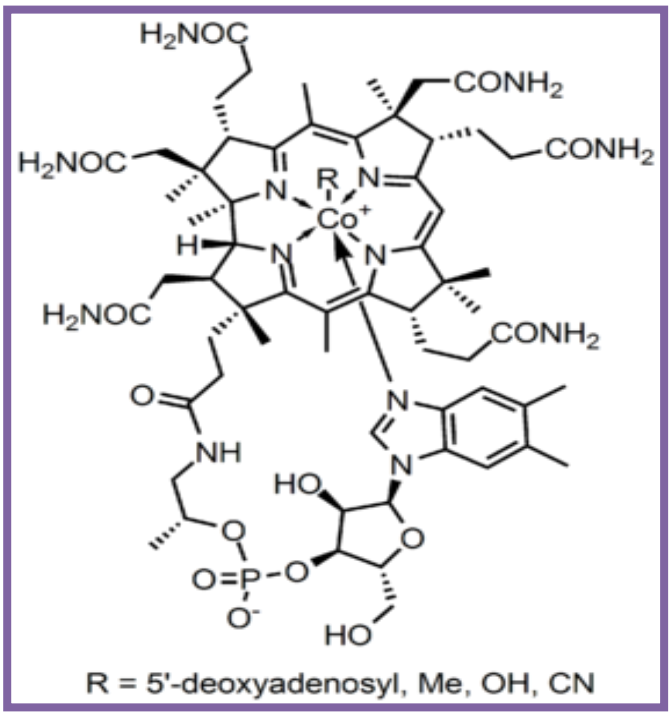


Fig (1-5) structure of Vitamin B_{12}

1.7.2. Vitamin B_2:

Riboflavin, also known as vitamin B_2 is an easily absorbed micronutrient with a key role in maintaining health in humans and animals. It is the central component of the cofactors flavin adenine dinucleotide (FAD) and Flavin mononucleotide (FMN), and is therefore required by all flavoproteins. As such, vitamin B_2 is required for a wide variety of cellular processes. It plays a key role in energy metabolism, and for the metabolism of fats, ketone bodies, carbohydrates, and proteins as shown as figure (1-6). It is also used as an orange-red food colour additive, designated in Europe as the E number E101, [41] Milk, cheese, leaf vegetables, liver, kidneys, legumes, tomatoes, yeast, mushrooms, and almonds are good sources of vitamin B_2, but exposure to light destroys riboflavin. The name "riboflavin" comes from "ribose" (the sugar whose reduced form, ribitol, forms part of its structure) and "flavin", the ring-moiety which imparts the yellow color to the oxidized molecule (from

Latin *flavus*, "yellow"). The reduced form, which occurs in metabolism along with the oxidized form, is colorless.Riboflavin is best known visually as the vitamin which imparts the orange color to solid B-vitamin preparations, the yellow color to vitamin supplement solutions, and the unusual fluorescent-yellow color to the urine of persons who supplement with high-dose B-complex preparations (no other vitamin imparts any color to urine). [42]

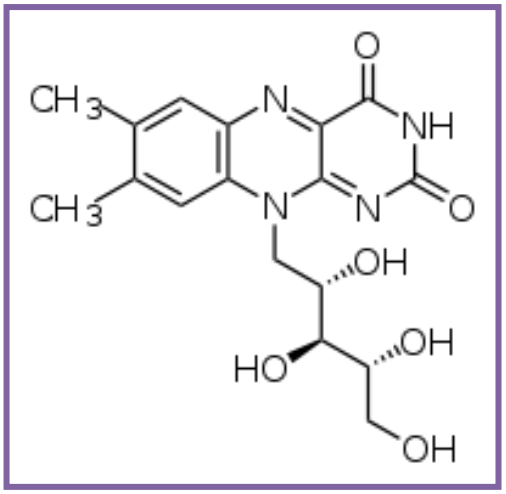


Fig (1-6) structure of Vitamin B_2

1.7.3. Vitamin C

Vitamin C or L-ascorbic acid or L-ascorbate is an essential nutrient for humans and certain other animal species. In living organisms ascorbate acts as an antioxidant by protecting the body against oxidative stress as shown as figure (1-7). It is also a cofactor in at least eight enzymatic reactions including several collagen synthesis reactions that, when dysfunctional, cause the most severe symptoms of scurvy. [43] In animals these reactions are especially important in wound-healing and in preventing bleeding from capillaries. Ascorbate (an ion of ascorbic acid)

is required for a range of essential metabolic reactions in all animals and plants. It is made internally by almost all organisms although notable mammalian group exceptions are most or all of the order chiroptera (bats), guinea pigs, capybaras, and one of the two major primate suborders, the Anthropoidea (Haplorrhini) (tarsiers, monkeys and apes, including human beings). Ascorbic acid is also not synthesized by some species of birds and fish. All species that do not synthesize ascorbate require it in the diet. Deficiency in this vitamin causes the disease scurvy in humans. It is also widely used as a food additive. [44]

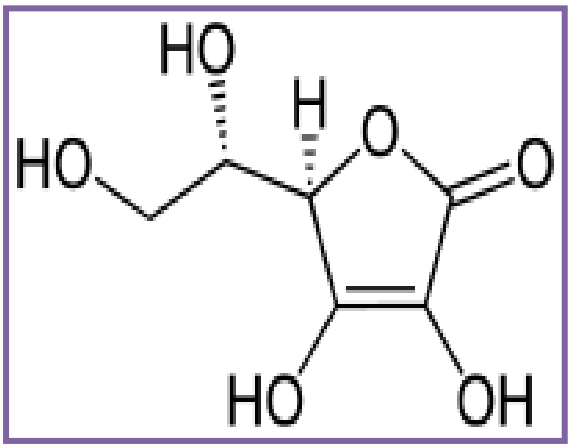
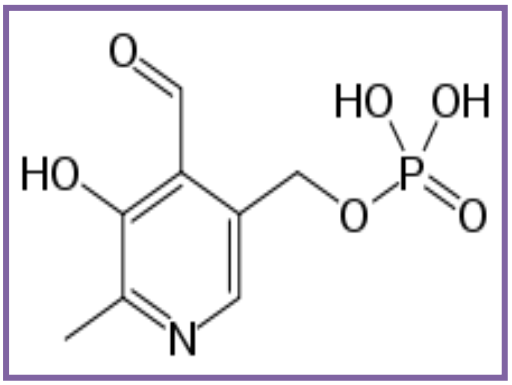


Fig (1-7) structure of Vitamin C

1.7.4. Vitamin B_6:

Vitamin B_6 is a water-soluble vitamin that was first isolated in the 1930s. There are three traditionally considered forms of vitamin B_6: pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM). The phosphate ester derivative pyridoxal 5'-phosphate (PLP) is the principal coenzyme form and has the most importance in human metabolism. (45)

Vitamin B_6 must be obtained from the diet because humans cannot synthesize it. PLP plays a vital role in the function of approximately 100 enzymes that catalyze essential chemical reactions in the human body as shown as figure (1-8). (46)



Vitamin B_6 Fig (1-8) structure of

1.8. Column Evaluation:

The knowledge of column evaluation is very necessary for any recently packed column. The measuring of column efficiency represents either by stating the number of theoretical plates in a column, N (the more plates the better), or by stating the plate height; the Height Equivalent to a Theoretical Plate (HETP) (the smaller the better). If the length of the column is *L*, then the HETP is:

$$\mathbf{HETP} = L / N \ldots\ldots\ldots \mathbf{(1\text{-}1)}$$

The number of theoretical plates that a real column possesses can be found by examining a chromatographic peak after elution. [8]

$$\mathbf{N=5.54\ (t_R/\ w_{1/2})^2 \ldots\ldots\ldots\ldots\ (1\text{-}2)}$$

Where $\mathbf{(t_R)}$ is the retention time, $w_{1/2}$ is the peak width at half-height. The equilibrium constant, *K*, is termed the distribution or partition coefficient; defined as the number of mole of analyte in the stationary phase divided by the number of mole in the mobile phase, where capacity factor **Ќ** was also recommended for column evaluation. [47]

The partition coefficient *K* is based on this equilibrium, and is defined by the following equation as shown below. [47]

$$K = \mathbf{N_s/\ N_m \ldots\ldots\ (1\text{-}3)}$$

This important relation can also be written:

$$K = \mathbf{(t_R - t_m)\ /\ t_m \ldots\ldots\ldots\ (1\text{-}4)}$$

Where **tm** represent the dead time.

Ќ is assumed to be independent of concentration, and can change if experimental conditions are changed, for example temperature is increased or decreased. As **Ќ** increases, it takes longer time for solutes to elute. For a column of fixed length and flow, the retention time (t_R) and retention volume (V_R) can be measured and used to calculate **Ќ**

Capacity factor between 1.5 and 5 are preferred. [48] Lower **Ќ** indicates no stationary phase interaction occurs and hence no chromatography and large **Ќ** values are accompanied by long analysis times.

A quantity called the separation or selectivity factor, α, is known as relative retention which describes the separation of two species (A and B) on the column;

$$\alpha = k_B / k_A \ldots\ldots (1\text{-}5)$$

A minimum value of 1.1 is required to achieve the separation of two adjacent peaks at any given experimental condition.

Peaks asymmetry is another important term, when the value of Asymmetry large 1.2 means that the quantitative peak area measurement will prone to error [2]

Resolution (**Rs**) is important parameter; it defines the degree of separation between two adjacent peaks [49]:

$$Rs = \tfrac{1}{2} (t_{R2}-t_{R1}) / (W_2+W_1) \ldots\ldots (1\text{-}6)$$

Where t_{R1} and t_{R2} are the retention times of peaks 1 and 2, W_1 and W_2 are the widths of the peaks at the baseline, respectively. The larger resolution is better the separation.

1.9. Aim of the Work:

The aim of this studied was to prepare a new HPLC stationary phase depended on reaction of silica gel with crystal violet indicator. The properties and characterizations of this new stationary phase were studied. The chromatographic performance was examined. Use of column packed with new resin for the separation and quantitation of some water soluble vitamins.

Chapter Two

Instrument and Equipments

2.1. Chemicals:

The materials and chemicals listed in Table (2-1) which was obtained from different solvents have been used in this work.

Compounds	***Supplied from***	**Purity**
Acetonitrile	Fluka	98.9%
Acetone	Fluka	99.9%
Benzene	Merck	99.9%
Chloroform	Hopkins and Williams	98.9%
Crystal Violet	BDH	molecular weight 407.979 g mol^{-1}
DMF	Fluka	99.9%
DMSO	Fluka	97.9%
DCM	Fluka	98.9%
Ethanol	Fluka	99.9%
Hexane	Merck	97.9%
Hydrochloric acid	GCC	98.9%
Methanol	Fluka	99.9%
n-heptane	BDH	98.9%
Nitric acid	Merck	99.9%
Petroleum ether	Merck	99.8%
Potassium hydroxid	Fluka	99.9%
Silica gel	Sigma	5µm
THF	Merck	99.9%
Vitamin B12	Samarra	99.8%
Vitamin B2	Sigma	99.9%
Vitamin B6	Samarra	99.9%
Vitamin C	Sigma	99.9%

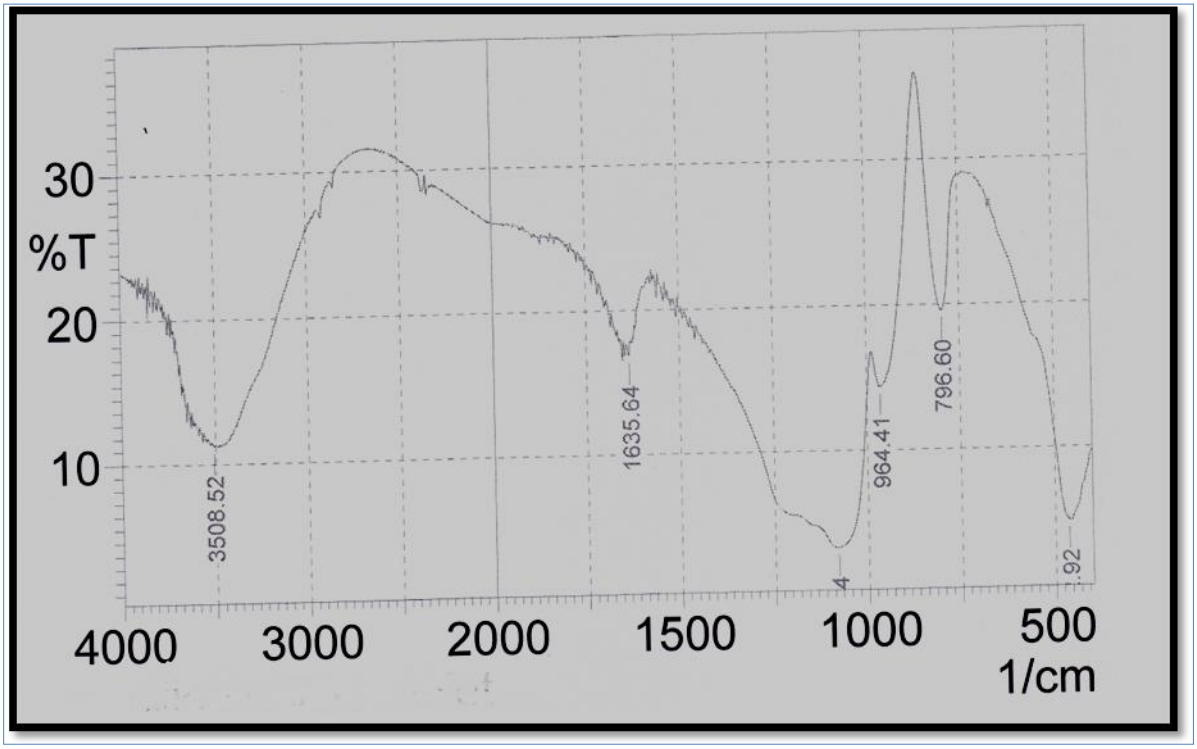
2.2. Instruments and Equipment

- High performance liquid chromatography used in this work was CECIL(Cambridge, England) which consisted of High performance variable wave length monitor model CE-1100 SERIES, a degasser model CE-1140, two liquid delivery pumps model CECIL -1100, 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 Resin.
- PH meter with Glass combination electrode was used to measure the pH of solutions (Germany).
- Shimadzu UV-Visible spectrophotometer model UV-1601(Kyoto, Japan) was used to measure the absorbance of solution.
- Blank stainless steel columns of dimensions 15 ×0.4 cm ID, was obtained locally.

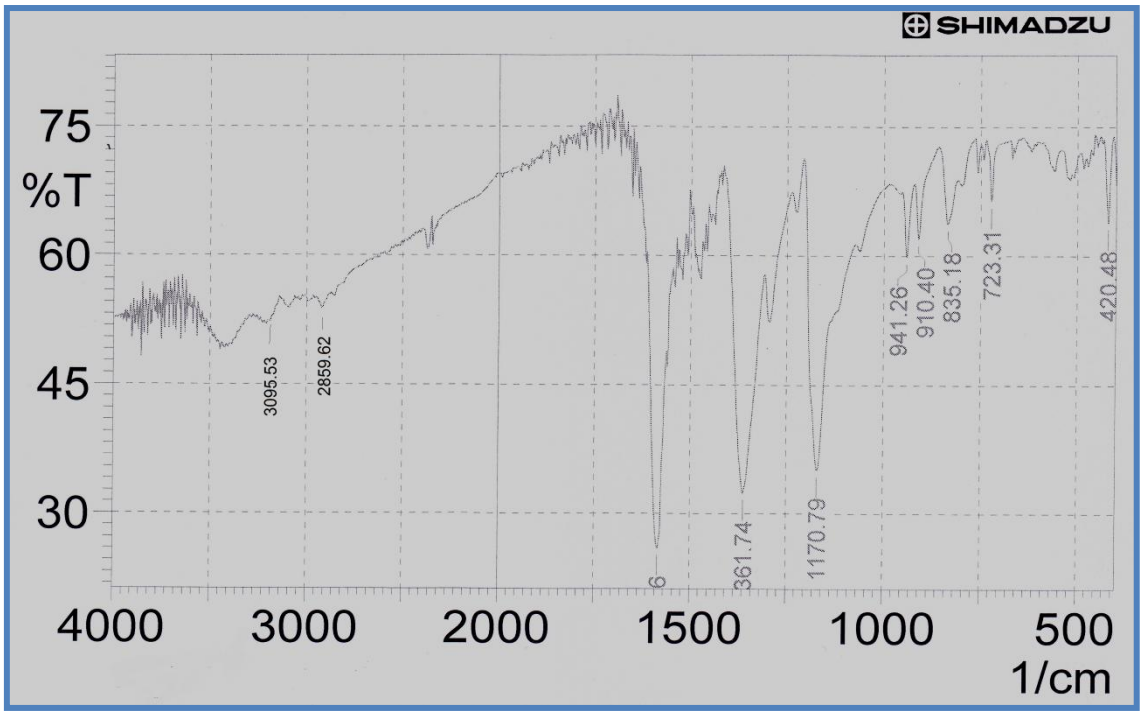
2.3. Preparation of the Stationary Phase

Four grams of Silica gel was first rinsed with 100ml hexan in a 250ml beaker and kept for about 24 hour so as to swell. A 10 ml of 1.5×10^{-3}M Crystal Violet was then add with stirring to the beaker. The color of the Silica has been changed from white to the deep violet. The content of the beaker was led to settle for 24 hours. The solution was then decanted and discarded.

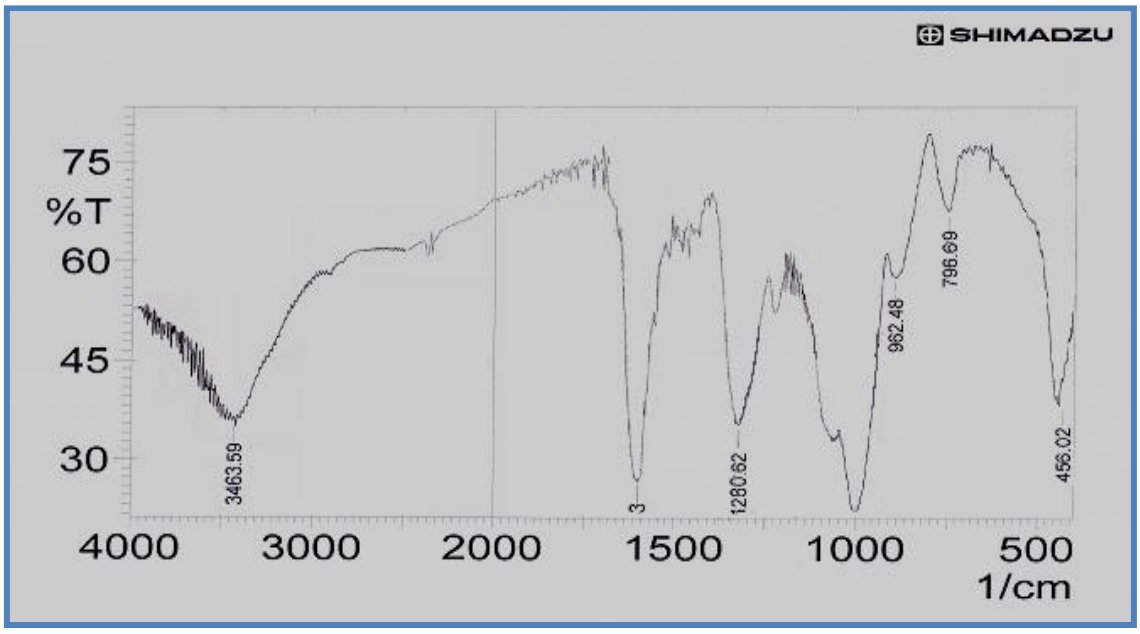
The silica was washed few times with methanol, then rinsed with hexane and dried in the oven at 80 $^{\circ}$C over night and kept in decicator for future work. The FTIR spectrum for the resulting Silica gel – Crystal violet , Crystal Violet , Silica gel , as shown in figures (2 -1).



(A)



(B)



(C)

Figure. (2- 1). FTIR Spectrum for (A) Silica Gel (B) Crystal Violet (C) Silica Gel-Crystal Violet .

2.4. Stability Tests For Silica gel –Crystal Violet:

The stability of the new product has been examined using different solvents and solutions. The product was first rinsed with 0.1M HCl and also treated with 0.1M KOH. The rinsed solution was measured spectrophotometrically at optimum wave length of the Crystal Violet which is at 595 nm .The resin was again washed three times with methanol and dried at 100°C. The dried product was then treated with solvents of different polarity such as benzene, THF, DMF, DMSO, Hexan, n-heptan, petroleum ether, acetone, chloroform, methanol, ethanol, and acetonitrile. 10 ml of each of solvent as described above has been added to 0.2 gm of dried product with continuous stirring for about 20 minutes. At the end it was found that new product was stable against those solvents.

2.5. SGV Swelling Determination:

Swelling measurements of the SGV was done by placing a small quantity of dry washed SGV sample in solvents such as Ethanol, Methanol, Acetonitrile, Acetone, and Water. The weight of the SGV after (48) hours was measured and the swelling of the SGV was calculated as percentage using the following equation [47].

$$\text{swelling}\% = \frac{m_t - m_0}{m_0} \times 100$$

m_o = weight of dry sample

m_t = weight of hydrate sample

2.6. Packing of the Column:

Packing of the column is considered very necessary to separation of materials by chromatographic system. In this work, a blank column tube was used after cleaning with 50% nitric acid then washed with water and dried. It is necessary, packing of blank column with a suitable stationary phase [48].

By our research team, the column was packed reasonable results new method device. The slurry was prepared by an ultrasonic bath and the dry SGV needed to fill up the column tubing with methanol was weighed. The column was replaced with the fitting from one side and hanged to a clamp near the open end. It was placed in the sonicator bath filled with water.

Four grams SGV was homogenized with 50ml deionized water. few milliliters (1-2ml) of the slurry were poured into the column using a dropper and the sonicator turned on. After about 15 minutes which was found to be enough to lead the SGV to settle down inside the column, another portion was poured in. This process was continued until the column was completely filled with the SGV as found by visual inspection. The column fitting was re-placed on the open side and connected to the liquid delivery pump of the HPLC. The pump was set at the beginning to deliver 0.5ml/min distilled water. The back pressure was monitored until it reached a stable value of 2.8 MPa. The flow rate was then increased by 1 ml increments until reaching 5ml /min. The recorded back pressure was 4.9MPa, 7.8MPa, 10MPa, 12.5MPa, respectively. After the compression process was completed, one side of the column opened (the one used for packing) and inspected for complete packing. More stationary phase was poured into the column when it was found not completely full. These steps, packing and compression were repeated

until having a good packed column. The column was connected to the HPLC system. The flow rate was set at 1ml/ min, using distilled water.

2.7. Sample Preparation:

A stock solution of 200ppm of the standard of each vitamins was prepared by dissolving 5mg of each of vitamin (C, B_2, B_6 and B_{12}) and diluted to 25 ml in methanol. The solvent used to prepare these solutions was the same as in the mobile phase employed for their separation. Also vitamin C, 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.

2.8. Sample Analysis:

HPLC using new stationary phase (silica gel– crystal violet) column with different mobile phase were employed to analyze all the prepared standard solutions of some vitamins. Phenylalanine, C, B_2, B_6 and B_{12} were chromatographed using different concentrations of each sample. Concentration of vitamins , ranging from 1 – 100 ppm, were injected and analyzed at least three times with best mobile phase. These samples were analyzed with different ratio from Dichloromethane and hexan. The retention time (**t***R*$_{\mathbf{R}}$*R*), Plate number (N) and height (H) of studied compounds was recorded and as listed in Table **(2-2)**. Different concentrations of vitamins and their mixtures were analyzed after optimizing the chromatographic conditions. Calibration curves of all studied analytes were constructed by ploting

the calculated peak area of each analyte versus concentration. The range of the linear calibration equation, regression, and detection limit were calculated. The same samples were analyzed with C-18 column using the literate parameter as listed in Table (2-3).

Table (2-2): t_R , N and H of vitamins at Dichloromethane and hexane(5:95, (v/v)) as the mobile phase., silica gel- crystal violet column (15× 0.4 cm (Id.)), flow rate 1ml/min, sample loop 50µl, detection wave length 230nm.

Name of compound	Retention Time t_R	Width (cm)	Plate number N	Height H(cm)
			$N=16(t_R/W)^2$	H=L/N
Vitamin C	1.365	0.31	310.21	0.048
Vitamin B_6	1.922	0.4	369.40	0.04
Vitamin B_2	2.309	0.5	341.21	0.043
Vitamin B_{12}	2.867	0.57	404.78	0.037

Note: - the parameter t_R , has been calculated by LC solution program while the N and H values has been calculated manually.

Table (2-3): t_R , N and H of vitamins Using C-18 column (25 x 0.4 cm (i.d.)), detection wavelength 230nm, flow rate 1 ml/min, sample loop 50µl and using Acetonitrile and 50Mm NaH_2PO_4 pH 2.5 as the gradient mobile phase.

Name of compound	**Retention Time t_R**	**Width (cm)**	**Plate number N**	**Height H(cm)**
			$N=16(t_R/W)^2$	H=L/N
Vitamin C	3.5	0.4	1225	0.020
Vitamin B_6	5.7	0.6	1444	0.017
Vitamin B_{12}	8.1	0.6	2916	0.008
Vitamin B_2	8.8	0.57	3813	0.006

Chapter Three

Results&Discussion

3.1. Preparation of SGV Stationary phase:

The addition of few milliliters of dilute crystal violet solution to silica gel has resulted in the conversion the white stationary phase to deep violet. This color change of the stationary phase may be attributed to the interaction between the positive charged quaternary ammonium groups of the crystal violet with negatively charged hydroxyl group on the silica gel. This interaction may be represented in figure (3 -1).

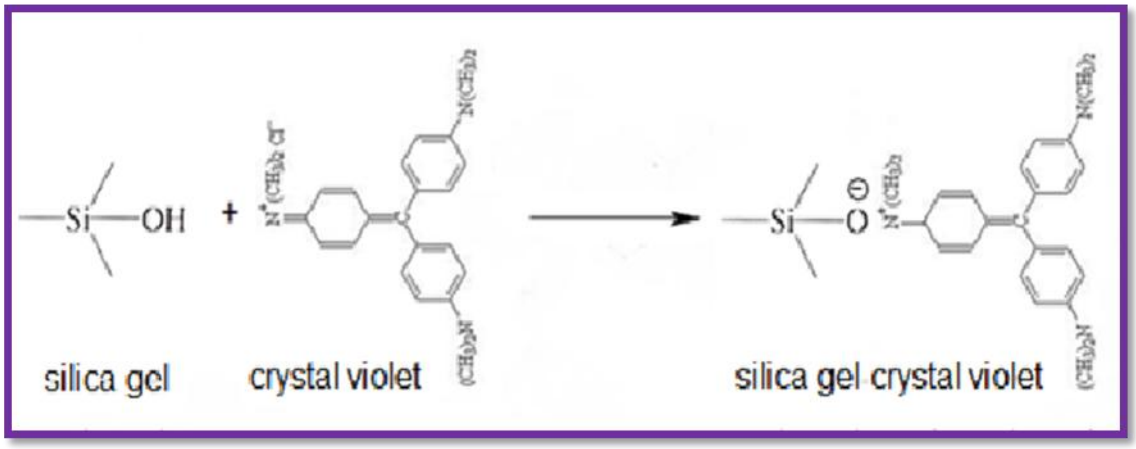


Figure (3 -1): Suggested interaction between silica gel and crystal violet.

The new SGV was washed few times with methanol in order to remove the excess crystal violet on the SGV. The final wash solution has been examined photometrically and has shown no absorbance and the baseline was stable at the absorbance wavelength range of the crystal violet (595) nm. The color of the SGV has become violet, which indicates that the SGV is still holding the crystal violet moiety. The addition of few milliliters of 0.1M HCl solution to this new stationary phase has turned the color of the SGV immediately to blue. It is, however, turned back to violet coloration when few milliliters of 0.1M KOH solution were added. This color change indicates that the one or both of the amine groups on the new stationary phase still having their acid-base characteristic. This

equilibria process maybe represent by the following suggested equation shown in figure (3 -2) As a consequence, the new stationary phase might act as uncharged SGV in basic solution, and positively charged in acidic solution.

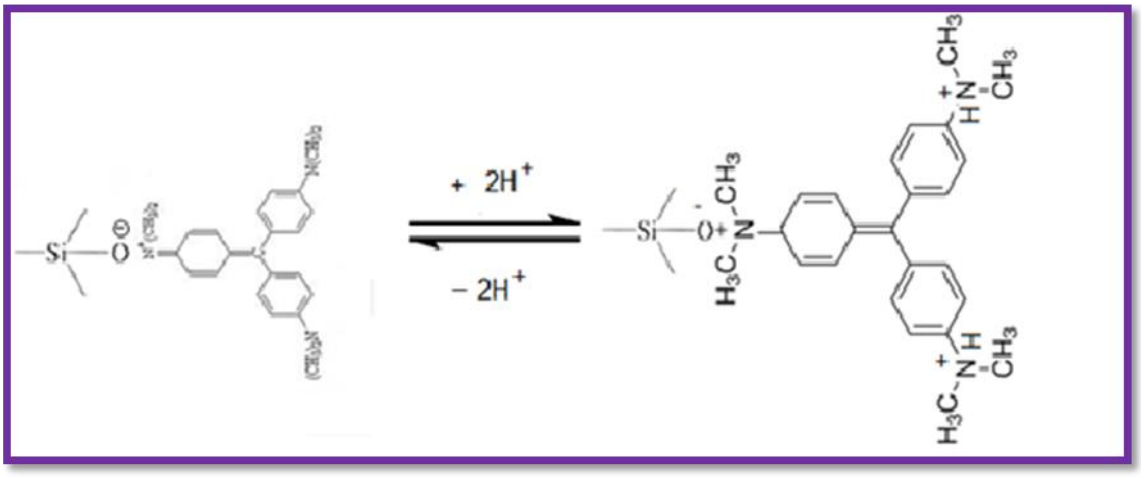


Figure (3-2): Suggested acid-base equilibria equation of the silica gel – crystal violet.

3.2. Stability Test of the Prepared SGV stationary phase:

The stationary phase was washed with methanol, dried in the oven, and then tested for its stability against several organic solvents with different polarities. These solvents include acetonitrile, ethanol, chloroform, acetone, hexane, petroleum ether, and benzene. 0.2 g of the new stationary phase was mixed with (10-15) ml of the solvent with continuous stirring for about 20 minutes. The SGV was led to settle after that period. The solvent was then decanted and the absorbance was measured at optimum λ max (595) nm of the crystal violet. No absorbance reading was noticed with all these solvents. These results showed that the crystal violet is strongly held by the silica gel. The **FTIR** spectra for each of crystal violet, silica gel , and silica gel – crystal violet are shown in Figure(2- 1). Figure (2 -1, A) shown a (O-H) at 3508 cm-1.

Figure (2 -1, B) shown a (C-N) at 1170.79 cm-1, (C-H) bending at 1361.74cm-1, (C=C) aromatic over lap (C=N) at 1583.56 cm-1, (C-H) aliphatic at 2859.62 cm-1, (C-H) aromatic at 3095.53 cm-1. The spectrum of the new prepared stationary phase as show in figure (2 -1, C) have some distinguished peaks of the original crystal violet. It is clear that the spectrum of the new stationary have (N-O) at 962.48 cm-1, (N-H) bending at 1085 cm-1, (C-N) at 1280 cm-1,(C=C) over lap (C=N) at 1593cm-1, (O-H) at 3463 cm-1. After comparing the spectrum of new stationary phase and silica gel. The new band may be representing the bond between quaternary ammonium group on the crystal violet and hydroxyl group on the silica gel. However, further spectrophotometric studies are required in order to suggest a proper structure of the new stationary phase and how it is attached to the silica gel and consequently the new functional groups on the prepared SGV.

3.3. Swelling of the modified stationary phase:

Swelling of the modified SGV to be used as stationary phase and its performance when packed in an HPLC column is of a considerable practical importance. In HPLC applications, resin particles are packed into a column; swelling will cause the packed bed to be tightened and subsequently will affect the flow of the mobile phase through the column producing excessive back pressure, and will cause stationary phase bed to fracture. The reverse, or stationary phase bead contraction, can be equally undesirable in column operation. In this case, the flow rate is sharply changed due to shrinking of the stationary phase particles, and channeling, particularly at the column walls. This has also a significant effect on how the mobile phase flows through the column [(48)].

Swelling test for prepared stationary phases was performed according to ASTM (Standard Test Method) procedure [(49)]. The degree of cross-

linking has been measured using polar, moderately polar and non polar that are usually used in HPLC such as solvents methanol, acetone, acetonitrile, and hexane. The results of the swelling tests are listed in Table (3-1) which indicates the swelling value. These results were theoretically expected; the values were ranged from 1.1-3.8% compared with the expected swelling of 3.6%**,** but that in water was unexpected because it was over 3.6% which could be attributed to the presence of hydrogen bonding forming moiety as the resin surfaces.

Table (3-1): Swelling of SGV stationary phase.

Stationary phase	*solvents*	***Swelling percentage (%)***
SGV	Water	3.8
	Acetone	1.4
	Hexane	1.1
	Acetonitrile	1.2
	Methanol	1.0

3.4. Solubility tests for SGV:

The solubility has been examined using different solvents such as acetonitrile, benzene, chloroform, DMF, DMSO, hexane, methanol and water. It is found that the stationary phase was insoluble and undecompose in all these solvents. It is found that these stationary phase was stable and remained unchanged. These results were attributed to the high cross-linking of the prepared stationary phase.

3.5. Column Packing:

The packing of column plays very important role in separation of materials. It has been often stated that the column is the heart of the liquid chromatography. On the other hand, the column packing has an influence on the quality of the chromatographic system.

There are several acceptable methods when packing a column. The slurry has to transport into the column very quickly by a high – pressure pump so as to prevent the solid substances that forms at the bottom of column, where these sedimentation will weaken the separation performance. In this work, we employed an ultrasonic bath for homogenizing the slurry before and during packing. The column was connected to the HPLC system and operated under a pressure for long period of time when the packing was finished. The packed column was operated at flow rate of 5 ml/min, which has produced a backpressure of 13.3 Mpa. At this maximum pressure, nearly 1000 ml deionized water was pumped from one end of column so that prevent a sudden fall of the packing.

Despite of operating the packed column for a long times, the recorded pressure was existing all the time, which indicating establish and uniform packing. Just then, the column was connected to the chromatograph and

the flow rate set at 1 ml/min with distilled water for two hour. So as to make certain if there was any removing of crystal violet to the silica gel, the detector wavelength was set as optimum wave length (595) and the baseline were recorded at this wavelength through that period. After this work, the pressure of the column was sustained as well as the baseline was very stable (at zero reading). To make clear if the column packing has been completed, visual testing for the column was made many times.

At end, there is no decrease or shortage in the packing material inside the column therefore there are very successful packing with this method.

3.6. Column Evaluation:

Since this work includes packing of a new column, its performance should be tested. This has been done by evaluation the plate number(N), height equivalent to theoretical plates HETP(H) , and capacity factor, K' , for different chromatographic runs. The plate number of column (N) *was calculated using the well-known equation* [8]*;*

$$N = 16(\frac{t_r}{w})^2 \quad \textbf{Or} \quad N = 5.54(\frac{t_r}{W_{1/2}})^2$$

Where $\mathbf{t_r}$ is retention time, (**W**) and ($\mathbf{W_{1/2}}$) are the peak width at baseline and half height, respectively. The $\mathbf{t_r}$, **W**, $\mathbf{W_{1/2}}$ and other subsequent parameters were calculated using the optimum flow rate. The optimum flow rate was measured by plotting (**H**) versus different flow rates. The **H** values were calculated from **H=L/N** (where **L** is the length of the column). Figure (3-3) shows the plots of **H** versus the mobile phase flow rate for new stationary phase.

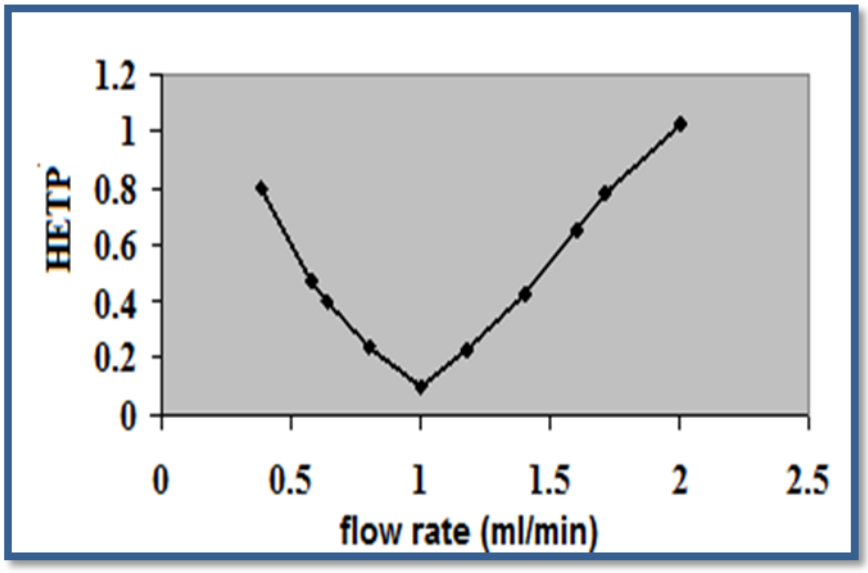
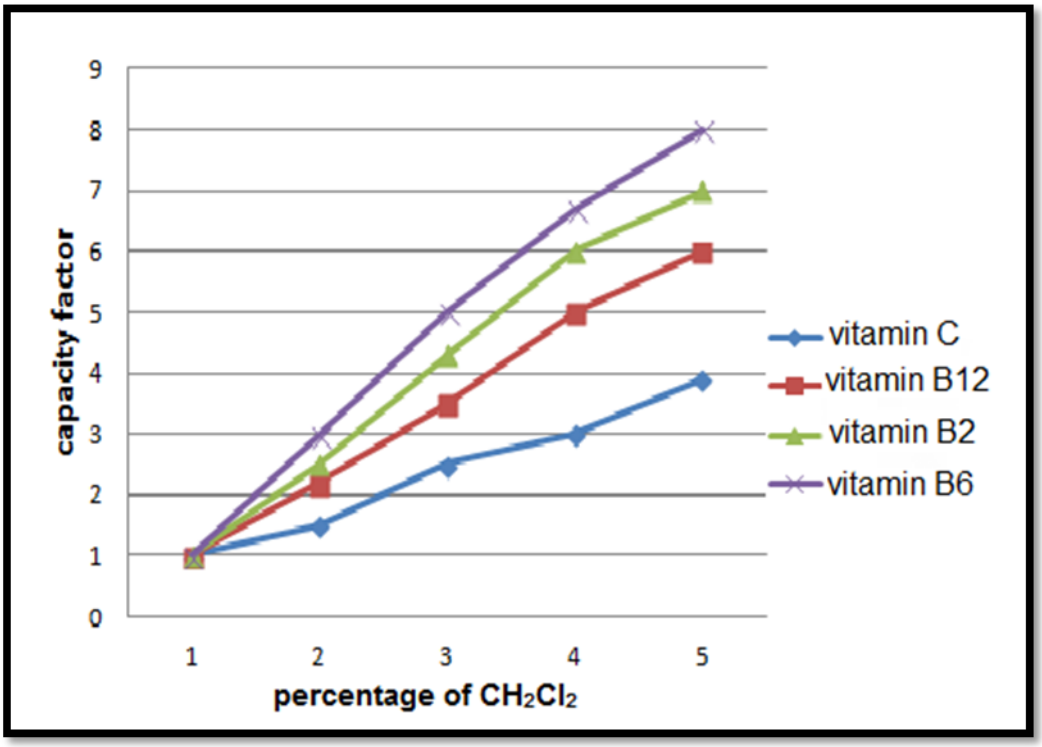


Figure (3-3): Plot of plate height H versus flow rate, silica-gel-crystal violet column (15×0.4 cm (id)). Eluent 5% dichloromethane -95% hexane, detection wavelength 230nm 25ppm of vitamin C.

The optimum flow rates were measured by analyzing vitamin C at different mobile phase flow rate ranging from 0.1-2.5ml/min. A plot of **H** versus flow rate has given minimum **H** near 1ml/min for the silica gel-crystal violet column. Some of the measured chromatographic parameters such as plate numbers **N**, plate heights **H**, capacity factor **Ќ**, separation factor **α**, and peak asymmetry for newly packed column were then calculated using this optimum flow rate. These parameters were measured by chromatographic analysis of vitamin C, B_2, B_6 and B_{12}. The ion ratio, which is commonly called capacity factor **Ќ**, is an important term that describes the interactions between the stationary phase and mobile phase. **Ќ** is independent on the column length, mobile phase flow rate and represents the molar ratio of the compound in the stationary phase, to that in the mobile phase. Capacity factor between1.5-5.0 is preferred [(92)].Lower **Ќ** value indicates no stationary phase. Interaction occurs and hence no chromatography. However, large **Ќ** value is accompanied by

long analysis times. The capacity factor for the samples chromatographic on silica gel-crystal violet column with hexane with different percentage of dichloromethane as eluent was calculated. Percentage dichloromethane has been found to affect the capacity factor **Ќ.** Changing the percentage of distilled water in methanol as a mobile phase from 2% to ≥10% has a great effect on Ќ and consequently on α when using silica gel-crystal violet as separating column as it is shown in figure (3-4) and listed in Table (3-2).



Figure(3-4): Plot of Capacity factor, versus present of Dichloromethane using silica gel–crystal violet column (15×0.4 cm (id)), flow rate 1ml/min, detection wavelength 230nm and 10ppm of vitamin C,B_2,B_6 and B_{12}

Table (3-2): 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 Dichloromethane in mobile phase***									
	1%		***2%***		***3%***		***4%***		***5%***	
	Ḱ	**α**	**Ḱ**	**α**	**Ḱ**	**α**	**Ḱ**	**α**	**Ḱ**	**α**
C	1.29	--	1.33	--	1.56	--	1.62	--	1.83	--
B_{12}	1.52	1.17	1.66	1.27	1.85	1.36	2.11	1.57	2.99	1.63
B_2	2.51	1.12	2.85	1.18	2.99	1.20	3.54	1.23	3.80	1.27
B_6	3.02	1.11	3.55	1.09	4.02	1.15	4.50	1.2	4.96	1.3

--Not detected

Capacity factor Ḱ was ranged from 1.29-3.02, 1.33-3.55, 1.56-4.02, 1.62-4.50 and 1.83-4.96 at 1%, 2%, 3%, 4% and 5% Dichloromethane. In the mobile phase. These results indicate that the best indicated competitive interaction was between these compounds in the stationary phase and the mobile phase at percentage 5% of Dichloromethane.

Separation factor α was ranged from 1.11-1.17, 1.09-1.27, 1.15-1.36, 1.2-1.57 and 1.27-1.63 at 1% 2%, 3%, 4% and 5% Dichloromethane in the mobile phase, respectively. At 5% Dichloromethane gave good separation factors were obtained, as it is shown in figure (3-5). These results are summarized in Table (3-2).

The chromatograms of vitamins C, B_2, B_6 and B_{12} with the silica gel–crystal violet column, using 50µl sample loop, with flow rate 1ml/min are shown in figure (3-5). The mobile phase was a mixture of 5% Dichloromethane and 95% hexane. The vitamins analyzed and had given well shaped peaks as well as good detector responses. The more polar

vitamin B_6 has a retention time at 2.867min and the nonpolar vitamin C has lower retention time at 1.365min. Table (3-3) shows the values of t_R, Ќ, Rs and peaks asymmetry of the studied water soluble vitamins.

This mean that the mechanism of interaction between vitamins and stationary phase depended on the polarity, and because appearance of nonpolar first means that the stationary phase was relatively polar and the interaction depended on the hydrogen bonding of N-H and O-H groups of vitamins with O-H groups of silica gel–crystal violet of Column.

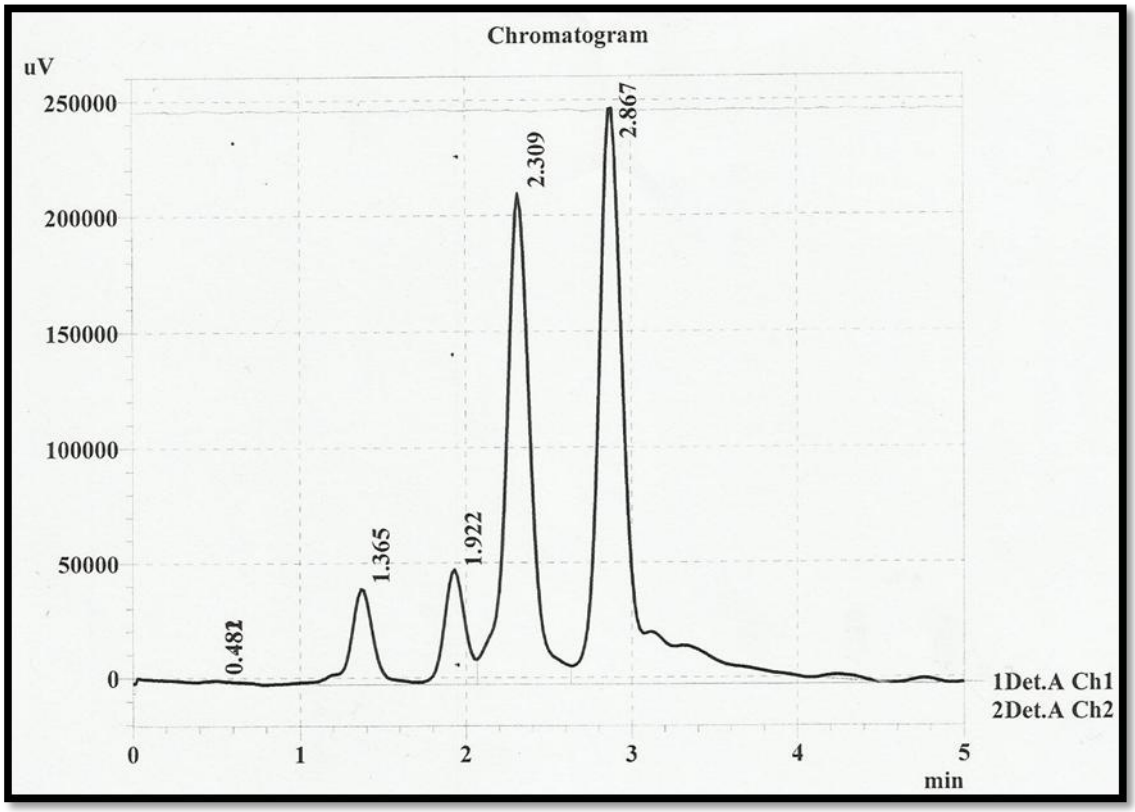


Figure (3 -5): Chromatogram of mixture vitamins (1) C 10 ppm , (2) B_2 25 ppm ,(C) B_{12} 15 ppm , (2) vitamin B_6 20 ppm. Using silica gel-crystal violet column (15 x 0.4 cm (i.d.)), detection wavelength 230nm, flow rate 1ml/min, Dichloromethane and hexane (5:95, (v/v)) as the mobile phase.

Table (3-3): Separation factor, peak asymmetry, and capacity factor for the separated vitamins compound using silica gel –crystal violet (15 x 0.4 cm (i.d.)). Dichloromethane and hexane (5:95, (v/v)) as the mobile phase.Flow rate 1 ml/min, sample loop 50 µl , detection wavelength 230nm..

Name of compound	Retention Time t_R	Capacity factor Ḱ	Separation Factor $_{(\alpha)}$	Resolution factor (Rs)	Peaks Asymmetry
		$k=(t_R-t_0)/t_0$	$\alpha=k_2/k_1$	$Rs = \frac{2(tR2 - tR1)}{W2 + W1}$	Tf= (a+b)/2a
Vitamin C	1.365	1.83	_	_	1.1
Vitamin B_{12}	1.922	2.99	1.63	1.56	1.02
Vitamin B_2	2.309	3.80	1.27	0.86	0.99
Vitamin B_6	2.867	4.96	1.3	1.04	1.18

Note: - tm= 0.481 by LC solution program.

Vitamin C, B_{12}, B_2 and B_6 samples were chromatographed using commercial C-18 column, with flow rate of 1ml /min. The mobile phase used was A)Acetonitrile B)50Mm NaH_2PO_4 pH 2.5, Gradient 3min 100%B, 6min 80%B,15min 60%B[(50)], As it is showed in Fig (3-12). These analyzed vitamins have given symmetrical peaks as well as good detector response. Table (3-4) shows the value of Separation factor, peak asymmetry, and capacity factor for the studied water soluble vitamins.

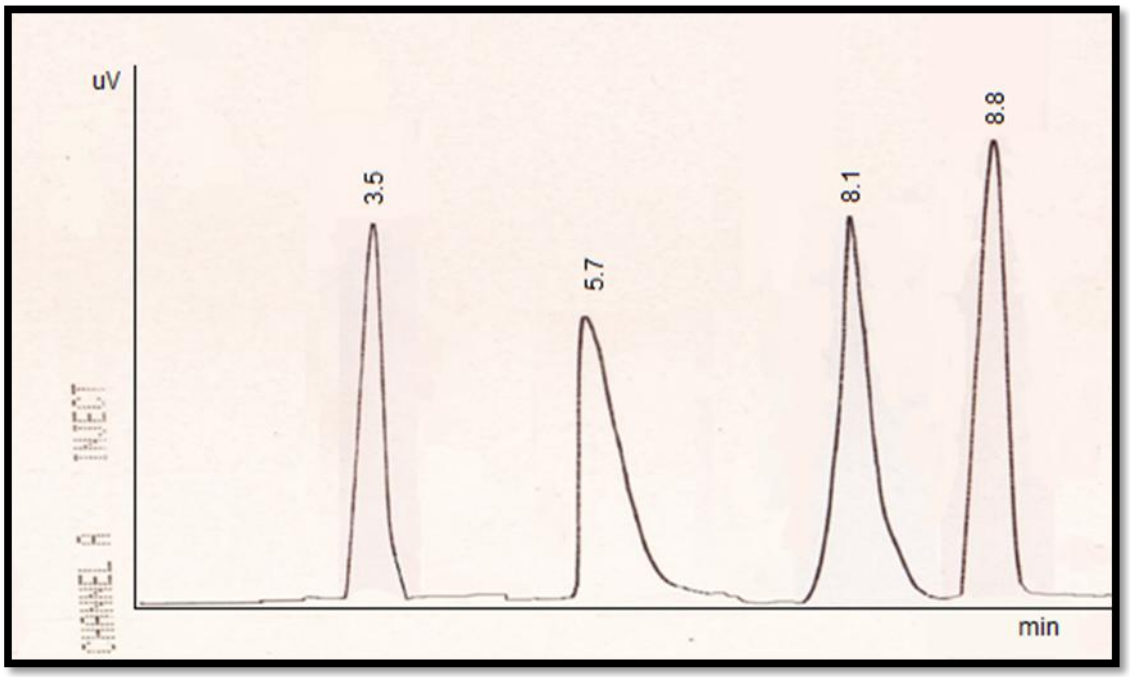


Figure (3-6): Chromatogram of mixture vitamins (1) C 30 ppm , (2) B_6 30 ppm ,(C) B_{12} 30 ppm , (2) vitamin B_2 30 ppm. Using column C18 (25*0.4mm (ID)), flow rate 1ml/min, detection wavelength 230nm, Acetonitrile and 50Mm NaH_2PO_4 pH 2.5 as the gradient mobile phase.

Table (3-4): Separation factor, peak asymmetry, and capacity factor for the separated vitamins compound Using column C18 (25*0.4mm (id)),detection wavelength 230nm, flow rate 1ml/min, detection wavelength 230nm, Acetonitrile and 50Mm NaH_2PO_4 pH 2.5 as the gradient mobile phase .

Name of compound	Retention Time t_R	Peak width (cm)	Capacity factor Ḱ	Separation Factor (α)	Resolution factor Rs	Peaks Asymmetry
			$k=(t_R-t_0)/t_0$	$\alpha=k_2/k_1$	$Rs = \frac{2(tR2 - tR1)}{W2 + W1}$	Tf= (a+b)/2a
Vitamin C	3.5	0.4	1.27	_	_	1.01
Vitamin B_6	5.7	0.6	2.7	2.1	4.4	0.98
Vitamin B_{12}	8.1	0.6	4.2	1.5	4	1.06
Vitamin B_2	8.8	0.57	4.7	1.1	1.2	1.14

Table (3-5) shows the comparison between the silica gel- crystal violet and C-18 columns in term of effective plate number, plate height, retention time, capacity factor and separation factor. F-test was also calculated at 95% C.L. and the values were ranged from (6.55-6.75). The calculated values greater than F values (6.39) This is due to the number of functional groups in silica gel- crystal violet stationary phase is more than the functional groups in C-18, this mean that the interaction of analytes with SGV was better.

Table (3-5): separation factor, capacity factors, and peak asymmetry for vitamins, (I) silica gel- crystal violet (15×0.4cm (i.d)) and (II) C-18column (25×0.4cm (i.d)).

Compounds			R_t	***N***	***H***	***Ḱ***	***Peak asymmetry***
#	***Name***	***Col***					
1	***Vit. C***	***I***	1.365	310.21	0.048	1.83	1.1
		II	3.5	1225	0.020	1.27	1.01
2	***Vit B_6***	***I***	1.922	369.40	0.04	2.99	1.18
		II	5.7	1444	0.017	2.7	0.98
3	***Vit B_{12}***	***I***	2.309	341.21	0.043	3.80	1.02
		II	8.1	2916	0.008	4.2	1.06
4	***Vit B_2***	***I***	2.867	404.78	0.037	4.96	0.99
		II	8.8	3813	0.006	4.7	1.14

3.7. Quantitative Analysis:

Calibration runs of vitamins compounds on silica gel – crystal violet stationary phase column, in Dichloromethane and hexane (5:95, (v/v)) as a mobile phase are shown in Figure (3-14). A linear dependence of the peak areas on the amount injected is evident for all compounds down to the detection limits. Vitamins have shown a linear response range extending from at least 50 ppm to the detection limit which was at low as 1 ppm for most analytes.

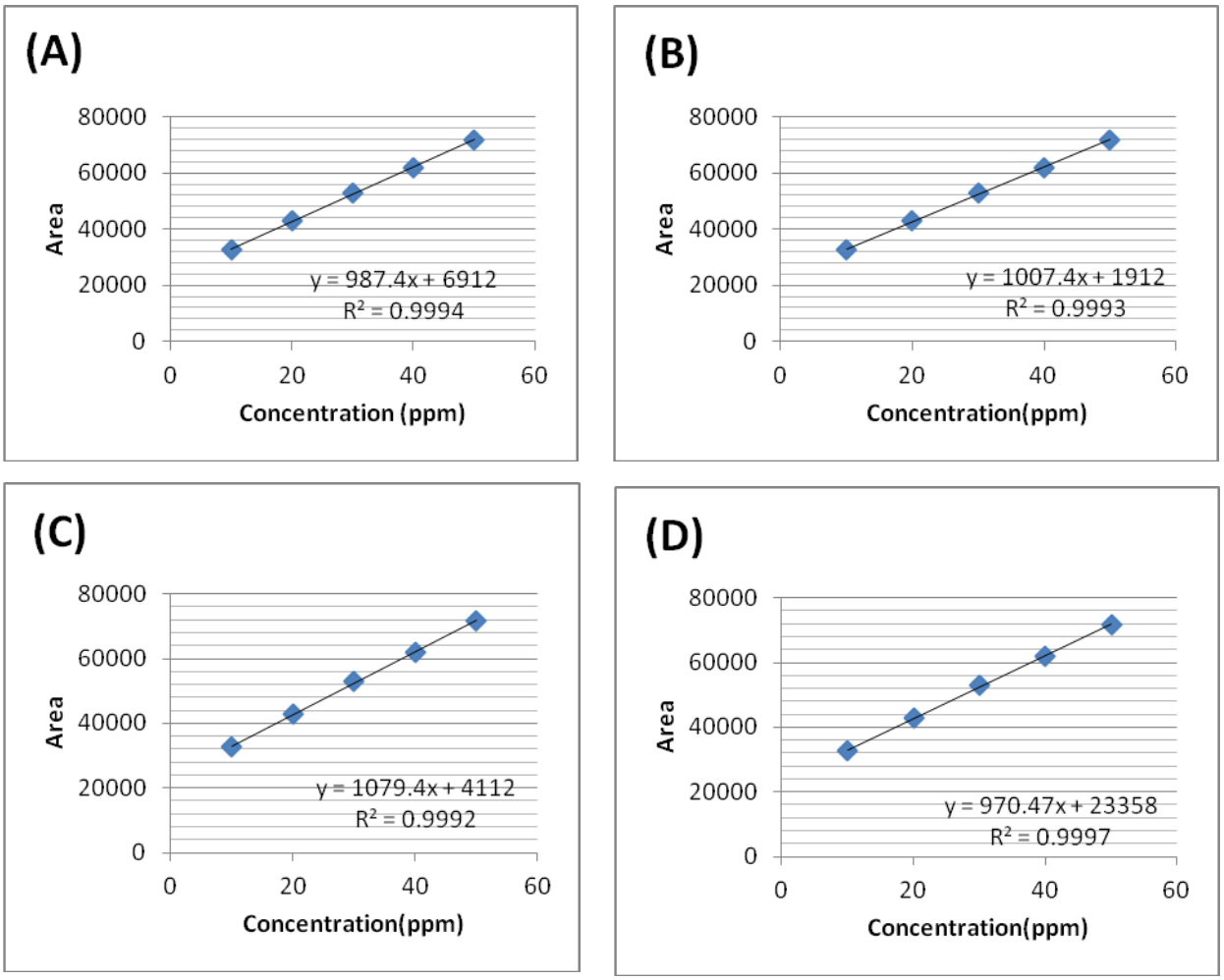


Figure (3-7): Calibration curve for (A) vitamin C, (B) vitamin B_2, (C) vitamin B_{12}, (D) vitamin B_6. Samples were analyzed Using silica gel - crystal violet column (15 x 0.4 cm (i.d.)), detection wavelength 230nm, flow rate 1 ml/min, and using Dichloromethane and hexane (5:95, (v/v)) as the mobile phase.

The slope values for the linear calibration curves were range from (970.47- 1079.4) depending upon kind of vitamin, the slope value as shown in Table (3 -6). The correlation coefficients for all calibration lines were ranged from (0.9992- 0.9997) with average value (0.9994). The detection limits of the vitamins are also shown in Table (3 -6). It ranged from (1- 3) ppm for all vitamins in Dichloromethane and hexane (5:95, (v/v)) as the mobile phase. These detection limits were calculated at S / N ratio of 3 or more.

Table (3 -6): linear equation , correlation coefficients R^2 , and detection limits , samples were analyzed Using silica gel – crystal violet column (15 x 0.4 cm (i.d.)) ,detection wavelength 230nm , flow rate 1 ml/min , and using Dichloromethane and hexane (5:95, (v/v)) as the mobile phase.

Compounds	***Linear Equation***	***R^2***	***Detection Limit (ppm)***
Vitamin C	y = 987.4x + 6912	0.9994	2
Vitamin B_2	y = 1007.4x + 1912	0.9993	3
Vitamin B_{12}	y = 1079.4x + 4112	0.9992	2
Vitamin B_6	y = 970.47x + 3358.3	0.9997	1

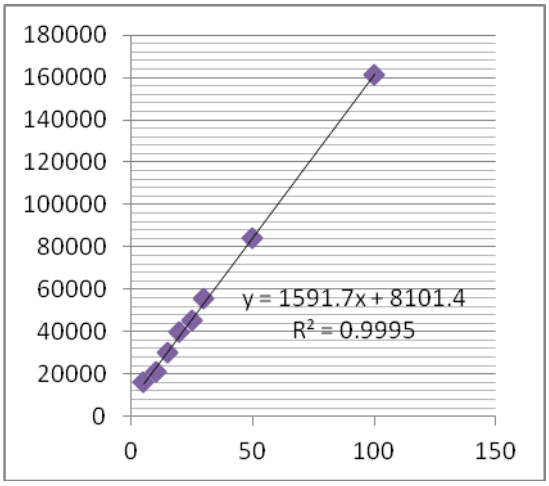
Prepared standard mixture solutions of the analyzed vitamins were injected for at least 3 times under the same condition and their concentration were calculated by measuring the peak area of each vitamin and using their respective the linear equation. The recovery were ranged from 96.44% to 97.96% with an average of 97.17%, the relative errors were ranged from (-2.04_-3.56) % with an average of -2.83% as shown in Table (3 -7).

Table (3-7): % R.S.D , %Recovery, and % Relative Error for the Analyzed vitamins Using silica gel – crystal violet column (15 x 0.4 cm (i.d.)) ,detection wavelength 230nm , flow rate 1 ml/min , and using using Dichloromethane and hexane (5:95, (v/v)) as the mobile phase.

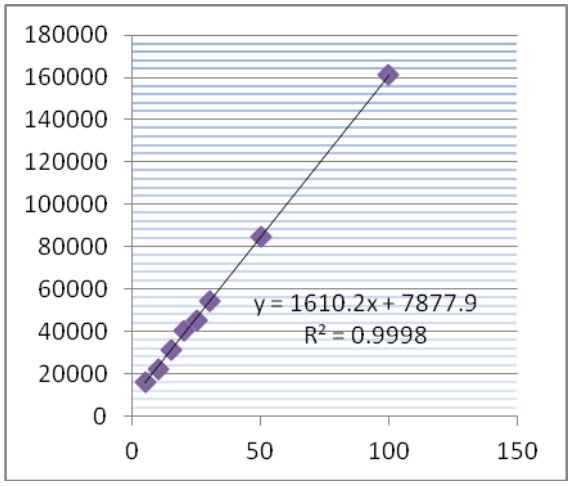
vitamins	Concentration injected (ppm)	Average concentration Found(calculated) * (ppm)	Absolute Error	Relative Error %	Recovery %	%R.S.D
Vitamin(C)	25	24.20	-0.8	-3.2	96.8	0.025
Vitamin(B_2)	25	24.37	-0.62	-2.52	97.48	0.04
Vitamin(B_{12})	25	24.11	-0.89	-3.56	96.44	0.065
Vitamin(B_6)	25	24.49	-0.51	-2.04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

***Average of three measurement.**

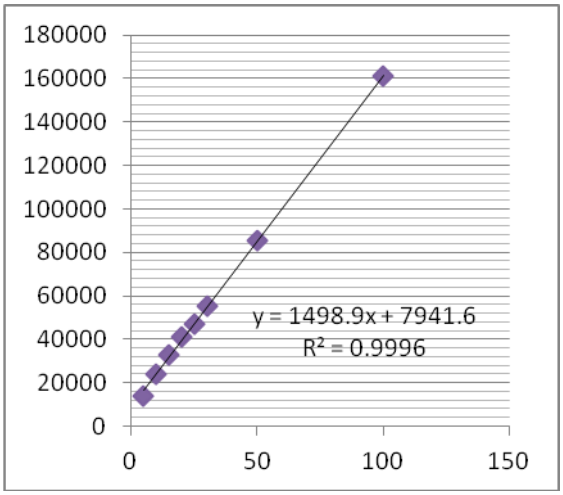
Calibration runs of vitamins compounds on C18column (25 x 0.4 cm (i.d.)), detection wavelength 230nm, flow rate 1 ml/min, and using Acetonitrile and 50Mm NaH_2PO_4 pH 2.5 as gradient mobile phase. are shown in Figure (3-8). A linear dependence of the peak areas on the amount injected is evident for all compounds down to the detection limits. Vitamins have shown a linear response range extending from at least 100 ppm to the detection limit which was at low as 1 ppm for most analytes as shown in Table (3 -8).



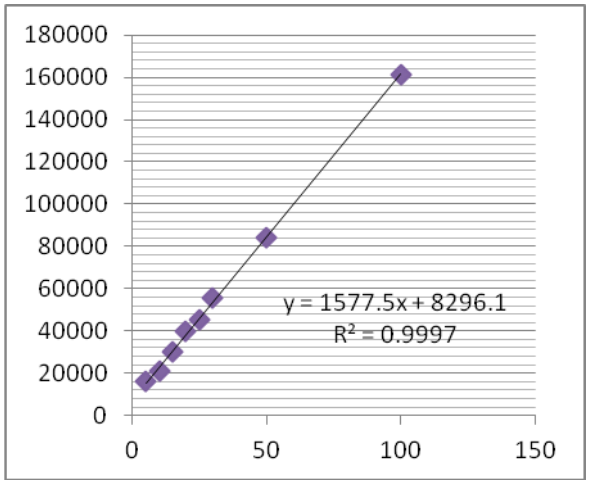
(A)



(B)



(C)



(D)

Figure (3-8): Calibration curve for (A) vitamin C, (B) vitamin B2, (C) vitamin B_{12}, (D) vitamin B_6. Samples were analyzed Using C-18column (25 x 0.4 cm (i.d.)), detection wavelength 230nm, flow rate 1 ml/min, and using Acetonitrile and 50Mm NaH_2PO_4 pH 2.5: as the gradient mobile phase.

Table (3 -8): linear equation , correlation coefficients R , and detection limits , samples were analyzed Using C18column (25 x 0.4 cm (i.d.)), detection wavelength 230nm, flow rate 1 ml/min, and using Acetonitrile and 50Mm NaH_2PO_4 pH 2.5 as the gradient mobile phase .

Compounds	***Linear Equation***	***R^2***	***Detection Limit (ppm)***
Vitamin C	y = 1591.7x + 8101.4	0.9995	1
Vitamin B_2	y = 1610.2x + 7877.9	0.9998	2
Vitamin B_{12}	y = 1498.9x + 7941.6	0.9996	1
Vitamin B_6	y = 1577.5x + 8296.1	0.9997	3

Prepared standard mixture solutions of the analyzed vitamins were injected for at least 3 times under the same condition 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 95.36% to 97.12% with an average of 96.3%, the relative errors were ranged from (-2.88_-4.46) % with an average of -3.7% as shown in Table (3 -9).

Table (3-9) % R.S.D , %Recovery, and % Relative Error for the Analyzed vitamins Using C18column (25 x 0.4 cm (i.d.)), detection wavelength 230nm, flow rate 1 ml/min, and using Acetonitrile and 50Mm NaH_2PO_4 pH 2.5 as the gradient mobile phase .

vitamins	Concentration injected (ppm)	Average concentration Found(calculated) * (ppm)	Absolute Error	Relative Error %	Recovery %	%R.S.D
Vitamin(C)	25	23.84	-1.16	-4.64	95.36	0.10
Vitamin(B_2)	25	23.96	-1.04	-4.16	95.84	0.06
Vitamin(B_{12})	25	24.22	-0.78	-3.12	96.88	0.10
Vitamin(B_6)	25	24.28	-0.72	-2.88	97.12	0.04

Table (3-10) shows the comparison between the silica gel- crystal violet and C-18 columns in term of Correlation coefficients R, detection limit, recovery and percentage relative error.

Table (3-10) Correlation coefficients R, detection limit, recovery and percentage relative error of vitamins using (I) silica gel- crystal violet (15×0.4cm (i.d)) and (II) C-18column (25×0.4cm (i.d)).

Compounds	*Column*	R^2	*Detection limit*	*Recovery*	*Relative error*
Vit. C	***I***	0.9994	2	96.8	-3.2
	II	0.9995	1	95.36	-4.64
Vit B_2	***I***	0.9993	3	97.48	-2.52
	II	0.9998	2	95.84	-4.16
Vit B_{12}	***I***	0.9992	2	96.44	-3.56
	II	0.9996	1	96.88	-3.12
Vit B_6	***I***	0.9997	1	97.96	-2.04
	II	0.9997	3	97.12	-2.88

Conclusion:

New stationary phase were prepared by the reaction of crystal violet solution with silica gel. The stability of the new attached functional group on the SGV was studied against different solvents. The new prepared stationary phase was found stable and no depletion of the crystal violet moiety from the SGV was noticed with the above materials. The new stationary phase has been packed into stainless steel column. This has been done by special technique. The chromatographic performance of the packed column was characterized. The number of plate numbers N, height equivalent of a theoretical plates H, capacity factors Ḱ, selectivity factors α, Resolution R_S and peaks asymmetry, were measured by analyzing different analytes on the new column using different mobile phase's compositions and flow rates. These parameters were measured from analysis four vitamins C, B_{12}, B_2 and B_6. The analysis of vitamins using silica gel – crystal violet column has performed with Dichloromethane and hexane (5:95, (v/v)) as a mobile phase and in flow rate 1 ml/min and detection wavelength 230 nm. This new stationary phase has dual characteristics of both ionic and hydrophobic properties which can be used in both directions by adjusting the condition of separation.

Future Work:

We strongly suggest the following to evaluate the new stationary phase And it's potential to separate wide varieties of samples.

1-Using smaller stationary phase particles.

2-Using gradient elution mode for the separation.

3-Analysis of other type of compound as well as poly aromatic compound.

4- Carry out an intense spectrometric and chemical analysis to better understand the functional groups on the resin that effecting the chromatographic separation.

5-Using other detectors such as refractive index, for example in order to detect the vitamins and other non UV – Visible absorbing species.

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الخلاصة:

تم تحضير طور ثابت جديد بطريقة كروموتوكرافيا السائل ذات الاداء العالي من ارتباط السليكاجل مع صبغة بنفسجيه (crystal violet) . وقد درست الاستقرارية للمجموعة الفعالة الجديدة المرتبطة مع مذيبات مختلفة مثل الميثانول,الايثانول,البنزين ,الكلوروفورم , الهكسان ,الاسيتونايترايل , الاسيتون والبترواليوم ايثر بالاضافة الى محاليل هيدروكسيد الصوديوم وحامض الهيدروكلوريك وقد وجد أن الطور الثابت الجديد مستقر ولم يلاحظ أي استنفاذ أو إفراغ. كما أثبتت تحاليل الأشعة تحت الحمراء ارتباط الصبغة مع الطور الثابت باستخدام المواد المذكورة أعلاه.تم تعبئة الطور الثابت الجديد في عمود من الفولاذ المقاوم للصدأ وذلك بسكب الطور الثابت في العمود الموضوع في حوض الموجات فوق الصوتية , وتم تعريض العمود للضغط اللازم للحصول على رص محكم بواسطة مضخة كروموتوكرافية. 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.

هذه المتغيرات تم قياسها بتحليل بعض الفيتامينات الذائبه في الماء مثل (B_6و C, B_{12}, B_2) تحليل هذه الفيتامينات بواسطة العمود الجديد تم باستخدام طور متحرك يتكون من95% هكسان و5% داي كلورو ميثان فكان معدل الصفيحات النظرية (N) يساوي(356.4) وكان معدل الارتفاع المكافئ يساوي (0.042) سم، عوامل الاستيعاب تتراوح بين (4.96 –1.83) ، العوامل الانتقائية تتراوح بين (1.27-1.63)، والتناظر يتراوح بين (0.99-1.18)، ودرجة الفصل تتراوح بين (0.86-1.56) ،الانحراف القياسي النسبي المئوي كانت قيمته ضمن المدى %(0.025-0.065)، والاسترداد المئوي كان بحدود المدى %(96.44-97.96)، والخطأ النسبي كان ضمن حدود المدى % (-3.56_-2.04). منحنيات المعايرة للمركبات المحللة كانت خطية من الحد الأدنى لكشفها الى 50 جزء بالمليون مع عوامل ترابط تتراوح بين (0.9992- 0.9997).

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
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قسم الكيمياء

تحضيرطور ثابت جديد لتقنية HPLC ودراسة أدائه بأتجاه فصل بعض الفيتامينات الذائبه في الماء

رسالة
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وهي جزء من متطلبات نيل درجة الماجستير في الكيمياء

من قبل

سيف سعد عبد الستار

بكالوريوس 2009 (جامعة النهرين)

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