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Chromatographic Separation of Some Amino Acids by High Performance Liquid Chromatography Method Using A New Stationary Phase

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By

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﴿وَ أَنْـــزَلَ اللـــهُ عَليـــكَ الكِتابَ
وَ الحِكُمَةَ و عَلمَكَ ما لَم تَكُـــن تَعلَم
وَ كانَ فَضلُ اللهِ عَلَيكَ عَظيـــماً﴾

صدق الله العظيم

سورة النساء (الآية 113)

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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.....وأحبلب قلبي.....

إخوتي وأخواتي

إلى كل من أحبني وأراد مودتي ..... أهدي ثمرة جهدي هذا .....

حبا" ووفاء"

عـلـي

Summary

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

The new stationary phase has been packed into stainless steel column. The pKa value for new resins has been calculated. The pKa for Weakly acidic – Neutral red resins were 7.15. 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 R_s 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 amino acids such as Histidine, Phenylalanine, Tryptophan, and Tyrosine. The analysis of amino acids using Weakly acidic – Neutral red column has performed using %10 acetonitrile in buffer phosphate as mobile phase.

The average value of plate numbers N was (316.59) . The plate heights H was averaged (0.085) cm , capacity factors Ḱ were ranged from (1.8 – 2.86). Separation factors ***α*** were ranged from (0.21-1.17), and peak asymmetry value were range from (0.99-1.09). Resolution R_s were ranged from (0.93 -3.77).

The analysis of amino acids using a new stationary phase has performed using buffer phosphate as mobile phase . The average value of plate numbers N was (234.79) , plate heights H was (0.107)cm , capacity factors Ќ were ranged from (1.13-4.73) , separation factors α were ranged from (0.23-1.22) , peak asymmetry value were range from (1.10-1.18) and resolution were (0.93).

R.S.D were ranged from(0.04 -1)% , recovery were ranged from (93.8-97.12)% , The relative errors were ranged from (-2.88 - -6.2) % .

Calibration curves for all analyzed compound were linear from their detection limits to at least 50 ppm with correlation coefficient ranged from(0.9991- 0.9996).The detection limits were ranged from(0.05-0.25) ppm at signal to noise ratio of three or more.

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List of Abbreviations

Abbreviation	Translation
BPC	Bonded Phase Column
FTIR	Fourier Transforms Infrared
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
HETP	Height Equivalent Theoretical Plate
i.d	inner diameter
LC	Liquid Chromatography
MPa	Mega Pascal
OTC	Open Tubular Columns
PAHs	Poly Aromatic Hydrocarbones
RBC	Reversed Bonded phase Chromatography
ODC	Octadecylsilane
RPC	Reversed Phase Chromatography
THF	Tetrahydrofuran
SFC	Supercritical-fluid chromatography
GPC	Gel-Permeation Chromatography
TAG	Triacylglycerols
DVB	Divinylbenzene
PLB	Porous Layer Beads
HILIC	Hydrophilic interaction

	chromatography
NP-HPLC	Normal phase HPLC
K	Distribution coefficient
Ḱ	Capacity factor
CPHX	Chlorophenoxamine
CAFF	Caffeine
CTHE	8-Chlorotheophylline
t_R	Retention time
N	Number of plate
HEPT	High plate

Chapter one

Introduction

1.1 Chromatography

Chromatography is a separation method that exploits the differences in partitioning behavior between a mobile phase and a stationary phase to separate the components in a mixture. IUPAC defines chromatography as: “a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase), while the other moves (mobile phase) in a definite direction. It may be liquid, gas or a supercritical fluid, while the stationary phase may be a solid, a gel, or a liquid.

Chromatography involves passing a mixture through a stationary phase, which separates the analyte to be measured from other molecules in the mixture and allows it to be isolated.[(1)] Chromatographic techniques are often named by listing the type of mobile phase, followed by the type of stationary phase. Thus, in gas–liquid chromatography the mobile phase is a gas and the stationary phase is a liquid. [(2)] The gas chromatography (GC) was found to be simple, fast and capable of producing separation of volatile materials that were impossible by distillation. It was natural to apply the successful results from GC to the older technique of liquid chromatography. [(3)]

Chromatographic separation occurs if the components of a mixture interact to different extents with the mobile and/or the stationary phases and therefore take different times to move from the position of sample introduction to the position at which they are detected. Therefore, the phases are chosen such that components of the sample have different solubility in each phase. The chromatographic methods can be classified as shown table (1-1). This classification is based on the type of the stationary-phase and equilibrium involved in separation. [(4)]

Table (1 -1). Classification of Chromatography Techniques. [4]

General classification	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	

A component which is quite interact in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase.

Components of a mixture may be interacted with the stationary phase based on charge, relative solubility or adsorption. Techniques such as High performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) use columns narrow tubes packed with stationary phase, through which the mobile phase is forced. The sample is transported through the column by continuous addition of mobile phase. This process is called elution. The average rate at which an analyte moves through the column is determined by the time it spends in the mobile phase. [(5)]

1.2. Liquid Chromatography

For compounds that cannot be volatilized readily, the liquid chromatograph (LC) can be used instead of the gas chromatograph.[(6)] Liquid chromatography(LC) refers to any chromatographic procedure in which the moving phase is a liquid, in contrast to the moving gas phase of gas chromatography. There are two major types of LC, classical and HPLC.

In classical LC a column is often used only once, then discarded. Therefore, packing a column has to be repeated for each separation, and this represents a significant expense of both manpower and material. Sample application in classical LC, if done correctly, requires some skill and time on the part of the operator. Solvent flow is achieved by gravity feeding of the column, and individual sample fractions are collected manually. Since typical separations require several hours in

classical LC, this is a tedious, time-consuming operation. Detection and quantitation are achieved by the manual analysis of individual fractions. Normally, many fractions are collected, and their processing requires much time and effort. Finally, the results of the separation are recorded in the form of a chromatogram: a bar graph of sample concentration versus fraction number.

Precise sample injection is achieved easily and rapidly in modern LC, using either syringe injection or a sample valve. Solvent flow is provided by high-pressure pumps. This has a decided advantage: controlled, rapid flow of solvent through relatively impermeable columns. Controlled flow results in more reproducible operation, which means greater accuracy and precision in LC analysis. High-pressure operation leads to better; faster separation, Detection and quantitation in modern LC are achieved with continuous detectors of various types. These yield a final chromatogram without intervention by the operator. The result is an accurate record of the separation with minimum effort. Repetitive separation by HPLC can be reduced to a simple sample injection and final data reduction, although the column and/or solvent may require change for each new application. From this it should be obvious that HPLC is considerably more convenient and less operator dependent than classical LC .[7]

The efficiency of an HPLC column should improve dramatically as the particle size is decreased, particularly when the column is operated at the optimum velocity. It is seen that reduction of particle size from 45 to 6 µm results in a ten-fold or more decrease in plate height. Each time the particle diameter is halved, the pressure drop required is raised by approximately a factor of four. Often, however, the column length can be shortened significantly. There are no practical operation conditions, when particles size are larger than 5 µm it would be desirable from the point of view achieving high plate number quickly. For a given column length, the plate count is increased 1.7 times for a 3 µm packing material compared with a 5

µm packing material. Eventually, however, the use of ever finer particles or longer columns of the same size particles requires pressures that exceed 5000 psi. the practical upper limit. Commercial columns are available with packing materials with particle diameters of 3, 5, and 10 µm.[4] HPLC has been used for analysis of wide variety of pharmaceutical products, body fluid samples, and environmental sample .[8]

1.3.Modes Of Liquid Chromatography

HPLC modes of separation may be broadly classified as gel-permeation (GPC), partition, ion exchange, normal phase and reversed-phase chromatography. Gel permeation (or size exclusion) chromatography separate compounds based on their molecular size and it is generally used for the separation of biological macromolecules. An overview on the separation technique of normal and reversed-phase chromatography follows in the next paragraphs. [9]

1.4. Normal Phase(Adsorption)Chromatography

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.[10] 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.[11]

Dessouky et al.[12] 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.[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. 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.[15] 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 an 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).[16]

1.5. Reversed Phase Chromatography

Reversed phase HPLC (RP-HPLC) consists of a non-polar stationary phase and a moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe_2SiCl where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. RP-HPLC operates on the principle of hydrophobic interactions which result from repulsive forces between a relatively polar solvent, the relatively non-polar analyte,

and the non-polar stationary phase. The driving force in the binding of the analyte to the stationary phase is the decrease in the area of the non-polar segment of the analyte molecule exposed to the solvent. This hydrophobic effect is dominated by the decrease in free energy from entropy associated with the minimization of the ordered molecule-polar solvent interface. The hydrophobic effect is decreased by adding more non-polar solvent into the mobile phase. This shifts the partition coefficient such that the analyte spends some portion of time moving down the column in the mobile phase, eventually eluting from the column. The characteristic of the analyte molecule plays an important role in its retention characteristics. In general, an analyte with a longer alkyl chain length results in a longer retention time because it increases the molecule's hydrophobicity. Very large molecules, however, can result in incomplete interaction between the large analyte surface and the alkyl chain. Retention time increases with hydrophobic surface area which is roughly inversely proportional to solute size. Branched chain compounds elute more rapidly than their corresponding isomers because the overall surface area is decreased. Aside from mobile phase hydrophobicity, other mobile phase modifiers can affect analyte retention. For example, the analyte-solvent interface is controlled by surface tension; the addition of salts tends to increase the retention time. Another important factor is pH since this can change the hydrophobicity of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate to control the pH. An organic acid such as formic acid or most commonly trifluoroacetic acid is often added to the mobile phase. These serve multiple purposes: They control pH, neutralize the charge on any residual exposed silica on the stationary phase and act as ion pairing agents to neutralize charge on the analyte. The effect varies depending on use but generally improve the chromatography. Reversed-phase columns are quite difficult to damage compared with normal silica columns, however, many reverse phase columns consist of alkyl

derivatized silica particles and should never be used with aqueous bases as these will destroy the underlying silica backbone. They can be used with aqueous acid but the column should not be exposed to the acid for too long, as it can corrode the metal parts of the HPLC equipment. Typical stationary phases for reversed phase, alkyl hydrocarbons are the preferred stationary phases; octadecyl (C_{18}) is the most common stationary phase, but octyl (C_8) and butyl (C_4) are also used in some applications. The designations for the reversed phase materials refer to the length of the hydrocarbon chain. The mobile phase is generally a binary mixture of water and a miscible polar organic solvent like methanol, acetonitrile or THF. Reversed phase is typically used for less polar species. **(10)**

Water is often described as the strongest elution medium for chromatography, but in fact this is only true for adsorption processes. Water may interact strongly with the active centers in silica and alumina, so that adsorption of sample molecules becomes highly restricted and they are rapidly eluted as a result. Exactly the opposite applies in reversed-phase systems: water cannot wet the non-polar alkyl groups and does not interact with them in any way. Hence it is the weakest mobile phase of all and gives the slowest sample elution rate. The greater the amount of water in the eluent, the longer is the retention time. **(17)** For biomolecules, RPC has been applied primarily for the separation of peptides, including large-scale preparative use.**(18)** Yet the retention mechanisms on reversed-phase are complex and not easy to understand.**(19)**

Christie **(20)** studied the retention of triacylglycerols using reversed phase column. He found that octadecyl column (C_{18}) is suitable more than octyl column (C_8) for retention of triacylglycerols. He considered from a theoretical standpoint , a stationary phase that similar in chain length to the fatty acyl chains will maximizes the interactions and should give the highest efficiency. A comparison of

normal (-CN) and reversed (C_{18}) phase chromatographic behaviour of polycyclic aromatic hydrocarbons has been achieved by Aygün and Özcimder.[21] They noticed gets higher for the same number of carbon atoms in reversed phase and separation was fast.

Yacob and Zinalibdin.[22] have determined of urinary hippuric acid and benzoic acid as indices for glue sniffer urine. The chromatographic separation was achieved using (3.9 x 150 nm) reversed phase column operating with isocratic elution of methanol: water: acetic acid (20 : 80: 0.2) , adjusted to pH 6.7 using sodium hydroxide was flowing at a constant flow rate of 0.8 $ml.min^{-1}$. The effluent was monitored by an UV detector set as 254nm. RPC can be used for the separation of neutral molecules in the lower molecular weight range.[23] The first RPC separations appeared in the late l940s when polar solutes were separated on chemically modified soft polymeric gels.[24] The use of RPC was applied to the purification of polypeptides in the late 1970s and has since then achieved considerable interest due to the high-resolving power of the technique.[25]

1.6. Ion Exchange Chromatography*(IEC)*

IEC is an electrostatic method for the separation of ionic species based on their differential migration on an ion exchange resin column. [26] A process in which a charged solute molecule in the mobile phase solution is electrostically attracted (the ion exchange interaction) to the oppositely charged stationary phase particles.[27]**I**on **C**hromatography (IC) was introduced in 1975 by Small[28]**,** as a new analytical method. Within a short period of time, ion chromatography evolved from a new detection scheme for a few selected inorganic anions and cations to a versatile analytical technique for ionic species in general. For a sensitive detection of ions via their electrical conductance, the separator column effluent was passed

through a “suppressor” column. This suppressor column chemically reduces the eluant background conductance, while at the same time increasing the electrical conductance of the analyte ions.

In 1979, Weiss. [29] Described an alternative separation and detection scheme for inorganic anions, in which the separator column is directly coupled to the conductivity cell. As a prerequisite for this chromatographic setup, low capacity ion-exchange resins must be employed, so that low ionic strength eluants can be used. In addition, the eluant ions should exhibit low equivalent conductances, thus enabling sensitive detection of the sample components. At the end of the 1970s [30], ion chromatographic techniques were used to analyze organic ions for the first time. In classical ion-exchange chromatography, a column (typically 10 cm to 50 cm long) was filled with an anion or cation exchange resin having a particle size between 60 and 200 mesh (0.075 □0.25 mm). After the sample was applied to the top of the column, it migrated down the column driven by gravitational force, and became more or less separated. Due to the high ion-exchange capacity of the columns, high electrolyte concentrations were necessary to ensure the elution of the sample ions from the column. In many cases, several liters of eluant had to be worked up.[31,32] The enormous improvement in the performance of modern ion-exchange chromatography is attributed to the pioneering work of Small. [28] Their major achievement was the development of low-capacity ion-exchange resins of high chromatographic efficiencies, which could be prepared reproducibly. The required injection volume was reduced to 10-100 µL, which resulted in an enhanced resolution with very narrow peaks. Another important improvement was that of automated detection, which allowed continuous monitoring of the signal. The introduction of conductivity detection for ionic species added a new dimension to ion-exchange chromatography. Styrene/divinylbenzenecopolymers,ethylvinylbenzene/divinylbenzene copolymers,

polymethacrylate, and polyvinyl resins are the most important of all the organic compounds that were tested for their suitability as substrate materials in the manufacturing process for polymer-based anion exchangers. Styrene/divinylbenzene copolymers are the most widely used substrate materials. Because they are stable in the pH range between 0 and 14, eluants with extreme pH values may be used. The copolymerization of styrene with divinylbenzene (DVB) is necessary to obtain the required mechanical stability of the resin.

Upon adding divinylbenzene to styrene, the two functional groups of DVB cross-link two polystyrene chains with each other. [33, 34] Cation or anion-exchanging properties are introduced into the resin by chemical modification after polymerization. Cation-exchangers can be subdivided into strong-acid types containing —SO_3H groups and weak-acid types containing —COOH groups. The former are produced by reacting the resin with chlorosulphonic acid which results in mainly para substitution of the benzene rings. The sulphonic acid groups are dissociated over a wide pH range and these resins will exchange their protons for other cations under both acid and alkaline conditions. The weak-acid type differs in that it is prepared by direct polymerization of DVB and methacrylic acid, CH3C(CH2)COOH. Protons are exchanged for other cations only above pH 5 as below this value the carboxylate groups are not dissociated.

Anion-exchangers comprise strong-base types incorporating quaternary ammonium groups (—N^+R^3) and weak-base types incorporating primary, secondary or tertiary amines. They are prepared by chloromethylating the resin followed by treatment with the appropriate amine. [35]

Naushad *et al* .[36] have used Amberlite IR-120 resin (H^+ form) coated with neutral red as a functional group for the separation of heavy metal ions from solution containing Cd(II), Zn(II), Cu(II), Ni(II), Al(II) and Fe(III) using classical

column and 0.01 M HNO_3 as eluent. Meyers[37] carried out a similar study for the separation of Ni(II),C_O(II) in waste treatment by using of weak acid cation resin .

Griessbach [38] was one of the first to separate amino acids with the aid of ion exchange resins. Weakly acidic resins are more suitable for separation of all types of amino acids.[39] Determination of tryptophan in proteins and feed stuff has also been carried out by ion exchange chromatography.[40] Free amino acids in wines were determined using ion exchange chromatography by Cosmos and Sarkardi.[41]

Ion exchange finds application in virtually all branches of chemistry. In clinical laboratories, it is used routinely to profile biological fluids and for diagnosis various metabolic disorders. [42] Ion exchange has long been used as the separation mechanism in automated amino acid and carbohydrate analyzers. More recently, ion exchange chromatography has been used to separate a wide range of biological macromolecules using special wide-pore low-capacity packing designed for this purpose. [43, 44]

1.7. 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.[45] 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 1–2 μm 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. [46]

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. [47] Porous packings have been in favor through out 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 H P LC packing materials are of this higher level of purity .[48]

There are two types of nonporous packings: nonporous silica and nonporous resin. The nonporous packings are very reminiscent of the older pellicular or porous - layer beads used in the early days of HPLC, but these materials are of much smaller particle sizes ,typically in the 1.5–2.5 µm range.[49] Porous Layer Beads are large particles with a diameter in the 30 µm range which allows to pack them dry. They consist of a non-porous core which is covered with a 1–3 µm thick

layer of a chromatographically active material. PLB's are rarely used nowadays but can be found in guard columns or as repair material for deteriorated columns with collapsed packing. [4]

Most polymer HPLC packings are polystyrene-divinylbenzene (PS–DVB) resins cross-linked at 10% or greater to ensure sufficient particle rigidity**.** PS–DVB HPLC packings are spherical in shape and 5 or 10 µm in size. The pore size and surface area of PS–DVB particles can also be controlled similar to the range of values given for silica. Functionalization of PS–DVB resins for use as ion-exchange packings is the other dominant use of these supports.

Both the dry-fill and wet-fill packing methods have been used to prepare HPLC columns. The dry-fill approach involves vertical tapping of the column with simultaneous rapping along its side to cause good consolidation of the column bed. This procedure is recommended for the packing of rigid particles with a diameter greater than20 µm. The wet-fill or slurry packing techniques are recommended for particles less than 20 µm in diameter. In this approach, the slurry is pumped quickly under high pressure into the column blank. [46]

1.8 Amino Acids Characteristic

Amino acids are biologically active substances, and a number of them are essential for living beings. Amino acids are found in living cells as well as in body fluids of higher animals, in amounts, which vary according to the tissue and particular amino acid. In addition to the amino acids of proteins, a variety occurs free naturally.[50] The aromatic amino acids contain resonancestabilized rings. In this group, only phenylalanine has strongly apolar properties. Tyrosine and tryptophan are moderately polar, and histidine is even strongly polar. The imidazole ring of histidine is already protonated at weakly acidic pH values. Histidine, which is only aromatic in protonated form can therefore also be

classified as a basic amino acid. Tyrosine and tryptophan show strong light absorption at wavelengths of 250–300 nm.[51] The charged functional groups of amino acids ensure that they are readily solvated by—and thus soluble in— polar solvents such as water and ethanol but insoluble in nonpolar solvents such as benzene, hexane, or ether. In ion exchange chromatography, amino acids interact with the stationary phase by charge-charge interactions. Amino acids with a net positive charge at a given pH adhere to beads with negatively charged functional groups such as carboxylates or sulfates (cation exchangers). Similarly, amino acids with a net negative charge adhere to beads with positively charged functional groups, typically tertiary or quaternary amines (anion exchangers).[52]

1.9. HPLC Detection System

The important role of the HPLC detector is to monitor the solutes as they are eluted from the column. The detector generates an electrical signal that is proportional to the level of some property of the mobile phase or solutes. Detector can be divided into two categories: bulk property detectors and solute property detectors. A detector that measures a property of both the solute and mobile phase**,** such as a refractive index detector, is a bulk property detector. Likewise , a detector that measures a property of the solute only , such as a U.V detector, is a solute property detector. Solute property detectors are significantly more sensitive than bulk property detectors, on the order of 1000 times or more. Some characteristics of a good HPLC detector are sensitivity , linearity , predictability in response , reliability , non destructiveness , ease of use , and low dead volume.[53] As with gas chromatography, numerous detectors have been developed for use in monitoring HPLC separations .[54] If sample components are very unlike and differ widely in their physical properties, it may be necessary to utilize two or more

detectors in series, to ensure that each component of interest is adequately measured. Detector noise level is defined as the maximum amplitude of the combined high-frequency noise and random baseline fluctuations arising from instrument electronics, temperature fluctuations, line voltage surges, and other effects not directly attributable to the solute. Variation of the detector signal can also result from flow changes, pump pulsations, and so on. Detector noise makes difficult the sensing of very small peaks; it is often given as the random baseline variation in units of detector response at a specified sensitivity. Detector drift is usually defined as a change in detector response over a period of time, usually several hours. Detector noise cannot be accurately determined unless the drift is small in relation to the magnitude of the noise. The absolute sensitivity of a detector is the total change in a physical parameter that is required for a full-scale deflection of the recorder at maximum detector sensitivity and at a specified noise level. Detection limit or detector sensitivity is generally taken as the concentration or mass of solute entering the detector per unit time that will provide a signal-to-noise ratio of 2, and this is usually considered the minimum concentration or mass of a solute that can be adequately detected. The minimum detection limit often is dependent on the chromatographic system. For instance, detectability with general detectors such as a differential refractometer is very much a function of the mobile phase. For other systems the minimum detection limit is a function of other variables such as column plate count, detector response to solute and so on. The cell should be designed with a minimum volume compatible with the other requirements of the detector. [7]

Although over the years a large number of LC detectors have been developed and described, over 95% of all contemporary LC analyses are carried out using one of four detectors, the UV detector in one of its forms, the electrical conductivity

detector, the fluorescence detector and the refractive index detector. In addition, some form of the UV detector probably accounts for 80% of those analyses.

1.9.1.The Refractive Index Detector

The first practical refractive index detector was described by Tiselius and Claesson in 1942[55], despite its limited sensitivity and its use being restricted to separations that are isocratically developed , it is still probably the fifth most popular detector in use today. Its survival has depended on its response, as it can be used to detect any substance that has a refractive index that differs from that of the mobile phase.

It follows that it has value for monitoring the separation of such substances as aliphatic alcohols, acids, carbohydrates, and the many substances of biological origin that do not have ultraviolet (UV) chromophores, do not fluoresce, and are nonionic. When a monochromatic ray of light passes from one isotropic medium to another it changes its wave velocity and direction. The refractive index of a substance is a dimensionless constant that normally decreases with increasing temperature; values are taken at 20°C or 25°C using the mean value taken for the two sodium lines of the spectrum. The optical systems that are used to exploit the refractive index for detection purposes are many and varied. One procedure is to construct a cell in the form of a hollow prism through which the mobile phase can flow. A ray of light is passed through the prism, which will be deviated from its original path, and is then focused onto a photocell. As the refractive index of the mobile phase changes, due to the presence of a solute, the angle of deviation of the transmitted light will also alter and the amount of light falling on the photocell will change. This method of refractive index monitoring is used by many manufacturers in their refractive index detector designs. The two beams are focused through the sample and reference cells, respectively. Light refracted from the mobile-phase/prism surface passes through the prism assembly and is then focused on two

photocells. The prism assembly also reflects light to a user port where the surface of the prism can be observed. The output from the two photocells is electronically processed and either passed to a potentiometric recorder or to a computer data acquisition system. Another variant on the refractive index detector arose from the work of Christiansen on crystal filters if a cell is packed with particulate material having the same refractive index as the mobile phase passing through it, light will pass through the cell with little or no refraction or scattering. If, however, the refractive index of the mobile phase changes, there will be a refractive index difference between the mobile phase and that of the packing.[56] Another detector that functions on the change in refractive index of the column eluent is the interferometer detector, which was first developed by Bakken and Stenberg in 1971 .[57]

1.9.2.The UV Absorption Detectors

UV absorption detectors respond to those substances that absorb light in the range 220 to 350 nm. A great number of substances absorb light in this wavelength range, including those substances having one or more double bonds (π electrons) and substances having unshared (un bonded) electrons, e.g. all olefins, all aromatics and compounds containing **(C = O, C = S, -N = N-)** groups. The detector normally consists of a short cylindrical cell having a capacity of between 2 µl and 5 µl through which passes the column eluent. The UV light also passes through the cell and falls on a photo–electric cell (or array) the output of which is conveyed to an appropriate signal modifying amplifier and thence to a recorder or data acquisition system. It is seen that the sensitivity of the detector as measured by the transmitted light will be directly proportional to the value of the extinction coefficient and the path length of the cell. Most modern UV detector sensors have

path lengths that range between 1 and 10 mm and internal radii that range from about 0.5 to 2 mm. UV detectors can be used with gradient elution providing the solvents do not absorb significantly over the wavelength range that is being used for detection. In reversed phase chromatography, the solvents usually employed are water, methanol, acetonitrile and tetrahydrofuran (THF), all of which are transparent to UV light over the total wavelength range normally used in UV detectors. In normal phase operation, however, more care must be taken in solvent selection as many solvents that would be appropriate as far as the chromatographic phase system is concerned are likely to absorb UV light very strongly. The n-paraffins, methylene dichloride, small quantities of the aliphatic alcohols (large quantities are likely to deactivate the stationary phase and produce very rapid elution of the components) and THF are useful solvents that are transparent in the UV and can be used with normal distribution systems.

The fixed wavelength detector, as its name implies, operates with light of a single wavelength (or nearly so) which is generated by a specific type of discharge lamp. The most popular lamp for this purpose is the low pressure mercury vapor lamp, which generates the majority of its light at 254 nm. There are a number of other lamps that could be used in fixed wavelength UV detectors, the low-pressure cadmium lamp which generates the majority of its light at 225 nm and the low pressure zinc lamp that emits largely at 214 nm. None of the lamps emits strictly monochromatic light and light of other wavelengths is always present but usually at a significantly lower intensity. It is seen that if a completely monochromatic source of light was required, then an appropriate filter would be needed. The low pressure mercury light source (wavelength 253.7 nm) is the lamp normally used in the fixed wavelength detector and provides the closest to true monochromatic light of all three lamps. However, it does provide light of significant intensity below 200 nm, but light of such wavelengths is often absorbed and eliminated by the mobile

phase. The zinc lamp has a major emission line at 213.9 but the emission line at 307.6 is also of comparable intensity and would probably need to be removed by a suitable filter if detection was required to be exclusively at the lower wavelength. The cadmium lamp has a major emission line at 228.8 but light is emitted at both lower wavelengths and at substantially higher wavelengths and so an appropriate filter would again be desirable. Suitable interference filters can be quite expensive to construct, which may account for the unpopularity of these two lamps. They do, however, emit light at wavelengths which would give an increased sensitivity to substances such as proteins and peptides, which might make their use worthwhile in the biotechnology field. Light from the UV source is collimated by a suitable lens through both the sample cell and the reference cell and then on to two photo cells. The cells are cylindrical with quartz windows at either end. The reference cell compensates for any absorption that mobile phase might have at the sensing wavelength. The output from the two photo cells are passed to a signal modifying amplifier so that output is linearly related to the concentration of solute being detected. The fixed wavelength UV detector is probably one of the most commonly used LC detectors; it is sensitive, linear and relatively inexpensive.[58]Cortes[59] have reported the separation of nitrate and nitrite as examples of ions that detected at 205 nm following their separation. Other ions that can be determined at 205 nm are acetate, formate, bromide, iodate, iodide, bromate, and thiocyanate. Direct UV detection is difficult when separation encountered one of the species is UV transparent. The UV/VIS detector may also used in an indirect mode. Small and Miller [60] reported this approach, as a detection technique for ionic species. Indirect photometric detection (IPD), in which transparent sample ions eluted with a light-absorbing ion. Janssen et al [61] have described a method for amino acid determination that was used phenylisothiocyanate (PITC) to form

phenylthiocarbamyl (PTC) derivatives of amino acids which can be separated by reversed phase HPLC and detected by UV at 254 nm .

1.9.3. The Fluorescence Detector

Compounds that fluoresce or of 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 (20 ml 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. The excitation wavelength can be selected in the more expensive models and the most advanced equipment has a monochromator for excitation and fluorescent light, providing a highly specific (but less sensitive) level of detection. Care should be taken to ensure that no compounds such as unsuitable solvent or oxygen in the mobile phase which could quench the fluorescence are present. The linear range depends on the system (sample, solvent and accompanying components) and may be relatively small. [(4)]

1.9.4.The Conductivity Detector

The conductivity detector was, at one time, considered to be an electrochemical detector and was included in the group of solute property detectors. 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 (5 mL) fitted with two or more electrodes constructed of platinum, stainless steel, or gold.

The conductivity detector actually measures the conductivity of the mobile phase and gives a small output when only water is present in the sensor. Such background signals from the mobile phase are backed off by suitable electronic adjustments. If the mobile phase contains buffers, the detector can give an output that completely overwhelms that from any solute being eluted, making detection impossible. It follows that the conductivity detector is not a solute property detector but a bulk property detector . The conductivity detector is a non-specific detector and used widely in ion chromatography where it occupies a unique and almost exclusive position.[58]

1.10. Aim Of The Work

The aim of this studied was to prepare a new HPLC stationary phase depended on reaction of Weakly acidic cation exchanger with Neutral red indicator. The properties and characterizations of this new stationary phase were studied. The chromatographic performance was examined. Us of column packed with new resin for the separation and quantitation of some amino acid.

Chapter two

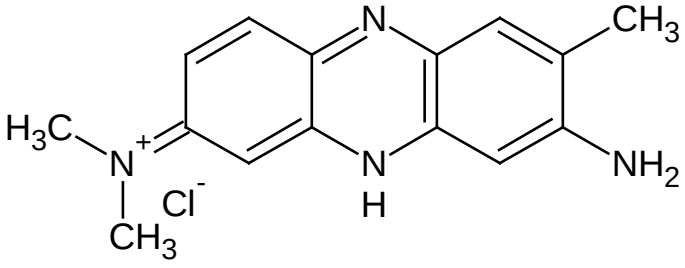
Experimental part

2.1. Instruments And Equipment

High performance liquid chromatograph used in this work was Shimadzu (Kyoto, Japan) which consisted of a system controller model SCL-10 AVP, a degasser model DGU-20As, a liquid delivery pump model LC-10AT VP, UV-visible spectrophotometer detector model SPD-10AV(Kyoto, Japan) , and manual injector model 7725 (USA), equipped with 20 µl sample loop. The HPLC system has been interfaced with computer via a Shimadzu LC-Solution chromatography data system program supplied by the manufacture; Canon printer model LBP 2900(Japan) , Sonicator Sonerex model Super PK 103H Mandolin (Germany), Glass combination electrode was used to measure the pH of solutions (Germany). Shimadzu Fourier transforms infrared model FTIR 8300 (Kyoto, Japan) was used to measure the IR spectra for resin. Blank stainless steel columns, dimensions 25cm x 0.4cm (i.d.) were obtained locally.

2.2. Chemicals

The following materials and chemicals were obtained from BDH (England), or otherwise as indicated next to the material, Cation exchange resin {weakly acidic capacity10 mequiv./g., type 500 mesh , particle size 58 µm} ,Neutral red(3-Amino-7-dimethylamino-2-methylphenazine hydrochloride), molecular weight 288.78 g/mol.



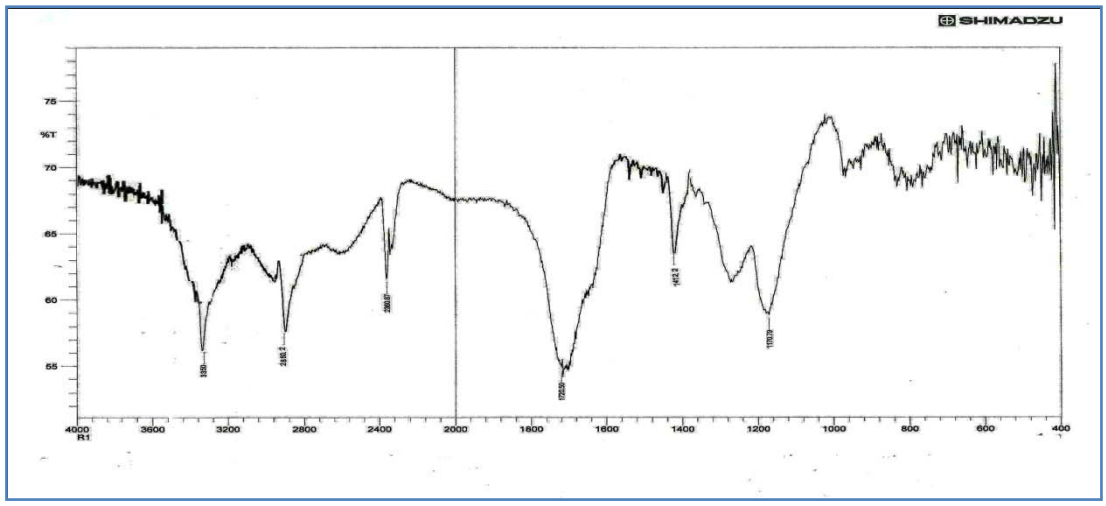
Neutral red

Amino acids Tyrosine, Tryptophan, Phenylalanine, and Histidine, buffer phosphate (Analar) , Methanol (Analar), Acetonitrile (Analar) , Ethanol obtain from Fluke,(Switzerland), and other chemicals and reagents NaOH, KOH , HCl, KCl were obtained either from Fluke or BDH companies.

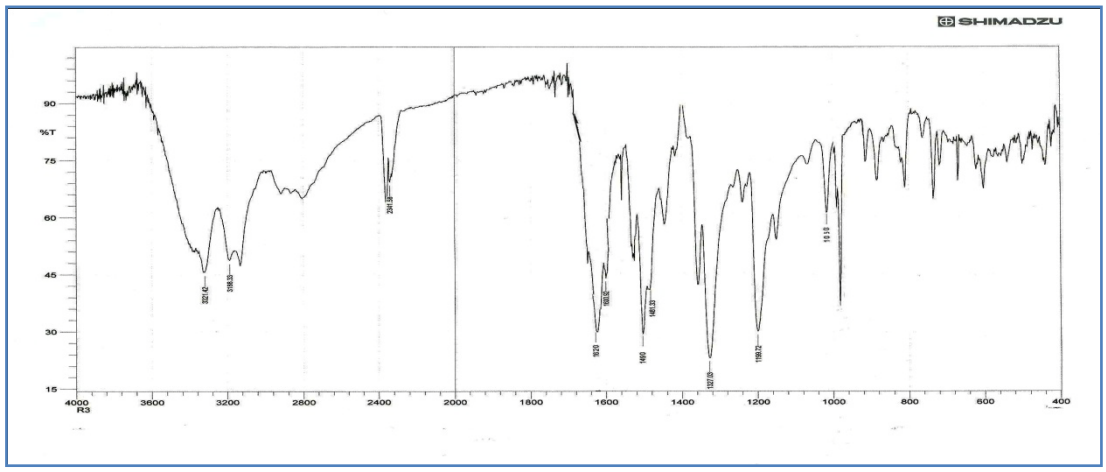
2.3. Preparation Of The Stationary Phase

Four grams of weakly acidic resin was first rinsed with 100ml deionized water in a 250ml beaker and kept for about 24 hour so as to swell . A 10 ml of 1.5×10^{-3} M neutral red was then add with stirring to the beaker. The color of the resin has been changed from white to the deep red. The content of the beaker was led to settle for a period of the time. The solution was then decanted and discarded.

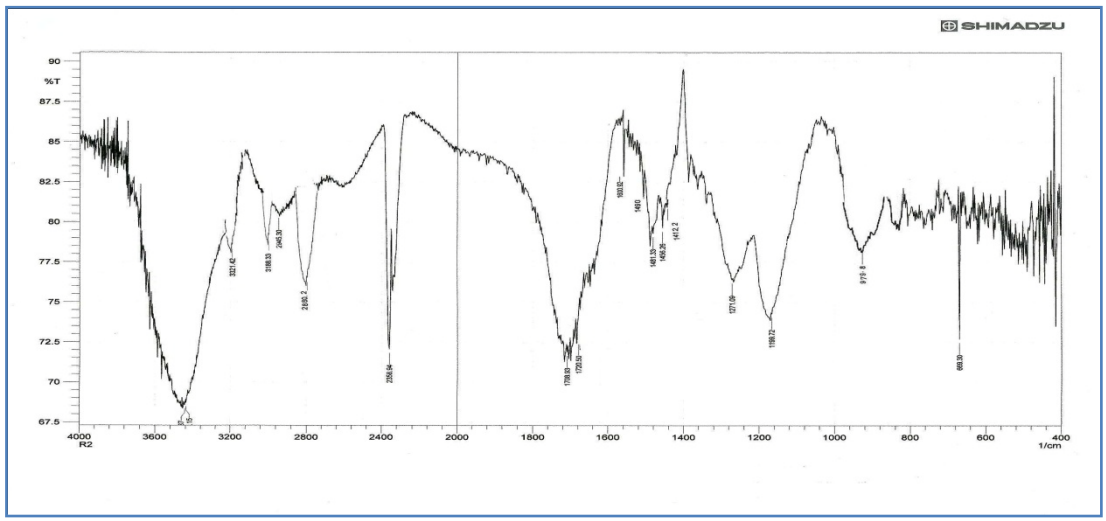
The resin was washed few times with deionized water, then rinsed with ethanol and dried in the oven at 80 C° over night and kept in decicator for future work. The FTIR spectrum for the resulting weakly acidic- Neutral Red resin, Neutral Red, weakly acidic resin, as shown in figures (2 -1).



(A)



(B)



(C) **Figure.(2- 1). FTIR Spectrum for (A) Weakly acidic (B) Neutral red (C) Weakly acidic – Neutral red**

2.4. Stability Tests For Weakly Acidic – Neutral Red Resin

The stability of the new resin has been examined using different solvents and solutions as described below. The resin was first rinsing with 0.1M HCl and also treated with 0.1M KOH. The rinsed solution was measured spectrophotometrically at the λmax of the neutral red which are (540)nm. The resin was again washed few times with water and dried at 100°C. The dried resin was then treated with solvents of different polarity such as benzene, petroleum ether, acetone, chloroform, methanol, ethanol, and acetonitrile. 10 milliliters of each of solvent as described above has been added to 0.2 gm of dried resin with continuous stirring for about 20 minutes. At the end we found that new resin was stable against those solvents.

2.5. Packing Of The Column

Packing of the column are considered very necessary to separation of materials by chromatographic system. In this work , the blank column tube has been used and it first cleaned with 50% nitric acid then washed with water and dried. It is necessary , packing of blank column with a suitable stationary phase.

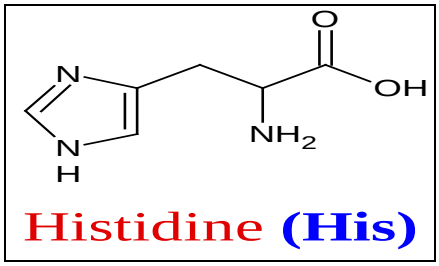
By our research team, the column was packed satisfactory results new method device. The slurry was prepared by an ultrasonic bath and the dry resin needed to fill up the column tubing with water was weighed. The column was replaced with the fitting from one side and hanged to a clamp near the open end. It was placed in the sonicator bath filled with water. Four grams resin was homogenized with 50ml deionized water. A few milliliters (1-2ml) of the slurry was 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 resin to settle down inside the column,

another portion was poured in. This process was continued until the column was completely filled with the resin 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 1ml/min distilled water. The back pressure was monitored until it reached a stable value of 3.5 MPa. The flow rate was then increased by 1 ml increments until reaching 5ml /min. The recorded back pressure was 5.8MPa, 8.4MPa, 10.2MPa, 13.3MPa, 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 processes, 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.

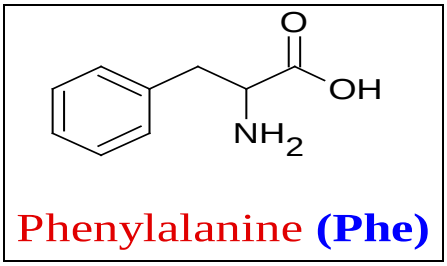
2.6. Sample Preparation

A stock solution of 100 ppm of each of Phenylalanine, Tryptophane, Tyrosine, and Histidine were prepared. A (0.01)gm of each of amino acid as described above were dissolved with stirring in 100 ml beaker of buffer phosphate. The solvent used to prepare these solutions were usually the same as the mobile phase employed for their separation. Other solutions were prepared 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.

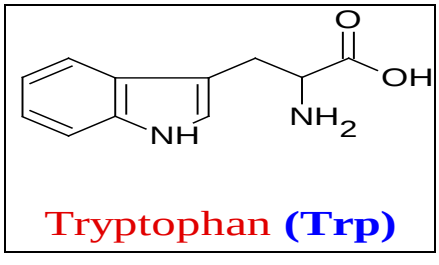
Structures Of Amino Acids Compounds



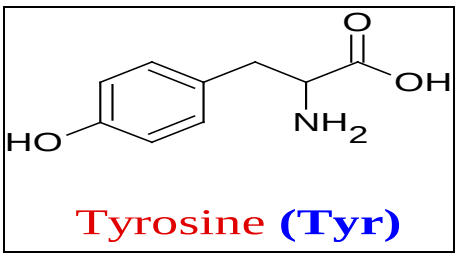
Histidine (His)



Phenylalanine (Phe)



Tryptophan (Trp)



Tyrosine (Tyr)

2.7. Sample Analysis

HPLC using new resin (weakly acidic – neutral red) column with different mobile phase were employed to chromatograph all prepared standard solutions of amino acid. The mobile phase at the beginning of analysis was buffer phosphate adjusted to pH 3 by adding few drops of 0.1 M HCl. This mobile phase was passed through the weakly acidic-neutral red column at 1ml/min for 1 hour until achieving a stable baseline. Phenylalanine, Tryptophane, Tyrosine, and Histidine were chromatographed using different concentrations of each sample. Concentration of amino acid, ranging from 0.05 – 50 ppm, were injected and analyzed at least three times with different mobile phase. These samples were analyzed after the pH of mobile phase was adjusted to

2,3,4,5,6,7,8, and 9. The retention times of each chromatographic run was recorded and average as listed in Table(2 -1).

Table (2 -1). Retention times of amino acids at different pH of the mobile phase; using weakly acidic-Neutral red column (25×0.4 cm) (I.d.). Eluent buffer phosphate at different pH, flow rate 1ml/min, sample loop 20µl, detection wave length 254nm, and concentration 10 ppm.

pH	Retention time (minutes)			
	Histidine	Tyrosine	Phenylalanine	Tryptophan
2	2.71	1.46	1.88	2.95
3	3.2	1.6	3.75	4.3
4	3.42	2.25	3.9	4.42
5	2.82	2.64	3.98	4.84
6	3.84	2.76	3.13	5.84
7	3.97	2.89	2.85	6.04
8	2.98	1.92	1.85	3.06
9	3.99	2.95	3.18	2.60

Different concentration of amino acids and their mixtures were analyzed after optimizing the chromatographic conditions of compounds. Calibration curves of all studied analytes were constructed by plotting the calculated peak area of each analyte versus concentration .The rang of the linear calibration equation, regression, and detection limit were calculated.

Chapter three

Results & discussion

3.1. Preparation Of Weakly Acidic-Neutral Red Resin

The addition of few milliliters of dilute neutral red solution to weakly acidic resin has resulted in the conversion the white resin to deep red. This color change of the resin may be attributed to the reaction between the positive charged quaternary ammonium groups of the neutral red with negatively charged carboxylate group on the weakly acidic resin. This reaction may be represented in figure (3 -1).

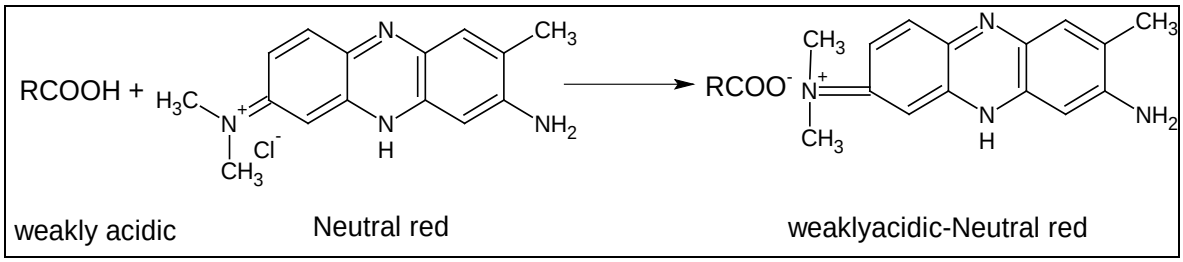


Figure (3 -1): Suggested reaction between weakly acidic and Neutral red.

The new resin was washed few times with deionized water in order to remove the excess neutral red on the resin. 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 neutral red (540)nm. [(62)] The color of the resin has become pinkish red, which indicates that the resin is still holding the neutral red moiety. The addition of few milliliters of 0.1M KOH solution to this new resin has turned the color of the resin immediately to yellow. It is, however, turned back to red coloration when few milliliters of 0.1M HCl solution were added. This color change indicates that the amine groups on the resin still having their acid-base characteristic. This equilibria process may

be represent by the following suggested equation shown in figure (3 -2).

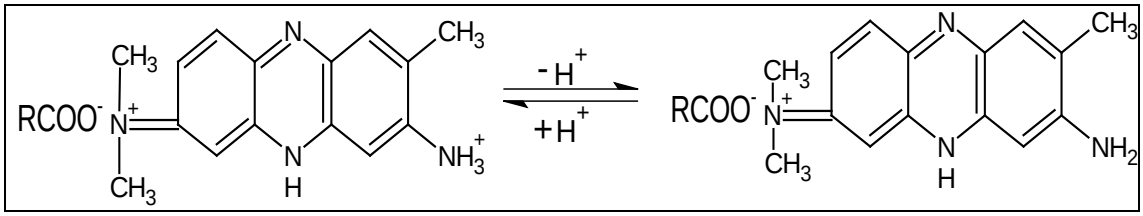


Figure (3 -2). Suggested acid-base equilibria equation of the weakly acidic- Neutral red.

As a consequence, the new resin might act as positively charged resin in the acidic solution, and as uncharged in basic solutions. The chromatographic behavior of this new resin may then be influenced by changing the pH of the mobile phase.

3.2. Stability Test Of The Prepared Resin

The resin was washed with distilled water, dried in the oven, and then tested for its stability against several organic solvents with different polarities. These solvents include acetonitrile, methanol, ethanol, chloroform, acetone, petroleume ether, and benzen. 0.2 g of the new resin were mixed with (10-15) ml of the solvent with continuous stirring for about 20 minutes. The resin was led to settle after that period. The solvent was then decanted and the absorbance was measured at λ max (540)nm of the Neutral red. [(62)] No absorbance reading was noticed with all these solvents. These results showed that the neutral red is strongly held by the resin.

The **FTIR** spectra for each of neutral red, weakly acidic resin, and weakly acidic-neutral are shown in Figure(2- 1). Figure (2 -1,A) showed a

(C-H) aliphatic stretching at 2860.2 cm^{-1} and bending at 1412.4 cm^{-1}, (C=O) at 1720.50 cm^{-1}, (O-H) at 3350 cm^{-1} .

Figure (2 -1,B) showed a (C=C) aromatic at 1490 cm^{-1} , (N-H) stretching aromatic at 3321.42 cm^{-1} , (C-N) stretching aromatic at 1199.72 cm^{-1} , (C-H) stretching aromatic at 3188.33 cm^{-1} , (C-H) bending at 1050 cm^{-1}, (N-H) bending aromatic at 1600.92 cm^{-1}, (N=C) aromatic at 1620 and quaternary ammonium bond at 1481.38 cm^{-1}. The spectra of the newly prepared resin as show in figure (2 -1,C) have some distinguished peaks of the original weakly acidic. It is clear that the spectra of the new resin have a splitting band (N-O) at 979.8 cm^{-1} after comparing the spectra of new resin and weakly acidic resin. The new band may be represent the bond between quaternary ammonium group on the neutral red and carboxylate group on the weakly acidic. However, further spectrophotometric studies are required in order to suggest a proper structure of the new resin and how it is attached to the weakly acidic resin and consequently the new functional groups on the prepared resin.

3.3. Characteristic Of The Prepared Newly Resin

Because of the chromatographic behavior of this new resin as positively charged in the acidic solution ,the new resin were titrated with KOH solution. the first and second derivative of these titration curves show one equivalence point for the new resin. The group of amine on the neutral red still having their acid-base properties as described in the above suggested acid-base equilibria equation , but of different acid strength , which is consistent with neutral red acidic strength (pk_1=6.7 , pK_2=7.4).[(62)]

The reaction between weakly acidic and neutral red in new resin has been caused shifting the pKa value of the new resin to (7.15).

3.4. Column Packing

Obviously , 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 chromatograph. On the other hand , the column packing have 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 ultarsonic 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 were 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 stableish 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 neutral red to the weakly acidic , the detector wavelength was set as (540) nm 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.

3.5. 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 :

$$\mathbf{HETP} = L / N \text{.....(3 -1)}$$

The number of theoretical plates that a real column possesses can be found by examining a chromatographic peak after elution .[10]

$$N = 5.54 \left(t_R / w_{1/2}\right)^2 \text{.....(3 -2)}$$

Where (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 **ḱ** was also recommended for column evaluation. [26] The parameters such as H , N , t_R , etc. have been used for measuring the optimum flow rate.

The value of minimum H should be calculated by Van deemter equation. This has been done by chromatographing on of the analyte at different flow rates and calculating the optimum flow rate at minimum **H** as shown in figure(3-3). A 10 ppm Histidine was chromatographed at flow rate ranging from 0.6 -2 ml/min using 10% acetonitrile in buffer phosphate. It was found that the optimum flow rate as equal to 1.4 ml/min as shown in figure (3- 3).

This flow rate was used to chromatographed other sample described below.

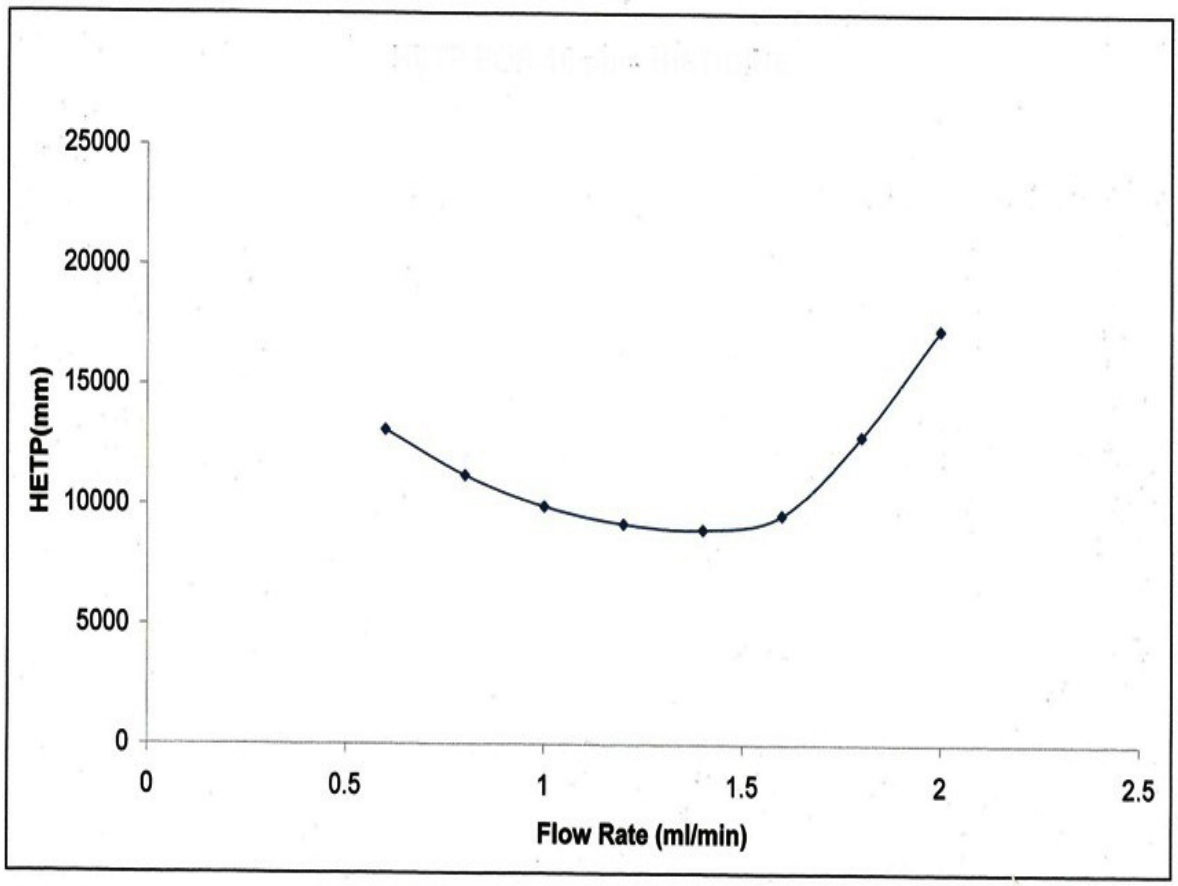


Fig (3 –3) Plot of plate height H, versus flow rate, using weakly acidic- Neutral red column, the mobile phase was 10% acetonitrile in buffer phosphate, and 10 ppm Histidine as a sample.

In the analysis of amino acids using weakly acidic- neutral red column with mobile phase 10% acetonitrile in buffer phosphate adjusted at pH 3, the plate number ranged from (192.48- 422.55) , with an average value of (316.59). The plate heights were ranged from (0.059-0.129) cm, with an average value of (0.085) cm.

However, for the same column but using buffer phosphate as mobile phase adjusted at pH 3 , plate numbers were ranged from (188.34-262.22) with an average value of (234.79),the plate heights were ranged from (0.097-0.132) cm, with an average value of (0.107) cm . These values showed that the analyte peaks obtained using 10% acetonitrile in buffer phosphate as mobile phase were sharper than that in buffer phosphate as mobile phase only. Table**(3 -1).** Shows some chromatographic parameters of the newly packed column.

Table(3 -1). Chromatographic parameters using weakly acidic – neutral red column (25× 0.4 cm (i.d.)). Eluant 10% acetonitrile in buffer phosphate &100%buffer phosphate. Flow rate 1.4 ml/min, sample loop 20 µl, detection wavelength 254 nm.

COMPOUND					
NAME	Mobile phase	Retention time t_R	Plate number N	Height H(cm)	Capacity factor Ḱ
Histidine	10% acetonitrile in buffer at pH 3	2.9	192.48	0.129	2.86
	100%Buffer at pH 3	3.2	188.34	0.132	3.26
Phenylalanine	10% acetonitrile in buffer at pH 3	2.1	355.65	0.070	1.8
	100 %Buffer at PH 3	3.75	233.24	0.107	4
Tryptophan	10% acetonitrile in buffer at pH 3	3.95	422.55	0.059	2.12
	100% Buffer at pH 3	4.3	255.36	0.097	4.73
Tyrosine	10% acetonitrile in buffer at pH 3	2.19	295.68	0.084	1.92
	100%Buffer at pH 3	1.6	262.22	0.095	1.13

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

Note :- t_m = 0.75 by LC solution program.

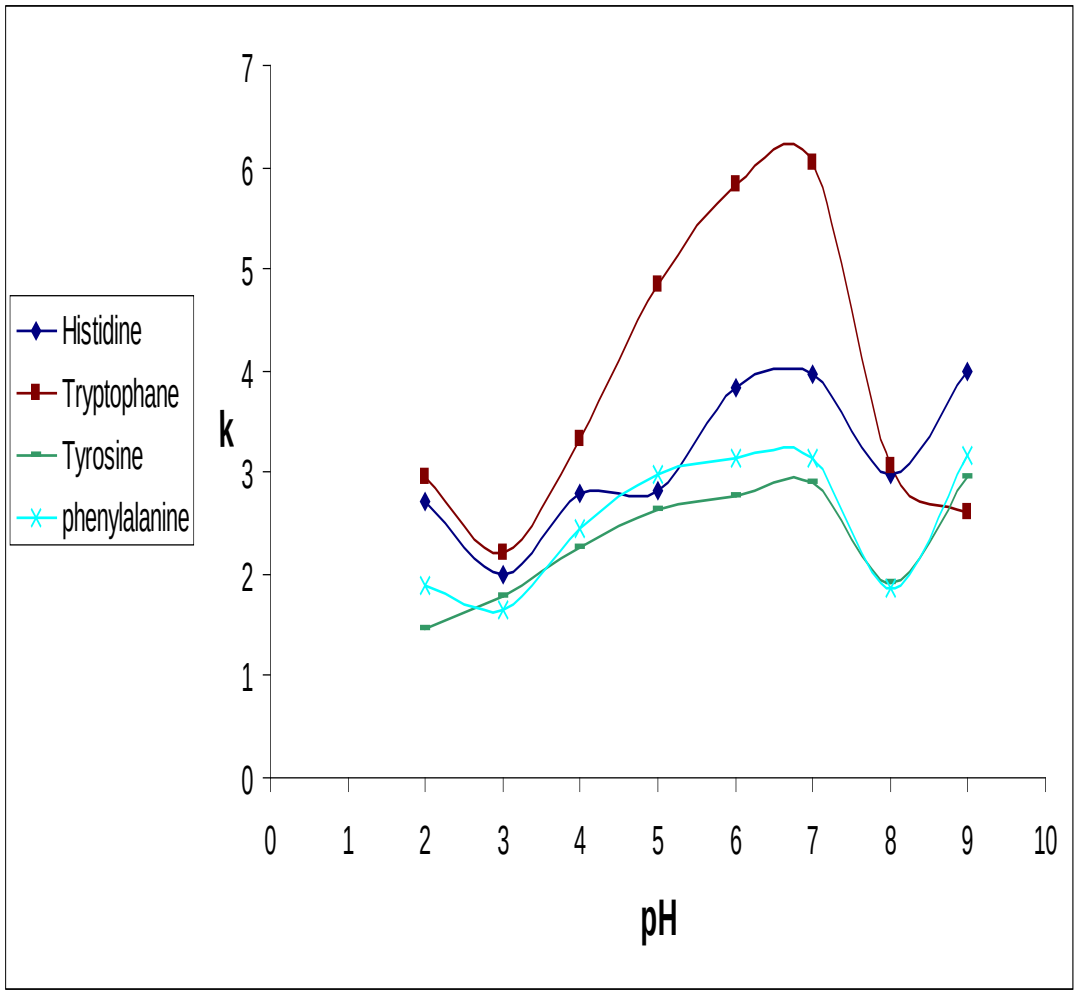


Fig (3-4) . plot of pH versus capacity factors using weakly acidic-neutral red column , mobile phase was buffer phosphate with different pH .

The partition coefficient *K* is based on this equilibrium, and is defined by the following equation as shown below. [26]

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

This important relation can also be written:

$$K = (t_R - t_m) / t_m \quad \ldots\ldots\ldots(3\text{-}4)$$

Where t_m represent the dead time.

Ḱ is assumed to be independent of concentration, and can change if experimental conditions are changed, for example temperature is increased or decreased. As Ḱ 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 Ḱ

Capacity factor between 1.5 and 5 are preferred. [63] Lower Ḱ indicates no stationary phase interaction occurs and hence no chromatography and large Ḱ values are accompanied by long analysis times. The capacity factor for the studied amino acids analyzed on the new column were ranged from (1.8 – 2.86) in 10% acetonitrile in buffer phosphate as mobile phase with average value (2.175) and (1.13 – 4.73) in 100% buffer phosphate as mobile phase with average value(3.28) as shown in Table **(3-1)**.

The capacity factor as well as retention time give smaller value using 10% acetonitrile in buffer phosphate compare to buffer phosphate as mobile phase which may be attributed to the hydrophobic behavior of the new neutral red moiety on the weakly acidic resin. This indicates that good competitive interaction between amino acids and the new stationary phase and the mobile phase. The Capacity factor for all studied amino acids were almost constant at acetonitrile percentage from (0-10) and became high at

high percentage of acetonitrile as show in figure(3 -4). This indicated that the interaction of these analytes with stationary phase is not chemical or hydrophobic it could be compilation of both of them.

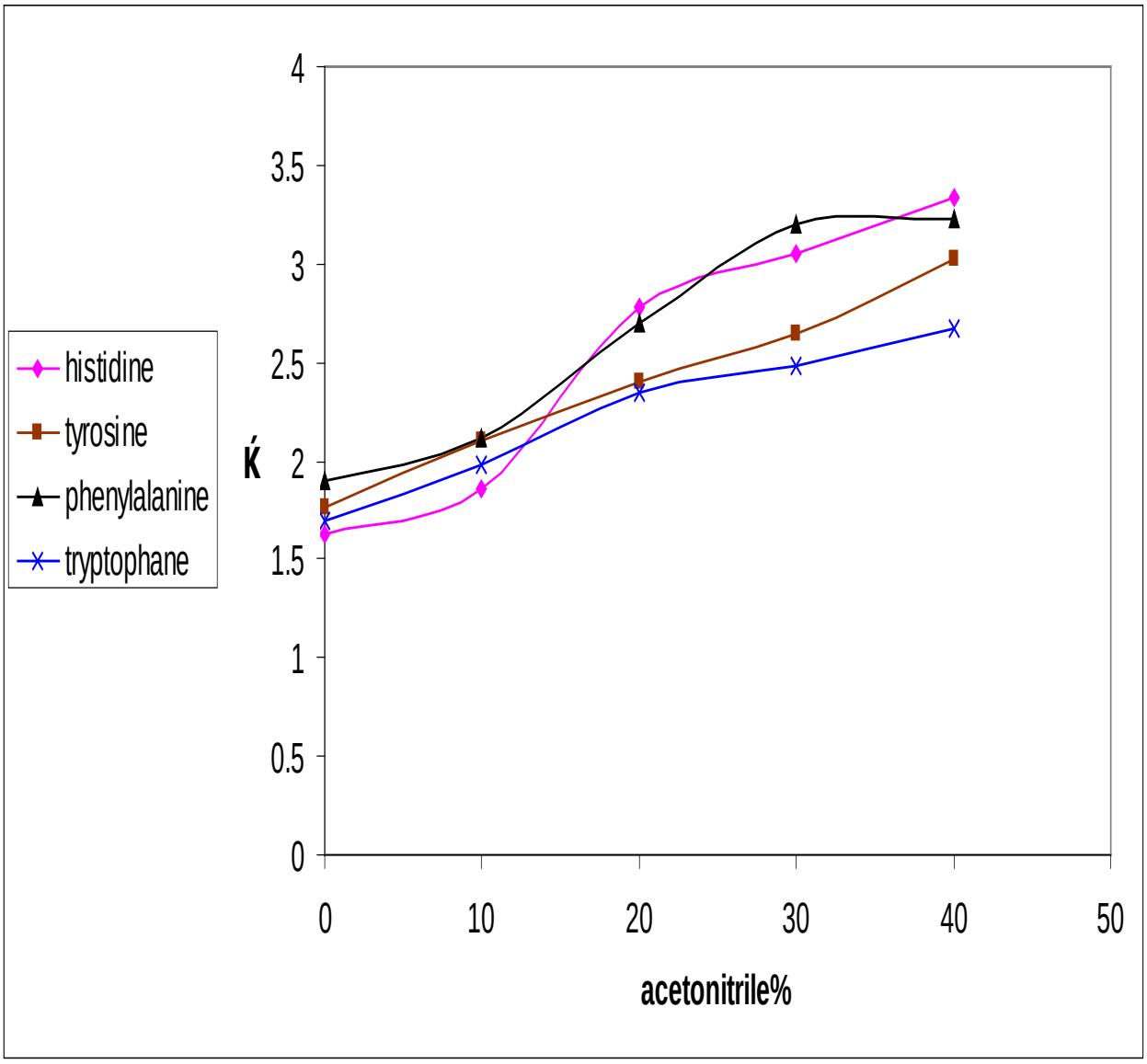


Fig (3 – 5). Plot of percentage of acetonitrile versus Capacity factor (Ḱ) , using Weakly acidic – Neutral red column , (25 x 0.4 cm (i.d.)) the mobile phase was buffer phosphate at pH 3 with different percentage of acetonitrile.

A value of 10% acetonitrile in the mobile phase give the best results in term of retention time and peak shape compare to low or high percentage of acetonitrile as show in figure (3 -5).

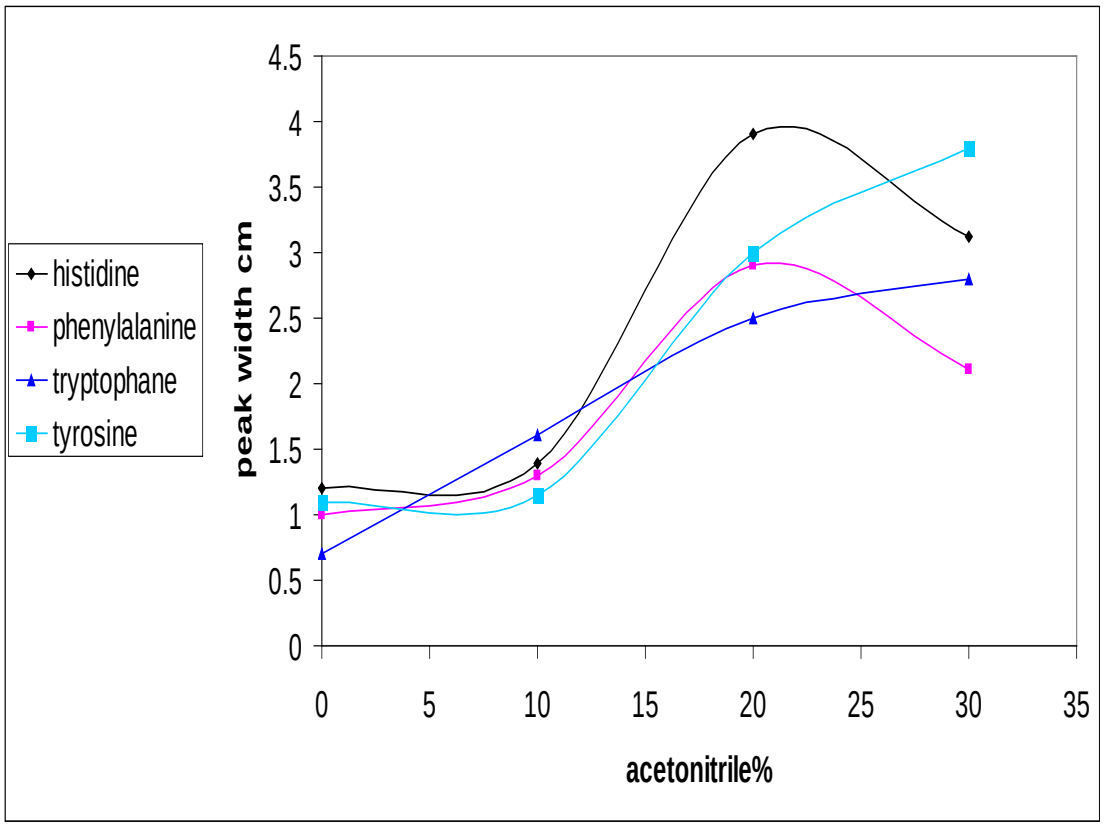


Fig.(3- 6). Plot of percentage of acetonitrile versus Peak width , using Weakly acidic – Neutral red column , (25 x 0.4 cm (i.d.)) the mobile phase was buffer phosphate at pH 3 with different percentage of acetonitrile.

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;

$$\alpha = k_B / k_A \ldots\ldots\ldots (3\text{ -}5)$$

A minimum value of 1.1 is required to achieve the separation of two adjacent peaks at any given experimental condition. Table (3 -2) show the **α** values for amino acids compounds chromatographed on the new column were range from (0.21-1.17) in 10% acetonitrile in buffer phosphate as mobile phase and ranged from (0.23-1.22) in 100% buffer phosphate as mobile phase . peaks asymmetry is another important term, when the value of Asymmetry large 2.5 means that the quantitative peak area measurement will prone to error. [23]

Table(3 -2) shows the value of the peak asymmetries for compounds chromatographed on new column were ranged from (0.99-1.09) with average value (1.04) in the best mobile phase.

Table(3 -2). Separation factor, peak asymmetry, and capacity factor for the separated amino acids compound using weakly acidic – Neutral red (25 x 0.4 cm (i.d.)). Elualt 10% acetonitrile in buffer & 100% buffer phosphate. Flow rate 1.4 ml/min, sample loop 20 µl , detection wavelength 254nm.

Compound	Mobile phase	Capacity factor Ḱ	Separation factor α	Peaks Asymmetry
Histidine	10% acetonitrile in buffer at PH 3	2.86	#	0.99
	100%Buffer at pH 3	3.26	#	1.1
Phenylalanine	10% acetonitrile in buffer at pH 3	1.8	0.63	1.06
	100%Buffer at pH 3	4	1.22	1.12
Tryptophane	10% acetonitrile in buffer at PH 3	2.12	1.17	1.09
	100%Buffer at pH 3	4.73	1.18	1.18
Tyrosine	10% acetonitrile in buffer at pH 3	1.92	0.21	1.02
	100%Buffer at pH 3	1.13	0.23	1.14

Not :- the peaks asymmetry has been calculated by LC solution program while the separation factor α and Ḱ has been calculated manually.

An organic modifier was tested , namely acetonitrile so as to enhance the peak shape and to obtain a stable baseline. The effect of acetonitrile on the capacity factors and peak widths for all amino acids has been studied. The influence of adding organic solvent to the eluent on the capacity factor (Ḱ) for amino acids was studied by using buffer phosphate as mobile phase at pH 3. The acetonitrile content has been found to affect the retention times of studied amino acids. In absence of acetonitrile , the retentions of the separated amino acids were large so as the capacity factor (Ḱ) for all analyzed amino acids compared to 10% acetonitrile in buffer phosphate at pH 3.

This can be explained by the relative decrease in the ionic strength of the eluent as the percentage of acetonitrile increase and more decreasing in the polarity of the eluent , so as increasing the percentage of acetonitrile has been caused increasing the retention time for all amino acids therefore , using of 10% acetonitrile in buffer phosphate was good enough to obtain a baseline separation for most of the analyzed amino acids as show in fig (3 – 4).

However , we found that addition of 10% acetonitrile to buffer phosphate will make the peak more sharp , while using of 40% acetonitrile in buffer phosphate will make the peak width is more broad as shown in fig (3 – 5).We found that using of 10% acetonitrile to buffer phosphate at pH 3 which give a better separation and peaks shapes compare to 100% buffer phosphate at PH 3 as shown in figures (3-6) , (3 -7) , (3-8) and (3- 9), depending on the above experiments .

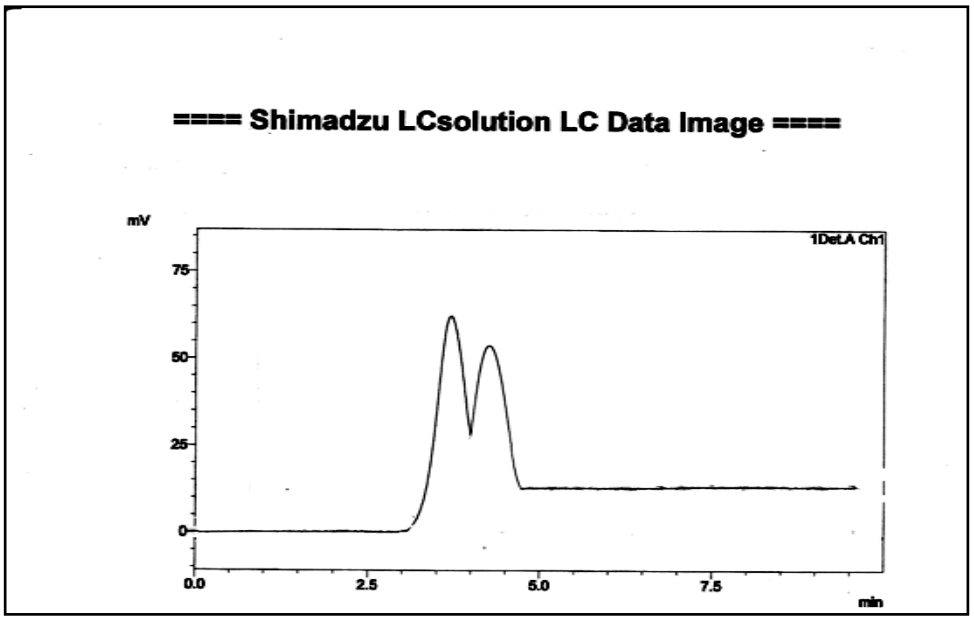


Fig (3 -7) . Chromatogram of amino acid (1) Phenylalaninie 10 ppm , (2) Tryptophane 10 ppm. Using Weakly acidic- Neutral red column (25 x 0.4 cm (i.d.)) ,detection wavelength 254nm , 100% buffer phosphate at pH 3 as mobile phase .

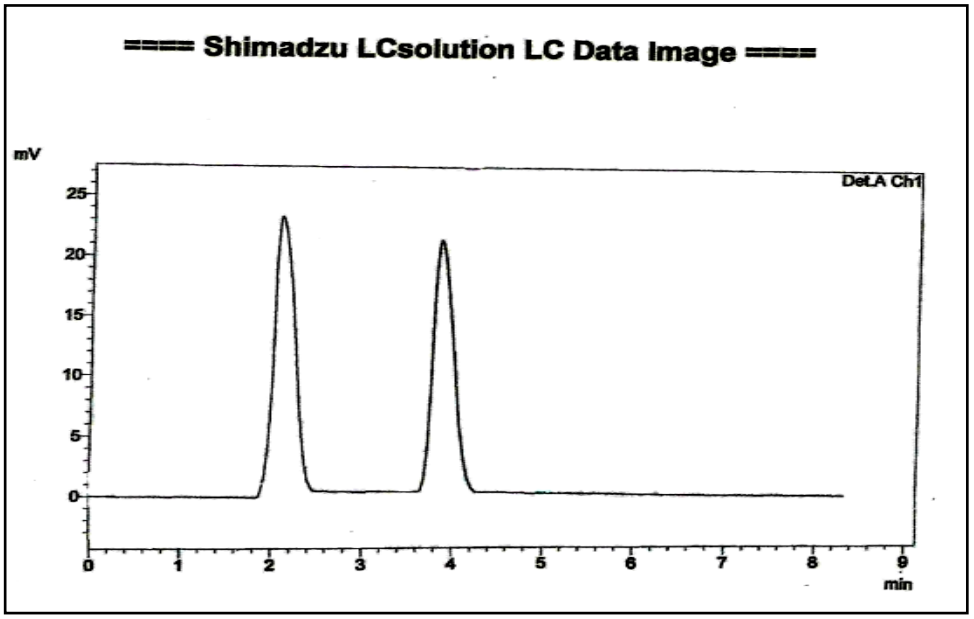


Fig. (3 -8). Chromatogram of amino acid (1) Phenylalaninie 10 ppm , (2) Tryptophane 10 ppm. Using Weakly acidic- Neutral red column (25 x 0.4 cm (i.d.)) ,detection wavelength 254nm , 10% acetonitrile in buffer phosphate at pH 3 as mobile phase.

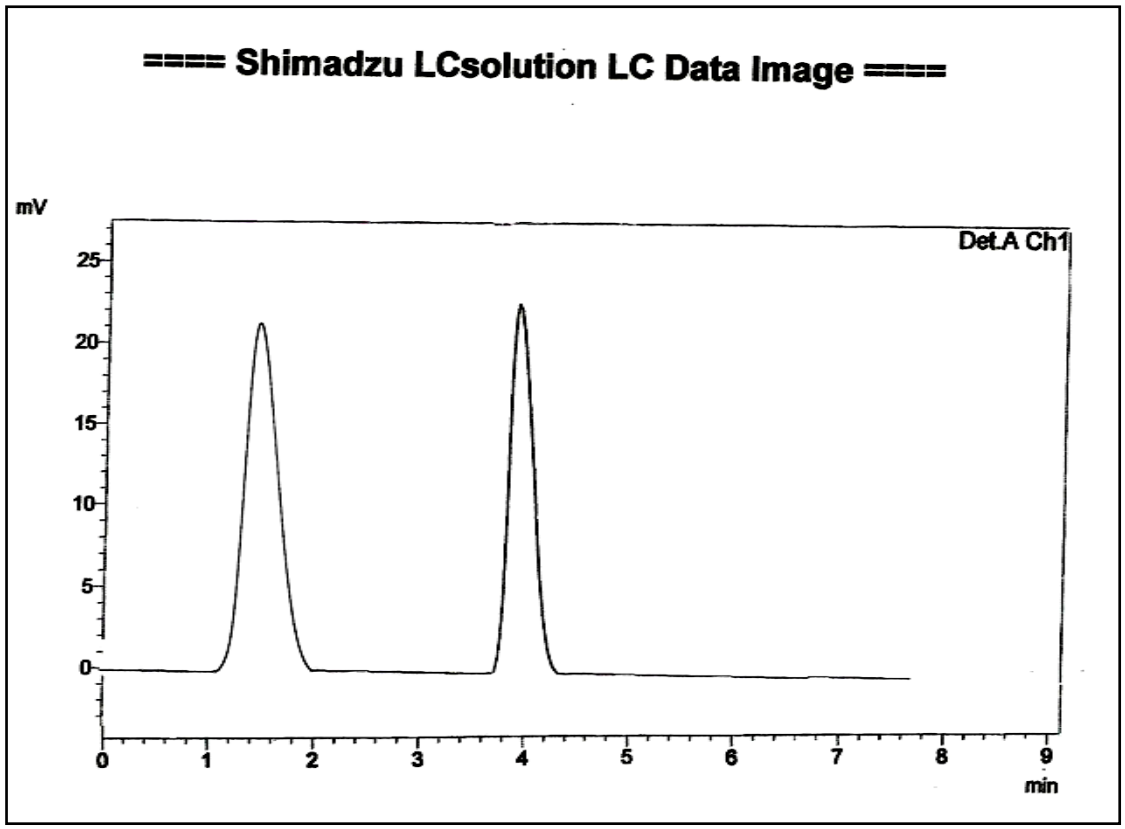


Fig. (3 - 9). Chromatogram of amino acid (1) Tyrosine 10 ppm , (2) Tryptophane 10 ppm. Using Weakly acidic- Neutral red column (25 x 0.4 cm (i.d.)) ,detection wavelength 254nm , 10% acetonitrile in buffer phosphate at pH 3 as mobile phase.

It is clear that the separation of Histidine and phenylalanine is not possible at experimental condition because the peak widths. This may be achieved separation using smaller particle size 5 or l0 micrometer. The separation of 10 ppm of Histidine, tryptophan, and tyrosine in a mixture was possible in less than 5 min. using 10% acetonitrile in buffer phosphate at pH 3 as shown in Figure (3- 9).

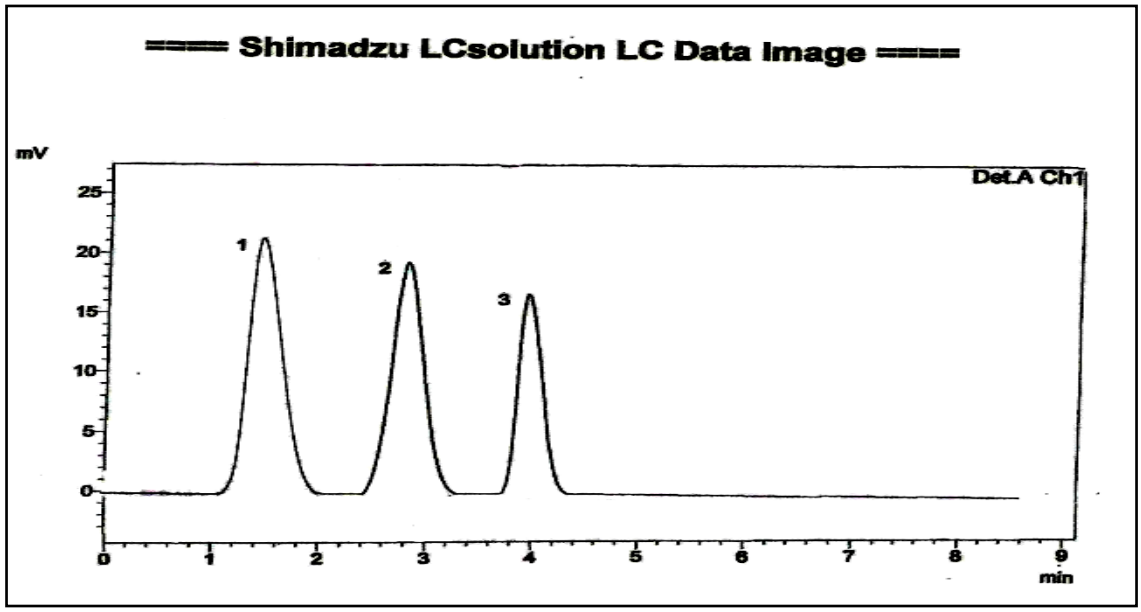


Fig. (3 - 10).Chromatogram of amino acid (1) Tyrosine 10 ppm , (2) Histidine10 ppm, (3) Tryptophane10 ppm . Using Weakly acidic- Neutral red column(25 x 0.4 cm (i.d.)) ,detection wavelength 254nm , and using 10% acetonitrile in buffer phosphate at PH 3 as mobile phase.

The resolution between two peaks is define as the time difference between adjustment two peaks maxima divided by the average peak width at baseline. [46]

$$R_S = \frac{2(TR2 - TR1)}{(w2 + w1)} \quad \text{.....(3 -6)}$$

Where t_{R1} and t_{R2} are the retention times of peaks 1 and 2 and W_1 and W_2 are the widths of the peaks at the baseline. When R_S=1, there is about 2% overlap between two peaks of equal size. A resolution of 1.25 is necessary to achieve baseline resolution of two peaks. The Resolution of mixtures were calculated and listed in table (3 -3).

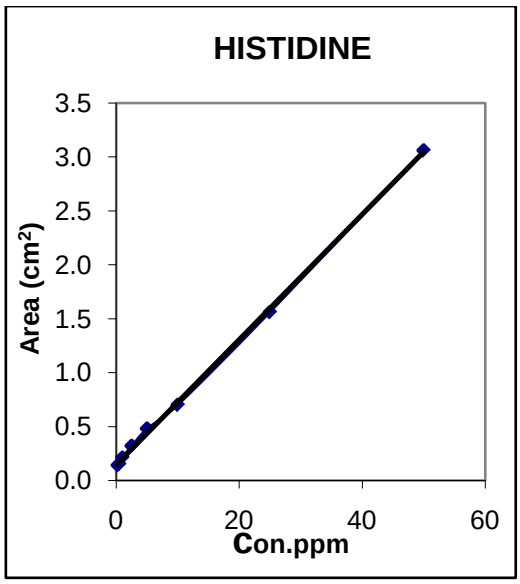
Table (3-3) Resolution (R_s) of mixture, using Weakly acidic- Neutral red column(25 x 0.4 cm (i.d.)) ,detection wavelength 254nm.

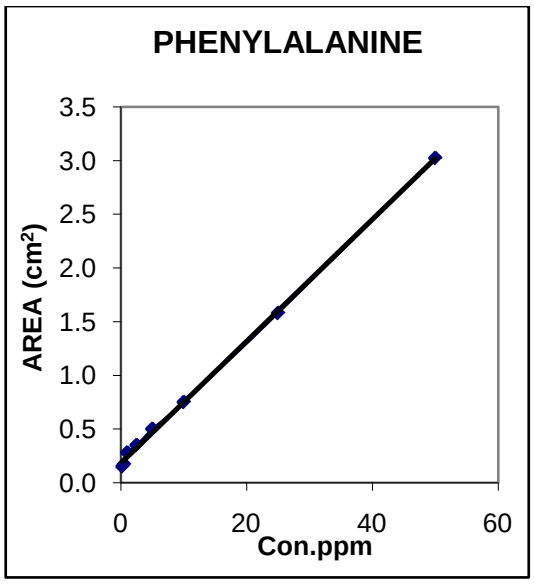
Mixture	Mobile phase	Retention time	Peak width cm	R_S*
(1)Phenylalanine 10ppm and (2)Tryptophane 10 ppm	%10 acetonitrile in buffer phasphate	(1)2.1	*0.57*	3.1
		(2)3.95	*0.6*	
(1)Phenylalanine 10ppm and (2)Tryptophane 10 ppm	%100 buffer phosphate	*(1)3.75*	*0.62*	0.93
		(2)4.3	*0.57*	
(1)Tyrosine 10 ppm and (2)Tryptophan 10 ppm	%10 acetonitrile in buffer phosphate	*(1)1.44*	*0.78*	3.77
		(2)3.95	*0.55*	
(1)Tyrosine 10 ppm , (2)Histidine 10 ppm , and (3) Tryptophan 10 ppm	%10 acetonitrile in buffer phosphate	*(1)1.44*	*0.8*	(1,2) 1.66
		(2)2.9	0.95	(2,3) 1.44
		(3)3.95	0.5	

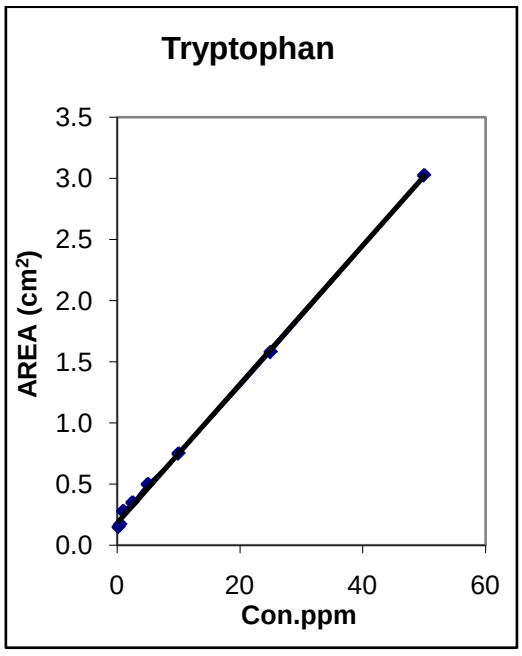
* **the R_S values were calculated manually by equation (3 -6).**

3.6. Quantitative Analysis

Calibration runs of amino acids compounds on weakly acidic-neutral red column, in 10% acetonitrile in buffer phosphate at pH 3 are shown in Figure (3- 10). A linear dependence of the peak areas on the amount injected is evident for all compounds down to the detection limits. Amino acids have shown a linear response range extending from at least 50 ppm to the detection limit which was at low as 0.05 ppm for most analytes.







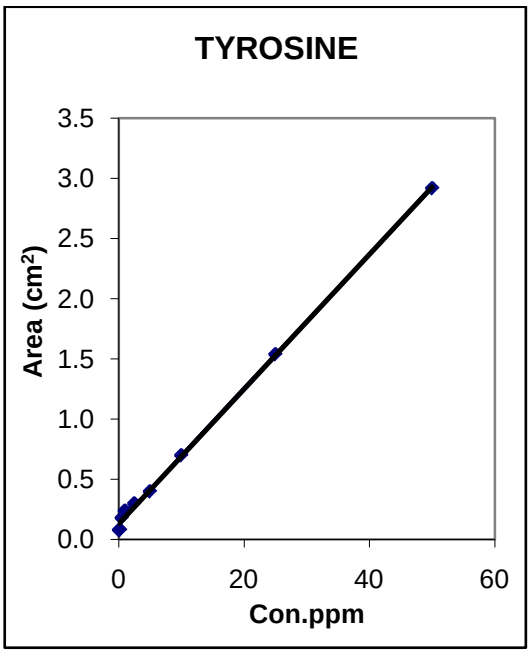


Fig. (3- 11). Calibration curve for Histidine. Tyrosine. Phenylalanine. Tryptophane. samples were analyzed Using Weakly acidic- Neutral red column (25 x 0.4 cm (i.d.)) ,detection wavelength 254nm , flow rate 1.4 ml/min , and using 10% acetonitrile in buffer phosphate at PH 3as mobile phase.

The slope values for the linear calibration curves were range from (0.040- 0.058) depending upon kind of amino acids, the slope value as shown in Table(3 -4**)**. The correlation coefficients for all calibration lines were ranged from (0.9991- 0.9996) with average value (0.9994). The detection limits of the amino acids are also shown in Table(3 -4). It ranged from (0.05- 0.25) ppm for all amino acids in 10% acetonitrile in buffer phosphate at pH 3. These detection limits were calculated at S / **N** ratio of 3 or more as show in figure (3 -11), (3 -12), (3 -13), (3 -14).

Table (3 -4). linear equation , correlation coefficients R , and detection limits , samples were analyzed Using Weakly acidic- Neutral red column (25 x 0.4 cm (i.d.)) ,detection wavelength 254nm , flow rate 1.4 ml/min . Eluent 10% acetonitrile in buffer phosphate at pH 3.

Compound	Linear Equation	R	Detection limit ppm
Histidine	**Y= 0.058X + 0.1442**	**0.9995**	**0.25**
Phenylalanine	**Y= 0.056X + 0.182**	**0.9994**	**0.05**
Tryptophan	**Y= 0.040X + 0.107**	**0.9996**	**0.1**
Tyrosine	**Y= 0.056X + 0.128**	**0.9991**	**0.08**

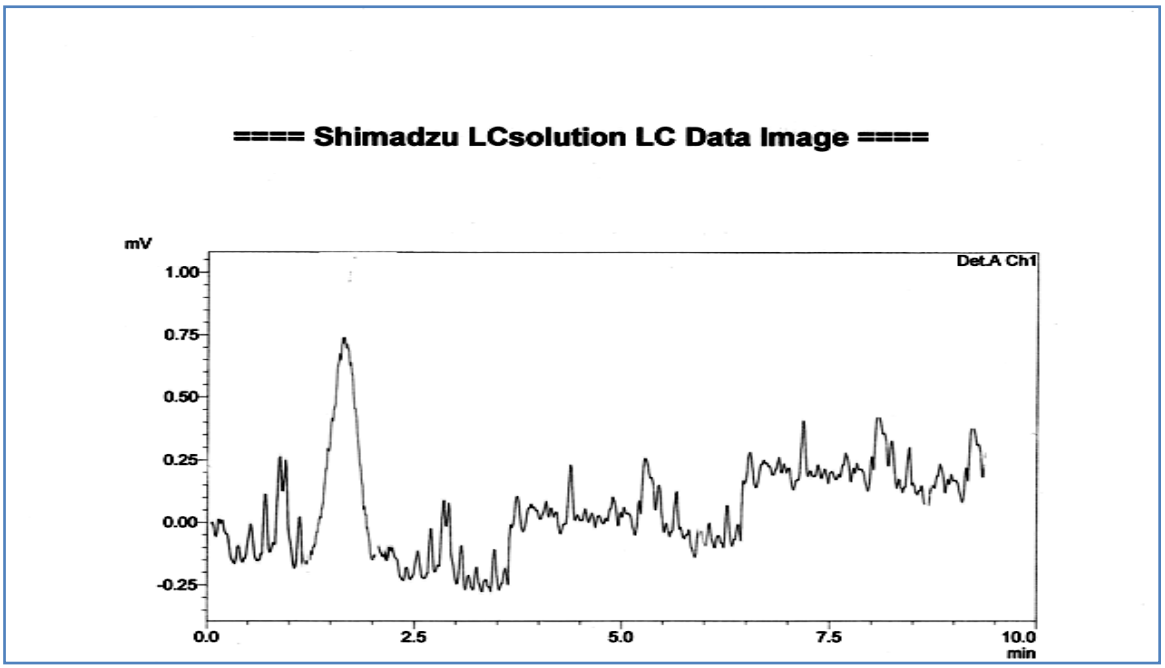


Fig.(3 -12). Detection limit of 0.25 ppm Histidine using Weakly acidic –Neutral red column (25 x 0.4 cm (i.d.)) , detection wavelength 254nm , flow rate 1.4 ml/min . Eluent %10 acetonitrile in buffer phosphate.

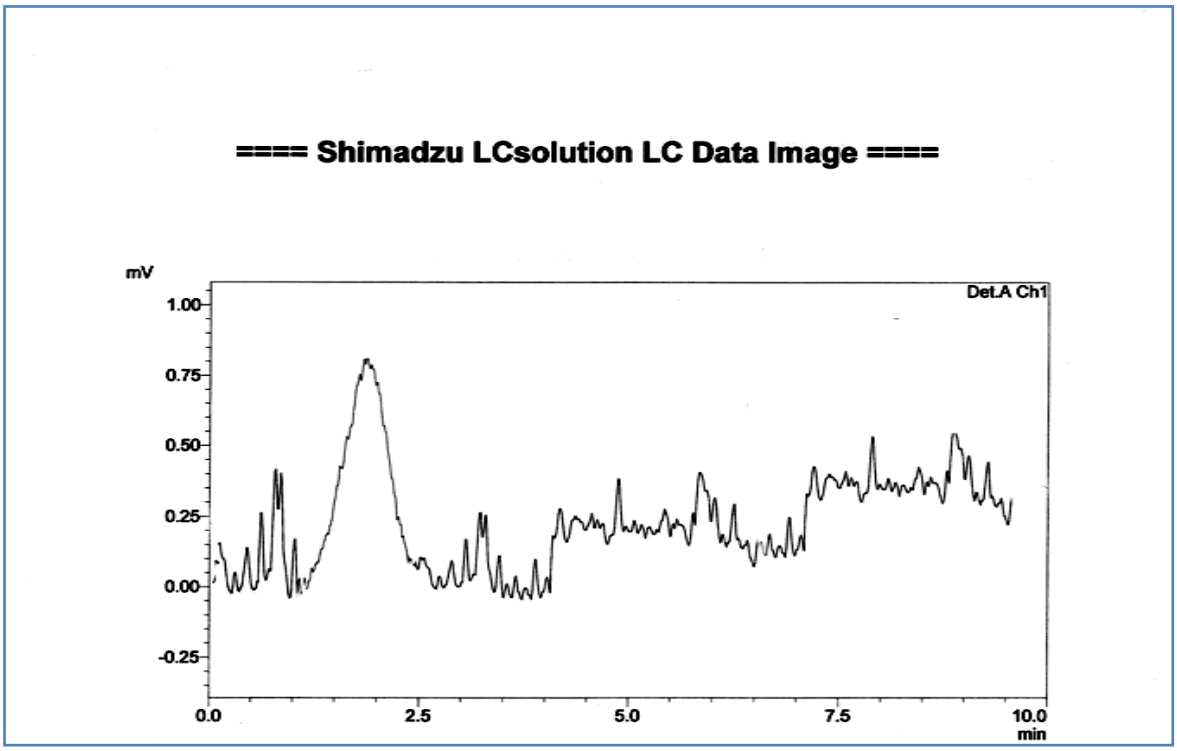


Fig.(3 -13). Detection limit of 0.05 ppm Phenylalanine using Weakly acidic – Neutral red column (25 x 0.4 cm (i.d.)) , detection wavelength 254nm , flow rate 1.4 ml/min . Eluent %10 acetonitrile in buffer phosphate.

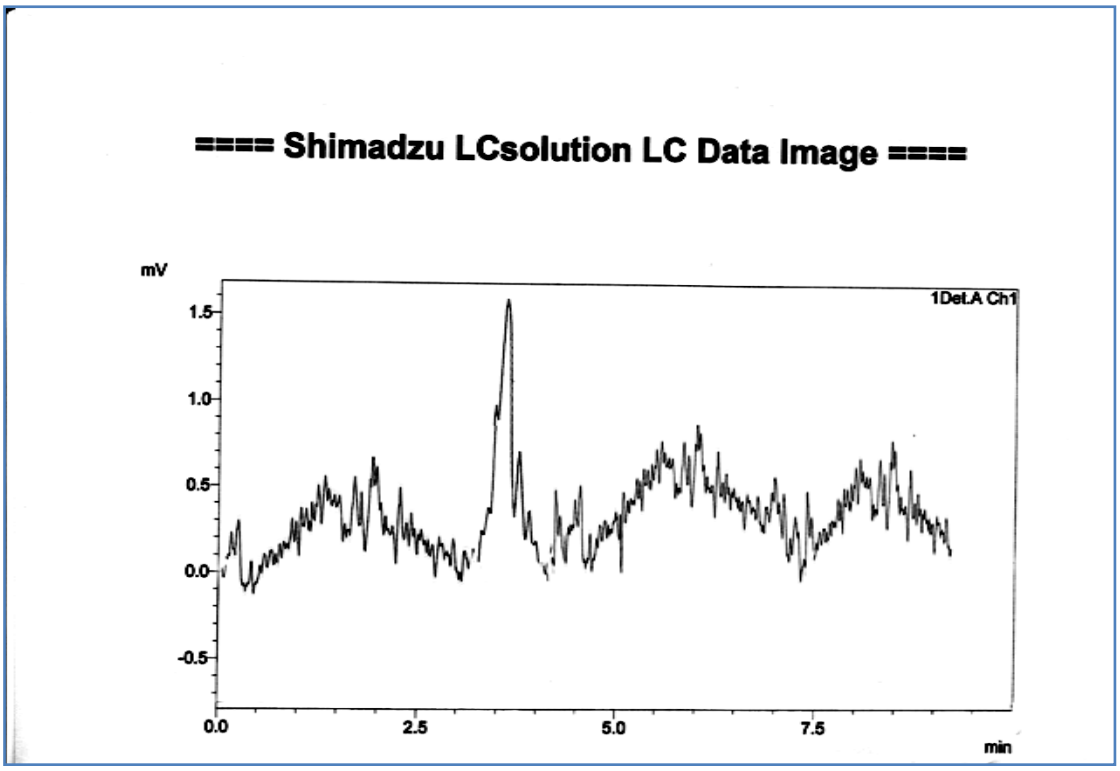


Fig.(3 -14). Detection limit of 0.1 ppm Tryptophan using Weakly acidic – Neutral red column (25 x 0.4 cm (i.d.)) , detection wavelength 254nm , flow rate 1.4 ml/min . Eluent %10 acetonitrile in buffer phosphate.

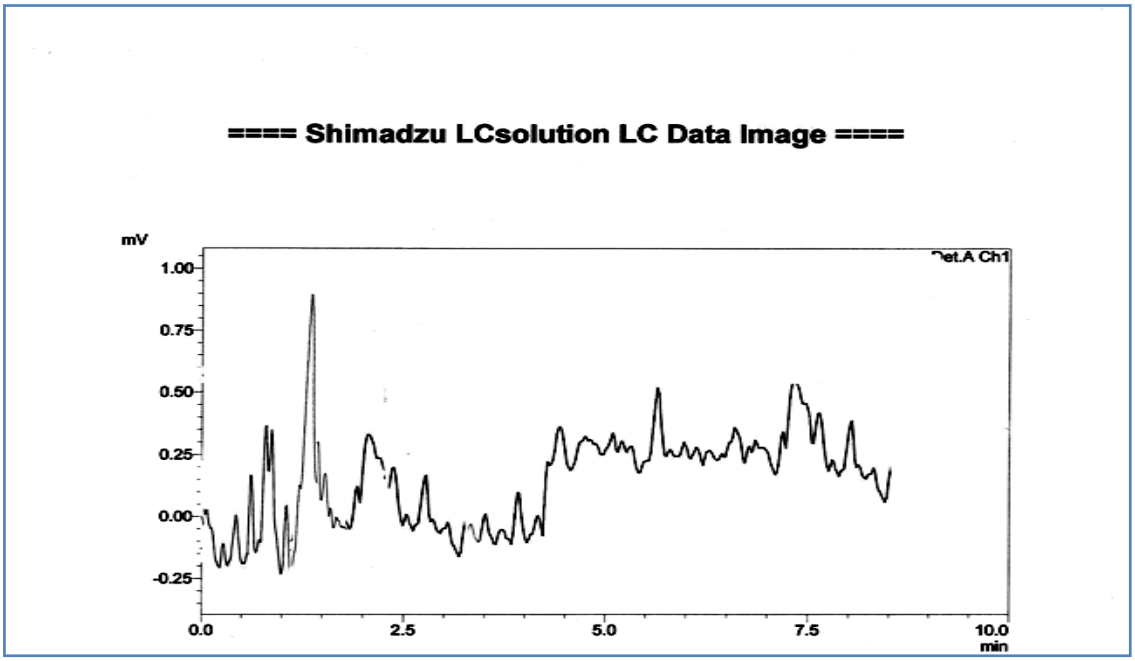


Fig.(3 -15). Detection limit of 0.08 ppm Tyrosine using Weakly acidic –Neutral red column (25 x 0.4 cm (i.d.)) , detection wavelength 254nm , flow rate 1.4 ml/min . Eluent %10 acetonitrile in buffer phosphate.

Prepared standard mixture solutions of the analyzed amino acids were injected for at least 3 times under the same condition and their concentration were calculated by measuring the peak area of each Amino acid and using their respective the linear equation. The recovery were ranged from 93.8% to 97.12% with an average of 95.23% , the relative errors were ranged from (-2.88—6.2) % with an average of -4.77% as shown in Table (3 -5).

Table (3 -5). Recovery and Percentage Relative Error for the Analyzed Amino acids using IPD method.

Amino acids	Concentration injected (ppm)	Average concentration Found(calculated)* (ppm)	Recovery%	Relative Error %	%R.S.D
Histidine	25	24.28	97.12	-2.88	0.04
Phenylalanine	10	9.52	95.2	-4.8	0.3
Tryptophan	5	4.69	93.8	-6.2	1
Tyrosine	2.5	2.37	94.8	-5.2	0.6

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

Conclusions

New stationary phase were prepared by the reaction of neutral red solution with weakly acidic cation exchanger. The stability of the new attached functional group on the resin was studied against different solvents. The new prepared resin was found stable and no depletion of the Neutral red moiety from the resin 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 $\acute{K}$, 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 amino acids Histidine, Phenylalanine, Tryptophan and Tyrosine. The analysis of amino acids using weakly acidic – Neutral red column has performed with 10% acetonitrile in buffer phosphate at pH 3 as mobile phase and also 100% buffer phosphates at PH 3 as mobile phase in flow rate 1.4 ml/min and detection wavelength 254 nm. This new resin 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 its 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 amino acid and other non UV – Visible absorbing species.

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الخلاصة

تم تحضير طور ثابت جديد بطريقة كروموتوكرافيا السائل ذات الاداء العالي من تفاعل صبغة الاحمر المتعادل مع راتنج المتبادل الكتيوني الحامضي الضعيف.

وقد درست الاستقرارية للمجموعة الفعالة الجديدة المرتبطة مع مذيبات مختلفة مثل الميثانول,الايثانول,البنزين,الكلوروفورم,الاسيتونايترايل,الاسيتون والبترولیوم ايثر, بالاضافة الى محاليل هيدروكسيد الصوديوم وحامض الهيدروكلوريك وقد وجد أن الراتنج الجديد مستقر ولم يلاحظ أي استنفاذ أو إفراغ.

كما أثبتت تحاليل الأشعة تحت الحمراء ارتباط الصبغة مع الراتنج باستخدام المواد المذكورة أعلاه.تم تعبئة الطور الثابت الجديد في عمود من الفولاذ المقاوم للصدأ وذلك بسكب عالق الراتنج 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 الاحماض الامينية مثل الهستدين, الفنيل أنيلين, التربتوفان, التايروسين.

تحليل هذه الاحماض الامينية بواسطة العمود الجديد تم باستخدام طور متحرك يتكون من 90% بفر فوسفيت و 10% اسيتونايترايل فكان معدل الصفيحات النظرية (N) يساوي 316.59 وكان معدل الارتفاع المكافئ يساوي 0.085 سم, عوامل الاستيعاب تتراوح بين 1.8-2.86 , العوامل الانتقائية تتراوح بين 0.21-1.17 , والتناظر يتراوح بين 0.99-1.09 ودرجة الفصل تتراوح بين 0.93-3.77 .

تم تحليل نفس الاحماض الامينية بنفس العمود المحضر باستخدام البفر فوسفيت كطور متحرك فكان معدل الصفيحات النظرية (N) يساوي 234.79 ,

ومعدل الارتفاع المكافئ يساوي 0.107 سم, عوامل الاستيعاب تتراوح بين 1.13-4.73 , العوامل الانتقائية تتراوح بين 0.23-1.22, والتناظر يتراوح بين 1.1-1.18 .

الانحراف القياسي النسبي المئوي كانت قيمته ضمن المدى 0.04-1.00 % والاسترداد المئوي كان بحدود المدى 93.80-97.12 % والخطأ النسبي كان ضمن حدود المدى -2.88- 6.2- .

أن منحنيات المعايرة للمركبات المحللة كانت خطية من الحد الأدنى لكشفها الى 50 جزء بالمليون مع عوامل ترابط تتراوح بين 0.9991-0.9996 وكانت حدود الكشف تتراوح من 0.05 الى 0.25 جزء بالمليون.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم /قسم الكيمياء

الفصل الكروموتوكرافي لبعض الاحماض الامينية بواسطة طريقة كروموتوكرافيا السائل العالية الاداء باستعمال طور ثابت جديد

رسالة

مقدمة إلى كلية العلوم- جامعة النهرين

كاستكمال جزئي لمتطلبات نيل درجة الماجستير في الكيمياء

من قبل

علي عباس رسول

بكالوريوس علوم كيمياء

جامعة الكوفة 2005

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