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Preparation of Monolithic Stationary Phase for Affinity-Based Separation of α- Amylase Enzyme

A Thesis

Submitted to the Council of the College of Science, University of Babylon as a Partial Fulfillment of the Requirements for the Degree of Master of Science / Chemistry

By

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بسم الله الرحمن الرحيم

﴿هوَ الَّذي جَعَلَ الشَّمْسَ ضِيَاءً وَالقَمَرَ نوراً وَقَدَّرَهُ مَنَازِلَ لِتَعْلَموا عَدَدَ السِنِينَ وَالحِسَابَ مَا خَلَقَ اللهُ ذلكَ إلاَّ بِالحقِّ يفصِّلُ الآياتِ لِقَومٍ يَعْلَمونَ﴾

صدق الله العظيم

سورة يونس (اية 5)

Supervisor's Certification

I certify that this thesis was carried out under my supervision at the department of chemistry in College of Science at University of Babylon, as partial fulfilment of the Requirement for the degree of Master in Analytical Chemistry

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Recommendation of Head of Chemistry Department

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Dedication

I dedicated this work to…..

my father and my mother…….

my supervisor, Professor Dr. Ahmed Ali Alkarimi …….

all the Professors, staff, and all my postgraduate colleagues…..

Khilowd Omran Ali

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Praise to Allah, Lord of the worlds, who helped me and gave me the strength, patience, and endurance to complete this thesis.

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I’m grateful to those who are the symbol of loyalty and my support in life, my brothers and sisters.

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List of abbreviations

Shorten	Full name
A	Absorbance
A.Ac	Acrylic acid
DVB	divinyl benzene
AMC	Affinity monolithic chromatography
DSC	disuccinimidyl carbonate
CDI	Carbonyl di imidazole
CNBr	cyanogen bromide
MIP	molecularly imprinted polymer
IMAC	immobilized metal-ion affinity chromatography
AFM	Atomic force microscopy
BSA	**bovine serum albumin**
HSA	**human serum albumin**
BIA	Biotechnology company focused on the production of methacrylate monolithic HPLC columns and developing industrial purification
BET	Brunauer-Emmett-Teller

CEC	Capillary electrochromatography
DAP	2,2- dimethoxy -2-phenyl Acetophenone
EDMA	Ethylene glycol dimethacrylate
FE-SEM	Field Emission Scanning Electron Microscopy
FT-IR	Fourier-transform infrared spectroscopy
GMA	glycidyl methacrylate
HPLC	High-performance liquid chromatography
hr	Hours
id	Internal diameter
min	Minute
od	Outer diameter
SEM	Scanning electron microscopy
C18	Silica column
°C	Temperature(Celsius)
TEM	Transmission electron microscopy
TSP	3-(trimethoxysilyl) propyl methacrylate
UV	Ultraviolet
IU/L	Unit of α-amylase activity
BPO	Initiater

Summary

Affinity monolith chromatography (AMC) is an effective technique for isolating, analyzing, or studying certain target chemicals in samples. Affinity monolithic support and an abiologically binding agent serve as the stationary phase in monolithic chromatography.

This study includes two parts:

First part: Preparation (A.Ac-co-EDMA-co-GMA) monolith.

The monolith consists of two monomers, glycidyl methacrylate (GMA), acrylic acid (A.Ac) and cross-linker ethylene dimethacrylate (EDMA) was prepared using U.V photo polymerization inside borosilicate columns (60 mm) with an inner diameter of (1.5 mm) and outer diameter of (3.0 mm), this borosilicate tube was salinized to prepare the inner surface of the tube for the polymerization process.

The prepared monolith was identified using FT-IR, ^{1}H-NMR , FE-SEM and BET techniques. Then the Sci-Finder program proved that the monolith(Acrylic acid –co-Ethylene dimethacrylate –co-Glycidyle methacrylate modified with starch) is new and prepared for the first time.

The optimum conditions for column preparation were investigated, such as the effect of the ratio between two monomers, the distance between the irradiation source and column, the impact of irradiation time, and the porogenic solvent types**.**

The column features were studied, such as the swelling percentage, permeability, porosity and injection flow rate.

Second part: application of prepared column by separating the α- amylase enzyme in human serum.

The prepared column was modified by adding starch solution (pH =10) to react with the epoxy group of GMA for 12 hours at room temperature to prepare an affinity column that can be used for separation and purification of α-amylase enzyme in human serum. The enzyme activity was calculated and compared with the direct method of the Bio Labo kit. The activity of the amylase enzyme was 53.496IU/L at (10µl / min) compared with the enzyme activity obtained by the direct method of 43.008IU/ L for the same serum sample.

It was found that the prepared affinity column can be used many times without changing the monolith's morphological properties. Additionally, It can be preserved for three months and 20 days without any change, besides the high efficiencies of this process.

CHAPTER ONE

INTRODUCTION

AND

LITERATURE

REVIEW

1. INTRODUCTION

1.1. General Introduction

Monolithic Materials: The term “monolith” is derived from the Greek term “monolithos”, which holds the meaning of “Monos”, which is (singular) and “lithos”, meaning (stone)[1]. As a result, monoliths are columns containing a single large stone; besides, they are porous materials with multiple pore sizes, such as (50nm), that can be found (flow-through or macropore pores)[2].In HPLC can easily replace conventional particle columns with monolith separation columns as a result of its comfort in manufacturing, versatility for many chemical surfaces, and suitable permeability for mass transportation with low back pressure regardless of the high flow rate [3].

Polymerization mixture contains functional monomer and cross-linker, initiator, and porogen that can be used to prepare monoliths. Monoliths can be investigated using various methodologies, including the most commonly used thermal polymerization, radical, and photo-initiated radical polymerizations[3,4].

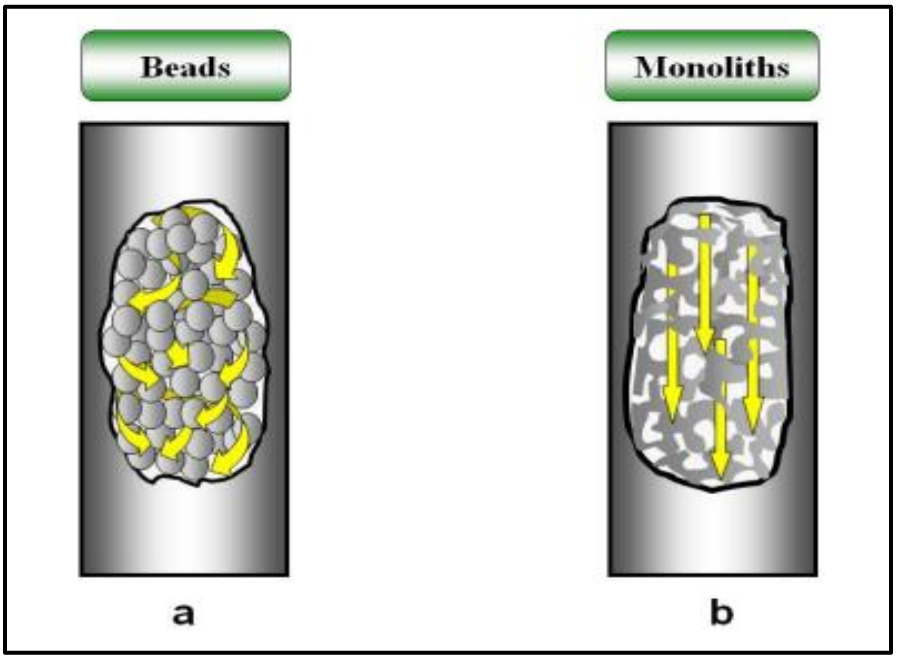
In 2000, Merck began producing monolith columns called "Chromolith Performance." This column is a monolith of C18 silica that is chemically bonded [5,6].

Merck then launched monolith columns of the second generation to develop further and improve previous monolith columns. The separation

was completed quickly and with high resolution while applying minimal pressure in this column[7–9].

The pores in monolithic columns are divided into three types: macropores or through-pores (> 50 nm), mesopores (2-50 nm), and micropores (2 nm). The macropores (through pores), when a high flow rate is provided, reduce the back-pressure of the column to manage the column permeability because they permit the solvent to transmit through the monolithic column quicker than the packed column, as shown in Figure(1.1) [10–12].

The continuous macroporous polymeric systems, an example of new materials, were prepared to reduce or minimize needless discontinuity and address the flow issue via inter-particle voids. Because the mobile phase must traverse the entire separation medium, these new "monolithic materials" have a higher capacity for transferring mass via convective transport in this case[13,14]. As a result, the flow rate does not affect efficiency, resolution or dynamic binding capacity. Figure (1.1) depicts the variation in the passage of the mobilephase.



Figure(1.1) Passage of the mobile phase through particulate media (macroporous particles) (a) and continuous support (macroporous continuous rods) (b[15]**.**

Monolithic columns produce a "single large particle" that completely envelopes the column, leaving no free space. A continuous skeleton of connected pores makes a continuous skeleton within this monolith, filled with interconnected pores which construct flow channels of consistent size [16,17].

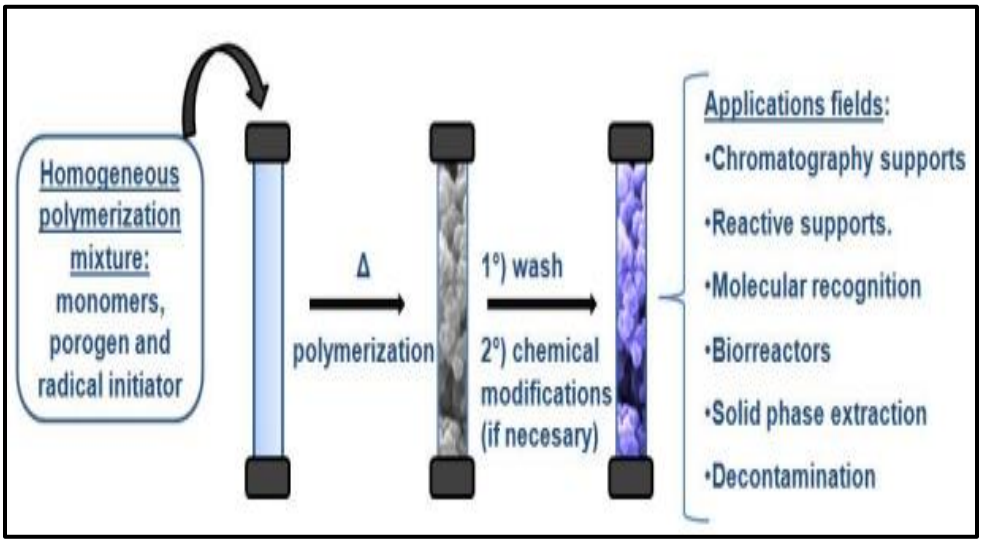
The monolithic column forms a network of channels in the continuous phase of a porous material[15], resulting in higher axial permeability, a larger internal pore surface area, and lower back pressure than conventional packed columns. As a result, these monoliths enable separation processes to be carried out at high flow rates and low back-pressures[15].

In addition, These channels improve the contact between the analyte and the stationary phase's active sites [13,16]. Furthermore, differences in hydrodynamic properties are discovered. In particle columns, the pores are only partly utilized, and diffusion is the significant constraint, whereas convection governs interphase mass transfer in monoliths, and the total pore volume is used.

Various techniques, such as scanning electron microscopy (SEM), transmission of electron microscopy (TEM), and atomic force microscopy (AFM), can be used to examine the physical properties of monolithic materials[13]. Significant data is provided by the three techniques on the morphology of monolithic materials and for measuring monolithic porous properties like pore size and determining the column's hydrodynamic features and mechanical strength[16].

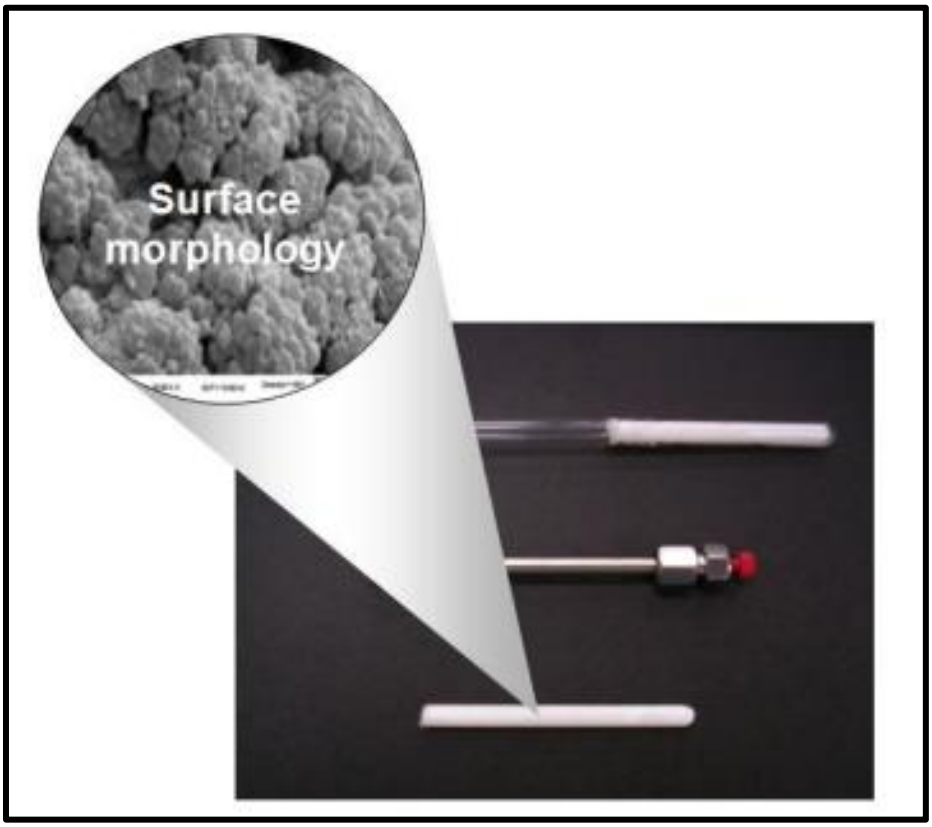
1.2.Continuous Macroporous Rods

All of the benefits of porous particle systems are present in these materials: they are stiff and retain their porous structure despite the solvent, even when they are dry. The radical initiator, a set of porogenic solvents with appropriate value relative to the polymer formed[18], and the necessary monomers [monovinyl monomer(s) and functional poly monomer(s) as cross-linking agent(s)], are all mixed in a homogeneous polymerization mixture to produce these supports, as produced to the copolymerization reactions in suspension that produce porous particles [13,19] A mould can stir up the polymerization reaction (which determines the shape of the support). Figure (1.2) depicts the steps involved in producing continuous macroporous polymer rods.



Figure(1. 2) Various steps for making continuous polymer rods[13].

The functional groups that appear on the monolithic surface after polymerization in a mould will vary depending on the monomer/s used[20]. The immobilization of biological catalysts or particular ligands as a separation media is then carried out using these groups for small and large molecules by affinity chromatography via HPLC. A glass tube filled with the polymerization mixture is commonly used as a mould for continuous porous polymer rods [21]. Figure (1.3) shows different moulds for the preparation of macroporous monolithic rods. Continuous porous polymer synthesis is versatile and can be carried out under different experimental conditions.



Figure(1. 3) Macroporous monolithic rods prepared in a glass tube and a column of stainless steel HPLC[21].

1.3.Types of monolith:

1.3.1.Inorganic Monoliths

Silica monolith is an inorganic monolithic column first used in chromatography as a stationary phase. The primary benefits of monolith silica columns over particle columns are accelerated analysis time and lower back pressure [13] . Sol-gel, (one pot) and other polymerization methods can be used for preparation[22]. The condensation reactions and hydrolysis of alkoxysilane are commonly in the sol-gel process.In addition to alkoxysilane, an additive is also used, poly(ethylene glycol) (PEG) or it's analog [23,24].

The steps used to produce silica monolith provide a monolith with Si-OH groups, which causes non-specific adsorption, and the pH range is limited[25]. The monolithic silica column can be prepared inside fused silica capillaries or mould as a polymeric coating material (column size comparable to conventional HPLC column). The preparation of the mould serves the goal of reducing the overall volume of the structure [9,24].

1.3.2. Organic Monoliths

Organic monolith or monolithic polymer columns are speedy to prepare, and the method is straightforward, with broad pH stability and rapid modification [26] . These monoliths are prepared inside capillaries, glass tubes, or microfluidic chips and contain polymerization bonds formed by polymer solutions, the polymerization solution contains monomers, cross linkers, initiators, and porogenPolystyrene, polymethacrylate, and polyacrylamide are common monolith column polymers[4,27].

Thermal polymerization, photo-initiated polymerization, microwave irradiation, and radiation-initiated polymerization can all be used to prepare organic monoliths[28]. Monolithic columns have been broadly utilized as a stationary phase for a variety of component separations, including proteins[3,29], oligonucleotides[30], peptides, and synthetic polymers[31].

As a result, there are three shapes of organic monoliths, the thin disk (up to 3 mm) is the first type, prepared in flat cylindrical moulds. The second type is the cylindrical monolithic rod-like column formed by in-situ polymerization methods in stainless-steel or glass tubing. This column

measures 30-50 mm in length and 1-8 mm in diameter. These columns are designed to separate proteins quickly [32–34].The monolithic capillary is the third and final type, and it is widely used in capillary electrochromatography and capillary HPLC[35,36].

1.3.3. Hybrid Organic-Inorganic Monoliths

Organic hybrid making materials possess several advantages over other monolithic manufacturing materials, including bending, low density, biocompatibility, excellent mechanical properties, and long shelf times. Composite materials are classified into two types based on their chemical composition: hybrid polymer-based monolith (HPM) and hybrid silica-based monolith (HSM) [25]. The sol-gel technique can be used to synthesize hybrid materials, in which the combination of the corresponding precursor alteration from the initial formation of the colloidal suspension (sol phase) to the organic-inorganic tissue arising in the stable xerogel [37].

1.4.Affinity Monolith Chromatography

The term "affinity chromatography" refers to a type of liquid chromatographic procedure that separates injected or applied compounds based on their attachment to a related mimic or immobilized biological agent (for example, the “affinity ligand”) [38].This technique takes advantage of strong and reversible interactions found in many chemicals and biological agents[39]. These interactions include antibody binding to an antigen and the binding of an enzyme to its substrate. Many forces or interactions constantly exist between the immobilized agent and its target, resulting in this binding's strength and selectivities, such as

coulombic forces, hydrogen bonds, dipole-dipole interactions, and steric effects [40,41]

Biochemicals like enzymes, antibodies, and other proteins may be isolated using affinity chromatography. This method also can be combined with different techniques for target compound separation and analysis [42].These characteristics have made affinity chromatography a famous method for isolating biochemical and chemicals, performing clinical or biochemical assays, biotechnology, and studying biological processes [40].Various supports can be utilized in conjunction with the immobilized binding agent in affinity chromatography. For this purpose, particulate-based materials like agarose beads, glass beads, silica particles and, particulate supports created by various organic polymers were previously used[42]. However, interest in employing affinity chromatography on monolithic supports is developing[42,43]. This method is called affinity monolith chromatography(AMC) [39,44]. Figure (1.4) depicts a typical scheme for compound isolation and measurement in AMC or traditional affinity chromatography[40,42].

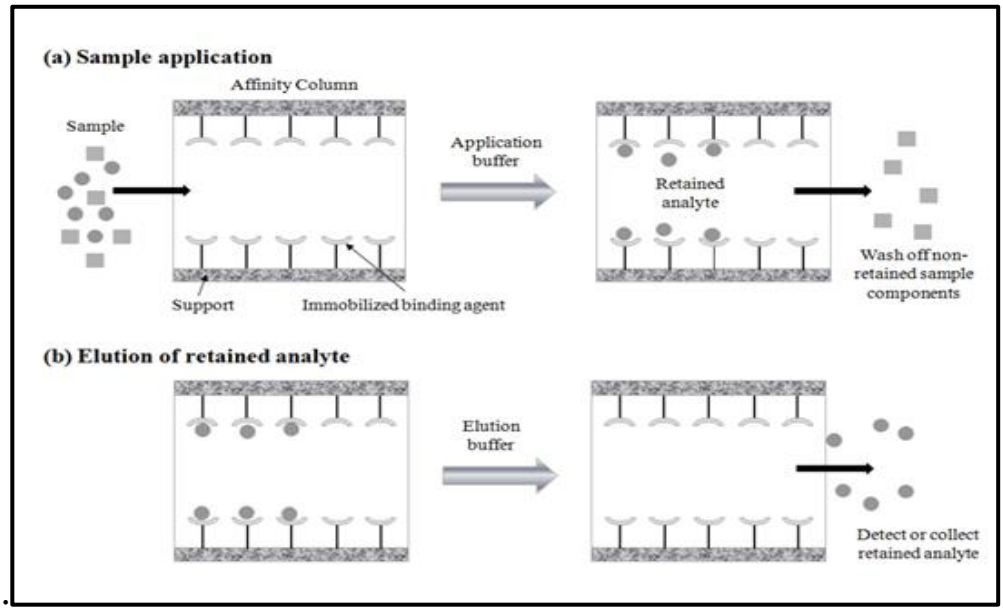


Figure (1.4) (a) sample application and (b) elution of the retained analyte or target compound[40]**.**

1.4.1. Monolithic Materials for Affinity Chromatography

A wide range of Monoliths for affinity chromatography was reported; one of these materials is monoliths based on glycidyl methacrylate (GMA)/EDMA copolymers, silica, agarose and cryogel[39].

1.4.1.1 GMA/EDMA monoliths

Copolymers of GMA and EDMA have been applied most frequently to create monoliths for affinity chromatography by comprising several supports available from BIA separations as convective interaction media (CIM)[45].

Figure (1.5) depicts a general scheme for preparing a GMA/EDMA monolith. In this instance, azobisisobutyronitrile (AIBN)

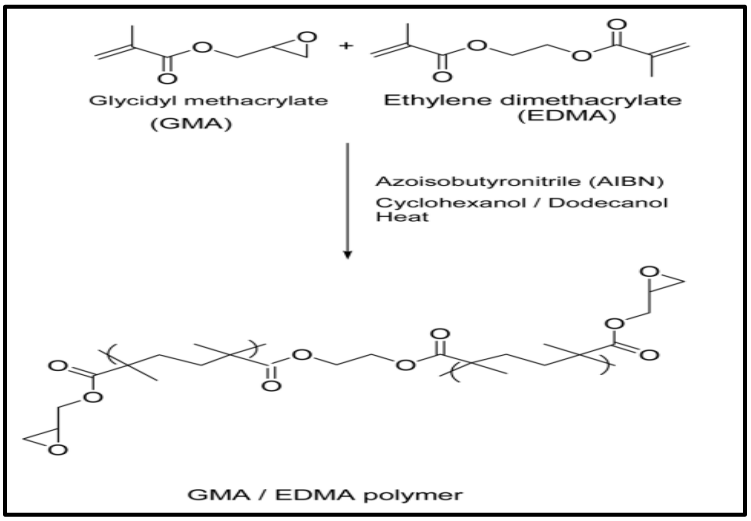


Figure (1.5) The formation of a GMA/EDMA copolymer. This illustration depicts only a portion of the final polymeric structure[46]**.**

The resulting monolith has epoxy groups, which may be used for ligand immobilization or turned into a diol form under acidic conditions [47,48].The diol form has a hydrophilic surface similar to that of more traditional affinity chromatography supports like diol-bonded silica or agarose[49]. Like the other affinity supports, this diol form can be activated later using several methods. After that, If the coupling approach calls for it, a ligand can be immobilized on this active support by passing it through a monolith with proper pH and buffer conditions.

Affinity chromatography was carried out using GMA/EDMA monoliths formed into rods [47,50],disks, and/or membranes[47,51–53]. Using a GMA/EDMA monolith with affinity ligands is beneficial because the GMA monomer contains epoxy groups. These groups allow for covalent immobilization, or other ways of coupling can be started using these groups or as a starting point. Furthermore, as with other affinity supports, the diol groups produced on this material for many biological agents tend to provide low-nonspecific binding support

[54].Other benefits of GMA/EDMA monoliths include their ease of preparation and ability to be manufactured with various surface areas and pore sizes [54],the composition of the porogen can be changed to control the average pore diameter and surface area. Other variables include the monomer-to-crosslinker ratio, the amount of porogen, and the polymerization time [53]. Modifying functional groups on the surface allows for control and optimization of the affinity ligand's total amount that can be placed on such supports [53]. However, compared to particulate silica monoliths or silica supports, GMA/EDMA monoliths have low surface areas, limiting the total amount of ligand that can be immobilized onto this material.

1.4.1.2. Agarose monoliths

Agarose monolithic supports are utilized in affinity chromatography; this is not shocking considering that the particulate form of agarose was always well-liked support for affinity separations[55,56]. An agarose emulsion is cast when preparing a monolith with enormous pores ranging from 20 to 200 µm, then poured into glass columns or forms that fit with a stopper at the bottom, the agarose gels changing into the proper shape when the temperature is reduced to 20 ^{0}C. Agarose can be produced as rods, discs, membranes, and threads, among other configurations.

The same chemical processes that are employed for agarose particles can be used to activate these materials and use them in ligand immobilization.

1.4.1.3. Silica monoliths

Silica-based monoliths are a different category of monoliths that are gaining popularity, these are interesting for several reasons, including the

widespread usage of silica particles, a material closely linked to them in HPLC. Sol-gel entrapment [56,57] and silica monoliths have both been utilized with affinity ligands. Commercially available silica monoliths, such as Chromolith-Si from Merck and Chromolith-NH2 from Applied Materials, have been used to prepare affinity columns [9,57,58].Even though bare silica monoliths do not naturally possess reactive groups that can be employed for the ligand's covalent Immobilization, they nonetheless can be transformed into diol [48].

The trapping of ligands in such support can be accomplished using the same sol-gel technique used to prepare silica monoliths[59,60]. Entrapment has a few benefits over covalent Immobilization for ligands, one of which is that the support and affinity monolith are created in the same process. Theoretically, because the ligands are neither chemically bonded to the support nor changed throughout the immobilization process, this technique should result in ligands that are still very active. Due to the sol-gel matrix's tendency to shrink, one drawback of sol-gel entrapment is that technology can only be used with columns up to a specific maximum diameter. Because of the creation of alcoholic byproducts during polymerization, another drawback of many sol-gel precursors is that they, like proteins, can denature ligands [61].

1.4.1.4.Cryogels

Cryogels have also been used to prepare affinity monoliths by using monomers that are dissolved in an aqueous phase and polymerizing at temperatures below -10 °C, cryogels are very porous media. , several macropores of 10to 100 µm in diameter are formed, the fact that cryogels

possess huge, intricate pores that are hydrophilic is a benefit. As a result, materials of varying sizes, such as proteins and material from microbial or animal cells, can pass through these pores[39,62] ,cryogels have substantially lower surface areas than other chromatographic supports, despite having low back pressures. When utilizing cryogels in affinity separations, this can lead to limited amounts of immobilized ligand [39].

1.4.2.Immobilization methods for affinity monolith

The manner in which the affinity ligand is inserted into or linked to the monolith in affinity monolith chromatography (AMC) is another crucial aspect to consider. Because using the incorrect methodology can cause ligand activity to change or disappear, the immobilization method should be carefully chosen. Through inappropriate orientation, multisite attachment, or steric hindrance [40], an immobilization technique should ideally not affect or disrupt the activity of the ligand. Figure (1.6) shows that many immobilization techniques have been considered for application in AMC [63]. Covalent methods, biospecific adsorption and other techniques based on entrapment, molecular imprinting, or coordination-based Immobilization are the most common components of these strategies[42,44].

1.4.2.1.Covalent immobilization

Affinity ligands are most frequently attached to monolithic supports through covalent Immobilization[44,64]. This technique involves activating the monolith and bringing it into contact with the wanted affinity ligand[64]. Either cycling reagents and ligands through the monoliths or dipping the monoliths into solutions of reagents and ligands

can be used to complete the activation and immobilization procedures [47,65].

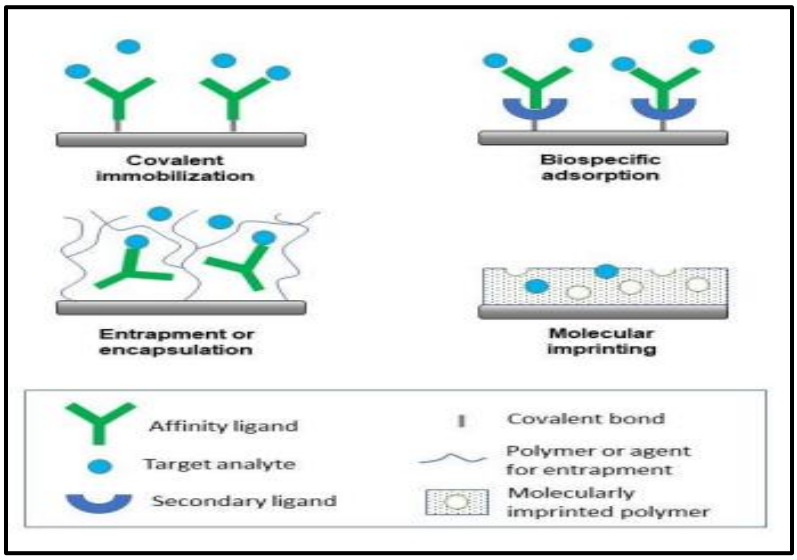


Figure (1.6) Strategies used to put or immobilize a binding agent within an AMC support. [66]

Affinity ligands were immobilized to monoliths using a variety of covalent techniques. The epoxy approach is a popular covalent immobilization technique. This approach often creates secondary amine bonds by coupling epoxy groups on monoliths and amine groups on affinity ligands[47,67]This approach is simple to apply to monoliths with epoxy groups built into their structure, like GMA/EDMA monoliths [42]Despite being straightforward and quick, this method tends to yield a less immobilized binding agent than other covalent techniques because the epoxy groups are prone to hydrolysis loss . Numerous AMC ligands have been immobilized using this method. This way, binding agents such as protein A, protein G, protein L, BSA, HSA, antibodies, amino acids, enzymes, synthetic dyes, boronates, and antibodies have been connected [65,68]. Another typical way of immobilization employed in AMC [67],

is the Schiff base method, often known as reductive amination[67].The activity of affinity ligands tends to be more assertive with this method than with the epoxy method. However, some activity may be lost if combined with a severe reducing agent [65,67].This technique has immobilised affinity ligands such as protein A, HSA, aptamers, enzymes, heparin, chondroitin sulfate A, boronates , and antibodies within the monoliths[39,40,69,70].Like the Schiff base approach, the glutaraldehyde approach uses amine-group-containing support. As with the Schiff base approach, the groups are combined with glutaraldehyde to create aldehyde groups, which are subsequently combined with amines on affinity ligands. Compared to the Schiff base method, this approach involves more stages [71,72].

Carbonyl di imidazole (CDI) is an alternative technique for covalent immobilization in AM[72]. By converting epoxy groups to diols beforehand, GMA/EDMA monoliths can be utilized for this procedure since they include alcohol groups used in this method[53]. The 1,1'-carbonyl di imidazole is then used to react with these alcohol groups and activate them. Then, a nucleophilic substitution between the activated groups of the support and the main amine groups on the ligand immobilizes an affinity ligand[73]. Although this procedure is easier to use than the glutaraldehyde and Schiff base procedures, it also typically yields lower ligand activity [40]. In the AMC, protein A, HSA, L-histidine, glycosaminoglycan, and antibodies have all been immobilized using this technique[58].

Another method for covalent Immobilization in AMC is the disuccinimidyl carbonate (DSC) technique [47].It employs a monolith

containing either alcohol or amine groups, which, after that, is then reacted with DSC to produce a succinimidyl carbonate-activated form [39,40] These groups can then react with affinity ligands' primary amine groups to produce a stable carbamate linkage, even though this technique is quick, it is susceptible to hydrolysis, reducing the amount of affinity ligand that can be immobilized[40] This method was used to immobilize HSA on (GMA/EDMA) monoliths and protein A on a silica monolith that contains amine[39].The hydrazide process is a covalent method for immobilizing glycoproteins and carbohydrate-containing ligands[38–40]. This procses requires a greater amount of steps than some of the other coupling methods discussed so far, but it does allow for the site-selective immobilization of glycoproteins via their carbohydrate chains. This technique has been used to immobilize antibodies within organic monoliths as well as to couple AGP in a silica monolith[73]. In AMC, the cyanogen bromide (CNBr) method was also used, particularly for carbohydrate-based materials such as agarose[74]. CNBr reacts with alcohol groups on the support in this technique. The activated support can be utilized to immobilize affinity ligands containing amine groups by forming an isourea linkage[38,42]. This process is straightforward and necessitates relatively mild conditions for the attachment of ligands[40].Anyhow, because CNBr is a toxic material, it must be used with extreme caution[72].

1.4.2.2 Bio-Specific Adsorption

A secondary binding agent immobilized to the support in bio-specific adsorption holds an affinity ligand in place[40,65].The secondary binding agent's covalent immobilization is the first step in this method. After

that, the affinity ligand is permitted to adsorb to this agent. Cross-linking the affinity ligand absorbed with the secondary binding agent can make it more stable in some cases[40].

Because they can bind the lower stem region of antibodies from distinct species, secondary agents like protein A and protein G are widely used in biospecific antibody adsorption[40,69]. Avidin and streptavidin are two other examples in which biotinylated affinity ligands can be captured and bound using them[69]. Biospecific adsorption can make it simple to regenerate the support by employing an elution step to remove the affinity ligand and an application step to reapply a new batch of the affinity ligand. However, because it requires multiple binding agents, the cost of biospecific adsorption may be higher than that of covalent immobilization. Because of the steric hindrance, the secondary binding agent's presence may also increase the likelihood of non-specific binding, lowering the affinity ligand binding capacity. In order to get monoliths ready for antibody capture by protein A or protein G, biospecific adsorption in AMC was utilised[40,50,75].

1.4.2.3. Other Immobilisation Methods

AMC has used various formats for immobilisation and the preparation of ligands. One example is entrapment, a noncovalent technique in which ligands are encased inside support [40].The support in this method typically contains pores or openings smaller than the affinity ligands that are entrapped but permit smaller targets to access those ligands[76].

Another method utilized in AMC to produce supports with target-specific binding regions is molecular imprinting[40,77,78]. This is

frequently accomplished by polymerizing a mixture of functional monomers and a cross-linking agent while a template molecule corresponds to the desired target [77]. A molecularly imprinted polymer (MIP) is the type of material produced by this process [77,78].This technique was utilized in AMC with a protein G required to isolate a recombinant version of this protein from cell lysates, with a cytochrome c imprint to recognize [79].

AMC affinity ligands have also been immobilized using coordination chemistry, this method is comparable to covalent immobilization and's commonly utilized alongside immobilized metal-ion affinity chromatography (IMAC)[44] ,this technique relies on the specific interactions among immobilized metal ions and particular amino acids in proteins and peptides [80].

1.5.Glycidyl Methacrylate, GMA

Glycidyl Methacrylate, also known as (2,3-Epoxypropyl Methacrylate), is a clear, colorless liquid with a potent ester and fruity aroma. It comprises a polymerisable methacrylate functional group on one end and a reactive epoxy group on the other. It is slightly miscible with water, soluble in most organic solvents, and volatile. Its vapor is heavier than air[81,82].In epoxy resin manufacturing, it is a standard monomer made of methacrylic acid with glycidol. It piqued the interest of researchers because pendant epoxide groups can participate in various chemicals [83,84],and the structure of GMA can show in figure (1.7).

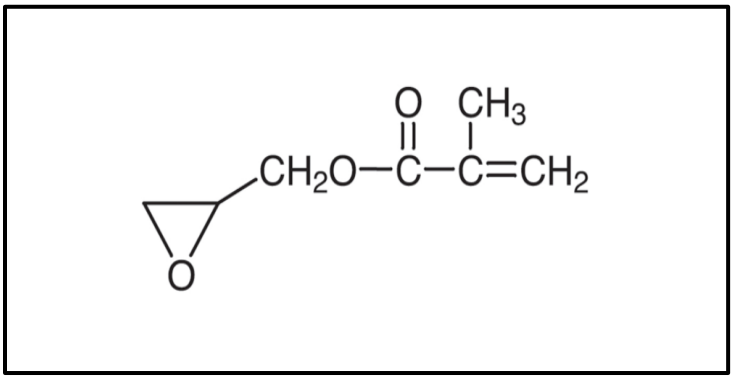


Figure (1.7) The structure of glycidyl methacrylate (GMA)

A strain in the ring's three members is thought to be the primary reason for the high reactivity of the epoxide group so that this group can be chemically modified for various applications. For example, glycidyl methacrylate was utilized to bind enzymes and other biologically active species[85].

The polymerization of glycidyl methacrylate to create varying polymers occurs in solution using a solvent that can dissolve the GMA monomer but, for the polymer, is a weak solvent. Many methods exist for synthesizing GMA copolymers[86].

Polymer surfaces can be altered by many reactions with the epoxy ring, preventing a wide range of chemical transformations after copolymer formation, as figure (1.8) depicts[87,88] .

Surface modifications may be attained through the introduction of specific ligands required for bio affinity chromatography; these reactions are depicted in figure (1.9)[89,90].

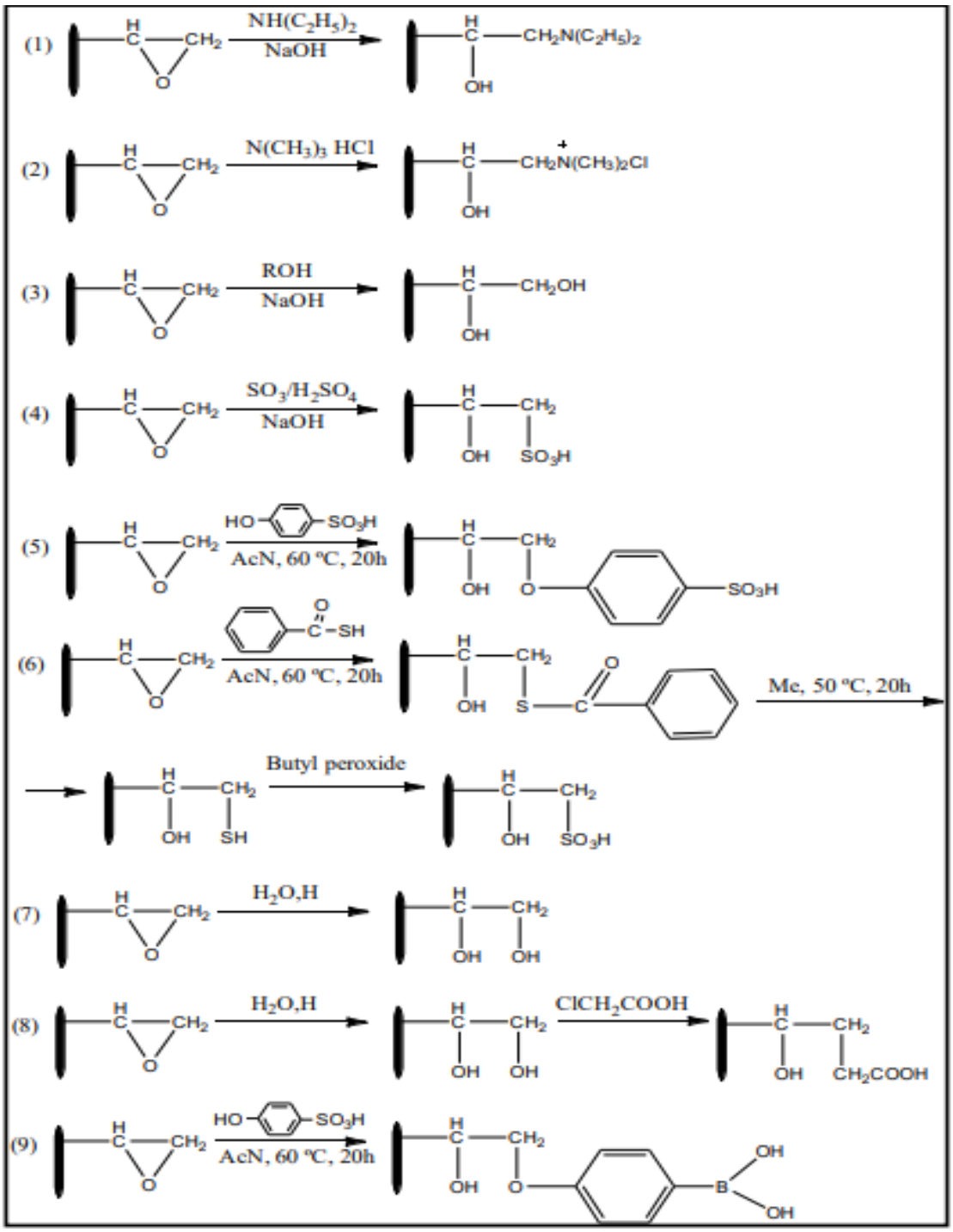


Figure (1.8) Chemical conversion of epoxy groups (1, 2) amination; (3, 4) alkylation; (5, 6). Sulfonation; (7) hydrolysis ; (8) carboxymethylation; (9) modification with p-hydroxy phenylboronic acid

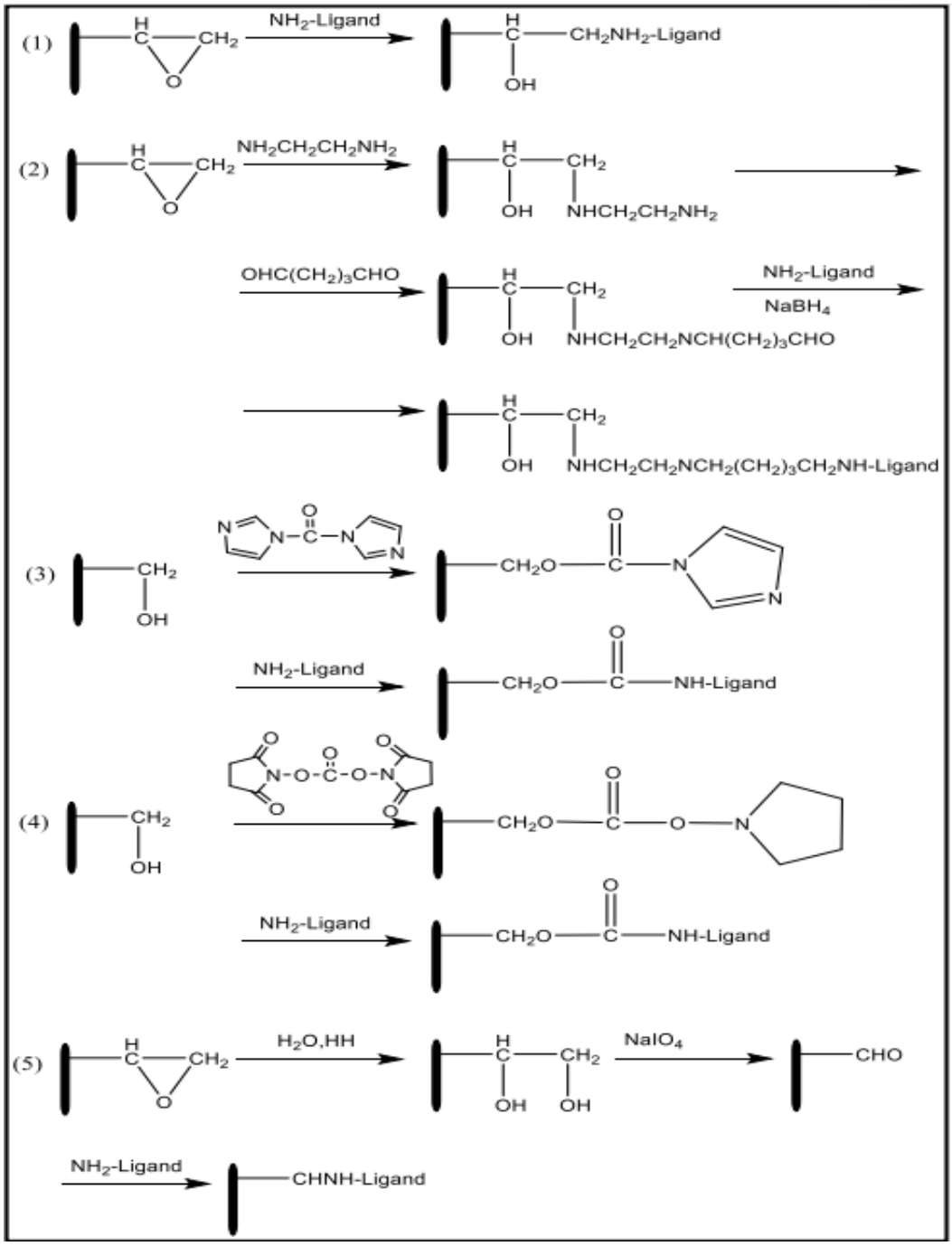


Figure (1.9) Several methods for affinity functionalisation (1) Direct Immobilisation via epoxy groups, Immobilisation via intermediate modifications: (2) with diamine and glutaraldehyde; (3) with carbonyl diimidazole; and (4) with disuccinimidyl carbonate. (5) Oxidation of hydroxyl-groups followed by a ligand attachment.

1.6. Acrylic acid (A.Ac)

Acrylic acid (IUPAC: propenoic acid) is an organic compound. The simplest unsaturated carboxylic acid comprises a vinyl group connected directly to a carboxylic acid terminus with the formula CH2=CHCOOH. This transparent liquid possesses a distinct acrid or tart odor. Water, alcohols, ethers, and chloroform are all miscible [91,92].The term "acrylic" was first used in 1843 to describe a chemical counterpart of acrolein, an oil with a pungent odor that is produced from glycerol [93],and the structure depicted in figure (1.10)

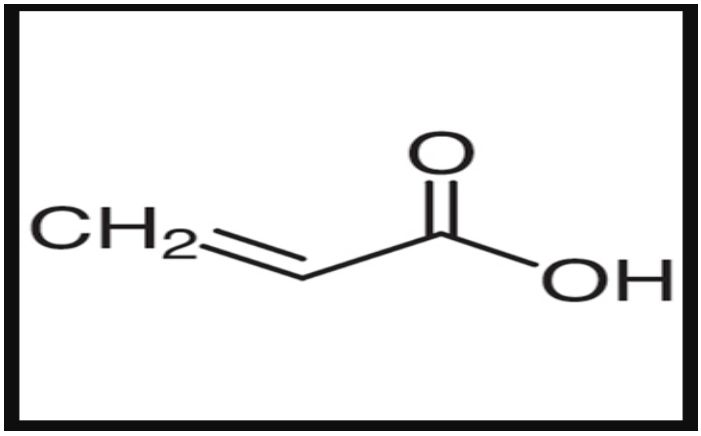


figure (1.10) the structure of Acrylic acid

Acrylic acid is created through the oxidation of propylene, a byproduct of the production of gasoline and ethylene[93].

$$2\ CH_2{=}CHCH_3 + 3\ O_2 \rightarrow 2\ CH_2{=}CHCO_2H + 2\ H_2O$$

Poly (acrylic acid) (PA.Ac) is widely utilized because of its excellent aspects of absorption and acid-base behavior. In polymers where the pH is less than 4.5, the carboxylic acid groups in the monomer permit the establishment of hydrogen bonds between polymer chains [94].

Acrylic acid and its esters rapidly react at their double bonds with other monomers (such as acrylamides, vinyl compounds, acrylonitrile, styrene, and butadiene) to create polyacrylic acid-making homopolymers or copolymers that are used to make a variety of plastics, floor polishes, adhesives, coatings, elastomers and paints[94].

Acrylic acid can react with ethylene glycol dimethacrylate (as a cross-linker), and the reaction[95],is shown in figure (1-11).

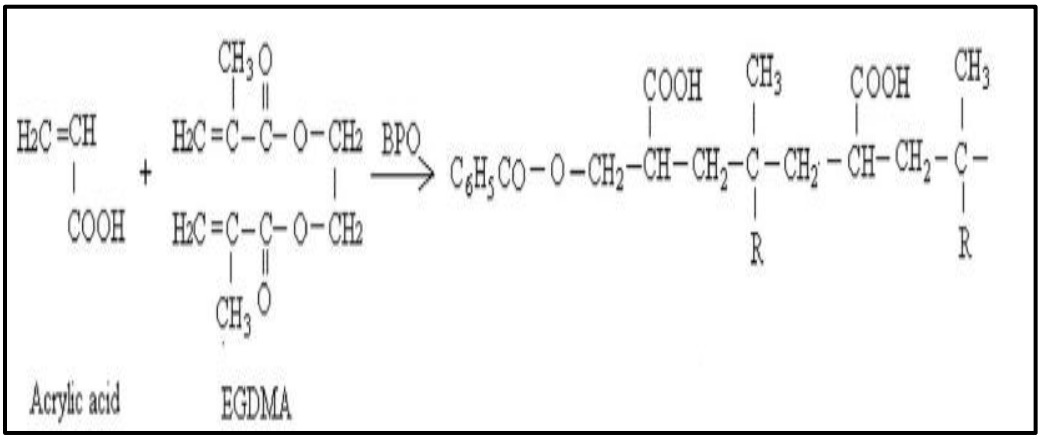
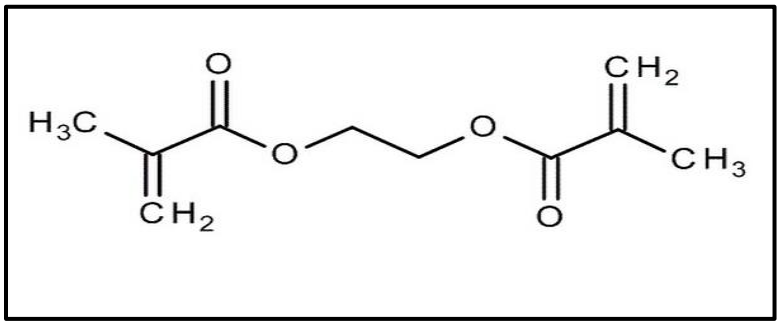


Figure (1-11)the reaction between acrylic acid and ethylene glycol dimethacrylate[94].

1.7. Ethylene glycol dimethacrylate(EDMA)

Ethylene glycol dimethacrylate is the enoate ester, the 1,2bis(methacryloyl) derivative of ethylene glycol. It is a cross-linking reagent, a polymerization monomer[96]. It derives from ethylene glycol and methacrylic acid. To form an elastic network, cross-links were introduced into the system using ethylene glycol dimethacrylate (EGDMA) as the cross-linker; it has two double bonds [97], as shown in figure(1.12)



Figure(1.12)The structure of ethylin glycol methacrylate[98].

In free-radical polymerization reactions, multi-functional monomers (also known as cross-linkers), for example, ethylene glycol dimethacrylate (EGDMA) and divinyl benzene (DVB), are commonly used. These monomers are copolymerized with modest amounts of monofunctional monomers such as styrene (STY) and methyl methacrylate, typically less than one to two percent of the total monomer (MMA). For example, the monomer structure of EGDMA exhibits symmetry; as monomers, the two types of vinyl have comparable reactivity and rate characteristics. However, after one of these vinyl groups is incorporated into a growing polymer chain, the second vinyl becomes attached to the macromolecular backbone via a sidechain. Of course, some of this pendant vinyl may prove helpful in the future by resulting in cross-linking and desired topological network development.[99]

This product is a cross-linking agent used to prepare resins, coatings and adhesives, a raw material for preparing cancer drugs[100], and is primarily utilized in the rubber and plastic industries as an ethylene-acrylic acid copolymer [101]

1.8.Human a-amylase and starch digestion

Erhard Leuchs named Ptyalin in 1831 to define the salivary component responsible for the chemical reaction that breaks down starch[102]. For the first time, an enzyme experiment has been documented. Payen and Persoz identified and named diastase, an enzyme from barley that decomposed starch in 1833[103]. As a result, enzymes are now known as amylases.

Glucose levels in the blood rise rapidly after a starch-containing meal has been digested since starch is the primary source of digestible carbohydrates in the human diet. Maltose, maltotriose, and limit dextrins are the end products of the first step of starch metabolism, which is catalyzed by a-amylase [104].There can be significant changes in postprandial blood glucose and insulin response to eating different foods containing the same amount of starch, though [105,106],evidence that starch digestion in the gastrointestinal tract is highly variable can be found by looking at these variances. [107–109]. Disorders that are related to lifestyles, like diabetes and cardiovascular disease, can be prevented and treated by reducing postprandial glycemia and insulinemia variations [110].Native starch is packaged in semicrystalline granules that are very large compared to the size of the a-amylase molecule. This is in contrast to the vast majority of intracellular metabolic enzymes, where the enzyme molecules are typically enormous compared to the metabolites on which they act. The size of the starch granules appears to be a promising target for amylase attack, with many potential sites for enzyme binding. Despite this apparent binding advantage, starch breakdown within an intact granule is slow. Crystalline areas are

generally unfavorable for enzyme attack[111]. A modest but variable amount of proteins and fats may also be present in the granules, which may impede starch-amylase interaction. Although most starch eaten by humans has been heated, processed, or exposed to numerous other processes during food preparation, raw starch is commonly used in animal diets. Amylase susceptibility is increased when granule integrity is disrupted and crystallinity is reduced during processing[112].

1.8.1. Types of Amylase :

1.8.1.1. α-Amylase

α-Amylase (E.C.3.2.1.1) is a hydrolase enzyme that catalyzes the hydrolysis of starch's internal 1-4,glycosidic linkages to produce glucose and maltose as shown in figure (1-13). It is a calcium metalloenzyme, which means that its activity depends on the metal cofactor's presence [113]. Endo-hydrolase and exohydrolase are the two types of hydrolases. Endo-hydrolases work on the interior of the substrate molecule, whereas exo-hydrolases work on the non-reducing terminal ends[114].As a result, residues of terminal glucose and α(1- 6)linkages are not sliced by α-amylase. Starch is a type of material on which α-amylase acts. The composition of the hydrolysate obtained after starch hydrolysis majorly depends on temperature’s effects, hydrolysis conditions, and enzyme origin. 7.0 is the ideal pH level for optimal activity[113]. α-Amylase's starch hydrolysis activity and the actions that can be carried out due to the hydrolysis have made it an essential enzyme. The production of glucose and fructose syrup from starch is one such activity, the utilization of enzymes in detergent formulations drastically increased. Enzymes are

non-toxic to the environment and help detergents get rid of stubborn stains. They are preferred to chemical catalysts because they are recyclable and operate at lower temperatures[115].

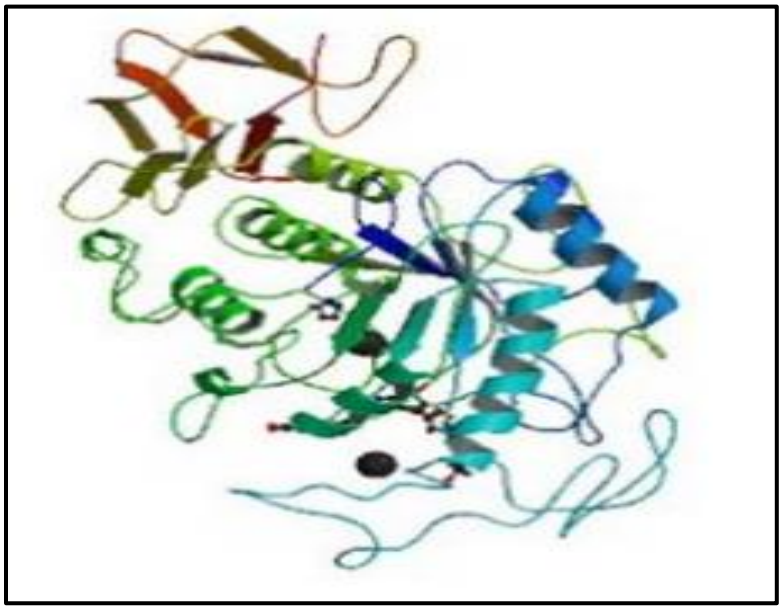


Figure (1.13) The structure of α-Amylase

1.8.1.2. β – Amylase

The exo-hydrolase enzyme β-amylase (EC 3.2.1.2) works from the polysaccharide chain's non-reducing end to produce successive maltose units by hydrolyzing α(1-4)glucan linkages. Branched polysaccharides like glycogen and amylopectin cannot be broken with this enzyme, resulting in partial hydrolysis with the dextrin units remaining[116]. The seeds of higher plants and sweet potatoes are the primary sources of β-amylase ,B-amylase breaks down starch into maltose during fruit ripening, resulting in the sweetness of ripened fruit. The enzyme's optimal pH ranges from (4.0- 5.5). β-amylase can be used for a variety of research and industrial applications. It can be used to investigate the

structural properties of starch and glycogen molecules, which, through various methods, are reduced[117].

1.8.1.3- γ – Amylase

γ-amylase (EC 3.2.1.3) cleaves α(1-6) glycosidic linkages as well as the last (1-4) glycosidic linkages at the non-reducing end of amylose and amylopectin, yielding glucose, unlike the other forms of amylase. γ-Amylase works best in acidic environments and has an optimal pH of 3 [117,118].

1.9-Literature Review

The high importance of the amylase enzyme and its high cost, the reason behind the repeated attempts to separate it and devise analytical methods to separate it with less cost, more accuracy, and higher concentration; this is confirmed by previous studies and researchers' attempts to reduce defects and reach the best result as shown in Table (1.1).

Table (1.1)The previous studies for separation α- amylase enzyme.

NO.	stationary phase	Technique used	Target ligand	result	Ref.
1	Cl-amylose column	Gel filtration chromatography	α –amylase enzyme	The recoveries of α –amylase was 65%, and The retention capacity of the stationary phase was 0.5 mg /ml of α –amylase bed volume.	[119,120]
2	CU^{+2}–bead embedded cryogel column or CU^{+2}BEC Column	Metal ion–affinity chromatography	α –amylase enzyme	Increased the optimal activity from 25 to a temperature range of 25–35 C^{o} ,	[121]
3	The silica column uses synthetic dyes	Dye-ligand affinity chromatography	α –amylase enzyme	Separation of enzymes and proteins	[122,123]
4	The silica column use substrate	HPLC	α –amylase enzyme	Particulate shell–core C18 columns (Kinetex, 2.6 m) performed the best, trailed by monolithic silica columns (Chromolith RP-18e) and standard C18 packings (Gemini, 5 m or 3 m). The performance of a polymeric monolithic column (ProSwift) was by far the worst.	[124,125]

5	synthetic dyes	Dye – ligand affinity chromatography	α –amylase enzyme	Separation of enzymes and proteins	[122,126]
6	column of dextran gel	Gel filtration	α –amylase enzyme	recovery of the enzyme was obtained, α-amylase values from 18 to 773 units, the enzyme was purified by a factor of 8 over the original sample	[127,128]
7	supermacroporous monolithic cryogel column	HPLC	α –amylase enzyme	Purification amylase because have interconnected macro pores	[62,129]
8	microporous glass spheres	adsorption	α –amylase enzyme	Column ,tubular reactor ,partial immobilization	[130,131]
9	(CNPG3) or 2-chloro-4-nitrophenyl Malto trioxide	spectroscopic methods (direct method)	α –amylase enzyme	Give the activity of α –amylase in human serum between 22-80 IU/ L at 37C° In 405 nm	[132]
10	(DNSA) 3,5-dinitro salicylic acid, Nelson-Somogyi (NS), Capper-bicinchoninate(CuBic) and the Park and Johnson (PJ)	Spectroscopic methods (reducing sugars)	α –amylase enzyme	The DNA test for assessing α-amylase activity demonstrated a nonlinear correlation between the quantity of colour produced and the number of hemiacetal reducing groups in the sample. NS is more accurate and trustworthy in terms of precision and dependability, but it takes a long time and hard work	[133–136]

				(PJ)have the same DNA problem and CuBic same the NS from where accuracy and sensitivity In general, the method of reducing sugar is not acceptable because of the toxic chemical residues that need time and cost to remove	
11	The starch-iodine and The turbidity method	Spectroscopic methods (amyloplasts methods)	α –amylase enzyme	Both methods quantify the unreacted starch in the assay medium to determine the amount of starch eaten in a reaction. The first colour is considered proportional to α-amylase activity, with wavelengths ranging from 550 to 700 nm. The decrease in turbidity is proportional to -amylase activity and wavelengths (mainly between 620 nm and 700 nm). Even though both amyloclastic procedures are relatively quick and straightforward, they only provide a qualitative indicator of α-amylase activity and are therefore unsuitable for quantitative investigation.	[137,138]
12	Enzyme glucose oxidase (GOD) and peroxidase (POD	Spectroscopic methods (enzymatic methods)	α –amylase enzyme	Expensive and inaccurate	[139–142]

13	Phadebas, Azo-starch or p-nitrophenyl glycosidesPNP	Spectroscopic methods (chromogenic methods)	α –amylase enzyme	It was distinguished by its ease ,accuracy and the lack of time spent but needs blocking agents to eliminate many interactions with other enzymes.	[143,144]
14	FTIR Spectroscopy	Spectroscopic methods	α –amylase enzyme	applied to the determination of a-amylase activity in human serum where a linear working range from 100 to 800 U/l (r.s.d." 11% for 150 U/l) was achieved	[145,146]
15	Infinite alfa by use of amylase reagent Gal G3-CNP.	kinetic method	α –amylase enzyme	Inaccuracy in measuring enzyme activity.	[147]
16	Diethyl amino ethyl (DEAE)cellulose column by use dextrin as a ligand	Affinity chromatography	α –amylase enzyme	Highly purified of the a-amylase enzyme was recovered Specific activity about 70 with 30 % recovery of all original enzymatic activity	[148,149]
17	Sepharose-α-cyclodixtrin column	Affinity chromatography	α –amylase enzyme	The sensitivity of this method is lower and needs improvement	[150]
18	DNS method by used starch agar plat	Spectrophotometer	α –amylase enzyme	The study is easy and low in cost, but the sample is vulnerable to bacterial contamination	[151,152]

19	CHA-Sepharose column	Affinity chromatography	α –amylase enzyme	This column give activity for a- amylase about 65%. Over 2 months of storage at 4 c°	[153]
20	Starch –column	Affinity chromatography	α –amylase enzyme	This experiment was rapid, reproducible and efficiently elutes the bond a-amylase from the starch –column . Strong affinity towards bacterial a-amylase.	[154,155]
21	Seralose 6B	Ion exchange chromatography	α –amylase enzyme	Separation a-amylase with high purity and high recovery.	[155,156]
22	Poly ethylene glycol/ fructose-1,6-biphosphate Two –phase system	Affinity chromatography	α –amylase enzyme	Purify a-amylase ,in this study used impalpable distinction of hydrophobicity between a-amylase and contaminating proteins.	[157]
23	High performance anion exchange chromatography – pulsed amperometric detection HPAEC-PAD	Ione exchange chromatography	α –amylase enzyme	Pulsed amperometric detection Simple, sensitive, less intrusive	[158]

24	Starch –iodine-sodium fluorescein (SIF)	Fluor metric method	α –amylase enzyme	a-amylase activity is determined using the increased fluorescence emission intensity following hydrolysis of the SIF complex by a-amylase	[159]
25	Affinity monolithic (A.Ac-CO-EDMA-CO-GMA modified with Starch)	Affinity chromatography	α –amylase enzyme	1- Only amylase specialist 2- Availability of its constituent monomers at a meagre cost 3- The column can be saved for a long time and reused more than 15 times. 4- The same coulomb can be used to separate another enzyme if the starch is replaced by the base material of the enzyme to be separated.	The current study

1.10- Aims of the Present Study

This study aims :

1 – Preparing and investigating of new monolithic columns using glycidyl methacrylate as the main component because of the significant ability of pendant epoxide groups to participate in many chemical reactions and modifications for numerous applications.

2-Modified the prepared monolithic column as a monolithic affinity column using starch as a substrate to separate α-Amylase enzyme

3- Identification of the prepared monolithic column using different techniques.

4- Studying the factors affecting the preparation of the affinity column to obtain the suitable surface area pores size for enzyme separation.

5- Investigating the optimum parameters to produce a monolithic affinity column that should have high efficiency in the bioseparation.

CHAPTER TWO

EXPERIMENTAL PART

2. Experimental Part

2.1. materials

Table (2.1) All chemicals used in this study

Chemical name	Molecular formula	Molecular weight g/mol	Producing company	Purity%
3-(trimethoxysilyl) propyl methacrylate TSP	$C_{10}H_{20}O_5Si$	248.35	Sigma-Aldrich Poole, UK	98.00%
Glycidyl methacrylate GMA	$C_7H_{10}O_3$	142.15	Sigma-Aldrich Poole, UK	97.00%
Ethylene dimethacrylate EDMA	$C_{10}H_{14}O_4$	198.22	Sigma-Aldrich Poole, UK	98.00%
acrylic acid A.Ac	$C_3H_4O_2$	72.06	BDH, Poole, England	99.00%
2,2- dimethoxy -2-phenyl Acetophenone DAP	$C_{16}H_{16}O_3$	256.30	Sigma-Aldrich Poole, UK	97.00%
starch	$C_6H_{12}O_6$	variable	Iraq	97.00%
amylase KIT with reagent			BIO LABO (France	
Aceto nitrile	C_2H_3N	41.05	Scharlau	99.85%
Acetone	C_3H_6O	58.08	Sigma-Aldrich Poole, UK	99.80%
Sodium hydroxide	NaOH	40.0	BDH	99.00%

Hydrochloric acid	HCl 36%	36.46	Fluka	Analar
2- propanol	C_3H_8O	60.1	Merek	99.70%
2-Butanol	$C_4H_{10}O$	74.12	Riedel-de Haen	99.50%
Methanol	CH_4O	32.04	GCC	99.50%
Ethanol	C_2H_6O	46.07	Chem-Lab NV	99.80%
Hexanol	$C_6H_{14}O$	102.17	Merck-Schuchardt	99.85%
Formic acid	HCO_2H	46.02	Thomas Baker	85.00%
Chloroform	CHC_{13}	119.38	Scharlau	99.00%

2.2. Instruments

Table (2.2) Instrument that used in **in this study**

Device name	**The manufacturing company**
UV-Visible spectrophotometer	UV-1700 double-beam Shimadzu, Japan
Electronic analytical balance with four decimal places	Denever Instrument Germany TP-214
Magnetic stirrer with heater	VWR West Chester, PA, USA
Sonicater	ultra sonic bath India
Irradiation device (220V-50HZ)	homemade
Syringe pump	Bioanalytical System Inc., USA
HPLC pump with isocratic	KD Scientific Holliston, MAU.S.A

elution system	
FT-IR 380 spectra	Bruker and Shimadzu
FE-SEM	TESCAN, Model: Mira3, Czech Republic
BET technique	BEL, Model: BELSORP MINI II, Japan

2.3. Fabrication of Monolithic Columns

Borosilicate tube with an inner diameter of (1.5 mm) and an outer diameter of (3.0 mm) Figure (2.1) was used to examine the production of monolithic columns, employing silanization and in-situ polymerization.

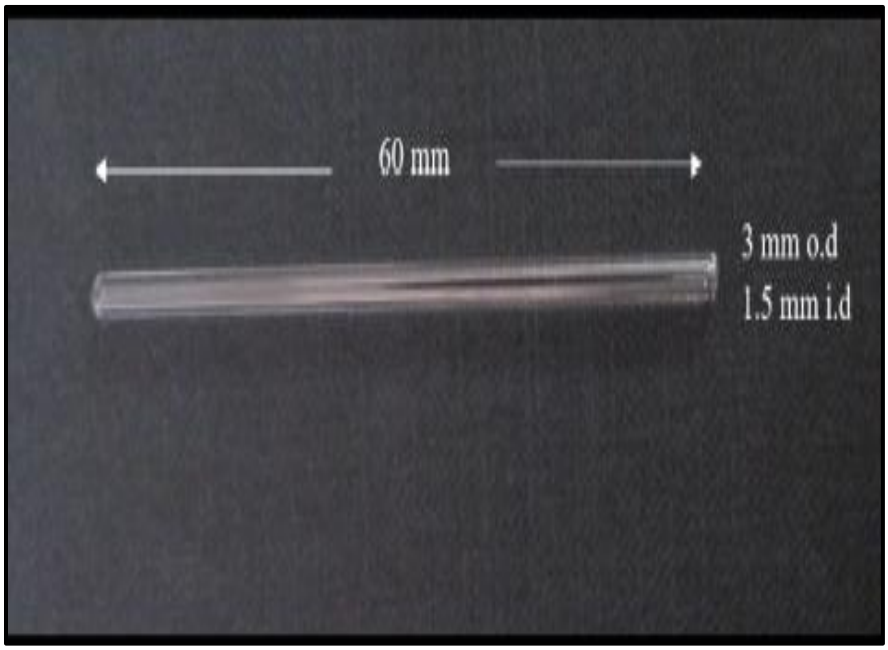


Figure (2.1) Borosilicate tube photographed before the silanization process was begun

2.3.1 .Silanizating step

This step was used to prepare the inner surface of the column, according to method was previously published in[9,160,161] including several steps:

1- The internal surface of borsilicat tube washed with acetone, and deionization water sequentially.

2- Activating the internal surface of borsilicat tube by washing the inner surface of the tube with a solution of sodium hydroxide at a concentration of 0.2M with flow rate 5µL.min^{-1} using a syringe for one hour and then washing with deionization water.

3- Using a concentrated hydrochloric acid solution of 0.2M to wash the tube's inner surface with a flow rate of 5µL.min^{-1} using a syringe pump for one hour, it follow wash with deionization water and ethanol sequentially to transform the inner surface of the (Si-OH) groups.

4- After that, TSP solution(20%3-Trimethoxysilyl propyl methacrylate in 80% ethanol in pH=5 controlled with acetic acid) was injected inside the borosilicate tube at flow rate 5µL.min^{-1} using a syringe for one hour.

5-Finally drying the borosilicate tube by using nitrogen gas and leaving for 24 hr.

2.3.2.Polymerizing step

Based on ***Ueki et al*** [162]**,** the free radical polymerization method was employed to prepare the monolith inside a borosilicate tube with some modifications are illustrated in next paragraph.

2.4.Preparation Glycidyl methacrylate (GMA) -CO-Ethylenedimethacrylate (EDMA)-CO- Acrylic acid (A.Ac) monolith

The polymer solution was prepared by (Uki *etal*)[162]with some modifications of 600µl GMA, 300µl A.Ac and 50µl EDMA with solvent(progen) contains 1050µl ethanol,600 µl hexanols, with1% DAP from monomers weight will be added to initiate polymerization precess

The initiator,progenic solvent, monomers and cross-linker were mixed well, ultrasonicated for 5min and later treated with N_2 gas to remove any O_2 bubbles.

The polymer solution was pumped inside a borosilicate tube using a glass syringe, and the inner surface of the borosilicate tube salinized according to the previously published method [161]. borosilicate tube with monolith mixture under U.V lamp for 4 min enough to polymerisation complete and prepare monoliths that showed in Figure (2.2)

A- **monolith before polymerization**

B- **monolith after polymerization**

Figure (2.2) Photograph of the monolith before and after photopolymerisation by UV lamp

Moreover, the steps to prepare the A.Ac –CO- EDMA-CO-GMA modifide with Starch monolithic column is shown in Figure (2.3).

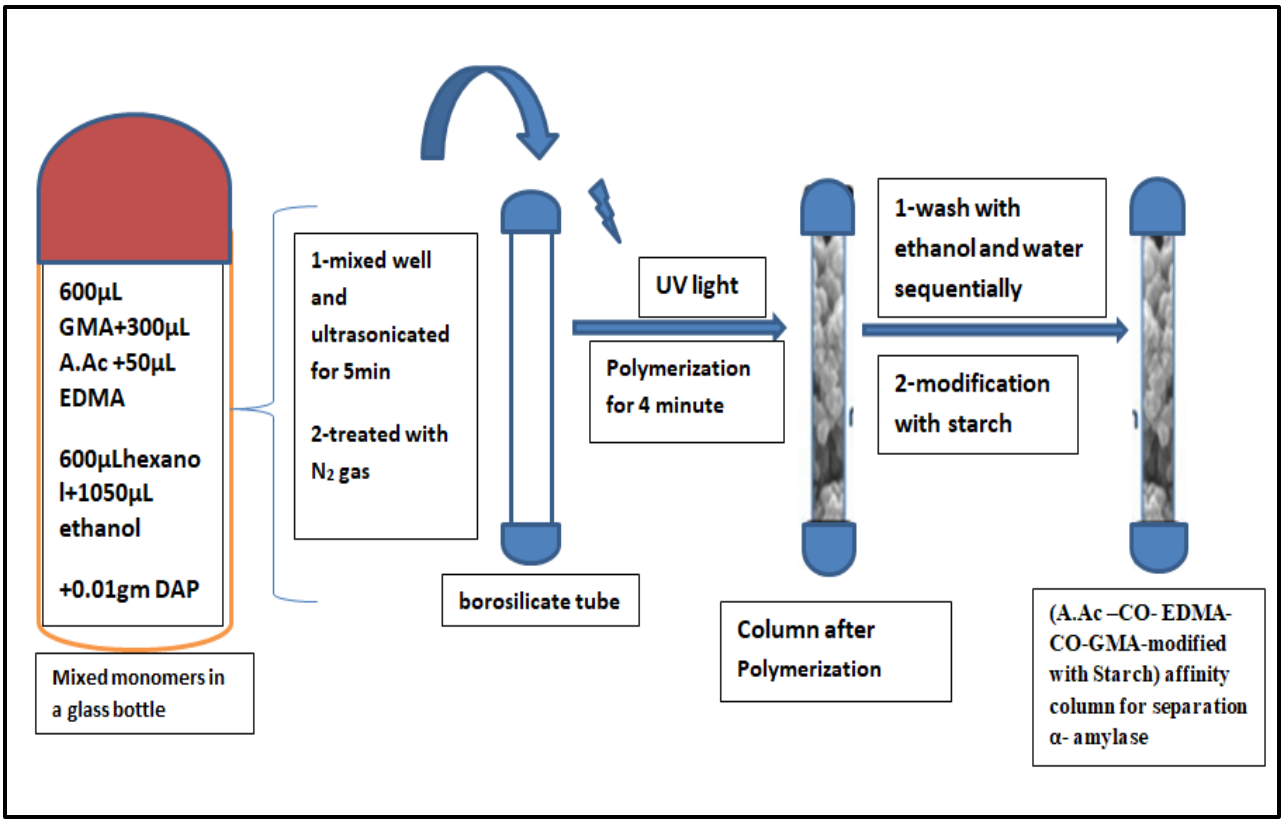


Figure (2.3) the steps of preparing a monolithic affinity column

2.5. Monolithic Column Modification with Starch

The monolithic column was modified with starch according [163]

incloding dissolve 2.5 g of starch in distilled water at 80 °c with stirring. After that, the temperature was reduced to 60 °c, and the pH was adjusted to 10 with 0.2M NaOH. This solution was pumped inside the monolithic column to open the epoxy group by reaction with starch at 10µl / min for 40 minutes; then, the starch solution was left in the column for 24 hr. After that, the column was washed with ethanol and distilled water for 40 minutes by pumping it at 10µl / min. The process of adding starch and washing it with deionization water and ethanol is explained in Figure (2.4).

Figure(2.4) Photograph of elution process for the monolithic after modification with starch

Furthermore, the step of modification with starch is shown in Fig (2.5).

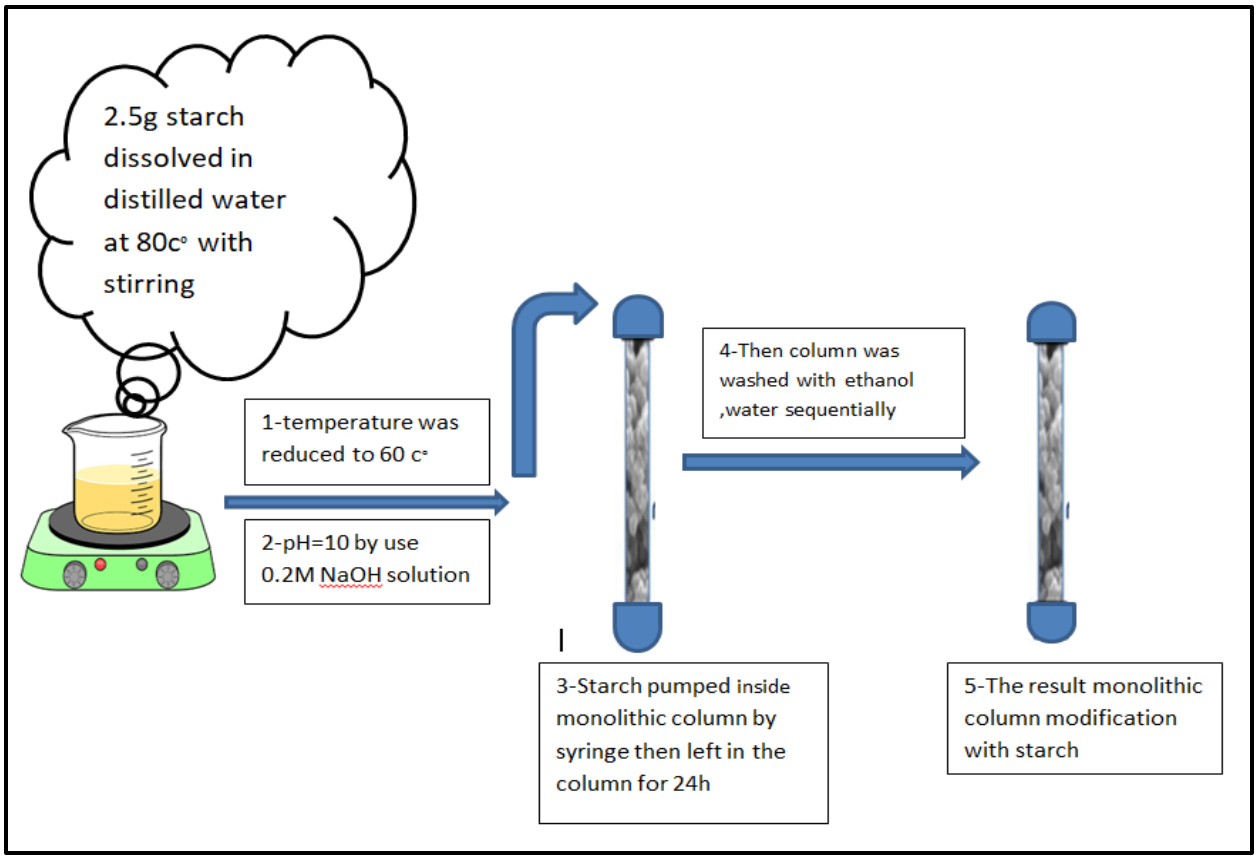


Figure (2.5) the modification with starch

2.6. The Column Used to Separate The α-Amylase Enzyme from Human Serum

The α- amylase activity in the serum was measured using the direct method by measuring the absorbance of the used BIOLABS kit [132].

200µl from serum was injected into a monolithic column at 10 µl / min. Then, the absorbance was recorded for the injected solution and excess solution outside the column after being treated with reagent and substrate.

Then the serum wase removed from the columnwashing with deionization water and acetonitrile solvent. The absorbance was measured for the rinsed serum after adding reagent and substrate solution to the outside serum from the column.

2.7. Irradiation Time Effect

Irradiation time effect on monolith column formation was investigated to obtain an adequate irradiation time so that monolith can be appropriately formed inside a borosilicate tube.

A time range (0.5-6) minutes was used, and the morphological properties of the column were tested using a scanning electron microscope (SEM)and Brunauer- Emmett- Teller(BET).

2.8. Effect of Porogenic Solvents

The progenic solvent plays a vital role in monolith formation, so the composition of the porous solvent was studied using ethanol with

different solvent to form the monolith by adopting the methods described in the previous two paragraphs (2.4) and using different solvents with a size 600µl for each solvent with ethanol in size1050 µl as shown in table(2.3)

table(2.3)different Porogenic solvent

Porogenic solvent		
1	Ethanol	Methanol
2	Ethanol	1-propanol
3	Ethanol	2- butanol
4	Ethanol	Hexanol

2.9.The Effect of The Ratio Between Two Polymers

The effect of concentration of each of the monomers, Acrylic acid (A.Ac) and glycidyl methacrylate(GMA), at room temperature was studied to reach the best polymer by measuring the return pressure.

2.10. Determine The Degree of Polymer Swelling

A specific weight was taken from the polymer prepared in the previous paragraph(2.4). Then it was placed in thirteen airtight glass cylinders, as different solvents were added as in the tables(3.5-3.7) for each cylinder separately to cover the monolith or the polymer completely with the solvent by adding 3 ml of these solvents to know the degree of polymer swelling.

2.11.Measuring Porosity

Fletcher method [164] was used to estimate the monolith's overall porosity by weighing the mass of the monolith upon drying and when filled with deionized water using the equation shown in(2-1)

$$Ø_t=(W_M-W_T)/\ dLR^2\pi \qquad (2\text{-}1)$$

$Ø_t$: porosity.

W_M: Weight of the monolith when filled with water.

W_T: Weight of the monolith when dried.

d: density of water(in 25C°=0.9975 $g.cm^{-3}$).

L: Monolith column length.

R :column radius

2.12. Monolith Permeability

Monolith permeability was investigated by evaluating the back pressure generated by the pump using distilled water at a different flow rate.

2.13. Flow Rate for Injection

To find out the best speed of the injection of the sample into the column, a speed test was carried out from (2,5,10,25,40,50)µl/min

2.14.Scanning Electron Microscope (SEM)

A TESCAN, Model: Mira3, Czech Republic microscope was used to characterize the morphology of the prepared monolith columns that have been prepared

2.15. Brunauer-Emmett-Teller (BET) Analysis

The monolith's surface area and average pore size were investigated using the Brunauer-Emmett-Teller (BET) model analyser BJH (Barrett-JoynerHalenda) model

2.16.Fourier Transform Infrared FTIR Spectroscopy

FT-IR380 spectra Shimadzu was used to study the prominent peaks of monolith before and after modifying with starch

2.17.Nuclear Magnetic Resonance ^{1}H-NMR Spectroscopy

H- NMR spectra for prepared monolith(A.Ac-co-EDMA-co-GMA-modified with Starch) were studied to confirm monolith formation.

CHAPTER THREE

RESULTS

AND

DISCUSSION

3.Results and Discussion

3.1. Preparation of the monolith column

The monolithic column was prepared inside a borosilicate tube with a length of 60 mm, an inner diameter of 1.5 mm, and an outer diameter of 3 mm were mention in the two paragraphs (2.4) and (2.3.1). The borosilicate tube used in this work is shown in Figure (3.1)

Figure (3.1) The shape of the column used in the work

3.2. Preparation the inner surface of the tube

Preparing the inner surface of the tube is the foremost step in forming the monolith inside the borosilicate tube. It includes interaction between (3-trimethoxysilyl propyl methacrylate) with silanol groups (Si-OH) on the inner wall of the borosilicate tube, the purpose of this step is to fix the monolith to the inner wall of the tube and to ensure that it does not come out or displace the polymer when using a high pumping speed. In

addition, it helps to prevent the effect of shrinkage during the polymerization process and avoid the interactions between the silanol group(Si-OH) and the target material to be analyzed[161].

Preparing the inner surface of the tube includes several steps; in each step, the solutions necessary for the conditioning process are pumped into the borosilicate tube using a glass syringe pump at a flow rate5µl.min^{-1} for one hour.

As the first step included washing the inner wall of the tube using acetone to remove any organic materials, then rinsing with distilled water to remove any acetone residue, then a solution of sodium hydroxide was used at a concentration of 0. 2 M to decompose the siloxane groups and increase the density of the silanol groups (Si-OH). It was washed with distilled water to remove any remaining basic solution[165]. Moreover, the hydrochloric acid solution was used at a concentration of 0. 2 M to remove residual alkali metal ions; then, the borosilicate tube was rinsed with distilled water to remove residual hydrochloric acid and then it was washed with ethanol to remove the distilled water.

3-trimethoxysilyl propyl methacrylate was pumped inside the borosilicate tube, and It was allowed to react with silanol groups for one hour. The tube was dried with nitrogen gas. After completing these steps, the inner surface of the tube was ready for the polymerization reaction and attaching the monolith to the inner walls of the glass tube[161]. The steps for preparing the borosilicate tube's inner surface are shown in Fig. (3.2[161].

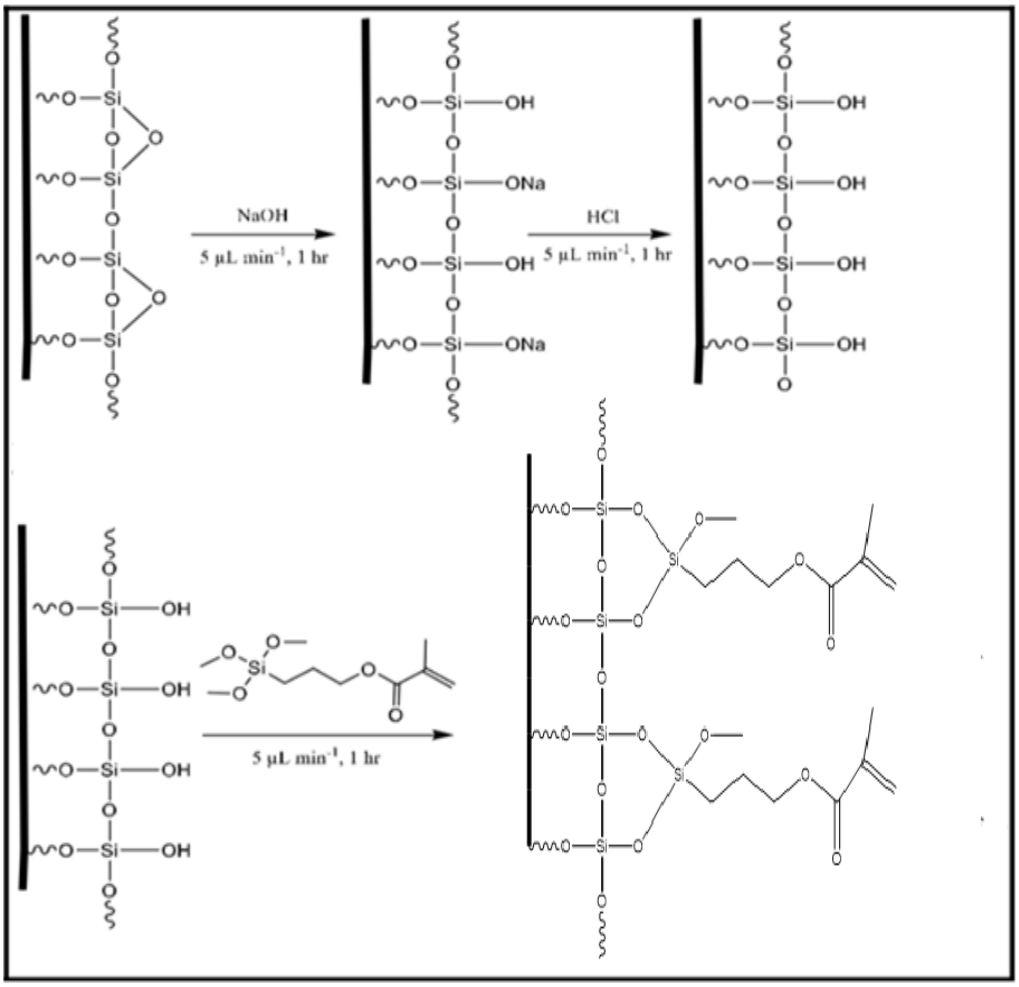


Figure (3.2) the steps of the Silanization process to prepare the inner surface of the borosilicate tube.

3.3. The polymerization process

The polymerization process of the organic monolith was prepared from a mixture consisting of glycidyl methacrylate (GMA) and acrylic acid (A.Ac) as shown in figure (3.3).

GMA was chosen because it contains two groups, the double bond of methacrylate that participates in the photopolymerization reaction and an

epoxide group that can be used in many chemical reactions as in post-polymerization modification reactions to produce different groups that can provide a separation mechanism[14], in addition, to cross-linker, progenic solvents, and initiators, each has a vital role in the formation of the polymer.

2,2- dimethoxy -2-phenyl Acetophenone(DAP) was used as a polymerization initiator instead of the most common initiator, 2,2 – azobisisobutyronitrile (AIBN). Because of some defects in (AIBN) including the formation of voids due to the rapid reaction and generation of N_2 gas during the polymerization process[166].

The cross-linker ethylene glycol dimethacrylate (EDMA) is a common cross-linking agent to prepare solid porous monolithic polymers The percentage of cross-linker to monomer must be constant because any change will affect the related properties; for example, if the percentage of cross-linker increases, the average pore size decreases due to the formation of highly cross-linked microspheres; this may be useful for obtaining a monolith that has a large surface area. However, a monolith with a high surface area will have limited permeability to solvents and increased back pressure. Therefore, the cross-linker to monomer ratio must be constant[167,168]

The solvents used are ethanol and hexanol in the polymerization mixture, so the prominent role of this solvent is to dissolve the monomers as well as the linker and initiator. At the same time, it does not dissolve the formed polymer[169] . The polymerization process was accomplished by photopolymerization using ultraviolet rays to start the polymerization

process by free radicals to form the monolith inside the borosilicate tube because it has many advantages such as controlling the pore size, short preparation time, avoiding high temperatures that lead to polymer breakage, controlling the position and length of the porous matrix, and high mechanical strength [170,171].

The structure of monemaers used in preparing monolithe can seen in figure (3.3)

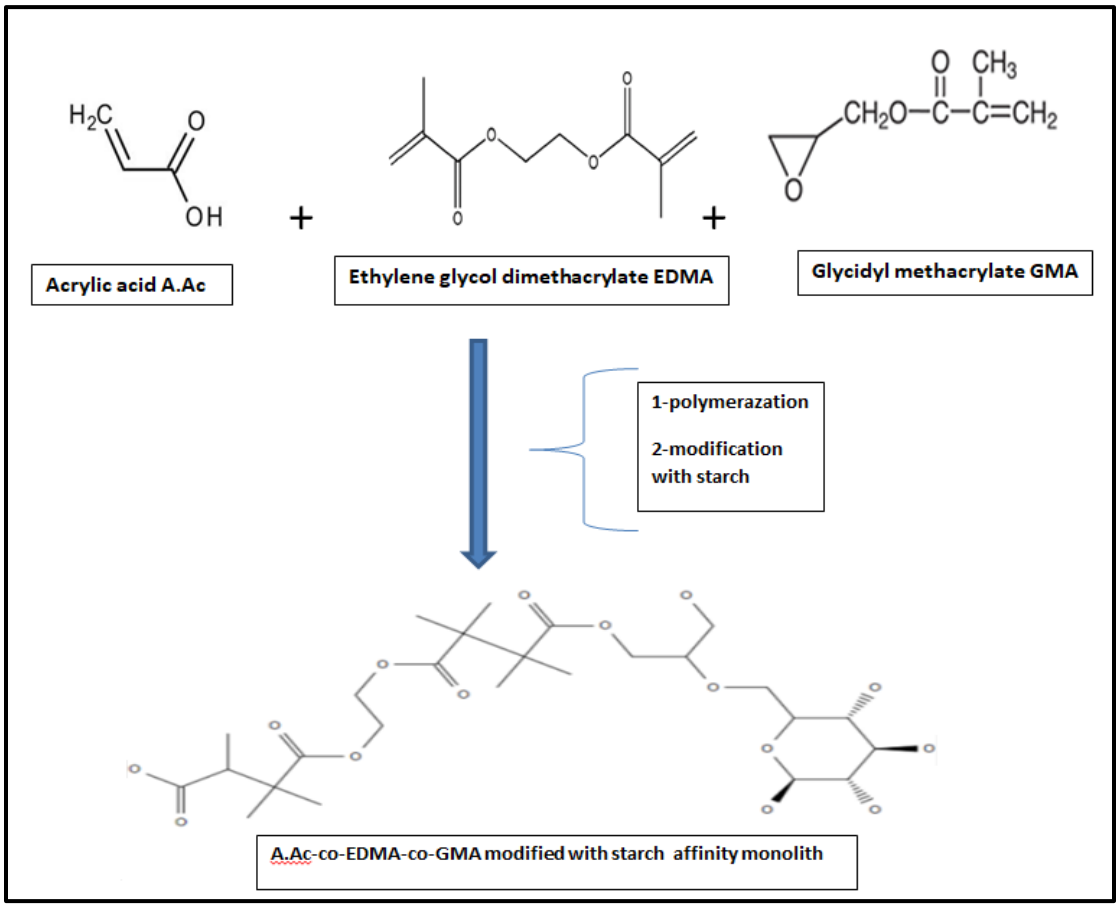


Figure (3.3) the stracture of monemars that used in prepared monolith .

The steps of the free radical polymerization process are explained as follows [172,173].

3.3.1. Initiator Splitting Step

This step consists of dismantling the initiator using ultraviolet light(UV) to form two benzoyl parts, which initiate the polymerization reaction, and an acetal part, which acts as an inhibitor to inhibit the polymerization process, as shown in Figure(3.4)

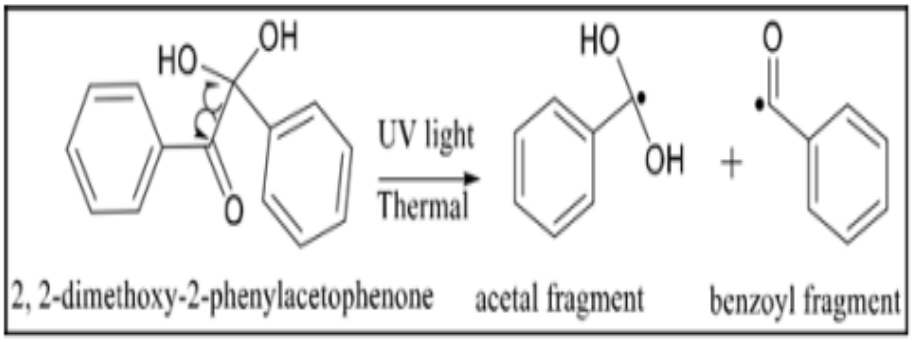
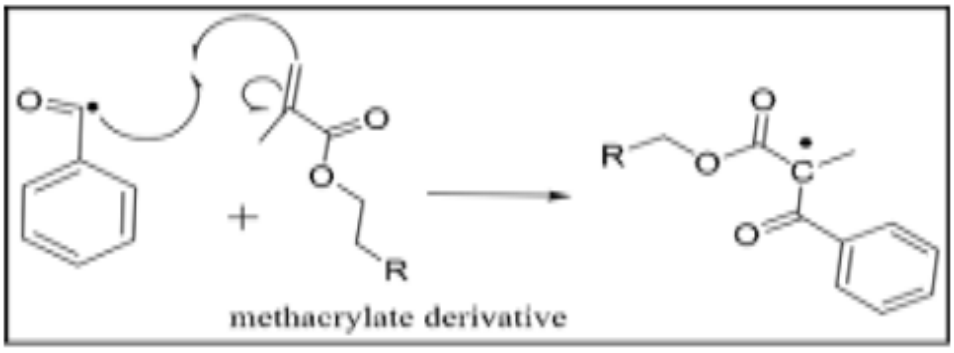


Figure (3.4) initiator splitting step

3.3.2. Starting Step

This step includes attacking the free radicals of the benzoyl double bond of the monomer to form a new free radical on the monomer that can be attacked before the double bond of the cross-linker or another monomer, as in Figure (3.5)



Figure(3.5) starting step

3.3.3. Growing step

The free radical interactions between the monomers and the cross-linker and growing of the polymeric chain as shown in Figure (3.6)

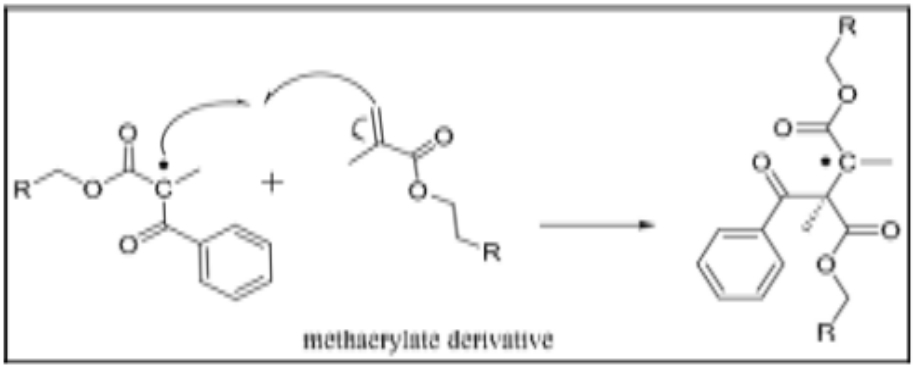
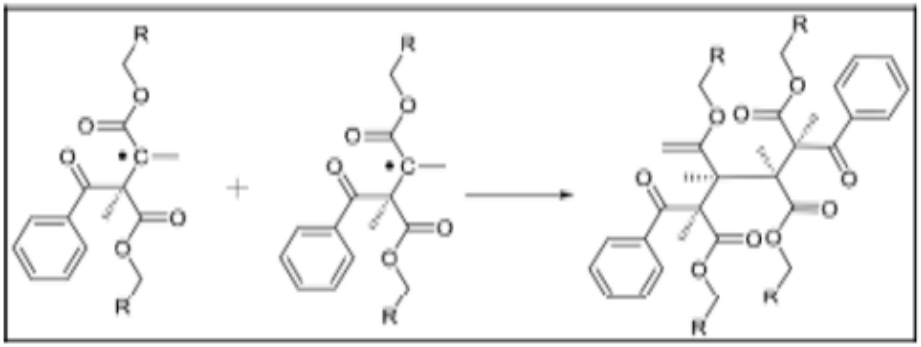


Figure (3.6) growing step

3.3.4. Terminating Step

In this step, the polymerization process is terminated due to the interaction of all free radicals with each other, as shown in Figure (3.7)



Figure(3.7) terminating step

3.4. Study The Effect of The Ratio Between Two Monomers

GMA was chosen as an essential component because it contains an epoxide ring that will be used to react with the appropriate substrate that can be affected by the enzyme to be separated; it also contains the double bond that will participate in the photopolymerization reaction. Acrylic acid was chosen to provide a surface area with hydrophilic hydroxyl groups.

The effect of the ratio of each of the monomers, Acrylic acid (A.Ac) and glycidyl methacrylate (GMA), at room temperature was studied to obtain the desired polymer by measuring back pressure; the results are shown in Table (3-1)

Table(3-1) the effect of the ratio between the two monomers

GMA µL	A.Ac µL	Monolithic formation time
450	450	The monolith formation after 13 minutes and back pressure was too low
500	400	The monolith formation after 9minutes with low back pressure
600	**300**	**The monolith formation after 4minutes with appropriate back pressure**
650	250	The monolith formation after 2minutes but back pressure very high

Table(3-1) indicates the ratio of 450 µL for GMA and 450 µL for A.Ac the polymer was formed after 13 minutes, where the size of the pores was large, and the back pressure was too low; this will cause a decrease in the interaction between the enzyme and the solid phase. With the ratio of 650 µL GMA and 250 µL A.Ac, The monolithic formed quickly, but the back pressure was very high due to the small pores, which led to the difficulty loading the enzyme through the monolithic.

The best ratio is 600 µL GMA and 300 µL A.Ac since the monolithic is formed after 4 minutes in an excellent shape, with appropriate back pressure, moderate pore size, suitable for loading the enzyme, and a suitable surface area.

3.5.Study The Effect of The Distance Between The Irradiation Source and The Separation Column

The distance between the irradiation source and the prepared separation column was studied to find out the best distance the polymer can form inside the separation column; the distance from the irradiation source ranged from 5 to 20 cm, and the results are shown in Table(3.2).

Table (3-2) Study the effect of the distance between the irradiation source and the separation column

The distance (cm)	The result
5	The polymer formed inside the column but was difficult to wash
8	The polymer formed inside the column but was difficult to wash
10	The polymer formed is inside the column and can be washed, but with high back pressure
12	**The polymer formed is inside the column and can be washed with good back pressure**
15	The polymer formed is inside the column but with low back pressure
18	The polymer formed is inside the column but incompletely
20	The polymer formed is inside the column but incompletely

Table (3-2) indicates that it is not possible to control the polymerization process when using a distance between 5 to 8 cm, which causes small gaps and the inability to wash the prepared column and use it to separate enzymes, as for the distance 10 cm the polymer was formed, but the process of washing it was complicated. The best distance was 12 cm, where the polymer formed and the pores are suitable for enzyme separation, and it could be washed with good back pressure. The polymer's distance from 15-20 cm was unsatisfactory and unsuitable for enzyme separation.

3.6. The Effect of Irradiation Time

After determining the proportion of monomer and the distance between the irradiation source and the prepared column, the most appropriate irradiation time for polymer formation was studied between 0.5-6 min; the results are shown in Table (3.3)

Table(3.3) The effect of irradiation time

irradiation time/min	The result
0.5	The polymer was not formed
1	The polymer was not formed properly
2	The polymer was formed with very low back pressure
3	The polymer was formed with low back pressure
4	The polymer was formed with reasonable back pressure
5	The polymer was formed with high back pressure
6	The polymer was formed with very high back pressure

From Table (3-3) can be observed that the irradiation time plays a vital role in changing the mixture of monomers into a solid polymer; so when using a higher irradiation time of (5min) and above, the polymer chains

grow. Thus the polymer branches grow rapidly and form a monolith with a small porous structure, as shown in Figure (3.8 C). On the other hand, when the irradiation time is reduced below 4 min, this may lead to the formation of less polymeric material inside the borosilicate tube, and the polymer will not form properly and affect the performance of the prepared monolith as shown in the Figure (3.8A), while when using irradiation time 4min It will give good back pressure and surface area as shown in the Figure (3.8 B)[174,175] .

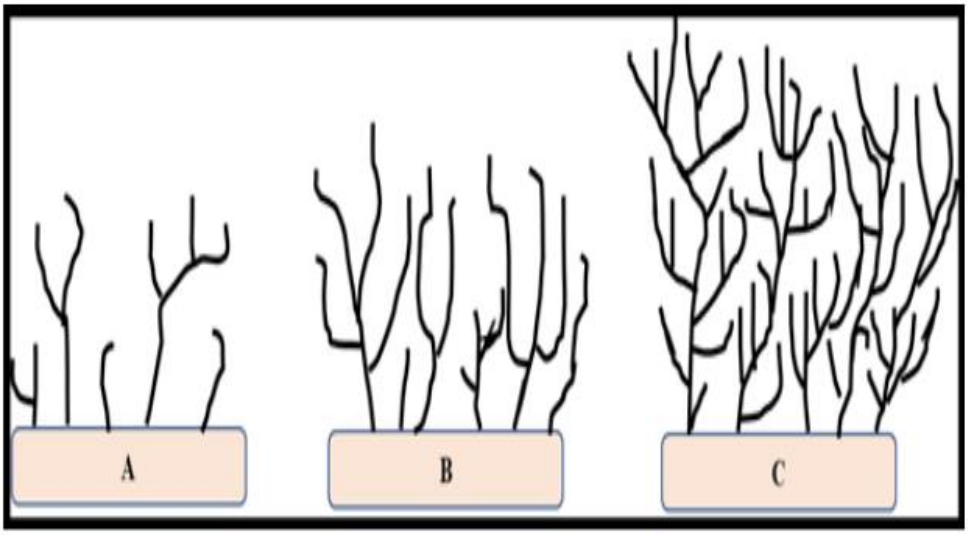


Figure (3.8) The effect of increasing the irradiation time on the branches of the polymer chains [174]

3.7.Effect of Porogenic Solvents

The purpose of studying the effect of the type of solvent on the polymer formation process is to find the best solvent that can be used alongside ethanol to form a polymer with a suitable surface area and por size by use 1050 µl for ethanol and 600µl for difirent solvint that used . The results of this study are shown in Table(3.4).

Table(3.4) effect of porogenic solvents

Porogenic solvent			The results
1	Ethanol	Methanol	The polymer was not formed
2	Ethanol	1-propanol	Polymer is formed with large pore size
3	Ethanol	2- butanol	The polymer is formed, but it is difficult to wash
4	Ethanol	Hexanol	The polymer is formed with a significant surface area and pores size

It can be concluded from Table (3.4) that the type of solvent (porogen) significantly affects the porous properties of the polymer. A high surface area and suitable pore size are obtained when ethanol and hexanol are used compared with the two solvents 1- propanol and 2-butanol as a monolith. However, it is not easy to wash due to the formation of a tiny, porous structure, affecting the prepared monolith's performance. A monolith with a large porous structure was also obtained using 1-propanol. In contrast, the monolith was not formed when methanol was used, these experiments were carried out at room temperature, and solvents with a low boiling point could be used[10].

3.8. Polymer Swelling Percentage

Cross-linked polymers swell because the solvent molecules diffuse within the crystal lattice of polymers with high molecular weight and cause a change in size, leading to the polymer's collapse during exposure to mechanical stress or high pressure. The high cross-linking polymer cause more excellent resistance and difficulty of swelling. The type of solvent could also affect and cause the dissolution of the polymer [176], the percentage of swelling was calculated by applying the (3-1) equation

$$swelling\ \% = \frac{mt - mo}{mo} * 100 \qquad (3\text{-}1)$$

As m_0: Weigh the polymer before swelling.

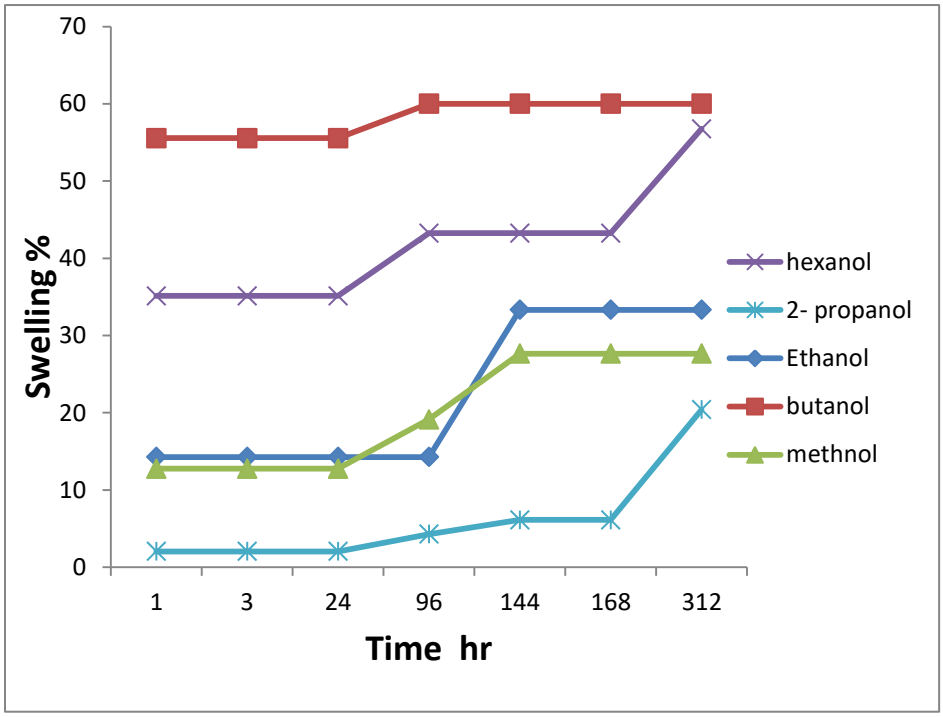
m_t : Weigh the polymer after swelling.

Different solvents with different properties were used to study the swelling effect. The results are shown in Tables(3.5 -3.7) and Figures (3.9 – 3.11).

Table (3.5) Percentage of polymer swelling using different alcohol solvents

Time/ hour	Swelling% Methanol	Swelling% Ethanol	Swelling% 2- butanol	Swelling% hexanol	Swelling% 2- propanol
1	12.76	14.28	55.55	35.13	2.040
3	12.76	14.28	55.55	35.13	2.040
24	12.76	14.28	55.55	35.13	2.040
96	19.14	14.28	60.00	43.24	4.28
144	27.65	33.33	60.00	43.24	6.122
168	27.65	33.33	60.00	43.24	6.122
312	27.65	33.33	60.00	56.75	20.40

Table(3.5) shows that the polymer swelling percentage is increased when using butanol followed by hexanol due to the polymer saturation with the solvent and its insolubility, as well as the lack of separation of the polymeric chain. Therefore, it causes small gaps, which reduces the mechanical properties when using high-pressure flow rates and the inability to separate the enzyme. The lowest degree of swelling is noticed when using propanol, as shown in Figure (3.9).



Figure(3.9) Percentage of polymer swelling using different alcohol solvents.

Table(3.6) Percentage of polymer swelling using different solvents of different polarity

Time/ hour	Swelling% H_2O	Swelling% Acetonitrile	Swelling% Acetone	Swelling% Formic acid	Swelling% Chloroform
1	27.87	8.130	17.796	33.766	2.70
3	27.87	8.130	17.796	33.766	2.70
75	27.87	8.130	17.796	33.766	7.20
123	27.87	12.195	17.796	37.66	7.20
147	27.87	12.195	18.64	38.90	7.20
291	27.87	12.195	18.64	38.90	8.108

The data from Table(3.6) shows that the high swelling percentage of the polymer when using formic acid is due to the polymer's saturation with the solvent. This may result in chemical decomposition or pressure to swell, which can lead to cracking and dissolution of the prepared monolith

While the lowest percentage of swelling occurs when using chloroform, which means that it does not cause clogging of the polymer and allows it to be used to separate the enzyme, the swelling effect is shown in Figure(3.10)

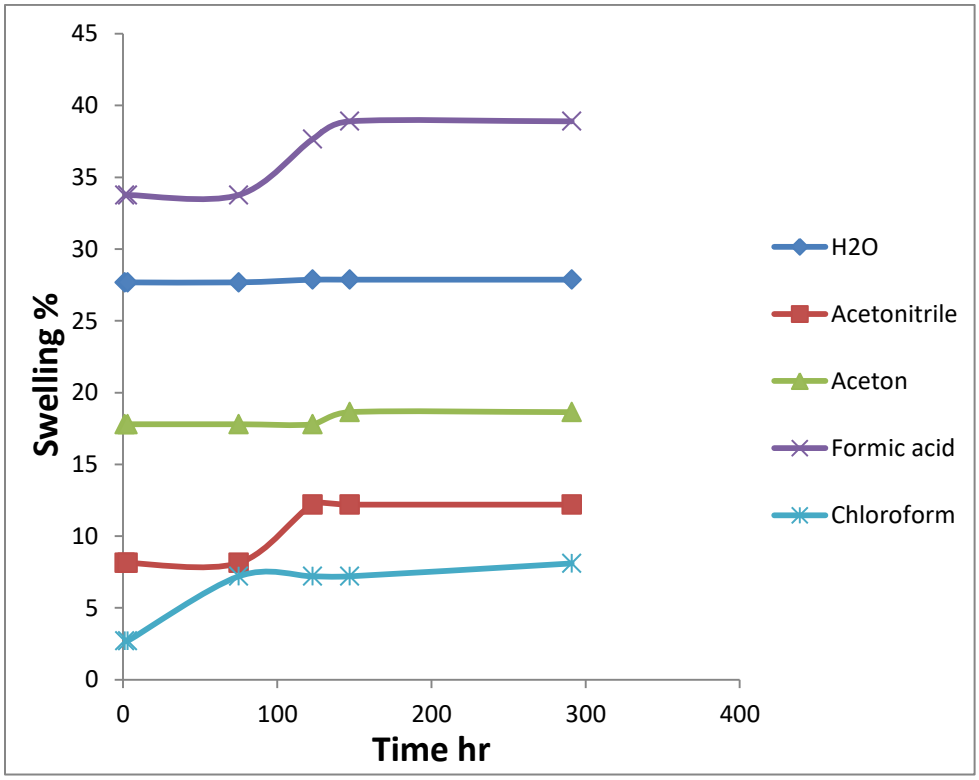
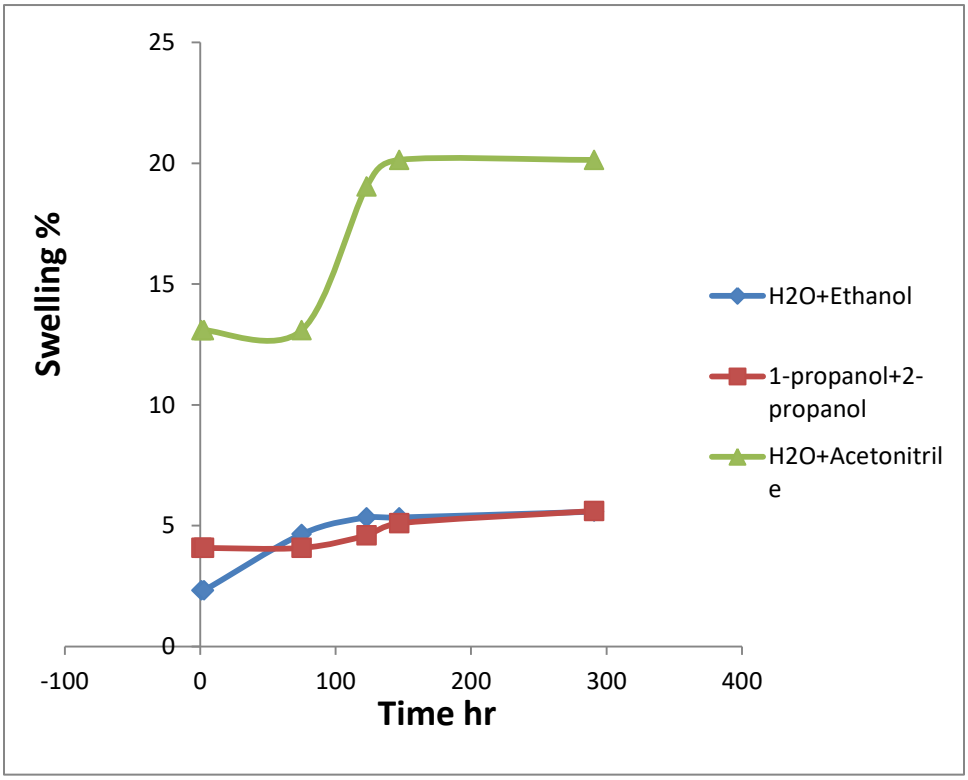


Figure (3.10) Percentage of polymer swelling using different solvents of different polarity

Table(3.7) Percentage of polymer swelling using a mixture of two different solvents.

Time/ hour	Swelling% H_2O+Ethanol (1:1)	Swelling% 1- propanol+2-propanol(1:1)	Swelling% H_2O+ acetonitrile (1:1)
1	2.32	4.08	13.09
3	2.32	4.08	13.09
75	4.65	4.08	13.09
123	5.34	4.6	19.04
147	5.34	5.1	20.14
291	5.58	5.58	20.14

Table(3-7) clarifies that the swelling of the polymer is to a high degree when using a mixture of two solvents(H_2O+Acetonitrile); this causes clogging of the polymer, while the degree of swelling is less when a mixture of two solvents (H_2O+Ethanol) is used which does not cause polymer blockage and is suitable for enzyme separation and can be used as a monolith preservation solution as shown in the Figure(3-11)



Figure(3.11) Percentage of polymer swelling using a mixture of two different solvents

3.9.Scanning Electron Microscope(SEM)

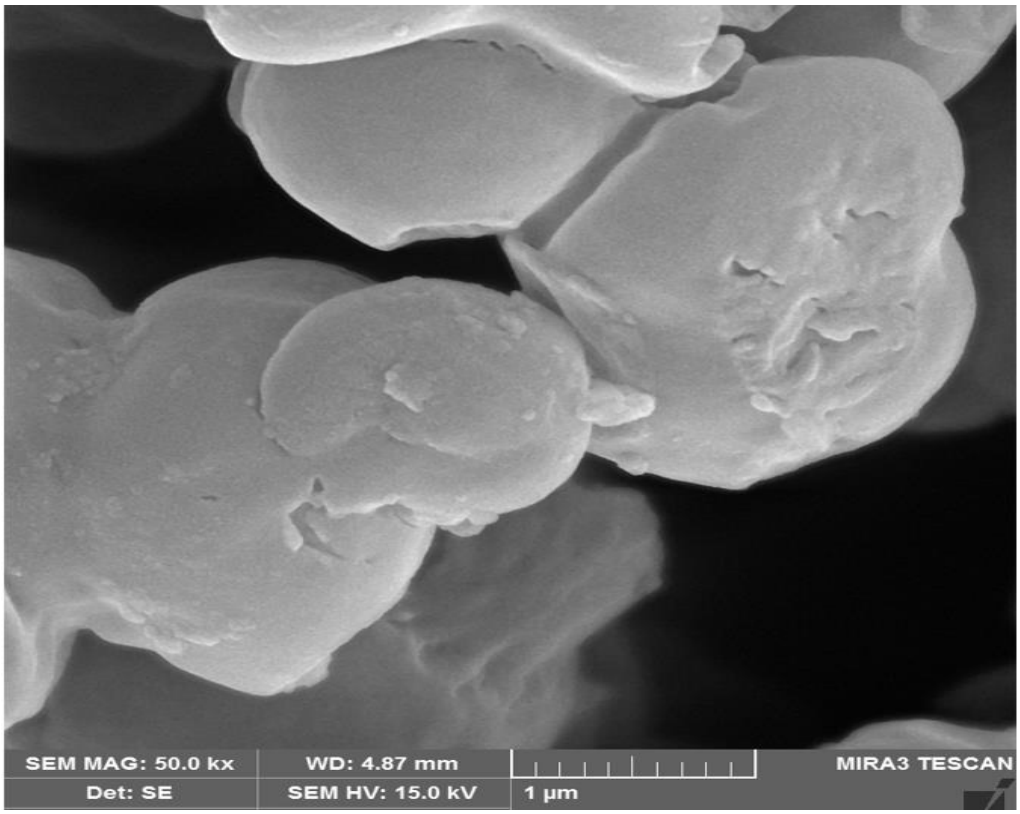
A scanning electron microscope was used to study the composition of the outer surface of the sample and gave a three-dimensional image [177].

The morphology of the prepared monolith column(A.Ac-co-EDMA-co-GMA modified with Starch) was photographed. It is made up of monomers acrylic acid (A.Ac), ethylin glycol dimethacrylate (EDMA), and glycidyl methacrylate (GMA). It has been modified by adding starch photographed by a scanning electron microscope(SEM)

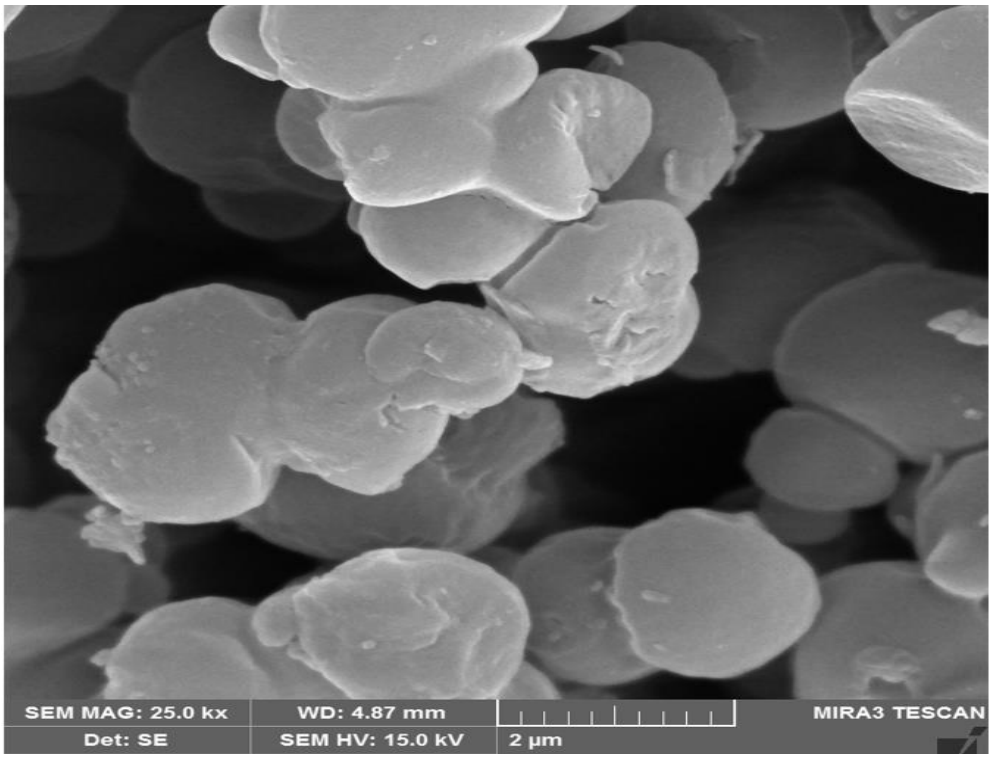
It can be seen from Figures (3.12 to 3.14) that the monolith composition

can be viewed as a network of large interconnected pores[171].

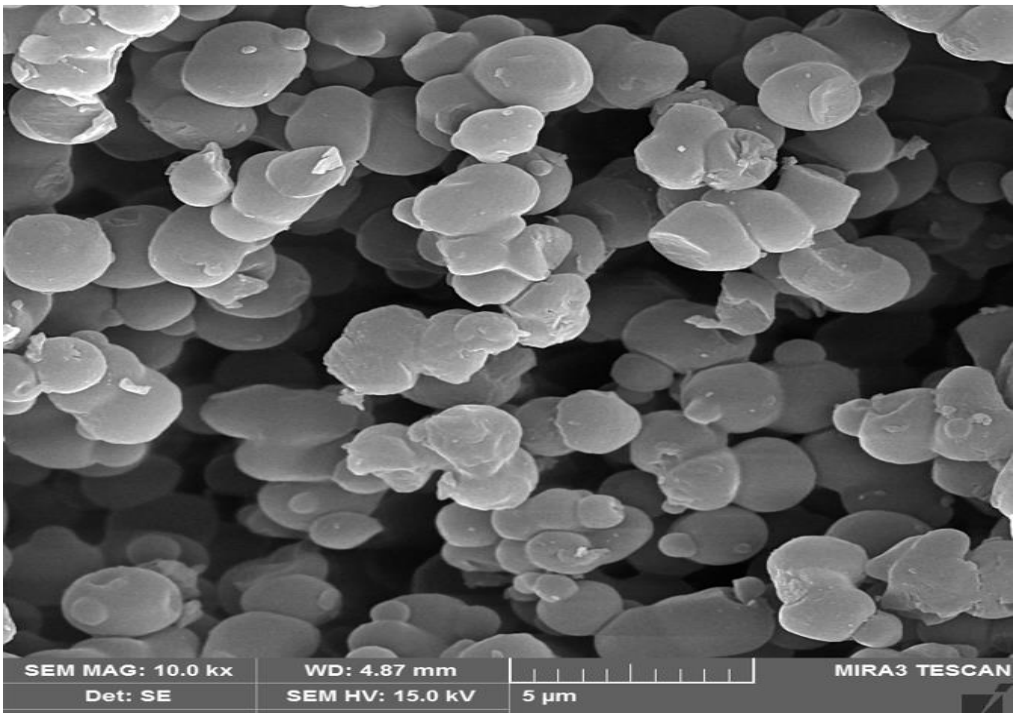
These pores allow the mobile phase to pass quickly through the monolith column, thus enhancing the permeability by reducing the back pressure. In addition, the monolith contains many medium pores and micropores. The composition of these pores is essential to increase the monolith block's surface area and the monolith's loading capacity of the monolith and thus leading to rapid extraction with a high flow rate and moderate back pressure[161].



Figure(3-12) The image of the prepared monolith(A.Ac-co-EDMA-co-GMA-modified with Starch) when enlarged 1µm.



Figure(3-13) The image of the prepared monolith(A.Ac-co-EDMA-co-GMA-modified with Starch) when enlarged 2µm.



Figure(3-14) The image of the prepared monolith(A.Ac-co-EDMA-co-GMA-modified with Starch) when enlarged 5µm.

3.10. Brunauer-Emmett-Teller (BET) analysis

Brunauer, Emmett and Teller found a method for calculating a specific surface area of a sample, including the pore size distribution of gas adsorption [178].

The results of the BET analysis for the prepared monolith found

that the surface area was(43.78 m^2/ g)and the pore size was

(8 nm).

3.11. Permeability and The Porosity of the Monolith

The permeability of the prepared monolith was studied by evaluating the back pressure generated by the electronic pump using distilled water with different flow rates through the monolith, as shown in Fig (3-15) .

Fig (3-15) Electronic pump(PU-980) for measuring pressure and flow rate.

The result is shown in Figure(3-16) by drawing the relationship between pressure and flow rate. Where was the porosity of the monolith calculated in the light of the (3-2) equation:

$$Ø_t = (W_M - W_T) / dL\ R^2\pi \qquad (3\text{-}2)$$

W_M= wet column weight- empty column weight

= 1.996- 0.6856 = 1.310 g

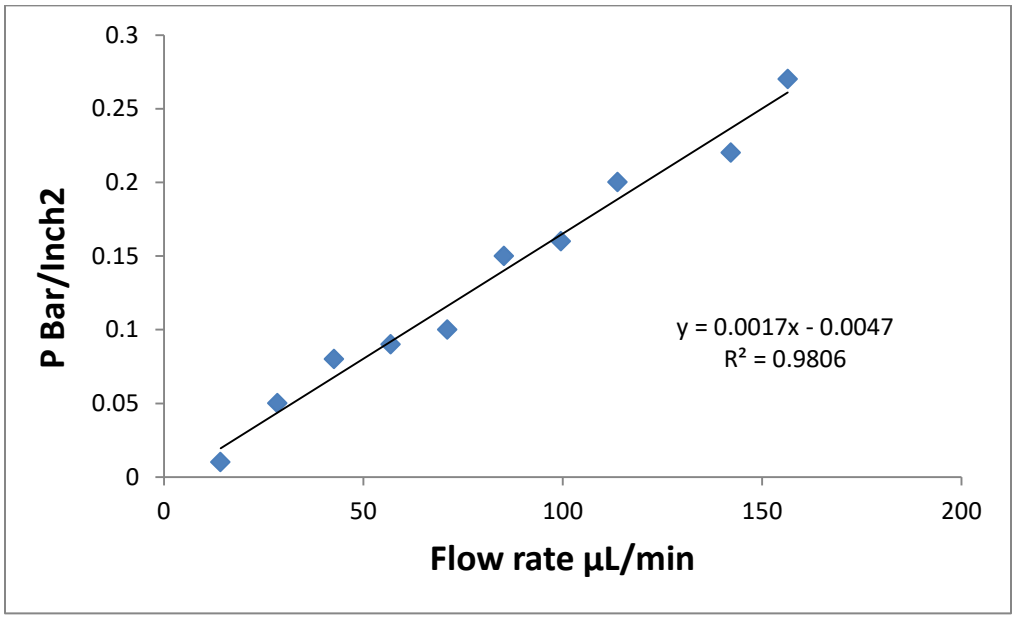
W_T = column weight after drying- empty column weight

=0.964-0.6856 =0.278g

d= 0.9975 L = 60 mm=6 cm

R=1.5/2=0.75 π =3.14

The result of the equation was that the porosity was (0.097)

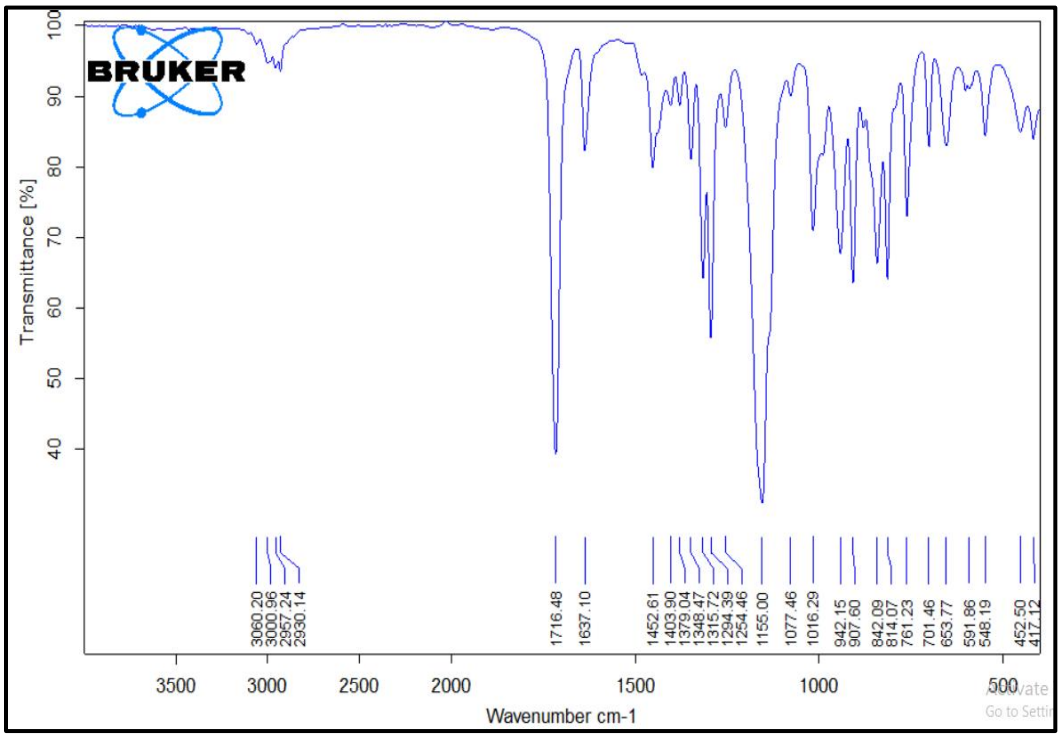


Figure(3-16) The relationship between pressure and flow rat.

3.12. FT-IR Technique to Prove Polymer Composition That Is Modified by Starch

3.12.1. FTIR for Glycidyl Methacrylate (GMA)

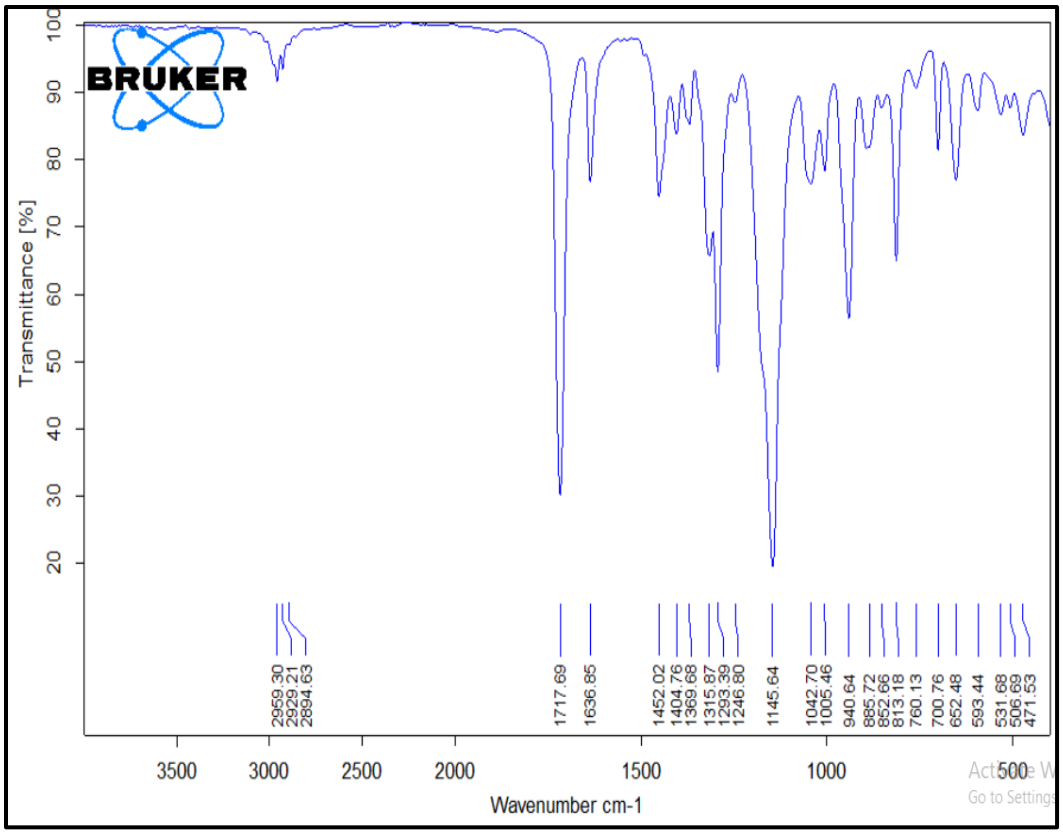
Glycidyl methacrylate (GMA) monomer characterization by FTIR spectrum as shown in Fig(3-17), shows many absorption bands, the most important of which is 1716cm^{-1}, which belongs to the carbonyl group(C=O), and an absorption peak at 1637.10 cm^{-1} for (C=C) and absorption peak belonging to(C-O-C) cyclic in position 907 cm^{-1} and 1254 cm^{-1} Since these peaks can be used to indicate (GMA) participation in the polymerization action.



Figure(3.17) FTIR of glycidyl methacrylate

3.12.2. FTIR for Ethylene Dimethacrylate (EDMA)

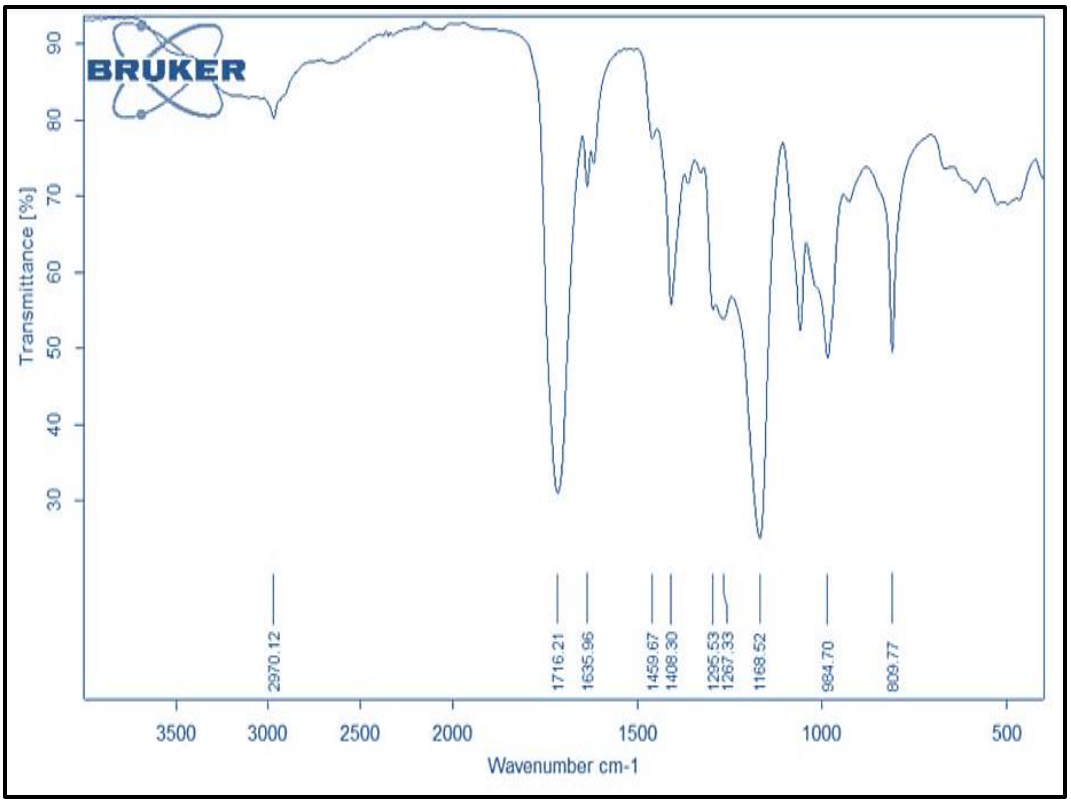
Ethylene dimethacrylate (EDMA) was Person to Monomer by Infrared Spectrophotometer(FT-IR). Figure (3-18) shows many absorption peaks, the most important of which is the absorption band at 1717.69 cm^{-1} for (C=O). Also, an absorption band appeared at 1636.85cm^{-1} for (C=C); this band indicates the participation of (EDMA) in the polymerization reaction.



Figure(3-18) FTIR of Ethylene dimethacrylate(EDMA)

3.12.3. FTIR for Acrylic Acid (A.Ac)

Acrylic acid(A.Ac) Infrared Spectrophotometer(FT-IR) is shown in Fig (3-19) It can be seen many absorption peaks, the most important of which is the absorption band at 1716.21 for (C=O), and peak at 2970.12 cm^{-1} for(OH) group. Also, an absorption band appeared at 1635.96cm^{-1} for (C=C); this band indicates participation (A.Ac) in the polymerization reaction.

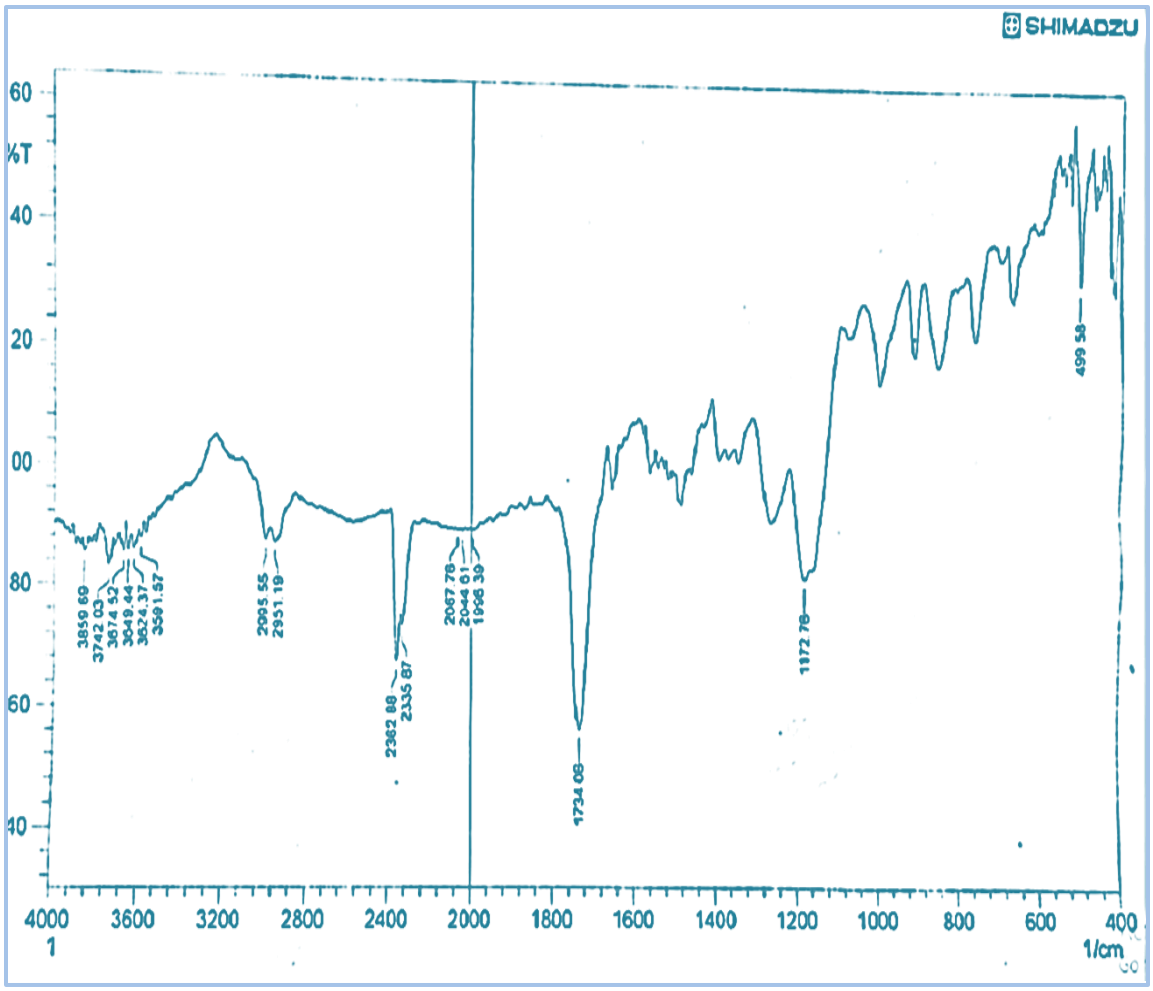


Figure(3-19) FTIR for acrylic acid (A.Ac)

3.12.4.FTIR for Monolith Before Modification With Starch

The FT-IR spectrum of the formed monolith is shown in Figure

(3-20), THIS shows that the (C=C) peaks in all the monomers and cross-linker spectrum disappeared due to the participation of these groups in polymer formation. The (C-O-C) cyclic group for the epoxide ring of the monomer glycidyl methacrylate in position 907 cm^{-1}, and 1250cm^{-1} is still unchanged, which is used for further modifications.



Figure(3-20) FTIR for monolith before modification with starch

3.12.5. FTIR AfterM With Starch

The FT-IR of the monolith after modification with starch is shown in Figure (3-21). It can be seen that the peak at 1150cm^{-1} for

(c-o-c) aliphatic ether between the carbon atom of the epoxide ring after opening and bonded with the sixth carbon atom in the sugar molecule to form a monolith modified with starch.

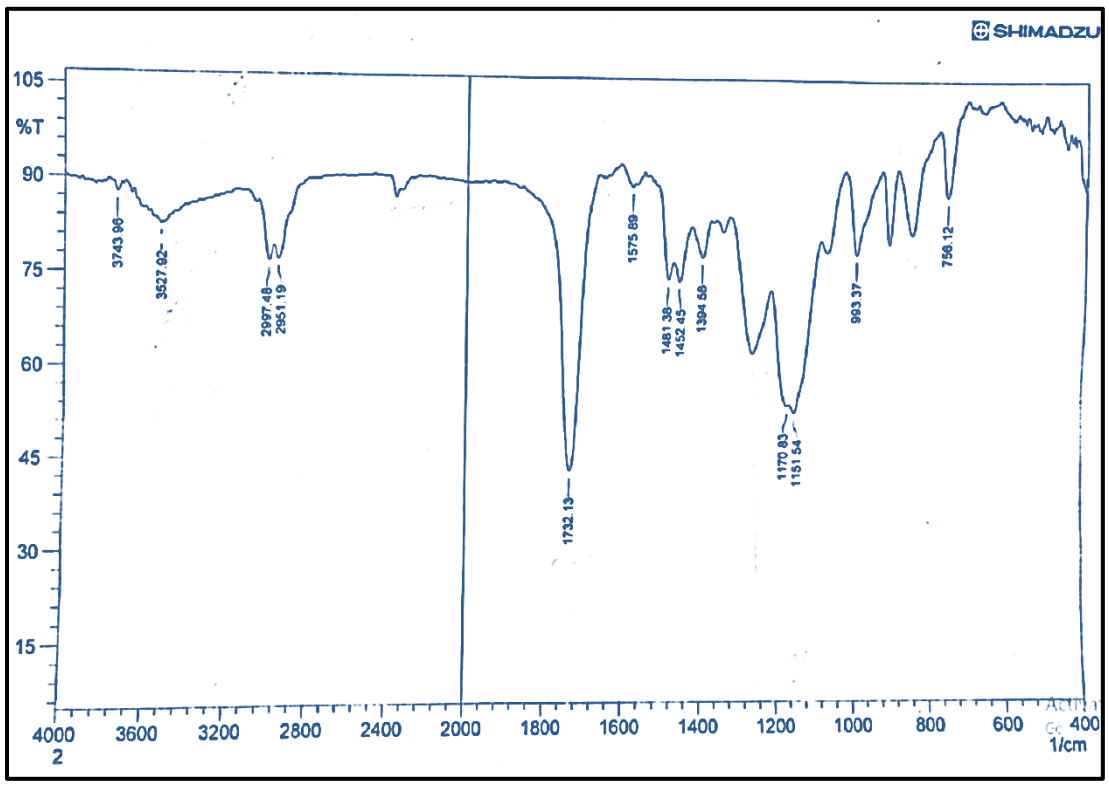


Figure (3-21) FTIR for monolith after modification with starch

Compared to the FTIR spectrum for free starch as shown in [179], the presence of the (C=O) cyclohexanone bond was observed in 1725cm^{-1}, which changes its position in the monolith spectrum after modification with starch to1720 cm^{-1}, which has a shifted.

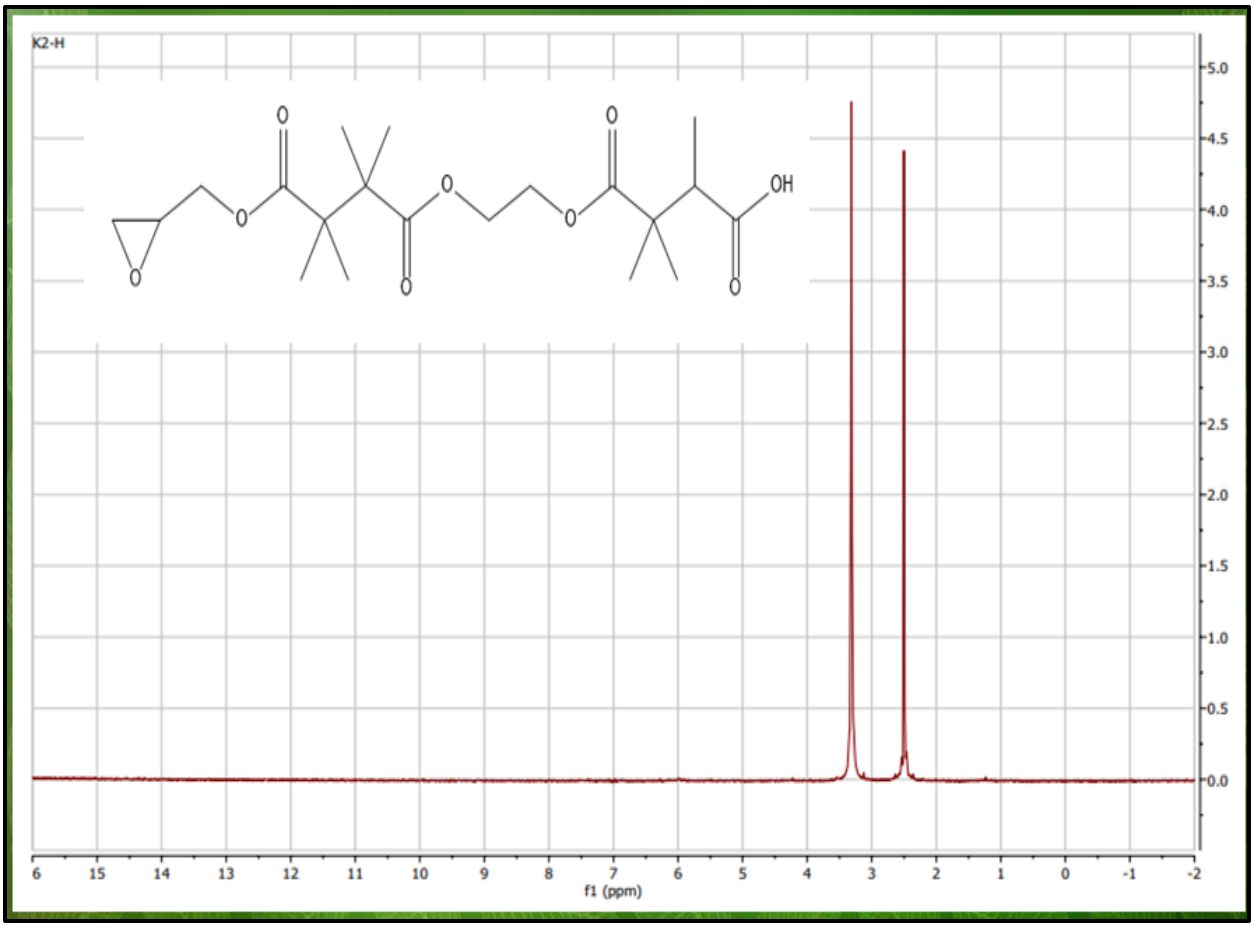
As for the (C=C) bond, It is present in both spectrums, but there is a slight deviation in the bond positions because of the difference in difference in the environment of the bonds.As shown in Table (3-8).

Table(3-8) The FT-IR data for the *main peaks* of the starch and the monolithic (co-polymer) before and after modification:

group name	starch	GMA	EDMA	A.Ac	Monolithic without starch	monolithic with starch
C-O-C (cyclic)	-	1254.46	-	-	1250	disappear
C=C	-	1637.10	1636.85	1635.9	disappear	disappear
C-H (bending)	1450	1452.6	1452.02	1459.67	1450	1481 (shifted)
O-H (bending)	1425				1440	1425
C=O (cyclohexanone)	1725	-	-	-	-	1720
C-O-C (aliphatic ether)	-	-	-	-	-	1150
C=O	-	1716.48	1717.69	1716.12	-	-

3.13. Nuclear Magnetic Resonance ^{1}H-NMR Spectroscopy

The prepared monolith (A.Ac-co-EDMA-co-GMA) was studied by H-NMR, where the peak (3.2 ppm) for the CH_3 group and (2.5 ppm) for the CH_2 group. Also can be noted that the disappearance of the CH_2 peak that belongs to the alkene group at(6-7 ppm) is a significant indication of polymer formation, As shown in Figure (3-22).



figure(3-22) ^{1}H-NMR for prepared monolith A.Ac-co-EDMA-co-GMA

3.14. Application of The Monolith in The Separation of α-Amylase Enzyme in Human Serum

The prepared monolith was used to separate and determine the amylase enzyme in human serum. On the other hand, the direct method was used to determine the amylase enzyme in human serum. The absorbance results obtained from monolith separation were compared with those obtained from the direct method.

3.14.1. Direct Method

The method attached to the Alpha-Amylase Kit test from Bio Labo Company used the direct method to measure the activity of the amylase enzyme of a serum sample [132].

The absorbance results were recorded after every 30 seconds at 37 ^{0}C at wavelength of 405 nm as follows:

A1=0.405 A_2=0.406 A_3=0.407

(A: Absorbance measured by direct method)

$A_1 - A_2 = \Delta A$

$\Delta Abc = A_3 - \Delta A$

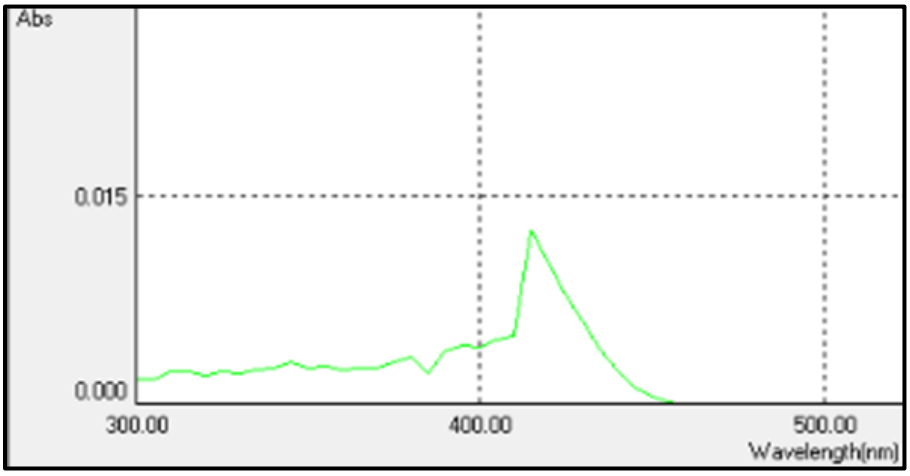
Activity(IU/ L)= (ΔAbc/30min) x factor (Factor=3178)

By applying the absorbances to the mathematical equation, the activity of the amylase enzyme was equal 43 .008IU/L It is within the normal range for the activity of the enzyme amylase from (22-80)IU/L.

3.14.2. Application by Using An Affinity Column

Firstly200 µl from the same sample of serum that was used in the direct method was pumped by glasses syringe inside the monolithic column; after the serum sample was passed through the column, then the excess was taken, and the absorbance was recorded to calculate the activity for amylase 1.377(IUL) by using same mathematical equation that used in the direct method

The result was much less than the expected value compared to the direct method, assuming that the person is healthy. This indicates that a higher percentage of amylase remained stuck to the starch inside the column. The absorbance spectrum is shown in Figure (3-23).



Figure(3-23) Absorbance for the excess.

Finally, the bonded α- amylase was removed by acetonitrile solvent that was pumped through the column and the sample was collected, the absorbance of the collected sample was recorded, and the activity was calculated by using the same mathematical equation that was used in the

direct method it found 53.496(IU/L) at 37 ^{0}C as flow

A_1= 0.503 A_2=0.504 A_3= 0.506

(A: Absorbance measured by prepared monolithic column)

By applying the same calculations above, the activity of the amylase enzyme was equal to 53.496 IU/L, which was within the normal range of a-amylase activity; the absorbance can showing in Figure (3-24) .

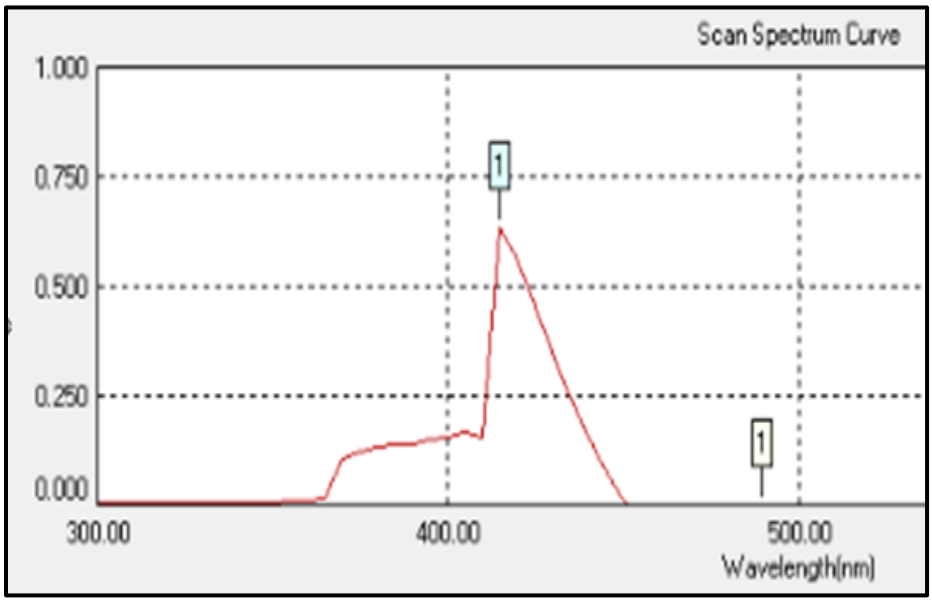


Figure (3-24) Absorbance of the bending amylase

The above work steps were repeated several times on different samples with the same column, which gave similar results with a higher amylase activity than the direct method.

The next table explains the efficiency of the prepared monolithic colum for determination of α-amylase enzyme for different samples

Tabel (3-9) **Measured activity of α- amylase enzyme in human serum using the prepared monolithic column for several different samples.**

NO.	Absorbance that Measured by monolithic column			Activity Measured by direct method in UI/L	Activity Measured by monolithic column in UI/L	RSD for activity Measured by monolithic column n=3
	A1	A2	A3			
1	0.502 0.503 0.503	0.503 0.504 0.504	0.504 0.505 0.505	43.89	53.284 53.390 53.390	0.129
2	0.460 0.474 0.503	0.464 0.475 0.504	0.466 0.476 0.506	43.008	47.88 50.318 53.496	0.016
3	0.502 0.610 0.501	0.503 0.615 0.502	0.504 0.620 0.503	43.99	53.284 65.149 53.173	0.154
4	0.501 0.503 0.502	0.504 0.504 0.503	0.509 0.505 0.504	44.855	53.602 53.390 53.284	0.030
5	0.302 0.303 0.304	0.303 0.304 0.305	0.305 0.305 0.306	22.90	32.203 32.203 32.209	0.021
6	0.415 0.420 0.424	0.420 0.425 0.425	0.424 0.426 0.426	37.76	44.386 44.597 45.021	0.005
7	0.503 0.504 0.505	0.505 0.505 0.506	0.506 0.506 0.507	43.090	52.602 53.496 53.602	0.0075
8	0.23 0.302 0.301	0.34 0.304 0.305	0.41 0.306 0.306	26.69	31.78 32.20 31.99	0.0065
9	0.507 0.601 0.602	0.508 0.602 0.603	0.509 0.603 0.604	43.010	53.814 63.771 63.877	0.094
10	0.506 0.503 0.503	0.507 0.504 0.504	0.508 0.505 0.505	44.021	53.708 53.390 53.390	0.013

3.14.3. Using an Unmodified Column with Starch

In order to make sure that amylase is affected by the hydroxide group of starch and does not bind with other hydroxide groups within the composition of the monolith, the monolith was used without modification with starch, and the results were:

A- By using the direct method, the absorbance result for a selected sample was 405 nm at 37°c wase (0.698) ,which is shown in Figure(3-25)

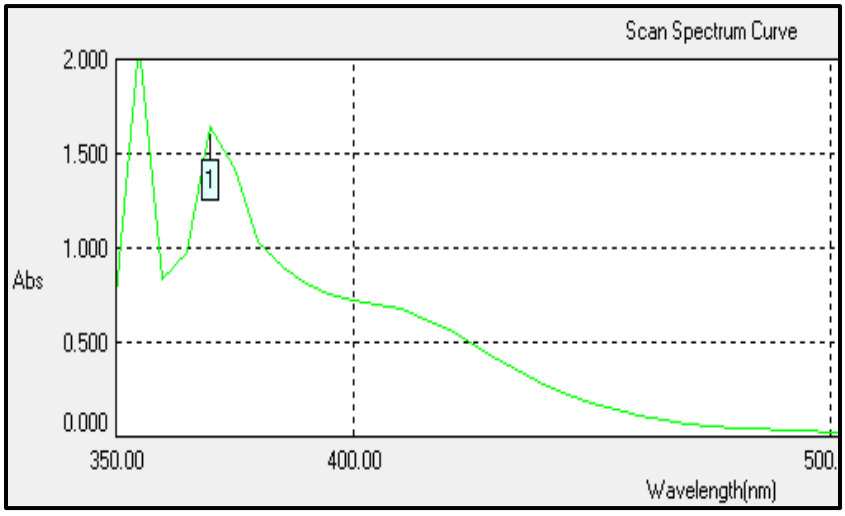


Figure (3-25) the absorbance for the serum by use direct method

B-200µl for the same sample was pumped by glasses syringe inside a monolithic column that was unmodified with starch; after the serum sample was passed through the column then, the excess was taken, and the absorbance was recorded (0. 692) as shown in figure (3-26)

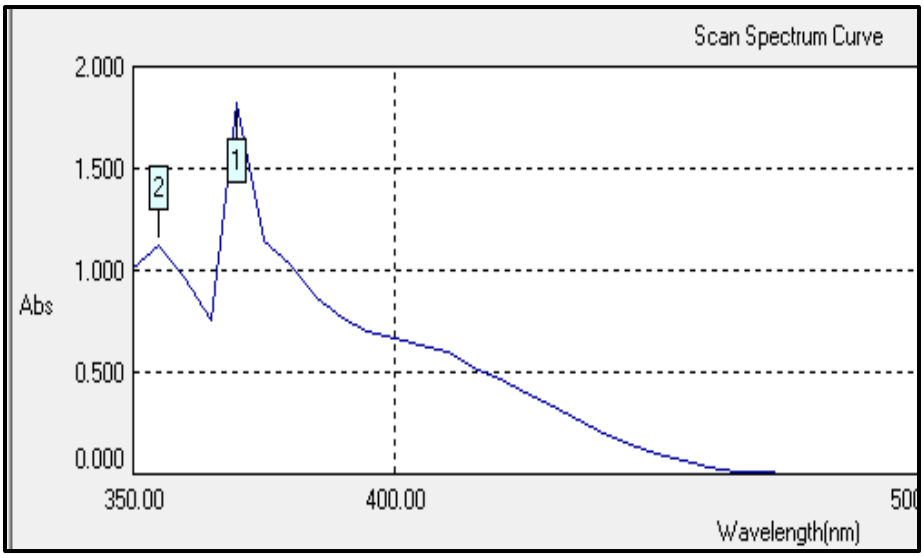


Figure (3-26) the absorbance for the excess and the outside from column.

C- - Finally, the serum inside the column was removed by acetonitrile solvent, and the absorbance of the collected serum was taken after treating it with regent and substrate; the absorbance is shown in Figure (3-27).

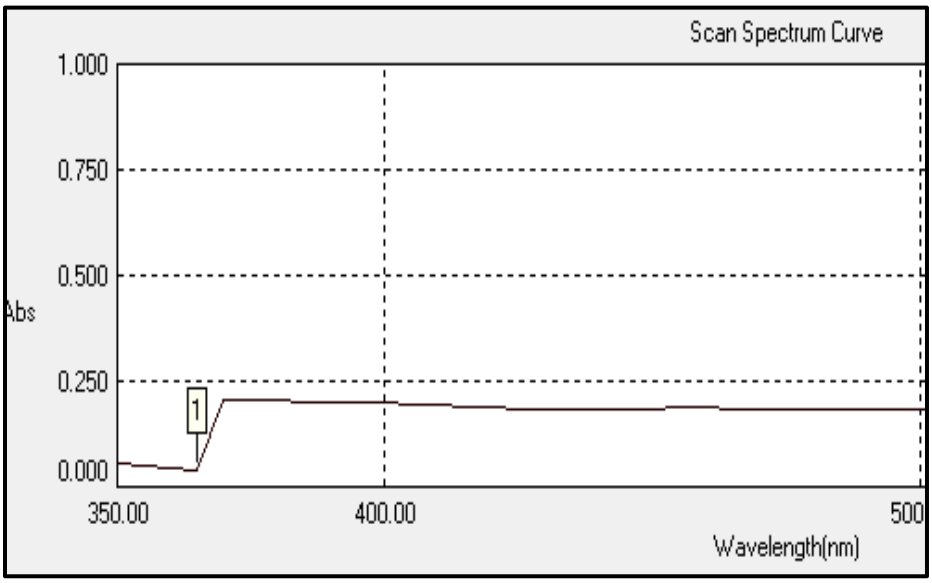


Figure (3-27) the absorbance for the serum that removing by acetonitrile.

When comparing the results with the direct method, it was noted that a high percentage of the enzyme is present in the excess and the outside of the column, which indicates that the enzyme is affected only by the hydroxide group in the starch

3.15. Investigation of Injection Flow Rate

The injection flow rate was adopted from (50,25,10,5) µl.min^{-1} to investigate the significant flow rate; the results are shown in Table (3-10).

Table(3-10) the flow rates used to remove the α- amylase enzyme

Speed µL/min	Absorption of excess after injection and out of the column			Absorbability of the serum removed by acetonitrile		
	A_1	A_2	A_3	A_1	A_2	A_3
50	0.083	0.132	0.173	0	0	0
25	0.07	0.071	0.073	0.283	0.326	0.341
10	0	0.007	0.009	0.178	0.397	0.544
5	0	0	0	0.675	0.691	0.735

The absorbance of the serum was measured by the direct method at 37 C° and a wavelength of 405 nm as follows:

A_1=0.202 A_2=0.215 A_3= 0.223

From Table (3-10), it can be seen that the best flow rate is 10 µl.min^{-1}, followed by 25 µl.min^{-1} in terms of the given absorbance results. Nevertheless, by comparing the time consumed for each speed compared to the volume used, the speed will be 25 better in terms of results, depending on the absorption results in the direct method. By drawing the relationship between the absorption of excess after injection and out of the column with the flow rate, the result is shown in Figure (3.28)

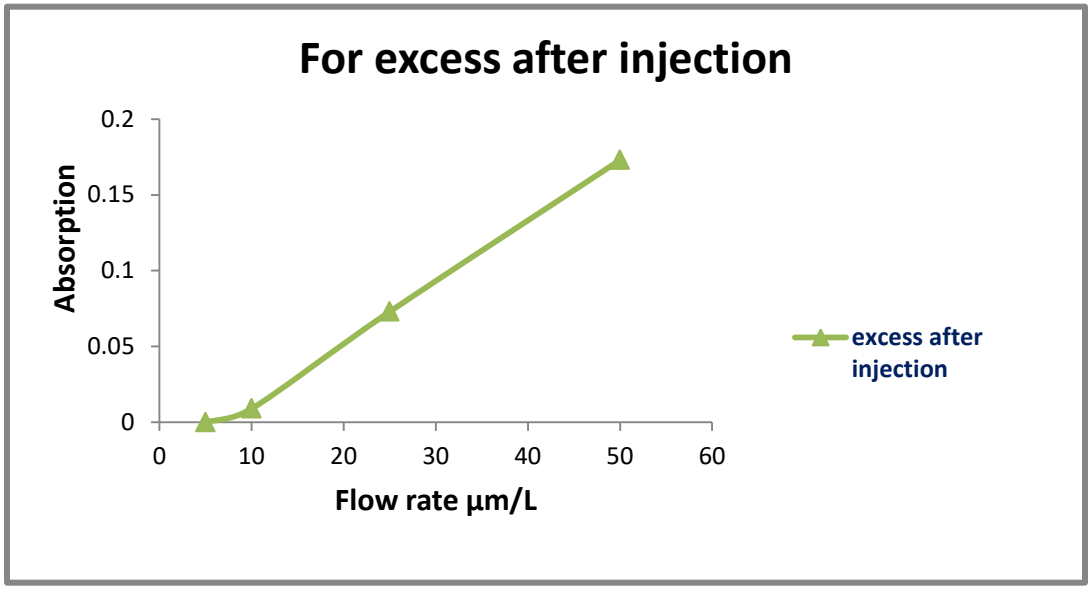


Figure (3-28) the relationship between flowrate and absorbance for excess after injection.

While the relationship between the absorption of the serum removed by acetonitrile and the flow rate is shown in Figure(3-29).

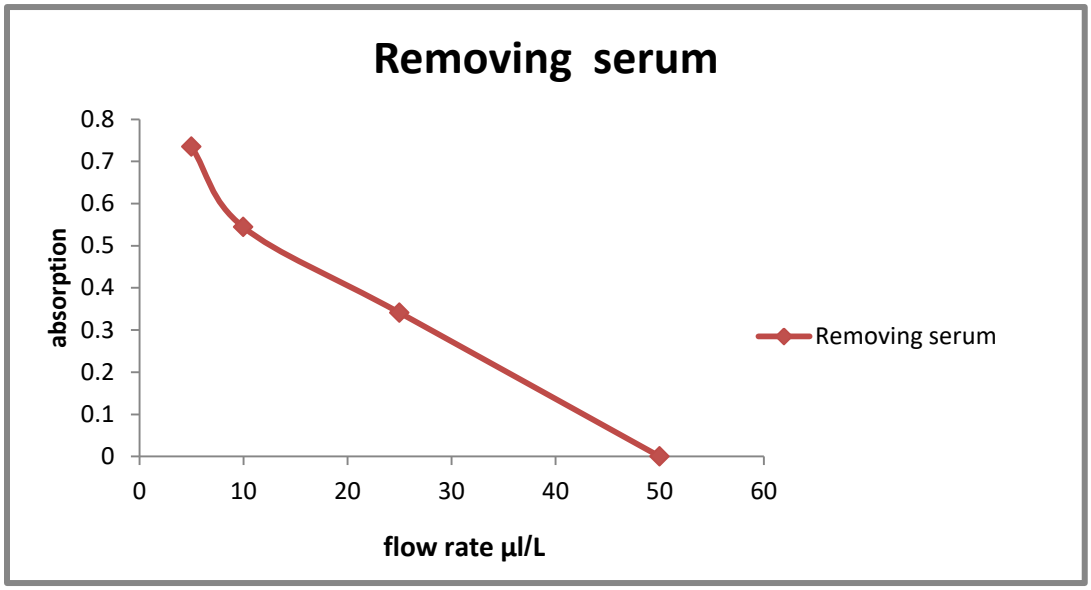
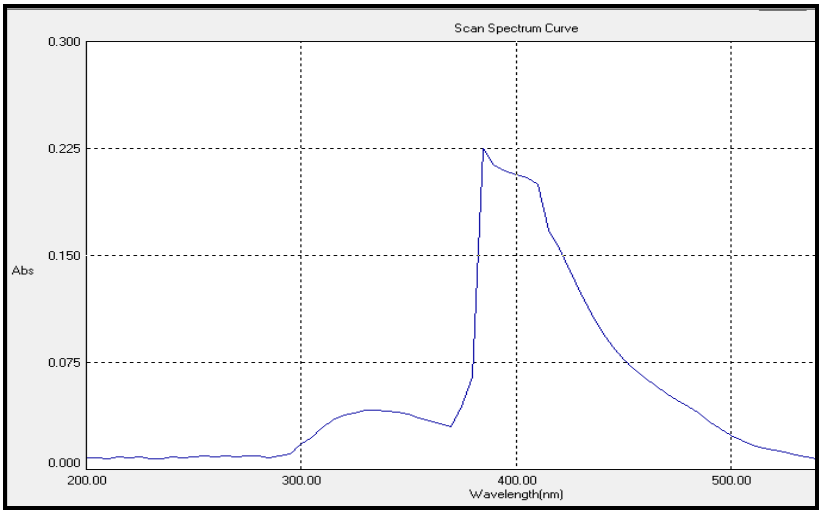
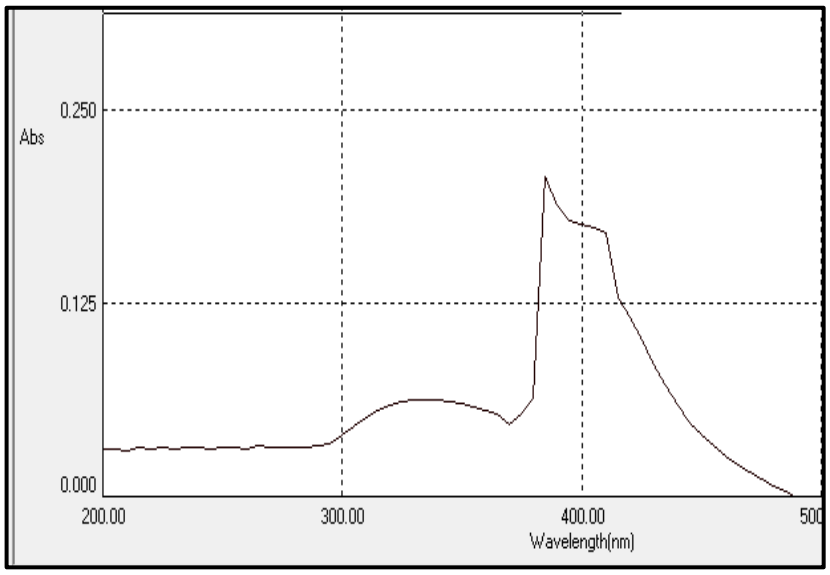


Figure (3-29) the relationship between flowrate and absorbance for removing serum

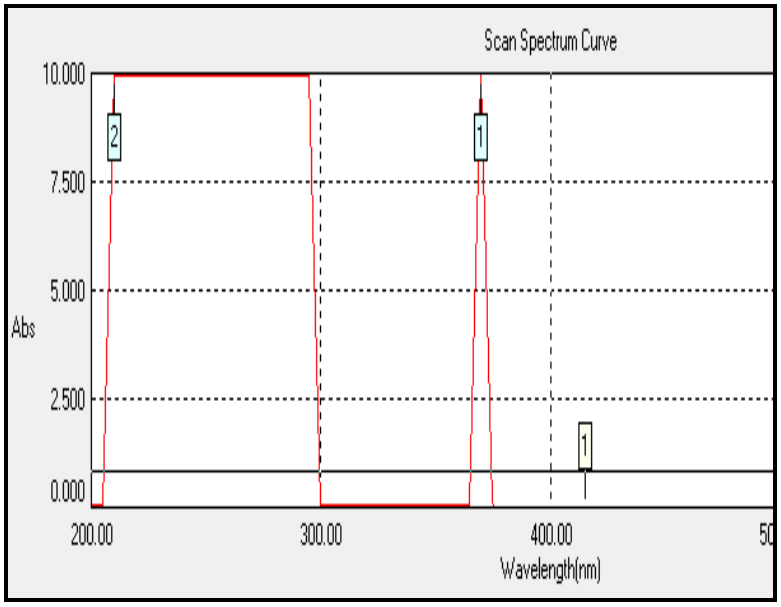
The next figure(3-30)show the highest absorbance for each flow rate mentain in the table(3-10).



Figure(3-30)The absorbance using the direct method

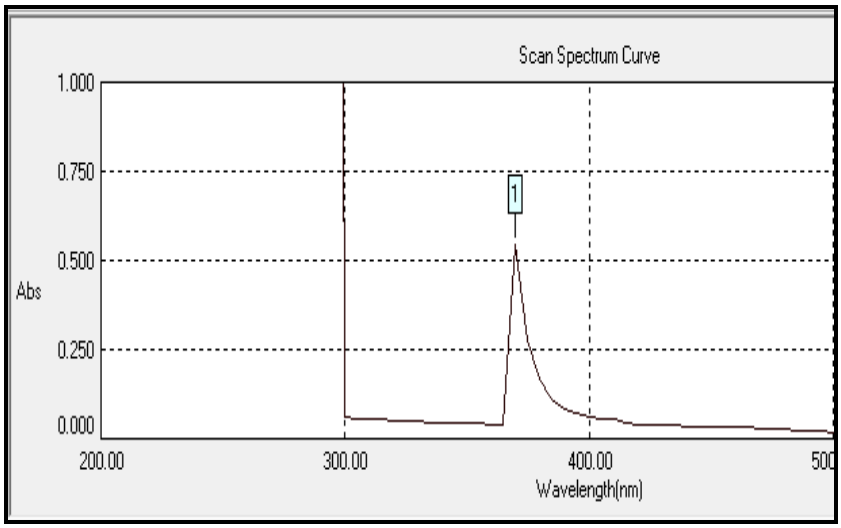


The excess after injection

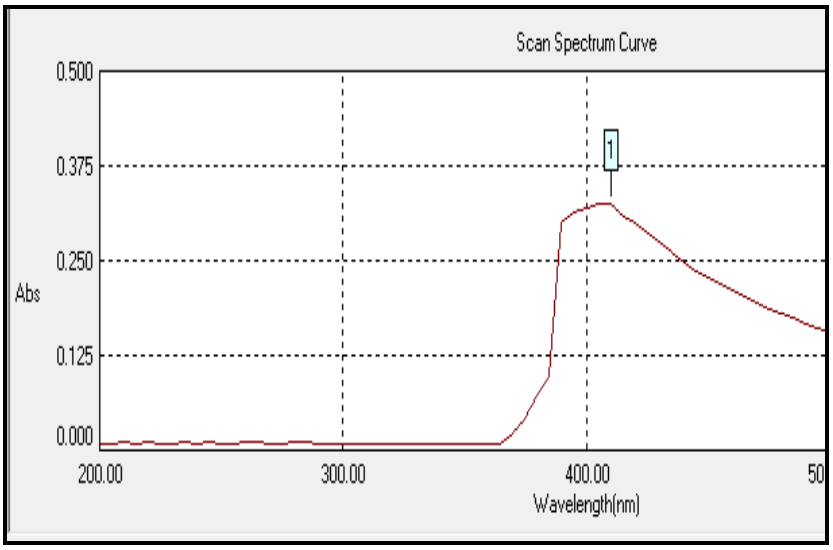


For serum removed with acetonitrile

Figure(3-31) Absorbence at 50 µl/ min flow rate

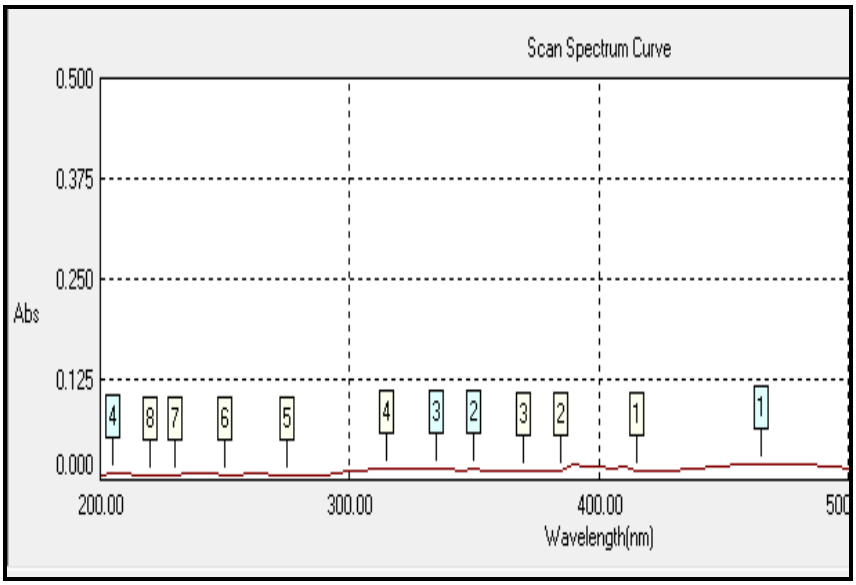


The excess after injection

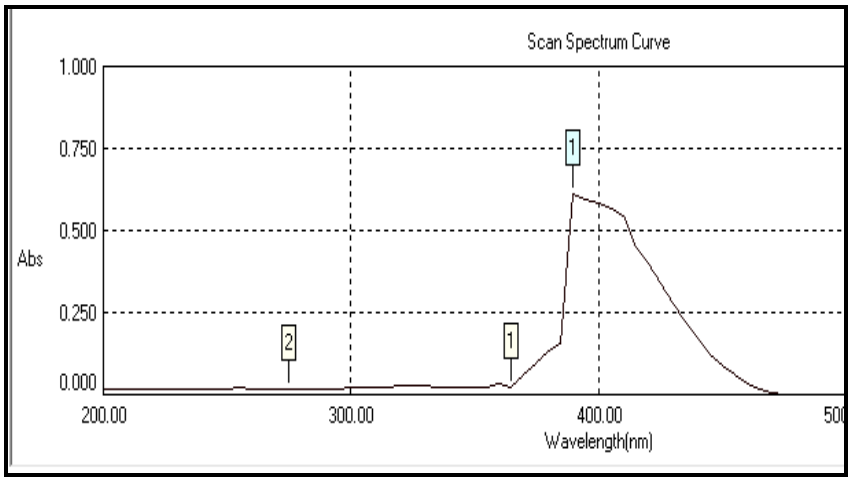


For serum removed with acetonitrile

Figure(3-32) Absorbence at 25 µl/ min flow rate



The excess after injection



For serum removed with acetonitrile

Figure(3-33) Absorbence at 10 µl/ min flow rate

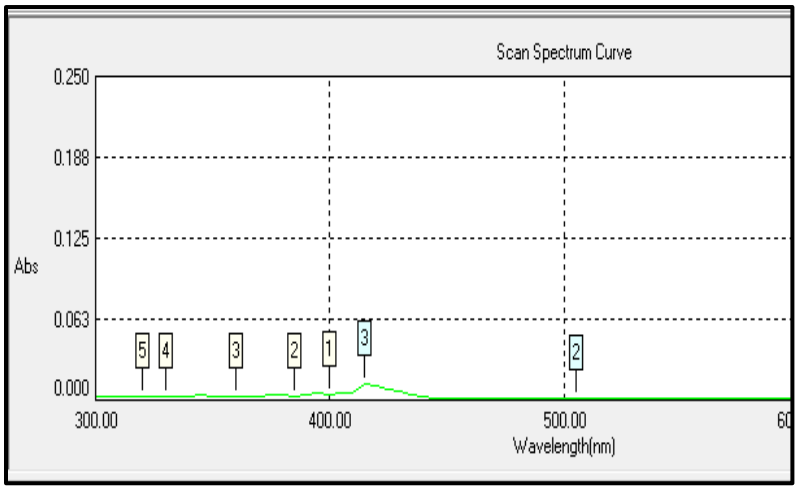
3.16. Another Type of Solvent That is Used to Remove α-Amylase from The Column

Depending on the principal lock and key, many different types of solvents were used to remove α-amylase from monolithic by competing with starch to bond with the α- amylase enzyme, so it was likely that dextrin and maltose solutions are the most suitable solutions to remove α-amylase from starch bound to the monolith, based on research published that[153,180]

However, it was found in this study that the most suitable solvent for removing the enzyme is acetonitrile, which gives higher activity and a higher concentration of the enzyme [181].

Whereas, removing the enzyme that bonded with starch using a solution of dextrin prepared with a buffer solution of pH =7 (buffer solution wase disolved 6.75g from Na2HPO$_4$ in 250ml distilld water and7.1g from NaH$_2$PO$_4$ in 250 ml distilled water then take 152 .5 ml from Na$_2$HPO$_4$ solution and 97.5ml from NaH$_2$PO$_4$ solution after that complete the final volume to 500ml by adding distilled water)we showed that could not remove the enzyme from the monolith, but the dextrin solution passed through the column and came out without affecting the bond between amylase and starch, as shown in the absorbance's for each solution as follows:

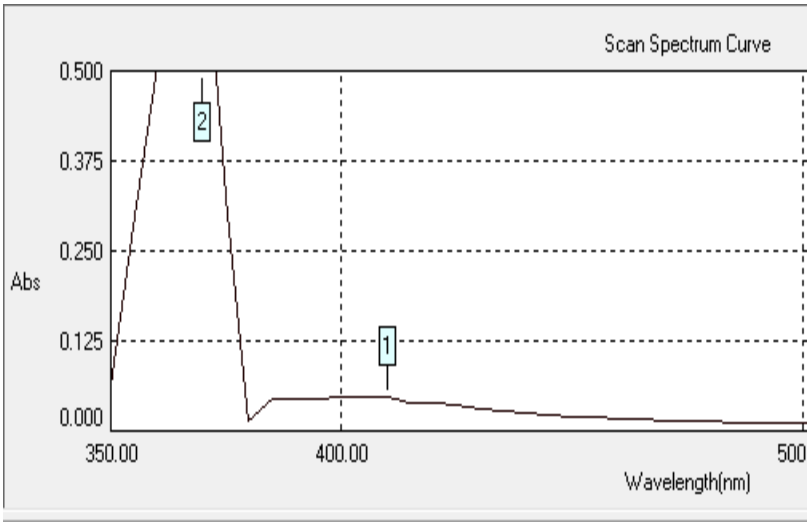
A- 200µl of serum injection inside the column at a 10 µl.min^{-1} flow rate, and after passing through the column, collect the excess from the injection serum and calculate the absorbance as showing in the (3-34)



Figure(3-34)For excess at 10 µl/ min flow rate

this means the highest percentage of the α- amylase enzyme remains inside the column

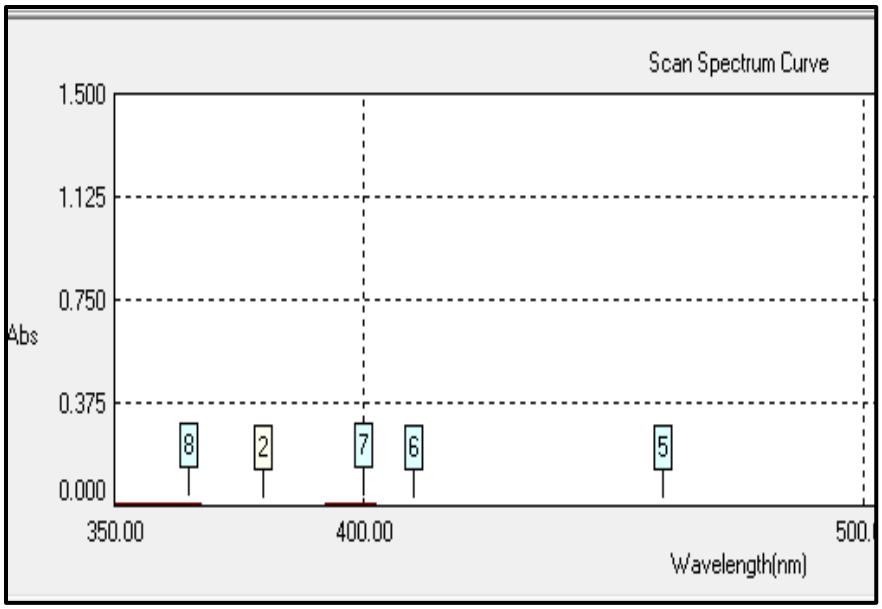
B- After washing the column with deionized water and the enzyme inside it, the washing water was collected and the absorbance was calculated for it, and it was as shown in the Figure (3-35)



Figure(3-35)absorbance for α.amylase after washing with deionized water

From the Figure(3-35), we notice the presence of a slight percentage of the enzyme in the washing water, which may indicate the unlinked enzyme residues present in the column, and it may not necessarily indicate the enzyme removed from the bond with the starch.

C- Finally 200µl from dextrin solution (2% w/v a buffer solution of pH =7) injection inside the column to removing the α-amylase that bonding with starch, then the absorbance for the collected after passing through the column and treated with regent and substrate, the absorbance was as shown in Figure (3-36)



Figure(3-36) Absorbance for α-amylase with dextrin

The wavelength of dextrin was 365 as shown in Figure (3-37)

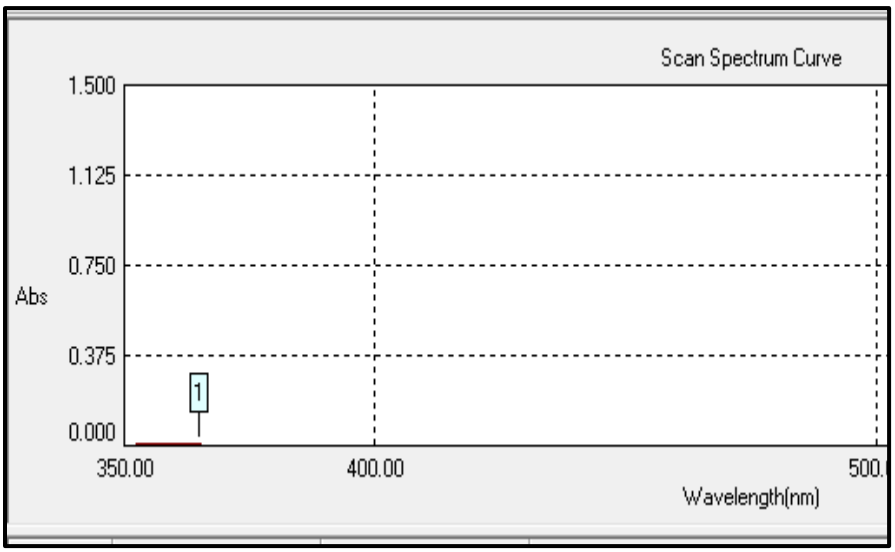


Figure (3-37) the absorbance of dextrin only

To explain this by absorbance, it indicates that the dextrin solution could not remove the enzyme bound with starch in column.

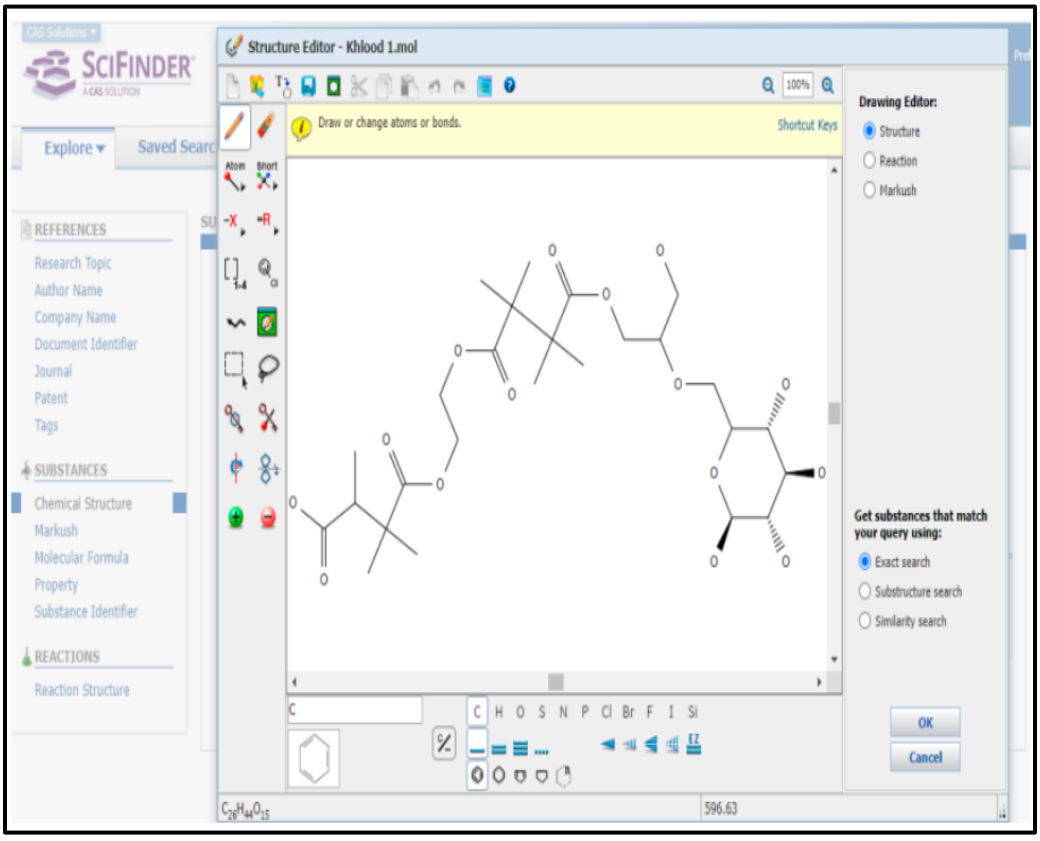
Table(3-11) summarize the result of the solvent that used to remove α-amylase from the bond with starch inside the monolithic column

Table (3-11) Explain the results from use various solvent to remove the α-amylase from column

NO.	Solvent	The absorbance	the activity (IU/L)
1	deionized water	0.056	5.826
2	Dextrin solution in buffer solution	No absorbance	-
3	Dextrin solution only	No absorbance	-
4	Buffer solution only	No absorbance	-
5	**acetonitrile**	**0.59**	**53.496**

3.17. Proof That the Compound is A New and not Previously Prepared

Depending on the SciFinder program and Through the foregoing and based on previous studies and the results of our research, we found that the compound is prepared for the first time, as shown in the Figures(3-38),(3-39),(3-40)and(3-41) which indicates the newness of this compound.



Figure(3-38) The structural formula of the structural unit of the polymer under study

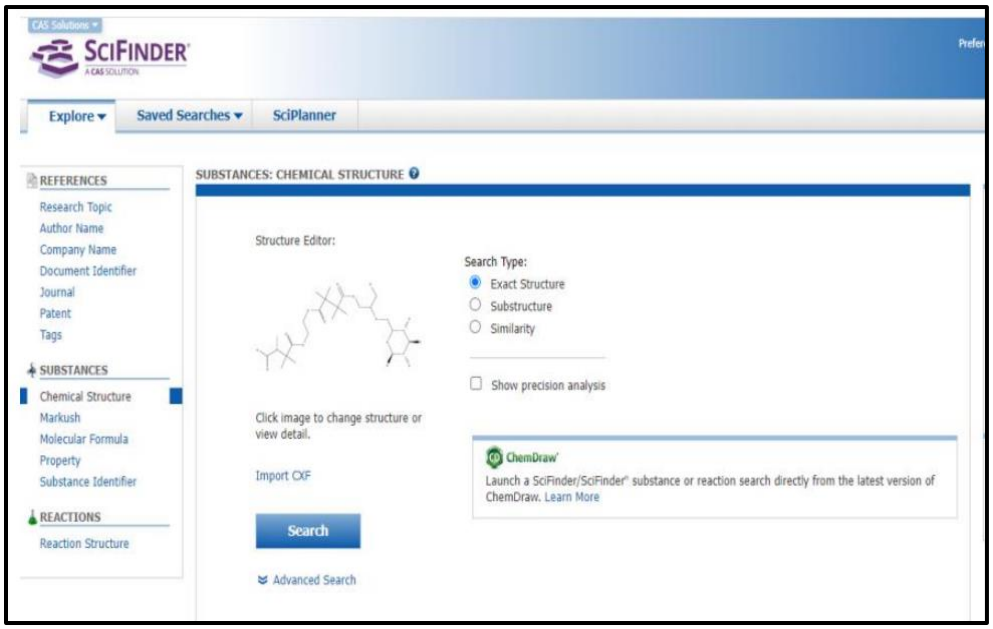
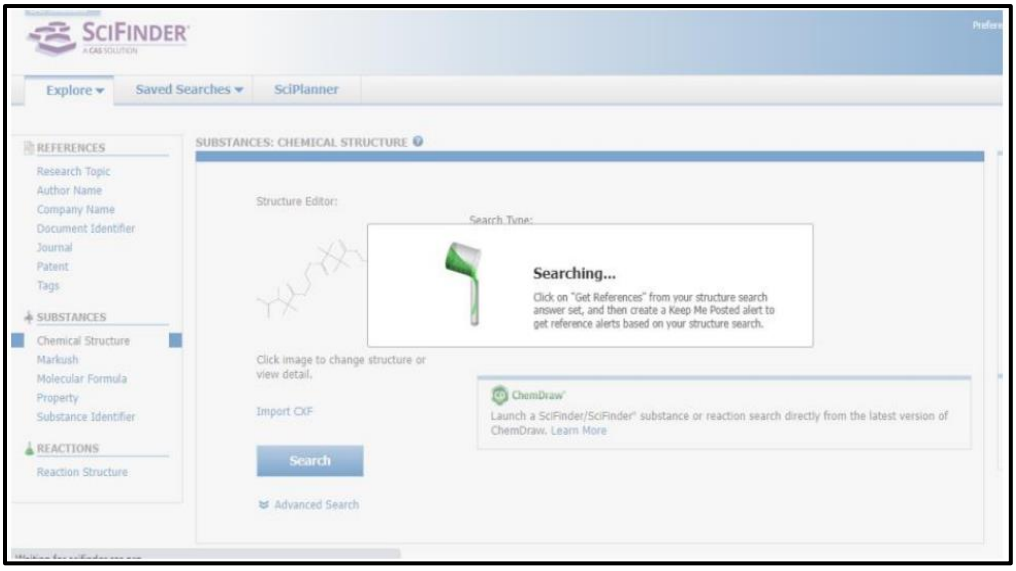


Figure (3-39) Synthesis output to supplement the sequence of the software used.



Figure(3-40) Supplement the search string in the program used.

Figure(3-41) The result of the research that accurately proves the novelty of the compound.

3.18. Conclusions

- Acrylic acid-co-ethylene dimethacrylate-co-glycidyl methacrylate modified with starch monolithic was prepared successfully and frugal inside a borosilicate tube using UV polymerization.
- The monolithic column was modified successfully to prepare an affinity column for separation and purification of α- amylase in human serum compared to the excessive direct method. In addition, the regent for the α- amylase enzyme can be prepared in the laboratory.
- The prepared monolith was identified using FTIR, ^{1}H-NMR, SEM and BET techniques. Then the Sci-Finder program proved that the monolith(Acrylic acid –co-Ethylene dimethacrylate –co-Glycidyle methacrylate modified with starch) is new and prepared for the first time.
- It was found that the affinity column can be used many times(about 15 times) without change in the skeletal structure of the monolithic.
- The lifetime of the prepared monolith was three months and 20 days with the high-efficiency process.
- The BET analysis showed that the surface area was (43.78 m^2/ g) and the pore size was (8 nm), which is suitable for proteins and enzymes analysis.
- Moreover, it can be concluded that the same column can be used to separate other enzymes if the starch is replaced with the appropriate substrate for each enzyme on the principle of lock and key.

3.19.Future Work Suggestion

- It is developing a monolithic affinity column by investigating other monomers that have different properties to separate different samples in a single run.
- Develop different methods to modify the epoxy ring with another substrate to separate another enzyme.
- It prepared monolithic columns with appropriate pore sizes by tuning the polymerization mixture to obtain monolithic columns that significantly enhance chromatographic performance for biomolecules.

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Publications

Original Article

Preparation of Affinity Monolithic Column for α-amylase Separation

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ABSTRACT

Affinity monolith chromatography) AMC(is an effective technique for isolating, analyzing, or studying certain target chemicals in samples . Affinity monolithic support and as abiologically binding agent serve as the stationary phase in monolithic chromatography . This study include monolithic column consists of co polymers from glycidyl methacrylate (GMA) , cross linker ethylene di methacrylate (EDMA), Acrylic acid(A.Ac) prepared by U.V photo polymerization inside borosilicate columns (60 mm) with an inner diameter of (1.5 mm), and an outer diameter of (3.0 mm) and then modified by adding starch solution (pH =10)to react with epoxy group of GMA for 12 hours in room temperature . Identification of the monolith using different techniques such as FTIR, UV- Light source,SEM and BET.The result affinity polymer can be used for separation and purification of α-amylase enzyme in human serum .

Keywords: Affinity monolith column, Glycidyle metharcrylate, Affinity chromatography, α- amylase.

Introduction

Traditional particle solid phases for capillary liquid chromatography have been replaced with monolithic columns, which represent an appealing option.[1,2] A monolith is a skeleton with massive through-pores that is continuous and linked.[1,3] The integrated construction increases mechanical strength, while the large through-pores (a few µm) reduce flow resistance.[4] This combination allows for monolithic columns with reduced diameters to be operated at higer flow rates, enhancing both sensitivity and throughput at the same time.[1,5] Polymeric monoliths were first described in the 1960s, However, the first effective ones for protein separations did not come until the late 1980s. In the 1990s, a growing number of alternative monoliths appeared.[6-8]

Organic monoliths are made by copolymerizing monofunctional and bifunctional molecules (uncommonly trifunctional), porogenic solvents and an organic precursor in the presence of a suitable initiator.[7,9,10] These porogens (solvents) resulted in the development of a polymer having linked holes that allowed both solutes and solvents to pass through, by selecting a proper mix of porogenic chemicals, the pore size distribution may be adjusted.[8,4,11]

Affinity chromatography uses specific interaction to identify and purify target molecules from mixtures for the ligand of interest.[12,6] As a result, the utilization of an appropriate affinity matrix and a sufficient technique are required for effective affinity chromatography. Here, An affinity matrix is a solid support (affinity support) on which a ligand is immobilized by a linker. Affinity matrices are now being utilized to purify molecules of interest from crude materials.[13] most significant factors to consider when designing ligands that include the affinity to molecules target; the specificity of the binding; the immobilization›s viability; the binding›s stability, washing, and circumstances of elution; and the ability to connect to the target following immobilization.[14] Generally, Covalent immobilization of a ligand onto a solid

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الخلاصة

الكروماتوغرافيا المتخصصة المونوليثية هي تقنية فعالة لعزل وتحليل بعض المواد الكيميائية المستهدفة في العينات قيد الدراسة، يحتوي الطور الثابت الصلب المونوليثي على مجاميع فعالة متخصصه لها القدرة على مسك مركبات كيميائية او حيوية معينة دون الاخرى

تتضمن هذه الدراسة جزئين:

الجزء الاول : تحضير العمود المونوليثي .

تم تحضير المونوليث من بلمرة كل من المونمرين (كليسيديل ميثاكريلات (GMA) وحامض الاكريليك (A.Ac) والرابط اثيلين ثنائي ميثا كريلات (EDMA) باستخدام البلمرة الضوئية بالأشعة فوق البنفسجية (UV) داخل انبوب زجاجي بطول (60mm) و بقطر داخلي (1.5mm) وقطر خارجي (3mm),و قد تم تنشيط سطحه الداخلي لتحضيره لعملية البلمرة .

تم تشخيص البوليمر المونوليثي المتكون باستخدام عدة تقنيات مثل

(FTIR , H-NMR,FESEM ,BET) واستخدم برنامج (SCi FINDER) لأثبات ان هذا المونوليث محضر لأول مرة .

تم دراسة الظروف المثلى لتحضير العمود كدراسة تأثير النسبة بين المونمرين ودراسة المسافة بين مصدر التشعيع والعمود ودراسة تأثير زمن التشعيع ودراسة تأثير انواع المذيبات المستخدمة .كما تم دراسة النسبة المئوية للانتفاخ والمسامية والنفاذية والسرعة المناسبة للحقن.

الجزء الثاني : التطبيق العملي للعمود المحضر .

تم اضافة مجاميع النشا الى سطح العمود المونوليثي المحضر للحصول على عمود فصل متخصص، تمت هذه العملية باستخدام محلول النشا عند داله حامضية (pH=10) ليتفاعل مع مجموعة الايبوكسي الخاصة بمونمر (GMA) لمدة 12 ساعة بدرجة حرارة الغرفة لتحضير كولوم متخصص لفصل انزيم الفا- اميليز من بلازما الدم البشري. تم قياس فعالية الأنزيم المذكور ومقارنتها مع الفعالية المحسوبة بالطريقة المباشرة باستخدام كت من شركة (BIOLABO) الفرنسية حيث كانت الفعالية لأنزيم الاميليز المقاسة باستخدام العمود المحضر 53.496 IU/L مقارنة بالفعالية للأنزيم المقاسة باستخدام الطريقة المباشرة لنفس العينة من البلازما والتي كانت 43.0081 IU/L .

وجد ان العمود المحضر (A.Ac-co-EDMA-co-GMA modified by starch) يمكن استخدامه عدة مرات دون اي تغيير بالخواص المورفولوجية او الهيكلية للمونوليث كذلك يمكن خزن هذا العمود المحضر لمدة ثلاثة اشهر وعشرون يوما دون اي تغير في كفاءة الفصل

وزارة التعليم العالي والبحث العلمي

جامعة بابل /كلية العلوم

قسم الكيمياء

تحضير طور ثابت متجانس كأساس في فصل انزيم الفا- اميليز

رسالة مقدمة

الى مجلس كلية العلوم – جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير في العلوم / الكيمياء

من قبل

خلود عمران علي مسربت

بكالوريوس علوم كيمياء – جامعة بابل – 2003

بأشراف

الاستاذ الدكتور

احمد علي عبد الصاحب الكريمي

2022م 1444 هـ