**DOKUZ EYLUL UNIVERSITY**
**GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES**

# REMOVAL OF SEROTONIN USING POLY (HEMA-MAT) NANOSTRUCTURES

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**IZMIR**

# REMOVAL OF SEROTONIN USING POLY (HEMA-MAT) NANOSTRUCTURES

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## ABSTRACT

Serotonin (5-hydroxytriptamin, 5-HT) which is an important neurotransmitter and involved in control and regulation of numerous functions of the central and peripheral nervous systems has been associated with several psychiatric disorders. Therefore, removal of serotonin is very important. Hence, poly(HEMA-MAT) nanostructures, novel affinity chromatography matrix for adsorption of serotonin synthesized and the optimum conditions for the adsorption of serotonin from aqueous solution were also determined in this study. Poly(HEMA-MAT) nanostructures were characterized by FTIR, SEM and elemental analysis. Maximum adsorption capacity was found to be 2901.36 mg/g and the adsorbed amounts per unit mass of nanostructures reached to a plateau value at about 500 ppm at pH 6.0. Up to 90% of the adsorbed serotonin was removed by using 1.5 M NaCl as elution agent. Poly(HEMA-MAT) nanostructures were also used for the adsorption of serotonin in banana and tomato. We concluded that poly(HEMA-MAT) nanostructures could be repeatedly applied for serotonin adsorption without significant losses in the adsorption capacity.

**Keywords:** Serotonin, Nanostructures, Adsorption, Hydrophobic Interaction Chromatography

## POLI (HEMA-MAT) NANO YAPILARI KULLANILARAK SEROTONIN UZAKLASTIRILMASI

## ÖZ

Merkezi ve periferal sinir sisteminin birçok fonksiyonunun kontrolü ve regülasyonunda yer alan ve önemli bir nörotransmitter olan serotonin (5-hydroxytriptamin, 5-HT), çeşitli psikiyatrik hastalıkların patolojisinde rol oynar. Bu nedenle, serotonin uzaklaştırılması önemlidir. Bu çalışmada, poli(HEMA-MAT) nanoyapıları, serotonin adsorpsiyonu için yeni afinite kromatografi matriksi olarak sentezlenmiş ve sulu çözeltiden serotonin adsorpsiyonu için optimum koşullar belirlenmiştir. Poli(HEMA-MAT) nanoyapıları FTIR, SEM ve elementel analiz ile karakterize edilmiştir. Maximum adsorption kapasitesi 2901,36 mg/g olarak bulunmuş ve nanoyapıların birim kütlesi başına adsorplanan miktar pH 6,0' da 500ppm derişimde plato değerine ulaşmıştır. Adsorplanan serotoninin %90'ından fazlası 1,5 M NaCl elusion ajanı kullanılarak uzaklaştırılmıştır. Ayrıca, poli(HEMA-MAT) nanoyapıları muz ve domates örneklerindeki serotoninin adsorpsiyonu için kullanılmıştır. Poli(HEMA-MAT) nanoyapılarının adsorpsiyon kapasitesinde önemli bir azalma olmaksızın serotonin adsorpsiyonunda tekrar tekrar kullanılabileceği sonucuna varılmıştır.

**Anahtar Sözcükler:** Serotonin, Adsorpsiyon, Hidrofobik Etkileşim Kromtografisi

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# CHAPTER ONE

# INTRODUCTION

## 1.1 Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is an indolic compound that is synthesised from the essential amino acid tryptophan. Biosynthesis of serotonin in the human body represents only a minor metabolic route for tryptophan. Under normal conditions it accounts for not more than 2% of ingested tryptophan, leading to a daily production of about 10 mg serotonin. The major part of tryptophan is utilised for the protein synthesis. Serotonin proved ubiquitously distributed in nature. In plants it was found in edible fruits, vegetables and nuts, whereas in the animal kingdom it was demonstrated in a variety of organisms from the worm to vertebrates. This diverse presence indicates that the serotonergic system is evolutionarily an ancient one. In lower organisms 5-HT mediates simple behaviors, for example, egg laying in C. Elegans (Waggoner et al, 1998). Important depots in mammals were enterochromaffin cells in the gastrointestinal mucosa, serotonergic neurones of the brain, pineal gland and platelets (Grahamme-Smith, 1988).

### *1.1.1 Biosynthesis*

Hydroxylation of tryptophan to 5-hydroxy-tryptophan (5-HTP) by tryptophan hydroxylase is the first and rate limiting step in serotonin synthesis. Antibodies against tryptophan hydroxylase demonstrated its presence in several tissues, including enterochromaffin cells of the gastrointestinal tract, serotonergic neurones of the brain and pineal gland (Tyce, 1985). Although platelets have been reported to contain low tryptophan hydroxylase activity their high serotonin contents originates from enterochromaffin cells of the gastrointestinal tract (Stoltz, 1985). Formation of serotonin occurs by decarboxylation of 5-HTP. The reaction is catalyzed by aromatic-L-amino acid decarboxylase (AADC) and uses pyridoxal-5-phosphate (the active form of vitamin B) as coenzyme. The enzyme has been detected in neoplastic

tissues such as carcinoid tumour and phaeochromocytoma (Ichinose et al., 1985). Specific types of carcinoid neoplasms may occasionally lack AADC, which gives rise to increased circulating and urinary levels of tumour derived 5-HTP, instead of serotonin (Kema, Vries & Muskiet, 2000).

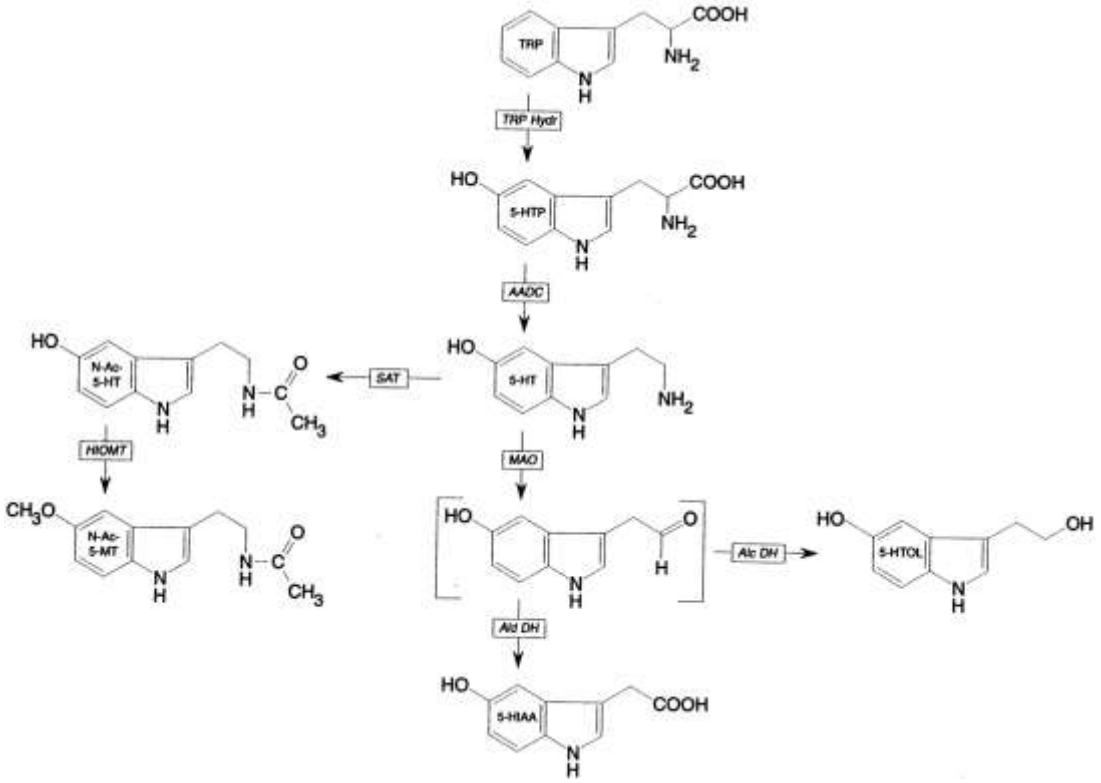


Fig.1.1 Biosynthetic and degradative routes in the metabolism of serotonin; Abbreviations: AADC, aromatic-L-amino acid decarboxylase; Alc DH, alcoholdehydrogenase; Ald DH, aldehydedehydrogenase; HIOMT, hydroxyindol-O-methyl transferase; MAO, monoamine oxidase; N-Ac-5-HT, N-acetyl-5-hydroxytryptamine; N-AC-5-MT, N-acetyl-5-methoxytryptamine, melatonin; TRP, tryptophan; TRP Hydr, tryptophan hydroxylase; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin, 5-hydroxytryptamine; 5-HT, 5-HTOL, 5-hydroxytryptophol; 5-HTP, 5-hydroxytryptophan.

### *1.1.2 Regulation of Synthesis*

Serotonin synthesis rate is obviously dependent on tryptophan hydroxylase, AADC activities and tryptophan availability. Since AADC activity is about 75-times higher than that of tryptophan hydroxylase, 5-HTP formation is considered to be the rate limiting step. Availability of tryptophan also influences serotonin synthesis rate.

High dietary intakes increase tissue serotonin contents, whereas tryptophan deficient diets cause reduced levels (Kema, Vries, & Muskiet, 2000). Processes that control plasma tryptophan uptake by peripheral cells have not fully been clarified as yet. Because of its amphipathic nature circulating tryptophan is partially bound to plasma albumin. Its uptake in brain is known to be dependent on the plasma free tryptophan concentration (Pardridge, 1998). Consequently, processes that influence the equilibrium between free and protein-bound forms of plasma tryptophan (e.g., free fatty acids, certain drugs). Modify its availability for uptake in brain and thereby affect serotonin synthesis rate (Blundell, 1992). Tryptophan uptake in brain cells is regulated by two saturable systems with high- and low-tryptophan affinities, respectively). Serotonin synthesis in brain is also regulated by mechanisms that activate or inhibit tryptophan hydroxylase. Calcium-induced phosphorylation renders the enzyme active, whereas an intra-neuronal serotonin pool inhibits it via negative feedback. Fluctuations in tetrahydrobiopterin concentrations may also be involved in the control of tryptophan hydroxylation activity (Kema, Vries, & Muskiet, 2000)

### *1.1.3 Metabolism*

Serotonin is metabolized via oxidative deamination (monoamine oxidase; MAO), conjugation with sulfuric and glucuronic acids, *N*-acetylation, 5-*O*- methylation and their combinations (Fig. 1.1). The enzymes are differently distributed among the various organs. The majority of serotonin is metabolized by the flavoprotein MAO. This enzyme is mainly located in mitochondria and catalyses oxidative deamination of several monoamines to their corresponding aldehydes, ammonia and hydrogen peroxide. At least two forms of MAO, differing in substrate affinities and inhibitors, are known. MAO has highest affinity for serotonin and norepinephrine, whereas MAO-B is more active in deaminating benzylamine and phenylethylamine (Kopin, 1985). 5-Hydroxyindoleacetaldehyde, the product of oxidative deamination of serotonin, can either be oxidised to 5-hydroxyindoleacetic acid (5-HIAA) or reduced to 5-hydroxytryptophol. The former reaction is catalyzed by aldehyde dehydrogenase with NAD as coenzyme. Aldehyde dehydrogenase has been detected in many tissues, including brain and liver (Kopin, 1985). The enzyme also catalyses oxidation of

catecholamine aldehyde intermediates to their corresponding acidic metabolites. Reduction of 5-hydroxyindoleacetaldehyde to 5-hydroxytryptophol is catalyzed by aldehyde reductase. This enzyme uses NADH as coenzyme. Conversion of an intermediate aldehyde to the corresponding alcohol also takes place in catecholamine metabolism (Kopin, 1985). Formation of 5-hydroxytryptophol represents a minor route of serotonin metabolism. Under normal conditions urinary 5-hydroxytryptophol accounts for about 1% of serotonin turnover. In alcoholism and (other) liver pathology the proportion of 5-hydroxytryptophol may considerably increase by a metabolic shift from 5-HIAA to 5-hydroxytryptophol (Halander, Beck, & Borg, 1992). Conjugation of serotonin with sulfuric acid (formation of serotonin-*O*-sulfate) or glucuronic acid (serotonin-*O*-glucuronide) represents a minor metabolic pathway (Tyce, 1985). Both types of conjugates have been detected in urine of healthy subjects and patients with carcinoid tumours (Kema, Vries, & Muskiet, 2000). Serotonin and 5-HIAA are predominantly excreted in the free form, whereas 5-hydroxytryptophol is mainly excreted as conjugate (Tyce, 1985; Halender, Beck, & Borg, 1992). Serotonin-containing foods can substantially increase urinary excretion of serotonin conjugates (Kema et al., 1992). Two highly specific enzymes that subsequently catalyse serotonin *N*-acetylation and 5-*O*-methylation are involved in the synthesis of melatonin (5-*O*-methyl-*N*-acetylserotonin) in the pineal gland.

Melatonin is a hormone implicated in of circadian rhythm, reproductive development and behaviour (Kema, Vries, & Muskiet, 2000). Serotonin-*N*-acetyltransferase has been found in both brain and liver. In the pineal gland it shows typical ircadian rhythmicity that causes considerably higher plasma melatonin levels at night (Kema, Vries, & Muskiet, 2000). Hydroxyindole-*O*-methyltransferase catalyses the second step in melatonin formation. This enzyme gives rise to 5-*O*-methlation of *N*-acetylserotonin with *S*-adenosylmethionine as methyl donor (Kema, Vries, & Muskiet, 2000).

Serotonin is involved in a variety of physiological processes, including smooth muscle contraction, blood pressure regulation and both peripheral and central nervous system neurotransmission. Its exact role in most processes is still to be

elucidated (Grahame-Smith, & Med, 1988). Synthesis occurs in brain, spinal cord, enterochromaffin cells of the gastrointestinal tract, bronchi, thyroid, pancreas and thymus. Circulating serotonin does not enter the brain by crossing the blood–brain barrier. In the central nervous system it acts as a neurotransmitter–neuromodulator that is implicated in sleep pattern regulation, appetite control, sexual activity, aggression and drive (a driven tendency or instinct, especially of sexual or aggressive origin) (Muhlbauer, 1985). Central nervous system serotonin exerts its actions in concert with other neurotransmitters (Grahame-Smith, & Med, 1988; Siever et al., 1991). In the periphery serotonin acts as a vasoconstrictor and proaggregator when released from aggregating platelets, as a neurotransmitter in the enteric plexuses of the gut and as an autocrine hormone when released from enterochromaffin cells from the gut, pancreas and elsewhere (Grahame-Smith, & Med, 1988; Farthing, 1991). Its precise function in the gastrointestinal tract is not fully understood (Gerson, & Tamir, 1985).

Abnormalities of serotonin-related processes give rise to various pathological conditions. Aberrations in its central nervous system function are thought to be involved in anorexia, anxiety, depression and schizophrenia, whereas degeneration of serotonergic neurones have been noted in Alzheimer's and Parkinson's diseases (Grahame-Smith, & Med, 1988; Blundell, 1992; Siever et al., 1991; Halliday et al., 1990). Peripheral aberrations in serotonin-related processes have been implicated in drug-induced emesis, hypertension, migraine, and genesis of cardiac arrhythmias, Raynaud's disease, fibrotic syndromes and some symptoms of carcinoid syndrome (Grahame-Smith, & Med, 1988; Maton & Med, 1988; Seibold, 1985; Wymenga et al., 1999). The quantitatively most pronounced aberration in serotonin production and metabolism is encountered in patients with carcinoid tumours. Carcinoid tumors are neuroendocrine tumors derived from enterochrommafin cells. These cells are able to produce serotonin, and when a carcinoid tumor develops, excessive amounts of serotonin are usually secreted. In order to diagnose carcinoid tumors, various serotonin markers have been used, with urinary excretion of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) being the one most frequently used (Oberg,

1998). However, the urinary 5-HIAA levels may be within the reference interval in approximately 30% of patients with carcinoid tumors (Feldman, & O'Dorsio, 1986).

Hence, determination of serotonin is very important. In literature, there have been a lot of studies about serototin determination. In the study of Pussard et al., serotonin level was measured in platelet-poor and rich plasma, platelets and urine. Platelet-poor plasma and platelets were injected after a single deproteinization step with perchloric acid. Addition of sodium borohydride to whole blood avoids oxidation of serotonin during the deproteinization step without any chromatographic interference. İn this study, urinary serotonin was purified by two successive cationic and anionic extraction steps (Pussard et al., 1996).

In the other study, a three-step biochemical protocol was developed to analyze monoamine content within the cochlea. The cochlear monoamines and some of their metabolites were quantified, from homogenated cochlear tissue, by a new application of high performance liquid chromatography coupled to electrochemical detection of norepinephrine (NE), dopamine (DA), serotonin (5-HT) and some of their metabolites (3,4-dihydroxyphenylacetic acid, DOPAC; homovanillic acid, HVA; and 5-hydroxyindole-3-acetic acid, 5-HIAA) in the blood-free rat cochlea. (Gil-Loyzaga et al., 2002).

A microbore column liquid chromatographic method for the simultaneous determination of norepinephrine (NE), serotonin (5-HT), and 5-hydroxyindole-3-acetic acid (5HIAA) in microdialysis samples from rat brain was described by Yoshitake et al. The method is based on precolumn derivatization of norepinephrine (NE), serotonin (5HT), and 5-hydroxyindole-3-acetic acid (5HIAA) with benzylamine in the presence of potassium hexacyanoferrate (III) resulting in the corresponding highly fluorescent and stable benzoxazole derivatives. It was concluded that the new fluorescence derivatization method in combination with microbore column liquid chromatography allows the simultaneous determination of NE, 5HT, and 5HIAA in the microdialysis samples at higher sensitivity, providing

easier maintenance in routine use than that achieved by high-performance liquid chromatographic methods with electrochemical detection (Yoshitake et all., 2002)

The response of planarians to dopaminergic ligands and to the effects of cocaine and opioids was studied by Raffa et al. To correlate behavior (specifically, drug withdrawal) with neurotransmitter levels, Raffa et al. developed a method to quantify 5-hydroxytryptamine (5-HT; serotonin) in Planarians. 5-HT was measured using HPLC with fluorescence detection. N N-methyl-5-HT was used as an internal standard (IS) (Raffa et al., 2004).

A rapid, sensitive and selective method for the determination of tryptophan (Trp), serotonin (5-HT) and psychoactive tryptamines (PATs) by thin-layer chromatography (TLC) with fluorescence detection was proposed by Kato et al. These compounds form fluorophores on the developing plate by heating after spraying with sodium hypochlorite, hydrogen peroxide or potassium hexacyanoferrate(III)-sodium hydroxide reagent. Fluorescent spots (vivid blue) were observed by irradiation with ultraviolet light (Kato et al., 2007)

As can be seen from the brief of literature above, the methods used for the determination of serotonin may require some extra processes and methods such as solid phase extraction, derivazation and quantification by GC-MS. These methods are time consuming and expensive. Therefore, it may be useful to determine serotonin by affinity chromatography techniques.

### 1.2 Affinity Chromatography

Affinity sorption is already a well established method for identification, purification and separation of complex biomolecules. This may be achieved by a number of traditional techniques such as gel permeation chromatography, high performance liquid chromatography, chromatofocusing, electrophoresis, centrifugation, etc., in that the process relies on the differences in the physical properties (e.g., size, charge and hydrophobicity) of molecules to be treated. In contrast, affinity sorption techniques exploit the unique property of extremely

specific biological recognition (Chaiken, 1983; Gribnan, 1982; Scopes, 1982). This is due to the complementarity of surface geometry and special arrangement of the ligand and the binding site of the biomolecule. All biological processes depend on specific interactions between molecules. These interactions might occur between a protein and low molecular weight substances (e.g., between substrates or regulatory compounds and enzymes; between bioformative molecules-hormones, transmittors, etc., and receptors, and so on), but biospecific interactions occur even more often between two or several biopolymers, particularly proteins. Affinity chromatography enables the separation of almost any biomolecule on the basis of its biological function or individual chemical structure. Examples can be found from all areas of structural and physiological biochemistry, such as in multimolecular assemblies, effector-receptor interactions, DNA-protein interactions, and antigen-antibody binding.

Affinity chromatography owes its name to the exploitation of these various biological affinities for adsorption to a solid phase (Jonson, 1998; Wilcheck, 1984). One of the members of the pair in the interaction, the ligand, is immobilized on the solid phase, whereas the other, the counterligand (most often a protein), is adsorbed from the extract that is passing through the column. Examples of such affinity systems are listed in Table 1.1.

Affinity sorption requires that the compound to be isolated is capable of reversibly binding (i.e., sorption-elution) to a sorbent which consists of a complementary substance (i.e., the so-called ligand) immobilized on a suitable insoluble support, i.e., the so-called carrier.

Table 1.1 Examples of Biological Interactions Used in Affinity Chromatography.

| Ligand | Counter ligand |
|---|---|
| Antibody | Antigen, virus, cell |
| Inhibitor | Enzyme<br>(ligands are often substrate analogs or cofactor analogs) |
| Lectin | Polysaccharide, glycoprotein,<br>cell surface receptor, membrane protein, cell |
| Nucleic acid | Nucleic acid binding protein (enzyme or histone) |
| Hormone,<br>vitamin | Receptor, carrier protein |
| Sugar | Lectin, enzyme, or other sugar-binding protein |

The term affinity chromatography has been given quite different connotations by different authors. Sometimes it is very broad; including all kinds of adsorption chromatographies based on nontraditional ligands, in the extreme all chromatographies except ion exchange. Often it is meant to include immobilized metal ion affinity chromatography (IMAC), covalent chromatography, hydrophobic interaction chromatography, and so on. In other cases it refers only to ligands based on biologically functional pairs, such as enzyme-inhibitor complexes. The term not only to include functional pairs but also the so-called biomimetic ligands, particularly dyes whose binding apparently often occurs to active sites of functional enzymes although the dye molecules themselves of course do not exist in the functional context of the cell. Thus chromatography based on the formation of specific complexes such as enzyme-substrate, enzyme-inhibitor, etc., i.e on biological recognition, is termed bioaffinity or biospecific chromatography and the respective interaction-biospecific adsorption or bioaffinity (Porath, 1973). The original term “affinity chromatography” acquired a broader meaning also including hydrophobic chromatography, covalent chromatography, metal-chelate chromatography, chromatography on synthetic ligands, etc., i.e chromatography procedures based on different, less specific types of interaction. The broad scope of the various applications of affinity has generated the development of subspecialty techniques, many of which are now recognized by their own nomenclature. Table 1.2

summarizes some of these techniques. As can be seen from Table 1.2, some of these subcategories have become accepted useful techniques (Wilcheck, & Miron, 1999).

Table 1.2 Subcategories of affinity chromatography.

| | |
|---|---|
| Affinity Chromatography | 1. Hydrophobic Chromatography |
| | 2. Immunoaffinity Chromatography |
| | 3. Covalent AC |
| | 4. Metal-Chelate AC |
| | 5. Molecular Imprinting Affinity |
| | 6. Membrane-Based AC |
| | 7. Affinity Tails Chromatography |
| | 8. Lectin Affinity |
| | 9. Dye-Ligand AC |
| | 10. Reseptor Affinity |
| | 11. Weak AC |
| | 12. Perfusion AC |
| | 13. Thiophilic Chromatography |
| | 14. High Performance AC |
| | 15. Affinity Density Pertubation |
| | 16. Library-Derived Affinity |
| | 17. Affinity Partitioning |
| | 18. Affinity Electrophoresis |
| | 19. Affinity Capillary Electrophoresis |
| | 20. Centrifuged AC |
| | 21. Affinity Repulsion Chromatography |

Principle of affinity chromatography is schematically shown in Figure 1.2. A wide variety of ligands may be covalently attached to an inert support matrix, and subsequently packed into a chromatographic column.

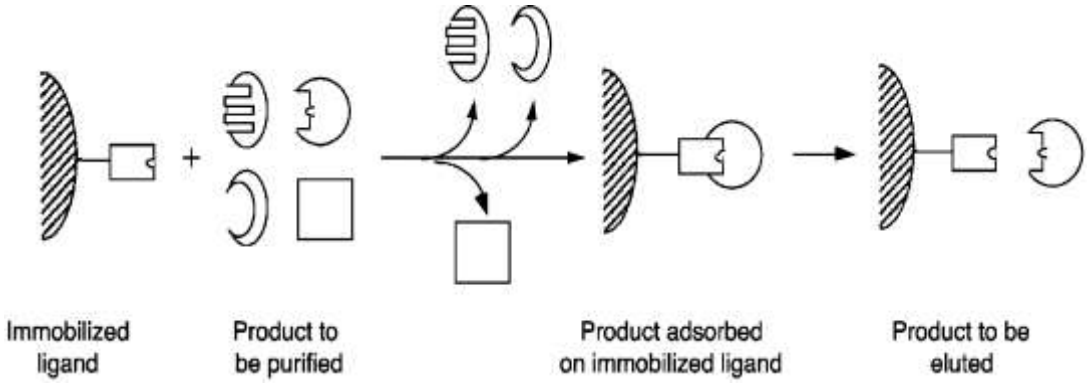


Figure 1.2 Principle of affinity chromatography

In such a system, only the protein molecules which selectively bind to the immobilized ligand will be retained on the column. Washing the column with a suitable buffer will flush out all unbound molecules. There are several techniques permit to desorb the product to be purified from the immobilized ligand. Because affinity chromatography proper relies on the functional properties, active and inactive forms can often be separated. This is however, not unique to affinity methods. Covalent chromatography can do the same thing when the activity depends on a functional thiol group in the protein. By affinity elution, ion-exchange chromatography is also able to separate according to functional properties. These are, however, exceptions to what is a rule for the affinity methods.

Very often the use of affinity chromatography requires that the investigator synthesizes the adsorbent. The methods for doing this are well worked out and are also easily adopted for those not skilled in synthetic organic chemistry. To further simplify the task, activated gel matrices ready for the reaction with a ligand are commerically available. The immobilization of a ligand can, in the best cases, be a very simple affair. In addition, immobilizations are just as easy for proteins as for small molecules.

A property that needs special consideration is the association strength between ligand and counterligand. If it is too weak there will be no adsorption, whereas if it is too strong it will be difficult to elute the protein adsorbed. It is always important to find conditions, such as pH, salt concentration, or inclusion of, for example,

detergent or other substances, that promote the dissociation of the complex without destroying the active protein at the same time. It is often here that the major difficulties with affinity methods are encountered. Ligands can be extremely selective, but they may also be only (group) grouping specific. The latter type includes glycoprotein-lectin interactions, several dye-enzyme interactions, and interactions with immobilized cofactors. However, these interactions have also proved to be extremely helpful in solving many separation problems. Good examples are ligands that are group selective against immunoglobulins (e.g., staphylococcal protein A or streptococcal protein G) (Janson, & Ryden, 1998).

### *1.2.1 Hydrophobic Interaction Chromatography*

Biomolecules are purified using chromatography techniques that separate them according to differences in their specific properties. Hydrophobic interaction chromatography (HIC) separates biomolecules, under relatively mild conditions, according to differences in their hydrophobicity. HIC is widely used in protein purification as a complement to other techniques that separate according to charge, size or biospecific recognition.

Hydrophobic interaction chromatography (HIC) is an alternative to reversed phase chromatography for exploiting the hydrophobic properties of proteins. In theory, HIC and reverse-phase chromatography (RPC) are closely related LC techniques. Both are based upon interactions between solvent-accessible non-polar groups (hydrophobic patches) on the surface of biomolecules and the hydrophobic ligands (alkyl or aryl groups) covalently attached to the gel matrix. In practice, however, they are different. Adsorbents for RPC are more highly substituted with hydrophobic ligands than HIC adsorbents. HIC is becoming popular for protein purification, often in combination with ion exchange or gel filtration chromatography, because it employs a more polar, less denaturing environment than reverse phase liquid chromatography (RPLC).

The commercial availability of well characterized HIC matrices offers new possibilities for purifying a variety of biomolecules, such as serum, membrane, recombinant, and nuclear proteins, and receptors. The technique is sufficiently sensitive to be influenced by nonpolar groups normally buried within the tertiary structure of the protein, but which are exposed if the peptide chain is incorrectly folded or damaged. This sensitivity is useful for separating the native protein from other forms. The protein adsorption selectivity of an HIC medium is primarily determined by the type of immobilized ligand (protein-binding molecule) on the support. In general, alkyl ligands exhibit only hydrophobic character, while aryl ligands exhibit mixed mode behavior – both aromatic and hydrophobic interactions are possible. Very hydrophobic proteins are generally applied to the least hydrophobic media; hydrophilic proteins are purified on the most hydrophobic media. The appropriate HIC medium can reduce salt consumption, and thus lower cost. The lowest possible salt concentration should be used to bind the protein to the ligand. This often depends on the salt chosen, for example, compared to ammonium sulfate or sodium sulfate, an up to four times higher concentration of sodium chloride may be needed to obtain the same binding. The salt concentration should be below that which will precipitate the proteins in the crude feed stock. 1 M solution of ammonium sulfate is most commonly used. A decreasing salt gradient can be used to increase protein resolution. Also, pH is an important separation parameter in the optimization of hydrophobic interaction chromatography.

### *1.2.2 Solid Matrix Support*

An essential prerequisite for affinity chromatography is the availability of appropriate chromatographic matrices withe the covalently bound specific ligand. Unlike the classic chromatographic procedures, which require only a few basic types of matrices, such as dextrans varying degrees of cross-linking or moleculer sieves or ion exchanger of different charge or ionic strenght, a matrix for affinity chromatography, with regard to the substance to be seperated or purified, is adapted to the given purpose by correspondingly selected ligand. In many cases the matrix

can be used for a specific purfication step only; that is it's tailored to the purfication of the corresponding substance.

The working steps of afinity chromatography –adsorption, washing and elution – are mostly simple to perform without major problems. The time-consuming and frequently limiting step is search for an appropriate complex partner and the preparation of bioaffine matrix. At present a variety of bioffine matrices is commercially available as so-called ready-to-use adsorbents. In many cases it is necassary and appropriate to have a homemade matrix for affinity chromatography. The synthesis of a matrix for affinity chromatography requires:

1. A matrix support suitable for chromatography
2. A substance with specific or selective affinity to the substance to be purified.
3. A chemical reaction for covalent linkage of the two partners

Table 1.3 Some specific properties of supports for the affinity chromatography

| | | |
|---|---|---|
| Hydrophilic | but | insoluble in water |
| Macroporus | but | mechanically stable |
| Chemically stable | but | easily chemical modified |
| Great surface | but | inert, not nonspecific adsorption |

*1.2.2.1 General Properties of Solid Matrix*

Up to now, a variety of matrix supports has been used with more or less success for affinity chromatography. The correct choice of matrix support and the covalent linkage between the matrix and the bioaffine ligand may be essential for the success of the chromatography. The supporting matrix plays not only a passive role as the solid-phase component, it can also have considarable effect on the stability of the complex formed, or it may even the bioaffine ligand itself. Furthermore, the manner and site of linking with the bioaffine ligand may have an essential influence on the effectivity of affinity chromatography.

The matrix should have properties generally required for a chromatograpic matrix and, furthermore, qualities that derive from specific of affinity chromatography. The general properties of matrix should be adequate particles size and shape, sufficient stability and surface. Increasing particle size reduces flow resistance and seperation power. On the other hand, matrices with very low particle size in consequence have too high a flow resistance and soon become clogged. Irregularly shaped particles lead to unequal path lenghts fort he substance to be seperated and, consequently, to band broadening. Best suited is spherical shape.

Also important are mechanical and chemical stability and resistance against microorganisms. A large surface is desirable which can be reached best by usin a porous and swellable matarial with a highest possible ratio of the iner to the outter surface. A matrix for affinity chromatography should posses several specific properties, some of which appear contradictory but associated with nature of the substances to be purified and with the kind of interactions to be utilized. The aqueous milieu is an essantial condition for structure and/or function of biologically active compounds. In some cases water molecules are a part of the native configuration of a biomacromolecule. This is why affinity chromatography is generally performed in the aqueous phase. Thus, the matrix must not only be water insoluble but also hydrophilic, swellable, or, at least, well wetable with water (the matrix itself or at least its surface). The matrix should be either macroporous or consist of a wide-wesh network to allow free diffusion of the bio macromolecules (proteins, enzymes, nucleic acids, antigens and the others) to the binding site and not prevent biospecific complex formation by a moleculer sieve effect. High porosity or wide-wesh network generally decreases the mechanical stability of the matrix material. Furthermore, the matrix should be chemically stable in a wide range (pH 2-12). On the other hand chemical modification must be possible in a simple way.

The supporting material should have the greatest possible surface and at the same time completly inert and have no unspecific interactions with the substance to be purified, such as electrostatic or hydrophobic interactions. But depending on the

nature of the matrix, this never can be completly exclueded and have been observing increasingly after covalent fixtion of the bioaffine ligand either through the incorporation of dissociable groups as result of the functionalization or hyrophobic spacers. This must not necessarily be a disadvantage. These ‘’nonspecific interactions’’ may amplify, for example, complex formation. As hydrophobic chromatography shows, such interactions may in extrem case lead to a new principle of seperation. Up to the present time there is no supporting matrix that has all the requirements in an optimal way. Thus, various supporting materials have been used more or less succesfully (Table 1.4).

Table 1.4 Types and chemical structures of various supporting materials

| **Type** | **Chemical structure** |
|---|---|
| Biopolymers (polysaccharides) | Agarose,cross-linked dextran, cellulose |
| Synthetic polymers | Polyacrylamide;poly(hydroksietilmetacrylate); polystyrene |
| Inorganic material | Porous glass , Iron Oxide (magnetogels) |
| Biopolymers/synthetic copolymers | Agarose polyacrylamide |
| Inorganic materials/synthetic copolymers | Silica/hydrophilic polymers |

*1.2.2.1.1 Biopolymers.* Hydrophilic biopolymers play a dominating role as supporting materials for affinity chromatography. Starting materials are natural polysacharides, such as agarose, dextran, cellulose, and, to a lesser extent, starch. The modification necessary for affinity chromatography can be carried out in a relatively simple way via the OH groups. In spite of many common features, there are distinct differences in properties and applications relating to the chemical structure of the biopolymers.

The conformations of the chains and the interactions between them have an influence on the use of polysaccharide as chromatographic support. The geomerty of the chains determined by the relative orientation of adjacent sugar residues around the glycosidic bonds. But in aquaeous solutions a disordered random coil form dominates, because of the flexibility of the chains. Under definiable conditions, however non-bounded energy-terms (hydrogen bridge, ionic interactions and others) can compensate for comfirmation entropy and fix the polysaccaride chains in an ordered configuration. Of great practical importance for the optimization of the polysaccarides as chromatographic support is that it's possible to influence the factors responsible for the equilibrium between an ordered and non-non ordered configuration in aquaeous solution. On the basis of their covalent structure, the polysaccarides can be divided into two classes:

1. Simple periodic structures
2. Interrupted periodic structures

In the first class the chains contain only identical sugar residues which are linked through identical positions and configurations.

The second class is charactarized by chains containing periodic sequences with ordered conformations, which are interrupted by deviations from regularity. The blance between ordered and soluble regions leads to highly hydratized gels with manyfold applications. Agarose is representative of the interrupted periodic type. Other types such as alginates and pectins interrupted ribbon configurations are of great interest in the application of immobilized whole cells but not for chromatographic supports.

Agarose is a linear water-soluble polysaccharide composed of alternating 1,3-linked β -D-galactose and 1,4-linked 3,6-anhydro-α-L-galactose units. That aquaeous solutions gel spontaneously when cooled below $50^0$C has led to the development of agarose gels in bead, pellet, or spherical form.

Dextran is a polysaccharide produced by microorganisms of genus Leuconostos. Its composed of 1,6-linked α-D-glucose units (over %90) and can be bruched out by 1,2 1,3 or 1,4 binding. Dextran cross-linking with epichlorohydrin in alkaline solution is one of the classic supports for gel permeation chromatography. Depending on chain lenght of dextran and the degree of cross-linking, gel with well-defined molecular screening effects can be produced.

Since the introduction of the chromatographic procedures, cellulose has been one of the standard supporting materials. Cellulose, a vegatable pollysaccharide, is composed of linear 1,4-β-D-glucose units. The polysaccharide chains aggregated to fibers separeted by amaorphousgions.

The first examples of affinity chromatography were made with insoluble starch without receognizing the importance of this method, such as the complex formation between α-amylase and insoluble starch or the seperation of α- and β- amylase. Today starch plays a secondary role as matrix.

*1.2.2.1.2 Inorganic Supports*. Like cellulose, inorganic materials are usually applied to many chromatographic methods (adsorption, distribution, gas chromatography, and others). Their advantages include high mechanical and chemical stability (particle and pore size) and resistance against microorganisms. At present, inorganic matrices are predominantly used in the field of immobilized enzymes, but as a result of the studies by Weetall and co-workers it has been possible to use porous glass as a matrix for affinity chromatography (Weetall, 1973; Weetall and Filbert, 1974).

Table 1.5 Some Functional Monomers for Coating

| |
|---|
| Allylamine |
| N-Allyl-1-bromoacetamide |
| 6-Acrylamidohexanoic acid |
| N-Hydroxysuccinimidyl-6-acrylaminohexanoic acid ester |
| N-Hydroxysuccinimidylacrylic acid ester |

*1.2.2.1.3 Synthetic Polymers.* Besides supports based on biomacromolecules, synthetic polymers have been explored as a potential supporting material for affinity chromatography. This development proceeded in close connection with the investigation of immobilized enzymes. To date, a variety of materials of varying composition has been described but none have attained the universal application of agarose. In most cases they are cross-linked macroporous vinyl polymers in beaded or spherical form with defined size and porosity. The properties can be obtained by proper choice of monomers and conditions of polymerization. The chemical structures of these supports are characterized by the polyethylene backbone, which influences chemical stability, and physical or structural stability, and the modifiable side chains.

The most important synthetic polymers are polyacrylamide gels and hydroxyalkylmethacrylate supports. Polyacrylamide gels are copolymerizates between acrylamide and N,N-methylene-bis-acrylamide as cross-linking agents containing a hydrocarbon framework with carboxamide side chains:

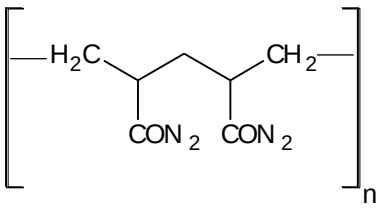


Figure 1.3 N,N-methylene-bis-acrylamide containing a hydrocarbon framework with carboxamide side chains

Polyacrylamide gels mainly are offered by Bio-Rad (Richmond, California) under the trade name Bio-Gel-P as a carrier for gel permeation, which then can be functionalized for affinity chromatography.

Another well-known matrices of this type are hydroxyalkylmethacrylate supports. In the early 1970s this type was synthesized by Coupek and coworkers (Coupek et al., 1973) and introduced to affinity chromatography. Spheron and Separon are prepared by heterogeneous suspension copolymerization of hydroxyethylmethacrylates and ethylenedimethacrylates in aqueous solution in the

presence of inert solvents. This gives a neutral, hydrophilic gel from heavily cross-linked microparticles with micropores, aggregating to macroparticles with macroporous structure. The inner structure, pore size, and distribution, specific surface, and quantity of reactive OH groups can be varied, with a molecular weight exclusion limit from 20,000 to 20,000,000. In contrast to other hydrophilic types of matrix, the macroporous structure is kept in dry state. The gel does not change in volume with changes of pH or in organic solvents and is not attacked by microorganisms as are other synthetic polymers. The gel can be used with good results in organic solvents. This is a great advantage for the modification of the matrix by polymer-analogous reactions. The mechanical and chemical stability is higher than that of matrices based on acrylic acid derivatives. Because of their high rigidity, this matrix shows excellent flow properties.

The nanomaterials level is the most advanced at present, both in scientific knowledge and in commercial applications. Nanosized particles can produce larger specific surface areas and, therefore, may result in high immobilization capacity for biomolecules. Therefore, it may be useful to synthesize nanosized particles with large surface areas and utilize them as suitable carriers for the adsorption of biomolecules.

*1.2.2.1.4 Nano-polymers.* The word nano is from the Greek word “dwarf”. Its prefix refers for units of $10^{-9}$. Nanobiosystem science and engineering is one of the most challenging and fastest growing components of nanotechnology. The confluence of biology and nanoscience will contribute to unifying concepts of science, engineering, technology, medicine, and agriculture. The nanomaterials level is the most advanced at present, both in scientific knowledge and in commercial applications.

Nanoscience is concerned with the study of unique properties of matter at its nano level exploits them to create novel structures, devices and sysytem for variety of different uses. Particles having size less than 100nm are generally called nanoparticles. This has strikingly different properties due to their small size and thus

is found useful in many applications. A decade ago, nanoparticles were studied because of their size-dependent physical and chemical properties (Murray, Kagan & Bawendi). Now they have entered a commercial exploration period (Mazzola, 2003; Paull, Wolfe, Hebert & Sinkula, 2003; salata, 2004). Nanoparticles have been investigated intensively due to their potential applications in many areas, such as biology, medicine and environment, because nanoparticles can produce larger specific surface area due to their nanoscopic size and, therefore, may result in high binding capacity and also they are highly strong and cheaper. Therefore, it may be useful to synthesize nanosized particles with large surface areas and utilize them as suitable carries for biological molecules.

In literature, there is no studies used nanoparticules as a affinity matrix for the adsorption of serotonin. Therefore, we aimed to synthesize Poly(HEMA-MAT) nanostructures, novel affinity chromatography matrix and to determine the optimum conditions for the adsorption of serotonin. Serotonin concentrations will also be analyzed by using HPLC with UV detection.

# CHAPTER TWO

# EXPERIMENTAL METHODS AND MATERIALS

## 2.1 Materials

HEMA (Sigma Chem., St. Louis, USA) and ethylene glycol dimethacrylate (EGDMA, Aldrich, Munich, Germany) were distilled under vacuum (100 mmHg). Serotonin purcased from Sigma Chem. All other chemicals were of the highest purity commercially available and were used without further purification. All water used in the experiments was purified using a All water used in the adsorption experiments was purified using a Millipore S.A.S 67120 Molsheim-France facility whose quality management system is approved by an accredited registering body to the ISO 9001. Before use the laboratory glassware was rinsed with deionised water and dried in a dust-free environment.

## 2.2 Preparation of Poly(HEMA-MAT) Nanostructures

For the synthesis of nano-HEMA-MAT the following experimental procedure was applied: N-methacryloyl-(l)-tryptophan methyl ester (MAT) was prepared using the method in the study of Akgöl et al. (2007). The stabilizer, polyvinyl alcohol (PVAL) (0.5 g), was dissolved in 50 mL deionized water for the preparation of the continuous phase. Then, the comonomer mixture was added to this dispersion phase, which was mixed in an ultrasonic bath for about half an hour. Before polymerization, initiator was added to the solution and nitrogen gas blown through the medium for about 1–2 min to remove dissolved oxygen. Polymerization was carried out in a constant temperature shaking bath at 70°C, under nitrogen atmosphere for 24 h (Table 2.1). After the polymerization, the polymeric nanospheres were cleaned by washing with methanol and water several times to remove the unreacted monomers. For this purpose, the nanospheres were precipitated at the rate of 18,000 g for 2 h in a centrifuge (Zentrifugen, Universal 32 R, Germany), the collected precipitate was resuspended in methanol and water several times. After that poly(HEMA–MAT)

nanospheres were further washed with deionized water. Poly(HEMA) nanospheres were produced by same formulation without MAT comonomer. In this study poly(HEMA–MAT) nanospheres having the size range of 100 nm were used as a solid matrix for the adsorption of Serotonin. Figure 2.1 shows that the hypothetic structure.

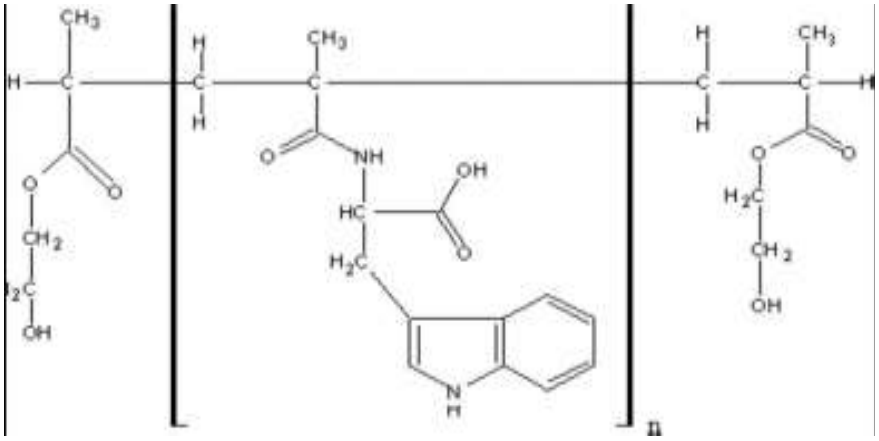

Figure 2.1 Hypothetic structure of poly(HEMA–MAT) nanospheres

Table 2.1a Component of polymerization

| **Dispertion Phase** | **Organic Phase** |
|---|---|
| 50 mL deionized water | 0,01 mL MAT |
| 0,0198 g Potassium persulfate (KPS)/45 mL water | 0,6 mL HEMA |
| 0,5 g PVAL/45 mL water | 0,3 mL EGDMA |

Table 2.1b Conditions of Polimerization

### *2.2.1 Characterization of Poly(HEMA-MAT) Nanospheres*

FTIR spectra of the nanospheres were obtained by using FTIR spectrophotometer (Perkin Elmer) The dry nanospheres (about 0,1 g) were thoroughly mixed with KBr (0,1 g, IR Grade, Merck, Germany), and pressed into a tablet form, and the spectrum

was then recorded. The particle size, the size distribution, and the surface charge were determined by Zeta Sizer (Malvern Instruments, Model 3000 HSA, England). The surface morphology of the poly(HEMA–MAT) nanospheres was examined by using SEM. The samples were initially dried in air at 25°C for 7 days before being analyzed. A fragment of the dried bead was mounted on a SEM sample mount and was sputter coated for 2 min. The sample was then mounted in a scanning electron microscope (SEM, Phillips, XL-30S FEG, Germany). The surface of the sample was then scanned at the desired magnification to study the morphology of the nanospheres. The degree of MAT incorporation in the synthesized poly(HEMA–MAT) nanospheres was determined by elemental analyser (Leco, CHN932, USA).

### 2.3 Serotonin Adsorption Studies From Aqueous Solution

Serotonin adsorption studies were performed in a batch system with 1h period at 25$^{o}$C while stirring continuously in the adsorption medium. Some variables such as time, pH, initial serotonin concentration, temperature and ionic strength were studied to optimize adsorption conditions. To determine effect of time, adsorption was completed at different times such as 15, 30, 45 and 60 min. To observe the effects of pH, pH of the solution was changed between pH 2.0-8.0 using different buffer systems (0.1 M citrate buffer was used for pH 2.0-4.0, 0.1 M acetate buffer was used for pH 4.0-5.0, 0.1 M phosphate buffer was used for pH 6.0 – 8.0). Initial serotonin concentration was changed between 1-100 ppm. To determine the effects of temperature, values were changed between 4$^{o}$C and 45$^{o}$C. To observe effect of ionic strength, concentration of NaCl was changed between 0.1-1.5 M. The amount of adsorbed serotonin per unit mass of the nanospheres was calculated by using the following expression.

$$q = \frac{C_0 - C_e}{M} V \qquad (1)$$

Where;

q: adsorbed serotonin amount (mg/g)

$C_0$: initial serotonin concentration (mg/L)

$C_e$: serotonin concentration at equilibrium (mg/L)

V: volume of serotonin solution (L)

M: amount of nanospheres used (g)

The adsorption experiments were conducted for 30 min which was the equilibrium period for the adsorption of serotonin at room temperature. Initial and final serotonin concentrations were determined by high performance liquid chromatography. This method also applied on determination of serotonin in banana and tomatoes samples. Banana and tomatoes were purchased at local commercial outlets. Cross sections (0.25 g) of the edible part were homogenized in 0.01 mol/L acetic acid, containing 10 g/L each of EDTA, ascorbic acid, and $Na_2SO_5$. After centrifugation for 40 min at 10000 x g, the supernatant was applied on HPLC under the condition of standard serotonin analyses.

### *2.3.1 High Performance Liquid Chromatography (HPLC) Analyses*

HPLC analysis of serotonin was performed using a Hewlett Packard Agilent 1100 series HPLC system (HP Corporation, Germany): isocratic pump (G1311A), degasser (G1322A), manual injector (G1328B), ultraviole diode array dedector (UV-DAD) (G1315B), 25 cm x 4.6 i.d C4 column (ACE) and system controller with a PC control program. Mobile phases were 0.1% acetic acid in double deionized water (A) and 0.1% acetic acid in acetonitrile (B). The conditions were as follow: A 90%, B 10% during the analyses. Other chromatographic conditions were as follows: flow rate, 0.5 mL/min; injection volume, 20 μL and run time, 30 min. Spectral data was collected at 280 nm and also (254–600 nm) for all samples.

### *2.3.2 Food Sample Studies*

Serotonin adsorption studies from food samples such as banana and tomatoes, were performed in a batch system with 1h period at 25$^o$C while stirring continuously in the optimized adsorption medium. Cross sections (0.25 g) of the edible part of banana and tomatoes were homogenized in 0.01 mol/L acetic acid, containing 10 g/L

each of EDTA, ascorbic acid, and $Na_2SO_5$. After centrifugation for 40 min at 10000 x g, the 600 μL of supernate was taken and divided in two ependorphs in the volume of 200 μL and 400 μl. 200 μl of sample was applied on HPLC under the same condition of standart serotonin analiyzes for measuring initial concentration. 100 μL of poly(HEMA-MAT) solution was added on the 400μL of food samples and then the mixture was shaked for 1h at $25^0$C. After shaking, samples were centrifuged for 40 min at 10000 x g, then supernatant was taken and applied to the HPLC for serotonin measurement. The adsorption capacity was calculated by the equation (1).

# CHAPTER THREE

# RESULTS AND DISCUSSION

## 3.1 Characterization of Poly (HEMA-MAT) Nanostructure

In this study, we suggest a novel method of introducing a hydrophobic group into the polymeric nanospheres via polymerization of HEMA and MAT. The distinctive feature of this method is the elimination of the activation and ligand coupling steps during the preparation of the affinity matrices. Some other advantages over other methods include the use of a known amount of ligand in the polymer preparation mixture and the good reproducibility of the affinity matrix. Figure 3.1 shows the FTIR spectra of poly (HEMA) and poly (HEMA–MAT) nanospheres. As seen in Figure 3.1, stretching vibration of hydroxyl groups of both poly(HEMA) and poly (HEMA–MAT) nanospheres was observed at 3531 $cm^{-1}$. The peak in this wavenumber of poly (HEMA–MAT) nanospheres is sharper than the peak of the poly (HEMA). It may be possible because the peak also contains N-H stretching vibrations in the poly (HEMA–MAT) nanospheres. The peak around 749.8$cm^{-1}$ indicates the aromatic characteristic of the MAT structure. These data confirmed that the poly (HEMA–MAT) nanospheres were formed with MAT functional groups.

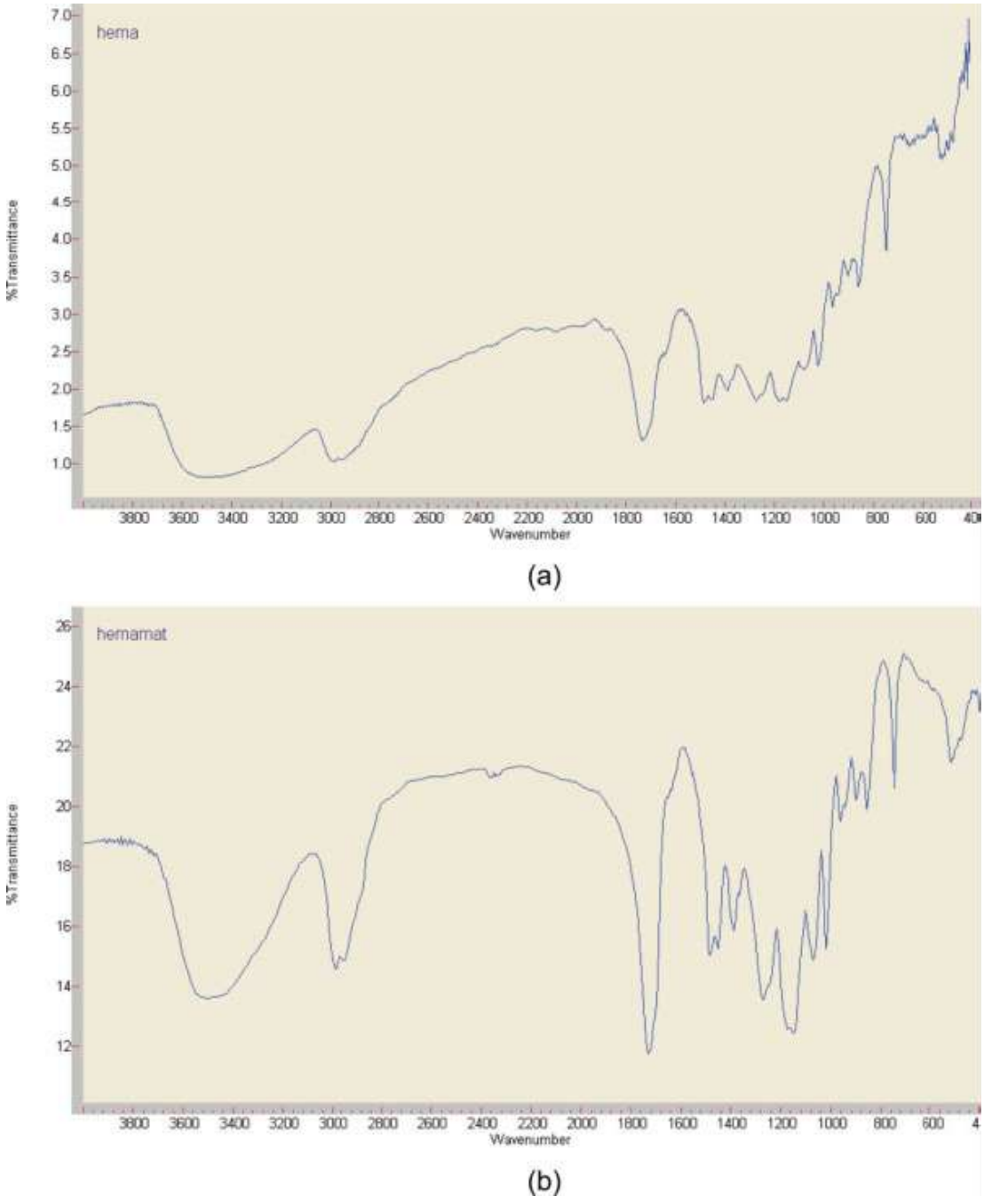


Fig. 3.1 FTIR spectrum of; (a) poly(HEMA), (b) poly (HEMA–MAT) nanospheres

As seen in Figure 3.2, particle size of the poly (HEMA–MAT) nanospheres were measured by Zeta Sizer and determined about 100 nm with 1.189 polydispersity. The particle size was an average of minimum 30 measurements, and the size distribution was recorded automatically by the software of these repeated measurements. To evaluate the degree of MAT incorporation into the polymeric structure, elemental analysis of the synthesized poly(HEMA–MAT) nanospheres was performed. The

incorporation of the MAT was found to be 1.95 mmol/g polymer using nitrogen stoichiometry. Nanospheres can produce larger specific surface area and therefore may result in high loading. The specific surface area was calculated as1856 $m^2$/g for poly(HEMA) and 1914 $m^2$/g for poly(HEMA–MAT) nanospheres.

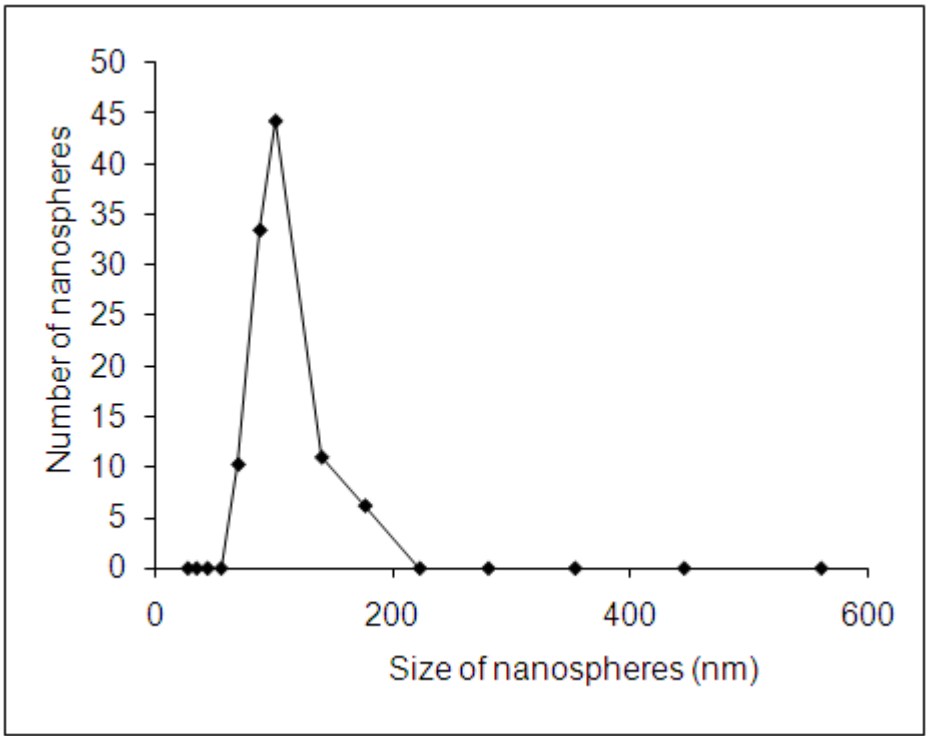


Figure 3.2 Size analysis of poly(HEMA–MAT) nanospheres.

As seen in Figure 3.3, the SEM micrographs clearly show the spherical character of poly(HEMA–MAT) nanospheres. In addition, the total monomer conversion was determined as 98.6% (w/w) for poly (HEMA–MAT) nanospheres.

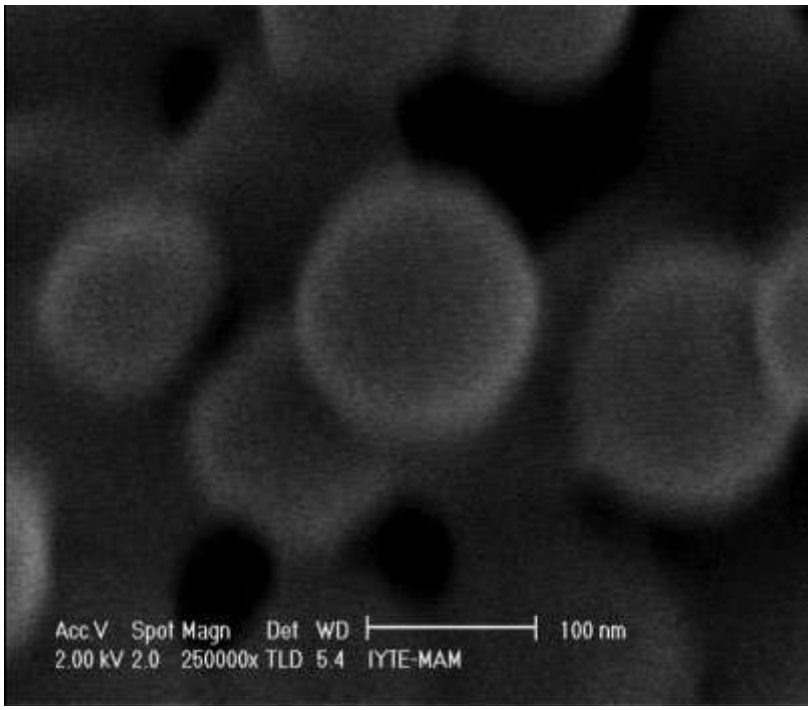


Figure 3.3 Microscopic observations; SEM micrographs of poly (HEMA–MAT) nanospheres.

### 3.2. HPLC Analysis

Many different mobile phases were tested and the one giving the best chromatography performance in terms of peak shape and retention time was selected. Mobile phases were determined as 0.1% acetic acid in double deionized water (A) and 0.1% acetic acid in acetonitrile (B). The conditions were as follow: A 90%, B 10% during the analyses. Optimal conditions for separation of the serotonin were established by evaluating commercial HPLC columns and evaluations of mobile phase. The HPLC profiles of standard and food samples (tomatoes and banana) were shown in Fig 3.4, Fig 3.5 and Fig 3.6, respectively. The retention time of the serotonin was 9.6 min at flow-rate of 0.5 mL/min. Run time per sample were 25 min.

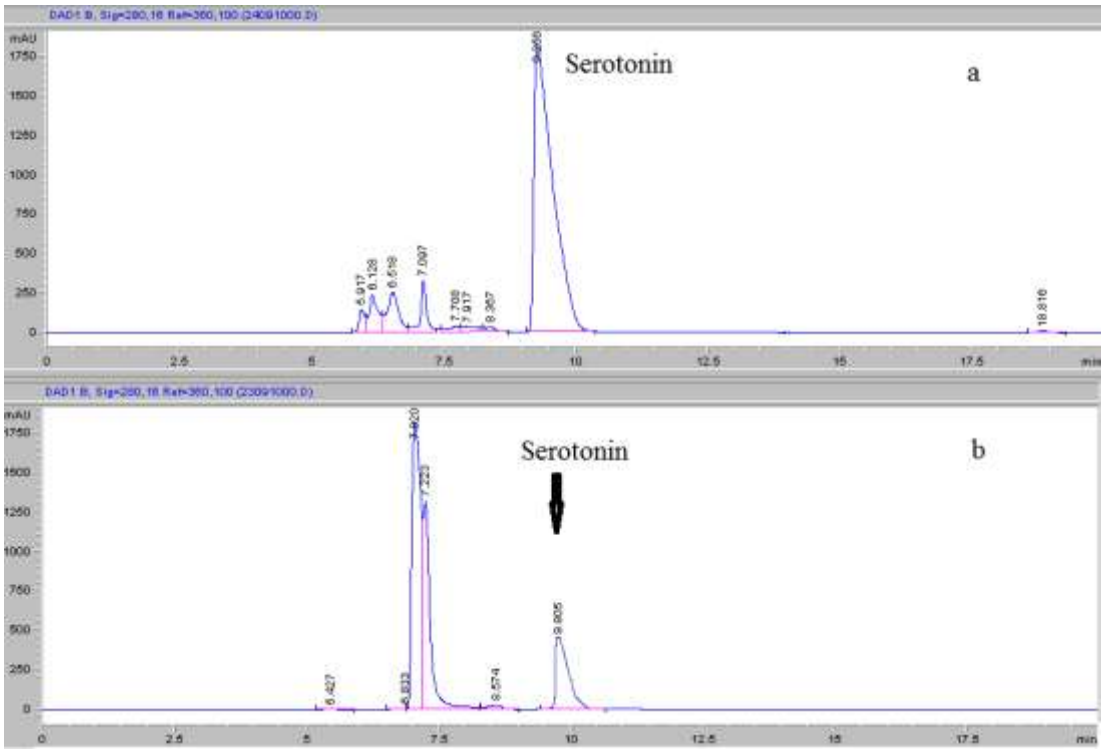


Figure 3.4 a) Chromatogram of standart solution b) Chromatogram of standart solution after adsorption (c: 10 ppm; t: 25 $^{0}$C; flow rate: 0.5 mL/min; pH: 6.0).

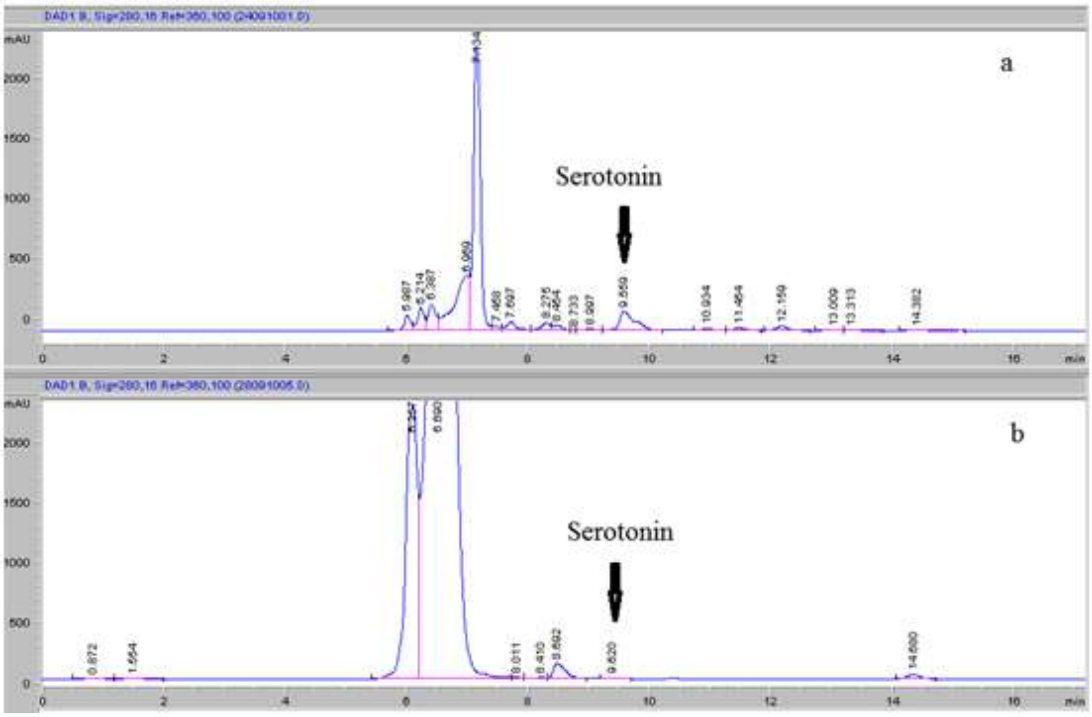


Figure 3.5 a) Chromatogram of tomatoes samples b) Chromatogram of tomatoes sample after adsorption ( t: 25 $^{0}$C; flow rate: 0.5 mL/min; pH: 6.0).

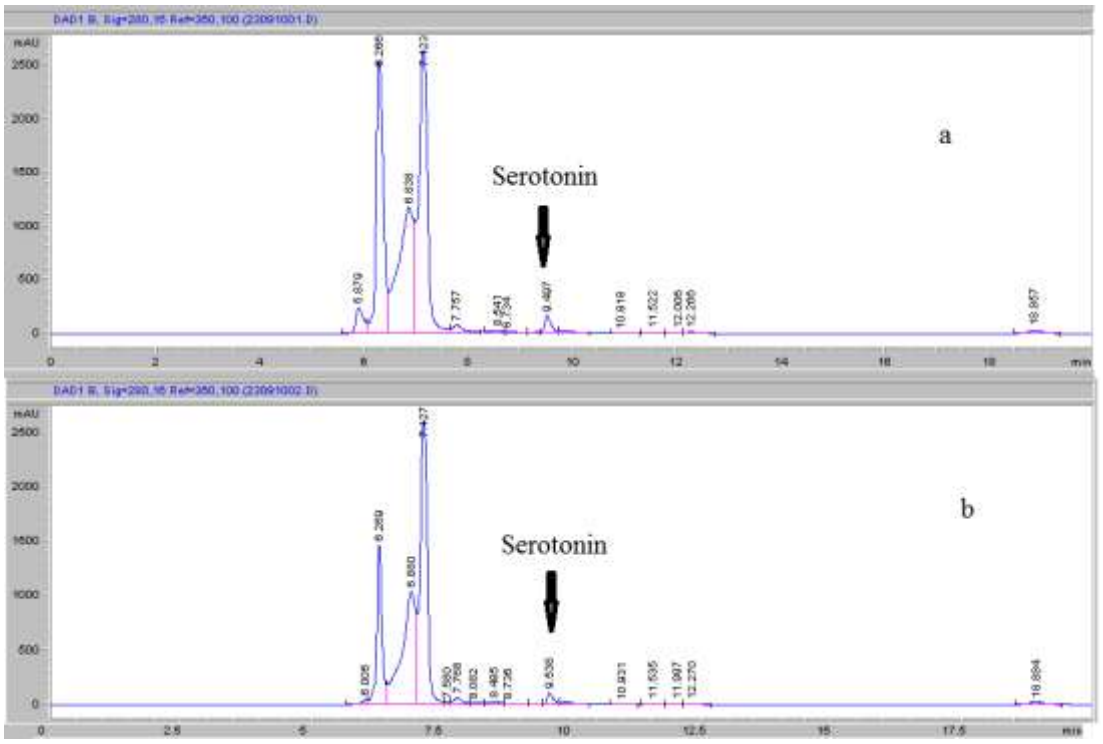


Figure 3.6 a) Chromatogram of banana samples b) Chromatogram of banana sample after adsorption (t: 25 $^{0}$C; flow rate: 0.5 mL/min; pH: 6.0).

The figure 3.4 shows typical chromatogram for a standard solution and standart solution after adsorption process. The chromatographic peaks of the samples were quantified by peak area measurement and compared with the peak area for the standard solution. To verify the identity of the serotonin peak, we spiked 5 ppm serotonin solution and found the expected area at the same retention time. And then we enject the sample after adsorption and compared the retention times of the peak found, it was the same with the standart solution. There was a significant decrease between two areas. It shows that the adsorption capacity of Poly (HEMA-MAT) nanostructures is high.

## 3.3 Optimization of Serotonin Adsorption

### *3.3.1 Effect of Contact Time*

Effect of contact time on serotonin adsorption onto poly (HEMA-MAT) nanostructrures is shown in figure 3.7. Adsorption increased with time and serotonin adsorption reached a plateau at 30 min which shows the saturation. Hence, adsorption studies were performed for 30 min.

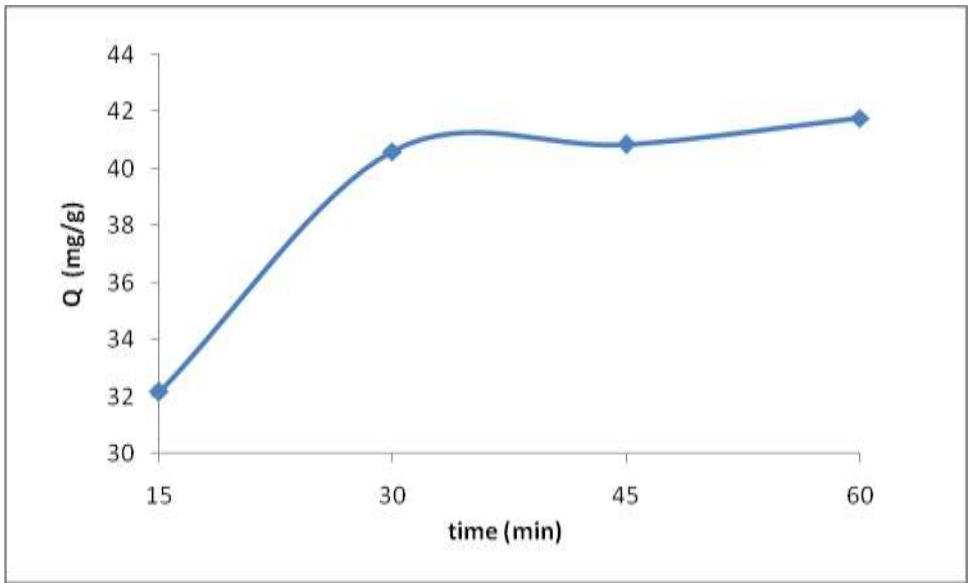


Figure 3.7 Effect of contact time on the serotonin adsorption (C: 10ppm, T: $25^0$C, pH:6 )

### *3.3.2 Effect of pH*

The effect of pH on the adsorption of serotonin onto poly (HEMA–MAT) nanospheres was studied in the pH range 2.0–8.0 and the effects of pH on adsorption are presented in Figure 3.8 The decrease in the serotonin adsorption capacities in more acidic and more alkaline regions can be attributed to electrostatic repulsion effects between the opposite charged groups. In this study, the maximum adsorption capacity was observed at pH 6. Notably the affinity between serotonin and MAT group containing nanospheres stems primarily from hydrophobic interaction. So, specific interactions at this pH may also contribute to the specific interactions. Also,

the reason of low adsorption capacity in acidic pH may be due to degradation of serotonin.

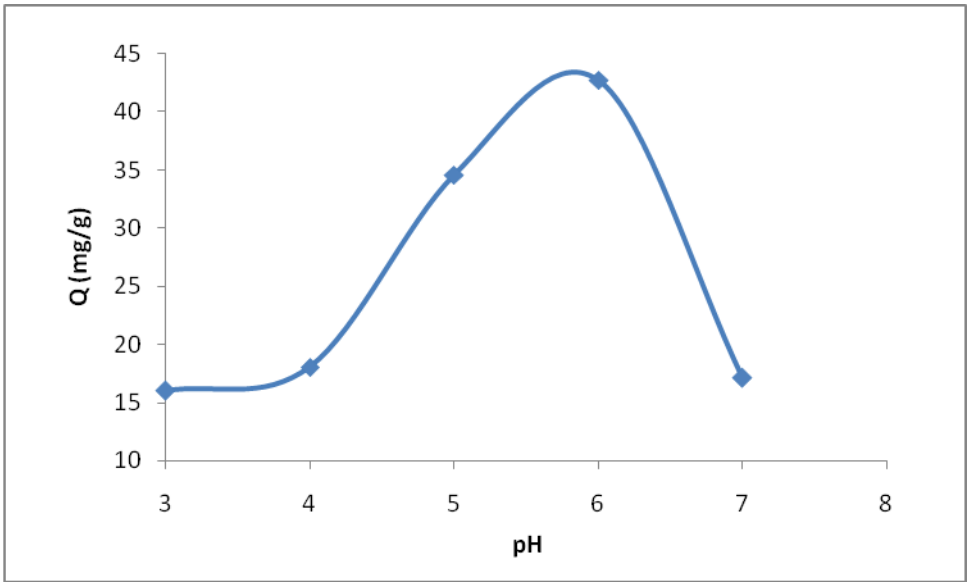


Figure 3.8 Effect of pH on adsorption of serotonin ( C: 10 ppm, T: 25 $^{0}$C )

### *3.3.3 Effect of Initial Serotonin Concentration*

Figure 3.9 shows the effect of initial serotonin concentrations on adsorption. With increasing serotonin concentration in solution, the amount of serotonin adsorbed by the nanostructures increases and then approaches saturation. This adsorption isotherm represents a high affinity between serotonin and MAT groups. It becomes constant when the serotonin concentration is about 500 ppm. A negligible amount of serotonin is adsorbed nonspecifically on the poly(HEMA) nanostructures (13.82 mg/g). Incorporation of MAT groups significantly increases serotonin adsorption capacity of the nanostructures (up to 2901.37 mg/g). This is due to a strong specific interaction between serotonin and MAT groups.

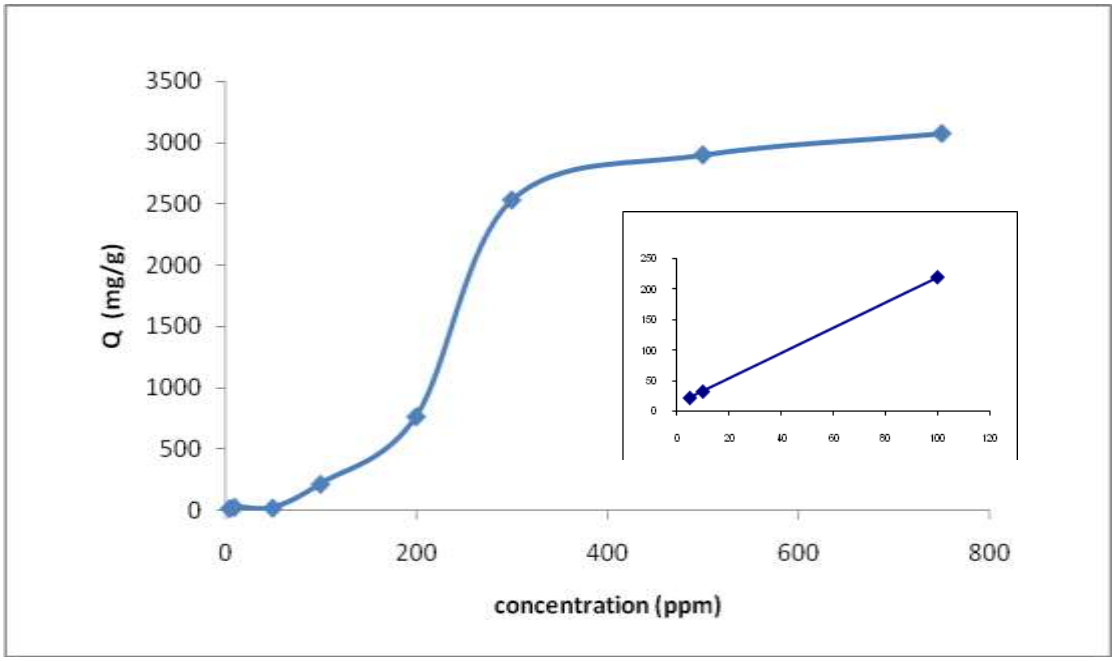


Figure 3.9 Effect of initial serotonin concentration (pH: 6 T: 25$^0$C)

### *3.3.4 Effect of Temperature*

Temperature is a crucial parameter in adsorption reactions. According to the adsorption theory, adsorption decreases with increase in temperature and molecules adsorbed earlier on a surface tend to be desorbed from the surface at elevated temperatures. The tests are usually performed at approximately 25-30ºC. Only very few reports has been given in the range 4 to 55$^o$C (Tsezos & Volesky, 1987; Kuyucak & Volesky, 1989; Aksu & Kutsal, 1991).

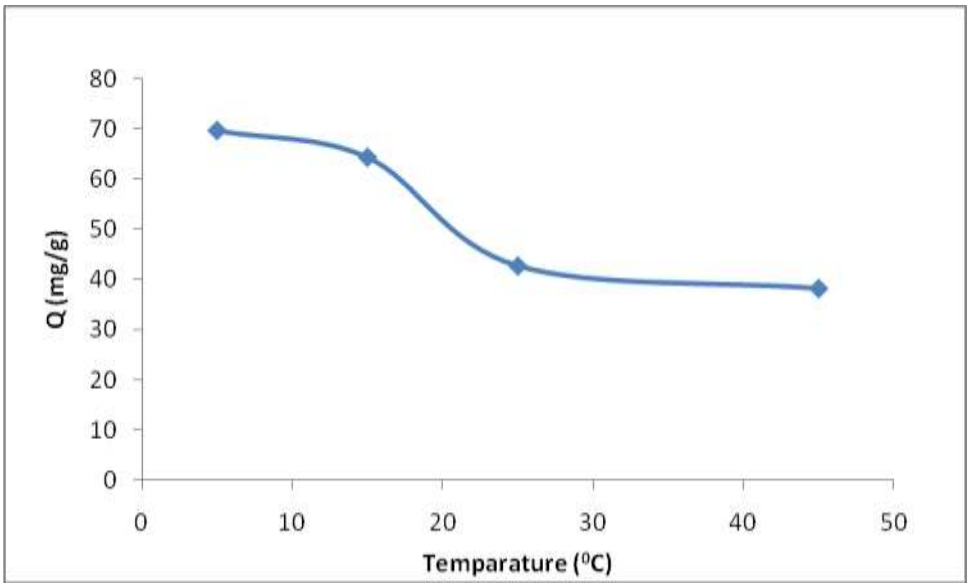


Figure 3.10 Effect of temperature ( pH:6 , C:10 ppm, t: 60 min)

Effect of temperature on serotonin adsorption was studied in the range of 4-45 $^{0}$C. As seen in Figure 3.10 from 4 $^{o}$C to 45 $^{o}$C, the adsorption capacities of the poly (HEMA-MAT) nanostructures decreased about 45.06%. Based on theories developed for the interaction of hydrophobic solutes in water, it was proposed that the binding of proteins to HIC adsorbents is entropy driven [$\Delta G = (\Delta H - T\Delta S)$], which implies that the interaction increases with an increase in temperature. However, an opposite effect was reported indicating that the role of temperature in HIC is of a complex nature. These decreases observed in the adsorption capacities were probably due to the differential effects exerted by temperature on the conformational state of different biological molecules and their solubilities in solutions.

### *3.2.5 Effect of Ionic Strength*

Effect of ionic strength on serotonin adsorption is given in Figure 3.8. As shown in Figure 3.11, the amount of serotonin adsorbed onto poly(HEMA-MAT) nano-structures decreased about 26.2% as the NaCl concentration changes from 0.1 to 1.5 M. The observed decrease of adsorption may be due to that an increase in NaCl concentration result in the reduction of electrostatic interactions (Yavuz, & Denizli, 2004).

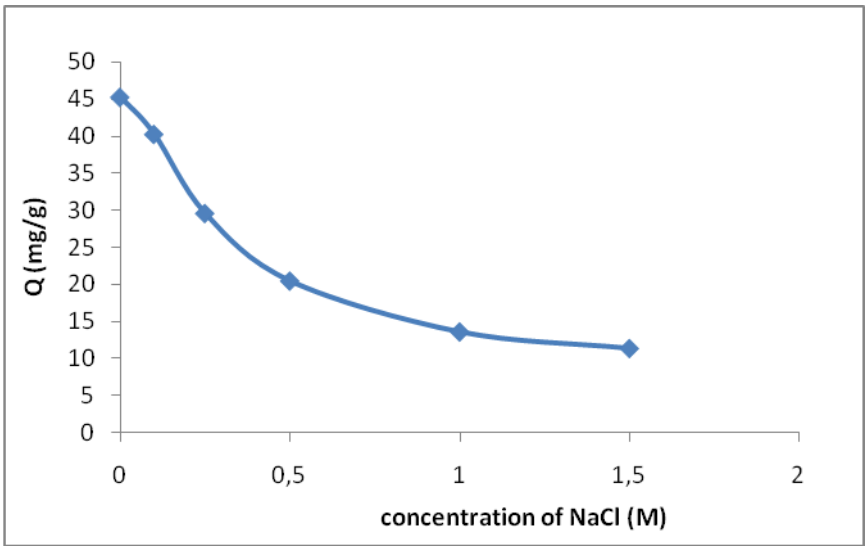


Figure 3.11 Effect of ionic strenght ( pH:6 , C:10 ppm, t:60 min)

### *3.2.6 Desorption Studies and Repeated Use*

Desorption of serotonin from the poly(HEMA-MAT) nanostructures was also carried out. Three different desorption agents such as, NaCl, NaSCN and triethylamine (TEA) were used for desorption studies. Serotonin adsorbed poly (HEMA-MAT) nanostructures were placed within the desorption medium containig 0.5-1.0-1.5 M of NaCl, NaSCN and 1%TEA solutions, respectively (25 $^{0}$C and 6h). Up to 90% of the adsorbed serotonin was removed by 1.5 M NaCl as elution agent.

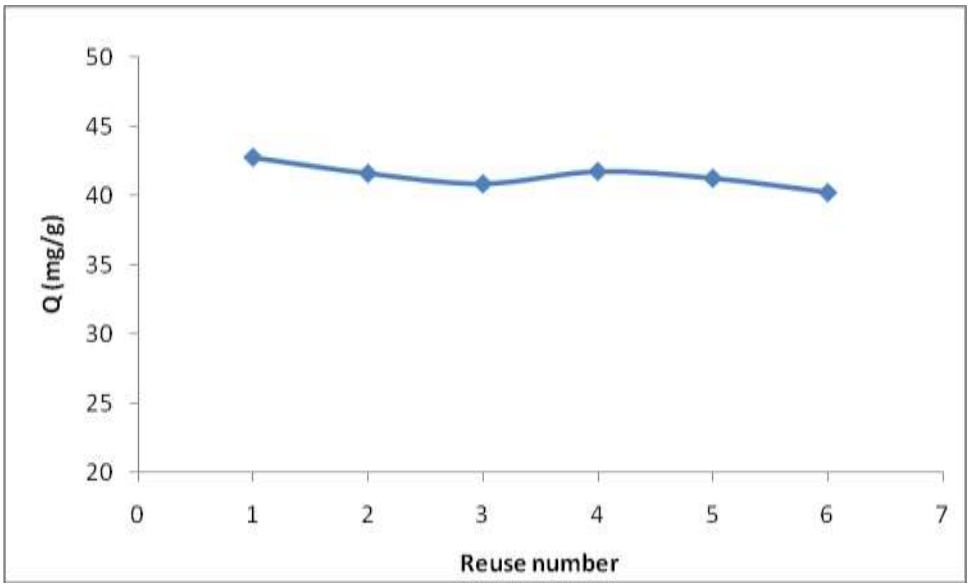


Figure 3.12 Repeated use of Poly(HEMA-MAT) nanostructures ( pH:6 , C:10 ppm, t:60 min)

In order to show the reusability of the poly (HEMA-MAT) nano-structures, the adsorption-desorption cycle was repeated six times using the same nanostructures. There was no remarkable reduce in the adsorption capacity of the nanostructures. (Figure 3.12). This result showed that the poly (HEMA-MAT) nanostructures can be repeatedly used in serotonin removal without detectable losses in its initial adsorption capacity.

### *3.3 Removal of Serotonin from Food Samples*

Serotonin adsorption studies from food samples such as banana and tomatoes were performed in a batch system with 1h period at 25$^{o}$C while stirring continuously in the optimized adsorption medium. The adsorption experiments were performed in replicates of three. For each set of data present, standard statistical methods were used to determine the mean values and standard deviations. Confidence intervals of 95% were calculated for each set of samples in order to determine the margin of error.

Adsorption capacities of the poly (HEMA-MAT) nanostructures were determined as 86.26 mg/g for banana samples and as 36.02 mg/g for tomatoe samples, respectively. With these results, it is concluded that the poly (HEMA-MAT) nanostructures can also be effectively used for the removal of serotonin from biological samples.

# CHAPTER FOUR

## CONCLUSION

The efficiency of separation of biomolecules can be improved by reduction in the size of carrier materials. Smaller particles can provide a larger surface area for the attachment of biomolecules. Therefore, it may be useful to synthesize nanosized particles with large surface area and utilize them as suitable carriers for the adsorption of biomolecules. Adsorption of biomolecules via hydrophobic interaction is based on the association of hydrophobic groups on the surface of biomolecules with hydrophobic groups on the support surface. The type of hydrophobic group incorporated into the support is an important variable that would influence the adsorption of biomolecules on the support. In this study, hydrophobic tryptophan containing nanostructures were prepared by polymerization of HEMA and MAT. The adsorption of serotonin on hydrophobic nanostructure was investigated using various reaction conditions. Very high adsorption amounts were determined due to the relatively larger surface area of the nanostructure used in this work. The reusability of the adsorbent is likely to be a key factor in proving process economics. It was also determined that desorption ratios are high (up to 90%) and nanostructures prepared in this work are suitable for repeated use without noticable loss of capacity.

Finally, it can be concluded that the hydrophobic tyrptophan containing nanostructures revealed good adsorption properties as a nano-support and will be useful in the removal of serotonin from different sources.

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