Cranfield University
Institute of BioScience and Technology

PhD Thesis
April 2002

Sreenath Subrahmanyam

Design of molecularly imprinted polymers for sensors and solid phase extraction

Supervisors: Professor Anthony P.F. Turner
Dr. Sergey A. Piletsky
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This thesis is submitted in fulfilment of the requirement for the degree of Doctor of Philosophy

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Dedicated to my parents

Loka Samastha Sukino Bhavantu
Loka Samastha Sukino Bhavantu
Loka Samastha Sukino Bhavantu
Om Shanti, Shanti, Shanti

May all the beings in all the worlds be happy
May all the beings in all the worlds be happy
May all the beings in all the worlds be happy
Om, Peace, Peace, Peace

Vedas world peace prayer

Abstract

This thesis presents broadly the applications of molecularly imprinted polymers in sensors and solid phase extraction. Sensors for creatine and creatinine have been reported using a novel method of rational design of molecularly imprinted polymers (MIPs), and solid phase extraction of aflatoxin-B1 has also been described in the thesis.

A method for the selective detection of creataine and creatinine is reported in this thesis, which is based on the reaction between polymerised hemithioacetal, formed by allyl mercaptan, o-phthalic aldehyde, and primary amine leading to the formation of fluorescent isoindole complex. This method was demonstrated for the detection of creatine using creatine-imprinted MIPs. Since MIPs created using traditional methods were unable to differentiate between creatine and creatinine, a new approach to the rational design of a MIP selective for creatinine was developed using computer simulation. A virtual library of functional monomers was assigned and screened against the target molecule, creatinine, using molecular modeling software. The monomers giving the highest binding score were further tested using simulated annealing in order to mimic the complexation of the functional monomers with template in the monomer mixture. The result of this simulation gave an optimised MIP composition. The computationally designed polymer demonstrated superior selectivity in comparison to the polymer prepared using traditional approach, a detection limit of 25 μM and good stability. The *'Bite-and- Switch'* approach combined with molecular imprinting can be used for the design of assays and sensors, selective for amino containing substances.

MIP for the selective binding properties for aflatoxin-B1 was prepared using the computational approach. The results obtained demonstrate that the MISPE offers a simple, convenient and a rapid methodology for solid phase extraction of aflatoxin-B1 even at very low concentrations of 2 ppb. The commercially available C-18 cartridges were able to recover only about 52% of aflatoxin-B1 at concentrations of 2 ppb when compared with almost complete recovery by the MIP. We have proved here that, MIPs as a solid phase extraction materials offer important and practical advantages with respect to other solid phase extraction methodologies.

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List of Notation/Abbreviations

Notation

e	electron
E	Potential (volts,V)
I	current (amps, A)
w	Weight (grams, g)
ppm	parts per million, (mg/l, ug/ml)
ppb	parts per billion, (ug/l, ng/ml)
ppt	parts per trillion, (ng/l, pg/ml)
Kd	dissociation constant
UV	ultra violet
IR	infra red
mg	milligram
ug	microgram
ng	nanogram
pg	picogram
nM	nano mole
mM	milli mole
uM	micro mole
Da	Daltons
MW	molecular weight
MHz	mega hertz
GHz	giga hertz
V	volts
nm	nano meter
AU	arbitrary units
U/mg	units per milli grams
ΔF(MIP)	response for imprinted polymer
ΔF(Blank)	response for blank polymer
I	imprinting factor

Abbreviations

ACC	cyclohexane carbonitrile
ACh	acetylcholine
AM	allyl mercaptan
AOAC	Association of official analytical chemists
BAW	bulk acoustic wave
BCD	before column derivatization
BLM	bilayer lipid membranes
BOD	biological oxygen demand
CA	chloramphenicol
CAF	caffeine
cAMP	cyclic amino phosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribo nucleic acid
DVB	divinyl benzene
EGDMA	ethylene glycol dimethacrylate
EGF	epidermal growth factor
ELISA	enzyme linked immuno sorbant assay
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid
GDP	glucose di-phosphate
GEO	geosmin
Glu	glutamic acid
GluR	glutamate ion-channel receptor
GPCR	*G- protein-coupled receptors*
GTP	glucose tri-phosphate
HCl	hydrochloric acid
HEM	hydroxyethyl methacrylate
HPLC	high performance liquid chromatography
HPLC/MS	high performance liquid chromatography/mass spectrometry
HTS	high-throughput screening
IL-6	interleukin-6,
iPoPD	electrosynthesized poly (o-phenylenediamine)

Kcal/mol	kilo calories per mole
LBM	langmuir-Blodgett Monolayer film
LC	liquid chromatography
MA	methacrylic acid;
MeOH	methyl alcohol
MIB	2-methylisoborneol
MIP	molecularly imprinted polymer
MISPE	molecularly imprinted solid phase extraction
MMA	methyl methacrylic acid
mRNA	messenger RNA
n-AChR	nicotinic acetyl choline receptor
NIP	non imprinted polymer (same as blank polymer)
NPLC	normal phase liquid chromatography
OPA	o-phthaleic dialdehyde
OPPy	overoxidized polypyrrole films
ORL1	opiate receptor -L1
PA	phenyl alanine
PAN	polyacrylonitrile
PC	personal computer
PCD	post column derivatization
PQC	piezo quartz crystal
PVC	poly vinyl chloride
QCM	quartz crystal microbalance
RNA	ribo nucleic acid
RPLC	reversed phase liquid chromatography
SH	sulfhydryl
SPE	solid phase extraction
SPR	surface plasmon resonance
TFMAA	2-(trifluoromethyl) acrylic acid
TLC	thin layer chromatography
TSM	thickness-shear-mode
Tyr-P	tyrosine phosphate
4-VP	4-Vinylpyridine

Chapter 1

Introduction

1.1 Biosensors

According to the modern definition, biosensors are analytical devices comprising a biological or biologically-derived sensing element either integrated within or intimately associated with a physicochemical transducer (Turner *et al*, 1987; Turner, 2000) (**Figure 1.1**). The two broad classes of sensing elements are *catalytic* (enzymes, microorganisms, tissue slices and biomimetic catalysts) and *affinity based* (antibodies, nucleic acids, receptor proteins and synthetic receptors) (Scheller *et al*, 1989; Griffiths and Hall, 1993). These highly selective and sensitive sensing elements yield continuous or discontinuous electronic signals reflecting the concentration of an analyte or group of analytes, when combined with electrochemical, optical, piezoelectric, magnetic or thermometric transducers.

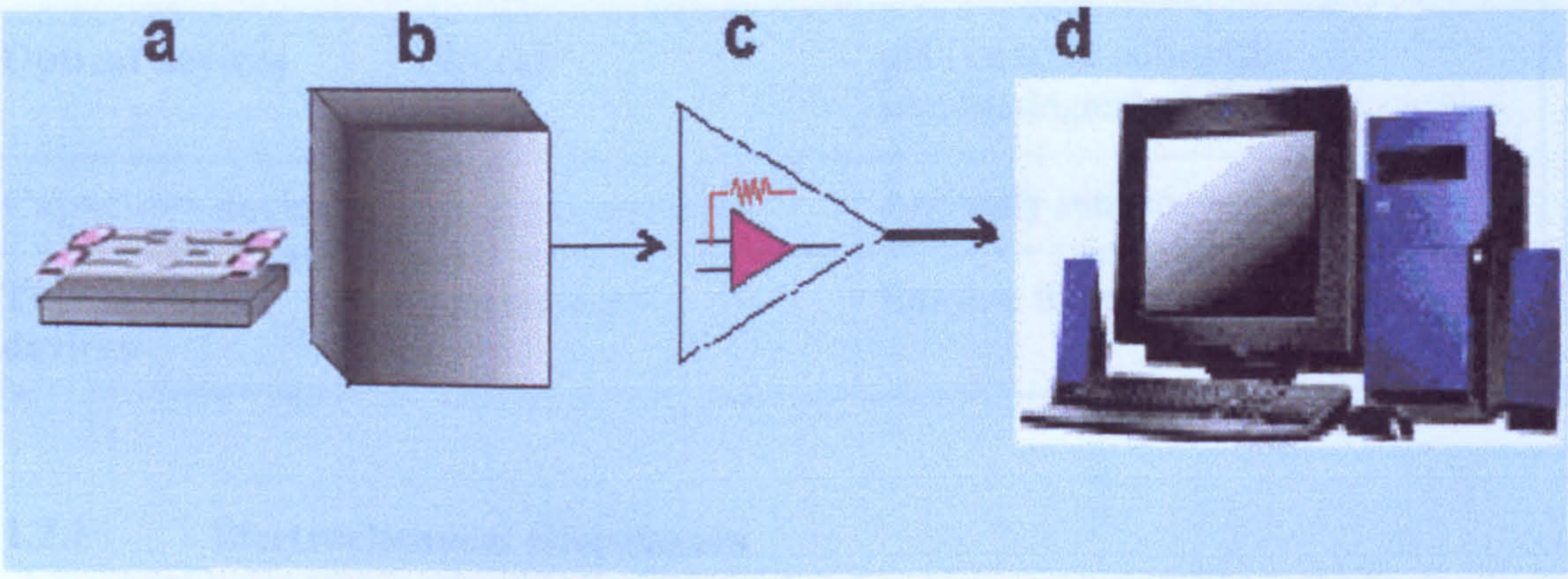


Figure 1.1 Schematic diagram showing the main components of a biosensor
(a) A bio-component, (b) a transducer which converts the biochemical reaction into a physical signal, (c) an amplifier which converts a physical signal into an electrical signal, which is processed and displayed by a recorder or PC (d).

1.2 The transducer

The transducer is an important component in a biosensor through which the measurement of the target analyte(s) is achieved by selective transformation of a biomolecule-analyte interaction into a quantifiable electrical or optical signal. A wide range of optical and electrochemical instruments have been employed in conjunction with biological sensing. Transducers can be of several types. General types of transducers used in sensor applications have been given in **Table 1.1.**

Table 1.1 General types of transducers used in sensor applications

Transducer system	Mode of measurement	Typical applications
Electrochemical devices	Conductivity	Enzyme-based reactions
Electrode	Amperometric	e^- from Enzyme substrates
	Potentiometric	H^+ from Enzyme substrates
Field effect transistors	Potentiometric	Ions, can be selective with Ion selective electrodes
Impedimetric	Impedance	Enzyme -based reactions, affinity sensors)
Piezo-electric crystals	Mass change	Vapours, immunoelectrodes
Optical devices	Optical	pH , enzyme substrates immunological analytes
Capacitive devices	Dielectric constant	Antibody sensors
Thermometric devices	Temperature	Enzyme thermistors

1.2.1 Electrochemical transducers

Electrochemical transducers detect an electrochemical signal that is generated by the interaction between the analyte and the receptor. It could be a change of a redox potential (potentiometric), the conductivity of the solution (conductometric) or the production of redox active molecules that generate a current (voltammetric, amperometric, coulometric). Potentiometric methods are based on the concentration dependence of the potential at reversible electrodes. The most widely used is the pH electrode. Other examples include ammonium (using PVC membranes with neutral carriers), ammonia and CO2 (with gas electrodes, a modification of the pH electrode) can be measured as products of the receptor-analyte complex formation.

A new generation of ion-selective devices is the ion-selective field effect transistor (ISFET), based on semiconductor devices. The advantage of the ISFET is the elimination of the electrical disturbance caused by the high resistivity of the sensor,

this was achieved by integrating the impedance transformer in the electrode body (Scheller and Schubert, 1992).

Conductometric sensors measure the change in the solution conductivity due to the increase in the concentration of ionised species. The application of the conductometry to real samples however is difficult, because of the differences in the composition of the samples such as pH and ionic strength that affect the reproducibility.

In amperometry, the current generated by the oxidation or reduction of a redox active species at the surfaces of an electrode is proportionally related to the concentration of the analyte. In order to facilitate the transfer of electrons between redox species and electrodes and to diminish the potential applied to the electrode, redox mediators have been used.

1.2.2 Optical transducers

In optical transducers, an optic fibre is a glass or plastic fibre through which light rays are guided back and forth due to total internal reflection. The end of the optic fibre is treated by immobilising the receptor on its surface. The formation of the complex receptor-analyte produces a change in a parameter that can be measured by a spectrometer placed at the opposite end of the fiber. The parameters that can be measured are refractive index, absorption, fluorescence, reflection and scattering (Goswami et al., 1990, Pharmacia Biosensor AB, 1995; Goddard et al., 1994).

1.2.3 Calorimetric transducers

Calorimetric transducers detect the enthalpy changes due to enzymatic reactions and relate them to the amount of substrate conversion. The main advantages of these devices are their wide applicability, robustness, suitability for continuous measurement. They are also not dependant of the optical properties of the sample. Unlike most of the other sensors, those based on thermal transducers can be mounted in a way that prevents fouling (Kroger and Danielson, 1997), however their use has been restricted mainly to laboratory research (Scheller and Schubert, 1992).

1.2.4 Acoustic transducers

Two types of acoustic devices have been used for the construction of biosensors: piezoelectric crystals (PZ) and surface acoustic wave devices (SAW). Piezoelectric crystals are generally, thin quartz or lithium niobate wafers that can be excited by an AC voltage, producing mechanical oscillations. The crystal has a characteristic resonance frequency that depends on its mass. Additional mass deposited on its

surface will cause a change in the resonance frequency, and this change will be proportional to the mass accumulated (Minummi et al., 1995; König and Grätzel, 1993).

SAW devices are based on the propagation of surface acoustic waves along a space in which the receptor is immobilised. The recognition between receptor and analyte can influence both the phase velocity and the propagation loss of the acoustic wave. Most of the devices described are based on the measurement of the changes in the phase velocity (D'Amico et al., 1997).

Selection of an appropriate analytical system for a given analyte depends on the specific requirements such as analytical performance, detection limits, precision, reproducibility and cost of the measurement. The sensitivity of a device depends on the affinity and/or catalytic properties of the biological component and the sensitivity of the physical transducer. An inherent advantage that can be exploited in biosensor technology is the high specificity that is achieved as a direct result of biologically optimised, molecular recognition (Byfield and Abuknesha, 1994). As a result biosensors have found applications in a wide variety of areas. A few examples of the application of biosensors have been shown in **Table 1.2.**

Table 1.2 General applications of biosensors

General Area	Example(s)
Healthcare industry	Blood glucose monitoring at home
In-vitro diagnostics	Renal failure monitoring
Environmental monitoring	Biochemical oxygen demand (BOD)
Food and drink industry	Fish freshness; sucrose content
Bioprocess monitoring	Fermentation monitoring and control; monitoring on-line production of ethanol, acetic acid.
Agriculture & related industries	Pesticide detection
Research & development	Real-time binding analysis of receptor-ligand interactions.
Military	Biological and chemical warfare (sensors for toxic gases, potent bacteria and viruses)

1.3 The biological/biomimetic component

The biological component of a biosensor can be enzymes, whole microbial cells, tissue slices, antibodies, natural and artificial receptors.

1.3.1 Enzymes

Enzymes are the most widely used biological components and a wide range of enzymes has been successfully used in biosensors. The advantages of enzymes over microbial cells are principally a combination of selectivity and sensitivity, achieved through the amplification effect provided by catalytic reaction. Enzymes allow a wide range of transduction technologies to be used and hence have found very wide applications in biosensors. Successful commercial enzyme sensors include:

a) glucose sensors by MediSense/Abbott, Bayer Diagnostics/Kyoto Daiichi/Menarini, Boehringer (Roche Diagnostics), LifeScan/Inverness Medical (Johnson & Johnson), YSI, Eppendorf, Omron-Tyobo, Novo Biomedical;

b) lactate sensors by YSI, Eppendorf and Omron-Tyobo;

c) urea, and creatinine sensors by Omron-Tyobo and Novo Biomedical (Newman *et al.*, 2001).

1.3.2 Natural receptors

Receptors are cellular, typically membrane proteins which bind specific chemicals (ligands) in a manner that results in a conformational change in the protein structure. The conformational change triggers a cellular response, for example opening an ion channel or secreting an enzyme. Receptors provide interesting opportunities for the development of biosensors principally for three reasons. Firstly, they possess high affinity and specificity refined by evolutionary process. Secondly, they are natural targets for toxins and mediators for physiological processes and due to this they can be used for monitoring these compounds in clinical and environmental analyses. Thirdly, receptors can be used for real-time elucidation of receptor-ligand interactions. The first part of ***Chapter 2, Review of literature,*** elaborates the application of natural receptors in sensors and assays.

1.3.3 Antibodies

Antibody proteins are produced by the immune system of higher animals in response to the entry of 'foreign' materials into the body, for example, viruses and bacteria. In contrast to enzymes, antibodies do not (usually) catalyse chemical transformations, but rather undergo a physical transformation. For example they bind tightly to the foreign material (the antigen) that provoked the response and mark it for attack by other elements of the immune system. Antibodies are also very specific in recognising and binding to the foreign substance. This specificity is capitalised on for the development of biosensors. It is also possible to prepare and use monoclonal antibodies against virtually any desired analyte.

1.3.4 Whole microbial cells

These are often used when the desired enzyme or receptor is unstable or difficult to purify. Use of whole microbial cells results in increased stability but decreased selectivity. This can either be an advantage or a disadvantage, for example, one can effectively detect a range of analytes in environmental monitoring, however micro-organisms frequently have slow response times and the microbial sensors need frequent recalibration. Usual practice is to preincubate the sensor with the analyte of interest before the measurement thus allowing enough time for induction of the necessary enzyme systems. Whole cell biosensors have been constructed to analyse, for example, alcohols, ammonia, antibiotics, biological oxygen demand (BOD), enzyme activities, mutagenicity, nitrates, organic acids, peptides, phosphate, sugars and vitamins.

1.3.5 Tissue slices

Sections of mammalian or plant tissue can also be used in biosensors. The wide variety of tissues that the human body uses for detection and defence of toxins could be used as physiologically-based biosensors that functionally respond to known and unknown biological, chemical, or physical stimuli. Cells that could form the basis of tissue-based biosensors could be from a variety of sources including neurons, immune cells, endothelial cells, fibroblasts, myocytes, primordial and peripheral stem cells. This means that different tissue samples could generate different signals due to co-operative action. This could result in biosensors with a wide range of selectivities.

1.4 Problems associated with biological components in biosensors

Despite advantages which biosensors have in terms of specificity and selectivity, they suffer from the serious disadvantage of low stability of biological molecules, which makes their storage and operation in harsh chemical environments problematic. The search for possible solutions to this problem associated with the use of natural molecules in sensors has lead researchers to the development of stable synthetic analogues of natural receptors and antibodies.

One of the most promising generic methods that, in theory, should be applicable for the design of affinity material for any type of analytes is ***molecular imprinting***. This thesis discusses the synthesis of molecularly imprinted polymers (MIPs) and their application in sensors and solid-phase extraction with the examples of the development of recognition materials for creatine and creatinine and adsorbent for aflatoxin-B1.

1.5 The aim of the thesis

The aim of the thesis is to study the development and application of MIPs with specific references to sensors and solid phase extraction.

1.6 Objectives of the thesis

The objectives of this thesis are as follows.

1. To develop a general procedure for the design of MIPs with improved affinity and selectivity;
2. To develop a generic approach applicable for the transformation of the MIP-template binding event into detectable optical/electrical signal.
3. To demonstrate the possibility of using these approaches for the development of MIP sensors/assays specific for analytes of clinical/environmental relevance such as creatine, creatinine and aflatoxin-B1.

1.7 Thesis structure

The thesis comprises six chapters and each of these chapters has been detailed below.

Chapter 1 (***Introduction***) gives definition to biosensors together with descriptions of transducers and biological/biomimetic components of a biosensor. It also describes the objectives of the thesis and briefly discusses other chapters of this thesis.

Chapter 2 (***Review of literature***) first reviews the applications of natural receptors in sensor technology, then presents the fundamental problems that limits the use of natural biomolecules in sensors, and presents the various advantages of artificial receptors in comparison to the natural biomolecules in their application in sensor technology. It also presents the technique of molecular imprinting and its application in the development of electrochemical, optical and quartz crystal microbalance sensors.

Chapter 3 (***Materials and methods***) presents in detail, the materials and instruments used in this work. It also presents the methodology and discusses the use of the computational method developed for the rational design of MIPs and presents the methods adopted for synthesis of MIPs and methods for their analyses.

Chapter 4 (***Results and Discussion***) is subdivided into two sections and presents the results of the work and their analysis with relevance to:

a) development of MIP sensors for creatine and creatinine;
b) development of MIPs for solid phase extraction (SPE) of aflatoxin B1.

Chapter 5 (***Conclusion and future work***) looks into the holistic aspects of the work that was undertaken and also suggests what needs to be done in the future to broaden this work and take maximum advantage from the new methods developed.

Chapter 6 (***References***) cites all the published papers, book articles and other resources that were referred in this thesis.

Chapter 2

Review of Literature

2.1 Application of Natural Receptors in Sensors and Assays

Receptors are unique molecules of biological origin, which provide important opportunities for the development of biosensors for three principal reasons. Firstly, receptors possess high affinity and specificity refined by the evolutionary process. Secondly, receptors are natural targets for toxins and mediators of physiological processes and due to this they can be used for monitoring these compounds in clinical and environmental analyses and in the development and screening of drugs. Thirdly, receptors are as important area of research in themselves and novel sensors are constantly required for real-time elucidation of receptor-ligand interactions. There are a number of reviews published on the development and application of biosensors (Guilbault and Schmid, 1991; Harwood and Pouton, 1996; Wijesuriya and Rechnitz, 1993; Turner and White, 1999; Bilitewski and Turner, 2000; Newman and Turner, 2001; Turner, 1999), however there is very little information on receptor-based sensors. This chapter (**Chapter 2**) of the thesis, ***Review of literature***, analyses and discusses broadly the application of natural receptors as bio-recognition elements in sensor technology.

2.2 Receptors and their classification.

Molecular receptors are cellular, typically membrane, proteins which bind specific chemicals (ligands) in a manner that results in a conformational change in the protein structure. The conformational change triggers a cellular response, for example opening an ion channel or secreting an enzyme. Membrane receptors are diverse due to their different structure and functions in the cell and this serves as a basis for their classification. According to modified Haga's classification (1995) four different classes can be categorised, which are ion-channel receptors, G-protein linked receptors, receptors with single transmembrane domain and enzyme linked receptors (**Table 2.1**).

Table 2.1 Classification of membrane receptors

Characteristics	Ion - channel receptors	G-protein-linked Receptors	Receptors with a single transmembrane domain	Enzyme-linked receptors
Endogenous ligands	Neurotrans-mitters	Neurotransmitters, hormones, Autoacoids, Chemotactic factors	Growth factor hormones, Cytokines	Atrial natriuritic peptide ligands, growth factors
Structure	Several proteins with a pore	1-2 proteins	1-2 proteins with catalytic domain	Individual protein linked with enzyme
Transmemb-rane segments	Four	Seven	One	Single pass transmembrane proteins
Function	Regulation of ion transport	Activation of G proteins Regulation of cellular functions and expression of proteins	Catalytic	Suppress proliferation, stimulate synthesis of extracellular matrix, stimulate bone formation, attract cells by chemotaxis,
Cellular responses	Depolarisat ion/hyperpo larisation	Depolarisation/ Hyperpolarisation	Regulation of cellular functions, Proliferation and differentiation	Regulation of cyclase, production of cyclic GMP, cell signalling and regulation of cell cycle

2.2.1 Ion-channel receptors

Ion-channel receptors are mainly oligomers composed of heterogeneous subunits, incorporating an ion-channel function into the oligomeric structure. Their primary function is rapid communication in the nervous system. The mechanism of ligand binding involves amino-terminal portions of α and other subunits, which lie outside the cell membrane, four hydrophobic domains in the carboxy-terminal part of each subunit (M1-M4), and five M2 segments lining the ion channel (**Figure 2.1.**). Ligands specific for this class of receptors include the endogenous chemicals such as neurotransmitters. Typical examples of these include γ-aminobutyric acid (GABA), glycine, serotonin, and ATP.

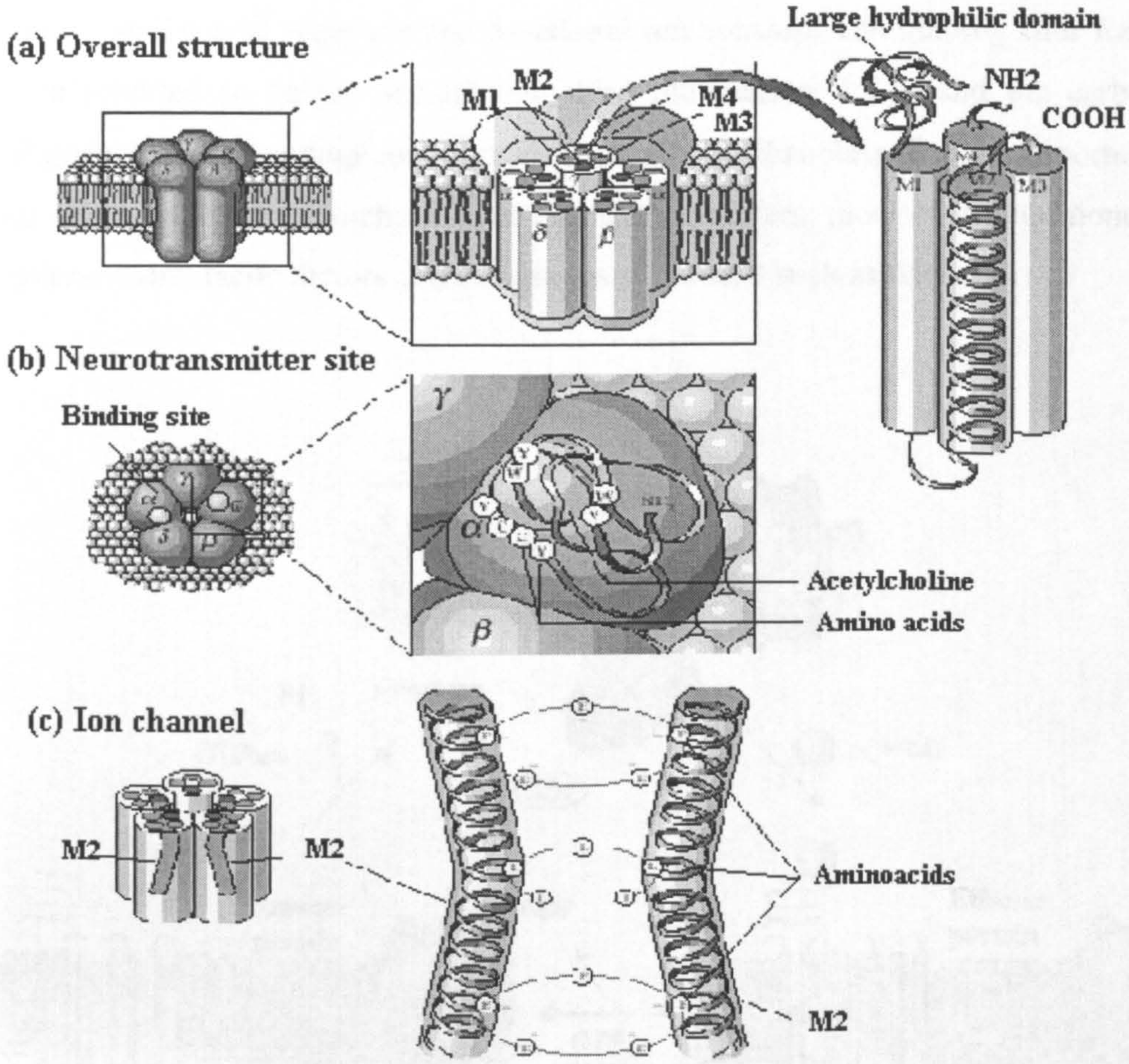


Figure 2.1 **The schematic structure of electric organ nicotinic receptor.** (a) The nicotinic receptor is made up of four glycoprotein subunits designated α, β, γ, δ. Subunits are clustered together in the membrane to form a pentameric structure (inset). Each subunit possesses an extracellular hydrophilic domain as well as four hydrophobic domains (M1-M4) that presumably form helical membrane-spanning coils. (b) The ACh binding site is believed to consist primarily of amino acids in the α subunit hydrophilic domain. (c) The receptor-associated ion channel is formed by the M2 segments from each subunit (inset). (Modified and reproduced with permission)

2.2.2 G – protein linked receptors

G-protein linked receptors mediate the cellular responses to an enormous diversity of signalling molecules, including hormones, neurotransmitters and local mediators, which are as varied in structure as they are in function. They consist of a single polypeptide chain which threads back

and forth across the lipid bilayer several times. The members of this receptor family have similar amino acid sequence and functional relationship. The binding sites for G proteins have been reported to be the second and third intracellular loops and the carboxy-terminal tail (**Figure 2.2**). The endogenous ligands belonging to this class that are important target analytes for sensor technology include all the neurotransmitters, most of the hormones and autocoids, several chemotactic factors and exogenous stimulants such as odorants.

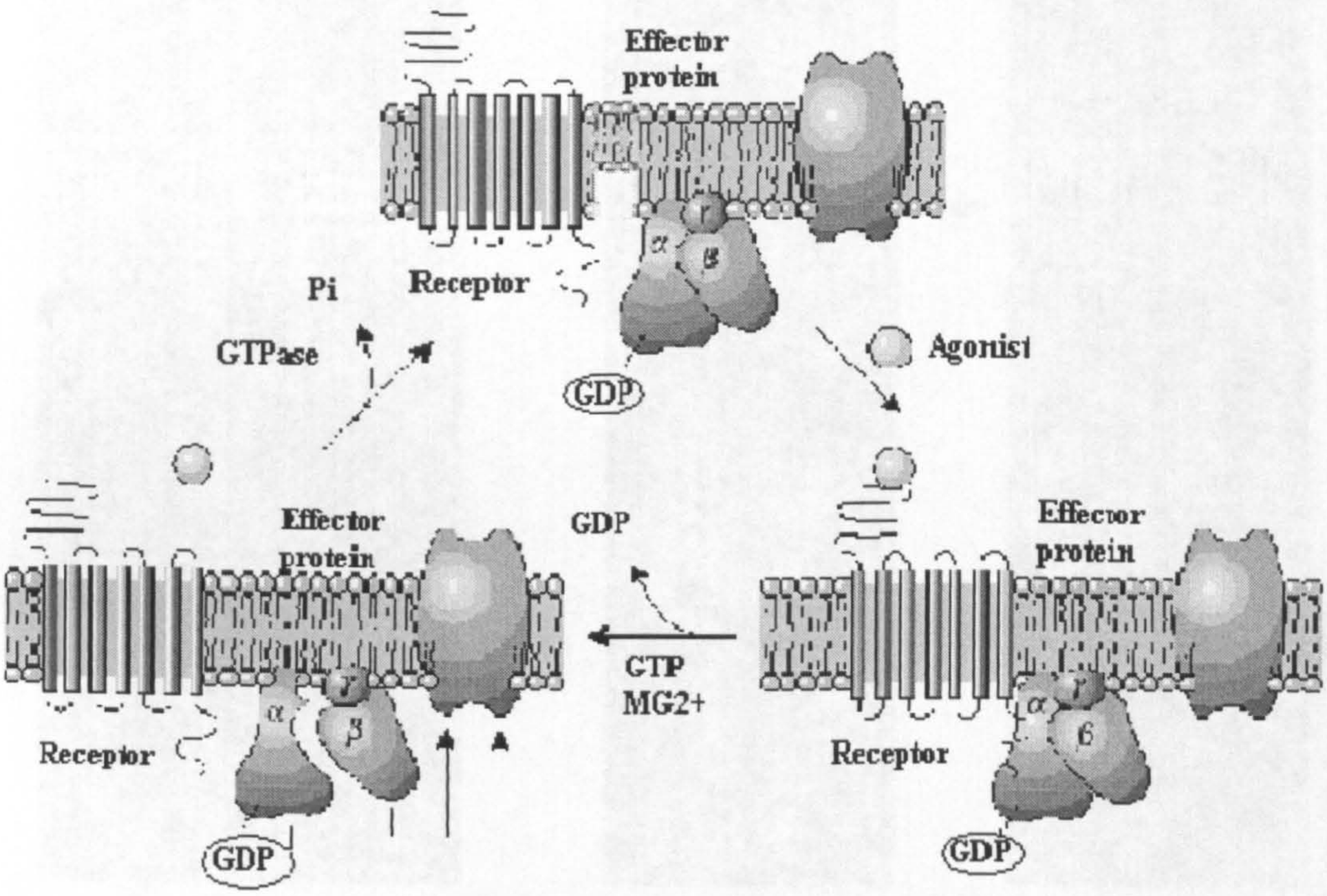
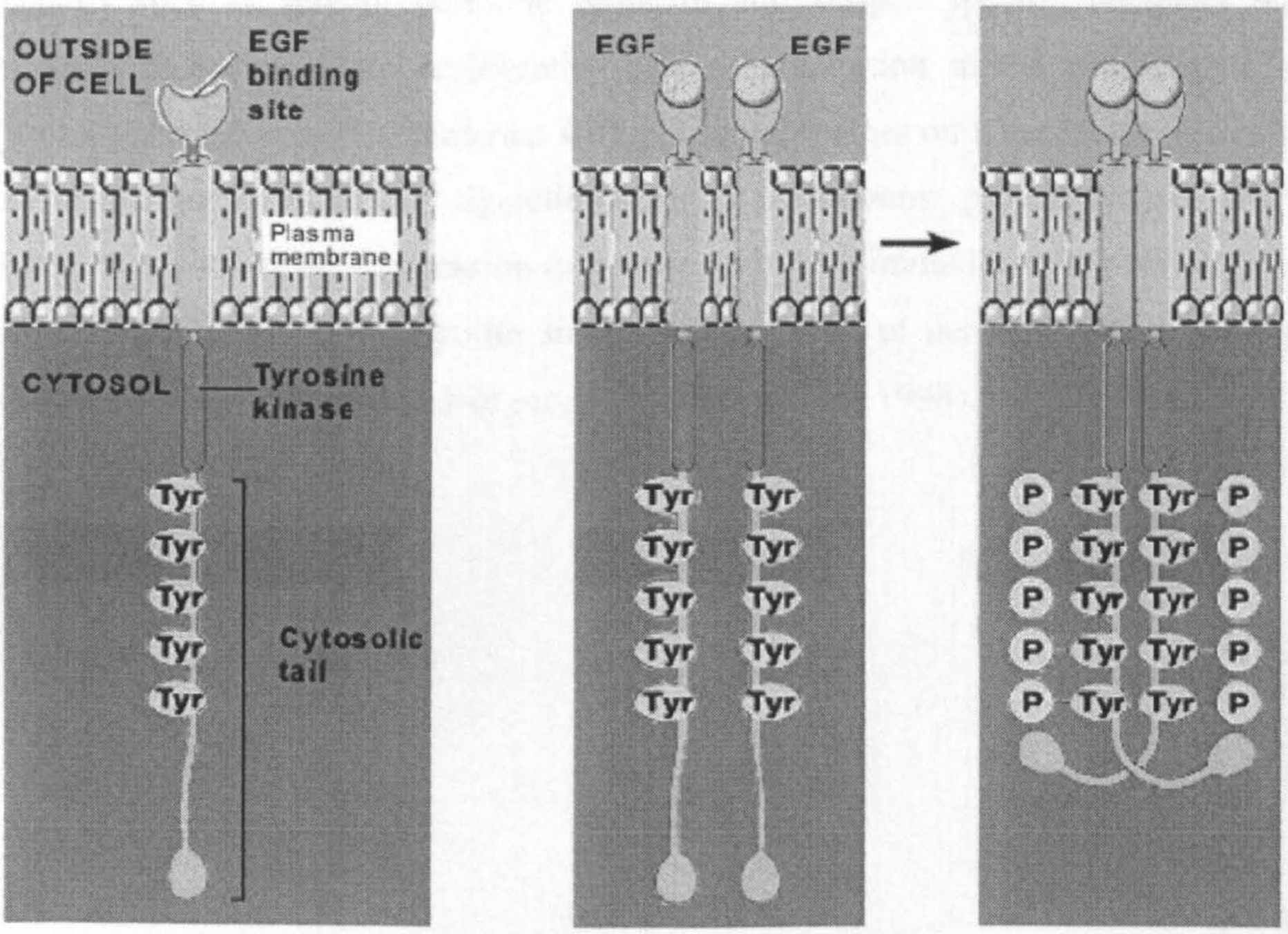


Figure 2.2 **The schematic structure of G protein receptor.** G proteins consist of α, β, and γ subunits. The α subunit possesses a binding site for guanyl nucleotides. Interaction of the G protein with an agonist-stimulated receptor (bottom right) leads to the replacement of GDP with GTP and dissociation of the α subunit from the remaining β γ dimer (bottom left). The system returns to the resting state by α subunit-mediated hydrolysis of GTP to GDP, followed by a reassociation of all three subunits. (Modified and reproduced with permission)

2.2.3 Receptors with single transmembrane segments

A group of receptors with single transmembrane segments include growth factors, such as epidermal growth factors, platelet-derived growth factors, fibroblast growth factors, and nerve growth factors. These receptors are composed of three domains, an extracellular domain responsible for ligand binding, a single-transmembrane segment and cytoplasmic domains (**Figure 2.3**). The typical ligands for this class of receptor are proteins such as phosphorylase-C, GTPase-activating protein, and phosphatidylinositol 3- kinase.



(a) Structure of epidermal growth factor (EGF) receptor

(b) Activation of epidermal growth factor receptor

Figure 2.3 (a) The schematic structure of epidermal growth factor (EGF) receptor, typical of many receptor tyrosine kinases. These receptors often have only one transmembrane segment. The extracellular portion of the receptor binds to the ligand. Inside the cell, a portion of the receptor has tyrosine kinase activity. The remainder of the receptor contains a series of tyrosine residues that are substrates for the tyrosine kinase. (b) The activation of receptor tyrosine kinases starts with the binding of a messenger (EGF, in this case), causing receptor aggregation or clustering. Once the receptors aggregate, they cross-phosphorylate each other at a number of tyrosine amino acid residues. The formation of tyrosine phosphate (Tyr-P) residues on the receptor creates binding sites for cytosolic portions that contain SH2 domains. (Modified and reproduced with permission)

2.2.4 Enzyme-linked receptors

Enzyme-linked receptors are transmembrane proteins with their ligand-binding domain on the outer surface of the plasma membrane (**Figure 2.4**). There are five sub-classes of enzyme-linked receptors, (i) receptor guanylyl cyclase with specificity for peptide hormones secreted by muscle cells in the atrium of the heart; (ii) receptor tyrosine kinase, a large transmembrane protein with a glycosylated extracellular portion that binds to epidermal growth factor; (iii) receptors such as growth hormone prolactin and antigen specific receptors on T and B-lymphocytes that regulate proliferation and differentiation in the hemopoietic system; (iv) tyrosine phosphatases which interact with phosphotyrosines on a particular type of proteins and play significant roles in cell signalling; (v) serine/threonine protein kinases which performs various functions from suppression of proliferation to stimulation of synthesis of extracellular matrix. Phosphacan, a chondroitin sulfate proteoglycan of nervous tissue, is a typical recent example of ligand for this class of receptors (Milev *et al.*, 1998).

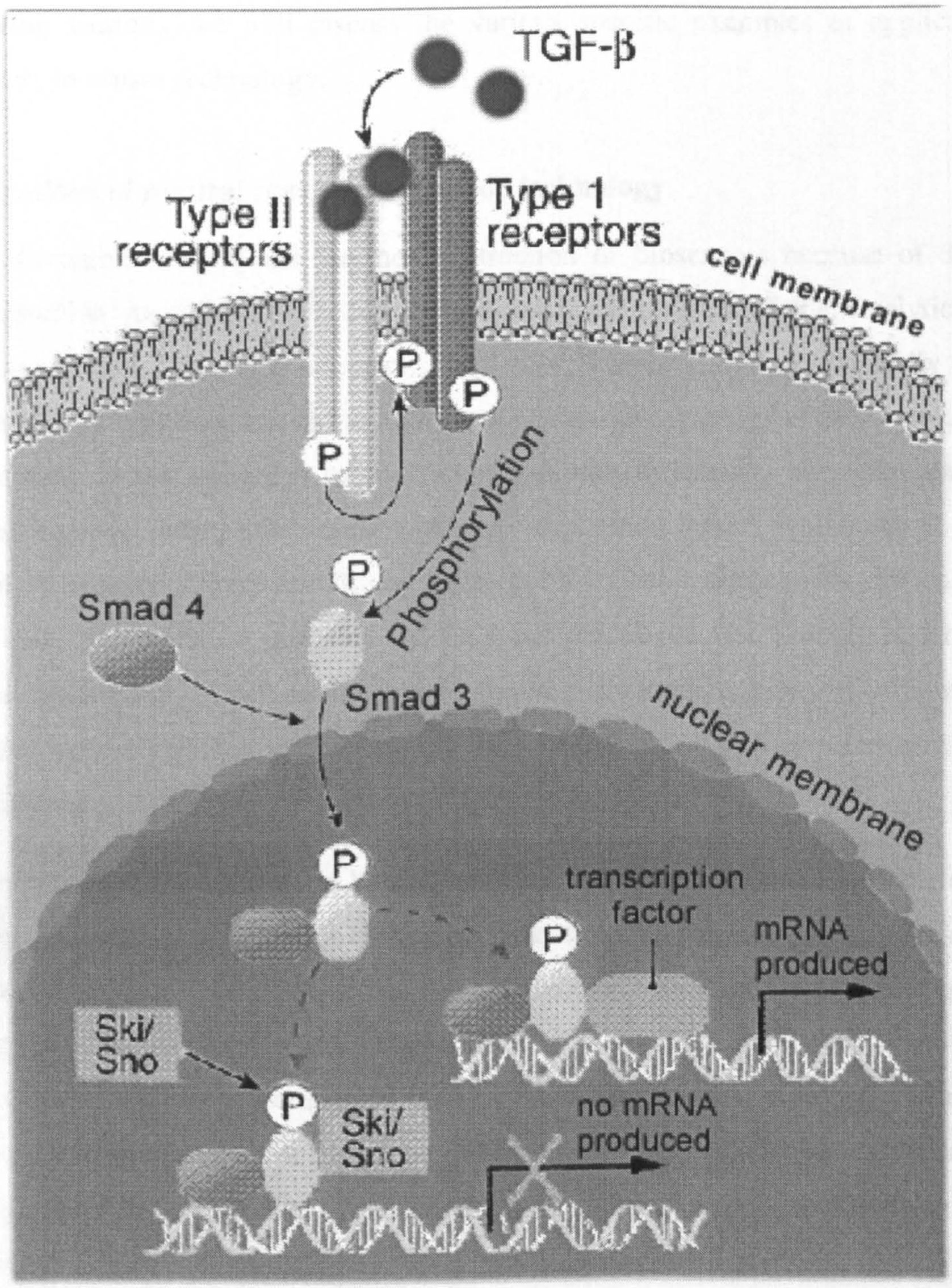


Figure 2.4 **The schematic structure of an enzyme-linked receptor-** TGF-ß signals through heteromeric complexes of types I and II transmembrane serine/threonine kinase receptors. In the absence of TGF-ß the type II receptor kinase is active and undergoes autophosphorylation on at least three serine residues that regulate receptor activity. Binding of TGF-ß1 induces hetero-oligomerisation with type I receptors and trans-phosphorylation of the type I receptor the by type II receptor kinase. (Modified and reproduced with permission)

In the following sections, we will discuss the various specific examples of applications of natural receptors in sensor technology.

2.3 Applications of natural receptors in sensor technology

Enzymes are favoured components for the construction of biosensors because of the broad range of measurable parameters that can be generated as a result of the catalytic process including protons, ions, heat, light, electrons and mass (Lowe, 1989). Additionally enzymes provide an amplification effect due to the high level of catalytic turnover of these molecules. In contrast, receptors do not (usually) catalyse chemical transformations but rather undergo a physical transformation during interaction with a corresponding ligand, which can be detected by a physical transducer. Direct monitoring of receptor-ligand interaction is difficult due to absence of signal amplification provided by the biochemical reaction present in the case of enzyme-based biosensors. Direct monitoring of binding demands equipment with very high specificity. One well established technology capable of monitoring such interactions is Surface Plasmon Resonance (*SPR).*

SPR is a phenomenon that occurs when light is totally internally reflected at a thin metal film or other suitable materials coated onto a dielectric. A fraction of the light energy incident at a sharply defined angle can interact with the delocalised electrons in the metal film (plasmon) thus reducing the reflected light intensity (**Figure 2.5**). The precise angle of incidence at which this occurs is determined by a number of factors, most important of which is the refractive index close to the back of the metal film, where the binding between immobilised receptor and ligand takes place. If binding occurs the local refractive index changes, leading to a change in SPR angle, which can be monitored in real-time. The magnitude of the change in SPR signal is directly proportional to the mass bound to the surface. Signals are easily obtained from nanogram quantities of material. Since the SPR signal depends only on binding to the immobilised receptor, it is also possible to study binding events in extracts, i.e. it is not necessary to have highly purified components.

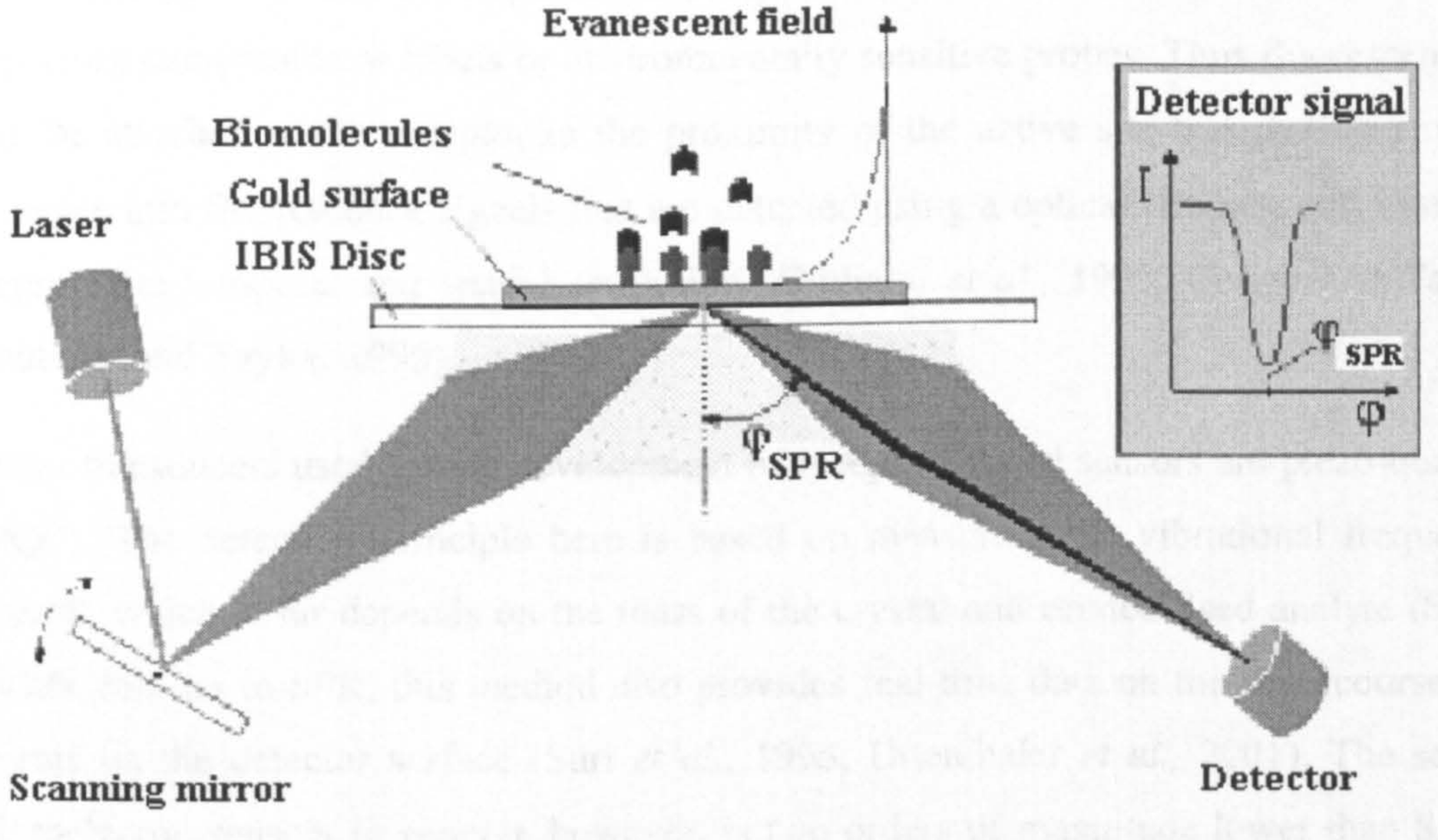


Figure 2.5 **The principle of the SPR biosensor.**

The ability of SPR to detect and quantify biospecific interactions from complex fluids, cell lysates, conditioned media and a variety of other sources makes these affinity biosensors a good method for specific ligand screening (Williams, 2000). Many applications of this technique in combination with natural receptors have been published (Davis *et al.,* 1996; Sakano *et al.,* 1996; Lackmann *et al.,* 1996; Williams, 2000). The ability of SPR biosensors to measure the quantity of complex formed between two molecules in real time without the need for radioisotopic labels makes these instruments amenable to characterising unmodified biopharmaceuticals, studying the interaction of drug candidates with macromolecular targets and identifying binding partners during ligand fishing experiments (Myszka *et al.,* 1996; Atwell *et al.,* 1997; Choulier *et al.,* 1999; Hudson, 1999; Mangold *et al.,* 1999; Thomas and Surolia, 1999; Myszka and Rich, 2000). A combination of sensor with mass spectrometry provides immediate molecular weight identification of the analytes that bind to the immobilised receptor (Nelson *et al.,* 1999, Nelson and Krone, 1999, Williams and Addona, 2000, Kronte *et al.,* 1997, Sonksen *et al.,* 1998).

Other examples of the development of receptor-based sensors include the use of fluorescent reporting compounds as labels or environmentally sensitive probes. Thus fluorescent molecules can be attached to the receptor in the proximity of the active site transducing environmental changes into fluorescence signals that are detected using a optical (fluorescent) system with an appropriate temporal and spatial resolution (Giuliano *et al.,* 1995, Gough and Taylor, 1993, Giuliano and Taylor, 1995).

Other transducers used for the development of receptor- based sensors are piezo-quartz crystals (PQC). The detection principle here is based on measuring the vibrational frequency of the crystal, which in air depends on the mass of the crystal and immobilised analyte (Shons *et al.,* 1972). Similar to SPR, this method also provides real-time data on the time course of binding events on the detector surface (Suri *et al.,* 1995, Uttenthaler *et al.,* 2001). The sensitivity of piezoelectric sensors in general, however, is two orders of magnitude lower than SPR and due to this reason this method has been used less frequently for the development of receptor- based sensors.

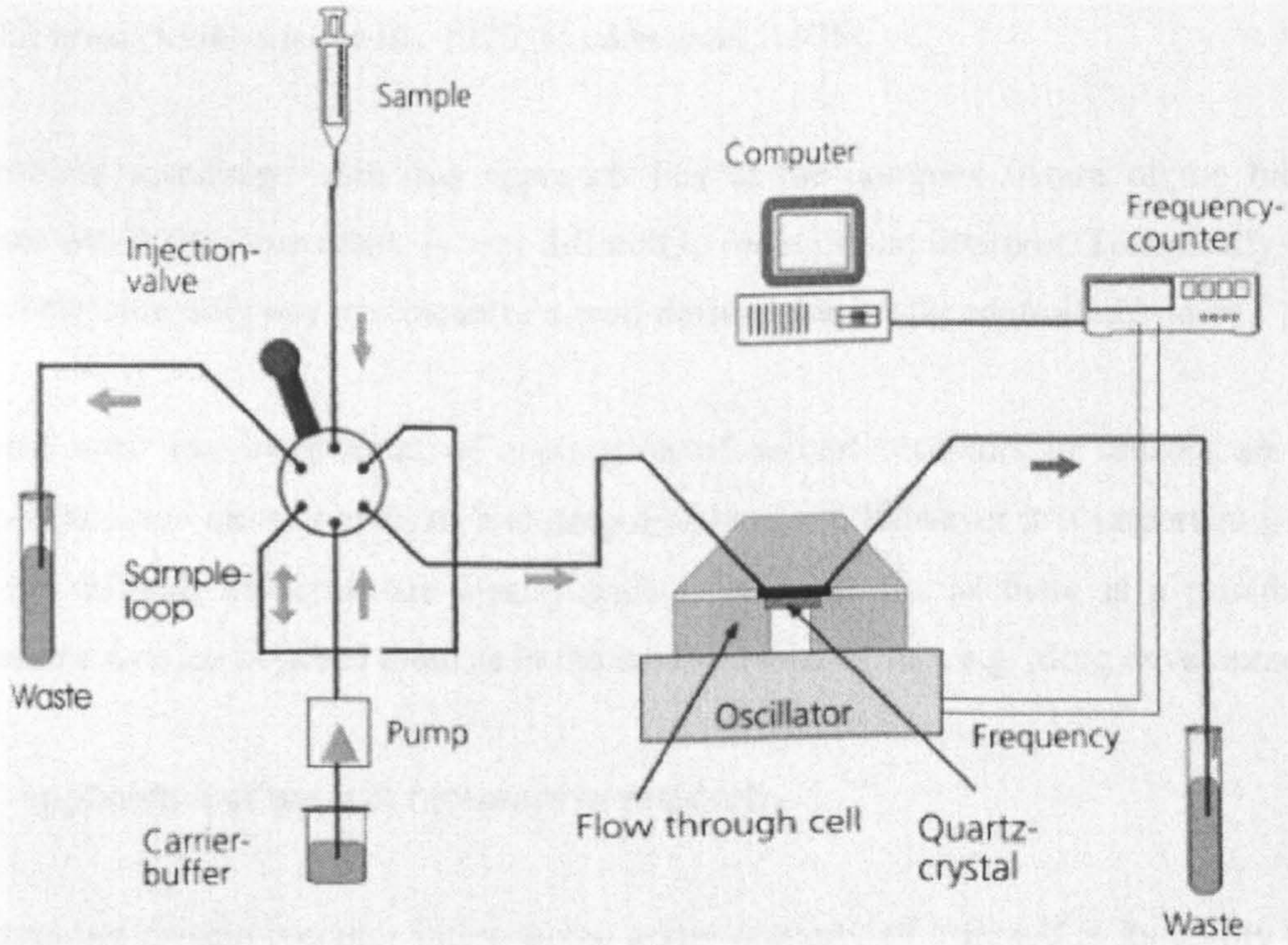


Figure 2.6 Schematic of a piezoelectric sensor, (Uttenthaler *et al.,* 1998) (Reproduced with permission)

An attractive possibility, which has not yet been broadly explored for construction of receptor-based sensors, is monitoring secondary functions of these molecules which follow binding of the ligand (Wingard, 1990). In the G protein-coupled receptors, the interaction of an activated receptor with the heteromeric G protein releases bound GDP and replaces it with GTP, with concomitant liberation of the activated GRP-associated Gα subunit. This activated subunit then interacts with a number of effector systems, including phospholipase C, adenylyl cyclase and ion channels. The cascade of biochemical reactions, which follows receptor-ligand interaction, can be monitored by measuring chemical or physical changes in the environment. In the case of ion-channel receptors their interaction with ligand opens a transmembrane ion channel and this reaction will inevitably lead to, e.g., change in membrane potential which can be recorded by a sensor (Triggle, 2000). This approach is attractive, first of all because of the possibility of providing amplification of the sensor signal similar to that present in enzyme-based sensors. Additionally, monitoring of the biochemical function might be more informative than simple binding measurement. Thus β-adrenergic receptor binds equally well both agonists and antagonists and they cannot be differentiated without analysis of their biochemical functions on a cellular level (Mukherjee *et al.*, 1975, Harden *et al.*, 1975).

The problem associated with this approach lies in the complex nature of the biochemical responses which, in some cases, is very difficult to monitor and interpret. Technically it is much easier to measure only one parameter of a well-defined and easily controlled system.

The three most important areas of application of natural receptors in sensors are research, environmental and clinical analysis and drug development. However it is important to note that it is very difficult to demarcate clearly each of these areas, as there is a possibility of a considerable overlap between them as in the case of research and, e.g., drug development.

2.3.1 Application of natural receptors in research

When probing protein-receptor interactions, a key property of a ligand is its ability to elicit a specific cellular response. As we already mentioned, direct binding assays and sensors are limited in that they do not discriminate between agonists and antagonists. In this respect cell

sensors have an advantage since they provide a way of measuring whole cellular responses by detecting, e.g. minute changes in extracellular acidification (Owicki and Parce, 1992; McConnell *et al.*, 1992; Chen and Tashjian., 1999). Hence they offer a good technique for the characterisation of receptor signalling pathways and classification of receptor subtypes (Coldwell *et al.*, 1999; Starback *et al.*, 1999). The problem, however, lies in the complex nature of cellular functions. Thus measurement of a general response will not identify which components of the cell are affected by a particular stimulus. This problem can be resolved by the use of receptor sensors, since they permit the interaction of individual receptor molecules with corresponding ligand to be analysed. The other advantage of receptor sensors is the possibility to provide methodology for real-time binding analysis of receptor-ligand interaction.

A large number of publications related to receptor sensors describe the development of sensors themselves rather than their practical application. One key reason for this is the potentially high value of practical applications specifically in drug discovery, which leads to a restriction in the flow of information from research groups in companies.

One example that has been published is a fluorescent sensor for the investigation of the binding specificity of nicotinic acetyl choline receptor (n-AChR) (Rogers *et al.*, 1989). Agonists and antagonists of the nAChR such as α-bungarotoxin, α-Naja toxin, and α-conotoxin-G1, d-tubocurarine and carbamylcholine effectively quenched the optical signal generated by fluorescein isothiocyanate (FITC)-labelled toxin bound to the nAChR-coated fibre. The response was specific since most of the tested agonists and antagonists of muscarinic, cholinergic, adrenergic, glutametergic, serotonergic, dopaminergic or GABAergic receptors produced no changes in the fluorescent signal.

In a related development, ligand binding to the n-AChR was studied by SPR (Kroger *et al.*, 1999; Sevin-Landais *et al.*, 2000). In the first study, biotinylated bungarotoxin immobilised on a streptavidin-coated gold film was bound to nicotinic acetylcholine receptor, integrated into lipid vesicles. This competition assay was used for the determination of competing ligands such as carbamoylcholine and decamethonium bromide and for the analysis of the dissociation constants of their complexes with acetylcholine receptor. In the second study, the affinity of

nAChR receptor for small ligands was determined in a competition assay using monoclonal antibodies directed against the ligand-binding site. Deckert and Legay (2000) reported the application of a SPR sensor to study the interaction between interleukin-6, a pleiotropic cytokine and specific IL-6 receptor at the surface of the T lymphocytes.

A light-addressable potentiometric sensor was used for the analysis of nAChR agonists (acetylcholine, carbamylcholine, succinylcholine, suberyldicholine, and nicotine) and competitive antagonists (d-tubocurarine, α-bungarotoxin and α Naja toxin) (Rogers *et al.*, 1991). The complex of receptor with α-bungarotoxin, labelled with biotin and urease, was captured on a biotinylated nitrocelluose membrane via a streptavidin bridge, and the subsequent change in pH due to the enzymatic reaction was detected by a silicon-based sensor. The assay explored the competition between biotinylated α-bungarotoxin and free ligands for the quantification of free ligand concentration. The sensor sensitivity was very high (2 ng/ml) and comparable with radioactive ligand binding assays.

The major potential area for the application of receptor sensors in research is the identification of ligands and physiological functions of new, previously uncharacterised receptors ("orphan" receptors). Over 70 orphan receptors in the steroid receptor family and at least 140 orphan G protein receptors have been identified from the human genome and these represent a major target for many therapeutic agents. Neither endogenous ligand nor physiological action has yet been defined for most of these receptors (Triggle, 2000).

Receptor-based assays and sensors have been used for identification of the endogenous ligands for newly discovered G protein-coupled opiate receptors (Soontjens *et al.*, 1996; Robertson and Willy, 1997; Civelli *et al.*, 1998; Wilson *et al.*, 1998 and Triggle, 2000). Davis and co-workers used SPR for screening ligands for a tyrosine kinase orphan receptor and described in detail specific approaches for the discovery of secreted and membrane-bound ligands (Davis *et al.*, 1996). A receptor sensor was also used for identification of a new opiate receptor ORL1 which interacts with a specific endogenous ligand nociceptin that has a widespread role in the mediation of nociception and stress reduction (Triggle, 2000).

Other specific examples of application of receptor sensors in research include studying receptor functions, orientation in cell membrane etc. Thus Barak *et al.*, (1997) used a β-arrestin2/green fluorescent protein conjugate to monitor activation of G protein-coupled receptors (GPCR) and identification of pharmacologically distinct GPCRs.

Surface plasmon resonance has been used to study the interaction between influenza virus hemaglutinin and its receptor – sialic acid containing glycoprotein fetuin (Tokemoto *et al.*, 1996). By using this sensor, important information about single and multivalent interactions existing between receptor and ligand was obtained. This device could be used to measure 1-10 nM of hemaglutinin. In similar work SPR detection was used for the analysis of biochemical parameters affecting the interaction of bovine rhodopsin with corresponding ligands (Bieri *et al.*, 1999).

Sevin-Landais *et al.*, (2000) have studied the orientation and structural integrity of the surface-reconstituted nicotinic acetylcholine receptor immobilised in tethered lipid membranes. The receptor was probed in a SPR study by using monoclonal antibodies and demonstrated that approximately 65% of the receptors present their ligand-binding site towards the lumen of the flow cell and that at least 85% of these receptors are structurally intact.

The structure and mode of assembly of subunits for voltage-sensitive K^+ channels have been elucidated and a variety of small disulphide cross-linked polypeptide toxins have been identified which bind with high affinity to K^+ channel proteins including two bee toxins (peptide MCD and apamin), a snake toxin (dendrodotoxin from the eastern green mamba) and two scorpion toxins, charybdotoxin and scyllatoxin (Lazdunski, 1993).

2.3.2 Application of natural receptors in clinical and environmental analysis.

A number of attempts have been made to detect toxins in environmental and clinical samples using receptor sensors. Toxins are a very heterogeneous group of chemical substances with molecular weights ranging from 200 to 150,000 Da. They are capable of affecting different biochemical processes including membrane function, ion transport, transmitter release, DNA

and protein synthesis. In many cases specific details of the site and mode of action of a toxin at the molecular level are not known.

The most characterised receptor site for toxins is the nicotinic acetyl choline receptor. It is an aggregate of five protein subunits (MW 256,000) approximating a rosette in appearance with a pore structure (ion channel), which may be open or shut and acts as a conduit for the passage of ions. The physiological activator, acetylcholine, triggers electrical activity by binding to this receptor, thus activating the pore structure and allowing Na^+ ions to flow into the cell. Toxic action results from the binding of toxins (bungarotoxin, α-naja naja toxin, etc), which prevent the opening of the associated ion channel by ACh. Both the specific binding property and the ion channel property of the n-AChR offer possibilities for the development of biosensors (Paddle, 1996).

In one attempt, nAChR extracted from *Torpedo* electric organ was adsorbed on the surface of quartz optic fibres to form the biological-sensing element. Specific binding of toxins to the receptor-coated fibre was detected by displacing FITC-labelled analytes. The real-time response of this optical sensor allowed kinetic measurements of receptor-ligand interactions (Valdes *et al.*, 1990).

Eray *et al.*, (1985) showed how a stable Langmuir-Blodgett Monolayer film (BLMs) with incorporated n-AChR can be formed over micromachined polyimide apertures on a silicon substrate. Such a device has the potential to detect a wide range of toxins. Channel activity of the receptor complex in the presence of substrate was measured.

A wide spectrum of toxins has been detected using other ion-channel proteins. Voltage sensitive Na^+ channel proteins from rat brain, mammalian skeletal muscle, chicken heart and electric eel respectively have been reconstituted in planar membranes and shown to retain their electrophysiological and pharmacological properties (Gennis, 1989; Lazdunski *et al.*, 1987). The individual toxins were identified potentiometrically by measuring the effect produced by these compounds on the transport of ions through the lipid membrane with an integrated receptor. In another approach an impedance sensor with immobilised ion channels has been developed to measure picomolar concentrations of specific peptide ligands (Cornell *et al.*,

1997). The approach mimics biological sensory functions and can be used with most types of receptors. The authors suggest that the dimensions of the impedance element can be reduced to become an integral component of a microelectronic circuit of a biochip used for the detection of drugs.

Sensors based on glutamate ion-channel receptor (GluR) were developed for the detection of L-glutamate and other agonists presented in nanomolar concentration (Collingridge and Watkins, 1994; McBain and Mayer, 1994; Uto *et al.*, 1990; Minami *et al.*, 1991a,b; Sugawara *et al.*, 1997). GluR isolated from rat synaptic plasma membranes was incorporated into planar bilayer lipid membranes (BLMs). The integrated multi-channel current, corresponding to the sum of the total amount of ions that had passed through the multiple open channels, was used as a measure of agonists' affinity and concentration.

G-protein-coupled receptors (GPCRs), specific for various drugs and toxins, are of high clinical and pharmacological interest and due to this they have been used extensively in sensor development. Compared to ion-channel receptors, G-protein-coupled receptors-based sensors could, in principle be more sensitive (although to the authors' knowledge this has not yet been realised in practice due to complexity of such a system) since the activation of one receptor can lead to cascade activation of many G-proteins and hence provide an amplification of the signal.

Recently GPCR, combined with an optical waveguide sensor was used for the detection of cholera toxin (Kelly *et al.*, 1999). The optical sensor was based on fluorescent-labelled glycolipid receptors incorporated within a fluid phospholipid bilayer membrane formed on the surface of a planar optical waveguide. The binding of the multivalent toxin initiates a fluorescence resonance energy transfer resulting in a distinctive spectral signature that is monitored by measuring emitted luminescence above the waveguide surface. The sensitivity of this device was 2-20 nM of toxin measured in biological samples.

Odour sensing is another application of this family of receptors. Olfactory receptor protein isolated from *Rana sp.*, was coated onto the surface of piezoelectric electrode which acted as a signal transducer (Wu, 1999). The sensor showed relatively fast (7 minutes), reversible and

long-term (up to 3 months) responses to volatile compounds such as n-caproic acid, isoamyl acetate, n-decyl alcohol, β-ionone, linalol, and ethyl caproate. This is actually the best example of stable receptor sensors. In the majority of other cases the operational stability and shelf life of receptor-based sensor devices was substantially lower and did not exceed 1-7 days.

2.3.3 Application of natural receptor in drug development

The application of receptor-based sensors in drug discovery and screening is, arguably, the most significant area of application, considering the huge size of the drug market and the opportunities it creates for the development of new analytical instrumentation. It is estimated that, 22% of the US$750 million on average required for the development of new drug (Myszka and Rich, 2000) is spent on screening assays and toxicity testing (Michelson and Joho, 2000). When applied to target validation and lead optimisation and screening, receptor sensors could become essential tools in strategies for overcoming bottlenecks in the drug development process.

The most popular targets for drug development are G-protein-coupled receptors associated with neuronal and endocrine pathways due to their widespread occurrence and practical significance. Thus there is currently a massive amount, over 700 sequences of G-protein coupled receptors, subdivided into the rhodopsin-like family, the metabotropic glutamate family, and the calcitonin receptors. The receptor angiotension and ß-adrenergic receptors mediate hypertension, andrenergic and serotonin receptors mediate depression, cannabinoid and dopamine receptors mediate intoxication, histamine h1 and histamine h2 receptors mediate allergy and ulcer respectively, and prostaglandin receptor mediates inflammation. Because of their integral role in cellular signaling, GPCR dysfunction can lead to different illnesses such as inflammation and asthma. Reversal of these aberrant effects can often contain, if not cure, many forms of disease. The GPCRs are therefore the primary target for a great number of drugs, which modulate effects varying from platelet aggregation and thrombosis to control pain and appetite (Dowell, 2001). Recent estimates suggest that up to 60% of the modern pharmacoepia is targeted on GPCRs (Guderman *et al.*, 1995).

There is as yet no high-resolution structure of G-protein coupled receptors, which limits possibilities to use computer modelling for drug design (Bikker *et al.*, 1988). Due to both this and the large number of existing receptors, the development and use of binding assays and sensors for screening of lead compounds is critically important.

Additionally, the most important targets in drug development are ion-channel receptors. Recent nAChR-targeted drug discovery programs have focused most prominently on (1) cognitive enhancing agents; (2) compounds stimulating nAChR-mediated dopamine release for Parkinson's disease; and (3) analgesic agents (Holladay *et al.*,1997). These therapeutic targets have in common the existence of established behavioural models, which have doubtless played an early central role in the characterisation of compounds as potential therapeutic agents. Behavioural evaluation of analogues has the advantage of identifying agents at a more advanced level of preclinical characterisation and also provides preliminary feedback on pharmacokinetic properties. However, behavioural assays are compound intensive, suffer from modest throughput and present difficulties in interpretation of results because there may be several simultaneously variable parameters intrinsic activity, pharmacokinetics, metabolism, and in some cases, behavioural effects of animal handling, changes in testing environment, etc.). An alternative strategy is to rely more heavily on binding assays and sensors, whereby possible therapeutic function can be hypothesised based on the evaluation of the strength and specificity of the receptor-ligand interactions (McDonald *et al.*, 1996).

In addition to being more informative than behavioural assays in the sense that they are capable of providing information on interactions at a molecular level, binding assays are more economical. A rigorous pre-selection of lead compounds by *in vitro* binding assay prior to using the drug candidates reduces the cost and time of the ensuing time-consuming and expensive stage, i.e., animal testing (Closse *et al.*, 1984). It is important to note that since the functional activity and the binding affinity differ in many receptor systems, screening using a binding assay cannot substitute for biological testing and should be followed by functional assays.

The screening of hundreds of thousands of chemical entities against a biological target in a short time frame requires the development of high-throughput screening (HTS) systems.

Several parameters need to be optimised in order to make HTS system effective. In order to minimise the cost of screening, the volume of sample to be screened should also be reduced. This in turn requires the miniaturisation of HTS technology as a whole. Miniaturisation requires new technologies and strategies for compound handling, assay/sensor development, assay/sensor adaptation, liquid handling and automation, in addition to refinement of the technologies used for detection systems and data management. Several groups are working on the development of different microelectronic multisensors or biochips for a multiparametric high throughput screening of potential drug candidates (Ehret *et al.*, 2001; Kotaki *et al.*, 2000; Cox, *et al.*, 2000). As array technology has developed, a variety of formats have become available such as patterned arrays, three-dimensional pads, flat-surface spot arrays or microfluidic chips (Walter *et al.*, 2000). A good example of practical work has been the development of protein chips for high throughput analysis of biochemical activities (Zhu *et al.*, 2000).

2.4 Problems associated with the use of natural receptors in sensor technology and the future perspective

The concept of putting together the selective catalytic or binding properties of receptors together with specific transducer devices for signal generation to form small, portable and highly sensitive detector systems for a wide variety of applications has proved to be very difficult to achieve in practice (Hall, 1992). The major problems limiting the development and the use of receptor sensors are the high price of biological receptors, their low stability and difficulty with the transformation of the binding event into a processable signal, as well as difficulties in interpretation of the connection that exists between signal formation and the biochemical function of the receptor.

Stability, in particular is an important factor, which limits commercialisation of biosensors based on receptors. Receptors often comprise several subunits, which are subject to denaturation. The preservation of receptor integrity is a major challenge that needs to be researched in future for its application in sensor technology (Sevin-Landais *et al.*, 2000). Design of receptor sensors relies heavily on highly sensitive equipment suitable for direct monitoring of receptor-ligand interactions, such as SPR. The equipment produced by companies such as

BIAcore is very sensitive and reliable but it is too expensive for use as a mass produced receptor sensor. An additional problem related specifically to the application of receptor sensors in drug development is need to reduce the cost of testing which, in many cases, is linked with a need for sensor miniaturisation. Diagnostic and pharmacological screening will increasingly be based on microfabricated devices. The demand for high throughput screening will catalyse the development of miniaturised sensor formats with a capacity to handle many diverse samples (Vetter, 1998).

In conclusion, the largest market for receptor-based sensors would be in drug development. The reason for this lies in the fact that for a prolonged period of time our knowledge about the structure and function of natural receptors will remain relatively limited. Due to this, the practical testing of chemicals for their binding to a receptor will be a necessary step in drug design and hence natural-receptor based sensors will be needed for drug development, however the use of natural receptors for clinical and environmental analysis will be limited by two major problems; high price and low stability of these molecules and difficulty with the transformation of the binding event into a processable signal, as well as difficulties in interpretation of the connection that exists between signal formation and the biochemical function of the receptor. Hence research efforts have been greatly focused on the development of synthetic receptors and their application in sensor technology, which will be the subject of discussion in the next part of this chapter on Review of literature.

2.5 Application of artificial receptors in sensor technology

Sensor technology in general is a mature, fast-developing area, as evidenced by the large number of patents issued every year (**Figure 2.7**). Biosensor patents, however, constitute a rather small but increasing fraction of the total number of patents related to sensor technology.

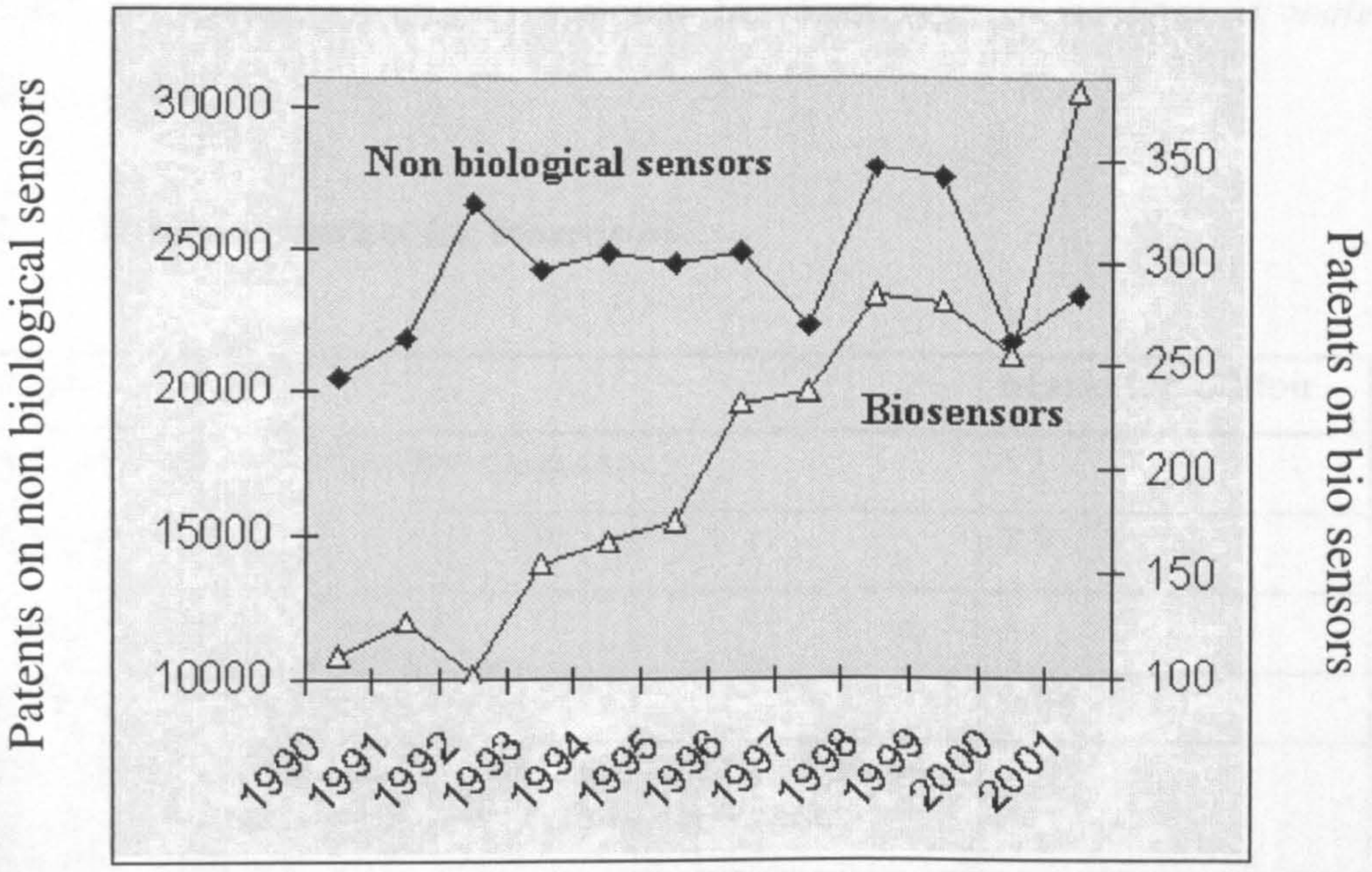


Figure 2.7 The number of patents issued worldwide in the sensor area during last decade.

The majority of biosensor-related patents describe innovations in materials science rather than new types of transducers or detection principles. Particular attention has been paid to solving the most critical problems related to the application of biological molecules in sensing like:

(a) low stability;

(b) high price of enzymes and receptors;

(c) poor performance of biomolecules in organic solvents and at low and high pHs, and at high temperature;

(d) absence of enzymes or receptors that are able to recognise certain target analytes;

(e) problems with immobilisation of biomolecules;

(f) poor compatibility with micromachine technology, miniaturisation;

(g) difficulties with design of multisensors.

The underdevelopment of the potentially huge biosensors market **(Table 2.2)** can be attributed to the above-mentioned problems. At present less than 10% of this market has been exploited with 2/3 of sales accounted for by glucose and BOD sensors (Anonymous, 1998; Marshall, 1998). The search for possible solutions to the problem of the low stability of biological molecules has lead scientists to the development of stable synthetic analogues of natural receptors and antibodies. One of the most promising methods that, in theory, should be applicable for design of affinity material for most type of analytes is ***molecular imprinting.***

Table 2.2. Potential market for biosensors.

Application	**Market, $ billion**
Microsystems market for in-vitro diagnostics	19
Drug testing	1.9
Drug delivery	1
Military (warfare agent detection)	0.64
Electronic noses and tongues (health care, testing of product quality, to establish authenticity of perfumes and wines)	4
DNA chips	1

Sources: The Economist (1988) and Marshall (1999).

2.6 Molecular imprinting

2.6.1 Introduction

The molecular imprinting approach involves formation of a complex between a given target (template) molecule and functional monomers in appropriate solvent, which is fixed by polymerisation into a growing polymer chain (**Figure 2.8**). Subsequent removal of the template leaves binding sites within the polymer possessing both shape and the correct orientation of functional groups, which are capable of selectively recognising the imprint species (Wulff, 1995; Mayes and Mosbach, 1997).

Collective weak intermolecular forces that act over complementary surfaces of the polymer and the template together with shape specificity contribute to the molecular recognition in a manner similar to natural receptors and enzymes. MIPs have a number of advantages in comparison with natural biomolecules **(Table 2.3).**

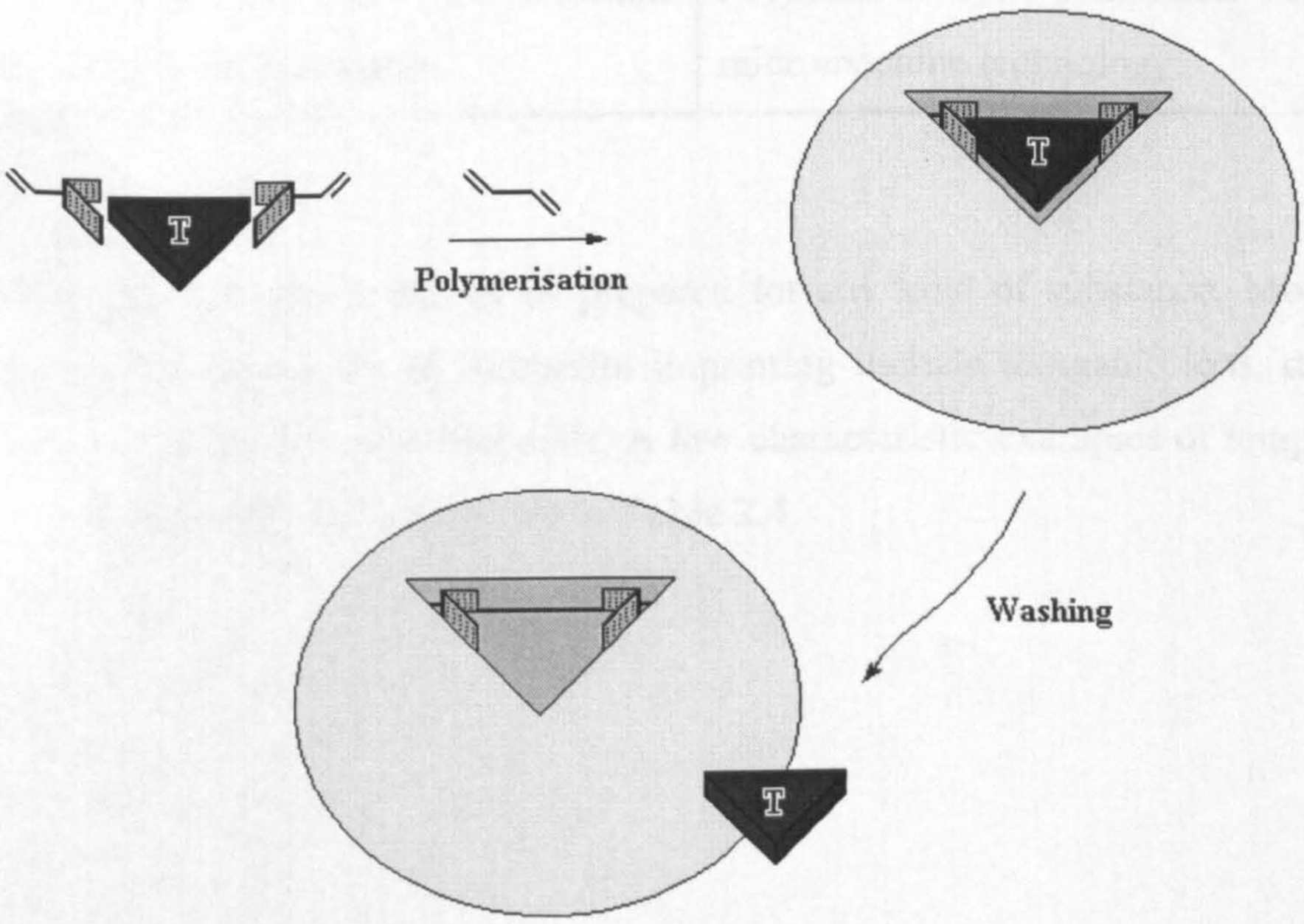


Figure 2.8 Scheme illustrating molecular imprinting polymerisation.

Table 2.3. Comparison of natural biomolecules used in sensors (enzymes, receptors, antibodies) and MIPs.

Natural biomolecules	MIPs
Low stability	MIPs are stable at low/high pHs, pressure and temperature
High price of the enzymes and receptors	Inexpensive and easy to prepare
Poor performance in non-aqueous media	MIPs can work in organic solvents
Integration of natural biomolecules in multisensor unit is difficult due to different operational requirements of these molecules (pH, ionic strength, temperature, substrate)	Due to minimal operational requirements of MIPs, the design of MIP-based multisensor is relatively easy
Natural receptors and enzymes exist for limited number of practically important analytes	MIPs could be prepared for practically any compound
Poor compatibility with micromachine technology and miniaturisation	Polymers are fully compatible with micromachine technology

Imprinted polymers can in theory be prepared for any kind of substance. More than three hundred reported examples of successful imprinting include inorganic ions, drugs, nucleic acids, proteins and even microbial cells. A few characteristic examples of templates used in molecular imprinting have been given in **Table 2.4**.

Table 2.4. Examples of various templates used in molecular imprinting

Template	Application	Reference
Amino acids and derivatives	Separation, sensors	Kempe and Mosbach, 1995; Vidiasankar *et al.*, 1997; Piletsky *et al.*, 1998; Andersson *et al.*, 1990; Panasyuk *et al.*, 1999;
Aniline, phenol and derivatives	Sensing	Vinokurov and Grigoreva, 1990; Morita *et al.*, 1997
Drugs	Separation, sensing	Levi *et al.*, 1997; Wang *et al.*, 1997; Mirsky *et al.*, 1999; Andersson, 2000; Kriz and Mosbach, 1995; Morita *et al.*, 1997;
Flavanoids	Sensing	Suárez-Rodríguez and Díaz-García, 2000;
Herbicides	Separation, sensing	Kroger *et al.*, 1999; Sergeeva *et al.*, 1999, 2001
Inorganic ions	Separation and sensing	Hutchins and Bachas, 1995; Yoshida *et al.*, 2000; Kimaro *et al.*, 2001; Murray *et al.*, 1997
Micro-organisms	Recognition	Alexander and Vulfson, 1997; Dickert *et al.*, 2001
Nucleic acids and derivatives	Separation, sensing	Piletsky *et al.*, 1990a, 1990b; MathewKrotz and Shea, 1996; Boyle *et al.*, 1989; Spurlock *et al.*, 1996
Polynuclear aromatic hydrocarbons	Sensing	Dickert *et al.*, 1998
Proteins	Separation, recognition	Hjerten *et al.*, 1997; Shi *et al.*, 1999
Steroids	Separation, detection	Hishiya *et al.*, 1999; Rachkov *et al.*, 2000
Sugars, sugar derivatives	Separation, sensing	Wulff and Haarer, 1991; Piletsky *et al.*, 1998; Chen *et al.*, 1997; Cheng *et al.*, 2001
Toxins and narcotics	Separation, sensing	Kriz and Mosbach, 1995; Matshui *et al.*, 1996; Takeuchi *et al.*, 2001.
Volatile compounds	Sensing	Ji *et al.*, 200; Dickert *et al.*, 2001.

The high specificity and stability of MIPs render them promising alternatives to the enzymes, antibodies, and natural receptors used in sensor technology (Piletsky *et al.*, 1999; Jenkins *et al.*, 1997). However, there are limitations associated with the development of MIP sensors:

(a) absence of a general procedure for MIP preparation;
(b) difficulty in integrating them with transducer;
(c) difficulty in transforming the binding event into an electric signal;
(d) problems in their performance in aqueous solution.

The development of a general procedure MIP design is one of the most challenging problems in molecular imprinting. Thermodynamic calculations and combinatorial screening approaches have been successfully used to identify the best monomer candidates for imprinting (Takeuchi *et al.*, 1999). This work became difficult with increasing size of monomer libraries, which include now thousands of polymerizable compounds. One possible solution to this problem is by the use of molecular modelling software and searching algorithms traditionally applied in drug design, which we recently adopted for the design of affinity polymers (Piletsky *et al.*, 2000) and which is discussed in detail in **Chapter 3** under ***Materials and Methods***. The polymers designed using a computational approach very often have affinity and specificity, which match those of natural receptors.

The second challenging problem of a general nature, which hinders the development of MIP sensors, is the difficulty of transformation of the polymer-analyte binding event into detectable electrical signal. To address this problem we have developed in our laboratory, a new "bite-and-switch" approach, which can be used for analysis of broad variety of amino containing compounds. This thesis addresses this problem more elaborately in **Chapter 4**, under ***Results and Discussion.***

Other problems, such as the development of immobilisation protocols are also under intensive investigation (Haupt, 2001), and a current review provides an analysis of approaches described in literature.

2.6.2 MIP design

The development of MIPs is not an easy task despite their moderately long history. One of the major problems is the choice of an optimal polymerisation protocol for the development of MIPs. This is mainly because of the availability of several variable parameters such as monomers (more than 4 thousands polymerisable compounds are commercially available), solvent, temperature and pressure etc. Some excellent materials have been designed in the past using rational approaches, where the selection of polymerisation conditions was based on rational design. However, better results could potentially be achieved using combinatorial approaches, where thousands of polymers can be synthesised and tested in order to select the best one (Lanza and Sellergren, 1998; Takeuchi *et al.*, 1999).

In this thesis, we report a new method that was developed recently by us, which we believe could be a general solution for MIP design (Piletsky *et al.*, 2000; Subrahmanyam *et al.*, 2001). The method includes computational screening of a virtual library of functional monomers against a target molecule followed by selection of those able to form the strongest complex with the template. In many cases, the computationally designed polymers possess affinity comparable with antibodies (**Table 2.5**).

Table 2.5. Affinity and sensitivity range of computationally designed molecularly imprinted polymer in comparison with antibodies for the analyte microcystin.

Receptor	K_d, nM	Sensitivity range ($\mu g\ l^{-1}$)
Computational MIP	0.3 ± 0.08	0.1-100
Monoclonal antibody	0.03 ± 0.004	0.025-5
Polyclonal antibody	0.5 ± 0.07	0.05-10

2.7 Principal types of MIP sensors

Detection of binding can be realised using electrochemical, optical and piezoelectric transducers. It is out of the scope of this review in the thesis to discuss the general characteristic features of the transducers, as they have been reviewed in detail elsewhere

(Baird and Myszka, 2001; Stenger *et al.*, 2001; D'Souza, 2001; Scheller *et al.*, 2001; Wang *et al.*, 1997; Karube 1992; Pinkerton, 1982; Brecht and Gauglitz., 1995; Higson and Vadgama, 1994).

Based on the three transducers mentioned above, two principal types of MIP sensors could be developed:

(a) affinity sensors;

(b) catalytic sensors.

Affinity sensors could be further subdivided onto two groups: i) immunosensor and ii) receptor type sensor devices.

Immunosensor type devices (i) are the most common type of MIP sensors. In this case the detection is based on the measurement of the concentration of template adsorbed by the MIP immobilized on the detector surface. Receptor sensors (ii) explore MIP's ability to change conformation upon binding with the template that leads to change in a measurable property, such as conductivity, permeability or surface potential (Piletsky *et al.*, 1998). Alternatively, sensors may be designed which use the ability of a functional monomer to change its property upon interaction with template, most frequently fluorescence (Rathbone *et al.*, 2000). Each of these three types of MIP sensors will be discussed in the following sections of this review

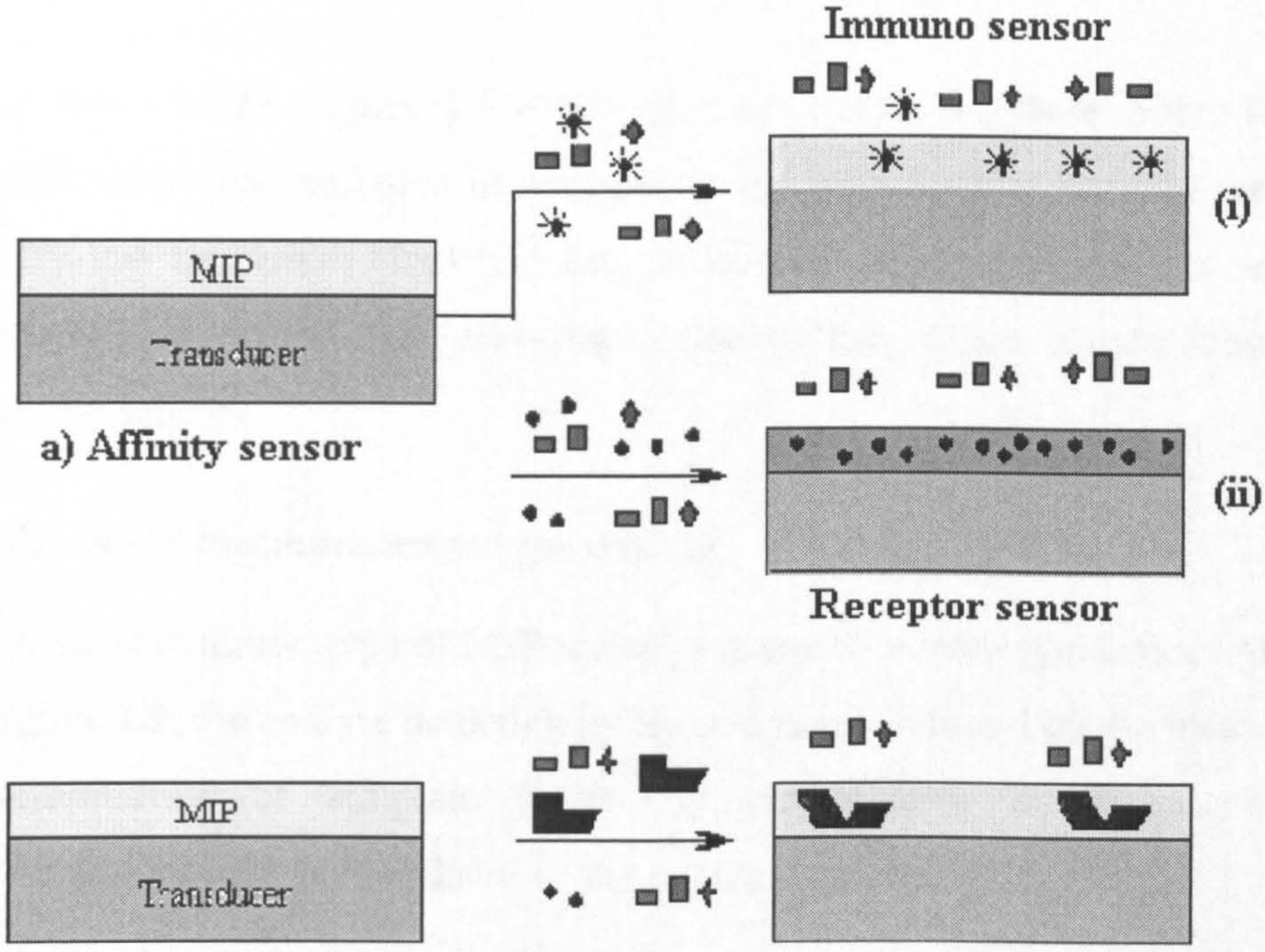


Figure 2.9 Two principal types of MIP sensors:

a. Affinity sensor, where the response is produced by accumulation of electroactive template on the MIP surface, and **b. Catalytic sensor** which responds to change in the environment induced by a MIP-catalysed reaction. **Affinity sensor** can be further classified into i) **Immuno sensor** and ii) **Receptor sensor**, where the response is generated by changes in polymer characteristics induced by its interaction with template.

2.7.1 Development of affinity sensors based on MIPs

As shown in the **Figure 2.9** above, affinity sensors are those where the response is produced by accumulation of template on the MIP surface. Affinity sensors based on MIPs can be further classified into a) immunosensor-type devices and b) receptor sensor-type devices. The following sections of this review discuss these two types of affinity sensors.

MIP-based immunosensor-type devices

The most common type of MIP sensor is immunosensor-type device. As shown in the **Figure 2.9,** the analyte detection by these devices is based on the measurement of the concentration of template (preferably electroactive or fluorescent) selectively adsorbed by MIP immobilised on the detector surface.

Tabushi *et al.* (1987) developed the first affinity, immunosensor-like devices using molecular imprinting. The group performed chemosorption of octadecylchlorosilane in the presence of n-hexadecane, (inert template) onto tin dioxide or silicon dioxide. After the removal of the hosts (n-hexadecane), vitamin K1 (phylloquinone), vitamin K2 (menaquinone), vitamin E (tocopherol), cholesterol and adamantane yielded strong electrochemical signals and hence were able to be detected. A similar approach was followed for the development of a two-dimensional MIP sensor for vitamin K1 (phylloquinone) (Andersson *et al.*, 1988). Here, this group used ellipsometry to measure the concentration of template absorbed on the detector surface. This preliminary work demonstrated the possibility for direct detection of a template adsorbed by an imprinted monolayer, although it suffered from lack of appropriate controls.

This method was expanded further for the preparation of monolayers imprinted with water-soluble templates. Indium oxide electrodes specific for phenyl alanine was developed (Starodub *et al.*, 1993). The methodology comprised two steps: a) adsorption of the template on the InO_2 surface and b) treatment of the electrode with adsorbed template by trimethyl chlorosilane from the gas phase. This method was successfully used recently for the development of sensors for nucleic acids, cholesterol (Piletsky and Starodub, 1992) and catechol derivatives (Morita *et al.*,

1997). Despite some advantages, including fast sensor response and easy preparation, these 2-D systems suffer from the lack of stability associated with imprinted monolayers.

Mirsky *et al.*, (1999) proved that the stability of the sensor could be improved by co-immobilisation of the template in an imprinted layer. They used new approach called "spread bar architecture design" by which it was possible to develop a stable monolayer, consisting of template (thiobarbituric acid) and functional monomer (hexadecylmercaptane). The template formed depressions in the hexadecylmercaptane layer, and these depressions were able to accommodate barbituric acid, changing the electrode/monolayer capacitance in the process of binding.

Conventional 3-dimensional polymers have been used more frequently for the design of sensors. Different electrochemical, piezoelectric and optical sensors have been developed using MIPs. The simplest MIP immunosensor-type device exploits the gravimetric detection principle. Piezo quartz crystal (PQC) sensors have advantages such as:

a) relative simplicity of the sensor design;

b) easy interpretation of the sensor response and

c) compatibility with variety of solvents and variety of templates.

A piezoelectric MIP sensor for the determination of L-menthol in the liquid phase was reported by Percival *et al* (2001). A highly specific noncovalently imprinted polymer was cast *in situ* onto the surface of a gold-coated quartz crystal microbalance (QCM) electrode as a thin permeable film. Selective rebinding of the target analyte was observed and quantified as a frequency shift by QCM. The detection level of L-menthol was 200 ppb. The sensor was able to distinguish between the D- and L-enantiomers of menthol owing to the enantioselectivity of the imprinted sites. A number of other terpenes (limonene, menthone, citronellol, citronellal, D-menthol) were examined in order to investigate the selectivity of the MIP. No change in resonant frequency was observed upon the addition of up to 2 ppm for all terpenes that did not contain an OH moiety (**Figure 2.10.**).

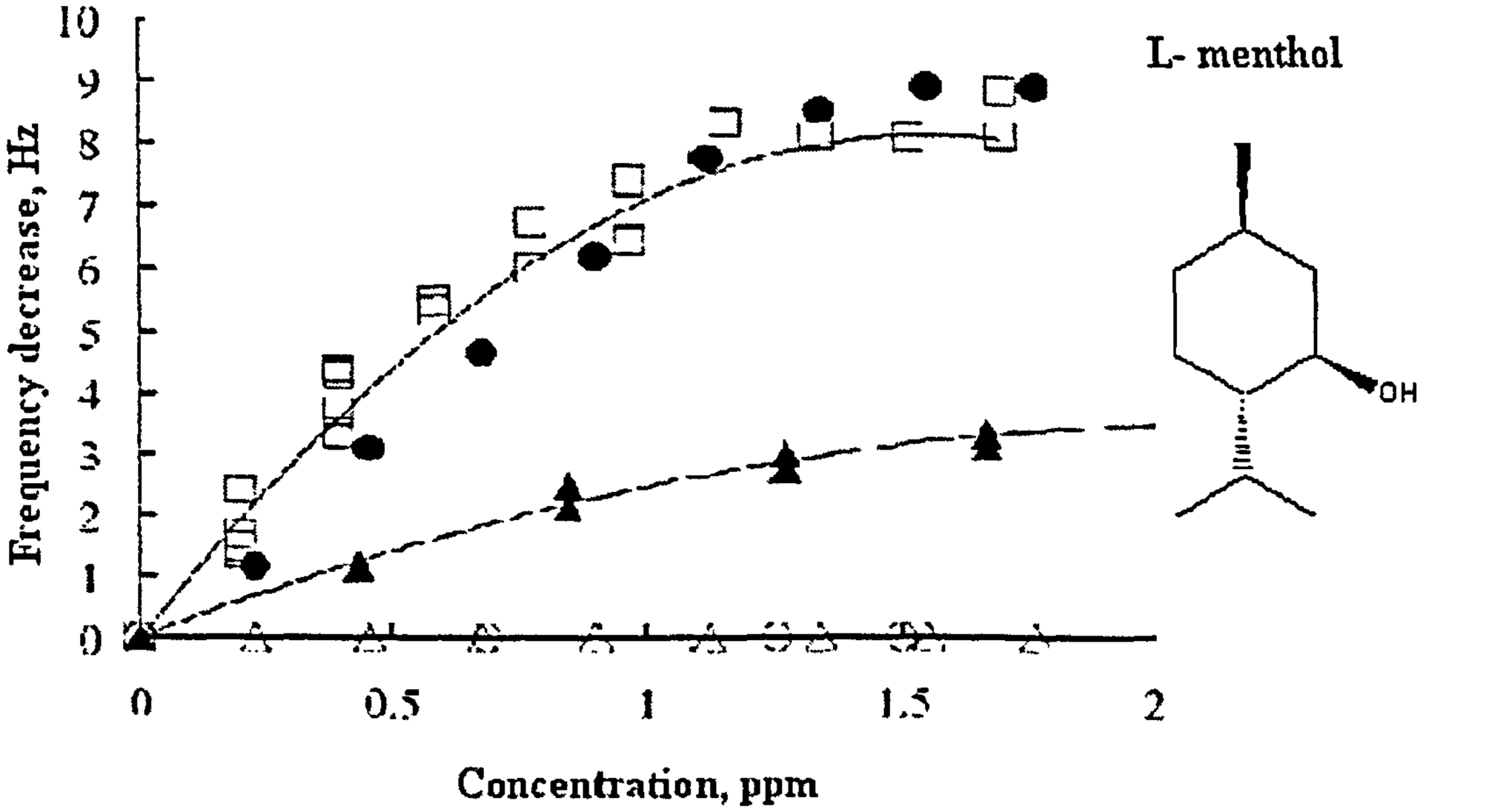


Figure 2.10 **Range of monoterpene analogues that the selectivity of the MIP-coated quartz crystals were tested toward L-menthol (squares), D-menthol (solid triangles), citronellol (solid circles), citronellal (triangles), and menthone (circles) (Percival *et al.*, 2001).**

In principle, the sensitivity of the QCM sensors can be enhanced in two ways; firstly it could be possible to increase the concentration of binding sites in the MIP by optimising the monomer composition and polymerisation conditions, and secondly, by increasing the mass sensitivity of the acoustic device. This may be achieved by increasing the resonant frequency of the sensor, as the sensor response to mass loading is proportional to the square of the operating frequency. The upper limit for QCM operating at the fundamental frequency is ~10 MHz; the higher the frequency, the thinner the crystal. Alternatively, surface acoustic wave devices may be employed that will extend the range of operating frequencies to over 1 GHz. (Ji *et al.*, 1999; Jacoby *et al.*, 1999).

In another example overoxidized polypyrrole films (OPPy) imprinted with L-glutamate were used for enantioselective detection of L- and D-glutamic acid (see

Figure 2.11 a, b) (Deore *et al.*, 2000). It was found that the template binding was ~30 times higher than the binding of opposite enantiomer.

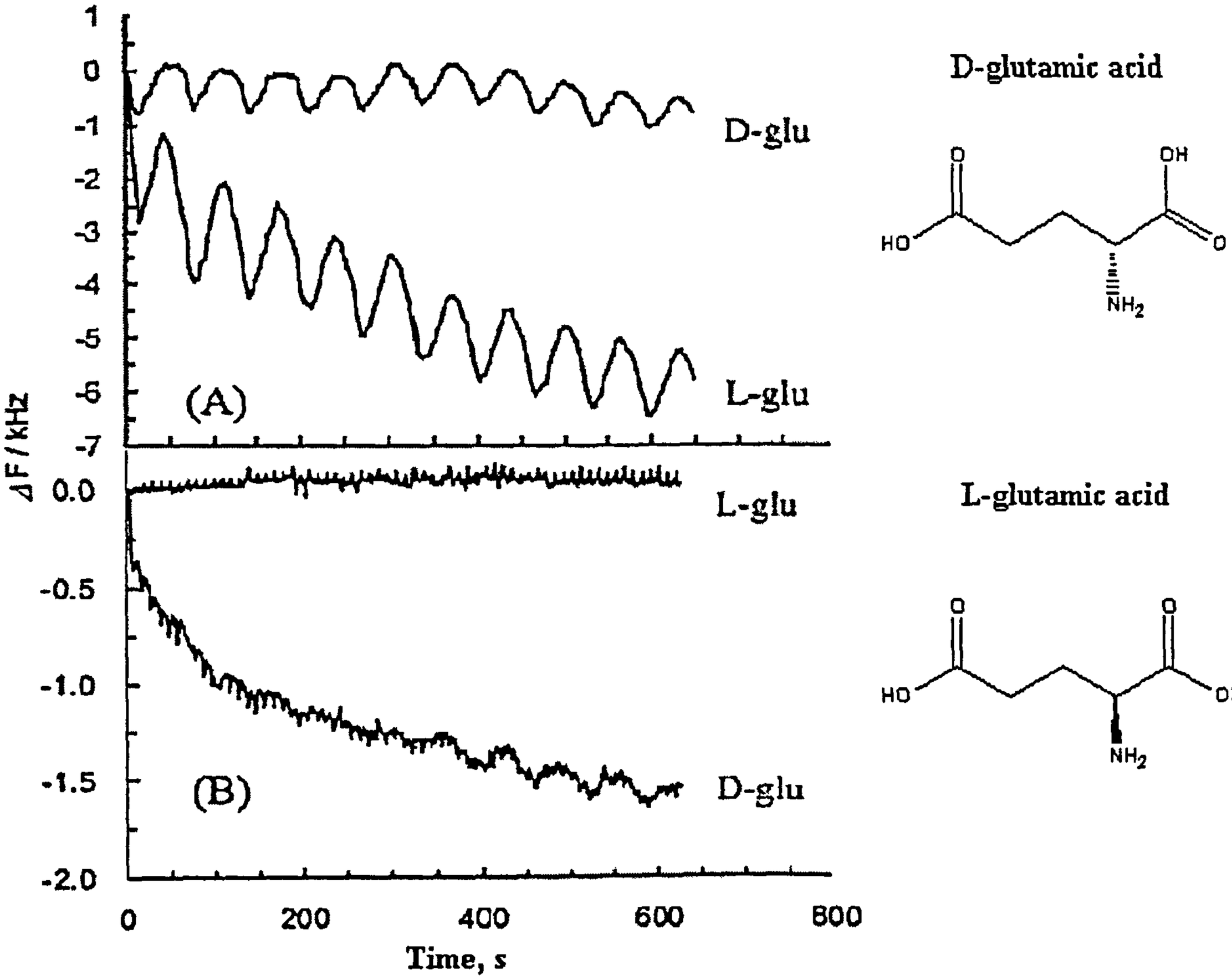


Figure 2.11 **Time dependence of the EQCM resonance frequency under potentiodynamic polarization of (a) OPPy (L-Glu) and (b) OPPy (D-Glu) films. The films were polarized in 10 mM D- and L-Glu solutions (pH 1.7). Each maximum and minimum in the figures corresponds to the frequencies at about +0.6 and 0.0 V. Film preparation condition: 0.050 mA cm^{-2} for 2.00 h (Deore *et al.*, 2000).**

A sensor templated with L-glutamic acid also has been reported to exhibit excellent selectivity over several other L- and D-amino acids. The applied potential of 0.0 V for

the uptake of Glu and solution pH of 1.7 were found to be optimum for chiral separation (**Figure 2.12**).

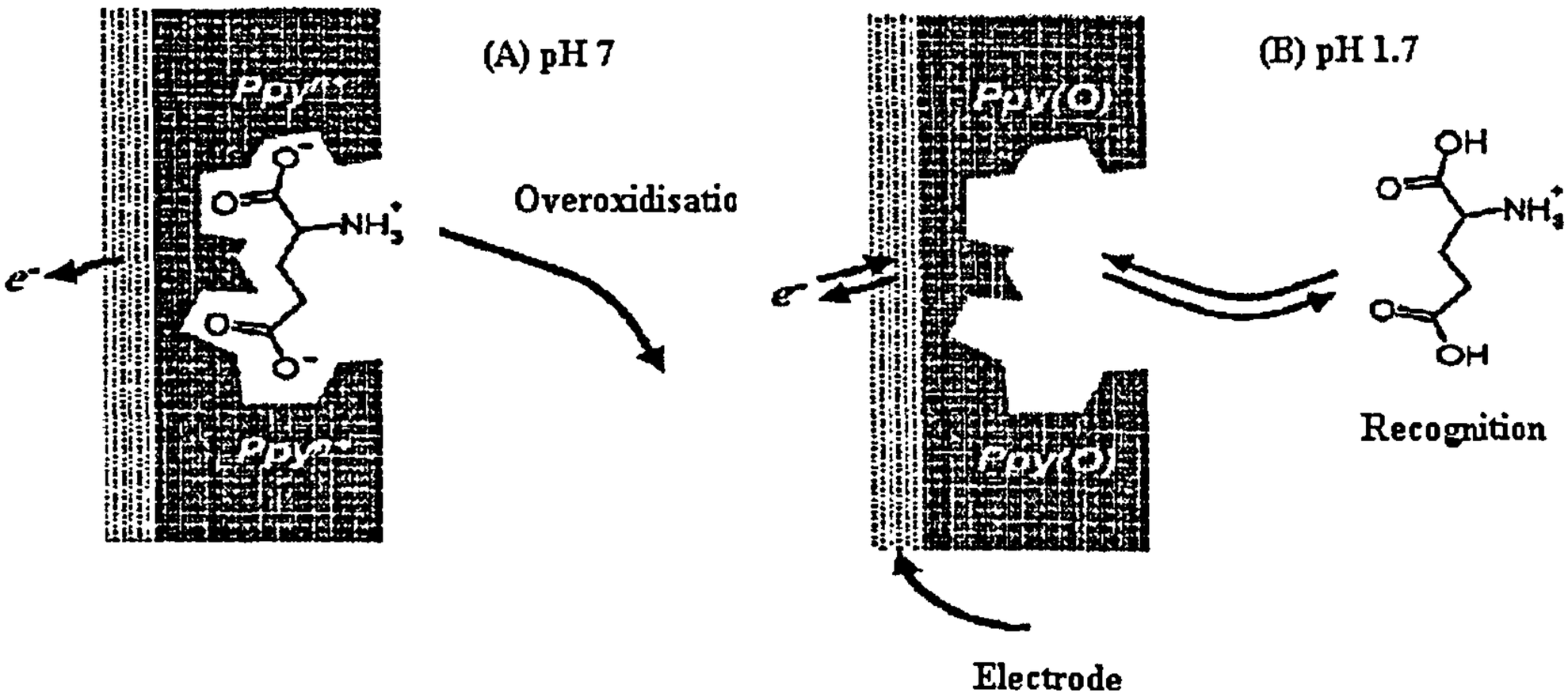


Figure 2.12 **A model for enantioselective uptake of glutamic acid into an overoxidized polypyrrole: (A) cavity formation by overoxidation, and (B) selective uptake/release of Glutamic acid upon charging/discharging the film (Deore *et al.*, 2000).**

Among various types of conducting polymers so far studied, polypyrrole has many attractive features as a molecular recognition system, since it can be used in a neutral pH region and its stable films can conveniently be polymerized on various substrate materials (Evanns *et al.*, 1990; Cadogan *et al.*, 1992; Hulanicki and Michalska, 1995; Migdalshi *et al.*, 1996). These features make polypyrroles attractive materials for application in sensors.

A new bio-mimetic bulk acoustic wave (BAW) sensor for measuring paracetamol in real samples with a high selectivity and sensitivity, was fabricated using a MIP as the sensing material (**Figure 2.13**) (Tan *et al.*, 2001a). Non-covalent MIPs were synthesized simultaneously using two different functional monomers: 4-vinylpyridine (4-VP) and methacrylic acid (MAA). These MIPs had improved recognition capability as compared with the polymers that were synthesized using only one of the functional monomers. The sensor was stable and exhibited good reproducibility.

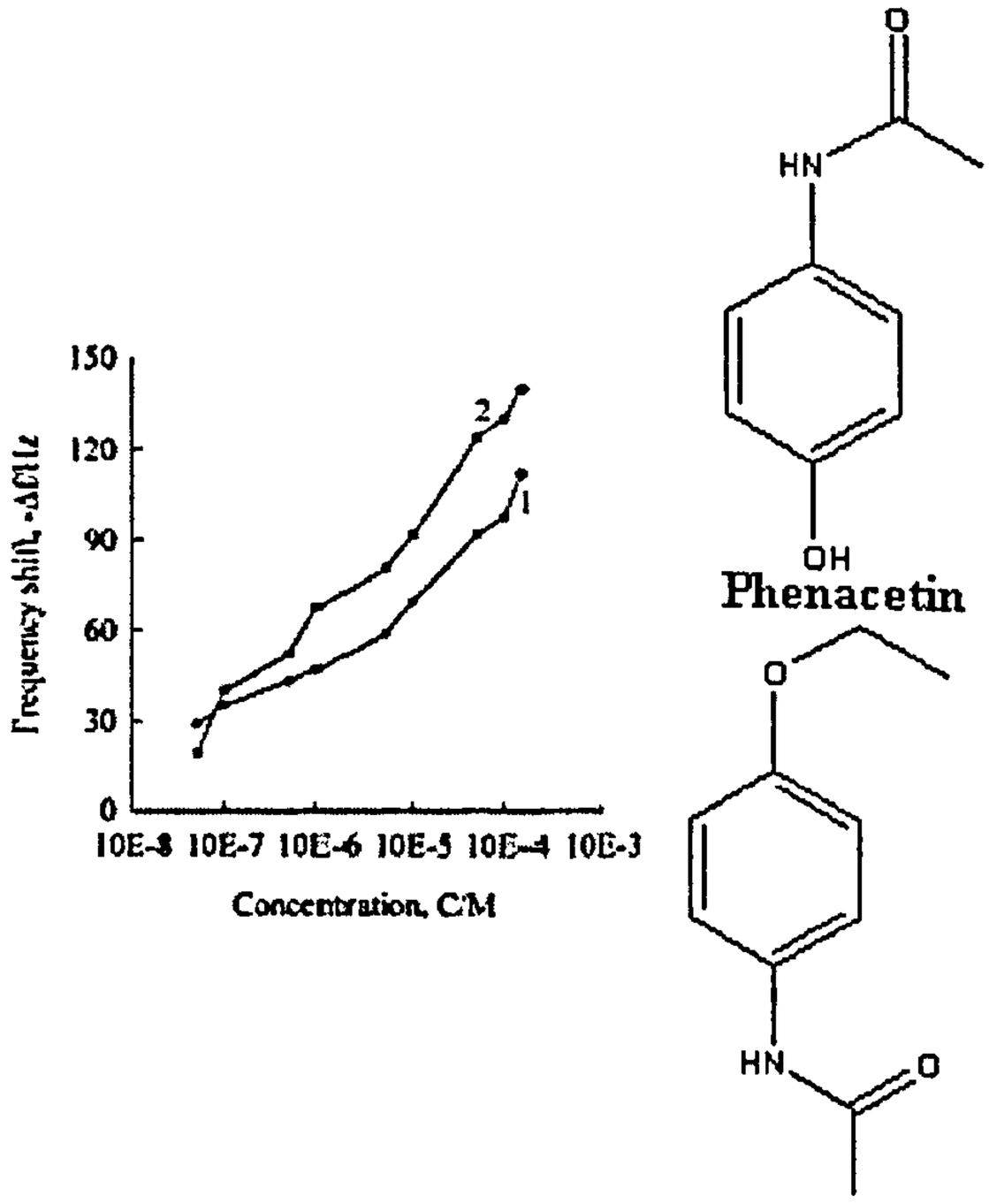


Figure 2.13 Response curves of the PVC-BAW sensor to phenacetin (1) and to paracetamol (2) (Tan *et al.*, 2001).

In a related work the same group showed high binding selectivity and sensitivity for phenacetin in ethanol. (Tan *et al.*, 2001b) (**Figure 2.14).**

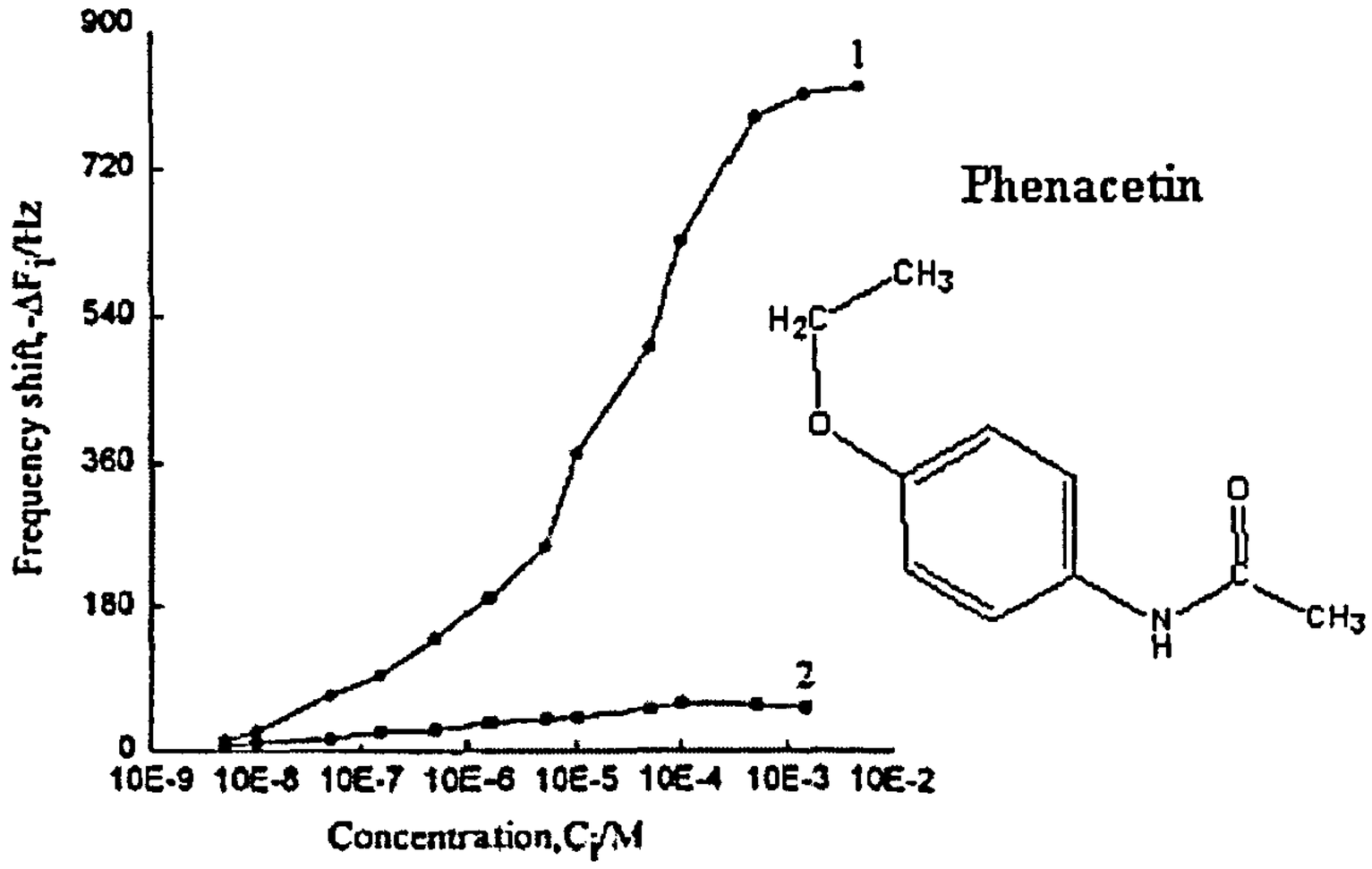


Figure 2.14 **Typical response of the PQC sensor. An aliquot of standard phenacetin in ethanol with different concentration was injected into the detection cell: (1) the sensor based on MIP; (2) the sensor based on NIP (Tan *et al.*, 2001).**

Other interfering compounds such as paracetamol, antifebrin and phenetole could also generate the signal although with much smaller amplitude (**Figure 2.15**).

A quartz crystal thickness-shear-mode (TSM) sensor combined with a MIP was used for the determination of nicotine in human serum and urine (Tan *et al.*, 2001a). The MIP was synthesized using nicotine as the template and MAA as the functional monomer. The sensor showed high selectivity and a sensitive response to nicotine in an aqueous system.

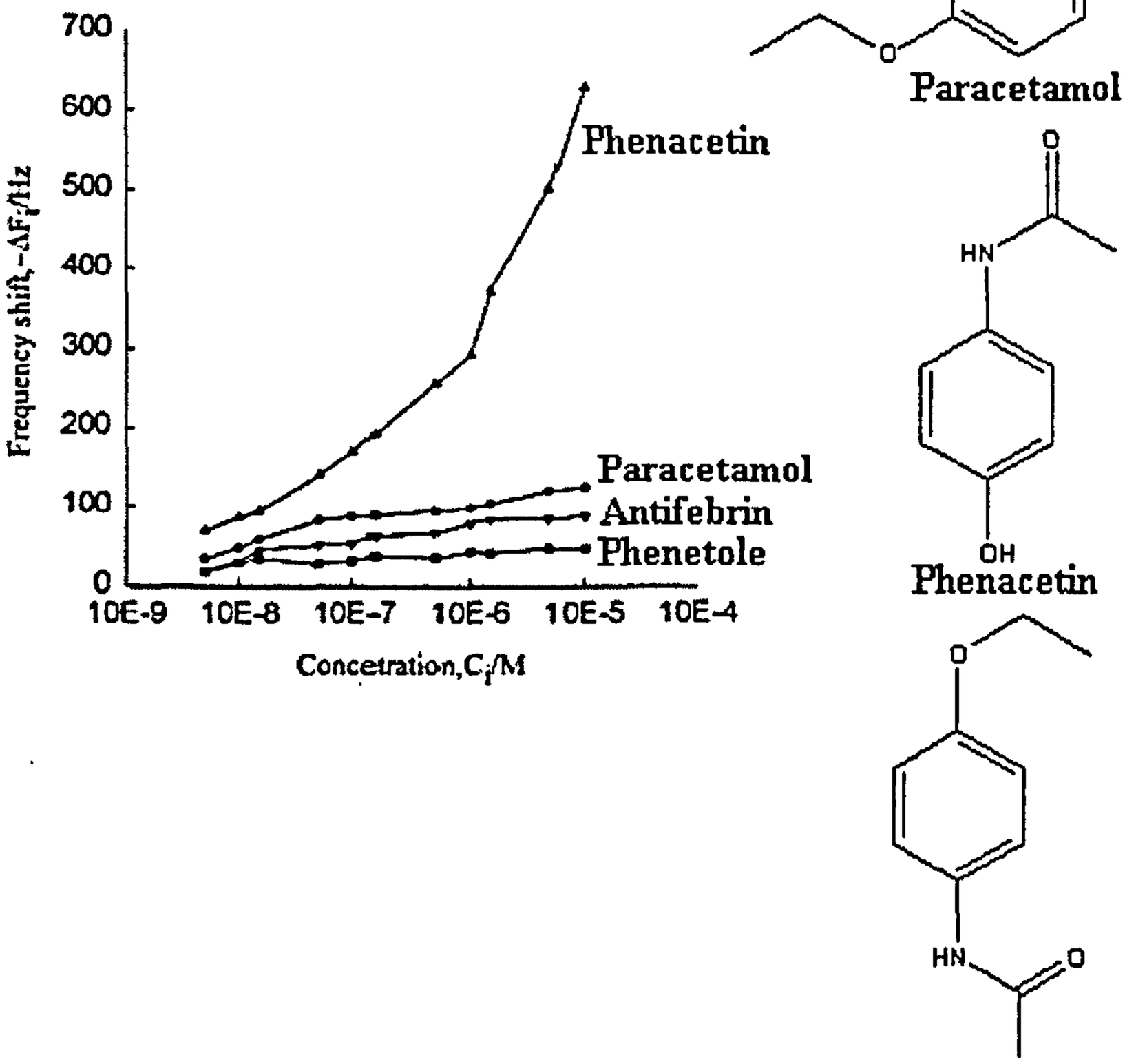


Figure 2.15 **Response of the MIP sensor to phenacetin (curve 1), paracetamol (curve 2), antifebrin (curve 3), phenetole (curve 4). The same electrode was used for all the detection in 10 ml aqueous system (Tan *et al.*, 2001).**

Kobayashi *et al.*, (2000) reported recognition of caffeine (CAF) using a MIP combined with 6 MHz QCM device (**Figure 2.16**). Polyacrylonitrile (PAN) copolymers with pyridine and styrene moieties were used for CAF imprinting. To produce the imprint, polymer solution containing the template molecule was coagulated in water. It was observed that PAN copolymer having 4-vinylpyridine (4-Py) moieties showed a small difference in frequency decrease with the MIP and with non-imprinted polymers. The selectivity of the CAF-imprinted polymers was

investigated using CAF analogous molecules and unfortunately there was no appreciable selectivity (**Figure 2.17**.).

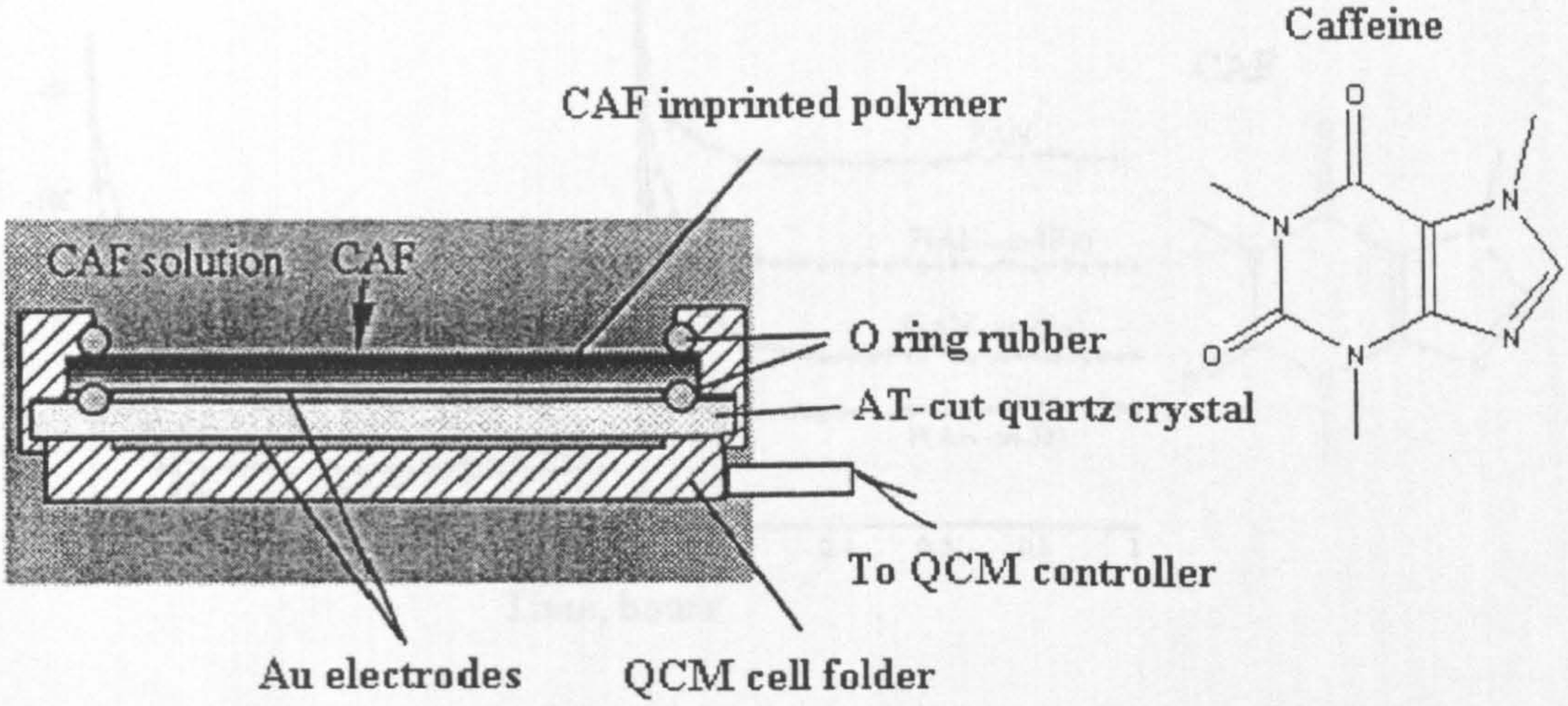


Figure 2.16 Schematic representation of an experimental set-up for QCM attached with imprinted polymer (Kobayashi *et al.*, 2000).

The recognition properties of the CAF-imprinted polymers were demonstrated by observing the frequency change for various MIP and non-imprinted polymers. It was observed that PAN copolymer having 4-vinylpyridine (4-Py) moieties showed a small difference of the frequency decrease between the MIP and non-imprinted polymers (**Figure 2.17**).

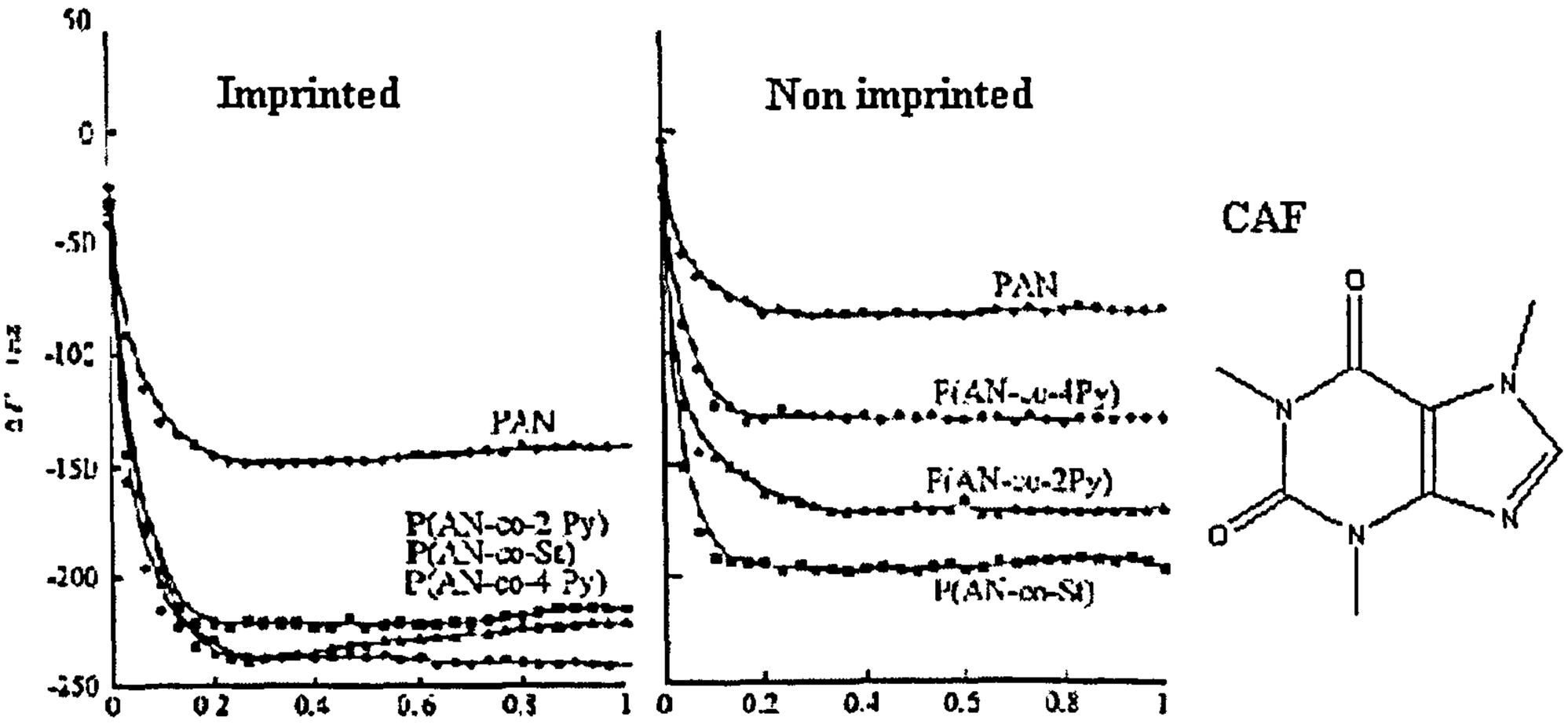


Figure 2.17 **Time courses of frequency change of QCM for (a) CAF-imprinted polymers and (b) blank polymers. Measurements of frequency change were carried out in 1 M CAF aqueous solution at 30°C (Kobayashi *et al.*, 2000).**

Cao *et al* (2001) reported a MIP based QCM sensor for dansylphenylalanine (PA) enantiomers. The polymer imprinted with dansyl-L-PA was immobilised on a gold electrode. The fabricated sensor was able to discriminate between L- and D-dansyl-PA enantiomers in solution owing to the enantioselectivity of the imprinted sites. The fabricated sensor could quantitatively determine the enantiomeric composition of L- and D-enantiomeric mixture. The detection limit was 5 μg /ml with a response range of 5-500 μg/ ml, measured in buffer, pH 10.0. A convenient regeneration process was proposed to increase the reproducibility and reusability of the sensor by flushing the polymer with pH 2.0 buffer. The method presented in this work provides a novel means of preparing highly sensitive chemical sensors via self-assembly and molecular imprinting techniques.

Ji *et al.* (2000) designed a sensor selective for the odorant 2-methylisoborneol (MIB) by utilizing a MIP as the recognition element. Piezo quartz crystals were first coated with a layer of nylon to provide increased sensitivity, and then a layer of a polymer imprinted with MIB was applied to endow the device with selectivity. The QCMs

were mounted in a thermostated chamber in a stream of flowing nitrogen (**Figure 2.18**). The resonant frequency of the sensors was monitored by a quartz chemical analyser and the data was processed.

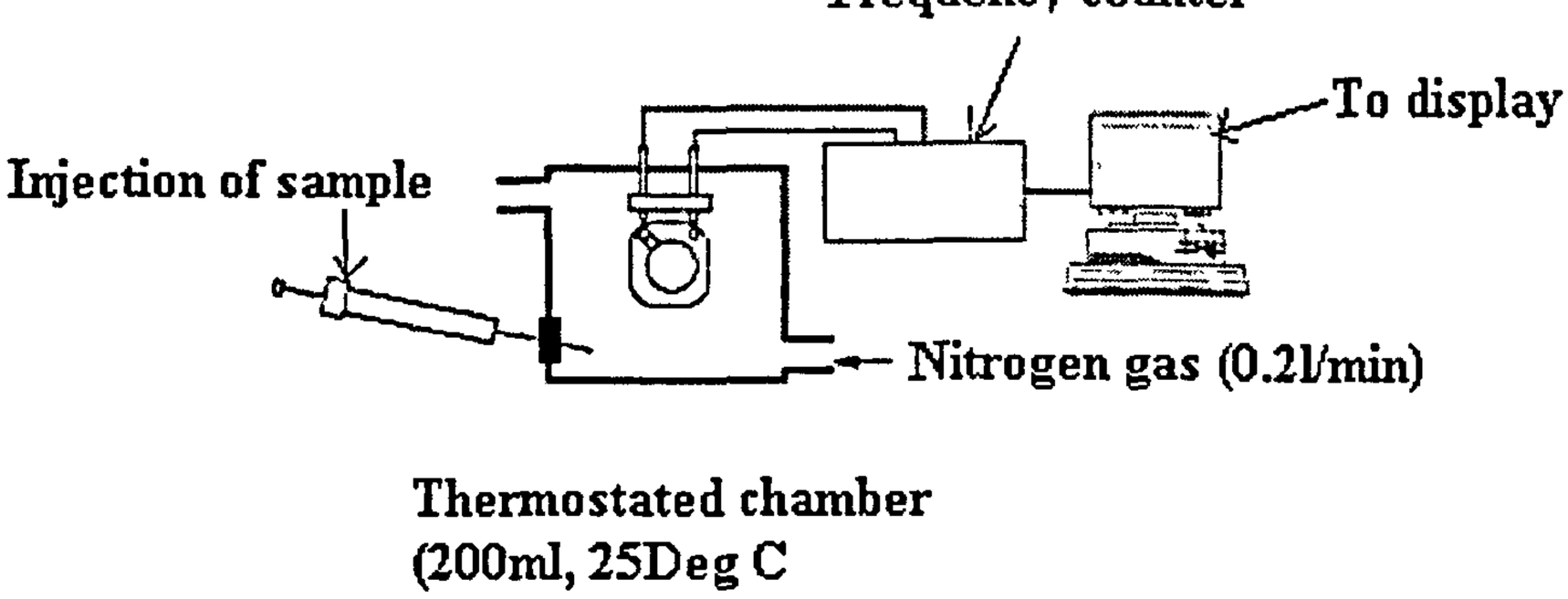


Figure 2.18 A schematic diagram of the odour sensor system (Ji *et al.*, 2000).

The responses of the imprinted and non-imprinted sensors to solutions of the template MIB and to GEO are shown in **Figure 2.19.** Although it has been claimed that the response of the imprinted sensor was significantly higher than the response of the non-imprinted sensor at MIB concentrations above 10 ppb, as shown in the **Figure 2.19**, there was actually a very small difference in response of the imprinted sensor in comparison to the non-imprinted sensor. At concentrations greater than 10 ppb, the response of the non-imprinted sensor to geosmin (GEO), another odorant often accompanying MIB, was slightly higher than the response of the imprinted sensor.

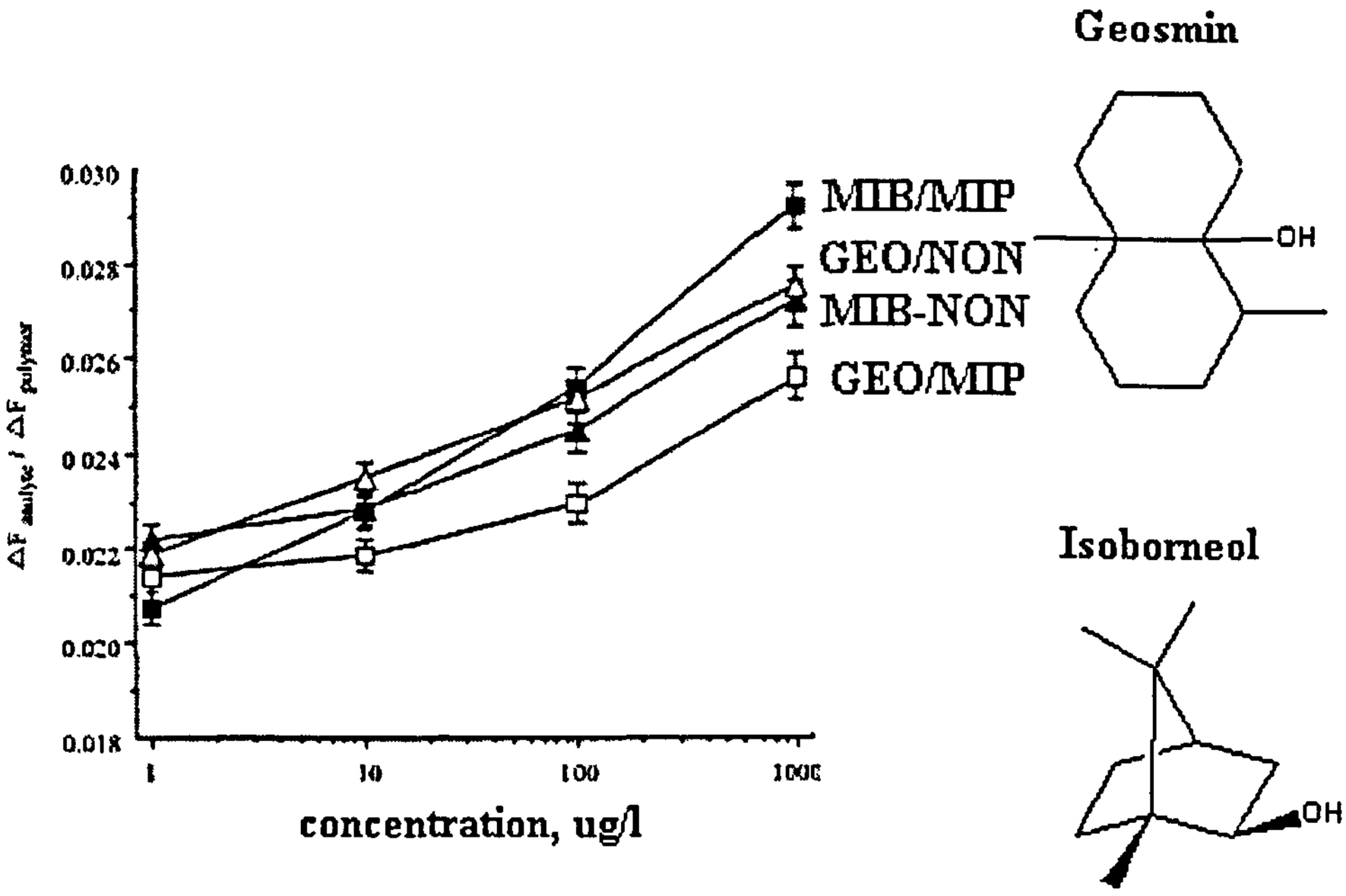


Figure 2.19 **Responses of imprinted and non-imprinted sensors to solutions of MIB and GEO in hexane (Ji *et al.*, 2000).**

The preparation and characterization of electrosynthesized poly (o-phenylenediamine) (iPoPD) as a molecular imprinting material were studied by an *in situ* quartz crystal impedance method (Peng *et al.*, 2001). The thickness shear mode (TSM) acoustic sensor modified with this material exhibited molecular recognition ability to the template molecule of DL-phenyl alanine. The detection was linear in the range of 2-20 mM. Using this electropolymerization technology, the preparation of the sensor was very easy and the reproducibility of preparation was very good.

Molecularly imprinted polyurethanes were used as sensor materials for monitoring the degradation of automotive engine oils (Dickert *et al.*, 2000). Imprinting with characteristic oils permits the analysis of these complex mixtures without accurately knowing their composition. Mass-sensitive QCMs coated with such layers exhibit mass effects in addition to frequency shifts caused by viscosity, which can be compensated by an uncoated quartz or a non-imprint layer.

Amperometric and optical sensors in general possess higher sensitivity than the piezoelectric sensors. Amperometric sensors for aniline and phenol were developed using MIPs based on imprinted polyaniline by Piletsky *et al.* (1994) and

potentiometric sensors specific for metal ions by Murray *et al.* (1997). The later group has proved that MIP sensors are suitable for practical analysis of lead and uranil ions in complex media. This is because of enhanced sensitivity and selectivity obtained with MIP (**Figure 2.20**).

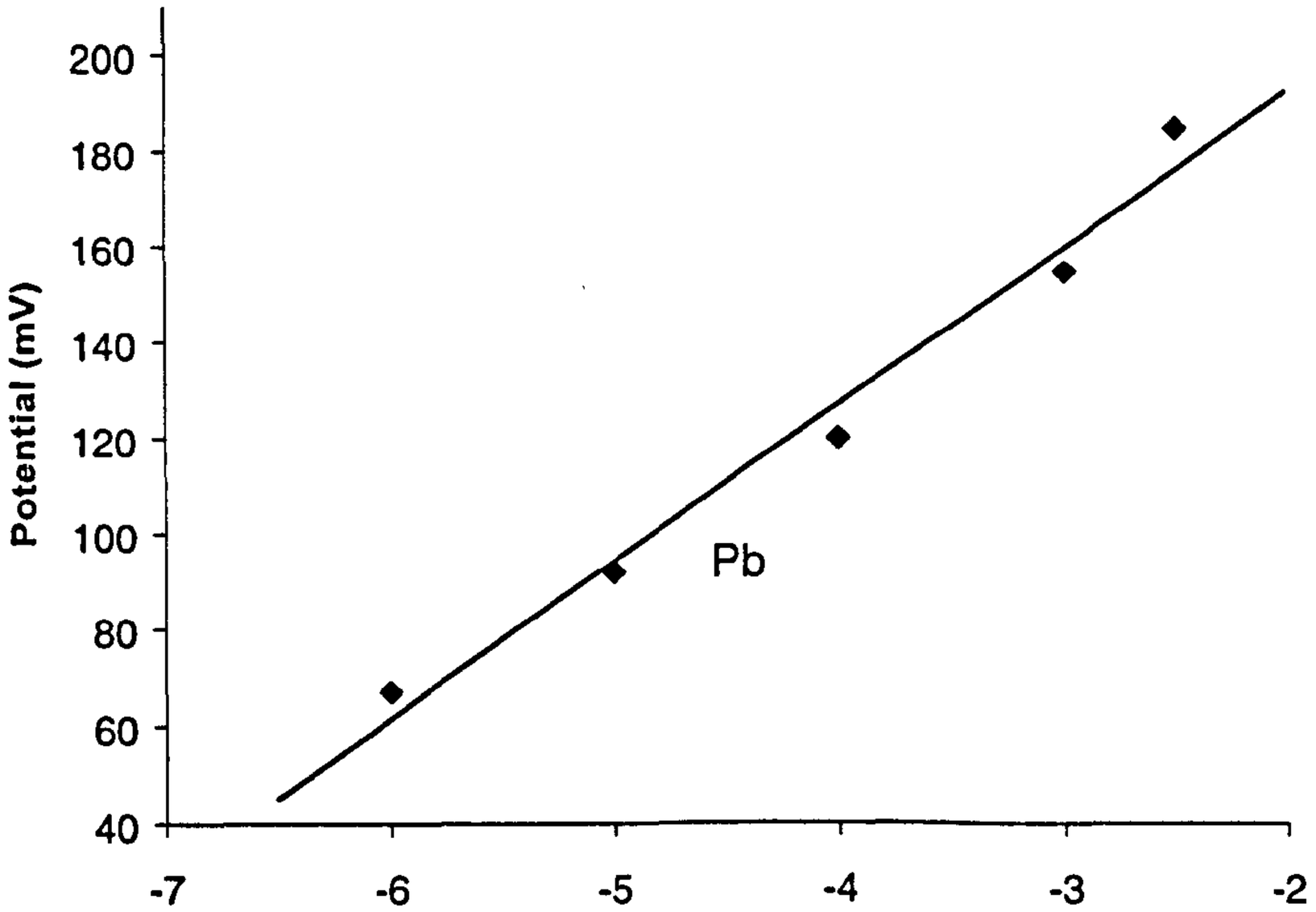


Figure 2.20 **Response of lead (II) ion imprinted electrode. Ions such as Ca, Cd, Cu, Ni, Zn and La had negligible interference (Murray *et al.*, 1997).**

Kriz and Mosbach (1995) showed that morphine could be adsorbed selectively by an agarose membrane with morphine-imprinted polymer beads. The competition between an electroinactive competitor (codeine) and a morphine sample led to release of the adsorbed morphine which generated a sensor response due to electrooxidation. The sensor showed reasonable sensitivity for morphine (0.1-10 mg/l), and good stability with time. It was shown recently that a similar approach can be used for the detection of clenbuterol in bovine liver samples using differential pulse voltammetry (Pizzariello *et al.*, 2001). The electroinactive analogue- isoxsuprine competitively displaced clenbuterol that was specifically adsorbed by MIP (**Figure 2.21**).

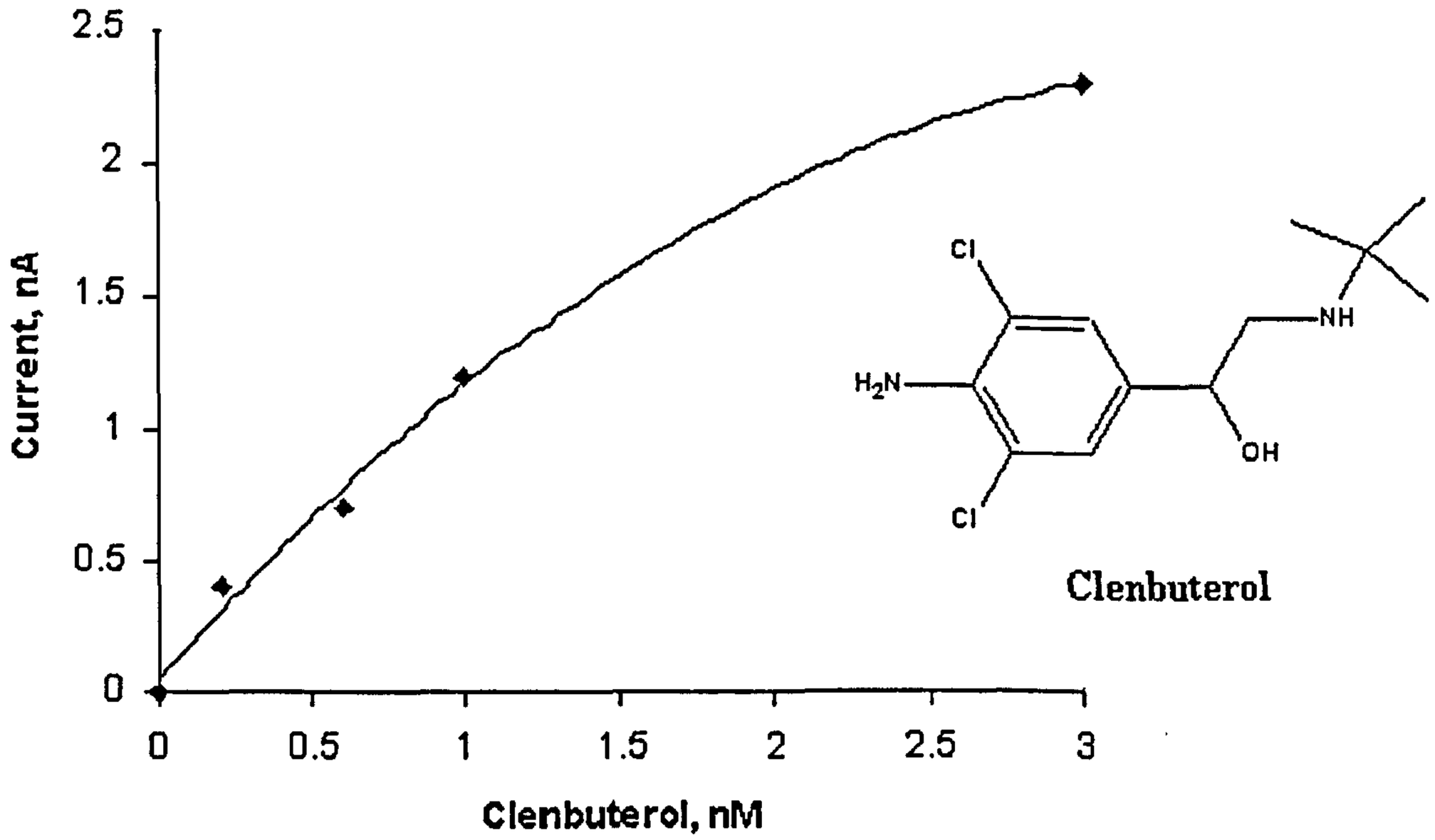


Figure 2.21 Calibration curve for clenbuterol, displaced by excess of electroinactive isoxsuprine. Measurements performed in 50 mM perchloric acid containing 10% ethanol (background electrolyte) (Pizzariello *et al.*, 2001).

An optical sensor specific for the fluorescent substance dansyl-L-phenylalanine using dansyl-L-phenylalanine-imprinted polymer was developed by Kriz *et al.* (1995). This optical sensor was based on a fibre-optic sensing device. Accumulation of fluorescent template in the polymer matrix resulted in an increase in fluorescence that could be used to detect 10 mg/l of substrate within 4 h.

An interesting variant of an optical sensor based on MIP was proposed by Steinke *et al.*, (1996). The MIPs prepared had anisotropic properties and provided specific orientation of bound template molecules. Therefore the MIPs showed a pronounced dichroism in UV light. This enabled recognition of specific binding. Hence this work could be applicable specifically for the detection of optical isomers.

In another example Dickert and Tortschanoff, (1999) demonstrated the detection of fluorescent polycyclic aromatic hydrocarbons using optical sensors with imprinted polyurethanes (**Figure 2.22**).

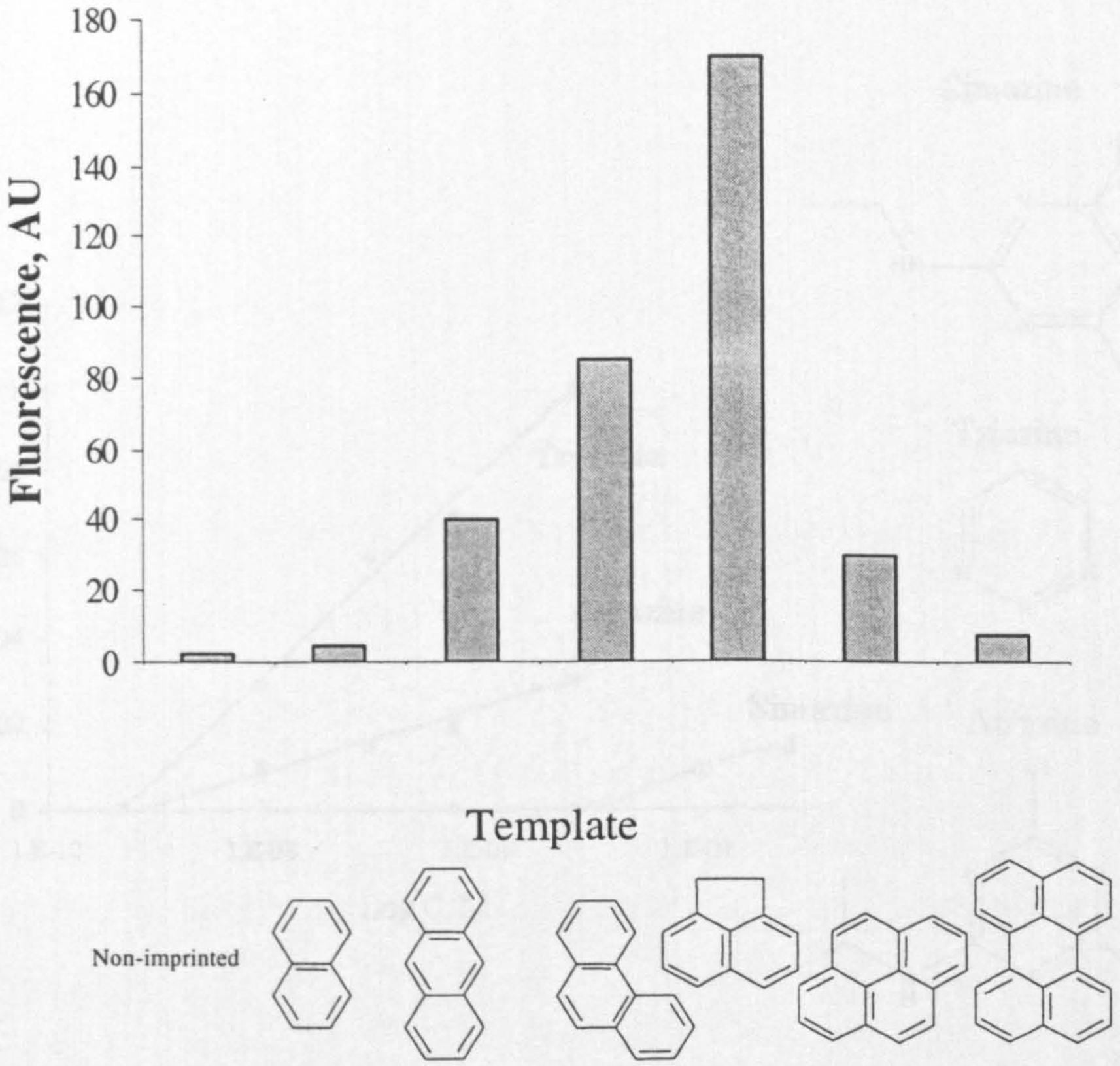


Figure 2.22 **Selectivity pattern of pyrene detection by fluorescence, using polyurethanes imprinted with polyaromatic hydrocarbons of different sizes (Dickert and Tortschanoff, 1999).**

A serious limitation to the broad applicability of immunosensor type devices is the limited number of practically important electroactive or fluorescent substances, which can be used as templates. A solution to this problem is the development of sensors that can operate in a competitive mode. In order to implement this, it is important to know if the binding sites in imprinted polymers are capable of recognising template molecules that are labelled with a fluorescent dye or enzyme. Successful demonstration of this possibility was performed for polymer imprinted with triazine. Here the competition between fluorescein-labelled and unlabelled template was used to measure 10^{-8} – 10^{-5} M concentration of free template dissolved in ethanol (Piletsky *et al.*, 1997) (**Figure 2.23**). The polymer was able to discriminate between triazine,

atrazine and simazine. Competitive assays with enzyme-labelled templates were developed later for epinephrine (Piletsky *et al.*, 2000b) and 2, 4-dichlorophenoxyacetic acid (Surugiu *et al.*, 2001).

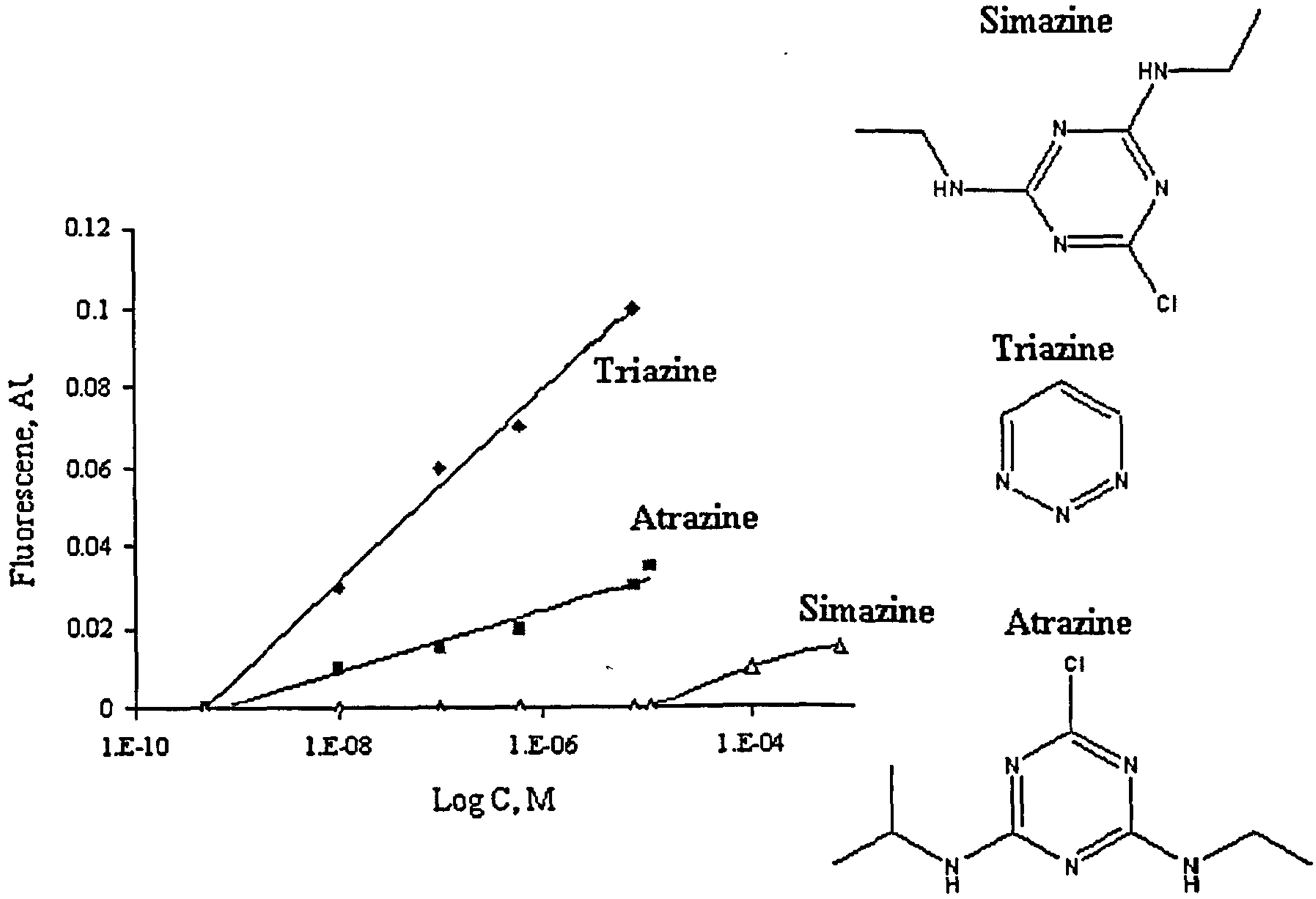


Figure 2.23 Displacement of fluorescein-triazine from triazine imprinted polymer by analytes (Piletsky *et al.*, 1997)

A MIP sensor for chloramphenicol (CA) was reported by Levi *et al.* (1997). The sensor was based on a displacement format and included a HPLC column with CA-specific MIPs. A constant flow of dye-labeled CA (chloramphenicol-Methyl Red) was run through column at a concentration of 0.5 µg/ml. Analyte containing free CA displaced the adsorbed conjugate, giving a peak with an area that was proportional to CA concentration (**Figure 2.24**). CA in blood serum samples was analysed successfully.

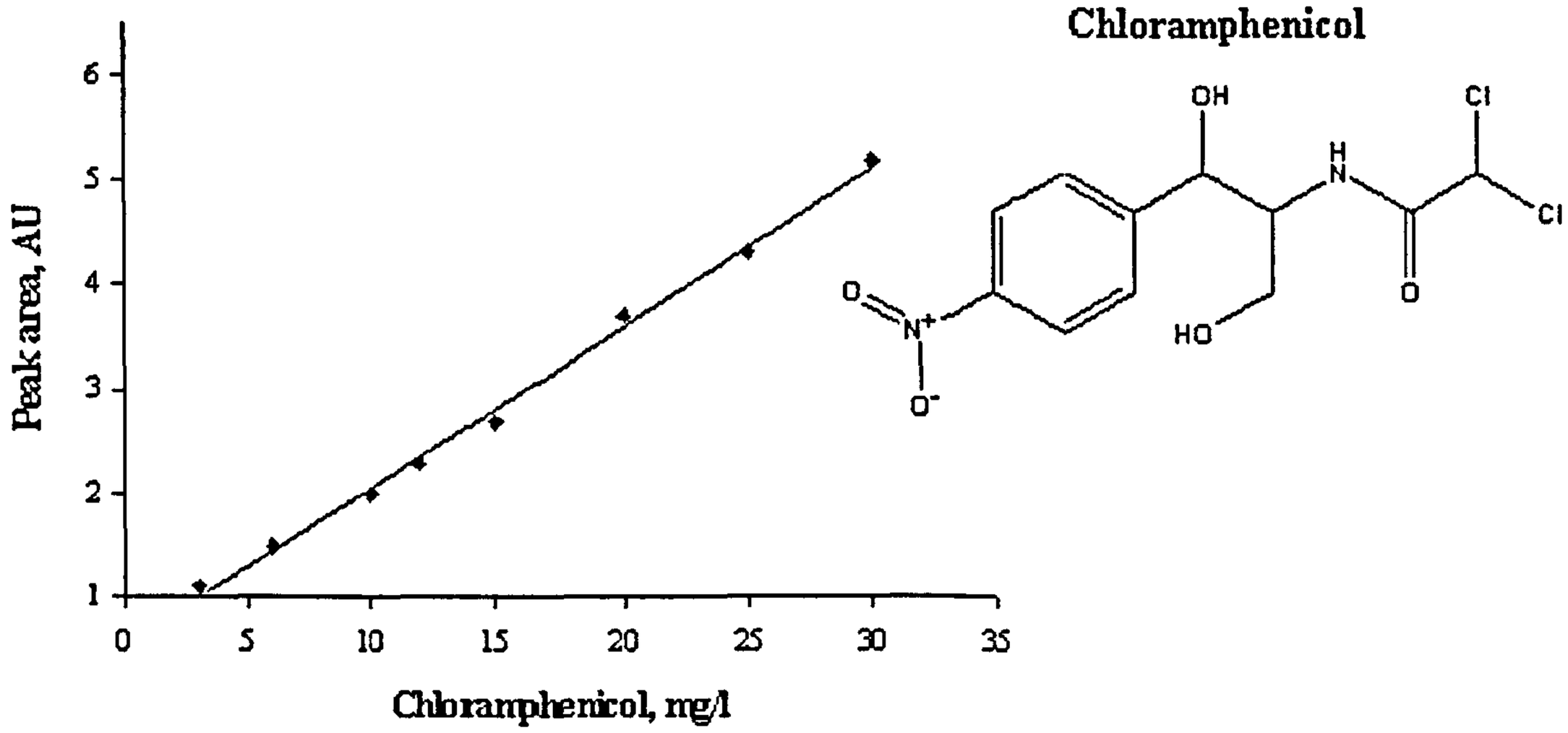


Figure 2.24 Displacement of chloramphenicol-methyl red from a chloramphenicol-imprinted polymer upon injection of template (Levi *et al.*, 1997)

Unfortunately, modification of all analytes with fluorescent or electroactive label is not possible and in some cases the modification itself can change the affinity of analyte. The solution to this problem was proposed recently by Piletsky *et al.*, (1999b). They reported the displacement of non-specific dyes from a MIP and the subsequent detection of the displaced dye for the quantification of ligand-polymer binding events. The dye solution of rhodamine-B was passed through a MIP-HPLC column specific to L-Phe-amide. When template was injected, part of the dye was competitively replaced by the analyte from the MIP. This displacement peak was three times higher for the template than for the opposite enantiomer. Kroger *et al.*, (1999) reported a similar displacement principle in combination with electrochemical measurements for template detection. In electrochemical sensors this method can be used for the detection of analytes that cannot be easily oxidized or reduced. The displacement of non-specific indicator molecules from a set or array of MIPs throws open possibilities to develop multisensors, as shown by both these groups.

Examples of electrochemical, optical and PQC immunosensor-type devices based on MIPs are shown in **Table 2.6.**

Table 2.6 Electrochemical, Optical and PQC sensors based on MIPs.

Template	Polymer	Detection	Reference
Amino acids and derivatives	PM Polyphenol Polypyrrole	Potentiometry Capacitance QCM Optical	Andersson *et al.*, 1990 Panasyuk *et al.*, 1999 Deore *et al.*, 2000 Piletsky *et al.*, 1999b; Sergeeva *et al.*, 1999; Wang *et al.*, Liao *et al.*, 1999; Steinke *et al.*, 1996
	MA	QCM Optical	Cao *et al.*, 2001 Kriz *et al.*, 1995
	Phenylenediamine	QCM	Peng *et al.*, 2001
Aniline, phenol and derivatives	Polyaniline SAM	Amperometry Amperometry	Kriz and Mosbach, 1995 Morita *et al.*, 1997
Anions and cations	Polypyrrole Polyvinyle	Potentiometry	Hutchins and Bachas, 1995 Murray *et al.*, 1997
Atrazine and other triazines	MA PM	Optical Optical	Piletsky *et al.*, 1997; Sergeeva *et al.*,, 1999
Automotive engine oils	Polyurethane	QCM	Dickert *et al.*, 2000
Barbituric acid	SAM	Capacitance	Mirsky *et al.*, 1999
Carboxylic acids		Optical	Zhang *et al.*, 2001
Chloramphenicol, Thiamphenicol		Optical	Levi *et al.*, 1997
Cholesterol	PM	Conductometry Optical	Piletsky *et al.*, 1992 Sergeeva *et al.*, 1999
Cinchola alkaloids			Matsui *et al.*, 2000
Clenbuterol	PM	Amperometry	Pizzariello *et al.*, 2001
2,4-D		Optical Amperometry Potentiometry	Haupt *et al.*, 1998a; 1998b, Haupt, 1999, Surugiu *et al.*, 2001 Kroger *et al.*, 1999 Lahav *et al.*, 2001
Epinephrine		Optical	Piletsky *et al.*, 2000b
β-Estradiol		Optical	Rachkov *et al.*, 1998; Haginaka and Sanbe, 1998; Rachkov *et al.*, 2000
Flavinol		Optical	Rodriguez and Garcia, 2000
Fluorescein		Optical	Lulka and Chambers 27
Gases		Electrochemical	Kodakari *et al.*, 1998; Hernandez and Bachas, 1998.
2-MIB (methyl isoborneol)	PM	QCM	Ji *et al.*, 1999a, 1999b, 2000
Morphine	PM	Amperometry	Kriz and Mosbach, 1995
Nicotine	PM	QCM	Tan *et al.*, 2001
Nitrobenzene	PP	Amperometry	Panasyuk *et al.*, 1998

Nucleic acid and their derivatives	PM Polypyrrole	Electrochemical Optical Conductometry Potentiometry	Piletsky *et al.*, 1998 Piletsky *et al.*, 1996; Turkewitsh *et al.*, 1998 Piletsky *et al.*, 1990 Boyle *et al.*, 1989; Spurlock *et al.*, 1996
o-Xylene PAH	Styrene-DVB	QCM Optical	Dickert *et al.*, 1998 Dickert and Tortschanoff, 1999; Dickert *et al.*, 1999
		Optical	Dickert *et al.*, 1998
Paracetamol	4-VP	QCM	Tan *et al.*, 2001
Pb^{2+} ion		Optical	Murray *et al.*, 1997
Phenactin	PM	QCM	Tan *et al.*, 2001
(S)-propranolol		Optical	Ye and Mosbach., 2001
Phenols		Electrochemical	Morita *et al.*, 1997
Sarin and soman		Optical	Jenkins *et al.*, 1998
Sugars	Allylamine	Optical	Piletsky *et al.*, 1996; Sergeeva *et al.*, 1999; Wang *et al.*, 1999
	PM	Conductometry	Piletsky *et al.*, 1998
	o-Phenylenediamine	Potentiometry Capacitance	Chen *et al.*, 1997 Cheng *et al.*, 2001
Terpene	PM	QCM	Percival *et al.*, 2001
Testosterone		Optical	Cheong *et al.*, 1997; Cheong *et al.*, 1998
Thephylline, caffeine, xanthine,	MMA	Optical	Lai *et al.*, 1998; Vlatakis *et al.*, 1993
Caffeine	Polyacrylonitrile with pyridine and styrene moieties	QCM	Kobayashi *et al.*, 2000
Vitamin- K1	Octadecylsilane	QCM	Andersson *et al.*, 1988

2.7.2 Development of MIP based receptor sensors

The second class of MIP affinity sensors, receptor sensors, can be further subdivided into two classes. The first sub-class is related to the MIP's ability to change conformation upon binding with template, leading to change in a measurable property, such as conductivity, permeability or surface potential (Piletsky *et al.*, 1998). The second sub-class is based on the ability of a functional monomer to change its property upon interaction with template, most frequently, fluorescence (Rathbone *et al.*, 2000).

Piletsky *et al.* (1992b) first reported the receptor properties of imprinted materials. In their earlier works, they showed that the templates such as amino acids, nucleic acids and cholesterol increase the transport of ions passing through the imprinted membranes (Piletsky *et al.*, 1994b; Piletsky *et al.*, 1998). This "gate effect" has been used for quantification of the concentration of templates. Since then, there has been a number of publications related to this work (Hedborg *et al.*, 1993; Piletsky *et al.*, 1994; Yoshimi *et al.*, 2001; Cheng *et al.*, 2001).

Specific interaction of MIPs with template molecules has been explored in MIP sensors that measured alternations in membrane electroconductivity. Sensors, specific for sugars (Piletsky *et al.*, 1998), L-phenylalanine and cholesterol (Pileltsky *et al.*, 1994) and atrazine (Sergeyeva *et al.*, 1999) show high selectivity and sensitivity at the micromolar-nanomolar range (**Figure 2.25**).

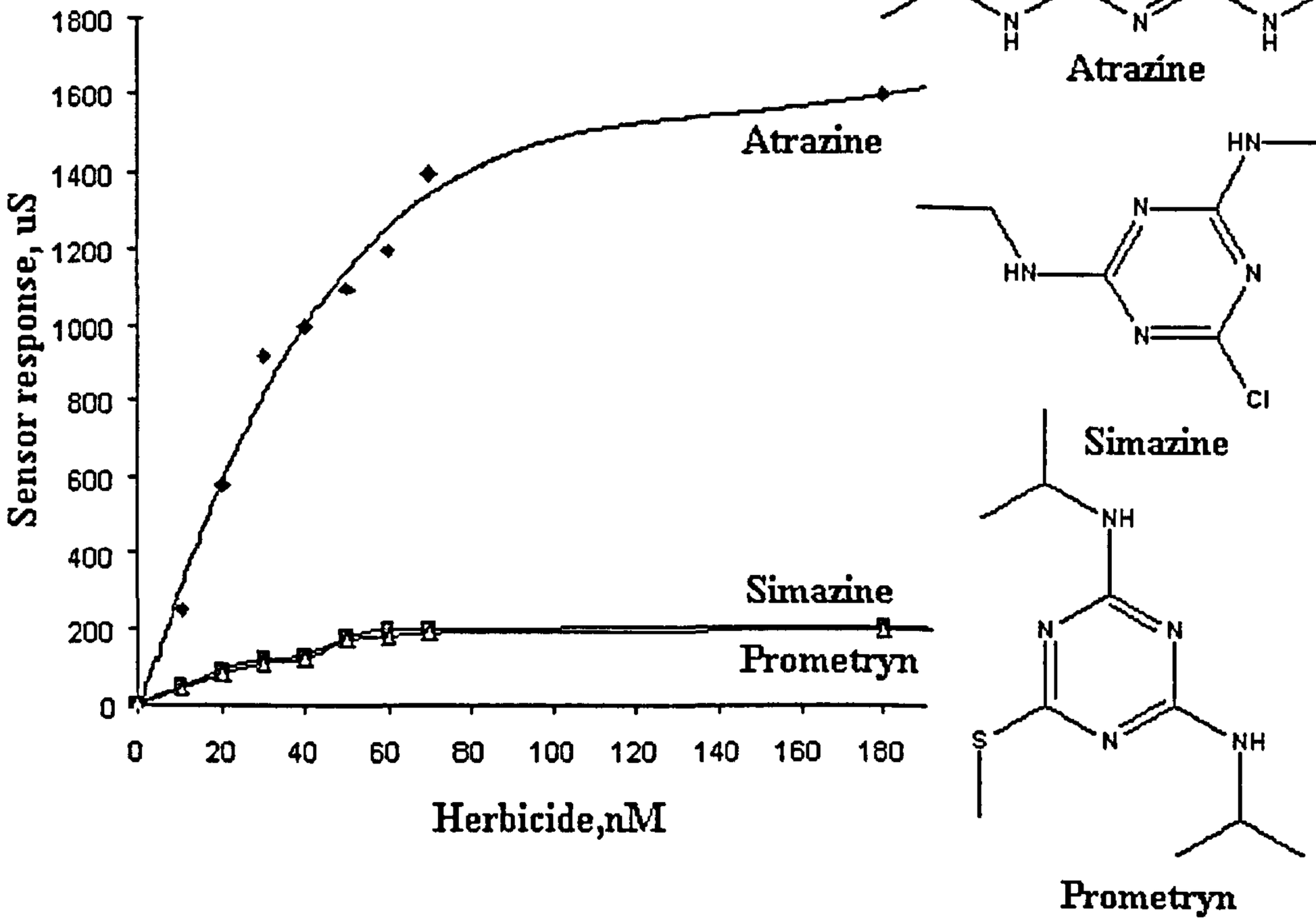


Figure 2.25 Sensor response for herbicides obtained for a polymer imprinted with atrazine (Sergeyeva *et al.*, 1999).

In another piece of work, MIP membranes were used as a sensing layer in capacitance sensors. This was a field effect device consisting of silicon wafers with SiO_2/MIP coating. Specific binding of the original template to the MIP resulted in the decrease in membrane capacitance (Hedborg *et al.*, 1993).

Preparation of potentiometric MIP sensors, based on electropolymerised materials was first described by Vinokurov (1992). Here the monomers themselves reacted as templates forming specific polymers. Sensors for pyrrole, aromatic amines and substituted phenols, based on polypyrrole, polyaniline and aniline-p-aminophenol copolymers, respectively, have been developed. A successful potentiometric sensor was later reported for the detection of glucose (Chen *et al.*, 1997). This was based on the measurement of concentration of protons released during interaction of metal-

complexing imprinted polymer with glucose. In this work, clinically relevant range of measurement of glucose concentration was reported (0 to 25 mM) in plasma.

Although the majority of published papers related to MIP sensors deal with electrochemical or piezoelectric devices, we believe that optical devices in general and fluorescent sensors in particular will play a major role in the future. This argument is based on the fact that the fluorescent detection offers high flexibility to MIP technology. The following section of this review elaborates current achievements and future prospects for commercialisation of receptor based MIP sensors, which use optical detection for template recognition.

Piletsky *et al.* (1996) proved that the "gate effect" could also be probed using optical detection. Here MIP based on allylamine was imprinted with sialic acid. A fluorescent complex was formed when polymer suspension was brought into contact with OPA reagent, a mixture of o-phthaleic dialdehyde and mercaptoethanol. The kinetics of complex formation depended on the presence of the template (sialic acid), which modulated the diffusion of soluble components to the reactive sites (**Figure 2.26**). The polymer was able to discriminate sialic acid from other sugars such as glucose and mannose.

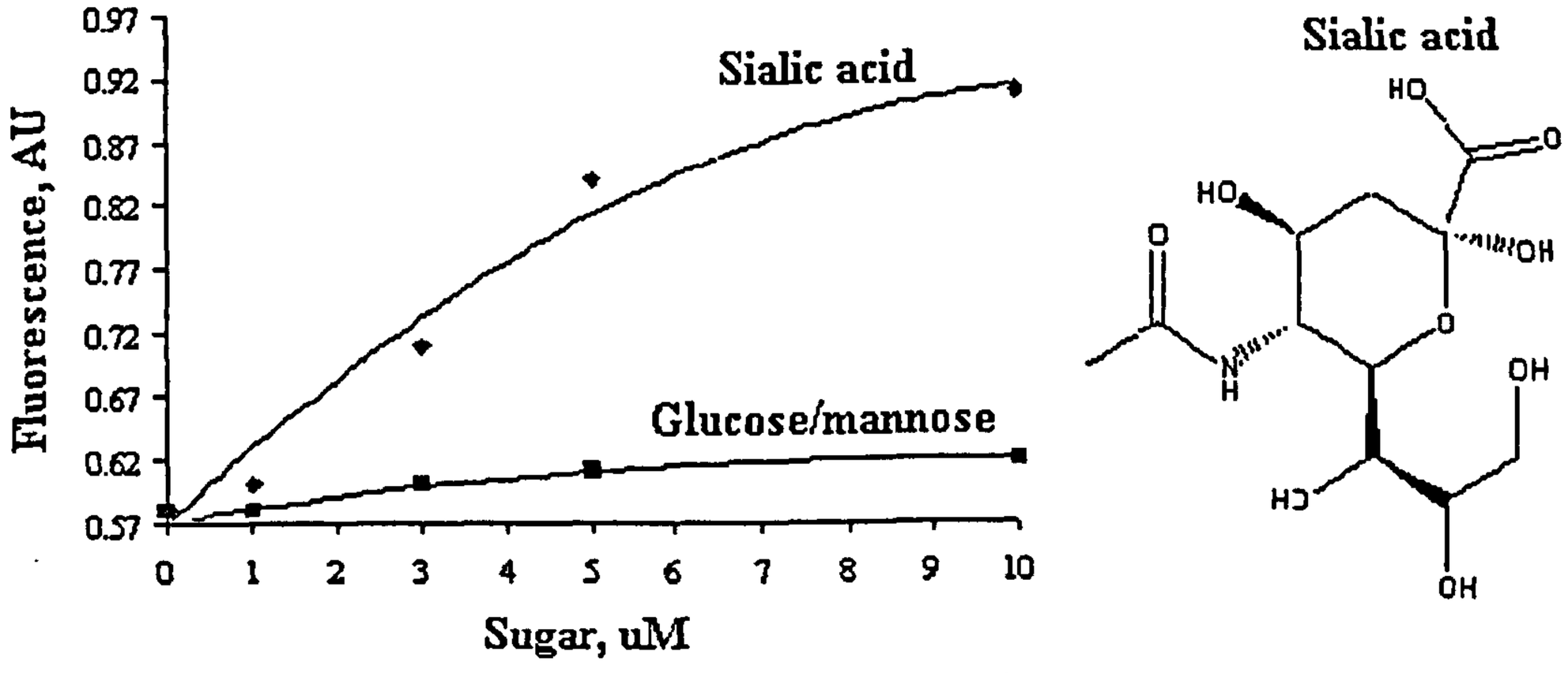


Figure 2.26 **Influence of sugars on the formation of fluorescent isoindole complex between OPA reagent and amino-functionality in the polymer imprinted with sialic acid (Piletsky *et al.*, 1996).**

The second class of receptor sensors explores the ability of fluorescent functional monomers to demonstrate dual functions: recognition and signalling. Cooper and co-authors proposed an interesting approach for the design of signalling polymers and their use in sensors (Cooper *et al.*, 1997). They integrated a functional monomer into a cross-linked matrix, which was able to change its fluorescence properties in the presence of proton donor compounds.

A fluorescent sensor for cAMP detection was constructed using environment-sensitive dye (Turkewitsch *et al.*, 1998). In this work (fluorescent sensitive dye), trans-4-[p-(N,N-dimethylamino)styryl]-N-vinylbenzylpyridinium chloride) was co-polymerised with cross-linker and template. The resulting polymer displayed both functions of template recognition and sensing (**Figure 2.27**).

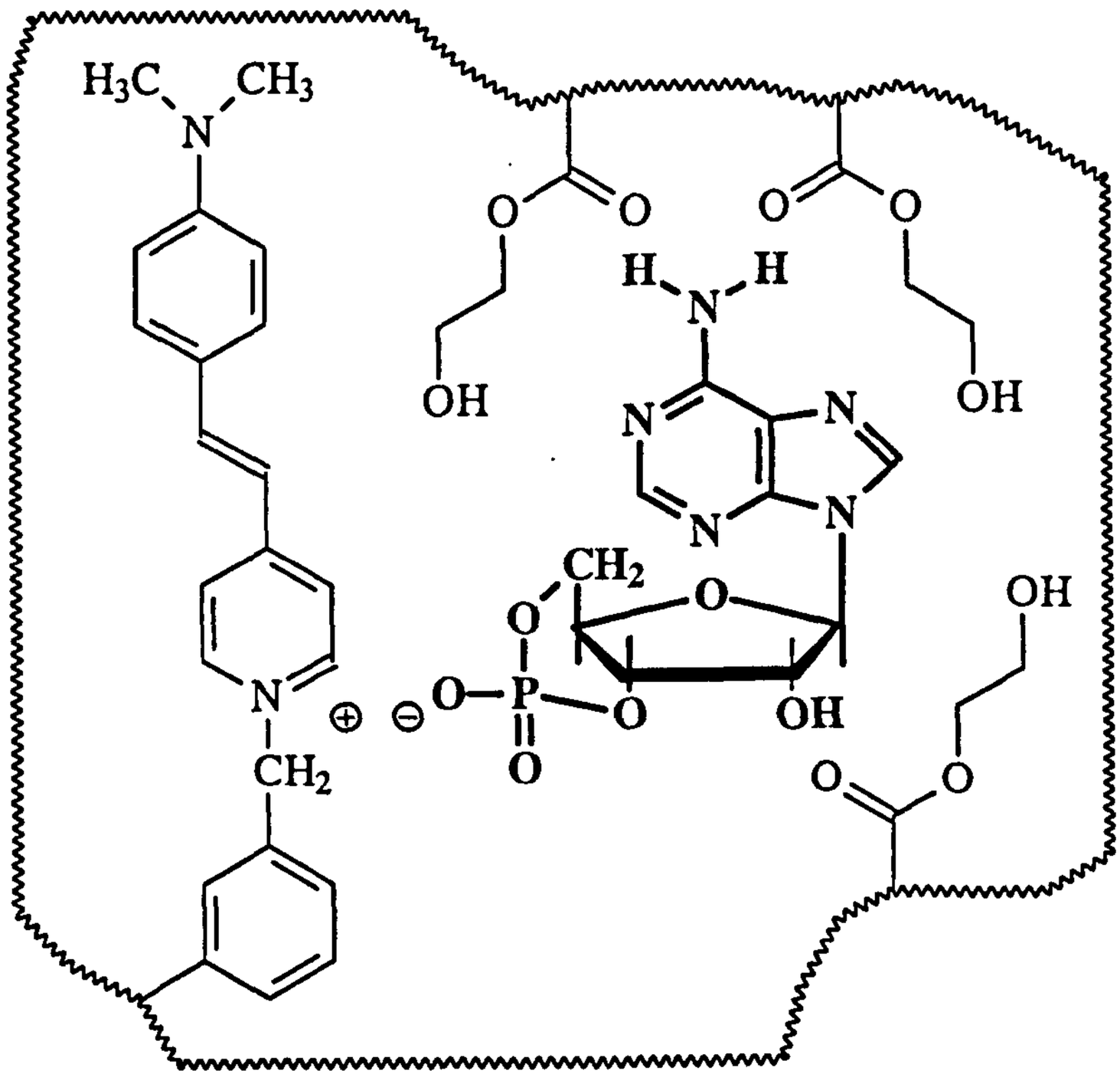


Figure 2.27 **Schematic representation of polymer binding site for cAMP with signalling trans-4-[p-(N,N-dimethylamino)styryl]-N-vinylbenzylpyridinium monomer (Turkewitsch *et al.*, 1998).**

Ye and Mosbach (2001) reported a proximity scintillation assay for (S)-propranolol. During the imprinting reaction they covalently incorporated a scintillation monomer (4-hydroxymethyl-2,5-diphenyloxazole acrylate) into MIP microparticles. This

monomer was capable of transforming β-radiation from the bound tritium-labelled template, into a fluorescent signal. To maximise the signal authors used small particles (0.6-1 μm) with reporter groups randomly distributed throughout the polymer matrix, which ensure their positioning in close proximity to the MIP binding site necessary for effective signal generation.

Matsui *et al.* (2000) used another approach where the signal was generated by an environment-sensitive template. Polymer particles containing acidic monomer - 2-(trifluoromethyl) acrylic acid (TFMAA) shift their fluorescent spectra when bound to cinchona alkaloids (**Figure 2.28**).

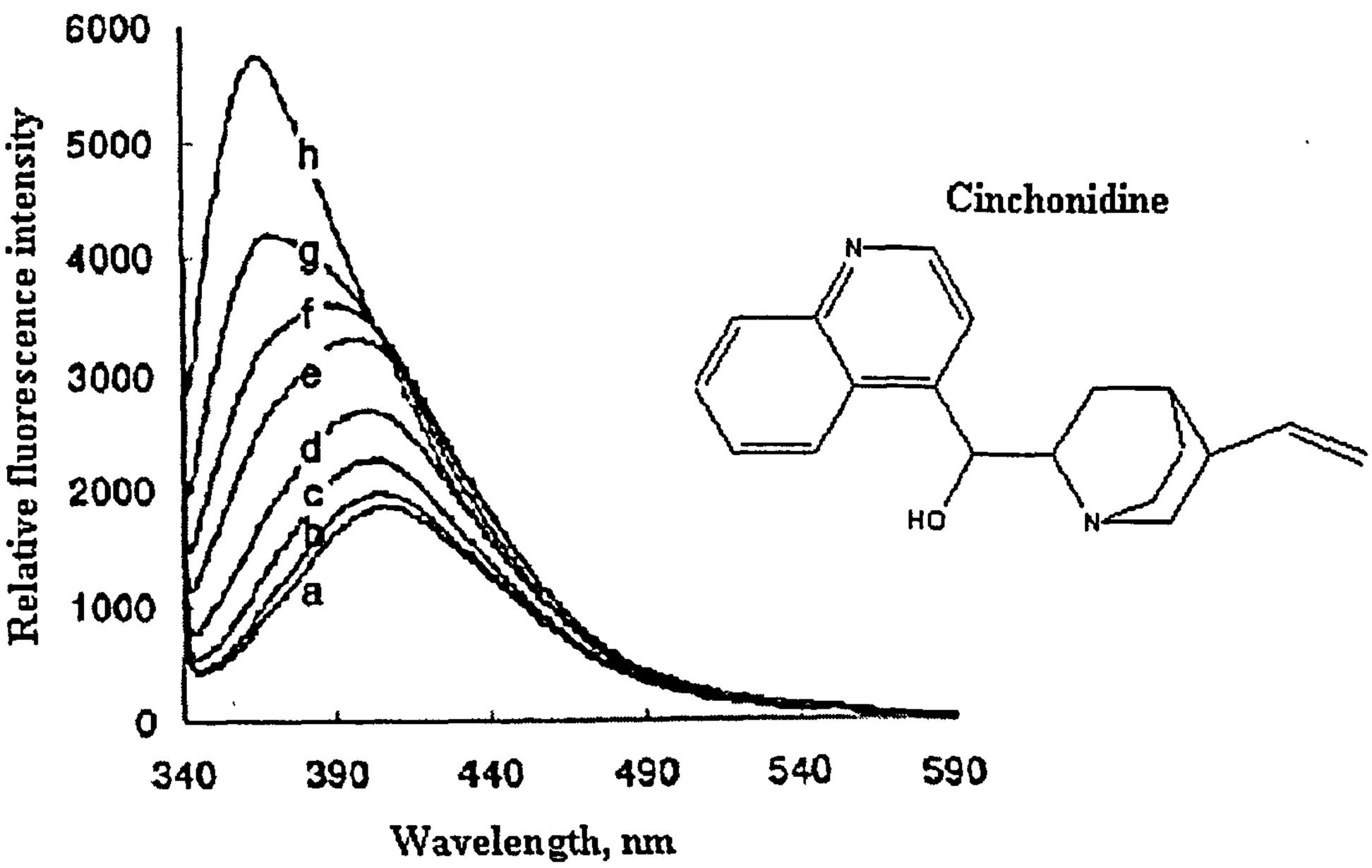


Figure 2.28 **Fluorescence spectra of MIP suspension incubated with cinchonidine (a, 0 b, 0.005; c, 0.02; d, 0.05; e, 0.10; f, 0.15; g, 0.25; h, 0.50 mM) in chloroform/acetonitrile (ex, 330 nm) (Matsui *et al.*, 2000).**

Highly specific MIPs based on group specific fluorescent reporters have been reported for sugars (Wang *et al.*, 1999), carboxylic acids (Zhang *et al.*, 2001) and primary amines (Subrahmanyam *et al.*, 2000). A detailed discussion of the MIPs designed to be selective for primary amines is presented in **Chapter 4** of this thesis.

The principal limiting factor in the operation of practically all MIP sensors is the slow diffusion of substances in MIPs, which complicates the regeneration of the polymer and slows the response time (to up to 40 min). This has been a problem for both, 2-dimensional and 3-dimensional MIPs (Mirsky *et al.*, 1999). The major reason for this is that the sensor response of imprinted sensors is controlled by template-polymer interaction rather than in-film diffusion (Spurlock *et al.*, 1996). These long detection times make it necessary to use a kinetic mode for applications where rapid measurement is important.

2.7.3 Development of catalytic sensors based on MIPs

Although immense progress has been made in the field of MIP catalysts (Ohkubo *et al.*, 2001; Motherwell *et al.*, 2001), there has been virtually no published work on catalytic sensors based on MIPs. Recently, in what could be claimed as the first example of catalytic MIPs, Piletsky *et al.* (2002) integrated MIPs with sensors to produce a synthetic enzyme electrode. They synthesised artificial tyrosinase using molecular imprinting of a transition stage analogue representing a complex of polymerisable imidazole, copper and catechol (**Figure 2.29**). The resulting polymer exhibited catalytic turnover, Michaelis-Menten kinetics and competitive inhibition, which were all similar to those of the natural enzyme (**Table 2.7**).

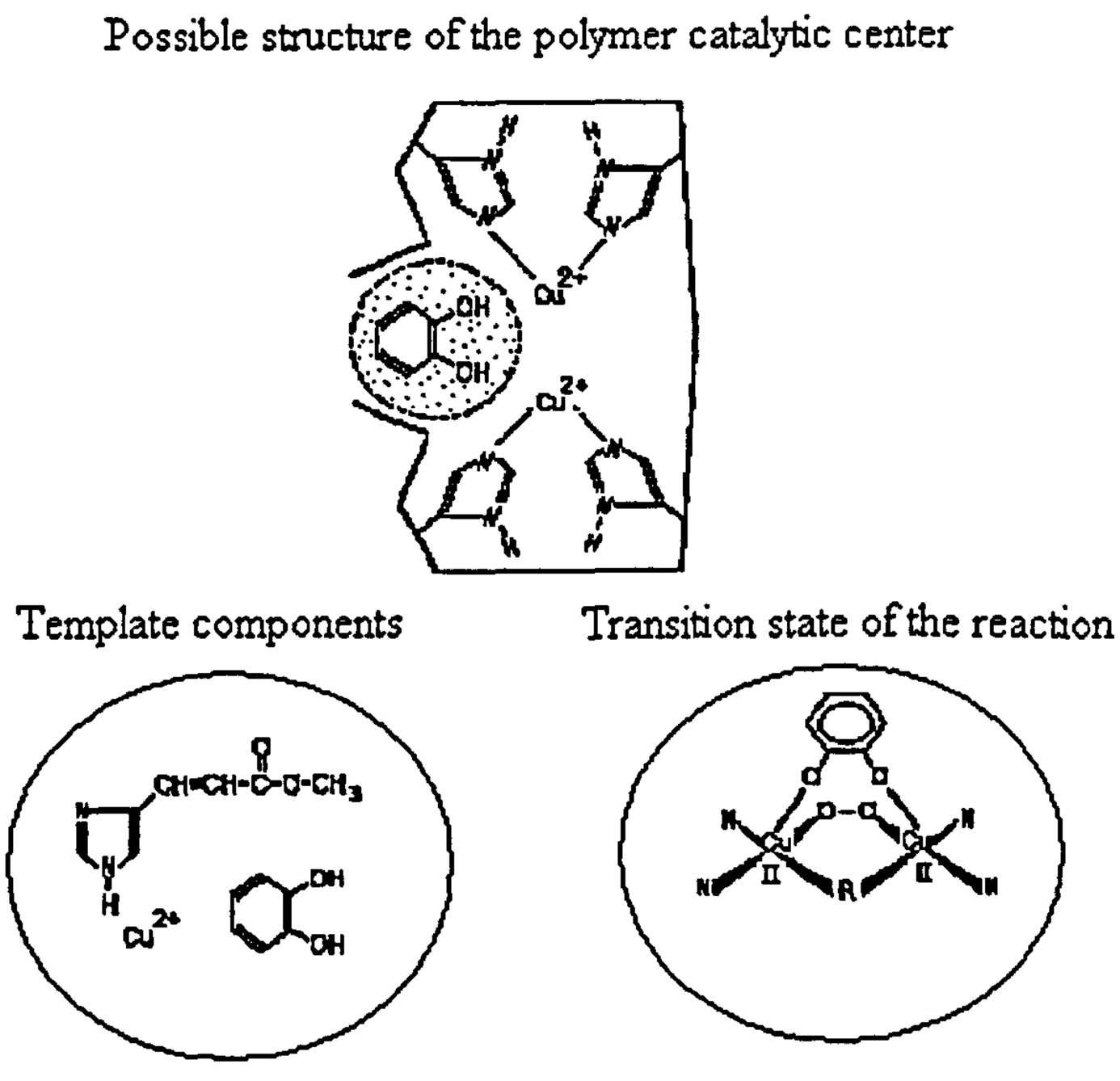


Figure 2.29 Possible structure of the catalytic site of MIP mimicking tyrosinase and properties of synthesised polymers (Piletsky *et al.*, 2002).

Table 2.7 **Catalysis of catechol oxidation by natural and synthetic enzymes. Reaction conditions: 5 mM CuCl solution in 100 mM Tris-HCl, pH 8.0. For comparison mushroom tyrosinase (2000 U/mg) was used in 30 µg/ml concentration (Piletsky *et al.*, 2002).**

Catalyst	Michaelis constant Km, mM	Vmax x 10^{-7} Ms^{-1}
MIP (template – catechol)	4.3 ± 1.1	21.0 ± 2.6
Control (template – resorcinol)	3.9 ± 1.4	4.0 ± 0.8
Tyrosinase	0.9 ± 0.4	8.5 ± 2.1

Further developments in catalytic receptors based on MIPs will depend largely on success in the preparation of MIP-based catalysts which can react with water-soluble and practically important analytes. We believe that this will improve the feasibility of developing a range of enzyme-mimicking MIP sensors or synthetic enzyme sensors.

2.7.4 Linked technology - solid phase extraction (SPE).

Sample pre-treatment contributes to 90% of analysis costs and forms the most time-consuming and least automated step in an analysis (Majors, 1995). Analyte enrichment and removal of interfering matrix compounds is required to achieve suitable low detection limits. Clean-up and concentration steps include liquid-liquid partition (Valenta, 1998) solid phase extraction with reversed-phase (Zollner et al., 2000) and ion-exchange materials (Biancardi and Riberzani, 1996). Since these techniques rely on relatively unselective interactions (Jodlbauer et al., 2002) the resultant clean-up level may be insufficient for some challenging matrices. As a result, sample preparation with antibody-based immunoaffinity columns became increasingly popular as a selective, simple and time-saving tool for toxin analysis (Scott and Trucksess, 1997; Visconti *et al.*, 1999; Zimmerli and Dick, 1995).

Specific MIPs can be designed for fast recovery of environmental pollutants and clinical analytes from real samples. It is also possible to produce inexpensive polymers that can

recover a group of chemicals, which allows for rapid, large scale and accurate screening of samples. The robustness of MIPs allows them to be reusable.

The basic concept for MIP application in SPE is to tune the chromatographic parameters of the adsorbent so that the MIP-SPE column traps the analyte, or a group of structurally related compounds, whereas matrix components are not retained (Anderson, 1998). After washing these off the MIP-SPE column, the compound of interest is eluted for further analysis.

The influence of the matrix on the MIP-SPE is particularly important for analysis of biological samples. In order to achieve good reproducibility and increase detection limits, matrix components present in solvent extracts of, e.g., bovine liver (Muldoon and Stanker, 1997) and human plasma (Andersson et al., 1997) should not interfere with template recognition by the polymer. Recently, the direct MIP-SPE of propranolol from crude samples, including human urine, dog plasma, and rat bile, has been demonstrated (Martin et al., 1997). In additon, the feasibility of MIP based SPE approach has been described in several applications (**Table 2.8**).

Table 2.8 Some examples of biological, pharmaceutical and environmental templates used in MISPE

Analyte	Sample	References
4-aminopyridine (K^+ channel blocker)	Human serum	Mullett *et al.*, 2000
Atenolol (anti-hypertensive and anti-arrhythmic)	Methanolic or acetonitrilic solution	Stevenson, 1999
Atrazine and other triazines (herbicides)	Beef liver extracts	Muldoon and Stanker, 1997
	Tap water	Ferrer and Barcelo, 1999; Matsui *et al.*, 2000
	Human urine	Bjarnason *et al.*, 1999
	Apple extracts	Matsui *et al.*, 1997
Bupivacaine (anaesthetic)	Human plasma	Andersson, 2000
Bentazone (pesticide)	Aqueous sample	Baggiani *et al.*, 1999
Clenbuterol (growth promoter)	Calf-urine	Berggren *et al.*, 2000
Darifenacin (to treat urinary incontinence)	Human plasma	Venn and Goody, 1999
7-hydroxycoumarin (coumarin)	Human urine	Walshe *et al.*, 1997
Indole acetic acid (plant hormone)	Chloroform	Kugimiya and Takeuchi, 1999
4-nitrophenol (phenolic compound)	Natural waters	Masque *et al.*, 2000
Nicotine	Chewing gum	Zander *et al.*, 1998
Pentamidine (to treat AIDS-related pneumonia)	Human urine	Sellergren, 1994
Propranolol (anti-hypertensive and anti-arrhythmic)	Dog plasmas, rat bile and human urine aqueous solution	Martin *et al*, Olsen *et al.*, 1999
Sameridine (local anaesthetic and analgesic)	Human plasma	Andersson *et al.*, 1997
Tamoxifen (anti-oestrogenic)	Human plasma and human urine	Rashid *et al.*, 1997
Theophilline (bronchodialator)	Human serum	Millett and Lai, 1998, 1999a and 1999b

2.7.5 Niche areas for application of MIP sensors.

Three particular properties make the commercial application of MIP sensors attractive:

a) MIPs are highly stable and can be autoclaved;

b) they are fully compatibile with microfabrication technology, and

c) the low cost of the materials and easy processes of polymer preparation in comparison with natural and other artificial receptor systems.

The key areas of MIP sensor applications are:

a) chemical and pharmaceutical manufacturing: using MIP sensors in extreme conditions (high and low pH's, toxic solvents and high temperature, pressure and radiation);

b) medicine and pharmaceuticals: application of MIPs mimicking natural receptors for drug screening and for in vivo monitoring;

c) environment: remote sensing, continuous emissions sensors and point-source monitors;

d) defence: rapid detection of chemical and biological warfare agents under battlefield and civil conditions;

e) deep and space exploration: sensors for analysis of extreme environments.

Arguably the most promising of these is in the chemical and pharmaceutical industry. It is estimated that pharmaceutical firms spend over $4 billion/year on sensors. On-line monitoring of industrial processes remains elusive for biosensors. However, MIP sensors could be used directly in the control of industrial processes due to their high stability and ability to withstand extreme pHs, pressure, temperature and organic solvents,

Clinical analysis and *in vivo* sensing is the second largest potential market for MIP-based sensors. The market for biochips for blood testing in hospitals and clinics alone is worth about $10 billion. MIPs do not induce an immune response like natural biomolecules and they can be autoclaved and hence are ideal materials for the development of implantable sensor devices. An important point in consideration of MIP sensors is the low cost of the

materials and the relatively easy preparation processes, which make them very competitive in mass production in comparison with natural receptor-based systems.

The third largest area for the application of MIP sensors is in environmental monitoring and in particular unattended sensing at remote sites. Although this is not a big market, it is important because of the absence of competition from traditional biosensors, which have too short a lifetime for this application. Both industry and environmental agencies have a need for remote MIP sensors, continuous emissions sensors and point-source monitors.

A particularly important application of MIPs in environmental monitoring could be the development of sensors for various toxins. Examples of these include algal toxins, e.g. microcystin, domoic acid, fungal toxins, e.g. aflatoxins, ochratoxins and man-made substances (pesticides and herbicides). The traditional methods of analysis of toxins are based on HPLC/MS or enzyme linked assays (ELISA). The raising of antibodies for ELISA is affected by the inherent toxic nature of these compounds on the system used. In contrast to antibodies, MIPs can be easily prepared for any toxin.

MIPs can also be used for the development of multisensors such as chemical "noses" and "tongues". The market for intelligent multisensors could be worth as much as $21 billion (Piletsky *et al.*, 2001). It holds enormous potential in a wide variety of industries and products such as health care, testing of quality of products, authenticity of designer perfumes and wines, and in landmines testing. Although this technology does not require high specificity, there are exceptions, for example drug testing and high throughput screening where artificial receptors based on MIPs could substitute their natural counterparts.

An interesting combination can be achieved by linking MIP-based solid phase extraction (SPE) materials with existing analytical equipment in order to increase its sensitivity and specificity. The second section of the **Chapter 4, (*Results and discussion*)** presents and discusses the results of experiments on solid phase extraction of aflatoxin-B1 using MIPs.

Despite the fact that many successful examples of the development of MIP-based sensors exist, as shown in this review, the current level of commercial activity related to their development and marketing remains low. With further progress in polymer science and engineering we expect the development of new generation of MIP sensors, which will gradually replace traditional biosensors in many areas of biotechnology, environmental, clinical and food analysis. Several key problems associated with MIP development need to be addressed, however, before successful commercialisation can commence. The issues include:

(i) development and validation of a general protocol for MIP design;

(ii) development of MIPs capable of effective functioning in water;

(iii) the need for a substantial increase in polymer affinity and improvement of the ratio between specific and non-specific binding;

(iv) development of effective immobilisation protocols.

Chapter 3

Materials and Methods

3.1 Materials

Ethylene glycol dimethacrylate (EGDMA) and 1,1'-azobis (cyclohexane-carbonitrile), were purchased from Aldrich (Poole, Dorset, UK). The urocanic acid ethyl ester was a gift from Institute of Molecular Biology and Genetics, Kiev. Ukraine. Dimethyl sulfoxide (DMSO) was purchased from Merck (Poole, Dorset, U.K.). Creatine, creatinine and aflatoxin-B1were purchased from Sigma- Aldrich, (St Louis, USA).

Empty solid phase extraction cartridges (1ml filtration tube cartridges) were purchased from Supelco (Bellefonte, PA, USA).

3.2 Methods

3.2.1 MIP for creatine

Synthesis of methylated creatine

Creatine (114 mg) was dissolved in 96 ml of methyl iodide and the mixture was stirred for 8 hours. The product was evaporated in vacuum and used for the MIP preparation.

Synthesis of MIP for creatine

o-Phthalic dialdehyde (134 mg) was dissolved in the 2 ml of DMSO. 82 mg of allyl mercaptan, 114 mg of the methylated creatine and 50 mg azobis (cyclohexane carbonitrile) were added to the solution and left for 1 hour at room temperature. The reaction mixture was incubated overnight at 80°C. The preparation of MIP for creatine is detailed in **Table 3.1.** The resulting polymer was ground, washed with 50% methanol solution containing 10 mM HCl, sieved and sedimented in acetone giving a suspension with average particle size of 5 μm. Judging from the elemental analysis, 10% of the template remained trapped in the polymer. The structures of creatine and methyl creatine are given in **Figure 3.1.**

Fluorescence measurements

Fluorescent measurements were carried out in a 4 ml cuvette, using a Fluoromax-2, Jobin Yvon-Spex instruments S.A., Inc (USA). A typical fluorescence experiment was as follows. Analyte (200 µl) ranging in concentration from 0.1 to 1 mM, was added to 2 ml of polymer suspension (3 mg/ml) in 100mM sodium phosphate or sodium borate buffer. The suspension was stirred and polymer fluorescence measured over 1 hour. Excitation and emission wavelengths were 320 nm and 410 nm, respectively.

3.2.2 MIP for creatinine

Synthesis of methylated creatinine

Creatine (114 mg) was dissolved in 96 ml of methyl iodide and the mixture was stirred for 8 hours. The product was evaporated under vacuum and used in the preparation of the MIP.

Synthesis of MIP for creatinine

O-phthalic dialdehyde (134 mg) and allyl mercaptan (82 mg) were dissolved in 2 ml of DMSO and mixed with methylated creatine (113 mg) (MIP A) or methylated creatinine (131 mg) (MIB B). Functional monomers, 258 mg of methacrylic acid (MIP B) or 138 mg of urocanic acid ethyl ester (MIP C) and 50 mg azobis (cyclohexane carbonitrile) were dissolved in reaction mixture, the solution was purged with nitrogen and left for 1 hour at room temperature. The polymerisation was initiated by heating overnight at 80°C. Polymer composition for MIP A, MIP B and MIP C are given in **Table 3.1.** The resulting polymer was ground, washed with 50% methanol solution containing 10 mM HCl, sieved and sedimented in acetone yielding a suspension with average particle size of 5 µm. The structures of creatinine and methylated creatinine are shown in **Figure 1.**

Fluorescence measurements

Analyte (200 µl) ranging in concentration from 10 to 2000 µM was added to 2 ml of polymer suspension (3 mg/ml) in 100 mM sodium phosphate or sodium borate buffer pH 7.0. The suspension was stirred and polymer fluorescence measured over 1 hour. Excitation and emission wavelengths were 320 nm and 410 nm, respectively. All measurements were made in triplicate. To measure low concentrations of analyte, polymer suspension was immobilised onto the cuvette surface under agarose gel.

Table 3.1 Composition of polymers

Polymer	AM	OPA	MA	Urocanic acid ethyl ester	Creatinine	Creatine
MIP A	82mg (1µmol)	134mg (1 µmol)	-	-	-	131mg (1 µmol)
Blank A	82mg (1µmol)	134mg (1 µmol)	-	-	-	-
MIP A*	82mg (1µmol)	134mg (1 µmol)			113 mg (1 µmol)	
Blank A*	82mg (1µmol)	134mg (1 µmol)			-	
MIP B	330 mg (4 µmol)	563 mg (4 µmol)	258 mg (3 µmol)	-	113 mg (1 µmol)-	-
Blank B	330 mg (4 µmol)	563 mg (4 µmol)	258 mg (3 µmol)	-	-	-
MIP C	330 mg (4 µmol)	563 mg (4 µmol)	-	138 mg (1 µmol)	113 mg (1 µmol)	-
Blank C	330 mg (4 µmol)	563 mg (4 µmol)	-	138 mg (1 µmol)	-	-

AM – allyl mercaptan

OPA - o-phthalic dialdehyde;
MA – methacrylic acid;
All the monomer mixtures contained 2 ml of EDMA – ethylene glycol dimethacrylate and 2 ml of DMSO – dimethyl sulfoxide with 50 mg of ACC – azobis (cyclohexane carbonitrile).

3.2.3 MIP for aflatoxin-B1

Synthesis of MIP

Aflatoxin-B1 (10umol), allylamine (20umol) hydroxyethyl methacrylate (20umol) were dissolved in 2 ml of the solvent DMF. Then 2ml of crosslinker EGDMA, and azobis (cyclohexane carbonitrile) (50 mg) were added to the monomers, the solution was purged with nitrogen and left for 1 hour at room temperature. The polymerisation was initiated by heating overnight at 80 °C. The resulting polymer was ground and washed many times thoroughly with water and methanol. The polymer was then sieved and polymer with a particle size 30 to 100 microns was collected and packed into the cartridge.

Fluorescent measurement of aflatoxin-B1

After elution from the MIP-SPE cartridges, the sample was then measured using a fluorimeter. Excitation and emission wavelengths were 360 nm and 435 nm, respectively.

Calibration curve for aflatoxin-B1

A calibration curve for different concentrations of aflatoxin-B1 was plotted and is shown in **Figure 3.1.**

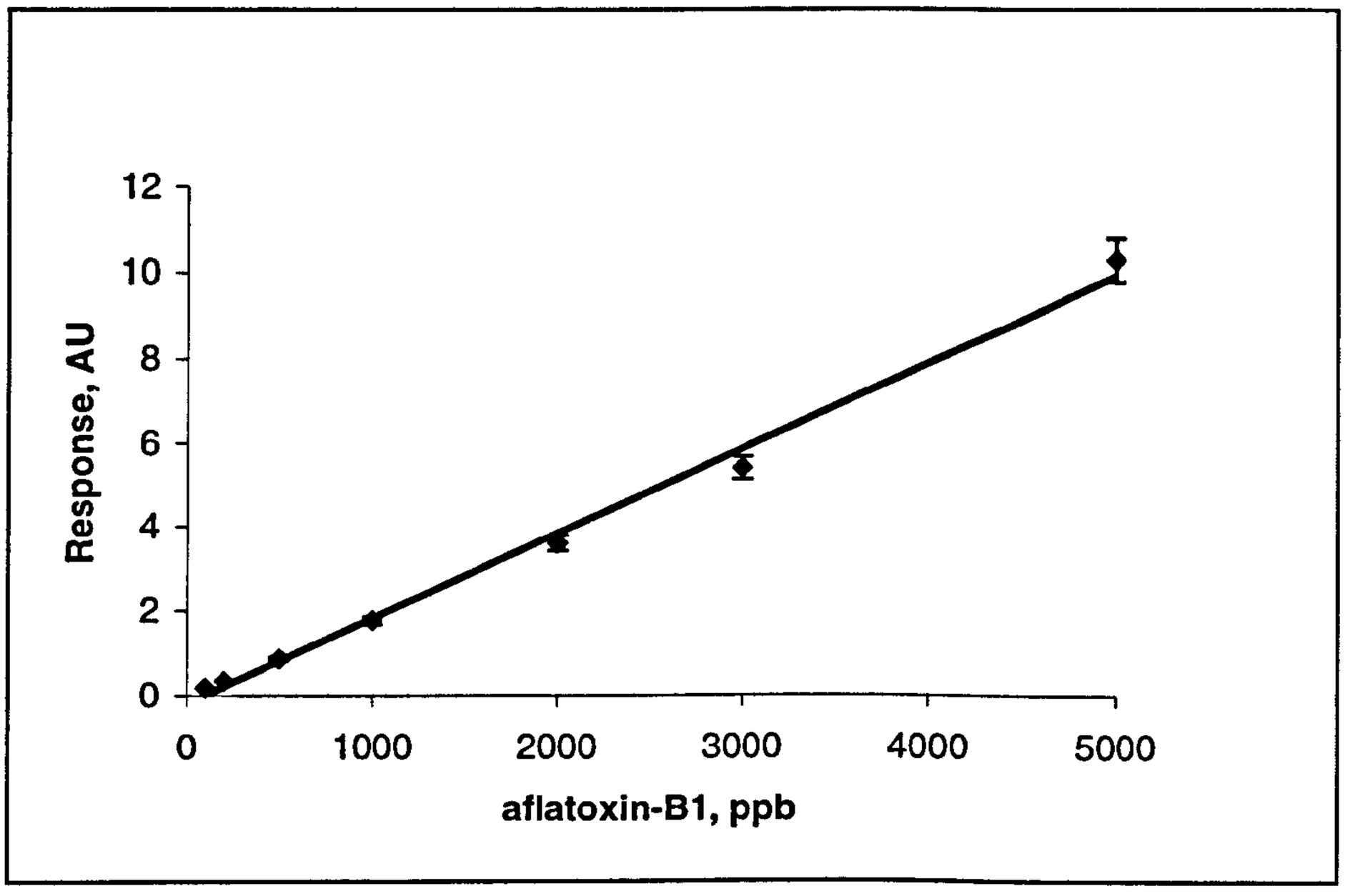


Figure 3.1 Calibration curve for the detection of aflatoxin-B1.

Solid phase extraction of aflatoxin-B1

Commercial SPE cartridges tubes and polyethylene frits (placed above and below the adsorbent) were packed with 2, 5 and 10 mg of polymer (MIP and Blank) and thoroughly washed with water and methanol. The cartridges were connected to a vacuum manifold.

A stock solution of 500 ppm of aflatoxin-B1 was prepared in methanol. From this stock, various dilutions ranging from 2 ppb to 5000 ppb of aflatoxin-B1 were prepared in appropriate buffer for further analysis. In a typical experiment, aflatoxin-B1 was loaded on to the cartridge under reduced pressure. The bound analyte was eluted from the cartridges with methanol as shown in **Figure 2** and analysed further using a fluorimeter.

Extraction of aflatoxin-B1 from corn samples

The extraction of aflatoxin from corn flour was performed as described elsewhere (AOAC official methods of analysis, 1990).

Corn sample (10 g) was weighed and mixed with 25 ml methanol-water mixture (55:45). To remove the fats from the corns, 10 ml of hexane and 0.4 g of NaCl were added to the mixture, which was then shaken for 1 hour and filtered through Whatman 4 filter paper. The methanol-water mixture was separated, mixed with chloroform added in equal proportion and shaken well in a separator. The chloroform extract was collected and dried. The aflatoxin-B1 was recovered in methanol and loaded onto a MIP cartridge for subsequent purification/preconcentration as described above.

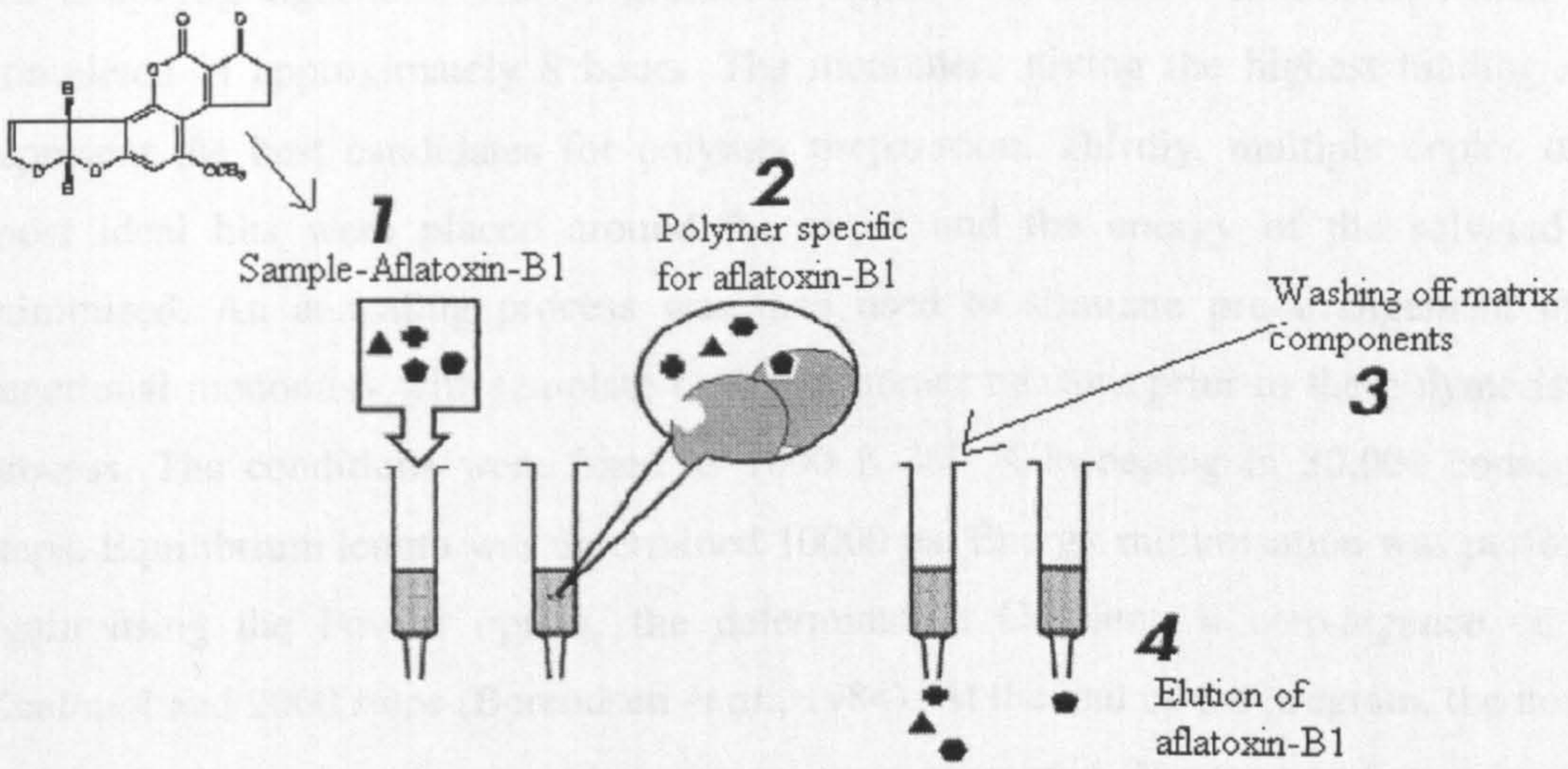


Figure. 3.2. **General method of aflatoxin extraction.** The sample is loaded on solid-phase extraction cartridge containing MIP (1), the analyte aflatoxin-B1 is specifically bound to the imprints of the polymer (2), and after washing off matrix components (3), the analyte aflatoxin is eluted (4).

3.2.4 Computer-aided design of MIPs

The computational work was performed using the molecular modelling software, Sybyl (Tripos Inc., St. Louis, USA), version 6.5, running on the Octane Silicon Graphics workstation, configured with 712 MB memory and 12 GB fixed drive.

The procedure of monomer selection included several stages. Firstly, a virtual library of molecular models of functional monomers and a molecular model of the template molecule were produced (Figure 3.3). Charges for each atom were calculated using Gasteiger-Huckel method, and the structure of the template and monomers refined using molecular mechanical methods (Press *et al.*, 1988). Secondly, each of the entries in the virtual library was probed for their possible interaction with the template molecule using the LeapFrog algorithm. The program was applied for 100,000 iterations, which were completed in approximately 8 hours. The monomers giving the highest binding score represent the best candidates for polymer preparation. Thirdly, multiple copies of the most ideal hits were placed around the target and the energy of the solvated box minimised. An annealing process was then used to simulate pre-arrangement of the functional monomers with template in the monomer mixture prior to the polymerisation process. The conditions were fixed as 1000 K-300 K sweeping in 30,000 consequent steps. Equilibrium length was determined 10000 ps. Energy minimisation was performed again using the Powell option, the determination Gradient, a convergence of 0.01 Kcal/mol and 2000 steps (Berendsen *et al.*, 1984). At the end of the program, the number and the position of the functional monomers were examined. The type and quantity of the monomers participating in the complex with template indicated the type and ratio of the template and monomers in optimised MIP composition.

Computational design of the MIP specific for creatinine

The LeapFrog analysis made for creatinine identified the following list of monomers which have reasonably high binding score: m-divinylbenzene (-6.66 kCal Mol^{-1}), urocanic acid ethyl ester (-6.48 kCal Mol^{-1}) and hemithioacetal (-6.31 kCal Mol^{-1}). These

monomers were then "packed" around the template using a solvation experiment. The monomers participating in the complex with template were chosen for the preparation of the MIP.

Computer aided design of the MIP specific for aflatoxin-B1

For aflatoxin-B1 the LeapFrog analysis identified the following list of monomers which have the highest binding scores for the template: allylamine (-26.72 kCal Mol $^{-1}$) and hydroxy ethyl methacrylate (-14.61 kCal Mol $^{-1}$).

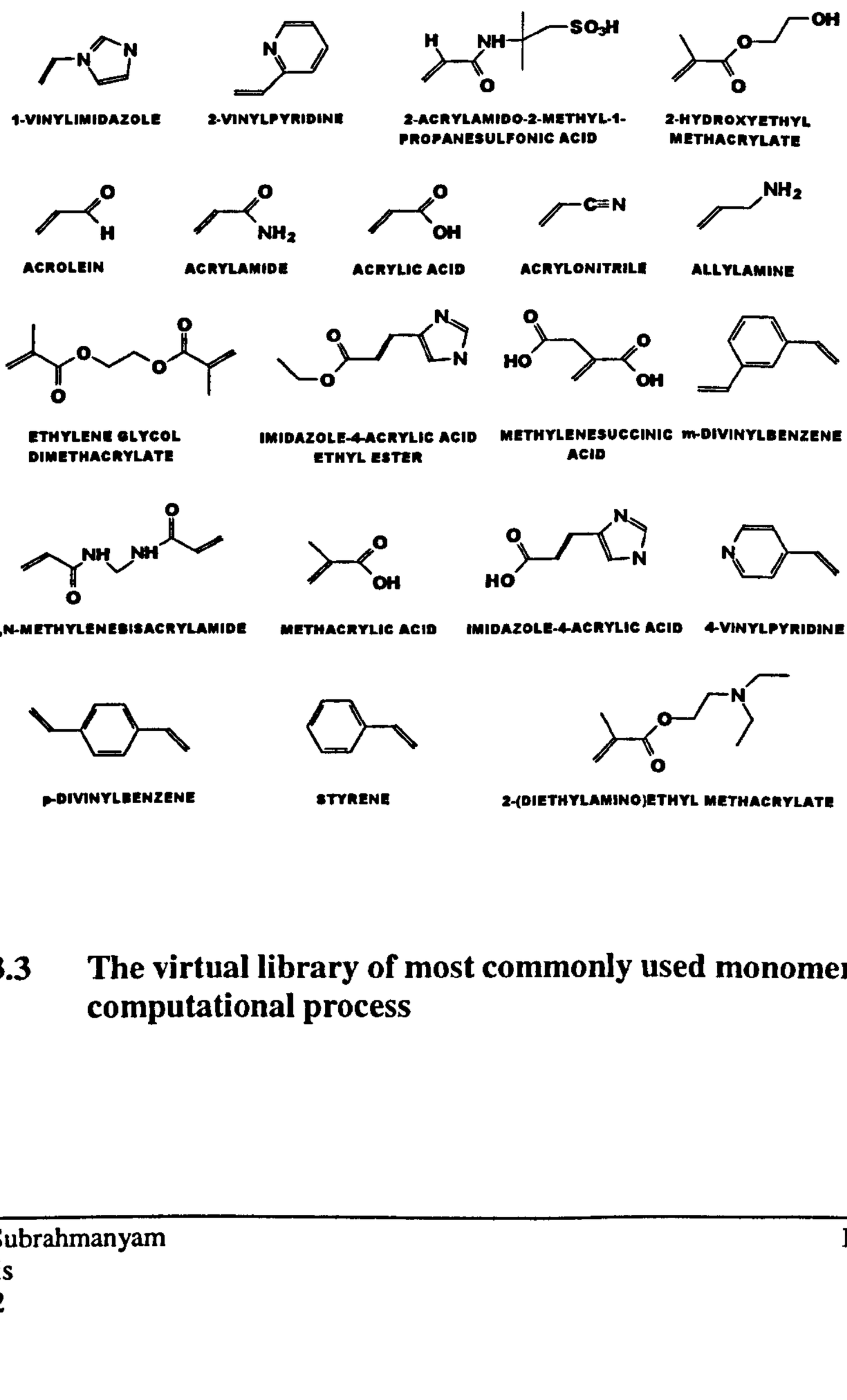


Figure 3.3 The virtual library of most commonly used monomers used for the computational process

Chapter 4

Results and Discussion

Two applications of MIPs, sensors for creatine and creatinine and solid phase extraction of aflatoxin-B1, are discussed in this chapter.

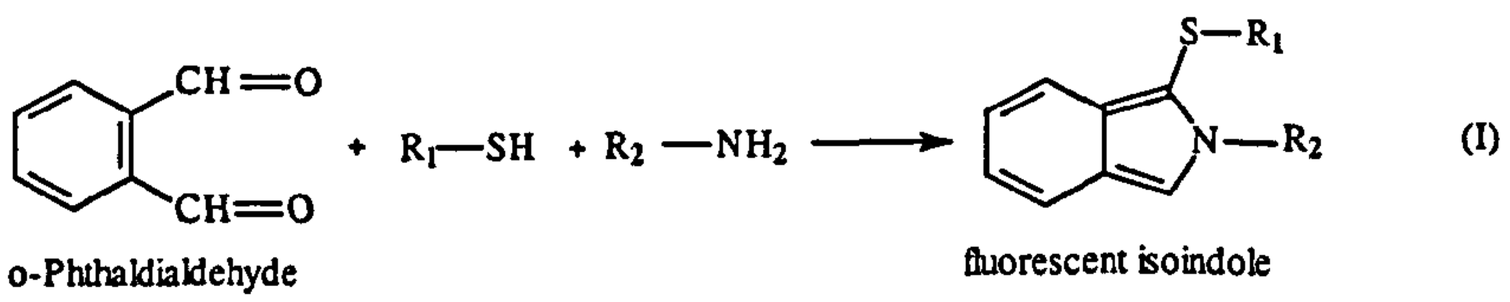
4.1 MIPs for sensing creatine

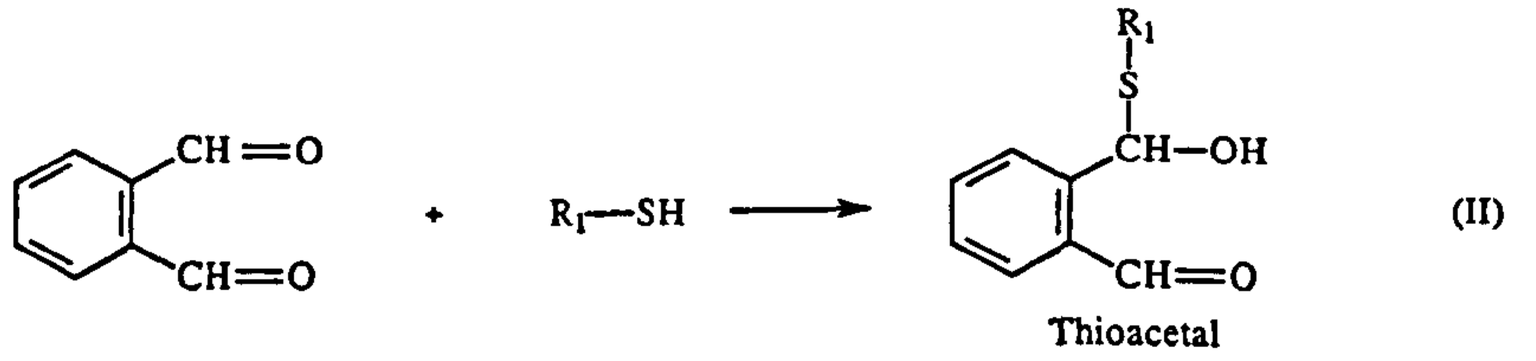
Despite the progress achieved in the area of MIP sensors, one of the greatest challenges remains the difficulty in transducing the binding event into the detectable signal. The first part of this chapter reports a new *"bite-and-switch"* approach for the design of recognition material for creatine and creatinine sensors based on imprinted polymers. The practical significance of this work lies in the fact that creatine has become one of the most popular nutritional supplements for resistance-trained athletes and body builders, and its detection is necessary in sports medicine (Williams and Branch, 1998). Additionally, large elevations in serum and urinary creatine and creatinine levels are basic markers of tissue degradation and/or kidney stress (Mihic *et al*, 1998; Peeters *et al*, 1999). The "bite-and-switch" approach combined with molecular imprinting can be used for the design of assays and materials for sensors specific for any amino containing substances and therefore has general significance for analytical chemistry.

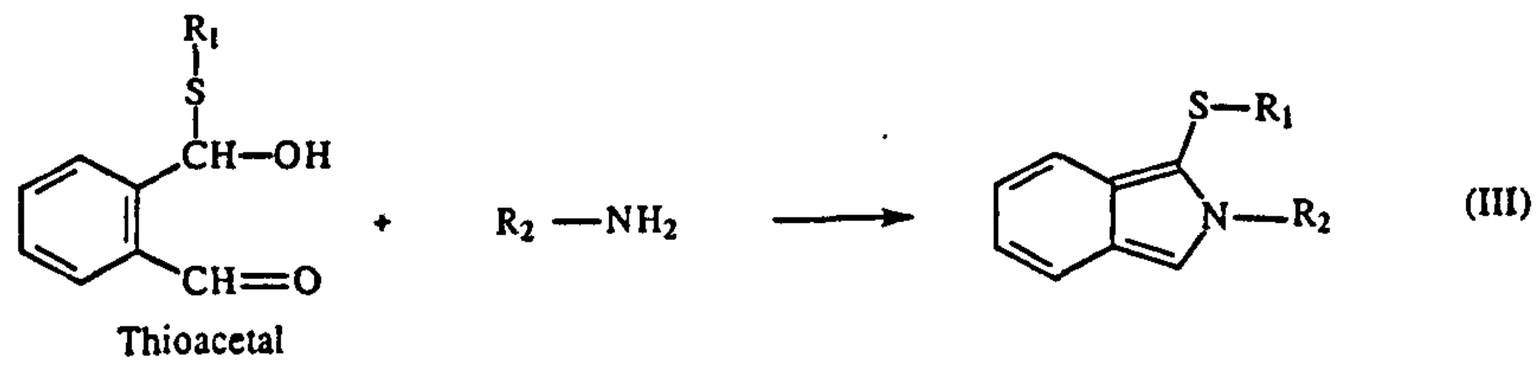
In this work we explore the ability of polymerisable hemithioacetal formed by allyl mercaptan and o-phthalic dialdehyde (OPA reagents) to react with primary amines and form a fluorescent isoindole complex. To make this reaction specific for creatine we used imprinting with a methylated creatine analogue that can form a reversible complex with hemithioacetal.

Interaction between phthalic dialdehyde, mercaptan group (OPA reagents) and primary amine

The reaction between o-phthalic dialdehyde, mercapto group (OPA reagents) and primary amines is well known and used in practice for detection of amino acids (Piletsky *et al*, 1996). It was shown recently that polymerisable thiols, such as allyl mercaptan can form stable hemithioacetal with o-phthalic dialdehyde (see **Figure 4.1**) (Piletsky *et al.*, 1999). This hemithioacetal can be co-polymerised with cross-linker into highly crosslinked rigid polymer, which retains the same ability as non-polymerised monomers or soluble OPA reagents to bind primary amines forming fluorescent isoindole. Generally this reaction is non-specific because any compound containing a primary amino group can react with OPA reagents or polymerised hemithioacetal.







R_1-SH –allylmercaptan; R_2-NH_2 – creatine

(I) interaction between phthalic dialdehyde, mercaptan group (OPA reagents) and primary amine.
(II) thioacetal formation.
(III) formation of fluorescent complex between hemithioacetal and primary amine.

Figure 4.1 The reaction between o-phthalic dialdehyde, mercapto group (OPA reagents) and primary amines

To make a polymer specific for creatine we used molecular imprinting. Creatine itself was expected to be an ideal template in our case. Unfortunately we found that this molecule reacts irreversibly with polymerised hemithioacetal and the template cannot be removed from the synthesised polymer. Isoindole, which is the product of this reaction, could not be hydrolysed back to the original components even when concentrated hydrochloric acid is used. This problem was overcome by using methylated analogue of creatine with blocked primary amino groups as a template (see **Figure 4.2**).

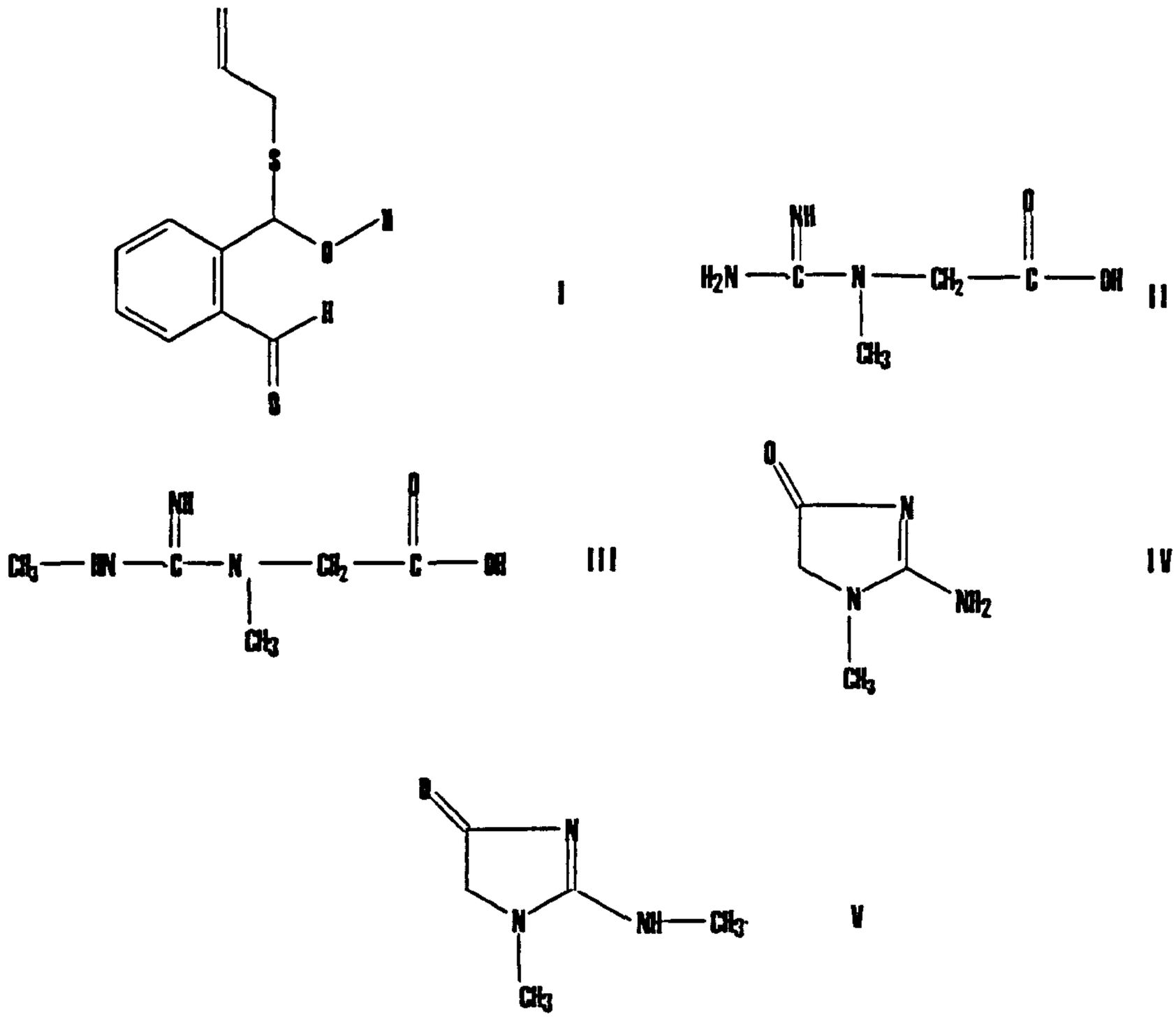


Figure 4.2 Structures of templates used for imprinting.
i) hemithioacetal ii) creatine iii) creatinine iv) methylated creatine v) methylated creatinine

Methylated creatine still possesses a structure similar to that of creatine, but can be easily removed from the polymer leaving specific recognition sites. It should be noted that this approach differs from non-covalent imprinting (Senholdt *et al*, 1997) since the

hemithioacetal-based polymers cannot be used repetitively for the rebinding of primary amines. A computer generated picture of the methylated creatine and polymerizable hemithioacetal complex is shown in **Figure 4.3**.

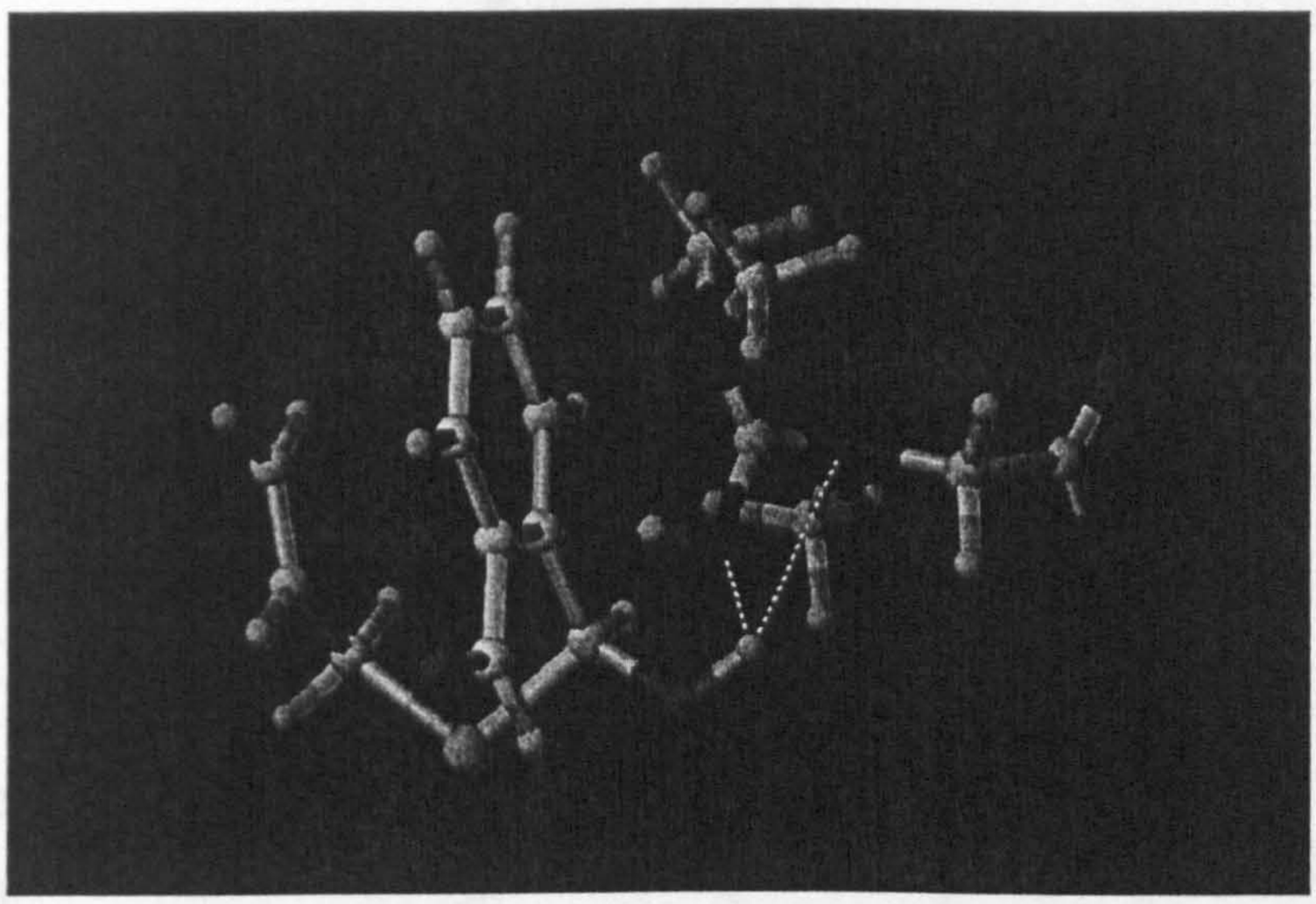


Figure 4.3 Complex formation between polymerisable hemithioacetal and methylated creatine.

Synthesis of MIPs for creatine

Highly cross-linked imprinted polymer was prepared with methylated creatine, (the concentration of cross-linker was approximately 77%), allyl mercaptan and o-phthalic dialdehyde using ethylene glycol dimethacrylate as cross-linker and dimethyl sulfoxide as solvent. DMSO was chosen as a solvent because it is able to solubilise all components of the monomer mixture and at the same time its polarity is very close to the polarity of aqueous environment of typical samples. The resulting polymer was ground, sieved and washed out from the template giving a suspension with an average particle size of 5 μm. The background fluorescence of the polymer suspension was low, but increased dramatically in the presence of creatine (see **Figure 4.4**). This reaction depends on pH, time and ionic strength, which we discuss in the next few sections.

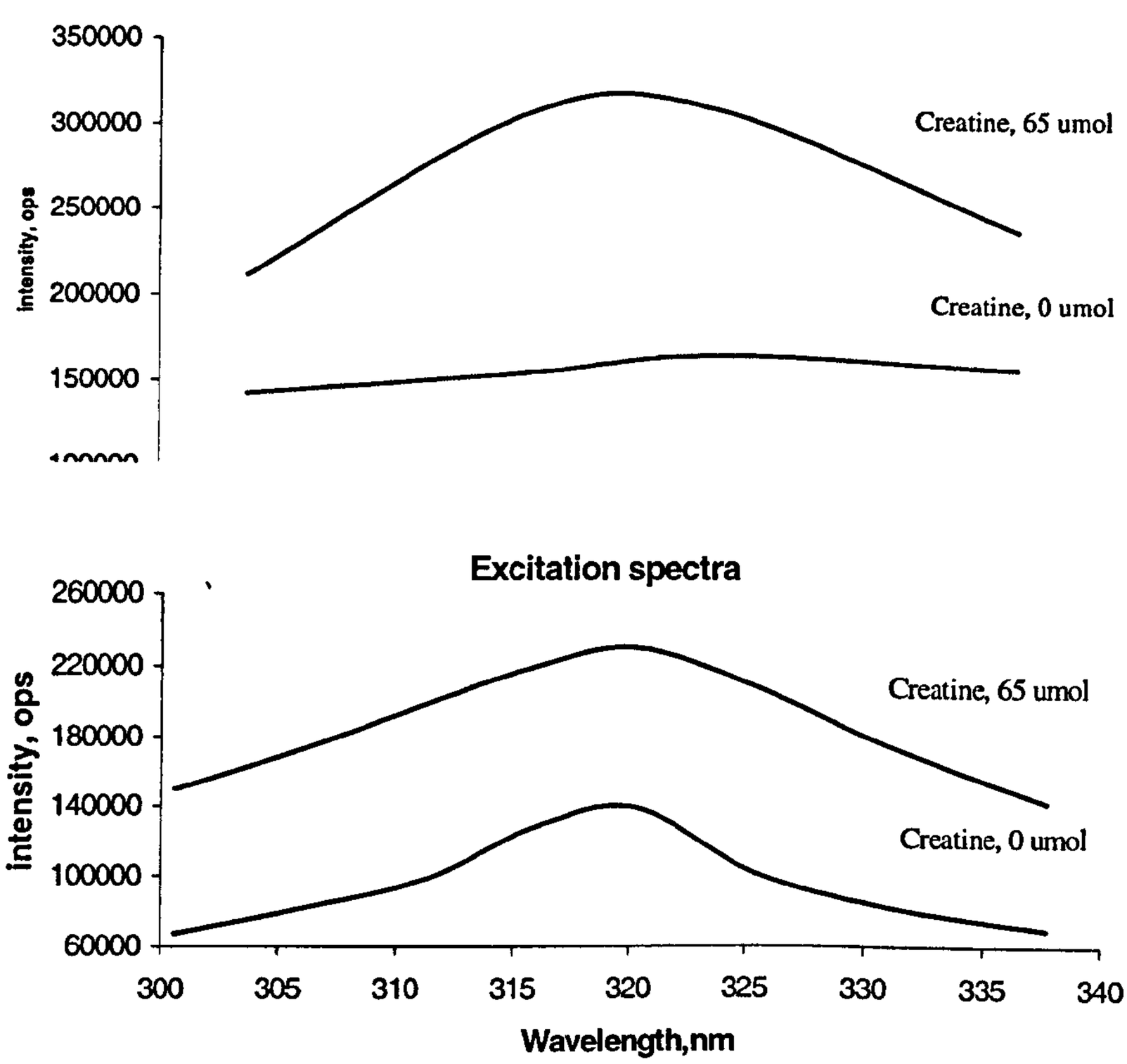


Figure 4.4 **Excitation and emission spectra of the MIP in the presence and absence of creatine. Ex = 320 nm; Em = 410 nm.**

Optimisation of pH

In the process of optimisation of the measuring conditions we found that the optimum pH of the reaction is 7.0 (for the pH's ranging from pH 6.0 to pH 9.0) (**Figure 4.5**). This is a rather unexpected result because it has been shown (Benson and Hare, 1975) that the reaction between OPA reagents and primary amines demands a more basic solution. We can assume that at higher pH, the complementarity between creatine and functional

groups in the polymer binding sites is distorted, probably as result of ionisation processes or hydroxyl anion interference with the recognition process. All the following experiments were carried out at pH 7.0.

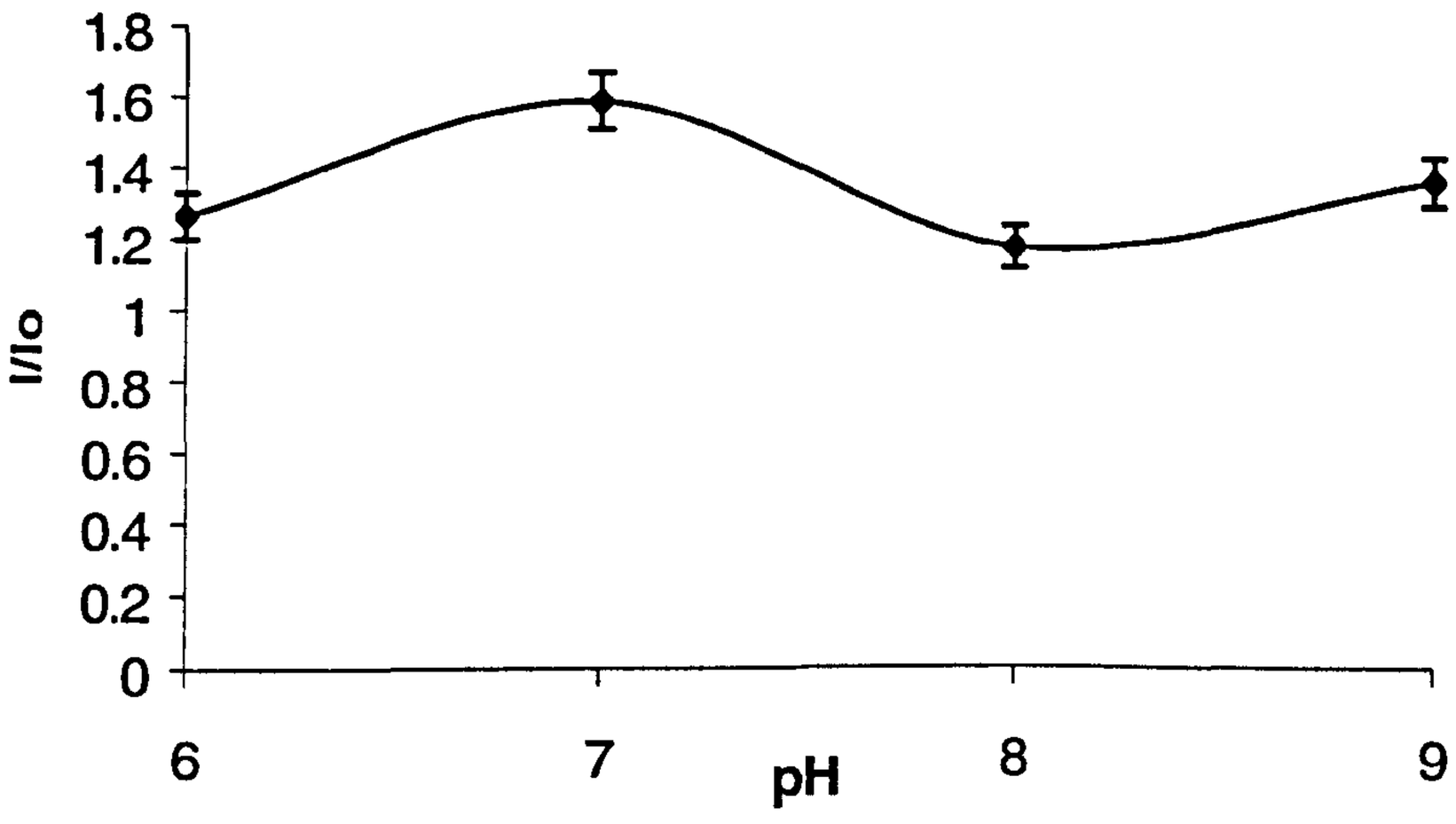


Figure 4.5 Optimal pH for the detection of creatine.

Influence of buffer concentration

The influence of buffer concentration on sensor response for creatine was much less pronounced and the response obtained in 100 mM sodium phosphate buffer (optimal conditions) was only 10% larger than the sensor response obtained in 5 mM buffer (**Figure 4.6**). The sensor response was fast, reaching 80% of absolute magnitude in 5 minutes. Maximum response was obtained in 45 minutes.

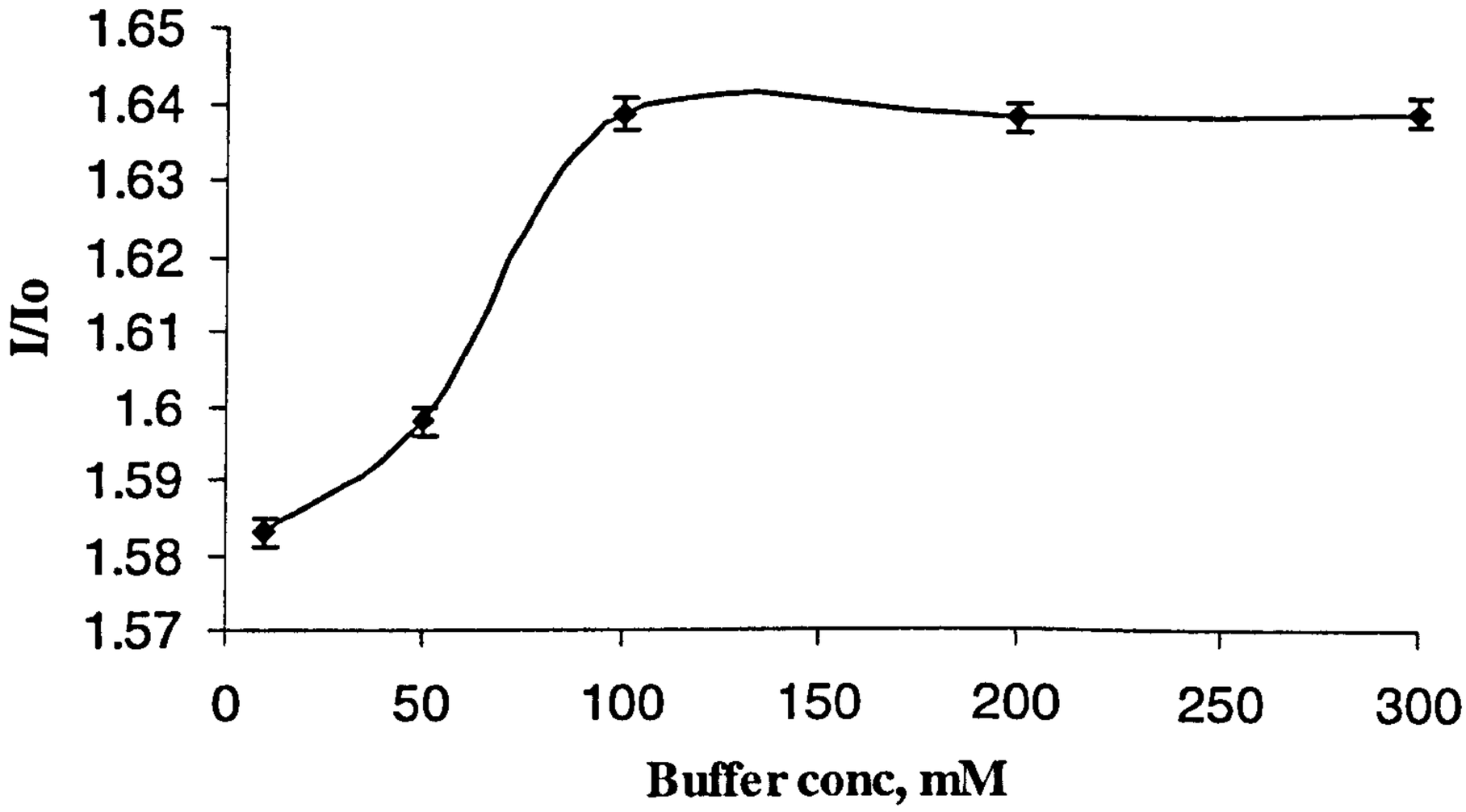


Figure 4.6 Optimal buffer concentration for the detection of creatine.

The sensor response was measured in several solvents such as tetrohydrofuran, dimethylformamide, methanol, dimethylsulfoxide and water. It is interesting that its magnitude was largest in the water, which makes this approach ideally suited for the detection of biological molecules where an aqueous environment is preferable.

Calibration curves for creatine

The calibration curves for creatine and structurally related creatinine are presented in **Figure 4.7**. The sensor responses for these two molecules generated by non-imprinted (blank) polymer were almost identical. Material synthesised in the absence of template had no specificity. On the contrary, highly crosslinked imprinted polymer was able to discriminate between the creatine and creatinine. Since it possessed higher sensitivity, imprinted polymer obviously had a larger number of binding sites available for the interaction with creatine than blank polymer. The larger response for creatine in

comparison with creatinine, indicates that MIP binding sites are less available for the interaction with creatinine than for creatine. All these features are strong indications of the effectiveness of the imprinting.

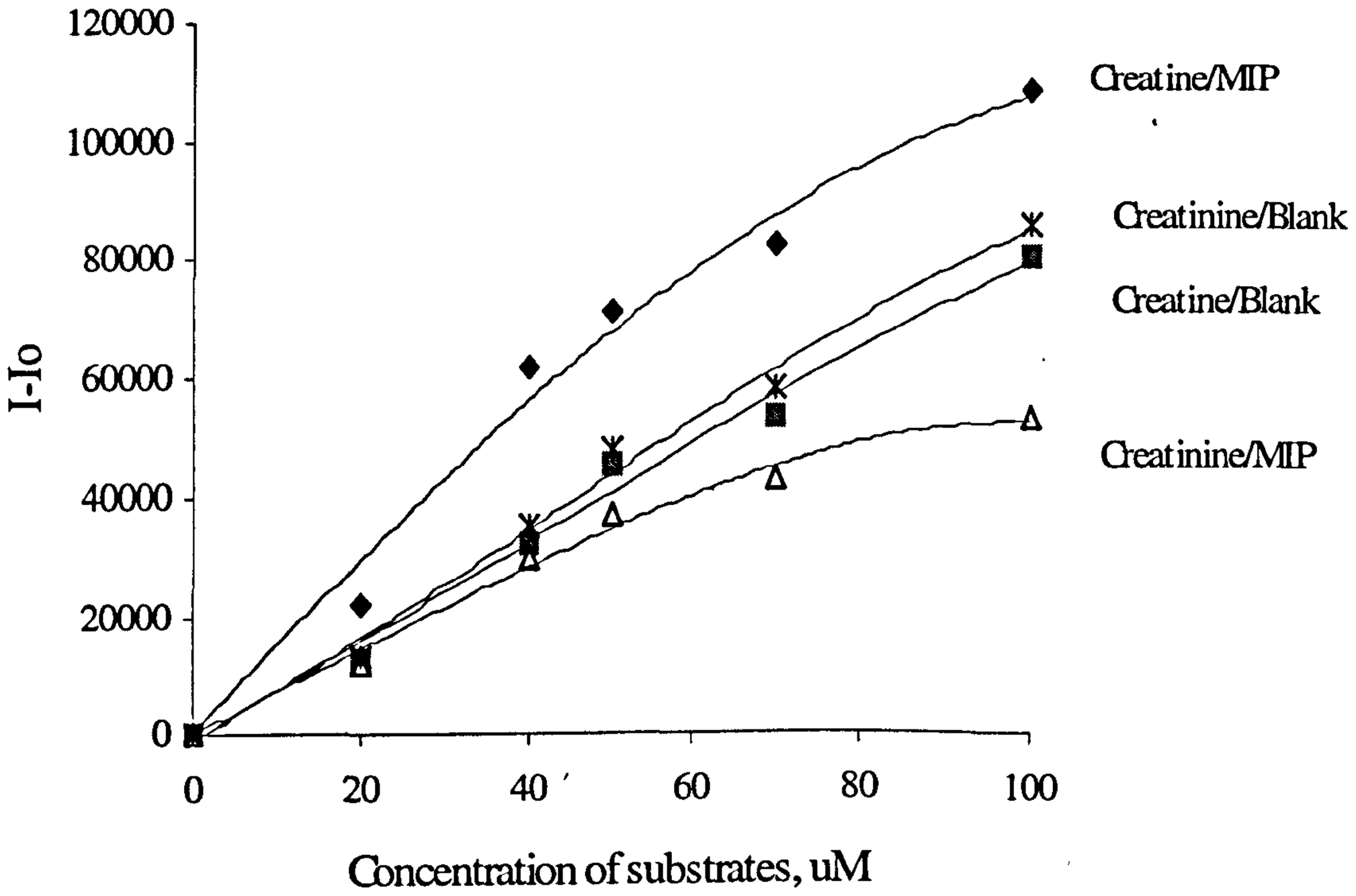


Figure 4.7 **Calibration curves for creatine and creatinine. Measurements were made in 100 mM sodium phosphate buffer, pH 7.0.**

Cross reactivity studies

The specificity of the imprinted and blank polymers was also demonstrated and compared in a cross-reactivity study. Sensor responses for several different molecules such as creatine, creatinine, phenylalanine, tyrosine, tryptophan, ammonia and urea are presented in **Figure 4.8**

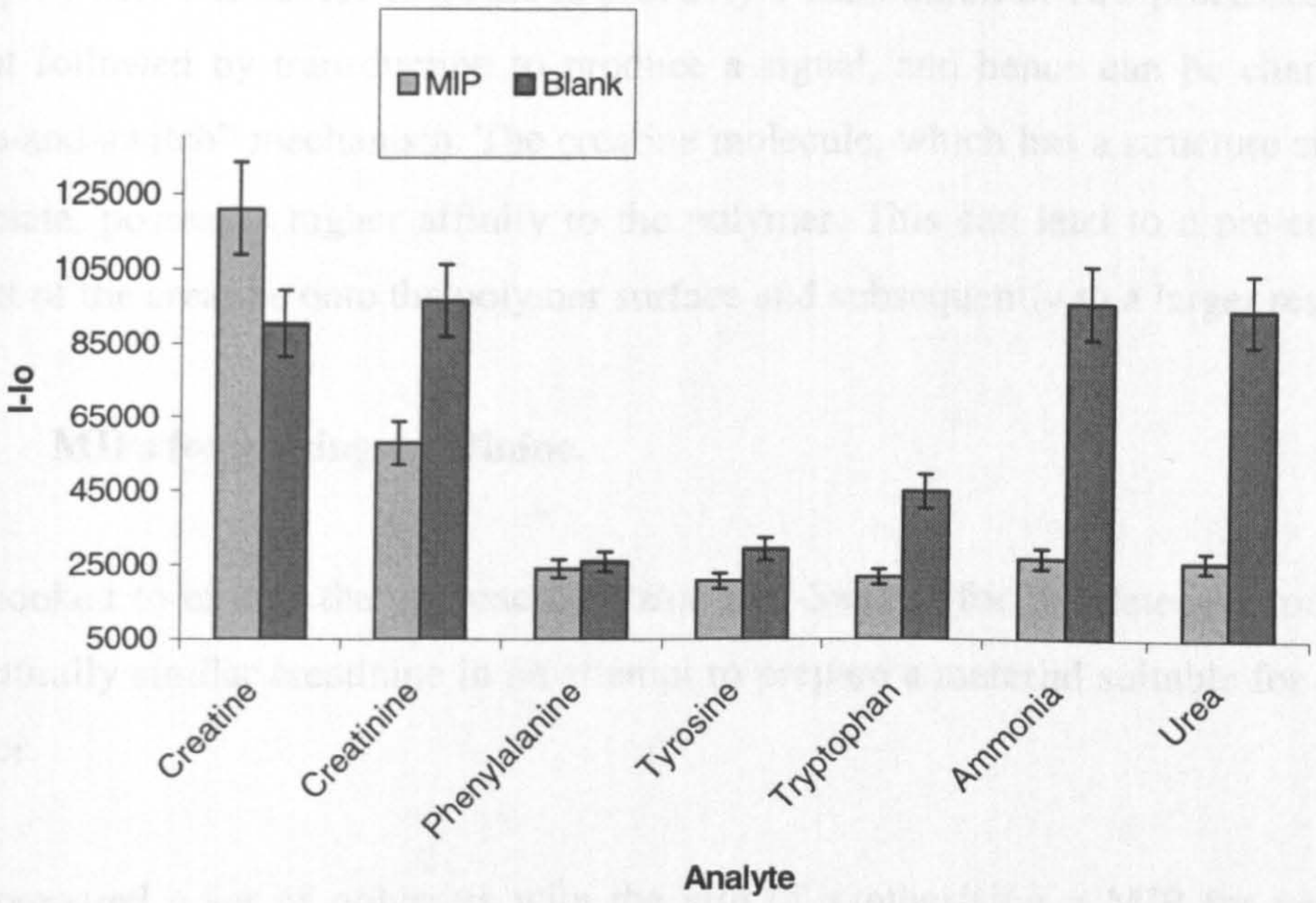


Figure 4.8 Sensor response for interfering molecules. Measurements were made in 100 mM sodium phosphate buffer, pH 7.0.

MIP- Sensor response for analytes obtained using imprinted polymers
Blank- Sensor response for blank obtained using blank polymer

In the case of blank polymer several molecules such as creatine, creatinine, ammonia and urea produced similar large responses. Smaller responses for phenylalanine, tyrosine and tryptophan can be explained by two factors, the relatively large size of those molecules as well as their high hydrophobicity, which prevents them from entering into hydrophilic pores. In the case of MIP the largest response was obtained for creatine, the molecule with the closest resemblance to the template. Creatinine, a molecule with a less similar structure gave a response of half that obtained for creatine. All the other molecules under investigation generated significantly smaller responses than those two molecules. It is interesting that even ammonia, which is small enough to "fit" to virtually any binding site, did not produce a response of comparable magnitude to creatine. We can assume that

it is not only the size of the molecule which governs the interaction between the analyte and polymer. The sensor response is probably a summation of two processes, a binding event followed by transduction to produce a signal, and hence can be characterised as "bite-and-switch" mechanism. The creatine molecule, which has a structure similar to the template, possesses higher affinity to the polymer. This can lead to a pre-concentration effect of the creatine onto the polymer surface and subsequently to a larger response.

4.2 MIPs for sensing creatinine.

We looked to extend the approach of '*Bite-and-Switch*' for the detection of creatine to structurally similar creatinine in an attempt to prepare a material suitable for a creatinine sensor.

We prepared a set of polymers with the aim of synthesising a MIP for creatinine,. A polymer with composition identical to MIP A, but imprinted with a creatinine analogue instead of creatine MIP (MIP A^*) was tested for sensing of creatinine. (Refer to **Table 3.1** under **'Materials and methods'** for details of the compositions of MIPs). It was found that polymer MIP A^* was unable to differentiate between creatine and creatinine **(Figure 4.9)**.

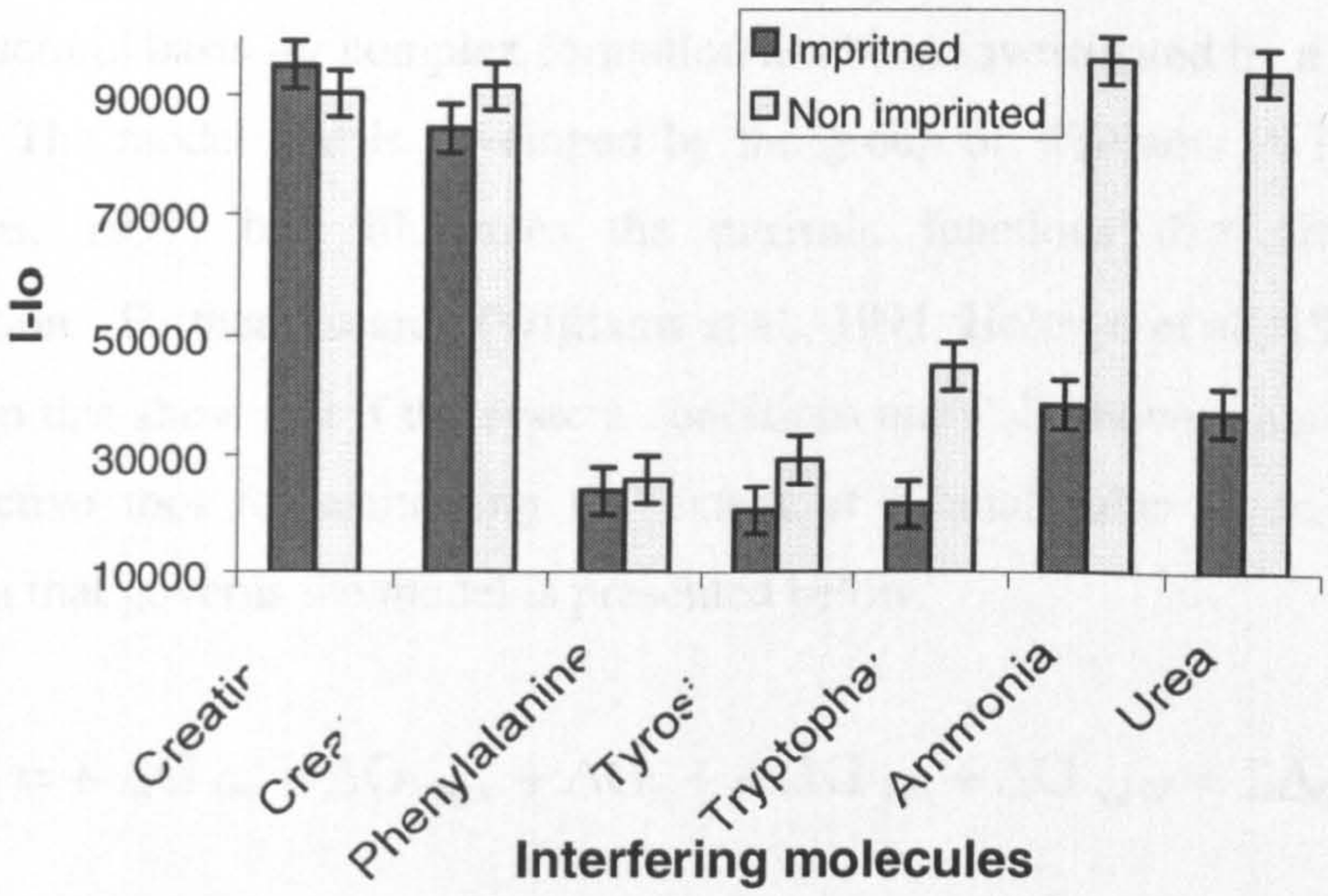


Figure 4.9 Response of MIP A* to creatine, creatinine and other interfering molecules.

In our attempt to generate a more selective MIP for creatinine (MIP B) we added to the monomer composition a functional monomer, methacrylic acid, which is capable of reacting with the template. Fluorescent studies performed under the same conditions indicated that the polymer MIP B, imprinted with methylated creatinine, had a similar sensitivity to MIP A and MIP A* (detection limit 5-10 μM) but was also unable to differentiate between creatinine and its structural analogue and biochemical precursor, creatine. To improve the polymer's affinity for creatinine, efforts were made to develop a rational method for the selection of functional monomers, which would be able to recognize creatinine better than the methacrylic acid traditionally used for MIP preparation.

Molecular modelling

The structural basis for complex formation has been investigated by a number of research groups. The model that is developed by the group of Williams (Williams et al., 1990; Williams, 1991) best illustrates the intrinsic functions that affect intermolecular associations. Further research (Williams et al., 1991; Holoryd et al., 1993; Williams et al., 1993) on this show that if the system conditions are well known, this model can serve as a predictive tool for estimating the extent of biomolecular association. The general equation that governs the model is presented below.

$$\Delta G_{bind} = + \Delta G_{t+r} + \Delta G_{rotor} + \Delta G_h + + \Delta G_{vib} + \Delta G_{vdW} + \Sigma \Delta_{Gp}$$

where ΔG_{t+r} denotes the free energy loss associated with translational and rotational freedom, for example, the loss in entropy from the restricted movement in space as related to the host. $\Delta_{G\ rotor}$ is the energy loss when internal rotors are 'immobilised or frozen'; it is also mainly an entropic effect. ΔG_h is the energy gain from hydrophobic interactions (favourable for interaction). ΔG_{conf} is the result of adverse conformational changes necessary for binding and ΔG_{vib} comprises residual soft vibrational modes. ΔG_{vdW} is the energy penalty resulting from unfavourable van der Waals interactions, and $\Sigma \Delta_{Gp}$ is the sum of interacting polar group contributions that is favourable for interaction.

A virtual library as shown in the previous chapter **(Chapter 3, Materials and methods, Figure 3.3)** of the 21 functional monomers most frequently used for MIP preparation was tested for their ability to form a complex with a molecular model of the template. In addition to hemithioacetal, two other monomers, m-divinylbenzene and urocanic acid ethyl ester gave the highest binding scores. The complexation between these monomers and template was analysed using simulated annealing. As a result, the optimised composition for the polymer was calculated giving the ratio of 1 (template) : 4 (hemithioacetal) : 1 (urocanic acid ethyl ester). A possible structure of the monomer-template complex predicted using the computational approach for creatinine is shown in **Figure 4.10**

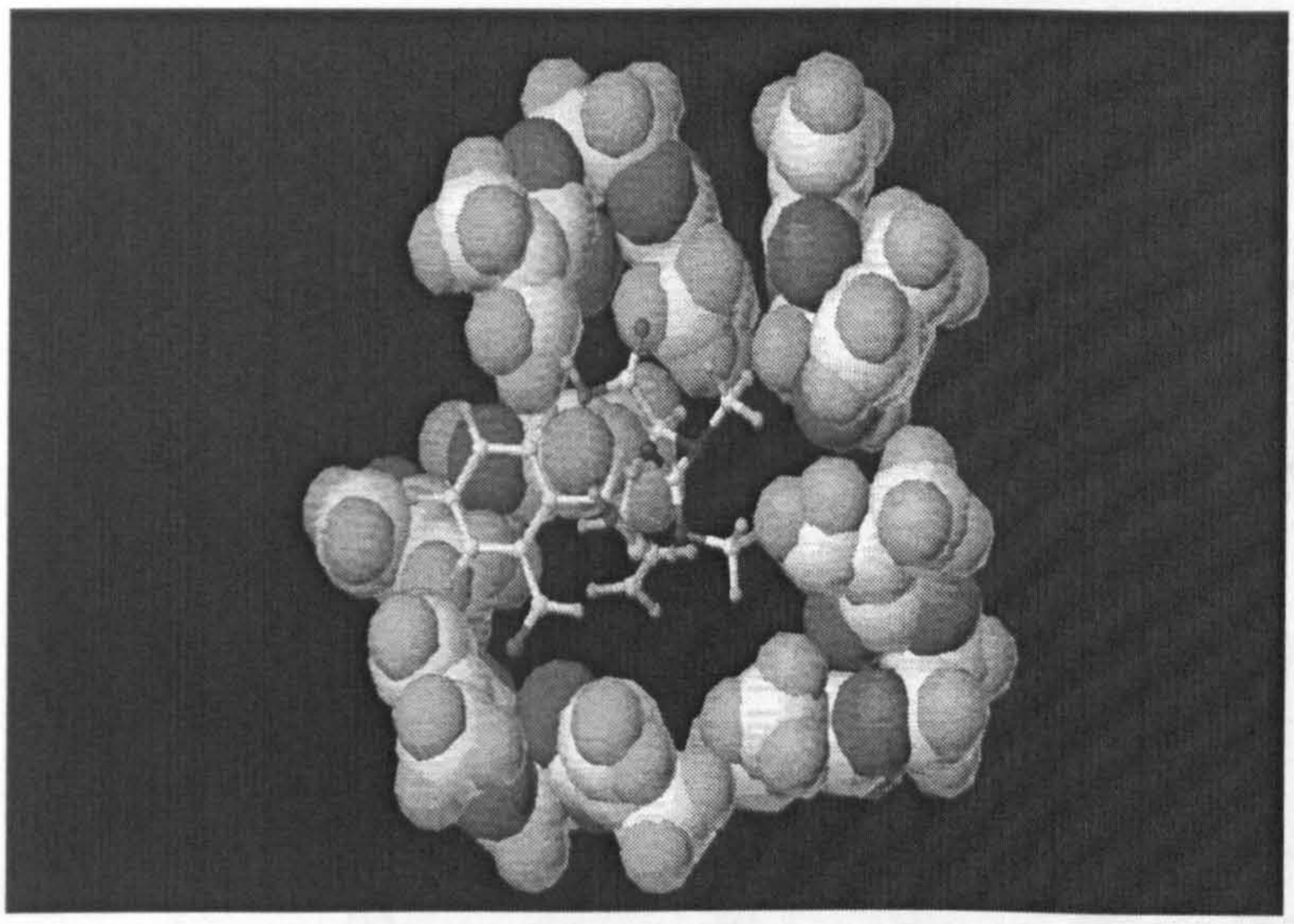


Figure 4.10 Possible structure of the monomer-template complex predicted using the computational approach for creatinine.

The polymer was then synthesised and studied further employing the same method that was followed for creatine as described under **Chapter 3, Materials and methods.**

Calibration curve for creatinine

The calibration curve for creatinine is presented in **Figure 4.11**. Sensor responses for creatinine and creatinine generated by non-imprinted (blank) polymers were identical.

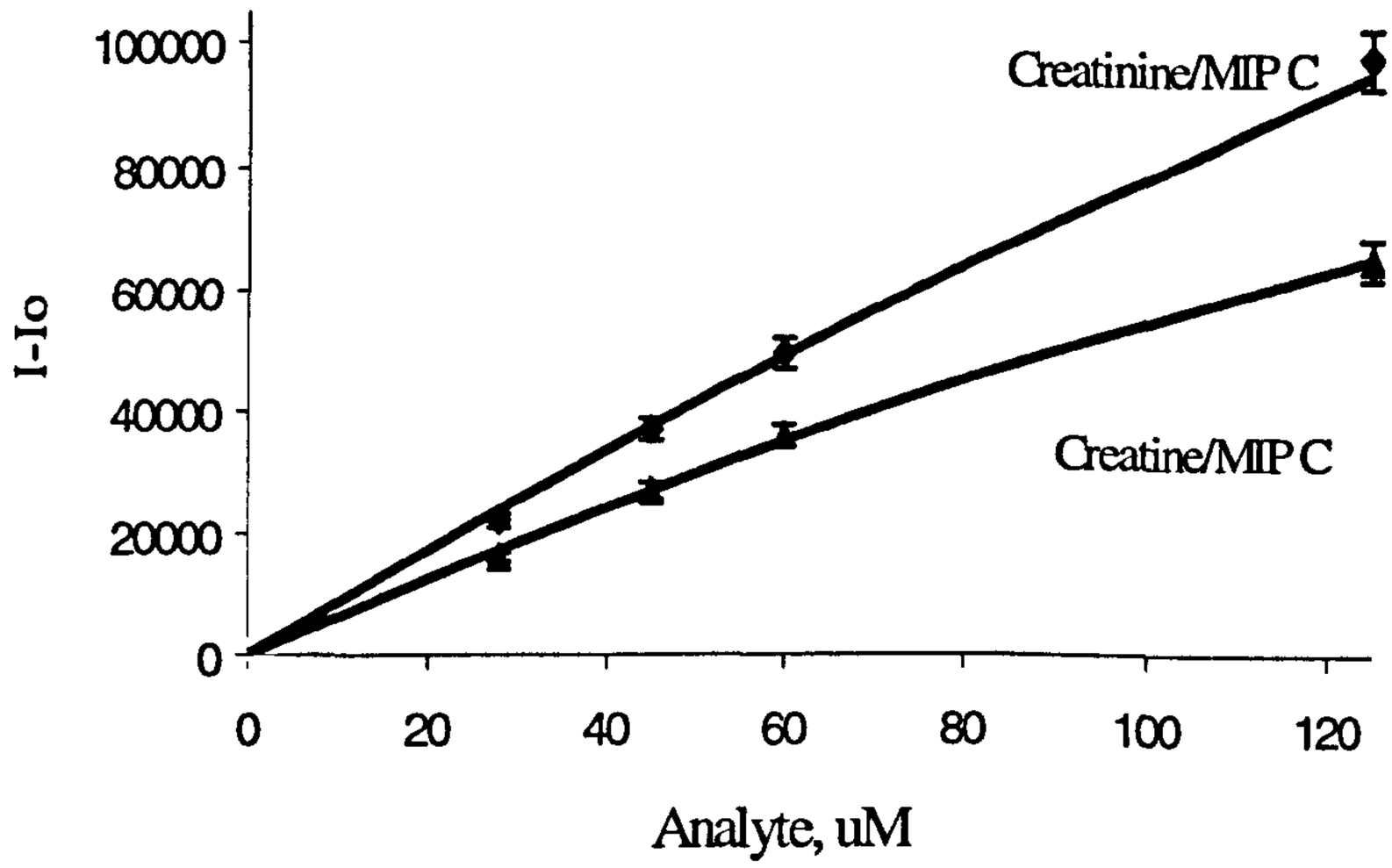


Figure 4.11 Calibration curve creatine and creatinine using computationally designed polymer. The measurements were made in 100mM sodium phosphate buffer at pH 7.0. All measurements were made in triplicate.

Imprinting factor

The response for computationally designed MIP C was higher than that obtained for the blank polymer. It was not possible to calculate the binding constants and evaluate the affinity of the synthesized polymers due to irreversible binding of the creatinine by the polymer, however we were able to calculate the imprinting factor using the following formula:

$I = \Delta F (MIP) / \Delta F (Blank)$

where I = imprinting factor,

$\Delta F(MIP)$ = response for imprinted polymer, and

$\Delta F(Blank)$ response for blank polymer.

The imprinting factors calculated for MIP A, MIP B and MIP C selective for creatine and creatinine clearly indicate the superior selectivity of MIP C in comparison with polymer synthesized by the traditional approaches (MIPA, MIP A* and MIP B) (**Figure 4.12**). (MIP A* is a polymer selective for creatinine using AM and OPA only) These results confirm the effectiveness of the imprinting process and illustrate the possibility of its improvement through the rational selection of functional monomers.

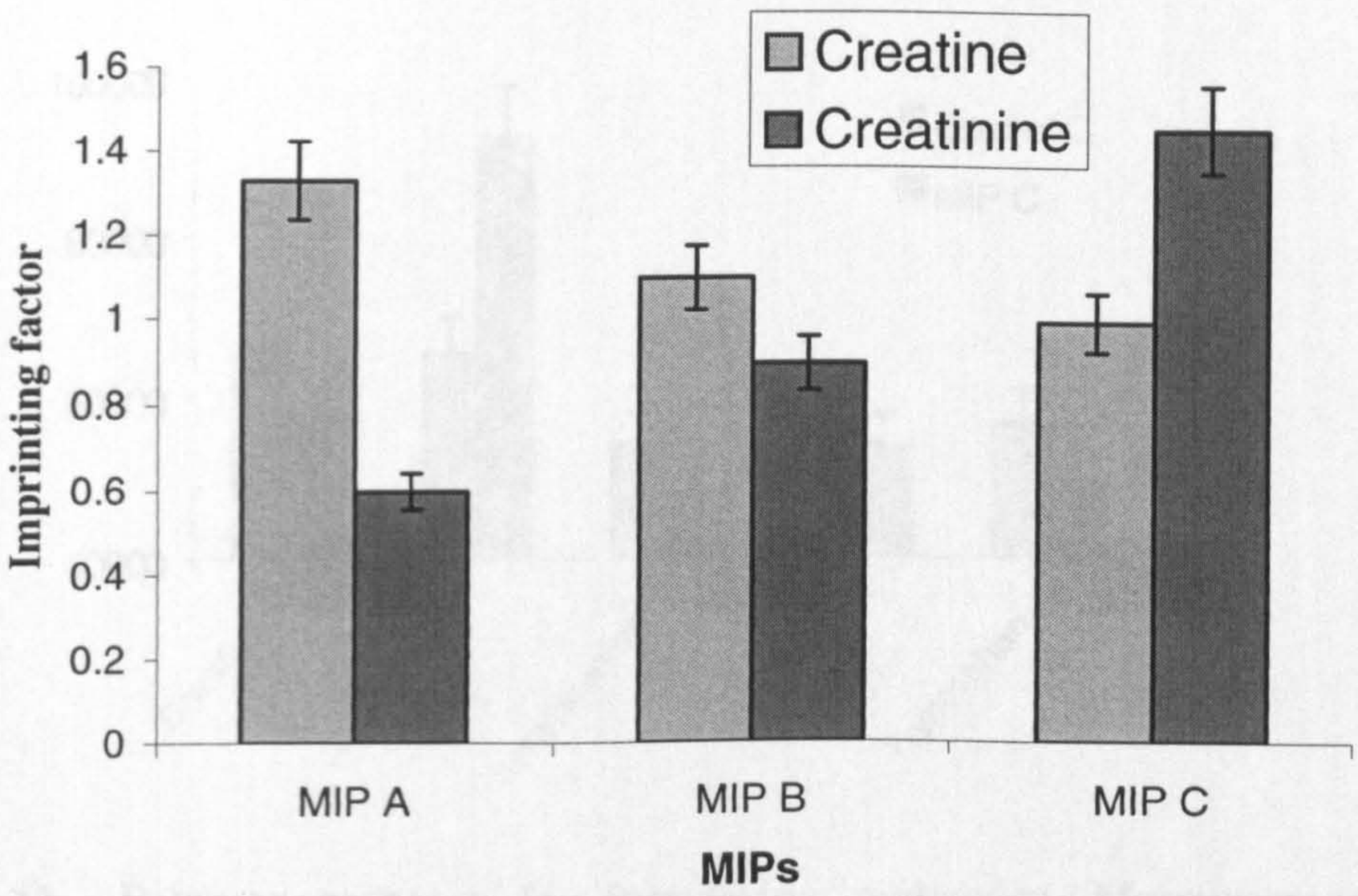


Figure 4.12 Imprinting factors of the polymers MIP A, MIP B and MIP C calculated for creatinine and creatine. All measurements were made in triplicate. The standard deviation was 7%.

The response of MIP C for creatinine was repeatedly studied over a period of three months. Virtually no changes in the sensitivity and selectivity of the polymer were observed over this period of time.

Selectivity of MIP A and MIP C

The selectivity of the MIP A and MIP C is presented in **Figure 4.13**. The response to templates and different interfering molecules such as phenylalanine, tyrosine and tryptophan were studied and compared. In the case of MIP A, creatine gave a higher

response as it closely resembles the template (methylated creatine). MIP C, the polymer prepared based on the computational approach, was more sensitive for creatinine than for creatine and amino acids such as phenylalanine, tyrosine and tryptophan. Much smaller responses for phenylalanine, tyrosine and tryptophan could be due to the large size of the molecules and their higher hydrophobicity, which prevents them from entering into hydrophilic pores. It is also possible that creatine and creatinine contain the guanidine group, while phenylalanine, tyrosine and tryptophan are primary amines and hence they might form different fluorescent complexes with OPA reagents.

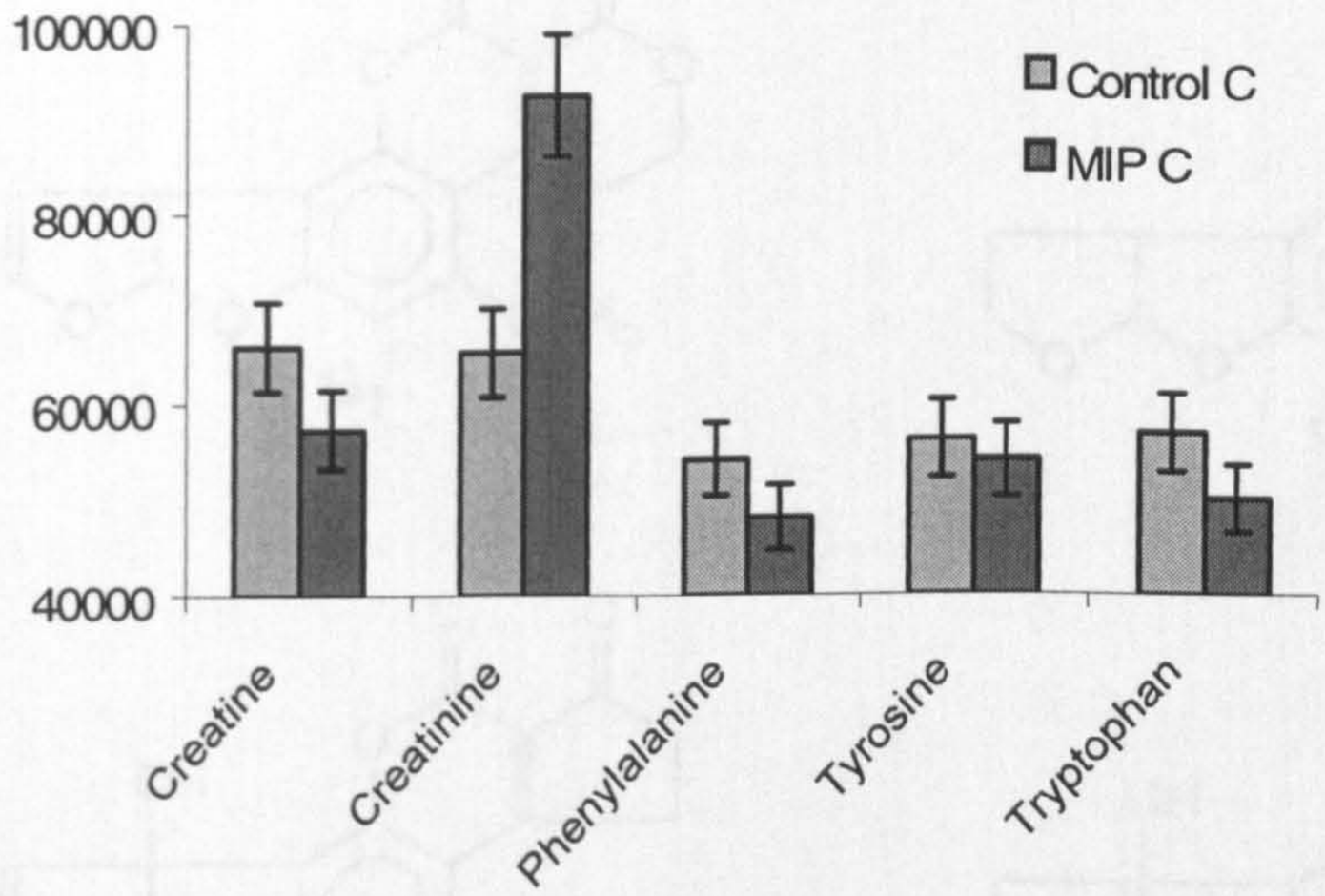


Figure 4.13 Polymer response for interfering molecules. Measurements were made in 100-mM sodium phosphate buffer, pH 7.0. All measurements were made in triplicate.

4.3 Solid phase extraction of aflatoxin-B1

Aflatoxins are substituted coumarins with molecular weight 312–330, secreted by several mould species i.e., *Aspergillus flavus*, *A. Parasiticus, A. nomius, A. niger, Penicillium* and *Streptomyces*. These toxins have closely similar structures and form a unique group of highly oxygenated, naturally occurring heterocyclic compounds.

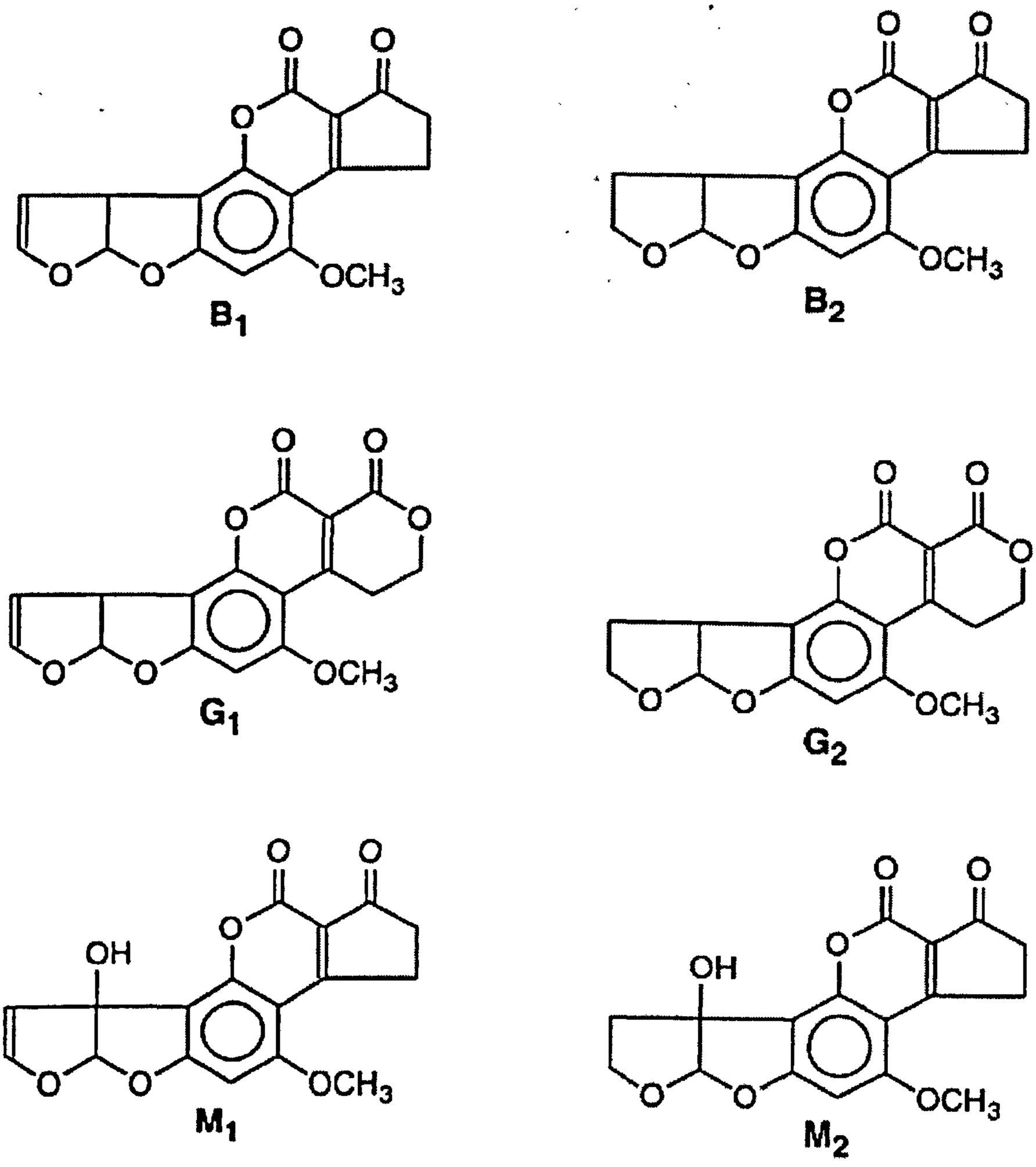


Figure 4.14 Structure of aflatoxins.

Aflatoxins are often found in peanuts, soybeans, wheat, barley, maize, rice, cocoa beans, potato, cheese and dry milk. Four major aflatoxins - B1, B2, G1, G2 and two additional metabolic products, M1 and M2, are of significance in food analysis as direct contaminants of foods and feeds (Figure 4.14.). Contamination of food products with aflatoxins is recognised as a worldwide problem. They can contaminate food products at the stages of their preparation and storage and can not be destroyed during thermal

treatment (Brown *et al.*, 1999). Among these compounds aflatoxin-B1 (**Figure 4.14**) is recognised to be the most active and the most widespread. Prolonged aflatoxin-B1 injections even in very low concentration often cause liver and kidney tumours with lung metastases in many animal species (mammalians, birds, and fishes). According to World Health Organisation Directive (1966) no more than 30 µg/kg of aflatoxin in food products is allowed (Wogan and Shank, 1971; Neal *et al.*, 1979; Neal *et al.*, 1998; Dorner *et al.*, 1998). There is a need for analysis of aflatoxin-B1 at concentrations, as low as 4 ppb (Wogan and Shank, 1971; Neal *et al.*, 1979; Neal *et al.*, 1998; Dorner *et al.*, 1998).

Traditional methods for aflatoxin-B1 detection are mainly based on estimation of necroses of the duckling liver using light microscopy (Goldblatt, 1969). Biological testing, however, is a time-consuming and labour-intensive method. A number of relatively newer methods have been developed as detailed below, however each of the methods has its own disadvantage. Among these methods are thin layer chromatography (TLC) and liquid chromatography (LC). Liquid chromatography methods for the determination of aflatoxins in foods include normal-phase LC (NPLC), reversed-phase LC (RPLC) with pre-column derivatization (BCD), RPLC followed by postcolumn derivatization (PCD) and RPLC with electrochemical detection. However, these methods are expensive and time consuming. The main problem for the application of these methods in food analysis lies in the need for sample pre-treatment. In practice the sample pre-treatment consumes 90% of time and contributes to the 85% of the analysis cost. Sampling and sample preparation remain a considerable source of error in the analytical identification of aflatoxins. Thus, systematic approaches to sampling, sample preparation, and analysis are absolutely necessary to determine aflatoxins at parts-per-billion levels. Solid Phase Extraction (SPE) is the most common procedure used for toxins removal from the environmental or food samples. In most cases SPE cartridges are non-specific (for example hydrophobic C_{18} adsorbents). The use of more specific immunoadsorbents in SPE is limited because of the difficultly in handling antibodies and the costly production procedure. The following section of this Chapter will present the results of the development and testing molecularly imprinted polymers for solid phase extraction of aflatoxin-B1.

The MIP specific for aflatoxin-B1 was designed and synthesised as described in the **Materials and methods.** The monomers for the MIP for aflatoxin-B1 were chosen based on the computational approach**.** The possible structure of the monomer-template complex predicted using the computational approach is presented in the **Figure 4.15**

Polymer was ground well and 1 mg was packed into a SPE cartridge. The influence of loading and elution conditions on the polymer performance was investigated.

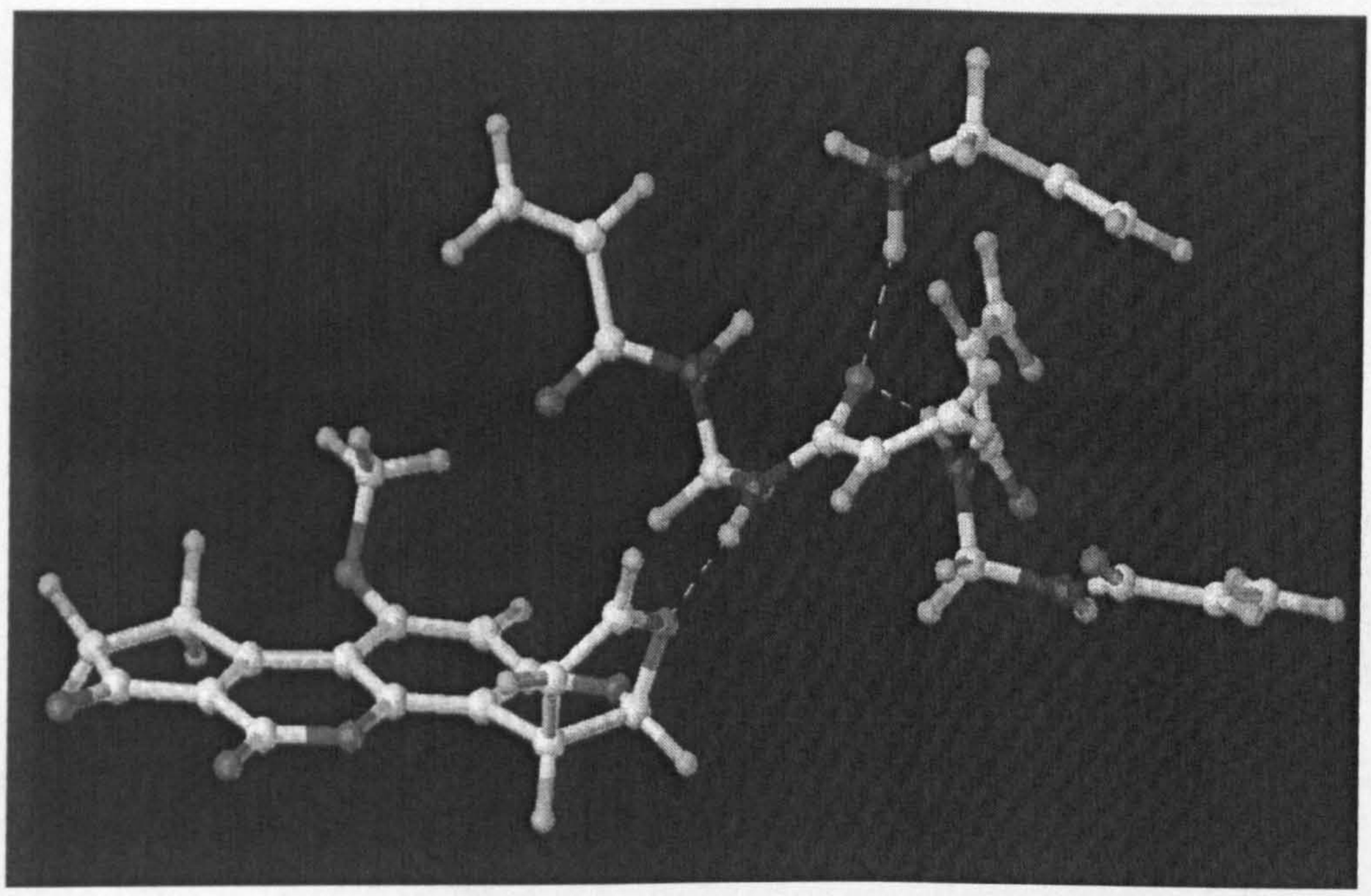


Figure 4.15 Possible structure of the monomer-template complex predicted using the computational approach for aflatoxin-B1.

Effect of pH on recovery of aflatoxin- B1

The effect of pH on the binding of aflatoxin-B1 was investigated using buffer with pHs ranging from pH 4.0 to pH 11.0. As shown in **Figure 4.16,** aflatoxin-B1 underwent almost complete recovery for this wide range of pH, while the recovery of the analyte on the blank polymer was low. This proves that imprinted polymer was capable of binding aflatoxin-B1 more strongly than the blank polymer. The extraction was nearly complete in buffers with pH 6.0- pH 9.0. This shows the effectiveness of the imprinting process.

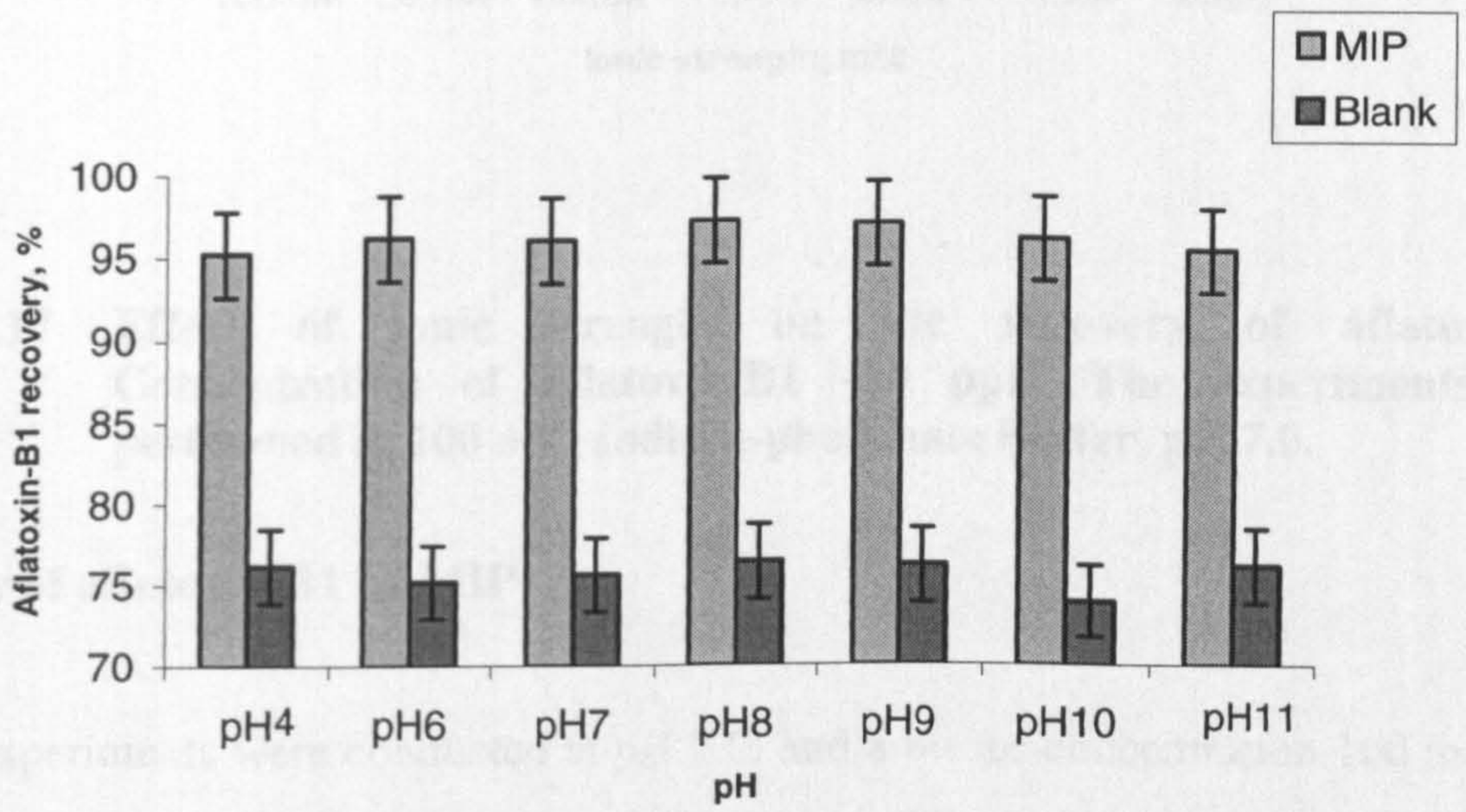


Figure 4.16 Effect of pH on the recovery of aflatoxin-B1. The experiments were made in 100 mM sodium-phosphate buffer. Concentration of aflatoxin- B1- 50 ppb.

Effect of ionic strength on the recovery of aflatoxin-B1

The study of the effect of the ionic strength on the recovery of aflatoxin-B1 showed that there was no appreciable difference in the recovery of aflatoxin-B1 in buffers with concentration between 10mM - 100mM. However at lower ionic strengths (0-5mM buffer) there was reduced recovery of the template. This is shown in **Figure 4.17.**

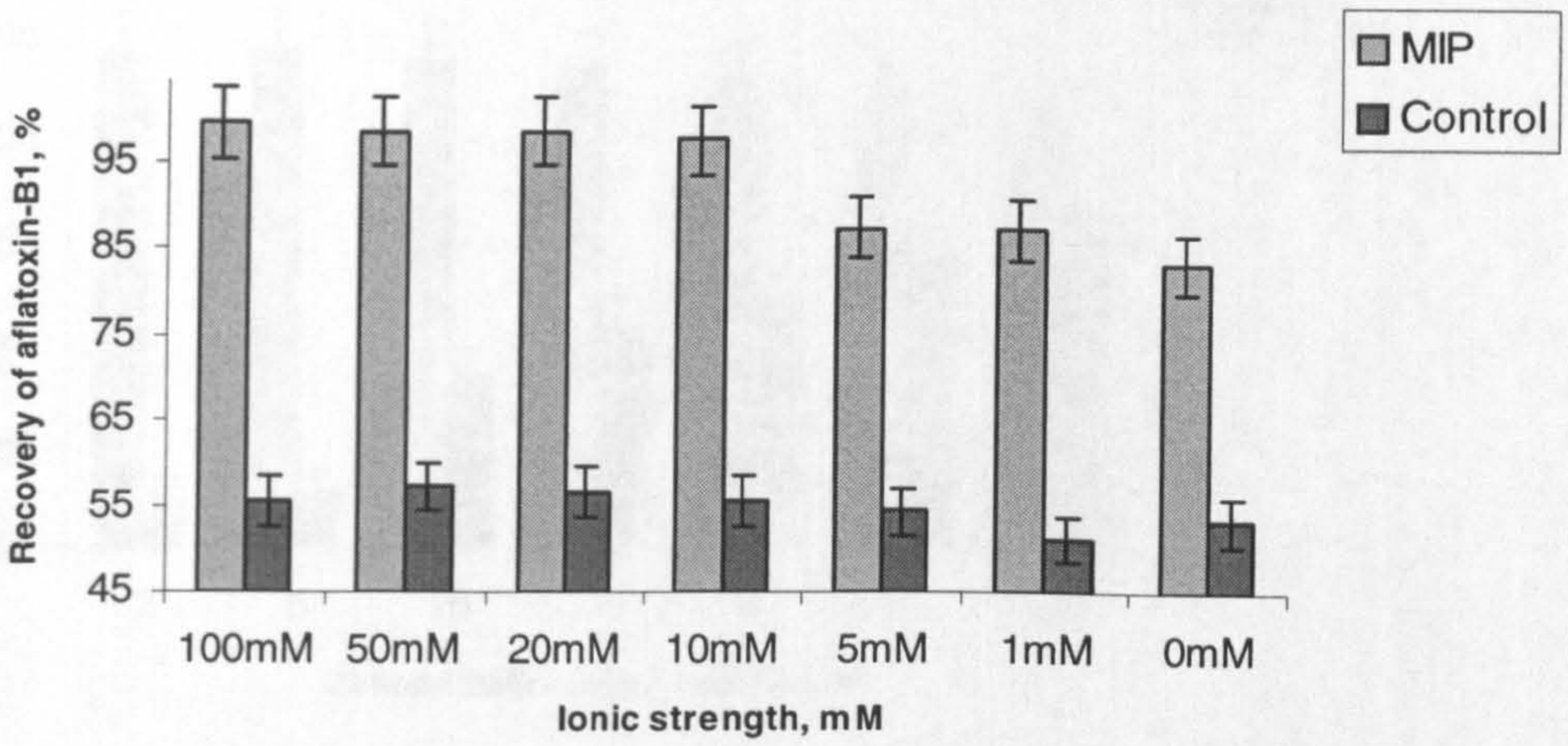


Figure 4.17 Effect of ionic strength on the recovery of aflatoxin-B1. Concentration of aflatoxin-B1 -50 ppb. The experiments were performed in 100 mM sodium-phosphate buffer, pH 7.0.

Recovery of aflatoxin-B1 by MIP

Further experiments were conducted at pH 7.0, and a buffer concentration 100 mM. The role of the concentration of analyte on the efficiency of its recovery using MIP and Blank polymers is presented in **Figure 4.18.** For the MIP, as the concentration of the template decreased, the percentage of the recovery of the template increased. A very opposite trend was seen for the control polymer. This is one of very strong indication of the effectiveness of the imprinting process. It is also important to mention that even at a concentration of as high as 5 ppm of the template, the recovery was greater than 90% for the MIP. Relatively lower recovery of aflatoxin B1 from the MIP at higher concentrations of the analyte could be due to the relatively low concentration of high affinity binding sites in the polymer. Relatively higher recovery of aflatoxin B1 by the control polymer for higher concentrations of the template can be attributed to the binding of the template under these conditions to the non-specific binding sites of the control polymer.

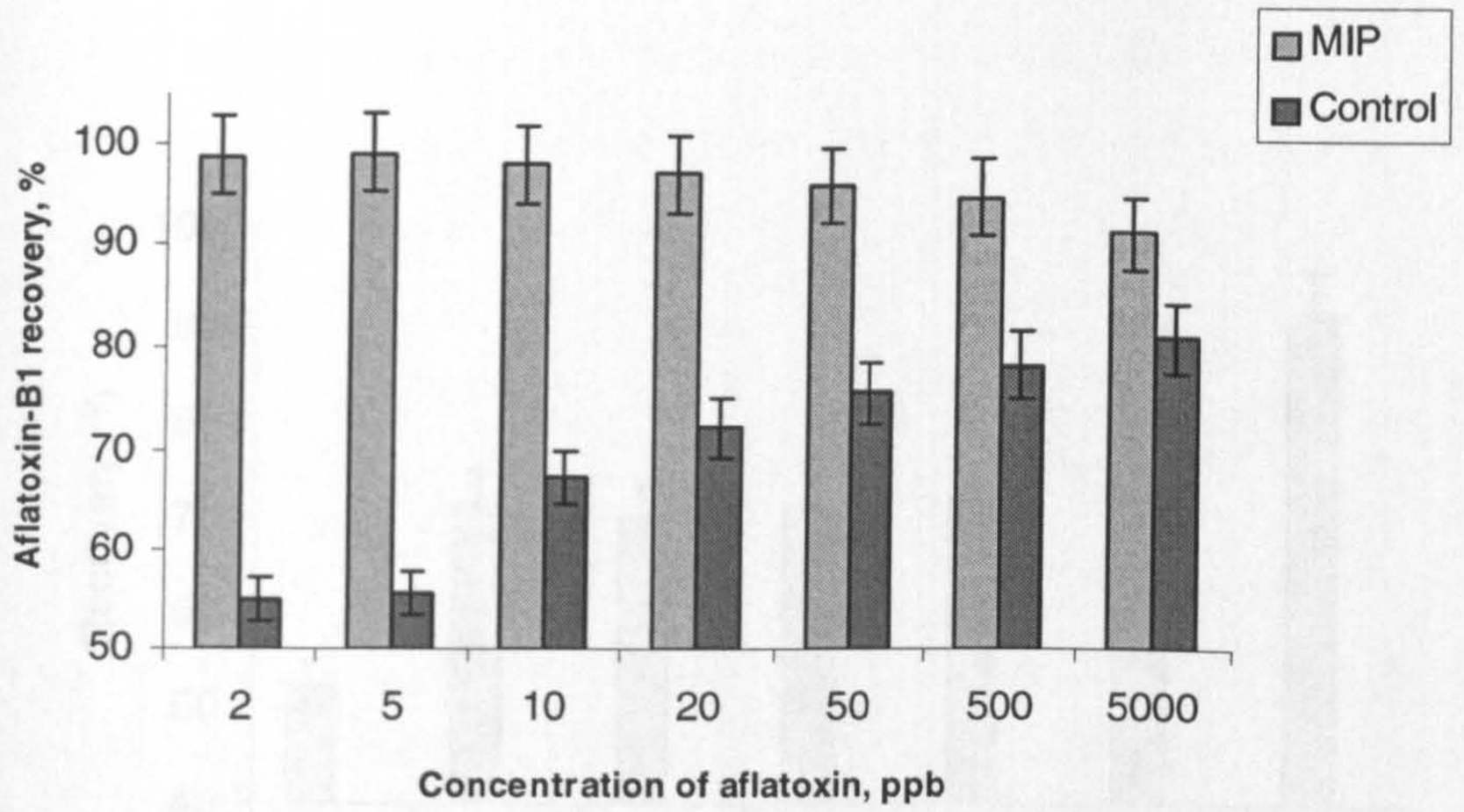


Figure 4.18 Recovery of aflatoxin-B1 by MIP and control polymer- The experiments were made in 100 mM sodium-phosphate buffer, pH 7.0.

Recovery of aflatoxin B1 from corn samples.

To validate our approach, the efficiency of recovery of aflatoxin B1 from MIP and Blank polymers was compared with that obtained using commercially available C18 bonded silicas. Although these materials have been very successfully applied to SPE, the presence of residual silanols has often complicated the extraction of basic compounds because of ionic interactions (Ruane and Wilson., 1987; Roberts *et al.*, 1989; Martin *et al.*, 1996; Martin *et al.*, 1997). The recovery of aflatoxin-B1 using a C-18 cartridge is shown in **Figure 4.19.** As illustrated in the figure, at lower concentrations (<50ppb of aflatoxin-B1), the recovery of the template was very low. Only 52% of the template could be recovered at concentrations of 2 ppb when compared with almost complete recovery by the MIP. In this context, MIP as a solid phase extraction material offers important and practical advantages with respect to both, blank polymer and the C18 cartridge.

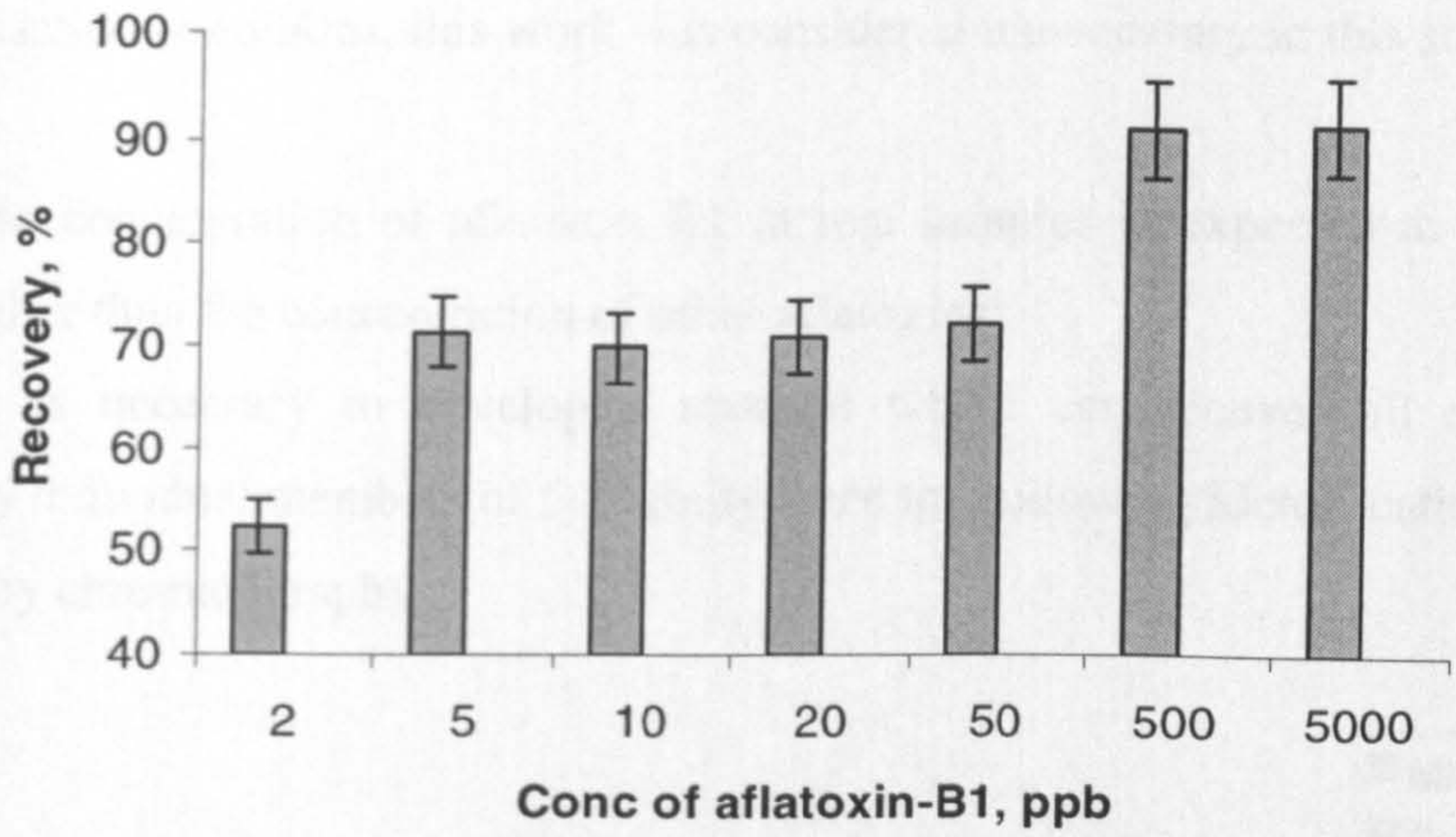


Figure 4.19 Recovery of aflatoxin-B1 by C18 cartridge.

Cross reactivity of aflatoxin B1 analogues

The selectivity of the imprinted polymer was evaluated by comparing the percentage recoveries of structurally similar analytes such as aflatoxin-B2, and aflatoxin G2 with the recovery of the template. The same results were compared for MIP and blank polymers. As can be seen from the **Figure 4.20,** the imprinted polymer showed greater recovery of the analyte, aflatoxin-B1, when compared with recovery using the blank polymer. Clear and substantial difference between the recovery of analytes from MIP and blank polymer was seen only in the case of aflatoxin-B1. Hence, it could be concluded that the selectivity of the MIP is greatly attributed to the imprinting process. The increased retentivity and selectivity showed by the MIP is due to the formation of the cavities that had shape and the arrangement of interacting sites complementary to the template aflatoxin-B1.

It is also possible to conclude that the polymer has group specificity towards the family of aflatoxins. Although in principle the selectivity of a MIP for individual aflatoxins could be improved by further optimisation of the monomer mixture and the polymerisation conditions, this work was considered unnecessary at this stage due to two facts:

a) the concentration of aflatoxin B1 in real samples is expected to be at least 10 times higher than the concentration of other aflatoxins;

b) it is necessary to develop a material which can recover all aflatoxins, not necessary individual members of the family since the following identification of them can be done by chromatography.

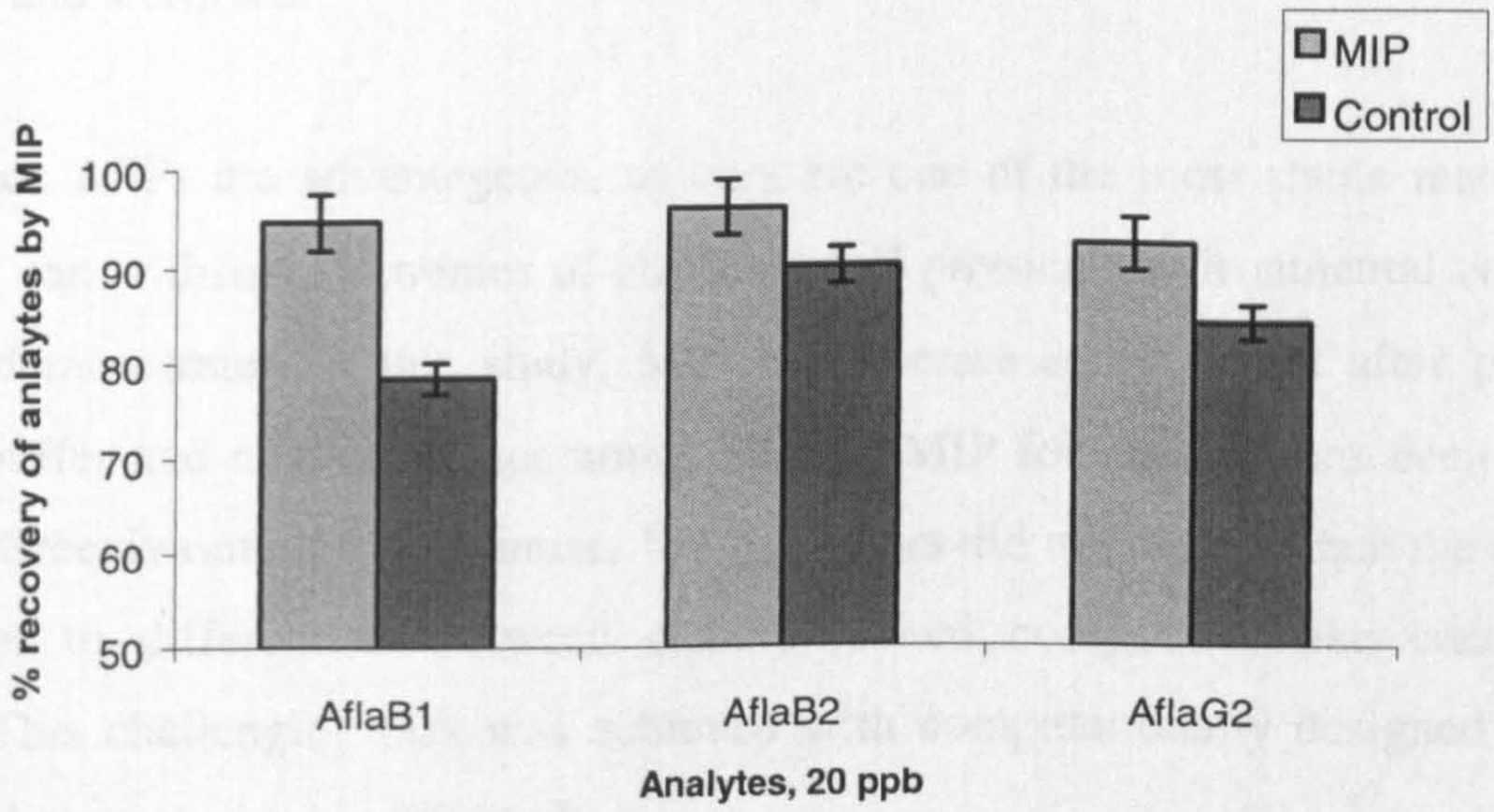


Figure 4.20 Cross reactivity of interfering analytes. The experiments were performed in 100 mM sodium-phosphate buffer, pH 7.0 and 0.01% in tween- 20.

The results obtained demonstrate that the MIP-SPE offers a simple, convenient and a rapid methodology for solid phase extraction of aflatoxin-B1 even at very low concentrations of 2 ppb.

4.4 Creatine and creatinine sensors- a comparative discussion.

Sensors for creatine and creatinine reported so far have almost always been based on amperometric enzyme sensors (Shin et al., 2001; Tombach et al. 2001; Killard and Smyth, 2000; Khan and Wernet, 1997; Renneberg et al., 1996, Madaras and Buck, 1996; Koncki et al., 1996). There have been also reports of development of creatinine ELISA (Benkert et al., 2000), where creatinine-specific antibodies have been generated for immunochemical creatinine determinations. There are several problems associated with biosensors and assays for creatine and creatinine. Among these problems are (i) high level of interference between creatinine, creatine and other molecules presented in the sample and (ii) low stability of the enzymes used in these devices which can not be easily regenerated and sterilised.

In this aspect, MIPs are advantageous, as they are one of the most stable materials for sensors that can withstand extremes of chemical and physical environmental conditions. As it was demonstrated in this study, MIP can operate equally well after prolonged storage in buffer and at room temperature. Though MIP for creatine has been reported previously (Sreenivasan and Sivakumar, 1997), authors did not demonstrate the ability of this polymer to differentiate between closely related compounds like creatine and creatinine. This challenging task was achieved with computationally designed polymer prepared and characterised in this study.

In addition to high stability and selectivity, the computationally designed MIP explored the unique combination of two functions important for sensor development: analyte recognition and signalling. Although signalling MIPs have been described previously, the polymer developed in this study also has group specificity for primary amines, which in principle can enhance the specificity of measurements by removing the interference from other groups of chemicals presented in the sample.

Chapter 5

Conclusion and future work

General conclusions

- A general procedure for the computational design of MIPs with improved affinity and selectivity has been developed and shown with examples which includes the development of MIP for creatinine and aflatoxin B1.

- One of the serious problems associated with the development of MIP sensors and which lies in absence of generic procedure for the transformation of polymer-template binding event into detectable optical/electrical signal has been addressed by the developing of "Bite and Switch" approach which can be used in the recognition of primary amines.

- The application of MIP in sensor-linked technology, specifically in solid phase extraction has been evaluated using example of developing MIP for recovery of aflatoxin-B1 from model and real samples.

Development of a general procedure for the design of MIPs

A procedure of computer aided rational design technique for the rapid development and optimisation of MIPs was developed. This method included screening of virtual library of the functional monomers for their interaction with a template molecule and selection of these monomers giving complex with the template for polymer preparation. Then the method included placing of the functional monomers around the template and used molecular mechanics to simulate pre-arrangement of the functional monomers around the template in the monomer mixture. At the end of the program, the number and the position of the functional monomers were examined. Simulated annealing thus gives us the type and quantity of the monomers participating in the complex with template indicating the type and ratio of the template and monomers in an optimised MIP composition.

Transformation of MIP binding event into a measurable signal

In this work, we also have developed a new 'Bite and Switch' sensors for the detection of creatine and creatinine. We explored the ability of polymerisable hemithioacetal formed by allyl mercaptan and o-phthalic dialdehyde to react with primary amines and form a florescent isoindole complex. We have hence proved that that the 'bite-and-switch' approach combined with molecular imprinting can be used for the design of assays and materials for sensors, specific for any amino containing. Hence this method has general significance for analytical chemistry.

Application of MIPs in sensors

We have shown in this work a new method for the selective detection of creatine and creatinine. The MIPs created using traditional methods were unable to differentiate between creatine and its structural analogue creatinine. We developed a new approach to the rational design of MIPs selective for creatinine using computer simulation. The computationally designed polymer demonstrated superior selectivity in comparison to the polymer prepared using traditional approach, a detection limit of 25uM and good stability. The 'bite-and-switch' approach combined with molecular imprinting can be used for the design of assays and sensors, selective for amino containing substances.

Application of MIPs in solid phase extraction

MIP for the selective binding properties for aflatoxin-B1 was prepared using the computational approach. The results obtained demonstrate that the MISPE offers a simple, convenient and a rapid methodology for solid phase extraction of aflatoxin-B1 even at very low concentrations of 2 ppb. The commercially available C-18 cartridges were able to recover only about 52% of aflatoxin-B1 at concentrations of 2 ppb when compared with almost complete recovery by the MIP. We have proved here that, MIPs as a solid phase extraction materials offer important and practical advantages with respect to other solid phase extraction methodologies.

Future work

There are a number of areas that need attention in the future if new generation of MIPs will replace traditional biosensors and chemical sensors. They have been detailed below.

- **General technology for the design of MIPs**

The development of a general procedure for the design of MIPs is one of the most challenging aspects in the filed of MIP technology. Thermodynamic calculations and the combinatorial screening approaches have been successfully used to identify the best monomer candidates for imprinting (Takeuchi *et al.*, 1999), however this work became difficult with the increase in size of monomer libraries, which now include thousands of polymerizable compounds. A potential solution to this problem is the use of molecular modeling software and searching algorithms traditionally applied in drug design. Our group recently adapted this for the design of affinity polymers (Piletsky *et al.*, 2000) and to some extent this work is described in this thesis. Future work should be concentrated on rectifying this approach by linking together modelling data with thermodynamic analysis and testing of variety of real systems.

- **Signal generation**

It has always been very difficult to transform the binding event into a measurable signal. This is one of the major problems that hinder the development of MIP sensors. Although we demonstrated the possibility to use a new "Bite and Switch" approach for a successful detection of binding event between the polymerisable thioacetal and primary amines, the needs in additional derivatisation of the template for MIP preparation and possibility to use this method only for amino containing analytes severely restricts its application. It is important to expand this method for variety of other analytes.

- **Poor performance in aqueous solution**

One of the serious problems of MIPs is their poor performance in aqueous environments, so there is a need in the development of water compatible MIPs. The following aspects would be emphasis of future research on this problem

a) Selection of monomers and cross-linkers soluble in water and analysis of their recognition properties

b) Computer simulation of polymer-template interaction in aqueous environment

c) Development of polymerisation procedure compatible with aqueous environment

d) Design of water-protective coatings for MIP

- **Difficult integration of MIP with detector**

The immobilisation of MIPs onto the detector surface remains difficult. Critically important is developing new approaches for grafting and photodeposition of MIPs to the surface of physical transducers. The work in this direction is in progress in our laboratory.

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Chen B., Piletsky S. A., Piletska E. V., Chianella I., Subrahmanyam S., Turner A. P. F. The design of artificial receptors for sensor applications using a novel computational and combinatorial molecularly imprinted polymer approach. *Biosens. Bioelectron.*,

Patent

Piletsky S. A., Day R. M., Chen B., Subrahmanyam S., Piletska E. V., Turner A. P. F. Rational design of MIPs using computational approach. (PCT/GB01/00324).

Refereed conference presentations

Subrahmanyam S., Piletsky S. A., Piletska E. V., Chen B., Day R., Turner A. P. F., 'Bite-and-switch' sensors for creatine and creatinine based on molecularly imprinted polymers, *The sixth world congress on biosensors*, Hyatt Regency, San Diego, USA, 24-26 May 2000.

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