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Detection of atrazine and 2,4,6-TCP in wine and other matrices

Cranfield Health

MPhil Thesis

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Abstract

Introduction

GOODFOOD aimed to develop new generation micro- and nanoscale devices for safety and quality assurance in the food chain and agrofood industry. Cranfield University, along with other partners in Worpackage 2, was responsible for developing immunosensors for the determination of pesticides in wines and fruit juices at analytically valid levels.

Methods and Materials

The immunosensor format was based on the use of antibodies with binding specificity directed towards the target analytes. These were chiefly supplied by CSIC in Barcelona. The protocol and reagents supplied were used for detection of atrazine and 2,4,6-Trichlorophenol in various matrices. Screen Printed Electrodes were produced, comprising carbon working and counter electrodes and Ag/AgCl working electrode. Screen printed electrodes can be mass-produced at low cost, and so are disposable after a single use. A macro-scale flow-cell system was used with the screen-printed electrodes for detection of horseradish peroxide (HRP) which is the enzyme label for the assay (Figure 3). As part of system optimisation, three electron acceptors were compared. Reaction time between substrate and enzyme was also examined as a factor. As a step towards miniaturisation, HRP was immobilised onto magnetic beads and then dilutions of the beads were measured in order to help calibrate the system.

Activated Sepharose 4B ^{TM} from Pharmacia was also used as a solid support. Small-scale Polydimethylsiloxane (PDMS) and adhesive-layer cells

were manufactured for optical analysis of immunoassay product. Optical analysis was performed by an RGB reader developed in-house.

Results

The detection of atrazine and 2,4,6-TCP at nanomolar concentrations in various matrices, using the supplied reagents was demonstrated. Of the three electron acceptors tested, ABTS was found to be most effective. Increased reaction time was found to increase the signal. Dilutions of magnetic beads coated with immobilised HRP gave a proportional response as anticipated.

Data obtained by optical analysis of assay product using the RGB-reader followed the expected trend and showed signal intensity reduction with increasing analyte concentration.

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List of Abbreviations

Abbreviation

2,4,6-TCP   2,4,6-trichlorophenol

Ab   Antibody

ABTS   2,2''-Azino-Bis(3-ethylbenzthiazoline-6-sulfonic acid)

Ag   Antigen

ATR   Attenuated Total Reflectance

BSA   Bovine Serum Albumen

CE   Capillary Electrophoresis

CME   Chemically Modified Electrode

DCM   Dichloromethane

DMF   Dimethyl Formamide

EC   European Commission

EC   Electrochemical

ECD   Electron Capture Device

ELISA   Enzyme Linked Immunosorbent Assay

EPA   Environmental Protection Agency (US)

EU   European Union

FIA   Flow Injection Analysis

GC   Gas Chromatography

HPLC   High Performance Liquid Chromatography

HRP   Horse Radish Peroxidase

HSV   Hue, Saturation, Value

IA   Immunoassay

IgG   Immunoglobulin G

ISE	Ion Selective Electrode
LC	Liquid Chromatography
LME	Liquid Microextraction
LOD	Limits of Determination
LSPR	Localised Plasmon Resonance
MCL	Maximum Contamination Level
MIP	Molecularly Imprinted Polymers
MRL	Maximum Residue level
MS	Mass Spectroscopy
OPD	o-Phenylenediamene
PAH	Polycyclic Aromatic Hydrocarbons
PBS	Sodium Phosphate Buffer
PBST	Sodium Phopsphate Buffer with Tween 20
PC	Personal Computer
PCP	Pentchlorophenol
PDMS	Polydimethylsiloxane
QCM	Quartz Crystal Microbalance
RAM	Restricted Access Materials
REDOX	Reduction Oxidation reaction
RGB	Red, Green, Blue
RIFS	Reflectomatic Interference Spectroscopy
RM	Resonant Mirror
SCF	Supercritical Fluid
SDS	Sodium Dodecyl Sulphate
SFE	Supercritical Fluid Extraction
SHE	Standard Hydrogen Reference Electrode
SPE	Screen Printed Electrode

SPE   Solid Phase Extraction

SPME   Solid Phase Micro Extraction

SPR   Surface Plasmon Resonance

SPW   Surface Plasma Waves

SQUID   Superconducting Quantum Interference Device

SWOT Diagram Strengths, Weaknesses, Opportunities, Threats Diagram

TCP   Trichlorophenol

TMB   3,3", 5,5' Tetramethylbenzidine

WHO   World Health Organisation

WP2   Work Package 2 (of the GoodFood Project)

WSPR   Waveguide Surface Plasmon Resonance

Chapter 1

Introduction and literature review

1.1 BACKGROUND TO THE PROJECT

The GoodFood project was a multidisciplinary integrated EU project, co-funded within the 6th Framework. It was implemented in order to address the issue of pesticide contamination in food and to develop improved methods of pesticide detection, and proposed to *'bring the lab to the foodstuff.'* The aim was to develop a new generation of analytical methods, based on micro- and nanotechnology. Techniques developed by the Project should enable people with less specialist training to quickly and simply test foodstuffs for chemical contaminants in the field rather than having to send samples to a central laboratory for extended analysis. The widespread introduction of simple, quick food-safety tests should improve quality standards across the EU. The project ran for 42 months from 01/01/04 until 01/06/07.

Aims and Objectives

A set of detection targets was identified by the relevant food industries and assigned to the different work packages of the project. Work Package 2, to which Cranfield University was a partner, was concerned with the development of novel immuno-diagnostic systems for the detection of pesticides. Wine and fruit-juice were chosen as a preliminary model foodstuff, and 2,4,6 Trichlorophenol, the herbicides atrazine and simazine, and the miticide bromopropylate were targeted.

The aim of this study was to produce a prototype small-scale sensing platform for detection of the target analytes. The following objectives were developed in the course of the study:

- Demonstrate IgG sensitive enough to meet the required standard
- Examine Electrochemical and Optical detection
- Compare electron acceptors
- Manufacture 2 small scale cells

1.2 THE WINE INDUSTRY

The wine industry in Europe is important economically (see Figure 1.1). In terms of area, production (with 3043072.00 Ha under vines in 1999-2000 (http//europa.eu.int)) and consumption, Europe is the world leader (www.europa.com CAP reform). In 1996, the EU had a 50.1% share of the world trade in wine (www.europa.com) and in 1997 the external wine trade was worth nearly €3,500,000. The US imported $3,663,931,000 worth of wine and beer from the EU in 2003, out of a total value of $5,976,702,000 worth of wine and beer imports (www.fas.usda.gov), which was the highest import level since 1970 (www.fas.usda.gov). Figure 1.1 shows the EU wine trade, with selected partners, 1995-2000.
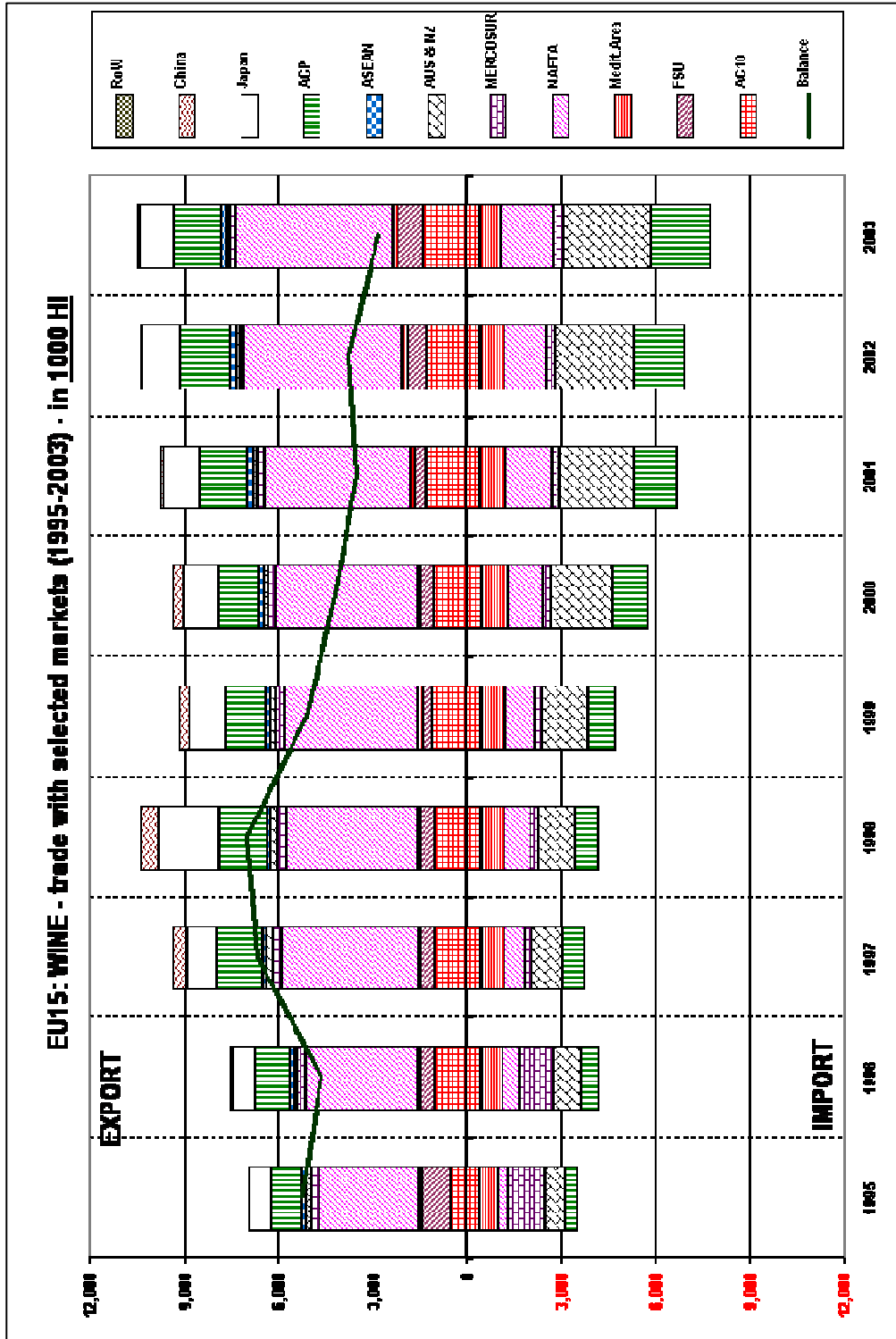
Figure1.1: EU wine trade 1995-2003 Source: http://europa.eu.int

Most of the grape production in the EU is destined for wine-making. As the wine trade is of great economic importance, it is vital to obtain a good quality grape. In order to do this it is necessary to protect the vine from parasite attacks and minimise weed competition. Applications of pesticides and herbicides are commonly used to achieve this.

1.3 TARGET ANALYTES

Pesticides enter the environment by various diverse routes, contaminating food materials during agricultural production, food processing, packaging and storage. Due to the volume of usage and to their ubiquity, environmental persistence and toxicological properties, pesticides represent a major hazard to global public health. In order to guarantee consumer safety, strict standards must be maintained. In the EU, several directives and regulations have been laid down, for example EC Directive 91/414; EEC Regulation 92/2078; European Council Regulation 99/1257; EC Directive 76/464/EEC and Directive 90/642/EEC, which now fixes the maximum residue levels for each pesticide in fruits and vegetables (see Section 1:3iv).

1.3.1 The chlorophenol problem

Chlorophenols (see Figure 1.2 for chemical structure) are ubiquitous in the environment. They are present in drinking water as a result of the chlorination of phenols, as by products of the reaction of hypochlorite with phenolic acids, as biocides, or as degradation products of phenoxy herbicides (www.who.int). 2,4,6-TCP has been reported to induce

lymphomas and leukemias in male rats (Blackburn et al 1986) and hepatic tumors in male and female mice (www.who.int)   IARC has classified 2,4,6-TCP as a possible human carcinogen (IARC 1987). Chlorophenols are used as fungicides and also in bleaching. Due to health and environmental concerns, they are being replaced in these roles with peroxides.

A recent issue has been the use of contaminated corks. Chlorine bleaching is used to remove natural fungi and bacteria which are present in the cork.   The hypochlorite solution used may not be sufficient to kill all fungi and bacteria as some are resistant. In addition, residual natural phenolic materials in the cork are chlorinated to form chlorophenols and biomethylated by resistant fungi to chloroanisoles. These processes can lead to formation of a set of congener substituted chlorophenols and chloroanisoles (2,4,6 trichloroanisole, 2,3,4,6 trichloranisole and pentachloroanisole) which may impart a strong, musty "corked" flavour to wine. Residues of the corresponding unmethylated chlorophenols can also add an antiseptic note to the flavour. This may affect consumer behaviour and lead to economic effects.

An additional source of contamination is the use of polychlorophenolic biocides in cork-oak forests.

There is also a risk of contamination by chlorophenols and chloranisoles from the use of hypochlorite to wash barrels prior to filling and from the use of chlorophenolic biocides for the preservation of softwood products such as wooden pallets and storage racks, also cardboard cartons and other packaging material. The semi-volatile nature of chlorophenols and chloranisoles provides a significant vapour pressure which may cause atmospheric contamination, leading to the translocation of contaminants within the winery.

There is also evidence to suggest that chlorophenols can be synthesised in the environment by the reaction of free phenols in water sources reacting with the chlorine which is routinely added to water supplies. These free phenols in the environment are contaminants resulting from the manufacture of paints, resins and plastic products. Common end products include 2,4-dichlophenol, 2,6-dichlorophenol and 2,4,6-trichlorophenol (www.who.int)
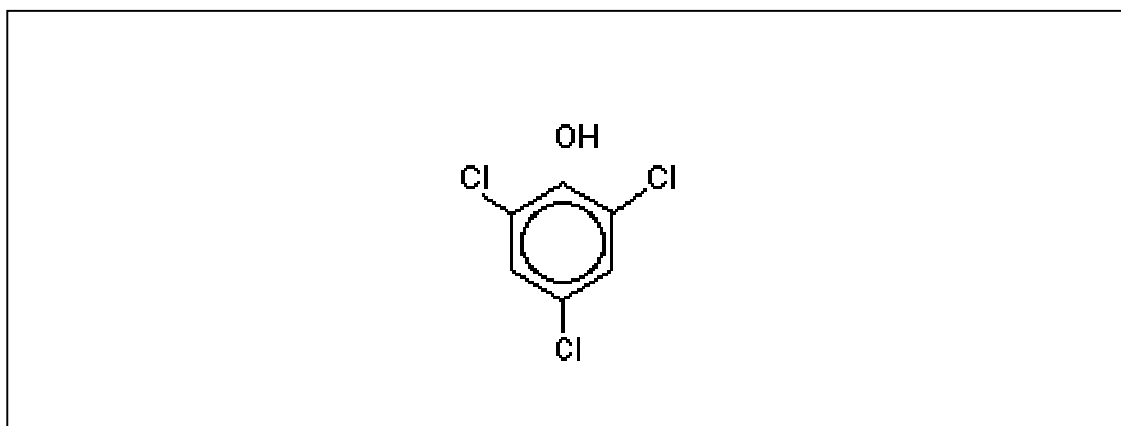
Figure 1.2 Chemical structure of 2,4,6-Trichlorophenol: Source http://www.chemguide.co.uk/basicorg/conventions/tcp.GIF

1.3.2 The Atrazine and Simazine Problem

Atrazine (6-chloro-N-ethyl-N'-(1-methyl)-1,3,5-triazine-2,4-diamine) Simazine (6-chloro-N,N'-diethyl-1,3,5-triazine-2,4-diamine)

Atrazine and simazine are both triazine herbicides, used for the pre- and post-emergence control of broad-leaf weeds and some grassy weeds. They are systemic herbicides that are absorbed through the root and inhibit the photosynthetic electron transport. They are accumulated in the apical meristems and leaves.

Atrazine

Atrazine (see Figure 1.3 for chemical structure) is one of the two most widely used herbicides in the world (www.epa.gov). It was first registered for use in the US in 1959 and its annual use in the US was estimated to be 64-75 million lbs (29-34 thousand tonnes) in 1996 (www.epa.gov). Due to its widespread use, atrazine is often considered an indicator compound for pesticide pollution. Atrazine has recently been refused re-registration in the EU (2004/248/EC), although some specified use is allowed for seven years. It has been re-registered in the US (www.epa.gov). Due to its widespread use outside the EU, atrazine may enter the EU on imported foodstuffs. An example of this route is the importation of soya or soya-based animal feed from Argentina and Brazil where Atrazine is used in the control of self-sown "*Roundup Ready*" soya. The use of atrazine in the cultivation of maize may also pose a problem.

Exposure to triazine herbicides was found by Kettles *et al* (1997) to significantly increase the risk of breast cancer in women. Taets *et al* (1998) found that Atrazine caused chromosomal damage in Chinese hamster ovary cells at concentrations of 0.003µg/ml and 0.018µg/ml (the maximum contamination level (MCL) permitted for drinking water as determined by the US EPA, and the maximum level recorded in Illinois water supplies respectively). Swan *et al* (2003a) found lowered sperm concentration and motility in mid-Missouri men compared to New York and Minneapolis men. This was later linked (Swan *et al* 2003b) to exposure to three agricultural herbicides, of which atrazine was one. Filipov *et al* (2007) found evidence that exposure to atrazine decreased striatal tissue levels of dopamine,

suggesting that atrazine is a dopaminergic toxin and perhaps a contributory factor to Parkinsons disease.

Due to its high water solubility, weak adsorptivity and persistence in ground water (www.epa.gov) (Barth *et al* 2007 found no decrease in atrazine and deethylatrazine contamination of the Brevilles sandy aquifer 5 years after agricultural inputs stopped) atrazine is a frequent contaminant of natural waters by infiltration, spills, spray-drift or surface run-off. The findings of Gfrerer *et al* (2002) indicated a homogenous distribution in Eastern Chinese Rivers and suggested that a mass transport of 57.5 kg per day of atrazine may be taking place in these rivers even at less than the EU drinking water limit of 100ng/l. The environmental persistence of atrazine may also lead to "carry over" and damage in subsequent crops, particularly if they are sensitive to atrazine (such as alafalfa or soya). Leavitt *et al* (1991) found that (in their study after the 1988 drought in the US) the greatest persistence of atrazine correlated with soil cation exchange activity, suggesting that environmental factors such as soil moisture and temperature were important factors in the breakdown of atrazine in the soil. With reduced rainfall in some regions probable due to climate change, this may become a larger issue than it currently is.

ATRAZINE (Aatrex®)

Cl

2-chloro-4(ethylamino)-6-(isopropylamino)-*s*-triazine
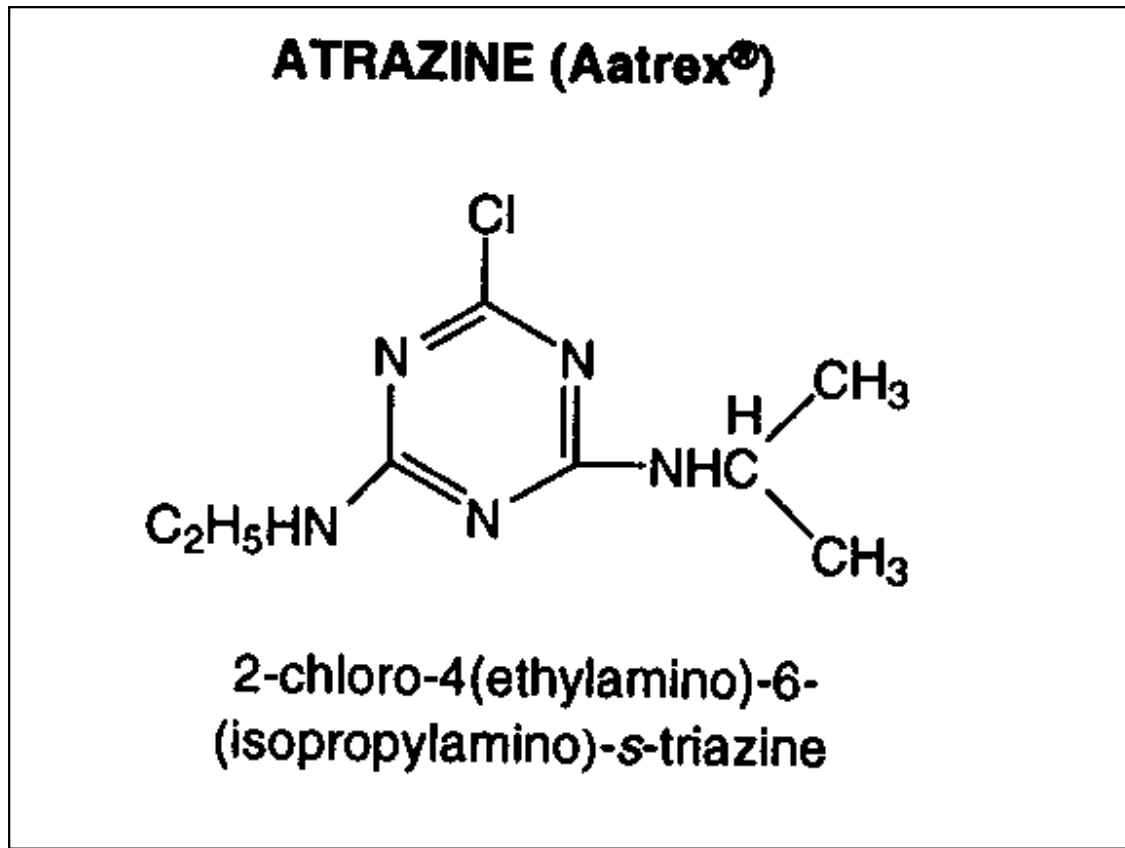
Figure 1.3: Chemical structure of Atrazine

Source: www.ipmworld.umn.edu/chapters/wareherb.htm

Simazine

Like atrazine, simazine (see Figure 1.4 for chemical structure) is a chlorinated triazine herbicide used for the pre-emergence control of broad-leaf, and some grassy, weeds. Simazine, like atrazine, is absorbed through the roots and accumulates in the leaves and meristem of plants. It also works by inhibition of photosynthesis. It is not absorbed through the leaves and is susceptible to wash-off by rain. Simazine can remain active in the soil for over a year (http://infoventures.com/e-hlth/pesticides/simazine/html) with a half-life of 8-12 weeks. It can be broken down by microorganisms or chemically in water. It has a low water-solubility, but is highly mobile in

certain soil types and has been found in groundwater and in surface water (www.epa.gov). Simazine, along with atrazine, has been linked to an increased risk of breast cancer (Kettles *et al* 1997), and was found to induce whole-cell clastogenicity in Chinese hamster ovary cells (Taets *et al* 1998).
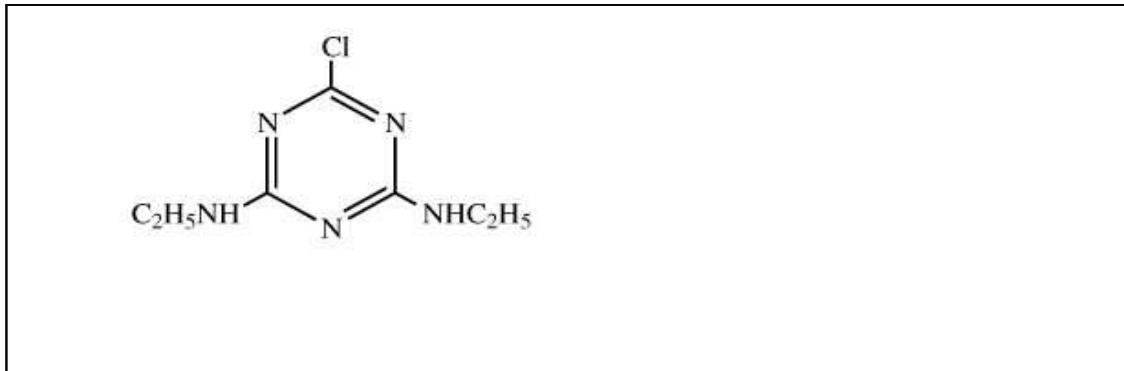
Figure 1.4: Chemical structure of Simazine

Source: www.generalchem.cn/images/crop/Simazine.jpg

1.3.3 The Bromopropylate problem

Bromopropylate (see Figure 1.5 for chemical structure) is a non-systemic araricide used on pome-fruit, citrus, grapevines, cotton and hops. It is also used on vines to control mites. It affects early egg development and kills newly hatched larvae by contact with the deposit on the foliage. It is a white crystalline powder which is fairly stable in neutral or slightly acid media.

It does not penetrate into the fruit-pulp but remains on the peel. Multiple applications have an additive effect and result in higher residues. The dispersion and dissipation of the residue is mainly due to the action of

weathering and dilution by plant growth. Bromopropylate has a half-life of three weeks on most fruits.

Bromopropylate has no known teratogenic or carcinogenic effects and a low acute toxicity in rats and rabbits (LD50>2000mg/kg). It has therefore been classified as unlikely to present an acute hazard in normal use. The estimated acceptable daily intake for humans is 0.03mg/kg. (GoodFood document: Deliverable D7-WP2D2).
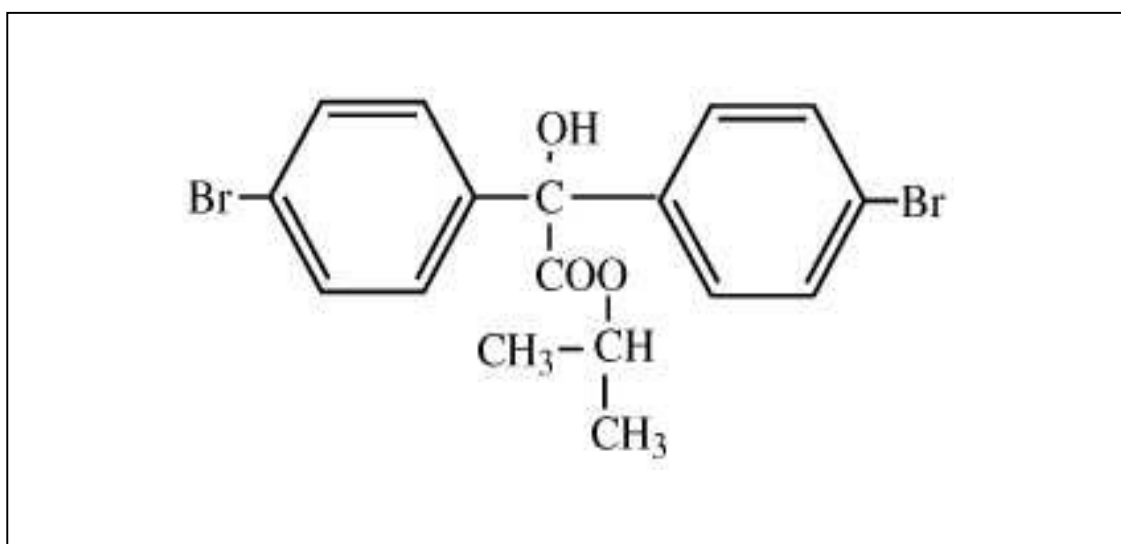
Figure 1.5: Chemical structure of Bromopropylate

Source: www.generalchem.cn/images/crop/Bromopropylate.jpg

1.3.4 Maximum Residue Levels

The EU has recently fixed Maximum Residue Levels (MRLs) of chemicals permitted in various commodities. Each member state is also allowed to fix its own MRLs but there is an ongoing process of harmonisation.

Table 1.1: Maximum Residue Levels permitted in grapes & wine and the EU directives governing them Source: European Union website (http://europa.eu)

Substance	EU Directive	MRL mg/kg
Atrazine	93/58/EC	0.1
Simazine		Not yet established
Bromopropylate	04/59/EC	0.2

This means, for example, that the maximum amount of atrazine permitted in grapes and wine intended for consumption is 0.1 milligrams in every kilogram of grapes or wine.

In addition to MRLs, the EU has established Limits of Determination (LODs) (946/46/EC). These represent the lowest concentration which can be measured with acceptable precision.

Table 1.2: Limits of Determination for Atrazine, Simazine and Bromopropylate   Source: Directive 946/46/EC

Substance	LOD mg/kg
Atrazine	0.1
Simazine	Not yet established
Bromopropylate	0.05

This means, for example, that while it is possible to accurately detect the presence of atrazine in dilutions as small as 0.1 milligrams per kilogram of matrix, it is not possible to be sure that it is absent from matrices where the atrazine may be more dilute.

These LODs determine the required sensitivity for the sensor. This has led to the proposal of an appropriate sensitivity and detection range being circulated within Workpackage 2 of the project; these targets are derived from the LODs in EC/946/46.

Table 1.3: Appropriate Sensitivity (from GoodFood WP2 micro-fluidics system requirements/discussion document 2/11/04)

Compound	Detection Limit	Range of Interest
2,4,6-Trichlorophenol	0.1µg/L	0.5-200µg/L
Atrazine	10ng/L	20-200ng/L
Simazine	0.1µg/L	0.3-200µg/L
Bromopropylate	2mg/kg	

So, for example, since it was impossible to reliably detect Atrazine at dilutions below 10 nanograms per litre of matrix, it was decided that Workpackage 2 would aim to design sensors that detected Atrazine at between 20 and 200 nanograms per litre of matrix.

1.4 STANDARD LABORATORY TECHNIQUES

Techniques for detecting target analytes are normally preceded by a sample preparation/ pre-concentration step. This is especially important when dealing with trace analysis in real wine-samples due to the complexity

of the matrix and the possibility of cross-binding of antibodies. Pre-concentration may also be necessary to detect dilute trace analyte.

1.4.1 Methods of Extraction

Traditional methods of extraction of analytes from samples include the following.

Liquid-liquid extraction

This is a well tested method based on the distribution of a solute in two immiscible liquids due to their partition coefficients. It has a variable efficiency with individual triazines and involves slow and often complex procedures. Its major disadvantage however is the use of large volumes of expensive, toxic, high purity solvent with the consequent health risks to the analyst and costs of safety equipment and disposal.

The miniaturisation of this technique by Jeannot & Cantwell (1996) where solvent extraction from water took place into an 8µl organic solvent droplet at the end of a Teflon rod and the further development of the technique using a microsyringe (e.g. Ligor & Buszewski 2000), has helped to reduce some of these problems. Liquid microextraction (LME or LPME) also eliminates the possibility of sample contamination due to carryover, which can be a problem with SPE or SPME, as a separate drop is used each time.

Solid Phase Extraction (SPE)

This provides an alternative to simple solvent extraction for sample clean-up and concentration. It is widely used and may sometimes eliminate

the need for solvent completely. Extraction columns are packed with a variety of bonded silica absorbent particles, which selectively retain specific chemicals from the surrounding matrix. The material trapped on the column can then be eluted and analysed. Alternatively, the silica particles can be embedded in a polytetraflouroethylene micronet to form a membrane which can extract analytes from large, dirty samples.

Solid Phase Micro Extraction (SPME)

In SPME (see Figure 1.6) a short length (approx 2cm) of a thin (15-150µm) silica fibre coated with adsorbent is mounted on a thin steel rod and contained within a chromatographic syringe needle. The end of the fibre support rod is fastened to the plunger normally used to fill the syringe with the sample. When the plunger is raised, the fibre is withdrawn inside the hypodermic needle. When the needle is inserted through the septum of a sealed solution sample, the fibre can be lowered into the sample by depressing the plunger. After a suitable time has elapsed, the fibre is withdrawn into the needle which is then withdrawn from the sample. It can then be injected directly into a gas-chromatograph, where the analyte is thermally desorbed. SPME can also be used for headspace analysis of volatile species above a dirty sample.
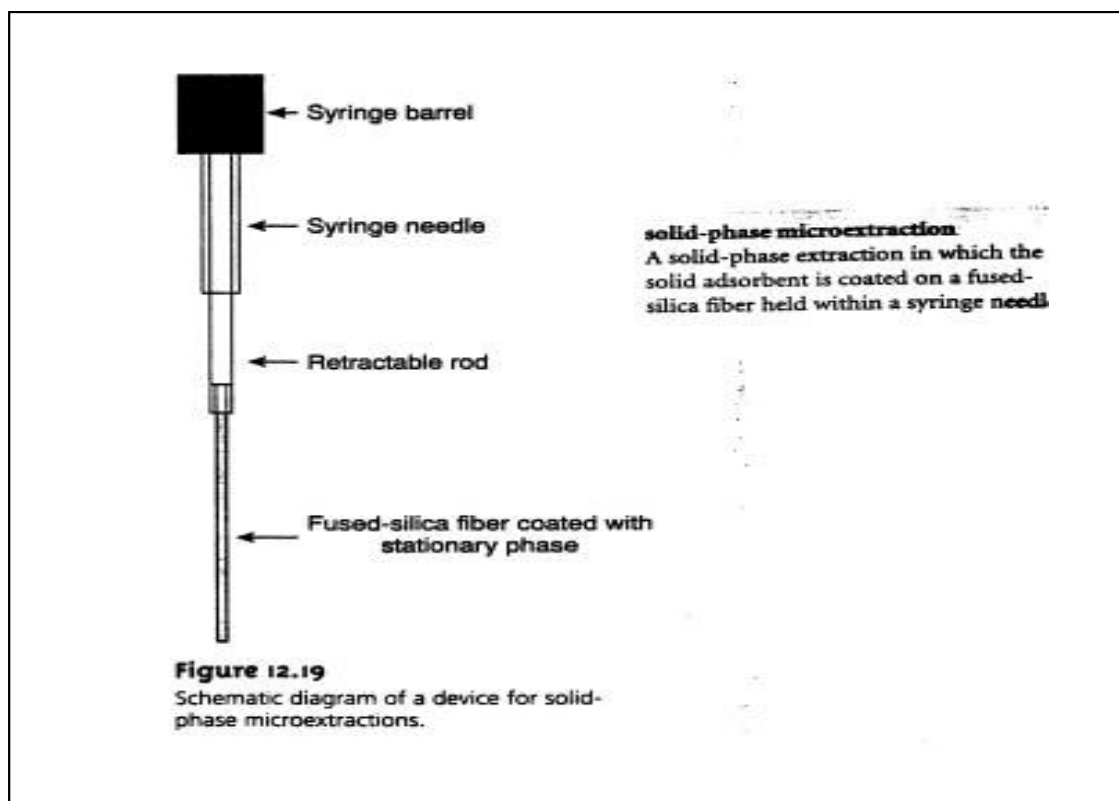
Figure 1.6: Schematic of a SPME device.

 (Source: http://electrochem.usak.ca)

Molecularly Imprinted Polymers (MIP)

Molecularly Imprinted Polymers are synthetic materials, which mimic molecular recognition by biological receptors. In MIP technology, macromolecular structures are prepared (often with the aid of computer aided design) by a polymerisation process in which sites are introduced by use of a ligand, as a template in a casting procedure. The selected ligand or *print molecule* is first allowed to establish bond formations with polymerizable functionalities and the resulting complexes or adducts are then copolymerized with cross-linkers to form a rigid polymer. After

extraction of the ligand, specific recognition sites are left in the polymer. The spatial arrangement of the complementary functional entities of the polymer network, together with the shape image, correspond to the imprinted molecule.

There are two basic approaches to preparation of MIPs:-

- The Non-covalent or Self Assembly approach. The prearrangement between print molecule and functional monomers is formed by non-covalent or metal coordination interactions.

- The Covalent or Pre-organised approach. The aggregates in solution prior to polymerisation are maintained by (reversible) covalent bonds

MIPs are relatively stable against mechanical stress, high temperatures and pressures, and are also resistant to treatment with acid, base or metal ions. They have a long shelf-life. However, because MIPs are often made in organic solvents, their optimal binding conditions often require an organic-based medium. Additionally, when used as a recognition element, the sensitivity of MIPs may not be as great as that of some antibodies. There can also be a problem of contamination of the sample by the ligand, which has been retained and then is released during elution, or else can give a high background signal if analysing by flourescence.

MIPs have been used successfully for extraction and sample pre-concentration. Molinelli *et al* (2002) directly applied red wine onto a MIP-based SPE cartridge, and achieved a recovery rate of 98.2% for Quercetin in the sample. Chapius *et al* (2003) obtained an 89% extraction of triazines and metabolites in mineral and tap water using MIPs.

Supercritical Fluid Extraction (SFE)

This highly efficient method of extraction is mostly used for extraction from solid samples. It is based on the fact that, at critical temperature, a substance cannot be condensed into its liquid state by the application of pressure.

Super-critical fluids (SCFs) have different properties than either gases or liquids. They are capable of dissolving large non-volatile molecules. SCF carbon dioxide, for example, can dissolve polycyclic hydrocarbons (both aromatic and aliphatic) and alkyl pthalates. SCF CO_2 is widely used as it has a super-critical temperature around ambient, has a low reactivity and toxicity, and can be obtained at high purity for relatively little cost. Analysis is often by Flame Ionisation, as the SCF can be allowed to expand naturally into the hydrogen flame, where conductivity changes result from the presence of the analyte ions.

1.4.2 Current methods for detection of target analytes

The main methods for the detection of the target analytes are gas chromatography (GC) or liquid chromatography (LC), especially High Performance Liquid Chromatography (HPLC). Since chromatography is essentially a separation technique with limited quantitive detection capability, a confirmatory detection mechanism is often added e.g. Mass Spectroscopy (MS) or Electron Capture Detection (ECD). A very brief explanation of GC and LC follows.

Gas Chromatography

In gas phase chromatography the material which is to be analysed is inserted into a column which is packed with an inert support coated with a non-volatile stationary phase. The analytes are vapourised in the the hot environment and carried through the column on a stream of inert carrier gas (often helium or argon). The analyte is distributed between the inert carrier gas mobile phase and the stationary phase. The analyte does not react with the mobile phase, so the rate of elution is therefore dependant on the volatility of the analyte and the interaction between the analyte and the stationary phase.

Liquid phase chromatography

Liquids may be used as the mobile phase in column and planar chromatography systems. The requirements for liquid systems are similar to those for gas systems, although higher pressures are needed to force the liquid through the system and solvents must be de-gassed in order to avoid gas bubble formation.

Column systems can be divided into 4 classifications depending on the nature of the stationary phase:-

1. *Partition liquid-liquid chromatography*: The liquid is retained on the column either by physical adsorption or formation of covalent bonds with the support.

2. *Adsorption liquid-liquid chromatography*: The analyte competes with the mobile phase for sites on the surface of the packing. Variables are flow rate and molarity of the mobile phase.

3. *Ion exchange*: The stationary phase is an ion exchange resin, often beads. Ions are introduced at the top of the column either via a

pneumatic valve or via electromigration from source (this offers
sample cleanup) and adsorbed onto stationary phase by ion exchange.
They are later eluted with excess like-charged ions. *Ion capillary
electrophoresis* (ICE) is based on the mobility of ions under an
applied electric field and uses this property to discriminate the analyte.

4. *Size exclusion or gel permeation*: this depends on the differentiation
of molecules on the basis of molecular size. Fractionation is directly
related to molecular size and shape.

Further details are available in many good textbooks (for example Ahmad ,
Cartwright & Taylor, Eds: 2001) and further explanation is beyond the
scope of this thesis.

Standard laboratory techniques

Trichlorophenol

Trichlorophenol does not appear in isolation; other chlorophenolic
compounds (mono-, di-, tetra- and penta- chlorophenols and chloroanisoles)
are also generally present. Thus separation and quantification of the separate
compounds is necessary.

The principal method of analysis of chlorophenols is gas
chromatography (GC) with varying detection mechanisms (flame-ionisation,
electron capture and mass spectrometry).

As chlorophenols are highly polar and have a relatively low vapour
pressure, it can be difficult to measure them directly using GC, therefore
chlorophenols are usually converted to less polar derivatives before analysis.
This improves peak shapes and prevents some adsorption problems.

Sample pre-treatment and analysis

Liquid-liquid extraction:

TCP is protonated in an acidified solution, so that it can then be transferred to organic solution. Wine is acidified with sulphuric acid and the TCP is extracted with an organic solvent such as petroleum-ether, pentane or mixtures of dichloromethane and pentane. This yields TCP and Chloroanisoles.

The extracted TCP can then be directly analysed by concentration under a nitrogen stream followed by GC-MS detection (LOD 10ng/L).

Instead of direct analysis at this stage, the TCP could be further separated from the chloroanisoles with an alkali solution of potassium carbonate. The TCP is deprotonated and extracted to the aqueous phase, while the chloroanisoles remain in the organic layer. It is then acetylated with acetic anhydride and re-extracted with hexane. This solution is then concentrated under a nitrogen stream and analysed by GC-MS (LOD=0.5-2 µg/L (in hexane solution)).

Solid-phase extraction (SPE):

Wine is applied to the SPE cartridge (C_{18}) and the TCP is eluted with an appropriate organic solvent (dichloromethane (DCM)), the extract is then concentrated and analysed by GC-MS without derivatisation (LOD=0.7ng/L Recovery: 82-103%, LOQ=4ng/L: Imprecision 1.7-3.9%)

Solid-phase micro extraction (SPME) and stir bar sorptive extraction:
These are both based on the principal of partition between a coating and the aqueous matrix. The coating is usually Polydimethyl-siloxane (PDMS) on a fibre (SPME) or stir bar.

The TCP is extracted by introduction of the PDMS phase into a volume of sample. After a specified time, the PDMS phase is removed, washed and transferred into a thermal desorption tube and analysed by GC-MS. (LOD=10-20pg/L, LOQ=40-100pg/L: Imprecision 2-3%)

Atrazine and Simazine

Methods for analysis of triazines normally involve an extraction and clean-up step in order to remove co-extracted inteferants and to pre-concentrate the analytes. Analysis by liquid and gas chromatography is widely used; use of a mass spectrometer gives high sensitivity and selectivity.

The extraction procedure is variable, dependant on the sample characteristics. The former practice of liquid-liquid extraction, with organic solvents such as ethyl acetate or methyl tert butyl ether mixtures of dichloromethane and isopropanol, has been replaced with solid phase extraction (SPE) and, more recently, solid phase microextraction (SPME) for the extraction and preconcentration of triazines (Pacakova *et* al 1996). This reduces sample handling, solvent use and labour.

Bromopropylate

Chromatographic methods (HPLC and GC) are widely used in the determination of bromopropylate. Sample pre-treatment involving the use of SPME followed by determination by gas chromatography –electron capture detection (GC-ECD) is reported by Correia *et al* (2001).

Advantages and disadvantages of standard techniques

The standard techniques described above are widely used. Capillary gas chromatography is used for the detection of over 300 pesticides (van der Hoff & van Zoonen 1999). Liquid chromatography, especially when recent developments in column packing material (immunosorbent, MIP and Restricted Access Materials (RAMs)) are considered (Hogendoorn & van Zoonen 2000), offers a good method for detection of polar and acidic, less volatile, pesticides. When the appropriate detection system is attached e.g. -MS, -ECD, -UV the sensitivity and selectivity of these systems is excellent. However, these techniques are mainly available only at centralised laboratories (with the consequent need for sample transport) and require trained personnel. The time required for analysis can be extensive and there may be a delay before results are reported. While these techniques are reference techniques, the sensitivity of antibody techniques and the growth in the availability of assay test-kits and their acceptance by regulatory authorities such as the US EPA has led to the widespread use of immunochemical techniques as screening tests.

1.5 IMMUNOCHEMISTRY

1.5.1 The immune response

Immunochemistry, including immunoassay techniques, is based on the highly specific recognition of target analytes exhibited by antibodies, produced by the mammalian immune system.

The immune system's principal function is to protect animals from infectious organisms and their toxic products. The immune response in vertebrates can be divided into two functional responses:

1. Non-adaptive immunity:

This is a first-line defence, by a fixed manner, through production of internal defensive cells (phagocytes, lysozymes, and interferon). It does not improve with repeated exposure.

2. Adaptive immunity:

This is the second-line defence and is called on when the first-line is compromised. It is enhanced by repeated exposure. Adaptive immunity is characterised by two forms of response: cellular and humoral defences.

Cellular immune responses are effective against parasites, viral cells and cancers. The humoral immune response is effective against extracellular phases of bacterial and viral infection. When a foreign molecule or antigen enters vertebrate tissue, humoral immune response is responsible for producing B lymphocyte cells that are stimulated, divide and differentiate. The result is the production of plasma cells which secrete proteins called antibodies (Abs). The antibodies are highly specific for the antigen (Ag) which caused the initial B lymphocyte response.

The produced antibodies can recognise and attach to the antigen to form a complex. This tagging identifies the target for immunologic attack and helps activate non-specific immune responses that can destroy the target by various mechanisms, including phagocytosis, followed by removal from the circulating body fluid.

1.5.2 Antibody structure and function

Antibodies (Ab) or Immunoglobulins (Ig) are a group of glycoproteins. They are divided into several classes; in vertebrates, five classes are usually recognised (IgA, IgG, IgD IgE and IgM designated respectively α, γ, δ, ε, and μ). These differ in function, size, charge, amino acid composition and carbohydrate content from each other. The IgG class is the most common immunoglobulin class found in normal vertebrate serum and accounts for 70-80% of the total immunoglobulin pool. It is the main class utilised in immunoassays. The IgG molecule is an aggregate of 4 polypeptide structures, with a molecular weight of 164 000 Da. IgG molecules are the most commonly found in serum.

Antibodies have more than one antigen-combining site. Most human antibodies are bivalent. Some bivalent antibodies can combine to form multimeric antibodies with an increased number of binding sites.

All immunoglobulin molecules have a basic structure composed of four polypeptide chains connected to each other by disulfide bonds (see Figure 1.7). There are 2 light chains and 2 heavy chains. The light chains consist of approximately 220 amino acids and have a mass of about 25 kDa. The heavy chains consist of approximately 440 amino acids and have a mass of about 50-70 kDa. The heavy chains are structurally distinct for each immunoglobulin class or subclass.

Both light and heavy chains contain two different regions. Constant regions have amino acid sequences that do not vary significantly between antibodies of the same class. The variable regions from different antibodies do have different sequences. It is the variable regions that, when folded together, form the antigen combining sites.

The four chains are arranged in the form of a flexible Y with a hinge region. This hinge region allows the antibody to assume a T shape. The stalk of the Y is called the crystallisable fragment (Fc) and contains the site at which the antibody can bind to a cell. The arms of the Y consist of two antigen binding fragments (Fab). These bind with compatible epitopes (or antigenic determinant sites). The Fc fragments are composed only of constant regions whereas the Fab fragments have constant and variable regions.

Both the heavy and light chains contain several homologous units of about 100-110 amino acids. Within each unit (which is called a domain) disulfide bonds form a loop of approximately 60 amino acids. Inter-chain disulfide bonds also link the heavy and light chains together.
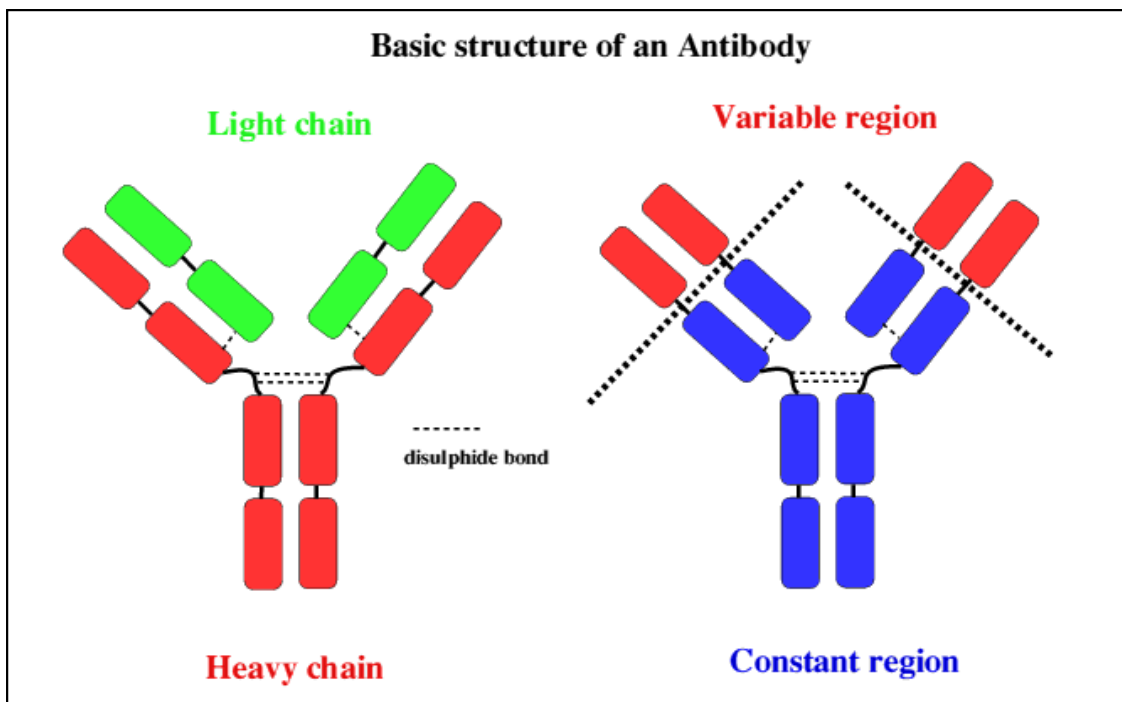
Figure 1.7: Basic structure of an antibody: source www-immuno.path.cam.ac.uk

1.5.3 Antibody production

The route for producing polyclonal and monoclonal Abs, which are used in IAs, is the same up to the immunization. Compounds of low molecular mass (<100 Da) are unable to evoke an immune response. As many pesticide molecules are below this they need modification before immunization, by binding to a larger molecule called the carrier, which is often a protein. Carrier design is an important step in the production of Abs.

Polyclonal antibodies are produced by immunizing animals. In immune response, a single B-lymphocyte produces a single type of Ab in response to an immunoconjugate. Different B-lymphocytes produce different Ab molecules which are specific for different parts of the immunoconjugate.

A polyclonal antiserum is a mixture of Abs produced by various B-lymphocytes, this means that polyclonal Abs are heterogenous, with various affinities.

Polyclonal Abs have some limitations, because polyclonal antiserum can vary from one animal to another, and the supply ends when the animal dies. Serum pools which are carefully characterised can reduce this problem, but a consistent source of product Abs cannot be guaranteed.

Monoclonal Abs are produced by hybridoma technique, first developed by Kohler and Milstein (1975). The spleen of an animal is extracted, and the B-lymphocytes are fused with a tumerogenic B-lymphocyte as myeloma cells. This gives them "immortality" and allows production of Abs with constant affinities and cross-reactivities.

Antibodies can also be fragmented with enzymes such as pepsin or papain to yield fragments that can be used in assays.

Recently, recombinant technology has been used to produce lines of antibodies of extremely high specificity. Dual-purpose antibodies and the use of modified antibodies to deliver a "charge" to a specific target site, for example a radioactive charge to kill tumors, and "superantibodies", which can penetrate the cell and bind to a specific target within (see figures 1.8 & 1.9), have grown from this technology (Zhao *et al* 2002 and Zhao & Kohler 2002).
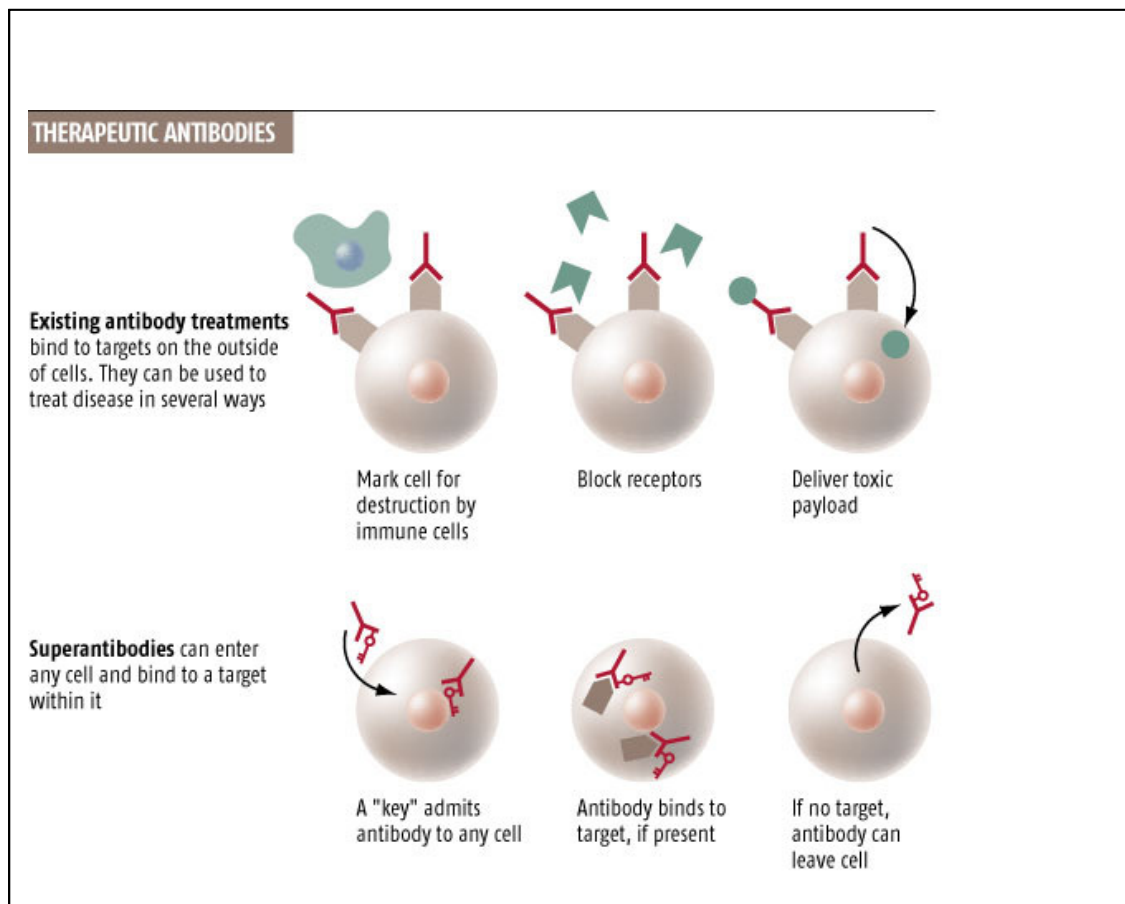
Figure 1.8: Therapeutic and Superantibodies Source: New Scientist 19/04/04
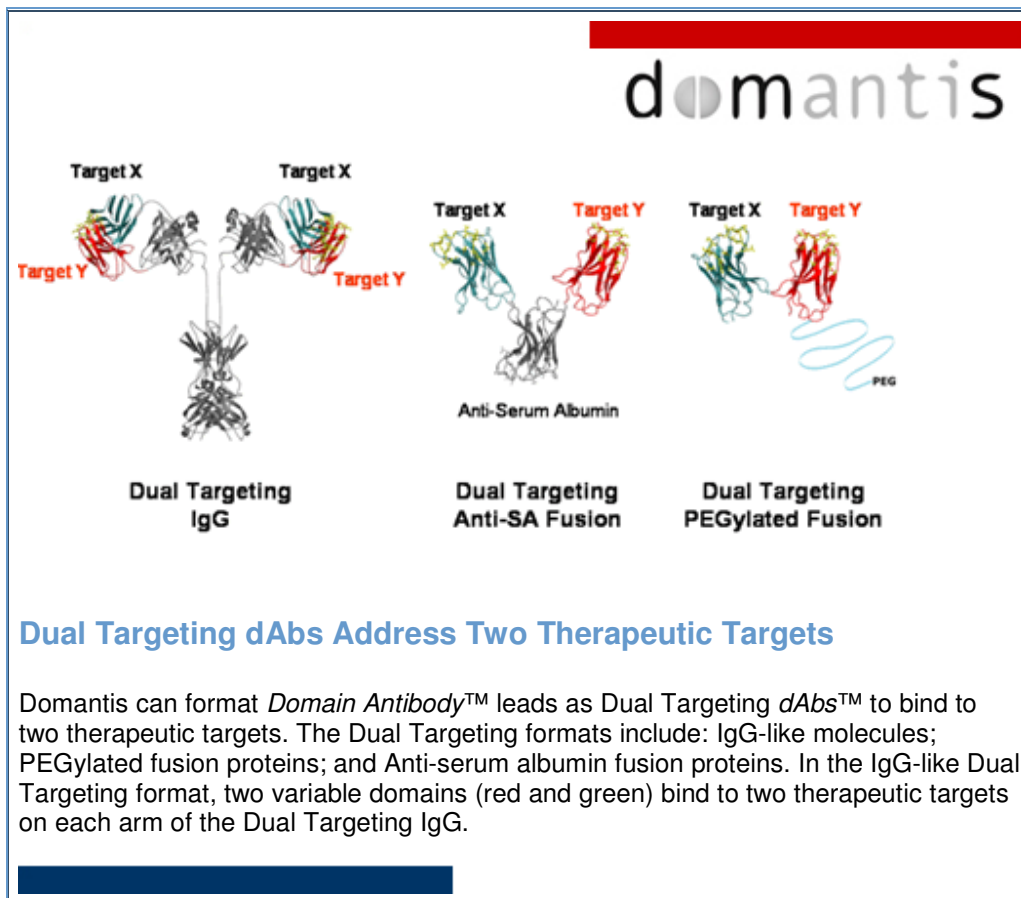
Dual Targeting dAbs Address Two Therapeutic Targets

Domantis can format *Domain Antibody*™ leads as Dual Targeting *dAbs*™ to bind to two therapeutic targets. The Dual Targeting formats include: IgG-like molecules; PEGylated fusion proteins; and Anti-serum albumin fusion proteins. In the IgG-like Dual Targeting format, two variable domains (red and green) bind to two therapeutic targets on each arm of the Dual Targeting IgG.

Figure 1.9: Dual purpose antibodies developed by Domantis: Source http://www.domantis.com/dual.htm

1.5.4 Immunoassay

Immunoassay techniques have a fairly short history. The first immunoassay was developed for the determination of insulin in blood by Yalow & Berson (1960). Since then, immunoassays (IAs) have been used to detect a wide range of clinical compounds, such as hormones, drugs and viruses. They are relatively simple to run and have become a popular tool for the screening of organic pollutants in the environment. Information on quality control and data interpretation has helped to improve the reliability

of IAs. This is shown by their increasing acceptance by regulatory authorities, such as the US EPA.

The key components of all immunoassays are antibodies. Antibodies are characterised by specific recognition sites in their structures, which enables highly specific interactions with the antigens. The generation of antibodies for small molecules, such as those for pesticides, is difficult because small molecules do not produce an immune response. This may be one reason for the slower introduction of immunoassay techniques into the environmental field, as opposed to the clinical. Rational hapten-carrier design (the carrier is the larger molecule which is bound to the antibody to promote an immune response) has resulted in a recent increase in use.

An IA depends on the interaction of antibody and antigen. In most IAs the antibody (or the antigen) is immobilised on a solid support, and a measurement of the binding sites by either the antibodies or the antigens is made which reflects the concentration of the analyte in the medium. A tracer is often added to facilitate measurement, by measuring the tracer signal. These tracers can be fluorescent, chemiluminescent, electrochemically active, enzymes or radioisotopes. Enzyme Linked Immunosorbent Assays (ELISAs), which are an indirect, heterogenous assay where the signal is magnified by an enzyme "label", are especially sensitive and highly specific to the target analyte.

Recent developments of interest include the use of paramagnetic micron-sized or nanobeads as a label which can be read by superconducting quantum interference device (SQUID) (Chemla *et al* 2000), or based on CMOS technology (Turgut, Ishikawa & Boser 2004) by monitoring a change in the magnetic field (Foley *et al* 2005). Morozov & Morozova (2003) developed an electrophoresis-assisted active immunoassay, where a dialysis

membrane was attached to the bottom of the wells and an electric field was applied perpendicularly to the membrane surface, which enabled rapid transportation and concentration of charged analytes.

Classification of immunoassays

Homogenous immunoassays

A homogenous immunoassay system does not require a separation of the free and bound antigen. The system relies on an alteration of the properties or function of the label, when the antibody-antigen (Ab-Ag) complex is formed. For example the Ab-Ag interaction may inhibit or enhance the enzyme label used.

These assay systems are easy to automate and relatively simple; they are therefore often used in the diagnostic industry.

Heterogenous immunoassays

In a heterogenous immunoassay, there is a separation step to remove unbound reagents before the tracer is determined. This is a more sensitive approach, less prone to interference and is more commonly used in test kits. Morber, Weil & Neissner (1993) developed a dipstick format, heterogenous, competitive assay for atrazine, which used TMB as indicator label.

Direct detection

The antigen-specific antibody is labelled and used to bind the antigen; this binding is then detected.

Indirect detection

The antigen-specific antibody is not labelled and its binding to the antigen is detected by a secondary agent, for example, labelled anti-Immunoglobulin antibodies.

The choice of detection method depends on the test. Direct detection involves fewer steps and is less prone to background interference; however it is less sensitive than indirect detection and requires a new labelling step for each analyte to be tested. In addition to the aforesaid advantages, indirect detection methods also utilise labelled reagents which are widely available commercially and can be used to test a wide range of analytes. Also, since the primary antibody is not modified by the label, there is no loss of activity.

Common configurations of Immunoassays

Competitive immunoassays

Competitive IAs (see Figure 1.10) are often used for analysis of small molecular weight compounds, which are too small to permit the simultaneous binding of two Abs. This includes many analytes of environmental importance, for example many pesticides. In an Ab coating format, these tests work on the principle of competition between the analyte and a labelled form (analogue) of the analyte, for limited number of binding sites on an antibody. Equilibrium is established between the Ab bound to the solid support, the analyte, and the labelled tracer (which are in solution). After the incubation period, the unbound reagents are washed away and the amount of enzyme bound to the solid phase by the Ab is measured. A reduction in the enzyme activity is directly proportional to the amount of analyte present. In an Ag coated format, there is competition between the

immobilised Ag and the analyte for a fixed amount of antibody-enzyme tracer (see Figure 1.10).

Competitive IAs (especially competitive ELISAs) are the most common commercial format. There is a wide range of commercially manufactured test-kits available for a variety of analytes. Envirologix, for example, manufactures a test-kit for atrazine.
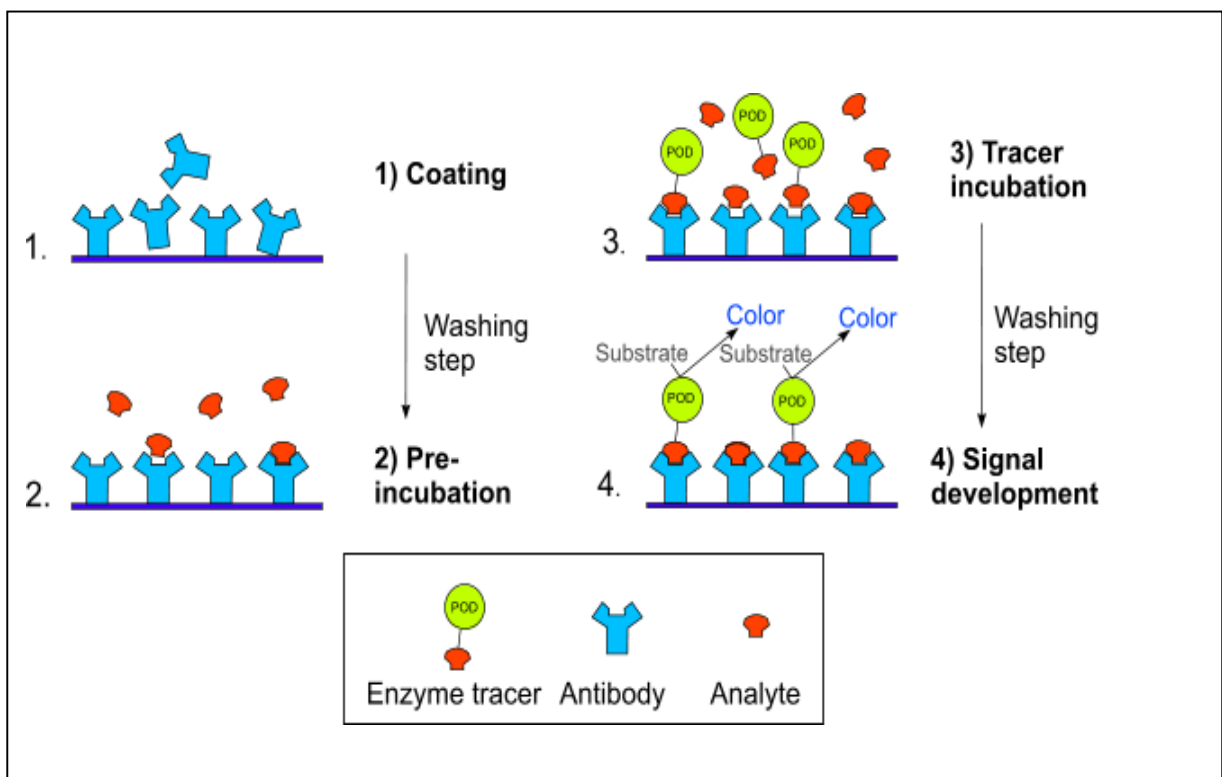
Figure 1.10: Direct competitive immunoassay using a peroxide tracer.
Source: http://www.ch.tum.de/wasser/weller/immunoassay_img_2.htm

Sandwich Immunoassay

Sandwich Immunoassay (see Figure 1.11) is used to detect analytes with a molecular weight that allows the simultaneous binding of two antibodies.

This format differs from the competitive format in several ways:

- Two antibodies are utilised
- The antibodies are present in excess, not limiting concentrations
- One of the antibodies is labelled with the enzyme tracer

There are many variations of this format; the most common uses one antibody immobilised onto a solid support, e.g. well, tube or supramagnetic bead. This antibody recognises one site on the analyte and binds to it after the sample is added. The second antibody is then added, which binds to a second site on the antigen and so forms the "sandwich." The signal generated is proportional to the amount of analyte.
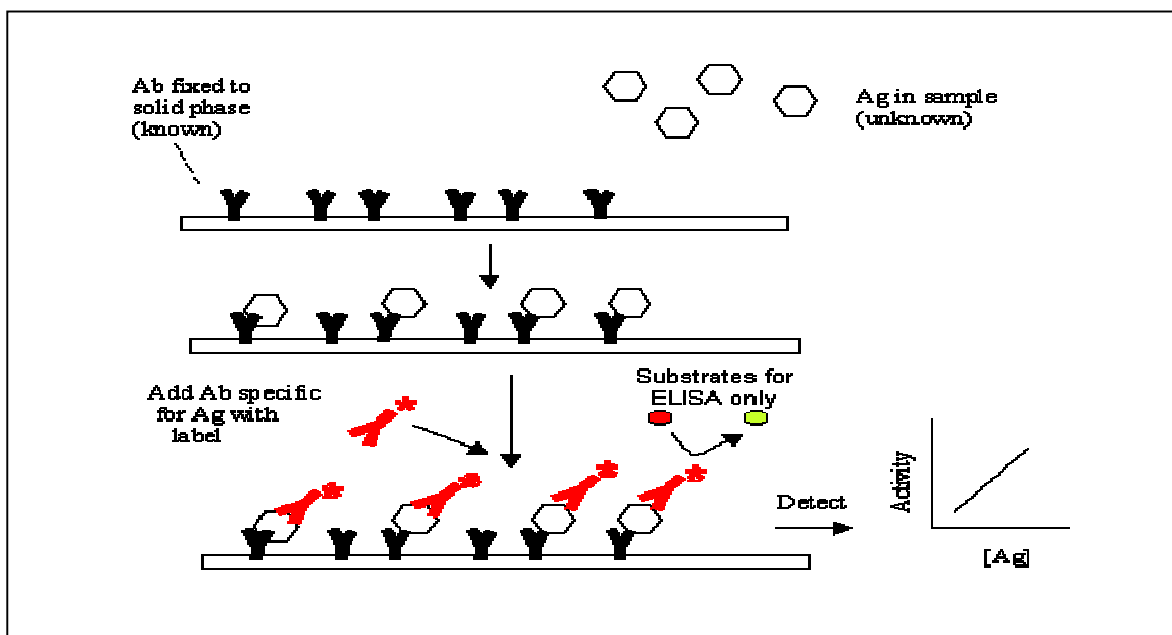
Figure 1.11: Sandwich immunoassay

Source: http://www.np.edu.sg/~dept-bio/biochemistry/aab/topics/immuno/sandwich.htm

Displacement Immunoassay

In this format, the antibody is immobilised onto a solid support and the antigen is labelled. At the start of the immunoassay, all of the available binding sites on the immobilised antibodies are occupied by labelled antigen. On addition of the sample (unlabelled antigen) there is a displacement of the labelled antigen. Under appropriate conditions the extent of this displacement will be proportional to the amount of analyte.

This format has recently been utilised in an MIP assay for the detection of Pentchlorophenol (PCP) to avoid contamination by elution of residual ligand used in the manufacture of the MIP (Colin Nichols in press Biosensors & Bioelectronics).

1.5.5 Advantages and Disadvantages of Assay techniques

The widespread use and availability of assay kits, their utility and on-site capacity are all clear advantages of assay techniques. Gruessener, Shamburgh and Watzin (1995) demonstrated the sensitivity, finding a correlation of 96% with GC-MS in the detection of atrazine in 217 surface water samples. The commercially available magnetic bead assay kit used for this project showed an internal variation of <3% between 156 duplicate standards, produced no false negatives and few false positives and had a detection limit of 0.05µg/L. Giersch (1993) developed a dipstick format assay test which permitted a quick yes/no visual detection for atrazine above 0.1µg/L. The sticks showed no deterioration when stored in the refrigerator for 8 months.

The disadvantages of assay techniques, such as the instability of some biological reagents (for example the enzyme tracer) are also inherent in an

indirect immunosensor. A macro-scale biosensor appears to offer no real advantages over an assay-kit, and due to the wide acceptance and previously established presence of assay kits in the market, commercial establishment of such a device would be difficult. However a miniaturised device offers some considerable advantages, as summarised in Table 1.4.

Table 1.4: Advantages of miniaturised system

Advantages of Micro-/Nano-scale sensor	**Disadvantages of Macro-scale sensor**
Faster reaction time	Slower reaction time not much greater than assay
Less solvent use	Greater solvent use
Enhanced sensitivity	Sensitivity not enhanced

Capillary Electrophoresis (CE) offers great potential for miniaturization, offering fast and efficient separation capability. Capillary electrophoresis separation with electrochemical (amperometric) detection (CE-EC) is highlighted in a recent review of microscale electrochemical analytical systems (Wang 2002).

1.6 BIOSENSORS

1.6.1 Overview

A biosensor can be defined as a device, having a biological receptor molecule and a transducer (optical, electrochemical, piezoelectric etc), for the conversion of the recognition of the analyte by the receptor to a signal that may be amplified. Figure 1.12 is a representation of the sensing elements used in biosensors.
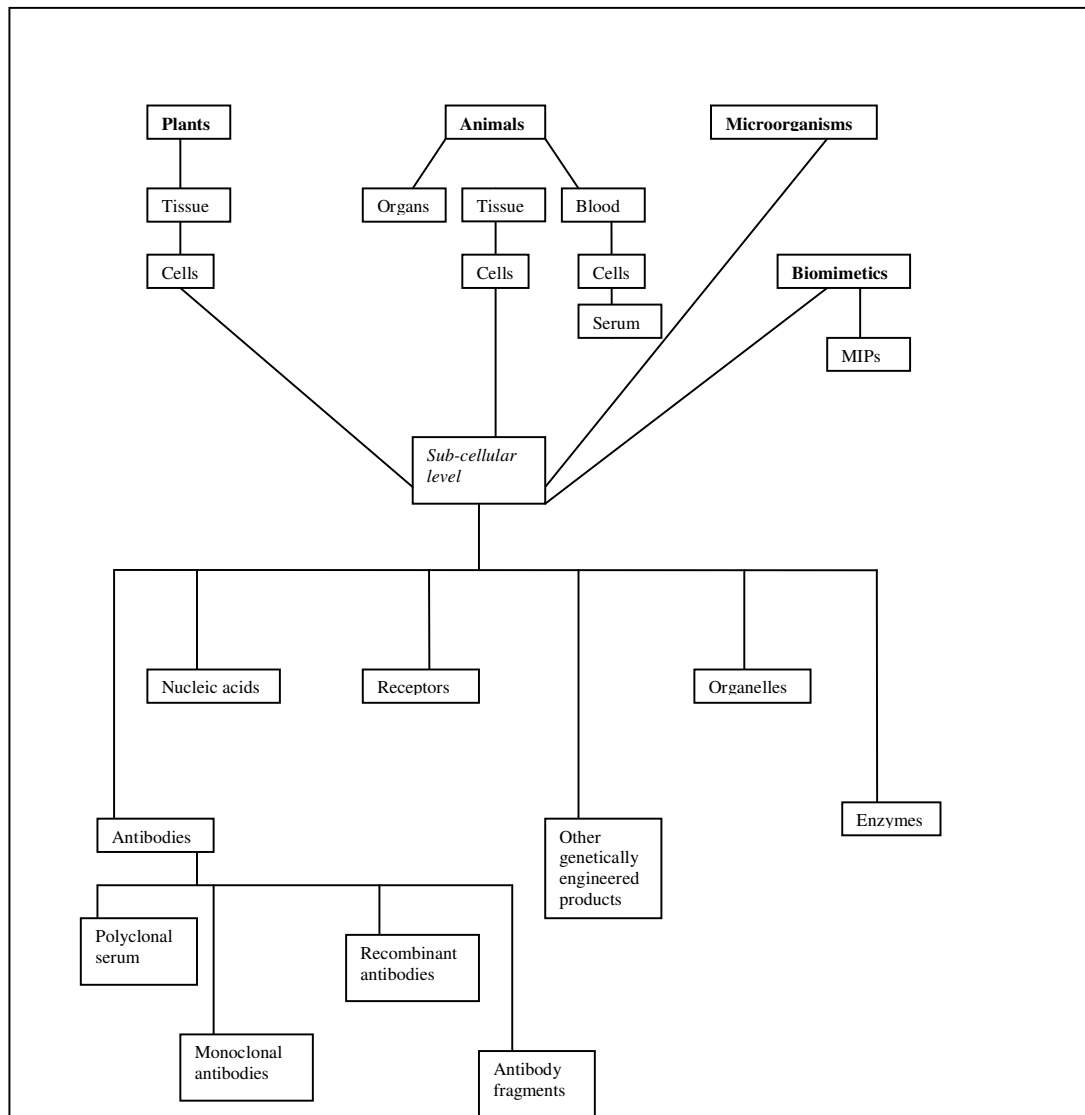
Figure 1.12: Sensing elements for biosensors

Biosensors are defined as analytical devices incorporating a biological material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products etc.), a biologically derived material (e.g. recombinant antibodies, engineered proteins, aptamers etc) or a biomimic (e.g. synthetic catalysts, combinatorial ligands, imprinted polymers) intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical. Biosensors usually yield a digital electronic signal which is proportional to the concentration of a specific analyte or group of analytes. While the signal may in principle be continuous, devices can be configured to yield single measurements to meet specific market requirements (definition from Biosensors & Bioelectronics).

In practice, many sensors measure a secondary signal, the product of an enzymatic reaction or a fluorescent compound. Many sensors are disposable (single use) and are not fully reversible. In some formats the recognition element may not be in 'direct spatial contact' with the transducer. Ease of use and portability are important factors.

The first biosensor was developed by Clark & Lyons (1962). Since then a multitude of biosensors and associated techniques have been developed and studied. Commercially, the markets for biosensors are vast. In 2002, the market for biosensors in the US alone was estimated at $563 million (Alocilja & Radke, 2002). With increased awareness for pathogenic organism and toxin safety, particularly in respect to food safety, medical applications and security, it seems likely that pathogen detection and therefore the use of biosensors will increase (Alocilja & Radke, 2002).

Biosensors and applications have recently been reviewed by various authors e.g. Marazuela & Moreno-Bondi (2001), Trojanowicz (2001), Homola & Bacumer (2003), Dzyadevich et al (2003) and Nakamura & Karube (2003).

Biosensors offer the advantage of simplicity, permitting use by untrained personnel. They allow real-time data to be obtained, which contrasts with periodic sampling and traditional laboratory methods of analysis.

Most modern biosensors allow electronic storage of data which can be utilised for remote monitoring, with the signal being transmitted to a central facility via telemetry (though this may of course be of limited use due to the "life-span" of the biological element). Perhaps the major advantage of biosensors lies in their specificity, through their utilisation of highly specific biological recognition elements such as enzymes and antibodies.

Biosensors can be classified by their Molecular Reception Element, by the reaction of the biological element or by transduction method.

Figure 1.13 summarises some common transduction methods for biosensors.

Independently of their transducing element, biosensors can be split up into three main classes, depending on the interaction of the biological element with the analyte: Metabolic sensors, Inhibition sensors and Affinity sensors. The next four sections deal with these classifications and a fourth, "biomimetic" sensors.

1.6.2 Metabolic Sensors

Metabolic sensors commonly rely on the availability of an enzyme or an organism capable of utilising the analyte as a substrate. For pesticides, this is unusual. It is not impossible, but pesticides are designed to inhibit rather than form a substrate. Micro-organisms have the ability to adapt and to metabolise almost anything found in their environment; this ability is commonly exploited in environmental toxicity testing rather than food-safety testing.

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┌─────────────────────────────────────────────────────────────────┐
│              ┌────────────────────────────────┐                  │
│              │    Transducers for Biosensors   │                  │
│              └────────────────────────────────┘                  │
│                                                                   │
│   ┌──────────────┐  ┌──────────┐  ┌──────────┐  ┌──────────────┐ │
│   │Electrochemical│  │ Optical  │  │ Acoustic │  │ Calorimetric │ │
│   └──────────────┘  └──────────┘  └──────────┘  └──────────────┘ │
│                                                                   │
│   ┌──────────────┐  ┌──────────┐  ┌──────────┐  ┌──────────────┐ │
│   │ Amperometry  │  │Direct    │  │Bulk      │  │Heat          │ │
│   └──────────────┘  │light     │  │acoustic  │  │conduction    │ │
│   ┌──────────────┐  │measurement│ │wave      │  │calorimetry   │ │
│   │ Potentimetry │  └──────────┘  └──────────┘  └──────────────┘ │
│   └──────────────┘                                               │
│   ┌──────────────┐  ┌──────────┐  ┌──────────┐  ┌──────────────┐ │
│   │ Impedimetry  │  │Evanescent│  │Surface   │  │Isoperibol    │ │
│   └──────────────┘  │field     │  │acoustic  │  │calorimetry   │ │
│                     │measurement│ │wave      │  └──────────────┘ │
│   ┌──────────────┐  └──────────┘  └──────────┘  ┌──────────────┐ │
│   │Conductimetry │  ┌──────────┐                │Isothermal    │ │
│   └──────────────┘  │Interferometry│            │calorimetry   │ │
│                     └──────────┘                └──────────────┘ │
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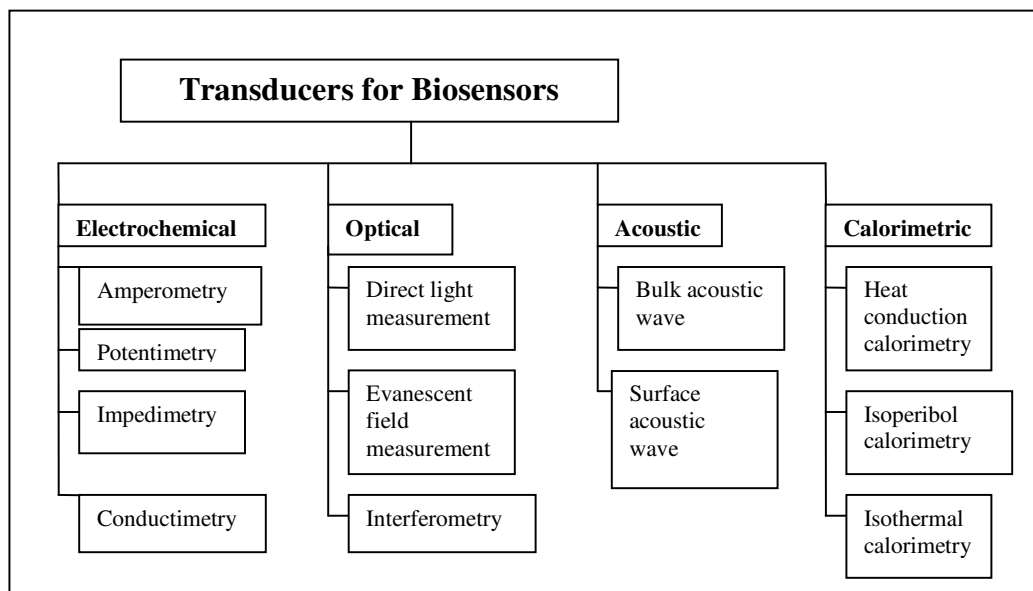
Figure 1.13: Transduction methods for biosensors (after Kroger)

1.6.3 Inhibition Sensors

The biological sensors in inhibition sensors range from whole organisms to isolated enzymes, combined with various transducers which are able to detect a decrease in some biological parameter (e.g. growth, respiration, photosynthesis or luminescence). Nakamura *et al* (2002) used

the excised photosynthetic reaction centre from the purple bacterium *Rhodobacter sphaeroides* as a sensing element for atrazine: the atrazine acted as an inhibitor on the photosynthetic ability of the reaction centre. Giardi *et al* (2001) used a Photosystem II-based biosensor to detect atrazine and heavy metals, but the most commonly used biological sensing elements are enzymes. They are usually employed in inhibition sensors, where the activity of the enzyme is inhibited by the analyte. In some cases their catalytic action can be used to transform the analyte into a measurable product (in a metabolic sensor). In many enzymes, the mode of action involves oxidation or reduction, which can be measured electrochemically. Enzymes are therefore commonly used to modify electrodes.

Enzymes have several advantages:-

- they bind to the substrate
- they are highly selective
- they have catalytic activity which can improve sensitivity
- they are fairly fast-acting
- they are the most widely used biological sensing elements, so there is a wide range of well-defined products and literature

However, they have some disadvantages.

- They are expensive. The cost of extracting, isolating and purifying enzymes is high.
- There can be a loss of activity when immobilised on a transducer.
- They may lose activity due to deactivation after a relatively short time.

Enzymes have been used in the analysis of wine many times. Campanella *et al* (2004) used a superoxidase dismutase biosensor to measure the

antioxidant capacity of red and white wines and compared the results with 2 spectrophotometric methods and a spectroflourometric method. They found a strong correlation between the biosensor and the spectrophotometric method. They also analysed the polyphenol, sulfite and ascorbic acid contents of the wines, using tyrosinase, sulfite oxidase and ascorbate acidase biosensors respectively.

Cummings *et al* (2001) compared three commercially available screen-printable inks and then used a screen-printed carbon electrode modified with tyrosinase for the amperometric analysis of phenolic compounds in beers. The biosensor was unable to distinguish between the various phenolic compounds present but showed a good sensitivity to phenols generally.

Lupu, Compagnone & Pelleschi (2004) also used screen-printed electrodes, mediator modified with Meldola Blue and Prussian Blue for the detection of malic acid and glucose in wine: the detection limits were 10^{-5} M for malic acid and 10^{-6} for glucose.

De Prada *et al* (2003) used a graphite-teflon composite electrode modified with alcohol oxidase and horseradish peroxidase, with ferrocene as a mediator, for the amperometric determination of ethanol in beer and wine. The only sample preparation used was dilution.

1.6.4 Affinity Biosensors

Affinity biosensors are based on the biological sensing element specifically recognising and binding to an analyte. The receptor element can be, for example, a lectin, a membrane receptor protein or, most commonly, an antibody. There has been recent growth in the field of chemically or

biochemically derived "biomimetic" sensors, and these will be discussed in a separate section.

Biosensors which use the antibody/antigen interaction for their analyte recognition are termed immunosensors (North 1985). Immunosensors are generally used due to their high specificity. Depending on the extent to which the chosen antibody displays cross-reactivity towards structurally related compounds, the signal will be caused by a single analyte or a small number of related chemicals. Monoclonal antibodies are generally more specific than polyclonal antibodies.

Because most pesticides are haptens, and therefore too small to elicit an immune response, they need to be linked to a carrier. Care has to be taken during antibody production as hapten-carrier design exerts crucial influence on the sensitivity and selectivity of the envisaged assay. As previously discussed in section 1.5.3, recombinant technology holds much promise for the development of highly specific, tailored antibodies and nucleic acid sensors.

Immunosensors can be classified as direct or indirect measurement devices. Direct devices detect the recognition event between immunoreagent and analyte. Indirect devices rely on a label such as an enzyme to create the signal.

The direct approach appears to be more desirable and simpler, but is less frequently used. Examples of direct optical sensors are the BIAcore system from Pharmacia, which employs surface plasmon resonance (SPR), and the IAsys (resonant mirror) system from Affinity Sensors (see figures 1.14 & 1.15). Due to the complexity of the equipment, and its high cost and the size, these have been mainly used in centralised laboratory settings. Another group of direct immunosensors are acoustic/piezoelectric devices

such as the quartz crystal microbalance (QCM) and surface acoustic wave (SAW) devices.

As yet, the majority of immunosensors for pesticide analysis are based on indirect measurements using enzyme labels.

1.6.5 Biosensor detection methods

The next 3 sections deal with Electrochemical, Optical and other detection methods for biosensors

Electrochemical biosensors

IUPAC has recently defined electrochemical biosensors as a *'biosensor with an electrochemical transducer. It is considered to be a chemically modified electrode (CME) as electronic conducting, semiconducting or ionic conducting material is coated with a biochemical film'* (IUPAC 1999).

Due to the high specificity and sensitivity required for this project, the format chosen was an immunochemical device with electrochemical reporting.

Introduction to Electrochemistry

Electrochemical analytical techniques are concerned with the interconnection between electricity and chemistry and the measurement of electrical parameters such as voltage, potential and charge. Its origins can be traced back to Galvani's experiments with frog leg muscles in the 18th Century (Galvani, L (1791), *De viribus electricitatis in motu musculari commentarius*, Bologna). Michael Faraday's work showed that there was a

relationship between the amount of charge and the quantity of electrolysis product.

Electrode reactions are heterogeneous and occur in the interfacial region between electrode and solution. In this region, charge distribution differs from that of the bulk phase. The process involves an oxidation/reduction (redox) reaction which involves the transfer of electrons from one reactant to another. This redox reaction can be presented as

Oxidised form (O) + ne^- $\leftrightarrow$ Reduced form (R)

This reaction takes place at the working electrode, where the applied potential is the parameter which is controlled. This causes the analyte in solution to be oxidised or reduced at the electrode surface.

Potential is a separation of charge and can be considered the driving force behind an electrochemical reaction. For a given electrochemically active molecule at an electrode surface, shifting the potential of the electrode in a more negative direction (relative to the reference electrode) will favour reduction, shifting the potential in a more positive direction will favour oxidation.

The Nernst equation, developed by Walter Nernst (1864- 1941) relates the potential (E) which is developed at an electrode to the standard electrode potential (Eº) and to the activities of the oxidised and reduced species O and R at the electrode surface.

$$E = E^o - RT/nF \ \text{In} \ aR/aO$$

Where E is the actual electrode potential produced at the electrode surface when the reactants O and product R are at the concentrations designated in the equation. Eº is the standard electrode potential of the couple vs a standard hydrogen reference electrode (SHE), which is characteristic for each half-reaction and is the hypothetical potential which would be
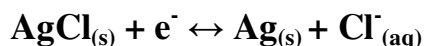
generated if all the reactants had an activity of 1 at standard temperature and pressure. R is the gas constant (8.31441 J K^{-1} mol^{-1}), T is temperature (K) n is the number of electrons, F is Faraday constant ($96,485$ C), In is natural logarithm (2.303 log), aR and aO are the products of the chemical activities of all the species which occur on the reduced and oxidised sides of the electrode equation.

The Nernst equation is true at an electrode where no current is allowed to flow, such as a reference electrode. In Voltammetry and Amperometry, the working electrode has an applied potential, which is relative to the reference electrode. The circuit is completed with a counter electrode, which allows current to flow by acting as a source or sink of electrons (the working and counter electrodes must be conducting). The reference electrode does not have a potential applied and achieves equilibrium in solution. The potential developed by the reference electrode is monitored by the potentiostat, which allows the working electrode to be poised at the correct potential relative to the reference electrode.

A common reference electrode, and the one used in the SPEs employed in this project, is the silver/silver chloride system (Ag/AgCl).

$$\textbf{Ag/AgCl(s)/Cl}^{-}\textbf{ (aq)}$$
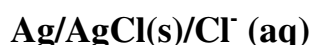
The half-cell electrode reaction is:

$$\textbf{AgCl}_{(s)} + \textbf{e}^{-} \leftrightarrow \textbf{Ag}_{(s)} + \textbf{Cl}^{-}_{(aq)}$$

The electrode potential is governed by the chloride ion concentration of the solution. As the chloride ion activity changes, the electrode potential will change. The reference potential deviates most significantly from the standard potential at low chloride ion concentrations (<0.1 mol l^{-1}) and it is therefore important to maintain the necessary concentration of chloride ion in the sample when using an Ag/AgCl SPE.

In order to understand voltammetry or amperometry it is first necessary to have some idea of the faradaic and non-faradaic components of an observed current.

Faradaic processes:

For a reaction to take place at an electrode surface, the applied potential must exceed a particular value. It can be useful to see the voltage as the electron pressure which forces a chemical moiety to gain or lose an electron. This is how reduction or oxidation of any electroactive species can be achieved. The voltage required depends on many factors such as solution pH, ionic strength, electrode material and of course the chemical species.

The current, which flows as a result of the transfer of electrons during electrochemical redox reactions, is a measure of the rate of reaction and is related to the concentration of analyte,

$$O + ne \leftrightarrow R$$

where O and R are the oxidised and reduced forms of the redox couple. The current resulting from the change in oxidation state is called the Faradaic current because it obeys Faraday's law

$$N=Q/nF$$

Where N is the number of moles of reactant, Q is the total charge passed (coulombs) and F is Faraday's constant (96487 C mol^{-1}).

The electrode reaction rate depends on a number of factors, of which two are prominent:

1. The mass transfer rate of electroactive species to the electrode
2. The electron transfer rate between electrode and redox species

The slower of these two processes controls the magnitude of the current at the working electrode.

Non-Faradaic processes:

The excess charge on the electrode in voltammetric reactions causes the formation of an electrical double layer close to the electrode surface. Since the interface must be neutral, a counterlayer of ions which are oppositely charged to the electrode forms. This layer consists of several regions, more compact closer to the surface of the electrode and more diffuse towards the bulk solution. When a charge is applied, or the potential of the working electrode is changed, the charges in the counterlayer reorganise. This reorganisation causes a current to flow. This is referred to as the non-Faradaic current. It is independent of the concentration of analyte and is an undesirable background "noise", especially at low concentrations of analyte.

Voltammetry

In voltammetry one is usually interested only in the reactions occurring at the working electrode. A three electrode cell is normally used.

Reference electrode: used to give a fixed reference point

Counter electrode: acts as a source or sink of electrons

Working electrode: where the electrochemical reaction of interest takes place.

The surface area of the counter electrode is usually larger than the working electrode, so that the reaction rate and hence the current is controlled by the working electrode.

In voltammetry and polarography (a subclass of voltammetry using a mercury drop as the working electrode), an initial potential is selected where no reaction occurs, the potential is slowly scanned in a negative or positive

direction (depending on the electrode material and the analyte) while the current is measured. At a sufficiently negative or positive electrode, the reaction of the analyte commences. The current then increases to a limiting (diffusion controlled) value. The resulting voltammogram will show a sigmoidal curve and the half wave potential

$$(E_{1/2} = E_o + RT/nF \ln (D_R/D_o)^{1/2})$$

gives qualitative information, i.e. species identification and concentration.

Amperometry

   Amperometric systems use the same equipment and three-electrode cell as voltammetric systems, with a working electrode where the reaction of interest takes place, a counter electrode as a source or sink for electrons and a reference electrode through which no current passes as a reference point against which the potential of the working electrode is poised. A potentiostat monitors and controls the potentials of the working and reference electrodes to keep them poised, and an output device, normally a PC, records the data.

   Amperometry measures the current produced by the oxidation or reduction of an electroactive compound at the working electrode while constant potential is applied to this electrode relative to the reference electrode. This is carried out with a static electrode in an unstirred solution. The current produced is measured. Faraday's law describes the measured current (I) as a direct measurement of the electrochemical reaction rate.

$$I = zF * dn/dt$$

where dn/dt is the oxidation or reduction rate in mol s^{-1}, z is the number of electrons transferred between each molecule of the analyte and the electrode,

and F is the Faraday constant. The rate of reaction depends on the rate of electron transfer at the surface of the electrode. The mechanism of electron transfer at an electrode can be said to consist of 6 steps (Brett & Brett 2004).

1. Diffusion of the species to where the reaction occurs (described by mass transfer coefficient k_d).

2. Rearrangement of the ionic atmosphere

3. Reorientation of the solvent dipoles

4. Alterations in the distances between the central ion and the ligands

5. Electron transfer

6. Relaxation in the inverse sense

Steps 2-5 are included in the charge transfer rate constant k_a (for an oxidation) or k_c (for a reduction) and include adsorption of the reagent on the surface of the electrode, which, if the product is soluble, will de-adsorb.

Steps 2-4 can be considered as a type of pre-equilibrium before the electron transfer. During the electron transfer itself, all positions of the atoms are frozen, obeying the Franck-Condon principle.

The formulation of electrode kinetics derived by Butler & Volmer leads to the equation

$$I=nFA(k_a[R]_* - k_c[O]_*)$$

where A is the electrode area, $[O]_*$ and $[R]_*$ are the concentrations of O and R next to the electrode, F is the Faraday constant, n is the number of

electrons transferred, I is the measured current and k_a and k_c are the charge transfer rate constants for an oxidation and a reduction respectively.

This leads to the conclusion that:-

On changing the potential applied to the electrode, k_a and k_c are influenced exponentially, showing the electrode as a catalyst. However, $k_c[O]_*$ and $k_a[R]_*$ are limited by the transport of species to the electrode. When all the species that reach it are oxidized or reduced, the current cannot increase further. If there are no effects from migration, diffusion limits the transport of electroactive species close to the electrode. The maximum current is known as the *diffusion-limited current*; if the applied potential is sufficiently positive or negative, the maximum current will always be reached (Brett & Brett 2004).

Amperometric sensors offer quantative data, as the current measured is directly proportional to the concentration of the analyte. Amperometric biosensors are widely reported in the literature e.g. Killard *et al* (2001) (for atrazine), Grennan *et al* (2003) who used recombinant single-chain antibody fragments to detect atrazine at 0.1µg/l, and Fahnrich *et al* (2003), who developed an amperometric immunosensor for Polycyclic Aromatic Hydrocarbons (PAHs). Gomes *et al* (2004) developed an amperometric biosensor for polyphenolic compounds in red wine. As mentioned in section 1.6.3, many enzyme modified electrodes use amperometry as the transduction mechanism.

Potentiometry

Potentiometric systems differ from amperometric and voltametric systems in that only two electrodes are used. In an ion-selective electrode (ISE) system, for example, two electrodes are connected across a high-impedence voltmeter. One of the electrodes is a reference electrode such as an Ag/AgCl electrode, while the other is selective towards a particular ion. The two electrodes may be in a single housing, referred to as a combination electrode.

In the simplest method of measurement, the pair of electrodes is immersed in the sample solution, and an electrothermodynamic equilibrium is established. Ions move across the electrode-surface interface, driven by their activity in solution. Because ions carry a charge, this creates a difference in potential which opposes further motion. The equilibrium voltage is therefore directly related to the activity of the ions in solution. Yulaev *et al* (2001) developed a potentiometric immunosensor for simazine based on detection of peroxidase, using gold planar electrodes. The total assay time was 14 minutes including electrode regeneration, with an LOD of 3ng/ml. This sensor has been used to detect simazine in meat extracts, milk, tomatoes, potatoes and cucumbers (Yulaev *et al* (2001).

Optical

Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is an example of a direct detection biosensor. SPR biosensors are optical sensors which exploit special electromagnetic waves – surface plasmon polaritons – to probe interactions

between an analyte in solution and a biomolecular recognition element immobilised on the SPR sensor surface.

The detection principle relies on surface plasmon resonance, an electron charge density wave phenomenon, which arises at the surface of a metallic film when light is reflected at the film under specific conditions. The resonance results from the energy and momentum being transformed from incident photons into surface plasmons, and is sensitive to the refractive index of the medium on the opposite side of the film from the reflected light.

The biomolecular recognition element (often an antibody), on the surface of the metal recognizes and captures analyte in a liquid sample. This produces a local increase in the refractive index at the metal surface, which gives rise to an increase in the propogation constant of Surface Plasma Waves (SPWs) propagating along the surface of the metal. Since the optimum angle for SPR generation is sensitive to the dielectric properties of the adjacent medium, analyte-antibody binding at the metal surface can be directly followed in real-time by monitoring the optimum angle (see Figure 1.14).

In an SPR sensor, an SPW is excited by a light wave and the effect of this interaction on the characteristics (such as amplitude, phase, polarisation and spectral distribution) of the light wave is measured. It is from these measurements that changes in the propagation of SPW can be determined. Excitation of an SPW can only occur if the component of the light's wave vector that is parallel to the metal surface matches that of the SPW. This is commonly achieved by use of 3 configurations:-

Prism coupling

A light wave passes through a high refractive index prism and is reflected at the prism-metal layer interface, generating an evanescent wave which penetrates the metal layer. This wave propagates along the interface with a propagation constant that can be adjusted to match that of the SPW by controlling the angle of incidence (Attenuated Total Reflectance or ATR method) (Reather 1983).

Waveguide coupling

The light wave is guided by an optical waveguide and, when it enters the region with a thin metal layer, it creates an evanescent field at the metal-solution interface that penetrates up to 200nm into the surrounding medium.
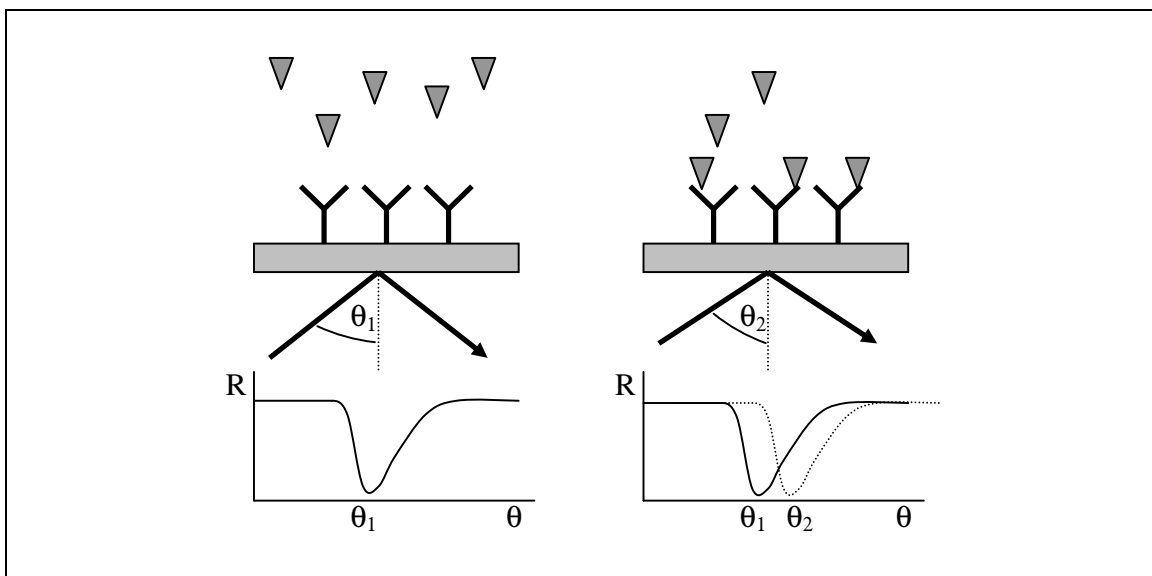
Figure 1.14: SPR immunosensor (Source: Setford 2000)

As illustrated in Figure 1.14, '*when light of an appropriate wavelength impacts at a specific angle (θ_1) upon a metal conductor, a*

surface plasmon (evanescent electro-magnetic field) is generated at the surface.

This effect manifests itself as a decrease in reflected light intensity (R) on varying the angle of incidence. Since the optimum angle for SPR generation is sensitive to the dielectric properties of the adjacent medium, analye-antibody binding at the metal surface can be followed directly in real time by monitoring the optimum angle (θ_2) change' (Setford 2000).

Diffraction grating

The component of the wave vector of the diffracted waves parallel to the interface is diffraction-increased by an amount which is inversely proportional to the period of the grating, and can be matched to that of an SPW (*Huntley* 1982).

The main formats used in SPR biosensing are direct detection, sandwich assay and inhibition assay.

Direct detection:

The refractive index change resulting from the interaction of the analyte in the sample and the biorecognition element immobilised on the sensor surface is directly proportional to the concentration of analyte.

Sandwich assay:

As the name suggests, this method has two stages:

Step 1. Sample containing analyte is brought into contact with sensor and binding takes place between biorecognition element (antibody) and analyte.

Step 2. Sensor surface is incubated with a secondary antibody. This binds to the previously captured analyte which further increases the signal.

Inhibition assay:

This is an example of a competitive assay. The sample is initially mixed with specific antibodies. This mixture is then brought into contact with the sensor surface coated with analyte molecules, so the unoccupied antibodies bind to the analyte molecules on the sensor surface. The signal is then measured (inversely proportional to analyte concentration).
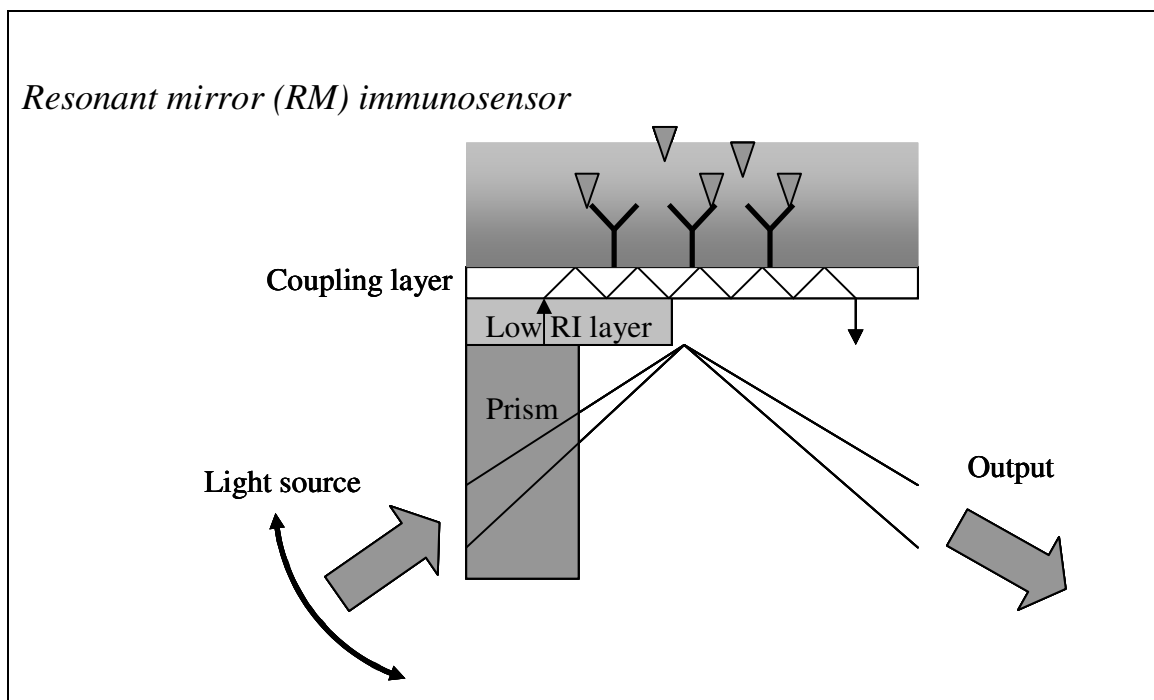
Figure 1.15: RM Immunosensor (Source: Setford 2000)

As illustrated in Figure 1.15, '*RM immunosensors also register antibody-antigen binding through changes in sensor surface refractive index (RI). Light radiation is totally internally reflected except at one specific*

angle where a component of the light can couple through a low RI spacer layer and propagate by multiple total internal reflections along a waveguide. The light couples back out and a characteristic phase change upon resonance is converted into an intensity change via polarising optics measured at a detector. Only molecules bound to the external surface of the waveguide will interact with the evanescent field arising in the optically less-dense surrounding medium. Analyte binding causes a change in RI, hence a shift in the resonant angle' (Setford 2000).

Hayes & van Duyne (2003) developed a nanoscale optical biosensor, based on localised plasmon resonance (LSPR), using biotytinylated surface-connected Ag nanotriangles to detect < picomolar up to micromolar concentrations of strepavidin.

Optical immunosensors

Indirect optical techniques are well established. The chromogenic enzyme labels from ELISA techniques carry over to optical biosensors. Optical biosensors can be used in combination with different types of spectroscopy – absorbance, refraction, luminescence, Raman etc and different light properties can be used for sensor development –amplitude, energy polarization, phase or decay profile. Fibre-optic biosensors are reviewed by Marazuel & Moreno-Bondi (2002) and offer several advantages.

- Enormous range of optical methods
- Fibres can transmit light over long distances (the bioreceptor need not be in intimate contact with fibre)

- Possibility of surface-specific spectroscopy (difficult to adjust)

- Multiplex capability of fibres

- Immune to magnetic or electrical interference

- Easily miniaturised at low cost

They also have several disadvantages:

- Interference of ambient light

- Possible photobleaching or indicator washout

- Possible long response times

- Limited range of commercial accessories

Other optical biosensors include a direct optical immunosensor developed by Brecht *et al* (1995) for atrazine, based on reflectometric interference spectroscopy (RIFS). This was sufficiently sensitive to detect atrazine at 0.5 ppb (500ng/ml antibody). Mouvet *et al* (1997) used waveguide surface plasmon resonance (WSPR), a direct detection technique, to determine simazine in natural surface water and spiked ground water samples. They found high correlation with HPLC results for samples only pre-treated by filtration. The use of nanoparticle labels in optical immunosensing is reviewed by Seydack (2005) and seems to offer great possibilities in improvement of immunoassay and immunosensor techniques.

Colour Theory

RGB Colour theory:

The RGB colour model is an additive model, i.e. one which involves light emitted directly from a source, where the primary colours (in the RGB model Red, Green & Blue) are combined to produce other colours. It should

be noted that additive colours are based on the way that the eye detects light and not an absolute property of light.

Combining one of the primary colours with another in equal amounts produces the additive secondary colours cyan, magenta and yellow. Combining all three primary colours in equal intensities produces white light. Varying the luminosity of each light will eventually reveal the full gamut of those three lights (colours).

The RGB colour model does not itself define what is meant by "red", "green" or "blue" and the results of mixing them are not exact unless the exact spectral make-up of the primaries are defined. The colour model then becomes an absolute colour space, such as sRGB or Adobe RGB.
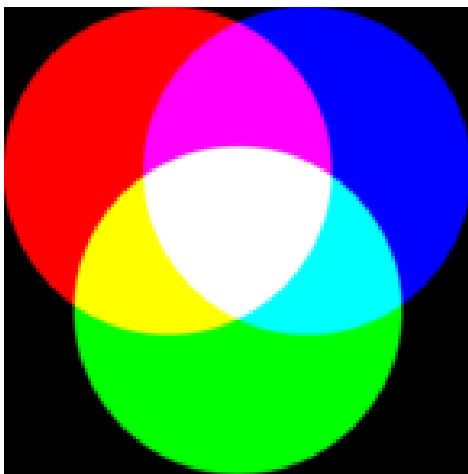
Figure 1.16 An example of Additive colour mixing from wikipedia

The HSV Colour Model:

The HSV (Hue, Saturation, Value) model (aka HSB or Hue, Saturation & Brightness) defines a colour space in terms of three constituent components:

- Hue: the colour type such as red, blue or yellow, this ranges from 0 to 360, but in some applications is normalised to 0-100%

- Saturation: the "vibrancy" or "intensity" of the colour, this ranges from 0-100%. This value is sometimes called the "purity" of the colour by analogy to the colorometric quantities of excitation purity and colorometric purity. The lower the saturation of a colour, the more "greyness" is present and the more faded the colour will appear. Therefore "desaturation" is useful to define the qualitative inverse of saturation.

- Value: the brightness of the colour, this ranges from 0-100%.

The HSV colour model was created in 1978 by Alvy Ray Smith. It is a non-linear transformation of the RGB colour space and may be used in colour progressions. The definition of the HSV colour space is not device independent. HSV is only defined relative to RGB intensities, without physical definitions of their chromaticities and white point.

The HSV model is commonly used in computer graphics applications. In various application contexts, the user must choose a colour to be applied to a particular graphical element. When used in this way, the HSV colour wheel is often used.
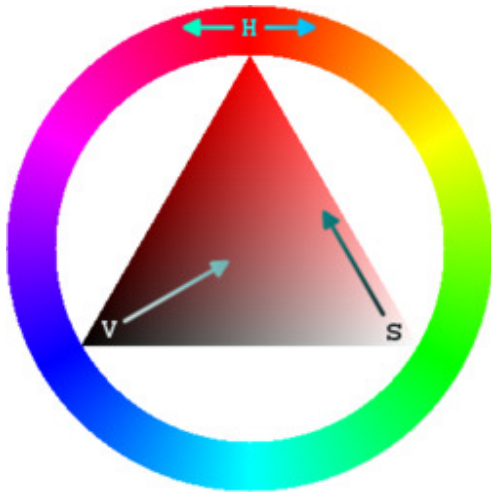
Figure 1.17: An HSV colour wheel Source: www.wikipedia.com

In the HSV colour wheel, the hue is represented by a circular region and a separate triangular region may be used to represent saturation and value. Typically the vertical axis of the triangle indicates saturation, while the horizontal axis corresponds to value. In this way, the colour can be chosen by first picking the hue from the circular region and then selecting the the desired saturation from the triangular region.

A conical representation of the HSV model can be used to visualise the entire HSV colour space in a single object. In the conical visualisation, the saturation is represented by the distance from the centre of a circular cross-section of the cone and the value is the distance from the point of the cone.
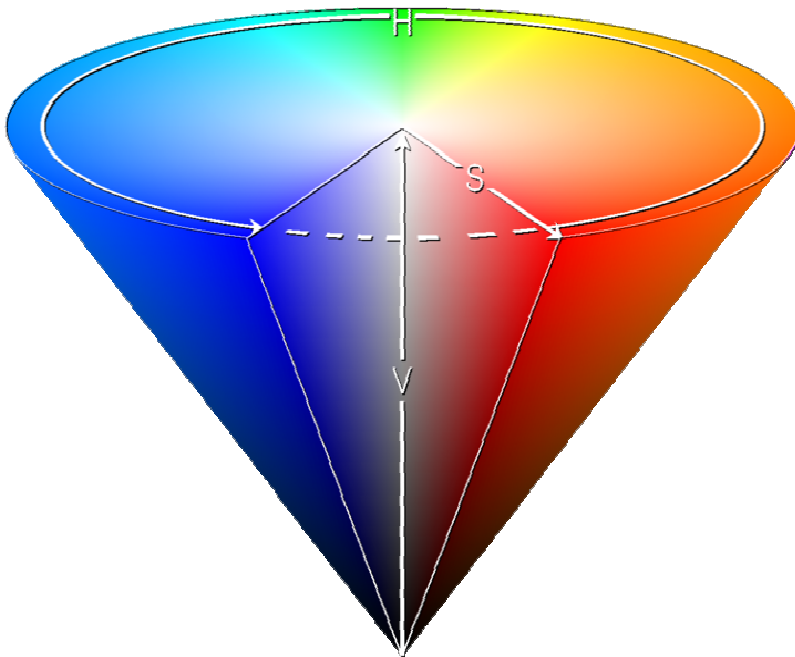
Figure 1.18: Conical representation of the HSV colour space from www.wikipedia.com

Other Methods

Acoustic/Piezoelectric sensors

Piezoelectric sensors work on piezoelectricity (first discovered in 1880 by Pierre and Jacques Curie), which is the electric polarization caused by mechanical strain in certain classes of crystals. This polarization is proportional to the strain and changes sign with it. The effect is explained by the displacement of ions in crystals that have a non-symmetrical unit cell. When the crystal is compressed, the ions in each unit cell are displaced, causing the electric polarisation of the unit cell. The converse effect, where an applied potential produces a mechanical deformation was predicted by Lipmann in 1881 and observed experimentally the same year by the Curie

brothers. Crystals which acquire a charge when compressed, distorted or twisted are said to be piezoelectric. This provides a transducer effect between electrical and mechanical oscillations. Quartz does this and is very stable and so is often used in piezoelectric devices. Most biological applications of piezoelectric devices use Bulk Acoustic Wave or Surface Acoustic Wave devices. In practical terms, the interest lies in generating specific forms of mechanical resonance in the substrate resulting in acoustic waves in specific directions and oscillation at specific frequencies. In practice, the analyte is deposited on the surface of the crystal. The oscillation frequency of the crystal then changes proportional to the concentration of the analyte.

In order to detect a specific analyte, the crystal must be coated with a selective coating that will bind to the analyte. Due to their high selectivity, antibodies are often used. One possible disadvantage of piezoelectric sensors, such as the Quartz Crystal Microbalance (QCM) is that very small or low-mass molecules may need binding to a larger molecule to cause enough of a change in oscillation to register. Pribyl *et al* (2003) developed a piezoelectric immunosensor for competitive direct detection of atrazine, with an LOD of 0.025ng/ml, which demonstrates the sensitivity of the technique.

Biomimetic sensors

This is a vast field which needs a review all to itself. Molecularly Imprinted Polymers (MIPs), which have been mentioned in Section 1.4, can be used as the sensing element in biomimetic sensors and may be spin-coated or dip-coated onto the transducer.

Haupt & Mosbach (2000) reviewed the use of MIPs in sensors, and electrochemical sensors based on MIPs were reviewed by Blanco-Lopez et al (2004) who concluded that in relation to preparation of films for sensors the flexibility (controlled by the ratio of functional monomer to cross-linker or by the addition of oligouerthanes) directly relates to the performance of the sensor. Lavignac et al (2004) reviewed the use of MIPs as alternatives to antibodies in sorbent assays and suggested that they could be a viable alternative.

Ye & Mosbach (2001) synthesized MIP microspheres and utilized them as antibody binding mimics in an assay for the herbicide 2-4-dichlorphenoxyaceetic acid (2-4-D). Chianella et al (2003) developed a MIP based SPE and piezoelectric sensor to detect Microcystin-LR with an LOD of 0.35nM toxin. Kitade et al(2004) developed a potentiometric sensor based on MIPs for serotonin and Weetall & Rogers (2004) developed molecularly imprinted electropolymerized carbon electrodes which were selective for flourescein, rhodamine and 2-4-D. Valina-Saba et al (1999) used gold clusters, monolayer-coated with boronate to bind hexose, opening up potential for glucose monitoring; and Mascini et al (2005) used gold based quartz crystals modified with synthetic oligopeptides as the sensing element of a piezoelectric sensor for dioxins in poultry, eggs and milk.

In connection with the food industry artificial tongues and artificial noses must be mentioned. These are sensor arrays, which are networked and mimic "taste" and "smell" They are used for quality control. Buratti et al (2004) developed an amperometric electronic tongue and used it and a commercial electronic nose for the classification and characterization of Italian wines. Riul et al (2003) developed an artificial taste sensor based on nanostructured films of conducting polymers.

1.7 CONCLUSIONS OF THE LITERATURE REVIEW

The detection of the target analytes at nM sensitivities is reported in the literature. Antibodies offer the required sensitivity and amperometric transduction appears widely in the literature, offering the advantages of an established technology. Recent advances in micro-scale and nano-scale technology offer exciting possibilities for enhancement and eventual replacement of current assay and sensor formats. Biomimetic sensors will benefit greatly from this technology.

The next chapters will cover the materials and methods used in the course of the current research. The results obtained in the research so far will then be presented and discussed; finally conclusions will be drawn and future work will be indicated.

Chapter 2

Materials and Methods

2.1 INTRODUCTION

It was decided that biosensors were to be used for this project for the following reasons:

- They have the required sensitivity
- They involve a less complex analytical procedure than an assay kit, making the technology more easily accessible to untrained staff.
- Use of Screen Printed Electrodes (SPEs) minimises contamination between samples and reduces costs.
- De-centralisation is an important factor and the portability and utility of a biosensor address this well.

In the context of the GoodFood project, these factors will allow

- de-centralised analysis, hopefully on-site at the vineyards and factories, monitoring the winemaking process
- reduced cost and training-time in instructing individuals in the correct use of the monitoring device
- reduced time between analysis and result, as compared to a centralised laboratory.

A flow-cell system was used for the electrochemical measurements (see Figure 2.5) with a view to further development of a flow-system, as it offers the following advantages:-

- fairly continuous monitoring and analysis

- greater quantity of sample can be processed than in "spot check"

The need for the development of inexpensive, easily mass-produced, disposable electrodes for biosensors was clearly identified by Cardiosi & Turner (1990). Screen-printing has been employed as a viable production method (Bergveld and Turner 1993). The technique has found widespread application in biosensors and chemical sensor arrays.

Disposable SPEs rely on planar electrodes (working, counter and reference) on a plastic or ceramic substrate. These strips (illustrated in Figure 2.1) can be thought of as self-contained electrochemical cells.

Screen printing is the controlled application of a number of ink layers through specially designed screens onto a supporting material, commonly called a substrate. The conductive ink (most commonly carbon based for working and counter electrodes and silver based for the reference) is applied by placing the ink on the patterned screen and then forcing it through the screen with the aid of a squeegee.

The technique is conventionally used in the graphics industry and in the electronics industry for the deposition of solder pastes onto printed circuit boards. There is a wide range of substrate materials and inks commercially available for screen printing and they can often be combined to produce electrode systems suitable for a specific application.

Screen printed electrodes offer the following advantages

- single-use, thereby minimising contamination between samples
- easy to manufacture
- low-cost
- easy to use.

The electrodes used consisted of:

- Substrate: PVC sheet from VT Plastics, Bedford.

- Basal track: MCA 45R ink from MCA Melbourne, UK.

- Reference electrode Ag/AgCl (15% AgCl) from MCA Melbourne, UK.

- Insulation layer: Vinyl matt insulation ink from Apollo, London, UK.

The electrodes were manufactured using an automated screen-printing machine (DEK 248 Machine, DEK Printing Machines Ltd, Weymouth, UK: see Figure 2.2). This helped to minimise variability.

Between the application steps, the electrodes were dried at room temperature. The insulation layer was dried at 120°C for two hours in order to drive off solvents and cure the insulation layer.
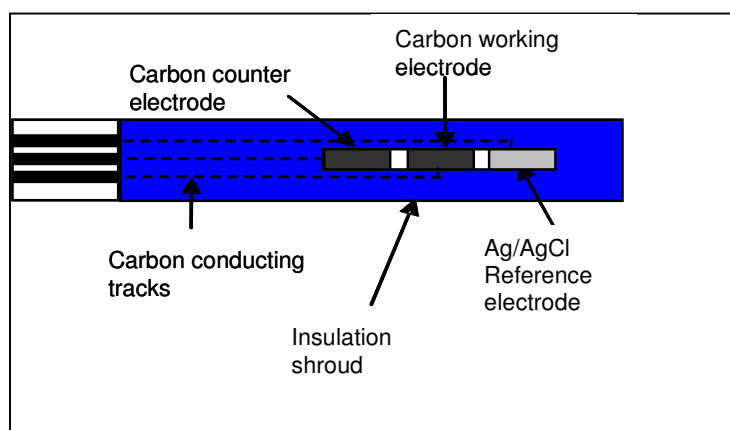
Figure 2.1: A screen printed electrode for FIA system. The direction of flow would be from right to left, so that the sample flows over the reference electrode first. The SPEs were manufactured on the DEK 248 (see Figure 2.2).

Figure 2.2: The DEK 248 Automated screen-printing machine used for manufacturing the SPEs.

2.2 ANALYTICAL METHODS

2.2.1 Calibration

Substrate Calibration

Serial dilutions of HRP were made up and allowed to react in microtitre-plate wells. The reaction was allowed to run for a range of times from 0 up to 60 minutes. 30 minutes is the reaction time allowed in the CSIC atrazine assay protocol. The plates were then read with a plate-reader at 405nm (ABTS) (Sigma product No. A 3219) and 450nm (TMB) (Sigma product No. T 0440) and 490nm (OPD) (Sigma product No. P5412). The results were compared.

Protein Calibration

To obtain information on the protein concentration of the 2a-HRP and hence the HRP Units, an assay was performed on the 2a-HRP (SIGMA Bicinchoninic Acid Protein Assay Kit BCA-1 and B9643). It was read on a Camspec 350 UV/Vis spectrophotometer.

Tracer Activity

To obtain information on the activity of the enzyme tracer:-

1. Solutions of known strength of HRP were made up.
2. 1000 and 100 fold dilutions of 2aHRP tracer were made up
3. These were reacted with ABTS.
4. The absorbance at 405nm was read using a Camspec 350 UV/Vis spectrophotometer.

2.2.2 Immunoassay Methods

The reagents and method protocol for immunoassays for atrazine and 2,4,6-trichlorophenol were supplied by CSIC Amrg in Barcelona (see appendices). Due to difficulties with several batches of reagents from CSIC, Anti-atrazine mouse antibodies (product code G13720M) and atrazine-S-HRP tracer were obtained from AMSBio (a uk subsidiary of Biodesign).

Atrazine

The assay for atrazine was a direct competitive assay and followed these steps.

A 1 in 2000 dilution of Ab10 in carbonate buffer was prepared. 100µl of Ab10 solution was added to each well of a Nunc F96 maxisorb 96-well immunoassay plate, and it was incubated overnight at 4∘C.

The following day, the plate was washed 4 times with PBST and 50µl of freshly prepared standards was added to each well.

It was then covered and incubated for 30 minutes at room temperature.

While the solution incubated, a 1/1000 dilution PBST enzyme tracer solution was prepared.

After the 30 minute incubation period, 50µl of enzyme tracer solution was added to each well, and the plate was covered and incubated for 6 minutes at room temperature.

Meanwhile, the substrate solution was prepared.

The plate was then washed 5 times with PBST, and 100µl of substrate solution added to each well. The plate was then covered and incubated at room temperature, protected from light, for 30 minutes.

After this period, 50µl of stop solution was added to each well to stop the enzyme reaction and the absorbances were read optically.

2,4,6-Trichlorophenol

The 2,4,6-TCP assay was an indirect competitive assay. The protocol was as follows.

A 0.6µg/ml solution of antigen (29-CONA 1:5 from CSIC) was prepared and 100µl of antigen solution was added to each well of a Nunc F96

maxisorb 96-well immunoassay plate. The plate was then incubated overnight at 4ºC.

2,4,6-TCP standards were prepared at 1000, 100, 25, 12.5, 6.25, 3.12, 0.78 & 0nM, and a 1:4000 solution of antiserum (Ab43 from CSIC) was prepared in 10mM PBST.

The plate was washed 5 times with 300µl/well of 10mM PBST.

50µl of standard solutions was pipetted into each well, followed by 50µl of antiserum solution. 100µl of PBST was added to the blank wells. The plate was then covered and incubated for 30 minutes at RT.

A 1:6000 solution of the secondary antibody (anti-rabbit IgG peroxidise conjugate, whole molecule from Sigma, cat no. A8275) was prepared in PBST.

The plate was washed 5 times with PBST, and 100µl per well of anti-IgG solution was added. The plate was covered and incubated at RT for 30 minutes.

The plate was then washed 5 times with PBST and 100µl of substrate solution was added to each well. It was covered, protected from light and incubated for 30 minutes at RT.

After this period, the reaction was stopped with 50µl/well of 4N sulphuric acid, and the absorbances analysed optically by UV/vis.

Other Matrices

The immunoaasys for atrazine and 2,4,6-TCP were performed in 0.1M PBST pH 7.4. In order to investigate the possibility of "real-world" analysis, the atrazine immunoassay was performed in white wine (diluted x10 in PBST) and grape pulp (diluted 20x in PBST), in addition both the

atrazine and 2,4,6-TCP assays were performed in groundwater obtained from the boreholes on site (diluted 10x in PBST).

Magnetic bead assay

Superparamagnetic beads were obtained from Dynal (Dynabeads M-270 Epoxy). Following the protocol provided by Dynal, the beads were then coated with 400U of HRP by the following process.

M-270 Epoxy dynabeads were re-suspended for storage in 2ml of DMF to give a bead concentration of $2x\ 10^9\ ml^{-1}$. They were removed from the fridge as required and placed in a vortex for 2 minutes to re-suspend the beads.

1 ml of the suspension was then pipetted into an eppendorf vial and placed on a magnet for 4 minutes.

The supernatant was pipetted off, and 2ml of 0.1M Sodium Phosphate buffer pH 7.4 was added to the eppendorf vial, to give a bead concentration of $10^9\ ml^{-1}$. This was placed in a vortex for 30 seconds.

The beads were subsequently incubated with mixing at RT for 10 minutes, then placed on a magnet for 4 minutes and the supernatant pipetted off.

They were then re-suspended in the same volume buffer and placed in a vortex.

The beads were replaced on the magnet for another 4 minutes and the supernatant was pipetted off.

To obtain the recommended bead concentration and ammonium sulphate concentration after the addition of the ligand solution, the beads were re-suspended in 400µl of buffer. 800µl of the ligand solution was added to the beads and they were placed in a vortex.

600µl of ammonium sulphate concentration was added, and the beads were incubated with rotation at 4ºC for 48 hours.

The eppendorf was then placed on a magnet for 4 minutes and the supernatant was pipetted off. The beads were subsequently washed 4 times with PBS and re-suspended in 800µl PBS.

Serial dilutions of the bead stock were prepared and the assay was performed in eppendorf vials. The results were analysed optically and electrochemically.

Activated Sepharose sandwich immunoassay

Due to problems concerning assay repeatability with the reagents supplied by CSIC, alternative reagents were investigated. Anti-atrazine Mouse-IgGs (catalogue no G13720M) were sourced from AMS Bio (a UK subsidiary of Biodesign), along with BSA-Atrazine conjugate. Secondary antibodies were purchased from Sigma (Anti-mouse IgG (whole molecule) peroxidise conjugate: product no. A 9044) and Activated CH Sepharose 4B from Pharmacia was selected as the solid-phase (this is a pre-activated medium for spontaneous covalent immobilisation of proteins and other ligands containing primary amino groups).

To coat the Sepharose the following method was used (Taylor J.A & Allen M.E. *in prep*).

0.5g of sepharose was washed with 100ml of 1M HCl over a Buchner vacuum flask for 15 minutes. The sepharose was then suspended in 500µl of BSA-Atrazine conjugate in 0.1M NaHCO3 pH8.0 containing 0.5M NaCl to a final concentration of 5.5mg/ml (coupling buffer). The resulting suspension was rotated for two hours at room temperature.

The sepharose was washed with 3ml of coupling buffer to remove excess ligand and suspended in 0.5ml of Tris 0.1M-HCl-pH8.0 and rotated at room temperature for 1 hour. It was subsequently washed over a Buchner vacuum flask 3 times alternately with 3mls of 0.1M acetic acid/ sodium acetate pH4.0 containing 0.5M NaCl and 3mls of 0.1M Tris-HCl-pH8.0. Finally, it was re-suspended in PBS.

A sandwich immunoassay was then performed using the method below.

50µl of the coated sepharose was placed in each of 6 eppendorf vials; the suspension was allowed to settle and the supernatant was pipetted off.

75µl of the primary antibody (at a 1:100 or 1:500 concentration) was mixed with 75µl of each of the atrazine standards (0nM 10,000nM, 1000nM, 100nM 50nM). 150µl of the atrazine-antibody solution was then added to each vial and the eppendorfs were rotated at room temperature for 1 hour.

The sepharose was allowed to settle and the supernatant was pipetted off, then the sepharose was washed with 100µl PBST. The settling, pipetting and washing steps were performed three times in all.

75µl of secondary antibody (at 1:1000 or 1:10000 concentration) was subsequently added to each vial, and the vials were rotated at room temperature for 30 minutes.

The supernatant was allowed to settle and was pipetted off, and then the sepharose was washed 3 times with 100µl per vial of PBST.

100µl of TMB substrate solution was added to each vial; the vials were protected from light and rotated at room temperature for 30 minutes. 50µl of 4M H2SO4 was added to each vial to stop the enzyme reaction.

Analysis was variously by UV/Vis spectrography at 450nm, RGB reader and chronoamperometry.

## 2.2.3 Comparison of optical and electrochemical assay measurement

In order to compare the electrochemical and optical measurements, the liquid in the three replicates of each standard concentration was taken from the assay plate wells and mixed. 150µl of this was then injected into the flow-cell system and measured amperometrically.

2.2.4 Electrochemical Methods

Amperometry

Amperometry measures the current at a fixed applied potential. In application, the reduction or oxidation potential used is characteristic of the species being analysed.

The enzyme label Horseradish Peroxidase (Sigma P-8250) was used to increase sensitivity and for signal amplification. It is widely used and easily available. It works (in the case where peroxide is used as the substrate) by reducing peroxide substrates to water with the concomitant oxidation of an electron donor (mediator), the reducing equivalents being transferred to the peroxide via the enzymes active site. The mediator is re-reduced by accepting electrons from a suitably poised electrode. The resultant current is related to the amount of HRP and hence the amount of analyte present. A diagram of this is shown in Figure 2.4, using ABTS as the example.
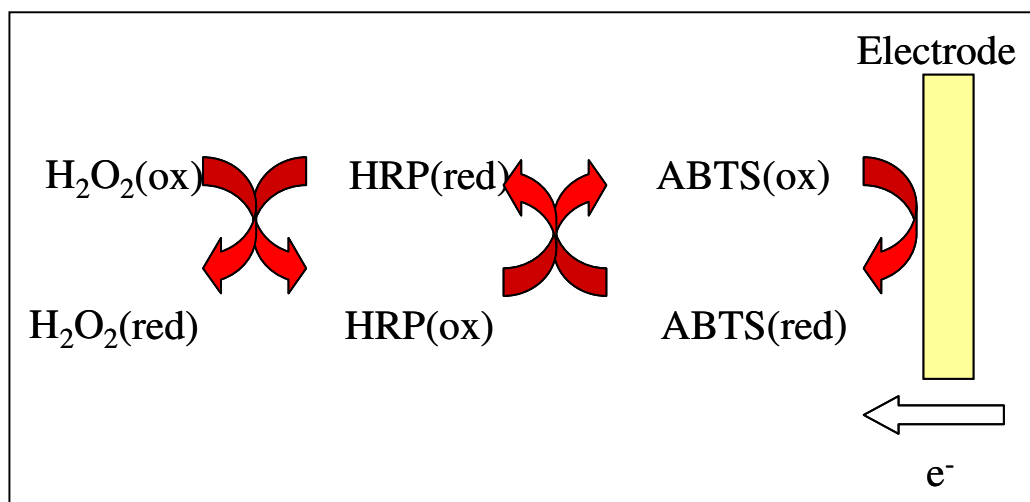
Figure 2.3: Illustration of REDOX reaction at working electrode using Horseradish Peroxidase as enzyme label and ABTS as the Mediator

Electron acceptors for this enzyme, ABTS (2,2'-azinobis[3-ethylbenzthiaoline-sulphonic acid) (Sigma A3219), TMB (Sigma) and OPD (Fisher 32797-2000) were chosen. All are chromogenic, can be read optically using UV/vis spectroscopy (405nm, 450nm and 490nm respectively) and are electrochemically active.

TMB, OPD and ABTS were compared.

Electrochemical detection

The substrates were also compared electrochemically with a computer controlled potentiostat-10 Autolab Electrochemical Analytical workstation running a general purpose electrochemical software operating system (GPES3) (Ecochemie, Utrecht, Netherlands).
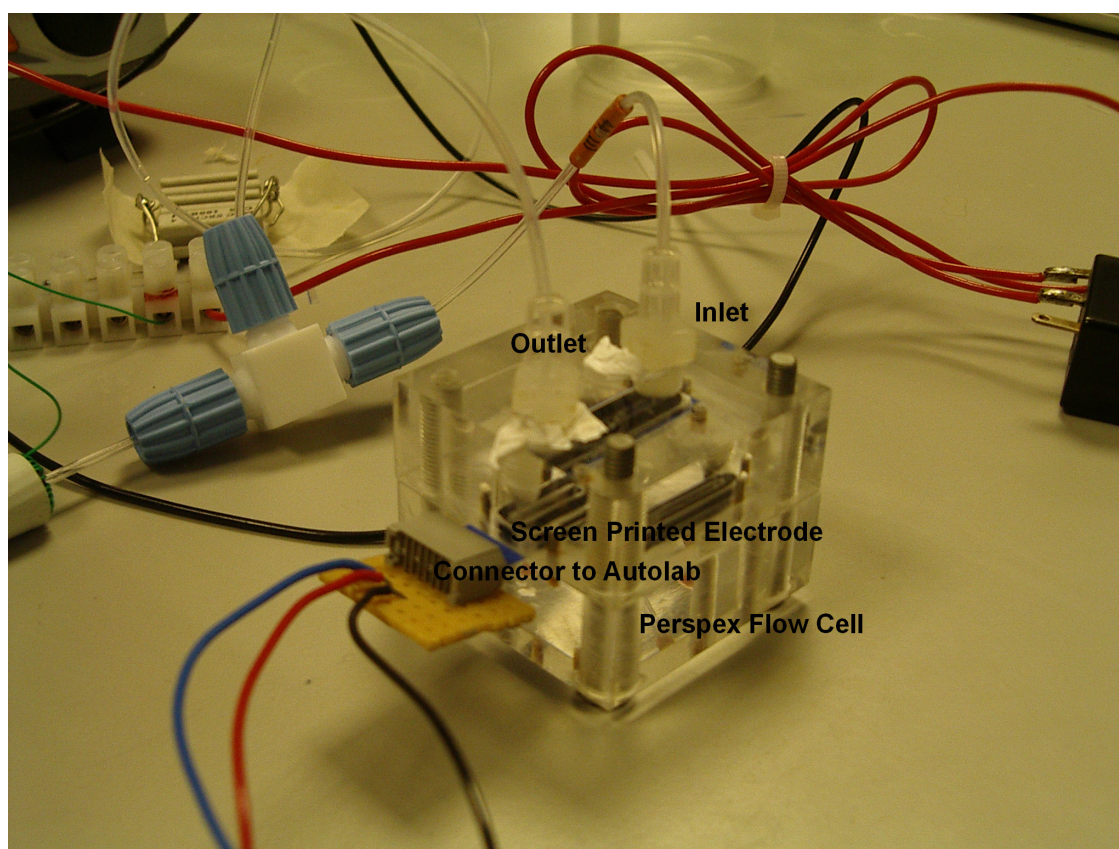
Figure 2.4: Flow-cell.

The methodology used for this was identical to that used for the optical experiments. However, instead of taking an optical reading, this protocol was followed:

250µL of the relevant product was taken from the well, injected into a Rheodyne valve and passed through a flow cell. The flow rate was controlled by an Ismatec peristaltic pump set at 250µL/minute.

The solutions were measured amperometrically at +150mV (ABTS) and -400mV (TMB) vs Ag/AgCl.

The 2aHRP analogue which was used as the label for the assay was serially diluted and reacted with both substrates.

The solutions were analysed optically and amperometrically and compared with the calibration obtained from the earlier data in order to see which substrate gave the best signal at the working concentration and to assess what the working concentration of 2a-HRP was in terms of free HRP units.

Repeatability of the electrode was measured by repeated injection of HRP and ABTS on one electrode and analysis of peak variability; electrode variability was assessed by analysis of 0.1U HRP and ABTS on separate electrodes, and assessment of the differences between the results.

The effect of flow rate on peak area was assessed by reacting 150µL of 5U of HRP with 150µL of ABTS substrate for ELISA (Sigma) for 5 minutes and then injecting 250µL. The flow-rate was controlled to 500µL, 250µ and 125µL per minute. The effect of reaction time on signal was assessed by reacting concentrations of HRP with ABTS or TMB. Analysis was then performed at timed intervals.

2.2.5 Magnetic Bead work

In order to calibrate the system and assess its sensitivity, preliminary magnetic bead work was commenced using Dynabead M-270 Epoxy coated beads. The following method was used.

The beads were suspended in DMF for storage at 4ºC.

Aliquots, containing 2×10^9 of stored beads, were taken; these were washed and re-suspended in 0.1M PBS. The beads were then coated with 400U HRP.

Serial dilutions of the beads were analysed optically with a UV/Vis plate reader, and electrochemically by "spotting" on to an electrode.

2.2.6 System Development

Macro-scale multi-well system

Firstly a macro-scale multi-channel system (see Figure 2.6) was attempted and secondly two smaller scale methods were developed.

For the macro-scale system, holes were drilled into the bottom of wells from "breakaway" immunoassay plates.   PTFE tubing was flanged and threaded through the holes. The underside of the flange was glued to the inside bottom of the well using an epoxy glue, as shown in Figure 2.6. The assay was performed in the well.

In order to prevent "clogging" of the electrode by the beads, the magnetic beads were held to the side of the well using a magnet, and the product was then pumped over the electrode.
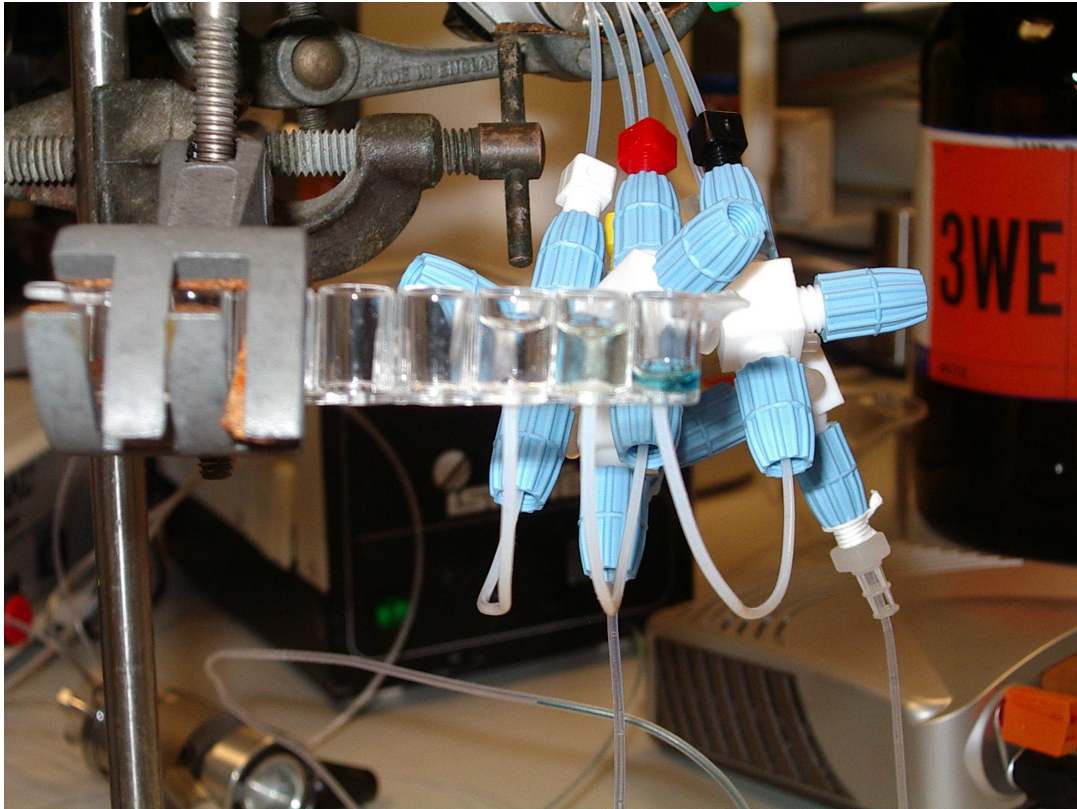
Figure 2.6 The macro-scale multi-channel system

Switching between the wells was accomplished through use of a mobile-phase selection valve.

Due to problems with air bubbles when switching between wells, and as a step towards a microdevice, a smaller-scale approach was tried.

Small-scale Systems

Materials and Equipment

PDMS has several advantages for the manufacture of small-scale and microsystems, especially compared to silicon and glass. PDMS is:-

- a relatively inexpensive material
- flexible and optically transparent down to 230nm

- Compatible with many biological studies, as it is impermeable to water, non-toxic to cells and permeable to gas

- offers ease of fabrication and bonding to other surfaces, which aids rapid prototyping and development.

Samples in the PDMS cell were interrogated optically by the use of a device created in-house, the RGB Reader (see Figure 2.7). The RGB Reader is based on a TCS230 programmable colour light-to-frequency converter device manufactured by Texas Advanced Optoelectronic Solutions (TAOS). This device reads an 8 x 8 photodiode array. The photodiodes are split into 4 groups of 16, which have Red, Green, Blue and Clear filters.

The RGB Reader was connected to a PC running devsys32 software.
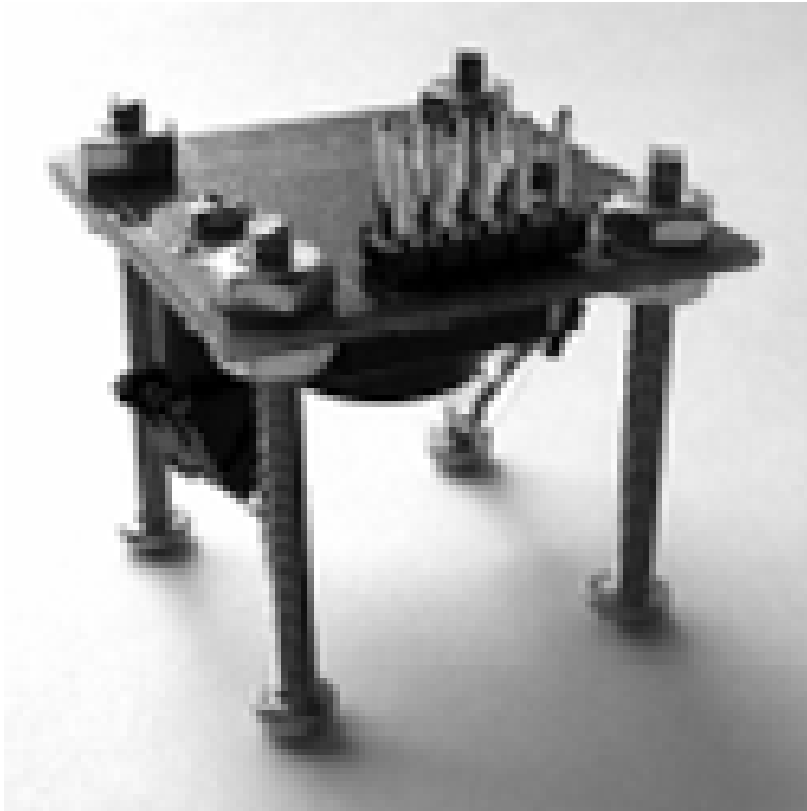
Figure 2.7: The optical RGB Reader

Methods

Two different methods were used to manufacture a smaller scale device. The patterns for both were created on a PC, using Synrad WinMark software and cut by a Fenix Laser Marker, CO_2 Laser marker, with a wavelength of 10510-10650nm (see Figure 2.8).

Figure 2.8: the Fenix CO_2 Laser Marker

Method 1: Adhesive Layer Cell

Adhesive layers from Adhesive Research Inc (Dublin) were used to build an electrochemical cell, using SPE as the basal layer (see Figure 2.9). This cell was manufactured in several layers.

- The base layer was the screen printed SPE.

- ARClad 8901 was used to cover the carbon tracks (there was no cover layer on the SPE).

- 4 layers of ARClad 8458 were then stacked onto the base layer.

- A 250μm thick polyester sheet was then placed on top.

- The whole was sealed with PDMS.
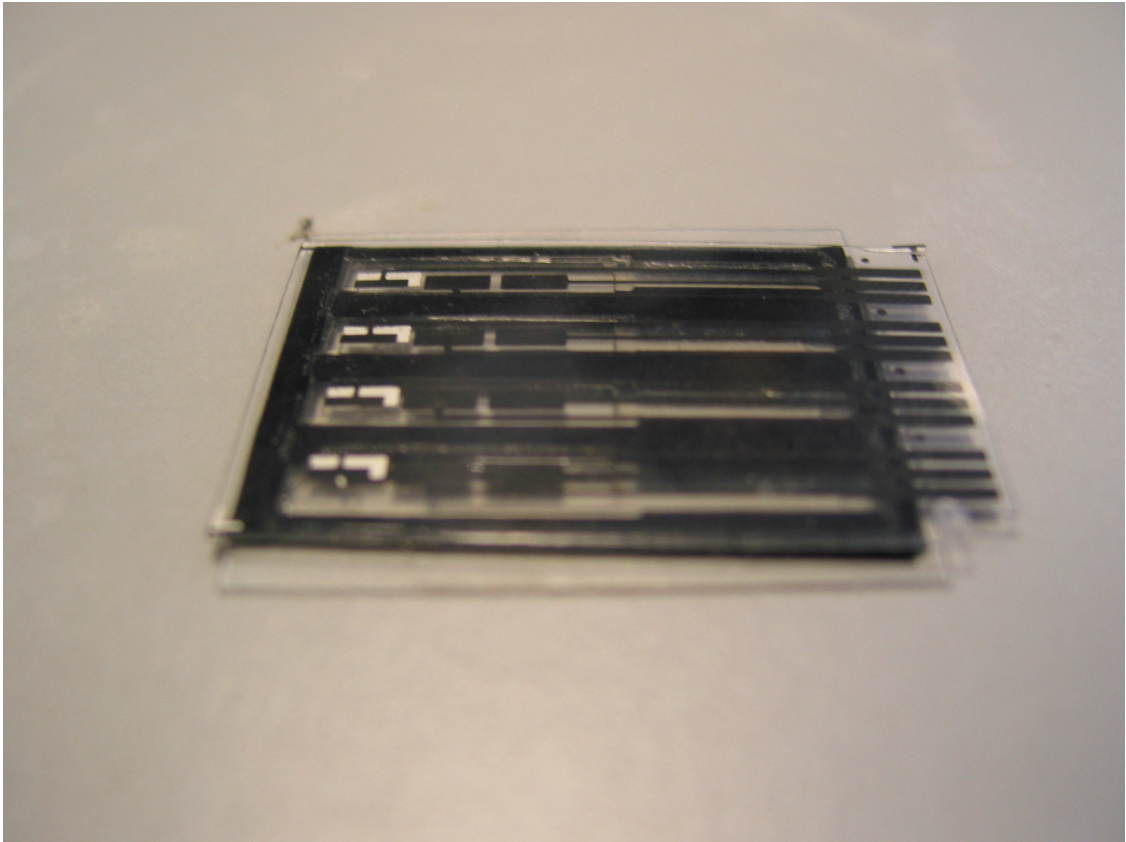
Figure 2.9: The adhesive-layer electrochemical cell

Method 2: PDMS Cell

- Formers were ablated from Perspex.

- Sylgard 184® (an optically transparent PDMS which permits spectroscopic analysis) was poured over them (see Figure 2.10).

- After curing, the PDMS was removed from the formers to obtain two halves of a cell.

- The two halves of the PDMS cell were sealed irreversibly together by plasma oxidation in an Emitech K1050X Plasma Asher using 73 Watts power for 20 seconds at 6×10^{-3} vacuum. This created a "leakprooof" cell which meant that liquids could be pumped through it and analysed.

Figure 2.10: The PDMS cell.

As a preliminary study, serial dilutions of HRP were made. A 50µl aliquot of each concentration was placed in a weighing boat and 50µl of ABTS substrate solution (Sigma Cat No. A3219) was then added to the HRP in the weighing boat. This was then covered with a sheet of PDMS and allowed to incubate for 5 minutes at room temperature. The RGB Reader was placed over the sample with its LEDs switched on. The device recorded the reflected light as Red, Green, Blue and Clear values.

The RGB Reader was also used to analyse HRP in the PDMS cells and to analyse product from the activated sepharose immunoassay in adhesive layer cells.

In order to mitigate the hydrophobicity of the surface of the channels in the PDMS cells use of the surfactants Tween 20 and Sodium dodecyl sulphate (SDS) was investigated,

50µl of HRP solution (0.1U) in PBS and 50µl of ABTS liquid substrate were incubated for 5 minutes with 2µl of either a 5% solution of Tween 20 or a 0.1M solution of SDS. The product was injected into the PDMS cell and the signal was analysed using the RGB reader.

The optical RGB cell

Problems of cross contamination of the signal from other channels in the multi-channel setup were encountered because of the optical transparency of PDMS. It was therefore decided to manufacture an optical cell (see Figure 2.11).

In order to speed up the process, these were made using many of the materials previously used in the adhesive-layer electrochemical cell. However, in the manufacture of the optical cells, the screen-printed SPE

base layer was replaced with a microscope slide. This resulted in reasonably fast assembly of cells, which could be read optically using the RGB reader.

Method:

- Immunoassays were performed.
- The product was pipetted from the wells into an adhesive layer cell and read directly.
- The RGB values were then converted to HSV values.

The eventual aim was to pre-load reagents into the cell and perform the assay in the cell.
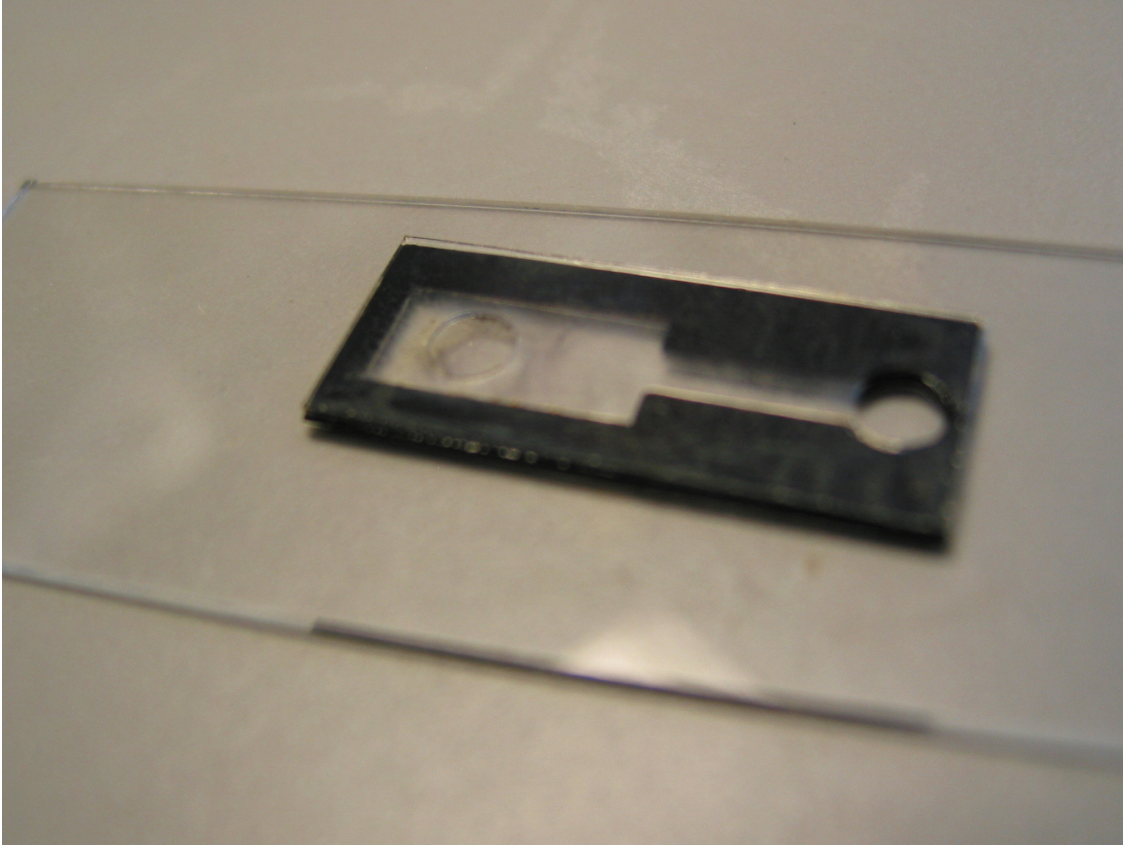
Figure 2.11 The adhesive layer optical cell

Chapter 3

Results and Discussion

3.1 INTRODUCTION

This chapter will show the results obtained in the course of the project. The results will be divided into the following sections:-

1. Immunoassay results obtained both at CSIC in Barcelona and at Cranfield University. Unless otherwise specified, the data was obtained at the Silsoe campus of Cranfield University.

2. Preliminary magnetic bead assay data

3. Substrate analysis results

4. Protein assay results

5. Electrochemical results

6. Results from PDMS cells

3.2 RESULTS

3.2.1 Immunoassay Results

The results from the immunoassay showed that the assay format and reagents provided by CSIC were able to detect Atrazine and 2,4,6-TCP at nanomolar concentrations. The atrazine immunoassay was performed in various matrices (grape pulp, white wine & ground water). The method used is as described in the previous chapter. As four of the five batches of antibodies supplied by CSIC were inactive (perhaps due to problems during transit), some antibodies and tracer for atrazine (product number G13720M) were bought in from AMSBio (a UK subsidiary of Biodesign, a US company) and the assay was performed to validate the method. The results for this assay are shown in Figure 3.3. Unless otherwise specified, the data

for Figures 3.4 - 3.8 was gathered using the third batch of reagents supplied
by CSIC.
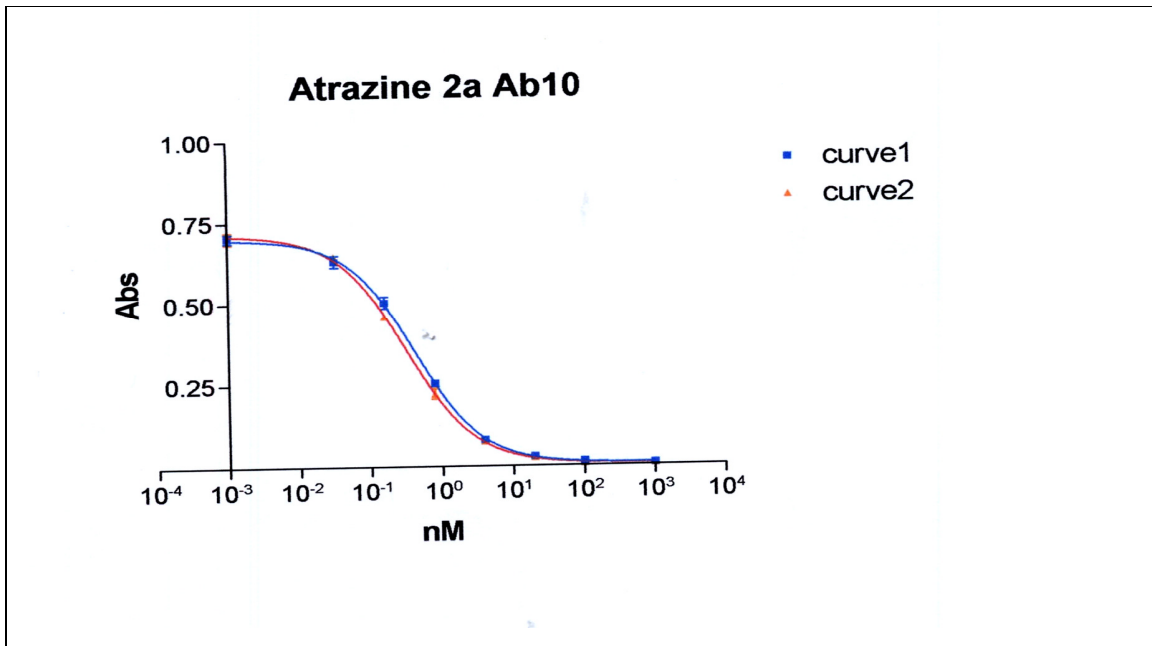
Figure 3.1: Atrazine ELISA: absorbance vs concentration. (results obtained
at CSIC, Barcelona)

The results obtained, as demonstrated in Figure 3.1, indicated that all
the standards can be detected and show the expected trend of signal
reduction with increased atrazine concentration.

The 2 curves shown in Figure 3.1 are a result of the way that the assay
plate was divided up. The data obtained at CSIC was adjusted and displayed
using the software package GraphPad Prism[TM]. As this was unavailable at
Silsoe, the data gathered there has been displayed by calculating the %B/B0
of each concentration (This is where the mean absorbance of a particular

concentration is shown as percentage of the mean absorbance of the blank). The error bars represent the mean standard deviation of the data for each concentration.
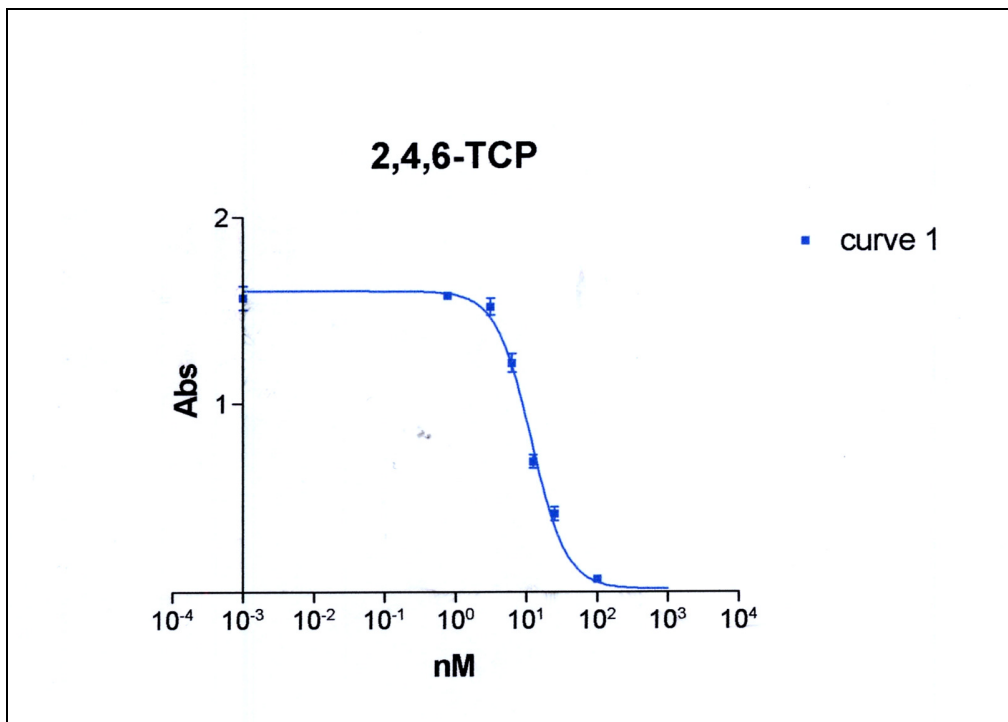
Figure 3.2: 2,4,6-TCP ELISA: absorbance vs concentration (assay performed at CSIC, Barcelona)

The results obtained, depicted in the Figure 3.2, indicate that 2,4,6-TCP can be detected at 0.78nM concentration.
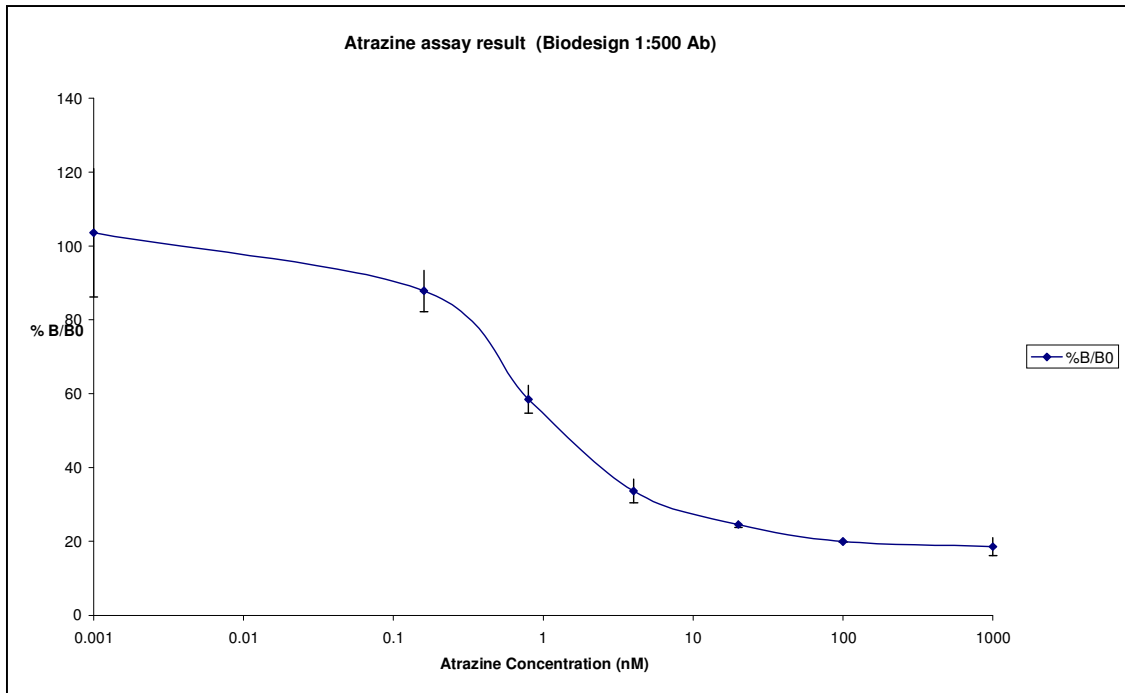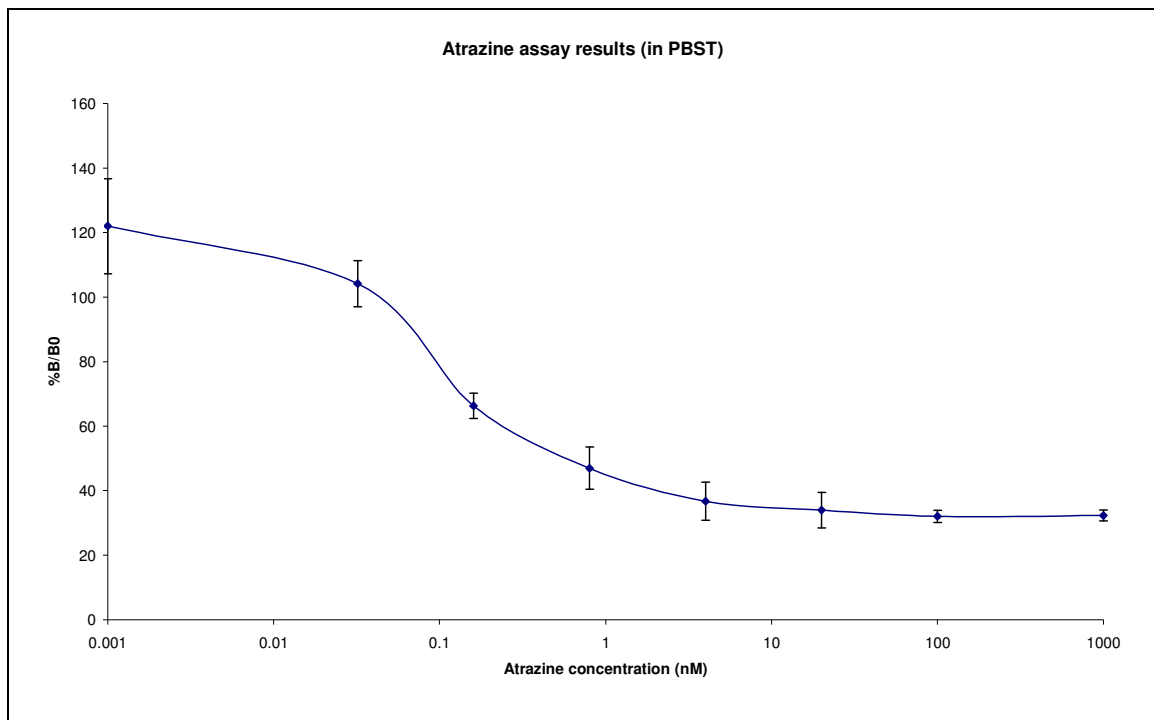
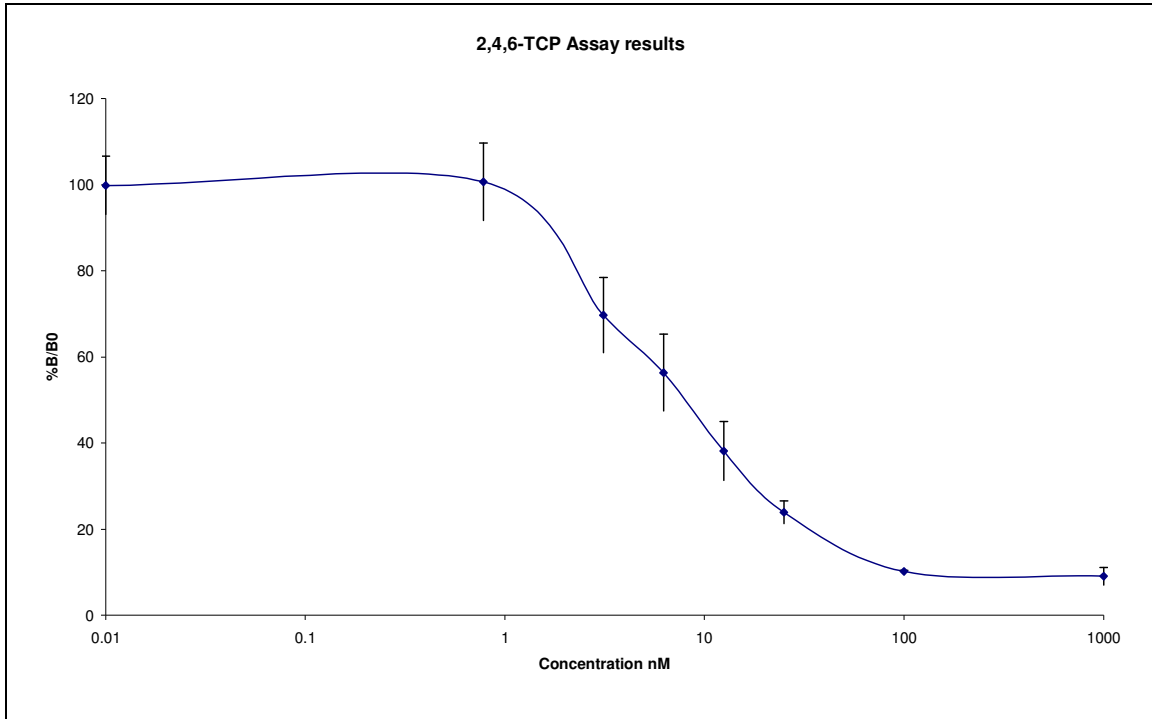**Figure 3.3**: Atrazine immunoassay results (in PBST) obtained using Biodesign reagents.

As demonstrated in the Figure 3.3, the lowest standard detected with the Biodesign antibodies (product no.G13720M) was the 0.16nM standard. The 0.032nM standard was not detected. The CSIC antibody Ab10 was able to detect the 0.032nM standard in the various matrices that were used (see Figures 3.1, 3.4, 3.6-8) however the batch variation encountered could limit its utility.

The detection of the 0.1nM standard indicates that the required regulatory Maximum Residue Level, set by the EU, of 0.1mg/kg of atrazine permitted in grapes and wine (about 50nM) can be easily detected. The high cost of such sensitive antibodies (£810 per ml for the Biodesign IgG) when

this sensitivity is not needed in order to meet the required standard, may enable the lowering of costs by substitution with less costly products.

Atrazine assay results (in PBST)

Figure 3.4: Atrazine immunoassay results in PBST using antibodies from CSIC. The 0.032nM standard was detected.
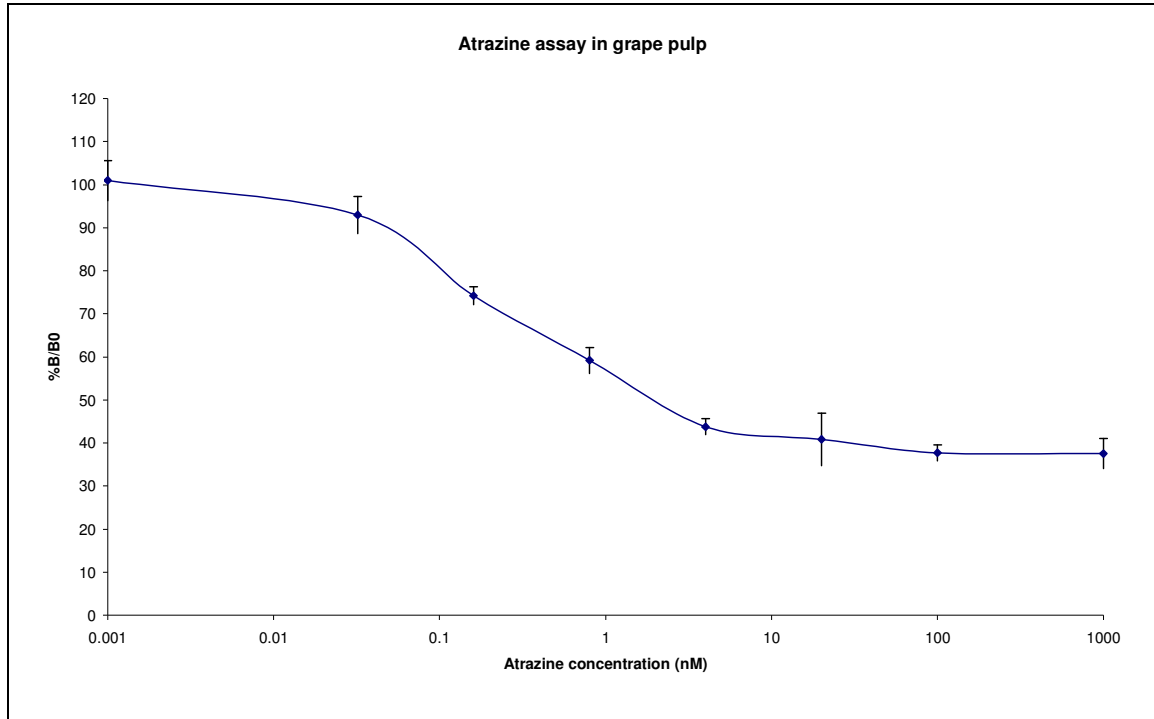
Figure 3.5: 2,4,6-TCP immunoassay results in PBST

The results of the immunoassay depicted in Figure 3.5 indicated that 0.78nM of 2,4,6-TCP can be detected in PBST. It demonstrates the expected trend of signal reduction with increasing concentration characteristic of a competitive assay.
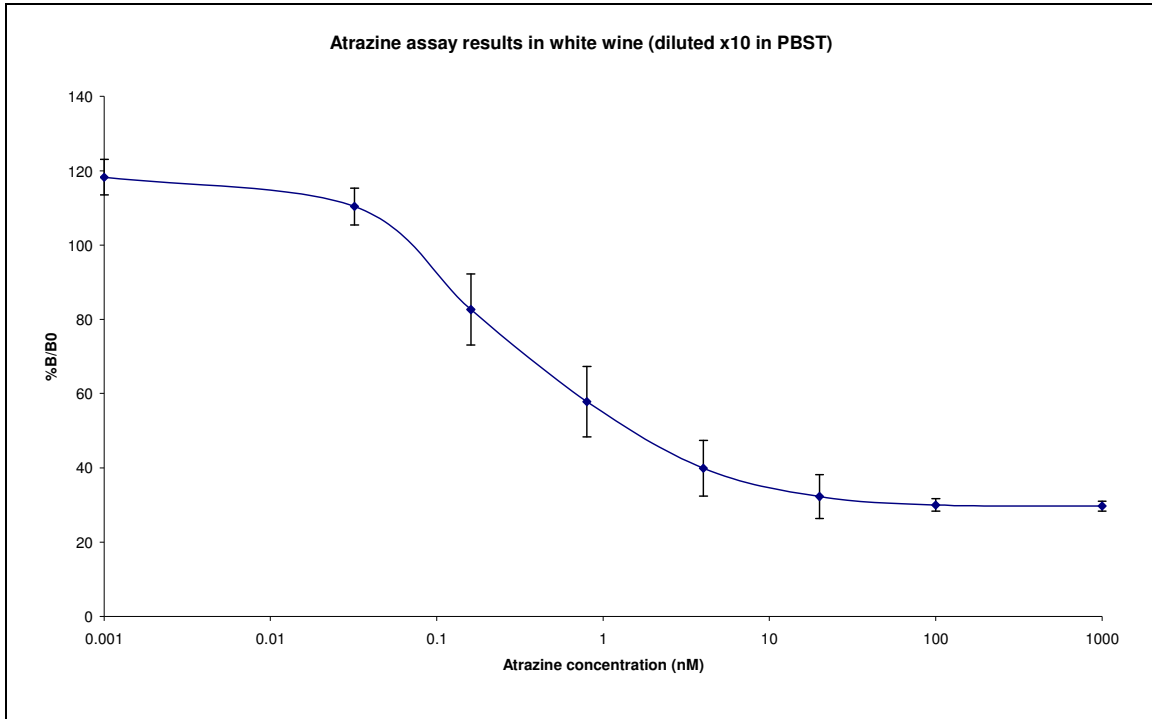
Figure 3.6: Atrazine immunoassay in white wine (diluted 10 times in PBST)

As white wine is a more complex matrix than PBST, it is more difficult to detect contaminants in this matrix. The samples were prepared by dilution in PBST at a ratio of 1:10. The results (depicted in Figure 3.6) demonstrated that it was possible to accurately detect the presence of atrazine in white wine at concentrations as low as 0.032nM.
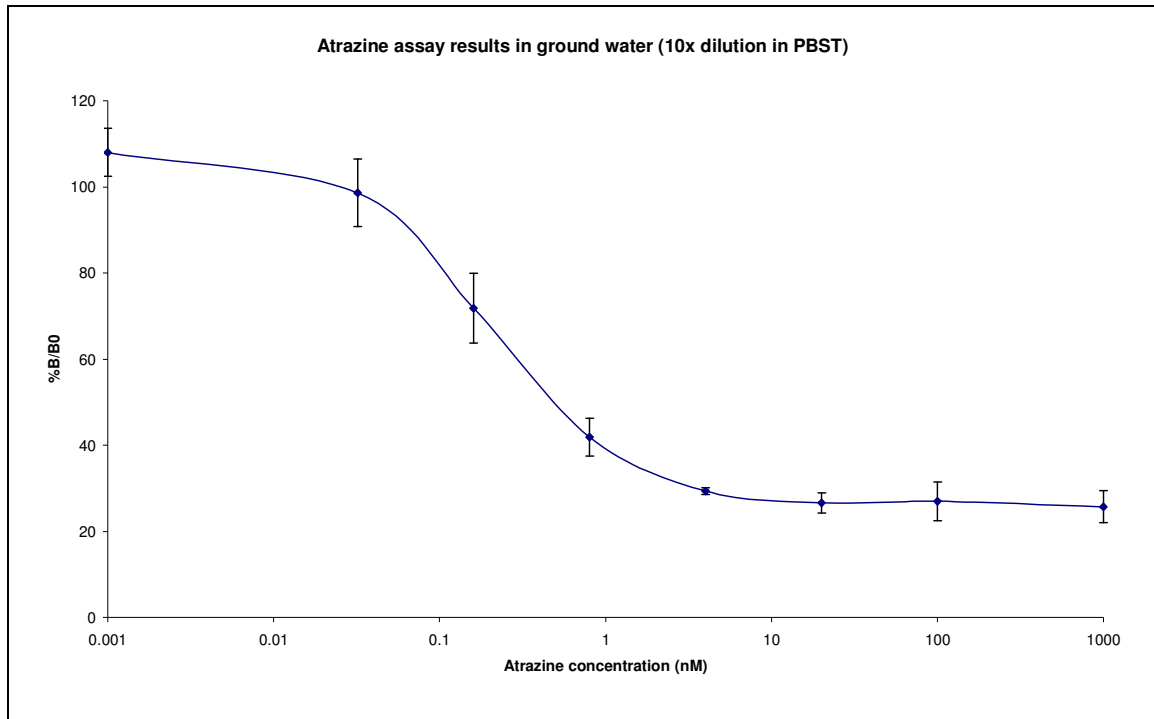
Figure 3.7: Atrazine immunoassay results in grape pulp (20x dilution in PBST)

The accurate detection of atrazine in grape pulp has two main benefits:

1. Many people consume grapes and grape juice, as well as wine. In terms both of food safety and of consumer confidence it is of paramount importance that retailed foods do not exceed the safe limits for contaminants established by the EU

2. The potential for testing grapes for contaminants in the field before commencing expensive harvesting, transportation and processing procedures has clear economic benefits for producers of wine and grape juice.

The results depicted in Figure 3.7 indicate that atrazine can be reliably detected in grape pulp at concentrations below 0.1nM. This detection sensitivity exceeds the EU Maximum Residue Level for atrazine in grapes.

Figure 3.8: Atrazine immunoassay results in groundwater (diluted 10x in PBST)

As documented in Chapter 1, Atrazine is often a contaminant of groundwater. Therefore it was decided to run an immunoassay to establish the limits at which atrazine could be detected in this matrix. The results obtained (see Figure 3.8) demonstrate that using only dilution as a preparatory technique, very low concentrations of atrazine can be detected in this matrix (below 0.1 nM).
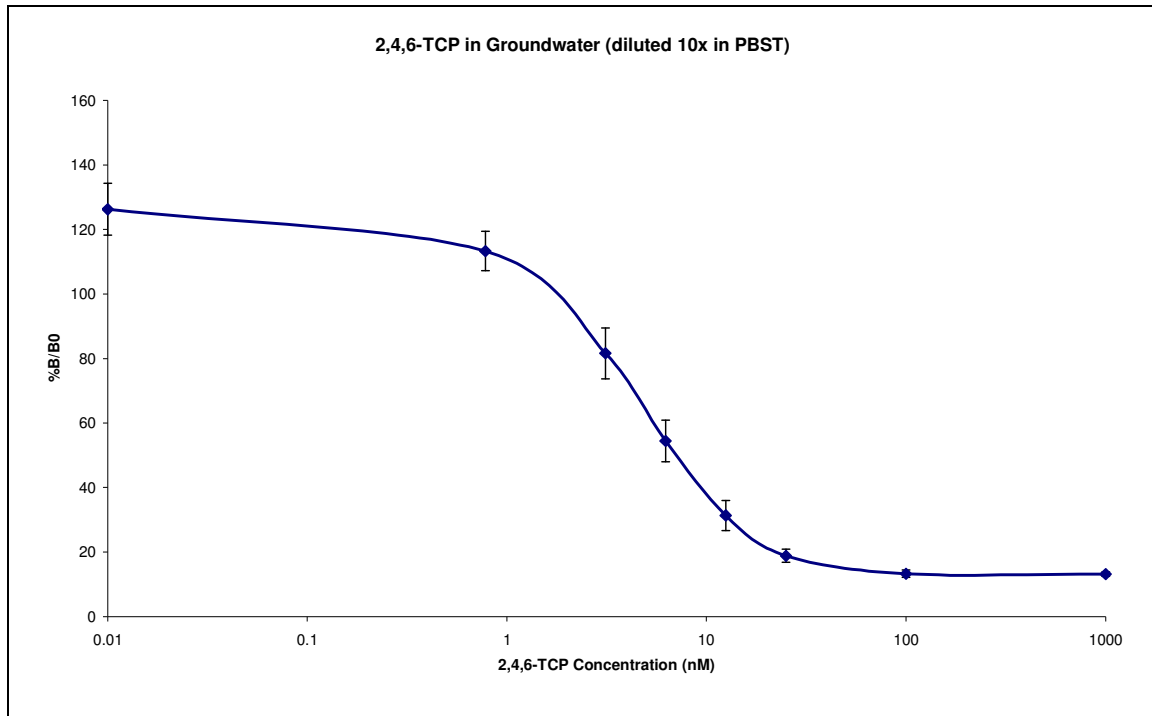
**Figure 3.9**: 2,4,6-TCP assay results in groundwater (diluted x10 in PBST)

As depicted in Figure 3.9, TCP could be detected in samples of groundwater at concentrations of 1nM. The detection of such low concentrations of 2,4,6-TCP in groundwater should be seen in the light of the World Health Organisation guidelines of a safe limit for drinking water of 0.2mg/litre. According to the WHO, concentrations in drinking water are generally less than 1µg/litre (WHO 2003) and the guideline value exceeds the lowest reported taste threshold. In wine the concentration of 2,4,6-TCP may be higher due to the use of chlorophenols in cork sterilisation. It may also be more difficult to analyse wine using antibodies due to cross reactivity with polyphenolic compounds in the wine.

3.2.2 Magnetic bead preliminary assay work

The reagents for a magnetic bead immunoassay for atrazine were supplied by CSIC. An attempt was made to utilise this technique. As the magnetic tube holder had only six niches, only six concentrations of atrazine were tested in each experiment.

The assay was first performed at the recommended bead concentration of 12µg/mg. However the results did not follow the expected trend of signal reduction with increased concentration of atrazine and there was strong colour development in the waste, indicating the presence of enzyme tracer in the waste.

The assay was subsequently performed at a bead concentration of 120µg/mg, in order to increase the likelihood of the tracer being bound. The washes of this assay were analysed and the data is presented in Figure 3.10 below.
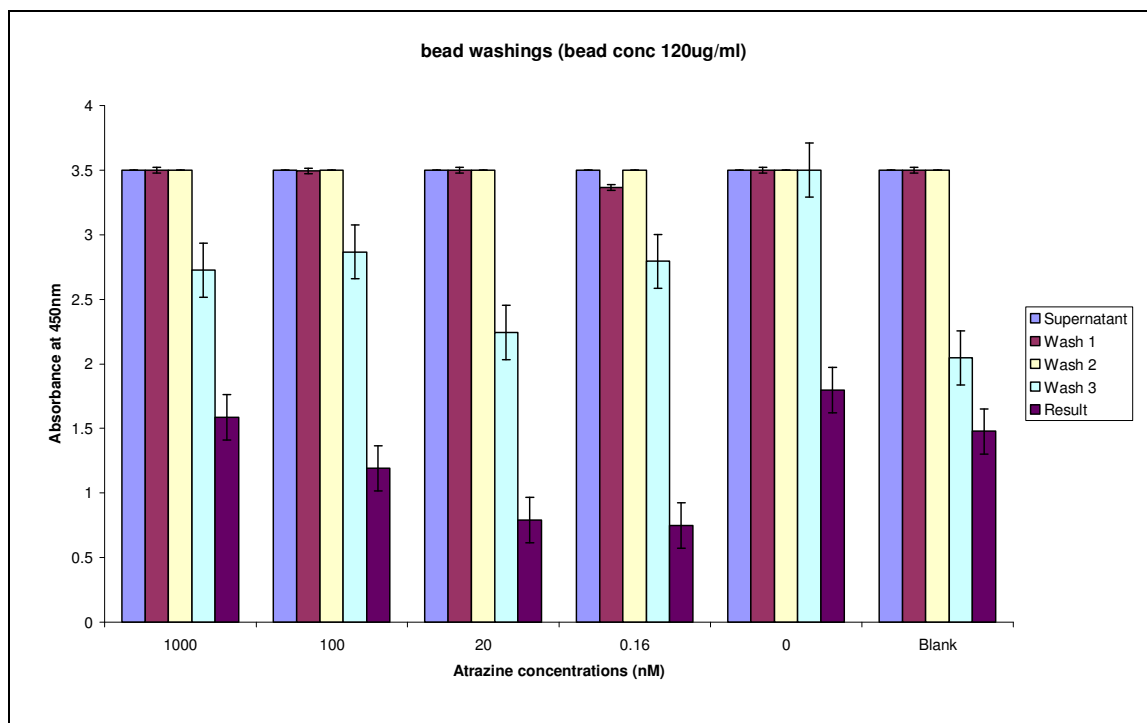
Figure 3.10: Data from magnetic bead assay for atrazine (reagents supplied by CSIC).

The results of the magnetic bead assay for atrazine, depicted in Figure 3.10, were surprising. The supernatant and first two washes demonstrated very similar levels of absorbance, decreasing only on the third wash. It was expected that absorbance would reduce with each wash, and also that the blank would demonstrate higher absorbance than the other samples. That this was not the case suggests that either the 2aHRP tracer was not being bound or else that the antibodies were becoming detached from the bead. Preliminary data from HRP coated beads from Dynal showed a signal reduction with increased dilution of bead stock as expected (see Figures 3.11 and 3.12). This suggests that the magnetic bead technique is a viable one.

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However, preliminary testing of each batch should be undertaken to ensure their viability. This may reduce the utility of the technique in a field situation.
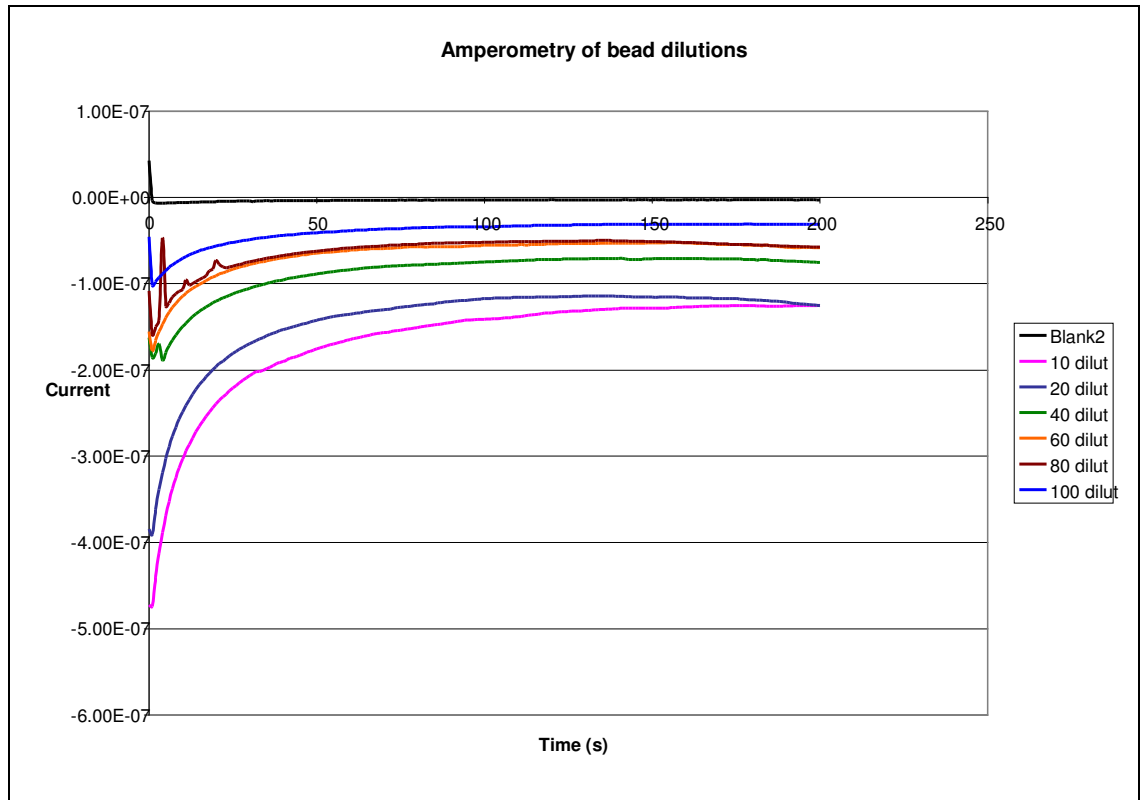
Figure 3.11: Amperometry of dilutions of HRP coated beads.

Dilutions were 10x, 20x, 40x, 60x, 80x, 100x of $2x10^9$ bead stock.
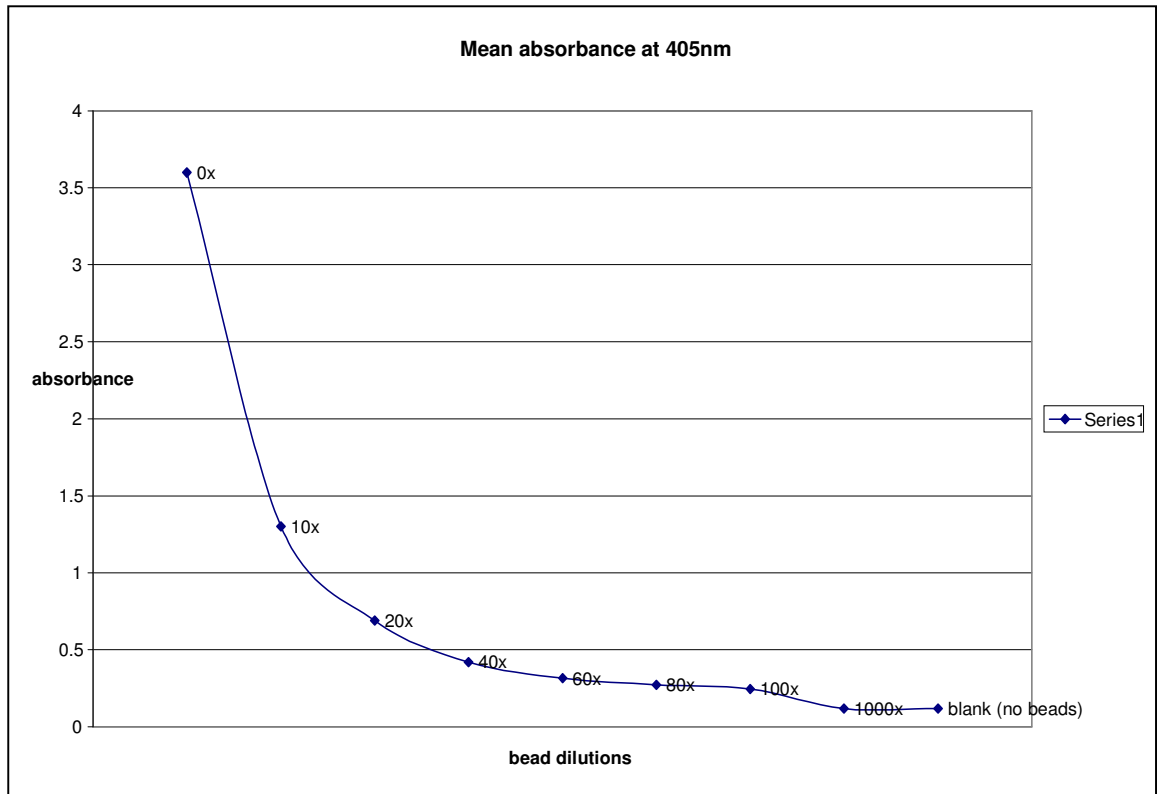
Figure 3.12: Optical analysis of HRP-coated bead dilutions

Dilutions were 10x, 20x, 40x, 60x, 80x, 100x of $2x10^9$ bead stock

These results shown in Figures 3.11 & 3.12 are strongly correlated: both demonstrate decreased absorbance with increased dilution of bead stock. This indicates the viability of this technique as an analytical method.

**Activated Sepharose atrazine immunoassay results**
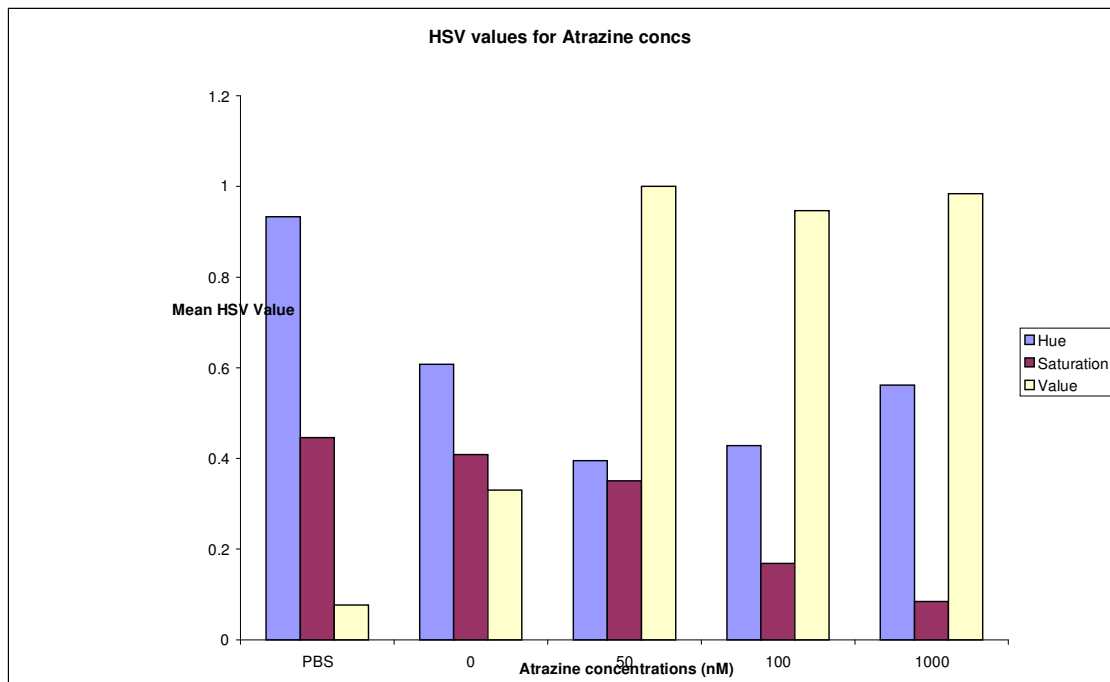
Figure 3.13: HSV values for atrazine immunoassay.

The activated sepharose atrazine immunoassay was performed; the assay product was then pipetted from the eppendorf and injected into an adhesive layer cell and analysed with the RGB device. The RGB values were subsequently converted to HSV values.

Of particular interest in Figure 3.13 is the saturation value, indicating colour intensity, which decreases as atrazine concentration increases, as would be expected in a competitive assay where signal is inversely proportional to analyte concentration.

3.2.3 Results of substrate analysis

The optical analysis (UV/vis) of ABTS, TMB & OPD suggests that ABTS is the better mediator. TMB performed better than OPD initially, but over time the signal decreased to below that of the OPD. This discrepancy may, in part, be accounted for by oxidation of the blank by the hydrogen peroxide used in making up the OPD solution. The oxidation of the blanks in this experiment is illustrated in Figure 3.15.
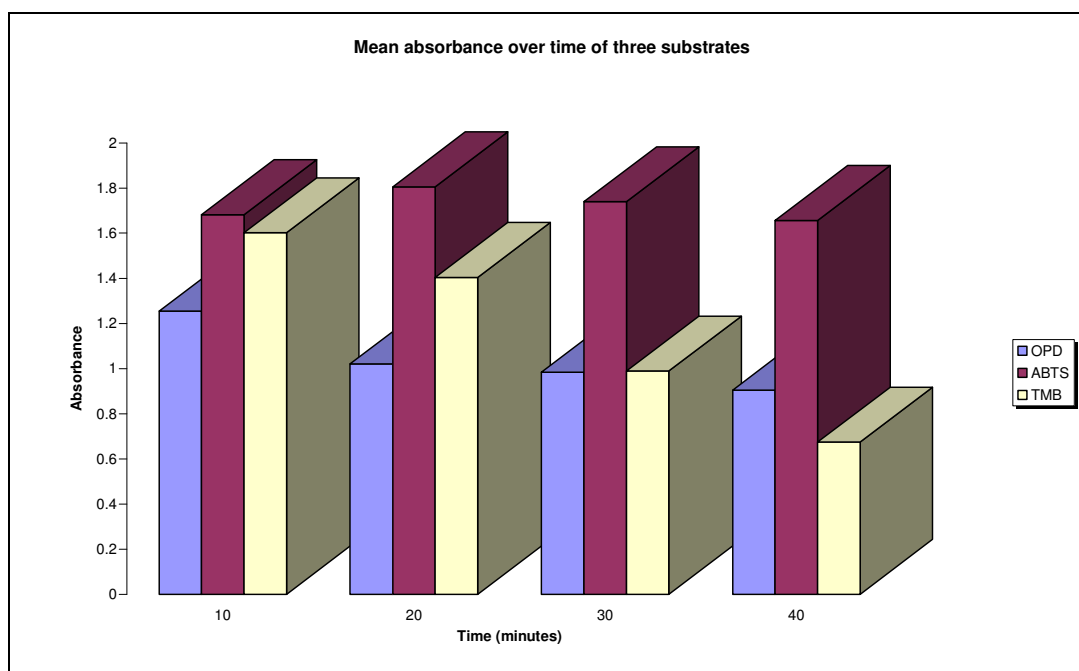
Figure 3.14: Plot of means (blank corrected): signal vs reaction time: HRP 0.1U with ABTS, TMB and OPD

As demonstrated in figure 3.14, ABTS is a more reliable mediator than either TMB or OPD, this would be particularly critical in a field situation where conditions are less than optimal.
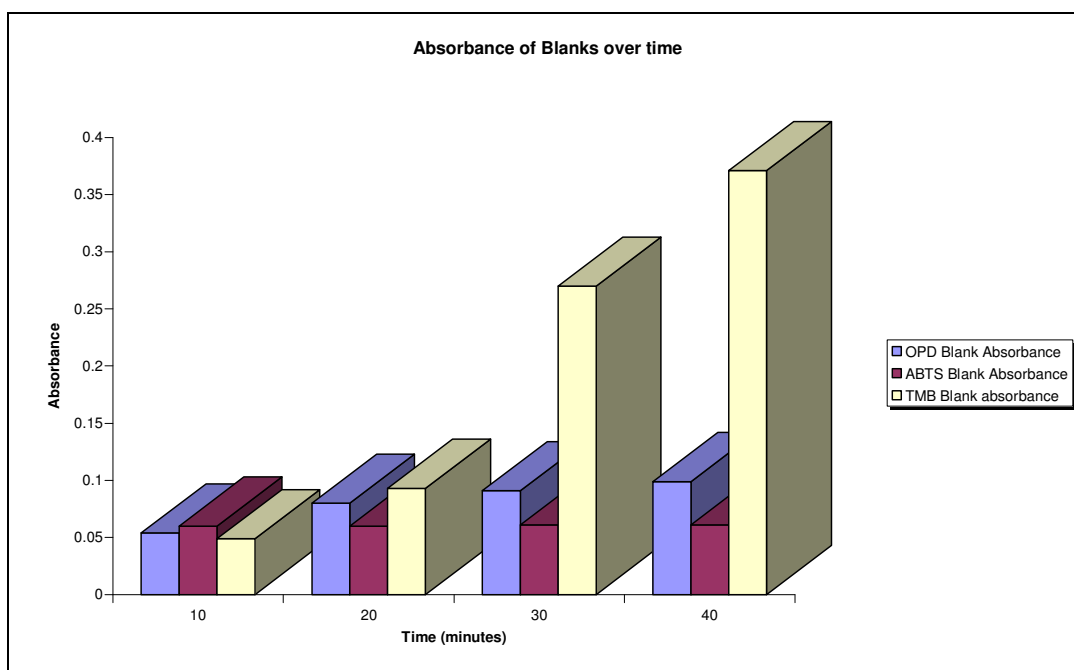
**Figure 3.15**: Absorbance of blanks over time

In order to accurately assess the stability of the three electron acceptors, blank solutions of each were made up and absorbance was measured at set times. The ABTS solution showed less absorbance after thirty minutes than either the OPD or the TMB. Electron acceptors will oxidise at varying rates over time even in the absence of analyte. The challenge is to distinguish between this oxidation and that caused by the analyte. Failure to establish an accurate baseline value for oxidation will result in flawed data production during analysis.

In performing the immunoassay, plates containing analyte and electron acceptor are incubated for thirty minutes. The results depicted in Figure 3.15 indicate that the ABTS blank solution showed less absorbance after thirty minutes than either the OPD or the TMB indicating that it was the more stable solution for the purposes of this experiment

3.2.4 Results of Protein analysis

As stated in Chapter 2, a protein assay was carried out in order to obtain information on the amount of protein and hence HRP in the 2aHRP used in the immunoassay. The results of the protein assay on the 2aHRP were at first inconclusive when analysed at the dilution used in the assay (1:1000), and at 1:100 contained below the minimum standard of 20µg/ml protein. Therefore it was not possible to accurately quantify it. When diluted 1:1 with PBST, 2aHRP was seen to show the same absorbance as the BSA 100ug/ml standard (as illustrated in figure 3.16).
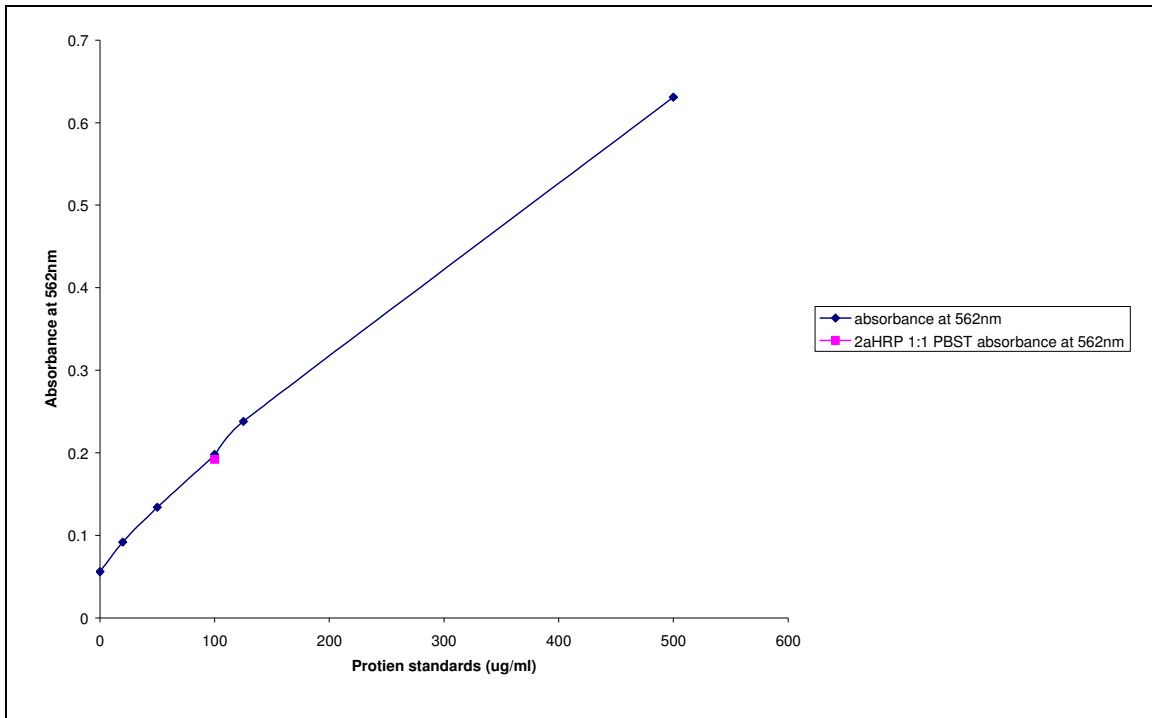
Figure 3.16: BSA protein standards and 2aHRP absorbance at 562nm vs protein concentration

The 2aHRP when diluted to the working concentration of 1:1000 showed an absorbance similar to 0.1U (1.031 compared with 1.108 for 0.1U after 20 minutes). This is illustrated in Figure 3.16.
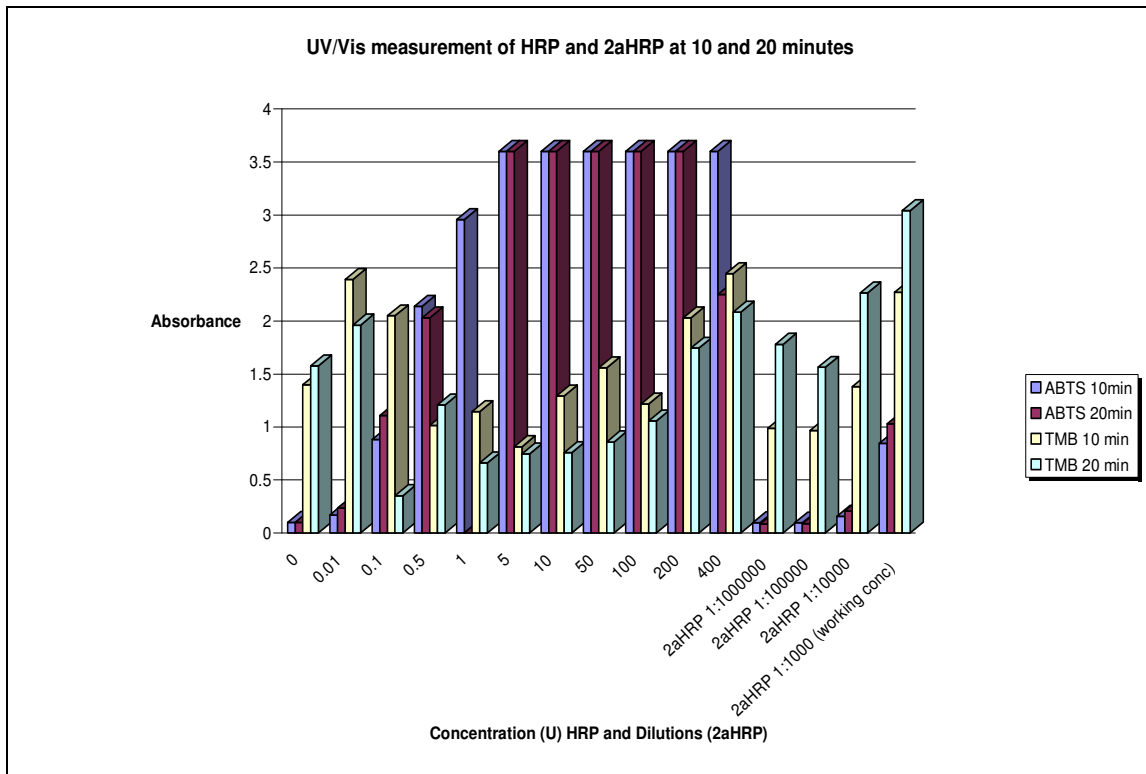
Figure 3.17: UV/Vis absorbance of HRP and 2aHRP with ABTS and TMB.

The "over" limit for the plate reader is 3.5, so the 5-400U have been set at 3.6 rather than >3.5. This experiment was to compare ABTS and TMB as substrates and also to compare the signal obtained from the tracer 2aHRP supplied by CSIC at various concentrations, at 10 and 20 minutes incubation time. The results depicted in Figure 3.17 indicate that the working concentration of 2a-HRP tracer, when incubated for 20 minutes with ABTS gives a similar signal to the 0.1U of HRP.

3.2.5 Electrochemical optimisation process

The aim of these experiments was to optimise the electrochemical detector system. Sensitivity, repeatability and variables such as time and flow-rate were examined.

The optical data shown in Figure 3.14 and 3.15 indicated that, for this purpose, ABTS was a better electron acceptor than TMB. Consequently, for most of the experiments, ABTS was used. OPD was not used electrochemically as the optimum potential was not known and the ABTS solution was considered more stable than OPD.
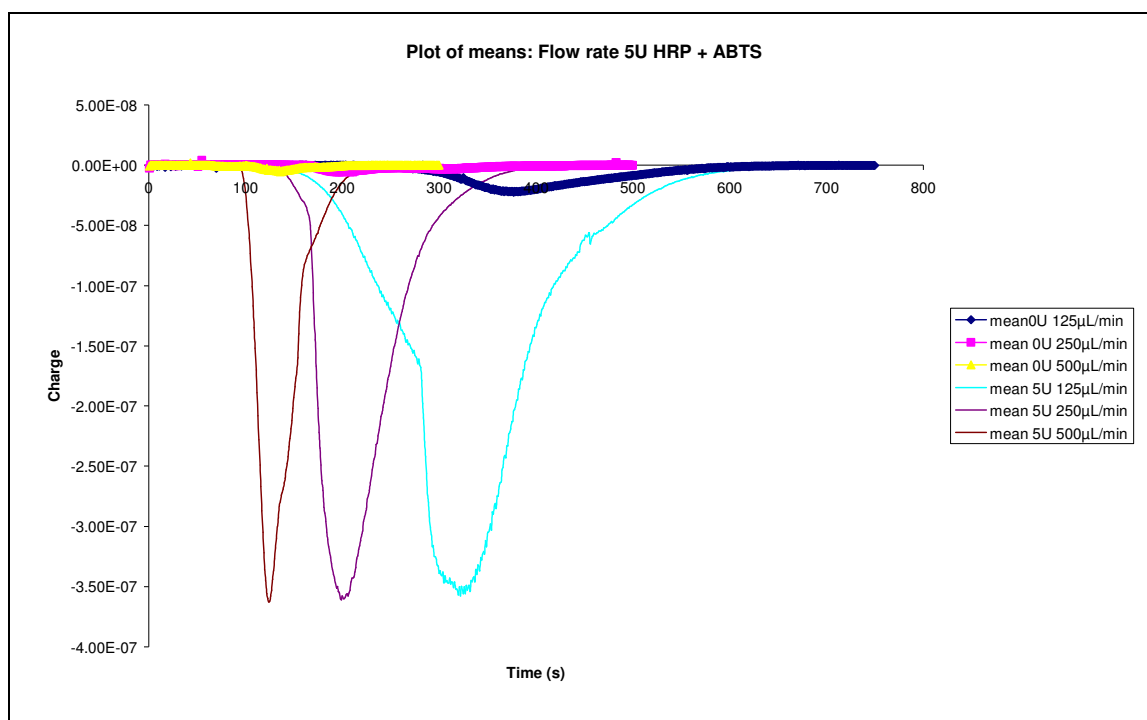
Figure 3.18: Amperometry of 5U HRP + ABTS at varying flow rates (125, 250 and 500µl/minute).

In order to establish an optimal flow rate for the macro-scale flow cell, 5U HRP and ABTS were pumped through the cell at varying flow rates. The flow rate of 250µl/min produced the most reliable results of those tried. Therefore, a flow rate of 250µl/min was subsequently used in all experiments for the macro-scale system.

To look at repeatability and variability of signal over time, 0.01 and 1.0 Unit dilutions of HRP were made up and left to react with ABTS. 250µl aliquots of these dilutions of HRP were then injected into the flow-cell system over a period of time and analysed.
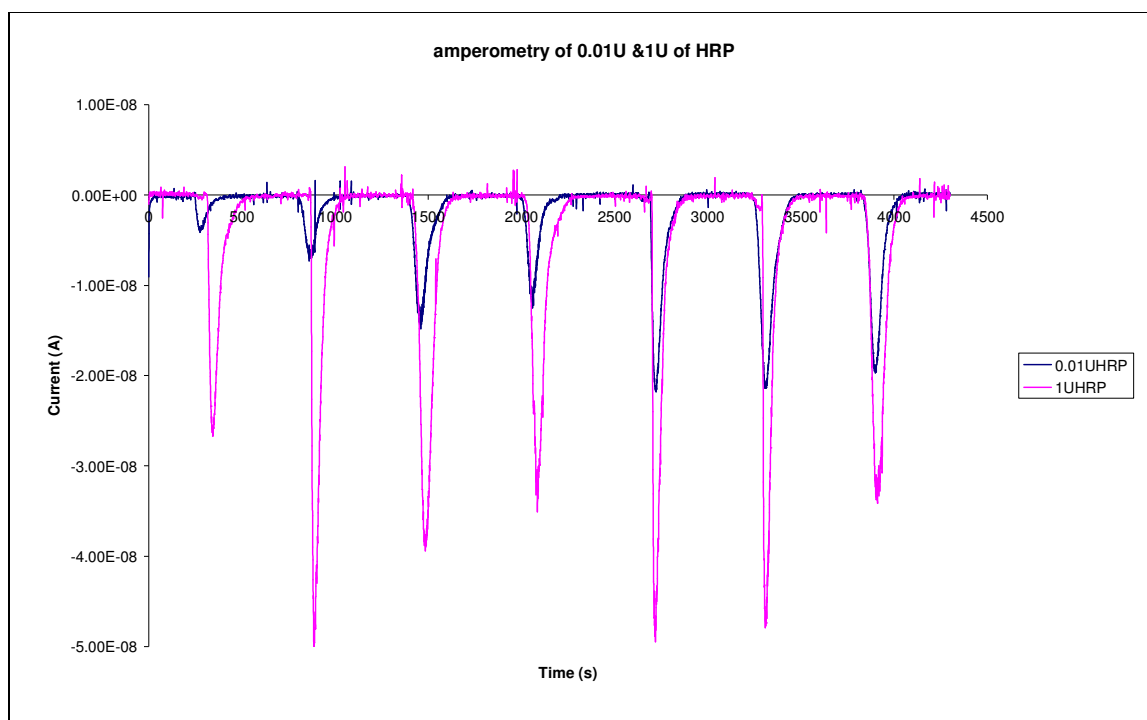
Figure 3.19: Comparison of 0.01 & 1U HRP.

Figure 3.19 demonstrates that 0.01 and 1 unit of HRP can be detected and shows the signal variability over time.
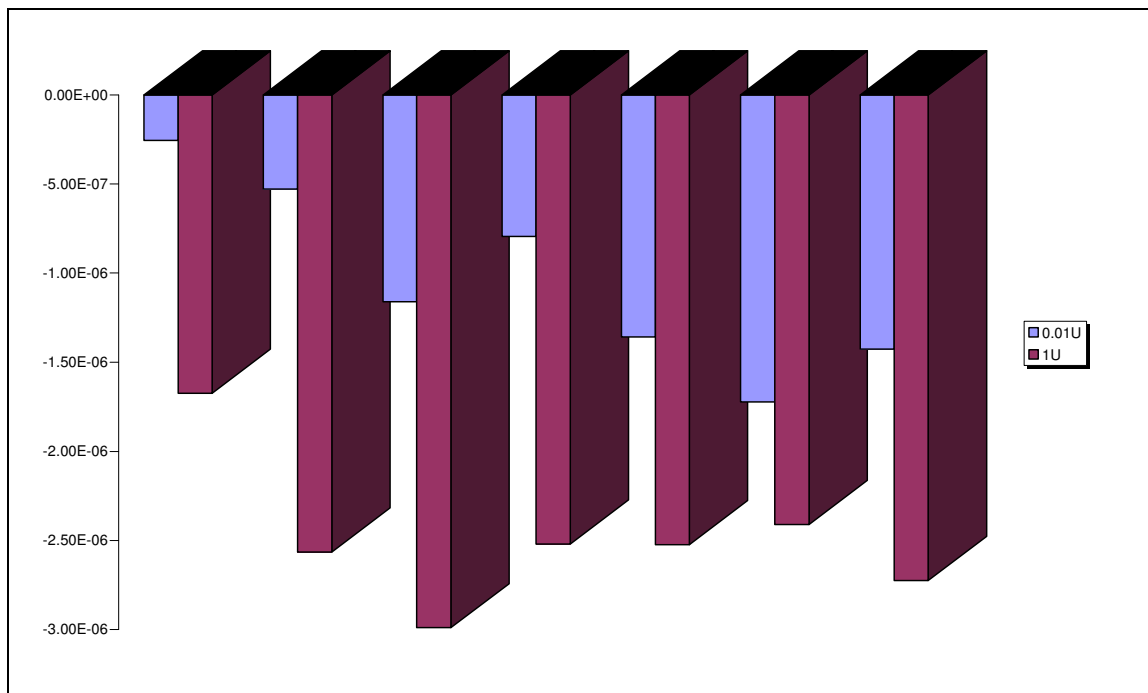
Figure 3.20: Integrated peak area of peaks in Figure 3.19.

The integrated area under each peak shown in Figure 3.19 was calculated and is shown graphically. The 0.01U and 1.0U concentrations can be distinguished and there appears to be some trend to signal increase with time. Further experiments using ABTS and TMB also showed an increase in signal over time, as illustrated in Figures 3.21 and 3.22.
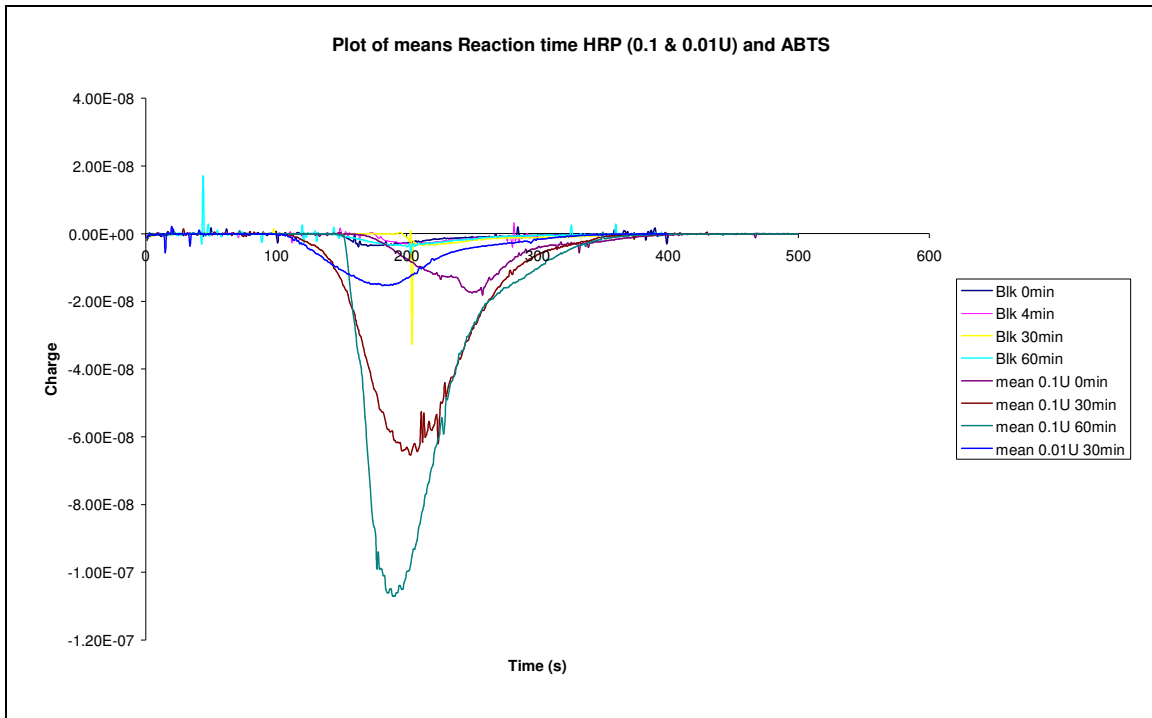
Figure 3.21: Increasing signal with longer contact time
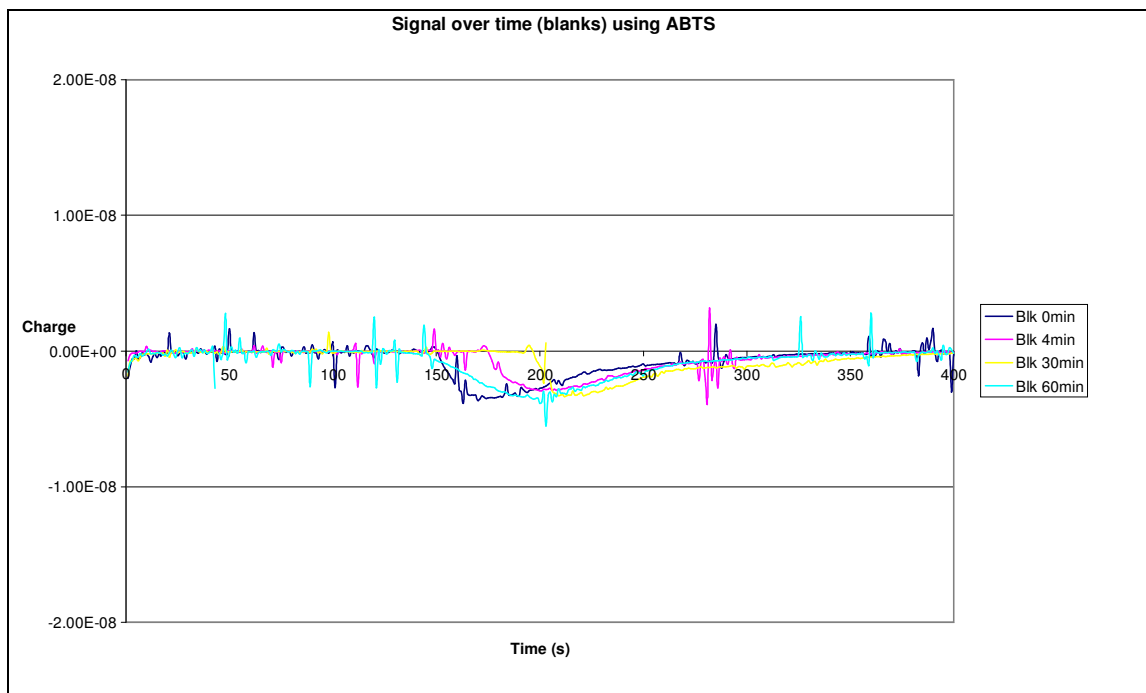
Figure 3.22: Blanks: charge vs contact time using ABTS.

Figures 3.21& 3.22 depict the results obtained when ABTS is held in contact with the surface of the electrode for varying lengths of time. The signal of the blanks does not seem to increase greatly over time, indicating that very little oxidation is taking place. This supports the findings of the optical experiments, which also indicated very little oxidation of ABTS over similar time spans. This confirms that ABTS is a stable electron acceptor, and indicates its suitablity for use in a biosensor.
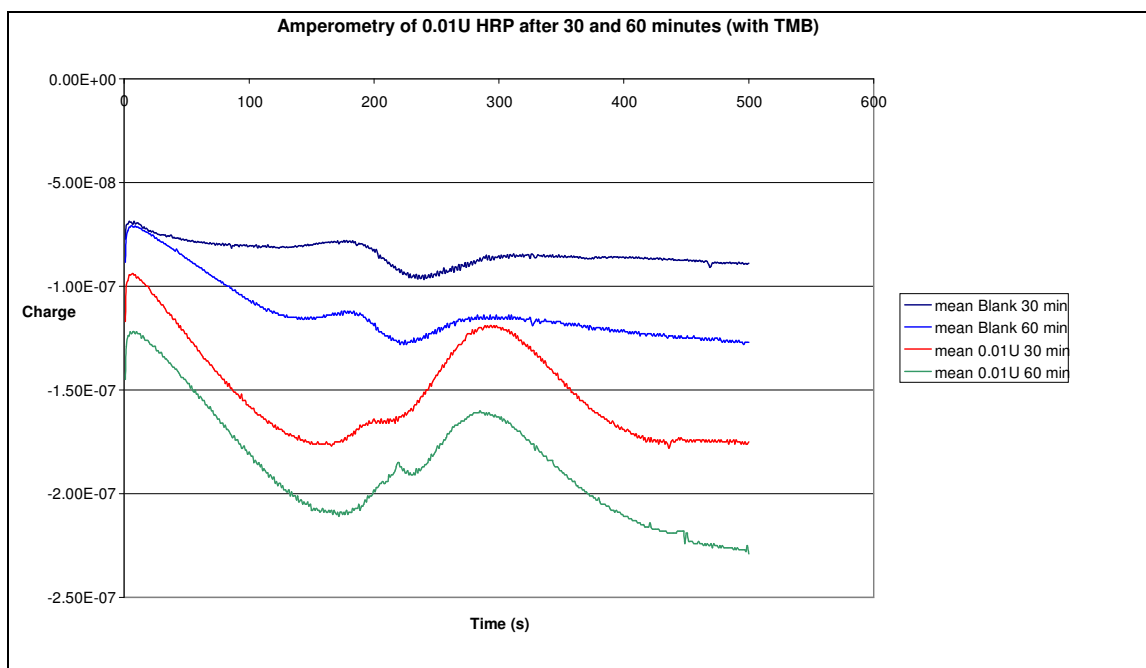
Figure 3.23: Amperometry of 0.01U HRP with TMB

Figure 3.23 illustrates the charge increasing in time when using TMB and HRP. There is a greater signal variation over time when using TMB than when using ABTS (see Figure 3.22).   This suggests that TMB is a less stable electron receptor than ABTS under these conditions.

Having looked at flow-rate and reaction time, experiments to examine sensitivity were conducted.   Figure 3.24 illustrates the results of some of these experiments.
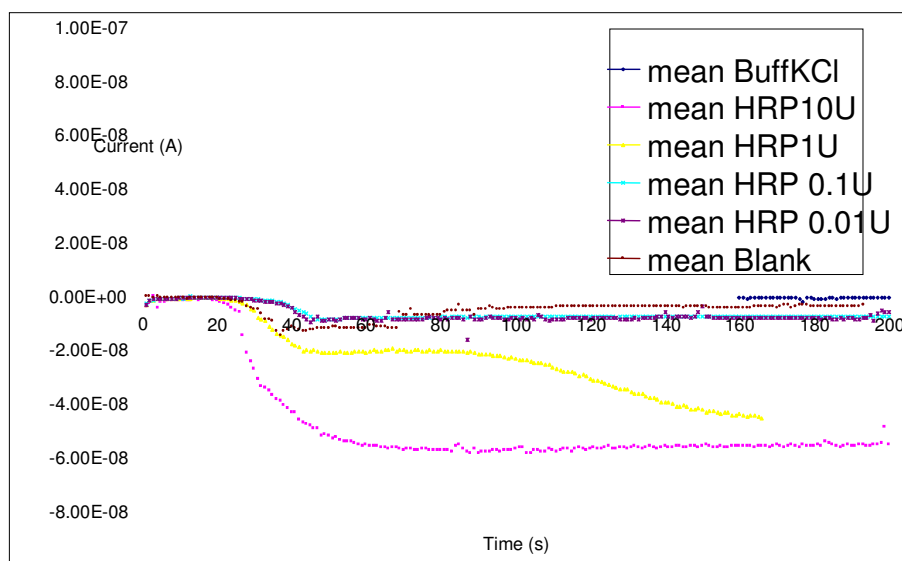
Figure 3.24: Amperometry of 0.01U, 0.1U, 1U and 10U HRP with ABTS.

Figure 3.24 demonstrates that between 10 and 0.01U HRP with ABTS can be detected and distinguished amperometrically.

3.3 GENERAL DISCUSSION

The overall aim of this study – to develop a small-scale sensor for the target analytes – was not fulfilled. However, some of the objectives towards fulfilling that aim have been successfully implemented.

The results for the immunoassays show that Atrazine and TCP can be detected with the required sensitivity (MRL of 0.1mg/kg for atrazine is c50nM). A clear reduction in signal with increase in analyte concentration was demonstrated in both the results obtained at CSIC and those obtained at Cranfield University. The HSV values obtained with the activated Sepharose and in-house RGB reader also follow this trend. This is especially interesting as the use of an RGB reader as an analytical detection tool

appears to be a novel use of this technology. Atrazine and 2,4,6-TCP could also be detected in various matrices, with simple dilution in PBST as a sample preparation step.

As documented in Chapter 1, atrazine is often found in groundwater, so that detection in this matrix can be seen as a realistic test. The detection in grape pulp can also be seen in this light. 2,4,6-TCP may be found as a contaminant in water supplies so its detection in groundwater seems realistic as well.

In the electrochemical experiments, the signal was similar for both: between -1.5E-08 & -2.00E-08 V with 0.01U HRP after 30 minutes incubation time. ABTS is widely available commercially as a ready-made product and was used most of the time.

The protein concentration of the enzyme tracer can be calculated, which allows calculation of the HRP content in the assay. The protein content of 2aHRP, when diluted 1:1 with PBST, was 100µg/ml, or 100µg/mg. The undiluted 2aHRP would therefore have a protein concentration of 200µg/mg. The HRP used in the experiments (Sigma P-8250) had 181Units (U)/mg. 1U of the Sigma HRP therefore weighs 5.5248 x 10^{-6}g (roughly 5.53µg). To calculate the units of activity in the 2aHRP, therefore, we can divide 200µg by 5.53µg, which is 36.2U/mg. The atrazine protocol formulated by CSIC, which was used in these experiments, uses a 1/1000 dilution of 2aHRP, therefore 0.2µg/ml of protein is present in the assay. If there are 36.2U in 1mg of 2aHRP, there should be 36.2/500 in 0.2µg solid. As 6ml of tracer solution is used in the assay we should multiply the result by 6.

$$36.2/500 = 0.0724$$

$$0.0724 * 6 = 0.4344$$

So there are about 0.4 Units of activity in the 12µg solid of 2aHRP in the assay.

As demonstrated, 0.1 and 0.01 Units can be detected electrochemically, so 0.4 Units is well within the range of detection.

Several experiments have shown the signal increasing with time (even in the blanks, which give c. 2×10^{-8} A) until about 20 minutes (see Figures 3.21 and 3.23). This is possibly due to the oxidising effect of the hydrogen peroxide in the liquid substrate system. This effect may make it difficult to rapidly detect the low concentrations required, and an incubation time may be required. This problem may be circumvented when the system is miniaturised, as reaction speed is likely to increase. However, the competitive format of the assay results in an inversely proportional signal, so what is important is to assess the repeatability of detection at the lowest concentration of atrazine. The effect of the acidification of the assay product needs further investigation, in the light of a downstream detection system.

Chapter 4

Conclusions and Future Work

4.1 CONCLUSIONS

The approach discussed and used in this study has both advantages and disadvantages, as does the data presented. These are illustrated in the two SWOT (window) diagrams in Figures 4.1 & 4.2.

Combining immunochemistry and electrochemistry appeared to offer the advantages of both techniques, as described below, however, it also combined the drawbacks with telling effects on the course of the study.

In general, immuno- techniques offer high sensitivity. The detection of <1nM concentrations of atrazine and 2,4,6-TCP have been shown in this study. Unfortunately the biological nature of the reagents has resulted in problems with the transport, storage and innate variability of said reagents.

Electrochemical techniques offer accurate, quantative data and lend themselves to low-complexity and miniaturised devices, facilitating end-user utility. The possibility of obtaining real-time data from a miniaturised system and the increased sensitivity and reaction time consequent on miniaturisation may minimise some of the problems encountered in this study. The use of the RGB reader as an analytical tool merits further investigation and is (based on my literature review) a novel method and the results obtained indicate that it may be sensitive enough.

Sample cleanup and pre-concentration of analyte is vital to minimise cross-reactivity in such a complex sample as wine. Simple dilution has proven effective for white wine, grape pulp and groundwater (see Figures 3.6 and 3.9) this is useful in reducing complexity and costs.

Of the various electron acceptors investigated, ABTS showed less oxidation of blanks and was more stable than TMB and OPD (see Figures 3.14 & 3.15) and has the further advantage of being commercially available in ready-made form. However in later work using the IgGs obtained from Biodesign, TMB was recommended as better suited for the purpose by the manufacturers.

Strengths	*Weaknesses*
Less Complexity than lab-based systems	Instability and variability of biological
High selectivity of Abs	components (eg IgG, enzyme tracer)
Assay → Electrochemical: Confirmatory	Difficulty of electrochemical detection at low
Disposability: Minimising contamination	concentrations
Optimisation of system	Operator inexperience
Opportunities	*Threats*
Screening test	Competition with established techniques and test-
Miniaturised Device for on-line analysis	kits
	Time required for completion

Figure 4.1: SWOT diagram of approach

Strengths	*Weaknesses*
Sensitivity of Ab demonstrated	Further analysis and data processing needed
Electrochemical data broadly accurate	Variability due to operator error and biological
Optimised conditions established	nature of reagents
RGB data broadly accurate	
Opportunities	*Threats*
Microfluidic system → increased sensitivity	Time
Operator experience → decreased variability	

Fig. 4.2: SWOT diagram of data

4.1.1 Outcomes of SWOT diagrams

The Figures 4.1 & 4.2 present:-

- **S**trengths

- **W**eaknesses

- **O**pportunities and

- **T**hreats

of the approach used and data gathered in the course of the research

presented in this report.

Strengths and Opportunities

Common strengths and opportunities focus on the sensitivity and selectivity of immuno-techniques and the optimisation of the flow-system for future research. The accuracy and confirmatory approach of electrochemical analysis is also important, as is the key opportunity presented by miniaturisation.

Weaknesses and Threats

The main weaknesses and threats presented are:-

- Instability due to biological nature of reagents,
- Operator-inexperience and, connected to this,
- Time required for completion.
- Competition with established technologies (such as assay test-kits) is also a potential problem.

The instability of biological reagents can be countered to some extent by the use of stabilisers such as Trehalose, Di-Glycerol Phosphate (DGP) or Hydroxyection (HydE) which are all commercially available. Optimisation of experimental conditions as well as storage and transport are also important factors.

Operator-inexperience can be countered with training and time. Time required for completion is a universal research problem and must be faced. Miniaturisation may circumvent competition, with the advantages of reaction-speed and smaller sample volumes required. The lower process-complexity of a sensor compared with the wash-steps of an assay may also be advantageous.

4.2 FUTURE WORK

The major problem encountered in the course of this project was the non-repeatability and variation in results due to the biological nature of the reagents. This may to some extent be countered by the use of non-biological reagents such as MIPs or through the use of stabilising materials. Due to limitations in the time available these options were not explored; however further research in this area seems well merited.

The aim of the project – to pursue novel, micro- and nano-scale devices – appears to be the correct focus. The magnetic bead assay format offers easy miniaturisation.

The use of HSV reading as an analytical tool also merits further investigation.

4.2.1 Clean-up and Pre-concentration

Sample cleanup and preparation is essential to eliminate matrix effects when analysing real samples. Molecularly Imprinted Polymers (MIPs) appear to be a very viable option as a cleanup and pre-concentration mechanism.

Sergeyeva *et al* (2003) manufactured a MIP membrane for atrazine which demonstrated high selectivity and binding capacity. MIPs show promise as a clean up/pre-concentration tool to reduce matrix effects. This could be envisaged originally as an SPE cartridge, and eventually as a much smaller "snap-in" component of a miniaturised system.

4.2.2 Supramagnetic Dynabeads^{TM} System

A system discussed during the course of the study, and which seemed promising, was one using supramagnetic dynabeads ^{TM} as a solid support for the antibodies (see Figure 4.3). This would permit signal buildup and separation of the bound analyte. The predicted protocol would be divided into two major steps:-

Immunoreaction:

    1. The magnetic beads (with immobilised antibody) are introduced.

    2. The beads are trapped by magnet.

    3. Sample addition (allows pre-concentration and interferent removal).

    4. Incubation/washing.

    5. Stop flow (allows product generation/signal amplification).

Measurement:

    1. Only the product contacts the electrode.

    2. Continuous flow, giving superior data to "stop" flow.

Advantages:

- Permits extraction of the beads and bound analyte from the sample.
- The draw-down of beads to the magnet will permit concentration of the analyte.
- Stopping the flow and allowing product build-up will increase the signal. Re-starting the flow while keeping the beads on the magnet

will mean that only the product is analysed and help to minimise fouling of the electrode surface.

- The magnetic beads can be flushed from the incubation module to waste, the incubation module can be re-used.

- The correct valving system will allow sequential multi-analyte detection on one electrode element.
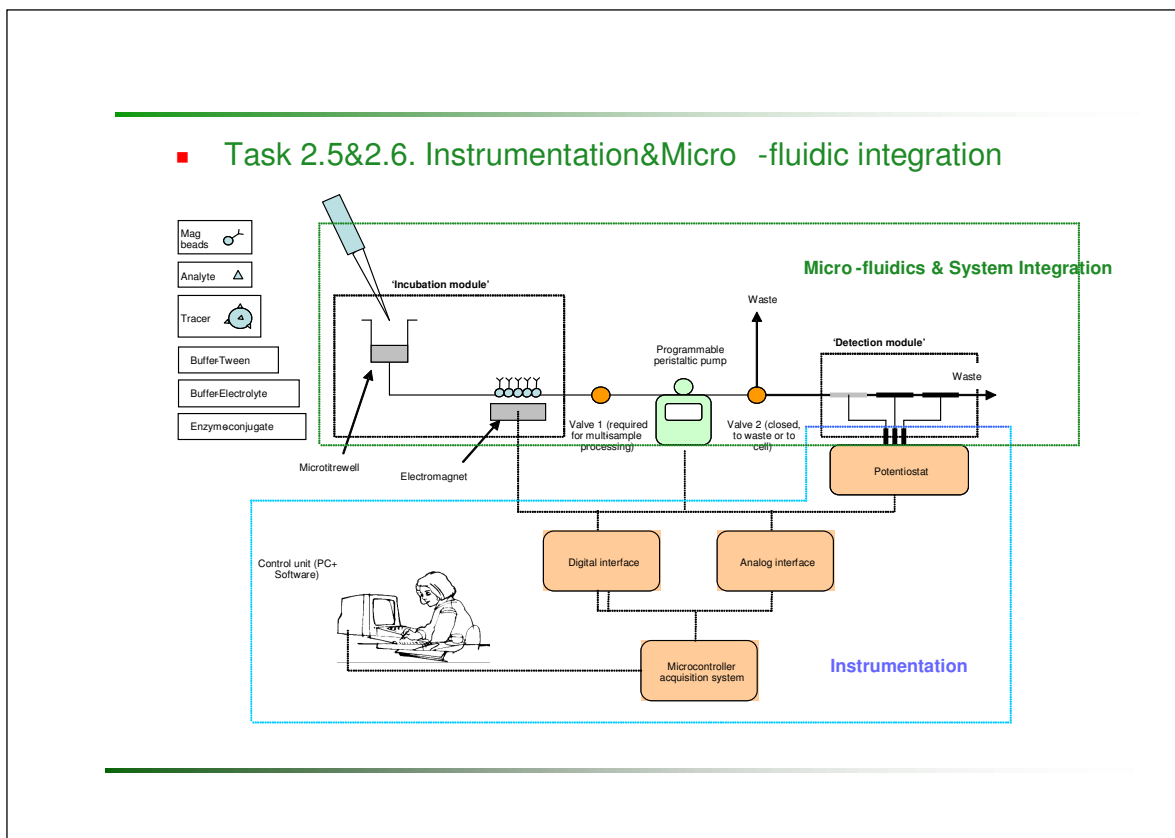
Figure 4.3: Diagram of possible magnetic bead system. This system, if developed, would allow multi-analyte analysis and is a step towards miniaturisation.

4.2.3 Further Possibilities

The potential of using carbon nanotubes to increase the surface area of the electrodes merits further investigation. This could be achieved by incorporating them into the carbon screen-printing ink with a view to use in a miniaturised system. This may also reduce reaction time helping to minimise signal contamination through oxidation of blanks.

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