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Variation In Immunoassay Components to Lower Sulphathiazole Detection Limits and Fluorescence Polarization Immunoassays for Potato Glycoalkaloids

BY

© **Carrie Ann Thomson**

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Variation Of Immunoassay Components To Lower Sulphathiazole Detection Limits And Fluorescence Polarization Immunoassays For Potato Glycoalkaloids** submitted by **Carrie Ann Thomson** in partial fulfillment of the requirements for the degree of **Doctor Of Philosophy** in **Food Chemistry**.

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ABSTRACT

This study first concentrated on decreasing the detection limit of an indirect competitive enzyme-linked immunosorbent assay (ELISA) for the detection of sulphathiazole (ST) through manipulation of some of the basic components of the test. A thorough evaluation of chromogenic substrates for horseradish peroxidase (HRP) led to the selection of 3,3',5,5'-tetramethylbenzidine (TMB). Use of TMB and a 2M sulphuric acid stopping reagent permitted the reduction of serum and coating conjugate levels, leading to the development of an assay with a "c" value (inflection point of the sigmoidal standard curve) of 4.4 parts per billion (ppb). Urease was tested as an alternative to HRP as the marker enzyme; conductimetric measurement of urease activity compared unfavourably with the HRP-TMB-sulphuric acid combination measured spectrophotometrically.

ST was covalently linked to HRP using the two step glutaraldehyde and periodate cleavage methods for use in direct competitive ELISAS. The periodate cleavage method produced a superior conjugate in terms of enzyme activity and performance in an ELISA. The direct competitive ELISA using a Protein A pre-coating step for antibody (Ab) immobilization was slightly more sensitive and faster and easier to perform than its indirect counterpart and resulted in "c" values in milk and honey as low as 39.5 ppb and 88.0 ppb, respectively.

In the final part of this study, fluorescence polarization immunoassays for toxic potato glycoalkaloids (GAs) were developed. Using a fluorescein-labelled solanidine derivative (AMF-SOL), all major GAs in commercial potato cultivars could be detected. Polyclonal serum was found to have the highest affinity for α-chaconine, while a monoclonal antibody was more sensitive to changes in α-solanine levels. Both polyclonal serum and a monoclonal Ab could detect all major *Solanum tuberosum* GAs (α-chaconine, α-solanine, and solanidine) at levels of 20-100 nM. The affinity constant of the polyclonal serum for AMF-SOL was estimated at 4.2×10^8 M^{-1} while that for the monoclonal antibody for AMF-SOL was 4.7×10^7 M^{-1}.

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LIST OF ABBREVIATIONS

"a" value	Upper asymptote of competitive enzyme immunoassay standard curve
Ab	Antibody
ABTS	2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid)
AFB	American Foulbrood
Ag	Antigen
4'-AMF	4'(Aminomethyl)fluorescein
AMF-SOL	4'-AMF-Solanidine conjugate
5-AS	5-Aminosalicylic acid
"b" value	Slope of competitive EIA standard curve
B_o	Upper asymptote of competitive EIA standard curve
B-M	Bratton-Marshall
BSA	Bovine Serum Albumin
bw	Body Weight
"c" value	Inflection point of competitive EIA standard curve
"d" value	Lower asymptote of competitive EIA standard curve
DAB	Diaminoazobenzol
DHFR	Dihydrofolate Reductase Inhibitor
DMAB	3-(Dimethylamino)benzoic acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
EMIT	Enzyme-Multiplied Immunoassay Technique
F_{ab}	Fraction (Antibody Binding) (of an Ab)
F_c	Fraction Crystallizable (of an Ab)
FI	Fluorescence Intensity
FP	Fluorescence Polarization
FPIA	Fluorescence Polarization Immunoassay
GA	Glycoalkaloid
GC	Gas Chromatography
GI	Gastrointestinal
HAT	Hypoxanthine-Aminopterin-Thymine
HGPRT	Hypoxanthine-Guanine Phosphoribosyl Transferase
HPLC	High Performance Liquid Chromatography

HRP ..... Horseradish Peroxidase

I_{50} ..... Analyte concentration required to reduce B_o by 50%

IA ..... Immunoassay

Ig ..... Immunoglobulin

K_{aff} ..... Affinity constant for Ab-Ag reaction

KLH ..... Keyhole Limpet Hemocyanin

K_m ..... Michaelis Constant

LPH ..... *Limulus polyphemus* Hemolymph

MAb ..... Monoclonal Antibody

MBTH ..... 3-Methyl-2-benzothiazolinone hydrazone

mP ..... Millipolarization Units

MRA ..... Microbial Receptor Assay

MRL ..... Maximum Residue Level

MS ..... Mass Spectrometry

MTX ..... Methotrexate

MW ..... Molecular Weight

NED ..... α-(Naphthyl)ethylenediamine

OPD ..... *o*-phenylenediamine

P ..... Degree of polarization

PG ..... Protein G

P_o ..... Limiting fluorescence polarization

ppb ..... Parts per billion

PA ..... Protein A

PAb ..... Polyclonal Antibody

PABA ..... *p*-Aminobenzoic acid

PBS ..... Phosphate-Buffered Saline

PBST ..... Phosphate-Buffered Saline containing Tween 20

PG ..... Purpurogallinzahl

PS ..... Polystyrene

Q ..... Rotational relaxation time

R ..... Gas constant

RIA ..... Radioimmunoassay

RP-HPLC ..... Reverse-Phase HPLC

RZ ..... Reinheitszahl

S_o ..... Ground level energy of a molecule

SA ..... Sulphonamide

SMR ..... Sulphamerazine
SMZ ..... Sulphamethazine
ST ..... Sulphathiazole
T ..... Absolute Temperature (K)
TGA ..... Total Glycoalkaloid
TMB ..... 3,3',5,5'-Tetramethylbenzidine
TMB-d ..... 3,3',5,5'-Tetramethylbenzidine dihydrochloride
V ..... Molar volume
η ..... Viscosity
τ ..... Fluorescence lifetime

CHAPTER 1. LITERATURE REVIEW

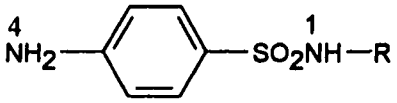
1.1 SULPHONAMIDES

Sulphonamides (SAs) are a family of N^1-substituted derivatives of sulphanilic acid (Figure 1.1) which possess antibiotic activity. SAs do not conform to the strict definition of antibiotics, however, since they are chemically synthesized; they are more appropriately referred to as chemotherapeutics (Hays, 1986). SAs differ from true antibiotics in that their action is largely bacteriostatic, and sulphonamide-initiated recovery from bacterial infections requires active phagocytosis (Anand, 1975).

Chemically, SAs display very limited solubility in cold water, and are sparingly soluble in ethanol or acetone. More generally, they are soluble in polar solvents, but relatively insoluble in non-polar solvents (Horwitz, 1981). Many SAs contain an acidic moiety (-NH-SO_2) and a basic *p*-amino group; thus, most are amphoteric compounds and display characteristic pK_a values (Bell and Roblin,1942; Horwitz, 1981). The nature of the R group determines the ionizability and hydrophobicity of the SA, and thus its biological activity (Anand, 1975; Bell and Roblin, 1942).

The antibiotic properties of SAs stem from their structural similarity to *p*-aminobenzoic acid (PABA), which is essential for the synthesis of folic acid (Anand, 1975; Richards *et al.*, 1991). The competitive blocking of PABA utilization in bacteria results in folic acid deficiency, and ultimately a thymineless death due to the prevention of incorporation of PABA into dihydropteroic acid (Richards *et al.*, 1991). SAs generally affect the synthetic processes of micro-organisms by inhibiting the biosynthesis of tetrahydrofolate, which is involved in 1-carbon transferase action (Anand, 1975). Furthermore, it has been suggested that SAs may cause partial inhibition of dihydrofolate reductase, in addition to their ability to compete with PABA (Richards *et al.*, 1991).

SAs are often used in combination with other antibacterials, especially dihydrofolate reductase inhibitors (DHFRs) such as trimethoprim, to become "potentiated SAs" due to the synergism between the two types of drugs (Anand, 1975; Rehm *et al.*, 1986; Richards *et al.*, 1991). The synergism arises from the ability of SAs and DHFRs to prevent folic acid synthesis, but at different sites in the biosynthetic pathway (Anand, 1975; Richards *et al.*, 1991). This synergistic relationship is also exploited in an attempt to avoid resistance development (Anand, 1975).



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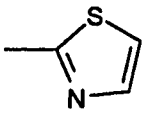

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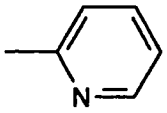

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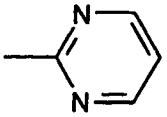

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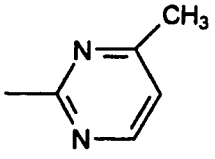
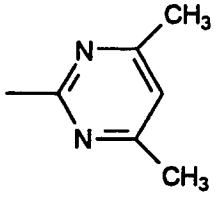


sulphamerazine



sulphamethazine

Figure 1.1 Structures of Some Common Sulphonamides

SAs exert bacteriostatic activity against many Gram-positive and Gram-negative cocci and bacilli, as well as mycobacteria. Only actively growing micro-organisms are affected, with the bacteriostatic action being preceded by a lag phase, during which cellular stores of PABA are metabolized (Anand, 1975).

The nature of the R group is principally responsible for the differences among SAs in their antibacterial activity, as well as their absorption, distribution, and excretion rates in animals to which they are administered (Anand, 1975). N^1-heterocyclic SAs are the most clinically useful, as they display a wider range of antibacterial activity, decreased toxicity, and more favourable pharmaceutical properties than, for example, N^1-alkyl SAs. Among the most active 5-membered heterocyclic SAs are the thiazoles, which, if methylated, possess increased hydrophobicity, which enhances their protein binding (and perhaps folate-enzyme binding) properties (Anand, 1975). Increasing the electronegativity of the $-NHSO_2$ group allows the SA to more closely resemble PABA, both geometrically and electronically, increasing its biological activity (Bell and Roblin, 1942).

With the discovery of their antibacterial activity in the 1930's, SAs gained widespread use in both human and veterinary medicine (Anand, 1975); however, they fell out of favour when penicillin became available in the early 1940's (Roudaut and Moretain, 1990). SAs underwent a resurgence in popularity when evidence of increasing bacterial resistance to antibiotics as well as adverse reactions to true antibiotics in humans became available (Schipper and Eveleth, 1959). Favourable properties of SAs include their low cost, broad spectrum of activity, and ease of administration (Anand, 1975; Gustafson, 1986; Roudaut and Moretain, 1990). SAs for veterinary use are also readily available without prescription (Agriculture Canada, 1982; Charm *et al.*, 1988), with a wide range of absorption and excretion rates (Anand, 1975).

SAs proved to be useful in the treatment of bacterial infections in mammals (including man) without affecting the growth of the host organism, since animals require pre-formed folic acid (Anand, 1975). Consequently, high doses have been used in the treatment of sick animals, while lower doses are used prophylactically, and sub-therapeutic doses improve feed efficiency and growth (Hays, 1986; Kaneene and Miller, 1992).

SAs, in particular sulphathiazole (ST), were introduced to apiculture in the 1940's for the prevention of American Foulbrood (AFB), a disease considered to be the number one enemy of beekeepers (Argauer, 1986; Eckert, 1947; Haseman, 1953). AFB, caused by *Bacillus larvae*, is a highly communicable disease which affects the early larval stage of honeybee development. Extensive economic losses are incurred

when a colony is infected and ultimately destroyed (Argauer, 1986; Gojmerac, 1980). ST is thought to have bacteriostatic or bactericidal action on *B. larvae*, which explains its effectiveness in controlling AFB (Reinhardt, 1947).

ST, either as its sodium salt or N^4-formyl derivative, is generally the SA of choice in combating AFB (Belliardo, 1981; Haseman, 1953; Horie *et al.*, 1992; Neidert *et al.*, 1986; Schwartz and Sherma, 1986). ST is easily administered dissolved in a 50% sucrose syrup or pollen supplement, or by dusting bees with a mixture of ST dispersed in powdered sucrose (Belliardo, 1981; Haseman, 1953; Landerkin and Katznelson, 1957). Conventional feeding rates are 1 g ST/3.785 L sucrose syrup (Argauer *et al.*, 1982). Through such feeding practices, there is potential for ST-contamination of the honey crop (Belliardo, 1981; Horie *et al.*, 1992). ST is extremely stable in honey (Argauer, 1986). Belliardo *et al.* (1981) demonstrated that 85% of ST added to honey at a level of 100 ppm remained after 11 months of storage at 4°C.

Despite the fact SAs are an inexpensive, effective, and easy means of controlling AFB, their use in both Canada and the US is illegal (Argauer, 1986; Argauer *et al.*, 1982; Neidert *et al.*, 1986; Schwartz and Sherma, 1986) because of the potential for contamination of honey. There is the possibility of surreptitious usage of ST in apiculture by unscrupulous beekeepers (Schwartz and Sherma, 1986). In addition, not all honey-producing nations of the world ban SA usage in honey (Diaz *et al.*, 1990; Horie *et al.*, 1992); hence, imported honey could be adulterated according to Canadian standards.

Similar to the possibility of honey pollution with ST from medicated bees, the tissues and body fluids of animals and the eggs of poultry treated with SAs can become contaminated since SAs are distributed throughout body tissues (Anand, 1975; Horwitz, 1981; Rehm *et al.*, 1986). In Canada, SAs were identified as a residue problem in 1976 (Agriculture Canada, 1982). To avoid the occurrence of violative residues in tissues and fluid milk destined for human consumption, withdrawal periods are established in order to allow the drug level to decrease to within acceptable levels by the processes of excretion and metabolism. Withdrawal times increase with increasing concentration (dose) and lipophilicity of the administered drug (Huber, 1986).

The principal causes of residues in foods include: (1) feed additive usage (and failure to keep medicated and non-medicated feeds segregated); (2) non-adherence to prescribed withdrawal times; (3) misuse of drugs (illegal, improper, or extra-label usage); and (4) treatment of bovine mastitis (the main route for SA contamination of milk) (Agarwal, 1992; Agriculture Canada, 1982; Kaneene and Miller, 1992). It should be noted that only certain SAs are approved for use within certain species (Agriculture

Canada, 1982; Horwitz, 1981); therefore, residues at any level of an unapproved SA are considered violative.

Testing for SA residues in meat, eggs, milk, and honey products is necessary for legal, processing, and public health reasons. As indicated, not all SAs are permitted for use in animal husbandry; therefore, tolerances have been established for various drugs in various products. Values in excess of these tolerances are considered in violation of regulations. Current Canadian regulations stipulate a maximum residue level (MRL) of 10 ppb sulphadimethoxine in milk (Canadian Gazette, 1991). While Canadian regulations specify zero tolerance for ST residues in honey (Neidert *et al.*, 1986), the enforcement level is set at 200 ppb.

From a food processing standpoint, SA residues in fluid milk are undesirable since they can interfere with starter cultures in the manufacture of dairy products (Brady and Katz, 1988; Rehm *et al.*, 1986; Schiffmann *et al.*, 1992). Schiffmann *et al.* (1992) demonstrated inhibition of *Lactococcus lactis* ssp. *cremoris* cultures by sulphamethoxine and sulphamethazine.

With the use of SAs, as with true antibiotics, there is concern that target micro-organisms may develop resistance, and that the transmission of these resistant strains to the human bacterial reservoir could lead to serious infections in humans (Van Poucke *et al.*, 1991). The development of antibiotic-resistant bacteria in humans is usually ascribed to the administration of antibiotics to people. It is widely believed that such resistance is not likely to be caused by exposure to low levels found as residues in foods (DuPont and Steele, 1987; Katz, 1983). However, it has been shown that resistance can develop in humans exposed to an antibiotic (nourseothricin) which had only been used in veterinary medicine (Hummel *et al.*, 1986). The main concern regarding the sub-therapeutic use of antibiotics in animal husbandry is the potential for the selection of multi-resistant organisms (Levy, 1987).

There is also some question regarding the safety of SAs. SAs have been shown to cause thyroid hyperplasia (goitre) in rodents, and sulphamethoxazole has induced thyroid carcinoma in the rat (Woodward, 1991, 1992). Rats, along with dogs, are highly sensitive to interference with thyroid hormone synthesis by SAs (Horwitz, 1981; Rehm *et al.*, 1986); however, thyroid dysfunction due to SAs has not been demonstrated in humans (Rehm *et al.*, 1986). Thus, the debate as to whether SAs act as carcinogens in humans continues (Shaw *et al.*, 1990; Woodward, 1991, 1992). Abnormal mitogenic activity in tissue culture has been linked to certain SAs (sulphadiazine, sulphamethazine, and sulphaphenazole), indicating potential mutagenicity (Badr, 1982).

Possibly the most contentious issue surrounding the question of the safety of SA residues is their alleged allergenicity. One of the commonly cited reasons for the avoidance of SA residues in foods is the possibility of idiosyncratic reactions in ultrasensitive consumers (Brady and Katz, 1988; Cribb *et al.*, 1991). Reports on the proportion of the general population allergic to SAs range from 1% (Cribb *et al.*, 1991) up to 5% (Rieder *et al.*, 1992), with a mean value of 3.5% often being quoted (Charm *et al.*, 1988; Rehm *et al.*, 1986). Humans display variable hypersensitivities to SAs (Huber, 1986) due to differences in the capacity of cells to detoxify the reactive products of the oxidative metabolism of SAs (Rieder *et al.*, 1992). The occurrence of adverse reactions is quite unpredictable and symptoms can be severe (Cribb *et al.*, 1991).

The exposure levels of humans to SAs when these residues occur in foods are quite low, and are generally below the minimum threshold necessary to produce acute allergic reactions (Collins-Thompson *et al.*, 1988; Rehm *et al.*, 1986). However, SAs are among the drugs considered to have a high potential for sensitizing susceptible individuals (Huber, 1986), and the triggering of an allergic reaction is not always related to dose (Rehm *et al.*, 1986). It is unknown whether a SA-hypersensitized individual who ingests an occult SA residue in food can develop an allergic reaction (Huber, 1986), or if persistent exposure to low levels can lead to toxicity problems (Collins-Thompson *et al.*, 1988). Hence, the potential for health problems arising from even low levels of SAs in foods cannot be ignored.

In order to control the exposure of consumers to excessive levels of SAs in their diets, it is necessary to analyze food products for SAs when they may be derived from animals treated at some point with SAs. It has been estimated that up to 75% of all US dairy cattle receive antimicrobial drugs at some point in their lives (Kaneene and Miller, 1992), and 60-80% of all animal protein consumed in the US originates from animals fed medicated feeds at some time during their lifetime (Hays, 1986). This is not surprising, considering 40-50% of the antibiotics manufactured in North America are used in animal feeds (Friend and Shahani, 1983; Levy, 1987).

Parks (1982) suggested that screening methods for SAs must be relatively rapid, sensitive at the violative level of the drug in question, reproducible over a wide range of concentrations, and be free of false positives. It should also tentatively identify the contaminating drug. As with any method of analysis, it is also desirable that the method be economical, and safe for the experimenter.

Analysis methods for SAs may be grouped as follows: (1) colorimetric; (2) chromatographic; (3) polarographic; (4) microbiological; (5) electrochemical; and (6) immunological (Table 1.1).

The first method for quantifying SAs appeared in 1939 and was developed to measure sulphanilamide in the blood and urine of patients receiving SA therapy (Bratton and Marshall, 1939). This "Bratton-Marshall" (B-M) colorimetric method is useful for determining diazotizable primary aromatic amines, which forms the basis of the test. The diazotized *p*-amino group is coupled to α-(naphthyl)ethylenediamine (NED) to produce a highly coloured stable azo product that can be measured spectrophotometrically in the visible range (Bratton and Marshall, 1939; Horwitz, 1981; Rehm *et al.*, 1986). Despite the ability of the B-M test to detect and quantify SAs, it is not without its limitations. First, the coupling agent (NED) is carcinogenic, as well as unstable (Schwartz, 1982). Since the molar absorptivities of the resultant azo products from all SAs are very similar, with absorption maxima at about 545 nm, identification of individual SAs is precluded (Horwitz, 1981; Rehm *et al.*, 1986). Parent drugs with free *p*-amino groups are readily available for reaction; N^4-acetylated metabolites may require rigorous hydrolysis before they can be detected (Horwitz, 1981). Since the major metabolic products in mammals are the N^4-acetylated derivatives (Anand, 1975; Horwitz, 1981; Rehm *et al.*, 1986), use of the B-M method could result in a significant underestimation of total SA levels. Furthermore, the limit of detection is 1 ppm (Horwitz, 1981).

The Tishler method (Tishler *et al.*, 1968) was developed for the isolation of SAs from tissues and milk such that they could be quantified using the B-M reaction. The elaborate extraction scheme, designed to be selective for SAs, serves to complicate the analysis, leading to lengthy analyses, often with poor recoveries (Horwitz, 1981; Tishler *et al.*, 1968). Since detection is based on the B-M reaction, the Tishler method is also plagued by the drawbacks noted above. Generally, it can be said that colorimetric methods lack specificity when the SAs occur in a complex matrix (Belliardo, 1981).

Chromatographic methods offer better selectivity than colorimetric methods, but recoveries can be low and reproducibility poor (Horwitz, 1981). HPLC and GC methods require extraction of SAs and generally extensive sample clean-up, which is laborious and time-consuming, resulting in low through-put rates (Agarwal, 1992; Horwitz, 1981). Furthermore, equipment is costly, and considerable technical expertise is required to perform the analyses.

Table 1.1 Methods for Analysis of Sulphonamides

TYPE OF METHOD	SELECTED LITERATURE REFERENCES
Colorimetric	Bratton and Marshall, 1939; Mohamed *et al.*, 1991; Schwartz, 1982; Tishler *et al.*, 1968
Chromatographic	Abián *et al.*, 1993; Agarwal, 1992; Argauer *et al.*, 1982; Barry and MacEachern, 1983; Horie *et al.*, 1992; Jürgens, 1982; Larocque *et al.*, 1990; Parks, 1982; Roudaut and Moretain, 1990; Takeda and Akiyama, 1991, 1992; Van Poucke *et al.*, 1991; Weber and Smedley, 1989; Zomer *et al.*, 1992
Electrochemical	Ng and Wong, 1993
Microbiological	Charm and Chi, 1988; Hawronskyj *et al.*, 1993; Read *et al.*, 1971; Vermunt *et al.*, 1993
Immunological	Assil *et al.*, 1992; Dixon-Holland and Katz, 1988, 1989, 1991; Fleeker and Lovett, 1985; Hoffmeister *et al.*, 1991; McCaughey *et al.*, 1990; Sheth and Sporns, 1990, 1991; Sheth *et al.*, 1990; Singh *et al.*, 1989;

Most HPLC systems for SAs cited in the literature operate in the reverse-phase mode, and rely on UV detection (Horwitz, 1981; Takeda and Akiyama, 1991, 1992). Fluorescamine-derivatization of SAs enables fluorescence detection to be used; however, it complicates the analysis, and only the conjugated, fluorescent SAs are detected (Takeda and Akiyama, 1991, 1992; Zomer *et al.*, 1992). However, HPLC methods can often allow for discrimination between the parent drug and metabolites (Rehm *et al.*, 1986).

GC methods, while sensitive and specific, are not widely applicable to the analysis of SAs (Agarwal, 1992). SAs have extremely low vapour pressures, and the preparation of suitably volatile derivatives is difficult (Horwitz, 1981). Samples for GC analysis must also be extracted and subjected to sample clean-up prior to analysis (Agarwal, 1992). GC/MS systems can separate, quantitate, and identify individual SAs, but are complicated and cumbersome (Zomer *et al.*, 1992).

TLC and paper chromatography can be used to separate SAs, and generally rely on colorimetric, fluorescence or fluorescence quenching detection. Extensive clean-up procedures are still required, and most methods are only qualitative, rendering them suitable only for screening (Agarwal, 1992; Horwitz, 1981).

Microbiological assays can be either of the inhibition or receptor-binding type (Charm and Chi, 1988; Katz, 1986; Rehm *et al.*, 1986). In inhibition assays, the presence of a SA is indicated when inhibition of a sensitive test micro-organism is observed (Rehm *et al.*, 1986). Microbial inhibition tests carry the disadvantage that they quantify only the biologically active form of the drug, and do not measure non-reactive metabolites. Such methods also tend to be slow and laborious (Katz, 1986).

The microbial receptor assay (MRA), more commonly known as the "Charm Test", relies on the competition between ^{3}H-sulphamethazine (SMZ) and SMZ in a test sample for binding sites on a microbial receptor specific for the *p*-amino group of SAs. The limits of detection are reportedly 10 ppb (as SMZ equivalents); however, the method will only measure SAs with an unblocked *p*-amino group (Charm and Chi, 1988). As earlier indicated, the major metabolic pathway of SAs involves N^{4}-acetylation (Rehm *et al.*, 1986); these metabolites would not be detected by the Charm Test. Thus, an hydrolysis step would be required to free the parent drug. Nonetheless, the test is rapid. (Charm and Chi, 1988).

Zomer *et al.* (1992) combined HPLC separation of SAs with detection using an MRA. This method allowed for positive identification and quantitation of SAs, and hence increased the selectivity of MRA and sensitivity of HPLC as compared to their singular usage.

A variety of immunologically-based tests for SAs has been cited in the literature for the analysis of tissues, milk, and animal feeds (Table 1.1). These tests rely on the specificity of an Ab for the Ag which stimulated its production. These tests are therefore quite specific, and depending on conjugation methods used for the immunogen and competitor conjugates, can quantify both the active metabolite/parent drug, and microbiologically inactive species. Immunoassays using enzymes as "detectors" are also generally quite safe. Enzyme-linked immunosorbent assays (ELISAs) are covered in Section 1.5 of this manuscript.

1.2 PEROXIDASE

Horseradish peroxidase (HRP) (Hydrogen-peroxide oxidoreductase, EC 1.11.1.7) is the most widely used marker enzyme in EIA procedures (Gosling, 1990; Porstmann and Kiesigg, 1992; Tijssen, 1985). The reasons for this preference for HRP include the following: (1) it is less costly than alternative enzymes, such as alkaline phosphatase and ß-galactosidase (Tijssen, 1985); (2) it is of plant origin, making it especially suitable for use in the assay of components in mammalian systems (Laman *et al.*, 1991); (3) it is easily conjugated to antigen, hapten, or antibody (Tijssen, 1985); (4) it accommodates a wide range of hydrogen donors in its reaction mechanism; (5) its activity is readily detectable, as it produces coloured products with certain compounds; (6) it exhibits excellent stability to extremes of temperature and pH; (7) it is readily available in highly purified forms; (8) it has a high substrate turnover number; and (9) it has a relatively low MW and hence does not cause steric hindrance (Gosling, 1990).

HRP is a hemoprotein which catalyzes the transfer of hydrogen from hydrogen donors (AH_2) to hydrogen peroxide (Dunford, 1991):

$$H_2O_2 + 2AH_2 \xrightarrow{\text{PEROXIDASE}} 2H_2O + \bullet AH \text{ (+ polymerized products)}$$

This is an oversimplification of the reaction mechanism for HRP, which actually involves at least three distinguishable steps, each with its own velocity constant (Dunford, 1991):

$$\text{HRP} + H_2O_2 \xrightarrow{k1} \text{COMPOUND I} + H_2O \quad (1)$$

$$\text{COMPOUND I} + AH_2 \xrightarrow{k2} \text{COMPOUND II} + \bullet AH \quad (2)$$

$$\text{COMPOUND II} + AH_2 \xrightarrow{k3} \text{HRP} + \bullet AH + H_2O \quad (3)$$

Step 3 is the rate-limiting step since *k2* is usually 40-100 times larger than *k3* (Marklund *et al.*, 1974). The generation of oxidized forms of the hydrogen donor allows for spectrophotometric analysis of enzyme activity (Tijssen, 1985).

Peroxidases are widely distributed in nature, particularly in plants. While they appear to catalyze the same reaction, they differ markedly in their physico-chemical and kinetic properties (Shannon *et al.*, 1966). Electrophoretic and chromatographic analyses of HRP have demonstrated several isozymes (Marklund *et al.*, 1974). Peroxidases may be classified into three main groups on the basis of their isoelectric pH (pI) values:

(1) acidic (pI<4); (2) peroxidases with pI values near neutrality; and (3) basic (pI>11), (Marklund *et al.*, 1974 ;Tijssen, 1985). The "C" isozyme of HRP predominates in EIA techniques (Tijssen, 1985).

HRP consists of 308 amino acids linked to a planar ferriprotoporphyrin IX ring. Two calcium ions and an average carbohydrate content of 18% result in an average MW for HRP of 42,100 (Dunford, 1991). At the centre of the porphyrin ring is an Fe^{3+} which is essential for enzyme activity (van Huystee *et al.*, 1992). The calcium ions appear to be involved in conferring thermal stability to the enzyme, although they are not essential for activity. Since the removal of calcium ions does not abolish all enzyme activity, it appears that they may assist in stabilizing the structure of the enzyme (van Huystee *et al.*, 1992), as well as contribute to its thermal stability (Dunford and Stillman, 1976).

The initial step in the reaction of peroxidases is their interaction with hydrogen peroxide or related compounds to form Compound I (HRP-I), the key catalytic intermediate, which is a derivative of native HRP in which the active site of the enzyme is oxidized (Davies *et al.*, 1976; Jones and Dunford, 1977). Compound I is reduced to the initial state of HRP by two successive univalent interactions with hydrogen donors. Compound II (HRP-II) is the one-electron oxidized intermediate form of the enzyme (Tijssen, 1985). The oxidized forms of hydrogen donors polymerize to give intense colour, which makes HRP a useful marker in visual histochemical and EIA techniques (Masulam, 1978).

The classical measure of HRP purity is the RZ (Reinheitszahl) number, which is the ratio of the absorbance values at 403 nm and 275 nm, which measures haemin content relative to aromatic amino acid content (Dunford, 1991). However, this is not a true measure of purity, as different isozymes display different RZ numbers, even in pure form. While commercially "pure" HRP generally has an RZ of at least 3.0, such preparations may contain many isozymes, with the major component being isozyme C, which has the highest activity and an RZ of about 3.5 (Tijssen, 1985). In addition, the RZ number refers only to purity as a protein, and not as an enzyme (Whitaker, 1972). Thus, published RZ values can be used only as a guideline in judging the purity of a preparation.

Classically, the reaction of HRP has been indirectly determined by measuring the appearance of the oxidized form of the hydrogen donor (Tijssen, 1985). The traditional test for HRP activity is the formation of purpurogallin from pyrogallol, developed in the early 1900's by Willstätter and Stoll. Using this method, the activity of the HRP preparation is expressed as the purpurogallin number, PZ (Purpurogallinzahl),

which is the number of mg purpurogallin formed by 1 mg enzyme under standard conditions. The method suffers from poor reproducibility, since it was developed before a more precise mechanism for HRP had been elucidated, and hence without knowledge of some of the rate-limiting steps, such as the combination of HRP-II with AH_2 (Chance and Maehly, 1955; Whitaker, 1972). Also, purpurogallin continues to be formed after the disappearance of HRP-II, which is therefore not reflective of the enzyme mechanism (Tijssen, 1985). Hence, this method has been largely abandoned.

A more common means of assessing HRP activity is the guaiacol assay, which measures the rate at which a coloured reaction product (tetraguaiacol) is formed by HRP in the presence of hydrogen peroxide (Chance and Maehly, 1955). This method suffers from two disadvantages. First, tetraguaiacol fades quickly after its formation (Tijssen, 1985). Second, it has been found that the "freshness" of the guaiacol solution used in the assay can affect results (Mäkinen and Tenovuo, 1982; Taurog *et al.*, 1992). Thus, the guaiacol test is not recommended for quantitative analyses. This test is used in the food industry to qualitatively test for peroxidase activity to determine the efficacy of blanching treatments (Kramer and Twigg, 1976).

There is a wide variety of hydrogen donors available for reaction with HRP to form spectrophotometrically measurable, coloured products. With reference to EIA techniques, several desirable characteristics of a hydrogen donor for use with HRP may be listed: (1) it should exhibit negligible oxidation rate in the absence of HRP; (2) it should have a much slower reaction rate between the hydrogen donor and HRP-I than between HRP and hydrogen peroxide; (3) it should display little pH dependence of the oxidation reactions; (4) the oxidized donor should have a stable physical property in a single form which can be readily quantified; (5) both the reduced and oxidized forms of the donor should be soluble in the reagents used in EIA; (6) the donor should not be toxic (Tijssen, 1985); (7) the enzyme (HRP-I) should have a high turnover number with the hydrogen donor; (8) the hydrogen donor should be readily available and inexpensive; (9) the oxidized form of the donor should have a high extinction coefficient, preferably with a broad absorption maximum; and (10) both the oxidized and reduced forms of the donor should be photostable (Porstmann and Porstmann, 1988).

Before discussing the various hydrogen donors available for use with HRP in EIA methods, it would be prudent to examine some of the inherent sensitivities of HRP which may influence its reaction rate and hence the ultimate results of any test in which it is employed. The true substrate of HRP is hydrogen peroxide, which, when in excess, becomes an inhibitor of the enzyme, leading to the formation of HRP-III or IV, which possess no catalytic activity (Dunford, 1991). Therefore, optimal results are

obtained only within defined concentration limits for hydrogen peroxide (Tijssen, 1985). The optimum concentration of hydrogen peroxide differs widely with the chromogen used, and thus the rate of formation of HRP-III can be influenced by the hydrogen donor (Porstmann and Porstmann, 1988). In addition, the optimum level of hydrogen peroxide for immobilized HRP (e.g., on a polystyrene microtitre plate) may differ from that of the free enzyme, with the immobilized enzyme suffering more from excess hydrogen peroxide than its soluble counterpart (Tijssen, 1985). The inclusion of Tween 20 in buffer solutions for HRP is necessary as HRP is inactivated in the presence of polystyrene in the absence of this detergent (Berkowitz and Webert, 1981). Detergents such as Tween 20 also serve to delay thermal inactivation of HRP, but this effect is dependent upon the nature of the hydrogen donor used. Sodium azide is also inhibitory to HRP and should therefore be excluded from EIA buffer systems. Furthermore, different HRP isozymes exhibit different activities toward different hydrogen donors. Cyanide or sulphide are reversible inhibitors of HRP at concentrations of 10^{-6} to 10^{-5} M, while fluoride, azide, or hydroxylamine inhibit at concentrations above 10^{-3} M (Tijssen, 1985).

A list of some hydrogen donors available for use in measuring HRP activity (and, therefore, quantitation in EIA methods) is given in Table 1.2, and their structures are presented in Figure 1.2. Several of these compounds, while they allow excellent quantitation of the immunological reaction, put the user at risk due to their carcinogenic, mutagenic, or irritating natures (Tijssen, 1985). It is desirable to use a hydrogen donor which affords an intensely coloured product, and is non-toxic. However, the risk to the experimenter can be reduced by using substrates in the tablet form (Sigma ImmuNotes, 1991); therefore, lack of toxicity may not be the only suitable criterion for evaluation of hydrogen donors for EIA quantitation.

Ortho-phenylenediamine (OPD) is a commonly used chromogenic substrate in EIA techniques. In its oxidized form it is yellow to orange in colour. It can be measured at low concentrations at 445 or 492 nm, depending on pH. In addition to possibly being mutagenic, OPD is photosensitive, developing colour in the absence of enzyme upon exposure to light (Tijssen, 1985). OPD is oxidized by HRP and condenses to 2,2'-diamino-azobenzol (2,2'-DAB), as shown in Figure 1.3. The end product, 2,2'-DAB shows an absorbance maximum at 450 nm at a pH of about 5, while its absorbance maximum shifts to about 490 nm (with a concomitant increase in extinction coefficient) at a pH of about 1. The affinity of HRP for OPD is also pH dependent (Porstmann and Porstmann, 1988).

Table 1.2 Toxic Effects of Common Hydrogen Donors for Horseradish Peroxidase[1]

HYDROGEN DONOR	KNOWN TOXIC EFFECTS
dicarboxidine	none[2]
o-dianisidine	irritant, carcinogen
m-phenylenediamine	carcinogen
o-phenylenediamine	irritant (eyes), mutagen
ABTS	mutagen[3]; safe[4] (?)
5-Aminosalicylic acid	none
DMAB/MBTH	none
TMB	none[5]

[1]Adapted from Tijssen, 1985
[2]Paul *et al.*, 1982
[3]Schall *et al.*, 1978; Hosoda *et al.*, 1986
[4]Sigma ImmuNotes, 1991
[5]Bos *et al.*, 1981; Holland *et al.*, 1974; Liem *et al.*, 1979
ABTS, 2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid); DMAB, 3-(Dimethylamino)benzoic acid; MBTH, 3-Methyl-2-benzothiazolinone hydrazone; TMB, 3,3',5,5'-Tetramethylbenzidine

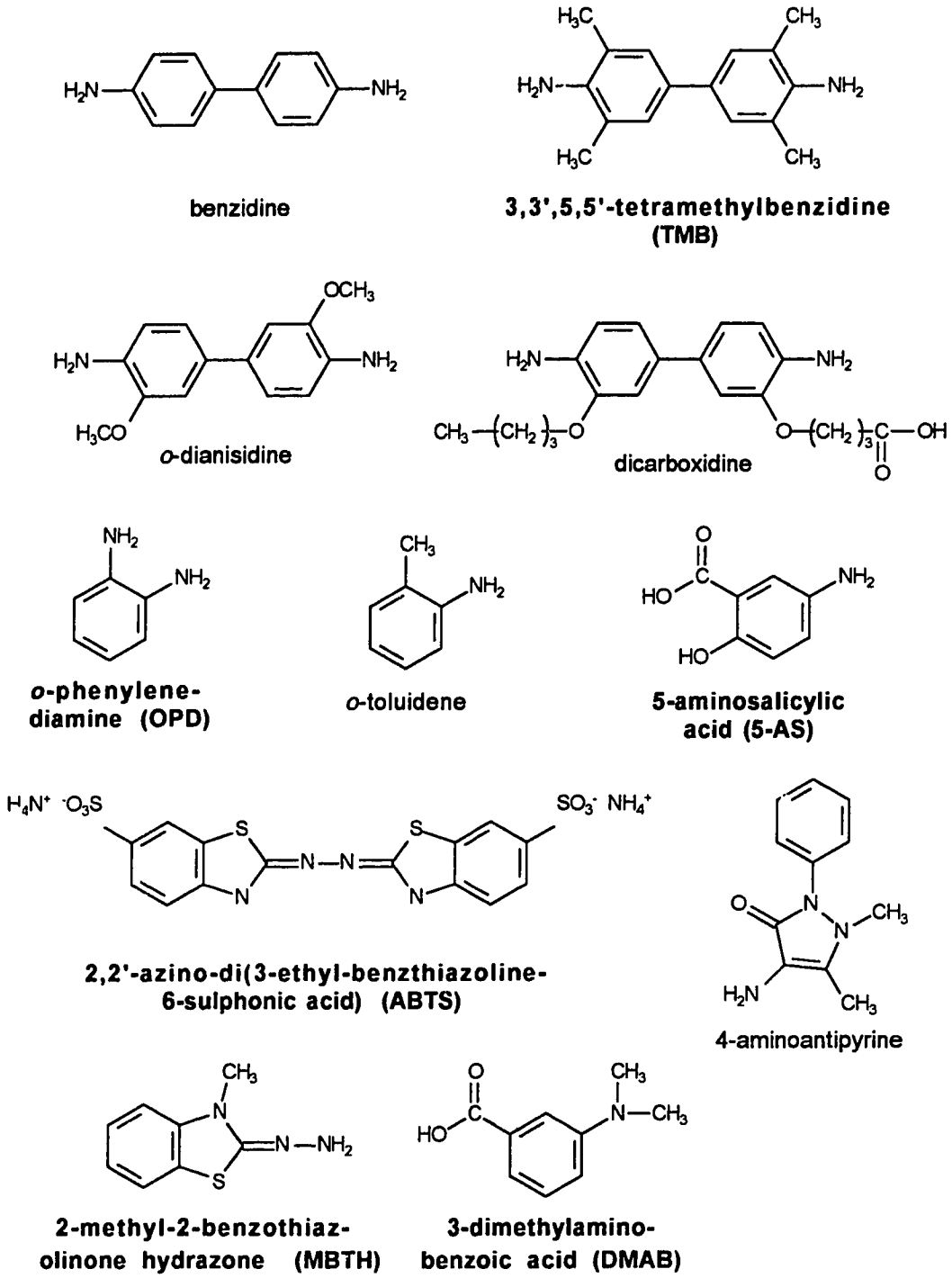


Figure 1.2 Structures of Common Hydrogen Donors for Horseradish Peroxidase

Structures with names in bold were evaluated in the current study.

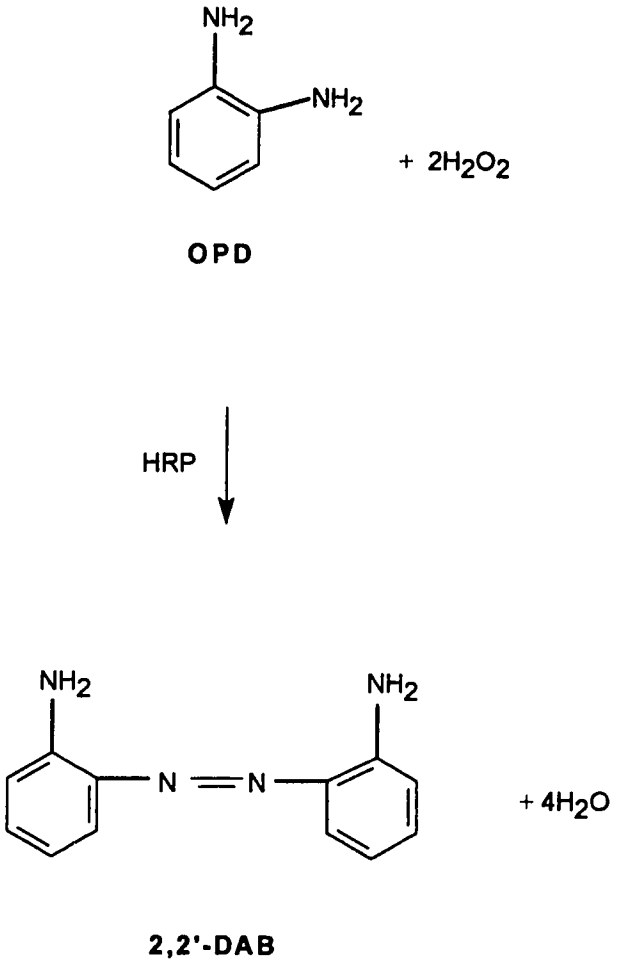


Figure 1.3 Reaction of HRP with Hydrogen Peroxide and OPD

(After Porstmann and Porstmann, 1988)

2,2'-Azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS) is a heterocyclic azine which allows for highly sensitive detection of HRP activity in ELISAs (Porstmann and Porstmann, 1988). The radical cation formed tends to disproportionate, as shown in Figure 1.4; however, this disproportionation can be mitigated by using approximately a 100-fold excess of azine. The radical cation, which is green in colour, is particularly suitable for the visual assessment of EIA results. The affinity of HRP to hydrogen peroxide and ABTS is pH-dependent. Although ABTS and its reaction products are purportedly safe, and the products intensely coloured, it is difficult to apply a stopping solution and obtain a stable reaction product, since many of the available solutions (e.g., sodium cyanide, ethylenediaminetetraacetic acid/fluoride) are toxic and/or promote disproportionation and a decrease in the reaction colour. Possibly the best solution is to use 5 mM azide to inhibit the enzyme, without encouraging disproportionation of the radical cation to a less intensely coloured compound (Porstmann and Porstmann, 1988).

5-Aminosalicylic acid (5-AS) is a non-hazardous hydrogen donor for HRP, giving a brown colour in its oxidized form (λ_{max}=450 nm) (Sigma ImmuNotes, 1991). One drawback of this substrate is that commercial preparations are not pure; however, it is relatively easily purified to a reportedly readily soluble and stable form, which exhibits low background values (Ellens and Gielkens, 1980; Tijssen, 1985). While extinction coefficients are higher for OPD than 5-AS, background absorbance levels are considerably lower when 5-AS was employed (Ellens and Gielkens, 1980). The poor colour yield obtained with 5-AS, however, does not allow for high sensitivity in EIA methods (Bos *et al.*, 1981).

The oxidative coupiing of 3-methyl-2-benzothiazolinone hydrazone (MBTH) with 3-dimethylaminobenzoic acid (DMAB) is also a safe alternative colorimetric system for measuring HRP activity (Ngo and Lenhoff, 1980). In the presence of hydrogen peroxide and HRP, MBTH acts as the hydrogen donor. The oxidized form of MBTH then reacts with DMAB to form a deep purple cationic indamine dye, with an absorption maximum at 590 nm (Figure 1.5) (Ngo and Lenhoff, 1980; Tijssen, 1985). The original method was favourable in that it afforded high detectability of HRP and lacked carcinogenicity; however, background absorbances tended to be high, the coloured product was photosensitive (Geoghegan, 1985; Tijssen, 1985), and sodium cyanide could not be used effectively to halt the reaction at the neutral pH used in the original assay of Ngo and Lenhoff (Geoghegan *et al.*, 1983). The use of a citric acid buffer was found by Porstmann and Porstmann (1988) to be of limited use in reducing background levels and sensitivity to light, despite the claims of Geoghegan *et al.* (1983).

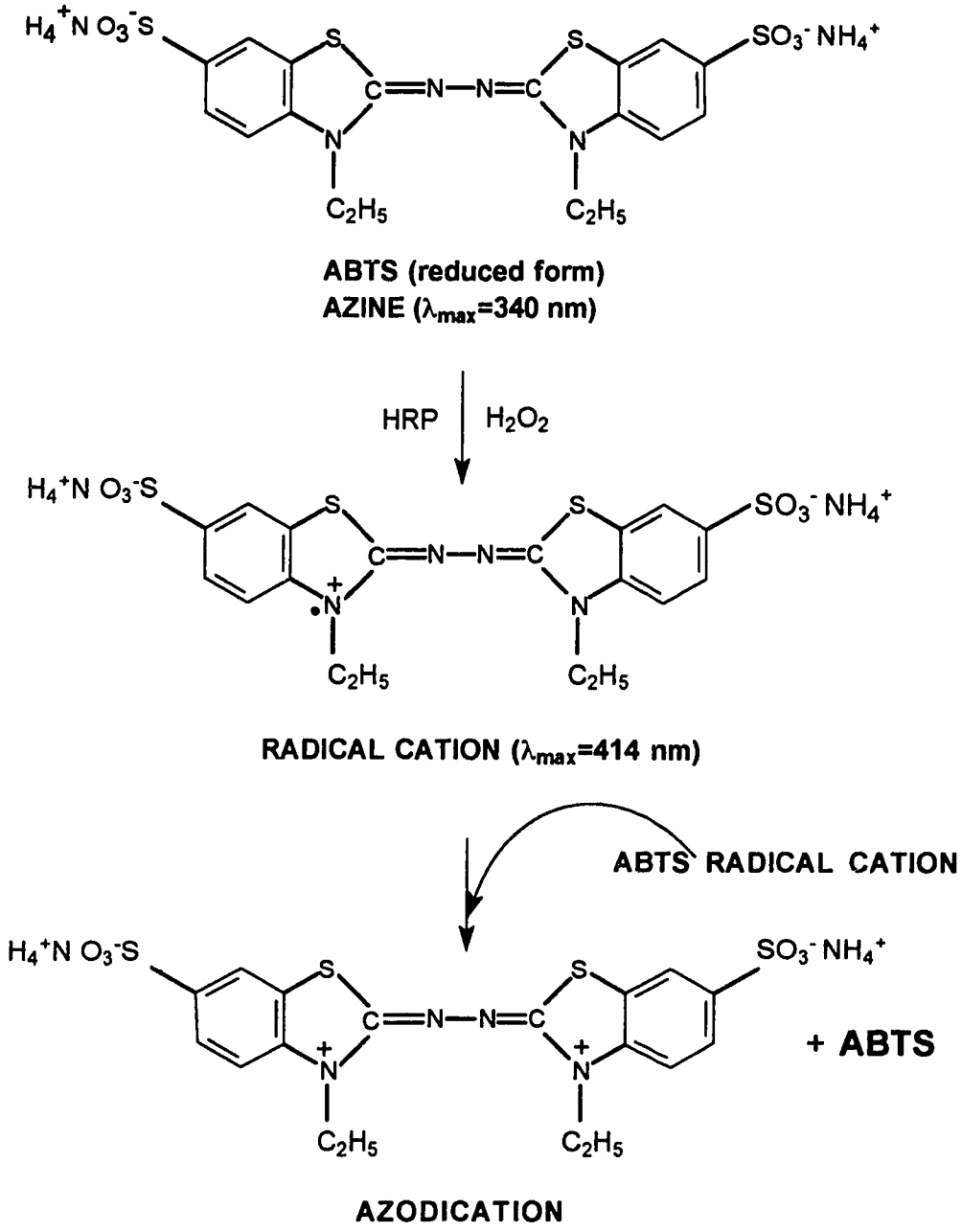
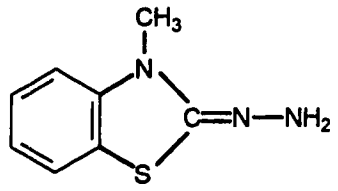
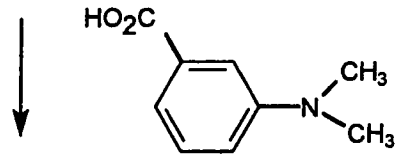


Figure 1.4 Reaction of HRP with Hydrogen Peroxide and ABTS

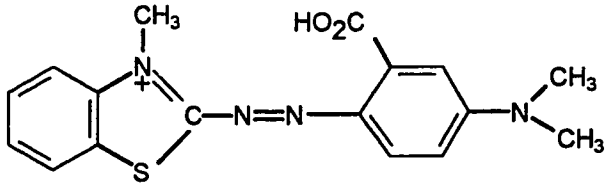
(After Porstmann and Porstmann, 1988)



MBTH



(DMAB)



INDAMINE DYE (λ_{max}=590 nm)

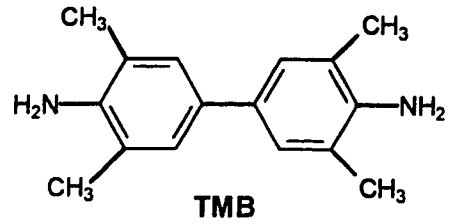
Figure 1.5 Reaction of HRP with Hydrogen Peroxide, MBTH AND DMAB

(After Ngo and Lenhoff, 1980)

The development and use of 3,3',5,5'-tetramethylbenzidine (TMB) in immunoassay techniques grew out of a need to replace benzidine in measuring haemoglobin in urine and plasma (Bos *et al.*, 1981; Holland *et al.*, 1974; Liem *et al.*, 1979) and the knowledge that blood shows considerable peroxidase-like activity and gives highly coloured products with certain substrates in the presence of hydrogen peroxide (Holland *et al.*, 1974). Although benzidine gives both a sensitive and specific test both for haemoglobin and HRP, it is carcinogenic, with its carcinogenicity purportedly stemming from its ability to become *o*-hydroxylated. Such *o*-hydroxylated derivatives of aromatic amines are suspected to have a role in carcinogenesis (Bos *et al.*, 1981; Holland *et al.*, 1974). While TMB is a structural analogue to benzidine, its extensive methylation at ortho positions precludes the possibility for hydroxylation, and hence renders it and its metabolites non-carcinogenic (Bos *et al.*, 1981; Catty and Raykundalia, 1989).

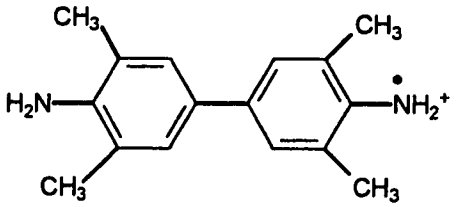
TMB in the presence of excess hydrogen peroxide and small quantities of HRP is oxidized to a radical cation which is blue in colour. Blue-coloured products are especially suitable for the visual assessments of EIA results, as along with green and red, blue is a colour to which the human eye is particularly sensitive (Porstmann and Porstmann, 1988). Two major absorption peaks (370 nm and 655 nm) and a relatively small one at 450 nm are observed in the reaction of HRP with hydrogen peroxide and TMB (Figure 1.6) (Bos *et al.*, 1981; Madersbacher and Berger, 1991). It has been shown that a linear relationship exists between the concentration of HRP and the absorbance value at 655 nm (Bos *et al.*, 1981).

The reaction mechanism described by Gallati and Pracht (1985) indicates that a blue radical cation is formed in the presence of excess TMB and hydrogen peroxide with small quantities of HRP. The oxidation of TMB is highly pH-dependent, and occurs optimally at pH 2.5 to 4.0, and more slowly at pH 5.0 (Mesulam, 1978). If the pH of the medium is lowered, the blue radical cation is converted to a yellow diphenoquinone, which has a higher extinction coefficient at 450 nm. This conversion is complete at pH 1.0 and can be effected by the addition of sulphuric acid. The addition of sulphuric acid effectively stops the enzyme reaction, while the resulting yellow colour remains stable for at least 90 min (Porstmann and Porstmann, 1988).



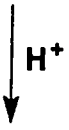
TMB

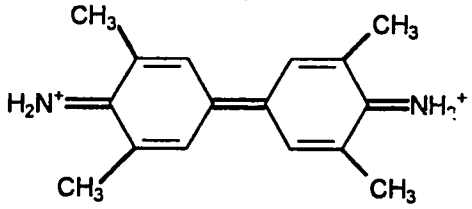
(λ_{max} = 210 nm, 286 nm)



TMB RADICAL CATION

(BLUE; λ_{max} = 210 nm, 370 nm, 655 nm)





3,3',5,5'-TETRAMETHYL-1,1'-DIPHENOQUINONE-

4,4'-DIIMONIUMION (λ_{max} = 210 nm, 450 nm)

Figure 1.6 Reaction of HRP with Hydrogen Peroxide and TMB

(After Gallati and Pracht, 1985)

The insolubility of TMB in aqueous solutions can be overcome by first dissolving it in aprotic solvents such as dioxane, dimethyl sulphoxide, or dimethylformamide (Bos *et al.*, 1981), or by using the dihydrochloride derivative, which dissolves slowly in water or citrate buffer (Liem *et al.*, 1979). Colour development in TMB solutions due to oxidation can be minimized by protecting them from oxygen and by storing them at 4°C, and by using freshly-prepared solutions (Liem *et al.*, 1979). The solutions are photostable (Sigma ImmuNotes, 1991). TMB can be purchased as a stable, ready-to-use reagent (Transgenic Sciences, Inc., Worcester, MA); however, its high cost is largely prohibitive.

As previously indicated, there is a colour change upon acidification of the blue oxidation product of TMB. The use of sulphuric acid has been shown to stop the enzyme reaction and result in a stable yellow-coloured product. In addition, the absorption values of the product when sulphuric acid is added increase two to four times as compared to those of the initial blue reaction product. Other acids such as trifluoroacetic and hydrochloric acid are successful in arresting enzyme activity but rapid, non-specific oxidation reactions continue to occur (Bos *et al.*, 1981). These changes are not uncommon, as chromogens, in their oxidized states, can effect bathochromic and hypsochromic shifts in absorption maxima when acids or bases are added due to the ability of many chromogens to act as pH indicators (Porstmann and Porstmann, 1988).

Several studies have indicated that TMB may be the chromogenic reagent of choice for EIAs employing HRP as the marker enzyme (Bos *et al.*, 1981; Hosoda *et al.*, 1986; McKimm-Breschkin, 1990; Roberts *et al.*, 1991). In these studies, TMB was found to allow for a more sensitive assay due to the generation of increased absorbance values as compared to other common hydrogen donors, including OPD and ABTS. McKimm-Breschkin (1990) found that the increase in sensitivity allowed antiserum to be diluted 10 to 100 times more as compared to that usable in an assay employing diaminobenzidine or chloro-1-naphthol as HRP hydrogen donors.

Madersbacher and Berger (1991) suggested that the working range of an ELISA employing TMB can be increased three-fold by employing a double-wavelength measurement of absorbance values. Their method involved measuring the absorbance of reaction products at both 450 nm (the maximum) and 405 nm after the addition of sulphuric acid. This allowed for the generation of a regression equation between A_{450} and A_{405}. The ratio of the absorbances at these two wavelengths was found to be 3.2 and was constant over the entire assay range. Thus, through mathematical manipulation, these authors felt they had afforded themselves an extended linear range for measurements of their ELISAs. However, these authors admit that the ratio cannot

be too large, since measuring errors of the ELISA reader could skew results, and multiplication by such a conversion factor may serve to magnify errors.

Many ELISA systems have been developed which employ TMB as the hydrogen donor for HRP; however, in many cases, it appears that these assays have not been "optimized" with respect to pH (Table 1.3).

Table 1.3 Reports of ELISAs Using TMB as Hydrogen Donor for Horseradish Peroxidase

LITERATURE SOURCE	pH USED IN ASSAY	TMB CONCENTRATION (M)
Roberts *et al.*, 1991	6.0	3.19×10^{-4}
Bos *et al.*, 1981	6.0	4.20×10^{-4}
Catty and Raykundalia, 1989	6.0	3.19×10^{-4}
Oku *et al.*, 1988	5.5	5.60×10^{-4}
Porstmann and Porstmann, 1988	4.0	1.00×10^{-3}
Madersbacher and Berger, 1991	5.5	3.19×10^{-4}
McKimm-Breschkin, 1990	5.0	3.19×10^{-4}

1.3 UREASE

Urease (urea amidohydrolase, EC 3.5.1.5) occupies a unique position in scientific history in that it was the first enzyme to be crystallized and shown to be a protein. Much controversy over the nature of enzymes and the existence and participation of metal cofactors ensued with the discovery of HRP in 1903 (Dunford, 1991). It was 50 years after the pioneering work of Sumner that urease was shown to be the first example of the nickel metalloenzymes (Zerner, 1991).

The action of urease on its substrate, urea, is generally formulated as given below:

$$H_2NC(O)NH_2 + 3H_2O \xrightarrow{\text{UREASE}} 2NH_4^+ + HO^- + HCO_3^-$$

However, the true hydrolytic action of urease converts urea to carbamate and ammonium ion (Zerner, 1991):

$$H_2NC(O)NH_2 + H_2O \xrightarrow{\text{UREASE}} H_2NCOO^- + NH_4^+$$

The breakdown of the carbamate ion to hydroxyl ion, bicarbonate ion, and more ammonium ion is non-enzymatic, and is dependent on the composition of the reaction buffer. The buffer also influences the activity of the enzyme and its stability with respect to temperature, pH, and product and substrate inhibitions (Huang and Chen, 1991). While a variety of compounds can serve as substrates for urease, the enzyme has a much lower K_m and higher V_{max} with urea (Zerner, 1991).

Urease is a hexameric molecule composed of six polypeptide units of MW 90,777 Da (based on amino acid sequence), which corresponds to a MW of 545,365 for the intact enzyme, which contains 12 g-atoms of nickel (Zerner, 1991). Commercial preparations are usually derived from the Jack Bean (Tijssen, 1985).

The reaction of urease on urea can be measured colorimetrically using bromcresol purple (Chandler *et al.*, 1982; Tijssen, 1985). The production of ammonia may be detected by a pH increase to the alkaline range which is indicated by a vivid colour change from yellow to purple of bromcresol purple. Because of the sensitivity to inhibition of urease by heavy metals, the enzyme reaction is readily stopped by the addition of small amounts of thimerosal (Chandler *et al.*, 1982; Lawrence and Moores, 1972). Urease activity is also decreased in the presence of protein-destroying substances; the enzyme is irreversibly inactivated by small amounts of hydrophobic

substances (Chin and Kroontje, 1961; Meyerhoff and Rechnitz, 1980). Therefore, the addition of detergents or other denaturing compounds could also serve as stopping solutions.

An alternative assay uses an ammonia gas-sensing electrode to measure urease activity. This assay is advantageous as it can be performed directly in enzyme solutions without the need for special colorimetric reagents or drastic changes in reaction conditions, such as pH. Activity can be measured in small volumes by following the initial rate of change in potential with time (Meyerhoff and Rechnitz, 1980).

The nature of the reaction of urease on urea affords a third possible means of measuring the enzyme's activity. The enzymatic generation of carbamate and ammonium ions, followed by their chemical breakdown to hydroxyl ion and bicarbonate ion results in the production of three types of charged species from an initially neutral urea molecule. The changes in concentration of these ions results in a change in the electrical conductivity of the solution, which can be measured conductimetrically (Schuler, 1983).

Conductivity is determined by measuring the current flow between two inert electrodes in a solution after the application of a fixed voltage (Holme and Peck, 1983; Thompson *et al.*, 1991). Such measurements are performed using AC voltage to avoid polarization of the electrodes, which occurs when a direct current is applied. Polarization, which is the formation of a potential opposed to the original voltage, can be reduced by increasing the surface area of the electrodes and/or by coating them with "platinum black" (platinized platinum) (Schuler, 1983).

Upon applying a voltage to the electrodes, an electric field, consisting of an anode and a cathode, is created. The current passing between the two electrodes is carried by the ions in the solution. The magnitude of the current is a function of the number of ions present in solution and the donation of electrons by anions to the anode and acceptance of electrons from the cathode by cations (Holme and Peck, 1983).

While ionic concentration is largely responsible for determining the conductivity of a solution, since the specific conductivities of components in the solution are additive, the overall conductance is also influenced by ionic mobility. Since different ions move at different speeds, the total transfer of electricity between ions and the electrodes is not shared equally by all ionic species (Holme and Peck, 1983). The ionic mobility increases with increasing temperature; thus, for reliable, accurate conductivity measurements, a constant temperature must be maintained (Chin and Kroontje, 1961; Schuler, 1983). Conductimetry values are also influenced by ionic concentration, since

with increasing concentration, the degree of dissociation decreases, and with increasing ion density, the ions mutually hinder each other (Schuler, 1983).

Individual ions have standard molar conductivity values, and their contribution to the conductance of a solution is the product of their ionic conductivity and concentration. Urease activity is conventionally expressed in terms of the hydroxyl ion concentration, since it possesses a much higher molar ionic conductivity than either ammonium or bicarbonate ions (Thompson *et al.*, 1991).

The use of urease as a marker enzyme coupled with conductimetric measurement of its activity may afford several advantages over colorimetric assay systems. Foremost in the list of problems associated with colorimetric assays is the narrow linear range of optical readers. While this range may span 2-3 OD units, the most reliable measurements are obtained in an even more limited range of 0.2-0.7 OD units (Skoog and West, 1976). Other advantages of the conductimetric measurement of urease activity include the fact that interferences due to the presence of coloured impurities are eliminated, the conductance values of opaque solutions can be measured, and the values of a standard curve at a particular temperature are constant. The method is also rapid and simple, and effective over wide concentration ranges of urea (Chin and Kroontje, 1961). In terms of EIA, urease may be useful as a marker enzyme because of its absence from mammalian cells (Chandler *et al.*, 1982) and ELISA readers employing conductimetric measurements are more rugged and inexpensive than their spectrophotometric counterparts

The conductimetric method of determining urease activity is not without its drawbacks, the major one being the tendency of urease to rapidly lose activity (Tijssen, 1985). Care must be taken in selecting buffer systems for several reasons: (1) the kinetics of the reaction, and inhibition of the enzyme by reaction products, is influenced by the type of buffer (Huang and Chen, 1991); (2) the enzyme is sensitive to heavy metals; therefore, common preservatives such as thimerosal must be avoided (Chandler *et al.*, 1982); (3) buffers should have a low conductance power to maintain low background values (Lawrence and Moores, 1972; Schuler, 1983); and (4) the nature of the buffer affects the sensitivity and linear range (Hanss and Rey, 1971). Samples which contain substances inhibitory to urease (e.g., heavy metals, protein destabilizers) cannot be analyzed conductimetrically using urease (Chin and Kroontje, 1961). Finally, conductimetry is a non-specific method in that it cannot discriminate between different reactions, and can produce artifacts. It lacks the specificity of spectrophotometric methods afforded through the selection of appropriate wavelengths (Lawrence and Moores, 1972).

Several immunoassay techniques are described in the literature employing urease conjugated to a protein antigen (Meyerhoff and Rechnitz, 1980), polyclonal serum antibodies (Chandler *et al.*, 1982), and a monoclonal antibody (Thompson *et al.*, 1991). Only one of these studies, however, (Thompson *et al.*, 1991) employed a conductimetric method of measuring urease activity.

In synthesizing urease conjugates, care must be exercised as the method of linking can affect the enzyme properties (Meyerhoff and Rechnitz, 1980). Changes in substrate affinity (K_m) and temperature sensitivity of urease have been found upon its immobilization to inert supports (Ramachandran and Perlmutter, 1976). While Ramachandran and Perlmutter (1976) found that urease immobilization by either the diazo method or a glutaraldehyde coupling technique did not significantly alter the enzyme's characteristics as compared to those of the soluble enzyme, other researchers have found that "immobilization" of urease on cyclic nucleotides by the carbodiimide method results in a great loss in enzyme activity (Meyerhoff and Rechnitz, 1980).

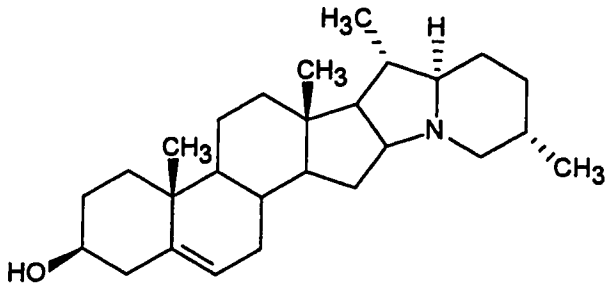
1.4 GLYCOALKALOIDS

Glycoalkaloids (GAs) are nitrogen-containing compounds based on a C_{27} steroidal skeleton, with an associated sugar moiety (Van Gelder, 1991). Various types and amounts of GAs are produced by plants in the family Solanaceae, which includes the cultivated potato, *Solanum tuberosum* (Morris and Lee, 1984). The composition of three common GAs is given in Table 1.4. While these GAs appear to offer a competitive advantage to plants by conferring disease and pest resistance (Gaffield *et al.*, 1991; Roddick *et al.*, 1988), they are known to be toxic to animals and humans (Baker *et al.*, 1991; Morris and Lee, 1984). Only GAs from potatoes have been recorded as causing human death, with the total number of fatalities estimated at 30 (Morris and Lee, 1984).

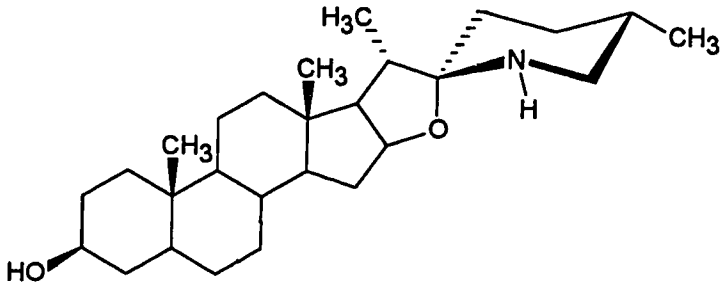
Table 1.4 Common Glycoalkaloids and Their Compositions

GLYCOALKALOID	ALKALOID	GLYCOSIDE	SUGARS
α-chaconine	solanidine	β-chacotriose	D-glucose, 2 L-rhamnose
α-solanine	solanidine	β-solatriose	D-galactose, D-glucose, L-rhamnose
α-tomatine	tomatidine	β-lycotetraose	D-galactose, 2 D-glucose, D-xylose

The GAs of potatoes include α-chaconine and α-solanine, the glycosylated derivatives of the aglycone solanidine (Figure 1.7), and account for at least 95% of the total GA (TGA) content of commercial potato cultivars (Jadhav *et al.*, 1981). These GAs can be demonstrated in freshly-dug tubers at levels of 1-15 mg/100 tuber (Morris and Lee, 1984). Since GA synthesis and ultimate levels are under genetic control, the "normal" level can vary significantly with variety (Morris and Lee, 1984; Slalina, 1990; Van Gelder, 1991). GA biosynthesis within the potato plant and its tubers can also be triggered by unfavourable conditions during cultivation, as well as post-harvest treatment and storage, which may result in levels much higher than those pre-programmed genetically (Jadhav *et al.*, 1981; Morgan *et al.*, 1985; Van Gelder, 1991). Processing operations, such as slicing, can also encourage the accumulation of GAs, and dehydration processes, such as deep frying, serve to increase the effective concentration of GAs in potato products (Morris and Lee, 1984; Sizer *et al.*, 1980). Furthermore, common cooking methods do not remove or destroy GAs, and flavourings



SOLANIDINE (Solanidane Skeleton)



TOMATIDINE (Spirosolane Skeleton)

Figure 1.7 Structure of Solanidane and Spirosolane Glycoalkaloids

can serve to mask the bitterness associated with high levels of GAs (>20 mg/100 g tuber) (Maga, 1980; Morris and Lee, 1984; Sizer *et al.*, 1980).

GAs are found in all organs of the potato plant, but are concentrated in areas of high metabolic activity (Van Gelder, 1991). In the tuber itself, GAs are formed in the parenchyma cells of the periderm and cortex, leading to a concentration gradient decreasing from the skin of the potato to its centre (Jadhav *et al.*, 1981). Consequently, small tubers tend to have higher GA levels than larger tubers, due to the increased surface area-to-volume ratio of the former (Maga, 1980). Peeling can significantly decrease GA levels, but if high GA concentrations are present, they can diffuse through the entire tuber, in which case peeling has a limited effect in reducing the potential toxicity of the tuber (Maga, 1980; Van Gelder, 1991).

Humans appear to be much more sensitive than other animals to poisoning by GAs, with poisoning symptoms having been observed at oral doses as low as 2 mg GA per kg body weight (bw) (Morris and Lee, 1984; Slalina, 1990). However, among humans, there is a broad distribution of sensitivity to GA poisoning (Morris and Lee, 1984), and susceptibility can depend on the nutritional status of the individual (Slalina, 1990), which makes establishing acceptable daily intake values difficult. Toxic doses have been estimated to be 2-5 mg TGA/kg bw, with lethal doses considered to be 3-6 mg TGA/kg bw (Morris and Lee, 1984; Slanina, 1990). These values are comparable to those for known poisons such as strychnine (5 mg/kg bw) and arsenic (8 mg/kg bw) (Morris and Lee, 1984); potato GAs are therefore very formidable toxins.

As earlier indicated, over 30 human deaths have occurred after the ingestion of tubers containing high levels of GAs. There are also over 2000 documented cases of GA poisoning linked to potatoes. This is considered to be a conservative estimate, as many cases are likely diagnosed simply as gastroenteritis and are never reported (Morris and Lee, 1984; Van Gelder, 1991).

Classic symptoms of GA poisoning may be grouped into neurological and gastrointestinal. Neurological symptoms, including drowsiness, dizziness, and trembling, are attributed to the anti-cholinesterase activities of α-chaconine and α-solanine (Gaffield *et al.*, 1991; Jadhav *et al.*, 1981; Morris and Lee, 1984). Gastrointestinal symptoms (diarrhea, vomiting, cramps) and headache arise from the disruption of membranes by GAs, which appears to be related to their saponin-like properties. The disruption of membranes of the gastrointestinal (GI) tract leads to haemolytic and haemorrhagic damage, and the accumulation of excess fluid in body cavities (Morris and Lee, 1984; Gaffield *et al.*, 1991). Deaths have often been attributed to the inhibition of cholinesterase, but may in fact be due more to shock from

fluid shifting and other events associated with membrane disruption (Gaffield *et al.*, 1991). It is assumed that the N-heterocyclic moiety is responsible for the various toxic effects (Gaffield *et al.*, 1991); however, there is evidence to suggest that the glycoside residue plays a role in toxicity, particularly in the disruption of membranes (Roddick *et al.*, 1990).

The generally accepted cut-off level between safe and potentially toxic potatoes is 20 mg TGA/100 g tuber; however, levels as low as 6-7 mg/100 g have been suggested to ensure safety (Slalina, 1990; Van Gelder, 1991). As pointed out by Slalina (1990), if the lowest dose inducing toxic effects is taken as 2 mg/kg bw, and 300 g of potatoes containing 20 mg TGA/100 g tuber were consumed by a 60 kg individual (total dose = 1 mg/kg bw), the effective safety factor would be 2. This compares poorly with the risk assessment for potato pesticide residues which commonly employ a safety factor of 100.

Although current US and European potato varieties characteristically contain 2-15 mg TGA/100 g (Slalina, 1990), such "safe" levels cannot be ensured as new cultivars emerge. Wild potato varieties are often used in breeding programs to impart disease and pest resistance, among other desirable characteristics, to resultant offspring. However, the introgression of genes from wild species into cultivated potatoes can be accompanied by a transmission of the genes which control the synthesis of high levels of GAs (Van Gelder, 1991).

This problem was encountered in 1970 when the newly developed cultivar Lenape was rapidly removed from commerce in Canada and the US due to exceedingly high GA levels (Lewis and Fenwick, 1991; Jadhav *et al.*, 1981). In Canada, TGA levels of 35 mg/100 g were measured, while values as high as 65 mg/100 g were found in American Lenape tubers. It was assumed that the wild species *S. chacoense* used in breeding had been the vector of such elevated GA levels (Van Gelder, 1991). The experience with Lenape focused attention on the need to screen all newly-developed cultivars for GA content (Jadhav *et al.*, 1981; Morris and Lee, 1984). A similar, but not well-publicized incident occurred in the UK after the use of the wild variety *S. vernii* in breeding to confer nematode resistance. Again, potentially commercially viable clones had to be withdrawn due to high GA levels (Lewis and Fenwick, 1991).

Many methods exist for the analysis of GAs, and have been reviewed by Coxon (1984). Common analytical techniques, such as gravimetry, colorimetry, and chromatography each had several drawbacks, but collectively required an extraction step and often an exhaustive sample clean-up, which usually resulted in inaccurate

analyses due to the loss of components or the inclusion of non-GA components in the measurements (Van Gelder, 1991).

Colorimetric methods utilize hazardous and toxic reagents, and are not always comprehensive in their measurement of both saturated and unsaturated GAs. Titrimetric methods exist which are safe and simple to perform, and which quantify all GAs; however, losses as high as 20% can occur during extraction (Jadhav *et al.*, 1981).

Colorimetric methods were largely superseded by chromatographic methods, which still require extensive sample preparation, analysis time, sophisticated equipment, and considerable technical expertise. Both HPLC and GC can be used to separate and quantify individual GAs and aglycones; however, each method suffers from certain limitations (Van Gelder, 1991). Although highly sensitive detectors are available for GC work, the method is less than ideal since the GA must first be derivatized. In addition, the high column temperatures required for separation reduce column life (Morgan *et al.*, 1985). HPLC suffers from the poor detectors available; UV or refractive index detectors are not sensitive to saturated GAs, and GAs in general do not have a suitable chromophore above 200 nm (Coxon, 1984; Morgan *et al.*, 1985; Van Gelder, 1991).

Immunological methods present a viable alternative to chemical and chromatographic methods since they are specific, sensitive, safe, and simple to perform. The large sample through-put capacity of immunological methods makes them suitable for screening large numbers of samples, as is required in plant breeding. Since the antigen-antibody reaction is so specific, there is little sample preparation, save for homogenization and dilution of potato material prior to analysis (Morgan *et al.*, 1983).

Vallejo and Ercegovich (1979) developed by the first immunoassay, a radio-immunoassay, using antiserum raised against a solanidine-BSA conjugate. However, poor cross-reactivity with the glycosides α-solanine and α-chaconine rendered the method unsuitable for TGA analysis. The low serum titre also limited widespread applicability of the method (Morgan *et al.*, 1983, 1985).

The first ELISA for potato GAs was successful in quantifying all the major GAs (Morgan *et al.*, 1983) and correlated well with established chemical methods (Morgan *et al.*, 1985; Hellenäs, 1986). However, the method of Morgan *et al.* (1983) for the conjugation of solanidine to the carrier protein netted poor solanidine-to-protein ratios, leading to a poor immunological response, resulting in low-affinity antibodies and high background absorbances in the ELISA (Plhak and Sporns, 1992).

In order to improve upon the method of Morgan *et al.* (1983), Plhak and Sporns (1992) prepared γ-chaconine and linked it to LPH by the periodate cleavage method.

This afforded an immunogen of a greater hapten-to-protein ratio, which netted high titres in the sera of immunized rabbits. The solid-phase (competitor) conjugate was synthesized by linking solanidine hemisuccinate to BSA. The test was successful in detecting the solanidine alkaloids, with I_{50} values similar for all compounds of this group tested. The test results for potato extracts were found to correlate well with HPLC methods. In addition, the ELISA was able to detect the unsaturated GA demissidine (Figure 1.7) which was not quantified by HPLC. However, as is common with ELISA methods, high coefficients of variation were observed both inter- and intra-assay.

1.5 IMMUNOASSAYS

Immunoassays (IAs) are based on the non-covalent binding between an antigen (Ag) and an antibody (Ab) to form an immune complex (Ag-Ab) in an immune reaction (Gazzaz *et al.*, 1992). Detection of the immune reaction may be achieved in a number of ways, including the use of radioactively labelled Ags; however, the relatively short shelf-life and inherent hazards associated with the use of radioisotopes has led to an increased use of enzymes as detection labels (Gosling, 1990; Sauer *et al.*, 1985). These enzyme-based methods are more broadly referred to as enzyme-immunoassays (EIAs) (Tijssen, 1985). The work described herein employed an immunoreactant immobilized on a solid phase; such assays are referred to as enzyme-linked immunosorbent assays (ELISAs).

An obvious prerequisite for an IA is a functional Ab molecule. Antibodies are members of a family of mildly glycosylated proteins called immunoglobulins (Igs) (Butler, 1980). Antibodies are formed in response to an Ag and can react specifically with that Ag (Coleman *et al.*, 1989). In terms of human health, the combination of Ab with Ag results in the elimination of a foreign, potentially harmful compound from the body; this is the premise behind active immunization of individuals. However, these two principles may be exploited to develop highly sensitive assays. By "challenging" a test animal, such as a rabbit, with a foreign Ag of interest, it is possible to stimulate the production of a population of Abs within the animal, a proportion of which will be specific for the Ag of interest. Following collection of the serum component of the blood, a test can be designed wherein the Ag of interest can be detected by the Abs produced due to the specificity of the Ab for its Ag.

Antigens are substances that are capable of combining with Abs or other cells of the immune system (i.e., T cells). An immunogen, however, is an antigen that is capable of inducing an immune response and reacting specifically with the products of such a response (Coleman *et al.*, 1989). Thus, all immunogens are Ags; however, not all Ags are immunogenic.

Significant Ags involved in infectious disease include bacteria, viruses, and parasitic organisms, all of which are inherently immunogenic to a certain degree. Generally, the most potent Ags have a MW > 100,000. Other immunogens include proteins foreign to the challenged animal, as well as certain carbohydrates, lipids, and nucleic acids (Coleman *et al.*, 1989; Nisonoff, 1982). In terms of toxicological research, however, the compounds to be analyzed are generally of low MW (Roe, 1991). Small molecules (MW < 1,000) are incapable of eliciting an immune response on their own, and are referred to as haptens (Erlanger, 1980; Nisonoff, 1982; Roe, 1991).

Fortunately, haptens become immunogenic when coupled to a larger carrier molecule, such as a protein. The Abs formed in response to such a hapten-carrier immunogen retain the ability to combine specifically with the uncoupled hapten (Coleman *et al.*, 1989).

Antibodies are serum proteins, generally present in the gamma globulin fraction of serum (Nisonoff, 1989). Although there are five major classes of human Igs (IgG, IgD, IgE, IgM, and IgA), distinguished on the basis of their heavy polypeptide chains, IgG is the most common, and can be used to discuss the basic molecular structure of all Igs (Coleman *et al.*, 1989).

Antibody molecules are comprised of two identical light (L) (MW ~22,500) and heavy (H) (MW 50,000-75,000) polypeptide chains linked by interchain disulphide bonds (Figure 1.8). The N-terminal 110 amino acid sequences are highly variable, while the rest of the protein is conserved and typical of the animal in which the Ab was produced (Butler, 1980; Coleman *et al.*, 1989; Nisonoff, 1982). The variable region sequences of the H and L chains are structurally associated with each other to form the Ab combining site, or paratope (Butler, 1980). This paratope, which is actually a hydrophobic "pocket" in three dimensions, is genetically programmed and is complimentary to a specific antigenic determinant or epitope. It is the paratope of the Ab that is responsible for the high specificity of Abs, and allows for discrimination between two epitopes (Coleman *et al.*, 1989).

The Ag-binding site of Abs can vary considerably in both shape and size; however, on average, most sites correspond in size to roughly 3-7 glucose molecules (Nisonoff, 1982). Epitopes are approximately 5-7 amino acids in size (~7x12x35 Å) (Gazzaz *et al.*, 1992). It should be noted that the actual area involved in binding can be small relative to the overall size of the Ag-binding pocket on the Ab.

The interaction between Ag and Ab is mediated by several types of non-covalent bonds, including hydrogen bonds, electrostatic attractions, van der Waals forces, and hydrophobic interactions (Coleman *et al.*, 1989; Nisonoff, 1982). These weak forces require that the epitope of the Ag and the paratope of the Ab be in close proximity. Nonetheless, interaction remains weak and can be broken by high salt concentrations, chaotropic ions, or extremes of pH (Gazzaz *et al.*, 1992).

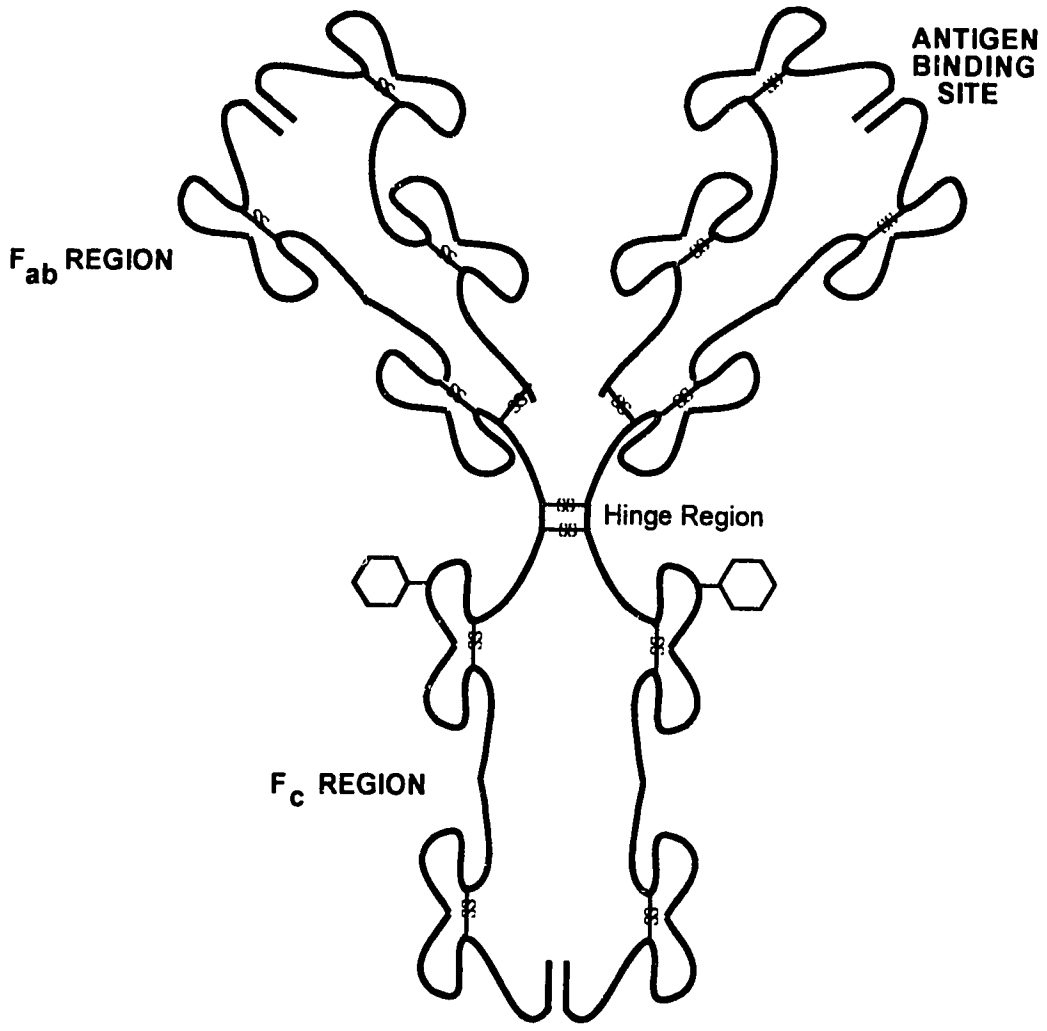


Figure 1.8 Basic Antibody Structure

F_{ab} = Fraction (Antibody Binding); F_c = Fraction Crystallizable

LEGEND

) = peptide chain -S-S = disulphide bond ⬡ = carbohydrate

As previously mentioned, haptens must first be conjugated to carrier molecules to become immunogenic. Common carriers include serum albumins of various species, keyhole limpet hemocyanin (KLH), thyroglobulin, ovalbumin, or fibrinogen (Tijssen, 1985). Better immune responses are achieved if the carrier protein used is recognized as foreign by the immunized animal (Nisonoff, 1982). No one carrier appears to offer a decided advantage in eliciting an anti-hapten response (Erlanger, 1980). The number of hapten groups conjugated per molecule of carrier protein must be optimized. Too few or too many hapten molecules per carrier molecule similarly lead to a poor immunological response. Although the exact degree of substitution varies with the hapten, an epitope density of between 8 and 25 results in a good Ab response (Erlanger, 1980).

The Ab produced in response to a hapten-carrier conjugate is directed mainly to the hapten portion farthest removed from the linkage (Tijssen, 1985). Thus, the site of linkage on the hapten is of paramount importance in achieving a useful immunogen. Conserved portions of groups or families of compounds (e.g., sulphonamides), may be left exposed to allow for a serum to be produced recognizing a broad range of similar compounds (Sheth and Sporns, 1991). Conversely, a highly specific serum (i.e., limited cross-reactivity with structurally similar molecules) may be desired, which influences the linkage site chosen.

A variety of chemical and physical methods is available for the conjugation of haptens to carriers. Chemical methods rely on the reaction between functional groups on the hapten and the carrier. On the carrier, the most reactive groups, and hence sites for hapten attachment, include ε- and α-amino groups, phenolic, sulphydryl, imidazole, and carboxyl groups. The reactive groups participating in the linkage are determined by the pH used in the conjugation procedure (Tijssen, 1985). The functional groups available on the hapten largely govern the choice of conjugation method (Erlanger, 1980). It is also possible to introduce suitable functional groups into hapten molecules. Among the most common reagents used in conjugation of haptens to carriers are carbodiimides, diazonium salts, or bifunctional bridging molecules such as glutaraldehyde (Tijssen, 1985).

It is important to recognize that the Abs produced in response to a haptenated carrier are directed to all parts of the conjugate, including the hapten, the "bridge" or "linking arm" between the hapten and the carrier, and the numerous epitopes on the carrier protein itself. In this way, a vast array of Abs with varying specificities will be produced in response to a haptenated carrier. Furthermore, such serum will contain the naturally-occurring Abs of the animal's immune repertoire. Such a serum is referred to as polyclonal; the actual proportion of Abs in that serum specific for the hapten of interest

may in fact be quite low (Assil *et al.*, 1992). In order to improve detectability using a polyclonal serum derived from a haptenated carrier, it may be necessary to change the type of bridge employed in synthesizing the immunogen and the conjugate used in the assay. It can also be helpful to use different carriers for the immunogen and the "detection" Ag (Tijssen, 1985).

Monoclonal Abs (MAbs), in contrast, are of a defined and single specificity. Köhler and Milstein (1975) devised a method to produce MAbs against any Ag of choice. By fusing an Ab-producing cell (B cell) with an appropriate myeloma tumour cell, an immortal cell line can be generated that secretes Ab characteristic of the B cell used in the fusion. The cell line can be perpetuated in cell culture or *in vivo* (Nisonoff, 1982), although the latter has fallen into disfavour due to its cruelty to host animals (usually mice) and the ultimate sacrifice of these animals.

The first step in the production of MAbs involves immunizing the test animal with the appropriate Ag or haptenated carrier. Splenocytes or lymphocytes, containing Ab-secreting B cells, are harvested from the immunized animal (most commonly a mouse) (Coleman *et al.*, 1989). These B cells are fused with a mouse myeloma cell line in the presence of a fusagen, such as polyethylene glycol, which induces cell fusion. It is important that the myeloma cell line be mutated such that it lacks the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT), necessary for purine and pyrimidine biosynthesis, and does not secrete its own Abs (Coleman *et al.*, 1989; Nisonoff, 1982).

Following fusion, it is necessary to select for the hybridomas (i.e., fused myeloma + B cell). To achieve this, culture conditions are chosen that allow hybridomas to thrive, while the parental myeloma and B cells die off. B cells have a finite life period; they die naturally in culture in a matter of days. Myeloma cells, on the other hand, can survive indefinitely under appropriate culture conditions (Nisonoff, 1982).

Cells are cultured in a medium containing hypoxanthine, aminopterin, and thymine (HAT medium). The aminopterin blocks *de novo* synthesis of pyrimidines and purines, which the myeloma cells require to survive. Hybridomas, however, are able to utilize hypoxanthine and thymine in HAT medium in an alternative pathway involving HGPRT for purine and pyrimidine synthesis. The HGPRT in the hybridoma cells is derived from the parental B cell (Coleman *et al.*, 1989; Nisonoff, 1982).

Viable hybridomas are screened by ELISA or RIA for their ability to produce the desired Ab. Cells testing positive for Ab production are cloned by limiting dilution until a clone producing the single, desired Ab is obtained (Nisonoff, 1982).

The beauty of MAb technology lies in its ability to produce a single, specific Ab from an impure Ag source. Furthermore, once established, a hybridoma cell line provides a permanent source of Abs (Nisonoff, 1982). This is in contrast to polyclonal Abs, which are of a finite supply. Although animals of the same species may be immunized with the same immunogen to produce more polyclonal serum, the immune response varies greatly from one animal to the next. Once a suitable polyclonal serum or MAb has been obtained, it is possible to develop an IA which utilizes the specificity of this MAb or Ab population for the Ag or haptenated carrier that stimulated the original immune response.

Immunoassays may take several forms, each with limitations and advantages. This discussion will concentrate on solid-phase based assays, in which either the Ag or Ab is immobilized by passive adsorption on a solid surface, most commonly in the wells of a microtitre plate. This adsorption is necessary to allow for quick and easy separation of immune complexes from unreacted immunoreactants (Clark and Engvall, 1980). Plastic is the most commonly used solid phase (Tijssen, 1985), with polystyrene (PS) being especially popular, since it is easily coated (Clark and Engvall, 1980; Zalazar *et al.*, 1992). Furthermore, the translucency of PS allows results to be read using a 96-well spectrophotometer (Harlow and Lane, 1988).

The first step in an ELISA protocol is the immobilization of one of the immunoreactants to the solid phase. Proteins are adsorbed to plastics by weak, mainly physical forces (Lehtonen and Viljanen, 1980). The precise nature of this non-covalent interaction is not well understood; it is generally attributed to non-specific hydrophobic interactions between the protein and the plastic polymer. This adsorption is characteristic of a given protein, but independent of the pI or charge of the protein (Tijssen, 1985). It has been shown (Pesce *et al.*, 1977) that the adsorptive binding of protein to PS occurs in two steps. The first step is first order with respect to protein concentration, and occurs much more rapidly than the second step, which proceeds as a pseudo-first-order reaction. Conversely, Cantarero *et al.* (1980) found that the proportion of protein bound to a fixed area of PS was independent of the amount added up to an input of ~1000 ng, with binding increasing with increasing temperature and incubation time. Beyond ~1000 ng of protein added, the amount of protein bound was inversely proportional to the MW of most proteins studied. Beyond the region of independence, it was suggested that protein-PS interactions were replaced by protein-protein interactions. It is likely that protein multi-layers are formed; therefore, the actual amount of protein exposed remains constant (Cantarero *et al.*, 1980).

The rate and extent of coating is dependent on a number of factors. These include the diffusion coefficient of the adsorbing material, the ratio of surface area to be coated to the volume of coating solution, and the concentration of the adsorbing material, as well as the temperature and incubation time (Clark and Engvall, 1980).

Non-specific binding of proteins can be discouraged by blocking with a protein, such as BSA or ovalbumin, following immobilization of the immunoreactant (Roe, 1991) to eliminate potential binding sites for other components in the assay. The inclusion of non-ionic detergents in subsequent steps can also minimize non-specific binding (Clark and Engvall, 1980); however, such detergents must be avoided during coating as they compete with protein for the solid phase and prevent the formation of hydrophobic bonds (Tijssen, 1985).

Because protein adsorption to plastics is principally physical in nature, it is not surprising that a significant amount of desorption from the solid phase can occur during the course of any assay. Furthermore, multiple layers of protein, involving protein-protein association, are unstable, and relatively easily desorbed (Tijssen, 1985). Engvall *et al.* (1971) indicated that up to 68% of adsorbed protein may become desorbed during an IA test.

The desorption of protein (either Ag or Ab) from the solid phase is undesirable as it lowers the sensitivity and specificity of the assay, since the released immunoreactants are able to participate in the competition (Engvall, 1980). Desorption can be minimized simply by using the immunoreactant at the appropriate concentration, and by washing extensively after immobilization and every step in the protocol. Covalent linkage of the immunoreactant to the solid phase improves binding, but may not result in increased detectability (Tijssen, 1985).

Several assay formats are available for the measurement of either Ag/hapten or Ab. Non-homogeneous methods require a separation step following the immune reaction; the use of a solid support greatly facilitates this separation, as discussed above. Within the realm of non-homogeneous assays, further differentiation may be made.

Competitive ELISAs are limited-reagent assays in that they use limited amounts of Ag and Ab (Kemeny and Chantler, 1988); there is a competition between immobilized and free hapten for a limited number of Ab binding sites. Soluble Ag-Ab complexes are removed, leaving behind only the immobilized immune complexes (Roe, 1991). Competitive ELISAs may be of the direct or indirect type. Indirect assays employ a secondary Ab-enzyme conjugate for quantitation, while in direct ELISAs, one of the principle immunoreactants is labelled with an enzyme.

The data from all competitive EIAs can be fitted to a sigmoidal standard curve (Figure 1.9), which is calculated using the 4-parameter equation:

$$y = \frac{a - d}{1 + (x / c)^b} + d$$

where

a = upper asymptote (=maximum signal with vanishingly small amounts of analyte)

d = lower asymptote ("background signal")

c = inflection point of curve

b = slope of curve

For purposes of comparing data, I_{50} or "c" values obtained from the standard curve can be used. The I_{50} value represents the concentration of analyte required to reduce the maximum signal ("a" value) by 50%. The "c" value, as indicated above, is the inflection point of the standard curve; therefore, the "c" value is a 50% reduction in the "a-d" value, with the background ("d" value) added back.

In indirect competitive ELISAs, Ag or protein-hapten conjugate is immobilized on a solid phase. Following a blocking step, a competition is established between the immobilized Ag and free Ag (e.g., in a test solution) for a limited number of Ab binding sites in solution (Figure 1.10). The amount of Ab bound to the immobilized Ag is a function of the level of free hapten in solution. That is, with greater amounts of free hapten, less Ab binds to immobilized Ag. After separation of unbound immunoreactants and soluble immune complexes, the bound immune complexes can be detected using a secondary Ab, to which an enzyme is conjugated . After the enzyme signal has been generated, quantitation is achieved through the use of a standard curve (Roe, 1991) (Figure 1.9). The intensity of the enzyme signal is inversely proportional to the level of free hapten.

The secondary Abs employed in indirect assays are raised against the Igs (most commonly IgG) of the species in which the first or primary Ab was raised (Morris, 1985). The secondary Ab binds mainly to the F_c fragment of the primary Ab (Kemeny and Chantler, 1988); therefore, several labelled anti-Ig Abs can react with the primary Ab (Tijssen, 1985), intensifying the signal achieved. Furthermore, secondary Abs are more universal in their application (Porstmann *et al.*, 1982). They can be used in assays for a variety of different Ags, provided the primary Ab was raised in the appropriate animal. This contrasts sharply with direct ELISAs which require the

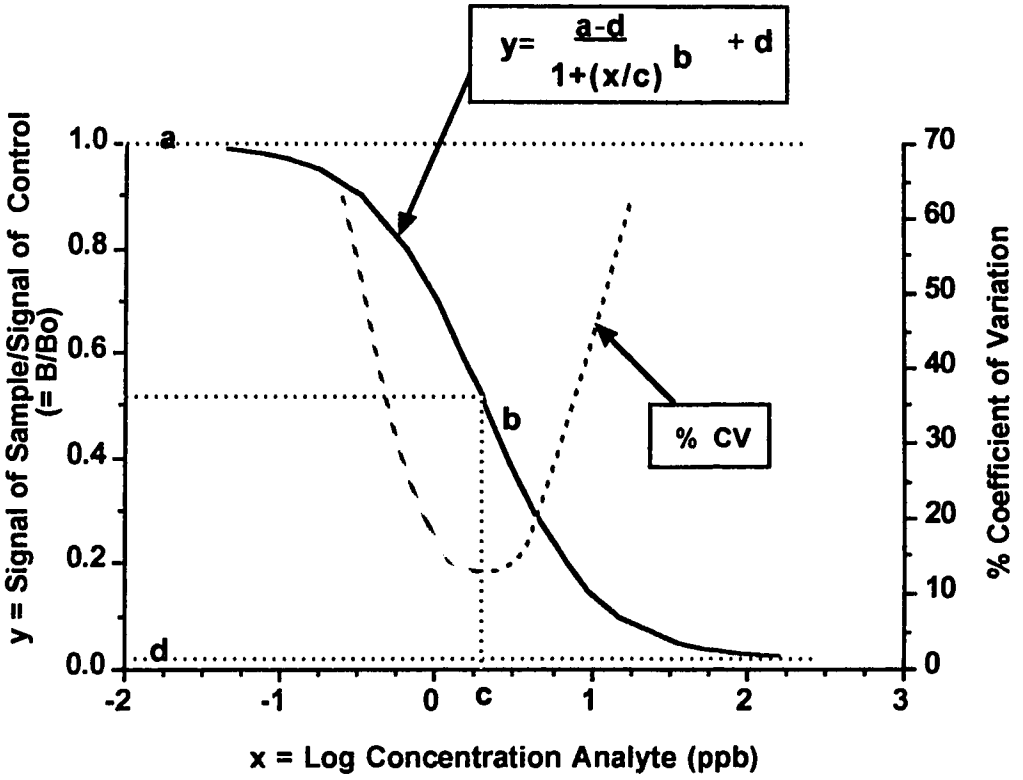


Figure 1.9. Standard Competitive Inhibition Curve For ELISAs

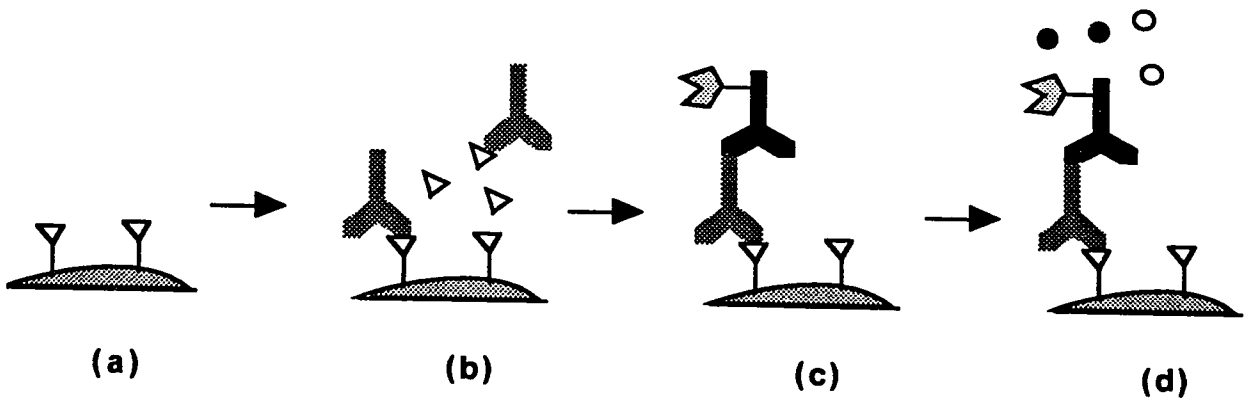
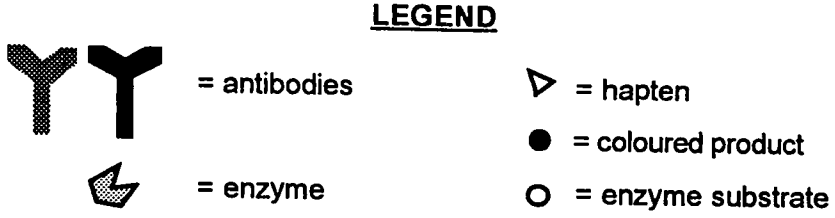


Figure 1.10 Indirect Competitive ELISA

(a) Coat microtitre plate with hapten-protein conjugate; **(b)** Add free hapten (i.e., standard or sample) and free anti-hapten Ab; **(c)** Add enzyme-conjugated secondary Ab; **(d)** Add enzyme substrate and develop colour.



preparation of the appropriate Ag-enzyme conjugate, the use of which is obviously limited to assays for that particular Ag.

Direct competitive ELISAs employ an enzyme-labelled immunoreactant. The research described in this thesis employed an enzyme-labelled hapten; therefore, this format will be the focus of this discussion. In such an assay, Ab is immobilized on the surface of a solid support (Figure 1.11). A competition is established between free (unlabelled) Ag in the sample and a known quantity of enzyme-labelled Ag for the limited amount of immobilized Ab. Washing removes unreacted components and soluble immune complexes, leaving behind immobilized immune complexes, some of which will have been formed by the union of Ab with an enzyme-labelled Ag. The intensity of the enzyme signal following incubation with its substrate is inversely proportional to the level of free Ag in the sample; quantitation is achieved using a standard curve (Clark and Engvall, 1980) (Figure 1.9).

The primary Ab in direct competitive ELISAs may be immobilized by simple adsorption to a plastic surface; however, the Ab may show a decreased Ag-binding capacity after coupling to a solid support (Ansari *et al.*, 1978; Sankolli *et al.*, 1987). The direct adsorption of Ab to the solid phase may also result in poor orientation of the binding sites. In particular, at low Ab concentration, there is a large potential for the Abs to interact with the support, leaving fewer paratopes exposed for combining with Ag. It is most desirable to have "ends-on" immobilization; that is, that Abs are immobilized via their F_C portions, allowing for full exposure of paratopes (Schramm and Paek, 1992). This is particularly true in competitive assays where Ab is limiting (Sankolli *et al.*, 1987).

Immobilization of Abs by their F_C portions may be encouraged by increasing the Ab density; however, higher levels of Ab can reduce the specificity of a competitive assay (Schramm and Paek, 1992) which may be due to steric hindrance of the Ag-binding site by surrounding Abs (Sankolli *et al.*, 1987). Pre-coating of plates with an intermediate protein or peptide that encourages proper Ab orientation can allow lower levels of Ab to be used, increasing assay sensitivity (Schneider and Hammock, 1992; Schramm and Paek, 1992).

Protein A, one of the most commonly used molecules for pre-coating solid phases, is derived from the cell walls of the Cowan strain of *Staphylococcus aureus* (Coleman *et al.*, 1989; Harlow and Lane, 1988). Its utility as a pre-coating molecule stems from its ability to bind specifically to F_C fragments of many mammalian IgG (Langone *et al.*, 1978) through extensive hydrophobic interactions with the second and third constant regions of the heavy chains (Harlow and Lane, 1988). Thus, the Ag-binding ability of the Ab is not impaired. Protein A has a particularly strong affinity for

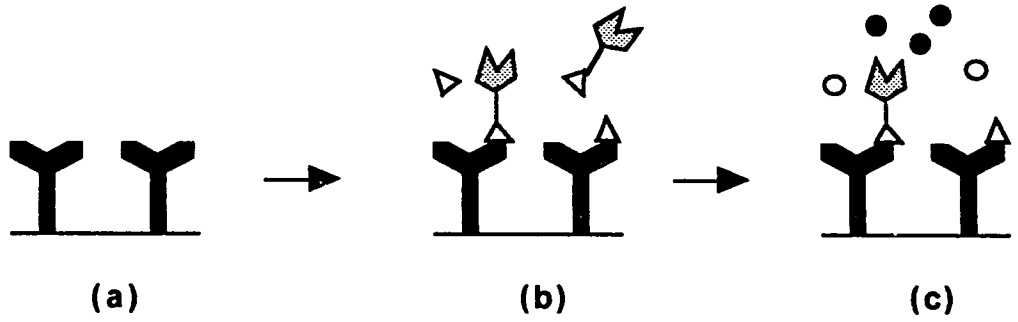


Figure 1.11 Direct Competitive ELISA

(a) Coat plate with Ab; **(b)** Add free hapten (i.e., standard or sample) and enzyme-conjugated hapten; **(c)** Add enzyme substrate and develop colour.

LEGEND

= antibodies

= enzyme

= hapten

= coloured product

= enzyme substrate

rabbit IgG (Harlow and Lane, 1988; Tijssen, 1985). Protein A pre-coating of solid phases can allow for more reproducible and stable immobilization of the primary Ab, since the dissociation of Protein A is usually negligible, although can increase with an excess of added IgG (Tijssen, 1985).

Protein G (PG), derived from the cell walls of β-hemolytic strains of *Streptococcus* (McGuire, 1989; Harlow and Lane, 1988), has similarly been used as a pre-coating molecule as it also binds selectively to the F_c region of Igs (Schramm and Paek, 1992). PG binds the F_c portion of only IgG Abs and possesses a different affinity profile with respect to the Abs of various species. PG tends to have a lesser affinity for rabbit IgG than does PA (Harlow and Lane, 1988; McGuire, 1989). PA possesses 5 binding sites for Abs, while PG has only 2 (Widjojoatmodjo *et al.*, 1993).

Another alternative is to employ "trapping" Abs. The injection of purified Abs from one species into a distant species often results in a strong immune response, as these Abs are recognized as foreign Ags (Harlow and Lane, 1988). Thus, Abs may be raised against the Igs of the species in which the primary Ab was raised, and may be directed toward the F_c region of these primary Abs, encouraging favourable orientation of Abs on the solid phase and high Ag-binding capacity (Sankolli *et al.*, 1987).

Schramm and Paek (1992) coated PS microtitre plates with poly(lysine), onto which Ab was subsequently immobilized by oxidizing carbohydrate residues on the F_c portion. The resulting reactive aldehyde groups reacted with amino groups of the poly(lysine); this was followed by reduction of the imine bonds.

One of the most powerful IA formats for the detection and quantitation of Ags is the two-site or "sandwich" non-competitive assay. The primary Ab is immobilized in excess on a solid phase. Test or standard solution of Ag is added and allowed to react with the immobilized Ab (Clark and Engvall, 1980). The sandwich is completed by the addition of a second Ab, which recognizes a different epitope on the multivalent Ag (Morris, 1985) (Figure 1.12). Detection of bound Ag can be achieved directly if the second Ab is labelled. Alternatively, the second Ab may remain unlabelled and can be detected indirectly using a third, labelled Ab. In order to avoid cross-reaction with the immobilized Ab, the labelled anti-Ig should be raised in a different species (Porstmann and Kiessig, 1992). Furthermore, the Ag-specific first and second Abs should be raised in different species, again to prevent reaction of the labelled anti-Ig with the immobilized capture Ab (Clark and Engvall, 1980). Although the indirect sandwich format is more complicated, it affords two advantages over the direct sandwich approach. First, the labelled anti-Ig enjoys universal usage and is readily purchased. Second, the

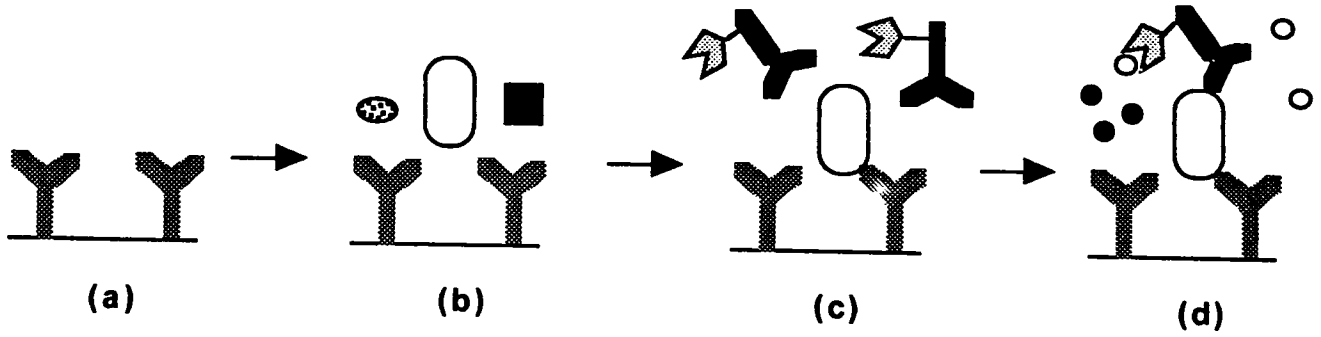
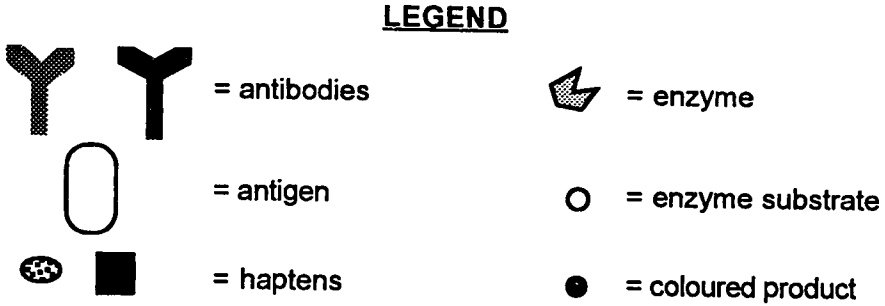


Figure 1.12 Two Site ("Sandwich") ELISA

(a) Coat plate with Ab specific for one epitope of Ag; **(b)** Add free Ag (i.e., standard or sample; **(c)** Add enzyme-conjugate secondary Ab specific for another epitope on Ag; **(d)** Add enzyme substrate and develop colour.



sensitivity of the indirect method is at least twice that of the direct format. This is due to an effect which arises from the fact that more than one anti-Ig can bind to the primary Ab (Porstmann *et al.*, 1982). In either case, the level of signal is directly proportional the Ag concentration (Clark and Engvall, 1980).

Despite the specificity of sandwich assays, their use is limited to the detection of large, multivalent Ags (Ali *et al.*, 1992). Since the Abs involved are specific for two different epitopes on the same Ag (Morris, 1985), it is necessary to employ either two MAbs, or affinity-purified polyclonal Abs (Harlow and Lane, 1988). Univalent Ags do not provide a site for attachment of the second Ab, while small haptens are engulfed by the Ag-binding site of the Ab, leaving no epitopes exposed for further combination with Ab. Sengupta *et al.* (1992), however, developed a sandwich method for small haptens wherein the effective size and valence of such haptens are increased by preparing bis-analogues or dimers. In an assay developed for testosterone, results using a dimer compared very favourably with those from RIA (Sengupta *et al.*, 1992)

All of the assay formats discussed above are of the heterogeneous variety since they require the separation of the bound immune complex from soluble Ab-Ag complexes and immunoreactants prior to quantitation (Morris, 1985; Voller, 1980). In heterogeneous EIAs, the enzyme on the labelled Ag or Ab retains full activity even while the Ab or Ag combines to form an immune complex (Voller, 1980).

Homogeneous IAs, in contrast, do not require a separation step prior to quantitation (Morris, 1985), since the signal intensity of the immunochemical label is increased or decreased upon binding to Ab or Ag such that bound and free label can be distinguished (Jenkins, 1992; Ullman and Maggio, 1980). The Enzyme-Multiplied Immunoassay Technique (EMIT) is the most widely used homogeneous enzyme immunoassay system, particularly for drug monitoring (Blake and Gould, 1984; Scharpé *et al.*, 1976). The most common variant of EMIT employs a hapten-enzyme conjugate in competition with free hapten for limited Ab. Conjugation of hapten to enzyme does not destroy enzyme activity; however, once the conjugate becomes bound to Ab, enzyme activity is significantly altered (Blake and Gould, 1984; Jenkins, 1992). The modulation of enzyme signal may be attributed to steric hindrance, which prevents access of substrate molecules to the enzyme active site. Alternatively, the interaction between the hapten-enzyme conjugate and anti-hapten Ab induces a conformational change in the enzyme, accompanied by a decrease in its activity (Jenkins, 1992; Mattiasson and Borrebaeck, 1980).

Homogeneous IAs are not limited to the use of enzyme markers. Similar protocols may be developed which make use of the moderation of signal from other

potential labels. Fluorescently-labelled haptens may be employed, and the change in fluorescence (e.g., degree of polarization, quenching) used in determining the extent of the immune reaction (Gosling, 1990) (Section 1.6).

All reactions in homogeneous IAs are performed in solution and do not require a separation step; such assays are therefore rapid and quite amenable to automation (Blake and Gould, 1984; Jenkins, 1992). Other advantages include better reproducibility of results than is realized with heteorogeneous systems, and easier modelling of the kinetics of the Ab-Ag interaction. However, there are limitations to the application of homogeneous EIAs. Generally, they are more effectively used for the quantitation of small haptens, since intimate interaction between Ab and enzyme is ensured (Porstmann and Kiessig, 1992). Furthermore, larger molecules decrease the advantageous stereochemical situation (Mattiasson and Borrebaeck, 1980). Homogeneous IAs are also inherently less sensitive than heterogeneous assays. Ideally, binding of the labelled Ag must effect a total (100%) modulation of the signal, which is difficult to achieve in practice. Homogeneous assays are, therefore, best suited to the analysis of compounds present at levels greater than 10 μM (Gosling, 1990). Components present in the sample matrix may also interfere, limiting the sensitivity of the assay (Jenkins, 1992). Such interference may be in the form of inhibitors or potentiators of enzyme label activity, or endogenous enzymes which may show some activity for the substrate used in quantitation (Smith *et al.*, 1981).

The ultimate objective of an IA is the quantitation of a substance of interest, often at very low levels. The detection limit of an IA procedure is determined by many factors, including the avidity of the Ag-Ab reaction, the quality of the tracer molecule, and the detection of the signal generated by the tracer. In the case of solid-phase IAs, the adsorption of the immunoreactant to the solid phase, the stability of this interaction, and the ratio of the surface of the solid phase to the volume of sample may be added to this list (Tijssen, 1985).

Of the factors listed above, a large amount of attention has been given to increasing the level of detectability of the tracer, which can be influenced by the assay conditions (e.g., pH), the inherent specific activity of the tracer (e.g., enzyme turnover number), and the amount of product which must accumulate before detection is possible (Tijssen, 1985). Research efforts have largely concentrated on reducing the level of detectability of the signal by (1) increasing the amount of label available for signal generation; (2) amplification of the enzyme signal; and (3) the use of a substrate intrinsically detectable with greater sensitivity (Bates, 1987; Kricka, 1993).

Multiple labelling involves maximizing the number of labels attached to one of the components of an assay (Kricka, 1993). One of the most common methods is the use of biotinylated Abs, which capture avidin or streptavidin molecules carrying multiple enzyme labels (Bates, 1987; Kricka, 1993). An alternate approach s to employ an enzyme/anti-enzyme complex such as peroxidase/anti-peroxidase (Bates, 1987; Tijssen, 1985).

Amplification of the enzyme signal can be achieved through substrate or cofactor cycling (Bates, 1987; Kricka, 1993). Many such systems are based on NAD^+/NADH redox cycling systems. NAD^+, formed through the dephosphorylation of $NADP^+$ by alkaline phosphatase, catalytically activates a specific redox cycle involving alcohol dehydrogenase and diaphorase. During each turn of the cycle, one molecule of a tetrazolium salt is reduced to an intensely-coloured formazan (Bates, 1987; Johannsson *et al.*, 1986; Kricka, 1993; Stanley *et al.*, 1985). In such systems, the availability, purity, and stability of the enzymes and their substrates can greatly affect the accuracy and reproducibility of the assay (Bates, 1987). Furthermore, the amplification effect is detected using colorimetry, which is inherently less sensitive than other modes of detection, such as fluorimetry (Jackson and Ekins, 1986).

The use of chemiluminescent substrates allows for ultrasensitive detection of enzymes (Kricka, 1993). Commonly used enzyme-substrate combinations are alkaline phosphatase and 1,2-dioxane-based substrates (Kricka, 1993) and HRP coupled with diacylhydrazides (e.g., luminol) in the presence of enhancers (Bronstein and Kricka, 1989).

Time-resolved fluorescence assays, employing long-lived Europium chelates, can increase the sensitivity of fluorescence methods by eliminating the interference caused by short-lived background fluorescence (Bates, 1987; Kricka, 1993). Alternatively, phosphorescent labels may be used which are resistant to changes in environmental conditions, such as pH and temperature (Kricka, 1993).

Adoption of any of the above methods can permit the detection of enzymes or other labels in the attomole or even zeptomole range (Kricka, 1993). Despite this advantage, several drawbacks must be considered. Most of the methods outlined require costly reagents and/or sophisticated, dedicated equipment. Reagents may also display limited stability. The additional steps involved in some protocols serve to complicate the analysis and increase the time required to generate results.

1.6 FLUORESCENCE POLARIZATION IMMUNOASSAY

Fluorescence polarization immunoassay (FPIA) is a rapid, homogeneous method based on the increase in signal resulting from the binding of a fluorescently-labelled hapten by Ab (Colbert *et al.*, 1991). It is a competitive assay that combines the specificity of an immunoassay with the speed and convenience of a homogeneous assay (Watanabe and Miyai, 1988), making it particularly attractive for therapeutic drug monitoring in matrices such as urine and blood serum (Varma-Nelson *et al.*, 1991). The only current application of fluorescence polarization (FP) in food science appears to be its use as a tool to determine microviscosity and structural order of components in food systems (Marangoni, 1992). However, the ability of FPIA methods to detect low MW compounds renders them potentially suitable for the assay of food contaminants and constituents of interest.

Luminescence describes the emission of light occurring when excited molecules return to the ground state. In photoluminescence, excitation energy is derived from photons of IR, UV, or visible light. A photon of appropriate wavelength excites a molecule from the ground state (S_o) to a higher electronic state in which the outer electrons remain paired, but one electron is promoted to a higher energy level. If the excited molecule emits light during its return to S_o in less than 10^{-8}s, the light is referred to as fluorescence. The emission wavelength is longer than that of excitation due to a loss of energy through processes which compete with fluorescence (Smith *et al.*, 1981).

The principles of FP were first published by Perrin (1926). In FP, light is resolved into rays with electrical vectors in a single plane using a fixed polarizing lens or prism. Rays are normally polarized with respect to the vertical plane, and propagation is along the horizontal axis (Watanabe and Miyai, 1988). If a solution containing randomly-oriented molecules is illuminated with plane-polarized light, molecules with an appreciable absorption oscillator component in the plane of the electrical vector of the exciting beam will be preferentially excited (Gutierrez *et al.*, 1989). If the position of the molecule remains essentially unchanged after excitation, its fluorescence will be polarized or partially polarized (Watanabe and Miyai, 1988). One of the underlying assumptions of FP is that the absorption and emission dipoles of the molecule are parallel and that the orientation of polarization is retained through excitation. This has been shown for fluorescein, a commonly used fluorophore (Entwistle and Noble, 1992; Jolley, 1981).

The emission from a fluorescent molecule is determined using a rotating polarizer. The degree of polarization (P) is given by:

$$P = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + F_{\perp}}$$

where $F_{\parallel}$ = fluorescence intensity with the emission polarizer positioned parallel to the plane of the excitation beam, and $F_{\perp}$ = fluorescence intensity with the emission polarizer positioned at right-angles to the plane of the excitation beam (Watanabe and Miyai, 1988). If excited molecules are not allowed to rotate during the time between absorption of light and the emission of fluorescence, they display a polarization value of 0.5, which is the "limiting polarization." If molecules are free to rotate between absorption and emission of light, polarization values will be less than 0.5 (Jolley, 1981).

The extent of polarization is determined by several factors, including the fluorescent lifetime (τ) and rotational relaxation time of the molecule (Q):

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{Q}\right)$$

where P = observed fluorescence polarization and P_o = limiting fluorescence polarization (Watanabe and Miyai, 1988).

The rotational relaxation time (Q) is the time required for a molecule to rotate through an angle of 68.5° due to rotational Brownian motion (Jolley, 1981). For spherical particles, Q is dependent on the solvent viscosity (η), the molar volume of the sphere (V), and the absolute temperature (T):

$$Q = \frac{3\eta V}{RT}$$

If the fluorescent lifetime (τ) of the molecule remains constant, the degree of polarization is dependent only on the rotational relaxation time of the molecule, Q. Since Q is much more dependent on the size of the molecule than on solvent viscosity or temperature, the degree of polarization gives an indication of molecular size (Jolley, 1981). Small molecules display rapid rotation and have small Q values in the order of less than 1 nsec. Consequently, they exhibit little polarization of fluorescence. Conversely,

large molecules are more restricted in their rotation; they have higher Q values (1-100 nsec), and thus cause the emitted fluorescence to be polarized to a certain degree in the plane of the excitation beam (Watanabe and Miyai, 1988).

The relationship between the molecular volume of a molecule and its ability to emit polarized fluorescence led to the application of FP to immunoassays. This was first described by Dandliker and Feigen in 1961 to measure the binding between ovalbumin and an anti-ovalbumin Ab.

From the previous discussion of the principles of FP, it is understood that the rotational velocity decreases as molecular size increases, which translates into an increase in polarization due to an increase in Q, the rotational relaxation time (Dandliker *et al*., 1964). This allows for the quantitation of the degree of binding of a small hapten or Ag to a large Ab molecule.

Small Ags or haptens (MW 1,000 - 10,000) have rapid rotational movement in solution (low Q values). Antibodies (MW ~ 150,000) rotate slowly and have large Q values. If the small Ag or hapten becomes bound to its specific Ab, however, the effective size of that Ag or hapten increases, resulting in an increased Q value for the immune complex (Watanabe and Miyai, 1988). If the Ag/hapten is labelled with a fluorescent molecule, its binding to Ab can be measured by the concomitant increase in polarization (Dandliker *et al.*, 1964; Dixon *et al.*, 1986; Smith *et al.*, 1981; Soini and Hemmilä, 1979). If free, unlabelled Ag/hapten is added to this system, the interaction between labelled analyte and Ab is inhibited. Thus, the rotational relaxation time of the labelled analyte remains low, and little polarization of fluorescence is observed (Watanabe and Miyai, 1988) (Figure 1.13). In such a competitive assay, the degree of polarization is inversely related to the level of free analyte in the standard or sample solution (Jolley, 1981). The increase in polarization that accompanies the binding of a fluorescently-labelled analyte to Ab permits discrimination between free and bound analyte in a homogeneous assay (Edwards, 1985).

Since few analytes of interest display natural fluorescent behaviour, it is usually necessary to label them with a fluorescent compound. In immunochemistry, the most commonly used fluorophores are derivatives of fluorescein and rhodamine.

Several conditions must be fulfilled in order for a fluorophore to serve as a useful label in FPIA applications. The fluorophore should exhibit a high molecular absorbance of excitation energy, and a high quantum yield under the assay conditions (Watanabe and Miyai, 1988). The quantum yield is a basic evaluation of fluorescence efficiency, and is a ratio of the number of quanta of energy emitted to the number of quanta of energy absorbed; the maximum theoretical value of 1.0 is not usually observed due to

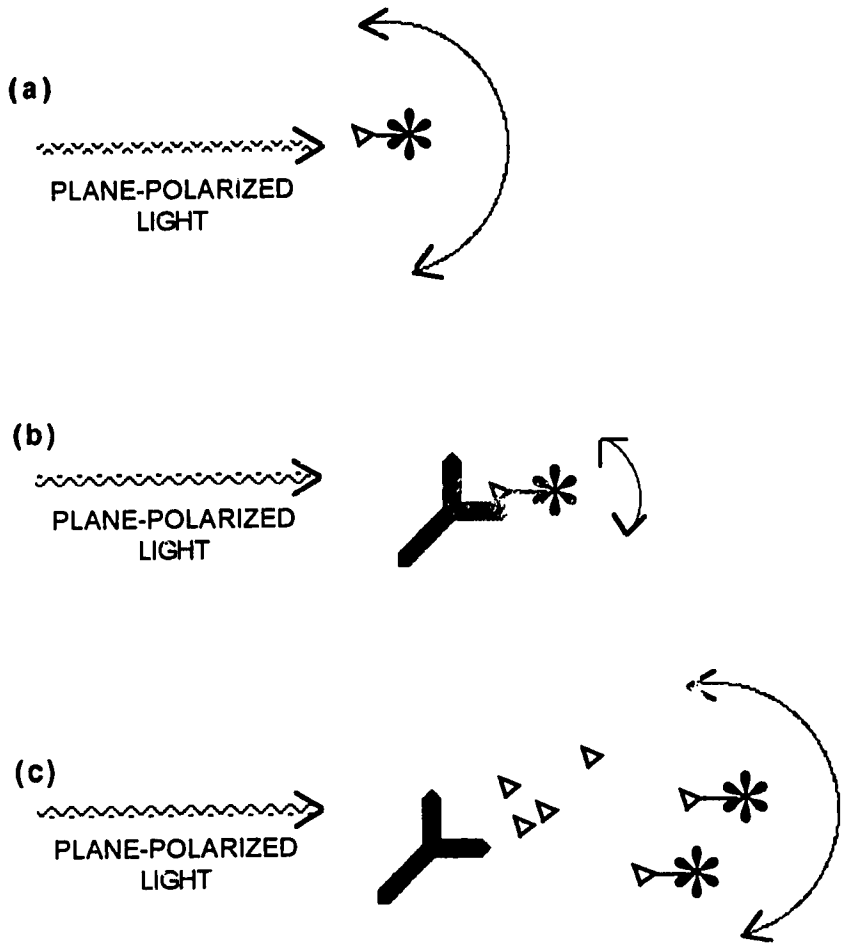


Figure 1.13 Fluorescence Polarization Immunoassay

(a) Free fluorescently-labelled hapten or Ag rotates quickly in solution (very limited FP); **(b)** Ab-Ag complex rotates slowly in solution (FP increases); **(c)** Addition of free Ag or hapten displaces label and FP values decrease.

LEGEND

= unlabelled analyte

= fluorescently-labelled analyte

= antibody

loss of energy to other processes that compete with fluorescence (Edwards, 1985). The emission wavelength should be in the 500-700 nm range, and the Stokes' shift should be greater than 50 nm, but preferably greater than 100 nm, to mitigate interference due to light scattering (Soini and Hemmilä, 1979; Watanabe and Miyai, 1988). The fluorophore should be water-soluble such that it does not significantly affect the solubility of the immunoreactant to which it is attached (Gutierrez *et al.*, 1989; Soini and Hemmilä, 1979; Watanabe and Miyai, 1988). The label should have a relatively long fluorescence lifetime (~20 nsec) that is suitable for the range of rotary Brownian diffusion constants likely to be encountered (Dandliker *et al.*, 1964; Soini and Hemmilä, 1979; Watanabe and Miyai, 1988). The fluorescently-labelled conjugates should be stable, and covalent linkage of the fluorophore to the immunoreactant should not significantly alter the immunoreactivity (Soini and Hemmilä, 1979; Watanabe and Miyai, 1988). Such conjugates should generate fluorescence that is clearly distinguishable from the background (Soini and Hemmilä, 1979).

Fluorescein and rhodamine derivatives have good chemical stability and are available in activated forms that allow for direct coupling to Ag or Ab (Edwards, 1985). While the longer emission wavelength of rhodamines relative to fluoresceins (585 nm versus 518 nm, respectively) is desirable, rhodamines exhibit lower fluorescence intensities than fluorescein, which has resulted in the latter being the label of choice in IAs (Gutierrez *et al.*, 1989; Soini and Hemmilä, 1979). The isothiocyanate form of fluorescein is particularly popular, as it retains strong fluorescence and can be coupled easily with immunoreactants containing primary or secondary amino groups at slightly alkaline pH (Edwards, 1985; Smith *et al.*, 1981; Soini and Hemmilä, 1979). Further advantages of fluorescein include the fact that the dianion form predominates in aqueous solution, resulting in good water-solubility (Gutierrez *et al.*, 1989; Smith *et al.*, 1981). Fluorescein exhibits a high quantum yield (approaching unity), and displays insignificant photolability (Gutierrez *et al.*, 1989). Fluorescein is also readily excited by common light sources, and fluoresces green, which is well-detected by conventional photomultipliers. The labelled product has a virtually indefinite lifetime and retains most of the quantum efficiency of the fluorescein molecule (Smith *et al.*, 1981).

The use of fluorescein as an immunochemical label is not without its drawbacks. With an excitation wavelength of ~490 nm and an emission wavelength of ~520 nm, it possesses a Stokes' shift of only ~30 nm, which can cause light-scattering problems. Fortunately, this can be alleviated to a degree using dedicated filter systems (Gutierrez *et al.*, 1989; Smith *et al.* , 1981). In principle, the sensitivity of a fluorimetric assay could be maximized by attaching many fluorescent molecules to the reagent of interest.

However, fluorescein is susceptible to concentration quenching in multi-substituted reagents, resulting in decreased fluorescence as labelling density increases (Bertram *et al.*, 1991; Dandliker and Feigen, 1961; Hassan *et al.*, 1979). Fluorescein-labelled analytes can also affect the sensitivity of an IA by binding non-specifically to proteins (Dandliker *et al.*, 1973); however, this appears to depend more on analyte structure than on the fluorescein molecule itself (Jolley, 1981).

The addition of a fluorescent label to a molecule must alter its chemical and physical properties to a certain degree. This may alter the immunoreactivity of the reagent, either by the labelling procedure, or simply due to the presence of a fluorescent label (Dandliker, 1977). Dandliker *et al.* (1973) found that in the case of a dansyl-labelled BSA (three dansyl groups per mole of BSA), the affinity constants of the anti-BSA Abs did not change appreciably for labelled versus unlabelled BSA; however, they postulated that the effect would be larger for smaller molecules.

Colbert *et al.* (1991) examined the effect of the proximity of the fluorescein label to a small hapten, methylamphetamine (MW 149). Several tracers were synthesized which differed in the length of the carbon bridge (from one to six carbons) between the hapten and the attached fluorescein label. These tracers were tested with an anti-methylamphetamine serum that had been raised against an immunogen with a four-carbon bridge to the carrier protein. Their results showed that a shorter bridge length resulted in a lower limit of detection and increased sensitivity

Homogeneous IAs are inherently less sensitive than their heterogeneous counterparts, as has been discussed. Interference from the sample matrix can be problematic in any homogeneous assay, but in the case of FPIA, is even more troublesome, since serum or antiserum components display a high fluorescence of their own. Moreover, high protein concentrations (>1 mg/mL) absorb excitation radiation, resulting in decreased fluorescence, while proteins and other macromolecules can cause light scattering, causing interference, particularly if the fluorophore has a small Stokes' shift (Soini and Hemmilä, 1979). Therefore, the practical limit of sensitivity depends on the magnitude of the background fluorescence arising from sample components (Dandliker, 1977) and most fluorimetric IAs are about ten times less sensitive than comparable RIA methods (Smith *et al.*, 1981). This is despite the fact that the intrinsic sensitivity of fluorescence detection is very high and much greater than that for the detection of radioactivity (Weider, 1978). The use of polarizers reduces light intensities and the potential sensitivity by about one order of magnitude (Gutierrez *et al.*, 1989).

The background signal, which is principally responsible for limiting the sensitivity of FPIA methods, arises due to both scattered light and intrinsic fluorescence, and may be reduced using labels with longer excitation and emission wavelengths than those of the background and large Stokes' shifts to avoid light scattering. Other remedies include the use of appropriate filter combinations to block scattered light, and the subtraction of a blank value (Gutierrez *et al.*, 1989).

The sensitivity of FPIAs may be increased using laser excitation of the fluorophore instead of the more conventional tungsten lamp (Soini and Hemmilä, 1979; Watanabe and Miyai, 1988). The use of lasers in excitation results in a high fluorescence emission due to a high excitation energy, leading to enhanced sensitivity in systems with negligible background. The excitation intensity of lasers also exceeds that of mercury arcs. Finally, laser tubes have long lifetimes as compared with relatively short-lived, expensive mercury bulbs (Schauenstein *et al.*, 1978).

Many of the advantages associated with FPIA methods stem from the fact that they are homogeneous assays. These advantages include speed and amenability to total automation, and the ability to perform either equilibrium or kinetic studies (Dixon *et al.*, 1986; Dandliker and de Saussure, 1970; Gutierrez *et al.*, 1989). Furthermore, it may be possible to assay several components almost simultaneously by employing differently-labelled probes or assay parameters (Spencer *et al.*, 1973).

FPIA methods are also attractive because they utilize stable, non-isotopic reagents that have indefinite lifetimes (Dixon *et al.*, 1986; Spencer *et al.*, 1973). The speed with which the fluorescent signal can be generated affords immediate end-point determination after completion of the immune reaction. This is in contrast with the use of enzymes, where enzyme activity must be determined, which increases the time, imprecision, and cost associated with the assay. Any amplification due to the enzyme will also magnify any error. Furthermore, enzymes display a greater sensitivity to changes in temperature than do fluorophores such as fluorescein (Smith *et al.*, 1981).

Perhaps the greatest advantage associated with FPIA methods is the potential long-term stability of standard curves, which enhances the between-batch reproducibility of results, as well as saving time and money (Jolley, 1981; Thomas *et al.*, 1991). Calibration curves have been shown to be stable for at least 30 days, with a projected stability of up to three months (Thomas *et al.*, 1991; Zaninotto *et al.*, 1992).

Despite the advantages attributable to FPIA, the success and widespread application of such techniques has been hampered by some associated disadvantages. Many of these disadvantages derive from the non-specific binding of

tracer to sample components and the intrinsic fluorescence of sample components, as has been discussed.

Attendant disadvantages of FPIA include its inherent lack of sensitivity due to the fact that only a small percentage of the total fluorescence intensity can be modulated (Khanna, 1988); its sensitivity is limited to the μmol/L and upper nmol/L range (Smith *et al.*, 1981). In addition, the method is limited to the measurement of small molecules with naturally low rotational relaxation times. The generally accepted upper limit is 20,000 MW (Gutierrez *et al.*, 1989; Khanna, 1988; Smith *et al.*, 1981). Colbert *et al.* (1993) imposed more stringent limitations on hapten size and suggested that FPIA techniques may not be suitable for haptens with MW greater than 400 to ensure rapid dissociation of the label from Abs to increase the speed of analysis.

The most significant impediment to the widespread application of FPIA methods has been the lack of simple, high performance instrumentation at a reasonable cost (Jolley, 1981; Smith *et al.*, 1981). The basic requirements are a fluorimeter equipped with both excitation and emission polarizers. The excitation polarizer is usually oriented to give polarization in the vertical plane, while the emission polarizer can be oriented with the polarization plane positioned either vertically or horizontally (Gutierrez *et al.*, 1989).

The problem of instrumentation has been successfully addressed by the Diagnostics Division of Abbott Laboratories (N. Chicago, IL), who developed a fully-automated system for performing FPIAs. The system is capable of analyzing 20 samples in 10 min, with coefficients of variation of 1-4%, and shows good correlation with other methods. The reagents are stable, and the standard curve can be stored in the instrument (Jolley *et al.*, 1981).

The majority of literature references regarding FPIA have described its use in therapeutic drug monitoring. Some of these studies included comparison of FPIA methods with more conventional drug monitoring methods, such as EIA, RIA, and chemiluminescent assays, as well as HPLC. Generally, results from FPIA correlate very well with those from more "standard" analytical procedures.

Najjar *et al.* (1992) compared RP-HPLC and FPIA for the analysis of methotrexate (MTX), and obtained a correlation coefficient of 0.979 in the range of 0.014 to 7.0 μg/mL MTX; however, the HPLC method was slightly more specific as it detected MTX metabolites. A monoclonal FPIA method correlated well with a monoclonal RIA method (r=0.96) for cyclosporine A (CsA) (Armijo *et al.*, 1992). In contrast to the study of Najjar *et al.* (1992), FPIA for CsA netted results 25% higher than those determined by RIA, presumably due to cross-reactivity of the MAb with CsA metabolites; this tende

to over-estimate the parent CsA value. However, coefficients of variation of less than 5% were observed for FPIA, which were 4-5 times lower than those associated with RIA and ELISA measurements. Furthermore, results could be generated in 1-2 h using FPIA, as compared with the 6-8 h required using RIA analysis.

Three anti-convulsant drugs (phenytoin, carbamazepine, and phenobarbitone) were analyzed by both FPIA and EMIT by Thomas *et al.* (1991). These authors found a correlation coefficient of 0.93 or better between the two methods for all three drugs. While FPIA displayed a longer dynamic range, both FPIA and EMIT displayed similar within- and between-batch reproducibilities.

CHAPTER 2. MATERIALS AND METHODS

2.1 REAGENTS

2.1.1 Chemicals

Solanidine, α-solanine, α-chaconine, α-tomatine, 4-dimethylaminopyridine, 25% antimony trichloride in chloroform, sodium sulphathiazole and sulphathiazole (free acid), *Limulus polyphemus* hemolymph (Type VII), glutaraldehyde (grade II, 25% aqueous solution), ammonium sulphamate, bromcresol purple (5,5'-dibromo-*o*-cresolsulphonphthalein), 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS), thimerosal, 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB-d), 3-methyl-2-benzothiazolinone hydrazone (MBTH), bovine serum albumin (fraction V) (BSA), peroxidase (Type VI, RZ=3.1), 3-dimethylaminobenzoic acid (DMAB), L-lysine, 5-aminosalicylic acid (5-AS), tetramethylbenzidine dihydrochloride tablets, and Tween 20 were obtained from the Sigma Chemical Co. (St. Louis, MO). Bromocresol green, sodium carbonate, electrophoresis grade urea, and dimethyl sulphoxide were obtained from Fisher Scientific (Edmonton, AB), while urea peroxide, protein A (PA) (from *Staphylococcus aureus*), goat anti-rabbit (GαR) IgG (Fc; Affinity-purified Ab), GαR horseradish peroxidase conjugate, and *o*-phenylenediamine (OPD) tablets were obtained from Calbiochem (San Diego, CA). Succinic anhydride was obtained from BDH, Inc. (Edmonton, AB), and 1,3-dicyclohexylcarbodiimide, N-hydroxysuccinimide, and N,N-dimethylformamide were obtained from Aldrich Chemical Co. (Milwaukee, MI). Sodium borohydride was from Anachemia (Edmonton, AB). GαR IgG (whole molecule) urease conjugate was obtained from Sigma Chemical Co. (St. Louis, MO), while urease conjugated GαR IgG (H+L) was obtained from Biodesign International (Kennebunkport, ME). Molecular Probes, Inc. (Eugene, OR) supplied the 4'-(aminomethyl)fluorescein.

All other chemicals used were of reagent grade or better. Water was purified using a Millipore Milli-Q ultrafiltration system (Millipore (Canada) Ltd., Mississauga, ON).

2.1.2 Standard Solutions

2.1.2.1 *Phosphate-Buffered Saline (PBS)*

Sodium chloride (18.0 g), disodium hydrogen phosphate (2.2 g), potassium dihydrogen phosphate (0.6 g) and thimerosal (0.2 g) were dissolved in ~1.9 L milli-Q water and the pH adjusted to 7.3 with 6 N sodium hydroxide. The total volume was made up to 2.0 L with milli-Q water.

2.1.2.2 *Phosphate-Buffered Saline with Tween (PBST)*

PBST was prepred as PBS, above, but 1.0 g Tween 20 was added before pH adjustment and dilution to final volume.

2.1.2.3 *Citrate Buffers*

Appropriate volumes of 0.1 M citric acid monohydrate and 0.1 M sodium citrate dihydrate were mixed. The pH was adjusted as needed using sodium hydroxide (6 M) or sulphuric acid (6 M).

2.1.2.4 *PBS/PBST Buffers for Urease Tests*

PBS/PBST buffers for urease tests were prepared as PBS/PBST, above, except thimerosal was omitted and 3.8 g tetrasodium ethylenediamine tetraacetate and 2.0 g sodium azide were added per 2.0 L solution.

2.1.2.5 *PBS for HRP Conjugation to Sulphathiazole (ST)*

Potassium dihydrogen phosphate (51 mL, 0.1 M), 49 mL 0.1 M sodium hydrogen phosphate and 90 mL 0.15 M sodium chloride were mixed and the pH was adjusted to 6.8 with 6 M sodium hydroxide. The mixture was diluted to 2.0 L with 0.15 M sodium chloride.

2.1.2.6 *o-Phenylenediamine (OPD) Substrate Solution*

Urea peroxide and OPD were combined in 0.1 M citrate buffer, (pH 4.75) to give final concentrations of urea peroxide and OPD of 1.0 mg/mL and 0.4 mg/mL, respectively. OPD solutions were prepared immediately prior to use.

2.1.2.7 *3,3',5,5'-Tetramethylbenzidine (TMB) Substrate Solution*

TMB-dihydrochloride (TMB-d) (0.01 g) was dissolved in 1.0 mL dimethyl sulphoxide and added to 100 mL of 0.1 M citrate buffer, pH 4.0, containing 0.10 g of urea peroxide.

2.1.2.8 *Stock Solution of AMF-SOL*

Fluorescently-labelled solanidine (AMF-SOL) was diluted to a concentration of 4140 nM in methanol and stored in the dark at 4°C. Aliquots of this stock solution were diluted as needed in PBST for use in FPIA procedures.

2.2 FOOD SAMPLES

2.2.1 Potato Samples

Potato samples A and B were greened *Solanum tuberosum* cv. Russett Burbank (harvested at soil surface) obtained from the Department of Plant Science, University of Alberta. Sample A was stored for 12 mo at 5°C in darkness, and Sample B was stored for 6 mo at 5°C in darkness then 6 mo at room temperature exposed to daylight. Sample C was a commercially obtained peeled *S. tuberosum* sample. After treatments, all samples were cut into 0.5 cm^3 pieces, freeze-dried, ground to pass through a 20-mesh screen, and stored at 5°C until needed (Plhak, 1993).

2.2.2 Honey Samples

Honey knowr to be free of ST was obtained from a local producer. Samples were diluted in milli-Q water on a w/v basis.

2.2.2.1 *Honey Dilution and Filtration*

Honey was diluted with milli-Q water on a w/v basis to dilutions of 1:2, 1:3, 1:5, or 1:10 and filtered by gravity through Whatman No. 1 filter paper.

2.2.2.2 *Reverse-Phase Treatment of Honey Samples*

Waters Sep-Pak C_{18} cartridges (Millipore Corporation, Milford, MA) were conditioned first with methanol and then water, as per the manufacturer's recommendation. Diluted ST-free honey samples (1:2, 1:10, w/v) were passed through the prepared cartridges and the eluate used directly in ELISAs.

2.2.2.3 *Preparation of Honey Analogue*

D-glucose (33.8 g) and D-fructose (38.8 g) were dissolved in milli-Q water with gentle heating, made up to 100 mL with milli-Q water, filtered through Whatman No. 1 paper, and diluted 1:2, 1:5, and 1:10 (v/v) with milli-Q water.

2.2.2.4 *pH Adjustment of Honey Samples*

The pH values of 15-mL aliquots of 1:3 aqueous honey dilutions were adjusted to approximately 4, 5, 6, 7, and 8 using 0.05 N or 0.1 N sodium hydroxide. Samples were mixed and allowed to equilibrate overnight. The pH values were checked and adjusted when necessary to approximate the target pH values indicated above. The actual pH values of the samples were determined immediately prior to ELISA analysis.

2.2.2.5 *pH Adjustment of a 1:10 Dilution of Honey*

A 1:10 dilution (w/v) of honey was prepared using a 40:60 (v/v) solution of 0.02 N sodium hydroxide/PBST and the pH adjusted to 7.4 using 1 N hydrochloric acid.

2.2.3 Milk Samples

Homogenized milk samples (3.5% butter fat) were obtained from a local supermarket at least 10 days prior to the "Best Before" date displayed on the carton. Milk was used directly as purchased in ELISA protocols.

2.3 EQUIPMENT

An Accumet 925 pH/ion meter was used to measure pH values (Fisher Scientific, Edmonton, AB). Dialysis tubing was Spectra/Por Membrane 1, molecular weight cut-off (MWCO) 6,000-8,000, and Membrane 2 (MWCO 12,000-14,000), manufactured by Spectrum Medical Industries, Inc. (Los Angeles, CA). Immulon 2 polystyrene microtitre plates were from Dynatech Laboratories, Inc. (Chantilly, VA) and were read either on an Automated Microplate Reader, Model EL309, from Bio-Tek Instruments, Inc. (Burlington, VT), or on a Conductance ELISA Reader, which was prototype property of Janus Technologies, Inc. (Lexena, KS), graciously loaned to our laboratory for urease studies. Samples were flash-evaporated using a Büchi Rotavapor RE 121 with a Büchi 461 water bath, both from Büchi, Switzerland and obtained through Brinkmann Instruments (Canada) Ltd. (Mississauga, ON). Samples were lyophilized using a Virtis 5L freeze-dryer from The Virtis Company, Inc. (Gardiner, NY). Fluorescence polarization measurements were performed using a Perkin-Elmer LS50B Luminescence Spectrometer equipped with a pulsed xenon lamp and operating in the L-format (Perkin-Elmer, Buckinghamshire, England). Data were analyzed using the fluorescence data manager package supplied with the luminescence spectrometer on a 486DX-33 cpu computer. Spectrosil quartz fluorescence cuvettes (10 mm path length; volume = 520 μL) were obtained from Starna Cells, Inc. (Atascadero, CA). Linbro acetate adhesive plate sealers were obtained from ICN Biomedicals (Costa Mesa, CA). Competitive inhibition curves from IA data were analyzed using *SOFTmax* software obtained from Molecular Devices Corporation (Menlo Park, CA). Silica gel Kieselgel $60F_{254}$ (0.2 mm thick) aluminum sheets were used to follow reactions, while silica gel Kieselgel $60F_{254}$ 20x20 cm, 1000 μm thick plates (both obtained from E. Merck, Darmstadt, Germany) were used for preparative TLC. Waters C_{18} Sep-Pak cartridges were obtained from the Millipore Corporation (Milford, MA). Spectophotometric

measurements were made using an HP8451A Diode Array Spectrophotometer (Hewlett Packard, Canada, Ltd., Mississauga, ON); samples were contained in UV semi-micro cuvettes (Fisher Scientific, Edmonton, AB). Homogenization was performed using a Kinematica PT10/35 Polytron homogenizer from Brinkmann Instruments, Rexdale, ON.

2.4 METHODOLOGY

2.4.1 Synthesis of Immunoassay Conjugates

2.4.1.1 *Synthesis of BSA-Sulphathiazole (BSA-ST) Coating Conjugates*

The BSA-ST coating conjugate was synthesized by the diazo reaction according to the method of Sheth *et al.* (1990). Sodium sulphathiazole (109 mg; 393 μmoles) was dissolved in 1.0 mL 3.5 N hydrochloric acid, to which 3.0 mL of a 1% solution of sodium nitrite was added. After 10 min stirring at room temperature, 50 mg ammonium sulphamate was added, and the reaction stirred another 10 min. This reaction mixture was added to 202 mg bovine serum albumin (BSA) dissolved in 0.5 mL milli-Q water and 1.0 mL 1.0 M sodium carbonate; 150 mg sodium carbonate was then added. The initial reaction flask was washed with 3x25 mL 1.0 M sodium carbonate; these washings were added to the BSA-ST reaction mixture. The entire reaction mixture was stirred overnight at 4°C. The following day, the reaction mixture was quantitatively transferred to dialysis tubing (MWCO 6,000-8,000) using 8.0 M urea, and dialyzed against 1 L of 8.0 M urea, followed by dialysis versus 4 L of 50 mM ammonium bicarbonate and 4 L of 25 mM ammonium bicarbonate. Dialysis against each solution was performed at room temperature for a minimum of 8 h. The dialyzed reaction mixture was freeze-dried and stored in a desiccator. A second, "lightly-loaded" BSA-ST conjugate was prepared as above using 9.34 mg (33.7 μmoles) sodium sulphathiazole and 202 mg BSA.

2.4.1.1.1 *Analysis of BSA-ST Conjugates*

Microanalyses of the conjugates were performed by the laboratories of the University of Alberta Chemistry Services. Carbon, nitrogen, and hydrogen were measured using a Perkin-Elmer 240 CHN Analyzer. Sulphur content was determined by barium perchlorate titration following sample pyrolysis in a nitrogen atmosphere.

2.4.1.2 *Synthesis of HRP-Sulphathiazole (HRP-ST) Conjugates*

2.4.1.2.1 *Two-Step Glutaraldehyde Method*

The general procedure of Dixon-Holland and Katz (1991) was employed. HRP was activated by dissolving 10.8 mg HRP in 200 μL of 1.25% glutaraldehyde in PBS

and stirring gently at 4°C for 17.5 h. The reaction mixture was transferred quantitatively to dialysis tubing (MWCO 6,000-8,000) and dialyzed at 4°C against 3 changes of 500 mL aqueous normal saline (NS) (8.5 g sodium chloride/L) for at least 3 h. ST conjugation solution was prepared by adding 5.7 mg of ST (free acid) (22.3 μmoles) to 0.5 mL NS, followed by the addition of 100 μL 1 N sodium hydroxide. This was added to the dialyzed mixture and the volume adjusted to 1.0 mL with NS. After the addition of 100 μL 1 M carbonate-bicarbonate buffer (pH 9.5), the reaction was stirred for 24 h at 4°C. At the end of 24 h, 100 μL of 0.2 M L-lysine were added, and the mixture incubated for 2 h at 4 °C. The reaction mixture was dialyzed as above against 4 changes (500 mL) of PBS. The contents of the dialysis tubing (1.0 mL) were recovered and stored in 0.25 mL aliquots at -20°C. In an attempt to alter the degree of conjugation, a total of three HRP-ST conjugates was synthesized using the glutaraldehyde method using the amounts of ST and HRP shown in Table 2.1.

Table 2.1 HRP-ST Conjugates Synthesized Using Glutaraldehyde

CONJUGATE	mg ST	mg HRP	Molar Ratio (ST:HRP)
G-Lite	3.0	11.3	46
G-Med	5.7	10.8	91
G-Heavy	11.5	11.7	169

2.4.1.2.2 *Periodate Cleavage Method*

The procedure employed is described below and was based on the method of Wilson and Nakane (1978). HRP (4.1 mg) was dissolved in 1.0 mL milli-Q water, to which was added 200 μL of 100 mM aqueous sodium periodate. This mixture was stirred for 20 min at room temperature, transferred to dialysis tubing (MWCO 12,000-14,000) and dialyzed overnight (~18 h) against 1 L of 1 mM sodium acetate buffer (pH 4.4) at 4°C. The next day, the contents of the dialysis tubing were transferred to a small reaction vessel, to which was added 20 μL of 200 mM carbonate buffer (pH 9.5) with two drops of 1 N sodium hydroxide to dissolve ST. This reaction mixture was stirred for 2 h at 4°C. After the addition of 100 μL of sodium borohydride (4 mg/mL in milli-Q water), the mixture was stirred for 2 h at 4°C. The contents of the reaction vessel were transferred to dialysis tubing, as above, and dialyzed against 3 changes

of PBS (without thimerosal) for a minimum of 3 h. The contents of the dialysis tubing (~2 mL) were recovered and frozen in 0.5 mL aliquots at -20°C. Similar to the conjugates synthesized by the two-step glutaraldehyde method, a total of three conjugates was produced by the periodate cleavage method (Table 2.2).

Table 2.2 HRP-ST Conjugates Synthesized Using the Periodate Cleavage Method

CONJUGATE	mg ST	mg HRP	Molar Ratio (ST:HRP)
P-Lite	2.4	4.6	90
P-Med	8.9	4.1	375
P-Heavy	81.5	4.6	3044

2.4.1.3 *Synthesis of Fluorescently-Labelled Solanidine*

2.4.1.3.1 *Synthesis of Solanidine Hemisuccinate*

A succinylated derivative of solanidine was synthesized according to the method of Plhak and Sporns (1992) (Figure 2.1). Briefly, solanidine (40.5 mg; 101.9 μmoles) was reacted with 422 mg (0.0042 μmoles) succinic anhydride in dry pyridine along with 26 mg (212.8 μmoles) 4-dimethylaminopyridine. The mixture was heated under reflux, with stirring, at 60°C for 119 h. The reaction was followed by TLC using an ethyl acetate/methanol/1% aqueous ammonia (80:20:1 v/v/v) solvent system. Glycoalkaloid products were visualized by spraying with 25% antimony trichloride in chloroform and heating gently. Unreacted succinic anhydride was visualized using 0.04% bromocresol green in ethanol, made green with 6 N sodium hydroxide. The reaction was halted by the addition of 20 mL water, and the reaction mixture extracted with 3X25 mL methylene chloride. The organic extracts were combined and rotoevaporated once. The reaction product was co-evaporated with 3x2 mL toluene. The product was taken up again in 30 mL methylene chloride and washed with 3x30 mL milli-Q water to remove unreacted succinic anhydride. The methylene chloride was removed under reduced pressure, and the residue dried under vacuum over phosphorus pentoxide. The 40.1 mg of solanidine hemisuccinate (MW 497.7, represented a 79% yield.

R = solanidine

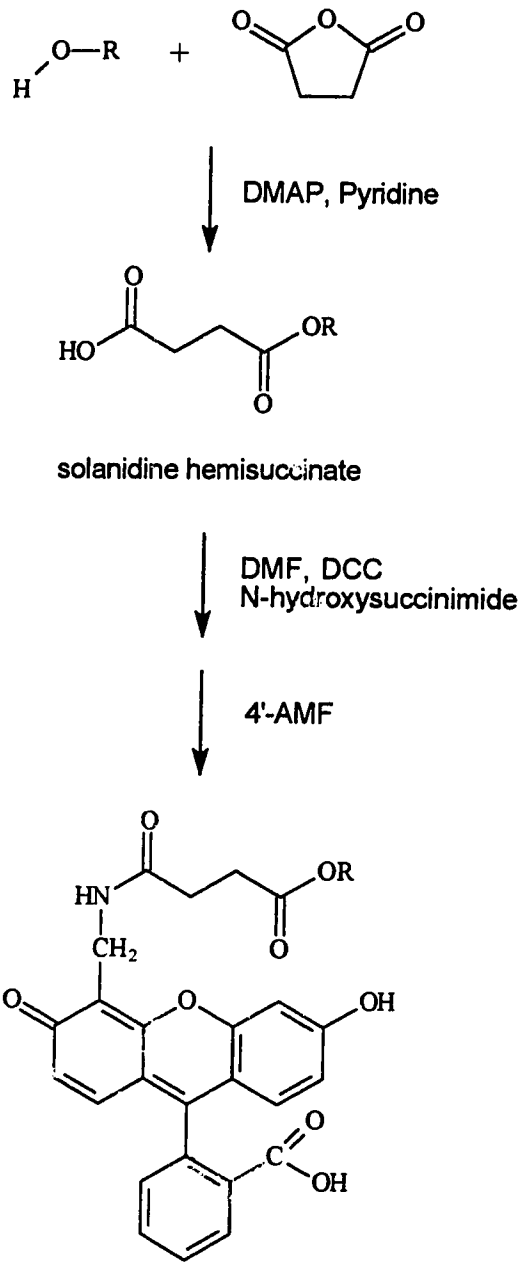


Figure 2.1 Reaction Scheme for the Synthesis of Fluorescently-Labelled Solanidine (AMF-SOL)

2.4.1.3.2 *Conjugation of Solanidine Hemisuccinate to Fluorescein Derivative*

The solanidine hemisuccinate was coupled to a fluorescein derivative using the active ester method of Shipchandler *et al.* (1987) (Figure 2.1). Solanidine hemisuccinate (21.1 mg; 42.2 μmoles) was reacted with 4.18 mg (36.3 μmoles) N-hydroxysuccinimide and 6.24 mg (30.2 μmoles) 1,3-dicyclohexylcarbodiimide in 625 μL dry N,N-dimethylformamide (DMF) at room temperature for 30 h. Urea formed during the reaction was removed by filtration. To the active ester was added 12.42 mg (31.2 μmoles) 4'-(aminomethyl)fluorescein dihydrochloride (4'-AMF), 12.5 μL triethylamine and 207.8 μL dry DMF. The reaction mixture was stirred at room temperature for 20 h, and the DMF removed under reduced pressure. The fluorescein-labelled solanidine was purified by normal-phase preparative TLC using a solvent system of ethyl acetate:methanol:1% aqueous ammonia (80:20:1 v/v/v). Fluorescently-labelled solanidine was scraped from the plate, dissolved in methylene chloride, and the solid phase removed by filtration. Methylene chloride was removed under reduced pressure, and the residue dried under vacuum over phosphorus pentoxide. The orange-coloured product (AMF-SOL) (15.3 mg; 18.2 μmoles) obtained was stored at -20°C.

2.4.2 Preparation of Antisera for IA Procedures

2.4.2.1 *Anti-Sulphathiazole Polyclonal Antibody Serum*

Polyclonal antiserum was raised against an ST-LPH conjugate in two female Flemish Giant X Dutch Lop Ear rabbits as detailed by Sheth (1991).

2.4.2.2 *Anti-Chaconine Sera*

2.4.2.2.1 *Polyclonal Anti-Chaconine Serum*

Polyclonal rabbit antiserum was synthesized by and obtained from Plhak and Sporns (1992). The immunogen was prepared by hydrolyzing α-chaconine under acidic conditions to γ-chaconine, which was linked through the remaining glucose residue to LPH using periodate cleavage followed by sodium cyanoborohydride reduction. Abs were raised in 2 Flemish Giant X Dutch Lop Ear male rabbits.

2.4.2.2.2 *Preparation of Anti-Chaconine Monoclonal Antibodies*

Monoclonal antibodies were prepared against a γ-chaconine-LPH conjugate by Plhak and Sporns (1994). The two MAb fractions used in this study were obtained from hybridoma grown in serum-free RPMI with 1% Nutridoma, and precipitated using 0-50% or 50-60% ammonium sulphate (Plhak, 1993). Frozen MAb preparations (in PBS) were thawed and centrifuged at 14,000xg for 20 min at 4°C to remove excessive

cloudiness, presumably resulting from denatured MAb. Protein concentration, as IgG, was determined by measuring absorbance values at 280 nm (A_{280}) and 350 nm (A_{350}) against a PBS blank, and using the following equation adapted from Harlow and Lane (1988):

$$[\text{PROTEIN}] = \frac{A_{280} - A_{350}}{1.35} (\text{mg / mL})$$

2.4.3 IA Protocols

2.4.3.1 *Standard Indirect Competitive ELISA Protocol*

Microtitre plates were coated with 200 µL/well of ST-BSA diluted to the appropriate concentration in PBS, covered with adhesive acetate plate sealers and incubated overnight (~18 h) at 4°C. All remaining incubations were performed at room temperature. The next morning, the BSA-ST solution was shaken from the wells by inverting the microtitre plates with a sharp turn of the wrist; microtitre plates were tapped dry on paper towelling. Wells were "blocked" using 1% BSA in PBS (200 µL/well). Microtitre plates were covered as before and incubated for 1 h. After removal of the blocking solution, microtitre plates were washed using a general washing procedure (3x200 µL/well aliquots of PBST). Serum diluted in 0.05% BSA in PBST (100 µL/well) and 100 µL/well of aqueous ST standard were added to microtitre plates, which were covered and incubated for 2 h, followed by a general washing. Enzyme-conjugated secondary Ab (GαR) was added to the microtitre plates (200 µL/well); incubation in covered microtitre plates proceeded for 2 h. Following a final general washing, enzyme substrate was added. Enzyme signal was read spectrophotometrically at the appropriate wavelength or using a conductance ELISA reader.

2.4.3.2 *Standard Direct Competitive ELISA Protocol*

Microtitre plates were pre-coated with 1.0 µg/mL Protein A (100 µL/well) (Schneider and Hammock, 1992), covered as in Section 2.4.3.1 and incubated overnight (~18 h) at 4°C. All further incubations were carried out at room temperature. The next day, the solution was removed from the wells as detailed in Section 2.4.3.1, and serum added (200 µL/well) at the appropriate dilution; microtitre plates were re-covered and incubated 2 h. After removal of serum, microtitre plates were blocked as in Section 2.4.3.1. The blocking solution was removed from the microtitre plates and a general washing performed. HRP-ST (P-Med) (100 µL/well) diluted 1:2,000 in PBST and

100 µL/well of aqueous ST standard were added to microtitre plates, which were covered and incubated for a further 2 h. After a final general washing, TMB substrate solution (Section 2.1.2.7) was added (200 µL/well) and the blue colour allowed to develop at room temperature. The reaction was stopped by adding 50 µL/well 2 M sulphuric acid; absorbance values were read at 450 nm.

2.4.3.2.1 *Pre-Coating with Goat-anti-Rabbit F_C-Specific Antibodies*

GαR F_C-specific Abs were diluted to a concentration of 0.5 or 1.0 µg/mL in PBS; 100 µL of this solution were added to the wells of a microtitre plate.

2.4.3.3 *Checkerboard Titration (Direct ELISA Format)*

Using the standard direct ELISA protocol (Section 2.4.3.2), microtitre plates were pre-coated (overnight incubation at 4°C) with either 100 µL of 1 µg/mL Protein A or GαR F_C-specific Abs. (In the case of addition of antiserum directly to the plate, 200 µL/well of anti-ST antiserum diluted in PBS were added to the wells at the same concentrations and the same configuration as noted below. Incubation proceeded overnight at 4°C). Serum was diluted in doubling dilutions (1:100 to 1:1,024,000); 100 µL of each concentration were added to the columns of a microtitre plate (i.e., concentration of serum decreased horizontally across the plate). Incubation proceeded for 2 h at room temperature. HRP-ST (G-Med), in doubling dilutions (1:500 to 1:32,000) was added (100 µL/well) to the rows of the microtitre plate; G-Med concentrations decreased vertically on the plate. (For plates receiving no Protein A or GαR Ab pre-treatment, 200 µL/well of G-Med were added). Incubation in covered microtitre plates proceeded for 2 h at room temperature. Following the final general washing, TMB substrate solution added (200 µL/well). After a suitable incubation time (5 to 30 min), the enzyme reaction was halted by adding 50 µL/well of 2 M sulphuric acid. Absorbance values were read at 450 nm. The levels of serum and G-Med in wells showing acceptable absorbance values (~0.2 OD) were further analyzed in direct competitive ELISAs (Section 2.4.3.2).

2.4.3.4 *Competition Step for ST Analysis in Milk and Honey Samples*

To avoid the influence of dilution on the performance of the direct ELISA in milk and honey, the competition step was established in a slightly different manner than that outlined in the standard protocol. Milk or diluted honey sample (75 µL) was added to the wells of the serum-treated microtitre plate, along with 25 µL of aqueous ST standards. To this was added 100 µL of P-Med diluted in PBST. Competition proceeded for 2 h at room temperature in covered microtitre plates. All other procedures

prior to and following the competition step were as outlined in the standard protocol (Section 2.4.3.2).

2.4.3.5 *Standard Competitive FPIA Protocol*

Equal volumes (usually 0.5 mL) each of 180 nM AMF-SOL in PBST, diluted MAb or PAb, and diluted glycoalkaloid or alkaloid standards were mixed in a test tube. Reaction proceeded for 30 min at room temperature. FP readings were measured using an excitation wavelength of 496 nm (2.5 nm slit width) and an emission wavelength of 518 nm (7.5 nm slit width).

2.4.4 IA Data Analysis

Absorbance values generated in competitive IA procedures were analyzed using *SOFTmax* to generate standard competitive inhibition curves (Figure 1.9). The *SOFTmax* program uses the highest and lowest analyte concentration values to establish the "d" and "a" parameters, respectively, of the standard curve. The "a" value refers to the maximum signal obtainable in the presence of vanishingly small amounts of analyte, while the "d" value denotes the signal in the presence of infinite concentrations of analyte. After fixing the values for "a" and "d", the program computes values for the slope ("b") and inflection point ("c") of the curve. The true values for "a" and "d" are computed by 100 iterations of the data. The program also generates a correlation coefficient which takes into account all of the values used in the raw data set to generate the curve.

A certain amount of variation is associated with the standard curve described above; the error is greatest at the extremes of the curve (highest and lowest analyte concentrations) and decreases as one moves inward toward the inflection point of the curve ("c" value). For this reason, the "c" value is a useful parameter to use in evaluating ELISA results. Furthermore, the "c" value accounts for background signal, while the popular I_{50} value does not. (The I_{50} value is the concentration of analyte required to reduce the maximum signal by 50%, regardless of the background signal). When "a" values are quite high (e.g., an optical density of 1.0), the "c" values and I_{50} values are quite similar. However, if the "a" value is limited to lower optical density values (e.g., 0.2), the background can account for a larger proportion of the "a" values; therefore, I_{50} values become misleading.

Considering that "a" absorbance values were purposely kept quite low in ELISAs using HRP and a chromogenic substrate in this study, it was felt that data could best be compared using the "c" values obtained from the *SOFTmax* program.

2.4.5 pH Profiling of HRP Chromogens and Evaluation of Stopping Reagents

Using the standard indirect ELISA protocol (Section 2.4.3.1), all chromogens were tested using a BSA-ST concentration of 0.25 µg/mL and a serum dilution of 1:150,000. Serum was added at 200 µL/well; no ST competitor was used. All substrate solutions contained 1.0 mg/mL urea peroxide.

2.4.5.1 *OPD*

OPD was dissolved at a concentration of 3.7 mM directly in the 0.1 M citrate buffer series (pH 3 to 6, in 0.5 pH unit increments). Absorbance values were measured at 450 nm for the unstopped reaction. Stopping reagents (6 N sodium hydroxide, 3 and 6 M sulphuric acid, and 1% SDS) were added at the rate of 50 µL/well. Wavelength maxima, as determined by an absorption spectrum on reaction products, are given in Table 2.3.

Table 2.3 Wavelength Maxima for Reaction Products of OPD with HRP and Hydrogen Peroxide

REACTION COMPONENTS	λ_{max} (nm)
OPD + Sodium hydroxide	420
OPD + SDS	450
OPD + Sulphuric Acid	490

2.4.5.2 *MBTH-DMAB*

MBTH was dissolved in methanol (37 mM) and diluted 1:100 into 0.1 M citrate buffers (pH 3.0 to 7.0 in 0.5 pH unit increments) to a final concentration of MBTH of 0.37 mM. DMAB was dissolved at a concentration of 185 mM in methanol; this solution was diluted 1:10 into the citrate buffer series to give a DMAB concentration of 18.5 mM. Equal volumes of 0.37 mM MBTH and 18.5 mM DMAB solutions in citrate buffers were mixed to give final concentrations of MBTH and DMAB of 0.185 mM and 9.25 mM, respectively. The wavelengths 540 nm and 660 nm were both tested to determine their suitability for monitoring the colour development with MBTH-DMAB and HRP. The reaction was stopped by the addition of 50 µL/well of 1% SDS or 6 M sulphuric acid. Absorbance values for the unstopped and stopped reaction products were measured at 540 nm.

2.4.5.3 *ABTS*

ABTS solutions were prepared in the citrate buffers (pH 3.0 to 7.0 in 0.5 pH unit increments) at a concentration of 3.7 mM; the reaction products were measured at 405 nm. The reaction was stopped by the addition of 20 µL/well of 1% SDS and absorbance values were read at 405 nm.

2.4.5.4 *TMB*

TMB (37 mM in dimethyl sulphoxide) was diluted 1:100 into the 0.1 M citrate buffer series (pH 3.0 to 6.0 in 0.5 pH unit increments) to give a final concentration of 0.37 mM. Unstopped reaction products were measured at 405 nm, 450 nm, and 660 nm in preliminary studies. Stopping reagents were 6 M and 2 M sulphuric acid and 1% SDS (added at 50 µL/well). After stopping with sulphuric acid, absorbance values were read at 450 nm. Reaction products resulting from the addition of SDS were read at 660 nm.

2.4.5.4.1 *TMB Tablets*

TMB-dihydrochloride tablets (1 mg) were dissolved in 0.1 M citrate buffer (pH 4.0). Urea peroxide was included at 1.0 mg/mL. Absorbance values were read at 660 nm before the addition of 6 M sulphuric acid (50 µL/well), after which they were read at 450 nm.

2.4.5.4.2 *Use of TMB as HRP Chromogen in a Soluble System*

A 37 mM solution of TMB-d in dimethyl sulphoxide was prepared and diluted (1:100) into 0.1 M citrate buffers (pH 3.0 to 6.0 in 0.5 pH unit increments); urea peroxide was added to a concentration of 1.0 mg/mL. Aliquots (5.0 mL) of the substrate solutions were dispensed into tubes, to which 50 µL of a 1:30,000 dilution of HRP conjugate in PBST was added. Once a suitable level of colour had developed, the reaction solutions were scanned spectrophotometrically (350-800 nm). SDS (1% solution, 50 µL/tube) was added to the mixtures to stop the reaction.

2.4.5.4.3 Comparison of OPD and TMB as Chromogenic Substrates for HRP in ELISA

The standard indirect competitive protocol was used (Section 2.4.3.1). Microtitre plates were coated with 0.25 µg/mL BSA-ST and serum dilution was 1:150,000. Microtitre plates were divided into two parts to accommodate the testing of OPD and TMB on the same plate. OPD was prepared in 0.1 M citrate buffer pH 5.0 (3.7 mM); TMB was prepared in pH 4.0 0.1 M citrate buffer (final concentration of 0.37 mM). After

10 min colour development, absorbance values of the contents of the wells were read at 450 nm (OPD) and 660 nm (TMB). Reactions were stopped by the addition of 50 μL/well 6 M sulphuric acid or 1% SDS; absorbance values were read at the wavelengths cited in Table 2.4. Absorbance values were read again after 60 min.

Table 2.4 Wavelength Maxima for OPD and TMB HRP Reaction Products with SDS and Sulphuric Acid as Stopping Reagents

STOPPING REAGENT	λ_{max} (nm)	
	TMB	OPD
Sulphuric acid	450	490
1% SDS	660	450

2.4.6 Optimization of Indirect Competitive ELISA for ST Using TMB as HRP Chromogen

A checkerboard assay was performed to select the levels of serum and coating conjugate to be used in the ELISA. BSA-ST was diluted in PBS to concentrations ranging from 0.016 μg/mL to 2.0 μg/mL in doubling dilutions and added to each of the wells (200 μL/well) in a single row of microtitre plates. Serum dilutions were prepared from 1:1,000 to 1:512,000 in doubling dilutions, and plated out horizontally (i.e., one concentration per column) on microtitre plates as per the standard indirect competitive protocol (Section 2.4.3.1). From these results, the coating conjugate concentrations and serum dilutions shown in Table 2.5 were selected and evaluated using TMB as the HRP chromogen. These combinations were tested using aqueous ST standards of 0, 0.5, 1, 5, 10, 50, 100, and 1,000 ppb. After the limiting concentration of coating conjugate had been established (0.031 μg/mL), serum dilutions of 1:500,000, 1:750,000, 1:100,000, and 1:150,000 were tested to determine the limiting level of serum. Microtitre plates were developed with TMB substrate and stopped by the addition of 50 μL/well of 6 M sulphuric acid. Absorbance values were read at 450 nm.

Table 2.5 Coating Conjugate Concentration and Serum Dilution Combinations Tested in Indirect Competitive ELISA for ST Using TMB

COATING CONJUGATE (μg/mL)	ANTISERUM DILUTION
0.03125	1:128,000
0.0625	1:256,000
0.125	1:512,000
0.0625	1:512,000

2.4.7 Indirect Competitive ELISA for ST Using HRP, TMB, and a Separate Incubation Step

Microtitre plates were prepared as per the standard indirect protocol (Section 2.4.3.1) using a coating concentration of 0.125 μg/mL BSA-ST. Blocked microtitre plates were divided into two parts, one-half to accommodate serum and ST standards according to the standard protocol, and one-half to accommodate serum pre-incubated with ST. The levels of ST used in both protocols were 0, 0.5, 1, 5, 10, 50, 100, and 1,000 ppb. A series of test tubes was set up, each tube containing 1.5 mL of serum (1:500,000 dilution). To this was added 1.5 mL of the aqueous ST standards, at the concentrations noted above. Tube contents were mixed, and the tubes covered and incubated at room temperature for 2 h, after which 200 μL aliquots of the Ab-ST solutions were dispensed into the wells of the previously washed and blocked microtitre plate. Analysis proceeded as per the standard protocol (Section 2.4.3.1). Microtitre plates were developed with TMB substrate for 20 min at room temperature, and stopped by adding 50 μL/well 2 M sulphuric acid. Absorbance readings at 450 nm were recorded.

2.4.8 Selection of HRP-ST Conjugate for Use in Direct Competitive ELISAs for ST

The selection of the HRP-ST conjugate for use in direct competitive ELISAs was based on absorbance units generated (using TMB as substrate and a 2 M sulphuric acid stopping solution) per mg of enzyme present. Microtitre plates were coated overnight (4°C) with 100 μL/well Protein A as per the standard direct competitive protocol (Section 2.4.3.2). The microtitre plate was divided such that 6 wells were dedicated to each treatment of HRP-ST. Each conjugate was tested at two dilutions (1:1,000 and 1:2,000). Following 2 h of incubation at room temperature with HRP-ST,

the microtitre plates were washed and developed with TMB for 10 min, and 50 µL/well of 2 M sulphuric acid added to stop the reaction. Microtitre plates were read at 450 nm. Using the amounts of enzyme employed in synthesizing the conjugate, and the dilution factor, it was possible to calculate the relative efficiency of the enzyme in each treatment.

2.4.9 Testing of HRP-ST for EMIT Effects

HRP-ST G-Lite was employed in the test for EMIT effects. A 1:1,000 dilution of HRP-ST was prepared in PBST. Doubling dilutions of antiserum (1:500 to 1:1,024,000) were prepared in 0.05% BSA in PBST. Diluted antiserum (50 µL) were added to the wells of a microtitre plate, one dilution per row. To each well was added 50 µL of diluted G-Lite. Some wells on the microtitre plate contained no Ab, but held only G-Lite. The microtitre plates were covered and incubated for 2 h at room temperature and developed using 100 µL/well TMB substrate. Colour developed immediately and the reaction was stopped by the addition of 50 µL/well of 2 M sulphuric acid. The contents of the microtitre plate were diluted 1:4 (v/v) with milli-Q water, and the absorbance values measured at 450 nm.

2.4.10 Determination of Optimum Serum Dilutions for Honey Analysis in a Direct ELISA

Protein-A-coated microtitre plates were treated with various dilutions of anti-ST PAb (1:6,250, 1:12,500, 1:18,750, and 1:25,000) to determine the lowest concentration of serum to be used in the direct ELISA using aqueous ST standards. The standard direct ELISA protocol was employed, in which 25 µL of aqueous ST standards were mixed in the wells with 75 µL of honey diluted 1:10 (w/v) in water along with 100 µL of P-Med diluted 1:2,000. Final concentrations of ST in the wells were 0, 0.5, 1, 4, 10, 31, 206, 413, and 2,063 ppb. After colour development with TMB (30 min) and 2 M sulphuric acid addition, absorbance values were read at 450 nm.

2.4.11 Analysis of Milk Samples for ST

2.4.11.1 *Direct Competitive ELISA for ST in Milk*

Using the direct competitive protocol for food samples (Section 2.4.3.4) and serum dilutions of 1:6,250, 1:12,500, 1:18,750, and 1:25,000, milk was analyzed using ST standard concentrations of 0, 0.5, 1, 4, 10, 31, 206, 413, and 2,063 ppb. HRP-ST conjugate was P-Med at a dilution of 1:2,000. Microtitre plates were developed for 30 min with TMB, after which 50 µL/well 2 M sulphuric acid was added. Absorbance values were read at 450 nm.

2.4.11.2 *Indirect Competitive ELISA for ST in Milk*

Using the standard indirect competitive protocol (Section 2.4.3.1) coating conjugate was tested at concentrations of 0.1, 0.5, and 1.0 µg/mL. The corresponding serum dilutions evaluated are shown in Table 2.6.

Table 2.6 Coating Conjugate Levels and Serum Dilutions Tested in an Indirect ELISA for ST in Milk

COATING CONJUGATE(µg/mL)	JM DILUTIONS
0.1	1:50[illegible]00, 1:[illegible]0,000
0.5	1:100,000, 1:200,000, 1:300,000,1:500,000, and 1:700,000
1.0	1:50,000, 1:100,000, 1:150,000,1:200,000, 1:300,000, and 1:400,000

Colour was developed (30 min) using TMB substrate. The reaction was stopped by adding 50 µL/well 2 M sulphuric acid; absorbance values were read at 450 nm.

2.[illegible] Evaluation of Urease as a Marker Enzyme in ELISA

[illegible]esting of Conductance Reader with Sodium Chloride

[illegible]rial dilutions of s[illegible]dium chloride in Milli-Q water were prepared (0.0061 mM to 100 m[illegible] in doubling dilu[illegible] and dispensed into the wells of a microtitre plate (200 µL/well). Conductance readings were obtained for each well. Detection limit was set as the mean background conductance value of Milli-Q water plus 3 standard deviations of the mean.

2.4.12.2 *Titration of Urease Conjugate*

The working dilution of the urease conjugate was determined using the standard indirect competitive protocol (Section 2.4.3.2). Serum was diluted 1:5,000 and BSA-ST concentration was 1.0 µg/mL. GαR urease-conjugated antibody (either from Sigma Chemical Company or Biodesign International, Inc.) was diluted in PBST in doubling dilutions from 1:200 to 1:6,400 and replaced the HRP conjugate in the standard protocol. Several wells received only Milli-Q wate[illegible]is point to serve as enzyme-free blanks. Following the final general washing with PBST, microtitre plates were washed 5 times

(200 µL/well) with Milli-Q water. Substrate solution (1.0 mg/mL urea in Milli-Q water) was added to each well (200 µL/well). After 30 min, the conductance value of each well was read using the conductance ELISA reader.

2.4.12.3 *Kinetics of Urease Reaction*

The linearity of the reaction of urease with urea over time was determined using a 1:1,000 dilution of GαR-urease conjugate in Milli-Q water (100 µL/well) with 100 µL/well of 1.0 mg/mL urea in Milli-Q water. Conductance readings were taken approximately every 5 min to 30 min.

2.4.12.4 *Inhibition of Peroxidase Conjugate Binding by Urease Conjugate Binding*

Microtitre plates were prepared according to the standard indirect competitive protocol (Section 2.4.3.1) using 1.0 µg/mL BSA-ST; serum dilution was 1:5,000. Following blocking and general washing, doubling dilutions of urease conjugate (1:200 to 1:6,400) were prepared in a 1:3,000 dilution of HRP-Ab in PBST and added to one-half of the microtitre plate (200 µL/well). At the same time, ten-fold dilutions of HRP-Ab in PBST (1:3,000 to 1:3,000,000) were added to the other half of the same microtitre plate (200 µL/well) to determine conjugate binding in the absence of urease conjugate. The rest of the steps were as for the standard protocol. It should be noted that "regular" PBST was employed (i.e., with thimerosal), as only the Ab fraction of the urease conjugate was being tested. Microtitre plates were developed (4 min) using TMB substrate; 50 µL/well of 2 M sulphuric acid were then added and the absorbance values read at 450 nm.

2.4.12.5 *Comparison of Urease- and HRP-Ab Conjugates in an Indirect ELISA for ST*

The performance of the urease conjugate was tested under conditions that had previously been found to be optimal for the HRP conjugate in an indirect ELISA for ST. The general indirect competitive ELISA protocol was used (Section 2.4.3.1). Microtitre plates were coated with 0.125 µg/mL of BSA-ST conjugate, and serum was diluted 1:500,000. Levels of ST used in competition were 0, 0.5, 1, 5, 10, 50, 100, and 1000 ppb. Urease conjugate was diluted 1:200 in PBST (without thimerosal) and added to one-half of the microtitre plate (200 µL/well). HRP conjugate was diluted 1:3,000 in "regular" PBST and added to the other half of the microtitre plate (200 µL/well). Following incubation for 2 h at room temperature, the appropriate enzyme substrate was added (TMB for HRP and urea for urease). The HRP reaction was stopped after 20 min by adding 50 µL/well of 2 M sulphuric acid, and the

absorbance values were read at 450 nm. The conductance of the solution in the urease wells was read after 20 min.

2.4.13 Fluorescence Polarization Immunoassays

2.4.13.1 *Determination of Excitation and Emission Spectra of AMF-SOL*

Excitation and emission wavelength maxima were determined using AMF-SOL diluted in PBST (final concentration 1,045 nM) by performing excitation and emission scans in which both excitation and emission slit widths were 2.5 nm.

2.4.13.2 *Effect of pH on the Fluorescence Intensity of AMF-SOL*

To assess the influence of pH on the fluorescence intensity of AMF-SOL solutions, 120 nM solutions of the label were prepared using PBST at either pH 6.5 or pH 7.3. Duplicate aliquots of each solution were diluted 1:1 (v/v) with PBST at the same pH to give a final label concentration of 60 nM to mimic the effect in a competitive assay. FP values were obtained in triplicate for each duplicate sample.

2.4.13.3 *Stability of AMF-SOL in PBST*

Initial studies outlined in this manuscript employed dilutions of the AMF-SOL label in PBST at pH 7.3. However, there was some concern regarding the stability of the label under these conditions, as other investigators have noted the potential for hydrolysis of the hemisuccinate linkage (Eremin *et al.*, 1991). To reduce the risk of label hydrolysis, the pH of the diluent (PBST) was reduced to 6.5, and the propensity for such hydrolysis evaluated at both pH values. AMF-SOL was prepared (180 nM) in PBST at either pH 7.3 or pH 6.5, and analyzed immediately by TLC using the system outlined in Section 2.4.1.3.2, with UV visualization. The solutions were stored at room temperature in the dark for 30 days, and re-analyzed by TLC.

2.4.13.4 *Equilibrium Study*

Equal volumes of reagents were combined to give a final Ab dilution of 1:200 and a label concentration of 60 nM in the FPIA reaction. FP values were read every 60 s (to 1 h) using a 10 s integration time.

2.4.13.5 *Antibody Dilution Curves*

2.4.13.5.1 Polyclonal Antibody Dilution Curve

PAb was diluted (v/v) in PBST (pH 7.3) 1:50, 1:100, 1:150, 1:200, 1:250, 1:300, 1:350, 1:400, 1:500, 1:600, 1:700, 1:800, 1:900, and 1:4,000. To 1.0 mL aliquots of

diluted PAb were added 1.0 mL aliquots of AMF-SOL diluted in PBST for a final concentration of 57.5 nM. Samples were covered and left to incubate overnight in the dark at 4°C. Samples were warmed to room temperature for at least 2 h the following day. FP measurements were made using and excitation wavelength of 496 nm (2.5 nm slit width) and emission wavelength of 518 nm (10.0 nm slit width). An integration time of 10 s was employed.

2.4.13.5.2 *Monoclonal Antibody Dilution Curve*

A MAb dilution curve was derived as above (Section 2.4.13.5.1) using doubling dilutions of MAb from 1:2.5 to 1:3,200 (0.62 to 0.002 μM) in the FPIA reaction. Label was included in the reaction mixture at a concentration of 60 nM. After tubes had equilibrated (30 min at room temperature), FP readings were taken, as described above (Section 2.4.13.5.1).

2.4.13.6 *Determination of the Effect of Antibody Binding on Fluorescence Intensity Values of AMF-SOL*

AMF-SOL was diluted in PBST (5, 10, 15, 20, 25, 30, 35, 40, 60, 80, and 100 nM) and fluorescence intensity (FI) values were measured using an excitation wavelength of 496 nm (2.5 nm slit width) and an emission wavelength of 518 nm (10.0 nm slit width). To test the influence of the binding of AMF-SOL to the Ab on FI values, samples were prepared by mixing equal volumes of a 1:20 Ab dilution and diluted AMF-SOL to give label concentrations identical to those evaluated in the absence of Ab.

2.4.13.7 *PAb Label Dilution Curve*

Polyclonal antiserum (1:100 dilution in PBST) was diluted 1:1 (v/v) with AMF-SOL to give final concentrations of 7.5, 10, 20, 40, 60, 80, 100, 125, 150, 175, 200, and 250 nM AMF-SOL. Samples were incubated overnight in the dark at 4°C and warmed to room temperature the following day. FP readings were made using an excitation wavelength of 496 nm (slit width 2.5 nm), an emission wavelength of 518 nm (slit width 7.5 nm), and a 10 s integration time. Data was analyzed using *SOFTmax* (Section 2.4.4).

2.4.13.8 *Competitive FPIA (Standard Curve Generation)*

Glycoalkaloids (α-solanine, α-chaconine, α-tomatine) and solanidine were diluted in methanol to final concentrations of about 100 nM. These stock solutions were

diluted as required in PBST to give final concentrations in the FPIA reaction of 5, 10, 15, 20, 40, 60, 80, 100, 125, 150, 175, 200, 250, 300, 1000, and 5000 nM. Competition was established as per the standard protocol (Section 2.4.3.5) to give a final Ab dilution of 1:200 and label concentration of 60 nM. FP values in the absence of analyte were obtained as above, except PBST substituted for analyte. Samples were incubated overnight in the dark at 4°C and warmed to room temperature prior to obtaining FP values. The fluorescein label was excited at 496 nm (2.5 nm slit width), and its emitted polarization detected at 518 nm (7.5 nm slit width).

2.4.13.9 *Specificity of PAb for AMF-SOL*

Aliquots of a 1:100 (v/v) dilution of Ab were incubated with equal volumes of either free (unconjugated) 4'-AMF or AMF-SOL at 4°C overnight in the dark. 4'-AMF stock solution was prepared at a concentration of 4,140 nM in methanol, and diluted in PBST to give a final concentration in the reaction of 60 nM. Similarly, the final concentration of AMF-SOL was 60 nM in the reaction with PAb. Polarization readings were taken after allowing the samples to warm to room temperature for at least 2 h.

2.4.13.10 *GA Extraction from Potato Samples*

GAs were extracted according to the method of Plhak and Sporns (1992) from 1.0 g of freeze-dried powder using 3X15 mL methanol and homogenizing for 1 min after each addition. An additional 1-2 mL methanol was used to rinse the homogenizer probe. Supernatants were pooled and made up to 50 mL in methanol. Duplicate crude potato extracts were made and stored at -20°C until required for analysis.

2.4.13.11 *Analysis of Potato Extracts by FPIA*

Potato extracts were diluted sufficiently in methanol such that FP values of extracts were in the range of the standard curves for the major potato GAs (α-solanine, α-chaconine, and solanidine) following a final dilution in PBST. All potato extracts contained 10% (v/v) methanol when analyzed. Diluted potato extract (0.5 mL) was mixed with 0.5 mL 180 nM AMF-SOL in PBST (pH 6.5) and 0.5 mL of a 1:66.7 dilution of PAb in PBST (pH 6.5). Following a 30 min incubation at room temperature, FP measurements were made. Individual GA contents were determined using the PAb FPIA standard curve for α-chaconine (Section 2.4.13.8).

2.4.13.12 *Affinity of PAb and MAb for GAs and AMF-SOL*

The relative affinities of the PAb and MAb preparations for a variety of GAs, as well as the AMF-SOL label and a hemisuccinate derivative of solanidine were assessed by comparing FP values for various levels of these compounds. GAs (α-chaconine, α-solanine, and α-tomatine), solanidine and solanidine-hemisuccinate were evaluated in a competitive assay by mixing equal volumes (0.5 mL each) of 180 nM AMF-SOL in PBST (pH 6.5), 1:66.7 PAb or 0.042 mg/mL MAb in PBST (pH 6.5), and standards diluted in PBST (pH 6.5). Standards contained 10% (v/v) methanol, and final concentrations were 0, 20, 40, 60, 80, and 100 nM. For the evaluation of Ab affinity for AMF-SOL, equal volumes (0.75 mL each) of 120 nM AMF-SOL in PBST, pH 6.5, and 1:100 PAb or 0.028 mg/mL MAb in PBST (pH 6.5) were mixed. Final label concentrations were 60, 80, 100, 120, 140, and 160 nM. FP measurements were made using an excitation wavelength of 496 nm (2.5 nm slit width), an emission wavelength of 518 nm (7.5 nm slit width) and an integration time of 10 sec..

2.4.13.13 *Stability of α-Chaconine Standard Curve Using PAb FPIA*

The stability of a standard curve for α-chaconine (0, 20, 40, 60, 80, and 100 nM) was evaluated using PAb FPIA (Section 2.4.3.5). Analysis was performed in duplicate using a stock dilution of PAb (1:66.7 in PBST at pH 6.5) and previously-prepared α-chaconine standards in PBST (pH 6.5) containing 10% (v/v) methanol. Label (180 nM in PBST at pH 6.5) was prepared fresh daily. All standards were assayed on two consecutive days (day zero and day one) , and at seven, 14, and 23 d to assess the reproducibility of FP values.

CHAPTER 3. RESULTS AND DISCUSSION

3.1 EVALUATION OF FACTORS AFFECTING THE SENSITIVITY OF AN INDIRECT ELISA FOR SULPHATHIAZOLE

The objective in the development of new IA methods for ST analysis was to increase the sensitivity and to allow for the detection of lower levels of sulphathiazole (ST) in such tests. Reduced detection levels allow regulatory agencies to conform to current governmental standards with respect to enforcement levels which are currently as low as 10 ppb for some sulphonamides (Zomer *et al.*, 1992). In addition, the ability to detect ST at very low levels increases the accuracy of results at higher levels of contamination. Finally, the development of tests with extremely low detection levels is a scientific challenge which deserves consideration.

A variety of techniques may be used to decrease the detection limit and increase the sensitivity of IAs, as was outlined in Section 1.5. However, as Section 1.5 highlighted, such procedures generally increase the cost, complexity, or time required to complete the analysis. Our approach was to examine more closely the basic elements of an indirect immunoassay to determine how they might be used more effectively to enhance the sensitivity and detectability of the tests. The factors specifically addressed in the present work included characterization of the competitor (coating) conjugate, a thorough evaluation of chromogenic substrates for HRP, a study to determine the usefulness of a separate competition step, and an investigation into the use of urease as a replacement for HRP in the quantitation of indirect competitive ELISA results.

3.1.1 Characterization and Evaluation of BSA-ST Coating Conjugates

The first step in the development of an indirect ELISA for ST is the synthesis of the plate-coating conjugate, as ST cannot be passively adsorbed to plastic. It has been demonstrated that the linking arm used in attaching a hapten to its carrier protein can influence the performance of an indirect ELISA (Garden, 1994; Wie and Hammock, 1984). Our lab has had considerable success with the use of diazo-linked coating conjugates, regardless of the linking arm present in the immunogen. The Abs produced in response to diazo-linked immunogens net a more sensitive test in conjunction with diazo-linked coating conjugates than with coating conjugates in which the linking arm in the coating conjugate has been changed (Garden, 1994). Nonetheless, the degree of hapten substitution on the carrier protein in diazo-linked

coating conjugates can affect the sensitivity and detection levels of the indirect ELISA (Garden, 1994). It is also known that excessive amounts of Ag on the solid phase (i.e., over-coating) can lead to a loss of sensitivity in an indirect ELISA (McLaren *et al.*, 1981).

The degree of ST substitution in the BSA-ST conjugates synthesized in this study was determined by comparing the percentage of sulphur in the products to that found in pure BSA. Individual elemental analysis of the conjugates and BSA indicated that pure BSA contained 1.41% S, while the conjugates contained 2.28% (heavily-loaded) and 2.90% S (lightly-loaded). These values corresponded to 36.0 moles ST/mole BSA for the heavily-loaded conjugate, and 17.4 moles ST/mole BSA for the lightly-loaded conjugate.

In early experiments where both the heavily- and lightly-loaded BSA-ST coating conjugates were employed at the same concentrations on microtitre plates, it was found that no significant analytical advantage was provided by one conjugate over the other; the heavily loaded BSA-ST coating conjugate was therefore used for all indirect competitive ELISA protocols. This was done for two reasons. First, at 36.0 moles ST/mole BSA, this conjugate was comparable to that used by Sheth and Sporns (1990), which contained 44 moles ST/mole BSA. While Garden (1994) reported that a reduction in the degree of substitution of BSA in the coating conjugate resulted in a more sensitive assay for sulphamerazine (SMR), this advantage was realized only at very low levels of SMR substitution on the BSA molecule (~4 moles SMR/mole BSA). Therefore, it was assumed that the slight difference in the rates of BSA substitution with ST in the conjugate of this work and that of Sheth and Sporns (1990) would not preclude the comparison of results from both studies. The second rationale for employing only one of the synthesized coating conjugates in this study was to simplify the interpretation of results by the elimination of the degree of substitution of the coating conjugate as a variable.

3.1.2 Evaluation of Chromogens for the Detection of HRP Activity

Many reports in the literature support the use of HRP for the quantitation of immunoassay results (Blake and Gould, 1984; Gosling, 1980, Porstmann *et al.*, 1985). Among the reasons given for the superiority of HRP over alternative enzyme markers is the range of chromogenic substrates that can be used with HRP; however, there appears to be no consensus as to which of the available chromogens is the best. This is despite the fact that the choice of chromogen for HRP can strongly affect the sensitivity of the ELISA (Porstmann *et al.*, 1981).

Few comprehensive studies have been carried out to determine which of the chromogenic substrates for HRP performs best. Even in studies where a variety of chromogens has been studied, attention to parameters which affect the performance of the chromogen, such as pH, has been lacking. Roberts *et al.* (1991) compared TMB to ABTS; their results favoured the use of TMB. Hosoda *et al.* (1986) tested six chromogens, and ranked them in the order of sensitivity attainable with each as TMB>OPD>ABTS>5-AS>MBTH>AEC (3-amino-9-ethylcarbazole). The validity of the study of Hosoda *et al.* (1986) is somewhat suspect, considering they measured the absorbance of a reaction product (AEC) which is reportedly insoluble (Sigma ImmuNotes, 1991). Porstmann *et al.* (1981) indicated that OPD gave higher absorbance values than ABTS, but that the latter afforded increased sensitivity. In a subsequent study by Al-Kaissi and Mostratos (1983), no difference was found among OPD, ABTS, and 5-AS in terms of assay sensitivity. Bos *et al.* (1981) indicated TMB was a superior chromogenic substrate to OPD. Ellens and Gielkens (1980) found no difference in the sensitivities attainable using OPD and 5-AS, but promoted the use of 5-AS, as it poses no toxicity problems. Saunders *et al.* (1977) found ABTS to be superior to 5-AS, while 5-AS was the preferred substrate of Bullock and Walls (1977). Ngo and Lenhoff (1980) developed the method employing MBTH-DMAB and strongly promoted its use. Geoghegan (1985) echoed the claims of Ngo and Lenhoff (1980) in a publication edited by Ngo and Lenhoff. Artiss *et al.* (1981) discouraged the use of MBTH-DMAB, while Stauffer (1989) indicated that MBTH-DMAB is probably the most sensitive chromogen for assaying HRP activity.

Some of the more recent literature advocates the use of TMB (Harlow and Lane, 1988; McKimm-Breshkin, 1990; Madersbacher and Berger, 1991). However, spectrophotometric measurements of the reaction product have often been made at a pH value other than the optimum (McKimm-Breshkin, 1990; Madersbacher and Berger, 1991), or at wavelengths other than λ_{max} (Madersbacher and Berger, 1991).

From the above discussion, it is evident that there is no conclusive evidence to support the use of one chromogen over another for detection of HRP . Our objective, therefore, was to determine the best chromogenic substrate for HRP to maximize the sensitivity of ELISAs.

OPD had been the chromogenic substrate of choice in our lab, and performed well in the immunoassays that had been developed. However, there was some concern over its alleged toxicity (Tijssen, 1985). Furthermore, OPD in solution develops colour readily when exposed to light, leading to high background absorbance

values, and its yellow product is not well-detected visually, which could limit its use in more "positive-negative" types of tests that might be developed for field use.

Four chromogens, in addition to OPD, were tested under ELISA conditions determined to be optimal for ST detection using OPD. These compounds were 5-AS, TMB, ABTS, and the MBTH-DMAB coupled reaction. These particular chromogens were selected to represent the different structural classes of chromogens available (Figure 1.2). Furthermore, adequate sensitivity had been reported using each of these compounds, as cited above, and each apparently poses no toxicity problem (Table 1.2).

Several criteria were used in the evaluation of the test chromogens. The test chromogen was required to exhibit good solubility and stability in a buffer system. More importantly, however, the chromogen was required to perform as well as or better than alternative chromogens in terms of the absorbance values attainable under conditions of limiting coating conjugate and Ab concentrations. Further considerations included the ability to stop colour development and maintain a stable product, and other factors which might affect the overall sensitivity of the assay, such as background absorbance levels.

3.1.2.1 *OPD*

OPD could be solubilized easily at a concentration of 3.7 mM in 0.1 M citrate buffer; pH was not observed to influence OPD solubility. The absorbance values of OPD reaction products with HRP and hydrogen peroxide were, however, significantly influenced by buffer pH (Figure 3.1). OPD reaction products showed a broad pH range for colour development, with a maximum at a pH value of about 5.0, which is in accordance with results published by Gallati and Brodbeck (1982).

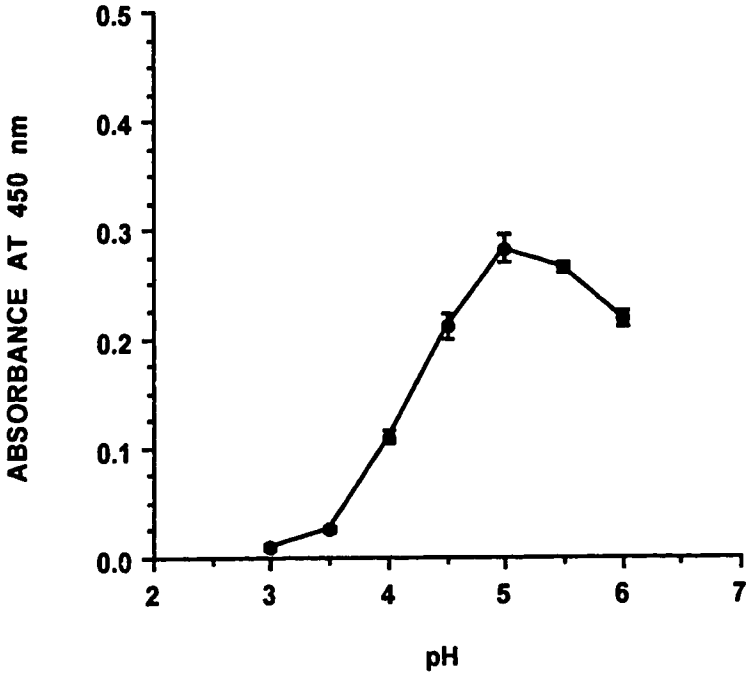


Figure 3.1 Effect of pH on the Absorbance Values of the Reaction Product of OPD with HRP and Hydrogen Peroxide

Each point represents the mean absorbance value of 5 wells; error bars indicate the standard deviation of the mean. Reaction time was 30 min at room temperature. Coating conjugate was 0.25 µg/mL; serum was diluted 1:150,000.

Several reagents were tested to determine their ability to stop colour development by HRP and produce a stable end-product. The use of sulphuric or hydrochloric acid (6 N each, 50 μL/well) as stopping reagents for OPD resulted in a shifting of λ_{max} values from 450 nm to 492 nm, while the addition of 6N sodium hydroxide (50 μL/well) decreased the λ_{max} to 420 nm, as determined by spectrophotometric scans of the reaction products. This is consistent with the results of Bovaird *et al.* (1982) who indicated that sulphuric acid slightly shifts and lowers the absorption of the spectrum. Porstmann *et al.* (1981) found that OPD exhibited poor stability in its oxidized state after stopping the reaction with sulphuric acid. In the present study, 6N hydrochloric acid was found to be effective in stopping the development of yellow colour with OPD; however, the resulting product initially had a pinkish hue, and turned dark brown over time.

The use of 6N sodium hydroxide as a stopping reagent for use with HRP and OPD was deemed unsuitable for two reasons. First, as noted above, the λ_{max} of the OPD reaction product in the presence of 6N sodium hydroxide was 420 nm, which could not be read on the available ELISA reader. Second, although the addition of 50 μL/well of 6N sodium hydroxide may have stopped the enzyme reaction, it caused a bleaching of the yellow colour, such that absorbance values were greatly reduced.

Sodium dodecyl sulphate (SDS) was tested as a stopping reagent for the HRP reaction with various chromogens, including OPD. SDS is an anionic detergent which disrupts nearly all non-covalent interactions in proteins (Stryer, 1988), thereby obliterating enzyme activity by causing an unfolding of the protein structure. In the case of OPD, 10 μL/well of 1% SDS did not produce a stable reaction product. Furthermore, the effect of SDS on absorption values varied with pH, with absorbance values actually decreasing at pH 5.0.

3.1.2.2 *5-AS*

Even after purification of 5-AS, according to the method of Ellens and Gielkens (1980), 5-AS could not be solubilized in any common solvent systems safe for use in ELISA, including 0.1 M citrate buffer, methanol, and dimethyl sulphoxide. Further evaluation of 5-AS was suspended.

3.1.2.3 *Oxidative Coupling of MBTH with DMAB*

The oxidative coupling of MBTH and DMAB in the reaction with hydrogen peroxide and HRP produces a purple colour which absorbs in the 575 to 600 nm region, with a maximum at 590 nm. In the reaction, it is necessary to use at least a 50-fold excess of DMAB (Ngo and Lenhoff, 1980).

The solubility of MBTH proved to be a problem. It first required solubilization in methanol, followed by a 100-fold dilution into the 0.1 M citrate buffer. The final concentration of the MBTH solution used was 0.185 mM, which was 20 times less than that used for OPD. This MBTH concentration was coupled with a 9.25 mM solution of DMAB; these concentrations were quite low, but were necessary to allow for the 50-fold excess of DMAB prescribed by Ngo and Lenhoff (1980).

The effect of pH on the absorbance of MBTH coupled with DMAB in the presence of hydrogen peroxide and HRP is shown in Figure 3.2. Absorbance values were shown to be minimal at pH 3.0, and increased with increasing pH values, leveling off as the pH approached 7.0, which agrees with the results of Ngo and Lenhoff (1980) who found an optimum at pH 7.0. Even at pH 7.0, however, maximum absorbance values were quite low when compared to those obtained for OPD under the same conditions (Figure 3.1). Furthermore, the time required to achieve such values was quite long (60 min).

The addition of 1% SDS (50 μL/well) to the MBTH-DMAB reaction products led to an increase in absorbance values, with the effect increasing as pH values increased. These values were stable for at least 60 min (Figure 3.2). In contrast, the use of 50 μL/well of 6 M sulphuric acid to stop the enzyme reaction did not afford any appreciable increase in absorbance values. A dilution effect on absorbance values would be expected with the addition of 50 μL/well of 6 M sulphuric acid; however, this was not observed, suggesting that absorbance values may have increased slightly with the addition of acid. The use of 6 M sulphuric acid was successful in maintaining stable absorbance values for at least 60 min (Figure 3.3).

Another difficulty associated with the use of MBTH-DMAB was the fact that the reaction product with HRP and hydrogen peroxide has an absorbance maximum at 590 nm. The closest wavelengths that could be employed with the ELISA reader used in this study were 540 and 660 nm. Measurement of the reaction product at 660 nm gave low and erratic absorbance values (OD<0.05). Depending on the shape of the absorption curve of MBTH-DMAB, it may be feasible to use 540 nm as the wavelength for measurement of the reaction product.

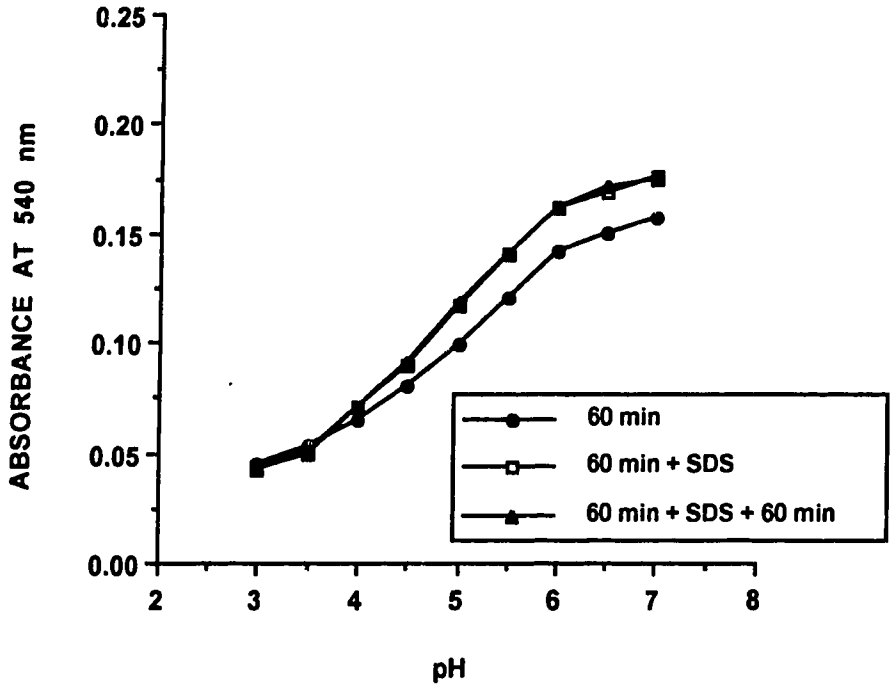


Figure 3.2 Effect of pH and SDS on the Absorbance of the MBTH-DMAB Reaction Product with HRP and Hydrogen Peroxide

Each point represents the mean absorbance value of six wells; the standard deviation of the mean was ≤ 0.007 for all determinations. Reaction time was initially 60 min (●), after which SDS was added (50 µL/well of 1% solution) (□). Plates stopped with SDS were read again after 60 min (▲)..Coating conjugate concentration was 0.25 µg/mL; serum was diluted 1:150,000.

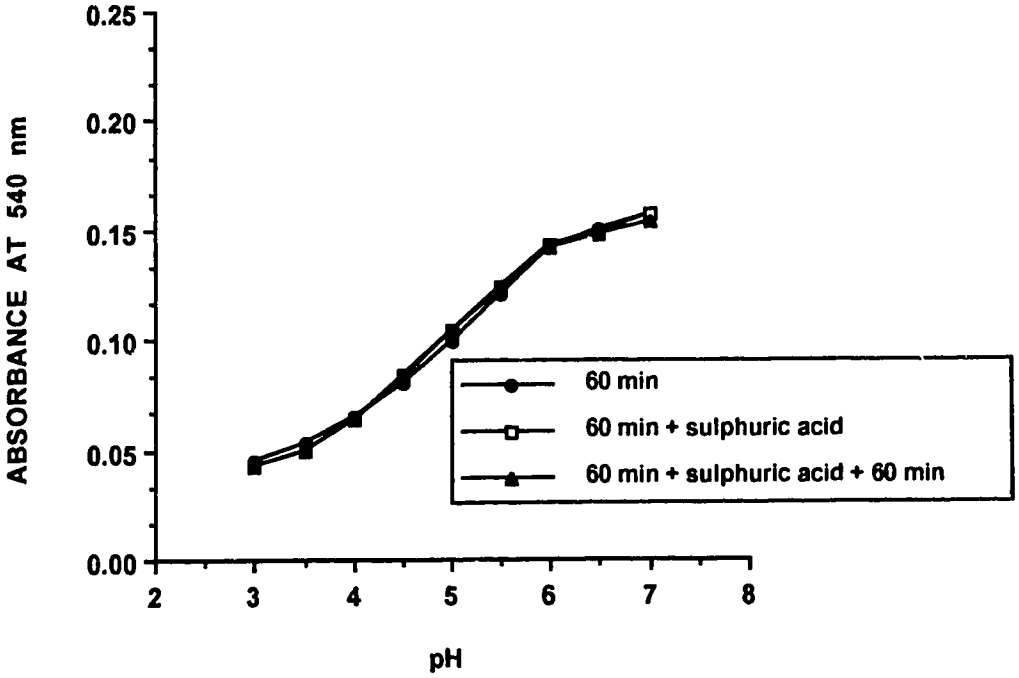


Figure 3.3 Effect of pH and Sulphuric Acid on the Absorbance of the MBTH-DMAB Reaction Product with HRP and Hydrogen Peroxide

Each point represents the mean absorbance value of six wells. The standard deviation of the mean was ≤ 0.006. Reaction time was initially 60 min (●), after which 2 M sulphuric acid was added (50 µL/well) (□). Plates stopped with 2 M sulphuric acid were read again after 60 min (▲). Coating conjugate concentration was 0.25 µg/mL; serum was diluted 1:150,000.

Tijssen (1985) indicated that an increase in the pH or a decrease in the ionic strength of MBTH-DMAB substrate solutions can lead to an increase in the absorbance values of blanks. While this was not specifically evaluated in this study, due to the relatively poor performance of MBTH-DMAB observed, this response of blanks to pH or ionic strength was another negative aspect considered. Moreover, MBTH and DMAB are both photosensitive reagents; therefore, blanks develop colour when exposed to light (Geoghegan *et al.*, 1983). This was yet another drawback considered in the evaluation of MBTH-DMAB.

3.1.2.4 *ABTS*

ABTS was readily solubilized in 0.1 M citrate buffer at a concentration of 3.7 mM, the same concentration as was used for OPD. The production of coloured products from ABTS by HRP in the presence of hydrogen peroxide was shown to be quite sensitive to buffer pH (Figure 3.4). A narrow pH range for best colour development was observed, with a maximum at pH 4.0; this agrees closely with the pH optimum of 4.2 quoted by Gallati (1979) for a 2 mM ABTS solution.

Figure 3.4 shows that the reaction of HRP with hydrogen peroxide and ABTS could be terminated by the addition of 20 µL/well of 1% SDS. Furthermore, there was an increase in the absorbance values upon addition of SDS; this effect was especially pronounced at pH 4.0, where absorbance values increased ~27% over those for the unstopped reaction. The absorbance values of the stopped reaction were also found to be stable for at least 60 min (Figure 3.4).

As indicated above, ABTS was tested at the same concentration as OPD, allowing the results of the evaluation of both ABTS and OPD to be compared. Under identical conditions in the immunoassay (i.e., equivalent concentrations of coating conjugate, primary and enzyme-conjugated secondary Ab), and using the optimal pH for each chromogen (pH 4.0 for ABTS and pH 5.0 for OPD), ABTS was found to give slightly higher absorbance values (Figure 3.4) than OPD (Figure 3.2). An added advantage accompanying the use of ABTS is that the reaction product with HRP and hydrogen peroxide is blue in colour, which is preferable for visual evaluation of results, as opposed to the yellow-orange colour of OPD. Moreover, it was possible to stop the HRP reaction with ABTS using SDS, whereas no suitable stopping reagent for the reaction with OPD was found.

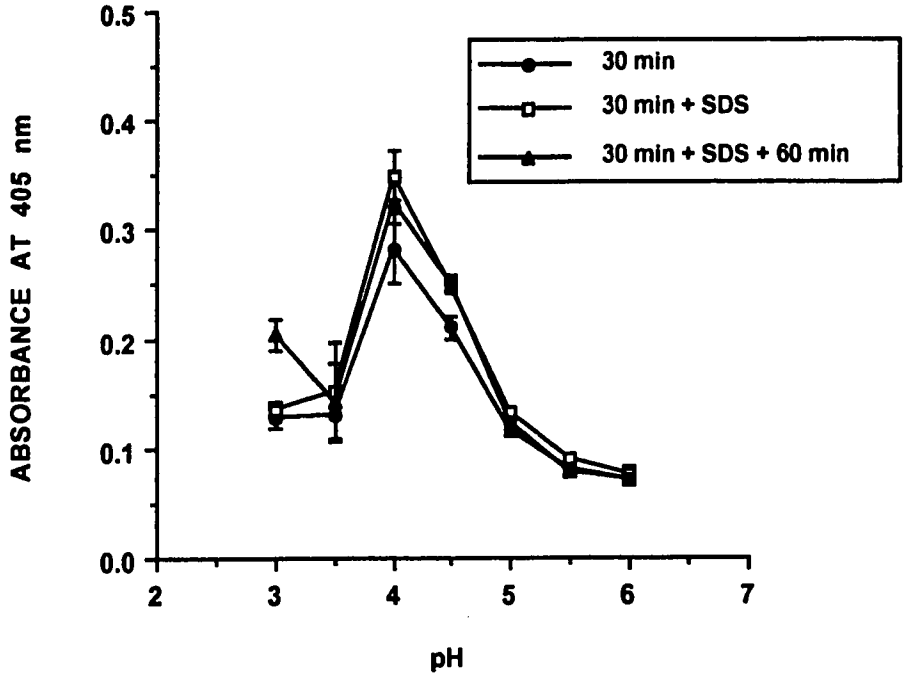


Figure 3.4 Effect of pH and SDS and the Absorbance of the ABTS Reaction Product with HRP and Hydrogen Peroxide

Each point represents the mean absorbance value of ten wells; error bars indicate the standard deviation of the mean. Reaction time was initially 30 min (●), after which SDS was added (20 µL/well of a 1% solution) (□). Plates stopped with SDS were read again after 60 min (▲). Coating conjugate concentration was 0.25 µg/mL; serum was diluted 1:150,000.

3.1.2.4 *TMB*

It was very difficult to solubilize TMB-dihydrochloride (TMB-d) in the 0.1 M citrate buffer series (pH 3.0 to 7.0) at a concentration of 3.7 mM. Solubility at this concentration was affected by pH; maximum solubility was observed at pH 3.0. Gentle heating was of little assistance in solubilizing TMB-d, and resulted in the development of blue colour, particularly at pH 3.0 and 3.5.

TMB-d could be dissolved in dimethyl sulphoxide at a concentration of 37 mM. Bos *et al.* (1981) reported similar difficulties in solubilizing TMB and suggested the use of a water-miscible aprotic solvent such as dimethyl sulphoxide to prepare TMB solutions. When this solution was diluted in a 1:10 (v/v) ratio with 0.1 M citrate buffer series, the solubility was still largely pH-dependent. At this concentration (i.e., 3.7 mM), the TMB-d remained in solution at pH values of 3.0, 3.5, and 4.0, but readily precipitated out of solution at the higher pH values. At a dilution of 1:100 of the TMB-d (in dimethyl sulphoxide) into the 0.1 M citrate buffers, for a final TMB-d concentration of 0.37 mM, solubility could be maintained at all pH values. Thus, the final concentration of TMB-d used in the ELISA evaluations was lower than that used for OPD by a factor of 10.

The solubility of TMB-d was found to be influenced by the addition of 1% SDS. When HRP was reacted with TMB-d and hydrogen peroxide in a soluble system (i.e., in test tubes in the absence of Ab), it was noted that the addition of 50 μL of 1% SDS to 5.0 mL reaction volumes promoted the precipitation of TMB at pH values below 5.0. The precipitate was blue in colour, leaving behind an essentially colourless supernatant, indicating that in this system, it was the product of the enzyme reaction that became insoluble, and was not denatured enzyme and Ab protein.

TMB develops a blue-green colour in the presence of HRP and hydrogen peroxide. This product reportedly has three absorption maxima: 370, 450, and 655 nm (Bos *et al.*, 1981; Madersbacher and Berger, 1991). The ELISA reader used in this work was limited to wavelengths of 405, 450, 490, 500, 540, and 660 nm. Therefore, the TMB reaction products with HRP and hydrogen peroxide at different pH values were read at the closest available wavelength on the ELISA reader to approximate the literature values. As shown in Figure 3.5, the highest absorbance values for the unstopped reaction were obtained at 660 nm, while relatively strong absorbances were recorded at 405 nm, which presumably measured the peak with an absorption maximum of 370 nm.

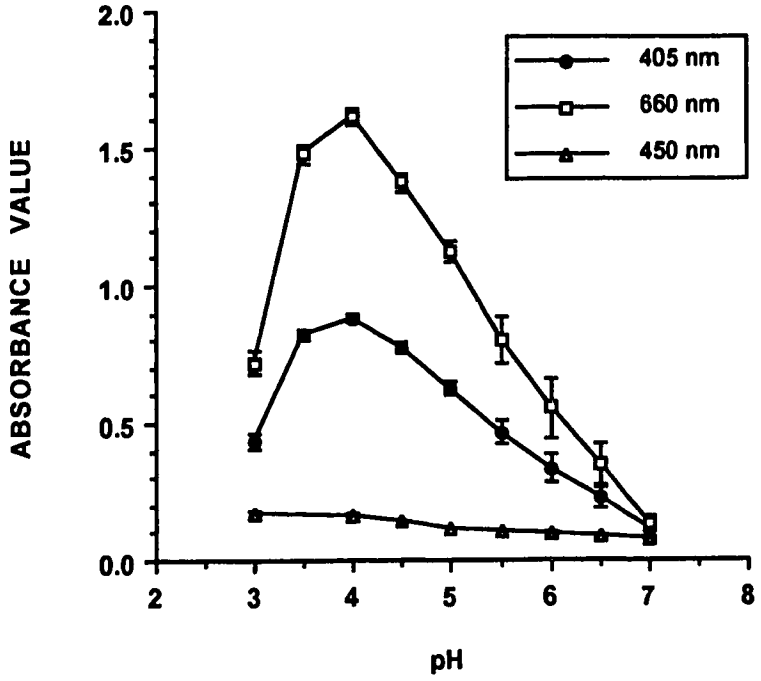


Figure 3.5 Effect of pH on Absorbance at Three Wavelengths of TMB Reaction Products with HRP and Hydrogen Peroxide

Each point represents the mean absorbance value of six wells; error bars indicate the standard deviation of the mean. Reaction time was 30 min at room temperature. Coating conjugate concentration was 0.25 µg/mL; serum was diluted 1:150,000.

The absorbance values of TMB following reaction with HRP and hydrogen peroxide were found to be influenced by buffer pH when measured at 405 nm or 660 nm; an absorption maximum was observed at pH 4.0 at both of these wavelengths (Figure 3.5). This value agrees well with the value of 3.95 reported by Gallati and Pracht (1985) as the pH optimum for the TMB reaction with hydrogen peroxide and HRP. Conversely, measurement of the unstopped TMB reaction product at 450 nm showed no obvious response to pH (Figure 3.5). Since maximum absorbance values for the TMB reaction product were recorded at pH 4.0 using a wavelength of 660 nm, these parameters were used in the further evaluation of TMB.

Although solubility of the TMB reaction product was a problem at pH values below 5.0 in the soluble system, as discussed above, no precipitation of TMB, regardless of pH, was observed using a solid-phase ELISA system which included Ab. Spectral scans of the reaction solutions recovered from the wells of a microtitre plate indicated that the addition of 50 μL/well of 1% SDS did not appreciably alter the λ_{max} at any pH values. At the optimum pH value of 4.0, λ_{max} was 666 nm; therefore, measurement of the reaction product at 660 nm after stopping with 1% SDS was justified.

A possible explanation for the precipitation of TMB in the presence of SDS in the soluble system may be that since SDS is very hydrophobic, it may ion-pair with the protonated form of the chromogen to form an insoluble complex. This would explain why precipitation was found to be a particular problem at pH values below 5.0. In the ELISA system, however, the hydrophobic nature of SDS may cause it to coat the plastic preferentially to complexing with the protonated chromogen allowing the reaction product to remain in solution.

Sulphuric acid (6 M) was also tested as a stopping reagent for the HRP reaction with TMB and hydrogen peroxide in an ELISA. As shown in Figure 3.6, appreciable absorbance values were measured for the unstopped enzyme reaction (λ=660 nm); however, upon the addition of 50 μL/well 6 M sulphuric acid the λ_{max} shifted to 450 nm and absorbance values at least doubled, with the increase being most significant at the optimum pH of 4.0. The absorbance readings were outside the range of peak performance of the spectrophotometric ELISA reader (i.e., 0.2-0.7) (Skoog and West, 1976); therefore, it was clear that serum and coating conjugate levels could be decreased even further than had been anticipated initially based on results for the unstopped TMB-HRP-hydrogen peroxide reaction.

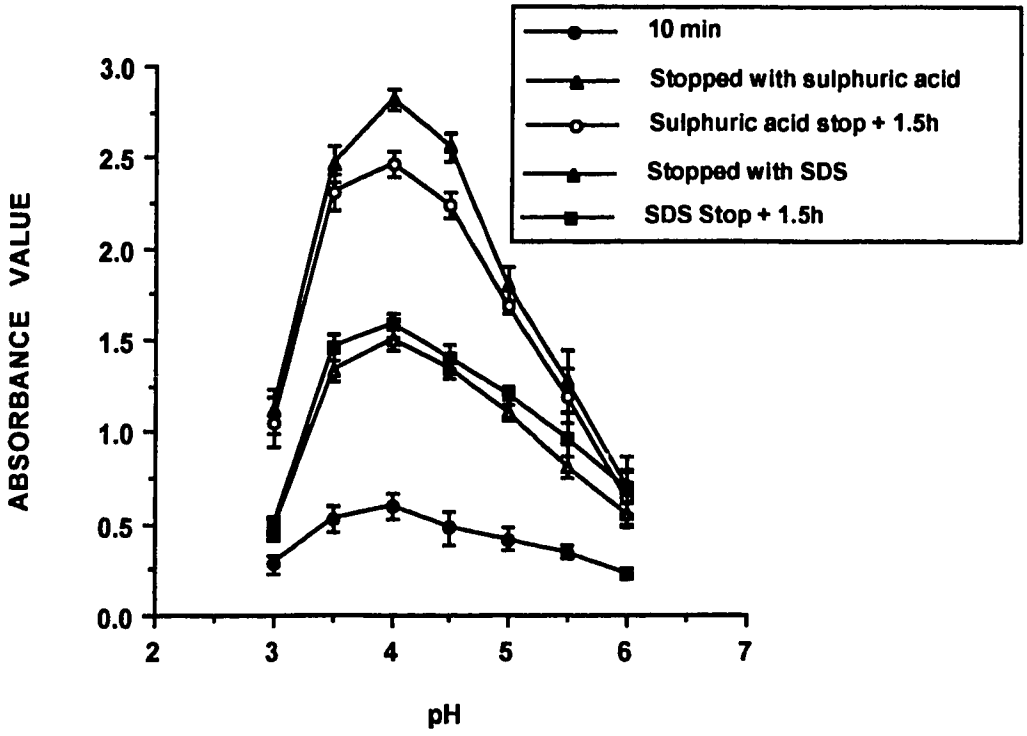


Figure 3.6 Effect of pH, Sulphuric Acid, SDS, and Time on the Absorbance of TMB Reaction Products with HRP and Hydrogen Peroxide

Each point represents the mean absorbance value of ten wells; error bars indicate the standard deviation of the mean. (●), λ=660 nm; (▲), 50 µL/well 2 M sulphuric acid (λ=450 nm); (△), 50 µL/well 1% SDS (λ=660 nm); (○), λ=450 nm; (■), λ=660 nm. Coating conjugate concentration was 0.25 µg/mL; serum was diluted 1:150,000.

Figure 3.6 also shows that both SDS and 6 M sulphuric acid could be used to successfully arrest colour development. These absorbance values remained relatively constant for at least 90 min.

It was later found that a 2 M solution of sulphuric acid could replace the 6 M sulphuric acid that had been used as the stopping reagent. By examining competitive inhibition curves derived under identical conditions, save for the strength of acid used to terminate the reaction, it was found that 2 M sulphuric acid netted the best results in terms of the maximum absorbance values attainable and the overall shape of the curve. This is in accordance with the recommendations of the Sigma Chemical Co. (St. Louis, MO).

The reaction of HRP with hydrogen peroxide and TMB was found to be fairly linear to about 30 min, after which absorbance values began to plateau (Figure 3.7). The relevance of these findings is that ELISA plates should be read no longer than 30 min following the addition of TMB substrate when end-point readings are used for the quantitation of ELISA results.

The evaluations of TMB discussed thus far were conducted using a powdered form of TMB-d. TMB-d is available in tablet form (Sigma Chemical Co., St. Louis, MO). The tablet form was found to be convenient to use, especially since it dissolved readily in 0.1 M citrate buffer at pH 4.0. However, the tablets are more than ten times more expensive than the powdered form of the substrate, using current market prices (Sigma Chemical Co., St. Louis, MO). Since absorbance values obtained using the tablets were comparable to those obtained using the powdered form of the substrate, the added cost could not be justified.

3.1.2.6 *Direct Comparison of OPD and TMB*

TMB was finally compared to OPD in microtitre plates under conditions that had previously been found to be optimal for OPD. That is, BSA-ST was used at a concentration of 0.25 μg/mL and serum was diluted 150,000 times. Both TMB-d and OPD were prepared in 0.1 M citrate buffers at the optimum pH values for colour development with each chromogen (pH 4.0 for TMB and pH 5.0 for OPD). It should be noted that the concentration of TMB employed (0.32 mM) was approximately 10% of that used for OPD (3.7 mM). The results depicted in Figure 3.8 show that under all conditions, (i.e., either the unstopped reaction, or by stopping with 1% SDS or 6 M sulphuric acid), TMB out-performed OPD. It also again highlights the advantage, in terms of absorbance values, afforded by the use of 2 M sulphuric acid instead of 1% SDS as a stopping reagent.

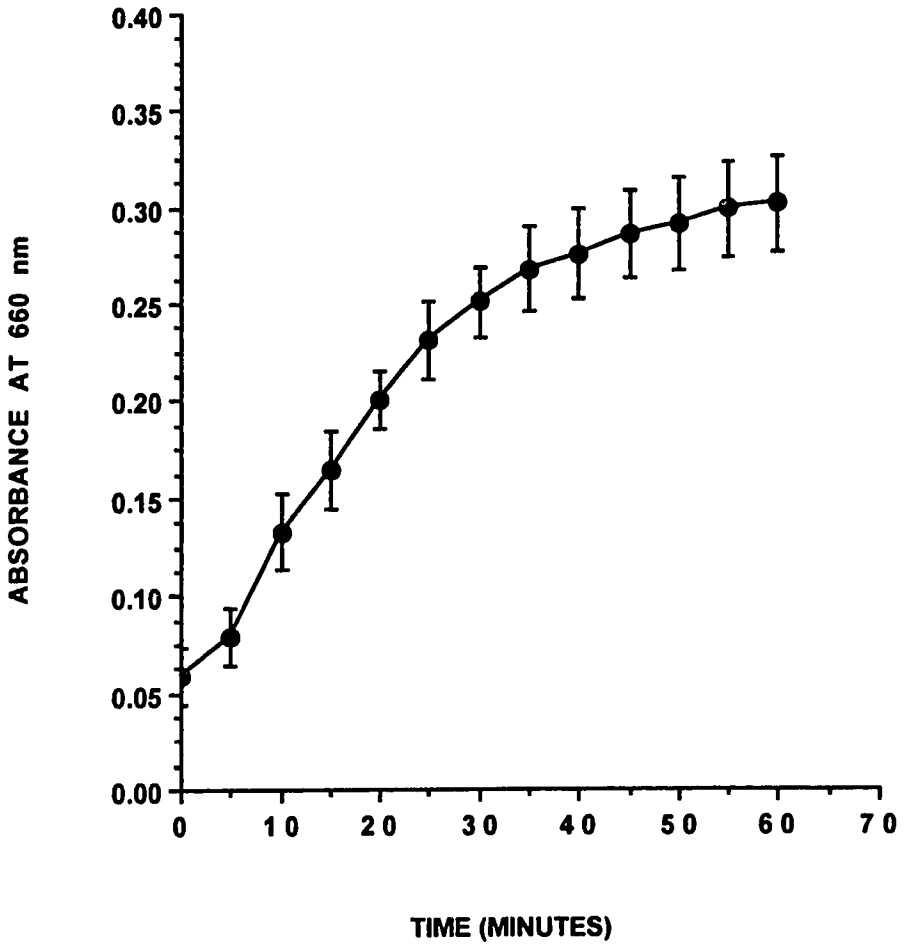


Figure 3.7 Effect of Time on Absorbance of TMB in Reaction with HRP and Hydrogen Peroxide

Each point represents the mean absorbance value of ten wells; error bars indicate the standard deviation of the mean. Coating conjugate concentration was 0.1 μg/mL; serum was diluted 1:500,000.

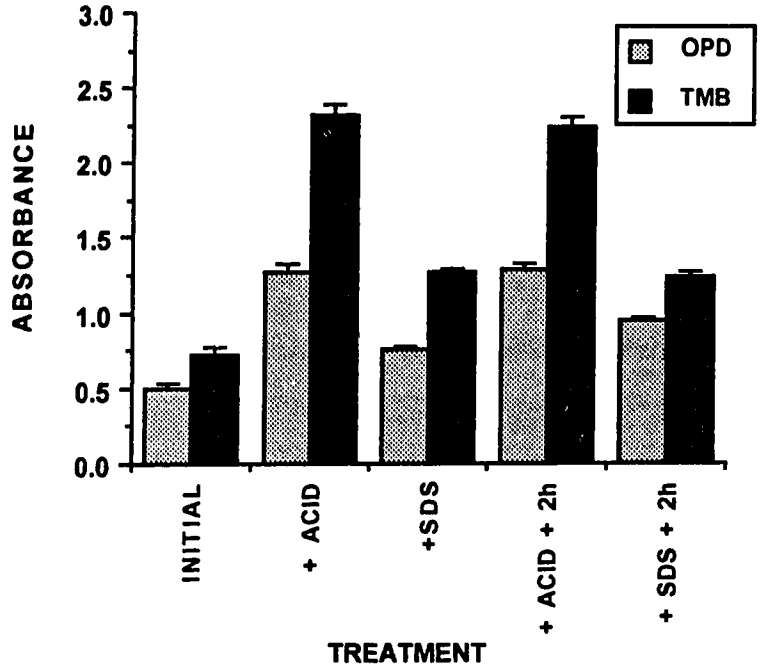


Figure 3.8 Comparison of TMB and OPD as Chromogens for HRP with Hydrogen Peroxide (Stopped and Unstopped Reactions)

Each point represents the mean absorbance value of 24 wells; error bars indicate the standard deviation of the mean. Initial reaction time was 10 min at room temperature, after which 50 µL/well of 1% SDS or 2 M sulphuric acid was added. Products of the stopped reactions were read again after 2 h. The wavelengths employed are listed in the table below. Coating conjugate concentration was 0.25 µg/mL; serum was diluted 1:150,000.

WAVELENGTH (nm)	SOLUTIONS READ
450	OPD(initial product); OPD (stopped with 1% SDS); TMB + sulphuric acid
490	OPD + sulphuric acid
660	TMB (initial product); TMB (stopped with 1% SDS)

Under conditions determined to permit optimal performance of the indirect ELISA system using OPD as the chromogen, as outlined above, absorbance values with TMB were well in excess of 1.0. Furthermore, there was a significant increase in absorbance values upon addition of the sulphuric acid used to stop the enzyme reaction and colour development. From these results, it was evident that serum levels and/or coating conjugate concentrations could be reduced further, to allow absorbance readings to fall into a more accurate range for spectrophotometric measurement (i.e., 0.2-0.7 OD units) (Skoog and West, 1976), which would reduce the level of detection.

Table 3.1 compares the parameters of the indirect competitive ELISA for ST employing either OPD or TMB as the HRP chromogen. The use of TMB permitted a dramatic reduction in the level of coating conjugate required as compared to that used by Sheth *et al.* (1990). It is likely that the 20 μg/mL of coating conjugate used by Sheth *et al.* (1990) was excessive, as in a subsequent publication by Sheth and Sporns (1991), the level of coating conjugate was reduced to 0.1 μg/mL. However, even with such a reduction in the coating conjugate level, the serum requirement remained 1:32,000 which was much higher than the 1:500,000 dilution of serum that could be employed using TMB as HRP chromogen.

It is important to note that suitable absorbance values could be achieved using TMB in only 10 min. This is a much shorter time as compared to the 30 min standard used by Sheth *et al.* (1990) with OPD.

The "a" values shown in Table 3.1 are quite different for OPD and TMB. Although a certain amount of error is associated with spectrophotometric measurements over a wide range of absorbance values in the visible range, the error in measurements of 0.2 OD is comparable to that of measurements of 1.0 OD (Skoog and West, 1976). Furthermore, the error due to well-to-well variation is generally larger than the error associated with absorbance values. The "a" value of 0.2 using TMB was generated using a reaction time of only 10 min; colour intensity could be increased by incubating for up to 30 min (Figure 3.7).

Table 3.1 Comparison of Assay Parameters in Indirect ELISA Using OPD and TMB as HRP Chromogenic Substrates

ASSAY PARAMETER	CHROMOGEN	
	OPD[1]	TMB[2]
Serum dilution	1:32,000	1:500,000
BSA-ST concentration (μg/mL)	20	0.0313
Maximum absorbance ("a")[3]	~1.0	~0.2
"c" value[4] (ppb ST)	100	4.4
Reaction time (min)	30	10

[1]Sheth *et al.*, 1990; OPD in 0.1 M citrate buffer at pH 4.75
[2]Competitive inhibition curve for this data is depicted in Figure 3.9
[3]Maximum absorbance in the presence of vanishingly small amounts of analyte
[4]From standard competitive inhibition curves (analyzed using *SOFTmax)*

The reduction in serum and coating conjugate levels resulted in a more sensitive assay for ST using TMB. Furthermore, the assay using TMB was more economical than that using OPD, particularly in terms of serum requirements. Polyclonal serum carries the disadvantage that it is available in a finite supply; therefore, it is preferable to use as little serum as possible in the ELISA procedure in order to increase the number of assays that may be performed using the serum of one test animal. Although more polyclonal serum may be produced, it is unlikely that such a serum would perform the same in an ELISA, as immune responses are quite variable among animals. Assays performed using TMB as the chromogen also required a shorter colour development period than those performed using OPD (Sheth *et al.*, 1990).

3.1.2.7 *Final Selection of HRP Chromogen*

From the data presented above, it was concluded that TMB is the best chromogenic substrate, of those evaluated, for the detection of HRP activity and quantitation of ELISA results. In addition to being non-toxic, TMB can be solubilized at the levels required for use in ELISAs. Absorbance values for the unstopped reaction far exceeded those obtained using a 10-fold higher concentration of OPD or ABTS under identical ELISA conditions, allowing for a dramatic reduction in coating conjugate and serum levels, as well as "c" values, as compared to a similar system

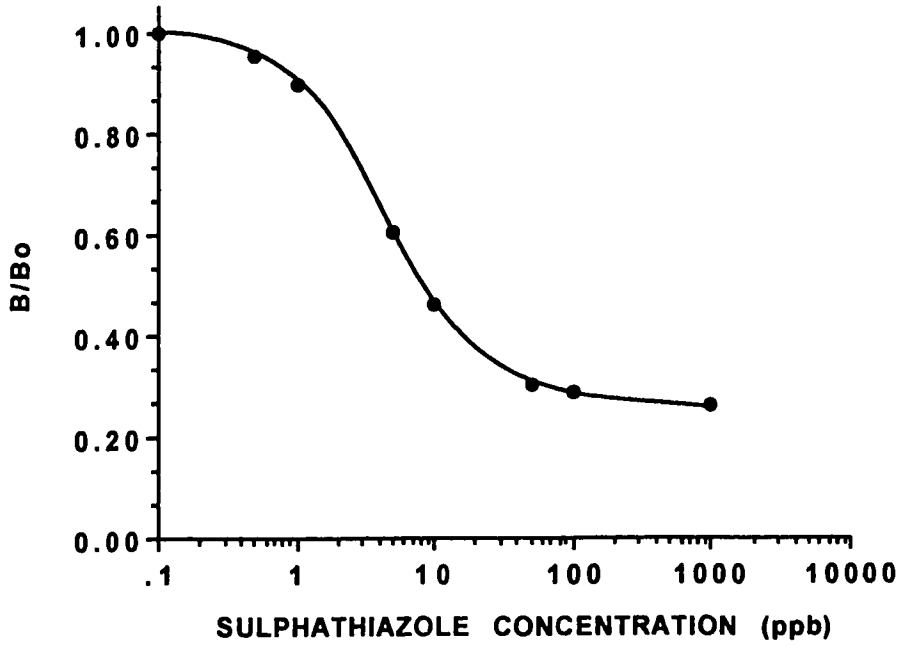


Figure 3.9 Competitive Inhibition Curve for Sulphathiazole Using an Indirect ELISA and TMB as HRP Chromogen

Each point was calculated using the mean absorbance value of 10 wells; the correlation coefficient for the curve (r) was 0.999 (generated using *SOFTmax*). Curve parameters: "a"=0.201; "b"=1.20; "c"=4.38; "d"=0.0526. Reaction time was 10 min at room temperature, followed by the addition of 50 µL/well of 2 M sulphuric acid. Coating conjugate concentration was 0.03125 µg/mL; serum dilution was 1:500,000.

using OPD (Sheth *et al.*, 1990). For these reasons, the ELISA methodology developed using TMB as the HRP chromogenic substrate was immediately adopted as standard protocol in our laboratory. Using the TMB reagent as described in this thesis, Garden (1994) obtained a "c" value of 1.4 ppb sulphamerazine in milk using PAb and an azo-linked SMR-BSA conjugate.

3.1.3 Use of a Separate Competition Step to Maximize the Sensitivity of an Indirect ELISA for ST

Several studies have included a separate incubation step between Ab and analyte prior to application of the solution to plates coated with protein-linked analyte (Karu *et al.*, 1991; Newsome *et al.*, 1993; Worobec *et al.*, 1985). It was hypothesized that such a format might increase the sensitivity of the competitive ELISA for ST by allowing the Ab to combine first with free ST followed by application to the BSA-ST coated plate.

It was found that carrying out the competition between Ab and free ST in a separate step (i.e., in a test tube), followed by allowing the pre-incubated serum to react with BSA-ST bound to microtitre plates did not afford any significant advantage over carrying out the entire competition within the wells of the microtitre plate. Although the use of a pre-incubation step between Ab and free ST resulted in a slight decrease in the "c" value as compared to that for the simultaneous competition between BSA-ST, Ab, and free ST in the wells of a microtitre plate (Figure 3.10), the advantage was not felt to be significant enough to warrant the adoption of the pre-incubation step owing to the extra preparation involved. Furthermore, the use of a pre-incubation step increases the total analysis time by at least 2 h.

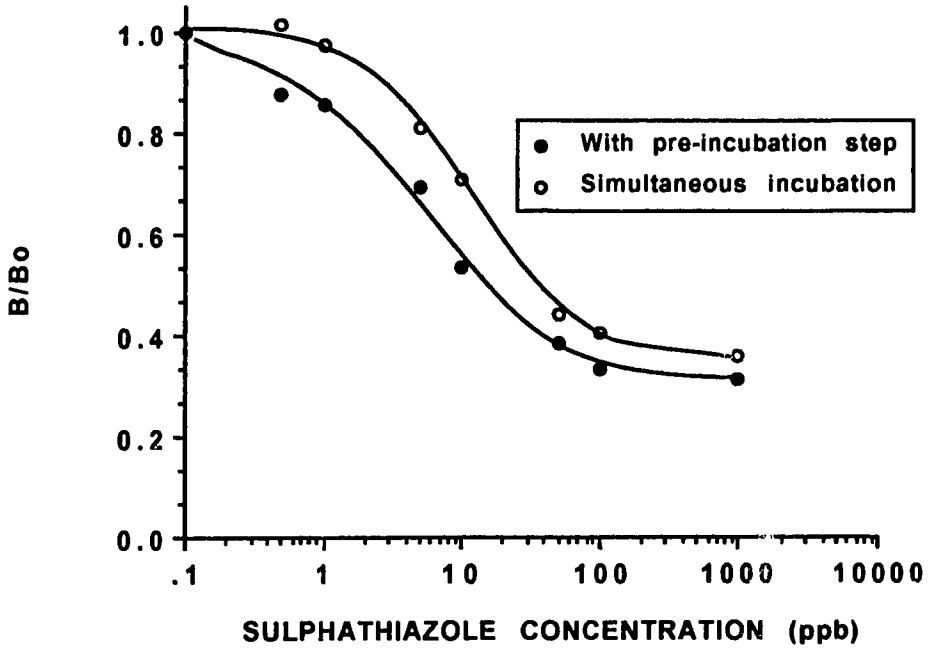


Figure 3.10 Competitive Inhibition Curves Produced With and Without a Pre-IncubationStep Between Antibody and Free Sulphathiazole

Each point was calculated using the mean absorbance value of five wells; correlation coefficient (r) for pre-incubation protocol=0.984 and for simultaneous protocol= 0.997 (generated using *SOFTmax).* Curve parameters (pre-incubation protocol): "a"=0.186; "b"=0.911; "c"=5.66; "d"=0.0565. Curve parameters (simultaneous incubation): "a"=0.160; "b"=1.19; "c"=10.6; "d"=0.0568. Reaction time with HRP, TMB and hydrogen peroxide was 20 min at room temperature. Coating conjugate concentration was 0.125 µg/mL; serum was diluted 1:500,000.

3.1.4 Evaluation of Urease as an Alternative to HRP as the Marker Enzyme in Indirect ELISAs

Urease has been used to a very limited extent in the development of EIAs (Chandler *et al.*, 1982; Meyerhoff and Rechnitz, 1980; Thompson *et al.*, 1991). We undertook this study to evaluate the potential for using Ab-conjugated urease as the marker in an indirect competitive ELISA for ST, using conductimetry to detect enzyme activity, for a number of reasons. As highlighted in Section 1.3, conductimetric measurements are not limited to as narrow a range of measurement as are optical readers. Meyerhoff and Rechnitz (1980) also indicated that commercial preparations of urease have considerably higher activity on a molar basis (international units per mole of enzyme) than other common marker enzymes, including HRP. Further anticipated advantages included the ability to monitor the extent of the immune reaction in coloured or opaque solutions, or in emulsions such as milk. Furthermore, ELISA readers based on conductimetry are less expensive to produce and more rugged than optical systems. The ruggedness of an ELISA reader is of particular importance if such units are to be adopted for field use.

In order for urease to replace HRP in indirect competitive ELISAs, it was necessary that the conductance reader available for monitoring the extent of the enzyme reaction (and ultimately quantitation of ELISA results) be of sufficient sensitivity. The ELISA reader used in the urease studies described herein was tested using aqueous solutions of sodium chloride, and was found to have a detection limit of 6.1 μM sodium chloride, which was calculated as the mean background conductance value of milli-Q water + three standard deviations of the mean (Figure 3.11).

The appropriate working dilution of urease conjugate was determined by titrating the conjugate at various dilutions against a constant level of Ab. In this series of experiments, plates were first coated with 1.0 μg/mL of BSA-ST, followed by a 1:5,000 dilution of Ab. These levels of both coating conjugate and serum were very high; however, earlier attempts with amounts previously found to be optimal for the HRP reaction with TMB in the indirect format met with failure in that no urease signal was detected. Therefore, it was necessary to significantly increase coating conjugate and serum levels in order to attain any signal. This was, in fact, an indication that the urease system could not out-perform the indirect competitive system already developed using HRP and TMB, where serum could be diluted 1:500,000 and a coating conjugate level of 0.0313 μg/mL could be employed (Section 3.1.2.6).

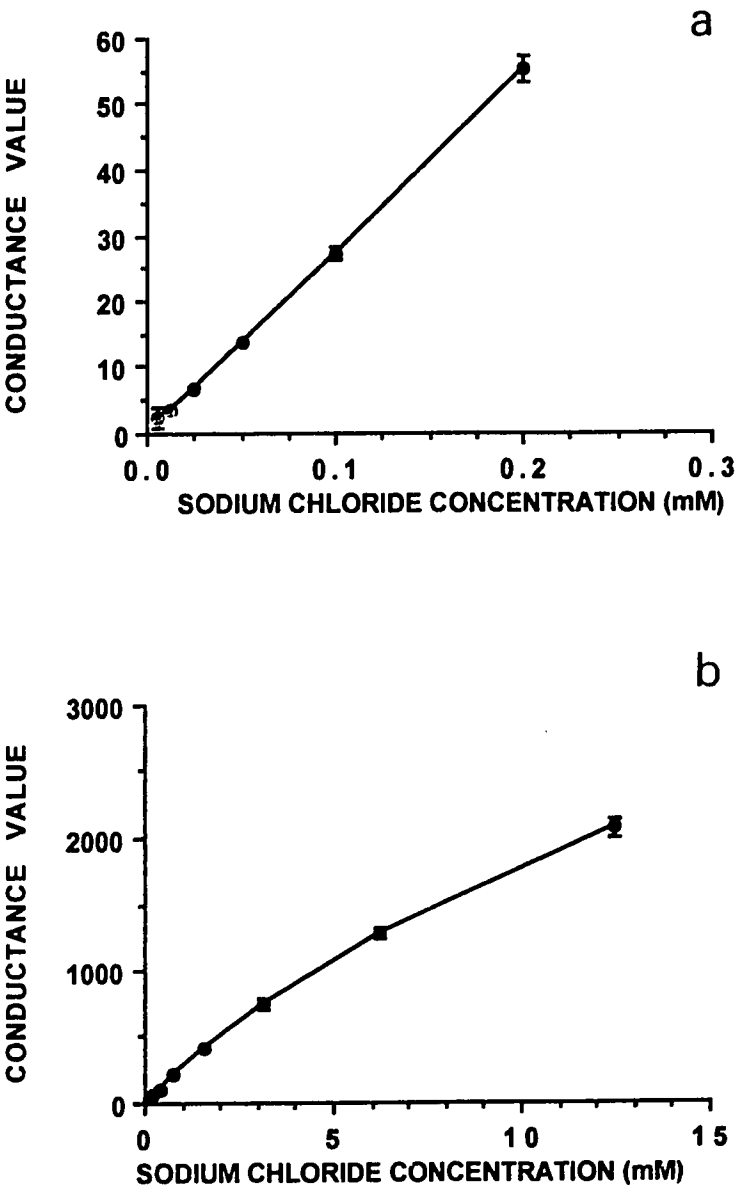


Figure 3.11 Conductance Values of Sodium Chloride Solutions

Points represent the mean conductance value of three wells; error bars indicate the standard deviation of the mean. Figure 3.11a shows the mean conductance values over the range 0.006 to 12.5 mM sodium chloride; Figure 3.11b shows the mean conductance values over the range 0.006 to 0.2 mM sodium chloride.

As shown in Figure 3.12, an appreciable signal from the urease reaction could be achieved only with a 1:200 dilution of the conjugate. While it is likely that a higher signal could have been achieved using a less dilute solution of urease conjugate, such a level of conjugate would be uneconomical. Even a dilution factor of 200 was quite low, considering that both manufacturers of the conjugates (Sigma Chemical Co., St. Louis, MO and Biodesign International, Kennebunkport, ME) suggested a 1:1,000 working dilution. Both suppliers based their working dilution requirements on results obtained using bromcresol purple as the indicator of the urease reaction with urea. The conjugates were tested in an indirect competitive ELISA format at a dilution of 1:1,000 using bromcresol purple as indicator; however, results were not encouraging as colour change was minimal.

The poor performance of the urease conjugate in the indirect competitive ELISA raised questions as to the reactivity of the enzyme portion of the conjugate. Another possible explanation was that the Ab portion was inactive. Both conjugates had been prepared using the one-step glutaraldehyde method of Avrameas *et al.* (1978). The possible effects of such a conjugation method on both the enzyme activity and immunoreactivity of the conjugate will be discussed below.

The activity of the enzyme portion of the urease conjugates was assessed by diluting urease conjugate 1:1,000 (v/v) in PBST and allowing it to react in solution with urea (1.0 mg/mL). The results shown in Figure 3.13 indicated that the enzyme was indeed active. After only six min reaction time, conductance readings were approximately double those obtained after 30 min incubation of a 1:200 dilution of the same conjugate in an indirect competitive ELISA. Furthermore, Figure 3.13 shows that conductance values continued to increase in a linear fashion with time up to at least 30 min, where conductance approached 350 units, representing a 5-fold increase over readings obtained in the same period of time in the ELISA.

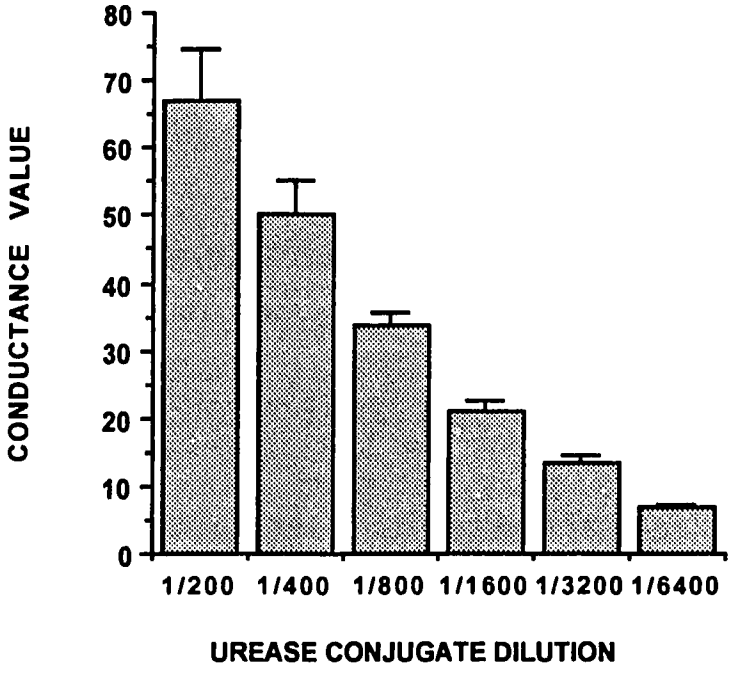


Figure 3.12 Effect of Dilution on Conductance Values Generated by Reaction of Bound Urease Conjugate With Urea

Points represent the mean conductance value of ten wells; error bars indicate the standard deviation of the mean. Reaction time was 30 min at room temperature. Coating conjugate concentration was 1.0 μg/mL; serum was diluted 1:5,000.

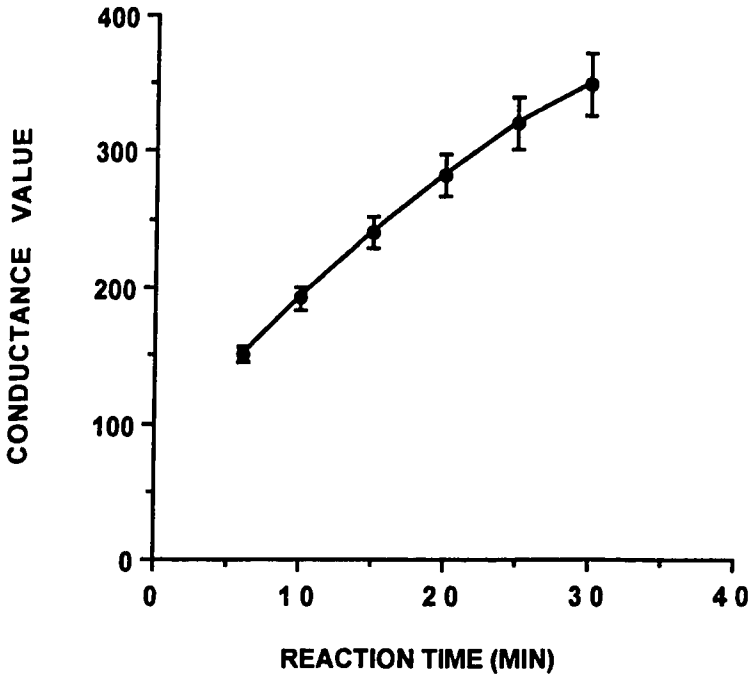


Figure 3.13 Reaction of Urease-Ab Conjugate in Solution

Each point represents the mean conductance value of 12 wells; error bars indicate the standard deviation of the mean. Wells contained 100 μL of 1:1,000 urease conjugate and 100 μL of 1.0 mg/mL urea.

Considering that the urease portion of the conjugate was, in fact, active, it was necessary to determine if the Ab fraction was functional. Loss of immunoreactivity of the Ab during the conjugation might account, at least in part, for the poor performance of the conjugate in the indirect ELISA. This was assessed by determining the ability of the urease conjugate to inhibit the binding of an HRP conjugate to Abs immobilized on a microtitre plate. The premise behind this experiment was that if both the urease and HRP conjugates possessed the same affinity for the primary (immobilized) Ab, the enzyme activity of the HRP conjugate, which was used for monitoring, would be reduced by 50% when the amounts of each conjugate bound were equivalent. The results shown in Figure 3.14 indicated that the urease conjugate was able to markedly (i.e., 50%) inhibit the binding of the HRP conjugate only at a dilution of 1:200 of the former. As the dilution rate of the urease conjugate increased, its ability to compete with the HRP conjugate for the primary Abs was impaired. At a dilution of 1:800, essentially none of the urease conjugate was bound to the primary Ab; instead HRP predominated, attesting to its greater affinity for and/or ability to bind to the primary Ab.

Tijssen (1985) commented that although glutaraldehyde is used widely to conjugate enzymes to other molecules, principally because the methods are applicable to a wide range of proteins and are technically undemanding, the methods generally result in inferior products. The use of the one-step glutaraldehyde method, in which both Ab (or hapten) and enzyme are simultaneously reacted with glutaraldehyde, can result in the formation of insoluble complexes of Ab (Clyne *et al.*, 1973; Hopwood *et al.*, 1970). Although HRP does not self-polymerize in the presence of glutaraldehyde (Clyne *et al.*, 1973), Hopwood *et al.* (1970) noted the polymerization and precipitation of other enzymes when reacted with glutaraldehyde. In addition to being wasteful of reactants, the one-step glutaraldehyde method can lead to the production of conjugates with compromised enzyme activity and immunoreactivity (Tijssen, 1985).

Given that the urease-Ab conjugates evaluated in this study were prepared using the one-step glutaraldehyde method, it is therefore not surprising that such conjugates displayed a more limited ability to combine with Ab than did the HRP-Ab conjugate used in quantitation of the indirect competitive ELISA. However, loss of immunoreactivity of the Ab portion of the urease conjugates may not have been the sole factor accounting for the poor performance of the conjugate. If the molecular weight of each of the enzymes is considered [urease = 545,365 (Zerner, 1991); HRP = 42,100 (Dunford, 1991)], it is possible that the greater size of the urease conjugate, even in the absence of any Ab polymerization, could lead to steric interference,

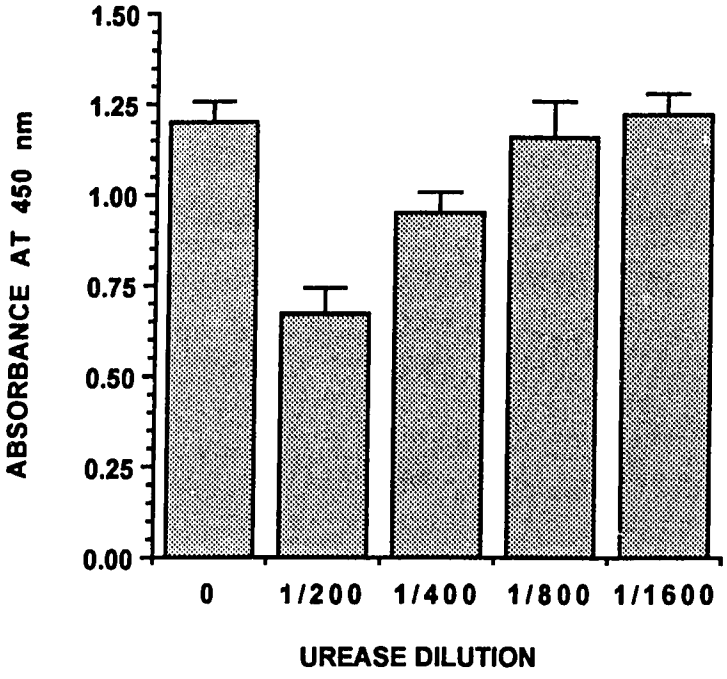


Figure 3.14 Inhibition of Peroxidase Conjugate Binding by Urease Conjugate Binding

Each point represents the mean absorbance value of 12 wells; error bars indicate the standard deviation of the mean. "0" urease dilution indicates no urease conjugate was added. Reaction time of HRP with TMB and hydrogen peroxide was four min at room temperature. Coating conjugate concentration was 1.0 μg/mL; serum was diluted 1:5,000.

particularly in an indirect ELISA. Quantitation of indirect ELISA results generally relies on enzyme amplification; that is, it is assumed that more than one secondary (enzyme-conjugated) Ab binds to the primary Ab (Porstmann *et al.*, 1982). Urease conjugates, by virtue of their large size, may be limited in their ability to bind to the primary Ab at a ratio of greater than 1:1. In fact, if the conjugate were large enough, the binding to adjacent primary Abs may be prevented, reducing the urease-Ab:primary Ab ratio even further.

It should also be noted that the use of conductimetry in immunoassays is likely to be thwarted due to the non-specific nature of the method. Conductimetry cannot discriminate between different reactions, and artifacts can be produced (Lawrence and Moores, 1972).

3.2 DEVELOPMENT OF A DIRECT ELISA FOR SULPHATHIAZOLE

Direct ELISA protocols employ an enzyme-labelled immunoreactant (Section 1.5); as such, they eliminate the requirement for enzyme-conjugated secondary antibody and thereby reduce the time required for analysis. In terms of commercialization, the direct format is preferred as the end user may obtain prepared plates (e.g., with Ab already immobilized) and need only add the sample to be analyzed and the enzyme-analyte conjugate, which is often supplied in a pre-diluted form.

A variety of commercial tests which operate in the direct format is available for the detection of SAs. These include the LacTek test (Idetek, Inc., Sunnyvale, CA), the CITE Sulfa Trio test (IDEXX Laboratories, Inc., Westbrook, ME), Agri-Screen ELISA (Neogen Corp., Lansing, MI) and the EZ-Screen test (Environmental Diagnostics, Inc., Burlington, NC). Although these tests can be quite sensitive (the CITE Sulfa Trio test can detect ST at a level of 10 ppb), they do not generally allow for accurate quantitation.

Considering the ease with which direct competitive ELISAs can be performed, along with the associated time savings, it was decided to undertake the development of a direct competitive ELISA for ST employing immobilized Ab and ST-labelled enzyme.

3.2.1 Synthesis of ST-Enzyme Conjugates

In order to develop a direct competitive ELISA for ST, it was necessary to synthesize a conjugate of ST and an enzyme marker. The choice of enzyme is important in labelled-hapten assays, since it determines the sensitivity of such assays to a large extent. Since it is unlikely that more than one enzyme-labelled analyte will bind to Ab, due to size and steric considerations, there is no amplification effect as is achieved in indirect ELISAs where more than one enzyme-labelled secondary Ab can bind to the primary Ab; therefore, it is desirable to use an enzyme with a high turnover number (Schneider and Hammock, 1992).

As was discussed (Section 3.1.2), HRP is generally considered to be the enzyme of choice for EIAs. For this reason, HRP was used as the marker enzyme for the detection of ST in the direct ELISA developed in this work. Our choice is supported by the results of Schneider and Hammock (1992) who showed that the sensitivity of the direct ELISA could be increased 100-fold when an HRP-hapten conjugate was used instead of an alkaline phosphatase-hapten tracer molecule.

3.2.2 Selection of HRP-ST Conjugate for Use in Direct ELISAs

Of the several methods of chemical conjugation available for HRP, two were selected and evaluated. These were linkage using the two-step glutaraldehyde method, a modification of the original method of Avrameas (1969), and the periodate cleavage method developed for the conjugation of Abs by Wilson and Nakane (1978). Although Tijssen (1985) indicated that the periodate cleavage is preferable to the two-step glutaraldehyde method for HRP conjugation, reports in the literature indicate that the latter of these methods continues to be popular (Dixon-Holland and Katz, 1991).

Our laboratory has had considerable success using immunization and coating conjugates of SAs synthesized using the diazo reaction. However, this method cannot be used for the conjugation of HRP to ST, since the enzyme becomes inactivated during the coupling procedure (McAdam *et al.*, 1992).

Three HRP-ST conjugates were synthesized using the two-step glutaraldehyde (G) method as outlined in Section 2.4.3.1, and designated G-Heavy, G-Med, and G-Lite, based on the degree of ST substitution on the HRP molecule. Similarly, three HRP-ST conjugates were prepared using the periodate cleavage method (P) described in Section 2.4.3.2, and designated P-Heavy, P-Med, and P-Lite. It would seem likely that the ideal conjugate would consist of a ratio of hapten to enzyme of greater than or equal to 1:1, as under-substitution of the enzyme may lead to poor presentation of the hapten to Ab; this may be influenced by the folding of the enzyme protein and steric considerations. In addition, under reaction conditions aimed at a low level of enzyme substitution with hapten, there may remain free, unconjugated enzyme which will not bind to Ab in the ELISA. Overloading of the enzyme with ST is to be avoided, as enzyme activity may be compromised at high substitution rates.

The six HRP-ST conjugates synthesized in this study were evaluated on the basis of enzyme efficiency in a direct ELISA format (Figure 3.15). From these results, it was evident that HRP-ST conjugate P-Med, synthesized using the periodate cleavage method using a molar ratio of ST to HRP of 375:1, resulted in the most efficient use of the enzyme, as shown by the high absorbance values obtained per mg enzyme protein.

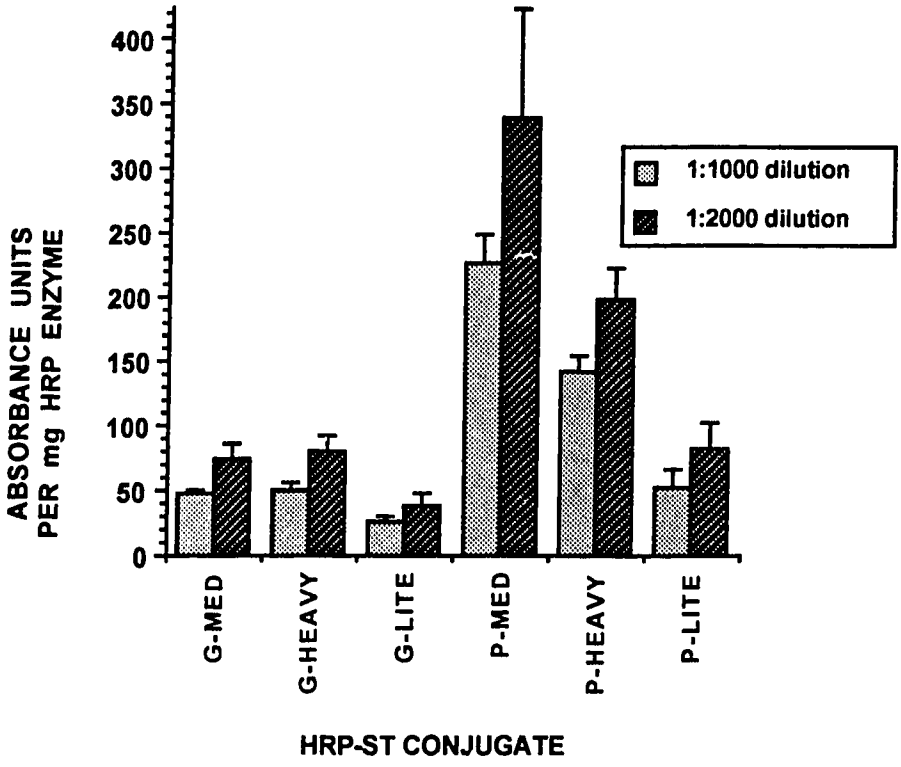


Figure 3.15 Efficiency of HRP-ST Conjugates

G-Heavy, G-Med, and G-Lite were the heavily-, mediumly-, and lightly-loaded HRP-ST conjugates, respectively, synthesized using the two-step glutaraldehyde method. P-Heavy, P-Med, and P-Lite were the heavily-, mediumly-, and lightly-loaded HRP-ST conjugates, respectively, synthesized using the periodate cleavage method. Each point represents the mean value for two determinations; error bars indicate the standard deviation of the mean. Reaction time between HRP, TMB and hydrogen peroxide was ten min at room temperature; reaction was stopped by the addition of 50 μL/well 2 M sulphuric acid. Serum was diluted 1:25,000.

The mid-range HRP-ST conjugates synthesized using either the glutaraldehyde or periodate cleavage method out-performed the more highly-substituted alternates. As indicated above, over-substitution of the HRP molecule with ST may adversely affect enzyme activity. For both types of conjugates, the most lowly-substituted ones proved to be the least efficient in an ELISA, possibly resulting from poor binding to the Ab due to steric factors and poor presentation of the hapten to the Ab.

Since the lowest-substituted HRP-ST conjugates performed the worst of both types of conjugates produced, it is likely that the optimum ratio of ST to HRP is greater than 1:1, since in the case of the lowest-substituted conjugates, it is known that at least one ST molecule was present on the HRP molecule; otherwise, no enzyme conjugate would have been bound by Ab, and no signal would have been detected. However, as indicated above, during the synthesis of this conjugate some enzyme may have remained free and unconjugated to ST. The results presented in Figure 3.15 were calculated assuming that all of the enzyme initially employed in the conjugation reaction became substituted with ST. Using the direct protocol as outlined in Section 2.4.2.2, any free (unconjugated) HRP is washed away prior to colour development and may account for the lower absorbance values. Assuming no non-specific binding of HRP, only Ab-bound ST-HRP accounted for the signal observed. Given the superior performance of the G-Med and P-Med conjugates, it also follows that they contained ratios of ST to HRP of greater than 1:1.

The results depicted in Figure 3.15 also indicated that the HRP-ST conjugates produced using the periodate cleavage method were generally superior to those produced using the glutaraldehyde cross-linking method. While the two-step glutaraldehyde method continues to be employed for the synthesis of HRP-hapten conjugates (Dixon-Holland and Katz, 1991), the method generally results in low coupling efficiency (Wilson and Nakane, 1978).

Although the exact mechanism involved in the coupling of protein moieties by glutaraldehyde appears to be incompletely understood (Molin *et al.*, 1978; Tijssen, 1985), it is known that the principal attachment sites on HRP are the primary amino groups of amino acids, particularly lysine (Molin *et al.*, 1978). HRP contains six lysine residues (Welinder, 1979). However, in commercial preparations of HRP, the majority of the ε-amino groups of lysine are blocked by allylisothiocyanate or by the carbohydrates surrounding the molecule (Nakane and Kawaoi, 1974), making them unavailable for coupling. This may account for the lack of difference in the efficiencies of the medium- and highly-substituted HRP-ST conjugates produced using the two-

step glutaraldehyde method in this study. It may be that in the medium-loaded HRP-ST conjugate, the maximum degree of substitution on the enzyme had been realized.

The periodate cleavage method is generally accepted as the best method for the conjugation of HRP to macromolecules (Madersbacher *et al.*, 1992, Tijssen, 1985) as it affords higher coupling efficiency than the two-step glutaraldehyde technique (Boorsma and Streefkerk, 1978). HRP is a glycoprotein; its carbohydrate content is estimated at 18% (Nakane and Kawaoi, 1974). The carbohydrate is attached at eight sites on HRP, particularly in the C-terminal half of the molecule (Welinder, 1979), and forms a "shell" around part of the HRP molecule (Wilson and Nakane, 1978). These carbohydrate moieties can be readily oxidized with sodium periodate to yield aldehyde groups without affecting the enzyme activity (Nakane and Kawaoi, 1974; Wilson and Nakane, 1978). The resulting dialdehyde groups on HRP can be coupled with free amino groups on the molecule to be conjugated. Subsequent reduction of the Schiff base with sodium borohydride stabilizes the linkage (Tijssen, 1985). The resulting conjugates show excellent stability when stored at -20°C (Nakane and Kawaoi, 1974).

To our knowledge, this is the first report of the use of the periodate cleavage method for the synthesis of HRP conjugates of sulpha drugs or of hapten molecules of any sort. The bulk of the literature concerns the use of the periodate cleavage method for the conjugation of Abs or other large proteins, containing an abundance of ε-amino groups of lysine, to HRP. From the work described here, it is clear that the method can be employed to conjugate haptens with only weakly basic amino groups. For example, the pK_a for the ε-amino group of lysine is 10.53 (Whitaker, 1972), while the pK_a of the N^4-amino group of sulphathiazole is 2.36 (Bell and Roblin, 1942). Moreover, the conjugates of ST and HRP prepared in this manner are superior to those produced using the two-step glutaraldehyde method.

All of the conjugates evaluated showed greater efficiency at a higher rate of dilution (Figure 3.15). Since only a fixed amount of HRP-ST can bind, depending on the level of Ab immobilized on the plate, the use of more concentrated solutions of HRP-ST is wasteful, since unbound HRP-ST is washed away prior to the addition of enzyme substrate. Furthermore, in order to attain maximum sensitivity in ELISAs, immunoreactants should be present at the lowest concentrations possible.

From these results, the medium-loaded HRP-ST conjugate produced using the periodate cleavage method (P-Med) was chosen for use in subsequent direct ELISA evaluations. The working dilution was set at 1:2,000.

3.2.3 Testing of HRP-ST for EMIT Effects

EMIT (Enzyme-Multiplied Immunoassay Technique) is a homogeneous immunoassay method which relies on a modulation of enzyme activity upon combination of the enzyme-labelled hapten with primary Ab in the immune reaction (Jenkins, 1992). The use of an enzyme-labelled ST molecule in a direct competitive ELISA necessitated the testing of the conjugate for EMIT effects. That is, it was necessary to determine if the signal generated by HRP was altered appreciably upon binding of the HRP-ST to the anti-ST Abs during the competition step.

Figure 3.16 depicts the results of the testing of the highly-substituted HRP-ST conjugate prepared by the two-step glutaraldehyde method (G-Heavy). Although there was a slight decrease in HRP activity in the presence of serum as compared to that in the blank (without Ab), the activity of the enzyme appeared to remain constant, regardless of serum level.

3.2.4 Selection of Direct ELISA Format

The format of the direct ELISA can greatly influence important parameters of the assay, such as sensitivity, the maximum absorbance attainable from enzyme reaction with its chromogenic substrate, and the analysis time required. If the proper strategy for incubation of the Ab, enzyme-analyte conjugate and free analyte is established, the free analyte can compete at very low levels, thereby increasing the sensitivity of the assay, provided the affinity of the Abs is slightly greater for the analyte than for the enzyme-analyte tracer (Schneider and Hammock, 1992).

The use of an enzyme-labelled hapten as the competitor conjugate necessitates the use of an Ab-coated solid phase. Key to the success of such ELISAs is the correct orientation of the Abs on the solid phase, such that their paratopes are left free to combine with hapten or tracer (Schramm and Paek, 1992).

To encourage the correct orientation of Abs on microtitre plates, Schneider and Hammock (1992) evaluated the use of Protein A and goat anti-mouse (GαM) Igs for the trapping of murine Abs, as well the use of a simple coating of murine Abs on the solid phase. These procedures were also evaluated in the present study.

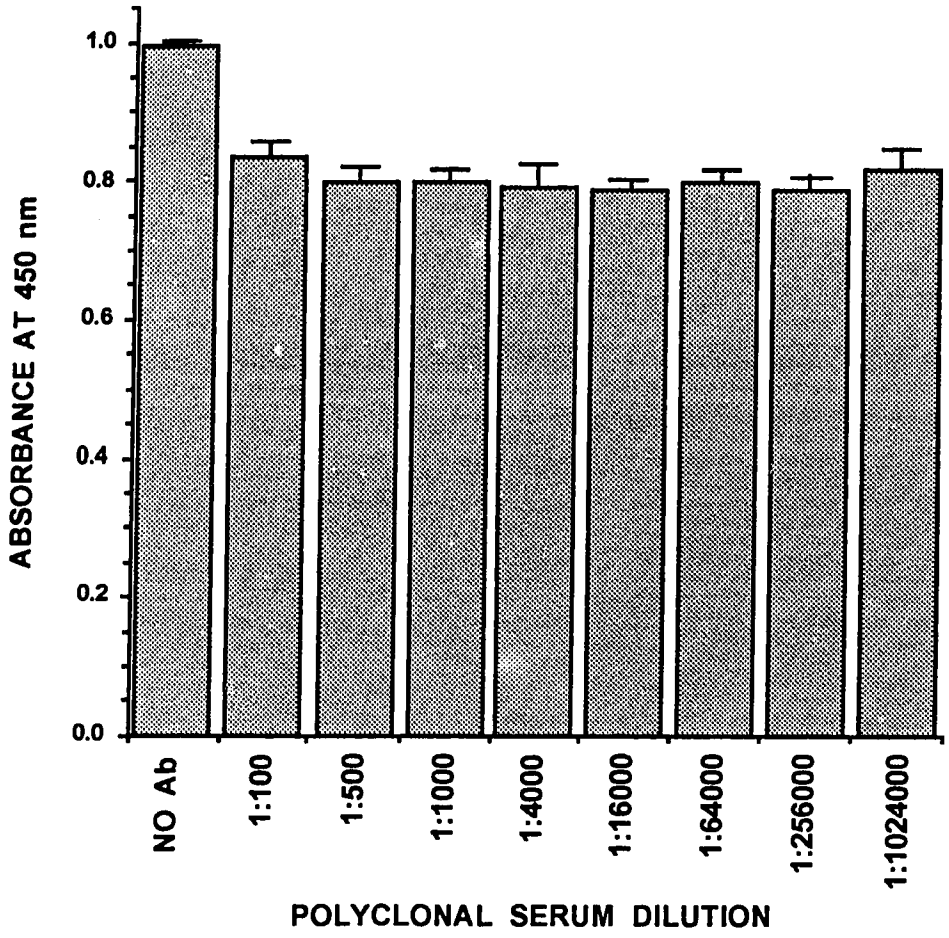


Figure 3.16 Effect of Serum Dilution on Activity of Enzyme Conjugate (HRP-ST) in Solution

Each point represents the mean absorbance value of 6 wells; error bars indicate the standard deviation of the mean. Reaction time for HRP with TMB and hydrogen peroxide was two min at room temperature; reaction was stopped with 50 μL/well 2 M sulphuric acid. HRP-ST conjugate (G-Heavy) dilution was 1:1,000.

The results of checkerboard ELISAs (Section 2.4.3.3) indicated that the use of Protein A in PBS was superior to the use of either GαR F_c-specific trapping Abs, or the simple adsorption of rabbit Abs to the microtitre plate in preparing plates with Ab for the competition step. Under identical conditions of serum and HRP-ST dilutions, higher absorbance values could be achieved on plates first treated with Protein A. Furthermore, these higher absorbance values could be reached in a shorter incubation time, indicating that by using a Protein A pre-coating step, serum levels could be reduced as compared to those necessary using either trapping Abs or no plate pre-treatment.

Figure 3.17 shows two representative competition inhibition curves derived using Protein A-coated plates and plates treated with trapping Abs. While the shape of the curves is comparable, as well as maximum absorbance values, it should be noted that background absorbance values were higher using trapping Abs. The B_0 values shown in Figure 3.17 for the Protein A pre-coating format were achieved in only five min, indicating that a further reduction in serum levels was permissible. Conversely, the use of trapping Abs necessitated longer incubation periods (20 min), even under conditions of higher levels of HRP-ST.

No competitive inhibition curves were derived for the treatment wherein plates were simply coated with the rabbit antiserum. Results of the checkerboard titration indicated that excessively high levels of serum (e.g., 1:1,600) were required to allow for the development of appreciable colour in the quantitation step. Moreover, even after extended periods of incubation (e.g., 30 min), the absorbance values remained quite low as compared to those obtained using either of the pre-coating steps. This is supported by the findings of Schneider and Hammock (1992). Therefore, further investigation of the simple adsorption direct assay format was suspended.

It was not surprising to find that the simple adsorption of rabbit Abs to microtitre plates in preparation for a direct competitive ELISA led to poor results in the checkerboard assay. First, the adsorption of the Abs directly onto the plastic surface can lead to a loss of ability to bind Ags (Ansari *et al.*, 1978; Sankolli *et al.*, 1987). Second, the non-specific nature of the binding of Ab protein to plastic also encourages a very random orientation of Abs; thus, some Abs may be immobilized through their F_{ab} portions, and affect their ability to capture Ags (Schramm and Paek, 1992; Widjojoatmodjo *et al.*, 1993).

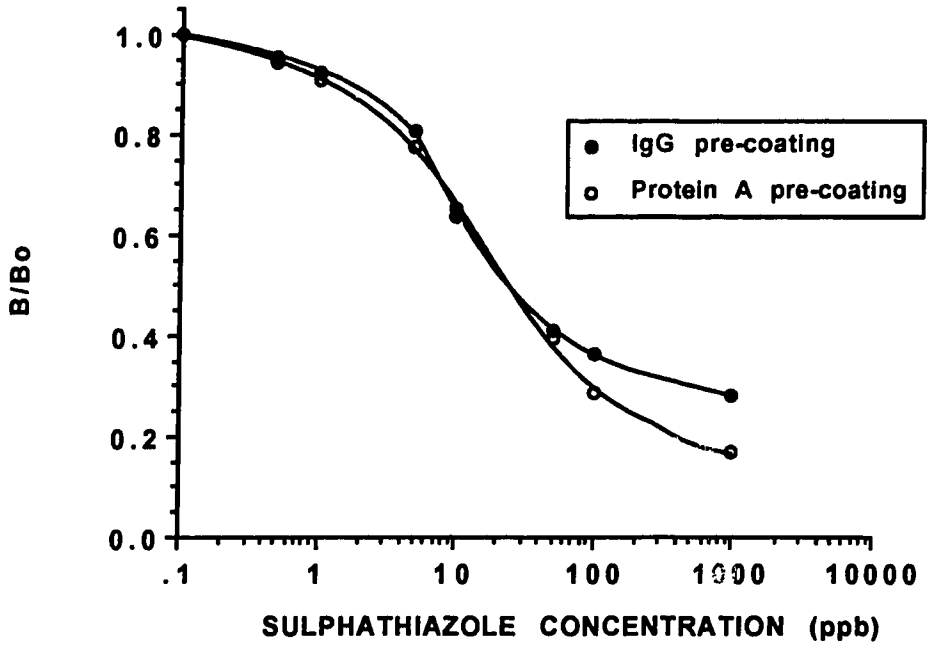


Figure 3.17 Comparison of Protein A and Anti-immunoglobulins asPre-coating Reagents in Direct ELISAs for Sulphathiazole

Each point represents the mean absorbance value of ten wells; curve parameters, as determined by *SOFTmax*, are given in the table below, where "a" and "d" are absorbance values at 450 nm. HRP-ST G-Med conjugate was used for both treatments. Wells were coated with 1.0 µg/mL Protein A or Anti-Ig (100 µL/well). Plates were developed using TMB as chromogenic substrate for HRP; reaction was stopped by the addition of 2 M sulphuric acid (50 µL/well); absorbance values were read at 450 nm.

PARAMETER (See equation page 42)	PROTEIN A TREATMENT	ANTI-Ig TREATMENT
Correlation coefficient (r)	0.998	0.991
"a" value (A_{450})	0.384	0.415
"b" value (slope)	0.849	0.973
"c" value (ppb ST)	16.3	11.5
"d" value (A_{450})	0.056	0.116
Reaction time (min)	5	20
Serum dilution	1:3,200	1:6,400
Conjugate dilution	1:4,000	1:1,000

In contrast, the use of either trapping Abs (in this case, GαR Abs specific for the F_c portion of the primary Ab) or Protein A as a preliminary step promotes a more favourable orientation of Abs on the microtitre plate by causing them to be bound through their F_c regions. Such an orientation leaves the paratopes of the bound Abs free to react with Ags (Schramm and Paek, 1992; Widjojoatmodjo *et al.*, 1993). Furthermore, since the Abs are not physically adsorbed to the plastic, they may retain more of their native Ag binding ability, quite apart from simply being oriented in the correct position. Schneider and Hammock (1992), who also found the pre-coating step to be advantageous to the performance of a direct competitive ELISA, suggested the enhanced sensitivity and reproducibility of such assays may stem from the low concentrations of Ab and enzyme tracer that can be used under such conditions.

Sankolli *et al.* (1987) similarly found that the use of an F_c-specific Ab was preferable to the simple coating of microtitre plates with primary Ab in promoting high binding capacity and low levels of well-to-well variation. They made no comparison regarding the ability of Protein A to serve in the same capacity as the trapping Abs.

While Schneider and Hammock (1992) did not find a significant difference between the use of Protein A or trapping Abs on the direct assay performance, Widjojoamodjo *et al.* (1993) found a distinct advantage to the use of Protein A as compared to GαM Abs for the immobilization of murine MAbs onto magnetic beads. They attributed the superior performance of the assay utilizing Protein A to the increased Ab-binding capacity of the Protein A-treated beads. The results of Widjojoamodjo *et al.* (1993) support the findings discussed in the present work.

3.2.5 Comparison of HRP-ST Conjugates In a Competitive ELISA for Sulphathiazole

The performances of the medium-loaded HRP-ST conjugates synthesized by the periodate cleavage and two-step glutaraldehyde methods were compared in competitive assays for ST (Figure 3.18). The higher efficiency of the conjugate produced by periodate cleavage permitted much higher absorbances ("a" values) than were attainable using the conjugate synthesized by the glutaraldehyde method after reaction with hydrogen peroxide and TMB in a comparable time frame. Also striking was the difference in the background absorbance values as a function of maximum absorbance (i.e., B/B_0 at infinite ST concentration).

The direct competitive ELISA for ST using aqueous standards was found to operate optimally using a pre-coating step with Protein A (100 μL/well of a 1 μg/mL solution), along with a serum dilution of 1:25,000, and a 1:2000 dilution of HRP-ST (P-Med) in the competition step, as shown in Figure 3.18.

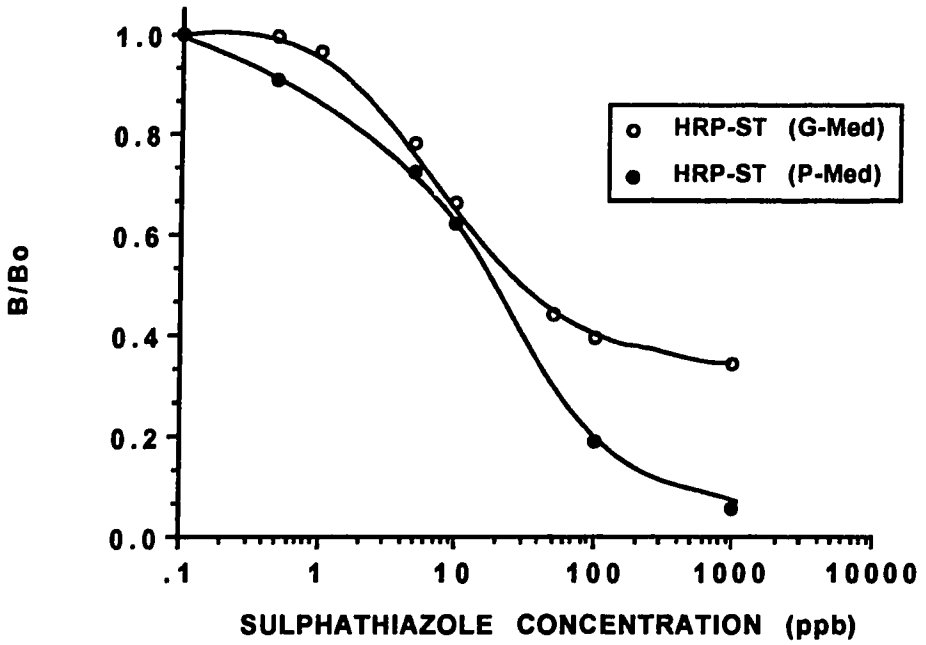


Figure 3.18 Comparison of Mediumly-loaded HRP-ST Conjugates Synthesized Using the Two-Step Glutaraldehyde and Periodate Cleavage Methods

Each point represents the mean absorbance value of ten wells; curve parameters, as determined by *SOFTmax*, are given in the table below. Wells were coated with 1.0 µg/mL Protein A (50 µL/well). Plates were developed using TMB as chromogenic substrate for HRP; reaction was stopped by the addition of 2 M sulphuric acid (50 µL/well); absorbance values were read at 450 nm.

PARAMETER (See equation page 42)	HRP-ST (P-Med) (PERIODATE)	HRP-ST (G-Med) (GLUTARALDEHYDE)
Correlation coefficient (r)	0.997	0.999
"a" value (A_{450})	0.927	0.183
"b" value (slope)	0.858	1.05
"c" value (ppb ST)	16.2	9.31
"d" value (A_{450})	0.027	0.061
Reaction time (min)	10	5
Serum dilution	1:25,000	1:25,600
HRP-ST dilution	1:2,000	1:2,000

As previously indicated (Table 3.1), the indirect competitive ELISA could be conducted using a serum dilution of 1:500,000. Thus, the direct system displayed a 20-fold increased demand for serum as compared to its indirect counterpart. This may be accounted for by the difference in the protocols at the point of serum addition. In the direct system, serum is added at a concentration such that the wells are completely coated with protein; however, this does not imply that all protein added becomes adsorbed to the solid phase, since the plastic surface or the Protein A or anti-IgG pre-coating has a finite capacity for protein attachment. Tijssen (1985) indicated that about 1.5 ng/mm^2 of BSA can be adsorbed, regardless of the input. It is likely, therefore, with respect to the present work, that of the 1:25,000 dilution of PAb used to coat the microtitre plates, only a small percentage was actually immobilized; the remainder may have been recoverable and useful for subsequent assays. This was found to be true for the Protein A solutions used in preparing the plates for serum addition.

3.3 DEVELOPMENT OF ELISAs FOR SULPHATHIAZOLE DETECTION IN MILK AND HONEY

The ultimate objective in the development of an enzyme immunoassay for ST was to apply such a test for the quantitation of ST in food products. As outlined in the literature review of this manuscript, ST is widely used in animal husbandry and as such may result in violative residues of ST occurring the milk of dairy cattle. ST is also effective in preventing AFB among honey bees, and may be used by unscrupulous beekeepers, resulting in ST residues in the honey crop.

3.3.1 Sulphathiazole Analysis of Milk Using Direct and Indirect ELISA

Using milk as the test medium, both the direct and indirect ELISA protocols developed for aqueous ST standards were evaluated. By manipulating the serum dilution in both types of ELISAs, and by altering the BSA-ST coating conjugate concentration for the indirect procedure, it was possible to fine-tune both assays with respect to the "c" values obtained. Results are included in Tables 3.2 and 3.3.

As discussed in Section 3.2.5, the direct ELISA had a much higher demand for serum than did the indirect assay; however, under optimum conditions for both approaches, "c" values could be reduced by about 150% using the direct system (i.e., from 54.4 ppb to 32.8 ppb). Although the magnitude of the improvement of the direct assay over the indirect was perhaps unimpressive in terms of the numbers involved, considerable time-savings may be realized through the use of the direct assay by eliminating the need for a secondary enzyme-conjugated Ab. Therefore, based on the results presented in Tables 3.2 and 3.3 and consideration of the time savings factor, it was concluded that the direct ELISA was superior to its indirect counterpart for the detection and quantitation of ST in milk.

The results of Table 3.3 clearly demonstrate the influence of serum concentration on the performance of a direct ELISA. Decreasing serum levels were accompanied by a reduction in the "c" values. The ultimate limiting dilution was determined by the ability to detect a signal from HRP. By employing an alternate label and detection system (e.g., chemiluminescence), it may be possible to further reduce the sensitivity of the direct ELISA from that demonstrated in Table 3.3.

Using an indirect PAb ELISA system and an azo-linked BSA-SMR coating conjugate, Garden and Sporns (1994) achieved a "c" value of 1.4 ppb for SMR in milk. The values presented in this thesis using a direct ELISA are higher than those of Garden and Sporns (1994); this may be due to a number of reasons. Possibly most important is the fact that these authors used a BSA-SMR coating conjugate with an

Table 3.2 Summary of Indirect ELISAs for Sulphathiazole in Milk

SERUM DILUTION	BSA-ST CONCN (μg/mL)	"a" VALUE (A_{450})	"b" VALUE (Slope)	"c" VALUE (ppb)	"d" VALUE (A_{450})	r	"c" VALUE IN MILK (c_{milk})[1] (ppb)
1:50,000	1.0	2.23	0.923	33.9	0.119	0.998	90.4
1:100,000	1.0	1.71	0.698	22.9	0.040	0.994	61.1
1:150,000	1.0	1.17	0.904	22.9	0.058	0.997	61.1
1:200,000	1.0	1.06	0.924	20.4	0.067	0.997	54.4
1:300,000	1.0	0.555	0.877	42.4	0.059	0.965	282.7
1:400,000	1.0	0.324	1.30	28.3	0.081	0.945	75.5
1:100,000	0.5	0.599	1.34	41.7	0.058	0.971	111.2
1:200,000	0.5	0.132	3.96	28.7	0.058	0.926	76.5
1:300,000	0.5	0.181	1.98	34.8	0.065	0.968	92.8

Values represent the mean values calculated from the absorbance values of six wells; curve parameters were generated using *SOFTmax*. Reaction time for HRP with TMB and hydrogen peroxide at room temperature was 30 min for all treatments; 50 μL/well 2M sulphuric acid was added to stop the enzyme reaction.

[1] c_{milk} = "c" x 2.667

Table 3.3 Summary of Direct ELISAs for Sulphathiazole in Milk

SERUM DILUTION	"a" VALUE (A_{450})	"b" VALUE (Slope)	"c" VALUE (ppb)	"d" VALUE (A_{450})	r	"c" VALUE IN MILK (c_{milk})[1] (ppb)
1:25,000	0.165	1.22	12.3	0.057	0.935	32.8
1:25,000	0.214	0.948	13.3	0.052	0.986	35.5
1:18,750	0.269	0.943	19.5	0.052	0.985	52.0
1;12,500	0.324	1.35	14.8	0.059	0.973	39.5
1:12,500	0.434	1.35	19.0	0.062	0.969	50.7
1:6,250	0.751	1.07	25.7	0.057	0.979	68.5
1:6,250	0.749	1.10	29.6	0.054	0.969	78.9
1:6,250	0.796	0.972	31.7	0.054	0.993	84.5

Values represent the mean values calculated from the absorbance values of six wells; curve parameters were generated using *SOFTmax*. HRP-ST conjugate (P-med) dilution was 1:2000. Reaction time for HRP with TMB and hydrogen peroxide at room temperature was 30 min for all treatments; 50 µL/well 2M sulphuric acid was added to stop the enzyme reaction.

[1] c_{milk} = "c" x 2.667

exceedingly low level of substitution (~4 moles SMR/mole BSA). Considering the high degree of substitution of ST in the BSA-ST coating conjugate used in the indirect ELISAs of the present study, it is possible that the sensitivity of this system may have been decreased further, and perhaps below that of the direct system, by employing a minimally-substituted BSA-ST conjugate.

The fact that the study of Garden (1994) and that presented here were performed using different haptens as well as different polyclonal sera cannot be overlooked when comparing the results of both studies. Since no kinetic studies were performed to assess the affinities of the PAbs in both studies for their respective target analytes (SMR and ST), no firm conclusions can be drawn regarding the superiority of one serum over the other. Given the success Garden and Sporns (1994) experienced using the indirect ELISA, it may be that the polyclonal serum used had a higher affinity for SMR than the anti-ST serum used in the present study did for ST.

3.3.2 Sulphathiazole Analysis of Honey Using Direct ELISA

A variety of methods has been developed for the detection of SAs in honey, the bulk of which have used chromatographic techniques (Argauer *et al.*, 1982; Barry and MacEachern, 1983; Belliardo, 1981; Diaz *et al.*, 1990; Horie *et al.*, 1992; Jürgens, 1982; Neidert *et al.*, 1986). In addition to the necessity for extensive sample clean-up procedures, most of the methods cited above had detection limits in the high ppb-low ppm range.

The first ELISA method for the analysis of ST in honey was developed by Sheth and Sporns (1990). This was an indirect ELISA which employed OPD as the detection reagent for HRP activity. The method suffered from the need for excessive amounts of serum (1:32,000 dilution) as well as very dilute honey samples (1:30, w/v). While the method eliminated the need for sample clean-up, it had a relatively high detection level (300 ppb). Assil (1991), who developed an indirect ELISA for the analysis of fumagillin in honey, indicated that more concentrated honey solutions are less likely to exhibit wide variability among different samples.

The objective of the present study was to develop an ELISA method, using HRP as the marker enzyme and TMB as the chromogenic substrate, to improve on the sensitivity for ST achieved by Sheth and Sporns (1990). It was also desirable that the assay permit the analysis of minimally diluted honey samples. Given the superior performance of the direct ELISA in detecting ST in milk, efforts were concentrated on maximizing the sensitivity of the direct ELISA for ST analysis in honey.

3.3.2.1 *Effect of Honey Dilution on Performance of Direct ELISA for Sulphathiazole*

Minimal dilution (i.e., 1:2 or 1:5, w/v) of honey samples with milli-Q water led to high background absorbances in the direct competitive ELISA for ST, resulting in poor competitive inhibition curves. By increasing the dilution rate of honey to 1:10 (w/v), the problem of high background absorbances was eliminated. As was observed in milk tests (Section 3.3.1), reduction of serum levels led to increased sensitivity of the test for ST (Table 3.4).

It was clear that at a low level of dilution, certain factors or substances in the honey samples were adversely affecting the assay. Since background absorbance values were quite high, it appeared as though more concentrated honey samples promoted a non-specific binding of the HRP-ST conjugate.

White (1975) indicated that honey is typically a concentrated solution of the monosaccharides glucose and fructose (Appendix I). It was therefore postulated that the high osmotic pressure of honey may have affected the performance of the ELISA. This hypothesis was proven false, however, when a honey analogue was prepared and assayed at dilutions comparable to those causing poor results using actual honey samples. Even at a dilution of 1:2 (v/v), the honey analogue could be assayed effectively in the direct competitive ELISA.

It was further postulated that minor constituents of honey samples, particularly hydrophobic substances such as wax and flavour volatiles, could interfere with the ELISA at low levels of dilution where such components would not be diluted out. To test this, concentrated honey samples (1:2 dilution, w/v) were pre-treated using C_{18} reverse-phase cartridges, which would retain the more hydrophobic organic components. High background levels remained a problem in the analysis of the C_{18} cartridge-treated honey; therefore, it was evident that minor organic components were not responsible for the interference in the ELISA.

3.3.2.2 *Effect of Honey pH on Performance of Direct ELISA for Sulphathiazole*

It was hypothesized that the naturally low pH of honey (~3.7) might have an influence on the immune reaction between Ab and HRP-ST, particularly at high concentrations of honey. It was thus necessary to study the effect of varying the pH of honey solutions on the results of a direct ELISA for ST determination.

Table 3.4 Direct ELISAs for Sulphathiazole Detection in 1:10 (w/v) Dilutions of Honey

SERUM DILUTION	"a" VALUE (A_{450})	"b" VALUE (Slope)	"c" VALUE (ppb)	"d" VALUE (A_{450})	r	"c" VALUE IN HONEY (c_{honey})[1] (ppb)
1:25,000	0.129	1.46	3.7	0.056	0.989	98.7
1:18,750	0.194	1.07	3.3	0.058	0.966	88.0
1:12,500	0.320	1.05	5.7	0.056	0.976	152.0
1:6,250	0.712	0.968	7.9	0.044	0.981	210.0

Values represent the means calculated using the mean absorbance values of six wells; curve parameters were generated using *SOFTmax*. HRP-ST conjugate (P-med) dilution was 1:2,000. Reaction of HRP with TMB and hydrogen peroxide was 30 min at room temperature; 50 µL/well 2M sulphuric acid was added to stop the enzyme reaction.

[1] c_{honey} = "c" x 26.7

It was ultimately found that the pH value of the honey had a profound effect on the performance of direct ELISAs for ST and was responsible for the high background absorbances observed in the direct competitive format. By increasing the pH of a 1:3 (w/v) dilution of honey in water from the native value (~3.7) to 8, in approximately 1 pH-unit increments, background absorbance values could be significantly reduced (Table 3.5). The results of Table 3.5 also indicate that the pH of the honey sample had a large effect on the "a" values in the assay; "a" values generally increased with increasing honey pH. The detection ability of the assay also improved significantly as honey pH increased, as indicated by the reduction in "c" values.

Given the physiological conditions under which Abs are produced, it was not surprising to find that a honey pH of ~7.1 permitted the best results to be obtained in a direct competitive ELISA for ST. The physiological pH of most mammals is slightly above neutrality (Pearson and Young, 1989); therefore, the immune response in which Ab production occurs in rabbits is in an approximately neutral environment with respect to pH. Not only will this pH value influence the ionization state of the Ag (if applicable), but also the ionization states of the individual amino acids that comprise the Ab protein. Therefore, the Ab formed in response to an Ag at physiological pH will have a distinct ionizability. The charges on the constituent amino acids also influence the three-dimensional structure of the Ab protein. Therefore, subjecting the Ab to pH levels outside the physiological value will promote protonation or deprotonation of amino acids (depending on pK_a values), which will in turn influence the three-dimensional structure of the Ab, including its binding site, and hence its ability to both recognize and combine with Ag. Destruction of conformational determinants, if present on the Ab, may also ensue under extremes of pH, leading to a loss of immunoreactivity with the target Ag.

Further to any conformational changes in Ab structure that may be promoted, changes in pH will also influence the ability of the Ab to form electrostatic bonds with the target Ag. The pH of the reaction medium will influence the charges on both the Ab and the Ag, which may promote or deter the interaction between the two (Roitt *et al.*, 1989; Coleman *et al.*, 1989). High and low pH values favour the dissociation of electrostatic bonds between Ag and Ab, leading to an increased rate of dissociation of immune complexes (van Oss and Absolom, 1984).

Table 3.5 Effect of pH of a 1:3 (w/v) Dilution of Honey on Direct ELISA Parameters

HONEY pH	"a" VALUE (A_{450})	"b" VALUE (Slope)	"c" VALUE (ppb)	"d" VALUE (A_{450})	r	"c" VALUE IN HONEY (c_{honey})[1] (ppb)
3.7	0.471	0.799	23.6	0.259	0.975	188.8
4.0	0.389	1.14	21.7	0.174	0.954	173.6
5.0	0.713	0.723	27.5	0.161	0.977	220.0
6.3	0.615	1.04	25.7	0.079	0.983	205.6
7.1	0.704	0.991	14.3	0.064	0.978	114.4
8.1	0.694	1.02	11.3	0.049	0.985	90.4

Values represent the means calculated using the mean absorbance values of 30 wells; curve parameters were generated using *SOFTmax*. Serum dilution was 1:6,250; HRP-ST conjugate (P-med) dilution was 1:2,000. Reaction of HRP with TMB and hydrogen peroxide was 30min at room temperature; 50µL/well 2M sulphuric acid was added to stop the enzyme reaction.

[1] c_{honey} = "c" x 8

The direct ELISA method discussed herein improved upon that of Sheth and Sporns in two key ways. First, the "c" value of the direct system represented approximately a three-fold reduction in the detection level as compared to that for the indirect system (Sheth and Sporns, 1990). This was largely attributable to the use of a superior chromogen (TMB), such that lower levels of HRP could be used, resulting in lower detection levels for ST. Second, using the direct ELISA, honey samples at their native pH needed only a ten-fold dilution to be analyzed, as compared to the 30-fold dilution used by Sheth and Sporns(1990). It is important to note that the reduction in "c" values demonstrated in the current study, as compared to those of Sheth and Sporns (1990) was a direct result of the protocol employed, since identical serum (i.e., from the same rabbit) was used in both studies.

It is likely that the "c" value for honey samples could be decreased even further using a 1:3 (w/v) dilution of honey, as the absorbance values in the presence of vanishingly small amounts of ST were quite high for the direct ELISA (Table 3.5). This suggested a further reduction in serum levels would be permissible.

The direct competitive ELISA method for ST detection described here is the first rapid test which allows for the quantitation at the 100 ppb level. Current Canadian regulations stipulate a zero tolerance for sulpha drug residues in honey; however, enforcement levels were set at 200 ppb due to the limitations of the available analytical methods. Furthermore, routine testing (by HPLC) by accredited honey testing facilities in Canada results in turnaround times of at least two weeks (Stecyk, 1987). The ELISA method described here will allow increased sample throughput and may afford a reduction in the enforcement levels for ST residues in honey in Canada.

3.4 DEVELOPMENT OF A FLUORESCENCE POLARIZATION IMMUNOASSAY FOR POTATO ALKALOIDS

Potato alkaloids and glycoalkaloids (GAs) are naturally-occurring toxins in potatoes with potencies rivalling those of strychnine and arsenic (Morris and Lee, 1984). Breeding programs are often aimed at increasing disease and drought resistance of new cultivars; this often involves the use of wild potato varieties which generally contain exceedingly high levels of GAs (Van Gelder, 1991). It is therefore imperative to develop rapid and sensitive GA analysis methods for the screening of emerging potato cultivars.

Plhak and Sporns (1992) developed an indirect PAb-based competitive ELISA for the detection and quantitation of the major GAs in commercial potato cultivars. A MAb was also developed and found to be sensitive and specific for potato GAs in an indirect competitive ELISA (Plhak and Sporns, 1994). Despite the success of these two methodologies in detecting GAs, accurate quantitation continued to be a problem due to the high degree of variation associated with solid-phase immunoassay results.

Much of the error in ELISA results arises from the lack of reproducibility in the coating of the solid phase with immunoreactant. In addition, since such methods are biphasic, steric factors can become very important in determining the extent of interaction between immunoreactants. Homogeneous IAs (Section 1.5) can eliminate these sources of error since the reaction occurs in a single (liquid) phase. Although the sensitivity of homogeneous IAs is inherently lower than that of heterogeneous IAs (Gosling, 1990), they are very reproducible and rapid, and thus well-suited to the analysis of compounds present in large amounts (Eremin *et al.*, 1991).

Owing to the increased reproducibility and speed associated with homogeneous IAs, an attempt to develop a fluorescence polarization immunoassay (FPIA) using a fluorescently-labelled solanidine conjugate was undertaken using both polyclonal rabbit serum and a MAb.

3.4.1 Synthesis and Characterization of a Fluorescently-labelled Solanidine Conjugate

The FPIA work described herein was performed using fluorescein-labelled solanidine as the tracer molecule. More specifically, 4'-(aminomethyl)fluorescein (4'-AMF) was linked to a hemisuccinate derivative of solanidine to give AMF-SOL (Section 2.4.1.3). The choice of fluorescein was based on literature reports of its stability, both in its free and conjugated forms, high quantum yield, and ready

excitability and detection of its fluorescence (Edwards, 1985; Gutierrez *et al.*, 1989; Smith *et al.*, 1981).

The 4'-AMF form of fluorescein was selected in order that a relatively short bridge could be made between the fluorescein molecule and the solanidine moiety. According to Colbert *et al.* (1991), positioning the fluorescent molecule more closely to the attached hapten results in a lower detection limit and increased sensitivity in the FPIA.

Dandliker (1977) indicated that the physical and chemical properties of a fluorophore can change upon its attachment to another molecule, such as a hapten. The results shown in Table 3.6 indicate that the excitation and emission wavelength maxima of the free and conjugated forms of 4'-AMF were slightly different. Therefore, all measurements were performed using an excitation wavelength of 496 nm and an emission wavelength of 518 nm.

Similar to the effect of conjugation of a fluorophore to a molecule on the excitation and emission wavelength maxima, the binding of the fluorescein-labelled hapten to its specific Ab can alter the fluorescence intensity (FI) of the fluorescein moiety. In the present work, the combination of AMF-SOL with its specific Ab was found to reduce the FI of the fluorescein by approximately 14%, based on the ratio of the slopes of the regression lines of the standard curves for free and Ab-bound AMF-SOL (Figure 3.19). This reduction in FI upon binding to Ab is attributable to changes in the microenvironment surrounding the fluorophore. Fluorescence is extremely sensitive to its physico-chemical environment, and is responsive to changes in pH, temperature, and solvent system (Schauenstein *et al.*, 1978). Jolley (1981) indicated that the FI of a tracer may increase, decrease, or remain unchanged upon binding to Ab; the observed effect is determined principally by the Ab.

Table 3.6. Excitation And Emission Maxima Of Free And Solanidine-Conjugated 4'-AMF

COMPOUND	λ_{ex} (nm)	λ_{em} (nm)
Free 4'-AMF	492	512
Solanidine-AMF	496	518

Each compound was measured at a concentration of 1000 nM in PBST,pH 7.3 Excitation and emission slit widths were both set at 2.5 nm.

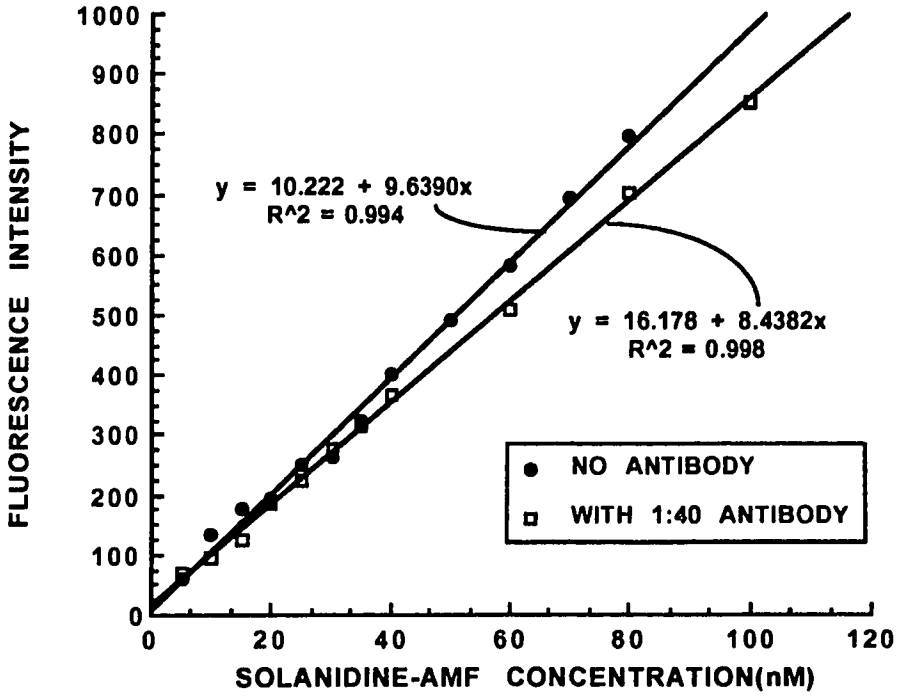


Figure 3.19 Standard Curve for AMF-SOL With and Without Antibody

Each point represents the mean value of six FI determinations. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (10.0 nm slit width). Samples containing Ab were incubated at least 30 min at room temperature prior to taking FI readings.

One final consideration regarding the use of the AMF-SOL label in FPIA measurements was its potential for non-specific binding to proteins. Dandliker *et al.* (1973) indicated that this can be particularly problematic when using fluorescein-labelled tracer molecules. At equivalent concentrations of 4'-AMF and AMF-SOL, coupled with equivalent PAb dilutions, there was no evidence of such non-specific binding in the present study (Table 3.7). The inclusion of Tween 20 in the buffer system also probably prevented the non-specific binding of the tracer and other immunoreactants to the test tube or cuvette walls.

Table 3.7. Fluorescence Polarization Values of Free and Ab-Bound 4'-AMF and AMF-SOL

COMPONENTS	Millipolarization (mP) UNITS
4'-AMF	19±1
4'-AMF + PAb	18±1
AMF-SOL	46±4
AMF-SOL + PAb	221±2

Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width). PAb was present in the reaction at a dilution of 1:200; AMF and AMF-SOL were measured at final concentrations of 60 nM in PBST. Reaction between components was at least 30 min at room temperature. All dilutions were in PBST, pH 7.3. Each value represents the mean value of three determinations ± standard deviation of the mean.

From the data presented in Table 3.7, it can be concluded that the Ab molecule was, in fact, recognizing the solanidine portion of the AMF-SOL conjugate. The relatively high fluorescence polarization (FP) value observed for free AMF-SOL as compared to that for free 4'-AMF was likely due to the large increase in molecular size of 4'-AMF upon linkage to solanidine, resulting in a complex with a molecular weight (MW~841) more than twice that of the free fluorophore (MW~398), rather than to non-specific binding to the Tween 20 component of the buffer system.

The concentration of AMF-SOL used in FPIA measurements was chosen to give an FI value of approximately 500 units when measured with a 2.5 nm excitation slit width and an emission slit width of 10 nm. From the standard curve for AMF-SOL

in the presence of PAb (Figure 3.19), this corresponded to a final AMF-SOL concentration in the FPIA reaction of about 60 nM. This concentration of AMF-SOL was therefore utilized in all FPIA reactions discussed in this manuscript.

The time required to reach equilibrium in the FPIA system was evaluated; however, the time required to generate FP values precluded determination of the exact reaction time. As closely as could be determined, equilibrium was reached in 1-2 min of mixing immunoreactants. At room temperature, therefore, 30 min was more than a sufficient period of incubation.

3.4.2 Polyclonal FPIA

The FPIA system for the detection of potato GAs was evaluated using a polyclonal serum raised against γ-chaconine linked to LPH (Plhak and Sporns, 1992). Periodate cleavage of γ-chaconine was followed by the coupling of resulting aldehyde groups to the amino groups of LPH. This was followed by selective reduction with sodium cyanoborohydride.

3.4.2.1 *Polyclonal Antibody Dilution Curve*

In order to determine the level of PAb to be used in FPIA for GAs, an Ab dilution curve was prepared (Figure 3.20). For analysis, it was desired to use an Ab concentration that netted a 20% reduction in the B_0 value achieved with 60 nM AMF-SOL, similar to the protocol used by Shipchandler *et al.* (1987) and García Sánchez *et al.* (1993). Under such conditions, the addition of analyte would cause a measurable decrease in the FP value. From the curve in Figure 3.20, a final PAb dilution of 1:200 was selected. Thus, all remaining FPIA evaluations used a final PAb dilution of 1:200 and label concentration of 60 nM.

3.4.2.2 *Kinetics of the PAb-(AMF-SOL) Reaction*

One of the drawbacks of working with a polyclonal serum is that it includes a population of different Abs, each with a different affinity for the analyte of interest. Moreover, it is exceedingly difficult to determine the concentration of Ab protein in such a preparation, as a variety of other non-Ig proteins is present in a polyclonal serum (Coleman *et al.*, 1989). Both of these factors constitute barriers to the determination of the kinetics of the immune reaction, and necessitate an altered approach. In this study, a label dilution curve was prepared using a final PAb dilution of 1:200 (Figure 3.21). Using this data, and a number of assumptions (discussed below), it was possible to estimate an average K_{aff} for the PAb population.

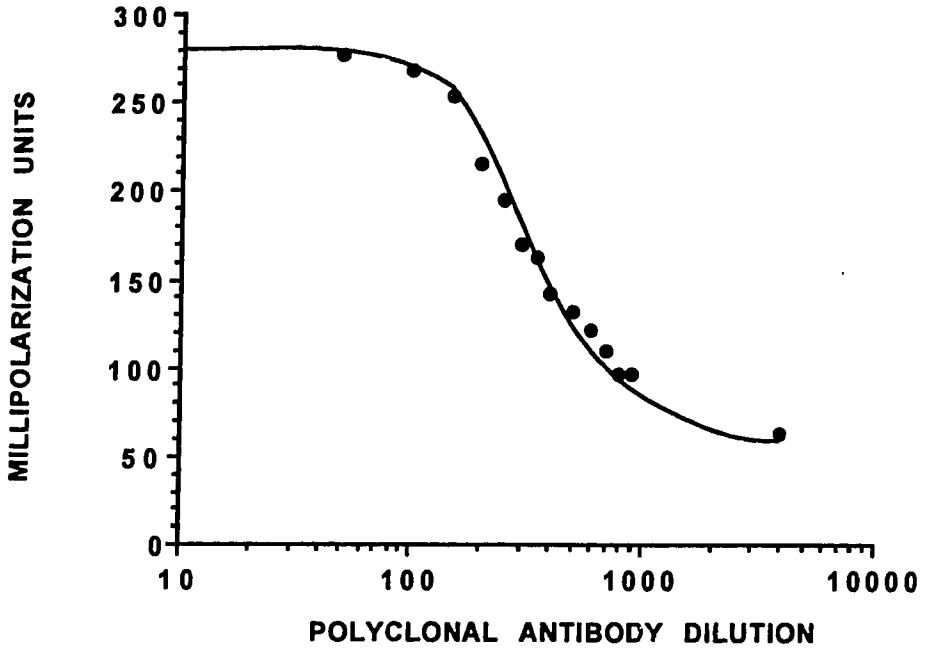


Figure 3.20 Polyclonal Antibody AMF-SOL Dilution Curve

Each point represents the mean value of six determinations. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width). Curve parameters, as determined using *SOFTmax* for original polarization data, were: "a"=0.294; "b"=1.71; "c"=299; "d"=0.062; r=0.991.

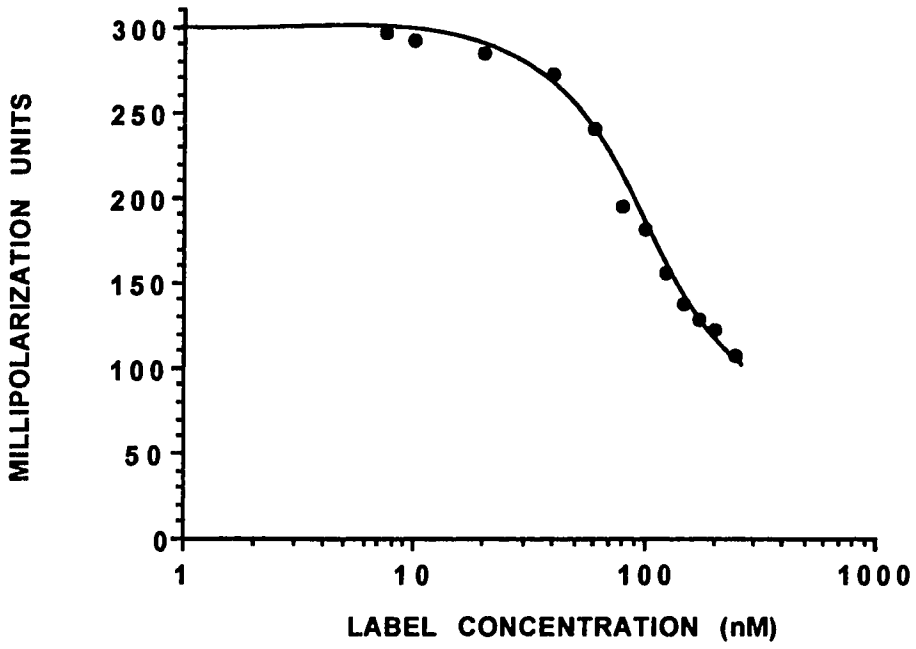


Figure 3.21 AMF-SOL Label Dilution Curve with Polyclonal Antibody

Each point represents the mean value of six determinations. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width). Curve parameters, as determined using *SOFTmax* for original polarization data, were: "a"=0.296; "b"=2.22; "c"=88.4; "d"=0.091; r=0.993.

Use of the Langmuir Plot (van Oss and Absolom, 1984) allows for the determination of the kinetics of Ag-Ab interactions when the concentration of Ab is unknown, provided the Ab concentration remains constant. The data obtained from the label dilution curve was used to generate a Langmuir Plot depicted in Figure 3.22. The percentage of label binding was calculated from

$$\frac{F_b}{F_f} = \frac{Q_f}{Q_b} \times \frac{(p - p_f)}{(p_b - p)}$$

where p = polarization reading, F = fraction bound (b) or free (f), Q_f= fluorescence of free tracer, Q_b = fluorescence of bound tracer, p_f = polarization value of free tracer and p_b = polarization value of bound tracer (Dandliker and DeSaussure, 1970). The last of these parameters (p_f and p_b) are fixed values and define the "a" and "d" values of the sigmoidal competitive inhibition curve.

In preparing the label dilution curve, it was not possible to determine the lower asymptote of the sigmoidal curve. This was due to the fact that at the exceedingly high label concentrations required to reduce FP values, the FI increased to beyond a level capable of being read by the fluorimeter. Furthermore, at such label concentrations, fluorescence quenching may occur, resulting in anomalous FP values. To circumvent this problem, a "background" level of 46 mP units was estimated by measuring the FP of AMF-SOL in the absence of Ab (Table 3.7). This assumption was applied in the generation of the Langmuir Plot.

In order to calculate a K_{aff} value, an assumption of the level of Ab participating in the reaction must be made. For the polyclonal serum used in this analysis, at a dilution of 1:200, a value of 33 nM was calculated using the assumptions given below.

The y-intercept of the Langmuir Plot is defined by

$$\textbf{y-intercept} = m/n$$

where m = valence of Ag and n = valence of Ab. In the case of a hapten, m =1. One of the underlying assumptions made in this analysis was that the majority of the Ab participating in the reaction was IgG, in which case, n=2. The y-intercept can therefore be fixed at a value of 0.5 for a PAb with a monovalent Ag or hapten. The slope of the Langmuir Plot is given by

$$\textbf{slope} = 1/nK_{aff}$$

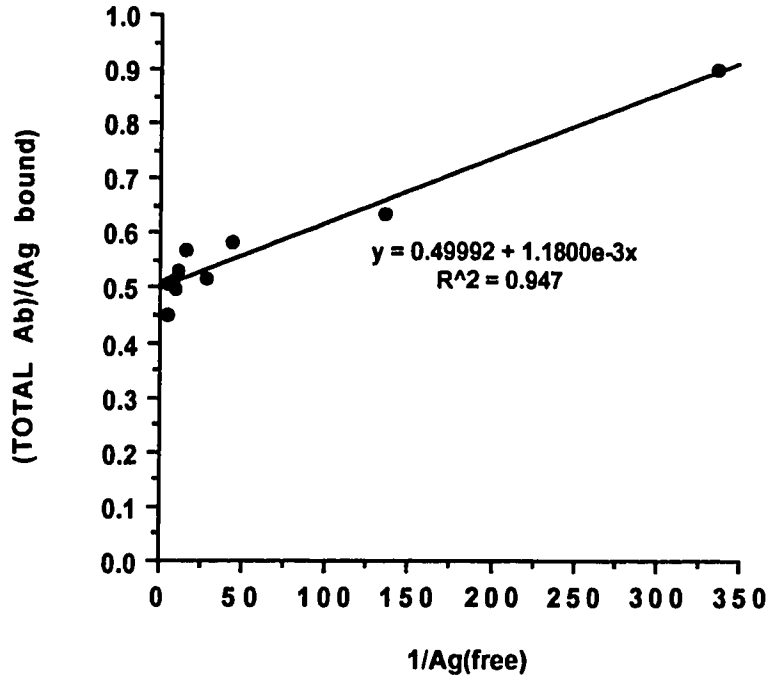


Figure 3.22 Langmuir Plot for Polyclonal Antibodies with AMF-SOL Label

Points represent the mean value of six determinations.

Again, for PAb, assuming a predominance of IgG for a mature serum (Coleman *et al.*, 1989), n=2, and K_{aff} can be calculated from

$$K_{aff} = 1/2xslope$$

From the Langmuir Plot generated for the polyclonal serum in this study, K_{aff} was estimated to be $4.2x10^{8}M^{-1}$.

Considering that rabbit serum generally contains 10 mg/mL IgG, 10% of which may be hapten-specific (Harlow and Lane, 1988), it can be calculated that, theoretically, 33 nM of Ab should be specific for the hapten in a 1:200 dilution (assuming an Ab MW of 150,000). This value for the concentration of Ab validated the assumption made in calculating K_{aff}, and thereby supported the K_{aff} value. It should be stressed that the value given for K_{aff} is a rough estimate of the general affinity of the polyclonal serum since it encompasses the affinities of a broad population of Abs for the AMF-SOL label.

3.4.2.3 *Detection of Potato Alkaloids and Alkaloid Derivatives Using PAb FPIA*

The major potato GAs were tested to assess the relative affinity of the PAb population for the individual GAs. The standard curves generated for α-chaconine, α-solanine, solanidine, and solanidine hemisuccinate are presented in Figure 3.23.

From the data in Figure 3.23, it was established that the PAb serum had the greatest affinity for α-chaconine. This was not surprising, considering that the polyclonal serum was raised against a derivative of chaconine. The PAb population had a similar, but slightly reduced, affinity for α-solanine, while the affinity of the serum for solanidine, the non-glycosylated potato alkaloid, was much reduced as compared to its glycosylated counterparts (Figure 3.23). These results parallel the findings of Plhak (1993) who examined the performance of the same PAb serum with GAs using a solid-phase EIA.

The slightly decreased affinity of PAb for solanidine may be explained by the absence of a glycosidic group in solanidine at the C_5 position. However, this difference does not significantly influence binding as it is distal to the portion of the molecule recognized by the Ab.

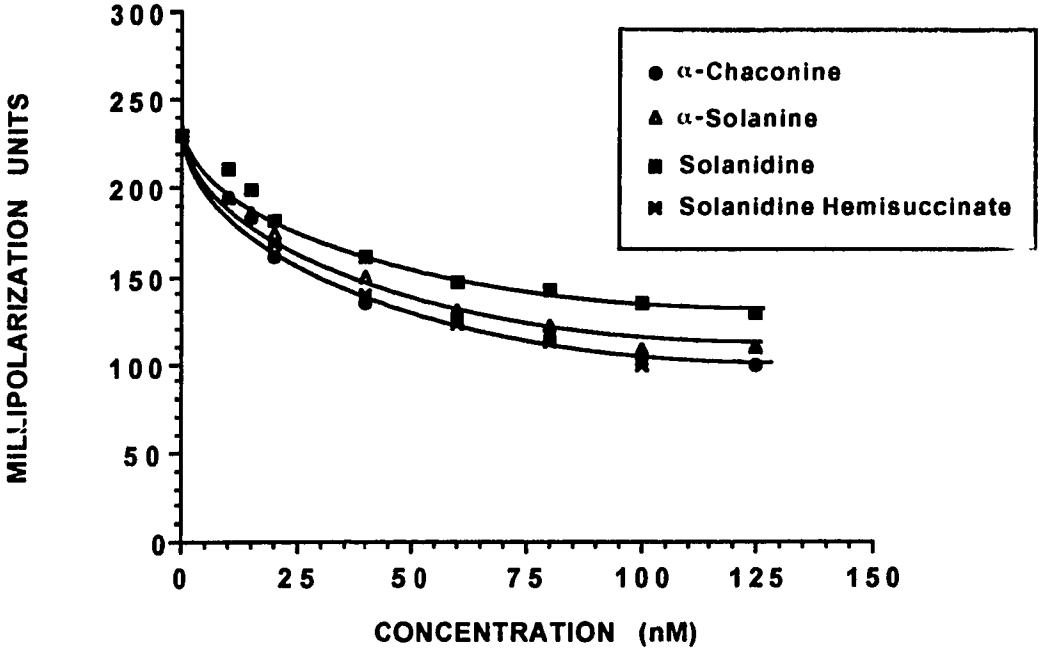


Figure 3.23 Standard Competitive Inhibition Curves for PAb with Potato Alkaloids in FPIA

Each point represents the mean value of six determinations; standard deviation of the mean was ≤9 mP units for all determinations. Label concentration (AMF-SOL) was 60 nM in the reaction; serum was diluted 1:200.

α-Tomatine, a GA with a spirosolane skeleton, as opposed to a solanidane skeleton (e.g., α-chaconine, α-solanine, and solanidine), (Figure 1.7) was not recognized by the polyclonal serum. This was not surprising, given the difference in chemical structure from solanidine, particularly in the portion of the molecule most responsible for recognition by the Abs.

The PAb showed a lower affinity for the AMF-SOL tracer than for any of the major potato GAs (Table 3.8). The data in Table 3.8 indicate that 20 nM of any of the glycoalkaloids or alkaloids resulted in a greater decrease in FP values than did 20 nM label. That is, in the reaction containing the standard 60 nM concentration of AMF-SOL, the analytes tested were more successful in displacing the AMF-SOL from the Ab than was an equal concentration of the label itself. While Dandliker *et al.* (1973) did not note such an effect for dansyl-labelled BSA recognized by anti-BSA Abs, they did postulate that the effect would be greater for smaller molecules. In the present work, however, the MWs of the GAs found to compete well with PAb (α-chaconine, MW 852; α-solanine, MW 868) were greater than that of the AMF-SOL conjugate (MW 841). Thus, it can be concluded that the lesser affinity of the PAb for AMF-SOL was not simply a function of molecular weight.

Table 3.8 Response of PAb to 20 nM Analyte or AMF-SOL Label as Competitor

COMPETITOR	FP (mP UNITS)
α-chaconine	161
α-solanine	174
solanidine	182
solanidine hemisuccinate	168
AMF-SOL[1]	210

Values represent the mean value of six determinations; standard deviations were ≤9 mP units for all determinations. All reaction mixtures contained 60 nM AMF-SOL label; serum dilution was 1:200. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width). Maximum FP (no analyte) was 296±12 mP units.

[1]Label concentration was actually 80 nM (i.e., 60 nM standard concentration + 20 nM for competition).

Results obtained using solanidine hemisuccinate (MW 498), used in the preparation of AMF-SOL, as the competitor molecule showed good recognition of the solanidine derivative by the PAb. In fact, the recognition of solanidine hemisuccinate by PAb was essentially the same as that for α-chaconine, as indicated by the superimposibility of the standard curves for both of these compounds (Figure 3.23). It therefore appears that it is the addition of the fluorescein molecule itself which reduces the affinity of PAb for AMF-SOL, as opposed to the presence of the hemisuccinate linkage. Since the Ab population was not produced in response to a compound containing fluorescein there are no complementary binding groups on the Abs for the fluorescein moiety. The binding of the label by Ab may be influenced by steric effects and repulsive forces, which may negatively affect the binding of AMF-SOL to the Ab. The fact that the FI of the label was decreased by 14% upon binding to Ab (Section 3.3.1; Figure 3.19) further suggests that there was some interaction between the Ab binding site and the fluorescein moiety of the AMF-SOL label.

Ekins (1974) indicated that the sensitivity of an IA is optimized when the Ab displays equal affinity for both the analyte and tracer molecule. By reducing the affinity for the tracer, as with a shorter bridge between the solanidine and fluorescein molecules of the AMF-SOL conjugate, the displacement of tracer by analyte was facilitated; this was supported by findings of Colbert *et al.* (1991). This ultimately results in a decrease in the detection level of analyte in the assay.

Another factor which may have influenced the affinity of PAb for the AMF-SOL tracer may have been the relatively flexible nature of the hemisuccinate linkage. Although the seven-atom chain joining the fluorescein to the solanidine would appear to be sufficient to allow the fluorescein moiety of the conjugate to remain outside the Ab-binding site (Ali *et al.*, 1992; Sengupta *et al.*, 1992), the flexibility of the hemisuccinate bridge may have allowed closer approach of the fluorescein to the Ab-binding site than would be calculated using bond lengths. Increasing the rigidity of the covalent bridge might increase its ability to be bound by Ab.

3.4.2.4 *Analysis of the Glycoalkaloid Content of Potato Samples*

Three different potato extracts were analyzed for their GA content using the PAb FPIA system. Results, calculated using the regression line for the standard curve for α-chaconine (Figure 3.24), are presented in Table 3.9.

Table 3.9 Glycoalkaloid Contents of Potato Samples

SAMPLE	DILUTION FACTOR	POLARIZATION VALUE[1] (mP units)	α-CHACONINE CONTENT[2] (mg/100 g, fresh weight basis)	
			PAb FPIA	PAb ELISA[3]
A-1	100	144±7	9.7	9.9
A-2	100	138±3	11.1	9.9
B-1	1000	149±5	87.1	56.0
B-2	1000	153±7	79.7	56.0
C-1	10	122±4	1.6	1.2
C-2	10	175±4	0.5	1.2

[1]Values represent the mean of six readings ± standard deviation of the mean.
[2]All values calculated assuming a 80% moisture content in fresh potatoes.
[3]As reported by Plhak and Sporns (1992).

The potato samples analyzed were chosen to cover a wide range of GA concentrations; this is reflected in the values presented in Table 3.9. Results obtained for samples A-1, A-2, C-1, and C-2 agree closely with those of Plhak and Sporns (1992) for the same potato samples. Samples B-1 and B-2, however, were found to have much higher GA levels when analyzed by FPIA as compared to ELISA (Plhak and Sporns, 1992). This could be a reflection of the extraction technique employed, rather than a shortcoming of either IA method. The methanol extraction does extract GAs; however, the reproducibility of the extraction method has not been demonstrated. Although the extraction procedures used in both studies were similar, they were carried out months apart. Furthermore, extraction of potato powder preparations with methanol is non-specific, and may therefore result in the extraction of non-GA components which may interfere with the FPIA and/or ELISA analysis.

The lack of reproducibility of the extraction procedure is echoed in the results for replicate extractions of the same potato samples. This was particularly true for the low-GA samples (C-1 and C-2). However, it must be borne in mind that these samples were analyzed using only a 1:10 dilution of the original extract. Therefore, there may have been interferences from components of the potato. Such components would have been diluted out in the other extracts, leading to better reproducibility between replicate samples.

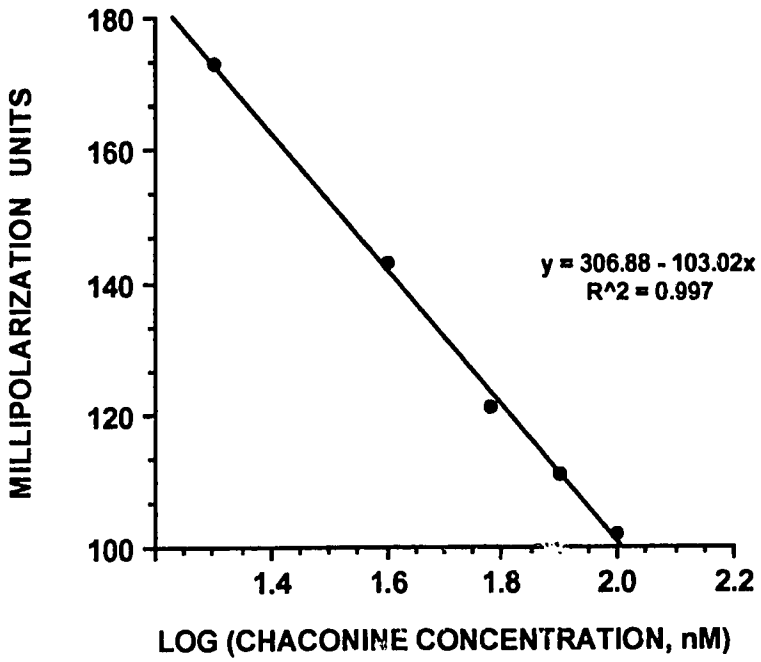


Figure 3.24 Standard Curve (Linear Regression) for a-Chaconine Using Polyclonal FPIA

Points represent the mean value of six determinations. PAb dilution in reaction was 1:200; label concentration in reaction was 60 nM. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width).

The cut-off value between safe and toxic potato tubers has been set at 20 mg/100 g tuber (Van Gelder, 1991). In terms of the screening of new potato cultivars, it would be prudent to establish a cut-off value of 16 mg/100 g tuber; that is, samples showing 16 mg/100 g tuber should be considered potentially unsafe and subjected to further analysis. Using this value and the FPIA system developed with PAb, sample extracts diluted 150 times and giving FP values ≤ 140 millipolarization units should be considered potentially toxic.

3.4.2.5 *Stability of PAb FPIA Standard Curve for α-Chaconine*

One of the advantages cited for FPIA analysis is its intrinsic reproducibility. This reproducibility extends to standard curves, which display long-term stability, thereby simplifying analysis and reducing reagent requirements (Colbert *et al.*, 1986; Thomas *et al.*, 1991; Zaninotto *et al.*, 1992) and the use of stored calibration curves can improve the between-batch reproducibility of results (Thomas *et al.*, 1991). In the present study, the stability of a standard curve for α-chaconine was monitored over 23 d (Figure 3.25). As indicated by the superimposibility of the curves, values did not change appreciably over the first two-week period; however, mid-range values (20-60 nM) had decreased when analyzed at 23 d.

3.4.3 Monoclonal Antibody FPIA

The evaluation of the MAb FPIA system was similar to that for the PAb FPIA, except that all evaluations were made at pH 6.5, as there was concern regarding the stability of the AMF-SOL label at pH 7.3. TLC analysis indicated a limited amount of hydrolysis (about 20%) occurred at both pH 7.3 and pH 6.5 after 30 d of storage. This would not have affected the results discussed herein, however, as label dilutions were prepared fresh daily. The label concentration in all reactions was maintained at 60 nM.

3.4.3.1 *Monoclonal Antibody Preparations*

Both MAb preparations used in this work were obtained from hybridomas grown in serum-free RPMI with 1% Nutridoma (Plhak, 1993), which is a serum replacement containing insulin, transferrin, BSA, low-density lipoproteins from bovine plasma, vitamins, amino acids, and other small organic molecules. IgG fractions were purified using 0-50% saturated ammonium sulphate (Fraction I) and 50-60% saturated ammonium sulphate (Fraction II) (Plhak, 1993). The protein contents of these

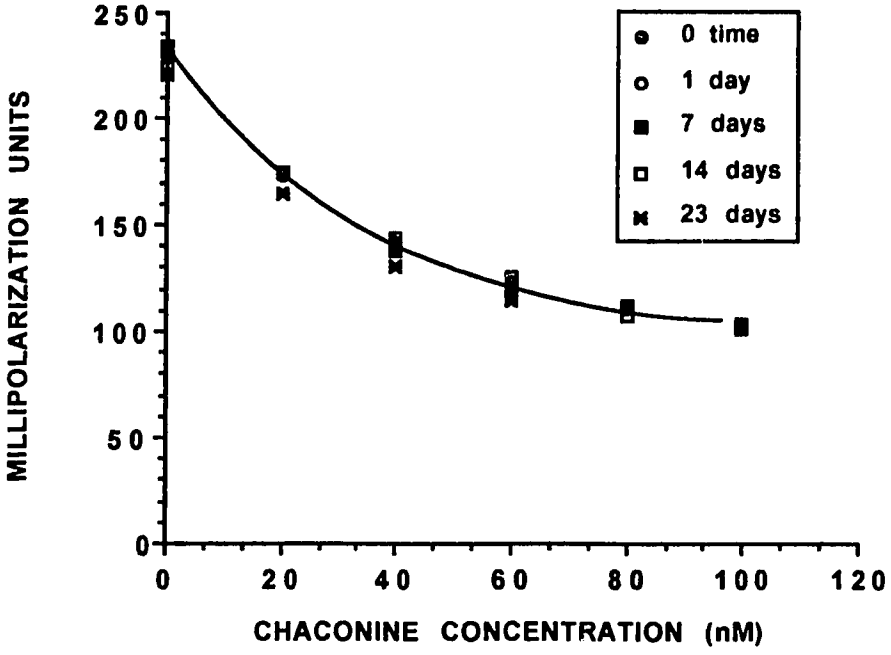


Figure 3.25 Stability of Standard Curve for α-Chaconine Using Polyclonal Antibody

Points represent the mean value of six determinations; standard deviation of the mean was ≤9 mP units for all determinations. Label concentration in reaction was 60 nM; serum dilution in reaction was 1:200. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width).

fractions, determined by UV analysis and expressed as IgG, are presented in Table 3.10.

Table 3.10 Protein (IgG) Content of MAb Fractions

FRACTION	[PROTEIN] (mg/mL)
I	0.359
II	0.465

3.4.3.2 *Effect of Buffer pH on Fluorescence Intensity of AMF-SOL*

In reducing the pH of PBST from 7.3 to 6.5, it was noted that while FP values were comparable to those obtained at the higher pH value, FI values decreased significantly; however, the wavelength maxima for excitation and emission were not affected. This was demonstrated with label preparations at both pH 7.3 and 6.5. Results are included as Table 3.11.

Table 3.11 Effect of pH on Fluorescence Intensity (FI) Values of 60 nM AMF-SOL Solutions

SOLUTION	FI VALUE
AMF-SOL, pH 6.5	161.075 ± 0.507
AMF-SOL, pH 7.3	362.157 ± 1.103

Values represent the mean of six readings ± standard deviation of the mean. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width).

The figures in Table 3.11 represent a 56% reduction in the FI of AMF-SOL with a decrease in pH from 7.3 to 6.5. It has been demonstrated that the excited species of a molecule can ionize at a different pH than the ground state. Bieniarz and associates (1994) synthesized a phosphorothioate fluorescein molecule and indicated it had a pK_a value of 6.4; therefore, it may be that the decreasing of pH from 7.3 to 6.5 changed the ionization state of the fluorescein moiety of the AMF-SOL label. Such a change in ionization state may affect the resonance structure of the molecule which may result in an altered ability to emit a photon, thereby influencing the fluorescent behaviour of the molecule (Pesce *et al.*, 1971). The greatest effects occur in a pH

range from 2 to 7; in this range, FI increases with increasing alkalinity. At pH values above 8, FI is relatively resistant to changes in pH (Schauenstein *et al*., 1978).

3.4.3.3 *Monoclonal Antibody Dilution Curve*

A MAb dilution curve was constructed using data obtained using Fraction II MAbs and is shown in Figure 3.26. For analysis, as noted for the PAb FPIA system, it was desirable to use a MAb concentration that gave approximately 80% binding of 60 nM AMF-SOL. For this MAb, the dilution selected corresponded to a MAb concentration of 0.014 mg/mL (93 nM) in the FPIA reaction.

The development of FPIA methods for GA analysis was contingent on the use of high levels of PAb or MAb, particularly when compared to the amounts required for a solid-phase assay (Plhak, 1993). High serum concentrations (i.e., a dilution factor of <1000) have been employed by other investigators (Eremin *et al.*, 1991; García Sánchez *et al.*, 1993; Kobayashi *et al.*, 1979; McGregor *et al.*, 1978; Turner *et al.*, 1991; Watson *et al.*, 1976), and appear to be a necessary element in FPIA protocols.

Notwithstanding the increased serum demands of the FPIA systems described for GAs, the use of FPIA affords several advantages over solid-phase IAs. Since FPIA is a homogeneous technique, its attendant advantages include speed and the lack of a separation step, permitting automation of the analysis (Jenkins, 1992). The fluorescently-labelled reactants also have a virtually indefinite shelf-life (Smith *et al.*, 1981), and the standard curves exhibit long-term stability, as has been discussed. Homogeneous assays, including FPIAs, are inherently less sensitive than heterogeneous assays (Eremin *et al*., 1991; Gosling, 1990). This was not specifically addressed in the current research, as extreme sensitivity is not essential in the analysis of potato samples as the levels of concern are quite high (Van Gelder, 1991).

3.4.3.4 *Kinetics of the Monoclonal Antibody-(AMF-SOL) Reaction*

The K_{aff} of the MAb was determined using a Langmuir plot, as was described for the polyclonal system. In the case of the MAb, however, the concentration of specific IgG was known. The combination of AMF-SOL with MAb resulted in a 17% decrease in the FI as compared to the FI value of free AMF-SOL. Taking this information into account, the K_{aff} for Mab was estimated at 4.7×10^7 M^{-1}, which was approximately one order of magnitude lower than that for the PAb for the same label. This lower K_{aff} value for MAb may account in part for the approximately three-fold increase in serum requirements for the MAb system (93 nM) as compared to the PAb system (33 nM).

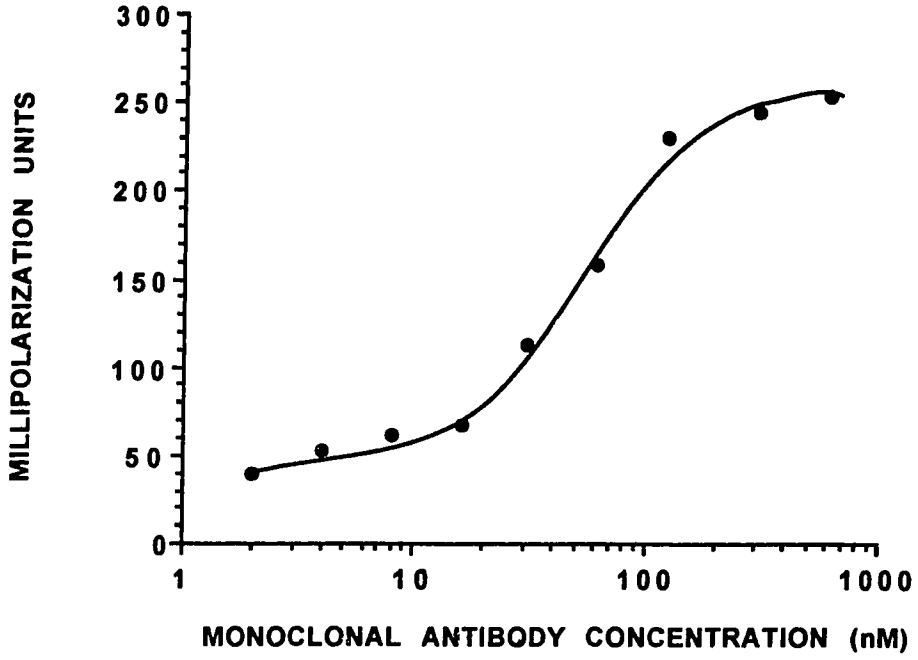


Figure 3.26 Monoclonal Antibody Dilution Curve with AMF-SOL

Points represent the mean values of six determinations; curve parameters, as determined using *SOFTmax* for original polarization data were: "a"=0.052; "b"=1.84; "c"=53.7; "d"=0.256; r=0.983. Label (AMF-SOL) concentration in reaction was 60 nM.

Plhak and Sporns (1992, 1994), using the same polyclonal serum and MAb as was used in the present study, found that the PAb had a slightly higher affinity for GAs than did the MAb, as indicated by I_{50} values. The 17% decrease in FI values of AMF-SOL upon binding to Ab, as compared to the 14% observed for PAb (Section 3.3.1) seems to indicate a greater interaction between the AMF-SOL label and the MAb, possibly due to differences in the configuration of the binding site. The steric hindrance due to the label results in an actual decrease in the affinity of MAb for the AMF-SOL label.

3.4.3.5 *Detection of Potato Alkaloids and Alkaloid Derivatives Using Monoclonal FPIA*

The relative affinity of MAb Fraction I for α-solanine, α-chaconine, solanidine, AMF-SOL, and solanidine-hemisuccinate was assessed at several levels of each competitor (Table 3.12). The results of Table 3.12 indicate that the MAb had the greatest affinity for α-solanine, and comparable but slightly reduced affinity for solanidine-hemisuccinate. The affinity for α-chaconine was slightly lower than that observed for α-solanine and solanidine-hemisuccinate; however, it was much greater than that observed for solanidine. These findings, save for the hemisuccinate data which was not evaluated by Plhak and Sporns (1992), agree closely with those of Plhak and Sporns (1992).

Table 3.12 FP Values with Various Competitors and MAb in FPIA

[ANALYTE] (nM)	FLUORESCENCE POLARIZATION VALUE (mP units)			
	α-CHACONINE	α-SOLANINE	SOLANIDINE	SOLANIDINE-H[1]
20	205±8	178±3	215±4	195±5
40	139±7	125±8	176±4	143±8
60	107±4	96±1	153±4	107±6

Values represent the mean values of six determinations±standard deviation of the mean. Monoclonal antibody concentration in reaction was 93 nM; label concentration in reaction was 60 nM. Excitation wavelength was 496 nm (2.5 nm slit width); emission wavelength was 518 nm (7.5 nm slit width). Maximum FP (no analyte) was 253±9 millipolarization units.

[1]Solanidine-H = solanidine-hemisuccinate.

CHAPTER 4. CONCLUSIONS

In the context of an indirect competitive ELISA for ST analysis, TMB was found to be superior to several alternative chromogens tested for the detection of HRP activity. Colour development and/or HRP activity were maximized at a pH of 4.0, a factor that has often been overlooked by other investigators (Bos *et al.*, 1980; McKimm-Breschkin, 1990; Madersbacher and Berger, 1991). The increased colour development with TMB permitted the reduction of serum and coating conjugate levels as compared with similar previously-developed protocols (Sheth and Sporns, 1990), leading to decreased detection levels. It may be possible to even further decrease the detection limit of the indirect ELISA described here for ST by decreasing the degree of ST substitution on the BSA molecule in the coating conjugate. Garden and Sporns (1994) adopted the TMB protocol described in this thesis and were able to achieve "c" values of 1.4 ppb sulphamerazine in milk. It is likely that the antiserum used in the study of Garden and Sporns (1994) was of a higher affinity than that used in the present study since they used an immunogen with a hapten-protein molar ratio of 8.4. The anti-ST serum used in the current study was produced in response to an ST-LPH conjugate with 28 moles ST per BSA equivalent, which is outside the range of 8 to 25 hapten molecules per carrier protein as prescribed by Erlanger (1980) for strong Ab titres.

Two types of linking methods were evaluated for the synthesis of HRP-ST conjugates for the development of a direct competitive ELISA for ST. The periodate cleavage method was found to produce more efficient conjugates in terms of enzyme activity, and performed better than those prepared by glutaraldehyde cross-linking when tested in the direct competitive assay.

The pre-coating of microtitre plates with Protein A prior to serum addition for the direct ELISA was preferable to the use of either an anti-Ig F_C-specific Ig or the simple adsorption of immune serum Abs to the plate. Serum levels in the assay could be reduced when Protein A was used to pre-coat microtitre plates as compared to the other two approaches; this was likely due to immobilization of Abs through their F_C portions, leading to superior orientation of Ab binding sites on the solid phase.

Slight differences in sensitivity and "c" values were observed between the direct and indirect competitive formats of ELISAs for ST. However, by preparing a minimally substituted BSA-ST conjugate, it may be possible to develop an indirect protocol to rival the direct assay with respect to sensitivity and detection levels.

Alternatively, a serum of higher affinity might be prepared, using a lower level of ST substitution on the injection conjugate. Using the serum available, the direct competitive ELISA was found to be slightly more rapid and easier to perform than its indirect complement. The direct format allowed for "c" values as low as 39.5 ppb ST to be determined in milk. The direct competitive ELISA was also able to detect ST in honey samples with a "c" value of 88.0 ppb, which represents the first rapid test capable of assaying ST at levels less than 200 ppb ST.

Attempts to replace the HRP-Ab conjugate with a urease-Ab conjugate using conductimetric detection of enzyme activity in the indirect assay were met with discouraging results. Urease conjugates were ultimately deemed unsuitable for use in such ELISAs for ST analysis. Signal amplification of the immune reaction was poor with urease, perhaps due to steric factors which prevented the binding of sufficient conjugate to the immobilized primary Ab. It may be more practical to use urease-Ab conjugates in two-site ("sandwich") ELISAs for larger Ags, where the size of the enzyme would be less critical in terms of steric effects. However, the non-specific nature of the conductimetric detection of the urease reaction may limit the application of such assays.

FPIAs for potato GAs were developed using polyclonal serum as well as a MAb. Both systems were capable of detecting the major potato GAs (α-chaconine, α-solanine, and solanidine) at levels of 20-100 nM, although the affinities of the MAb and PAb for individual GAs differed slightly. The tracer molecule used in these FPIA procedures was a fluorescein derivative linked via a hemisuccinate bridge to solanidine. The affinities of PAb and MAb for the tracer molecule (4.2×10^8 M^{-1} and 4.7×10^7 M^{-1}, respectively) were less than for the GAs tested; this lower affinity was shown to be a result of the presence of the fluorescein moiety. By increasing the length of the bridge between the solanidine and fluorescein moieties in the conjugate to reduce steric and/or electronic effects, it may be possible to increase the relative affinity of both Ab types for the tracer. Alternatively, the rigidity of the bridge may be increased, again to reduce steric effects. One or both of these approaches would increase the affinities of the Abs for the tracer, and bring them closer to those observed for GAs. The net result would be an increase in the sensitivity of the assay. It should be noted, though, that for GA analysis, extremely low detection levels are unnecessary.

Potato extracts containing low, medium, and high levels of GAs were successfully analyzed using PAb FPIA. Results agreed closely with those obtained for the same potato samples analyzed using the same PAb and a solid-phase EIA (Plhak

and Sporns, 1992). However, under optimal operating conditions (i.e., with a plate reading attachment for the fluorimeter), results could be obtained far more rapidly by FPIA than by using the solid-phase system. Moreover, the superior reproducibility of FPIA measurements, in addition to the stability of the standard curves and reagents, contribute significantly to time savings for FPIA analysis.

One of the limitations of the FPIA procedures described herein for potato GAs is the requisite high serum level. This is particularly true if polyclonal serum is used, as it is available in a finite supply. It would therefore be advantageous to concentrate on the development of FPIA protocols which employ a MAb.

The PAb FPIA system described here could be used to quantitate the major GAs in commercial potato cultivars; however, it could not detect α-tomatine, another toxic GA (Nishie *et al.*, 1975), which has been found in wild potato varieties (Barbour *et al.*, 1991). In addition, increased use of wild potato species in breeding programs may introduce other alkaloids into potato cultivars destined for commercial production (Gregory, 1984; Van Gelder and Scheffer, 1991). Further avenues of research would therefore include the development of additional MAb-producing cell lines for recognition of other potential GAs. Simultaneous FPIA analysis of potato extracts for solanidane and spirosolane GAs would be possible by using a second tracer molecule synthesized from α-tomatine and a different fluorescent label, such as a derivative of rhodamine.

The development of an FPIA system employing two or more MAbs and tracers would greatly facilitate the screening of many thousands of potato plants in the early stages of growth, which is a prerequisite to an effective breeding program (Lewis and Fenwick, 1991). The ground work for such a system has been described in this thesis.

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APPENDIX

AVERAGE COMPOSITION OF CANADIAN HONEY

COMPONENT	% BY WEIGHT
Glucose	33.8
Fructose	38.8
Sucrose	1.2
Maltose	6.1
Dextrin	1.3
Water	17.5

(White, 1975)