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Development of Immunoaffinity Columns and Immunoassays for the Collection, Partial Purification and Measurement of Sporidesmin A and Its Metabolites

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Doctor of Philosophy

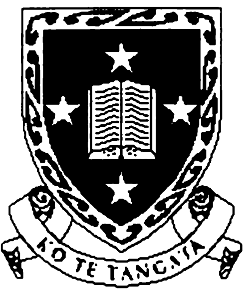
in Biological Sciences

at the

The University of Waikato

by

Lynette Ruth Briggs



The University of Waikato

1997

ABSTRACT

Monoclonal and polyclonal antibodies were produced for the development of competitive enzyme-linked immunosorbent assays (cELISA) for sporidesmin A, the mycotoxin that causes facial eczema in grazing livestock. Derivatives of sporidesmin conjugated to a number of proteins were used as immunising antigens and plate-coating antigens for cELISA. Two immunoassays, each detecting the presence of a different region of the sporidesmin A molecule have been developed. They allow the two major sporidesmins found in grass or in *Pithomyces chartarum* cultures to be distinguished. Organic solvent extraction of samples was avoided so that water-soluble metabolites could also be detected.

To assist in the selection of animals resistant to facial eczema, the cELISAs were applied to a study of the metabolism of sporidesmin A in sheep. The cELISAs distinguish between sporidesmin A and its metabolites, as one antibody detects only sporidesmin A and the other detects sporidesmin A and metabolites. After sheep were dosed with sporidesmin A it was possible to monitor the excretion of the toxin in bile and its metabolite(s) in both bile and urine using the appropriate cELISA. There were no differences between resistant and susceptible sheep in the cumulative totals or excretion rates of immunoreactive metabolite in animals dosed with sporidesmin A.

The detection of sporidesmin metabolites in sheep urine by cELISA was shown to have potential as a biomarker for measuring exposure to toxic pastures which could give early warning of the risk of facial eczema.

An immunoaffinity chromatography matrix was prepared by coupling antibody to cyanogen bromide-activated Sepharose. The matrix bound sporidesmin A and immunoreactive metabolites from urine, and bound analytes were eluted with 40% methanol in water. The matrix capacity for sporidesmin A was 900 ng of sporidesmin A bound per mL of matrix, and for sporidesmin metabolites from urine it was approximately 500 ng of sporidesmin A immunoreactive equivalents per mL of matrix.

A method was developed for the preparation of milk for immunoaffinity chromatography so that blocking of columns by milk fats was avoided. Sample concentration obtained using the immunoaffinity chromatography lowered the limit

of quantification for the previously developed cELISA for sporidesmin A in milk by a factor of 100.

A trial was carried out to determine whether sporidesmin residues appeared in milk following experimental ingestion of the toxin. Using the cELISA, sporidesmin metabolites were detected in milk samples collected after dosing. Further evidence for the presence of immunoreactive material was obtained by using immunoaffinity chromatography to recover the material from milk. Positive results in the sporidesmin A specific cELISA suggest the presence of free sporidesmin A at picogram levels. Purification from a much larger sample, and analyses by HPLC and mass spectrometry are needed to confirm this result.

It should now be possible to use the anti-sporidesmin antibodies in immunochemical technologies and formats which are suitable for on-farm use. These could be used to help minimise exposure to sporidesmin A and provide an important advance in animal health.

ACKNOWLEDGMENTS

I wish to thank my supervisors Peter Molan and Neale Towers for the opportunity to commence this project, for their encouragement, guidance and invaluable assistance during the course of it.

I gratefully acknowledge the following contributions made to this research by:

Rex Gallagher, Steven Eichler, Chris Miles, and Sarah Finch for production of haptens and of immunising and plate-coating conjugates;

Barry Smith and Peter Embling for surgical preparation of sheep and provision of bile and urine samples for the study of sporidesmin metabolism;

Allan Hawkes for HPLC analyses;

Trevor Watson for flock management, sheep immunisation and blood collection;

W. E. Jonas and A. F. Erasmuson for providing additional compounds for use in the cross-reactivity studies;

Roger Collin and Joan Fitzgerald for spore counting data;

Kees Wesselink for organisation of sheep trials and animal sampling;

Michael Bennenbroek and staff at No. 5 Dairy, DRC for the dairy cow trial;

Glenda Smith for maintenance of mice and rabbits for antibody production;

Jan Sprosen, Liz Fowke and Xandra Thompson for collecting sheep urine;

Pauline Hunt for draughting Figures 1.7.1, 2, 3.

Thanks to Barry Smith for thesis editing and to other members of the Toxinology and Food Safety Research Group, Ruakura, for your valuable contribution.

Thank you to fellow thesis writers Yvonne Kuys, Adrian Molenaar, Nick Waipara and Sarah Finch, for your encouragement.

I gratefully acknowledge the financial support of AgResearch throughout this project and funding provided by the Foundation for Research, Science and Technology.

Many thanks to Roger, Jenine and Craig, as always you were great, and finally to Sherpa for incredible patience.

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LIST OF ABBREVIATIONS

A_o	maximum ELISA absorbance measured in the absence of analyte
ACDMBA	2-amino-5-chloro-3,4-dimethoxy benzyl alcohol
anti-mouse-HRP	donkey anti-mouse immunoglobulin horseradish peroxidase conjugate
anti-rabbit-HRP	anti-rabbit immunoglobulin horseradish peroxidase conjugate
anti-sheep-HRP	rabbit anti-sheep immunoglobulin horseradish peroxidase conjugate
BSA	bovine serum albumin
C_{18}	octadecylsilica
cELISA	competitive enzyme-linked immunosorbent assay
%CR	percent cross-reactivity
CV	coefficient of variation
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FE	facial eczema
GGT	γ-glutamyltransferase
GLC	gas-liquid chromatography
Gy	Gray
hapten 10b	sporidesmin A 10b-hemisuccinyl
hapten 11	sporidesmin A 11-hemisuccinyl
HAT	hypoxanthine, aminopterin, thymidine
HG	hemiglutaryl
HGPRTase	hypoxanthine guanine phosphoribosyl transferase
HPLC	high-performance liquid chromatography
HRP	horseradish peroxide
HS	hemisuccinyl
HT	hypoxanthine, thymidine
I_{50}	concentration giving half-maximal inhibition
IAC	immunoaffinity chromatography
IEF	isoelectric focusing
IgG *etc.*	immunoglobulin G *etc.*
IM	intramuscular

IMDM	Iscoves modified Dubeccos medium
IP	intraperitoneal
IRE	immunoreactive equivalents
IS	intrasplenic
IV	intravenous
kDa	kilo dalton
MEM	minimum essential medium
NEAA	non-essential aminoacids
PBS	phoshate-buffered saline
PEG	polyethylene glycol
pI	isoelectric point
R	resistant to facial eczema
RIA	radioimmunoassay
S	susceptible to facial eczema
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SN	culture supernatant
Spdm A	sporidesmin A
TLC	thin-layer chromatography
TMB	3,3',5,5'-tetramethylbenzidine
Tris	Tris(hydroxymethyl)aminomethane
Tw. 20	Tween 20

Chapter 1: *INTRODUCTION*

CHAPTER 1

INTRODUCTION

1.1 Occurrence of facial eczema

Ingestion of spores of the saprophytic fungus *Pithomyces chartarum* by grazing livestock causes the disease commonly known as facial eczema in sheep, cattle, deer, and goats. Outbreaks of facial eczema occur frequently in New Zealand and they have been recorded periodically in Australia, South Africa (White *et al.*, 1977) and more recently in South America (Smith and Towers, 1984), France (Bézille *et al.*, 1984) and North America (Hansen *et al.*, 1994).

The disease occurs only under specific weather conditions when a long hot dry period is followed by warm, wet weather favouring the growth and sporulation of *P. chartarum*, typically in New Zealand during the late summer and autumn. This results in high *P. chartarum* spore counts in the dead vegetative material of the pasture litter. Facial eczema will occur when feed shortages and intensive grazing practices force animals to graze to the base of the pasture and into the zone where spore counts are high (Smith *et al.*, 1987). Severe outbreaks occur about every five years with moderate outbreaks occurring in the intervening years.

Facial eczema is responsible for significant reductions in animal growth, reproductive performance, and in wool and milk production. This mycotoxicosis is therefore of considerable economic significance to the agricultural industry, particularly in New Zealand where greatest production losses are associated with subclinical facial eczema that occurs annually.

1.2 Cause of facial eczema

The spores of *P. chartarum* contain the potent mycotoxin, sporidesmin A (Structure 1 in Figure 1.2.1), which causes extensive liver damage, particularly to the biliary system. The resulting impaired liver function often causes a secondary photosensitisation of exposed areas of skin around the eyes, ears and muzzle (Mortimer and Ronaldson, 1983). Nearly all sporing isolates of *P. chartarum* in

Figure 1.2.1 Chemical structures of sporidesmin A (**1**), sporidesmin B (**2**), sporidesmin C diacetate (**3**), sporidesmin D (**4**), sporidesmin E (**5**), sporidesmin F (**6**), sporidesmin G (**7**), sporidesmin H (**8**) and sporidesmin J (**9**) [according to White *et al.* (1977), Halder (1980) and Nagarajan (1984)].

New Zealand produce sporidesmin A, while isolates from other countries may not, *e.g.* from South Africa (Kellerman *et al.*, 1980) or South America (Brewer *et al.*, 1989). In some locations, therefore, the presence of *P. chartarum* spores does not necessarily indicate the presence of sporidesmin A. The hepatotoxicity of sporidesmin A has been attributed to the presence of the disulfide group, which in a cyclic reduction/autoxidation with glutathione and other cellular thiols, generates damaging 'active oxygen' species (Munday, 1984).

Sporidesmin A was initially isolated by Synge and White (1960), and later purified and characterised by Ronaldson *et al.* (1963). In general, the sporidesmins are unstable compounds which decompose easily under sunlight or ultraviolet irradiation (Clare and Mortimer, 1964). They belong to the epipolythiodioxopiperazines, a class of compounds which contain a common bridged polysulfide piperazine ring.

Other derivatives of sporidesmin A, sporidesmins B-J (structures 2-9 in Figure 1.2.1), have been isolated or synthesised, but in comparison to sporidesmin A are of minor significance in the production of the disease. Of the derivatives of sporidesmin A mentioned above, only sporidesmin B and E are known to be toxic. Sporidesmin B was found to be five times less toxic in sheep than sporidesmin A when dosed orally, while sporidesmin E had comparable toxicity to sporidesmin A (White *et al.*, 1977).

Sporidesmin A and D are the two major components of the sporidesmins which can be extracted from the spores of toxigenic strains of *P. chartarum* grown either in culture or on grass (A. D. Hawkes, AgResearch, Hamilton, New Zealand; personal communication, 1993). In the non-toxic analogue sporidesmin D (structure 4 in Figure 1.2.1) the disulfide bridge is broken and the two sulfur groups methylated.

1.3 Effects of sporidesmin A on farm animals

Sheep are highly susceptible to the toxic effects of sporidesmin A. Although it acts as a potent clastogen *in vitro* with Chinese hamster cells, results indicate that sporidesmin A is not a clastogen in sheep (Ferguson *et al.*, 1992).

The clinical pathogenesis and pathology of facial eczema in various species have been studied and reviewed by Mortimer and Ronaldson (1983). The disease occurs in two phases after toxin intake. In the first phase, two to four days following dosing, injury occurs at two main sites in the liver, one within the hepatocytes and

the other in the bile ducts and supporting tissues. The cytotoxic effects of sporidesmin A on the hepatocytes appears to be reversible. Within ten days of dosing, however, invasion of the lumen of the damaged bile ducts with granulative repair tissue can cause occlusion of these ducts and obliterative cholangitis occurs. This second phase of the disease is when the clinical signs of photosensitisation and jaundice may appear. Liver damage, initiated by sporidesmin A and followed by further injury resulting from the back-flow of bile acids from the occluded bile duct system, causes retention of phylloerythrin within the blood. Phylloerythrin, which results from chlorophyll degradation, absorbs ultraviolet light and induces tissue destruction near the surface of the skin, particularly in non-pigmented areas where the hair or wool cover is thin. Skin irritation may cause animals to rub the affected areas against fixed objects, and the resulting wounds often become infected or fly blown.

Death from facial eczema is not usual except when there is extreme stress brought about by the photosensitisation, and this can be reduced by provision of shade and accessible drinking water. Except in severe outbreaks, most animals suffer liver injury without showing the clinical signs of photosensitivity. Animals may never recover from the liver injury which reduces productivity and growth rates, and can cause weight loss (Towers, 1994).

1.4 Metabolism of sporidesmin A

1.4.1 Absorption, excretion and tissue distribution of sporidesmin A

Although many aspects of the absorption and excretion of sporidesmin A in sheep, rabbits, rats and guinea-pigs have been investigated, much of the metabolism of sporidesmin A remains unknown.

Worker (1960) used toxin-containing extracts of the fungus, then known as *Sporidesmium bakeri,* to confirm the portal route of entry of the toxin into the liver of rabbits. This was carried out by a comparison of the bile duct damage caused by fungal extracts administered by the portal vein and by the jugular vein. Information on the rate of uptake of the toxin from the circulation was also obtained. It was found that the first bile duct lesions began to appear approximately three to six hours after intraportal injection of toxic extract. The extent and severity of these lesions were confirmed histologically. Further experiments were carried out where following injection of toxic extract, bile was collected from animals for varying

intervals and the bile tested directly for toxicity. Toxicity was assessed by dripping the bile from toxin-injected rabbits into the top of the duodenum of successive 'recipient animals'. Appreciable amounts of toxic material were shown to be present in the bile in as little as half an hour after injection of extract and maximal output appeared to occur within 2 h of injection. Wide individual variation in the degree of liver damage between animals was observed in each experiment and it was not known whether the observed toxicity of bile was due to the presence of the toxin or to physiologically active degradation products of the toxin.

Mortimer and Stanbridge (1968) detected a highly cytotoxic substance in body fluids of sheep after oral dosing with a high dose rate of 1 mg of sporidesmin per kg of body weight. Using an *in vitro* cytotoxicity assay, the substance was shown to have the same cytopathogenic effect on tissue culture cells as did sporidesmin and was detected in the bile of two out of five sheep within 10 min of dosing. Peak concentrations of approximately 20 μg of sporidesmin per mL of bile were reached at between 2 and 8 h after dosing. Sporidesmin was recovered from the bile and was identified by chemical methods. Concentrations of substance(s) with sporidesmin-like activity up to 4 μg/mL were detected in urine and peak concentrations of 0.4 μg/mL were found in serum. In this study, sporidesmin or toxic sporidesmin metabolites were not detected in milk samples taken from dosed sheep.

Leaver (1968) carried out a similar investigation using the rabbit eye test and a tissue culture toxicity test for the quantification of sporidesmin. It was demonstrated that when sheep were given a single oral dose of 1 mg of sporidesmin per kg body weight, 3.3% of the total dose of sporidesmin was secreted unchanged in the bile, and the peak concentration of 10.5 μg of sporidesmin per mL of bile was reached between 2 and 3 h after administration. Active sporidesmin was also secreted in the bile when a single oral dose of 0.5 mg of sporidesmin/kg body weight was given, and there was a broad correlation between the amount of sporidesmin secreted and the severity of the liver damage.

Studies on patterns of excretion and tissue distribution of sporidesmin and any sulphur-containing metabolites were made possible by the availability of ^{35}S-sporidesmin. Such a study was undertaken by Towers (1970a) where a single intraperitoneal or oral dose of ^{35}S-sporidesmin was given to female guinea-pigs. ^{35}S-sulfur from ^{35}S-sporidesmin was rapidly excreted by both urinary and biliary routes. The major proportion of the radioactivity was excreted in the bile and eventually appeared in the faeces. Although radioactivity was continually excreted

throughout the experimental period, 70% had been excreted in the bile within 3 h of dosing.

Radioactivity from ^{35}S-sporidesmin was similarly distributed in the organs of animals dosed orally and intraperitoneally. The uptake of sporidesmin in the orally dosed animals, however, was lower than that in animals intraperitoneally dosed. The highest concentration per milligram of tissue was found in the liver, the organ most subject to tissue damage from sporidesmin. In individual animals the amounts in the liver ranged from 2 to 9 times the amount found in the kidneys, and from 2 to 5 times the amount in the blood. In these studies, up to 92% of the total blood radioactivity was associated with the red blood cells. Appreciable levels of radioactivity were also found in the spleen and lung, while the heart, brain, and adrenal and salivary glands each contained less than 0.1% of the administered counts. Generally, the maximum specific activity was reached at 12 h, after which the levels were maintained, or declined. The radioactivity in the blood, however, was initially high between 1 and 3 h, but at 6 h had fallen to a level that declined only slightly over the next few days.

Towers (1970b) also investigated the tissue distribution and excretion of radioactivity for 48 h after a single intra-peritoneal dose of sporidesmin in male rats. Biliary excretion of the radioactivity was rapid and accounted for 50 to 52% of the administered radioactivity, whereas urinary excretion accounted for only 4.1 to 4.5%. Results indicated that sporidesmin was excreted in both conjugated and non-conjugated forms. Tissue distribution of the radioactivity of ^{35}S-sporidesmin was similar to that previously found in guinea-pigs (Towers, 1970a). In rats highest levels were found in the liver and kidney, moderate levels in the lung and bloodstream, and low levels in other tissues. Fifteen percent of the administered radioactivity was found in the liver 3 h after dosing. All tissues contained a considerable proportion of the radioactivity 48 h after dosing.

To determine the factors responsible for the species difference in sensitivity to sporidesmin, Towers (1972) studied the absorption, and the biliary and urinary excretion of radioactivity in biliary fistulated rats and guinea-pigs given ^{35}S-sporidesmin. The absorption and excretion of radioactivity by both species was similar in both magnitude and rate. It was suggested that differences in the species' sensitivity to sporidesmin was due to variations at the enzyme level, rather than in differences in absorption and excretion.

Sheep bred for resistance or susceptibility to facial eczema were dosed with sporidesmin, and sporidesmin concentrations in bile were then determined (Fairclough and Smith, 1983). The animals were orally dosed at a rate of 0.08 mg of sporidesmin per kg per day over 3 consecutive days. A trace amount of ^{35}S-labelled sporidesmin was also given on the first and third day of dosing to allow quantification of sporidesmin appearing in the bile by HPLC fractionation and by scintillation measurement of the sporidesmin-containing fractions.

Results showed that sporidesmin concentration in the bile increased to a maximum in 1 to 3 h and then declined over the following 20 h. There was considerable individual variation among animals in the pattern of uptake and in the maximal concentration reached after dosing. In this study, peak concentrations between 10 and 80 ng of sporidesmin per mL of bile were found. An apparent effect of the first dose of sporidesmin on the concentration of toxin appearing in bile at the third dosing was also reported. In general, 'susceptible' rams showed a marked increase in maximum sporidesmin concentration in bile between the first and third dosing whereas 'resistant' rams showed no marked increase at these times. Animal resistance or susceptibility to sporidesmin was indicated by a liver damage score which was assessed at slaughter. It is known that the hepatocyte cell is primarily responsible for the metabolism and transport of toxins from the bloodstream to the bile ducts of the liver. These workers therefore suggested that it was possible that a sporidesmin-induced biochemical change within the liver was responsible for the observed variation in response among animals to a sporidesmin challenge.

1.4.2 In vitro studies of metabolism

Fairclough *et al.* (1978) studied the metabolism of sporidesmin by the drug-metabolising enzymes of the liver using ^{3}H-, ^{14}C- and ^{35}S-labelled sporidesmin. Microsomes, isolated from livers of sheep treated with hexachlorobenzene to induce the drug-metabolising enzymes were incubated with labelled and unlabelled sporidesmin in the presence of a NADPH generating system. At least eight different metabolites were found and isolated but only tentative structures have been assigned to the monohydroxy and dihydroxy derivatives of sporidesmin. An assay which measures the rate of metabolism of ^{35}S-sporidesmin was used to determine sporidesmin metabolism rates in a fraction prepared from liver biopsy samples of a number of Romney and Merino sheep. Results showed a 2- to 3-fold difference in the rate of metabolism of sporidesmin among sheep and a higher metabolism rate in Merino than in Romney sheep. Because Merino sheep are known to be more

resistant to sporidesmin dosing than Romney sheep, it was suggested that differences in the rate of metabolism of sporidesmin by hepatic drug-metabolising enzymes may be one of the factors which determine whether sheep are resistant or susceptible to facial eczema.

1.5 Methods for control of facial eczema

Several methods for the control of facial eczema are available and since none of these ensure absolute protection against severe outbreaks, a combination of measures is often used.

1.5.1 Reduction of toxin intake

Sporidesmin A intakes can be reduced by minimising the exposure of grazing animals to toxic pastures or by reducing fungal growth by the use of fungicides. One of the earliest methods of minimising exposure involved the implementation of a forecasting system designed to warn farmers in disease-prone areas of danger periods based on identifying climatic conditions which favoured rapid fungal growth and sporulation (Atherton *et al.*, 1974). During these periods the toxicity of pastures is assessed by grass sampling and spore counting, and the pastures with the lowest spore numbers are grazed. Spore intakes can be reduced by reducing grazing pressures and thus the risk of grazing down to the litter zone where spore numbers are highest. Grazing pressures can be reduced by spreading the stock over all available pastures, stock numbers can be decreased and alternative crops or supplements can be fed.

Fungicide spraying has proved to be a very successful method of controlling facial eczema when the fungicides are correctly applied to pastures before spore counts rise to dangerous levels (Oldman and diMenna, 1984). However, the cost of widespread spraying of fungicides is high.

1.5.2 Protection against ingested toxin

Dosing animals with zinc salts at very high dose rates before and while they are exposed to sporidesmin A can reduce both the number of animals suffering liver damage and the severity of the liver damage occurring (Smith *et al.*, 1977; Towers and Smith, 1978). The addition of zinc sulfate to the reticulated water and daily oral drenching of zinc oxide are now the most common facial eczema control methods

used in the New Zealand dairy industry. Dosing with zinc salts is not widely used in the sheep farming industry because high labour input is required for regular drenching and zinc cannot be administered effectively through trough water because sheep water intake is low and sporadic. This problem can be overcome by the use of slow release boluses which have been recently developed and are now available (Munday *et al.,* 1997).

Protecting animals against ingested sporidesmin A by production of anti-sporidesmin A antibodies has not been successful. Subcutaneous immunisation of sheep with sporidesmin A or the structurally related 2-amino-5-chloro-3,4-dimethoxy benzyl alcohol (ACDMBA) produced antibodies which bound to sporidesmin A (Fairclough *et al.*, 1984). Approximately 10 weeks after inoculation the animals were dosed orally with sporidesmin A at 0.1 mg per kg body weight per day for three consecutive days. The vaccinated sheep were more sensitive to the sporidesmin A and suffered more severe liver damage.

Gallagher *et al.* (1987) described the immunisation of sheep with sporidesmin A 11-hemisuccinate conjugated to bovine serum albumin (BSA) to produce anti-sporidesmin antibodies. The sheep were dosed orally with sporidesmin A at 0.24 mg per kg body weight and again it was found that the production of antibodies binding to sporidesmin A enhanced the hepatotoxic effects of sporidesmin A (Gallagher *et al.*, unpublished data, 1987).

Injections of hexachlorobenzene to stimulate production of detoxifying enzymes in the liver to metabolise and detoxify foreign chemicals provided protection throughout the toxic season. The possibility of residues in animal tissues makes this approach unacceptable for livestock destined for human consumption (Mortimer, 1977).

1.5.3 Breeding for increased resistance to facial eczema

In any outbreak of facial eczema it can be observed that some sheep are more tolerant to the toxin than others. Part of this variability is genetically controlled, and increased resistance to sporidesmin A has been shown to be a heritable trait in both sheep (Campbell *et al.*, 1981) and Jersey cattle (Morris *et al.*, 1990). Breeding for resistance can reduce the losses caused by facial eczema, and over several generations animals can be seen to tolerate increasing amounts of toxin before liver damage occurs. Testing potential sires by dosing with sporidesmin A, however, is

expensive and exposes valuable animals to the risk of liver damage. The identification of a genetic marker, such as a detoxifying enzyme, which could be detected in a blood sample or liver biopsy would therefore be extremely useful in avoiding this risk.

1.6 Analytical methods for sporidesmin quantification

The heritable differences known to exist among sheep with regard to their susceptibility to sporidesmin A intoxication may reflect differences in the rate of destruction of the toxic disulfide bridge. To facilitate studies of the metabolism of sporidesmin A in sheep resistant and susceptible to the toxin, methods for the analysis of the sporidesmins A and D and their metabolites in various body fluids are required. These studies could lead to the development of more effective and safer methods for control of facial eczema, as well as to the detection of sporidesmin residues in agricultural food products and feeds.

1.6.1 Biological methods

Biological assay systems can be used for screening for toxicity but are of limited usefulness because of their non-specific nature, low detection sensitivity and general imprecision. White *et al.* (1977) and Mortimer and Ronaldson (1983) have reviewed the three biological methods which have been used in the detection and estimation of sporidesmin A. Generally, chemical methods have now replaced these methods.

The guinea-pig test involved feeding recently weaned guinea-pigs for 3-4 weeks with test extracts which had been mixed with ground meal. The animals were then killed and the characteristic bile-ductule proliferation in the livers indicated the presence of sporidesmin A in the extract. This method was used for the detection of the mycotoxin in pasture samples and culture concentrates.

In the cytotoxicity test sporidesmin-containing extracts were added to the culture media at increasing dilutions, above sloped test-tube cultures of mammalian cells previously cultured for 24 h. Cytopathogenic effects of sporidesmin A appear after 24 to 48 h of further culture. The semiquantitative test is sensitive to concentrations of sporidesmin A as low as 0.4 ng per mL of culture medium (Mortimer and Collins, 1968). This method has been used for assays of extracts of fungal cultures and for detection of sporidesmin A in biological fluids, *e.g.*, bile, urine and serum.

In the rabbit corneal opacity test, the test fluids (50 μL) were placed into the conjunctival sac of the rabbit eye. A total sporidesmin A application of about 5 μg produces corneal opacity and oedema within 5 days, and 10 to 20 μg has the same effect within 2-3 days. This is an inaccurate assay method although it has been used successfully to detect sporidesmin A in biological fluids.

1.6.2 Chemical methods

White *et al.* (1977) reviewed the chemical detection of sporidesmin A in extracts. This was first done by paper chromatography with sporidesmin spots being detected by first spraying with azide-iodine solution, and then with a starch solution. The sporidesmin spots appeared white on a blue background. This led to the development of the azide-iodine titration (Russell, 1960), which was based upon the uptake of iodine by sporidesmin A in the presence of sodium azide, and was used extensively to measure sporidesmin concentration in extracts of *P. chartarum* spores and cultures. The test depends on the presence of a disulfide bond in the test substance and is therefore non-specific. Clare (1963) also showed that a number of factors could affect the results obtained, and that in order to obtain dependable results, the titration conditions for this test had to be strictly controlled.

White *et al.* (1977) also described several other chemical methods which included UV absorption and radioactivity measurement where *P. chartarum* is grown on a synthetic medium containing ^{35}S and the isotope appears in the sporidesmin. Later, TLC was used for the detection and estimation of sporidesmin A in contaminated pastures. Silica gel GF_{254} was used with benzene-ether (5:1) to separate sporidesmin A and sporidesmins B and E from D and G, or hexane-*t*-butyl alcohol (9:1) to separate sporidesmin A from sporidesmins B, D, E, and G.

Quantification by HPLC

Both assay sensitivity and reproducibility have improved considerably with advances in materials for packing of chromatography columns and improvements in instrumentation. HPLC has therefore become the method most widely used for the determination of sporidesmins. Extensive sample clean-up before injection, however, is required to remove interfering substances.

Lauren and Fairclough (1980) carried out the qualitative determination of sporidesmin A metabolites using HPLC. Sporidesmin A labelled with ^{3}H and with ^{35}S was incubated with liver microsomes, extracted with ethyl acetate and the crude

product fractionated on lipophilic columns (Lipidix 5000) and further examined using HPLC. At least eight different metabolites were detected. Halder (1980) described an HPLC method for the estimation of sporidesmin A in fungal cultures. The cultures were extracted with acetonitrile-benzene and the extracts evaporated, redissolved in methanol-water, partitioned with hexane and then with benzene. The benzene extract was dried and dissolved in a standard solution of naphthalene in methanol which was analysed by HPLC. Analysis of sporidesmin A in ovine urine and plasma was also carried out and required sample dilution in water and adjustment to pH 7.0. The aqueous phase was then extracted with benzene. Following evaporation, the residue was dissolved in the standard naphthalene solution and analysed by HPLC.

Mortimer and Ronaldson (1983) used HPLC to analyse fungal spores. Chloroform extracts of the spores were dried, redissolved in a methanol-water mixture, passed through a SepPak column and the bound toxin was eluted and analysed by HPLC.

Sporidesmin A levels in bile were determined by Fairclough and Smith (1983) using HPLC and a ^{35}S-labelled sporidesmin A tracer. Sheep were dosed with sporidesmin A which contained a trace of the radiolabelled toxin, and bile was collected and extracted. The extract was dried, redissolved and then fractionated by HPLC. The radioactive fraction containing sporidesmin A was counted and the results used to determine the concentration of sporidesmin A in the bile sample.

More recently fungal cultures have been extracted with methanol-water (Miles *et al.*, 1992) and the aqueous suspension extracted with diethyl ether. The extract was evaporated and the residue redissolved before analysis by HPLC.

Although HPLC is a sensitive, reproducible and specific technique, the existing HPLC methods for the analysis of sporidesmin A and its metabolites have some limitations. Samples require extensive clean-up, only one sample can be analysed at a time, running costs are high, and trained analysts are required. The assays are therefore expensive, time-consuming and inconvenient when handling a large number of samples.

1.7 Immunoassays for determination of mycotoxins

More recently another method has been developed for detecting mycotoxins which is suitable for use in large-scale screening of samples. This technique is the

immunoassay which involves the binding of the analyte by a specific antibody raised against that analyte. By quantifying the binding of antibody to analyte by labelling either the antibody or the analyte with a tracer, the analyte concentration can be determined. Immunoassays often require little or no sample clean-up, provide high sensitivity and rapid throughput, and depending on the specificity of the antibodies, will detect compounds with similar structures such as metabolites. Both polyclonal and monoclonal antibodies have been obtained that bind specifically with many mycotoxins and their metabolites (Chu, 1991a). Many of these have been used in the successful development of immunoassays for a number of mycotoxins, with detection limits at picogram and nanogram levels [reviewed by Chu (1984), Chu (1986), Pestka (1988), Candlish (1991), Chu (1991a), Pestka, (1994)], suggesting that immunoassay would be a suitable alternative method for sporidesmin analysis.

1.7.1 Enzyme immunoassays

Radioimmunoassay (RIA), where the tracer is a radioactive label, was initially used for mycotoxin analysis but more recently the enzyme-linked immunosorbent assay (ELISA), where the tracer is an active enzyme molecule, has proved to be more useful. The ELISA technique was first developed by Engvall and Perlman (1972) and one of the major advantages it has over RIA is that it does not use radioactive substances. This therefore avoids expensive instrumentation and problems associated with handling and disposal of radioactive materials.

Two types of enzyme immunoassays exist, the heterogeneous and homogeneous immunoassays (Candlish, 1991). The heterogeneous type involves the separation of free toxin in the aqueous phase from the bound toxin in the solid phase and includes ELISAs. Microtitre plates, Sepharose and polystyrol beads and Terasaki plates have been used as solid phase supports for ELISA. High protein binding, 96-well, polystyrene microtitre plates have been most widely used because of the availability of suitable support technology, which includes multi-well pipettors, automated washers, and spectrophotometers (Pestka *et al.*, 1995). Hydrophobic interactions between non-polar protein domains and the non-polar plastic matrix bring about non-specific protein binding to the microtitre plate surface (Engvall, 1980).

In the homogeneous immunoassay, enzyme activity is altered when antibody binds to enzyme-labelled analyte. It is not necessary to separate the free and bound enzyme-conjugate in the assay and all of the reagents required for the immunoreaction are added together in one stage. These assays have not been used

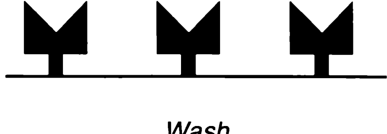
for routine mycotoxin analysis because of the limited availability of suitable enzyme conjugates and the restriction of the assay to samples which are free of substances such as enzyme inhibitors, activators and substrates, that might influence the activity of the indicator enzyme (Ward *et al.*, 1993).

1.7.2 ELISA formats applied to mycotoxin determination

The most common form of ELISA that has been applied to mycotoxin determination is the competitive type (Candlish, 1991), which is divided into two formats. One type, the direct competitive ELISA or cELISA (Figure 1.7.1), involves a mycotoxin-enzyme conjugate competing with the toxin in a sample for the same binding site on the mycotoxin-specific antibody. Conjugation methods used for immunogen preparation in antibody production can be applied to link mycotoxins to enzyme tracers for ELISA, providing that the reaction conditions do not denature the enzyme (Pestka, 1994). The enzyme most commonly used is horseradish peroxidase (HRP). The antibody is coated on a microtitre plate and the sample solution is added to the microtitre plate well and incubated with enzyme conjugate added at the same time. Alternatively the sample solution and enzyme conjugate are added separately and incubated in two steps. The plate is washed and the amount of enzyme bound to the plate is then determined by incubation with a substrate solution containing hydrogen peroxide and oxidisable chromogen (Chu, 1984). The resulting colour is measured spectophotometrically. Since the toxin-enzyme and antibody concentrations are constant, the colour intensity as a result of enzyme reaction is inversely proportional to the toxin concentration in the standard or sample, and the amount of toxin present can be calculated from a standard curve.

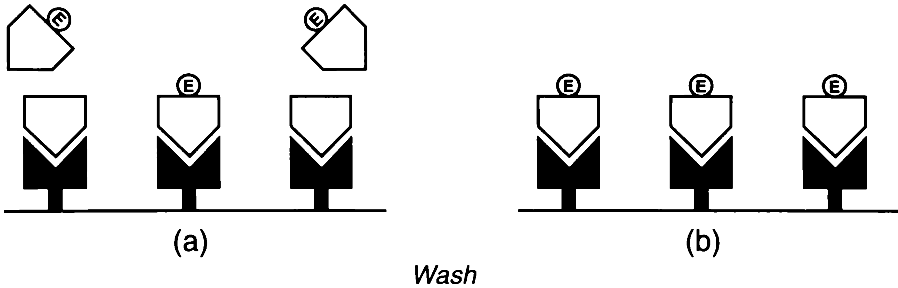
In the second format, the indirect competitive ELISA or cELISA, (Figure 1.7.2), a mycotoxin-protein or mycotoxin-polypeptide conjugate is first prepared and coated on a microtitre plate which is then incubated with a mycotoxin-specific antibody together with the mycotoxin-containing sample. The free toxin competes with the immobilised mycotoxin conjugate for binding to the soluble mycotoxin-specific antibody. A second antibody which binds immunoglobulin and is conjugated to an enzyme, is added to determine the amount of antibody bound to the plate by measuring the subsequent reaction with a substrate. Both HRP and alkaline phosphatase conjugated to the anti-immunoglobulin antibody are commonly used and the conjugates are available commercially.

1 Attachment of antibody to solid phase



Wash

2 Incubate with enzyme-labelled mycotoxin in presence (a) or absence (b) of standard or sample



3 Incubate with enzyme substrate (○) and measure product (●)

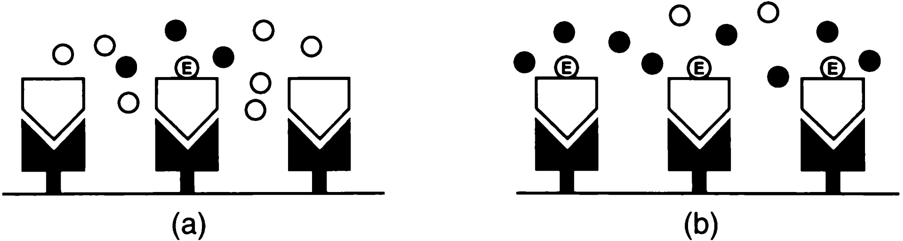
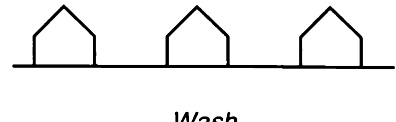


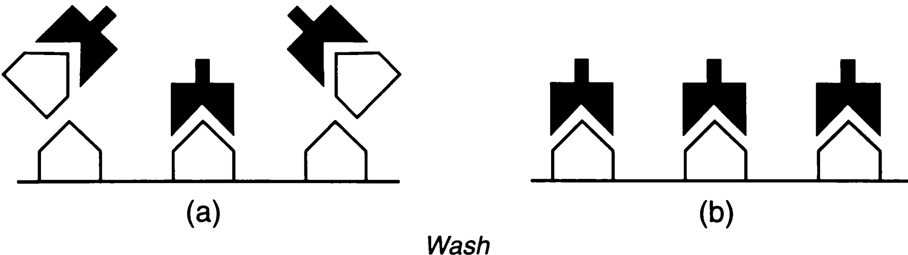
Figure 1.7.1 Direct competitive ELISA [adapted from Clark and Engvall (1987)].

1 Attachment of antigen to solid phase



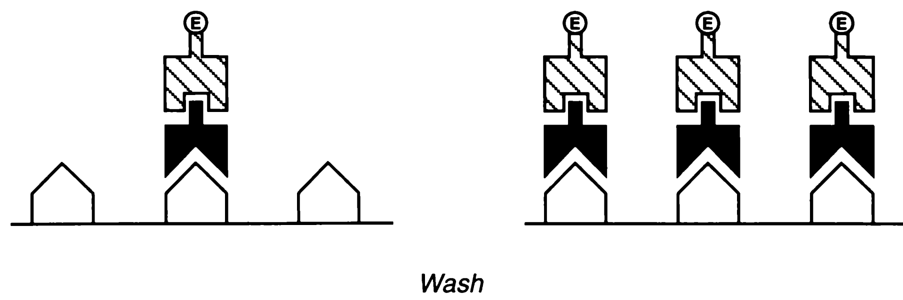
Wash

2 Incubate with mycotoxin-specific antibody in presence (a) or absence (b) of standard or sample



Wash

3 Incubate with enzyme-antiglobulin conjugate



Wash

4 Incubate with enzyme substrate (○) and measure product (●)

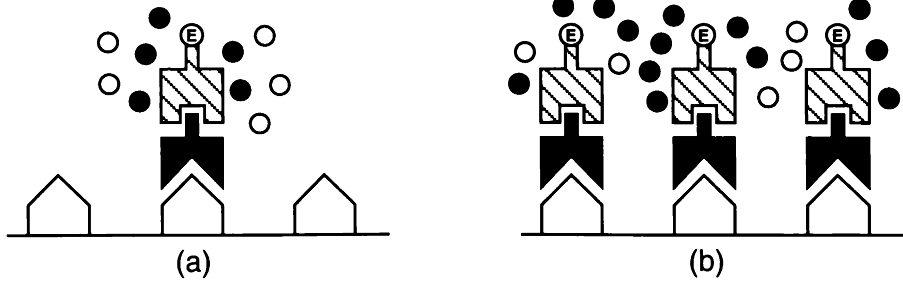


Figure 1.7.2 Indirect competitive ELISA [adapted from Clark and Engvall (1987)].

The indirect ELISA has a detection sensitivity that is comparable with or slightly better than the direct ELISA (Chu, 1986), and has the added advantages of using less antibody and not requiring the extra synthetic step of preparing a toxin-enzyme conjugate. The disadvantage of the method is that an additional incubation step is necessary in each assay which therefore requires a longer analytical time (Chu, 1991b).

1.7.3 Immunogen production

Compounds of low molecular weight (less than 1-10 kDa), such as mycotoxins, cannot by themselves produce an immune response in an animal. Such low molecular weight compounds are termed haptens. Anti-hapten antibodies, however, can be formed providing the hapten is chemically linked to an immunogenic macromolecule such as a protein or polypeptide. Preparation of such conjugates has been surveyed by Erlanger (1980) and Tijssen (1988). The conjugation of mycotoxins or their derivatives to carrier protein molecules has been achieved using water-soluble carbodiimide or mixed anhydride methods (Candlish, 1991), and the protein has usually been one of the following: globulin fractions, serum albumins from various species, keyhole limpet haemocyanin, ovalbumin, thyroglobulin, fibrinogen or polylysine (Chu, 1984; Candlish, 1991).

The functional groups of the hapten govern the selection of methods used to conjugate the hapten to the functional groups of the protein. Some mycotoxins, such as ochratoxins, patulin, penicillic acid and rubratoxin B (Candlish, 1991), contain reactive groups such as amino or carboxylic groups, which can be directly conjugated to a carrier protein or to a spacer molecule (Chu, 1991a).

Many mycotoxins, such as aflatoxins and the trichothecenes (Chu, 1984), need a reactive group such as a carbonyl group to be introduced by chemical synthesis. In general, an O-carboxymethyl oxime derivative can be prepared when the toxin molecule contains an available carbonyl group. Hydroxyl-containing mycotoxins, such as T-2 toxin, however, may be conjugated to a protein via hemisuccinate or hemiglutarate derivatives formed by reacting the mycotoxin with succinic or glutaric anhydride (Chu, 1986). The presence of a spacer alkyl chain, such as glutarate, between the protein and the mycotoxin may alter the way in which the hapten is presented to the immunised animal. This sometimes is an advantage, improving the quantity and quality of antibody production (Chu, 1991a).

Where mycotoxins do not contain a functional group suitable for conjugation, and one cannot be readily introduced chemically, it may still be possible to raise antibodies recognising the toxin by using analogues of the toxin which have strong structural homology and contain reactive groups suitable for conjugation.

The accuracy of immunoassay for mycotoxins is affected by the specificity of the antibody used and by the possible presence in the sample of other structurally related analogues of the mycotoxin that may react with the antibody. An immunogen may raise antibodies with a variety of different specificities. Many bind to regions of the mycotoxin, the mycotoxin and bridge group, the bridge group and adjacent regions of the carrier protein, or to the carrier protein only (Ward *et al.*, 1993). In addition, antibodies binding the toxin may also vary in the extent to which they bind to various mycotoxin analogues, *i.e.* vary in their cross-reactivity. Ultimately the specificity of the antibody primarily depends on the approaches used for raising antibodies (Chu, 1990), *i.e.* the mycotoxin or analogue used as the immunogen and the conjugation site in the mycotoxin molecule. Entities of the hapten molecule projecting most distally from the conjugation site typically induce the greatest antibody response and are therefore said to be "immunodominant". Least specificity will be observed for sites close to the point of conjugation (Morgan, 1989; Pestka *et al.*, 1995). Analogues, precursors, or metabolites of a mycotoxin that closely mimic the three-dimensional structure of the immunodominant region are likely to cross-react with the resultant antibodies (Pestka *et al.*, 1995). With careful design of the immunogen, specificity can be manipulated to produce antibodies possessing the required properties. These can be antibodies of broad specificity, capable of recognising groups or classes of molecules, or they may be highly specific, recognising one compound only amongst others of similar structure (Morgan, 1989; Candlish, 1991).

The ability of a mycotoxin-protein conjugate to produce mycotoxin-specific antibodies in an animal can depend on the amount of the mycotoxin conjugated to the protein molecule. A high or low molar ratio can often result in poor antibody production with the immunogen (Candlish, 1991). To avoid this, synthesised conjugates are often characterised in relation to molar ratios of mycotoxin to protein. This can be estimated by ultraviolet spectroscopy of the conjugate, since generally, the haptenic group has an absorbance spectrum that differs from that of the protein carrier (Candlish, 1991). Alternatively a small amount of radioactively labelled hapten can be incorporated into the conjugation mixture (Burrin and Newman, 1992).

1.7.4 Antibody production for use in ELISA development

(1) Immunisation

If polyclonal antiserum is to be produced, normally rabbits, goats, or sheep are immunised with the purified mycotoxin-protein conjugate. However, if monoclonal antibodies are to be formed, only mice (rarely rats) are immunised because these are the only species for which there are available tumour cell lines which fuse efficiently with added spleen cells to form antibody-secreting hybridomas.

Immunisation of animals can be achieved by injection of a mixture of conjugate and an adjuvant such as Freund's. Incorporation of these non-specific stimulators of the immune response optimises immunisation in two ways. Firstly, the adjuvant protects the antigen from rapid dispersal by trapping it in a local deposit. Secondly, it contains substances that stimulate the secretion of animal factors that increase phagocytosis in the region of the immunogen deposit (Harlow and Lane, 1988; Hefle, 1995). Polyclonal antiserum with a high titre can normally be produced within a few months of the initial immunisation, but in some instances repeated immunisation over a longer period may be required (Morgan, 1989). The factors that influence the immune response include: the nature of the immunogen, the dose of the immunogen, the choice of the animal species, the site of immunisation, the adjuvant, and the immunisation schedule (Wilkinson *et al.*, 1992).

(2) Antisera selection

Immunisation programmes yield a number of antisera from the immunised animals 1 or 2 weeks after a booster injection, and suitable antisera may be selected by ELISA for assay sensitivity and specificity. A method for selection has been described by McAdam *et al.*, (1992). In this a microtitre plate is coated with toxin-protein conjugate which preferably contains a protein that is different from the carrier protein in the immunogen, therefore enabling the selection of antibodies specific for the hapten rather than the immunogen carrier protein. Serial dilutions of the antisera are incubated in the plate wells, and antibody binding to the toxin-conjugate is measured with the appropriate anti-species immunoglobulin conjugated with horseradish peroxidase. The antisera dilutions which give approximately 50% of the maximum absorbance recorded with excess antibody are determined. These dilutions are used in cELISA to measure assay sensitivity and antibody specificity (cross-reactivity). Sensitivity is determined by calculating the I_{50}, which is the molar concentration of the analyte giving 50% inhibition of antibody binding to the coating conjugate in cELISA. Specificity can be determined by substituting

coating conjugate in cELISA. Specificity can be determined by substituting structurally related compounds for the target analyte in the cELISA (Pestka *et al.*, 1995). The molar concentration of the related compounds required to give a 50% reduction in the maximum absorbance is calculated, and this value is expressed as a percentage of the analyte concentration giving the same reduction in absorbance.

(3) Development of monoclonal antibodies

Although production of polyclonal antibodies is relatively simple, and the affinity of polyclonal antibodies is generally high, the antibodies are a complex mixture derived from multiple lymphocyte clones and are of different specificities, affinities, and isotypes. The composition of the antibody population may vary each time the immunised animal is bled (Pestka *et al.*, 1995). More recently, the demand for large amounts of antibodies with consistent properties for immunoassay has led to the production of monoclonal antibodies, although the major disadvantages to this approach are the requirement for tissue culture facilities, high cost and the labour input involved (Pestka *et al.*, 1995).

Monoclonal antibody production was first described by Kohler and Milstein (1975). The procedure involves repeated immunisation of mice which are bled from time to time and the titre and specificity of the polyclonal antibodies produced are evaluated by ELISA. When a mouse becomes hyperimmune and produces antibodies with the required specificity, its spleen cells are removed and fused with myeloma cells in the presence of polyethylene glycol. The myeloma cell line is both non-antibody secreting and deficient in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRTase). This enzyme is involved in the salvage pathway of nucleic acid metabolism, and provided hypoxanthine and thymidine are supplied, it is essential for cell survival when cells are cultured in the presence of aminopterin which blocks the main pathways of nucleotide synthesis (Littlefield, 1964). The fused spleen and myeloma cells, or hybridomas, are cultured in media containing hypoxanthine, aminopterin and thymidine (HAT) so that unfused myeloma cells which lack the enzyme and the unfused spleen cells, which do not have the immortality of the myeloma cell line, die within a few days. However, hybridoma cells, possessing both the enzyme from the spleen cells and the capability of immortal growth *in vitro* from the myeloma cells, are capable of prolonged growth and continue to grow and secrete antibodies. Culture supernatant fluids are screened for the presence of antibodies with the specificity required using methods similar to those used in polyclonal antisera selection. Hybridoma lines secreting suitable antibodies are selected and cloned by limiting dilution (Zola, 1987) to calculated cell

densities of approximately 0.5 to 2.5 cells per well. Cultures that originate from a single cell are generated, and antibodies produced from these subclones are monoclonal (Figure 1.7.3).

Production of antibodies can be carried out either in tissue culture or in the ascites fluids of mice. In theory an antibody-secreting hybridoma cell line can provide a perpetual supply of homogeneous antibody with the desired affinity, specificity, and ELISA performance characteristics, limited only by the ability to maintain the cell line.

(4) Antibody purification

Although some immunoassays employ crude antisera, purification of the immunoglobulins from other serum proteins usually helps improve assay sensitivity and reproducibility and reduces assay times (Rittenburg and Grothaus, 1992). Commonly used purification techniques include precipitation with caprylic acid and or ammonium sulfate, hydroxyapatite chromatography, gel filtration chromatography, ion exchange chromatography, and protein A or G affinity chromatography. Another technique is that of antigen-specific affinity chromatography where specific antibodies are purified from other polyclonal antibodies as well as the serum proteins (Harlow and Lane, 1988).

1.7.5 Establishment of ELISA protocols in complex matrices

When an ELISA for a mycotoxin in standard solution has been developed it is then applied to the determination of the mycotoxin in natural samples (Candlish, 1991). This involves the development of a suitable extraction technique for the mycotoxin in the sample of interest. If the components in the sample matrix do not interfere with the assay, however, some liquid samples may be assayed directly without extraction or alternatively after dilution in buffer to a concentration at which interfering substances do not affect the assay (Chu, 1990). Matrix interference may be brought about by large molecules causing steric hindrance of antibody binding to antigen or by non-specific binding of antibody to sample proteins, tannins and or saponins. Fatty acids, which have been reported to denature antibodies, and endogenous enzymes may also cause assay interferences (Hefle, 1995).

Dilution of the sample extract in buffer is often all that is required to remove this interference (Pestka, 1988), although this will decrease assay sensitivity. Interferences can also be minimised by routinely incorporating toxin-free sample

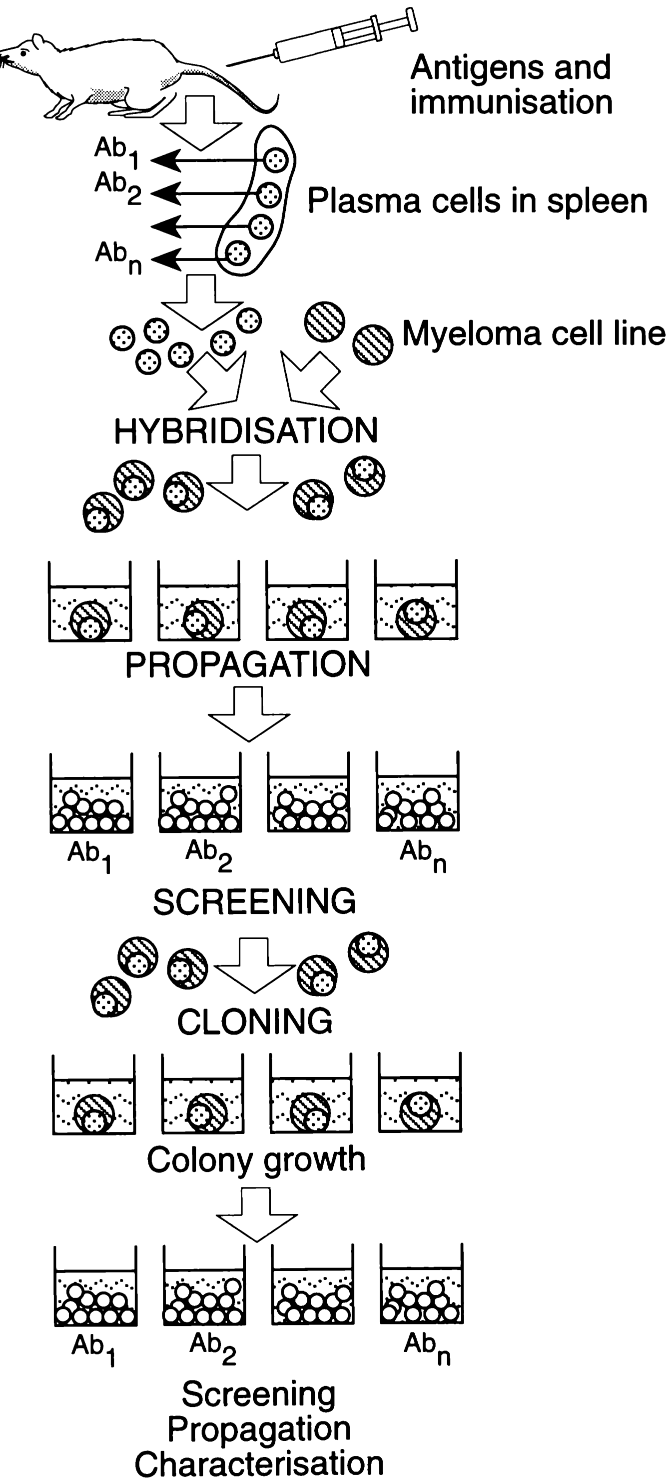


Figure 1.7.3 Schematic representation of hybridoma production [from Zola (1987)].

extracts during standard curve preparation (Pestka *et al.*, 1995). When extraction is necessary, existing techniques can normally be adapted. Selection of a suitable extraction procedure will depend on the nature of the sample matrix and the relative solubilities of the mycotoxin and sample components which may interfere with the assay (Ward *et al.*, 1993). The extraction efficiency can be assessed by an alternative analytical method such as HPLC and the compatibility of the extraction solvent with the ELISA is determined by comparing assay absorbances measured when the toxin-free sample extract is included and when it is excluded in the standard curve determination. Occasionally sample components that interfere with the assay are co-extracted with the toxin, *e.g.* peanut components. In more difficult situations such as these, a sample clean-up and concentration before analysis using C_{18} reverse-phase SepPak or silica gel SepPak cartridges (Chu, 1984), may be necessary. This may have an additional advantage of concentrating the toxin and extending the limit of detection (Pestka *et al.*, 1995).

A blocking agent, added to assay buffers to improve assay sensitivity, may also remove matrix interferences by inhibiting or preventing non-specific binding of sample components in the assay (Rittenburg and Grothaus, 1992). A variety of blocking agents have been used in immunoassays and these include bovine serum albumin (BSA), ovalbumin, casein, gelatin, non-specific serum and nonionic detergents including Triton X-100 and Tween 20.

The presence of matrix effects in ELISAs can be detected by preparing mycotoxin standards in a pooled sample extract which is toxin-free and the dose-response curve of these standards in the ELISA is then compared with that of standards prepared in buffer. Matrix interference in the ELISA is concluded to be absent when these two dose-response curves become superimposable (Candlish, 1991).

Validation of the ELISA can be determined by recovery experiments with spiked authentic samples. A quantity of sample is spiked with a known concentration of mycotoxin in a small volume of solvent. The sample is then subjected to the standard extraction and/or dilution procedure, and toxin recovery determined by ELISA. Recoveries of 90 to 110% can be expected (Candlish, 1991). Apparent high recoveries of toxin can indicate matrix interferences, while low recoveries can be attributed to inefficient extraction of the toxin from the sample.

1.7.6 Performance characteristics of ELISAs

Before an ELISA is used, the performance characteristics of the assay are fully evaluated so that the significance of the assay results can be established (Rittenburg and Grothaus, 1992). The important performance characteristics include assay sensitivity for detection (minimum limit) and the accuracy and precision of the assay results (Feldkamp, 1992).

Assay sensitivity is the least amount of analyte that can be detected under the conditions of the assay, *i.e.* the amount of analyte that provides an absorbance that is statistically different from the baseline absorbance of the toxin-free matrix (Candlish, 1991). Optimum sensitivity can be achieved by selecting an antiserum with very high affinity, and ideally high specificity, and using it at very low concentrations. Sensitive assays also require the availability of an enzyme tracer with high specific activity (Feldkamp, 1992). Dilution of a sample or sample extract to remove matrix interferences will also influence assay sensitivity.

Accuracy is the ability of an assay to give the correct result for the analyte of interest without interference from other cross-reacting molecules. The accuracy of an ELISA can be assessed by measuring a toxin standard added to test samples, and by correlating ELISA results with those obtained from another method such as HPLC, TLC, GLC, or RIA. Candlish (1991) reported such an evaluation with an ELISA for aflatoxin B_1 against TLC which showed a correlation coefficient of 0.97 for laboratory contaminated samples. A poor correlation coefficient of 0.74, however, against HPLC was reported for ochratoxin A.

Reduced accuracy of an ELISA can be caused by:
(a) non-specific adsorption of immunoreagents to the solid phase;
(b) non-specific assay interference by other substances in the sample or sample extract;
(c) specific cross-reactivity of the antibody with other antigenically (structurally) similar compounds such as mycotoxin metabolites (Candlish, 1991).

Precision is the property of an assay to give the same result for the sample when repeated within a run or between runs. The precision and reproducibility of an ELISA in measuring mycotoxins from a standard curve is normally defined as the coefficient of variation (CV) within assay (intra-assay) and between assays (interassay) (Candlish, 1991; Feldkamp, 1992). A CV of 10% or less is achievable

in most immunoassays (Ward *et al.*, 1993). A direct competitive ELISA for aflatoxin B_1 was reported by Candlish (1991) to have an intra-assay CV of 3 to 12% and interassay CV of 4 to 18% whereas an indirect competitive ELISA for ochratoxin A had an interassay CV of 4 to 18% and interassay CV of 9 to 22%. The increased variation of the indirect method was suggested to have been due to the extra pipetting step.

1.8 Immunoaffinity chromatography

When only trace amounts of toxins are present in complex matrices, extensive clean-up and concentration of samples are necessary to remove large amounts of impurities before any analytical determination can be carried out (Frémy and Chu, 1989). For example, sample impurities are likely to co-elute with the analyte(s) of interest during HPLC analysis, and in ELISA impurities may cause assay interferences. Concentration of dilute samples may be necessary, however, to extend the limit of assay detection. The successful production of anti-mycotoxin antibodies has led to the use of these antibodies in the development of immunoaffinity chromatography (IAC) to provide sample clean-up and concentration [reviewed by Candlish and Stimson (1993), Gilbert (1993)].

IAC is a technique where an immunoaffinity matrix is formed by covalently attaching an antibody to a chromatography support and then using this matrix to specifically and reversibly bind with compounds of interest, for example mycotoxins from a crude extract. The immunoaffinity matrix is packed into a column which is then loaded with filtered or centrifuged sample extracts. As the solution passes through the column the antibodies bind the target compounds. Other components in the solution, which are not bound by the antibody or the inert solid-phase, pass straight through. After washing with a buffer solution or distilled water to remove any remaining impurities, the compounds of interest are eluted with solvents under conditions which bring about dissociation from the antibody. The mycotoxin is eluted in a pure and concentrated form available for quantitative analysis and the column is regenerated by washing with starting buffer (Candlish and Stimson, 1993).

1.8.1 Immunoaffinity column development

The development of an immunoaffinity column involves the selection of an appropriate antibody and the immobilisation of this antibody onto a support matrix (Candlish and Stimson, 1993). The antibody used recognises only the compounds to

be purified or concentrated and if group-specific antibodies are used purification of related compounds or metabolites can be achieved. The antibody affinity is such that it forms a reversible complex with the compound of interest. The complex is sufficiently stable to not dissociate during application of the sample or washing buffers but readily dissociates during the elution step without the need of denaturing conditions. The antibody is stable to immobilisation, chromatography and regeneration.

The ideal support matrix would have negligible non-specific adsorption, lack charged groups, and be capable of binding antibody in a leak proof manner with full preservation of activity (Goding, 1986). There are several classes of support materials available for IAC, and among these the material most commonly used is agarose, commercially known as Sepharose or Bio-gel. Many different coupling procedures are available although agarose is usually first activated with cyanogen bromide which reacts with the free hydroxyl groups on the agarose (Goding, 1986). The antibody is then bound to the activated support by means of the antibody primary aliphatic or aromatic amino groups. Remaining unreacted active groups on the agarose are blocked with agents such as ethanolamine, glycine or glutamic acid to avoid non-specific binding of substances during chromatography.

Conditions under which analyte samples are loaded, washed and eluted from the column matrix are optimised (Goding, 1986). The ionic strength and pH of the buffer used, column flow rate and temperature, during both loading and washing to remove non-specifically bound substances, will all affect binding specificity and total binding capacity. Finally, conditions for eluting the mycotoxin bound to the IAC column are chosen. Elution is achieved using one or more different conditions such as change in ionic strength, pH change, protein deforming buffers, denaturants such as methanol, or polarity-reducing agents such as ethylene glycol (Candlish and Stimson, 1993).

The performance of IAC columns can be assessed by spiking samples with mycotoxin standards and determining the recovery of toxin from these samples using ELISA or HPLC (Azcona *et al.*, 1990). The repeatability of the recovery can be determined from replicate analyses and the capacity of a column determined as the maximum amount of toxin that is recovered from 1 mL bed volume of matrix when the column is loaded with excess mycotoxin (Bagnati *et al.*, 1990).

1.8.2 *Application of immunoaffinity chromatography to mycotoxin analysis*

IAC has provided a very effective clean-up and concentration step in the analysis of food samples, animal feed samples and biological fluids for mycotoxins (Candlish and Stimson, 1993). Some IAC columns have been sufficiently robust to allow re-use up to 100 times (Bagnati *et al.*, 1991). Depending on the requirement and facilities available, the method has been used before semi-quantitative screening of mycotoxins or it can be coupled with methods such as HPLC or ELISA for accurate quantification of mycotoxin (Groopman and Donahue, 1988; Sharman *et al.*, 1989; Candlish and Stimson, 1993). A rapid screening assay, for example, has been developed for aflatoxins using IAC columns which also contain a second chromatographic bed containing magnesium silicate (Florisil) (Candlish and Stimson, 1993; Cathey *et al.*, 1994). The mycotoxin which is fluorescent binds to the immunoaffinity matrix. The column is first washed and the mycotoxin is eluted and bound to the Florisil to form a fluorescent band. Increased amounts of aflatoxin increase the intensity of the fluorescence in the Florisil tip and this is estimated using an ultraviolet light box. For more accurate results estimation can be carried out using a fluorimeter.

IAC has been applied to a wide range of different mycotoxin-contaminated matrices such as grains, nuts, coffee and milk products, dried fruit, urine, serum and body tissues (Gilbert, 1993). Some materials, such as grains and nuts require grinding to a fine powder prior to extraction. Samples with high lipid content such as maize and milk will cause chromatography columns to block if the samples are not defatted by solvent extraction or centrifugation before IAC (Candlish and Stimson, 1993). Extraction of mycotoxin from a solid matrix usually employs organic solvents such as methanol, acetone, acetonitrile or chloroform, either alone or as mixtures that contain water. Antibody binding of mycotoxins, however, is reduced by high concentrations of organic solvents and this reduces the performance of IAC columns. Methods are required that result in the adequate extraction of the mycotoxins from the sample and provide sample extracts that can be loaded onto IAC columns without inhibiting antibody-antigen interaction. When satisfactory extraction can only be achieved by solutions that contain high percentages of organic solvent, dilution of the extract to a level where solvent concentrations are low enough to not inhibit antibody-toxin binding is necessary before column loading. Alternatively, solvent may be removed from the sample extract by evaporation.

IAC has the potential to be a valuable technique for the clean-up and concentration of sporidesmin A and its metabolites, particularly if only trace amounts occur in complex matrices such as body fluids and agricultural food products.

1.9 Anti-sporidesmin A antibody production

There are several reports of the production in animals of antibodies which bind specifically to sporidesmin A. In the past, antibody production was undertaken because it was seen as a possible strategy to provide protection from the effects of exposure to sporidesmin A. Jonas and Ronaldson (1974) derivatised sporidesmin A by opening the disulfide bridge across the dioxopiperazine ring of sporidesmin A, alkylating the sulfhydryl groups and then reacting this preparation with the ε-amino group of poly-L-lysine (structure 1 in Figure 1.9.1). The derivatised sporidesmin A was then coupled to keyhole limpet haemocyanin. Antibodies raised to this immunogen in rabbits were detected by an indirect haemagglutination test. The anti-sporidesmin A antisera had low titres and from cross-reactivity studies with structurally related compounds it was determined that the chlorine group was important in the antigenic structure of sporidesmin A.

Similarly, Jonas and Erasmuson (1977) suggested that the chlorine group may be an important determinant when they produced anti-sporidesmin A antisera with low titres using an immunogen which contained a synthetic hapten (structure 2 in Figure 1.9.1), similar to part of the substituted indole ring of sporidesmin A. In this study, guinea-pigs and rabbits were immunised with 2-amino-5-chloro-3,4-dimethoxy benzyl alcohol (ACDMBA) coupled to bovine gamma-globulin through azo linkages. Guinea-pigs were immunised with the same immunogen coupled to various heat-killed bacteria. Antibodies to ACDMBA were demonstrated by a complement fixation test and inhibition of the test showed that the anti-ACDMBA antibodies cross-reacted with sporidesmin A and some derivatives of sporidesmin A.

Anti-sporidesmin antibodies have also been produced in sheep. This was achieved by immunisation with sporidesmin A coupled to bovine thyroglobulin, ACDMBA coupled to heat-killed staphylococci or ACDMBA coupled to bovine gamma-globulin (Fairclough *et al.*, 1984). In this study plasma levels of antibody binding to sporidesmin A were estimated using ^{35}S-sporidesmin A and binding was found to be higher in sheep immunised with sporidesmin A conjugated to bovine thyroglobulin than in those given the ACDMBA-complexes.

(1) sporidesmin A poly-L-lysine conjugate

(2) 2-amino-5-chloro-3,4-dimethoxy benzyl alcohol (ACDMBA)

(3) sporidesmin A 11-hemisuccinate

Figure 1.9.1 Chemical structures of sporidesmin derivatives used in immunogens for anti-sporidesmin antibody production: (1) by Jonas and Ronaldson (1974); (2) Jonas and Erasmuson (1977) and (3) by Gallagher *et al.* (1987).

Gallagher *et al.* (1987) described the conjugation of derivatised sporidesmin A to a protein carrier to produce an immunogen which was injected into sheep and rabbits. The derivative was sporidesmin A 11-hemisuccinate (structure 3 in Figure 1.9.1) (Gallagher *et al.*, 1992). It was hoped that production of antibodies with high affinity for circulating sporidesmin A would bring about sporidesmin A inactivation before the toxin reached target sites, and the tissue damage causing facial eczema would therefore be prevented. Anti-sporidesmin antibody titres were determined by ELISA and values greater than 100 000 were obtained.

1.10 Objectives of the present study

The work described in this thesis was undertaken to facilitate further research into facial eczema by providing a range of sensitive analytical techniques for detecting sporidesmin A and its metabolites in body fluids.

The project aims were to:
(1) produce high-affinity antibodies with the required specificity against sporidesmin A, its metabolites and analogues, suitable for use in ELISA and immunoaffinity chromatography;
(2) develop ELISAs and immunoaffinity columns for the analysis of sporidesmin A and its metabolites in body fluids;
(3) apply the developed technologies to a study of the metabolism of sporidesmin A in sporidesmin A resistant and susceptible sheep, and determine ways in which the results may assist in the development of safer and more effective methods for the control of facial eczema.

Chapter 2:

MATERIALS & METHODS

CHAPTER 2

MATERIALS AND METHODS

All procedures involving animal experimentation were carried out only after approval from the Ruakura Animal Ethics Committee was given.

2.1 ELISA buffer solutions

The following solutions were used: plate coating buffer; carbonate-bicarbonate buffer, 0.05 mol/L, pH 9.6; phosphate-buffered saline (PBS); pH 7.4, NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.15 g), KH_2PO_4 (0.2 g), and distilled water (1 L); washing buffer, 0.05% Tween 20 (v/v) in PBS; sample buffer, 2% methanol (v/v) in washing buffer; standard buffer, 1% methanol (v/v) in washing buffer; antibody buffer, 1% BSA (w/v) in washing buffer; horseradish peroxidase substrate (Appendix I), 0.042 mol/L 3,3',5,5'-tetramethylbenzidine (TMB, obtained from Boehringer Mannheim, FRG) in dimethylsulfoxide (1 vol), 0.1 mol/L sodium acetate buffer, pH 5.5 (10 vol) and 1.3 mmol/L H_2O_2 (1 vol).

2.2 Sporidesmin analogues and sporidesmin-protein conjugates

Sporidesmin A, B and D were extracted and purified in our laboratories. Sporidesmin A derivatives were available from earlier work (Ronaldson, 1978) and additional compounds structurally related to sporidesmin A were obtained from AgResearch, Wallaceville, New Zealand. Sporidesmin A hemisuccinate isomers, available from earlier studies (Gallagher *et al.*, 1992) were conjugated to bovine serum albumin (BSA) and thyroglobulin to produce the immunising conjugates, or to ovalbumin and conalbumin to produce coating conjugates, using the active ester method of Bauminger and Wilchek (1980). Thyroglobulin, BSA, ovalbumin and conalbumin were obtained from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.).

2.3 Immunisation

2.3.1 Mice

Three groups of five BALB/c mice (4-6 weeks old, obtained from AgResearch, Ruakura) were each immunised with 120 µg of sporidesmin A 11-hemisuccinyl BSA, sporidesmin A 10b-hemisuccinyl BSA or sporidesmin A 11-hemisuccinyl thyroglobulin, in 100 µL of filter-sterilised PBS.

The primary injection was given intrasplenically (Spitz *et al.*, 1984). A mouse was anaesthetised by intraperitoneal injection of 10 µL/g body weight of the following anaesthetic mixture: nembutal (20 mg), saline (4 mL) and absolute ethanol (1 mL). The animal was placed on its right side on a surgical drape and held in place by taping the tail to the drape. The fur around the incision area was clipped with scissors and the skin swabbed with 70% ethanol. An oblique incision 1.5 cm long was made through the skin on the left dorsal side below the rib cage. The muscle and peritoneal layers were cut and the spleen was brought to the exterior using forceps. A 1/2 inch x 25 gauge needle fitted to a 1 mL syringe was inserted into the spleen and the immunogen injected at the same time as the needle was withdrawn in order to distribute the immunogen as much as possible through the spleen. The spleen was carefully pushed back into the peritoneal cavity and the incisions through the abdominal wall and skin were closed with sutures. The animal was left to recover under a heat lamp for about 2 h.

Booster injections were given intraperitoneally as an emulsion which contained 120 µg of immunogen in 100 µL of PBS and 100 µL of Freund's adjuvant (Sigma Chemical Co.). Complete adjuvant was used in the first boost and incomplete in subsequent injections. Five injections were given at 3 weekly intervals followed by injections at intervals that varied from 1-4 months over the next year. After seven immunisations, the first mouse was taken for hybridoma production and the remaining mice received further injections. One week after each immunisation blood samples (100 µL) were removed from the lateral tail vein and antibody titres of the resulting antisera determined by an indirect enzyme-immunoassay as described below. Three days prior to fusion, and 4 weeks after the last immunisation, 300 µg of the immunogen was dissolved in 300 µL of sterile-filtered PBS in a sterile microcentrifuge tube, and the solution was centrifuged at 10 000*g* for 5 min. Using a laminar flow cabinet, the supernatant was transferred to another

sterile microcentrifuge tube and then 100 μL was injected into the lateral tail vein and the remainder injected intraperitoneally .

2.3.2 Rabbits

Four New Zealand White female rabbits were each immunised with a subcutaneous injection of 100 μg of the sporidesmin A 11-hemisuccinyl BSA conjugate in 400 μL of PBS and adjuvant (1:2 v/v). The emulsion was injected into four different sites in the shoulder and hip region, *i.e.* 100 μL per leg. Complete Freund's adjuvant was used in the first injection and incomplete adjuvant in the following injections. A total of four immunisations at monthly intervals were followed by a rest period of four months and then three further monthly injections. Immunisations were followed one week later by the collection of 1 mL of blood from the marginal ear vein and the sera obtained monitored for antibody production and specificity. Two selected animals were bled by cardiac puncture and the antisera were prepared and stored at -20°C.

2.3.3 Sheep

Twenty sheep were each injected with 250 μg of sporidesmin A 11-hemisuccinyl BSA in 1 mL of PBS and Freund's adjuvant (1:4 v/v). The emulsion with complete adjuvant was first injected subcutaneously into two sites in the neck region, *i.e.* 0.5 mL into each site. Subsequent monthly immunisations were given with incomplete adjuvant, injected intramuscularly into two sites on the hind legs. One week after each immunisation a 10 mL blood sample was taken from the jugular vein. The antisera were monitored for antibody production. After seven immunisations, during which there was a rest period of 4 months after the fourth immunisation, 400 mL of blood was collected from selected animals into blood donor bags, antisera prepared and then stored at -20°C. The collection was repeated a month later.

2.4 Antibody screening by immunoassay

2.4.1 Indirect enzyme-linked immunosorbent assay (ELISA)

Antibody titres and binding of antibodies to plate-coating antigens were determined by ELISA. Microtitre plates (Maxisorp immunoplates obtained from Nunc, Roskilde, Denmark) were coated with 10b- or 11- derivatised sporidesmin A conjugated to ovalbumin (2 μg/mL) in coating buffer (100 μL/well). The plates

were sealed and incubated for 16 h. The coating solution was then removed and the wells were washed three times with PBS. Non-reacted sites were blocked for 75 min with 200 µL/well of PBS containing 1% BSA (ELISA grade, Sigma A-7030). This was followed by two washes with washing buffer. To each well, 50 µL of sample buffer was added followed by 50 µL of various dilutions of supernatant fluid taken from the cell cultures, or antiserum in antibody buffer. The plate contents were gently mixed and incubated for 1 h. After four washes, 100 µL of donkey anti-mouse immunoglobulin horseradish peroxidase-conjugate (anti-mouse-HRP), from Silenus Laboratories Pty. Ltd., Victoria, Australia, peroxidase conjugated rabbit anti-sheep IgG (anti-sheep-HRP) from USB Immunochemicals, Cleveland, Ohio, U.S.A., or anti-rabbit IgG peroxidase conjugate (anti-rabbit-HRP) from Sigma Chemical Co., was added. The antibody peroxidase conjugate used depended on the species from which the first antibody was obtained. The conjugates were diluted in antibody buffer as follows: anti-mouse-HRP, 1:2 500, anti-sheep-HRP, 1:10 000 and anti-rabbit-HRP, 1:4 000. The plates were incubated for 2 h. Wells were washed four times and then 100 µL/well of freshly-prepared substrate solution was added (Appendix I). After 25 min the reaction was stopped by adding 100 µL of 10% sulfuric acid. All procedures were carried out at 20°C.

The absorbance at 450 nm was determined in an ELISA reader (Bio-Rad Model 3550 microplate reader). Curve fits of log concentration versus linear absorbance were determined with a logit transformation using the Bio-Rad Microplate Manager data analysis software (illustrations of standard curves show an interpolated line).

2.4.2 Competitive indirect enzyme-linked immunosorbent assay (cELISA)

cELISA was used to determine sensitivity and specificity of antibody binding to sporidesmins A and D. Plates were coated and blocked as described for monitoring antibody titres. Sporidesmin A and sporidesmin D standards were prepared in methanol and diluted in standard buffer to give a methanol concentration of 2%. Polyclonal antisera were screened with standards ranging from 0.05 ng/mL to 1000 ng/mL while tissue culture supernants were screened with 1 µg/mL of sporidesmins A and D. Standard solutions (50 µL) were added to the wells followed by 50 µL of antibody at an appropriate dilution in antibody buffer. All samples were analysed in duplicate. The antibody dilution used was that which gave approximately 50% of the maximum absorbance recorded with excess antibody. cELISA was completed as described for ELISA.

2.5 Hybridoma production

2.5.1 Preparation of myeloma cells

Approximately 1×10^8 myeloma cells were required for fusion with one mouse spleen. The myeloma cell line P3/NS-1/1-AG4-1 (NS-1) (ATCC TIB 18) was obtained from The American Type Culture Collection (Rockville, Maryland, U.S.A.). Penicillin, streptomycin, 50x hypoxanthine and thymidine (HT), and 50x hypoxanthine, aminopterin, and thymidine (HAT) were obtained from Sigma Chemical Co. Iscoves Modified Dubeccos Medium (IMDM), Minimum Essential Medium (MEM), non-essential amino acids (NEAA) and foetal calf serum (FCS) were from Gibco Laboratories (Grand Island, New York, U.S.A.). Suitable batches of FCS were selected from the manufacturer's screening results for the ability to support hybridoma growth. FCS was thawed at 37°C, dispensed into 20 mL aliquots and heated for 30 min in a water bath at 56°C to inactivate complement. Tissue culture plasticware was purchased from Nunc (Roskilde, Denmark).

Approximately 2×10^6 myeloma cells were thawed from liquid nitrogen stocks 2 weeks before the fusion and grown in complete IMDM with 10-20% FCS (Appendix I), together with HT (100 µmol/L hypoxanthine, 16 µmol/L thymidine).

Cell thawing

A vial of cells was removed from the liquid nitrogen storage and thawed in a water bath at 37°C. When the last of the ice had just melted the cells were transferred to a centrifuge tube and diluted with 10 mL of IMDM with 15% FCS, which was added at 20°C over several minutes. The cell suspension was centrifuged at 200*g* for 5 min and the resulting cell pellet resuspended in another 5 mL of growth medium. The suspension was then delivered into four wells in a 24-well culture plate and placed in a humid atmosphere of 6% CO_2 at 37°C in a water-jacketed incubator.

Cells were grown to a maximum cell density of 2 to 5×10^5 cells/mL (Oi and Herzenberg, 1980) and leading up to fusion the cultures were kept in exponential growth phase and expanded into petri dishes (145 mm in diameter) or culture flasks (50 mL and 260 mL).

Cell counting

Cells were counted using a haemocytometer which has a special coverslip resting on supports that hold it 0.1 mm above the base of a slide which is engraved with a series of lines that outline 0.1 mm^3 chambers (Harlow and Lane, 1988). A 0.25% solution of trypan blue (Sigma Chemical Co.) was prepared in filter-sterilised PBS and a small aliquot of a cell suspension was added to an equal or greater volume of the dye. A drop of the dye mixture was transferred to the edge of the haemocytometer coverslip and the volume of the drop was sufficient for it to be drawn evenly into the haemocytometer chamber by capillary action. The haemocytometer was viewed under a microscope and the unstained cells within four of the 0.1 mm^3 chambers were counted. The number of cells were calculated according to the formula :

$$\text{number of cells/mL} = \text{total number of cells counted} \times 10^4 / 4$$

and an adjustment was made for dilution in the dye solution.

Every 2-3 days the cultures were split 1-in-5 by removing 80% of the culture and replacing this with fresh medium. The removed medium was centrifuged at 200*g* for 5 min and the cells discarded. The supernatant was retained frozen and was used as 'myeloma conditioned medium' in subsequent cultures. Cultures were diluted 1:5 in culture medium 2 days and again 1 day before fusion. On the day of the fusion, the myeloma cells were washed with a pipette from the bottom of the culture flasks and pooled into one flask.

2.5.2 Preparation of feeder cells

Peritoneal macrophages were prepared using the method of Fazekas de St. Groth and Schiedegger (1980) except the cells were collected into IMDM (Campbell, 1984) rather than into 0.34 mol/L sucrose. Two days before the fusion a 5 mL syringe fitted with a 25 gauge needle was filled with 4 mL of sterile, serum-free IMDM containing 50 units/mL of heparin and placed on ice. A BALB/c mouse was killed using carbon dioxide, sprayed with 70% ethanol and placed on a dissecting board. The abdominal skin was carefully removed to expose the peritoneal membrane which was also sprayed with 70% ethanol. Using forceps, a flap of the membrane in the middle of the abdomen was lifted and the cold IMDM was injected into the peritoneal cavity beneath the forceps. The needle was withdrawn and covered while the abdomen was gently massaged for a few minutes. The needle was again inserted into the peritoneum but this time at the sides of the abdomen and as much of the medium as possible was withdrawn back into the syringe.

In the laminar flow cabinet the needle was removed from the syringe and the contents transferred to a centrifuge tube. The cells were centrifuged at 200*g* for 3 min, resuspended in 10 mL of complete IMDM and stored on ice. An aliquot was taken for macrophage cell counting and the volume adjusted to give a cell count of 1×10^5/mL. The cell suspension was then plated out at 100 µL per well in 96-well microtitre plates to give 10^4 cells/well (Sugasawara *et al.*, 1985).

2.5.3 Preparation of spleen cells

On the day of the fusion, a selected immunised mouse was anaesthetised with ether and placed on a dissecting board. The scapular artery in the forelimb was severed, and the blood recovered by means of a transfer pipette was retained as a source of antibodies for use in subsequent ELISA development or as a positive control in hybridoma screening assays. The dead mouse was sprayed with 70% ethanol and the abdominal skin removed. The spleen was then removed aseptically, placed in 5 mL of sterile serum-free IMDM, and in a laminar flow cabinet it was transferred to a sterile petri dish containing another 5 mL of IMDM. Surface fat and connective tissue were dissected away using sterile forceps and scissors. A 1 mL syringe and needle and forceps were used to rupture and tease the spleen apart. The contents of the petri dish were strained through a sterile cell dissociation sieve grinder (Sigma Chemical Co.) into a 50 mL conical centrifuge tube. Any remaining tissue was rubbed through the 50-mesh screen using the glass pestle supplied with the sieve. Another 5 mL of IMDM was used to rinse the petri dish and sieve, and the centrifuge tube contents were left to stand for 3-5 min to allow any larger cell clumps to settle. The supernatant was aspirated, transferred to another centrifuge tube, centrifuged at 200*g* for 5 min, and the resulting cell pellet was resuspended in 20 mL of serum-free IMDM. An aliquot was taken for cell counting and the total number of spleen cells harvested was determined.

2.5.4 Cell fusion

The fusion of spleen cells to myeloma cells in polyethylene glycol (PEG) was carried out using the method described by Galfrè and Milstein (1981) with some modifications.

A vial of PEG (Sigma Chemical Co., P 2906), purchased in 5 g lots, was melted in a boiling water bath, and 5 mL of warm, filter-sterilised, serum-free IMDM was

added. This was mixed on the vortex mixer and left in the incubator at 37°C until required for the fusion.

A ratio of two spleen cells to one myeloma cell was used (Spitz, 1986). The volume of culture fluid containing the required number of myeloma cells was transferred into centrifuge tubes and centrifuged for 5 min at 200*g*, and the resulting cell pellets were resuspended in 10 mL of serum-free IMDM. The myeloma cells were then added to the spleen cells, the mixture centrifuged again for 5 min and the supernatant completely removed. The tube containing the cells was transferred to a Lab-line Multi-blok heater and maintained at 37°C in a laminar flow cabinet.

The PEG in IMDM, 25 mL of serum-free IMDM and 150 mL of complete IMDM-HAT (HT with 0.4 μmol/L aminopterin, Appendix I) were also transferred to the heating block. The cell pellet was loosened by gently flicking the base of the tube, and for every 1×10^7 cells present, 50 μL of PEG in IMDM was slowly added dropwise (1 drop/5 sec) over 1 min. For example, when a single spleen yielded 2×10^8 splenocytes and a total of 3×10^8 cells were present, 1.5 mL of the 50% PEG solution was required. While the PEG was being added the mixture was gently stirred with the pipette tip. The time was noted using a stopwatch.

The cells were then swirled in the tube for 1 min over the heating block.

Dilution of the PEG was carried out over 5 min by gradually adding the 25 mL of serum-free IMDM with gentle stirring.

1 drop/5 sec	(1 mL)	during the	1st minute
2 drops "	(2 mL)	" "	2nd "
4 " "	(4 mL)	" "	3rd "
8 " "	(8 mL)	" "	4th "
8 " "	(10 mL)	" "	5th "

The cells were left to stand at 37°C for another 5 min before centrifugation at 200*g* for 3 min. The supernatant was discarded and the cells gently resuspended in the complete IMDM-HAT to give a concentration of approximately 2×10^6 cells/mL based on the original number of cells taken for fusion.

2.5.5 Hybridoma culture

The fusion mixture was distributed into 96-well microtitre plates (100 µL/well), containing the mouse macrophage feeder cells (see Section 2.5.2), for selection of hybridomas showing growth in IMDM-HAT. Five days after the fusion, 100 µL of the culture fluid was removed and replaced by freshly prepared IMDM-HAT. The process was repeated every second day.

When the colonies covered approximately half of the well area, 100 µL of the culture supernatants were removed and screened by ELISA for the presence of antibodies binding to the sporidesmin coating antigens. Cultures giving positive results were sub-cultured into three wells. When the colonies had grown to fill the wells the culture supernatants were collected, pooled and tested by cELISA for sporidesmin A and sporidesmin D binding antibodies. After 2 weeks, HAT-IMDM was gradually eliminated by replacement with HT-IMDM containing 10-15% FCS. Hybridoma lines producing selected antibodies were expanded and a portion of the cells removed for freezing and storage at -70°C.

Freezing cells

The day before freezing, the cells were split 1:10 into fresh IMDM. The following day they were washed from the culture dishes or flasks and pooled in another flask. A small aliquot was taken for cell counting (see Section 2.5.1). The cells were centrifuged at 200*g* for 5 min, and as the supernatant was removed, the volume was measured. The total number of cells harvested was then determined and the appropriate volume of storage medium (Appendix I) added to give approximately 5×10^6 cells /mL. Once the medium containing DMSO was added, the process was completed as rapidly as possible since DMSO is toxic to cells in the liquid phase. The cells were immediately dispensed in 0.8 or 1.5 mL volumes into 1 or 2 mL cryovials, transferred to a polystyrene storage box and placed in a -70°C freezer. The next day the vials were transferred into liquid nitrogen storage.

The remaining cells were cloned by dilution to a calculated density of 2.5, 1.0 and 0.5 cells/well (Zola, 1987) and growth of the diluted cells was supported with macrophage feeder cells. Wells containing single colonies were identified and screened for antibody production by ELISA and cELISA. Hybridoma cells from wells giving positive results were expanded and subcloned again twice.

2.6 Antibody production and purification

2.6.1 Antibody isotype

The isotype classes of the monoclonal antibodies were determined following the manufacturer's instructions using a immunoglobulin subclass identification kit, Mouse Typer, which was purchased from Bio-Rad Laboratories (Richmond, California, U.S.A.) and is based on an enzyme-immunoassay method. The kit has a purified rabbit anti-mouse panel of antibodies specific to immunoglobulin isotypes and subisotypes IgA, IgM, IgG_1, IgG_{2a}, IgG_3, kappa chain and lambda chain.

2.6.2 Monoclonal antibody production

(a), In culture supernatant
Cell lines selected for antibody production were grown in 100 mL batch cultures to stationary phase, and the culture fluids were collected and stored at -20°C.

(b), In ascitic fluids
BALB/c mice, 8-10 weeks old, were injected intraperitoneally with 500 µL of pristane (Sigma Chemical Co.) 10 days before an intraperitoneal inoculation with 1-5 $\times 10^6$ hybridoma cells in 500 µL of serum-free IMDM. Prior to the injection of the hybridoma cells the mice were given one irradiation treatment of 2-3 Gy (cobalt source). This sub-lethal dose resulted in sufficient immunosuppression to permit the establishment of hybridoma cells in the peritoneum but allowed mice to be raised under normal housing conditions (Weissman *et al.*, 1985). When the ascitic fluid accumulated in the peritoneal cavity, the animals were humanely killed and the fluid drained. Immediately after collection it was centrifuged for 15 min at 2 000*g* and stored at -20°C.

2.6.3 Antibody purification

Monoclonal antibodies in culture supernatant and ascitic fluids and polyclonal antisera were purified using affinity chromatography with Protein G Sepharose 4 Fast Flow (obtained from Pharmacia LKB, Uppsala, Sweden) according to the manufacturer's instructions. The chromatography gel was packed into a glass column (Wright Scientific Ltd., Gloucestershire, England) with internal dimensions of 1.0 × 5.0 cm and a bed volume of 5 mL. Culture supernatants were filtered (0.2 µm) and processed in 500 mL batches. Ascitic fluids (8 mL) were diluted in 25 mL

of starting buffer and polyclonal antisera (4 mL) were diluted in 8 mL of starting buffer before they were loaded onto the column. The column was loaded using a Pharmacia LKB P1 peristaltic pump at a flow rate of 0.9 mL/min and was washed at 1.7 mL/min. Bound antibodies were eluted with 0.1 mol/L glycine buffer at pH 2.7 and the eluate was monitored at 280 nm using an Isco Model 1 UA-5 absorbance monitor. Fractions were 3.0 mL in volume and were collected by an LKB 2070 Ultrorac II fraction collector into tubes containing sufficient 1.0 mol/L Tris/HCl buffer, pH 8.0, to adjust the eluted antibodies to approximately pH 7.0. The antibody containing solutions were pooled and dialysed against three changes of PBS. Purified antibodies, at a concentration of no less than 1 mg of protein per mL of PBS, were stored in aliquots at -20°C.

2.6.4 Determination of total protein

The concentration of total protein concentration in the pooled antibody-containing fractions was determined using the Bio-Rad microplate procedure (Bio-Rad Laboratories, 1984) based on the Bradford method. The standard curve was determined using a bovine gamma globulin standard purchased from Bio-Rad Laboratories (Standard 1).

2.6.5 Electrophoresis and isoelectric focusing

Antibody purity was determined by electrophoresis using the automated electrophoresis system PhastSystem (Pharmacia LKB). SDS-PAGE was carried out using PhastGel homogeneous media 12.5. Silver stain SDS-PAGE low-range protein standards were obtained from Bio-Rad Laboratories. Samples and standards were adjusted to a protein concentration of approximately 10 ng/μL using the sample buffer recommended by Bio-Rad Laboratories. They were heated for 5 min at 95°C, cooled and centrifuged at 10 000*g* for 5 min. Isoelectric focusing was carried out using PhastGel IEF media 3-9 and a broad pI calibration kit containing Pharmalyte 3-10, also purchased from Pharmacia LKB.

The procedures used for separating and staining gels were those given in the handbook supplied with the PhastSystem. The PhastSystem Separation Technique file No. 111 SDS-PAGE in homogeneous media was followed for SDS-PAGE and gels were stained using the method given in Table 2 in Development Technique File No. 210, Sensitive Silver Staining. IEF was carried out according to Separation

Technique File No. 100 and gels were stained by following Table 1 in File No. 210, Sensitive Silver Staining.

2.7 Determination of antibody cross-reactivities

Cross-reactivities with compounds which were structurally related to sporidesmin A were determined from standard curves of the tested compounds in cELISA. Cross-reactivities were calculated according to the formula:

$$\% \text{ cross-reactivity } (\% \text{ CR}) = (I_{50} \text{ sporidesmin A} / I_{50} \text{ analogue}) \times 100$$

where I_{50} is the molar concentration of compound giving 50% reduction of the maximum absorbance obtained in the absence of sporidesmin A or analogue.

2.8 Immunoassay of body fluids

2.8.1 Bile

Hepatic bile was collected from cannulated sheep which were dosed orally with a single dose of sporidesmin A (0.2 and 0.4 mg/kg body weight). Samples were taken at intervals over 48 h after dosing and stored at -20°C before analysis.

Immunoassay reagents were diluted as listed in Table 2.8.1 and the protocol for bile analysis was as described for cELISA with the following modifications to remove matrix effects. Samples were diluted 1:49 and 1:99 in sample buffer in which the Tween 20 concentration had been increased from 0.05% to 1.0% (w/v). This was followed by mixing on a vortex mixer and incubation in a water bath for 30 min at 70°C. Standards were prepared in buffer containing 1% Tween 20 and were treated the same as the samples. After cooling to 20°C, 50 µL of the samples or standards were added to the plate and assayed as previously described (Section 2.4.2).

2.8.2 Urine

Urine was collected in the field from ewes by nasal occlusion and collecting urine into a large plastic beaker. Urine was also collected by catheter from sheep dosed with sporidesmin A and sporidesmin D (0.2 mg/kg body weight). The urine was collected at intervals over 110 h after dosing and stored at -20°C before analysis.

Urine samples were diluted 1:49 and 1:99 in sample buffer modified by the addition of 1% BSA. Standard buffers also contained 1% BSA.

Table 2.8.1 Immunoassay conditions for body fluid analyses

analyte	antibody group[a], (dilution)	body fluid	coating antigen[b], concentration	second antibody dilution
sporidesmin A	A, (1:375 000)	bile	hapten 11-ovalbumin, 2 μg/mL	1:2 000
sporidesmin A and metabolites	B, (1:60 000)	bile	hapten 11 or 10b-ovalbumin, 4 μg/mL	1:2 000
sporidesmin metabolites	B, (1:130 000)	urine	hapten 11 or 10b-ovalbumin, 4 μg/mL	1:2 500
sporidesmin A	A, (1:2 000 000)	milk	hapten 11-ovalbumin, 2 μg/mL	1:2 000
sporidesmin A and metabolites	B, (1:130 000)	milk	hapten 11 or 10b-ovalbumin, 4 μg/m	1:2 000

[a] Group A antibody is a monoclonal antibody specific for sporidesmin A and group B antibody is specific for sporidesmins A, D and metabolites. [b] Hapten 11 represents sporidesmin A 11-hemisuccinyl, and hapten 10b is sporidesmin A 10b-hemisuccinyl.

2.8.3 Milk

Milk samples (50 mL) were taken from farm vats and stored at -20°C. After thawing they were heated to 40°C for 5 min and vortexed before a sample was taken for immunoassay. These were diluted 1:10 in sample buffer (PBS containing 2% methanol and 1% Tween 20). A pooled milk sample, collected in the winter when pastures were free of sporidesmin A, was included in the standard buffer (diluted 1:9). Samples and standards were heated at 70°C for 30 min, cooled to room temperature and centrifuged at 10 000*g* for 5 min. The aqueous fraction (50 μL) was taken for cELISA. When skimmed milk was analysed by cELISA 1 mL of whole milk was taken as described above and centrifuged at 10 000*g* for 5 min before the aqueous fraction was diluted 1:9 in sample buffer. Pooled skimmed milk was included in the standard buffer.

2.9 Immunoassay validation

To evaluate any potential matrix effects, sporidesmin A standard curves were prepared in diluted body fluid samples and compared with standard curves prepared in the standard buffers alone. The optimised methods were validated by spiking body fluid samples with a standard solution of sporidesmin A in methanol by adding 10 µL of standard to 990 µL of the undiluted body fluid sample. Samples were spiked with three different concentrations of analyte. Three samples were spiked at each concentration and each sample was assayed in eight replicates duplicated each day on two plates. Recoveries were determined by comparing the cELISA results with the calculated sporidesmin concentrations. The intra-assay (intra-plate) variation was determined from the variation of the mean sporidesmin concentration of each of the three spiked samples for the three different concentrations on one plate. The inter-assay (inter-plate) variation was determined from the variation of the mean sporidesmin concentrations determined on each plate over 2 days.

2.10 Enzyme hydrolysis of sporidesmin metabolites

The hydrolyses of bile and urine with β-glucuronidase, Type B-1 from bovine liver (Sigma Chemical Co.), were based on the methods described by MacDougald *et al.* (1990) and Meyer and Hoffman (1987). Aryl sulfatase from *Helix pomatia* (Boehringer Mannheim) was reacted with body fluids according to Olsen *et al.* (1986). Protein conjugates were digested according to Duchatel and Maghuin-Rogister (1985), using lyophilised pronase from *Streptomyces griseus* (Boehringer Mannheim). The β-glucuronidase and sulfatase digestions were carried out overnight at 37°C, while digestion of body fluids with pronase were carried out over 24 h at 37°C. Reagent volumes used were as listed in Table 2.10.1 and enzymes were dissolved or diluted in the appropriate buffer as shown in Appendix I.

Table 2.10.1 Reagents for enzyme digestion of body fluids

enzyme	enzyme volume	body fluid	body fluid volume, μL	enzyme buffer	buffer volume, μL
β-glucuronidase	3 000 units in 10 μL	urine	500	acetate, pH 5.5	500
β-glucuronidase	1 500 units in 5 μL	bile	50	acetate, pH 5.5	450
sulfatase	200 units in 10 μL	urine	500	acetate, pH 5.5	500
sulfatase	100 units in 5 μL	bile	50	acetate, pH 5.5	450
pronase	0.5 mg in 25 μL	urine	250	Tris/HCl, pH 7.7,	225
pronase	0.5 mg in 25 μL	bile	50	Tris/HCl, pH 7.7,	425

2.11 Immunoaffinity chromatography

2.11.1 Sporidesmin A in buffer

Purified antibody (25 mg) was coupled to 2 g of freeze-dried, cyanogen bromide-activated Sepharose 4B (Pharmacia LKB), according to the manufacturer's instructions. The resulting immunoaffinity matrix was packed into a glass column (Wright Scientific Ltd.) with internal dimensions of 1.0 × 5.0 cm and a bed volume of 2 mL. The column was loaded and washed using a Pharmacia LKB P1 peristaltic pump and a flow rate of 0.8 mL/min. Sporidesmin standard was added in 20 μL of methanol to 1 mL of 0.05% Tween 20 in PBS and loaded onto the column using the standard flow rate of 0.8 mL per min. The eluate was monitored at 280 nm using an Isco Model 1 UA-5 absorbance monitor. Fractions (35 drops/fraction) were collected using an LKB 2070 Ultrorac II fraction collector.

Chromatography procedure:

0.05% Tween 20 in PBS	20 mL
sporidesmin A standard in 0.05% Tween 20 in PBS	
0.05% Tween 20 in PBS	4 mL
water	15 mL
5% MeOH in water	15 mL
10% MeOH in water	15 mL
20% MeOH in water	15 mL
30% MeOH in water	15 mL
40% MeOH in water	15 mL
50% MeOH in water	4 mL
water	4 mL
0.01% Thiomerosal in PBS - storage buffer	

Fractions containing methanol were placed in a centrifugal evaporator (Jouan RC10.10) to remove the organic solvent. All fractions were then freeze-dried and stored at -20°C. Later they were reconstituted in 200 μL of sample buffer before analysis by cELISA in duplicate.

The immunoaffinity gel was also packed into minicolumns which had a capacity of 1 mL and were fitted with upper and lower polyethylene filters. The columns (Mobicols) were purchased from Mo Bi Tec, Gottingen, Germany. Column loading and washing was carried out using the peristaltic pump at a flow rate of 0.8 mL/min. The absorbance of the eluate, however, was not monitored and the only fraction retained was that eluted with 40% methanol in water, which had been previously shown to contain sporidesmin A and/or metabolites.

Chromatography procedure:

0.05% Tween 20 in PBS	10 mL
sporidesmin A standard in 0.05% Tween 20 in PBS	1 mL
0.05% Tween 20 in PBS	15 mL
water	5 mL
10% MeOH in water	5 mL
20% MeOH in water	5 mL
40% MeOH in water	15 mL
water	10 mL
0.01% Thiomerosal in PBS - storage buffer	

The 40% methanol fraction was taken to dryness by rotary evaporation and reconstituted in immunoassay sample buffer and transferred to a volumetric flask (1 mL) and made up to volume. Fifty microlitres was taken for cELISA.

The column was stored in storage buffer at 4°C.

2.11.2 Sporidesmin metabolites in urine

To prepare urine for immunoaffinity chromatography, 1 mL of urine which had been centrifuged at 10 000*g* for 5 min, was diluted with 9 mL of 0.05% Tween 20 in PBS. The matrix was packed into a Wright column (0.5 mL) and equilibrated in 0.05% Tween 20 in PBS. The diluted urine was loaded onto the column at a flow rate of 0.9 mL per min.

Chromatography procedure:

PBS in Tween 20 (0.05%)	10 mL
urine in PBS in Tween 20 (0.05%)	10 mL
PBS in Tween 20 (0.05%)	30 mL
water	10 mL
5% MeOH in water	10 mL
10% MeOH in water	10 mL
30% MeOH in water	20 mL
40% MeOH in water	20 mL
water	10 mL
0.01% Thiomerosal in PBS - storage buffer	

The fractions recovered by elution with 30% and 40% methanol were pooled and taken to dryness by rotary evaporation. The material recovered in each pool was redissolved in 5% methanol, 100 µL retained for cELISA using the group B antibody, and the remainder dried under a stream of nitrogen to remove methanol. This was freeze-dried and stored at -20°C.

2.11.3 Sporidesmin A and metabolites in milk

Milk was prepared for immunoaffinity chromatography using the sampling technique used in cELISA (Section 2.8.5). A sample (5 mL) was diluted in 45 mL of PBS containing Tween 20 (0.5%) which had also been warmed to 45°C, mixed thoroughly and the mixtures kept at 45°C for 5 min before cooling in ice and

centrifugation at 2 000*g* for 20 min at 4°C. The aqueous fraction was recovered and frozen. Just before chromatography the diluted milk was thawed and centrifuged at 10 000*g* for 20 min at 4°C. The supernatant recovered was filtered through glass fibre filters, first 1.6 mµ (Whatmans GF/A) followed by 0.7 µm (Sartorius Minisart GF). After cooling in ice, a measured volume was loaded onto the IAC column (Mobicol, 1 mL bed volume) using a flow rate of 0.8 mL/min. Wash and elution steps were carried out at 2.0 mL/min.

Chromatography procedure:

0.05% Tween 20 in PBS	10 mL
milk diluted in 0.5% Tween 20	45 mL
0.05% Tween 20 in PBS	10 mL
water	20 mL
10% MeOH in water	5 mL
40% MeOH in water	15 mL
10% MeOH in water	5 mL
water	10 mL
0.01% Thiomerosal in PBS – storage buffer	

The fraction recovered by elution with 40% methanol was taken to near dryness (approximately 200 µL) by rotary evaporation at 40°C and transferred with several washes of sample buffer to a 1 mL volumetric flask. This was stored frozen at -20°C until later when it was thawed, further dilutions in sample buffer made and 50 µL of each was taken for analysis by cELISA using the group A antibody.

2.12 HPLC analysis

Bile and urine samples were analysed for sporidesmin A using the HPLC procedure reported by Miles *et al.* (1992). A 2 mL sample of bile was extracted twice with 2 mL of diethyl ether and 10 mL of urine was extracted twice with 10 mL of dichloromethane. The extracts were taken to dryness by evaporation under nitrogen, reconstituted in 500 µL of methanol and a sample volume of 10 µL injected into the HPLC column.

Chapter 3:

PRODUCTION OF ANTIBODIES

CHAPTER 3

PRODUCTION OF ANTIBODIES

3.1 Introduction

Previous attempts to produce anti-sporidesmin A antibodies resulted in either antibodies with low titres (Jonas and Ronaldson, 1974; Jonas and Erasmuson, 1977) or antibodies which bound to the sporidesmin A-protein conjugates used for plate-coating but did not bind to the free analyte in cELISA (Gallagher *et al.*, unpublished data, 1987). The latter antibodies were produced in rabbits and sheep and the immunogen used was sporidesmin A 11-hemisuccinyl BSA (Gallagher *et al.*, 1987). Further quantities of this immunogen together with sporidesmin A 11-hemisuccinate and sporidesmin A 10b-hemisuccinate were available for use in this project.

It was first considered whether polyclonal or monoclonal antibodies would be suitable for the research to be undertaken. Often banks of both monoclonal and polyclonal antisera are developed so that the advantages of each can be made use of (Dunbar and Schwoebel, 1990). Polyclonal antibodies are defined as the total population of antibodies present in animal serum. This complex population contains different antibody subclasses including IgG, IgM, IgE, IgA and IgD. Each antibody represents the secretory product from a single stimulated lymphocyte and its clonal progeny. The advantage of using polyclonal antisera is that it is possible to have multiple high-affinity antibodies with different specificities present in the antibody population. There is the disadvantage that the immunogen must be highly purified to obtain antibodies with a narrow range of specificities. Usually multiple antigenic sites (epitopes) are recognised because of the multiple antibody population present. Quantities of antibodies are limited to the amount of sera collected during the life of the immunised animal and different bleedings have to be characterised individually due to changes in antibody affinity, specificity and subclass.

Hybridomas on the other hand can provide a continuous source of antibody of pre-defined specificity. The major advantages of using monoclonal antibodies are the ability to obtain a single homogeneous antibody which recognises a defined antigenic determinant and large quantities of antibody can be obtained since theoretically immortal cell lines can be developed. Antibodies with low affinity binding can be

selected during the screening procedure and these antibodies may be suitable for immunoaffinity chromatography. The major disadvantages of monoclonal antibody production is that the procedure is expensive, time-consuming and needs well-equipped tissue culture facilities. Hybridoma cell lines are frequently unstable due to chromosome loss or may be lost because of tissue culture contamination and thus the 'unlimited supply' may not be realised.

The production of antibodies which could be used in cELISA to distinguish between toxic and non-toxic analogues of sporidesmin (*e.g.* sporidesmin A and D) would obviously be a major advantage in the study of the metabolism of sporidesmin A. To provide such specificity monoclonal as well as polyclonal antibody production was undertaken.

In order to increase the chances of producing suitable antibodies for use in cELISA and immunoaffinity chromatography, it was decided that mice, rabbits and sheep would be immunised with the already available immunogen (sporidesmin A 11-hemisuccinyl BSA) together with additional immunogens produced by conjugating sporidesmin derivatives to other carrier proteins.

3.2 Immunisation

Immunisation protocols followed were those described in Section 2.3.

3.2.1 Immunogens

The protein used in a coating conjugate used for antibody screening by ELISA was not the same as that used in the corresponding immunogen. This was to avoid the selection of antibodies which were raised against the protein carrier in the immunogen. Sporidesmin A hemisuccinate isomers, shown in Figure 3.2.1, were conjugated to BSA (Fraction V) and thyroglobulin (porcine) to produce the immunising conjugates, or to ovalbumin (Fraction V) and conalbumin to produce coating conjugates.

3.2.2 Immunisation programmes

Immunisation of sheep and rabbits was carried out as described in Sections 2.3.2 and 2.3.3, following schedules which are shown in Figure 3.2.2. Mice were immunised for monoclonal antibody production as described in Section 2.3.1. A schedule for the immunisations is shown in Figure 3.2.3.

(1) (2)

Figure 3.2.1 Sporidesmin A hemisuccinate isomers used in immunising or coating conjugates. Sporidesmin A 11-hemisuccinate (**1**) and sporidesmin A 10b-hemisuccinate (**2**).

3.3 Screening of sera

After immunisation was commenced it was necessary to establish methods to determine the presence and specificities of antibodies. An indirect ELISA, as described in Section 2.4.1, was used to determine antisera titres. The assay incubation times and temperatures used were those frequently used by other researchers in screening mycotoxin-specific antibodies. Microtitre plates were coated with sporidesmin A 11-ovalbumin (2 μg/mL) and serial dilutions of the antisera were taken for ELISA, the first dilution being 1:99. A second antibody which binds immunoglobulin and was conjugated to peroxidase, was added to determine the amount of anti-sporidesmin antibody bound to the plate by measuring the subsequent reaction with a peroxidase substrate. The second antibody conjugate was chosen to bind immunoglobulins from the species from which the first antibody was obtained. These conjugates were diluted in antibody buffer to give dilutions ranging from slightly less to slightly greater than those recommended by each manufacturer for use in ELISA, and dilutions giving maximum colour development in ELISA with excess first antibody, *i.e.* with antisera diluted at 1:99, were selected for routine screening.

(A) **Sheep**

20 sheep

* 1 SC → 2 IM * → 3 IM * → 4 IM * →

rested for 4 months

six selected sheep

* 5 IM → 6 IM * → 7 IM * ● Two sheep selected and retained for further immunisation

(B) **Rabbits**

four rabbits

* 1 SC → 2 SC → 3 SC * → 4 SC * →

rested for 4 months

two selected rabbits

5 SC → 6 SC * → 7 SC * ●

Figure 3.2.2 Four weekly immunisation of sheep (**A**) and rabbits (**B**) for polyclonal antibody production. Animals were bled for monitoring antibody production (*) and bulk bled for antisera collection (•). SC indicates subcutaneous and IM intramuscular immunisation.

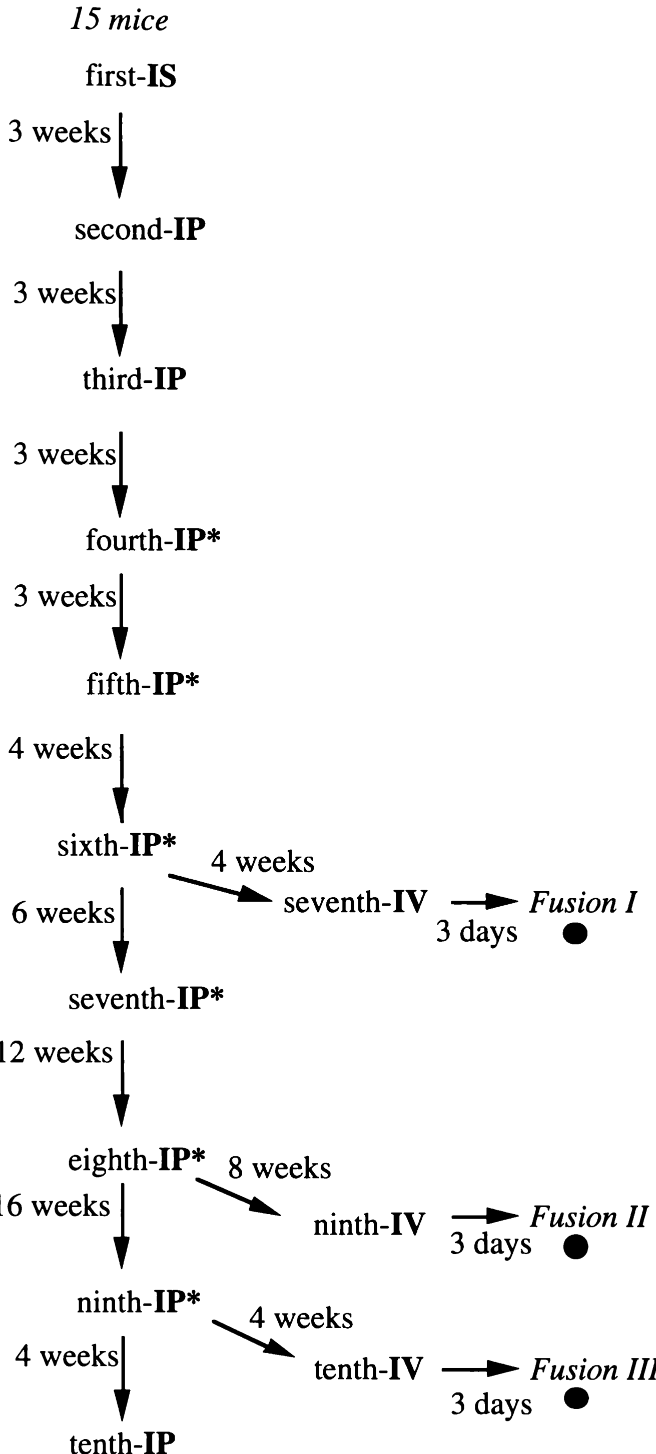


Figure 3.2.3 Immunisation of mice for monoclonal antibody production. Mice received up to 10 injections of immunogen at intervals shown in the figure. Animals were tail-bled (*) and bulk-bled (•) to monitor antibody production. IS indicates intrasplenic, IP intraperitoneal and IV intravenous immunisation. The immunogens used, each for a group of 5 mice, were: sporidesmin A 11-hemisuccinyl BSA, sporidesmin A 10b-hemisuccinyl BSA and sporidesmin A 11-hemisuccinyl thyroglobulin.

When it was shown by ELISA that antibodies binding to sporidesmin A 11-ovalbumin had been produced, competitive indirect ELISAs (cELISAs), as described in Section 2.4.2, were carried out to determine the sensitivity and specificity of antibody binding to sporidesmins A and D. The antisera dilutions used in cELISA were those which gave approximately 50% of the maximum absorbance recorded with excess antibody in ELISA. Sporidesmin A and sporidesmin D standards were prepared in methanol and diluted in standard buffer to give a methanol concentration of 2%. After the coating and blocking steps had been carried out, standard solutions (50 µL) were added to the wells followed by 50 µL of diluted antibody. When the antibody was bound to free toxin, the amount of antibody available to bind to the coating antigen was reduced. This in turn reduced the amount of second antibody-peroxidase bound and subsequently the amount of colour developed in the assay. Thus the colour intensity as a result of enzyme reaction was inversely proportional to antibody binding with the free toxin in the standard.

When antisera were monitored for their potential for polyclonal immunoassay and monoclonal antibody production, the highest titres and cELISA sensitivities were found in the sheep antisera (Table 3.3.1).

The sheep and rabbit antisera preferentially bound sporidesmin A while the majority of the mouse antisera had greater affinity for sporidesmin D than for sporidesmin A (Table 3.3.2).

Larger volumes of mouse antisera were collected at the time the spleens were removed for fusion, and this enabled more extensive screening to be carried out and the effect of the coating antigen used on the sensitivity of cELISA was investigated. The results (Table 3.3.3) suggested that sporidesmin A 11-hemisuccinyl ovalbumin was the most suitable coating antigen for screening hybridomas as it gave greatest sensitivities (*i.e.* the lowest I_{50} values) and gave competition assays with all antisera tested. This was not the case for all coating antigens and in some instances neither sporidesmin A nor sporidesmin D could inhibit binding of the antiserum to the coating antigen, *e.g.* sporidesmin A 10b-hemisuccinyl coating antigens with antisera from mice 1 or mouse 5.

Table 3.3.1 Immune response of animals immunised with sporidesmin A 11-hemisuccinyl BSA, measured by ELISA and cELISA

species	number of animals	number of immunis-ations	median titre[a]	titre range[a]	I_{50}[b] sporidesmin A, ng/mL
Sheep	17	4	1:5000	1:1000 - 1:20000	15
Rabbits	4	4	1:2000	1:1000 - 1:4000	1000
Mice	5	5	1:1000	1:500 - 1:1000	800

[a] Titre is the antisera dilution required to reduce ELISA absorbances to 50% of the maximum absorbance. [b] Concentration of sporidesmin A giving 50% inhibition of antibody binding to sporidesmin A 11-hemisuccinyl ovalbumin (coating antigen) as determined by cELISA. The lowest I_{50} obtained for the antisera screened in each species is recorded.

3.4 Monoclonal antibody production

As the immunoassays being developed were to be used in studies of the metabolism and excretion of sporidesmin A, it was desirable to have antibodies that specifically recognise sporidesmin A and others that would recognise metabolites. It was thought that the later objective would be best met by antibodies specifically recognising the non-toxic analogue sporidesmin D. Monoclonal antibody production was undertaken in an attempt to obtain antibodies and ultimately immunoassays with high sensitivity and the required specificity.

3.4.1 Fusion I

Mouse 12 was selected for hybridoma production because it produced antiserum which, together with a high antibody titre, had the highest sensitivity for sporidesmin A in cELISA. Spleen cells from this animal, which had been immunised seven times with sporidesmin A 11-hemisuccinyl BSA over 5 months, were fused with myeloma cells. The resulting hybridomas were screened by ELISA and 17 cell lines were selected as producing antibodies which bound to the sporidesmin A coating antigens. These antibodies, however, did not bind to free sporidesmin A when screened in

Table 3.3.2 Antisera specificity to sporidesmin A and D measured by cELISA

species	no. of animals	immunogen[a]	immunisation route	specificity to sporidesmin A[b]	specificity to sporidesmin D[b]
sheep	16	hapten 11-BSA	subcutaneous	++	+
	1	hapten 11-BSA	and intramuscular	++	–
rabbits	4	hapten 11-BSA	subcutaneous	++	+
mice	2	hapten 11-BSA	intrasplenic	+	++
	3	hapten 10b-BSA	and	+	++
	3	hapten 11-thyroglobulin	intraperitoneal	+	++
	1	hapten 11-BSA		++	+
	1	hapten 10b-BSA		++	++

[a] Hapten 11 represents sporidesmin A 11-hemisuccinyl and hapten 10b represents sporidesmin A 10b-hemisuccinyl. [b] Determined by measuring inhibition of antibody binding to coating antigen by the sporidesmin analogues A or D. This was determined by comparing the colour developed in the presence of sporidesmin A or D with the maximum colour development obtained in the absence of the toxins. ++ Indicates that antibody binding to coating antigen was inhibited by sporidesmins A and D at assay concentrations of < 1 µg/mL; + indicates inhibition of binding at 1 µg/mL, and - indicates no inhibition of binding at 1 µg/mL. In this study the microtitre plates were coated with sporidesmin A 11-hemisuccinyl ovalbumin (2 µg/mL).

Table 3.3.3 The sensitivity (I_{50})[a] of cELISA with different coating antigens, using mouse antisera

antisera	competing analogue[b]	coating antigen (2 µg/mL) hapten 11-[c] thyroglobulin	hapten 11-ovalbumin	hapten 10b-[d] conalbumin	hapten 10b-ovalbumin
		I_{50} (µg/mL)			
mouse 1[e]	Spdm A	>1 000[a]	78[a]	>1 000[a]	>1 000[a]
	Spdm D	25	3	>1 000	>1 000
mouse 5[e]	Spdm A	>1 000	87	>1 000	>1 000
	Spdm D	>1 000	>1 000	>1 000	>1 000
mouse 11[f]	Spdm A	155	378	105	159
	Spdm D	37	15	11	12

[a] I_{50} is the concentration of sporidesmin A giving 50% inhibition of antisera binding to the coating antigen as determined by cELISA and is expressed in nanograms per millilitre. [b] Spdm A represents sporidesmin A, and Spdm D sporidesmin D. [c] Hapten 11 represents sporidesmin A 11-hemisuccinyl. [d] Hapten 10b represents sporidesmin A 10b-hemisuccinyl. [e]Antisera were collected at fusion. [f]Antiserum was collected by bulk bleed under anaesthesia. The immunogen used to raise the antisera was sporidesmin A 11-hemisuccinyl BSA.

cELISA. Changes in assay conditions such as variation in pH, blocker, detergent, and substitution of Tris for phosphate buffer did not provide experimental conditions in which inhibition of the binding of monoclonal antibodies to the coating antigen by sporidesmin A could be demonstrated. To eliminate the possibility that the inability of these antibodies to bind free sporidesmin A arose from the selection of hybridomas producing antibodies which bound to the hemisuccinyl group rather than to the sporidesmin moiety, the plate coating was changed to sporidesmin A 11-hemiglutaryl ovalbumin. The antibodies still bound to the plates but again there was no evidence of ability to bind to free sporidesmin A in cELISA. Antibodies produced from this fusion were abandoned and a second fusion with spleen cells from another mouse undertaken.

3.4.2 Fusion II

The immunisation of the remaining mice had continued with greater intervals between injections while the first fusion was carried out and it was hoped that maturation of the immune response during this period would result in antibodies with greater affinity for free sporidesmin A than for the immobilised coating antigens. After nine immunisations with sporidesmin A 11-hemisuccinyl BSA over 10 months, mouse 1 was chosen for its high antibody titre and sensitivity for sporidesmin A in cELISA. Hybridoma cells were produced, screened as in Section 3.3 and 16 cell lines were found to secrete antibodies binding to free sporidesmin A and D in cELISA (see Appendix II). Six lines were selected for subcloning as described in Section 2.5.5. The antibodies produced had greater affinity for sporidesmin D than for sporidesmin A.

3.4.3 Fusion III

The mice used in Fusions I and II were chosen as having the highest anti-sporidesmin A titres but also had high anti-sporidesmin D titres. In an effort to produce monoclonal antibodies specific for sporidesmin A, spleen cells from the only mouse, *i.e.* mouse 5, producing antisera that showed a predominant specificity for sporidesmin A, were fused with myeloma cells. This mouse had received 10 immunisations with sporidesmin A 11-hemisuccinyl BSA over 14 months. After screening by ELISA and cELISA, 30 cell lines producing sporidesmin A-binding antibodies were selected (see Appendix III). Twenty hybridomas produced antibodies which preferentially bound sporidesmin A. Some of these did not cross-react with sporidesmin D even at assay concentrations of 1 μg/mL. Two of these cell lines were subcloned by limiting dilution as described in Section 2.5.5.

3.5 Antibody selection

3.5.1 Monoclonal antibodies

It had been shown earlier that the choice of coating antigen had an effect on the sensitivity of cELISAs using mouse antisera (Table 3.3.3). A study of monoclonal antibody binding to coating antigens was undertaken to determine which coating antigens could be used in cELISA. This was determined by ELISA using coating antigens diluted to 2 μg/mL and undiluted culture fluids which contained monoclonal antibodies. Thirteen antibodies specific for sporidesmin A (group A antibodies) and

nine antibodies specific for sporidesmin A and D (group B antibodies) were screened for binding to the following coating antigens: sporidesmin A 11-hemisuccinate conjugated to thyroglobulin and ovalbumin, and sporidesmin A 10b-hemisuccinate conjugated to conalbumin and ovalbumin. Group A antibodies did not bind to the sporidesmin A 10b-hemisuccinyl coating antigens *i.e.* there was no colour development in ELISA, whereas the group B antibodies bound to all four coatings and colour was developed in the assay. The sporidesmin A 10b-hemisuccinyl coating antigens were therefore not suitable for use in ELISA and cELISA with the group A antibodies.

cELISA was used to determine sensitivity and specificity of antibody binding to sporidesmins A and D as described in Section 2.4.2 and the two monoclonal antibodies with the highest sensitivities for binding with sporidesmins A and D were selected for use in cELISA. One was a group A antibody and was specific for sporidesmin A, whereas the other which was a group B antibody was less specific and cross-reacted with both analogues (Table 3.5.1).

Table 3.5.1 Monoclonal antibody reactivities with sporidesmins A and D

	group A antibody		group B antibody	
compound	$I_{50}{}^{a}$	%CR[b]	$I_{50}{}^{a}$	%CR[b]
sporidesmin A	21.9	100	15.5	100
sporidesmin D	>3 500	<0.6	3.2	484

[a] I_{50} values are expressed in picomoles per millilitre.

[b] Percent cross-reactivity (%CR) = (I_{50} sporidesmin A/I_{50} analogue) × 100, where I_{50} is the molar concentration of compound giving 50% inhibition of antibody binding to the coating antigen. cELISAs were carried out as previously described using the microtitre plates coated with sporidesmin A 11-hemisuccinyl ovalbumin (2 µg/mL) and diluted culture supernatant containing the group A or group B antibody. Antimouse-HRP conjugate was diluted 1:2 500.

3.5.2 Polyclonal antibodies

Anti-sporidesmin A antibodies were successfully raised in both sheep and rabbits after the first round of immunisation but sheep proved to be a better source of antibodies. The specificities of the sheep antisera to sporidesmins A and D were similar to those found in the rabbit antisera (Table 3.3.2), although the mean titres in sheep were higher and the most sensitive assays were obtained with sheep antisera (Table 3.3.1). It was therefore decided that further polyclonal antibody production would be continued in the sheep only.

Six sheep with the highest titres were selected for a second round of immunisations. The resulting antisera were studied for possible use in cELISA and the effect of the coating antigen upon assay sensitivity was examined (Table 3.5.2).

Table 3.5.2 The sensitivity (I_{50})[a] of cELISA with different coating antigens, using sheep antisera collected after seven immunisations

	spdm A 10b-HS ovalbumin[b]			spdm A 11-HG ovalbumin[b]		
sheep	antisera	competing analogue[c]		antisera	competing analogue[c]	
number	dilution	spdm A	spdm D	dilution	spdm A	spdm D
254	1:6 000	>1000[a]	>1000[a]	1:6 000	20[a]	>1000[a]
287	1:12 000	14	457	1:6 000	76	>1000
239	1:12 000	>1000	>1000	1:12 000	98	>1000
182	1:12 000	>1000	>1000	1:6 000	159	>1000
243	1:12 000	16	234	1:12 000	65	>1000
57	1:25 000	12	776	1:12 000	107	>1000

[a] I_{50} values are expressed in nanograms per millilitre. [b] Coating antigens were used at 2 μg/mL. [c] Spdm A represents sporidesmin A, and spdm D sporidesmin D. HS represents hemisuccinyl and HG hemiglutaryl. Sporidesmin A 11-hemisuccinyl BSA was the immunogen used to raise the antisera.

When sporidesmin A 11-hemiglutaryl ovalbumin was used as the coating antigen, antiserum from sheep 254 was highly specific for sporidesmin A and did not cross-

react with sporidesmin D even at assay concentrations of 1 000 ng per mL. Antiserum from sheep 243 bound to both sporidesmin A and sporidesmin D when sporidesmin A 10b-hemisuccinyl ovalbumin was used for plate coating.

Antisera from sheep 287 and 57 also bound to both analogues but the I_{50} values for sporidesmin D were approximately two and four times greater, respectively, than the I_{50} value obtained for sporidesmin D with antiserum 243.

Sheep 254 and 243 were retained for 12 months after which three further immunisations were given. At this time a total of 10 immunisations had been given over a 25 month period. The antisera were collected and analysed by ELISA (Figure 3.5.1) to determine if further immunisation had brought about an increase in antibody concentration and to determine a suitable dilution for use in cELISA. In both cases the titres after the first seven injections were higher than those measured after a further 12 months without immunisation. The titres after three additional immunisations, however, were slightly higher than those recorded at the end of the previous phase. The dilution chosen for use was that giving approximately 50% of the maximum binding to the respective coating antigen: for antiserum from sheep 254 it was 1:25 000 with sporidesmin A 10b-hemisuccinyl ovalbumin and 1:50 000 with sporidesmin A 11-hemisuccinyl ovalbumin. Dilution of antiserum collected from sheep 243 was 1:150 000 with sporidesmin A 10b-hemisuccinyl ovalbumin and 1:200 000 with sporidesmin A 11-hemisuccinyl ovalbumin. Assay sensitivities were determined in cELISA using two coating antigens (Table 3.5.3).

Table 3.5.3 The sensitivity (I_{50})[a] of cELISA with different coating antigens, using sheep antisera collected after 10 immunisations

		coating antigen (500 ng/mL)		
sheep antisera	competing analogue[b]	spdm A 10b-HS ovalbumin	spdm A 11-HS ovalbumin	specificity
254	spdm A	>1000[a] (1:25 000)[c]	3.4[a] (1:50 000)	spdm A
	spdm D	>1000	>1000	
243	spdm A	1.4 (1:150 000)	19 (1:200 000)	spdm A
	spdm D	19	>1000	and D

[a] I_{50} values are expressed in nanograms per millilitre. [b] Spdm A represents sporidesmin A, and spdm D sporidesmin D. [c] Values in brackets represent antiserum dilution used in cELISA for each coating antigen. Sporidesmin A 11-hemisuccinyl BSA was the immunogen used to raise the antisera.

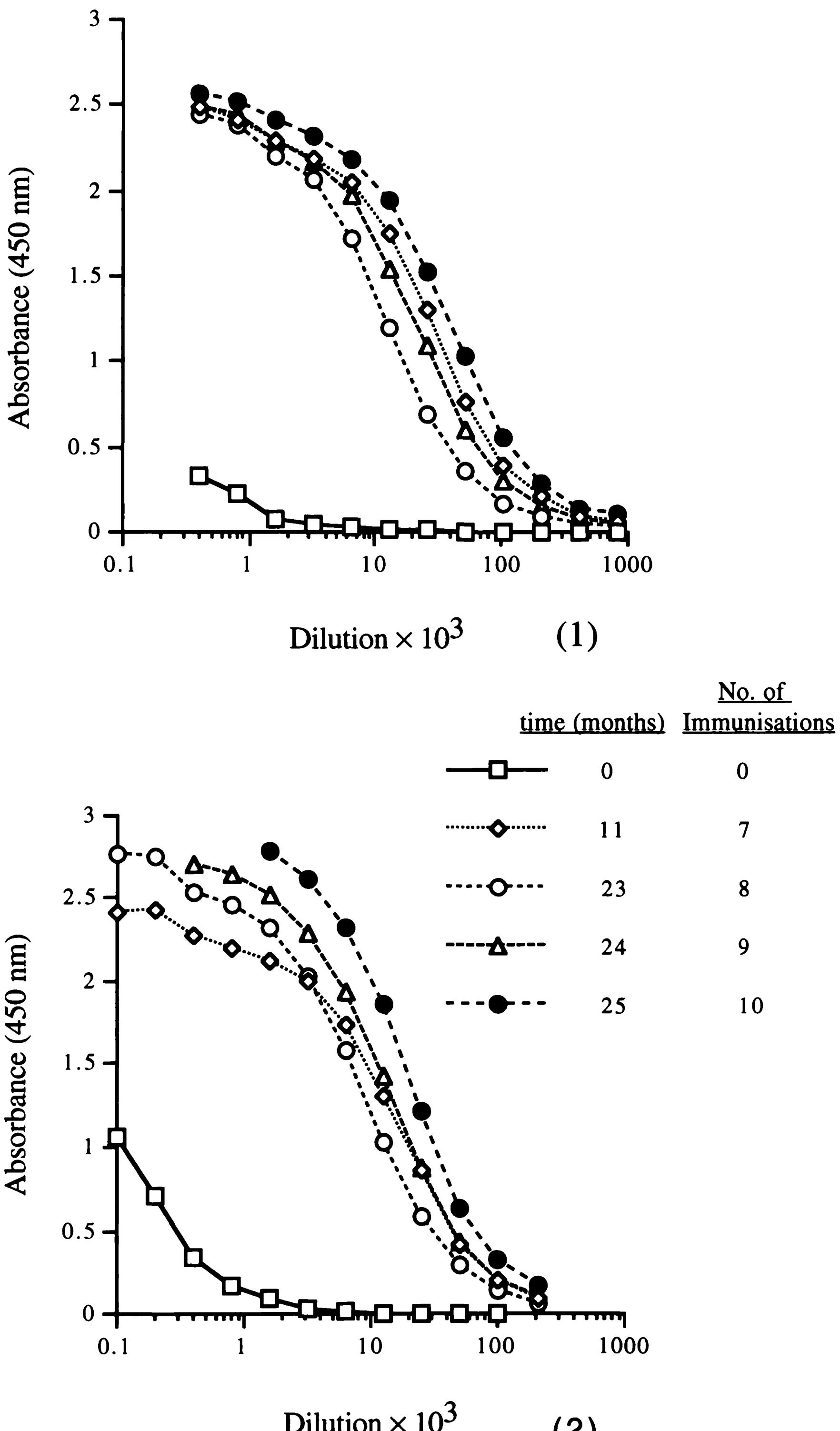


Figure 3.5.1 Increase in antibody concentration with increase in the number of immunisations administered. Absorbances recorded in ELISA, using sporidesmin A 10b-hemisuccinyl ovalbumin (2 μg/mL) as the coating antigen, are shown plotted against serial dilution of antisera collected from sheep 243 (**1**) and 254 (**2**) after 10 immunisations over 25 months.

Antiserum 254 showed high specificity for sporidesmin A in cELISA only when sporidesmin A 11-hemisuccinyl ovalbumin was the coating antigen. When sporidesmin A 10b-hemisuccinyl ovalbumin was the coating antigen, there was no specificity as competition with sporidesmin A or D in cELISA did not occur. Antiserum 243 gave the most sensitive assay for sporidesmin A and cross-reacted with sporidesmin D when sporidesmin A 10b-hemisuccinyl ovalbumin was the coating antigen. The assay sensitivities recorded (I_{50}) indicated that both of these antisera should be suitable for use in the analysis of sporidesmin A, and in the case of antiserum 243, also for the analysis of sporidesmin D and possibly other analogues and metabolites.

3.6 Large-scale monoclonal antibody production

To provide stock of antibody for both ELISA and immunoaffinity chromatography, monoclonal antibodies were isolated from both culture supernatant and ascites fluid.

3.6.1 Culture supernatants

Antibody was recovered from 4 L of supernatant prepared by culturing the selected hybridomas described in Section 3.5.1. The concentration of antibody in culture supernatant is reported by Harlow and Lane (1988) to be usually 20 to 50 µg per mL. Therefore, assuming a similar concentration in the supernatants, the 4 L was expected to yield at least 800 mg of antibody.

3.6.2 Ascitic fluid

Highly concentrated monoclonal antibodies can be obtained by injecting antibody-secreting hybridoma cells into the peritoneum of BALB/c mice. Ascitic fluid accumulates in the peritoneal cavity and can be collected after the animal is humanely killed. The antibody concentration is reported to be about 100 times that found in culture supernatant and may be as high as 10 mg per mL (Harlow and Lane, 1988). Some of the immunoglobulin (2-10%), however, may be the normal polyclonal antibodies from the animal.

Ascites fluid production can be extremely variable in the volume produced and the time taken for the fluid to accumulate in the mouse peritoneum. A study was therefore undertaken to determine if it was possible to enhance the production of ascites fluid and to achieve consistency in both establishing the cells within the animals and

reducing the time taken for fluid accumulation. Three protocols were tested on three groups each of 10 mice (8-10 weeks old). In protocol A mice were given an intraperitoneal injection of 0.5 mL of pristane followed by a further injection of 0.25 mL one week later. After another week $1\text{-}5\times10^6$ of the hybridoma cells in 0.5 mL of serum-free medium were injected into the peritoneum and ascites fluid collected as described in Section 2.6.2(b). Protocol B involved the same treatment as A except 0.25 mL and 0.25 mL of incomplete adjuvant was injected instead of 0.5 mL and 0.25 mL of pristane. Protocol C was the same as A except a sub-lethal irradiation treatment of 2-3 Gray (Campbell, 1985) was administered 1 day before inoculation with hybridoma cells.

The results obtained with cell line 6A5, which was selected as described in Section 3.5.1, are shown in Table 3.6.1.

Table 3.6.1 Ascites production from cell line 6A5

treatment	number of mice	priming agent	volume administered (mL)	irradiation (Gy)	total ascites volume (mL)
A	10	pristane	0.5^a, 0.25^b	0	8
B	10	incomplete adjuvant	0.25, 0.25	0	3
C	10	pristane	0.5, 0.25	2.5	61

^{a} represents the first priming dose administered 2 weeks before inoculation with hybridoma cells and ^{b} is the second dose given 1 week before inoculation.

The total volume of ascites obtained in treatment C was nearly eight times greater than that yielded by any other treatment group and was collected during the first 2 weeks after the introduction of the hybridoma cells into the mice. As results from this method were considerably better than those from treatments A and B, the method was used for all subsequent ascites fluid production. Protocol C was also followed to produce ascitic fluid from the cell line secreting group B antibody (see Section 3.5.1). This cell line (2F4) however, formed solid tumours and only a small volume (6 mL) of fluid was collected. Bulk production of the group B antibody was therefore restricted to production in cell culture supernatants.

3.7 Antibody purification

3.7.1 Antibody isotype

Structurally, antibodies are composed of one or more copies of a unit that can be visualised as forming a Y shape. Each unit contains four polypeptides – two identical copies of a polypeptide known as the heavy chain and two identical copies of a polypeptide called the light chain. The isotype (class and subclass) of an antibody is defined by its heavy chain. Determination of antibody isotype can give an indication of the properties of an antibody which are of assistance in selection of purification techniques. For example, as most mouse IgG_1 antibodies will not bind well to Protein A, purification with Protein A is not recommended for these antibodies (Campbell, 1991). Also, as IgG_{2b} antibodies are most susceptible to proteolytic enzymes (Sigma Chemical Co., 1991), particular precautions need to be taken during purification of IgG_{2b} antibodies to avoid proteolytic degradation. The isotypes of the monoclonal antibodies from the two cell lines selected (Section 3.5.1) were determined by means of an isotyping kit as described in Section 2.6.1. The group A antibody was found to be IgG_{2b}, and the group B antibody IgG_1

3.7.2 Purification with Protein G

Purification of antibodies for use in ELISA and immunoaffinity matrix preparation was carried out using Protein G as described in Section 2.6.3. Although it is possible to use unpurified antibodies in ELISA (Zola, 1987), it was decided that the preparation of a large single batch of purified antibody would be advantageous in immunoassay development. Batch variations would no longer be a problem and the possibility of assay interference from components in unpurified ascites fluid, culture supernatant or antisera, would be removed.

Several Gram-positive bacterial species have surface proteins that bind to the constant (Fc) region of immunoglobulin molecules in non-immune reactions. Protein G, which can be isolated from the cell wall of group G strains of *Streptococcus*, binds with IgG from most mammalian species depending on the origin and subclass of the IgG (Campbell, 1991). Protein G has been genetically engineered from recombinant strains of *Escherichia coli* to improve its performance in IgG-binding applications. The region of native Protein G responsible for albumin binding has been eliminated leaving the IgG-binding region intact and active (Pilcher *et al.*, 1991). Immobilisation of Protein G on supports such as Sepharose has led to the development of affinity

chromatography matrices which can be used for the purification of both monoclonal and polyclonal antibodies.

Purification of both monoclonal antibodies derived from hybridoma culture supernatants and mouse ascites fluid was carried out as described in Section 2.6.3. The antibody-containing solutions were combined into two pools, one derived from purified culture supernatant and the other from the ascites fluid and each dialysed against three changes of PBS. The protein concentration of each pool was determined using a procedure based on the Bradford method as described in Section 2.6.4 and aliquots (5 mL) of each were stored at -20°C at a concentration no less than 1 mg of protein per mL. The yield of purified antibodies is shown in Table 3.7.1.

As Protein G binds strongly with sheep IgG (Ohlson *et al.*, 1988), affinity chromatography with immobilised Protein G was also used to purify a portion of the sheep antisera for later use in immunoaffinity matrix preparation.

Table 3.7.1 Yield of purified antibodies

volume processed (mL)	antibody purified	source	volume of purified antibody (mL)	protein concentration (mg/mL)	total protein (mg)
4000	A, monoclonal	SN[a]	198	0.91	180
42	"	ascites	237	1.18	280
3700	B, monoclonal	SN	131	0.84	110
6	"	ascites	21	1.16	24
20	254, polyclonal	sheep	85	4.04	343
47	243, polyclonal	sheep	144	6.13	882

[a] SN represents hybridoma culture supernatant

3.7.3 *Electrophoresis and isoelectric focusing*

Antibody purity was monitored by SDS-PAGE and isoelectric focusing as described in Section 2.6.5. In SDS-PAGE, proteins are first dissociated into their polypeptide subunits by SDS and β-mercaptoethanol before electrophoresis. SDS which is strongly anionic binds to hydrophobic regions of protein molecules and removes the

original conformation and charge differences. Upon electrophoresis, these treated proteins migrate as anions and separate according to molecular weight (Zola, 1987).

Electrophoresis of the unpurified culture supernatants gave one major band corresponding to BSA which has a molecular weight of 66.2 kDa (Figure 3.7.1). Electrophoresis of samples of both monoclonal antibodies after Protein G purification gave bands at molecular weights of 23 and 50 kDa representing the light and heavy subunits from the monoclonal antibodies (Olsson *et al.*, 1988). A minor contaminant with a molecular weight greater than 100 kDa was present in the samples containing the monoclonal antibody purified from culture supernatants (lanes 3 and 6 in Figure 3.7.1). This may have been undissociated immunoglobulin.

Isoelectric focusing is a method used for the separation of amphoteric molecules and bioparticles which have different isoelectric points. In the presence of an electric field and a pH gradient, charged molecules migrate electrophoretically until they are condensed or focused at a position that corresponds with their isoelectric point (Laas, 1989).

The isoelectric patterns obtained from each sample were discrete bands focused within a distinct region (Figure 3.7.2). A band representing albumin at pH 4.5 (Reik *et al.*, 1987) was not observed. IEF of the group A antibody produced in culture supernatant appeared to give the same number of bands and band pattern as that produced in ascites. The band patterns obtained with the group B antibody, however, depended upon the method used for antibody production. The sample obtained from ascites fluid had one major band together with other minor bands, while the culture supernatant bands had the same number of bands but the relative intensities of each were similar.

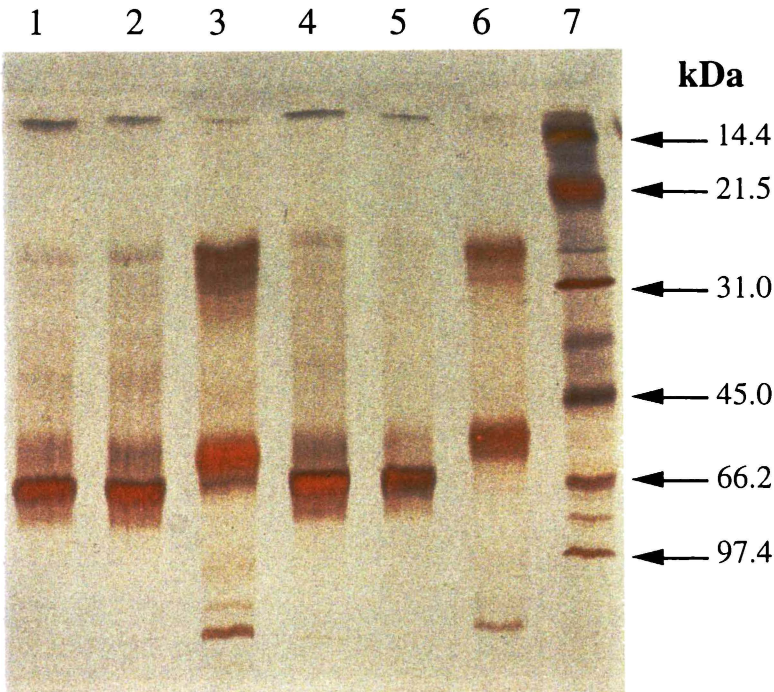


Figure 3.7.1 SDS-PAGE in PhastGel homogeneous media 12.5 of fractions from purification (using Protein G Sepharose 4 Fast Flow) of culture supernatants containing monoclonal antibodies. Samples are: (1) unpurified group A antibody, (2) unbound fraction from 1, (3) bound fraction recovered from 1, (4) unpurified group B antibody, (5) unbound fraction from 4, (6) bound fraction recovered from 4, and (7) silver stain SDS-PAGE low-range protein standards (Bio-Rad). For all six samples the amount applied to the gel was approximately 50 ng of protein.

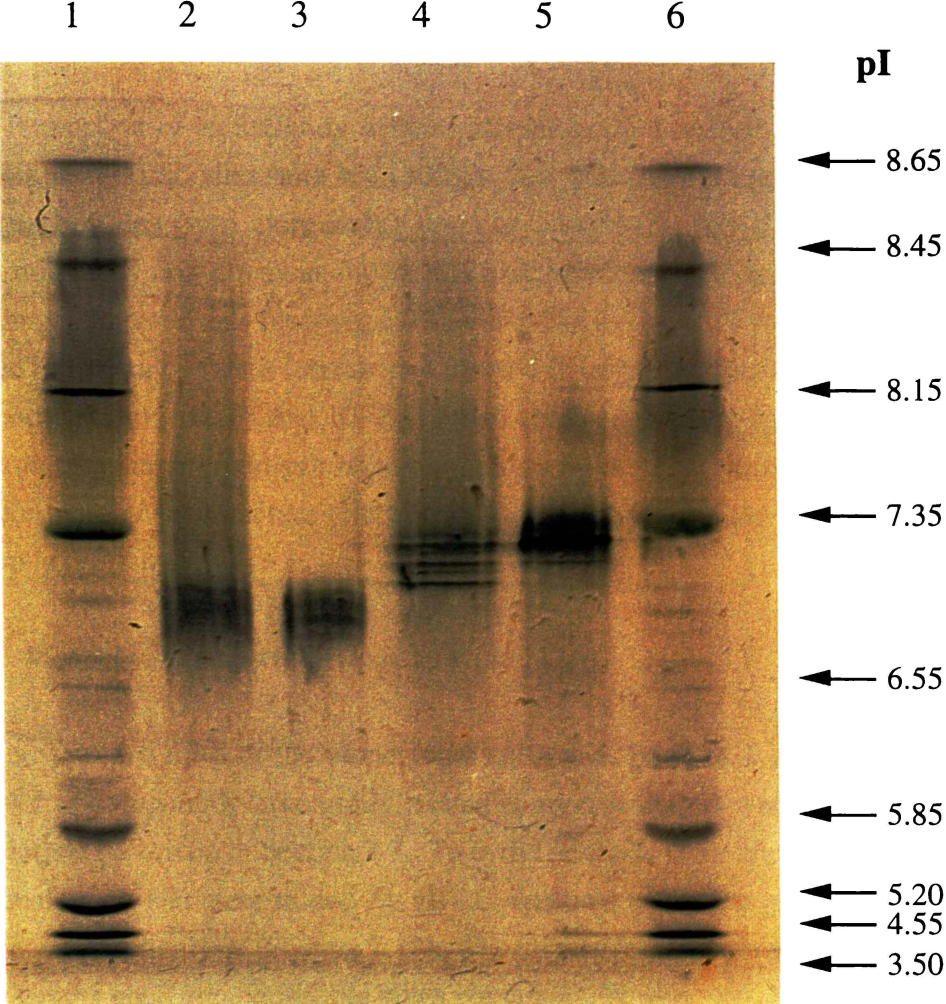


Figure 3.7.2 Isoelectric focusing in PhastGel IEF media 3-9 of monoclonal antibodies purified by affinity chromatography in Protein G Sepharose 4 Fast Flow. Samples are: (1) and (6) pI calibration kit containing Pharmalyte 3-10, (2) group A antibody from culture supernatant, (3) group A antibody from ascites, (4) group B antibody from culture supernatant, and (5) group B antibody from ascites. The gel was stained with silver stain.

3.8 Cross-reactivities

3.8.1 Introduction

The interaction of an antibody with an antigen is the basis of all immunochemical techniques. Antibodies with high affinity not only have a higher capacity for the antigen but also form more stable immune complexes. Binding of the antigenic determinant is non-covalent, reversible and involves hydrophobic and hydrogen bonding, van der Waals forces and coulombic interactions. The total binding energy is due to the sum of such interactions and the fit of the antigenic determinant in the antibody binding site (Kabat, 1980). Small changes in the antigen structure such as the loss of a single hydrogen bond can have a large effect upon the strength of the antibody-antigen interaction (Harlow and Lane, 1988) and thus large effects upon antibody cross-reactivity.

3.8.2 Monoclonal antibody cross-reactivities

The cross-reactivities of the monoclonal antibodies purified with Protein G with a number of compounds structurally related to sporidesmin A were determined using the method described in Section 2.7. To avoid artefacts from impurities the purity of the analogues was determined by HPLC analysis using the conditions used for sporidesmin A analysis (Section 2.12). The chromatograms showed one major peak representing each compound and when contaminants were present only minor peaks representing trace amounts were observed. However, in the case of highly cross-reactive contaminants it is possible for even a trace of these compounds to give significant interference in cross-reactivity studies.

Antibody cross-reactivities with these compounds were studied and calculated according to the formula:

% cross-reactivity (%CR) = (I_{50} sporidesmin A/I_{50} analogue) $\times$ 100.

The compounds that cross-reacted with the selected monoclonal antibodies fell into two groups, those bound by the group B antibody only and those that were bound by both antibodies (Table 3.8.1).

Table 3.8.1 Antibody cross-reactivities with compounds structurally related to sporidesmin A

compound	structure	%CR[a] group A antibody	%CR group B antibody
sporidesmin A		100	100
sporidesmin D		<0.1	1550
5-chloro-3,4-dimethoxy-2-azo -4'-phenylbenzyl alcohol		<0.1	3.6
		<0.1	2.2
2-amino-5-chloro-3,4-dimethoxy benzyl benzene boronate		<0.1	1.9
2-amino-5-chloro-3-methoxy benzyl alcohol hydrochloride		<0.1	1.7
3-chloro-4,5-dimethoxy-2-nitrobenzyl alcohol		<0.1	0.9
6,7-dimethoxyindole		<0.1	0.4
3,4-dimethoxy-2-nitrobenzaldehyde		<0.1	0.1
2-amino-5-chloro-3,4-dimethoxy-methylbenzoate hydrochloride		<0.1	0.1
5-chloro vanillic acid		<0.1	<0.1

[continued]

Table 3.8.1 (Continued)

compound	structure	%CR group A antibody	%CR group B antibody
2-amino-5-chloro-4-hydroxy-3-methoxy-benzaldehyde		<0.1	<0.1
5-chloro-4-hydroxy-3-methoxy-2-nitrobenzyl alcohol		<0.1	<0.1
5-chloro-4-hydroxy-3-methoxy-2-nitrobenzylaldehyde		<0.1	<0.1
5-chloro indole-2-carboxylic acid		<0.1	<0.1
tryptophan		<0.1	<0.1
sporidesmin A 11-hemisuccinate		50 000	34
sporidesmin A 11-methylglutarate		32 240	115
sporidesmin A diacetate		1 775	72
sporidesmin B		48	207
sporidesmin A 10b-hemisuccinate		32	9

[continued]

Table 3.8.1 (Continued)

compound	structure	%CR group A antibody	%CR group B antibody
gliotoxin		<0.1	<0.1

[a] % cross-reactivity (%CR) = (I_{50} sporidesmin A/I_{50} analogue) × 100, where I_{50} is the molar concentration of compound giving 50% reduction of the maximum absorbance obtained in the absence of sporidesmin A or analogue. Microtitre plates were coated with sporidesmin A 11-hemisuccinyl ovalbumin for assays using group A and group B antibodies (2 µg/mL and 4 µg/mL of coating antigen respectively). cELISAs were carried out as previously described with group A antibody diluted 1:300 000, group B antibody diluted 1:100 000, and antimouse-HRP conjugate diluted 1:2 500. Mean I_{50} of sporidesmin A with group A antibody and group B antibody were 33.7 and 16.7 ng/mL, respectively, and were determined from five different assays.

3.9 Discussion

Other researchers have previously produced anti-sporidesmin A antisera (Jonas and Ronaldson, 1974; Jonas and Erasmuson, 1977; Fairclough *et al.*, 1984; Gallagher *et al.*, 1992). Only Gallagher *et al.* (1992) reported the production of high titre antibodies, although these were not suitable for immunoassay development as they did not bind to free sporidesmin A in cELISA. In the research carried out here, however, polyclonal and monoclonal antibodies were produced which bound to sporidesmin A and its analogues in cELISA. Some of these antibodies were selected and purified for further use in the development of cELISAs for the quantification of sporidesmins and sporidesmin metabolites in body fluids, and the production of immunoaffinity matrices.

3.9.1 Immunisation

Specific antibodies with high affinity were required for this study. As production of anti-sporidesmin A antisera by earlier researchers had resulted in mainly low titre antibodies, it was essential that immunisation schedules used in this research

maximised the animal response to the immunogens. A commonly used method to increase the immune response to an antigen is to combine the immunogen with an adjuvant. Although there is a wide range of adjuvants available for use (Audibert and Lise, 1993) in this study Freund's complete and incomplete adjuvants were used as these are reported to be two of the most effective adjuvants (Dunbar and Schwoebel, 1990; Erhard *et al.*, 1991). Freund's adjuvant is often reported to cause site reactions such as inflammation and abscesses (Hurn and Chantler, 1980), but in this work site reactions were reduced to a minimum by administering only small volumes, *i.e.* 100, 200 and 400 μL per site, in the mouse, rabbit and sheep respectively.

In this research, the primary immunisations of mice were administered intrasplenically for monoclonal antibody production as described by Spitz (1986). The efficacy of the intrasplenic route for immunisation is reported to be greater than that of other *in vivo* methods of immunisation (Hong *et al.*, 1989). This is thought to be due to the deposition of the immunogen directly into splenic tissue to give a high concentration of antigen locally, easy access for the cells of the immune system to the unmetabolised immunogen and an increase in the duration of exposure of the these cells to the immunogen. Immobilisation of the immunogen within the spleen can be further increased by incorporating it into an inert and non-toxic carrier such as affinity beads of cross-linked agarose or small pieces of blotting membrane (Nilsson *et al.*, 1987). Various immunogens can be included in liposome membranes and the liposomes then used as carriers for intrasplenic immunisation (Gregoriadis, 1990; Nilsson and Larsson, 1990). These methods are also used particularly where the availability of the immunogen is limited. Since the availability of immunising conjugates was not an issue in this study, methods to increase the immobilisation of immunogen within the spleen were not used.

The immunisation programmes undertaken were shown to be effective in raising antibodies with suitable affinity and specificity. Species differences were shown to be important in the immune response. Sheep and rabbit antisera were more specific for sporidesmin A whereas the majority of mice antisera were more selective for sporidesmin D. This may be attributed to either the difference in the immunisation routes used or to species differences in immunogen metabolism resulting in different sporidesmin structures being presented to different species. The research also demonstrated the value of immunising a group of animals as antibody titres and specificities ranged considerably in each group.

Hyperimmunisation by repeatedly boosting the animals with the same immunogen was needed to produce antibodies which bound to sporidesmin A with high affinity. The antibodies resulting from such schedules are known to have many additional properties when compared to those obtained from sera from primary injections and the reasons for this are discussed by Harlow and Lane (1988). Sera from primary injections contain a high proportion of IgMs, whereas sera from hyperimmunised animals show a class shift to mostly IgG antibodies, most of which can be specific antibodies. The average affinity of antibodies for an antigen increases with repeated injections. The basis for such affinity maturation is understood to be due to clonal selection and somatic mutation. In the presence of small amounts of antigen those ß–lymphocytes with the highest affinity antigen receptors will compete most successfully for antigen. Clones of ß–lymphocytes secreting high affinity antibodies will therefore be selected for proliferation. This process is reinforced by somatic mutation of variable region genes and under continuous selective pressure, higher affinity antibodies result. The selection of high affinity clones often gives a response which becomes less diverse with repeated injections. This clonal dominance, observed at the later stages of hyperimmunisation protocols, is brought about by selection and proliferation of a few clones secreting antibodies to a limited number of epitopes.

3.9.2 Antibody production

Numerous myeloma cell lines are now commercially available (Harlow and Lane, 1988). NS-1 (P3/NS-1/1-Ag4-1), one of the most commonly used myeloma lines for production of hybridomas, was used because of its excellent growth characteristics and high fusion efficiency. NS-1 does not secrete functional antibodies but it does synthesise kappa light chain and therefore some hybridomas established from it may secrete immunoglobulin containing the myeloma light chain. In practice this does not cause a major problem as the efficiency of fusion with this line normally gives a sufficient proportion of hybridoma secreting antigen-specific immunoglobulin.

PEG was used for the fusion of mouse spleen cells with the NS-1 myeloma cells. PEG causes aggregation of the integral proteins in the cell membrane resulting in areas of lipid bilayer denuded of protein. Two closely associated areas of such denuded membrane will merge and allow the fusion of adjacent cells (Ahkong *et al.,* 1975). The molecular size of the PEG used for fusion is important (Fazekas de St Groth and Scheidegger, 1980). PEG is toxic to cells and low molecular weight PEG is more toxic than high molecular weight PEG, which is viscous and difficult to work with

(Campbell, 1991). The PEG used in this study was sufficiently fluid to be pipetted easily and had a molecular weight ranging from 3 000 to 3 700.

Peritoneal macrophages which provide growth-stimulating substances (Sugasawara *et al.*, 1985) were used to support hybridoma cells after fusion and cloning procedures. Thymocytes could have been used as an alternative (Campbell, 1984), however, macrophage cells were chosen as these removed much of the cellular debris present particularly after fusion, and this aided in microscopical observation of the hybridoma cultures.

Mice are normally chosen for monoclonal antibody production based on results from ELISA screening of circulating antibodies, although this is only a rough guide to the number and specificity of antibody-forming cells in the spleen. Generally animals are selected on the basis of high antibody titre. However, in this work success in obtaining antibodies of the required specificity was only achieved when a greater emphasis was placed on selection based on antisera specificity. It was also found that the coating antigen used was important as in ELISA it was found that the group A antibodies did not bind to the sporidesmin A 10b-hemisuccinyl conjugates and would not have been detected had these coating antigens been used for ELISA screening. This lack of binding may be explained by structural hindrance of antibody binding by the protein conjugated to sporidesmin A on the same face of the molecule as the disulfide group (Figure 3.2.1). Alternatively, the unmodified 10b-hydroxyl group could be required for antibody binding to coating antigens.

Polyclonal as well as monoclonal antibodies were produced primarily to maximise the chance of obtaining suitable antibodies. Polyclonal antibody production in sheep gave the added possibility of obtaining relatively large amounts of antiserum more readily than from hybridoma culture supernatant or mouse ascites fluid. Polyclonal antibodies were less expensive than monoclonal antibodies to produce. This was an advantage when larger volumes of reagent were required and when slight variation in quality did not cause difficulties. Sheep were preferred to rabbits because they live longer and can be bled repeatedly to yield larger volumes of antisera.

One of the claimed advantages of monoclonal antibodies is the readily obtained, unlimited supply of antibody produced by propagating hybridomas as ascites tumours although the results presented here show that this is not always the case. Although ascites were successfully produced for the group A antibody, group B antibody produced solid tumours and only a small amount of fluid could be drained. Other

researchers have reported similar problems (Hoogenraad and Wraight, 1986) and although no further production of the group B antibody in ascites was undertaken in this study, Hoogenraad and Wraight (1986) reported that when solid tumours form it is possible to drain a small amount of liquid from the peritoneal cavity and inject it into other mice which are immunohistocompatible and primed with pristane. This may lead to the propagation of ascites-forming cells in the second group of mice.

Ascites fluid was most successful in the group of mice which had been irradiated after being primed with pristane, less successful after pristane without irradiation, and least successful by priming with incomplete adjuvant. To ensure that the immunosuppressed animals could be raised under normal housing conditions without infection, the irradiation dose was limited to 2-3 Gray which approximated the 3-4 Gray recommended by Campbell (1984). Weissman *et al.* (1985) reported that by administering pristane and hydrocortisone followed by irradiation at 6 Gray to obtain immunosuppressed mice, large volumes of ascites could be obtained, although animals irradiated at this level must be kept in special facilities to avoid infection.

Incomplete Freund's adjuvant was the least effective priming agent for ascites fluid production. This result was not in agreement with the findings of Gillette (1987) and Mueller *et al.* (1986) who both reported that incomplete Freund's adjuvant was as good or better than pristane for the production of large amounts of ascites fluid containing monoclonal antibody.

3.9.3 Antibody purification

Purification of monoclonal antibodies from ascites fluid or culture supernatant is essential if non-specific effects due to proteins other than the immunoglobulin of interest are to be avoided (Reik *et al.*, 1987). Various methods have been reported for purification of monoclonal antibodies and the advantages and disadvantages of these have been extensively discussed (Jiskoot *et al.*, 1991). A relatively simple one-step procedure which is now used in many laboratories for the purification of both monoclonal and polyclonal antibodies was used to purify the immunoglobulin fraction from culture supernatant, ascites fluid and sheep antisera. This involved affinity chromatography with Protein G (Larsson, 1990). Although Proteins A and G have very similar applications in affinity chromatography, Protein G has some advantages over Protein A. It reacts with more IgG isotypes, reacts less with other immunoglobulin classes and seems to have the strongest and widest range of binding properties (Akerstrom *et al.*, 1985; Pilcher *et al.*, 1991).

As expected the major contaminant (Reik *et al.*, 1987) in unpurified monoclonal antibodies was albumin derived from foetal calf serum added to the culture media or from mouse serum in ascites. Monitoring the purified antibody with SDS-PAGE and IEF confirmed that purification by affinity chromatography with Protein G was successful, as this contaminant appeared to be removed. SDS-PAGE of the group A antibody from culture supernatant showed a minor band with a molecular weight equivalent to albumin. As no corresponding albumin band was present in IEF it was concluded that the contaminant was most likely not albumin.

Isoelectric focusing of the purified monoclonal antibodies gave more than one band for each antibody. Other workers have also reported usually two to four bands for each monoclonal antibody with isoelectric points ranging from 6.4 to 7.6 (Danielsson *et al.*, 1988). After synthesis, a homogeneous immunoglobulin molecule is very susceptible to alterations in its charge properties and the product becomes heterogeneous (Awdeh *et al.*, 1970). The microheterogeneity is most likely due to differences in glycosylation and to deamidation of aminoacids (Danielsson *et al.*, 1988). The isoelectric patterns obtained from group A and B antibody samples were discrete bands each focusing within a distinct region which can be taken as evidence of monoclonality (Reik *et al.*, 1987). The IEF pattern obtained with the group B antibody ascites sample, although focused within the same region, was different from that of the culture supernatant sample. This could have been due to the presence of IgG antibodies in ascites from the mouse repertoire or foetal calf immunoglobulins in culture supernatants which were co-purified with the monoclonal antibody.

3.9.4 Cross-reactivity studies

In the studies undertaken on monoclonal antibody cross-reactivity with compounds structurally related to sporidesmin A (Table 3.8.1), it was found that the group A monoclonal antibody was highly specific for sporidesmin A and recognised only compounds of similar structure with an intact disulfide bridge. The group B antibody had broad specificity and was capable of recognising sporidesmin-like molecules. Therefore, the group A antibody would be expected to bind relatively unmodified sporidesmin A-like derivatives, whereas the group B antibody may be expected to also bind sporidesmins and sporidesmin metabolites where the sporidesmin molecule is modified, so long as the distal region of the molecule containing the chlorine group is left sufficiently unchanged for binding to occur.

The group A antibody bound to compounds which contain the disulfide bridge, *e.g.* sporidesmin A and sporidesmin B and derivatives such as sporidesmin A 11-hemisuccinate, sporidesmin A 11-methylglutarate, sporidesmin A diacetate and sporidesmin A 10b-hemisuccinate. The group A antibody did not cross-react with sporidesmin D in which the disulfide bridge is broken and the two sulfur molecules are methylated. These results would suggest that the antibody is binding to a site that includes the intact disulfide group. However, it was found that the antibody did not cross-react with gliotoxin, a compound which contains the disulfide bridge but has the methyl group attached to the disulfide-containing ring structure replaced by CH_2OH. In addition, the substitution on the adjacent ring structures of gliotoxin is significantly different from those in the sporidesmin A molecule and the furthermost 5-membered ring structure is replaced by a 6-membered group. It was therefore concluded that when significant changes to groups adjacent to the disulfide bridge occur, as in gliotoxin, for example, the epitope is influenced in such a way that binding of group A antibody does not occur. Cross-reactivities were very much greater than 100 per cent with analogues containing an 11-ester grouping, as in sporidesmin A 11-hemisuccinate, sporidesmin A 11-methylglutarate, sporidesmin A diacetate. This suggested that the immunogen hemisuccinate bridge is an important part of the epitope for this antibody. The group A antibody did not cross-react with compounds containing groupings similar to regions of the sporidesmin molecule distal to the disulfide bridge, *e.g.* 2-amino-5-chloro-3,4-dimethoxybenzyl alcohol, which suggested that the group A antibody did not bind to this region of the molecule.

In sporidesmin B the 11-hydroxyl grouping is absent and cross-reactivity of the group A antibody with sporidesmin B was reduced. This would suggest that the presence of oxygen in the 11-hydroxyl group is important for antibody binding. Group A antibody binding to compounds was also reduced where the 10b grouping was derivatised as in sporidesmin A 10b-hemisuccinate. When the smaller acetate grouping was present in sporidesmin A diacetate, such a reduction was not observed. This result would suggest that substitution in the 10b position with a group such as succinate, can cause steric hindrance of antibody binding. The group A antibody did not bind to coating conjugates linked to protein carriers through the 10b position. Again this could also be explained by steric hindrance of antibody binding, in this case brought about by the large protein molecule.

The group B antibody not only bound to the compounds that the group A antibody cross-reacted with but also, albeit weakly, to analogues of the distal benzene ring and associated groups. This was greatest (3.6%) with compounds which contained the

chloride, methoxy, adjacent nitrogen and 10b-hydroxy groups, *e.g.* 5-chloro-3.4-dimethoxy-2-azo-4'-phenylbenzyl alcohol, whereas cross-reactivity was less (0.1%) with compounds such as 3,4-dimethoxy-2-nitrobenzaldehyde which contained fewer of these groupings. The group B antibody therefore appears to bind to a region which includes or interacts with regions of the sporidesmin molecule distal to the disulfide bridge. Esterification at the 11 position, *e.g.* sporidesmin A 11-hemisuccinate, sporidesmin A diacetate, sporidesmin A 11-methylglutarate, had relatively little influence on the antibody's ability to bind these compounds. Cross-reactivity of antibody B with sporidesmin A 11-methylglutarate was 115% while that of group A antibody was 50 000%, suggesting that the immunogen hemisuccinate bridge is not included in the epitope for the group B antibody.

Group B antibody binding was reduced when the 10b position was derivatised as in sporidesmin A 10b-succinate suggesting that derivatisation is causing steric hindrance or alternatively influence antibody binding in some additional way.

HO
O
HO
Cl
S
N
N
S
H_3CO
N
H
O
OCH_3

Figure 3.9.1 Regions of the sporidesmin A molecule important for antibody binding, for group A antibody binding (⟶) and for group B antibody binding (- - ➝).

Unexpectedly the group B antibody cross-reacted to a much greater extent (1550%), with sporidesmin D than it did with the hapten used in the immunogen, *i.e.* sporidesmin A. It is possible that the breaking of the disulfide bridge and methylation of the two sulfur groups influenced the binding site in such a way as to increase binding affinity. Alternatively, if sporidesmin A was rapidly metabolised to sporidesmin D or a sporidesmin D-like metabolite, it would be possible that an

immunised animal would be exposed to an immunogen that is predominantly sporidesmin D-like. The antibodies generated would therefore be expected to cross-react to a greater extent with sporidesmin D than sporidesmin A.

If additional analogues had been available, for example those containing structures similar to the two central ring structures in the sporidesmin A molecule, further information on the nature of the binding sites of the antibodies may have been obtained.

The antibodies produced were shown to have the specificities against sporidesmin A and its analogues required for the planned subsequent research. Sporidesmins A and D are the two major components of the sporidesmins which occur in pastures contaminated with *P. chartarum* and in cultures of *P. chartarum*. As the group A antibody does not cross-react with sporidesmin D, a cELISA using the group A antibody enables toxic sporidesmin A to be measured independent of the non-toxic sporidesmin D present. The group B antibody, however, which has broad specificity, might be expected to bind sporidesmin metabolites and could therefore be used in cELISAs for the measurement of sporidesmin metabolites (as yet unidentified) in body fluids of sheep dosed with sporidesmin A. In cELISA the assay sensitivities appeared to be acceptable and to warrant further development. Sufficient antibody had also been purified for the development of immunoaffinity chromatography matrices to be undertaken.

3.10 Summary

In order to maximise the chances of obtaining antibodies suitable for use in cELISA or immunoaffinity chromatography, monoclonal as well as polyclonal antibodies were produced in this study using a variety of immunogens in mice, sheep and rabbits.

When antisera were monitored by ELISA and cELISA the highest titres and cELISA sensitivities were found in the sheep antisera. Sheep and rabbit antisera were more specific for sporidesmin A whereas the majority of mice antisera were more selective for sporidesmin D.

It appears that the maturation of the immune response was important in the production of monoclonal antibodies suitable for use in cELISA measuring sporidesmin A, as only after a lengthy immunisation protocol including rest periods were high affinity antibodies obtained which would bind the free mycotoxin.

Careful selection of the coating conjugates was important. Not only did variation in the hapten and protein carrier used in the plate coating affect the sensitivity and specificity of the resulting assays, but it was also particularly important to the results obtained when screening hybridoma supernatant fluids.

Two sheep, producing antisera which gave the most sensitive assays for sporidesmin A, were retained and given further immunisations until a total of 10 inoculations were given over 25 months. At the end of this time antibody titres were still increasing with each immunisation. Antiserum from sheep 254 was highly specific for sporidesmin A while that from sheep 243 showed 7.8% cross-reactivity with sporidesmin D when sporidesmin A 10b-hemisuccinyl ovalbumin was used as the coating antigen.

Two groups of monoclonal antibodies were produced, those that bound to sporidesmin A (group A antibodies) and those that bound to both sporidesmin A and sporidesmin D (group B antibodies). Large-scale production of a group A antibody in ascitic fluid was successful in mice primed with pristane and given one sub-lethal irradiation treatment. The group B antibody was produced in culture supernatants only as when the antibody-producing cell line was injected into mice, solid tumours were formed and only a small volume of fluid containing antibody could be collected.

The group A antibody isotype was found to be IgG_{2b} and the group B antibody IgG_1. Since IgG_1 antibodies do not bind well with Protein A, purification was carried out using a one-step method with Protein G affinity chromatography. Antibody purity was monitored by SDS-PAGE and the albumin band, which was the major contaminant in both unpurified culture supernatant and ascites, was not visible in samples after purification using affinity chromatography with Protein G. The isoelectric patterns obtained from samples purified by Protein G were mainly discrete bands focused within a distinct region indicating monoclonality.

Cross-reactivity studies indicated that the group A antibody appears to have a binding site which includes the intact disulfide bridge and the epitope for this antibody may include the immunogen hemisuccinate bridge. Group B antibody appears to bind to a region which includes or interacts with regions of the sporidesmin A molecule distal to the disulfide bridge, for example the chloride, methoxy, adjacent nitrogen, 10b hydroxyl groups, and associated ring structures. Therefore, the group A antibody would be expected to bind relatively unmodified sporidesmin A-like derivatives containing the disulfide bridge and associated structures, whereas the group B

antibody may be expected to also bind sporidesmins and sporidesmin metabolites where the sporidesmin molecule is modified, so long as the distal region of the molecule is left sufficiently unchanged for binding to occur. The group A antibody allows toxic sporidesmin A to be distinguished from the non-toxic analogue sporidesmin D .

The selected polyclonal and monoclonal antibodies were retained for use in the development of cELISAs for quantification of sporidesmins and sporidesmin metabolites in body fluids and the production of immunoaffinity matrices.

Chapter 4:
IMMUNOASSAY DEVELOPMENT

CHAPTER 4

IMMUNOASSAY DEVELOPMENT

4.1 Introduction

ELISAs have been used as effective methods for analysing mycotoxins in predominantly aqueous samples and, because of their ease of use and high sensitivities, usually have major advantages over existing traditional procedures such as HPLC, particularly when assay times are compared.

The two monoclonal antibodies which were found to bind different regions of the sporidesmin molecule (Section 3.8.2), were available for the development of immunoassays for analysis of body fluids. Because of the differences in antibody specificities, each gave assays with the potential to detect the presence of compounds containing different regions of the sporidesmin molecule.

To establish ELISA protocols for the analysis of sporidesmin A and its metabolites in complex matrices, *i.e.* body fluids, the following studies were undertaken:
(1) selection of assay design and format to give the required sensitivity and specificity;
(2) investigation for the presence of matrix effects and correction of these if present;
(3) optimisation of the assays;
(4) validation of methods, if possible against another accepted method of analysis;
(5) determination of assay performance characteristics.

4.2 Immunoassay of bile

4.2.1. Sample collection

Hepatic bile samples were collected as described in Section 2.8.1. Samples of gall bladder bile were also obtained from seven sheep during the winter when they had not been exposed to sporidesmin A. The bile was collected at slaughter and used for initial assay development. Gall-bladder bile is more concentrated than hepatic bile and it was thought that any assay matrix effects occurring with these samples would be more severe than any observed with hepatic bile. When sheep were surgically

cannulated and hepatic bile became available, final assay optimisation and validation was carried out using this bile, as research into the metabolism of sporidesmin A required that cELISAs be developed for the quantification of sporidesmin A and its metabolites in hepatic bile.

4.2.2 Assay format

Although assay sensitivity is limited by the affinity of the antibody for the analyte (Tijssen, 1985), assay formats also greatly influence sensitivity (Schneider and Hammock, 1992) and there are many variations possible. As a selection of coating antigens was available for this research, the format chosen for assay development was an indirect competitive assay. This format is reported to have detection sensitivity which is comparable with, or slightly better than, the direct ELISA (Chu, 1986) and has the added advantage of requiring less antibody than the direct format. Furthermore, since there is no direct contact of the sample matrix with the detection system, indirect assays are also reported to be more rugged, sensitive and reliable than direct competitive formats (Krotzky and Zeeh, 1995).

Heterologous cELISA systems are defined as cELISA in which the coating hapten is structurally different from the immunising hapten. Although heterologous assay systems have been reported to yield more sensitive assays, homologous systems have also been used successfully (Székács and Hammock, 1995). The monoclonal antibodies used in the present work were obtained from mice immunised with sporidesmin hemisuccinyl 11-BSA, and with the availability of both sporidesmin A hemisuccinyl 11- and 10b-protein conjugates for coating plates, both homologous and heterologous formats were investigated in assays using the group B antibody. The group A antibody was used only with 11-conjugated coating antigens as previously it had been shown that this antibody did not bind the 10b-conjugates (Section 3.5.1).

4.2.3 Matrix effects

Matrix effects can be described as any change in assay performance that can be attributed to the presence of components, other than the analyte, in the sample. Sample components can bind or otherwise interfere with interactions involving antibodies and enzyme conjugates and give false positive or negative results. Matrix interferences were studied for each assay as matrix effects may depend on a number

of factors such as the antibody and coating conjugate used, the assay format or the nature of the method used for sample preparation.

The usual way of detecting matrix effects is to compare the standard curves generated with standards prepared in sample blanks (*i.e.* in diluted or extracted body fluids) known to be free of analyte, with those generated for the same set of standards prepared in defined assay buffer (Krotzky and Zeeh, 1995). The presence of matrix effects in the cELISAs for sporidesmin A in bile were investigated using this method. Sporidesmin A standards were prepared in methanol as described in Section 2.4.2, and spiked into gall bladder bile (sample 3) diluted in standard buffer to give dilutions ranging from 1:9 to 1:99. The buffer was PBS containing 1% methanol (final concentration 2%) and 0.05% Tween 20. The same standards were also prepared in standard buffer without bile. Standard curves for each set of standards were determined by cELISA using the format and method described in Section 2.4.1. Except where it was indicated otherwise, the analyses for all of the immunoassay development were carried out in duplicate. Replicate absorbance measurement which had %CV values greater than 10% were rejected.

The curves prepared in diluted bile were considerably different from those prepared in buffer (Figure 4.2.1) and it was concluded that the effect of bile on the assays was significant, even after the bile was diluted to 1:99.

It is possible to remove matrix effects in assays by sample pre-treatment such as dilution, addition of a suitable blocking agent or by sample clean-up. Alternatively the assay standards can be prepared in the raw matrix. The latter is acceptable as long as the level of interference between samples is constant. Another approach is to correct for the presence of interference by subtraction of blank matrix absorbance from the absorbance obtained with samples but this approach depends on there being no variation in matrix effects between different samples of bile.

To determine if the level of interference caused by each bile sample was constant, matrix interferences with A_0 values (maximum ELISA absorbance measured in the absence of analyte) were studied. Individual bile samples were diluted 1:99 in sample buffer (PBS containing 2% methanol and 0.05% Tween 20) and the absorbances measured in the ELISAs were compared with absorbances recorded for sample buffer without bile (Figure 4.2.2). The level of interference caused by each bile sample varied in ELISA using both antibodies so methods of removing these effects were investigated.

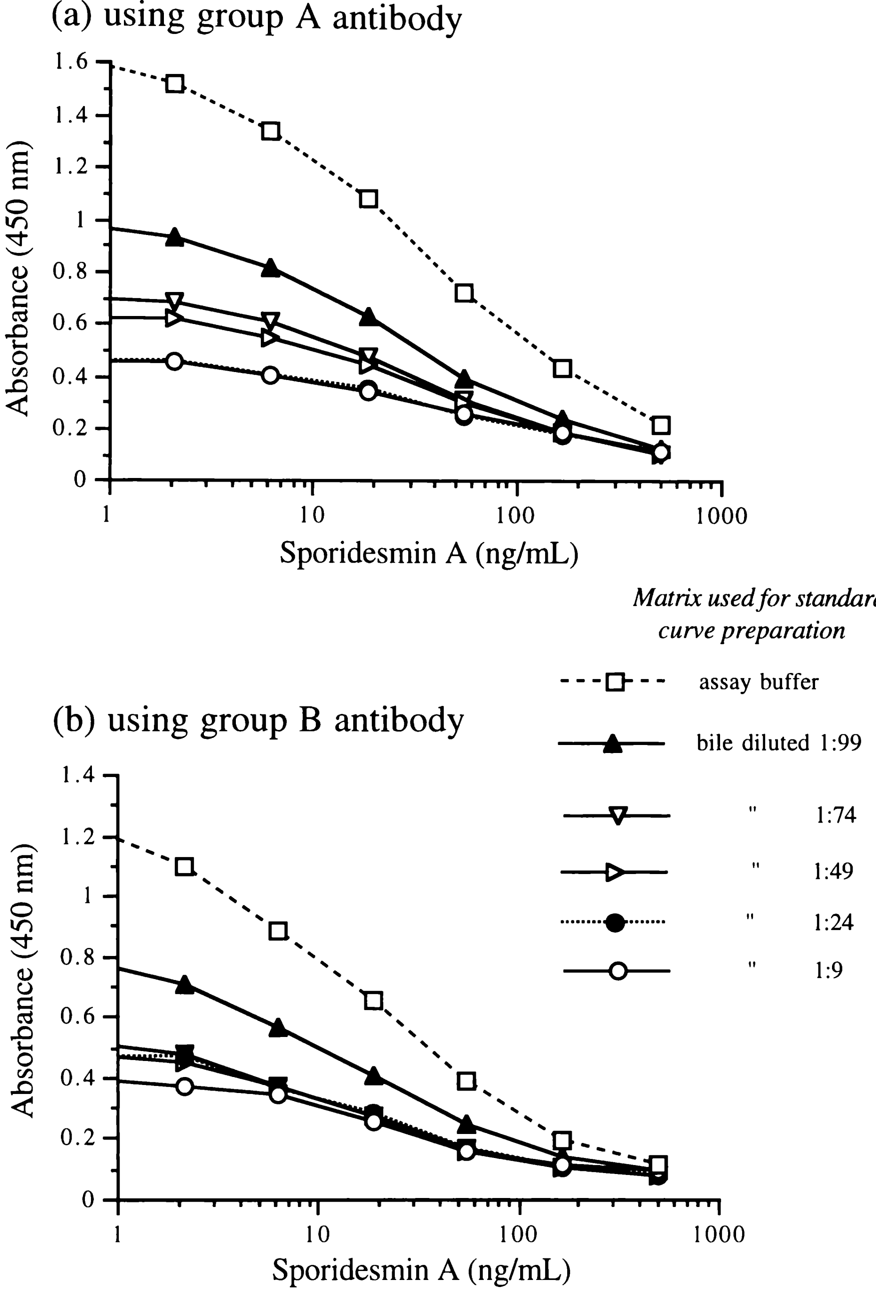


Figure 4.2.1 Effect of sample dilution on matrix interference caused by bile in the sporidesmin A cELISAs. Dilutions of gall bladder bile (sample 3) in standard buffer were made. Microtitre plates were coated with sporidesmin A hemisuccinyl 11-ovalbumin (for group A antibody at 0.33 µg/mL and for group B antibody at 0.67 µg/mL). Plates were blocked with 1% BSA for 2 h, incubated with sporidesmin A and group A or group B antibody (diluted 1:7 000 or 1:2 000 respectively) for 1.5 h. Incubation with second antibody (goat anti-mouse diluted 1:1 300, obtained from Boehringer Mannheim, FRG) was for 1.5 h. Tween 20 was included in washing and assay buffers at 0.05%. Except for coating at 4°C all other assay steps were carried out at 21°C. cELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

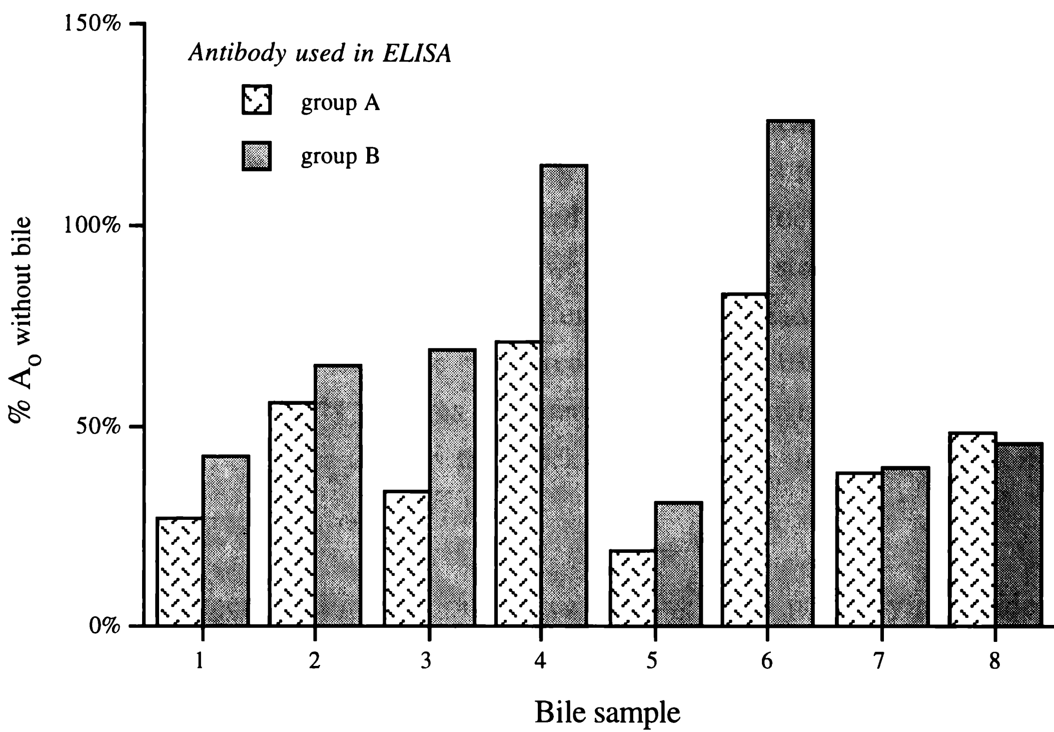


Figure 4.2.2 ELISA interferences caused by bile diluted 1:99 in sample buffer. The A_O values were determined from sample buffer without bile. The assay procedure was as described in Figure 4.2.1. $\%A_O$ values shown are the mean of duplicate determinations: standard deviation ranged from 0.3 to 4.6.

The effects of changes to assay conditions and sample pre-treatment procedures were investigated. It was planned that polar metabolites as well as free sporidesmin A would be measured together. Extraction of samples, therefore, with non-polar solvents was not included as, although sporidesmin A would be extracted, metabolites would be excluded from non-polar solvents.

4.2.4 Removal of matrix effects

(1) Use of alternative blocking agents

The use of alternative blocking agents in the ELISA as a means of removing bile matrix effects was investigated by measuring standard curves for sporidesmin A in assays where: (a) the usual 1% BSA was used as the assay blocker, and (b) 5% low fat milk powder was used. The standards were prepared in standard buffer and in bile (sample 3) diluted 1:99 in standard buffer, and the standard curves obtained in cELISA using both antibodies were compared. In both, cELISA bile displaced the standard curve significantly (Figures 4.2.3 and 4.2.4), although the effect was less noticeable when BSA was used as the blocking agent with the group A cELISA (Figure 4.2.3).

A further experiment was carried out to see if using 0.5% polyvinylpyrrolidone (PVP, Serva 33420) as the blocking agent gave better results. Bile samples (n = 7) were diluted (from 1:99 to 1:599) in sample buffer and analysed by ELISAs using both antibodies (Figure 4.2.5). As A_o values recorded in the presence of many of the bile samples were different from the bile-free control, even at high dilutions, matrix effects were still considered to be significant. As there was no advantage in changing to 5% milk or PVP, 1% BSA continued to be the blocking agent routinely used in the assays.

(2) Use of an alternative assay buffer

Substitution of TBS (20 mM Tris-buffered saline, pH 7.4) for PBS (10 mM phosphate-buffered saline, pH 7.4) in washing, sample and antibody buffers was investigated as a means of reducing matrix effects caused by bile. Each bile sample was diluted 1:99 in TBS or PBS sample buffer and analysed by ELISA. The A_o recorded was expressed as a percentage of that measured when bile was not included in the sample buffer. Second antibody purchased (from Boehringer Mannheim) was replaced with a similar reagent purchased from Silenus Laboratories. The latter was found to give a higher A_o in the ELISAs compared to the Boehringer reagent, and

(a)

1.4
1.2
1
0.8
0.6
0.4
0.2
0

Absorbance (450 nm)

1
10
100
1000

Sporidesmin A (ng/mL)

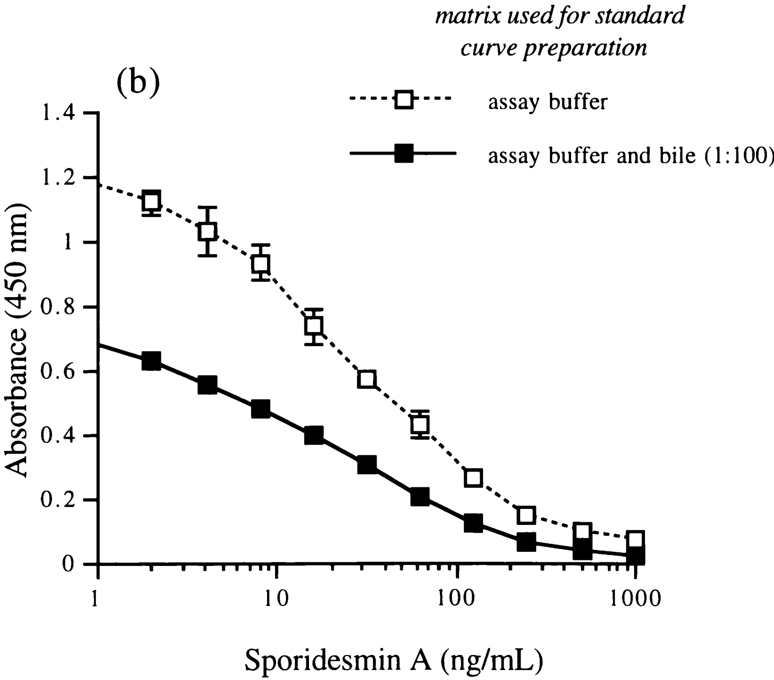
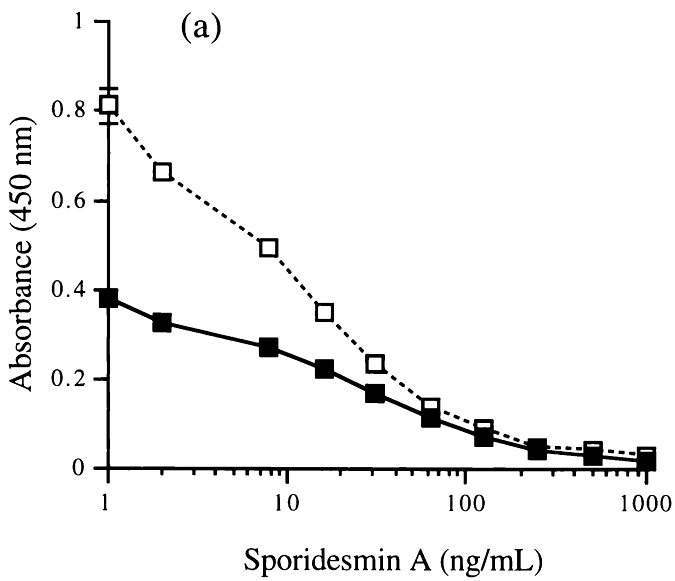


Figure 4.2.3 Effect of using (a) 1% BSA or (b) 5% low fat milk as assay blocking agents in cELISA on matrix effects caused by bile. Sporidesmin A standard curves were prepared in standard buffer or in standard buffer containing diluted bile (sample 3, diluted 1:99). The assay procedure was as described in Figure 4.2.1 using Group A antibody. The mean of duplicate determinations are shown with error bars showing the standard deviation.



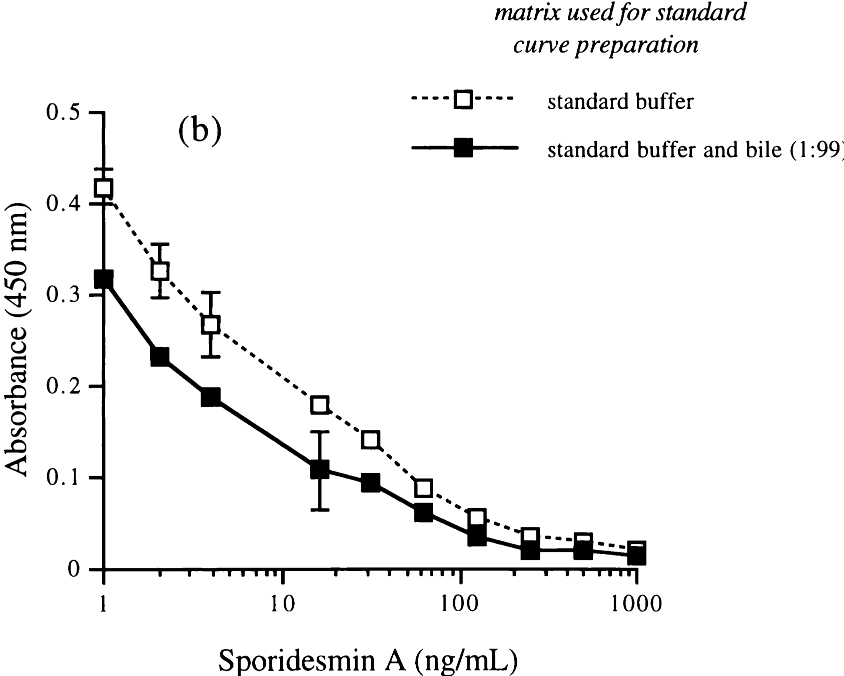


Figure 4.2.4 Effect of using (a) 1% BSA or (b) 5% low fat milk as blocking agents in cELISA on matrix effects caused by bile. Sporidesmin A standard curves were prepared in standard buffer or in standard buffer containing diluted bile (sample 3, diluted 1:99). The assay procedure was as described in Figure 4.2.1 using group B antibody. The mean of duplicate determinations are shown with error bars showing the standard deviation.

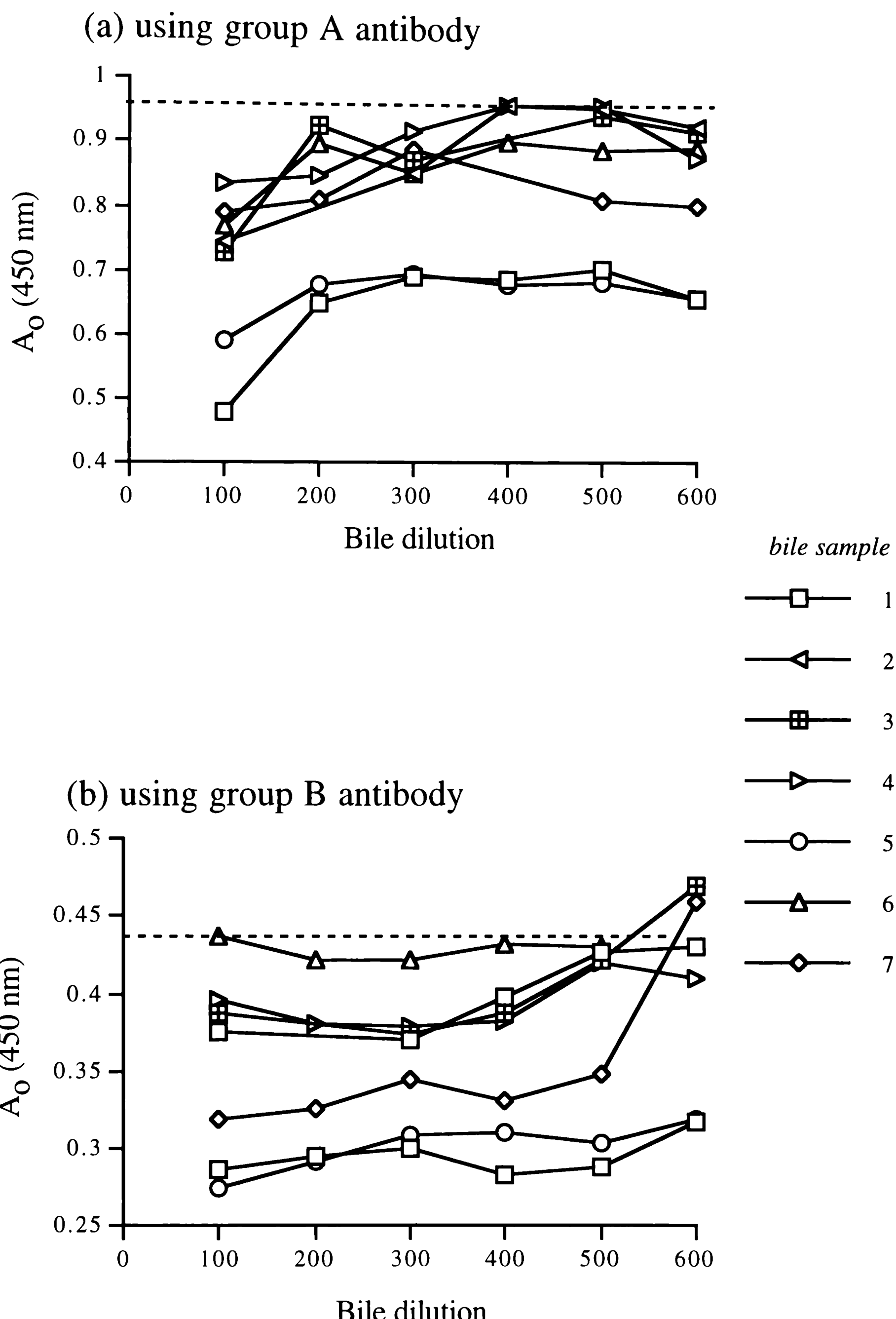


Figure 4.2.5 ELISA A_0 measured in the presence of diluted bile when 0.5% polyvinylpyrrolidine (PVP) was used as the assay blocking agent. Quadruplicate determinations of samples consisting of sample buffer only gave a mean A_0 marked ------. The assay procedure was as described in Figure 4.2.1. ELISA absorbances shown for samples containing bile are the mean of duplicate determinations (S.D. = 0.001 – 0.042).

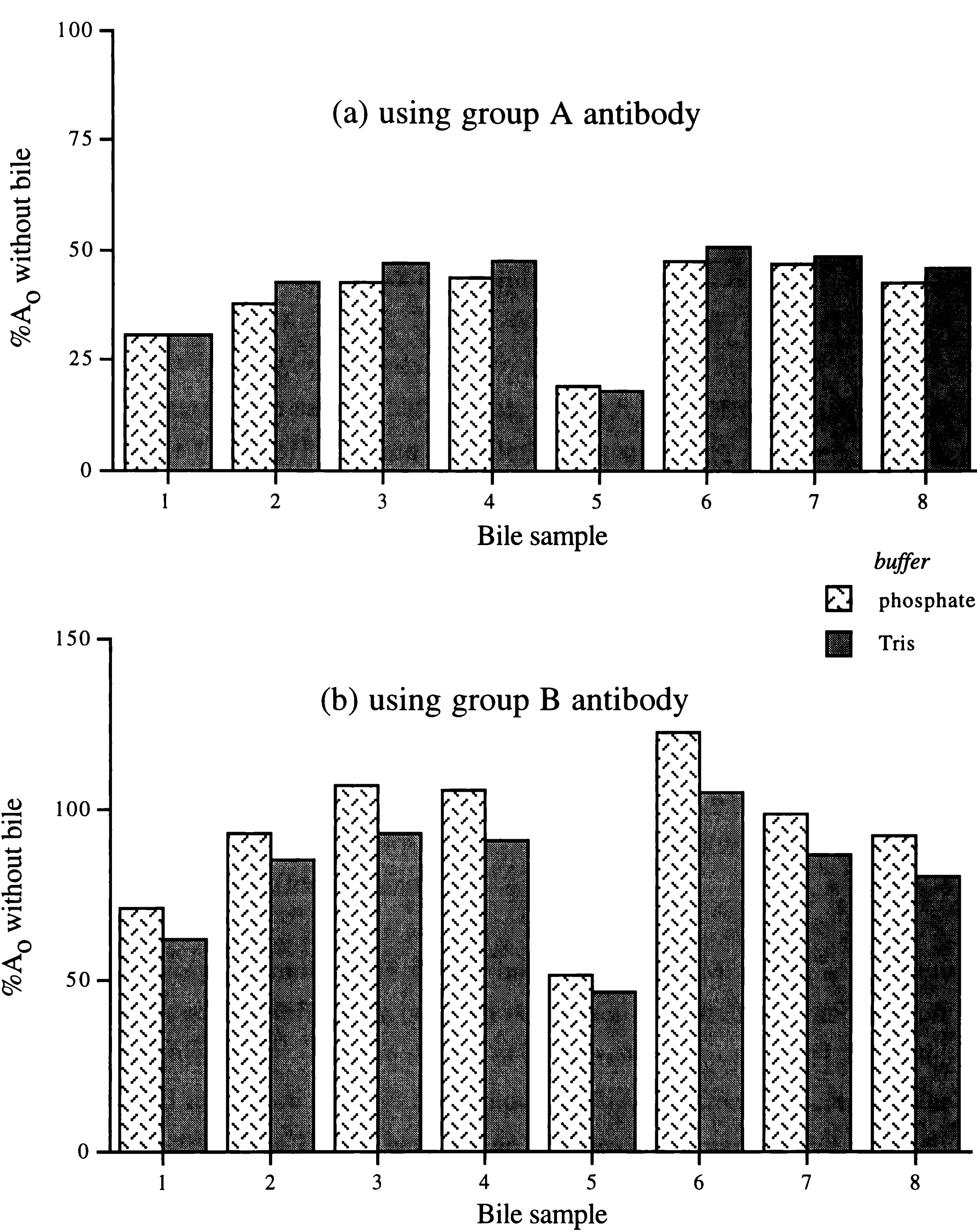


Figure 4.2.6 Effect of sample assay buffers containing PBS (10 mM, pH 7.4) or TBS (20 mM, pH 7.4) on A_O measured by ELISA of samples containing bile diluted 1:99 in sample buffer. Quadruplicate ELISA of samples consisting of sample buffer only gave the 100% absorbance values (A_O). The assay procedure was as described in Figure 4.2.1 except the second antibody (donkey anti-mouse-HRP, diluted 1:2 500) was obtained from Silenus Laboratories, Victoria, Australia. %A_O values shown for samples containing bile are the mean of duplicate determinations, (S.D. ranged from 0.2 to 3.7).

gave more sensitive assays as less first antibody was required. It was also less expensive.

The results (Figure 4.2.6) showed little difference between PBS and TBS in assays using group A antibody whereas in assays using group B antibody the %A_O percentages were slightly closer to 100% for PBS. For this reason PBS continued to be the assay buffer used in the assays for sporidesmin A.

(3) Changes to sample buffer pH and ionic strength

The effect of altering the sample buffer ionic strength and pH were also studied. Phosphate buffer (1 M, pH 7.4) and six dilutions of this were prepared with the lowest strength being 16 mM. Sodium chloride was also added to give a concentration of 150 mM and the buffers were used to prepare sample buffer which contained 2% methanol and 0.05% Tween 20. Bile was diluted 1:99 in each and analysed by ELISA. The A_O measured with each diluted sample was compared with that measured for the same buffer without bile.

The pH of PBS (10 mM, pH 7.4) was adjusted by adding NaOH or HCl to give buffers covering a pH range from pH 6.0 to pH 9.3. Bile was diluted in these buffers and analysed by ELISA and the A_O measured were compared with those measured for the same buffers without bile.

Changing the phosphate molarity in sample buffer from 10 mM, which is routinely used, did not reduce the difference between A_O values measured with and without bile present (Figure 4.2.7). Altering the pH of the sample buffer from pH 7.4 which is routinely used in ELISA also did not remove the matrix effects observed in the presence of bile (Figure 4.2.8). Therefore no changes were made to the routinely used pH or ionic strength of the sample buffer.

(4) Changes in concentration of Tween 20 in sample buffer

A range of sample buffers containing from 0.03% to 2% Tween 20 were prepared by two-fold serial dilution of PBS containing 2% Tween 20 and 2% methanol in PBS containing 2% methanol. Bile (sample 4) was diluted 1:99 in each buffer and analysed by ELISA and the A_O measured compared with that for the same sample buffer without bile (Figure 4.2.9).

In the assay using the group A antibody it was shown that absorbances were most similar when 2% Tween 20 was used in the sample buffer. With the group B

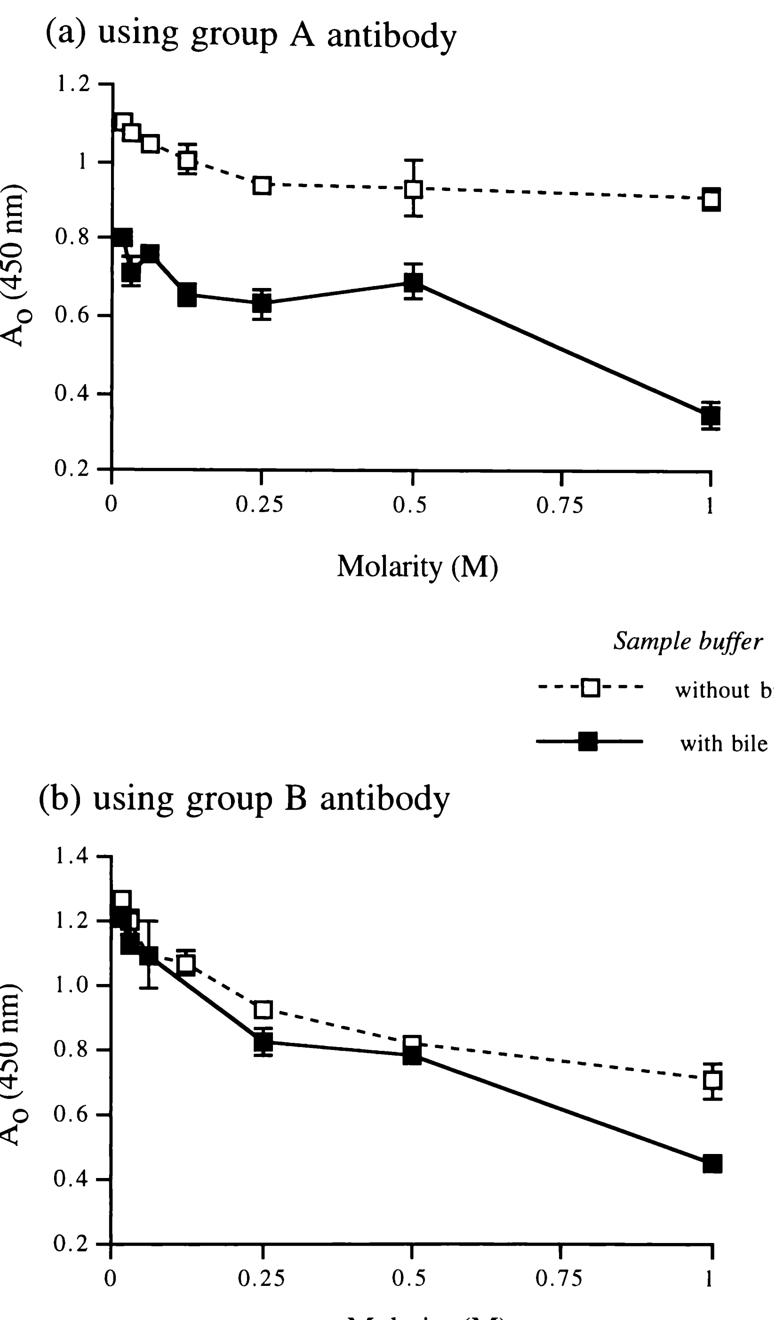


Figure 4.2.7 The effect of phosphate molarity in sample buffer on A_0 measured in ELISA in the presence and absence of bile (sample 4 diluted 1:99). The assay procedure was as described in Figure 4.2.6. ELISA absorbance values shown for samples containing bile are the mean of duplicate determinations with error bars showing standard deviations.

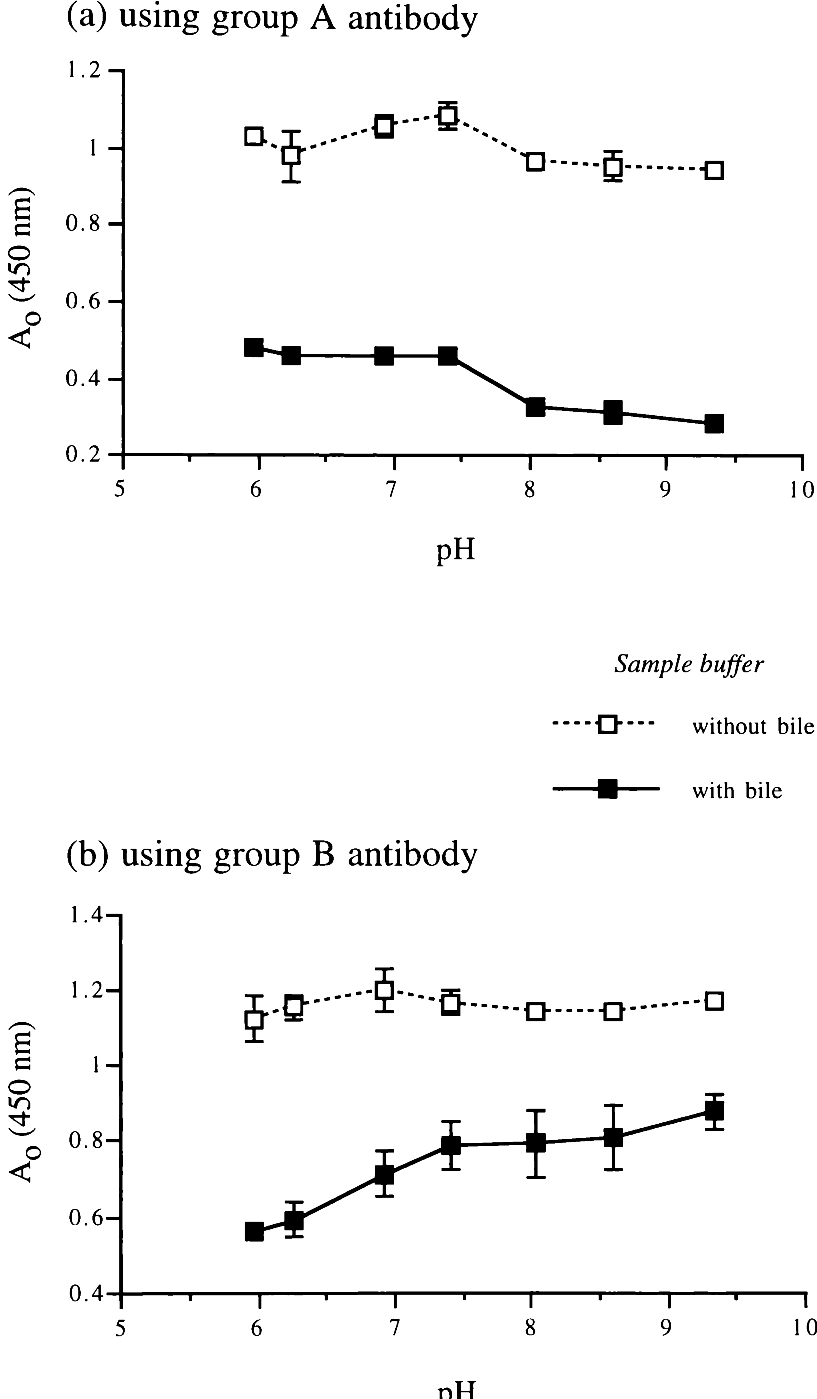
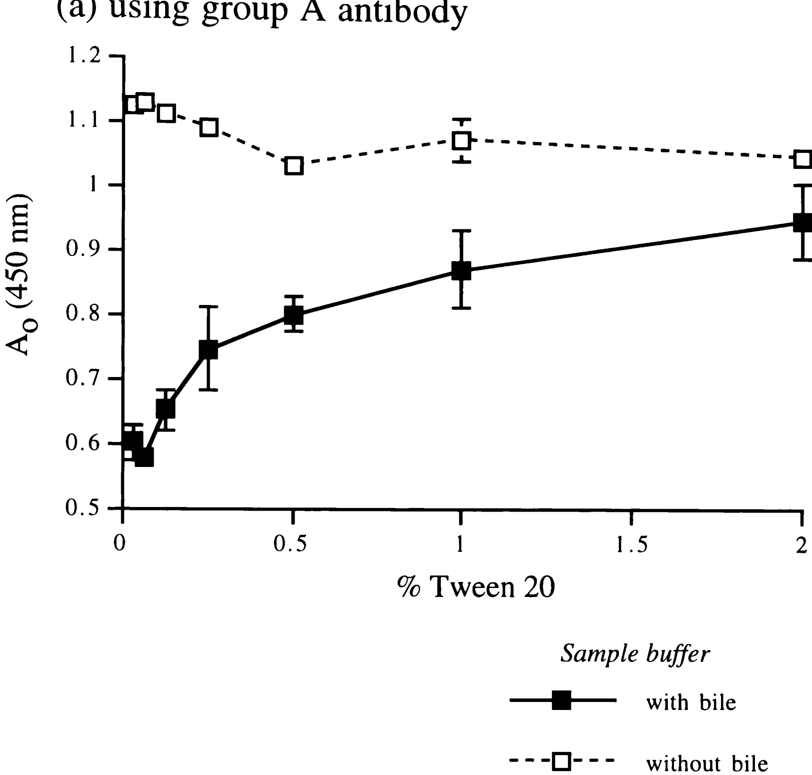


Figure 4.2.8 The effect of sample buffer pH on A_O measured in ELISA, in the presence and absence of bile (sample 4 diluted 1:99). The assay procedure was as described in Figure 4.2.6. ELISA absorbance values shown for samples containing bile are the mean of duplicate determinations with error bars showing standard deviations.



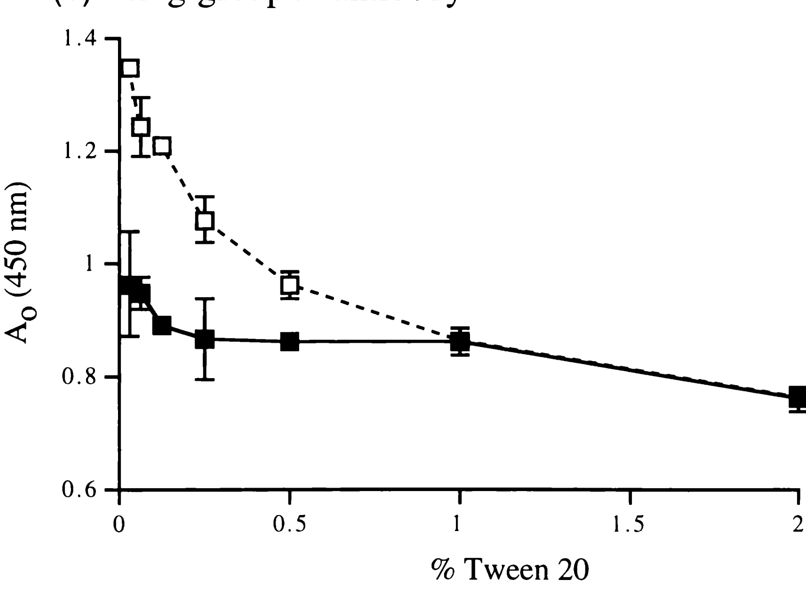


Figure 4.2.9 Effect of the inclusion of Tween 20 in sample buffers on the A_0 measured in ELISA in the presence and absence of bile (sample 4 diluted 1:99). The assay procedure was as described in Figure 4.2.6. ELISA absorbance values shown for samples containing bile are the mean of duplicate determinations with error bars showing standard deviations.

antibody 1% and 2% Tween 20 were found to give the best results although the absorbance was nearly halved by 1 to 2% Tween 20 in the assay using the group B antibody.

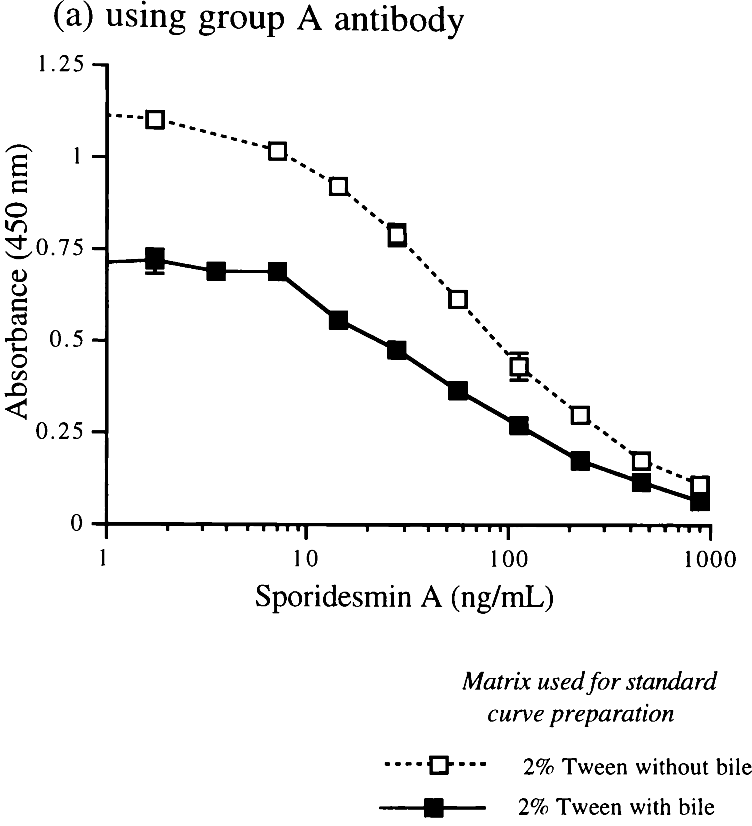
Sporidesmin A standards were prepared in (a) standard buffer consisting of PBS containing 2% Tween 20 and 2% methanol and (b) bile (sample 5) diluted 1:99 in standard buffer. Bile sample 5 was chosen for use as it had been shown in Figure 4.2.2 to cause the greatest assay interference in the ELISAs. These solutions were then used to obtain standard curves in cELISA. As the two sets of standard curves with and without bile were not superimposable (Figure 4.2.10), it was concluded that matrix effects were not removed by using 2% Tween 20 in the sample buffer. The higher concentration of Tween 20 also reduced the cELISA absorbances when using the group B antibody. This would reduce the assay precision. The A_O could be increased by increasing the amount of antibody, although this would reduce assay sensitivity. Increasing the dilution of bile beyond 1:99 would also decrease assay sensitivities so further dilution in 2% Tween 20 was not considered.

(5) Use of alternative detergents

As it had been shown that using increased concentrations of Tween 20 in sample buffers had a beneficial effect on assay interferences (Figure 4.2.9) the use of alternative detergents was investigated. The detergents studied were sodium deoxycholate, sodium dodecylsulphate (SDS), nonidet P40, Triton X-100, Tween 40 and Tween 80. Sample buffers containing 0.06 to 2% of detergent were prepared and bile sample 4 was diluted 1:99 in each. Each sample was measured in ELISA and the A_O recorded compared with that recorded using the same buffer without bile (Figure 4.2.11).

When some detergents such as sodium deoxycholate, SDS and Tween 40 were used with the ELISA using the group B antibody, the A_O for samples with and without bile were similar but they were low. Increasing the antibody concentration to overcome this problem was not considered as it would result in cELISA with reduced assay sensitivity. When 2% Triton X-100 was included in sample buffers, however, assay absorbances (A_O) with and without bile were similar and near to 1.0 absorbance unit.

Sporidesmin A standards were prepared in standard buffer containing 2% Tween 20 or 2% Triton X-100. They were also prepared in bile (sample 4 diluted 1:99) in the same buffers. When standard curves obtained in cELISA using both antibodies were



Matrix used for standard curve preparation

2% Tween without bile

2% Tween with bile

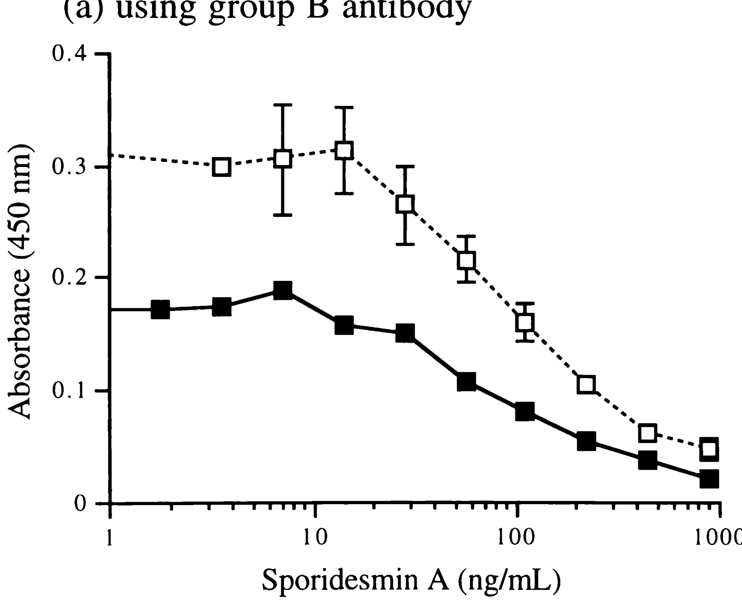


Figure 4.2.10 Effect of the inclusion of 2% Tween 20 in sample buffers on matrix effects caused by bile in cELISA. Sporidesmin A standard curves were prepared in (1) sample buffer and (2) bile (sample 5, diluted 1:149) in sample buffer. The assay procedure was as described in Figure 4.2.6. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

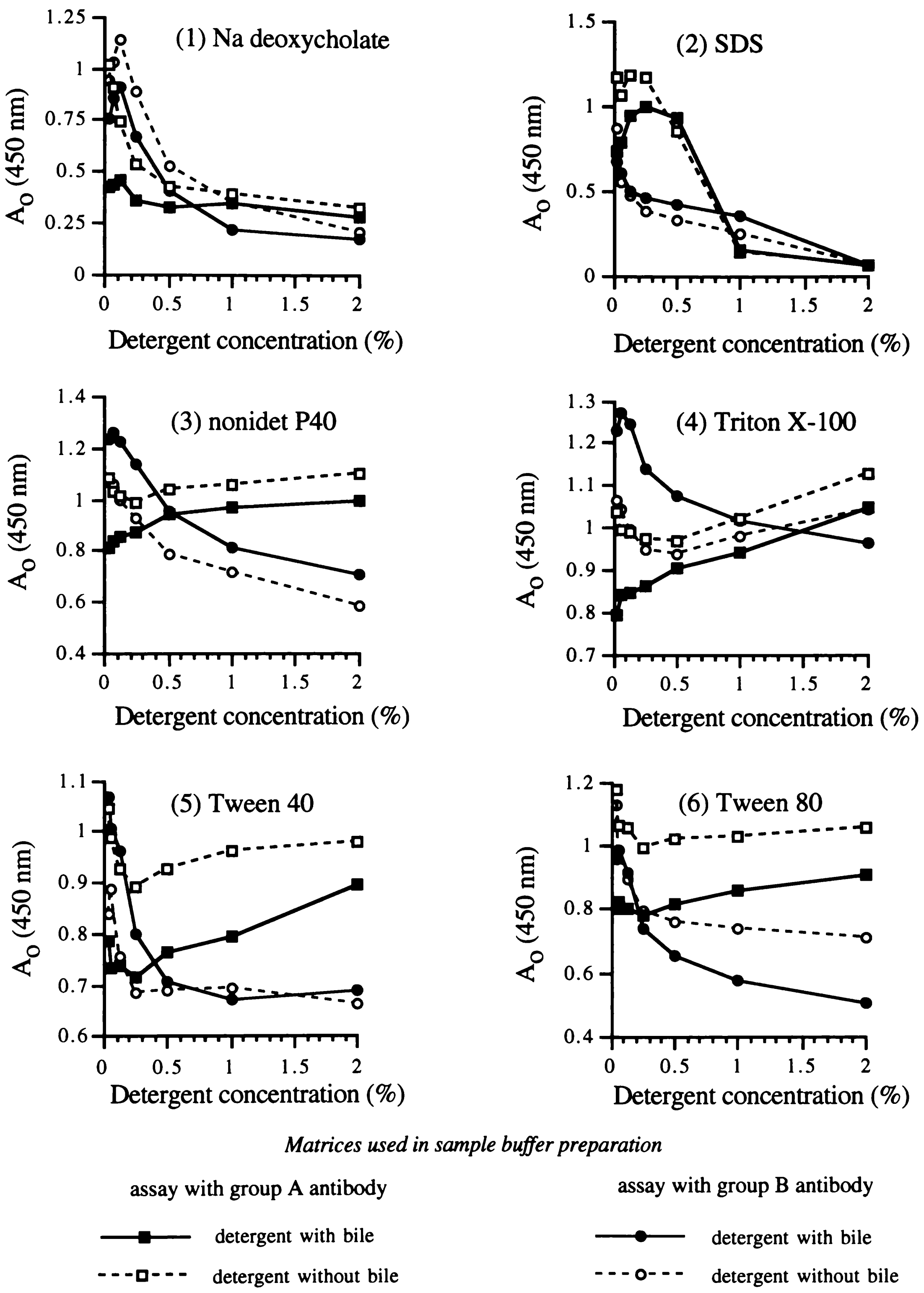


Figure 4.2.11 Effect of the inclusion of various detergents in sample buffers on A_0 measured in ELISA in the presence and absence of bile (sample 4, diluted 1:99). The assay procedure was as described in Figure 4.2.6. ELISA absorbance values shown are the mean of duplicate determinations. The standard deviation of the A_0 values ranged from 0.001 to 0.065 (mean = 0.018).

compared it was found that the curves with and without bile were not superimposable. Results obtained using 2% Triton X-100 were no better than those with 2% Tween 20 (Figure 4.2.12). Alternative methods of sample treatment were therefore investigated.

(6) Deproteinisation of bile with methanol

Varying proportions of bile (sample 5) and methanol, as outlined in Table 4.2.1, were mixed in microcentrifuge tubes (1.5 mL), left in an ice-salt bath for 15 min and centrifuged at 10 000*g* for 5 min. An aliquot of each supernatant containing 10 µL of the bile sample originally taken, was diluted to 500 µL in assay buffer (PBS containing 0.05% Tween 20) so that the final dilution of bile was 1:49. These samples were analysed in ELISA using the group A antibody. Control samples (PBS substituted for bile) were also prepared so that the effect of methanol on the assay could be taken into account.

Table 4.2.1 Reagent volumes used for precipitation of bile with methanol

bile (µL)	methanol (µL)	supernatant (µL) (containing 10 µL of bile)	sample buffer (µL)
10	0		490
80	20	12.5	487.5
70	30	14.3	485.7
60	40	16.7	483.3
50	50	20	480
40	60	25	475
20	80	50	450

The precipitates obtained after removal of all of the supernatant, were dissolved in a volume of PBS equal to that of the original volume of bile taken. Ten microlitres of each was added to 490 µL of assay buffer and analysed by ELISA using the group A antibody.

The results (Figure 4.2.13) indicated that as the proportion of methanol used in precipitation increased, the difference between the A_0 measured in ELISA of

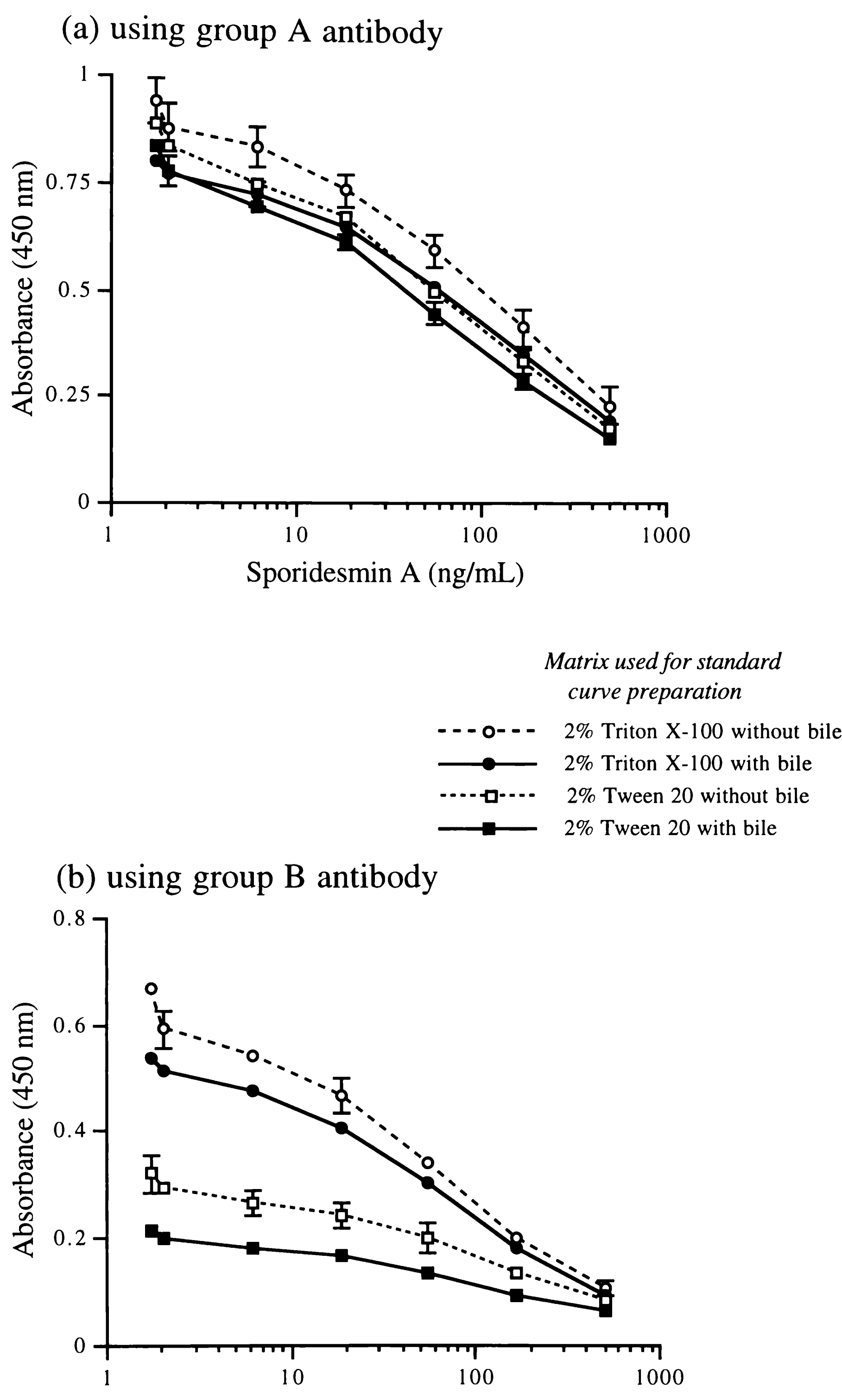


Figure 4.2.12 Effect of 2% Triton X-100 and 2% Tween 20 in standard buffers on matrix effects caused by bile in cELISA. Standard curves were prepared in (a) sample buffer and (b) bile (sample 4, diluted 1:99) in buffer. The assay procedure was as described in Figure 4.2.6. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

supernatant and that measured in the sample without bile, decreased. ELISA of the precipitates showed that assay interferences increased as the amount of methanol used for the precipitation increased. The component in bile which interfered with the assay appeared to be removed by precipitation with methanol, and 80% methanol was most effective in removing the interfering component. The experiment was repeated with acetone instead of methanol and only the supernatants were checked for assay interferences in ELISAs using both the group A and group B antibodies. The experiment was also carried out using ethanol. The results (Figure 4.2.14) indicated that precipitation of bile with 80% acetone or ethanol also significantly decreased ELISA interferences in the supernatants collected. The A_0 measured with bile in 80% solvent and the equivalent solvent blank without bile were compared for all three solvents (Figures 4.2.13 and 4.2.14). The difference between these measurements in assays using the group A antibody was least with methanol and acetone which were better than ethanol. Methanol was preferred to acetone, however, as greater variability in ELISA results was observed with some of the samples containing acetone.

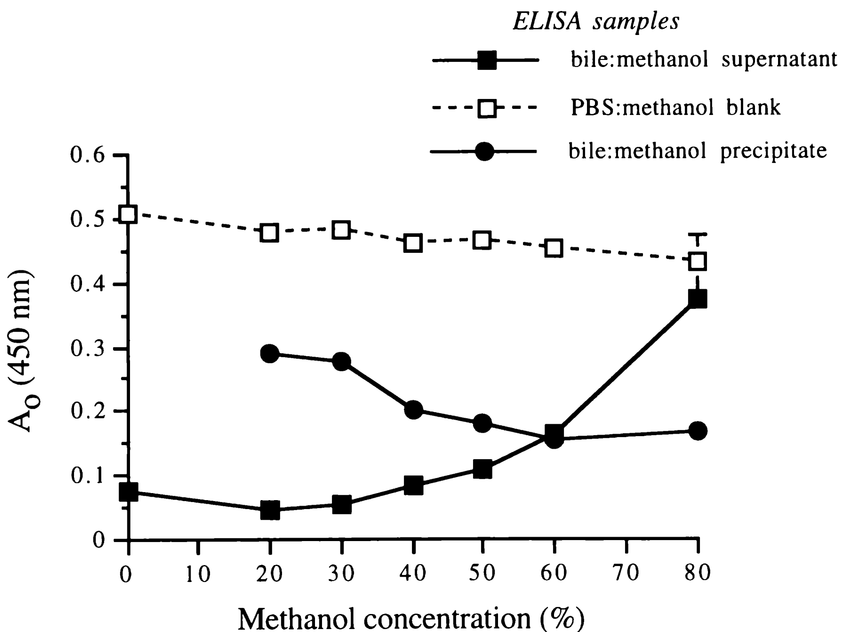
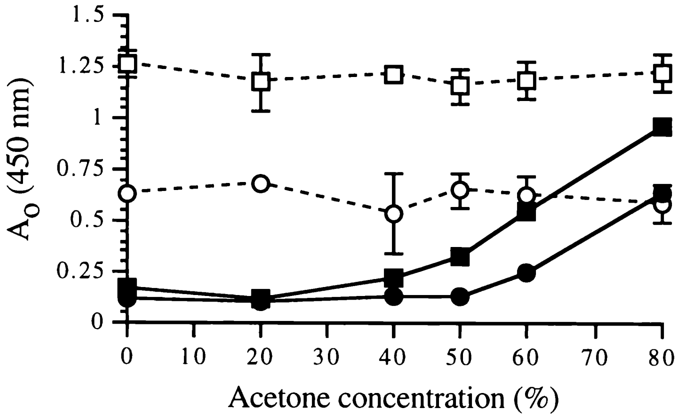


Figure 4.2.13 ELISA of supernatants and precipitates recovered from deproteinisation of bile with methanol. The final dilution of bile (sample 5) in the assay using the group A antibody, was 1:49. Assay and wash buffers contained 0.05% Tween 20 and the assay procedure was as described in Figure 4.2.6. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

(a) precipitation with acetone



ELISA samples

using group A antibody

—■— bile + solvent

- - -□- - - solvent blank

using group B antibody

—●— bile + solvent

- - -○- - - solvent blank

(b) precipitation with ethanol

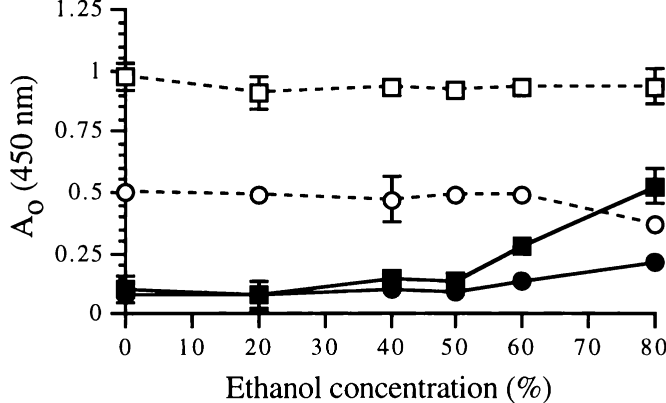


Figure 4.2.14 ELISA of supernatants recovered from deproteinisation of bile sample 5 with (a) acetone and (b) ethanol. The final dilution of bile was 1:49 in the assays using group A and group B antibodies. Tween 20 was included in washing and assay buffers at 0.05% and the assay procedures were as described in Figure 4.2.6. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

As methanol was the solvent of choice, 50 μL of bile samples (1-8) were diluted in 450 μL of methanol to give a mixture containing 90% methanol. After centrifugation at 10 000*g* for 5 min, the supernatant was collected and diluted 1:4 (final dilution 1:49) or 1:9 (final dilution 1:99) in PBS containing 0.05% Tween 20, and each supernatant analysed by ELISA using the group A or group B antibody. The A_O values were expressed as a percentage of those for the same concentrations of methanol in buffer and Tween 20 without bile (100% values) (Figure 4.2.15). Bile which had been deproteinised in methanol caused less matrix interference than that which had only been diluted 1:99 without methanol precipitation (Figure 4.2.2). A slightly better assay result was obtained for some of the samples when the supernatant from methanol precipitation was diluted to give a final dilution of 1:99 rather than to 1:49 and analysed using the group A antibody. The assay using the group B antibody gave similar percentages for both dilutions (Figure 4.2.15).

As it had been shown that bile treated with 90% methanol had reduced matrix effects, the effect of preparing standards in bile prepared by this method on cELISA standard curves was determined. Sporidesmin A standards were prepared in methanol to give concentrations ranging from 0.12 to 60 μg/mL. Each standard (15μL) was diluted in 210 μL of methanol and 25 μL of bile (sample 5) was added. After mixing and centrifugation, 50 μL of the supernatant was added to 450 μL of PBS containing 0.05% Tween 20. In a second set of standards the bile was replaced by PBS and 0.05% Tween 20. The standards were analysed by cELISA and the standard curves obtained with and without bile compared (Figure 4.2.16). The two sets of standard curves were not superimposable.

In an attempt to get a better result the standard curves were produced again, but this time with and without methanol-treated bile in the standard buffer containing 2% Tween 20 instead of 0.05%. As the maximum assay absorbances were expected to be reduced in higher Tween 20 concentrations, to overcome this the incubation time with sporidesmin A and group A or group B antibodies was extended from 2 h to overnight and at 4 °C instead of 20°C.

Although the two sets of curves obtained under these assay conditions (Figure 4.2.17) were similar they were not superimposable so an alternative method to remove assay interferences was still required.

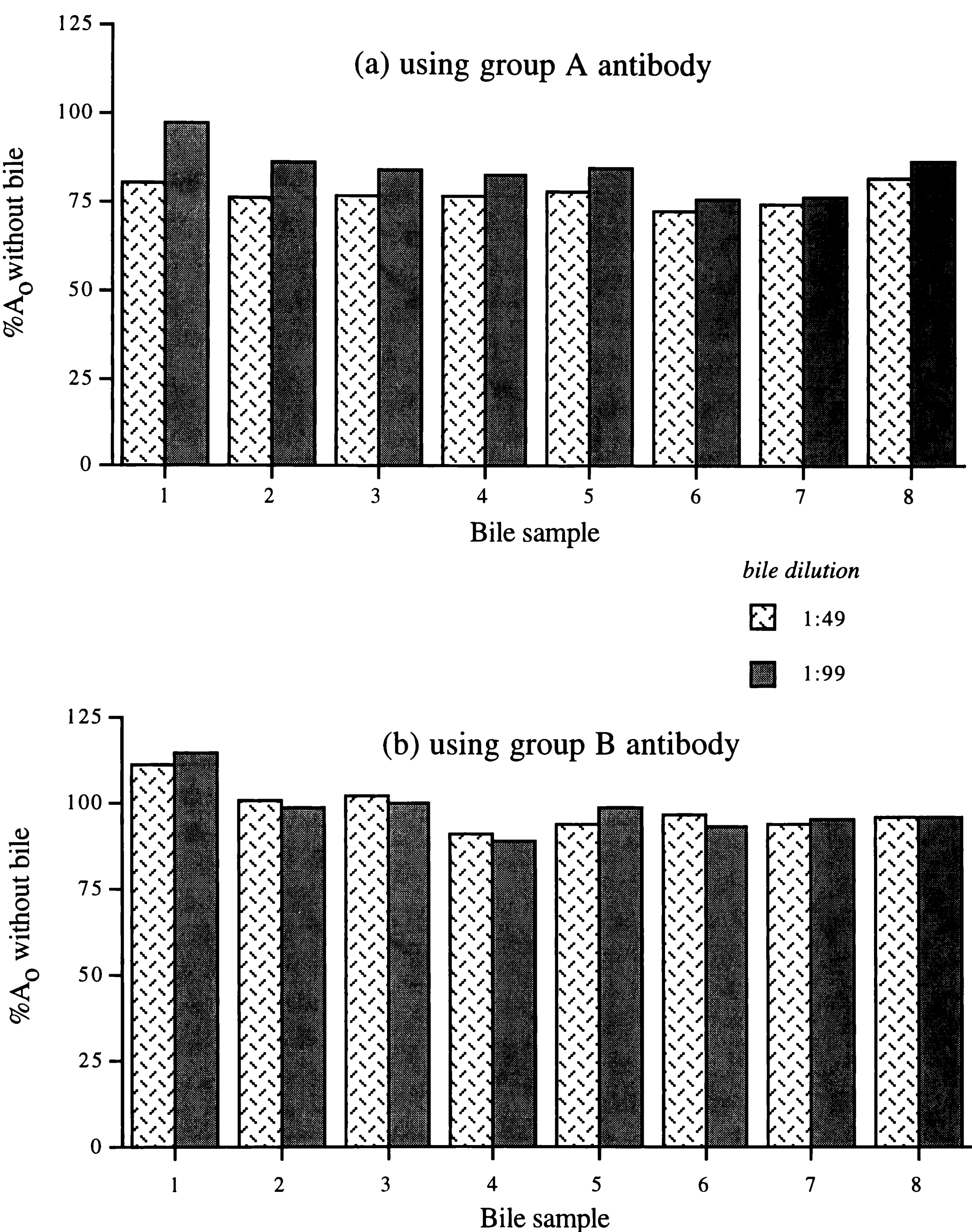


Figure 4.2.15 Effect of deproteinisation of bile with 90% methanol on matrix effects caused by bile in ELISA. Deproteinised bile supernatant was diluted 1:4 or 1:9 in PBS containing 0.05% Tween 20. The 100% A_0 values were obtained by ELISA of samples where PBS and Tween 20 was substituted for bile and were determined in triplicate. The assay procedure was as described in Figure 4.2.6. $\%A_0$ values shown are the mean of duplicate determinations. Standard deviation of $\%A_0$ values ranged from 0.1 to 6.9.

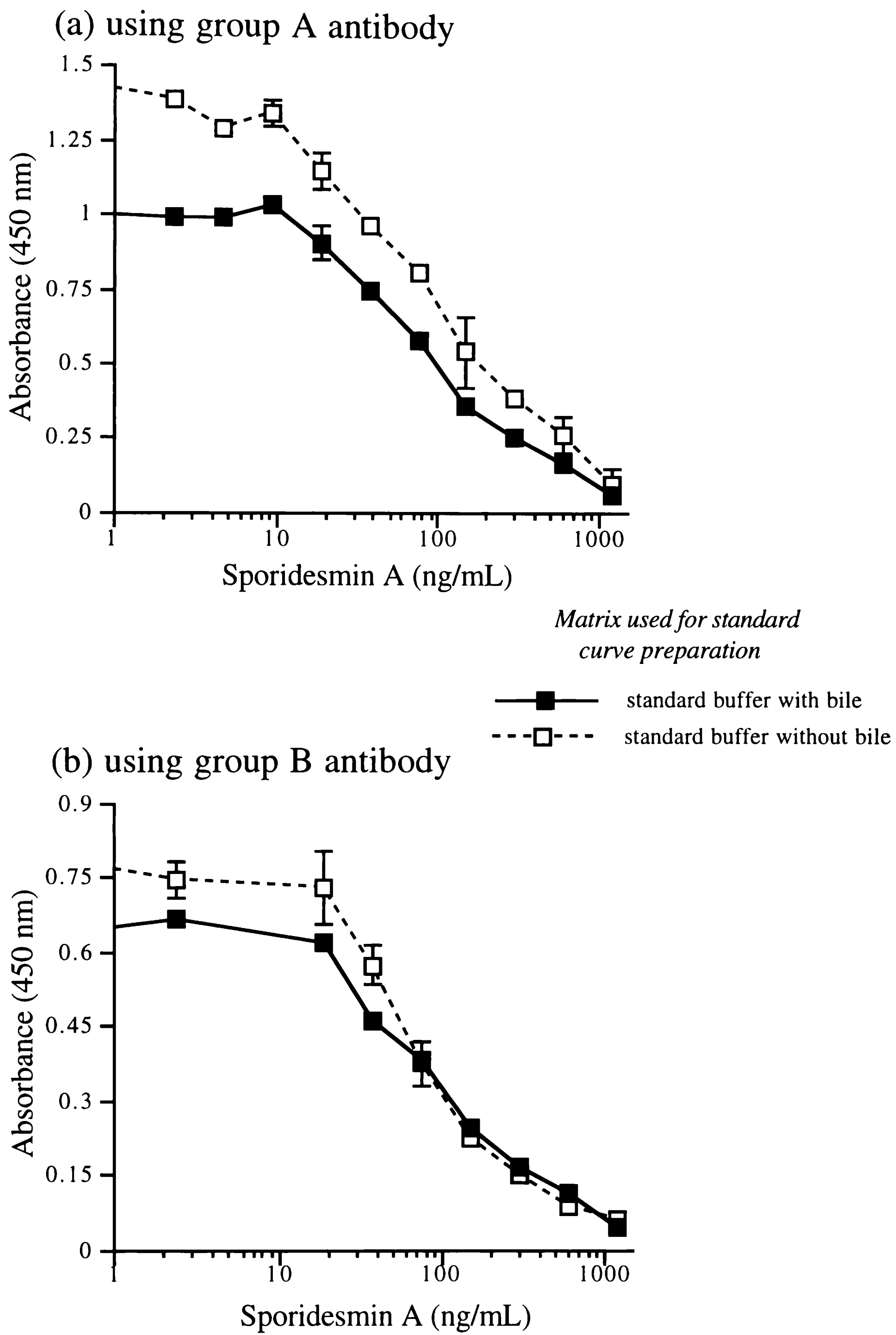


Figure 4.2.16 Effect of deproteinisation of bile with 90% methanol on matrix effects caused by bile in cELISA. Sporidesmin A standard curves were prepared in buffer containing methanol-treated bile (sample 5 final dilution 1:99). The buffer used was PBS containing 0.05% Tween 20 and 9% methanol. The assay procedure was as described in Figure 4.2.6. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

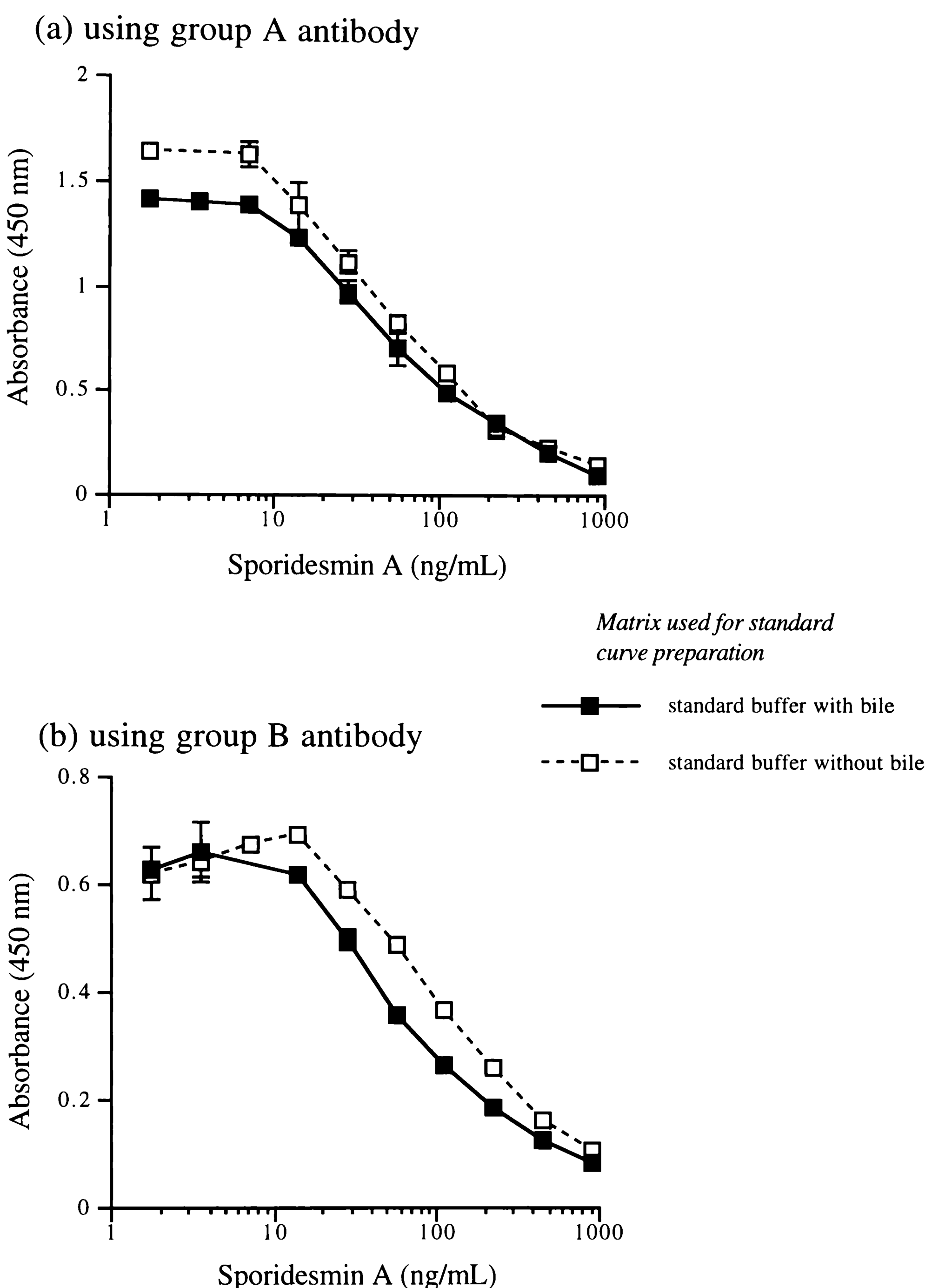


Figure 4.2.17 Effect of deproteinisation of bile with 90% methanol on matrix effects caused by bile in cELISA. Sporidesmin A standards were added to the assay (1) in buffer containing methanol-treated bile (sample 5 final dilution 1:99) and (2) in buffer without bile. The buffer used was PBS containing 2% Tween 20 and 9% methanol. The assay procedure was as described in Figure 4.2.6 except incubation of group A or group B antibody with standard was carried out overnight at 4°C. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

To simplify the investigation it was decided that matrix effects on one assay at a time would be studied, and the assay using the group B antibody was chosen because of the importance of developing a generic assay to detect metabolites and free sporidesmin A. It was also decided that as increased concentrations of Tween 20 in sample buffers had a beneficial effect on assay interferences caused by bile (Figure 4.2.9), the concentration of Tween 20 would be increased for routine use. Previously, however, increasing the Tween 20 concentration from 0.05% to 2% had reduced the A_O value (Figure 4.2.9). To overcome this problem the Tween 20 concentration was increased from 0.05% to 1% rather than to 2%. Also instead of using an extended assay incubation time to increase assay absorbances, which is less convenient for routine use, the coating antigen and antibody concentrations were re-optimised. The coating antigen concentration was increased from 0.67 to 3 µg/mL, and the optimum dilution of antibody required for the assay under these new conditions was determined by producing standard curves for sporidesmin A in cELISA with dilutions of the group B antibody ranging from 1:30 000 to 1:60 000 (Figure 4.2.18). From these results an antibody dilution of 1:60 000 was selected for routine use as it gave an A_O near to 1.0 absorbance unit.

(7) Dilution of sample followed by heating

Dhar and Ali (1992), in developing an ELISA for the quantification of testosterone in serum found that binding of the hormone to serum proteins was eliminated by heating diluted samples at 70°C for 30 min. This procedure was investigated as a method to eliminate non-specific binding to bile components in the ELISA and to remove matrix effects.

The effect of bile dilution followed by heating at 70°C for 30 min, on ELISA matrix effects was determined. This was achieved by taking three sets of two-fold dilutions of bile (sample 5) made in sample buffer consisting of PBS containing 2% methanol and 0.05% Tween 20 (a and c) or 1% Tween 20 (b). Samples in (a) and (b) were heated at 70°C for 30 min and cooled to room temperature while those in (c) were not heated. Each dilution was analysed in ELISA using the group B antibody and compared with the A_O determined using the respective buffer and treatment but without bile.

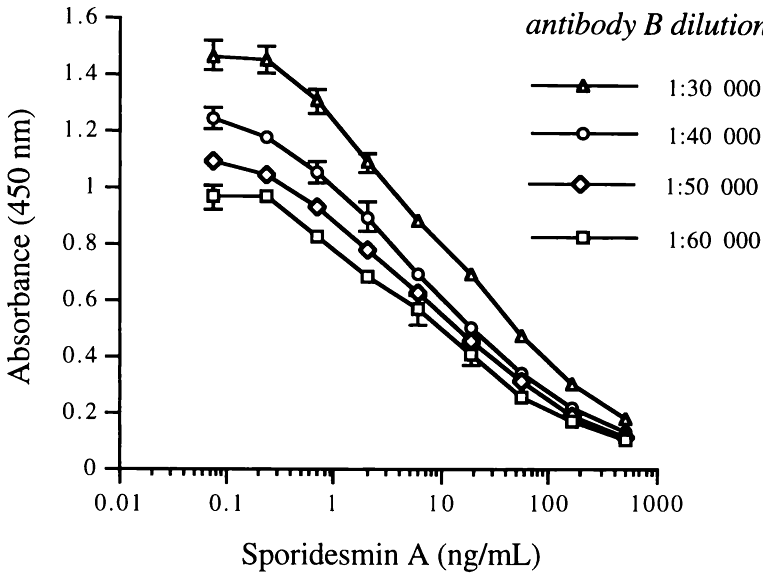


Figure 4.2.18 Standard curves for sporidesmin A determined by cELISA using varying dilutions of the group B antibody. Microtitre plates were coated with sporidesmin A hemisuccinyl 11-ovalbumin at 3 µg/mL. Plates were blocked with 1% BSA for 75 min, incubated with sporidesmin A and the group B antibody for 1.5 h. Incubation with second antibody (diluted 1:3 000) was for 2 h. The standard buffer consisted of PBS containing 1% Tween 20 and 2% methanol and Tween 20 was included in all other assay and washing buffers at 0.05%. Except for coating at 4°C all assay steps were carried out at 21°C. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

The results (Figure 4.2.19) indicated that absorbances for samples diluted 1:31 or more and then heated were close to the values obtained in the same assay without bile. The control samples which were diluted but not heated had much lower A_O values. Thus heating diluted bile samples at 70°C had a significant effect on the assay interferences caused by bile and this was the case when either 1% or 0.05% Tween 20 was included in the buffer, although assay absorbances were less when the sample diluent contained 1% Tween.

So far matrix effects had been studied using gall bladder bile collected from sheep at slaughter. However, when surgical cannulation of sheep bile ducts was carried out, hepatic bile became available. Final assay optimisation and validation was with this bile as research into the metabolism of sporidesmin A required that cELISAs be developed for the quantification of sporidesmin A and its metabolites in hepatic bile.

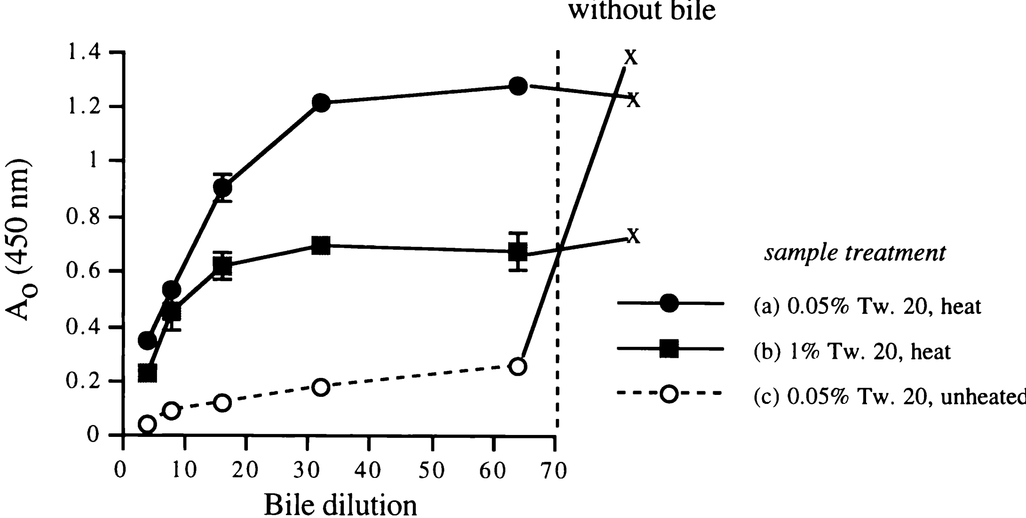


Figure 4.2.19 Effect of bile (sample 5) dilution on ELISA A_O with and without heat treatment of the sample. The assay procedure was as described in Figure 4.2.18 with group B antibody diluted 1:60 000. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

The effect of Tween 20 concentration on matrix effects caused by both hepatic and gall bladder bile in the ELISA using the group B antibody, was examined. Seven gall bladder and three hepatic bile samples were prepared for assay by dilution 1:29 in buffers containing (a) 0.05% and (b) 1% Tween 20, and were heated at 70°C for 30 min. The A_O values measured in ELISA were compared with those for the equivalent sample buffer without bile and these were taken as the 100% values. The absorbances were closer to the 100% values when 1% Tween 20 rather than 0.05% Tween 20 was used in the assay (Figure 4.2.20). The hepatic bile samples were closer to 100% than the gall bladder bile samples, *i.e.* assay interferences were less with the hepatic bile than with the gall bladder bile. This would be expected as the hepatic bile samples were less coloured and less concentrated than the gall bladder bile samples.

The influence of different incubation times on the bile matrix interference was also investigated. The gall bladder and hepatic bile samples were diluted 1:29 in PBS containing 1% Tween 20 and 2% methanol. Diluted samples were heated and analysed by ELISA. The A_O absorbances measured after different incubation times

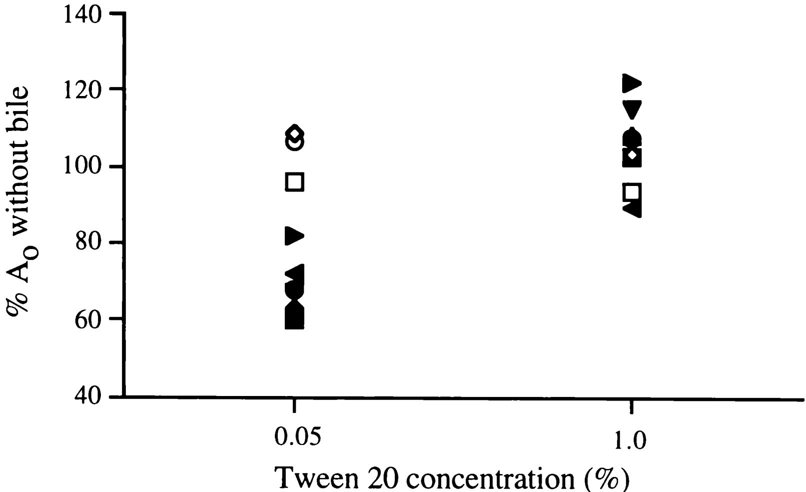


Figure 4.2.20 Effect of Tween 20 concentration in sample buffer on matrix effects caused by various bile samples in the ELISA using the group B antibody. Samples were heated at 70°C for 30 min before assay. Open symbols represent hepatic bile and closed symbols represent gall bladder bile. A_O values, determined for sample buffers without bile, were taken as the 100% values. The assay procedure was as described in Figure 4.2.18 with appropriate changes to Tween 20 concentration in sample buffer. $\%A_O$ values shown are the mean of duplicate determinations and the %CV of absorbance ranged from 0.5 to 7.3 (mean = 3.8).

were compared with those for sample buffer without bile incubated with the antibody for the same time (100% values). The results (Figure 4.2.21) indicated that the shortest incubation time gave a range of ELISA absorbances closer to 100% than those measured with longer incubation times, *i.e.* matrix effects were less with shorter incubation times. The absorbance values obtained with the hepatic bile samples were closer to the 100% values than those obtained with the gall bladder samples.

Having minimised the bile matrix effect on the A_O value the effects on the standard curve were determined to ensure that the position and shape of the curve were not changed by the presence of bile. For this the most concentrated hepatic bile sample available, *i.e.* the most coloured, was chosen. It was diluted 1:39 in buffer containing 1% Tween 20. Sporidesmin A standards diluted in methanol were added to give a final concentration of 2% methanol. The standards were then heated at 70°C for 30 min and cooled to room temperature before assay. The absorbance values for sporidesmin A standards with and without bile were compared. The

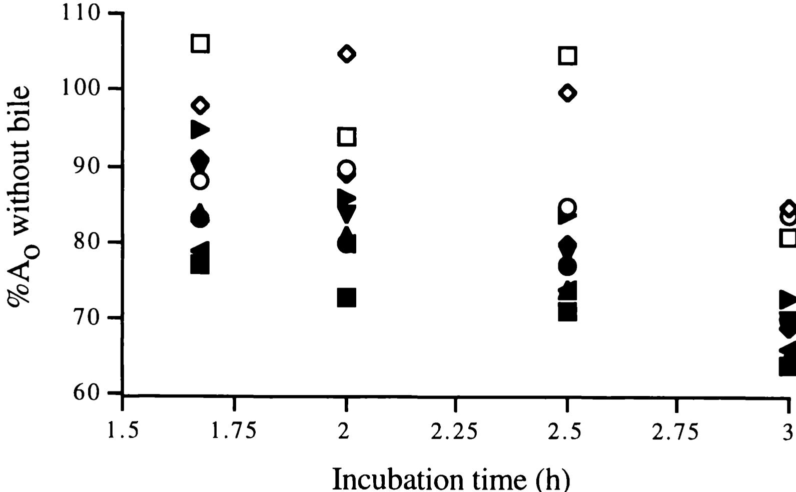


Figure 4.2.21 Effect of incubation time of bile with group B antibody on matrix effects caused by various bile samples in the ELISA. Samples were heated at 70°C for 30 min before assay. Open symbols represent hepatic bile and closed symbols represent gall bladder bile. A_O values, determined for heated samples but without bile, were taken as the 100% values. The assay procedure was as described in Figure 4.2.18 with appropriate changes to incubation times. %A_O values shown are the mean of duplicate determinations and the %CV of absorbance ranged from 0.1 to 8.4 (mean = 3.3).

standard curves appeared to be superimposable (Figure 4.2.22) which indicated that matrix effects had been removed by dilution and heat treatment.

To determine more precisely which bile dilution should be used in the assay a pooled bile sample was diluted 1:39 and 1:49 and spiked with sporidesmin A in methanol (final methanol concentration 2%). Bile-free diluent was spiked with the same sporidesmin A concentrations and both sets of samples were analysed by cELISA. The sporidesmin A spike concentrations (listed in Table 4.2.2) were within the working range of the standard curve which, by convention was taken to be the analyte concentration giving between 80 and 20% of the A_O. The results (Table 4.2.2) indicated that sporidesmin A measured in both dilutions of bile were within the limits of 100 (± 20)% (commonly taken as the minimum acceptable assay precision) of that measured in bile-free dilutent. Therefore, dilution of bile 1:39 and 1:49 in sample buffer followed by heat treatment removed cELISA matrix effects caused by the pooled hepatic bile.

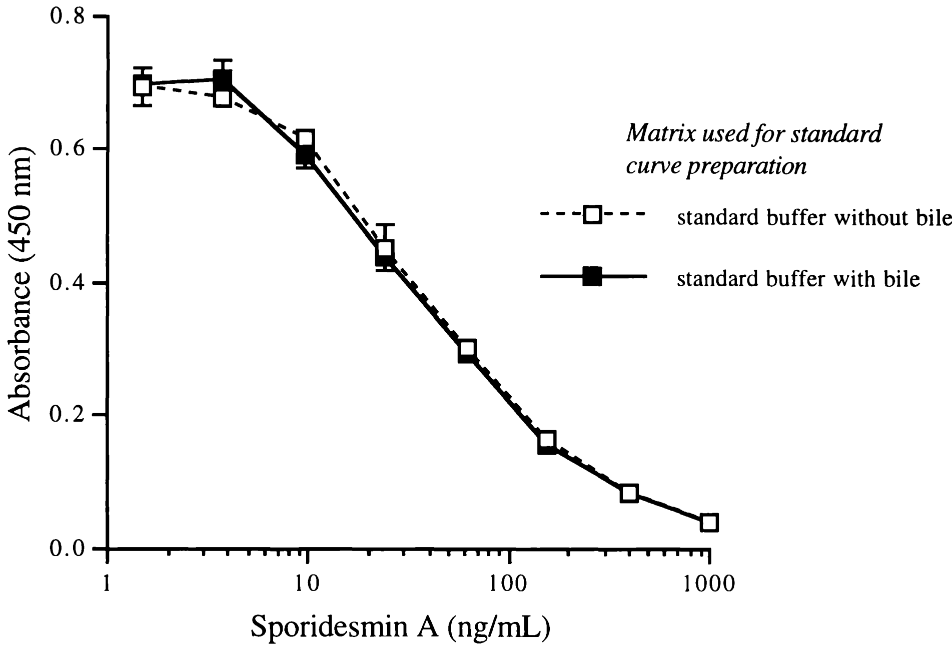


Figure 4.2.22 Effect of heat treatment of diluted bile on matrix effects in cELISA using the group B antibody. Sporidesmin A standards were prepared (a) in bile diluted 1:39 in standard buffer (PBS containing 1% Tween 20 and 2% methanol) and (b) in standard buffer without bile. Standards were heated at 70°C for 30 min and cooled to room temperature before assay. The assay procedure was as described in Figure 4.2.18 except incubation time for antibody with sporidesmin A was reduced from 1.5 to 1 h. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

Table 4.2.2 The effect of hepatic bile on sporidesmin A measured by cELISA using the group B antibody

sporidesmin A added	sporidesmin A measured with hepatic bile[a]					
	diluted 1:39			diluted 1:49		
ng/mL[b]	ng/mL[b]	%	%CV[c]	ng/mL[b]	%	%CV[c]
110.9	106.8	96	2.4	114.2	103	4.4
36.9	42.8	116	2.4	36.1	98	6.8
12.3	16.9	137	0.5	11.3	92	1.2

[a] Hepatic bile collected by cannulation was included in standards. [b] Represents the sporidesmin A concentration in the assay wells and values shown for bile are the mean of duplicate determinations. [c] Indicates the cELISA interwell %CV.

To determine if the same sample buffer and heat treatment was suitable for gall bladder bile, the experiment was repeated with three of the gall bladder samples studied earlier. The pooled hepatic bile sample was also included as a positive control. Bile was diluted 1:49 before heating and analysis by cELISA using the group B antibody. The sporidesmin A concentrations determined in the presence of bile was within the limits of 100 (± 20)% (Table 4.2.3) of those in the absence of bile indicating that the method was also suitable for gall bladder bile.

Table 4.2.3 The effect of hepatic and gall bladder bile on sporidesmin A measured by cELISA using the group B antibody

sporidesmin A added	% of sporidesmin A measured with bile (diluted 1:49)							
ng/mL[a]	GB2[b]	%CV[c]	GB3	%CV	GB4	%CV	hepatic[d]	%CV
62.50	103	6.1	101	8.4	114	0.5	88	3.3
15.63	110	1.3	120	3.7	105	0.9	89	2.5

[a] Indicates the concentration in the assay wells, and values shown for bile are the mean of duplicate determinations. [b] Gall bladder bile samples collected at slaughter. [c] Indicates the cELISA interwell %CV of duplicate analyses. [d] Represents a pooled hepatic bile sample collected by surgical cannulation.

It was therefore concluded that matrix effects in the cELISA using the group B antibody could be removed if hepatic or gall bladder bile was diluted 1:49 in sample buffer (PBS containing 1% Tween 20 and 2% methanol), heated at 70°C for 30 min and cooled to room temperature before analysis.

cELISA with group A antibody

The sample preparation method was then applied to the development of cELISA of hepatic bile using the group A antibody. Optimisation of plate coating concentration in the presence of 1% Tween 20 was first necessary. A plate was coated overnight with 100 μL of sporidesmin A hemisuccinyl 11-ovalbumin with concentrations ranging from 0.04-5.0 μg/mL. The plate was blocked with 1% BSA for 75 min. Hepatic bile was diluted 1:39 in sample buffer (PBS containing 1% Tween 20 and 2% methanol), heated at 70°C for 30 min and cooled to room temperature before addition to the plate. This was followed by the group A antibody diluted at 1:80 000. The results from ELISA (Figure 4.2.23) indicated that 2 μg/mL was the optimum concentration of coating antigen, as at this concentration maximum A_O were recorded.

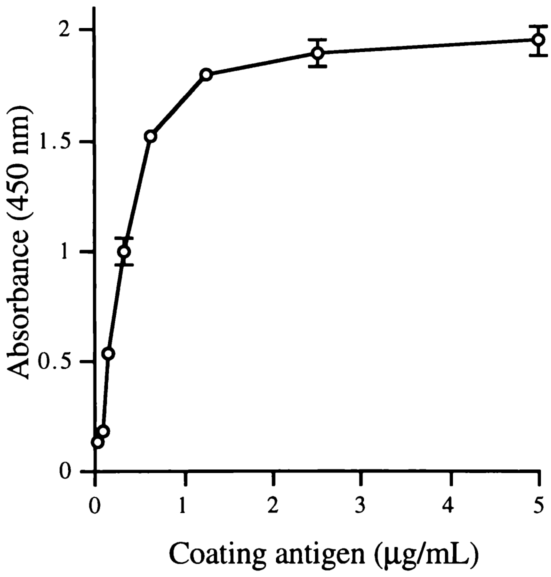


Figure 4.2.23 Effect of sporidesmin A hemisuccinyl 11-ovalbumin concentration on absorbances measured in ELISA using the group A antibody. The assay procedure was as described in Figure 4.2.18 except the group A antibody was diluted 1:80 000 and the antibody was incubated for 1 h with sample buffer containing heated bile. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

The optimum dilution of antibody required for the assay under these coating conditions was then determined. This was achieved by determining cELISA standard curves for sporidesmin A with dilutions of the group A antibody ranging from 1:200 000 to 1:400 000. Antibody dilution of 1:375 000 was selected for routine use as this gave maximum absorbances near 1.0 absorbance unit.

To determine if dilution of hepatic bile followed by heating was suitable for the cELISA using the group A antibody, standards were added in methanol to buffer (PBS containing 1% Tween 20 and 1% methanol) and to buffer containing pooled hepatic bile diluted 1:39, 1:49 and 1:99. The standards were heated at 70°C for 30 min before cELISA. The amounts of sporidesmin A measured in bile, which were in the working range of the assay, were compared with the amounts measured in buffer that were taken to be the 100% values (Figure 4.2.24). The means of the recoveries of sporidesmin A measured in bile diluted 1:49 and 1:99 (102.9 ± 11.5% and 94.4 ± 10.9 respectively) were closer to 100% than the mean measured for that recovered from bile diluted 1:39 (115.1 ± 2.5).

The results obtained with bile diluted 1:49 were within the limits of 100 (± 20)% and therefore had acceptable assay precision. It was concluded that matrix effects could be removed from hepatic bile by dilution 1:49 in sample buffer (PBS containing 1% Tween 20 and 2% methanol), heated at 70°C for 30 min and cooled to room temperature. This would enable sporidesmin A to be measured by cELISA using the group A antibody.

4.2.5 Assay optimisation

During the process of overcoming matrix effects the assay was frequently modified and individual steps optimised, but final optimisation was completed before assay validation began.

For group B antibody:

For optimisation of plate-coating in the presence of 1% Tween 20, a plate was coated overnight with 100 μL of sporidesmin A hemisuccinyl 11-ovalbumin with concentrations ranging from 0.28 to 10.0 μg/mL. The assay was as described previously (Figure 4.2.18) except that the hepatic bile, which was diluted 1:49 in sample buffer (PBS containing 1% Tween 20 and 2% methanol) and heated, was added to the plate before the group B antibody. The results (Figure 4.2.25) indicated that 10 μg/mL was the minimum coating antigen concentration giving maximum

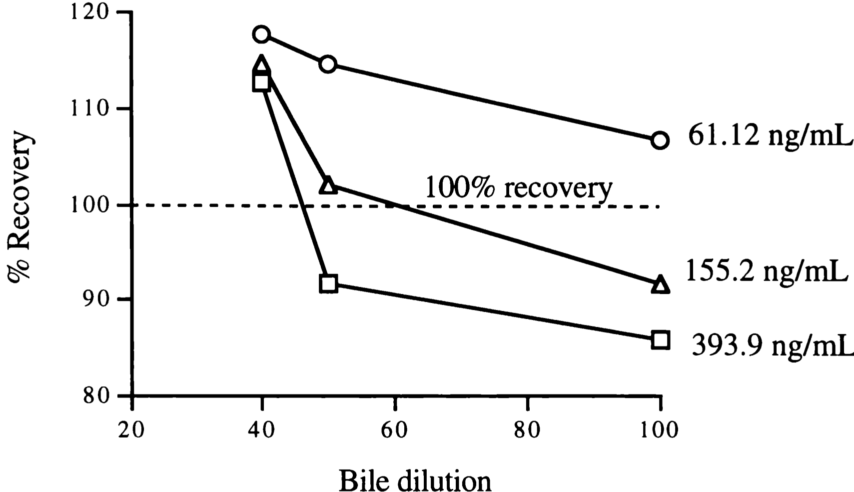


Figure 4..2.24 Effect of matrix dilution on the apparent recovery of sporidesmin A from bile determined by cELISA using the group A antibody. Values for standards measured in hepatic bile diluted in buffer (PBS containing 1% Tween 20 and 2% methanol) were compared with those measured in buffer. All samples were heated at 70°C for 30 min before cELISA. Microtitre plates were coated with sporidesmin A hemisuccinyl 11-ovalbumin at 2 µg/mL. Plates were blocked with 1% BSA for 75 min, and incubated with sporidesmin A and the group A antibody (diluted 1:375 000) for 1.0 h. Incubation with labelled second antibody (diluted 1:2 500) was for 2 h. Tween 20 was included in all other assay and washing buffers at 0.05%. Except for coating at 4°C all assay steps were carried out at 21°C. Values shown are the mean of duplicate determinations. cELISA interwell %CV ranged from 0.3 to 7.3 (mean = 3.4).

absorbance in the ELISA. However, as the availability of conjugate was limited, 4 µg/mL was the concentration chosen for routine use.

Initially, second antibody (anti-mouse-HRP) was diluted within the range recommended by the manufacturer, and therefore optimisation for the cELISA using the group B antibody was required. To do this standards were prepared in buffer containing hepatic bile, diluted 1:49 and heated at 70°C for 30 min. Four standard curves for sporidesmin A were determined using the cELISA and the second antibody diluted 1:2 000, 1:2 500, 1:3 000 and 1:4 000, respectively (Figure 4.2.26). The group B antibody was diluted to 1:75 000 so that with coating antigen concentration increased, the A_0 would be near to 1.0 absorbance unit and the assay sensitivity would be retained. Absorbance values recorded for the standard curve using second antibody at 1:2 000 were significantly higher than those for the standard curve using second antibody at higher dilutions, and therefore 1:2 000 was

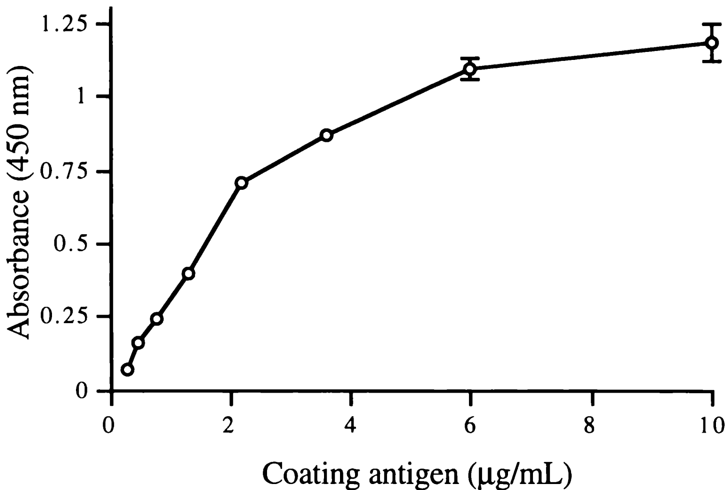


Figure 4.2.25 Effect of coating antigen concentration on absorbances measured in ELISA using the group B antibody. The assay procedure was as described in Figure 4.2.18 except the antibody (1:60 000) was incubated for 1 h with hepatic bile diluted 1:49 in sample buffer. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

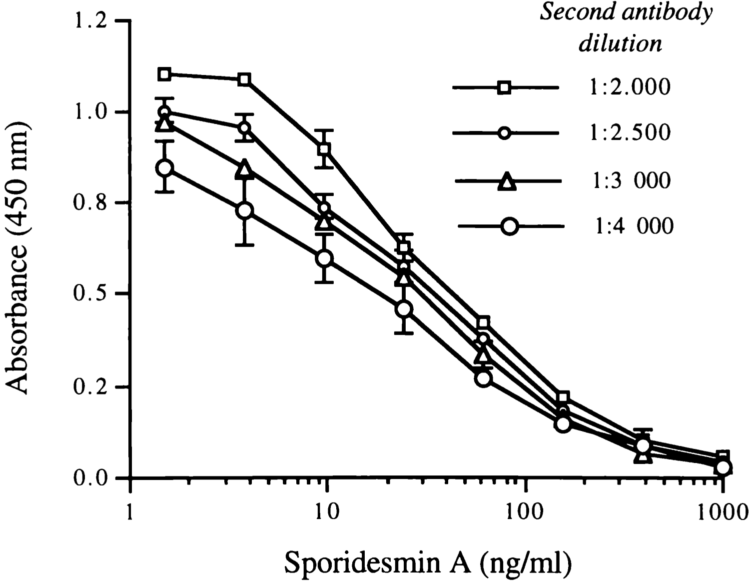


Figure 4.2.26 Effect of HRP-labelled second antibody concentration on absorbances measured in cELISA for sporidesmin A using group B antibody in the presence of hepatic bile (1:49). Assay procedure was as described in Figure 4.2.25 except coating antigen was used at 4 µg/mL and group B antibody diluted 1:75 000. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

chosen for routine use. It was also noticed that the result for the assay blank, where group B antibody was omitted from the assay and replaced with antibody buffer, was the same for all concentrations of second antibody, *i.e.* 0.05 absorbance unit. This indicated that non-specific binding of the second antibody to the plate, coating antigen and or blocker had not increased with the increased concentration of the second antibody.

When all of the assay conditions had been optimised, one final check was made to determine if the dilution of the group B antibody selected for use would give the required assay performance. To do this the binding curve for the group B antibody was determined so that the position of the selected dilution (1:75 000) on this curve could be examined. Ideally this should be where slight changes in the amount of antibody bound to coating antigen brought about by binding to sample analyte, gives maximum absorbance change and therefore maximum assay response and precision. The antibody binding curve was determined by measuring ELISA absorbances with two-fold serial dilutions of group B antibody ranging from 1:500 to 1:512 000. Assay absorbance values were expressed as a percentage of maximum absorbance measured in the presence of excess antibody (Figure 4.2.27). The A_O for the selected antibody dilution (1:75 000), was near to 30% of maximum absorbance and as this was still positioned on the steep part of the binding curve, minimum change in antibody concentration should result in maximum response in assay absorbance.

(2) Group A antibody

For convenience the dilution of labelled second antibody used was changed to 1:2 000 so that the dilution was the same for both assays. The dilution (1:375 000) of the group A antibody was also checked to determine if this would give optimal assay response by examining the binding curve for the antibody. ELISA absorbances (A_O) were measured for two-fold serial dilutions of group A antibody ranging from 1:8 000 to 1:4 096 000. The results (Figure 4.2.28) indicated that the A_O for the dilution chosen for routine use was near to 30% of maximum absorbance and was positioned on the steep part of the curve where small changes in antibody concentration with binding to analyte would give maximum change to assay absorbance.

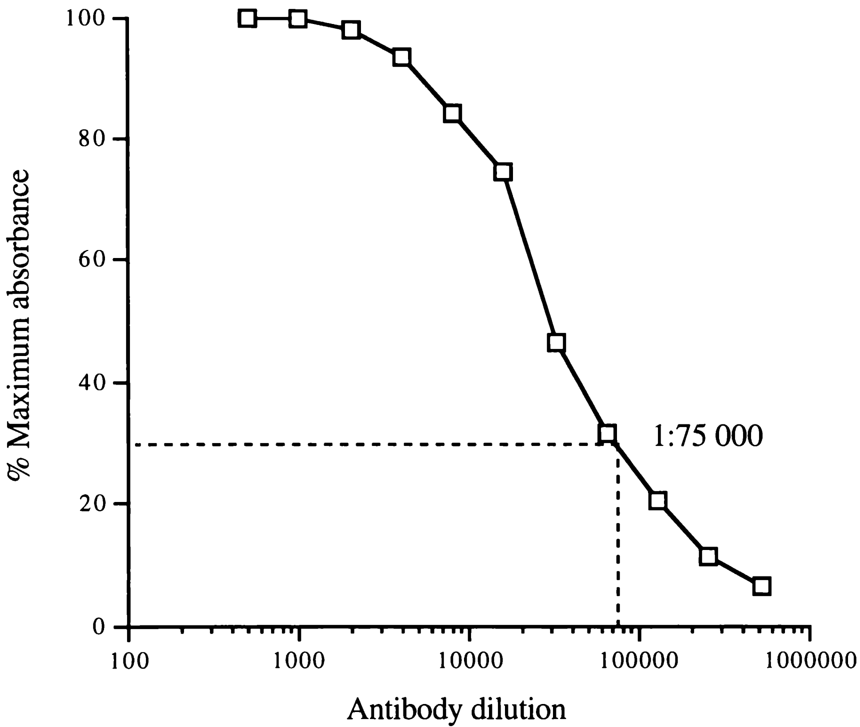


Figure 4.2.27 Group B antibody binding to sporidesmin A hemisuccinyl 11-ovalbumin (4 μg/mL) measured by ELISA. The assay procedure was as described in Figure 4.2.26 except sample buffer (PBS containing 1% Tween 20 and 2% methanol) and group B antibody were incubated for 1 h and second antibody was diluted 1:2000. ELISA absorbance values shown are the mean of duplicate determinations and the standard deviation ranged from 0.4 to 3.8% maximum absorbance (mean = 2.1%).

4.2.6. Assay validation and performance characteristics

Having optimised the assay conditions, the assays for determining sporidesmin A and metabolites in bile were then validated and characterised. Important assay performance characteristics include accuracy, sensitivity and precision. Accuracy is the ability of an assay to give the correct result without interference from other cross-reacting molecules and sensitivity is required for the quantification of low levels of compounds of interest in a specific matrix. Precision is the closeness of agreement between independent test results obtained from repeated measurements. A standard curve which has a relatively high A_0 and a steep slope will give an assay with high accuracy and precision, while a low I_{50}, will give an assay with good sensitivity.

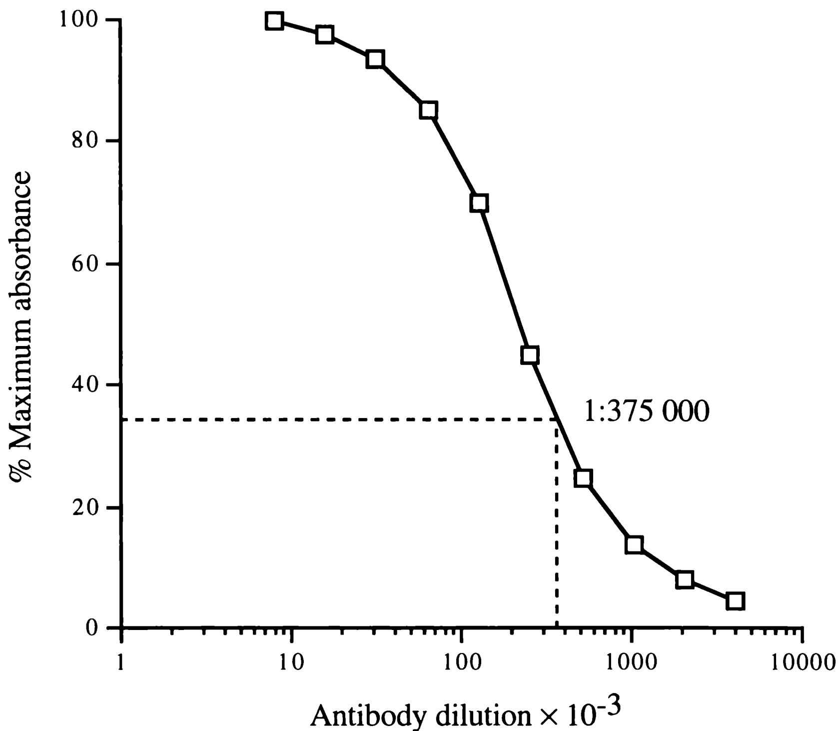


Figure 4.2.28 Group A antibody binding to sporidesmin A hemisuccinyl 11-ovalbumin (2 μg/mL) measured by ELISA. The assay procedure was as described in Figure 4.2.24 except sample buffer and group A antibody were incubated for 1 h and second antibody was diluted 1:2000. A single analysis of each sample was carried out.

The accuracy of a newly developed immunoassay method can be tested by comparison with an established reference method. Hepatic bile samples which were obtained from animal dosing experiments gave positive results in the sporidesmin A-specific cELISA, *i.e.* using the group A antibody. As there is an established HPLC method for the quantification of sporidesmin A (Section 2.12), the samples were analysed by both methods, the results plotted against one another and the correlation coefficient estimated by linear regression analysis (Figure 4.2.29). Quantification of sporidesmin A by cELISA was correlated with HPLC results giving a slope of 0.93.

Assay sensitivity was optimised in the preceding sections. Examination of the standard curve for the cELISA for bile using the group A antibody (Figure 4.2.30), provided information concerning assay sensitivity. The assay I_{50} for sporidesmin A, which indicates the assay sensitivity without taking sample dilution into account,

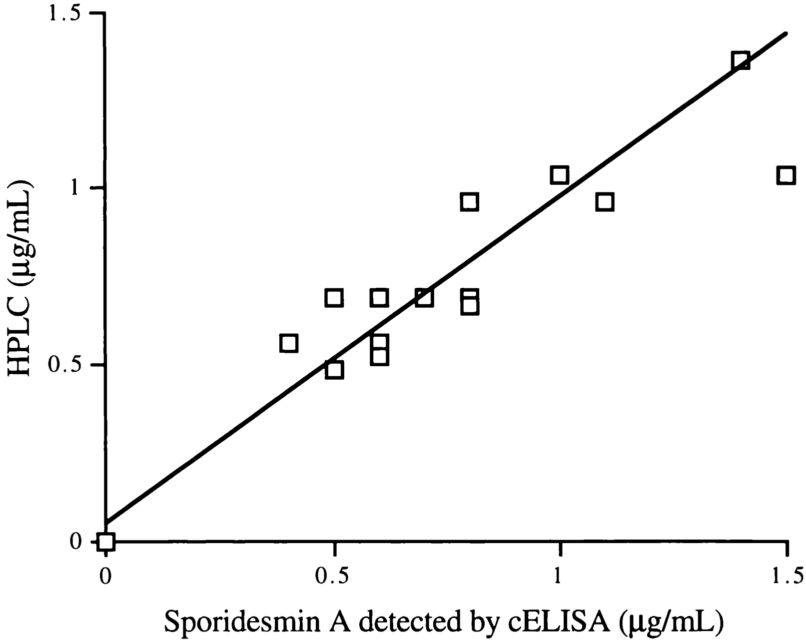


Figure 4.2.29 Correlation between cELISA (using group A antibody) and HPLC measurement of sporidesmin A in hepatic bile from sheep. Regression data: n = 14; r^2 = 0.95; slope = 0.93. cELISA results shown are the mean of duplicate determinations.

was 40 ng/mL. The effective working range (defined as the analyte concentration in the original sample equivalent to 20 and 80% of the maximum absorbance) was 0.5 to 20 μg of sporidesmin A per mL of bile.

The optimised assay was validated by spiking bile with sporidesmin A standards using the number of replicates and method described in Section 2.9. Percentage recoveries of sporidesmin A from bile spiked with 10, 5, and 1.5 μg/mL were 85.6 ± 5.2, 89.1 ± 3.8, and 114.6 ± 14.8, respectively, mean recovery 96.4 ± 15.8. The mean intra-assay coefficient of variation was 11.7%, while the mean inter-assay variation was 7.8%.

The standard curve for the cELISA of bile using the group B antibody (Figure 4.2.31) had an assay I_{50} for sporidesmin A and sporidesmin D of 30.0 ng/mL and 0.7 ng/mL, respectively. The effective sample working range was 500 to 10 000 ng of sporidesmin A/mL of bile, and with a sporidesmin D standard the range was 15 to 300 ng/mL.

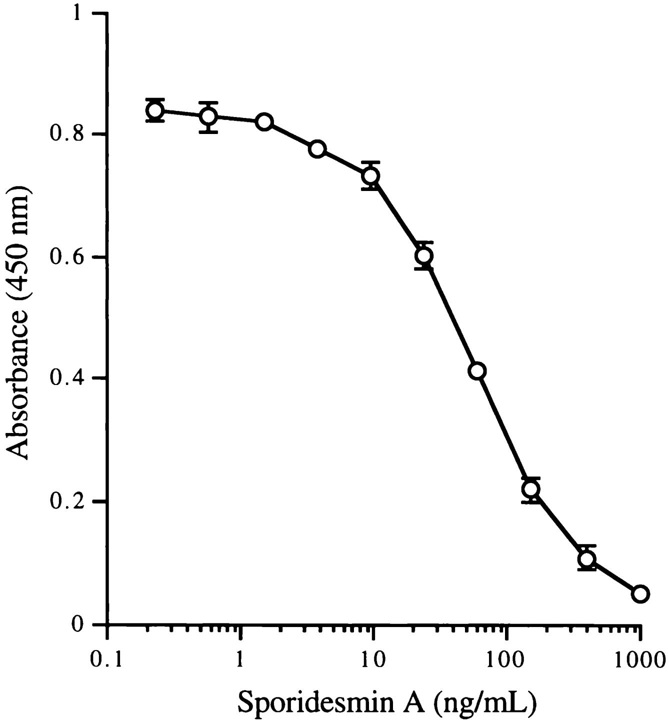


Figure 4.2.30 Standard curve for the quantification of sporidesmin A in bile by cELISA using the group A antibody. Microtitre plates were coated with sporidesmin A hemisuccinyl 11-ovalbumin (2 μg/mL) overnight. Plates were blocked with 1% BSA for 75 min and incubated with standard or sample and group A antibody (diluted 1: 375 000) for 1 h. Samples and standards were diluted 1:49 and 1:99 in PBS containing 1% Tween 20 and 2% methanol and heated at 70°C for 30 min before cELISA. Incubation with second antibody (diluted 1:2 000) was for 2 h. Tween 20 was included in all other assay and washing buffers at 0.05% and all assay steps were carried out at 21°C. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

Percentage recoveries of sporidesmin A from spiked bile containing 10, 5, 4, 3, 2.5, and 1.5 μg/mL were 95.6 ± 9.7, 85.7 ± 7.2, 105.9 ± 9.0, 106.9 ± 8.4, 79.8 ± 13.2 and 122 ± 7.8 respectively, mean recovery 99.3 ± 15.5. Mean intra-assay (intra-plate) coefficient of variation was 7.6%, while inter-assay (inter-plate) variation was 5.2%. A standard bile sample was assayed on every ELISA plate and the mean daily inter-assay coefficient of variation for this sample over five assays was 9.1%. The cELISA using the group B antibody detected both sporidesmins A and D and could detect metabolite(s).

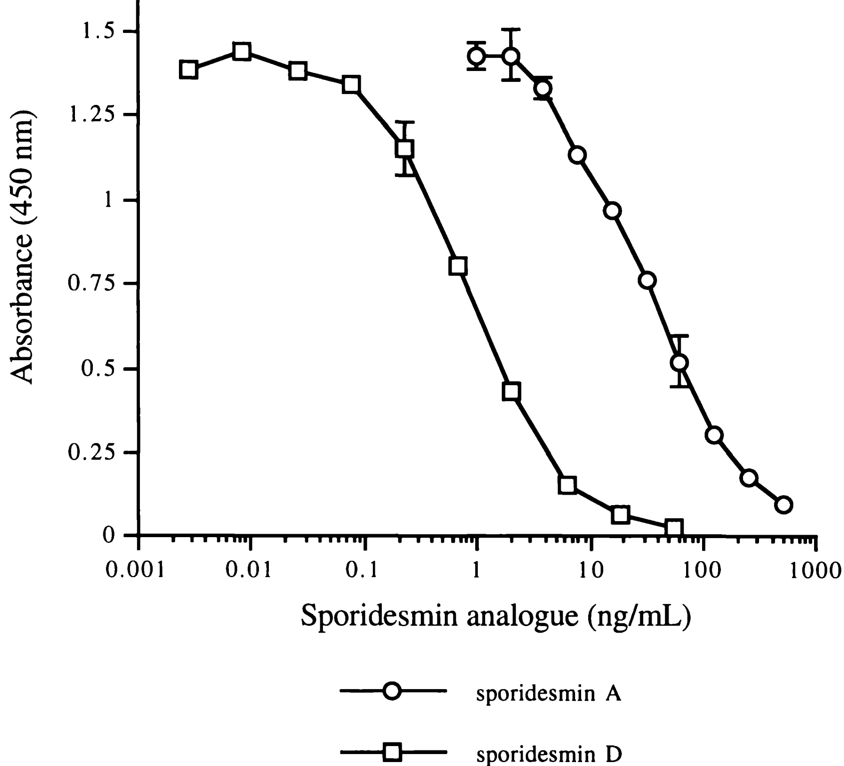


Figure 4.2.31 Standard curves for the quantification of sporidesmin A in hepatic bile by cELISA using the group B antibody. The assay procedure was as described for Figure 4.2.30 except microtitre plates were coated with 4 μg/mL of sporidesmin A hemisuccinyl 11-ovalbumin and group B antibody was diluted 1:75 000. ELISA absorbance values shown are the mean of duplicate determinations with error bars showing standard deviation.

4.3 Immunoassay of urine

Preliminary investigations with cELISA using the group A antibody indicated that unmetabolised sporidesmin A was not present in urine collected from sheep dosed with sporidesmin A (0.4 and 0.2 mg of sporidesmin A per kg of body weight). Sporidesmin A was also not detected by HPLC using the method described in Section 2.12 where the limit of detection was 45 ng per mL of urine. Sporidesmin metabolite, however, could be detected with the cELISA using the group B antibody. Therefore, only the cELISA using group B antibody was optimised and validated for use with urine samples.

4.3.1 Matrix effects

Assay development was carried out on urine samples collected from sheep which had not been exposed to contaminated pastures. To determine if the level of interference caused by each urine sample was similar, six samples were diluted in sample buffer (PBS containing 0.05% Tween 20) to give a range of dilutions from 1:9 to 1:639, which were then analysed by ELISA.

The absorbances (A_O) measured in ELISA, following the procedure described in Section 2.4.1, were compared with the absorbance measured with sample buffer alone (Figure 4.3.1). Except for sample 3, the assay interferences caused by each urine sample were similar and were greatly reduced by dilution. However, the absorbance was still less than that measured with sample buffer alone even after dilution to 1:639.

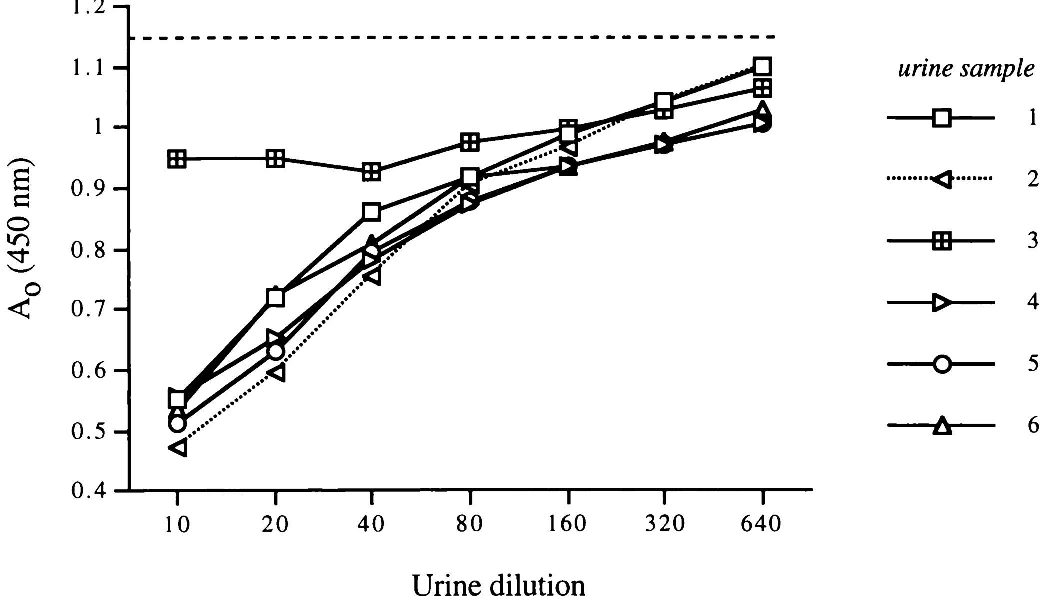


Figure 4.3.1 Effect of sample dilution on matrix interference caused by urine on the ELISA A_O using the group B antibody. Microtitre plates were incubated overnight with sporidesmin A hemisuccinyl 11-ovalbumin diluted (2 μg/mL) in coating buffer. Plates were blocked with 1% BSA for 75 min and incubated with sample buffer (PBS containing 0.05% Tween 20) and group B antibody (diluted 1:60 000 in antibody buffer) for 1 h. Incubation with second antibody (diluted 1:3 000 in antibody buffer) was for 2 h. Tween 20 was included in washing and assay buffers at 0.05%. Except for coating at 4°C all other assay steps were carried out at 21°C. Five replicate determinations (%CV = 4.9) of samples consisting of sample buffer alone gave the absorbance value marked (-----). Single analysis of each urine sample was carried out.

An investigation was undertaken to see if matrix effects could be removed by altering the assay conditions so that extensive sample dilution was avoided to retain assay sensitivity. Six samples were combined to give a pooled sample, and a pair of standard curves for sporidesmin A were generated, one in urine diluted 1:19 in buffer, and the other in buffer without urine. For each pair of curves a variety of changes were made to the composition of the standard buffer, that is to the Tween 20 concentration, methanol concentration and the inclusion of 1% BSA or 1% ovalbumin. The effect of heating the standards before assay was also investigated. Four plates were analysed, each having three pairs of standard curves using the assay conditions shown in Table 4.3.1.

Table 4.3.1 Effect of sample buffer composition and sample heating on matrix effects caused by urine in cELISA

matrix number	sample buffer components			sample heated at 70°C	standard curves superimposable
	Tween 20 (%)	2% methanol	1% BSA		
Plate 1:					
1a	0.05	✓			
1b	1	✓	✓		+
1c	1	✓			
Plate 2:					
2a	1	10% MeOH	✓		
2b	1	✓	✓		+
2c	0.05	✓	✓		+
Plate 3:					
3a	1	✓	✓	✓	
3b	1	✓	✓		+
3c	1	✓	1% OVA	✓	
Plate 4:					
4a	0.05	✓	✓	✓	+
4b	0.05	✓	✓		+
4c	1	✓	✓		+

+ Indicates assay conditions giving a standard curve generated in urine most similar to that generated in buffer using the same conditions. OVA represents ovalbumin.

Each standard was analysed in duplicate and the standard curves generated in urine were compared with those generated in the equivalent buffer without urine (Figure 4.3.2). Changes in assay conditions that improved the coincidence of the standard curves, especially over the working range A_{20}-A_{80}, were considered beneficial in removing matrix effects. The plates were run separately and the results from each were analysed before proceeding to using different conditions on the next plate.

Plate 1

Curves pairs determined with 1% Tween 20 and 1% BSA in standard buffers (Figure 4.3.2, plate 1b) were more similar than those with 0.05 or 1% Tween 20 without BSA (plate 1a, c).

Plate 2

Curves pairs determined with 2% methanol and 1% BSA, 0.05 or 1% Tween 20 in standard buffers (plate 2b, c) were slightly more similar than those determined with 10% methanol and 1% BSA, 1% Tween 20 (plate 2a).

Plate 3

Curves pairs determined with 1% BSA in standard buffer without heating the standards before assay (plate 3b), were more similar than those determined with 1% BSA or 1% ovalbumin in standard buffer and heating of standards at 70°C for 30 min before assay (plate 3a, c). The curve pair with BSA in standard buffer and heating (plate3a) were more similar than those with ovalbumin and heating (plate 3c). The three curve pairs were determined with 1% Tween 20 in standard buffers.

Plate 4

When curve pairs were determined with 0.05% Tween 20, 2% methanol and 1% BSA in standard buffers, curve pair similarity did not appear to be affected by standards being heated before assay (plate 4a *c.f.* 4b). When curve pairs were determined with 2% methanol and 1% BSA in standard buffers and without heating before assay, curve pair similarity was not affected by the Tween 20 concentration in the standard buffer (plate 4b *c.f.* 4c)

As the standard curve measured in 0.05% Tween 20 (Figure 4.3.2, Plate 4b) gave a more sensitive assay than the one measured in 1% Tween 20 (Plate 4c), *i.e.* the I_{50} was less for plate 4b, standard buffer which was PBS containing 0.05% Tween 20, 1% BSA and 2% methanol was selected for use in the urine assay.

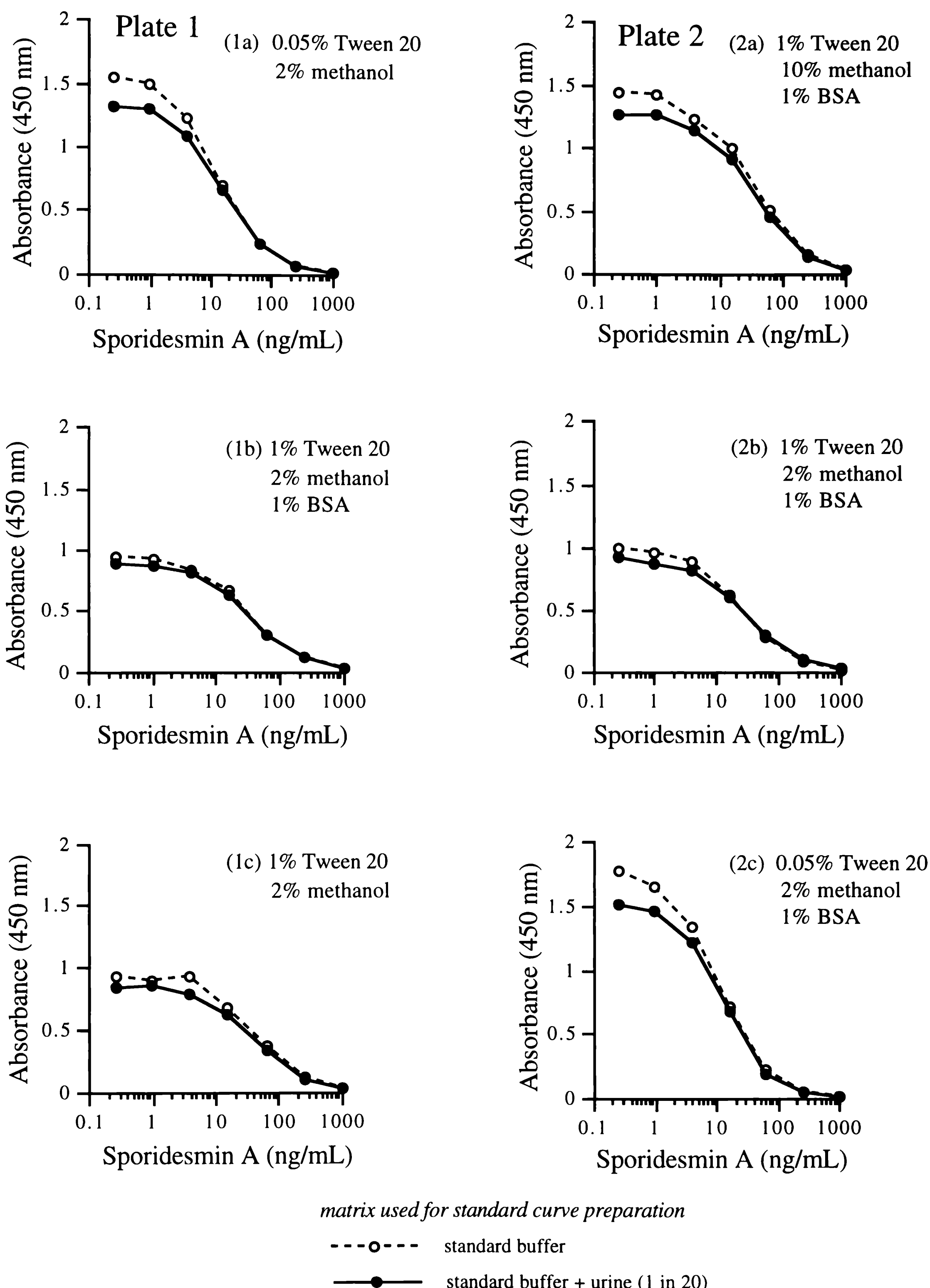


Figure 4.3.2 Effect of assay conditions on matrix effects caused by urine in cELISA. Standard curves for sporidesmin A were generated in pooled urine diluted 1:19 in buffer and compared with those generated in the same buffer without urine. Variations to standard buffer compositions are indicated on each graph *i.e.* (1a-c) and (2a-c). The assay procedure was as described in Figure 4.3.1.

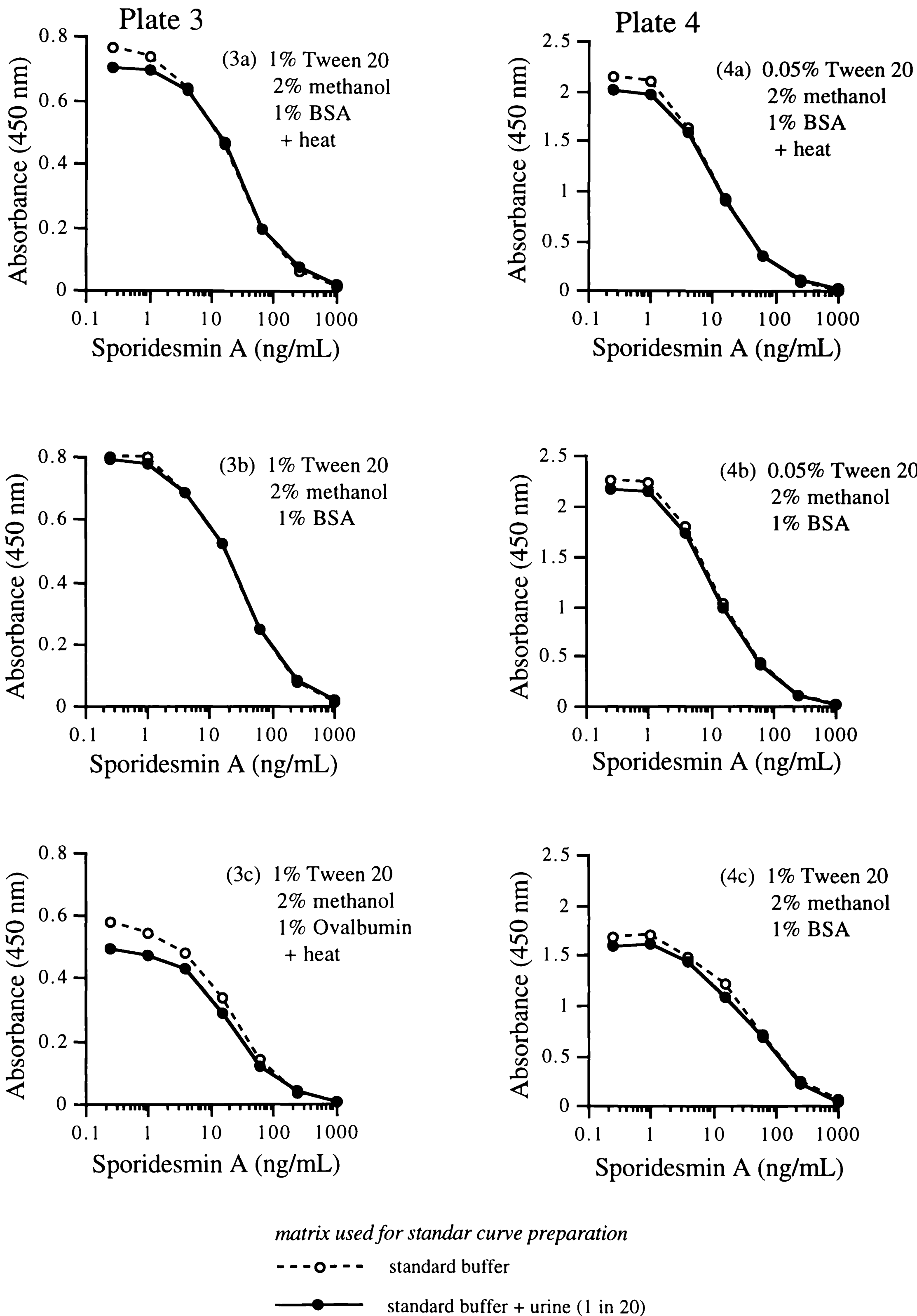


Figure 4.3.2 Contd. ELISA absorbance values shown are the mean of duplicate determinations and the standard deviation ranged from 0.001 to 0.066 (mean = 0.012).

In Figure 4.3.2 pooled urine had been diluted to 1:19 in sample buffer. In the selected assay conditions, standard curves prepared with and without urine were similar but not completely superimposable at the lower regions of the assay working ranges (Figure 4.3.2). A study was therefore undertaken to determine if dilutions of individual urine samples 1:29 instead of 1:19 would cause less assay interference. To do this sporidesmin A standards were spiked into the selected buffer and into individual urine samples diluted 1:29 in the same buffer. Urine samples were selected based on colour intensity to give a range of urine concentrations for testing in the assay. The concentrations of sporidesmin A (listed in Table 4.3.2) measured in urine were compared with the concentrations measured in buffer which were taken to be the 100% values. The results (Table 4.3.2) indicated that recovery at the lower end of the assay working range (*i.e.* for 3.74 ng/mL) varied greatly and exceeded the limits of 100 (± 20)%, commonly taken as the minimum acceptable assay precision. As this was unacceptable the experiment was repeated using urine diluted 1:49. This time the results (Table 4.3.3) indicated that except for one sample, recoveries were within the range of 100 (± 20)%. Therefore urine samples diluted 1:49 should be suitable for assay.

Table 4.3.2 The effect of matrix dilution (1:29) on the apparent recovery of sporidesmin A from various samples of urine determined by cELISA using the group B antibody

sporidesmin A added (ng/mL)[a]	% of sporidesmin A measured in urine samples (diluted 1:29)								
24.09	93	99	87	92	91	92.4			
	(7.1)	(2.8)	(6.7)	(3.6)	(0.4)	(2.8)			
9.49	102	100	95	98	95	87.8	102	102	100
	(1.4)	(2.4)	(0.6)	(2.9)	(1.7)	(0.2)	(5.0)	(0.4)	(5.8)
3.74	123	119	95	112	89	92	139	122	130
	(2.1)	(2.8)	(2.9)	(0.6)	(0.6)	(0.2)	(1.1)	(5.9)	(3.0)

[a] Represents the concentration in the assay. Values shown in brackets are the %CV of the duplicate absorbances measured in cELISA.

Table 4.3.3 The effect of matrix dilution (1:49) on the apparent recovery of sporidesmin A from various samples of urine determined by cELISA using the group B antibody

sporidesmin A added (ng/mL)[a]	% of sporidesmin A measured in urine samples (diluted 1:49)							
15.63	102 (1.6)	103 (3.4)	98 (5.4)	93 (2.4)	90 (0.3)	80 (2.5)	103 (2.2)	97 (0.1)
3.91	110 (2.2)	124 (4.1)	104 (3.8)	92 (0.7)	110 (1.3)	92 (0.1)	112 (1.1)	97 (1.0)

[a] Represents the concentration in the assay. Values shown in brackets are the %CV of the duplicate absorbances measured in cELISA.

4.3.2 Assay optimisation

As assay conditions to remove matrix interference had been determined, assay optimisation was undertaken. Previously optimisation of the concentration of coating conjugate was carried out with 1% Tween 20 in the sample buffer (Figure 4.2.25), therefore, optimisation of plate-coating in the presence of 0.05% Tween 20 was required for the urine assay. Two microtitre plates were incubated with 100 µL of sporidesmin A hemisuccinyl 11-ovalbumin at concentrations ranging from 0.28 to 10.0 µg/mL in coating buffer. Both plates were incubated overnight, one at 4°C and the other at 21°C. After blocking with BSA the plates were incubated with pooled urine diluted 1:49 in sample buffer containing 0.05% Tween 20 and the group B antibody diluted 1:30 000. Incubation with second antibody diluted 1:2 500 was for 1 h. Results (Figure 4.3.3) indicated that 10 µg/mL was the minimum coating antigen concentration giving maximum absorbance (A_O) in the ELISA, however, because of the limited availability of the conjugate, 4 µg/mL was the concentration chosen for routine use. When plates were coated at 21°C, A_O was approximately twice that measured when plates were coated at 4°C. Because a more sensitive assay would be obtained with the higher A_O, plates were coated at 21°C .

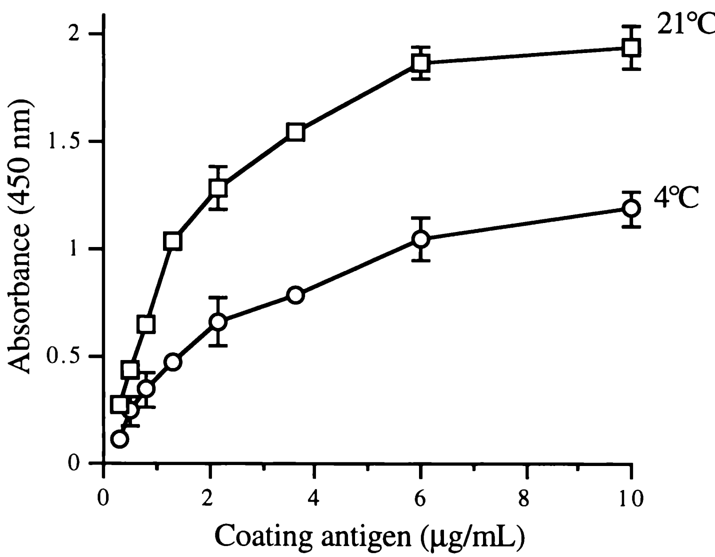


Figure 4.3.3 Effect of coating temperature and coating antigen concentration on absorbances measured in ELISA using the group B antibody. The assay procedure was as described in Figure 4.3.1, but with sample buffer consisting of PBS containing 1% BSA, 2% methanol and 0.05% Tween 20. Group B antibody was diluted 1:30 000 and second antibody 1:2 500. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

A second experiment was carried out to determine the effect of structurally different coating antigens on ELISA absorbance values. This experiment was carried out in a similar manner to the first except a microtitre plate was incubated overnight at 21°C with half the wells coated with sporidesmin A hemisuccinyl 11-ovalbumin and the other half with sporidesmin A hemisuccinyl 10b-ovalbumin at concentrations ranging from 0.28 to 10.0 μg/mL. Although the maximum absorbance measured for the 11-conjugate was greater than that measured for the 10b-conjugate, the curve shapes were similar for both antigens (Figure 4.3.4) and the concentration chosen for routine use for both conjugates was 4 μg/mL.

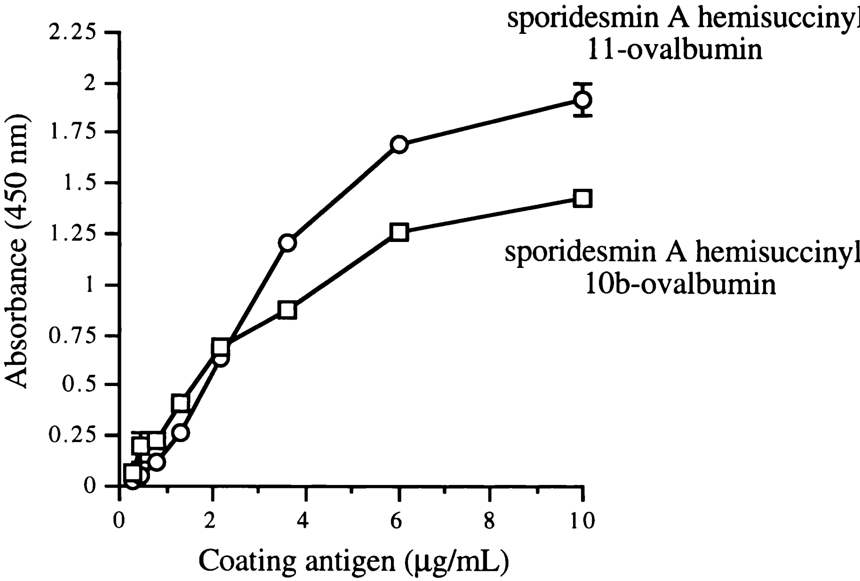


Figure 4.3.4 Effect of coating antigen on absorbances measured in ELISA using the group B antibody. The assay procedure was as described in Figure 4.3.3. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

The binding curve for the group B antibody to sporidesmin A hemisuccinyl 11-ovalbumin (4 μg/mL) was initially determined in sample buffer containing 1% Tween 20 (Figure 4.2.27). It was determined again but this time with sample buffer containing 0.05% Tween 20. A plate was coated with conjugate (4 μg/mL) and blocked as described in Figure 4.3.1. Sample buffer was added to the plate, followed by two-fold serial dilutions of the group B antibody ranging from 1:299 to 1:389 000 in antibody buffer. The binding curve (Figure 4.3.5) indicated that the antibody dilution giving 30% of the maximum absorbance was now 1:90 000 whereas in the presence of 1% Tween it was 1:75 000 (Figure 4.2.27). The dilution chosen for routine use was 1:90 000 as the ELISA A_0 was near to 1.0 absorbance unit and the assay was more sensitive than that obtained than when 1:75 000 dilution was used. The selected antibody dilution was positioned on the steep part of the binding curve which indicated that the assay should have acceptable precision.

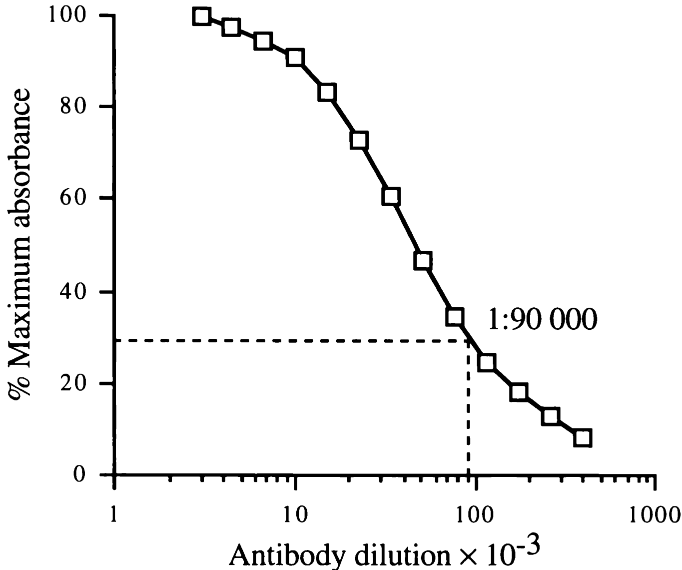


Figure 4.3.5 Group B antibody binding to sporidesmin A hemisuccinyl 11-ovalbumin (4 µg/mL) measured by ELISA. The assay procedure was as described in Figure 4.3.3. Samples were analysed in triplicate. Mean standard deviation of percentage maximum absorbance was 1.1 ± 0.6.

A further check on the urine dilution which should be used in the now optimised assay was carried out. Sporidesmin A standards were spiked into buffer and buffer containing the pooled urine sample diluted 1:39, 1:49 and 1:99. The amounts of sporidesmin A measured in urine were compared with the amounts measured in buffer which were taken to be the 100% values. The results (Figure 4.3.6) indicated that the means of the recoveries for sporidesmin A in urine diluted 1:49 and 1:99 (100.6 (± 16.9)% and 97.2 (± 7.6)% respectively) were closer to 100% than the mean of recoveries in urine diluted 1:39 which was 116.9 (± 19.4)%. This confirmed that dilution of urine 1:49 or 1:99 in sample buffer (PBS containing 1% BSA, 2% methanol and 0.05% Tween 20), should eliminate matrix effects in the cELISA using the group B antibody.

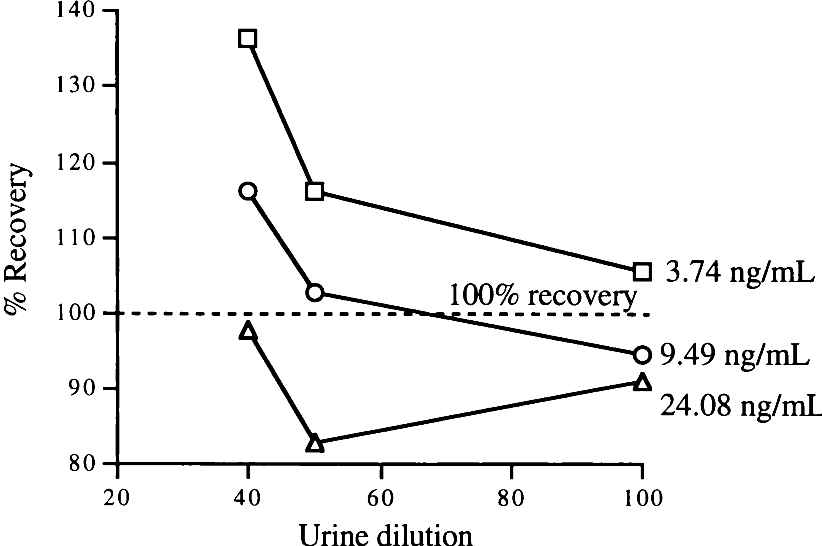


Figure 4.3.6 Effect of matrix dilution on the apparent recovery of sporidesmin A from urine determined by cELISA using the group B antibody. Microtitre plates were incubated overnight with sporidesmin A hemisuccinyl 11-ovalbumin (4 μg/mL) in coating buffer. Plates were blocked with 1% BSA for 75 min and incubated with sample buffer (PBS containing 0.05% Tween 20, 2% methanol and 1% BSA) and group B antibody (diluted 1:90 000) for 1h. Incubation with second antibody (diluted 1:2 500) was for 2 h. Tween 20 was included in washing and assay buffers at 0.05% and all assay steps were carried out at 21°C. Samples were measured in duplicate: the mean %CV of absorbance measured by cELISA was 1.9.

4.3.3 Assay validation and performance characteristics

Since the urinary metabolites as yet are unidentified there is no existing method for measuring sporidesmin metabolites and the immunoassay developed for urine could not be validated with an established reference method using real samples.

The assay was developed to measure immunoreactive metabolites in sheep urine after dosing with sporidesmin A or D. In the cELISAs sporidesmin A standards were used to measure immunoreactive equivalents when sporidesmin A was dosed, and sporidesmin D standards were used when sporidesmin D was dosed. The standard curves (Figure 4.3.7) were determined using the sporidesmin A 10b and 11-conjugated coating antigens. The cross-reactivities of the group B antibody with sporidesmins A and D using the assay conditions selected for urine analysis, were calculated as described in Section 2.7 and are shown in Table 4.3.4.

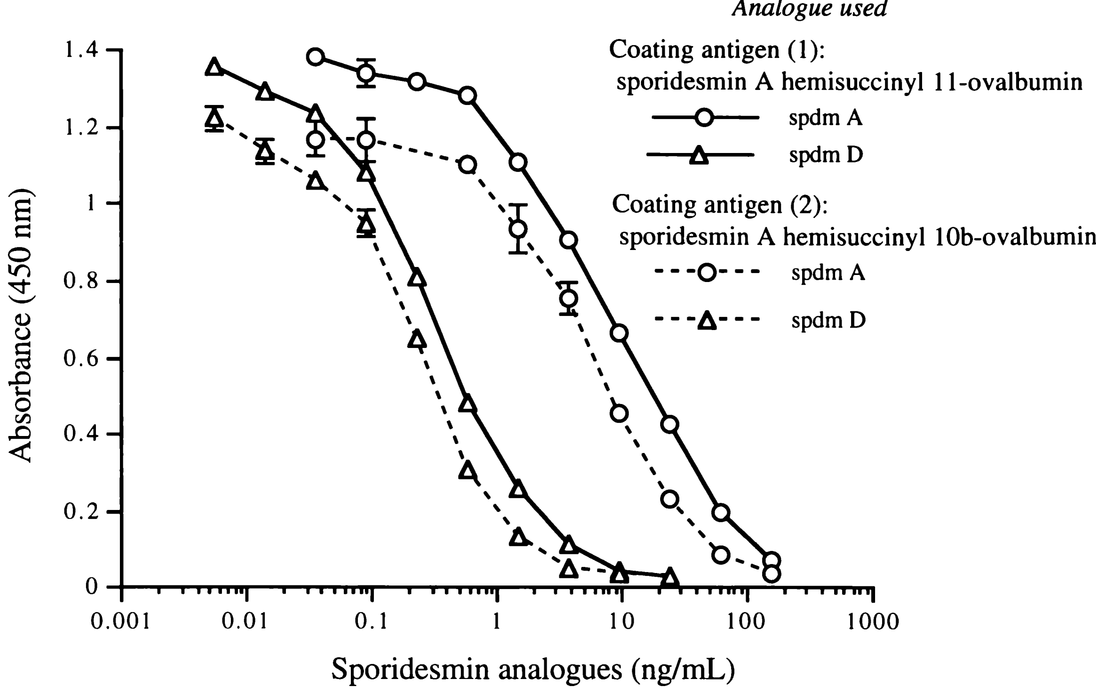


Figure 4.3.7 Standard curves used for the quantification of sporidesmins A or D using the group B antibody. Standards of both sporidesmin A (spdm A) and sporidesmin D (spdm D) were used to produce the standard curves. Coating antigens were diluted to 4 µg/mL in coating buffer. The assay procedure was as described in Figure 4.3.6. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

Table 4.3.4 The assay sensitivities (I_{50}), and cross-reactivities of sporidesmin A and D in the cELISA using group B antibody with different coating antigens

coating antigen	sporidesmin A		sporidesmin D	
(4 µg/mL)	I_{50}[a]	% CR	I_{50}[a]	% CR
spdm A HS 11-ovalbumin[b]	18.23	100	0.66	2 762
spdm A HS 10b-ovalbumin[c]	13.85	100	0.50	2 700

[a] I_{50} values are expressed in nmole per litre. [b] Represents sporidesmin A hemisuccinyl 11-ovalbumin. [c] Represents sporidesmin A hemisuccinyl 10b-ovalbumin.

The assay I_{50} values for sporidesmins A and D were 7.0 and 0.3 ng/mL, respectively. The effective sample working range was 200 to 3 500 ng of sporidesmin A/mL of urine (equivalent to an assay range of 2 to 35 ng/mL). With a sporidesmin D standard the sample range was 3 to 60 ng/mL (assay range 0.03 to 0.6 ng/mL). The optimised assay was validated by spiking urine with sporidesmin A standards using the number of replicates and method described in Section 2.9. Percentage recoveries of sporidesmin A from spiked urine containing 0.4, 1, and 3 µg/mL of urine were 93.0 ± 6.9, 89.1 ± 13.8, and 92.1 ± 7.0, respectively, with the mean recovery 91.4 ± 2.7. The mean intra-assay coefficient of variation was 8.1%, while the mean inter-assay coefficient of variation was 7.9%. A standard urine sample was assayed on every ELISA plate. The mean daily inter-assay coefficient of variation for the sample over 12 assays was 11.7%.

4.4. Immunoassay of milk

4.4.1 Milk sampling

Immunoassay development was carried out using a pooled milk sample obtained from a stirred farm vat during winter when cows were not exposed to sporidesmin A. This was divided into 5 and 20 mL aliquots and stored frozen at -20°C. Sub-sampling the milk after thawing, was difficult because freezing causes a breakdown of milk fat micelles, and an uneven distribution of fat globules resulted. To overcome this problem the thawed milk was heated to 40°C for 5 min and mixed on a vortex mixer immediately before sampling for immunoassay.

4.4.2 Matrix effects in ELISA using the group A antibody

An investigation was undertaken to determine if the milk matrix interfered with the ELISA using the group A antibody. The influence on matrix effects of sample dilution together with heating and sample buffer composition, were also investigated. Matrix interferences with the ELISA A_0 were determined first before studying the effects on the cELISA standard curves. Two sets of two-fold dilutions of the pooled milk, ranging from no dilution to 1:255, were prepared, one in 0.05% Tween 20 in sample buffer (PBS and 2% methanol) and the other in 1% Tween 20 in sample buffer. Each dilution was divided into two to give four sets of dilutions. The samples from one of each matching set were left at room temperature while the other set was heated at 70°C for 30 min. A_0 values measured in ELISA were compared with the A_0 recorded for the equivalent sample buffer with the same heat

The results (Figure 4.4.1) indicated that in all four treatment groups, dilution to 1:7 was sufficient to remove most of the matrix effect and the results were fairly similar for all treatments.

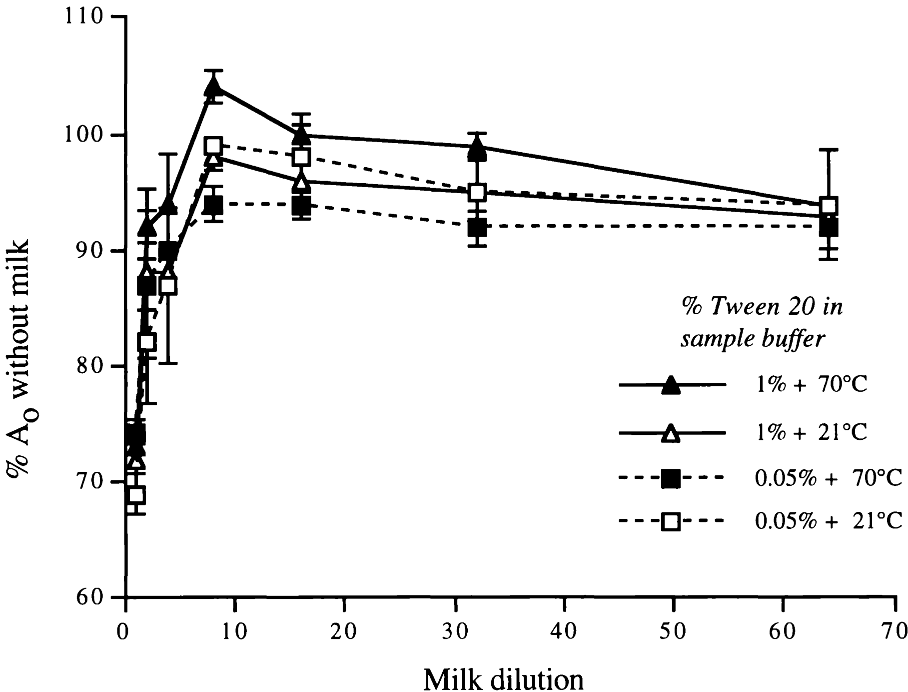


Figure 4.4.1 Effect of sample dilution on matrix interference caused by milk in the ELISA using the group A antibody with different concentrations of Tween 20 and heat treatment. Microtitre plates were incubated overnight with sporidesmin A hemisuccinyl 11-ovalbumin (2 µg/mL) in coating buffer. Plates were blocked with 1% BSA for 75 min followed by incubation for 1 h with milk diluted in sample buffer and the group A antibody (diluted 1:375 000). Incubation with second antibody (diluted 1:2 000) was for 2 h. Tween 20 was included in washing and assay buffers at 0.05% and all assay steps were carried out at 21°C. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

4.4.3 Removal of matrix effects

(1) Inclusion of BSA in standard buffer

In an attempt to remove matrix effects caused by milk, 1% BSA was added to the assay buffer. Sporidesmin A standards were prepared in standard buffer and in milk diluted 1:49 in standard buffer, and the standard curves obtained by cELISA were compared (Figure 4.4.2a). Another set of curves were obtained with 1% BSA included in the standard buffer (Figure 4.4.2b). The standard curves obtained with and without milk were more similar when BSA was not included in the buffer.

(2) Sample dilution

Results presented in Figure 4.4.1 indicated that dilution of milk 1:7 in sample buffer was sufficient to eliminate matrix effects on A_0 determined by ELISA. To see if this was true for the full cELISA standard curve, four sets of standards were prepared: (a) in standard buffer, (b) in standard buffer containing milk diluted 1:4 (c) 1:9 and (d) 1:24 and analysed by cELISA (Figure 4.4.3). Whereas the standard curves determined in the presence of milk diluted 1:9 and 1:24 were superimposable the standard curve determined in milk diluted 1:4 was displaced. All three curves differed from the curve prepared without milk. Thus it was concluded that optimum removal of matrix effects was achieved with 1:9 dilution and that additional dilution did not bring about any further reduction in assay interference and that dilution alone was not sufficient to overcome matrix effects.

(3) Effect of defatting milk

To determine if milk fats were responsible for the matrix effects standard curves determined in whole milk and defatted milk were compared. Sporidesmin A standards were prepared in milk diluted 1:9 in standard buffer containing 0.05% Tween 20. The standards were heated at 70°C for 30 min as it was found that the aqueous and fat phases were better separated after heating. This made it easier to recover a sample of the aqueous phase for analysis. After heating, the standards were divided equally and one set of standards was centrifuged at 10 000*g* for 3 min and the aqueous phase taken for assay, whereas the uncentrifuged standards were mixed on a vortex mixer and analysed whole. Similar sets of standards were also prepared with 1% Tween 20 instead of 0.05% in the standard buffer. Results (Figure 4.4.4) indicated that the standard curves obtained in the whole and in the aqueous fraction of centrifuged diluted milk were superimposable, and it was concluded that the insoluble milk fats in the diluted milk were not responsible for

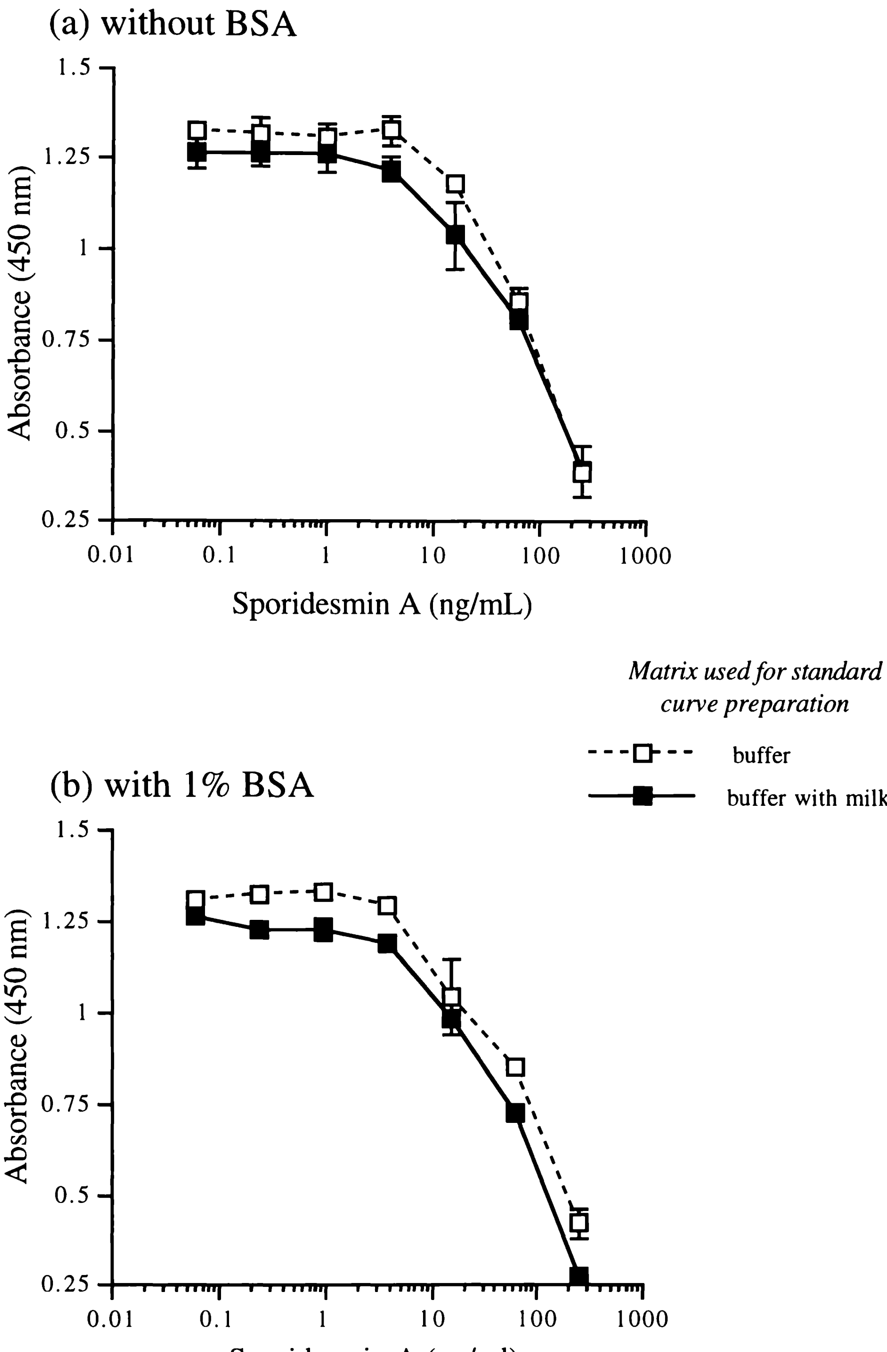


Figure 4.4.2 Effect of inclusion of BSA in sample buffer on matrix effects caused by milk in cELISA using the group A antibody. Standard curves determined with and without milk diluted 1:49 in sample buffer (PBS containing 2% methanol, 1% Tween 20). The assay procedure was as described in Figure 4.4.1. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

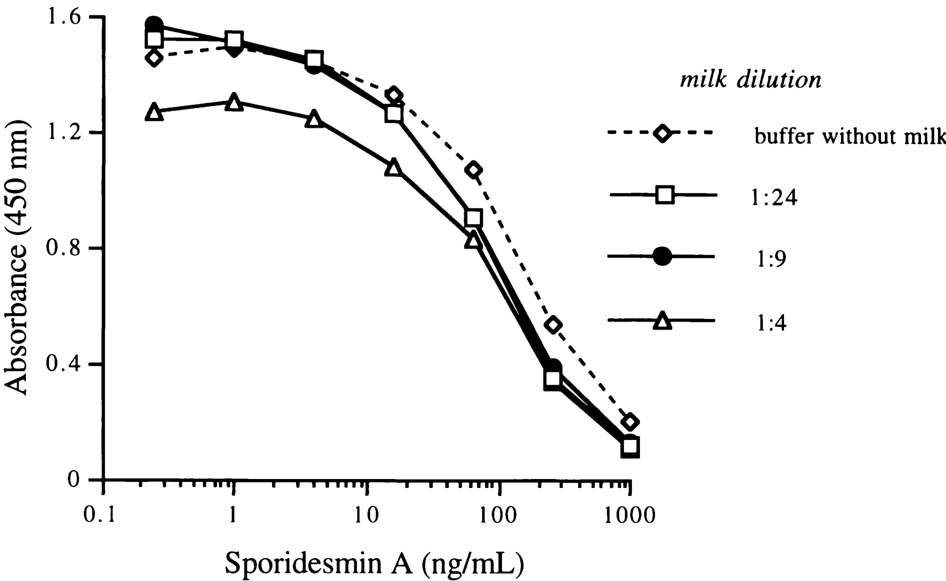


Figure 4.4.3 Effect of the dilution of milk (with standard buffer) on matrix effects in cELISA using the group A antibody. Standard buffer was PBS containing 1% Tween 20 and 2% methanol and the assay procedure was as described in Figure 4.4.1. ELISA absorbance values shown are the mean of duplicate determinations and the standard deviation in absorbance ranged from 0.001 to 0.052 (mean = 0.015).

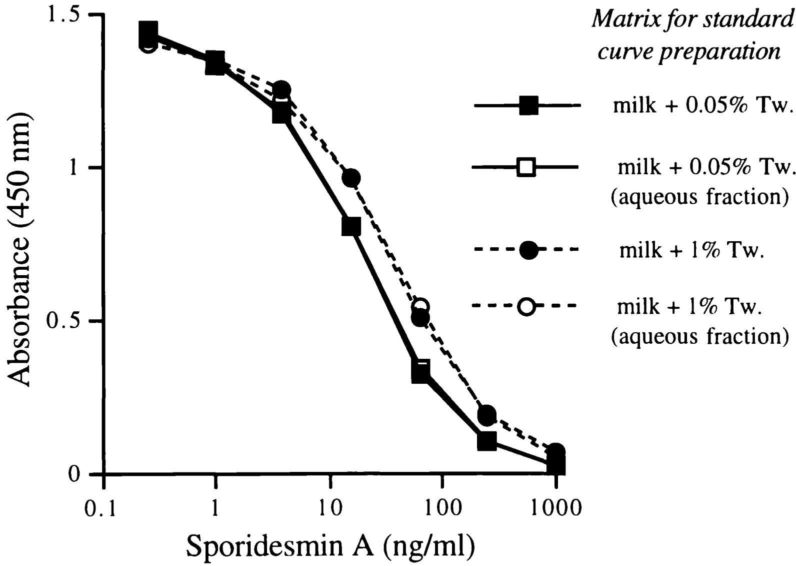


Figure 4.4.4 Effect of milk fat on sporidesmin A standard curves. Four sets of standards were prepared in whole milk diluted 1:9 in standard buffer with two sets containing 0.05% Tween 20 and two sets containing 1% Tween 20 in the standard buffer. After heating at 70°C for 30 min one standard from each buffer set was analysed whole while standards from the other set were centrifuged and the aqueous phase taken for cELISA. ELISA absorbance values shown are the mean of duplicate determinations and the standard deviation of absorbance ranged from 0.001 to 0.042 (mean = 0.013).

assay interference. Reducing the Tween 20 concentration from 1% to 0.05% resulted in a more sensitive assay but again there was no difference in the standard curves obtained with whole milk and defatted milk.

(4) Changes in concentration of Tween 20 in standard buffer

The effect of the concentration of Tween 20 in sample buffer on matrix effects caused by whole milk was further studied by comparing standard curves prepared with different concentrations of Tween 20. Standards were prepared with and without milk and standard curves determined by cELISA. One pair of curves was determined in standard buffer containing 1% Tween 20 and another containing 0.05% Tween 20. The displacement of the standard curve by the presence of milk was less for buffers containing 0.05% Tween 20 than for buffers containing 1% Tween 20 (Figure 4.4.5).

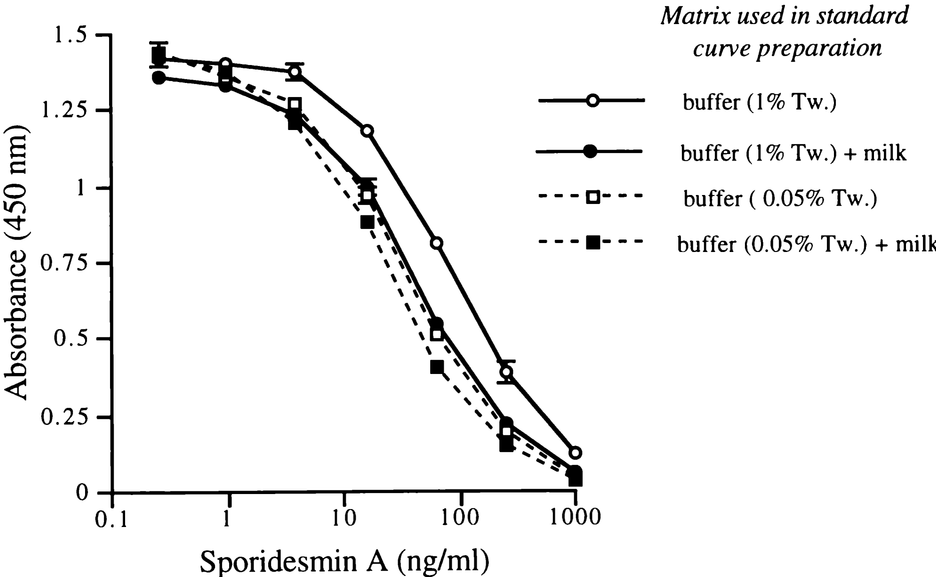


Figure 4.4.5 Effect of Tween 20 concentration in sample buffer on matrix effects caused by milk in cELISA using the group A antibody. Standard curves were determined with and without milk diluted 1:9 in sample buffer. The assay procedure was as described in Figure 4.4.1. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

(5) Dilution of milk followed by heating

Although sample dilution and heating had not eliminated the reduction in ELISA A_O due to the presence of milk (Figure 4.4.1), an experiment was carried out to see if this was the case for the full standard curves determined in milk. The effect of heating standards prepared in milk was studied by preparing standards using the methods outlined in Table 4.4.1. These standards were analysed by cELISA and the standard curves obtained are shown in Figure 4.4.6. As the curves determined in milk with and without heat were superimposable it was concluded that heat had no effect on matrix effects caused by milk. The curves generated in whole milk were neither the same as those generated in skimmed milk, nor the same as in buffer alone.

Table 4.4.1 Standard preparation to determine the effect of heat and separation of milk on matrix effects caused by milk in cELISA

	included in standard buffer:		
	whole milk	skimmed milk[a]	
treatment	(1:9)	(1:9)	heat[b]
a			✓
b	✓		
c	✓		✓
d		✓	✓

[a] Skimmed milk was prepared by centrifugation of whole milk at 10 000g for 5 min and the aqueous phase was withdrawn using a transfer pipette with the tip placed beneath the fat phase. [b] Diluted samples were heated at 70°C for 30 min.

As variations in sample buffer composition and heat did not eliminate the matrix effects it was decided that standards should be prepared in pooled milk diluted 1:9 in standard buffer. Preparation of standards in analyte-free matrix is a standard procedure when it is not possible to eliminate matrix effects by other means and the level of interference between samples is constant.

The applicability of this method to overcoming matrix effects was tested by determining the effect of milk sample variation on sporidesmin A recovery. Standards (listed in Table 4.4.2) were spiked into pooled milk diluted 1:9 in

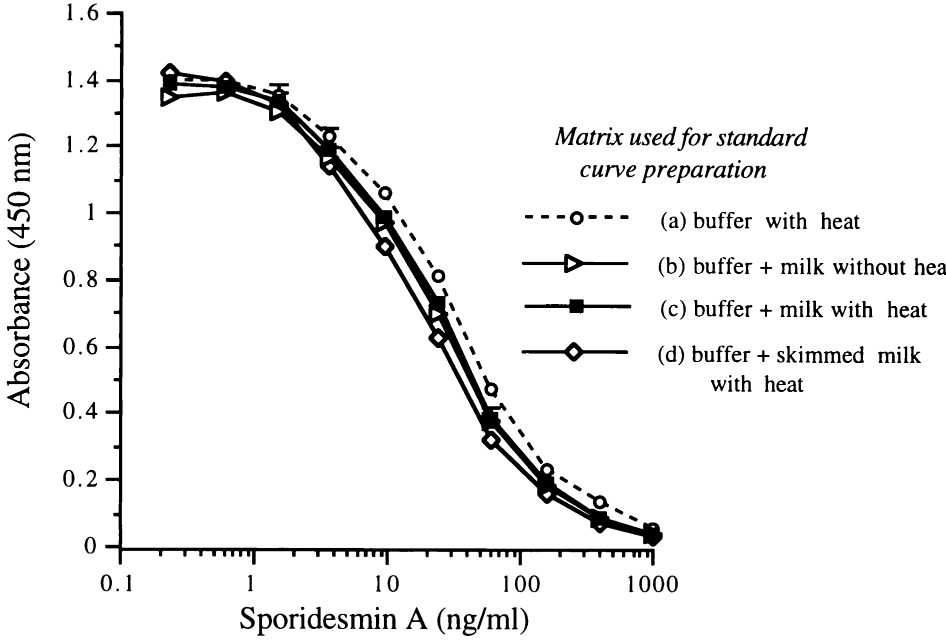


Figure 4.4.6 Effect of heat and separation of milk as outlined in Table 4.4.1, on matrix effects in cELISA using the group A antibody. Tween 20 was included in all assay buffers at 0.05%. The assay procedure was as described in Figure 4.4.1. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

standard buffer containing 0.05% Tween 20 and into diluted individual milk samples selected with a range of fat and protein concentrations. The spiked, diluted milk samples and pooled milk were heated at 70°C for 30 min before cooling to room temperature and analysis by cELISA. The sporidesmin A concentrations in the individual samples are presented as a percentage of the value determined for the pooled milk.

The results (Table 4.4.2) suggested that in the presence of 0.05% Tween 20 sporidesmin A concentration in the individual samples was both over-estimated and variable, especially at the lowest concentration. Recoveries fell outside the accepted range of 100 (± 20)% and as this was unacceptable the experiment was repeated using the amounts listed in Table 4.4.3 and 1% Tween 20 in standard buffers.

In the presence of 1% Tween 20 there appeared to be no significant bias between results for individual samples and those in pooled milk, and most results fell within the acceptable range of 100 (± 20)% (Table 4.4.3). Therefore 1% Tween 20 was included in the buffer for all subsequent milk analyses.

Table 4.4.2 Effect of milk sample variation and 0.05% Tween 20 on the apparent recovery of sporidesmin A determined in milk by cELISA using the group A antibody

sporidesmin A added (ng/mL)[a]	% of sporidesmin A measured in individual milk samples (diluted 1:9)								
62.50	121	96	100	117	94	122	102	121	
	(9.1)	(5.1)	(0.8)	(2.2)	(2.6)	(1.3)	(0.5)	(8.7)	
15.63	122	106	95	125	113	122	94	115	115
	(0.6)	(5.2)	(2.6)	(1.1)	(5.8)	(1.3)	(2.7)	(0.2)	(2.3)
3.91	146	113	124	152	127	137	104	145	148
	(6.0)	(5.0)	(2.2)	(2.4)	(1.8)	(3.8)	(0.9)	(2.4)	(5.1)

[a] Represents the concentration in the assay. Values shown in brackets are the %CV of the duplicate absorbances measured in cELISA.

Table 4.4.3 Effect of milk sample variation and 1% Tween 20 on the apparent recovery of sporidesmin A determined in milk by cELISA using the group A antibody

sporidesmin A added (ng/mL)[a]	% of sporidesmin A measured in milk samples (diluted 1:9)								
61.13	87	107	92	100	81	106	112	101	88
	(0.1)	(1.8)	(0.1)	(7.1)	(2.6)	(1.7)	(2.5)	(2.3)	(0.1)
24.08	84	95	96	105	84	119	107	104	77
	(2.9)	(8.0)	(2.2)	(0.1)	(0.8)	(0.5)	(1.5)	(2.8)	(2.3)
9.49	97	104	83	111	93	123	118	o/s[b]	o/s[b]
	(0.9)	(0.5)	(1.7)	(0.8)	(0.7)	(1.7)	(0.2)		

[a] Represents the concentration in the assay. [b] Indicates results which were out of the working range of the assay. Values shown in brackets are the %CV of the duplicate absorbances measured in cELISA.

4.4.4 Assay validation and performance characteristics

The optimised assay was validated by spiking milk with two different levels (400 and 800 ng/mL) of sporidesmin A on 2 days using the methodology and number of replicates described in Section 2.9. Three samples were spiked at each level and two dilutions of each sample were assayed in four replicates. Percentage recoveries of sporidesmin A from the spiked milk were 102.0 ± 4.4 and 96.2 ± 11.5 respectively and the mean recovery was 99.1 ± 4.1. Mean inter-well coefficient of variation was 8.7%. The mean intra-assay (intra-plate) coefficient of variation was 3.6% while the inter-assay (inter-plate) variation over 2 days was 8.8%.

The assay I_{50}, which indicated the actual assay sensitivity without taking sample dilution into account, was determined from the standard curve for sporidesmin A (Figure 4.4.7) and was found to be 46.0 ng/mL The assay range was 10 to 100 ng per mL which give an effective working range for sample concentration of 200 to 2 000 ng per mL of milk.

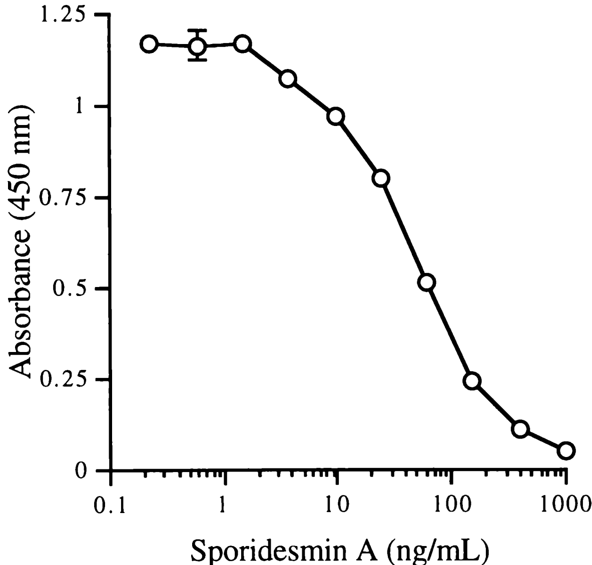


Figure 4.4.7 Standard curve for the quantification of sporidesmin A in milk by cELISA using the group A antibody. Microtitre plates were coated with sporidesmin A hemisuccinyl 11-ovalbumin (2 µg/mL) overnight. Plates were blocked with 1% BSA for 75 min, incubated with standard or sample and group A antibody (diluted 1: 375 000) for 1 h. Standards were prepared in pooled milk diluted 1:9 in standard buffer (PBS containing 1% Tween 20 and 2% methanol), and were heated at 70°C for 30 min. Incubation with second antibody (diluted 1:2 000) was for 2 h. Tween 20 was included in all other assay and washing buffers at 0.05%. All assay steps were carried out at 21°C. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

4.4.5 Matrix effects in ELISA using the Group B antibody

The cELISA using the group B antibody which detects both sporidesmin A and metabolites was modified for use with milk. To determine whether this cELISA was susceptible to interferences in the presence of milk a pooled milk sample, collected from cows that had not been exposed to sporidesmin A, was diluted and analysed by ELISA with the group B antibody. Four sets of two-fold dilutions of the milk, ranging from no dilution to 1:63 were prepared. Two sets were diluted in 0.05% Tween 20 in sample buffer (PBS and 2% methanol) and two in 1% Tween 20 in sample buffer. The samples from one of each matching set were left at room temperature while the other set was heated at 70°C for 30 min. The absorbances (A_0) measured in ELISA were compared with the absorbance recorded for the equivalent sample buffer with the same heat treatment without milk. The results (Figure 4.4.8) indicated that after all four treatments, assay interferences were significantly worse than those observed in the ELISA using the group A antibody (Figure 4.4.1). Generally dilutions greater than 1:7 were required to maximise A_0 but even these were only 60 to 80% of A_0 measured when milk was not included. Absorbances measured in milk dilutions kept at 21°C were less variable than those measured in samples heated to 70°C.

The effect of preparing sporidesmin A standards in milk on cELISA standard curves was determined. Standards were prepared (a) in skimmed milk and (b) whole milk, both diluted 1:9 in standard buffer consisting of PBS containing 2% methanol and 0.05% Tween 20. They were also prepared (c) in the aqueous fraction recovered by centrifugation (2 000*g* for 15 min.) from whole milk after dilution as in (b), and (d) control samples were prepared in buffer without milk. The standards were analysed by cELISA and the standard curves compared (Figure 4.4.9).

The standard curve generated from standards in buffer was considerably different from the three curves which had the various preparations of milk included. The two curves prepared with standards in whole milk and the aqueous fraction of milk, appeared to be superimposable which would suggest that insoluble fats were not responsible for assay interferences.

It was therefore decided that for the cELISA of milk using the group B antibody, sporidesmin A standards would be prepared in pooled milk diluted 1:9 in standard buffer (PBS containing 0.05% Tween 20 and 2% methanol). Using these standards a standard curve was obtained (Figure 4.4.10) with an assay I_{50} value of 8.8 ng of

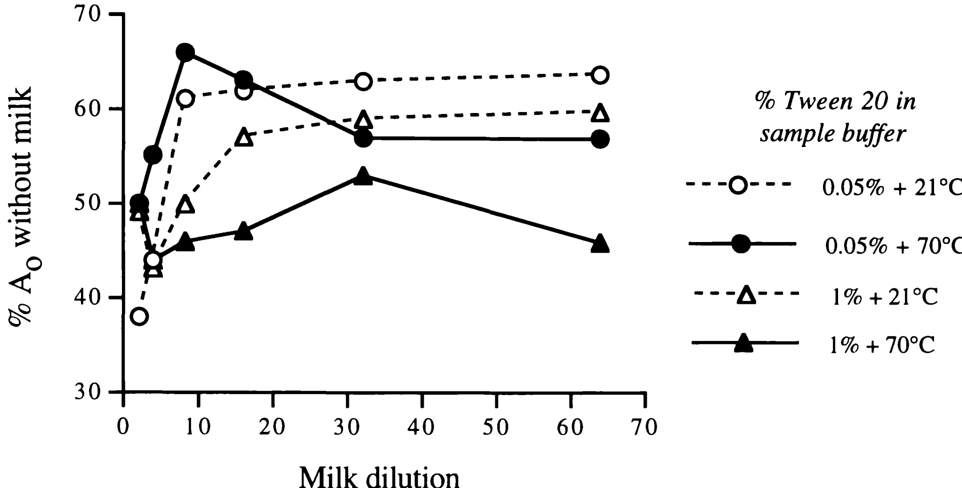


Figure 4.4.8 Effect of sample dilution on matrix interference caused by milk in the ELISA using the group B antibody. Microtitre plates were incubated overnight with sporidesmin A hemisuccinyl 11-ovalbumin (4 µg/mL) in coating buffer. Plates were blocked with 1% BSA for 75 min followed by incubation for 1 h with dilutions of milk in sample buffer and the group B antibody (diluted 1:65 000). Incubation with second antibody (diluted 1:2 500) was for 2 h. Tween 20 was included in washing and assay buffers at 0.05% and all assay steps were carried out at 21°C. ELISA absorbance values shown are the mean of duplicate determinations with standard deviation of $\%A_O$ ranging from 0.4 to 3.5 (mean = 1.5).

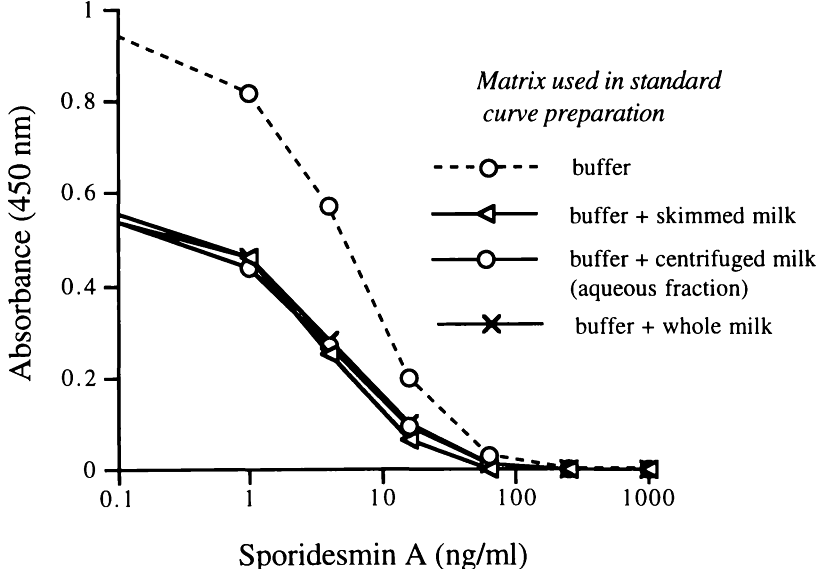


Figure 4.4.9 Effect of milk and milk fractions (diluted 1:9) on standard curves determined by cELISA using the group B antibody. Standard buffer was PBS containing 0.05% Tween 20 and 2% methanol. Prepared standards were not heated before analysis and the assay procedure was as described in Figure 4.4.8. ELISA absorbance values shown are the mean of duplicate determinations with standard deviation of the absorbance ranging from 0.001 to 0.020 (mean = 0.006).

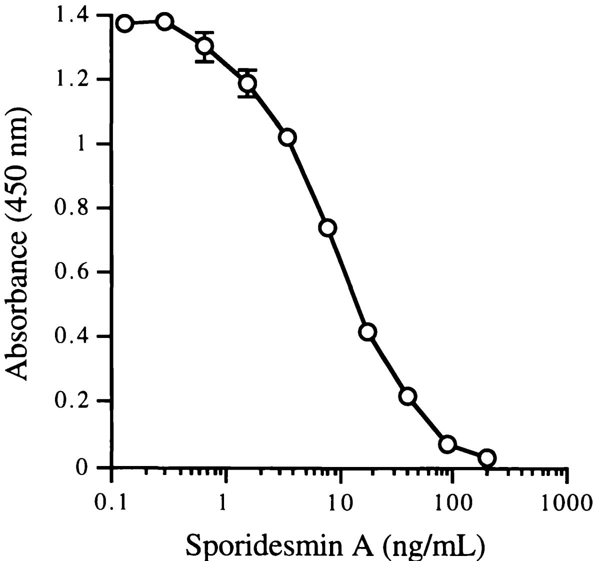


Figure 4.4.10 Standard curve for the quantification of sporidesmin A and sporidesmin metabolites in milk by cELISA using the group B antibody. Microtitre plates were incubated overnight with sporidesmin A hemisuccinyl 11-ovalbumin (4 μg/mL) in coating buffer. Plates were blocked with 1% BSA for 75 min, incubated with standard or sample and group B antibody (diluted 1:65 000) for 1 h. Standards were prepared in pooled milk diluted 1:9 in standard buffer (PBS containing 0.05% Tween 20 and 2% methanol). Incubation with second antibody (diluted 1:2 000) was for 2 h. Tween 20 was included in washing and assay buffers at 0.05% and all assay steps were carried out at 21°C. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

sporidesmin A/mL and a working range of 40 to 600 ng/mL of milk (equivalent to an assay range of 2 to 300 ng/mL).

At this time milk samples were obtained from three farms which had high spore counts and clinical cases of facial eczema were observed in the herds. When the samples were analysed using the immunoassays which had been developed, sporidesmin A and/or immunoreactive metabolite(s) were not detected. It was therefore concluded that if these substances were present in milk they were at levels less than 40 ng/mL and the immunoassay sensitivity was not sufficient for their detection. As sample pre-concentration by immunoaffinity chromatography or solid-phase extraction would be required before milk could be analysed by cELISA, the immunoassay using the group B antibody was not validated for routine use.

4.5 Discussion

The purpose of developing immunoassays was to provide analytical methods to screen for sporidesmin A, its analogues and metabolites in various ovine and bovine body fluids in research. Protocols which did not incorporate a solvent extraction step were developed so that any unknown water-soluble metabolites were not excluded, and if they cross-reacted with the antibody used in the cELISA they could be detected in the immunoassay. When the occurrence of these analytes has been established, more specialised immunoassays could be developed if required to support further research. Inclusion of solvent in extraction steps may reduce assay interferences observed in the presence of body fluids and this may allow sample dilution to be reduced or omitted and thereby provide increased assay sensitivity.

4.5.1 Assay format

The assays developed were predominantly homologous systems in an indirect competitive format. Heterologous formats were investigated, however, for the urine assay using the group B antibody. Heterologous formats are ELISA systems in which the immunogen and plate-coating antigens have different haptens as well as different carrier proteins. For the urine assay the immunising conjugate was sporidesmin A hemisuccinyl 11-BSA and the coating conjugate was sporidesmin A hemisuccinyl 10b-ovalbumin. The standard curves obtained for sporidesmin A and D were more sensitive using the 10b-conjugate (heterologous system) than those obtained using the 11-conjugate for plate-coating (homologous system), *i.e.* the I_{50} values were lower. Heterologous assay designs were classified by Carlson (1995) as: (1) hapten heterology where the competitor is structurally related but not identical to the hapten; (2) bridge heterology where only the linking chain of the hapten is varied and (3) site heterology where only the attachment site of the linking chain to the hapten is varied. Therefore, for the analysis of urine, site heterology of the linkage to the hapten gave a more sensitive assay than the homologous format.

Many workers have also demonstrated the advantages of incorporating bridge heterology in the production of immunogens and coating antigens, particularly with regards to increased sensitivity (Rose *et al.*, 1995). Although bridge heterology was not investigated as an alternative format, coating antigen with a hemiglutaryl bridging group is available for use (Section 3.4.1). Bridge heterology could therefore be investigated to see if it provides assay advantages over the homologous system used with the hemisuccinyl bridge.

4.5.2 Matrix effects

Matrix effects had a major influence on assay sensitivity as all of the body fluids required dilution before analysis to assist in overcoming sample interference in the cELISAs. In addition, increasing the Tween 20 concentration in the milk and bile assay also reduced sensitivity.

Matrix interferences were removed when bile was diluted and heated for 30 min at 70°C. A significant portion of non-specific interference in immunoassays is caused by hydrophobic interactions between matrix and ELISA components such as microtitre plate surfaces and antibodies. Eynard *et al.* (1992) demonstrated that thermal denaturation of proteins brought about changes in surface hydrophobicity. It is possible, therefore, that heat disrupted surface hydrophobicity of the interfering components in bile, and without non-specific interaction, the matrix interferences were removed from the ELISAs.

In some circumstances it was necessary to increase the Tween 20 concentration from 0.05% to 1% to reduce matrix effects, but this was at the cost of reducing assay sensitivity. Tween 20 is routinely used in immunoassay as a blocking agent to reduce non-specific binding and the reduced sensitivity may have been due to Tween 20 binding to specific as well as non-specific binding sites.

Deproteinisation of gall bladder bile with 90% methanol was shown to reduce matrix interferences in the cELISAs. With less concentrated hepatic bile this procedure may have been more effective in removing matrix effects, however, dilution and heat pre-treatment of samples was the preferred method for removing matrix interferences. This was because of the concern that in real samples sporidesmin A may interact with proteins possibly by hydrophobic interaction or by the sporidesmin disulfide bridge interacting with thiol groups on proteins as proposed by Waring *et al.* (1995). Evidence that such could occur came from the fact that Towers (1970a) found after oral dosing of guinea-pigs with ^{35}S-sporidesmin, 92% of the total blood radioactivity was associated with the red blood cells. If such an association should occur, deproteinisation with methanol would in effect remove sporidesmin A from the supernatant by precipitation. Dilution and heat pre-treatment of samples would enable sporidesmin A to be quantified by cELISA as long as the epitope was available for antibody binding and the sporidesmin A-macromolecule was immunoreactive.

4.5.3 Assay validation and performance characteristics

Before an assay can be used with confidence it is preferable that it be fully validated using naturally contaminated samples to demonstrate it meets essential quality control requirements of accuracy, sensitivity and precision. For some of the assays developed this was not possible as the only authentic samples containing sporidesmin A which could be used to check the cELISA for accuracy against an established reference method, were the bile samples which were obtained after dosing sheep with sporidesmin A. Recovery and precision in cELISA for other body fluids were determined using freshly spiked samples. There are limitations in using this procedure as the toxin in spiked samples may not be in the same phase as in naturally contaminated samples and therefore yield different results. In real samples, analyte is in contact with the matrix for considerably longer periods than the spiked samples. It may have undergone metabolic conversion or be concentrated in a particular tissue or cellular component in such a way as to be less available for measurement. However, in the absence of such naturally contaminated samples spiked samples are the only viable option.

Hepatic bile samples were obtained from animal dosing experiments and analysed using HPLC and ELISA. The results were plotted against one another and analysis by linear regression indicated that cELISA was correlated with HPLC results. The slope of the line plotted was 0.93. Although the accuracy was within the range of 100 (± 20)% which was taken to be acceptable, there was an over-estimation of sporidesmin A in the cELISA in comparison to that determined by HPLC. This could have been due to analytical error, or alternatively there may be biliary metabolites present in addition to free sporidesmin A which are able to cross-react with the group A antibody but which were not detected by HPLC. This cannot be confirmed until biliary sporidesmin metabolites have been isolated and cross-reactivity with the group A antibody determined.

Because of the dilution required to avoid matrix effects the cELISA limit of quantitation was 500 ng of sporidesmin A per mL of bile. The HPLC method was more sensitive (limit of detection: 150 ng/mL) than the cELISA method using the group A antibody. However, HPLC has not been used for routine measurement of sporidesmin A in body fluids because extensive sample clean-up is required, making the procedure time-consuming and inconvenient when handling large numbers of samples. Immunoassays, on the other hand, often require little or no sample clean-up, provide high sensitivity and rapid throughput, and depending on the specificity

of the antibodies, will also detect compounds with similar structures such as unidentified metabolites. Although in this case a higher sensitivity did not eventuate the assays have been useful in demonstrating the presence of water-soluble cross-reacting metabolites. Sensitivity for the parent toxins might be increased by incorporating solvent extraction, sample clean-up and concentration steps.

An important aspect of the methodology was the interpretation of data. Logit transformations were used to obtain sigmoid curves of log concentration versus linear absorbance. At the extreme ends of sigmoid curves, assay response is insensitive to small changes in analyte concentration which means that assay precision is reduced in these regions. This factor has an important influence on immunoassay sensitivity, *i.e.*, the limit of quantitation of the assay which is defined as the minimum concentration above which quantitative results may be obtained with a specified degree of confidence. The methodology for determining the sensitivity of immunoassays is not standardised and there are a variety of approaches used. In the assays described for this research the lower limit of the assay working range was the concentration providing 20% inhibition of maximum colour development (A_O). This is rather conservative in comparison to the limits used by some authors, for example 15% or 10% used by Skerrit (1995). Brady (1995) also considered 20% inhibition to be conservative as he reported several researchers accepting a less conservative value of 10%, while others have calculated the standard deviation of the mean of the blank measurement and selected two or three times that value respectively as the minimal detectable concentration. The limitation of these methods is that they address assay sensitivity determined from the perspective of the assay response variability only, and do not account for the variability in the measurement process (Brady, 1995). One way of taking this into account would have been to determine recovery of quantities spiked at the same level as those giving 20% inhibition of colour development in the assays. This would precisely define the level above which quantitative results may be obtained.

A further important performance characteristic is that of assay ruggedness, which is defined as the ability of the assay to remain unaffected by small deviations from the established protocol such as in the quantities of reagents used, and in incubation times and temperatures (Deshpande, 1996). Although assay ruggedness was not extensively studied during the assay development reported, the assays were optimised so that all reagents, with the exception of the anti-sporidesmin A antibodies, were added in excess, *i.e.* concentrations were such that in the absence of analyte, maximum absorbances (A_O) were obtained. This meant small errors in the

reagent volume had minimal effect on the assay performance. Although increased sensitivity can be achieved when the amount of hapten bound to the well of the microtitre plate is limited (Garden and Sporns, 1994), in this research it was considered that assay ruggedness and therefore optimal plate-coating was more important. During assay development plate "edge effects" were observed particularly in the assays with 1% Tween 20 concentrations. These effects were not evident, however, when the coating antigen and second antibody concentrations were in excess. All assay procedures were carried out in a constant temperature room which was also found to be important for keeping assay variation to a minimum.

The optimised and validated assays were applied to the analysis of samples generated in research studies. These assays could be further refined to meet other specific requirements, for example if a commercial potential was identified. In this case the assays would be tailored either to field or laboratory situations, and assay format, sensitivity or stability of reagents may need to be modified.

4.6 Summary

ELISA methods for the detection of sporidesmin A and its metabolites in ovine bile and urine and in bovine milk have been developed (Table 4.6.1) for the analysis of samples generated in research programmes. Assay protocols not incorporating solvent extraction were developed to facilitate the dilution of any water-soluble metabolites that might be present. Presence of the body fluids, particularly bile, gave reduced colour development in the standard cELISA leading to over-estimation of sporidesmin A. To overcome these matrix effects, the influence of a number of assay parameters including pH, ionic strength, presence of organic solvents, different blockers, buffers, detergents and heat treatment of samples were investigated, and assay conditions which allowed analysis of body fluids without matrix interference were determined.

Table 4.6.1 cELISAs developed for the quantification of sporidesmin A, sporidesmin D and sporidesmin metabolites in ovine bile and urine and bovine milk

body fluid	antibody		I_{50}[a]	sample	sample working	validated
	A	B	(ng/mL)	dilution	range (ng/mL)	
bile	✓		40[b]	1/50	500-20 000[b]	✓
		✓	30	1/50	500-10 000	✓
			(0.7)[c]		(15-300)[c]	
urine		✓	7	1/50	200-3 500	✓
			(0.3)		(3-60)	
milk	✓		46	1/10	200-2 000	✓
		✓	9	1/10	40-600	

[a] Expressed as the assay I_{50}. [b] Determined using sporidesmin A to generate the standard curve. [c]Determined using sporidesmin D to generate the standard curve.

Chapter 5:

APPLICATION OF IMMUNOASSAYS

CHAPTER 5

APPLICATION OF IMMUNOASSAYS

5. 1 Sporidesmin toxicokinetics in sheep

5.1.1 Introduction

Heritable differences are known to exist among Romney sheep with regard to their susceptibility to sporidesmin A intoxication (Campbell *et al.*, 1981). At present, selection of potential sires for resistance is achieved by dosing with sporidesmin A, which is expensive and exposes valuable animals to the risk of liver damage. As differences in susceptibility to sporidesmin A may be reflected in differences in the biotransformation of the toxin, studies of the metabolism of sporidesmin A in sheep resistant (R) and susceptible (S) to the toxin were undertaken in the hope of identifying differences that could lead to the development of more effective and safer methods for sire selection. Ultimately this could lead to more effective control of facial eczema.

To carry out such research, methods are required for the quantification of sporidesmin A and its metabolites in body fluids and tissues. Chromatographic methods have not been developed for routine use as the assays are expensive, time-consuming and inconvenient when handling large numbers of samples. A cytotoxicity test has been used by Mortimer and Collins (1968) to measure cytotoxic activity (presumed to be due to sporidesmin A) in body fluids after sporidesmin A administration to sheep. The methodology was of limited use because of the non-specific nature of the test. Fairclough and Smith (1983) dosed ^{35}S-labelled sporidesmin A and measured sporidesmin A in bile by combining HPLC and radioisotopic counting. The low specific activity of the material dosed limited the usefulness of this technique.

The cELISAs described in Chapter 4 were developed to overcome these limitations. They required little sample preparation, were rapid to perform and were suitable for use with large numbers of samples. In addition, one assay (using group A antibody) was specific for sporidesmin A and the other (using group B antibody) also detected unidentified metabolites. A study of the metabolism of sporidesmin A in sheep was undertaken using these cELISAs.

5.1.2 Occurrence of sporidesmin metabolites in body fluids after dosing with sporidesmin A

Female Romney sheep (20-35 kg) 6 to 15 months of age, selected from randomly bred animals or selected for either resistance or susceptibility to sporidesmin A intoxication were used. The sheep were obtained from trial flocks of sheep which had been selected by recording response to a standard dose of sporidesmin A (Morris *et al.*, 1995). The sheep were taken from pasture, adapted to a lucerne pellet diet for at least 10 days prior to experimentation and placed in individual metabolism cages. Two days after catheterisation (for urine collection) or 10 days after surgical cannulation (for bile collection), sheep were given sporidesmin A orally by an intraruminal tube. Urine samples were collected at appropriate intervals and 5 mL bile samples were also collected at increasing intervals after dosing. When bile samples exceeded 5 mL, surplus bile was returned to the biliary system to minimise interruption to the enterohepatic circulation. At appropriate intervals blood samples were taken, by jugular venipuncture for liver function tests, *i.e.* serum glutamate dehydrogenase and γ-glutamyltransferase (GGT) activity assays. The analyses were carried out at the Animal Health Laboratory, Ruakura.

(a) Analysis of bile after dosing

A sheep was administered a single dose of 0.4 mg of sporidesmin A per kg of body weight and hepatic bile samples were collected, stored and analysed by cELISA as described in Section 2.8.1.

After the sheep was dosed with sporidesmin A, it was possible to monitor sporidesmin A and metabolite output in bile samples using the appropriate optimised cELISA. The assay with group A antibody measured excreted sporidesmin A while the assay with group B antibody measured immunoreactive metabolites as well as sporidesmin A. Maximal levels of sporidesmin A and of total reactive material (sporidesmin A and/or metabolite) were observed at 2 h and 6 h after dosing respectively, and immunoreactive material could no longer be detected after 48 h (Figure 5.1.1).

In this experiment pre-dose serum GGT levels were elevated suggesting the presence of damage to the liver and/or the biliary system raising doubt about the normality of the surgically prepared sheep and the validity of the results on biliary secretion. It was decided therefore to concentrate on following urinary excretion of metabolites until the surgical approach to bile collection could be modified so that a

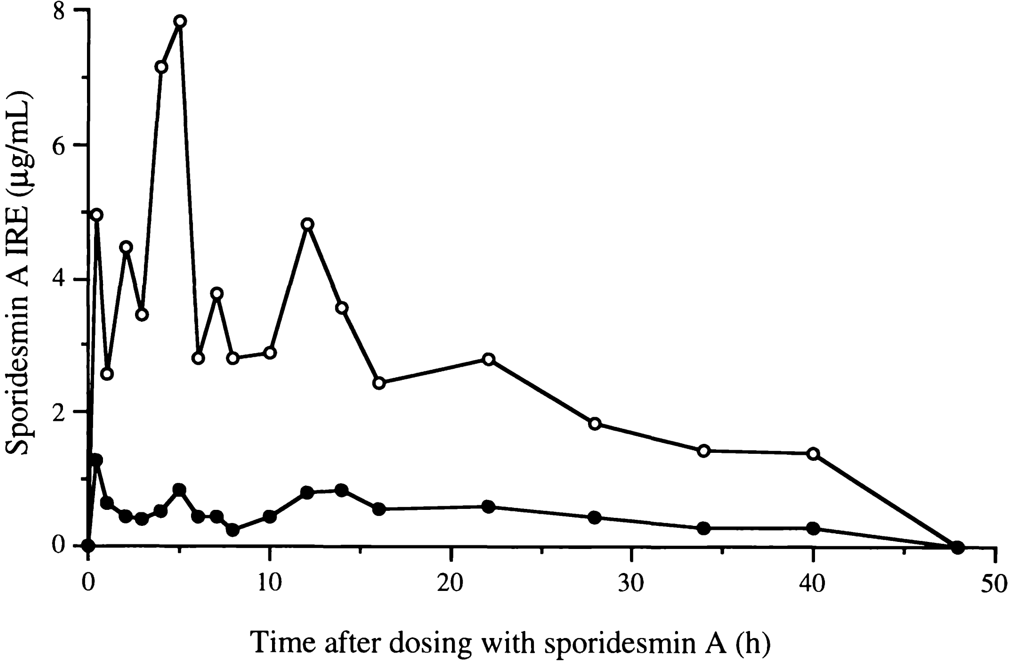


Figure 5.1.1 Biliary sporidesmin A and metabolite levels after dosing orally with sporidesmin A at 0.4 mg/kg of body weight. Open symbols show a combination of sporidesmin A and metabolites (expressed as the immunoequivalent amount of sporidesmin A) determined with the group B antibody, and the closed symbols show sporidesmin A alone, determined with the group A antibody. Concentrations shown are the mean of duplicate determinations. %CV of cELISA absorbance using group A antibody ranged from 0.6 to 8.6 (mean = 2.3), and %CV of cELISA absorbance using group B antibody ranged from 0.2 to 6.5 (mean = 2.8).

less invasive method could be developed and liver function tests were normal before dosing commenced. Since urine collection from female sheep requires a simple catheterisation procedure, it is unlikely to interfere with the normal metabolism of sporidesmin A and thus with the detection of differences between R and S sheep.

(b) Analysis of urine after dosing

Catheters were placed in the urethra of five female sheep dosed with a single dose of 0.2 mg of sporidesmin A per kg of body weight. Urine was collected into plastic bags, the volume measured and samples were taken for storage at -20°C until analysed by cELISA as described in Section 2.8.2. All urine samples were diluted 1 in 50 before analysis, although some samples which were shown to contain high levels of metabolite required further dilution (up to 1 in 500) to bring the analyte concentrations into the working range of the cELISA.

Because preliminary investigation with HPLC (Section 2.12) indicated that unmetabolised sporidesmin A was not present in the urine of the sheep dosed with 0.2 mg of sporidesmin A per kg of body weight, urine was analysed only using the cELISA with the group B antibody. The sporidesmin metabolite(s) found in the urine has not yet been identified, and the cross-reactivities of these compounds in the cELISA are not known. Therefore, results were expressed as sporidesmin A or D immunoreactive equivalents (IRE), depending on which sporidesmin analogue standard was used to generate the standard curve for the cELISA. Sporidesmin metabolites were first detected in the urine 4 h after dosing, reaching maximum levels during the first 35 h (Figure 5.1.2). After this they began to decrease although low levels could still be detected after 103 h.

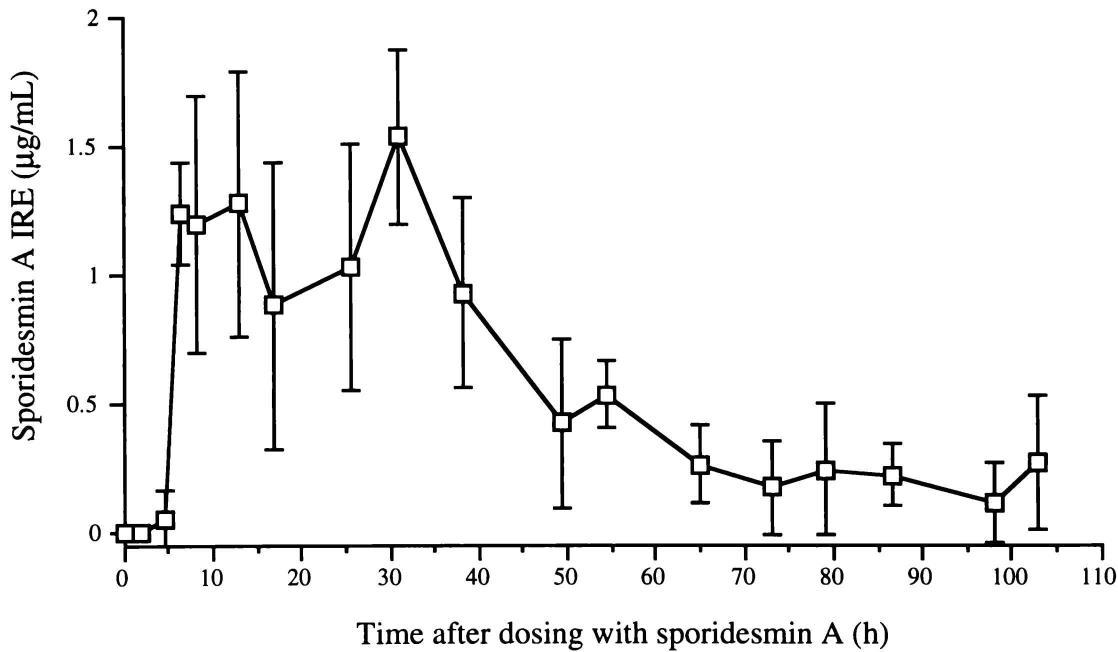


Figure 5.1.2 Urinary sporidesmin metabolite concentration (mean ± S.D., n = 5) after oral dosing with sporidesmin A at 0.2 mg/kg of body weight. Urine analysis was by cELISA with group B antibody. Sporidesmin A IRE indicates immunoreactivity measured using sporidesmin A standards to generate the standard curve for the cELISA.

5.1.3 Occurrence of metabolites in urine after repetitive doses of sporidesmin D

As a toxic compound may cause liver or kidney damage and thereby limit its own metabolism and excretion, the non-toxic analogue sporidesmin D was dosed so that the repeatability of results could be examined without the influence of toxic injury caused by sporidesmin A. Three sheep, from a flock not selected for resistance to facial eczema, were each dosed on three separate occasions with 0.2 mg of sporidesmin D per kg of body weight, with 42 and 137 day intervals. Urine was collected and analysed as described in Section 5.1.2b.

When the output of metabolite (concentration of immunoreactive material × volume of urine collected) was plotted against time (Figure 5.1.3) it was found that the results varied greatly between animals and also fluctuated for each animal. Since much of this variability was due to differences in urine volume and sampling interval, the procedure of Lee *et al.* (1990) and Olson and Chu (1993) where the data were presented as the cumulative urinary output plotted against time, was used to overcome the problem (Figure 5.1.4).

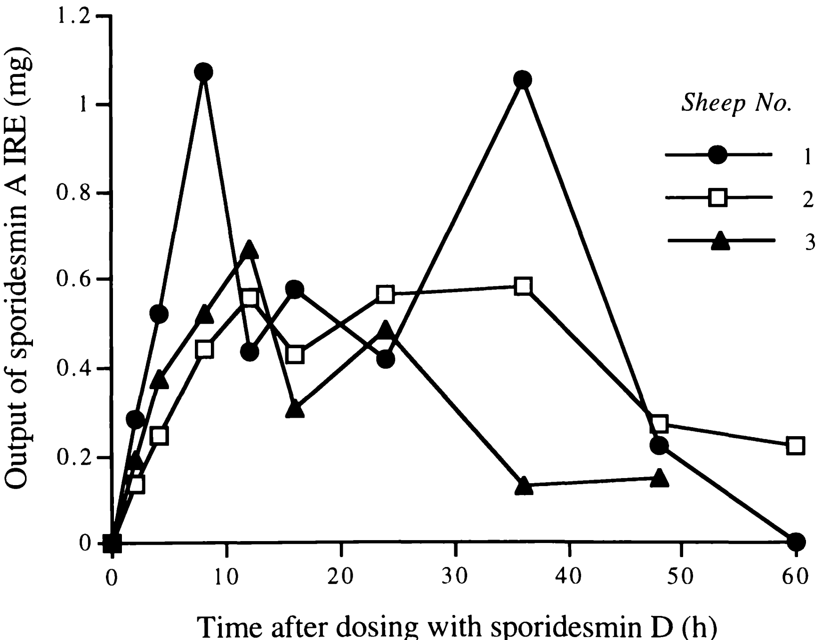


Figure 5.1.3 Urinary sporidesmin metabolite output after dosing orally with sporidesmin D at 0.2 mg/kg of body weight. Outputs were calculated by multiplying the volume of urine collected by the metabolite concentration which was determined by cELISA with group B antibody. The mean of duplicate determinations are shown: %CV of cELISA absorbance ranged from 0.2 - 8.9 (mean = 3.3).

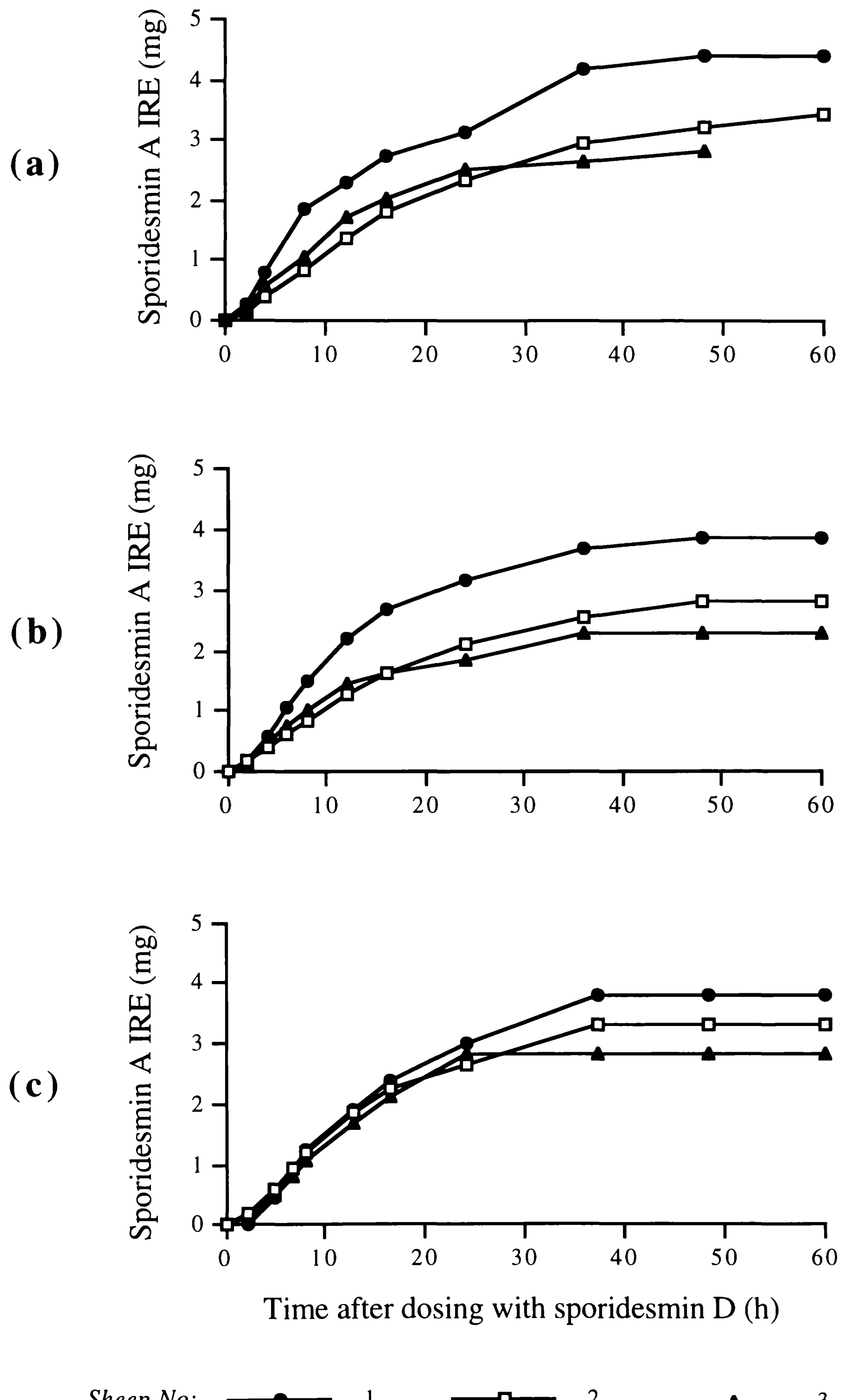


Figure 5.1.4 Cumulative excretion of immunoreactive metabolites in three sheep after dosing with 0.2 mg of sporidesmin D/kg of body weight on three separate occasions: (a) day 0; (b) day 42; (c) day 179. Results shown are the mean of duplicate determinations by cELISA using group B antibody: %CV for cELISA absorbances ranged from 0.2 to 9.7 (mean = 3.3).

The results from all three instances of dosing with sporidesmin D were determined and compared (Figure 5.1.4). The three consecutive doses of sporidesmin D showed that the three sheep maintained a similar relationship to one another each time in the manner in which they eliminated sporidesmin D metabolite via urine (Figure 5.1.4 a, b, c).

5.1.4 Comparison of the clearance of sporidesmin A and sporidesmin D metabolites

The excretion pattern of immunoreactive metabolite after dosing with sporidesmin A was compared with that previously obtained for animals with sporidesmin D.

Two of the sheep (animals 1 and 2) which had been dosed three times with sporidesmin D (Section 5.1.3) were dosed 21 weeks later with 0.2 mg of sporidesmin A per kg of body weight.

When the cumulative output of immunoreactive material after dosing with sporidesmin A was compared with the cumulative output of immunoreactive material for the sporidesmin D dose, it was apparent that sporidesmin D was cleared from the body into the urine more quickly than sporidesmin A (Figure 5.1.5). The cumulative output after the second dose of sporidesmin D was chosen as representative of the three instances of dosing with sporidesmin D. The maximum urinary excretion rates of immunoreactive metabolite occurred 2 to 8 h after dosing with sporidesmin D, and 15 to 30 h after administration of sporidesmin A. Excretion of metabolite was complete after 50 h in the case of sporidesmin D but continued up to 75 h in the case of sporidesmin A.

5.1.5 Urinary excretion of sporidesmin metabolites by sheep resistant and susceptible to sporidesmin A

Potentiation is a term used to describe an effect in which prior exposure of sheep to small doses of sporidesmin A exacerbates the effects of later toxic doses causing a more severe reaction to the dosed sporidesmin A. The metabolite excretion in potentiated R and S sheep was determined as it was thought that it may amplify any differences which may occur between the metabolism of sporidesmin A in R and S sheep, and subsequent urinary output. Seven S and seven R sheep were dosed orally with 0.03 mg of sporidesmin A per kg of body weight and 10 days later were dosed with 0.2 mg per kg of body weight. Urine was collected, the volume measured and samples taken for analysis as described in Section 2.8.2.

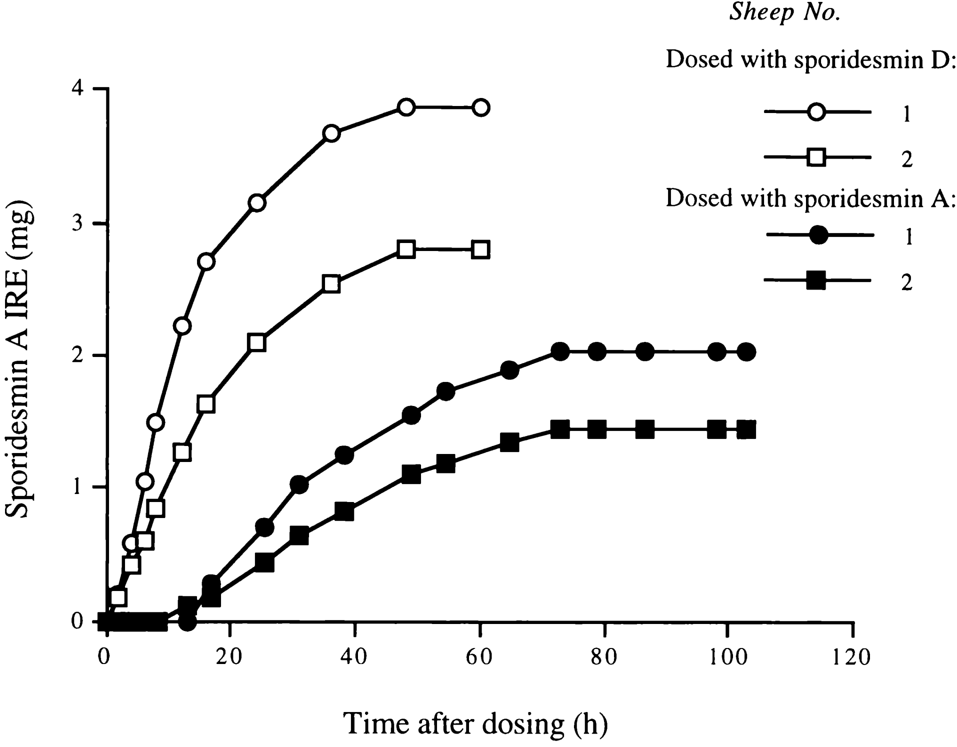


Figure 5.1.5 Cumulative output of immunoreactive metabolite in the urine of two sheep after dosing with sporidesmin D at 6 weeks (open symbols) and after dosing with sporidesmin A 21 weeks later (closed symbols). Both analogues were administered at 0.2 mg/kg of body weight. Samples were analysed in duplicate by cELISA using the group B antibody. Results shown are the mean of duplicate determinations: %CV for cELISA absorbances ranged from 0.2 to 9.0 (mean = 3.0).

There was no difference between the two groups in the cumulative totals or excretion rates of immunoreactive metabolite in the R and S sheep dosed with sporidesmin A after potentiation (Figure 5.1.6). Urinary excretion continued for 103 h with maximum rates of excretion occurring between 10 and 30 h. The large variance in the results was due to individual differences in the sheep.

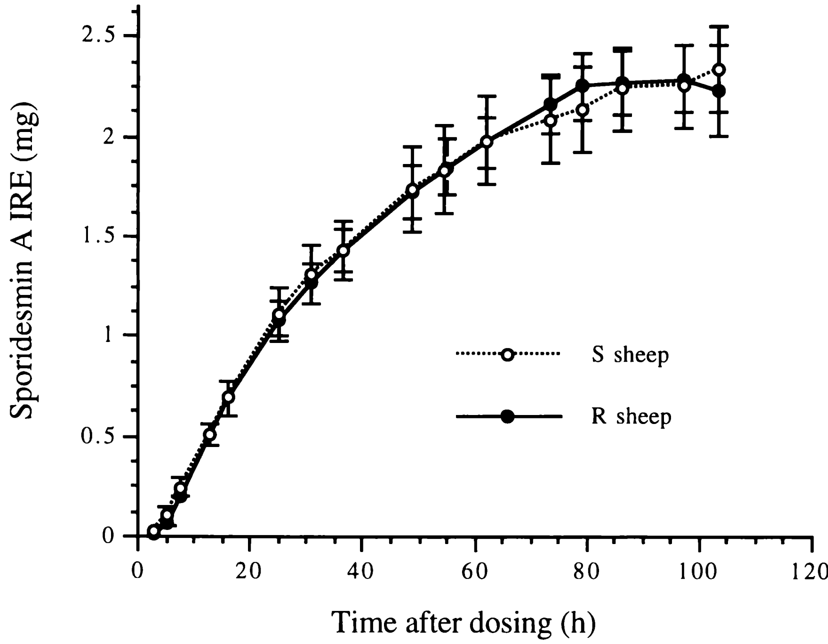


Figure 5.1.6 Comparison of cumulative output of sporidesmin metabolites in R and S sheep urine following dosing with sporidesmin A. Sheep were dosed with 0.03 mg of sporidesmin A/kg of body weight followed by 0.2 mg/kg 10 days later. Group means (± S.E.M.) of R and S sheep (n = 7) are shown for each group.

5.1.6 Discussion

The multipeaked pattern in the plot of biliary excretion of sporidesmin A and its metabolites (Figure 5.1.1) suggests that enterohepatic recycling of the toxin may have occurred in the sheep. Towers (1970a) demonstrated enterohepatic recycling of sporidesmin A or its metabolites in guinea-pigs and suggested it may have a role in the high toxicity of sporidesmin A to this species. Enterohepatic circulation in sheep would slow elimination of metabolites and possibly extend the duration of the toxic effect on the animal.

The urinary excretion of the immunoreactive material derived from sporidesmin A covered a longer time course than for the immunoreactive material derived from sporidesmin D (Figure 5.1.5). Although this may be an artefact of the assay method in that the group B antibody may not recognise the metabolites excreted in the later stages of the experiment after dosing with sporidesmin D whereas it does after dosing with sporidesmin A, it is more likely to represent differences in the metabolism of the two analogues. A more rapid excretion of sporidesmin D than of

sporidesmin A would be expected as sporidesmin D is less hydrophobic than sporidesmin A, so the non-toxic analogue would be more readily removed from the blood and ultimately more rapidly excreted (Timbrell, 1991). Furthermore, as sporidesmin A is known to bind to red cells (Towers, 1970a), uptake from the blood stream into the liver would also be expected to be slower for sporidesmin A than for sporidesmin D, and subsequent metabolism and excretion would cover a longer time course.

The similarity in the patterns for excretion of immunoreactive material in urine, suggests that there is no difference in the ability of R and S sheep (Figure 5.1.6) to metabolise sporidesmin A to metabolites that were excreted in urine. However, this interpretation is valid only if R and S sheep produce the same metabolites in the same proportions.

Although the cELISA with the group B antibody recognises a molecular epitope which may be common to a number of sporidesmin A metabolites, differences in the molecule outside the epitope may alter the antibody affinity for the compound. Therefore, the antibody may have different cross-reactivities with different metabolites. The possiblity that different mixes of metabolites could give the same apparent result in cELISA despite different masses and structures, cannot be excluded. In addition it is possible that the immunoreactive products detected in urine by the cELISA represent only a portion of the total metabolites generated after dosing sheep with sporidesmin A. This could mean that although the excretion of these metabolites was identical in R and S sheep, the excretion of additional, unrecognised metabolites could still differ between R and S animals.

The study has demonstrated some of the advantages (high sensitivity, ease of sample preparation, *etc.*) of cELISA and exposed some of the difficulties with uncertainty in the identity of the analyte detected and therefore uncertainty in quantitation. These deficiencies identify a need for independent analytical methods which can be used in conjunction with cELISA. For example, the use of radiolabelled sporidesmin A would result in labelled metabolites which could be located in HPLC fractions by scintillation counting. Parallel analysis of the samples by cELISA would enable the identification of metabolites that retained the intact antigenic epitope as against those that did not, and the relative cross-reactivities of different metabolites could also be determined. Ideally, labelled chlorine should be incorporated as this would lead to a label on the region of the molecule most likely to persist during metabolism. The cELISA provides an important tool in continuing research aimed

at the isolation and identification of the major metabolites to determine if these differ in R and S animals. From this information it may be possible to develop a test to distinguish R and S sheep so that sire selection could be achieved at a reduced cost with minimal risk to animals.

5.2 Detection of sporidesmin metabolites in the urine of sheep grazing on pastures containing P. chartarum.

5.2.1 Introduction

Following experimental dosing of sheep with 0.2 mg of sporidesmin A per kg of body weight, sporidesmin metabolites could be detected in the urine by cELISA, as soon as 6 h after dosing (Figure 5.1.2). This suggested that the presence of metabolites in urine might serve as a biomarker for exposure of grazing sheep to sporidesmin A. To test this hypothesis urine samples were collected from sheep grazing toxic pastures.

5.2.2 Field trial

Urine samples were collected from two groups of sheep grazing on pastures which had elevated counts of *P. chartarum* spores. One group consisted of 13 sheep from the flock resistant to facial eczema and the second group consisted of 13 randomly bred animals used as a control for another trial. Urine samples were collected at weekly intervals from the resistant animals for 2 weeks and from the second group for 6 weeks. Urinary sporidesmin metabolites were measured by cELISA as described in Section 2.8.2. On 5 sampling days urine samples were also sent to the Animal Health Laboratory, Ruakura, for creatinine analysis and the results were used as an indication of urine concentration. Blood samples were taken weekly and liver damage was monitored by plasma GGT analysis.

Spore counts from grass washes of the pastures were available as they were determined for another trial (Table 5.2.1). These were used as an indication of pasture toxicity. After one month, when some of the animals had significantly elevated GGT levels, the sheep were removed to pastures which had been sprayed with a fungicide and should therefore have been free of actively growing *P. chartarum* and contained minimal amounts of sporidesmin A.

The urinary metabolite concentration was expressed as a ratio to the creatinine concentration to help reduce the effects of variations in urine volume and concentration on the apparent sporidesmin metabolite concentration (Table 5.2.2). When the mean urinary sporidesmin metabolite:creatinine ratio was related to the spore counts (Figure 5.2.1) the sporidesmin metabolite:creatinine ratio increased as the spores counts increased from 30 to 94 000 spores per g of grass. Although this result looked promising there were insufficient data points to establish a relationship between urinary sporidesmin metabolite levels and pasture spore counts.

The number of sheep with detectable sporidesmin metabolites (> 6 ng/mL of urine) occurring in the urine, however, was shown to be related to the number of spores counted (Figure 5.2.2). 100% of the animals excreted detectable urinary metabolites when the spore counts were 420 000 per g of grass, whereas only 8% of the animals gave positive results at 30 000 spores per g of grass and none at 22 000.

After the animals had grazed on toxic pastures for 3 weeks the analysis of serum GGT levels showed that a number of the animals had suffered liver damage, *i.e.* had elevated GGT levels. The urinary metabolite levels in these animals were compared with those for the animals with normal GGT levels. There was no significant difference ($P > 0.05$) between urinary metabolite levels measured in animals which were not affected by grazing on pastures containing *P. chartarum* and animals which suffered liver damage as indicated by plasma GGT levels above 50 U/L (Table 5.2.2). Correction for variation in urine volume and concentration by transforming the data to the sporidesmin metabolite:creatinine ratio did not affect this result.

5.2.3 Discussion

Pastures can be screened for *P. chartarum* spores or sporidesmin A content (Collin *et al.*, 1995), although this does not give a measure of actual animal intake of sporidesmin A. Alternatively, animal ingestion of toxic pasture can be determined by monitoring liver damage by measuring plasma GGT levels. The major limitation of this method is that it may take up to 3 weeks after the initial intake of sporidesmin A before elevation of GGT occurs, which is too late to signal the need for preventative measures. Smith *et al.* (1987) showed that mild liver damage occurred when sheep were grazed for 10 weeks on pastures having maximum spore counts of 88 000 spores per g of grass. A sensitive assay that could detect the presence of urinary metabolite after grazing on pastures well below toxic levels (*i.e.* below

Table 5.2.1 The number of sheep with sporidesmin metabolites occurring in the urine of sheep grazing pastures contaminated with *P. chartarum*, determined by cELISA

paddock number	grazing time (days)	wash spore count $\times 10^{-3}$ (per g of grass)	spdm metabolite: creatinine $\mu mol\ L^{-1}/U\ L^{-1}$	number of sheep with urinary metabolites
35	5	240 (1[a])		
37	5	470 (2)		
21	4			
26	4	420 (2)		12/12 (1[a])
25	7	94 (1)	0.37 ± 0.22 (n = 26)	25/26 (2)
21	2	56 (1)		
22	1	30 (2)	0.02 ± 0.05 (n = 26)	2/26 (1)
23[b]	8	40 (4)	0.02 ± 0.04 (n = 11)	3/11 (6)
24[b]	11	50 (6)	0.05 ± 0.06 (n = 11)	5/11 (5)
21[b]		22 (3)		0/11 (1)

[a] Values in brackets indicate the number of days sheep were grazed in a paddock when grass samples were taken and *P. chartarum* spores counted, or when urine samples were collected for analysis by cELISA using the group B antibody (minimum limit of quantitation using sporidesmin D to generate the standard curve, was 6 ng/mL). [b] Indicates pastures which were sprayed with a fungicide to eradicate *P. chartarum*.

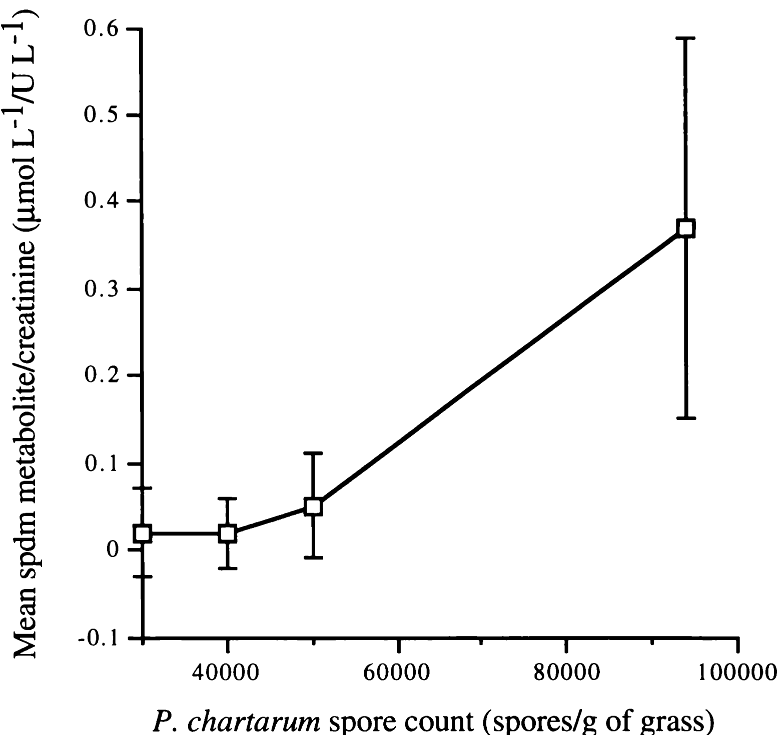


Figure 5.2.1 *P. chartarum* spore count and mean concentration of sporidesmin metabolites in sheep urine. Spore counts were determined from grass washes and sporidesmin metabolites detected in urine by cELISA using group B antibody. Mean concentrations of sporidesmin metabolites are shown with error bars showing standard deviation (n = 11, 11, 26, 26).

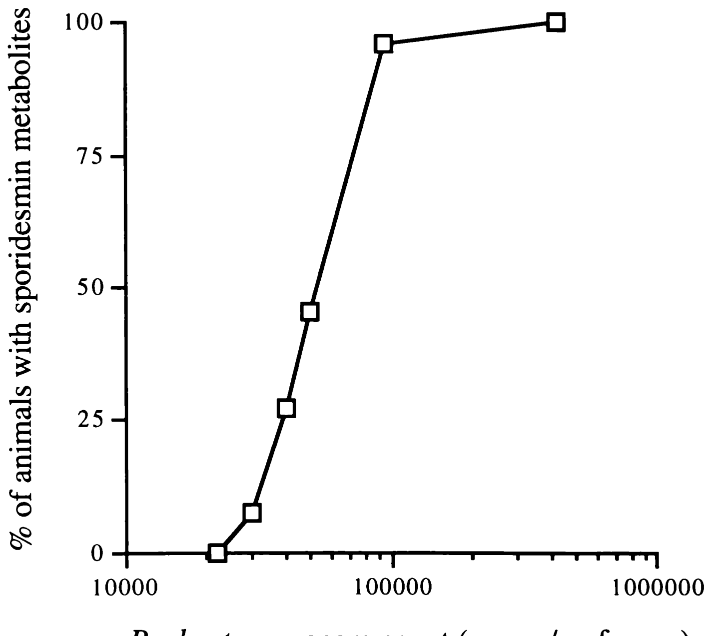


Figure 5.2.2 *P. chartarum* spore count and the number of sheep with detectable sporidesmin metabolites in sheep urine. Spore counts were determined from grass washes and sporidesmin metabolites detected in urine by cELISA using group B antibody.

Table 5.2.2 Urinary sporidesmin metabolite levels in sheep with and without elevated plasma GGT

plasma GGT	spdm metabolite[a] (ng/mL)	spdm metabolite:creatinine (μmol L^{-1}/U L^{-1})
normal:		
< 50 U/L (n = 13)	19.2 ± 8.3	0.37 ± 0.20
elevated:		
>50 U/L (n = 13)	25.5 ± 12.3	0.41 ± 0.23

[a] Sporidesmin metabolites measured in cELISA with sporidesmin D standard used to generate the standard curve. Values are means ± S.D.

88 000 spores per g of grass) would give early warning of the risk of facial eczema and lead to more effective control of the disease.

Results indicated that the level of urinary metabolite (mean of several sheep) was elevated when pastures had spore counts above 80 000 (Figure 5.2.1), although further research would be needed to establish the relationship and determine the mean urinary metabolite concentration indicative of a significant pasture spore count.

However, because the number of sheep with detectable sporidesmin metabolites (independent of absolute concentration) occurring in the urine was related to the number of spores counted, this study indicated that the detection of sporidesmin metabolites in sheep urine by cELISA could provide a method detecting exposure to toxic pasture. This would also provide a less invasive sampling method than blood collection for GGT analysis.

The concentration of metabolites in urine was much lower when animals grazed sporidesmin-contaminated pasture, than that measured after dosing with 0.2 mg of sporidesmin A per kg of body weight, and the sensitivity of the cELISA became a limiting factor in detecting metabolites, particularly when dilute urine was voided. The highest sample concentration recorded was only 49 ng of sporidesmin D immunoreactive equivalents per mL of urine (cELISA working range was 6 to 75 ng per mL). Future research should therefore involve developing a more sensitive

assay, possibly by extraction of the immunoreactive components with solvents or by concentration and purification of a pooled flock sample by solid-phase extraction. It may then be possible to measure sporidesmin metabolite in urine of sheep grazed on pastures with even lower spore counts.

5.3 Characterisation of immunoreactive metabolites in urine

Characterisation of the immunoreactive metabolites found in sheep urine would increase understanding of the metabolism of sporidesmin A. Furthermore, if the structure of the major component of these metabolites was known it could then be possible to prepare an immunogen for the production of metabolite-specific antisera with an aim to provide more sensitive immunoassays for the detection of sporidesmin A exposure. Essential to any attempt to isolate unknown compounds are methods for following them during the various separation procedures. The usefulness of the cELISA using the group B antibody for this purpose was demonstrated during a preliminary study of the nature of the urinary metabolites.

5.3.1 Fractionation of urine by HPLC

Urine samples were studied from two sheep dosed with sporidesmin A and from two others dosed with sporidesmin D. The samples were collected before and after dosing. The latter had been shown by cELISA using the group B antibody to contain immunoreactive material. Urine samples were fractionated by HPLC and fractions containing immunoreactive metabolite were located by cELISA using the group B antibody.

Samples were centrifuged at 10 000*g* for 5 min and without any further preparation, 10 µL of the supernatants were analysed by HPLC. The procedure was as described in Section 2.12 using a C18 reverse-phase column and an isocratic solvent system consisting of acetonitrile:water:methanol (45:45:10). Eight fractions were collected, each over 1 min. Solvent was removed from the fractions by evaporation under nitrogen, and any remaining water was removed by freeze-drying. The residue from each fraction was dissolved in 200 µL of PBS containing 0.05% Tween 20, 1% BSA and 2% methanol (dilution equivalent to 1:19) and analysed in duplicate by cELISA using the group B antibody.

When urine collected from sheep dosed with sporidesmin A was fractionated, the majority of the immunoreactivity occurred in the first two fractions near the solvent

front (Figure 5.3.1). In the reverse-phase system this indicated that the immunoreactive metabolites were polar. When urine was collected from animals dosed with sporidesmin D and fractionated by HPLC, most of the immunoreactivity occurred in the second fraction with small amounts in the following two to four fractions (Figure 5.3.2).

5.3.2 Enzyme-catalysed hydrolysis of sporidesmin metabolites in sheep urine and bile

Non-polar compounds are frequently converted to polar metabolites and excreted as glucuronides or sulfates. Antibodies raised against the parent compound often have much lower cross-reactivities for the conjugated derivatives than for the parent compound and direct analysis of the sample may grossly under-estimate metabolite levels in the sample. Deconjugation of these compounds with enzymes may release the free toxin resulting in greater responses in subsequent cELISA. The presence of zearalenone glucuronide in urine was demonstrated in this way by enzymatic hydrolysis with β-glucuronidase and cELISA of the hydrolysate (MacDougald *et al.*, 1990). To determine if sporidesmin A was metabolised to form conjugates, urine and bile containing immunoreactive metabolite were sampled before and after incubation with β-glucuronidase and sulfatase. In order to take into account the possibility of sporidesmin A or metabolites binding to proteins, samples were also treated with pronase. The samples were analysed by cELISA to determine whether a specific enzyme hydrolysis had increased the total immunoreactivity detected *i.e.* to see if deconjugation had released the toxin.

Hepatic bile and urine were collected from a sheep dosed with 0.4 mg of sporidesmin A per kg body weight, which had a biliary cannula and urinary catheter in place. Bile and urine samples were treated with enzymes as described in Section 2.10 and outlined in Tables 5.3.1, 5.3.2 and 5.3.3. After hydrolysis, samples were diluted in appropriate assay buffer (Sections 2.8.1 and 2.8.2) to give final dilutions of 1:39 for urine and 1:79 for bile, and were analysed by cELISA using group A and B antibodies.

Appropriate control samples were also analysed. These were urine and bile samples taken from the same sheep before dosing, and to which sporidesmin A was added to give an assay concentration of 100 mg per mL. Samples were incubated in the absence and presence of sulfatase or β-glucuronidase. There were no differences in the IRE measured in the samples by cELISA in the absence or presence of enzyme

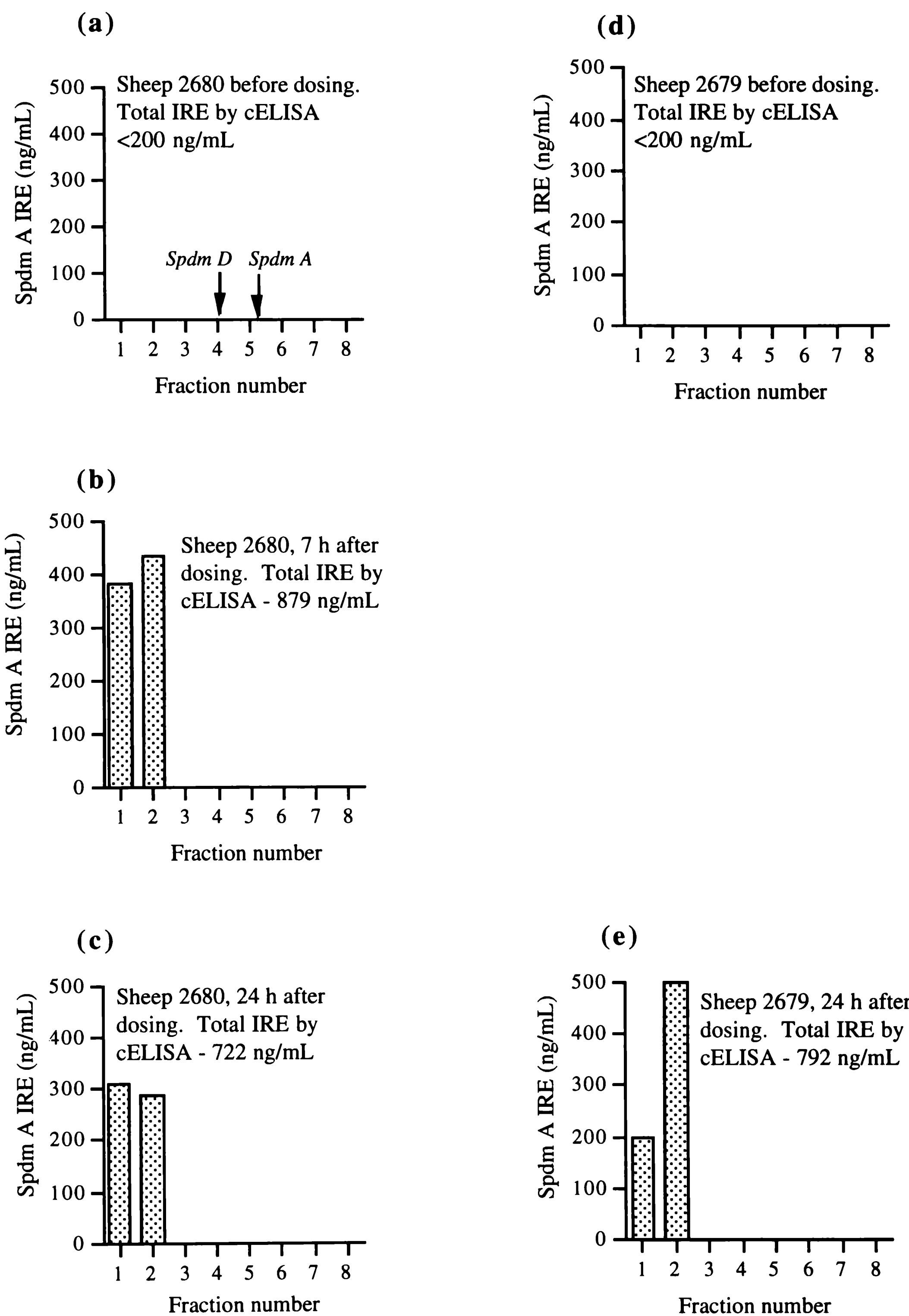


Figure 5.3.1 HPLC fractionation of urine from two sheep before and after dosing with sporidesmin A. Spdm A IRE indicates immunoreactive equivalents measured in each fraction by cELISA with group B antibody, using sporidesmin A standards to generate the standard curve. Retention time for sporidesmin D standard and sporidesmin A standard are shown in (a). Concentrations shown are the mean of duplicate determinations: mean %CV of absorbance ± 4.1.

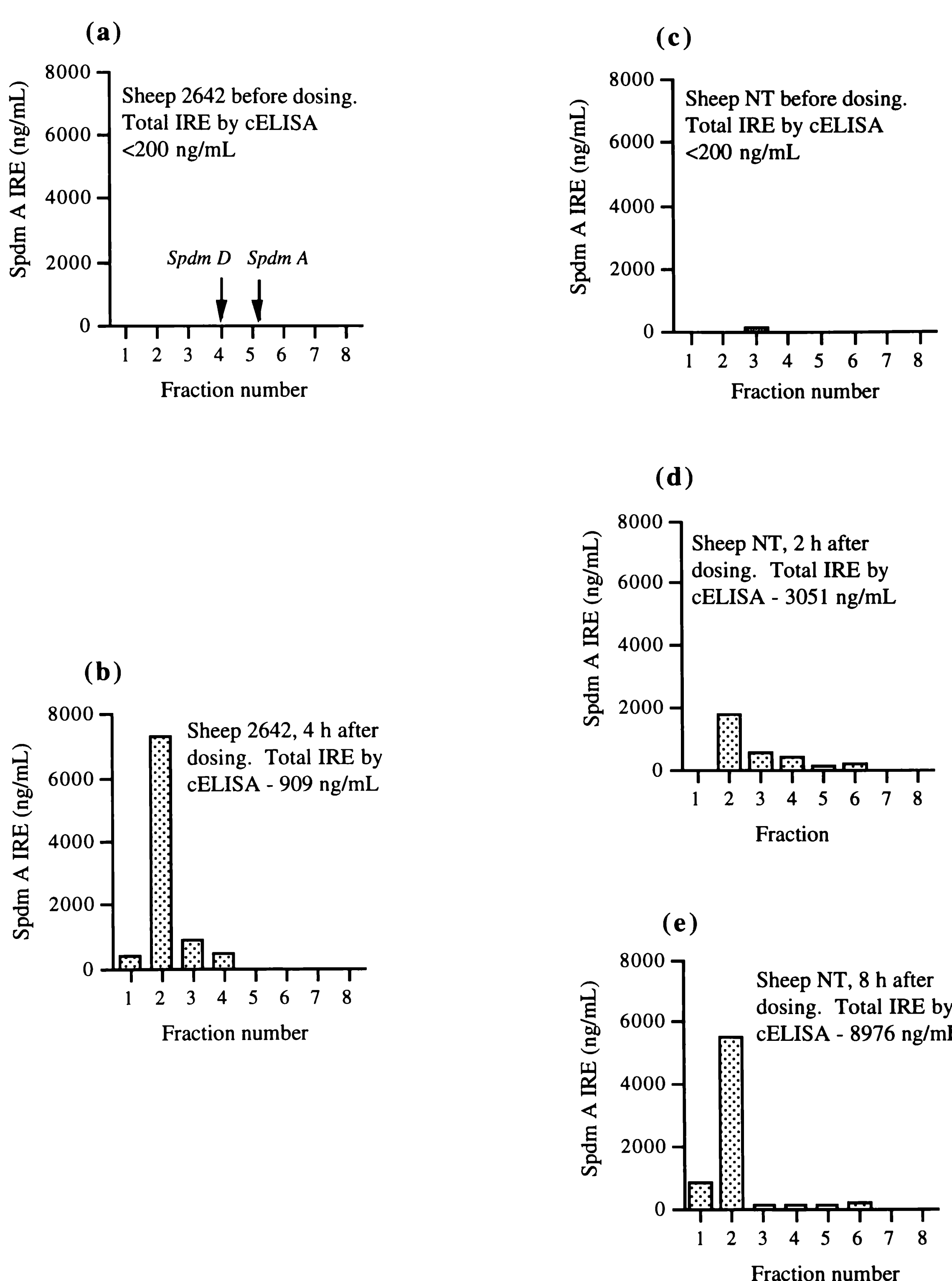


Figure 5.3.2 HPLC fractionation of urine from two sheep before and after dosing with sporidesmin D. Spdm A IRE indicates immunoreactive equivalents measured in each fraction by cELISA with group B antibody using sporidesmin A standards to generate the standard curve. Retention times for sporidesmin D standard and sporidesmin A standard are shown in (a). Concentrations shown are the mean of duplicate determinations: mean %CV of absorbance was ± 3.6.

Table 5.3.1 The effect of β-glucuronidase hydrolysis on immunoreactive metabolites in sheep bile and urine determined by cELISA

body fluid sample		spdm A added	β-glucuronidase	incubated overnight	IRE in cELISA (assay concentration – ng/mL)	
time after dosing (min)	treatment	(100 ng/mL)	added	at 37°C	group A	group B
	bile:					
0	1	✓	✓	✓	49	69
0	2	✓	–	✓	62	80
0	3	–	–	✓	<3	<4
0	4	✓	✓	–	81	87
120	5	–	✓	✓	33	98
120	6	–	–	✓	42	151
270	7	–	✓	✓	17	98
270	8	–	–	✓	24	151
	urine:					
0	9	✓	✓	✓	69	174
0	10	✓	–	✓	69	160
0	11	–	–	✓	<3	<7
0	12	✓	✓	-	97	132
345	13	–	✓	✓	<3	160
345	14	–	–	✓	<3	222
345	15	–	–	–	<3	210

Concentrations were determined by cELISA and the mean of duplicate determinations are shown. %CV for the cELISA absorbance using the group A antibody with bile was ± 5.1 and with urine ± 4.7. For the cELISA using the group B antibody, absorbance %CV with bile was ± 5.7 and with urine ± 4.5. Borders surround the results from sample treatments in the presence and absence of enzyme.

Table 5.3.2 The effect of sulfatase hydrolysis on immunoreactive metabolites in sheep bile and urine measured by cELISA

body fluid sample		spdm A added	sulfatase added	incubated overnight	IRE in cELISA (assay concentration – ng/mL)	
time after dosing (min)	treatment	(100 ng/mL)		at 37°C	group A	group B
	bile:					
0	1	✓	✓	✓	79	61
0	2	✓	–	✓	62	79
0	3	–	–	✓	<3	<4
0	4	✓	✓	–	97	112
120	5	–	✓	✓	36	79
120	6	–	–	✓	42	151
270	7	–	✓	✓	21	104
270	8	–	–	✓	24	151
	urine:					
0	9	✓	✓	✓	79	200
0	10	✓	–	✓	69	160
0	11	–	–	✓	<3	<7
0	12	✓	✓	-	99	131
345	13	–	✓	✓	<3	208
345	14	–	–	✓	<3	220
345	15	–	–	–	<3	210

Concentrations were determined by cELISA and the mean of duplicate determinations are shown. %CV for the cELISA absorbance using the group A antibody with bile was ± 5.1% and with urine ± 4.7%. For the cELISA using the group B antibody, absorbance %CV with bile was ± 5.7% and with urine ± 4.5%. Borders surround the results from sample treatments in the presence and absence of enzyme.

Table 5.3.3 The effect of pronase hydrolysis on immunoreactive metabolites in sheep bile and urine measured by cELISA

body fluid sample		spdm A added	pronase added	incubated overnight	IRE in cELISA (assay concentration – ng/mL)	
time after dosing (min)	treatment	(100 ng/mL)		at 37°C	group A	group B
	bile:					
0	1	✓	✓	✓	259	102
0	2	✓	–	✓	77	33
0	3	–	–	✓	<8	<8
0	4	✓	✓	–	94	87
120	5	–	✓	✓	166	230
120	6	–	–	✓	46	81
270	7	–	✓	✓	189	181
270	8	–	–	✓	30	57
	urine:					
0	9	✓	✓	✓	232	358
0	10	✓	–	✓	53	95
0	11	–	–	✓	<8	<8
0	12	✓	✓	-	357	223
345	13	–	✓	✓	88	216
345	14	–	–	✓	<8	79
345	15	–	–	–	<8	84

Concentrations were determined by cELISA and the mean of duplicate determinations are shown. %CV for the cELISA absorbance using the group A antibody with bile and urine was ± 4.8%. For the cELISA using the group B antibody, absorbance %CV with bile and urine was ± 4.3%. Borders surround the results from sample treatments in the presence and absence of enzyme.

(Tables 5.3.1, 5.3.2, treatments 1 and 2, 9 and 10), so it was concluded that the enzymes did not interfere with the cELISAs.

There were no increases in the IRE measured when bile and urine samples containing immunoreactive metabolite were hydrolysed with β-glucuronidase and sulfatase (Tables 5.3.1, 5.3.2, treatments 5 and 6, 7 and 8, 13 and 14). In all cases, however, the IRE measured by the cELISA using the group B antibody were decreased after incubation with the enzymes.

The IRE measured in samples taken before dosing, spiked with sporidesmin A and incubated with pronase, were significantly greater than those measured in the same samples without enzyme (Table 5.3.3, treatments 1 and 2, 9 and 10). Pronase therefore interfered with the cELISAs and no valid conclusions could be drawn from the results (Table 5.3.3) in determining whether sporidesmin metabolites may contain protein.

5.3.3 Fractionation of hydrolysate by HPLC

As there was no significant increase in the immunoreactivity measured by cELISA when urine was treated with enzymes, another experiment was undertaken to see if HPLC analysis of hydrolysates could detect a change in the polarity of the immunoreactive material, *i.e.* a change in the retention time with the formation of deconjugated metabolite.

Urine samples collected from sheep 2680, 7 h after dosing with 0.2 mg of sporidesmin A per kg of body weight, were incubated with β-glucuronidase, sulfatase and pronase as described in Section 2.10. Samples were also incubated with buffers but without enzyme. The hydrolysate (20 μL) from each reaction was fractionated on HPLC to produce eight fractions, each collected over 1 min. The chromatography conditions were those routinely used for sporidesmin analysis (Miles *et al.*, 1992). Solvent was removed from the fractions by evaporation under nitrogen, and after freeze-drying to remove remaining water, they were redissolved in 200 μL assay buffer and 50 μL was analysed by cELISA using the Group B antibody following the procedure in Section 2.8.2.

As observed previously (Figure 5.3.1b), the immunoreactive components of the fractionated sample at pH 7.4 and pH 5.5 were predominantly in the first two polar fractions (Figure 5.3.3). After treatment with β-glucuronidase, sulfatase and

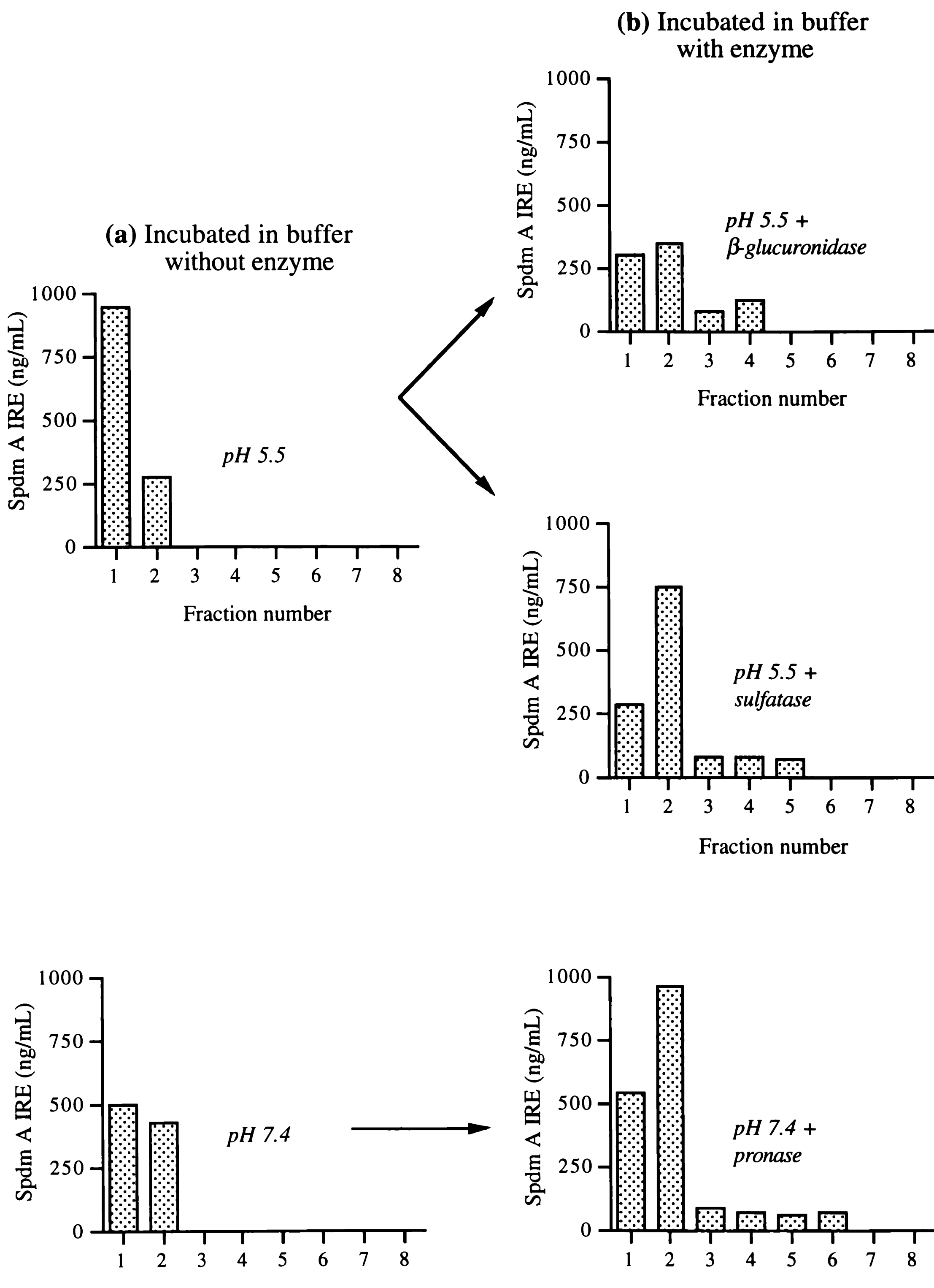


Figure 5.3.3 Immunoreactivity of fractions collected during HPLC of sheep urine after (a) incubation in buffer and (b) incubation in buffer and enzymes. Urine was collected from sheep 2680 7 h after dosing with sporidesmin A (0.2 mg/kg body weight). Fractions were analysed by cELISA using group B antibody and the concentrations shown are the mean of duplicate determinations. %CV of assay absorbance ranged from 0.1 to 9.37 (mean ± 3.3).

pronase the major portion of immunoreactivity remained in the first two fractions. As seen previously in Table 5.3.1, the total amount of immunoreactivity was reduced with β-glucuronidase treatment. Furthermore, there was an appearance of immunoreactive components which were non-polar, suggesting that conjugates had been present before hydrolysis. With sulfatase the amount measured in the first two fractions was slightly less than that measured in the absence of enzyme. There was an increase in the amount of immunoreactivity measured in the first two fractions after pronase treatment. This increase probably reflected the enzyme interference which had been observed earlier (Section 5.3.2). Significant amounts of immunoreactivity were not measured in fractions collected at retention times which coincided with any of the known sporidesmins. UV absorption peaks were also not recorded at these retention times.

5.3.4 Discussion

The cELISA proved to be useful in this preliminary investigation of the urinary sporidesmin metabolites in that the high sensitivity allowed the detection of immunoreactive material (metabolites) in HPLC fractions. These fractions either showed no UV absorbance peaks or the UV recording was obscured by absorbance peaks of other polar components in the urine (fractions 1 and 2 in Figures 5.3.1, 2).

HPLC of urine containing sporidesmin A and D metabolites indicated that most of immunoreactive metabolites were considerably more polar than the free sporidesmins (Figures 5.3.1, 5.3.2). This would agree with the assumption that to facilitate excretion metabolites would be expected to be more polar and less lipid-soluble than the free toxin (Timbrell, 1991). In the case of sporidesmin A metabolites, the majority of the immunoreactivity was associated with the first two fractions and with sporidesmin D metabolites it was associated with the second fraction.

Greater amounts of immunoreactivity were detected by the cELISA in the urine collected after dosing with sporidesmin D than in urine after dosing with sporidesmin A. However, as the identity and cross-reactivity of the metabolites are unknown, this may not reflect the true situation as group B antibody may have greater cross-reactivity with sporidesmin D metabolites than with sporidesmin A metabolites, resulting in lower assay absorbances (*i.e.* apparently higher concentrations). The apparently greater sensitivity of the cELISA for sporidesmin D metabolites could also possibly explain the presence of metabolites recorded by the

cELISA for fractions three to six from fractionated urine collected after sporidesmin D dosing (Figure 5.3.2). However, without further study, the possibility that these results are assay matrix effects cannot be ruled out as the fractions were diluted 1:19 rather than 1:49 which was routinely used in the urine assay (Section 2.8.2). Matrix effects could also explain the presence of metabolite in the third fraction from urine collected before sporidesmin D dosing (Figure 5.3.2c) as this pre-dose sample should be free of immunoreactive components. Further research using less polar chromatographic conditions to increase retention times and resolution of immunoreactive fractions at the solvent front will be required. This will depend on the cELISA for the location of the metabolites until sufficient material to yield reliable UV absorbance peaks is available.

It was not possible to demonstrate the conversion of conjugated metabolites to free sporidesmins by analysing hydrolysates using cELISA in a manner similar to the demonstration of the presence of zearalenone-glucuronide conjugates by (MacDougald *et al.*, 1990). This technique is dependent on the conjugate having lower cross-reactivity with the antibody than the free toxin and this has not been demonstrated. If cross-reactivities are similar there would be no significant change in apparent concentration. In this work there was a decrease in apparent concentration following incubation and cELISA using the group B antibody. Such a situation would arise if the conjugates had greater cross-reactivity than the parent toxin.

Evidence that some of the immunoreactive material is present as conjugates was provided by the change in the HPLC profile following hydrolysis with β-glucuronidase and sulfatase. The reduction in immunoreactivity after enzyme incubation was associated with a loss in IRE measured in the first two polar fractions (Figure 5.3.3) and the appearance of immunoreactivity in some of the less polar fractions. However, matrix effects could not be ruled out as the immunoreactivity was low and samples could not be diluted further before analysis. In this preliminary work the amount of immunoreactive material studied proved too low so further work with urine containing greater amounts of metabolite(s) is required. An additional control experiment consisting of a pre-dose urine treated with enzymes and the fractions generated by HPLC analysed by cELISA, is also required to determine if the immunoreactivity measured in fractions is a matrix effect from hydrolysed urine.

It is possible that the inability to conclusively demonstrate the conversion of conjugated metabolites to free sporidesmins by analysing hydrolysates using cELISA or HPLC-cELISA, was due to the hydrolysis conditions used. Although the conditions used have been found to be suitable for deconjugation of zearalenone conjugates (Duchatel and Maghuin-Rogister, 1985; Olsen *et al.*, 1986; MacDougald *et al.,* 1990), they may not be adequate for the deconjugation of sporidesmin metabolites.

5.4 Summary

After sheep were dosed with sporidesmin A it was possible to monitor sporidesmin A and metabolite in bile, and metabolite in urine using the appropriate cELISA with the group B antibody.

When repetitive doses of sporidesmin D were given, sheep maintained a similar relationship to one another in the rate at which they eliminated sporidesmin D metabolite via the urine.

The urinary excretion of immunoreactive material after dosing with sporidesmin A covered a longer time course than that following dosing with sporidesmin D.

There were no differences between R and S sheep in the cumulative totals or excretion rates of immunoreactive metabolite in animals dosed with sporidesmin A after potentiation.

The detection of sporidesmin metabolites in sheep urine by cELISA has the potential to provide a biomarker for measuring exposure to toxic pastures and give early warning of the risk of facial eczema.

The cELISA proved to be a useful tool for the immunodetection of sporidesmin metabolites in the chromatographic fractions of urine collected from sheep after dosing with sporidesmin A and D.

Urine and bile samples containing immunoreactive metabolites were treated with β-glucuronidase, sulfatase and pronase. Analysis of these samples before and after hydrolysis by cELISA and HPLC-cELISA did not provide conclusive evidence that the immunoreactive metabolites were glucuronides, sulfates or protein conjugates.

Chapter 6:

IMMUNOAFFINITY CHROMATOGRAPHY

CHAPTER 6

IMMUNOAFFINITY CHROMATOGRAPHY

6.1 Introduction

Biological fluids are complex matrices and often, because matrix components interfere with analytical methods, it is necessary to remove these materials from sample extracts before analysis. Clean-up and concentration of a wide range of mycotoxin-containing biological fluids have been achieved by many researchers by using immunoaffinity chromatography (IAC) (Candlish and Stimson, 1993). In this study IAC was investigated as a means of concentrating sporidesmin A because there was a need to extend the limit of detection in samples where only trace amounts were present. It was also anticipated that the methodology could be applied to the extraction and isolation of immunoreactive sporidesmin metabolites from body fluids and tissues. The principal prerequisite for IAC is the availability of relatively large amounts of suitable antibodies as several milligrams of antibody are needed for each column. Either polyclonal or monoclonal antibodies can be used (Shepherd and Carter, 1992). The polyclonal and monoclonal anti-sporidesmin A antibodies produced for use in ELISA were used to prepare IAC matrices which were investigated to determine if they were suitable for use in clean-up and concentration of samples containing sporidesmin A or sporidesmin metabolites.

6.1.1 Sample preparation

Immunoaffinity columns can easily become clogged if the sample is not free of precipitated material or lipids. Over time this effect can slow the flow rate. Therefore, fine particles and lipids in samples were removed by filtration or centrifugation to extend the life of the affinity matrix as it was proposed that the columns developed in this study should be reused several times.

6.1.2 Column flow rates

The flow rate through an affinity chromatography support is an important factor in producing optimal chromatography (Hermanson *et al.*, 1992), *i.e.* maximum capture of the analyte with minimum non-specific binding of other matrix components. Flow rates at four different chromatography steps are critical: during loading of the sample;

the wash step to remove non-specifically bound material; the elution phase and during regeneration of the matrix before application of the next sample (Ostrove, 1990). The capture of the target molecule from the sample becomes less efficient as the flow rate increases. Furthermore the rate at which the eluent moves through the support determines what access the eluent has to the IAC matrix. If the antibody affinity is low, a slow loading flow rate is used, whereas at the washing step it can be increased 2 to 5 times to remove non-specifically bound material from the matrix. The flow can be stopped after applying the eluent to allow the adsorbent to remain in the presence of the eluting agent before re-commencing elution. This may improve elution of specifically bound substances as dissociation can take place before elution. Regeneration of the matrix can be carried out at high flow rates.

6.1.3 Binding buffers and non-specific interactions

Materials can bind non-specifically to the IAC support but this can be minimised by altering the composition of the binding buffer. Non-specific binding may often be ionic, therefore sodium chloride is often included in binding buffers to prevent such interactions between the sample and IAC matrix. Binding of unwanted material brought about by hydrophobic interactions can be reduced by the addition of low levels of detergent (Pharmacia, 1986). Non-specific binding can also be limited by keeping the loading of IAC matrix to a level just below saturation (Harlow and Lane, 1988), therefore limiting the availability of excess binding sites to a minimum.

6.1.4 Methods of elution

The elution of the bound analyte in IAC depends on breaking the bonds between the immobilised antibody and sample analyte, ideally leaving both the analyte and antibody in active states. Most common elution procedures use a change in the composition of the mobile phase to alter the environment created during the binding phase, for example decreasing or increasing the pH of the eluting buffer (Hermanson *et al.*, 1992). Elution can also be achieved by denaturants such as methanol, change in ionic strength, protein deforming buffers, or polarity-reducing agents such as ethylene glycol (Candlish and Stimson, 1993).

6.1.5 Matrix capacity

The total binding capacity is a useful way to describe how well the support performs under ideal conditions and determines how much sample can be processed in a given

time. The more antibody immobilised during the coupling reaction, the greater the capacity of the support. The major limitation of this effect is that non-specific hydrophobic or ionic interactions become substantial when too much antibody is immobilised. The target molecule concentration as well as the concentrations of other sample constituents can dramatically alter the binding parameters of an IAC matrix (Hermanson *et al.*, 1992).

Flow rates through or past an immunoaffinity matrix also have a significant effect on the capacity. As the flow rate of a sample through a column is increased the capture of the target molecule from the sample becomes less efficient and the apparent capacity of the affinity support will drop. It is therefore important to overload the IAC matrix with target molecule to negate this flow rate dependency (Hermanson *et al.*, 1992).

6.2 Determination of elution conditions

Suitability of matrices for the recovery of sporidesmin A or its metabolites from body fluids was investigated first by determining if it was possible to recover these analytes from the columns and if so the most efficient methods of doing this.

6.2.1 Elution of sporidesmin A standard

(1) Polyclonal matrix

The immunoaffinity matrix was prepared as described in Section 2.11.1 by linking cyanogen bromide-activated Sepharose to ovine polyclonal anti-sporidesmin A antibody (animal 243) purified using Protein G Sepharose affinity chromatography. This antibody had been previously shown to bind sporidesmin A and to a lesser extent sporidesmin D and sporidesmin metabolites in sheep urine collected from sporidesmin A dosing trials. An IAC matrix composed of this immobilised antibody would be expected to bind sporidesmin A, sporidesmin D and sporidesmin A metabolites found in urine.

Trials were carried out to determine the optimal elution conditions required to dissociate sporidesmin A from this immunoaffinity matrix to obtain maximum recovery of sporidesmin A. Research carried out by Halder (1980) indicated the possibility that sporidesmin A may be unstable at extremes of pH and that maximum recovery of sporidesmin A from biological samples by solvent extraction was at pH 7.0. Therefore, as elution of sporidesmin A at extremes of pH could have reduced recoveries, elution with an organic solvent in water was investigated.

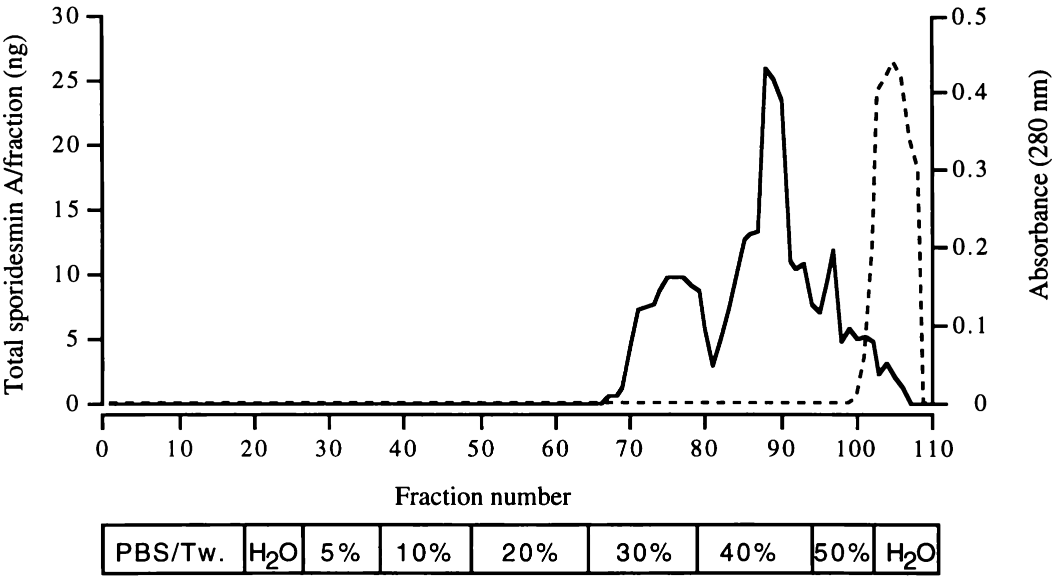
Two mL of the matrix was packed into a glass column and washed with 10 mL of 0.05% Tween 20 in PBS at a flow rate of 0.8 mL per minute. Twenty microlitres of sporidesmin A standard (500 ng) was diluted in 1 mL of 0.05% Tween 20 in PBS and loaded onto the column at the same flow rate.

Chromatography procedure:

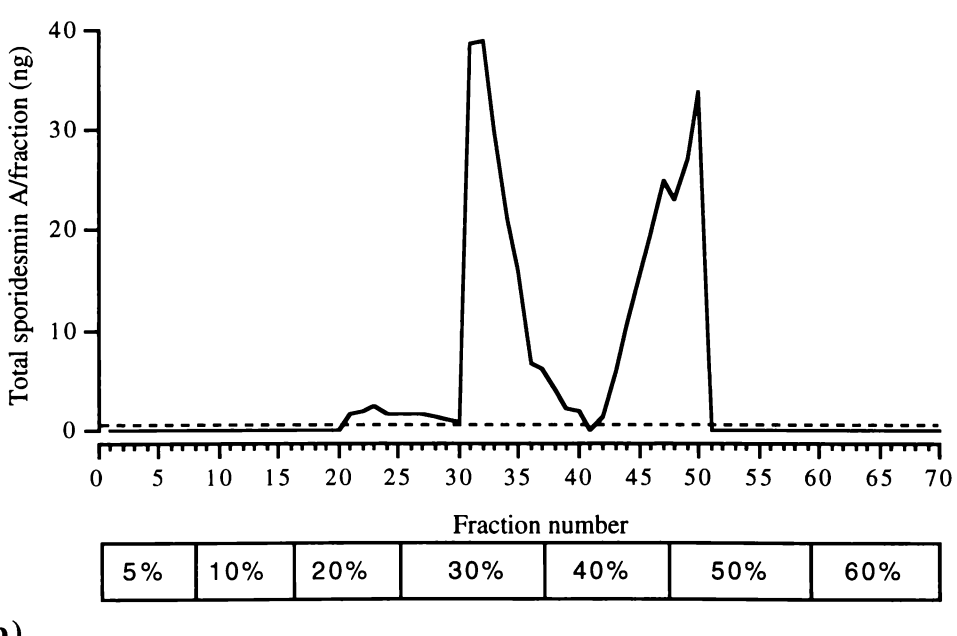
0.05% Tween 20 in PBS	20 mL
water	4 mL
5% MeOH in water	15 mL
10% MeOH in water	15 mL
20% MeOH in water	15 mL
30% MeOH in water	15 mL
40% MeOH in water	15 mL
50% MeOH in water	4.8 mL
water	4 mL
0.01% Thiomerosal in PBS	storage buffer

The eluate was monitored at 280 nm. Fractions (35 drops per fraction) were collected and those containing methanol were placed in a centrifugal evaporator to remove the organic solvent. All fractions were freeze-dried and then stored frozen. Later they were reconstituted in 0.2 mL of immunoassay sample buffer, and were analysed by cELISA to locate fractions containing the mycotoxin and therefore determine the conditions required for elution of sporidesmin A from the immunoaffinity column.

The elution profile is shown in Figure 6.2.1a. While some of the fractions eluted with 30% methanol were shown by cELISA to be immunoreactive, most of the immunoreactivity was eluted in 40% methanol and a residual amount in 50% methanol. After the column had been washed with 4.8 mL of 50% methanol the absorbance of the eluate measured at 280 nm began to rise. As it was considered that the increase in absorbance at this wavelength may be an indication that 50% methanol was stripping the antibody from the IAC matrix, the eluting solvent was immediately changed to water. After elution with 9 mL of water the absorbance returned to the baseline value. Freeze-drying yielded a white salt-like solid in the first 28 fractions and it was concluded that the volume of water (4 mL) used in the first water wash was insufficient to remove all traces of salts remaining from the first wash with PBS. The results suggested: (1) a larger volume of water should be passed through the column before elution of bound sporidesmin A so that the mycotoxin could be recovered free



(a)



(b)

Figure 6.2.1 IAC elution profile of sporidesmin A (500 ng) using (a) a polyclonal matrix and (b) a monoclonal matrix, with increasing concentrations of methanol in the eluting solvent. (---) Absorbance at 280 nm, (—) total sporidesmin A per fraction measured by cELISA.

of buffer salts; (2) it may be possible to recover all of the sporidesmin A bound to the column using a larger volume of 40% methanol, *i.e.* with 15 rather than 7.5 bed volumes; (3) sporidesmin A could also be recovered in a smaller volume of 50% methanol. However, as it was thought that this may remove the antibody from the matrix, the lower concentration of 40% methanol was chosen for subsequent use.

(2) Monoclonal matrix

A second matrix was prepared by linking the group B monoclonal antibody to the activated Sepharose. In cELISA this antibody bound sporidesmin D and urinary metabolites and cross-reacted to a lesser extent with sporidesmin A than sporidesmin D. One mL of the matrix was packed into a glass column. Column loading, chromatography and fraction recovery were carried out as described for the polyclonal matrix.

Chromatography procedure:

0.05% Tween 20 in PBS	10 mL
water	"
5% MeOH in water	"
10% MeOH in water	"
20% MeOH in water	"
30% MeOH in water	"
40% MeOH in water	"
50% MeOH in water	"
60% MeOH in water	"
water	"
0.01% Thiomerosal in PBS	storage buffer

The elution profile is shown in Figure 6.2.1b. A small amount of immunoreactivity was eluted with 20% methanol and the remainder with 30 and 40% methanol. Unlike the polyclonal matrix there was no increase in the eluate absorbance at 280 nm when the column was washed with 50% methanol.

6.2.2 Elution of urinary sporidesmin metabolites

As there was a large amount of polyclonal antibody available for IAC matrix preparation and only limited amounts of monoclonal antibody, method development and preliminary studies were undertaken using the polyclonal matrix. Urine samples collected from a sheep dosed with sporidesmin A were shown by cELISA to contain

sporidesmin metabolites and were used in the studies. One mL of pooled urine was centrifuged at 10 000*g* for 3 min and added to 1 mL of immunoaffinity matrix in 8 mL of 0.05% Tween 20 in PBS. The tube containing the gel and urine was mixed thoroughly by gentle rocking and left on its side for 30 min. The matrix was then packed into a glass column and the excluded urine solution was passed through the column and the retained material chromatographed using the procedures below.

Chromatography procedure:

0.05% Tween 20 in PBS	until the absorbance returned to baseline
water	10 mL
5% MeOH in water	10 mL
10% MeOH in water	10 mL
20% MeOH in water	10 mL
30% MeOH in water	10 mL
40% MeOH in water	10 mL
50% MeOH in water	10 mL
water	4 mL
0.01% Thiomerosal in PBS- storage buffer	

Fractions were collected and prepared for cELISA as described in Section 6.2.1 for the IAC of sporidesmin A standards. A minimal amount of immunoreactivity was shown to be eluted with 20% MeOH while most was eluted with 30% and 40% MeOH and a smaller portion was recovered with 50% MeOH (Figure 6.2.2). The absorbance of the eluate measured at 280 nm was increased above the baseline value when the column was washed with 50% MeOH which may have indicated that antibody was removed from the matrix by this concentration of methanol. It was therefore proposed that the immunoreactive material bound to the IAC column should be recovered by elution with a larger volume of 40% MeOH, *e.g.* 15 bed volumes.

6.3 Determination of matrix capacity

To determine the binding capacity of the immunoaffinity matrix (1 mL wet volume in PBS containing 0.05% Tween 20) was packed into a Mobicol column (1 mL capacity). Two sporidesmin A standards were used. The first approximated the expected capacity (*i.e.* 1 000 ng) while the second was greatly in excess of the expected capacity (*i.e.* 20 000 ng) to overcome any flow rate effects (Section 6.1.5). Each standard was added in 20 μL of methanol to 1 mL of 0.05% Tween 20 in PBS and loaded onto the column using the standard flow rate of 0.8 mL per minute. An

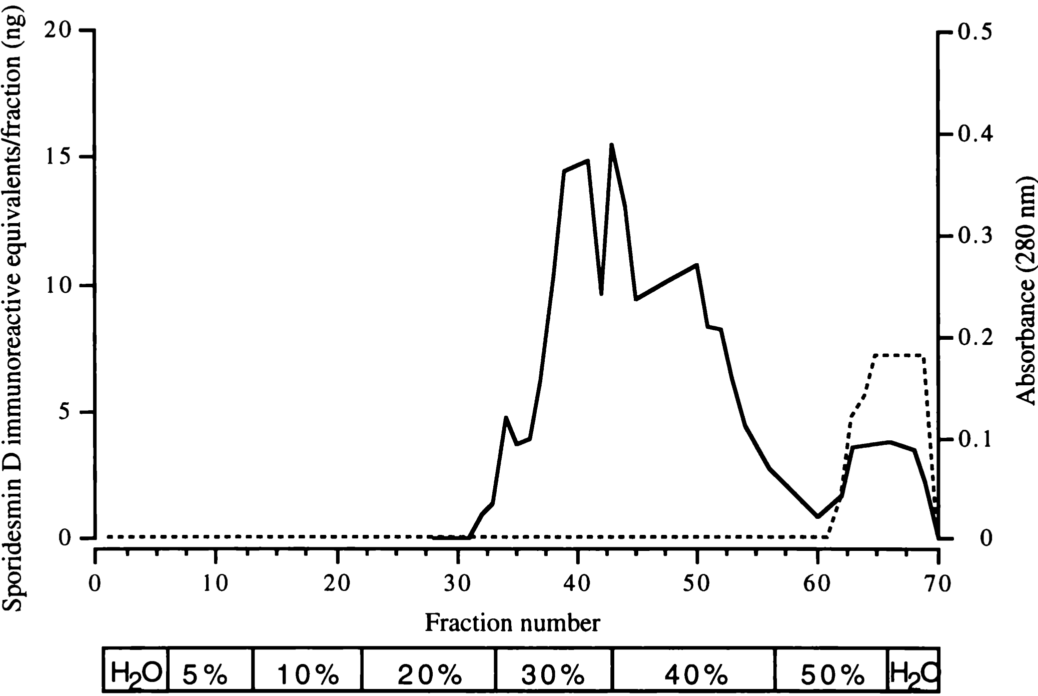


Figure 6.2.2 IAC elution profile of immunoreactive material from 1 mL of sheep urine containing sporidesmin metabolites. A polyclonal matrix was eluted with increasing concentrations of methanol in water. (---) Absorbance at 280 nm, (—) total immunoreactivity measured by cELISA as sporidesmin D immunoreactive equivalents per fraction. Sporidesmin D standards were used to generate the standard curve in the cELISA.

attempt to wash the column with water followed by elution with 40% MeOH failed because the heat generated by the mixing of these two solvents caused bubbles to form on the column. This did not occur when the column was washed with water followed step-wise with small amounts of 10 and 20% MeOH and then eluted with 40% MeOH. Therefore the procedure described below was used routinely.

Chromatography procedure:

0.05% Tween 20 in PBS	10 mL
sporidesmin A standard in 0.05% Tween 20 in PBS	1 mL
0.05% Tween 20 in PBS	15 mL
water	5 mL
10% MeOH in water	5 mL
20% MeOH in water	5 mL
40% MeOH in water	15 mL
water	10 mL
0.01% Thiomerosal in PBS - storage buffer	

The 40% MeOH fraction was taken to dryness by rotary evaporation and the flask containing the dried material was frozen at -20°C. Just before cELISA the contents of the flask were reconstituted in immunoassay sample buffer, transferred to a volumetric flask (2 mL), and made up to volume. Fifty microlitres was taken for cELISA.

The mean value for the maximum matrix binding capacity was determined to be 868 ng of sporidesmin A per mL of immunoaffinity matrix (Table 6.3.1).

Table 6.3.1 Recovery of sporidesmin A from IAC columns determined by cELISA

sporidesmin A (ng) applied	sporidesmin A (ng) recovered from IAC[a]
1 000	890
20 000	846

[a] Represents mean of values determined after reconstituted sample was diluted 1:9 and 1:24 in immunoassay sample buffer and 50 μL of each dilution analysed by cELISA in duplicate.

6.4 Recovery of sporidesmin A from buffer

The recovery of sporidesmin A standard was determined to assess whether the IAC methodology was acceptable for analytical use. Sporidesmin A (100 μg) was dissolved in 500 μL of methanol and dilutions of this stock solution were prepared to give amounts ranging from 5 to 1 000 ng in 10 μL of methanol. Ten microlitres of each standard was spiked into 1 mL of 0.05% Tween 20 in PBS and sporidesmin A was recovered by IAC using the chromatographic procedure described earlier (Section 6.3). The fraction obtained by elution with 40% MeOH in water was taken to dryness by rotary evaporation, reconstituted in immunoassay sample buffer and made up to volume in a 1 or 2 mL volumetric flask. Where required, a further dilution in immunoassay sample buffer was made and 50 μL was taken for analysis by cELISA.

The percentage recoveries of 5 to 1 000 ng of sporidesmin A, determined by cELISA, ranged from 72 to 124 with no apparent trend for different loadings (Table 6.4.1). The mean percentage recovery was 87, with the mean interwell %CV between replicates in cELISA being 5.0.

Table 6.4.1 Recovery of sporidesmin A from 0.05% Tween 20 in PBS by IAC

sporidesmin added (ng)	sample volume (mL)	ELISA dilution	recovery ng	recovery %	ELISA interwell CV%
5	1	1	4.4	89	1.0
10	1	1	12.4	124	1.1
20	1	1	15.1	75	4.3
62.5	2	1	46.9	75	1.0
125	2	1	90.0	72	13.7
250	2	1:1	191.0	76	2.7
500	2	1:9	430.8	89	9.4
500	2	1:9	453.6	91	2.3
1 000	2	1:9	890.0	89	9.8

6.5 Recovery of sporidesmin metabolites from urine

The IAC methodology was applied to the qualitative recovery of sporidesmin metabolites.

6.5.1 Investigation of cELISA interference by urine components

The immunoreactive sporidesmin metabolites detected by cELISA in sheep urine after natural exposure to, or dosing with sporidesmin A, have not yet been characterised. Since radiolabelled sporidesmin A is not available for animal dosing and consequent production of radiolabelled metabolites, the detection of these metabolites during purification can only be achieved by cELISA. Urine components may bind non-specifically to the IAC matrix and be co-purified with the metabolites. It was therefore essential that the effect on the cELISA of any non-specifically bound urine components present after IAC, be investigated.

Urine was collected from eight sheep which had not been dosed with, or exposed to, sporidesmin A. From a pool of these samples an aliquot (1 mL) was taken and centrifuged at 10 000*g* for 3 min, diluted in 9 mL of 0.05% Tween 20 in PBS and

loaded onto an IAC column (0.5 mL bed volume) at a flow rate of 0.9 mL per minute. The fraction eluted with 40% MeOH in water was collected and taken to dryness by rotary evaporation, reconstituted in immunoassay sample buffer and made up to volume in a 1 mL volumetric flask. Further dilutions in immunoassay sample buffer were made and 50 µL was taken for analysis by cELISA using the group B antibody.

Chromatography procedure:

0.05% Tween 20 in PBS	10 mL
urine in 0.05% Tween 20 in PBS	10 mL
0.05% Tween 20 in PBS	15 mL
water	5 mL
5% MeOH in water	5 mL
10% MeOH in water	5 mL
20% MeOH in water	5 mL
40% MeOH in water	15 mL
water	10 mL
0.01% Thiomerosal in PBS - storage buffer	

Results from cELISA of the IAC fraction diluted (1:1) showed a significant reduction in assay baseline colour development which suggested the presence of compounds that interfered with the cELISA (Table 6.5.1). Further dilution (1:4) eliminated this reduction indicating that the interference was non-specific.

Table 6.5.1 Effect on cELISA absorbances of components of urine extracted non-specifically by IAC

sample	ELISA dilution	ELISA absorbance (450 nm)[a]	% inhibition of absorbance
without sample	-	0.783	-
control urine[b]	1:1	0.637	19
"	1:4	0.796	0
"	1:9	0.786	0

[a] Absorbance determined in cELISA using the group B antibody. [b] A pooled urine sample which had been subjected to IAC.

6.5.2 Determination of matrix capacity for sporidesmin metabolites in urine

The matrix capacity for sporidesmin metabolites was measured to determine the efficiency of the polyclonal matrix in recovering immunoreactive metabolites from one particular sheep urine sample. The binding capacity is determined by the cross-reactivity and affinity of the immobilised polyclonal antibody for the metabolites but it is also influenced by any components in the urine which interfere with IAC matrix binding to the metabolites. Urine samples, collected from sheep after dosing orally with 0.2 mg of sporidesmin per kg of body weight, and shown by cELISA to contain immunoreactive metabolites, were combined to give a pool of 30 mL. This was divided into 1 mL aliquots and stored at -20°C in microcentrifuge tubes. Just before chromatography two tubes were thawed and centrifuged at 10 000*g* for 3 min. The supernatants from these were recovered and combined. Aliquots of the supernatant were made up to 10 mL in 0.05% Tween 20 in PBS as shown in Table 6.5.2. Each diluted sample was loaded onto the IAC column (0.5 mL bed volume) at a flow rate of 0.9 mL per minute and the chromatography procedure carried out as described in Section 6.5.1.

Before being subjected to IAC the pooled urine sample from the dosed sheep was analysed using cELISA optimised for urine samples (Section 2.8.2), and found to contain 1752 ng of sporidesmin A immunoreactive equivalents per mL. The amount of immunoreactive material recovered increased as the urine volume increased from 0.25 to 0.5 mL, but not from 0.5 to 1 mL, indicating that the maximum capacity was achieved with the metabolite contained in approximately 0.5 mL of urine, *i.e.* about 270 ng equivalents (Table 6.5.2). For this matrix and urine sample maximum recovery efficiency was achieved when the urine sample volume to bed volume was less than 1:1.

6.5.3 Isolation of sporidesmin metabolites from urine

Although the maximum recovery of metabolite was only 40% of that applied to the IAC column (Table 6.5.2), the IAC method developed was still suitable for scaling-up to extract a portion of the metabolites from pooled urine samples so long as it was recognised that the recovered metabolites represented only a selected portion of the spectrum of metabolites present. With this proviso it was proposed that IAC be scaled up and used for isolating metabolites for further characterisation.

Table 6.5.2 Percentage recovery of sporidesmin metabolites by IAC from various volumes of urine

urine volume (mL)	metabolites loaded (ng)[a]	recovery ng[a]	recovery %	ELISA interwell %CV
0.25	438	177	40	1.5
0.5	876	272	31	7.6
1.0	1752	260	15	4.4

[a] Represents sporidesmin A immunoreactive equivalents measured by cELISA using the group B antibody. The standard curve in the assay was generated using sporidesmin A standards.

The matrix was packed into a Wright column (2 mL) and equilibrated in 0.05% Tween 20 in PBS. Urine (6 mL) was diluted as described previously (6.5.1), and processed in six 10 mL lots to ensure a urine volume to bed volume ratio of 1:2.

Chromatography procedure:

PBS in Tween 20 (0.05%)	10 mL
urine in PBS in Tween 20 (0.05%)	10 mL
PBS in Tween 20 (0.05%)	30 mL
water	10 mL
5% MeOH in water	10 mL
10% MeOH in water	10 mL
30% MeOH in water	20 mL
40% MeOH in water	20 mL
water	10 mL
0.01% Thiomerosal in PBS – storage buffer	

The fractions recovered from the six runs by elution with 30% methanol were pooled and taken to dryness by rotary evaporation. Fractions eluted with 40% methanol were treated the same way. The material recovered in each pool was redissolved in 4.1 mL of 5% methanol, 100 µL taken for cELISA and the remainder divided into four 1 mL aliquots. Methanol was removed under a stream of nitrogen and the remaining aliquots freeze-dried and then stored at -20°C before further study.

Results from cELISA indicated that a total of 1.38 µg of sporidesmin A immunoreactive equivalents were recovered in the fraction eluted with 30% methanol

and 0.12 μg in the fraction eluted with 40% methanol. This was 14% of the total immunoreactivity contained in the 6 mL of urine as determined by cELISA before IAC.

6.5.4 Enzyme hydrolysis of sporidesmin metabolites

The question of whether sporidesmin metabolites were excreted in the urine as conjugates (*e.g.* as glucuronides, sulfates) was addressed by incubating samples with enzymes such as β-glucuronidase and sulfatase (Section 5 3). Samples were analysed by cELISA before and after the incubation to determine whether there had been an apparent increase in the total immunoreactive equivalents. To investigate the possibility that sporidesmin A or metabolites bind to protein, samples were also treated with pronase.

When urine samples, collected from animals dosed with sporidesmin A, were hydrolysed with enzymes and the hydrolysates analysed by cELISA, the results did not provide conclusive evidence to suggest the presence of sporidesmin glucuronide, sulfate or protein conjugates (Section 5.3.2). Olsen and Chu (1993) could not demonstrate an increase in apparent sterigmatocystin content when the incubated urine sample, prepared by filtration, by clean-up with a XAD-2 column or by C-18 SepPak, was hydrolysed with β-glucuronidase before analysis for sterigmatocystin. They did, however, observe the formation of free sterigmatocystin when urine samples were purified by IAC and hydrolysed with enzymes. Therefore the attempt to demonstrate hydrolysis of sporidesmin metabolites was repeated using the material which had been purified by IAC (Section 6.5.3) and reaction conditions described earlier in Section 2.10. Unpurified urine samples taken from three different sheep were also studied. These samples were collected before and after dosing, hydrolysed with β-glucuronidase, sulfatase and pronase and the hydrolysates analysed by cELISA. Experimental controls were: urine collected before the sheep were dosed plus enzyme; sample in buffer without enzyme and enzyme in buffer without sample. The IAC purified material (337 ng of sporidesmin A immunoreactive equivalents) was dissolved in 250 μL of PBS. Twenty μL was added to 230 μL of the appropriate buffer (Section 2.10), the enzyme added and the hydrolyses carried out. The hydrolysates (50 μL) were taken for analyses by cELISA using both group A and B antibodies and the assay absorbances obtained were compared with those obtained for unhydrolysed samples.

The presence of these conjugates would be indicated when cELISA of the hydrolysed sample gave less colour development than that developed in cELISA of the untreated sample. Sulfatase hydrolysates of the material purified by IAC gave similar assay results as the unhydrolysed samples with both antibodies (Table 6.5.3). When β–glucuronidase was used, the cELISA absorbances in the assays using the group A antibody were similar for both treated and untreated samples, whereas with the group B antibody they were not. cELISA of pronase hydrolysates of the urine samples collected before the sheep were dosed with sporidesmin A revealed the presence of substances which interfered with the assay using the group A antibody. Unlike earlier studies (Section 5.8.2) there appeared to be no interference with the assay using the group B antibody. Analysis of the pronase hydrolysates of all three unpurified urine samples as well as the IAC purified material gave lower results when the group B antibody was used. These slight reductions in cELISA results provided weak evidence that sporidesmin glucuronides and protein conjugates may be present in the urine sample studied.

Table 6.5.3 Effect of enzyme hydrolysates of sheep urine obtained after dosing with sporidesmin A, on cELISA

	% reduction of cELISA absorbance with:		
	β–glucuronidase	sulfatase	pronase
Group A antibody:			
IAC purified urine	0	0	no result[a]
unpurified urine	0[b]	0[b]	"
Group B antibody:			
IAC purified urine	30	0	80
unpurified urine	0[b]	0[b]	22, 29, 34

[a] Urine samples which were collected from sheep before dosing with sporidesmin A were hydrolysed with pronase. These hydrolysates were shown to interfere with the cELISA which indicated that the hydrolysates of samples collected after dosing with sporidesmin A would give invalid results in cELISA using the group A antibody. [b] Represents results from three animals.

As the pronase hydrolysates of urine collected before the sheep were dosed with sporidesmin A contained substances which interfered with the assay using the group

A antibody, the hydrolysates were ultrafiltered by centrifugation in Microcon 10 devices (Amicon Division, W. R. Grace & Co. MA, USA) to remove enzyme and other material above 10 kD. After ultrafiltration the diffusate sample no longer interfered with the cELISA which indicated that the hydrolysate component responsible for the assay interference had a molecular weight greater than 10 kD. cELISA using the group A antibody of the urine samples, with and without pronase hydrolysis and with ultrafiltration, showed no increase in the immunoreactive equivalents detected (Table 6.5.4). The assay using the group B antibody also showed no difference in immunoreactivity between hydrolysed and unhydrolysed IAC purified samples after ultrafiltration (Table 6.5.4), although the unpurified urine showed a slight difference after pronase hydrolysis and ultrafiltration.

When all the samples with and without β-glucuronidase hydrolysis were ultrafiltered and analysed by cELISA using both antibodies, the results (Table 6.5.4) were similar to those obtained when the samples had not been ultrafiltered (Table 6.5.3).

Table 6.5.4 Effect on cELISA of ultrafiltered enzyme hydrolysates of sheep urine obtained after dosing with sporidesmin A

	% reduction of cELISA absorbance with:	
	β–glucuronidase	pronase
Group A antibody:		
IAC purified urine	0	0
unpurified urine	0[a]	0[a]
Group B antibody:		
IAC purified urine	22	0
unpurified urine	0[a]	8[a]

[a] Represents the result from one animal sample.

6.6 Immunoaffinity chromatography of milk

As circulating sporidesmin A reaches the udder of lactating cows (Towers, 1978), it is important from both a veterinary health and a food safety perspective to determine whether free sporidesmin A is transferred into milk. IAC was investigated as a means

of concentrating any sporidesmin A and/or its metabolites present in milk to extend the limits of detection for the ELISAs which had been previously developed for the quantification of sporidesmin residues in milk (Section 4.4).

6.6.1 Preparation of sample

Methods developed for the preparation of milk samples for cELISA (Section 2.8.3) were used to prepare milk samples for IAC. Milk from 25 individual samples was pooled, then divided into 20 mL aliquots and stored at -20°C. An aliquot was thawed and warmed to 45°C, mixed thoroughly and 5 mL taken and diluted in 45 mL of 0.5% Tween 20 in PBS. A second 5 mL amount was taken and diluted in 45 mL of 0.05% Tween 20 in PBS. Both diluted samples were heated at 70°C for 30 min and centrifuged at 2 000*g* for 20 min at 4°C. The aqueous fractions were recovered.

The interface between cream and aqueous phases in 0.5% Tween 20 was clearly defined and it was possible to remove the solid cream layer with a spatula. With 0.05% Tween 20, the aqueous fraction had to be re-centrifuged after the cream layer was first removed because the separation between the two phases was not as well defined.

A similar experiment was carried out with:
(1) 5 mL milk + 20 mL of 0.5% Tween 20 in PBS to give a final concentration of 0.4% detergent;
(2) 5 mL milk + 5 mL of 1% Tween 20 in PBS to give a final concentration of 0.5% detergent.

In both cases the separation between the two phases was not as well defined as that achieved with 1 in 10 dilution in 0.5% Tween 20 in PBS.

Milk (10 mL) was taken and spiked with 1 μg of sporidesmin A standard in 50 μL of methanol. The milk was divided into two 5 mL samples and 45 mL of 0.5% Tween 20 in PBS was added to each. The diluted samples were heated at 70°C for 30 min and after cooling they were centrifuged at 2 000*g* for 20 min at 4°C. The FAT which had separated after centrifugation was removed and discarded and 45 mL of the aqueous fraction was loaded onto an IAC column (Mobicol, 1 mL bed volume) using a flow rate of 0.8 mL/min.

Chromatography procedure:

0.05% Tween 20 in PBS	10 mL
processed diluted milk in 0.5% Tween 20	45 mL
0.05% Tween 20 in PBS	15 mL
water	15 mL
5% MeOH in water	5 mL
10% MeOH in water	10 mL
20% MeOH in water	10 mL
40% MeOH in water	15 mL
water	10 mL
0.01% Thiomerosal in PBS - storage buffer	

The aqueous phase recovered from the second milk sample was frozen at -20°C overnight. On thawing it was noticed that a white precipitate had formed. After centrifugation at 2 000*g* for 15 min at 4°C the supernatant was removed and 45 mL loaded onto the column.

The fractions recovered by elution with 10, 20% and 40% methanol were concentrated by rotary evaporation to almost dryness, reconstituted in immunoassay sample buffer (2 mL) and analysed by cELISA for sporidesmin A. Sporidesmin A could not be detected in the 10 and 20% methanol fractions. Fifty-five percent of the sporidesmin A spike (500 ng) was recovered in the fractions eluted with 40% methanol from the first sample which had been centrifuged only once. Fifty-six percent of the spike was recovered from the second sample which had been frozen overnight and re-centrifuged. The recovery appeared to be similar for both samples and therefore not affected by the presence or absence of the material which was precipitated during freezing. It was assumed that sporidesmin A was not associated with this material as the recovery was not altered by the second centrifugation step and removal of the precipitate. Subsequently, freezing was incorporated as part of the standard procedure for preparing milk sample for IAC.

6.6.2 Optimisation of chromatography

In an attempt to increase the recovery of sporidesmin A from milk by IAC, a series of experiments were carried out to determine if this could be achieved by altering the chromatographic conditions. Sporidesmin A (6 μg/300 μL of methanol) was added drop-wise with mixing to 60 mL of milk and 5 mL aliquots (each containing 500 ng of sporidesmin A) were prepared for IAC as described in Section 6.6.1 using 0.5%

Tween 20 in the diluent. The aqueous phase recovered was frozen at -20°C overnight. After thawing and centrifugation at 2 000*g* for 15 min at 4°C, the supernatant was recovered and 45 mL loaded onto the column.

The following chromatographic conditions were varied: the flow rate during column washing and elution; the detergent concentration in the column pre-wash and wash; and the volume of 10% and 20% MeOH used in the column wash. When these experiments were completed 13 milk extracts had been processed on the same column with the best recovery of sporidesmin A being only 61% of that loaded onto the column (Table 6.6.1). Recovery initially ranged from 55 to 61% but after a total of 11 milk extracts had been processed it fell to 45%. It was also observed at this point that the flow through the column was reduced and when the gel was removed from the column it appeared to settle into clumps. It was concluded that the reduction in the recovery to 45% may not be due to the alteration in chromatographic conditions but instead be caused by decreased column capacity and efficiency brought about by the presence of particulate material from the milk extracts remaining on the column.

Table 6.6.1 Effect of alteration in chromatographic conditions on the recovery of sporidesmin A from milk using IAC

Expt.	flow rates used		pre-load wash (mL)		column washes (mL)				% recovery
					1	2	3	4	
No.	1[a]	2[b]	0.05% Tw.20	0.5% Tw.20	0.05% Tw.20	water	10% MeOH	20% MeOH	of 500 ng sporidesmin A
1	√		10		10	15	4	4	56, 57
2		√	10		10	15	4	4	59, 61
3		√	10		0	20	4	4	60, 61
4		√	10		10	15	0	4	55, 56
5		√		10	0	15	10	10	55
6		√	10		0	15	10	10	47
7		√	10		0	15	0	20	45

[a] Represents one flow rate of 0.8 mL/min used throughout the whole chromatographic procedure.
[b] Indicates a flow rate of 0.8 mL/min during column pre-wash and sample loading and thereafter 1.8 mL/min was used.

6.6.3 Clarification of samples before chromatography

Methods to improve the clarity of samples before IAC were investigated so that clogging of the matrix could be avoided and repeated use of the columns could be possible. Aliquots of milk (5 mL) were taken and processed as described earlier Section 6.6.1. It was found that increasing the second centrifugation speed to 10 000*g* and filtering the supernatant collected using a glass fibre filter (0.7 µm) improved the clarity of the samples loaded onto the IAC column.

6.6.4 Effect of chromatographic conditions on the recovery of sporidesmin A from milk

(a) Detergent concentration in sample diluent

As it was demonstrated that alterations made in the chromatographic conditions did not significantly increase the recovery of sporidesmin A from milk (Table 6.6.1), the effect of the concentration of detergent in the milk diluent on sporidesmin A recovery was investigated. Matrix, which had not been previously used in chromatography, was placed in a new Mobicol (1 mL bed volume). Three aliquots of milk (5 mL) each containing 400 ng of sporidesmin A were diluted in 45 mL of PBS containing 0.05, 0.25 and 0.5% Tween 20 respectively. The diluted samples were heated at 70°C for 30 min and centrifuged at 2 000*g* for 20 min at 4°C. The aqueous fraction from each sample was recovered, frozen overnight and re-centrifuged at 10 000*g* for 20 min at 4°C. After filtration through a glass fibre filter (0.7 µm), 45 mL of each fraction was cooled in ice and loaded onto the column using a flow rate of 0.8 mL per minute. The column was washed and sporidesmin A was eluted using a flow rate of 1.8 mL per minute. To check that the matrix performance was still good in the absence of milk, a reference sample containing 400 ng of sporidesmin A in 1 mL of 0.05% Tween 20 in PBS was loaded onto the column and recovered using the same method as that used for the milk samples.

Chromatography procedure:

0.05% Tween 20 in PBS	10 mL
processed diluted milk	45 mL
0.05% Tween 20 in PBS	5 mL
water	20 mL
10% MeOH in water	5 mL
40% MeOH in water	15 mL
10% MeOH in water	5 mL
water	10 mL
0.01% Thiomerosal in PBS - storage buffer	

The fraction recovered by elution with 40% methanol was taken to almost dryness by rotary evaporation, reconstituted in immunoassay sample buffer and made up to volume in a 2 mL volumetric flask. Further dilutions were made before analysis by cELISA for sporidesmin A.

The recovery of sporidesmin A from milk was better when the concentration of detergent used was 0.25 or 0.5% (Table 6.6.2), although the recovery from all milk samples was substantially less than from PBS containing 0.05% Tween 20. This sample without milk was chromatographed after the milk samples had been processed and it was concluded that the lower recoveries with milk could be due to interferences from milk components, or to the larger sample volume loaded onto the column.

Table 6.6.2 The effect of detergent concentration in sample diluent on sporidesmin A recovered from milk by IAC

		sample treatment				cELISA
	spdm A	heated at	% Tween 20	recovery		interwell
sample	added (ng)	70°C	in diluent	ng	%	%CV
milk	400	√	0.05	212.8	53.2	7.7
milk	400	√	0.25	247.6	61.9	6.8
milk	400	√	0.5	250.0	62.5	9.9
PBS	400		0.05	342.3	85.6	3.5

(b) Volume of sample loaded onto the column

To determine the effect of sample volume on recovery, milk (5 mL) was spiked with 500 ng of sporidesmin A in 25 µL of methanol. The filtered aqueous supernatant (45 mL) prepared for IAC was divided into three aliquots of 5, 15 and 25 mL each, and sporidesmin A recovered from each by IAC.

The percentage of sporidesmin A recovered from 5, 15, and 25 mL samples were similar (Table 6.6.3). It was concluded that for 5-25 mL, recovery of sporidesmin A (50-200 ng) by IAC was independent of sample volume loaded.

Table 6.6.3 Effect of sample volume loaded onto the immunoaffinity column on sporidesmin A recovery

volume of diluted milk (mL)	spdm A added (ng)	sample treatment: heated at 70°C	sample treatment: Tween 20 in diluent (%)	recovery: ng	recovery: %	cELISA interwell %CV
5	50	√	0.05	30.3	60.6	3.8
15	150	√	0.05	91.5	61.0	8.3
25	250	√	0.05	159.4	63.8	3.7

(c) Omitting heating the diluted sample before IAC

An experiment was also undertaken to determine what effect omitting heat treatment of the diluted milk had on sporidesmin A recovered by IAC. Two milk samples (5 mL) each spiked with 400 ng of sporidesmin A, were prepared for IAC as described in Sections 6.6.4a and 6.6.4b. One sample was diluted in 0.05% Tween 20 in PBS and the other in 0.5% Tween 20 in PBS. Heating the samples at 70°C for 30 min, however, was omitted. When the aqueous fraction recovered after centrifugation was not heated it was difficult to pass through the glass-fibre filter (0.7 µm) before IAC. This was overcome by passing the solution through a Whatman GF/A glass-fibre filter (1.6 µm) before the Sartorius glass-fibre filter (0.7 µm).

The recovery of sporidesmin A from milk diluted in 0.5% Tween 20 (Table 6.6.4) was similar to that obtained previously when the samples were heated (Table 6.6.2), *i.e.* 63% and 64% respectively. However, at the lower detergent concentration

omitting heating appeared to improve sporidesmin A recovery (53 to 61%) although the experiment was not repeated and this result may not be significant.

Table 6.6.4 Recovery of sporidesmin A by IAC without heating included in sample preparation

	spdm A	Tween 20	recovery		cELISA
sample	added (ng)	in diluent (%)	ng	%	interwell %CV
milk	400	0.05	244.6	61.2	7.4
milk	400	0.5	257.8	64.4	0.3

6.6.5 Investigation of cELISA interference by milk components

To demonstrate whether any milk components which were co-purified with sporidesmin A or its metabolites by IAC, interfered with the cELISA, milk samples were collected in winter when there was no chance of unknown background exposure to sporidesmin A. Fresh milk (100 mL) was taken from a farm vat and stored at -20°C. Later the frozen whole milk was thawed, warmed to 45°C, mixed thoroughly and a 5 mL aliquot taken. This was diluted in 45 mL of 0.5% Tween 20 in PBS which had also been warmed to 45°C, mixed thoroughly and the mixtures kept at 45°C for 5 min before cooling in ice and centrifugation at 2 000*g* for 20 min at 4°C. The aqueous fraction was recovered and frozen. Just before chromatography it was thawed and centrifuged at 10 000*g* for 20 min at 4°C. The supernatant recovered was filtered through glass fibre filters, the first with a pore-size of 1.6 mμ followed by another with a pore-size of 0.7 μm. After cooling in ice, a measured volume was loaded onto the IAC column (1 mL bed volume) using a flow rate of 0.8 mL per minute. Wash and elution steps were carried out at 2.0 mL per minute. Two other samples were prepared in a similar way except they were diluted in 45 mL of 0.05% Tween 20 in PBS.

Chromatography procedure:

0.05% Tween 20 in PBS	10 mL
milk diluted in 0.5% or 0.05% Tween 20	45 mL
0.05% Tween 20 in PBS	10 mL
water	20 mL
10% MeOH in water	5 mL

40% MeOH in water	15 mL
10% MeOH in water	5 mL
water	10 mL
0.01% Thiomerosal in PBS- storage buffer	

The fractions recovered by elution with 40% methanol were taken to near dryness (approximately 200 µL) by rotary evaporation at 40°C and transferred with several washes of immunoassay sample buffer to a 1 mL volumetric flask. This was stored frozen at -20°C until later when it was thawed, further dilutions in immunoassay sample buffer made and 50 µL of each was taken for analysis by cELISA using the group A antibody.

It was shown that when the eluate from IAC was reconstituted in sample buffer and added undiluted to the cELISA, the absorbance measured was similar to that recorded in the absence of sample (Table 6.6.5). Furthermore, the concentration of Tween 20 used in the milk diluent before IAC did not affect the result. It was concluded that any substances in milk which were co-purified with sporidesmin A, and eluted with 40% methanol from the IAC column, did not interfere with the cELISA using the group A antibody.

6.6.6 Validation of the optimised chromatography method for the recovery of sporidesmin A from milk

Sporidesmin A (100 µg) was dissolved in 500 µL of methanol and dilutions of this stock solution were prepared to give amounts ranging from 25 to 400 ng in 40 µL of methanol. Forty microlitres of each standard was spiked into 5 mL of milk and the recovery of sporidesmin A from IAC was determined by cELISA. The milk was processed and purified by IAC as described in Section 6.6.5 using 0.5% Tween 20 in the milk diluent and care was taken to see that the column was not compacted.

The percentage recovery of sporidesmin A from IAC determined by cELISA, ranged from 68.2 to 88.8 (Table 6.6.6). The mean recovery was 75.6 ± 9.8 and the mean interwell %CV in cELISA being 3.7. The recovery determined for 25 ng loading was approximately 20% better than those determined for 400 and 100 ng, suggesting that best recoveries were achieved only when low loadings such as 25 ng were used.

Table 6.6.5 Effect of eluates from IAC of milk on cELISA absorbances using the group A antibody

sample	dilution of sample for cELISA	cELISA absorbance (450 nm)	% inhibition of absorbance
without sample		0.870[a]	
1[b]	1:0	0.848	2.5
	1:1	0.834	4
	1:2	0.867	0
	1:4	0.854	2
without sample		1.241[a]	
2[c]	1:0	1.222	1.5
	1:1	1.201	3
	1:2	1.218	2
	1:4	1.235	0.5
without sample		1.241[a]	
3[c]	1:0	1.234	1
	1:1	1.255	0
	1:2	1.230	1
	1:4	1.193	4

[a] Maximum absorbance measured in cELISA in the absence of sample. [b] Sample 1 diluted with 0.5% Tween 20 in PBS before IAC. [c] Sample 2 and 3 diluted 0.05% Tween 20 in PBS before IAC.

Table 6.6.6 Recovery of sporidesmin A from milk by IAC

sporidesmin added (ng)	sample volume (mL)	sample dilution in cELISA	recovery		ELISA interwell
			ng	%	%CV
400	1.0	1:4	274.4	68.6	5.1
400	1.0	1:4	279.5	69.9	5.2
100	1.0	1:4	68.2	68.2	3.5
100	1.0	1:4	70.6	70.6	3.2
25	1.0	1:1	21.9	87.6	2.5
25	1.0	1:1	22.2	88.8	2.8

6.6.7 Recovery of sporidesmin residues in milk

A trial was carried out to determine whether sporidesmin residues appear in milk following experimental ingestion of the toxin. Three milking cows which had been selected for culling were used in the trial. Milk samples were taken before and after oral administration of two doses of approximately 0.3 mg of sporidesmin A per kg of body weight given on two consecutive mornings. Following dosing, the cows were milked twice daily for 4 days and then slaughtered.

Whole milk samples were analysed by cELISA for free sporidesmin A and sporidesmin metabolites using both the group A and group B antibodies as previously described in Section 2.8.3. Sporidesmin A was not detected by the cELISA using the group A antibody which had a working range of 130-6 500 ng/mL of milk. Sporidesmin metabolites, however, were detected in whole milk using the group B antibody although levels were just out of the working range of 66-560 ng/mL of milk for the assay. The working range for the assay of skimmed milk (Figure 4.4.9) is more sensitive (40-470 ng/mL) and this allowed the detection of immunoreactive substances peaking at 8 h in milk samples collected from two of the cows after the first dose of sporidesmin A (Figure 6.6.1). It was not possible to detect immunoreactive material in the milk from the third cow.

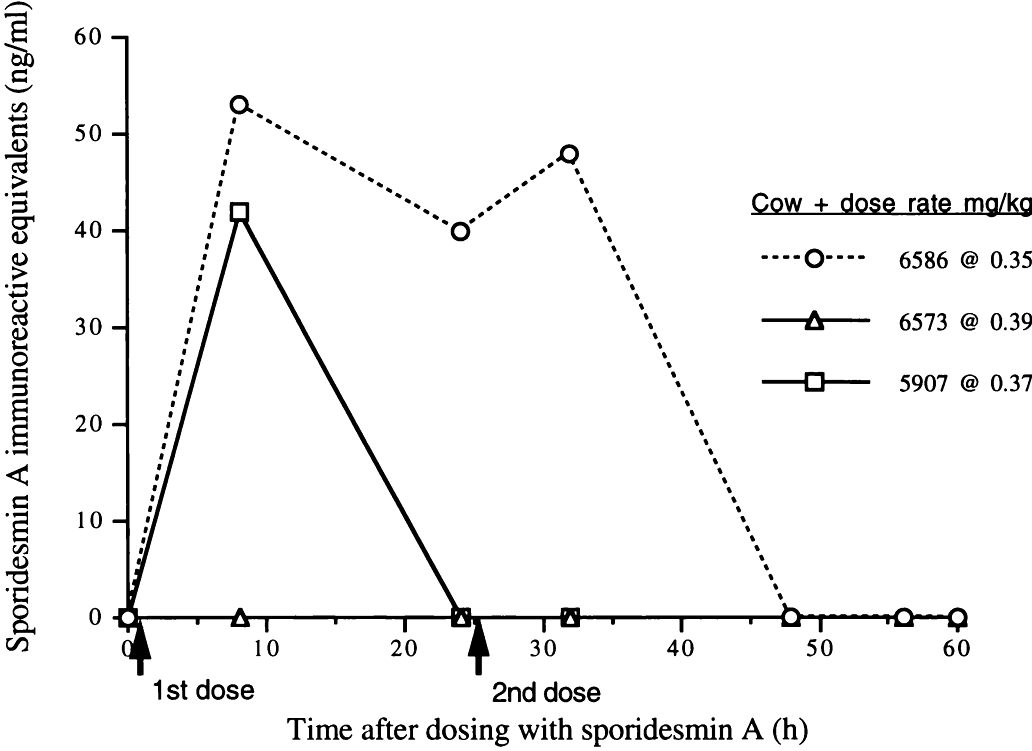


Figure 6.6.1 Sporidesmin metabolites in milk after experimental ingestion of sporidesmin A, measured by cELISA using the group B antibody. Samples were measured in duplicate: the mean %CV of absorbance measured by cELISA ranged from 0.3 to 6.8 (mean = 2.8).

To improve the limit of detection IAC was used to concentrate sporidesmin metabolites from a milk sample which was positive for metabolite in the cELISA. Five mL of the milk sample collected from cow number 6586, 8 h after the first dose of sporidesmin A, was prepared for IAC as described in Section 6.6.5 and loaded onto the column. The fraction eluted with 40% methanol was taken to near dryness by rotary evaporation and the residue recovered reconstituted to 500 μL in immunoassay sample buffer. Two-fold serial dilutions were made and 50 μL of each were analysed by cELISA using both the group A and group B antibodies.

The concentrations of immunoreactive material measured in the two assays (Table 6.6.7) were the same in diluted samples when they were corrected for dilution. This indicated that there were no matrix effects interfering with the assays, confirmed the previous detection of sporidesmin metabolites and suggested the presence of sporidesmin A. The amount of sporidesmin A measured was near to the lower limit of the working range for the cELISA using the group A antibody. The result therefore

needs to be confirmed by purifying a much larger volume of milk and analysing the extract for sporidesmin A using an independent methods such as HPLC and mass spectrometry.

Table 6.6.7 Sporidesmin residues recovered by IAC of milk sampled after experimental ingestion of sporidesmin A

antibody used in cELISA	dilution of sample in cELISA	sporidesmin A immunoreactive equivalents (ng/mL)[a]	cELISA interwell %CV
A	1:0	0.6	0.5
"	1:1	0.5	3.6
B	1:1	7.1	3.8
"	1:3	6.4	2.8
"	1:7	6.5	2.8
"	1:15	6.2	1.1

[a] Immunoreactive equivalents measured by cELISA using sporidesmin A standards to generate the standard curve and corrected for dilution.

6.7 Discussion

6.7.1 Introduction

IAC was investigated as a means of concentrating sporidesmin A and sporidesmin metabolite(s) from urine and milk samples because there was a need to extend the limit of detection in samples where only trace amounts were present. This was the case with many of the samples generated in this research. The methodology was also applied to the qualitative extraction and isolation of immunoreactive sporidesmin metabolites from urine and milk. For quantitative detection, recoveries of analyte from IAC need to be reproducible and preferably greater than 80%, whereas in qualitative studies, recoveries less than 80% may be adequate to provide sufficient material for characterisation so long as column size can be scaled-up or columns can be used repeatedly.

6.7.2 Matrix performance

The performance characteristics of the polyclonal matrix were determined and the results compared with those for matrices used in the purification of other small molecules. The maximum matrix capacity determined for the ovine antibody bound to CNBr-activated Sepharose was approximately 900 ng of sporidesmin A per mL of gel. This was similar to that determined by Bagnati *et al.* (1990), who obtained a column capacity of 700 ng of trans-diethylstilboestrol per mL of gel. Martlbauer *et al.* (unpublished results, 1995) obtained column capacities ranging from 250-1 050 ng per mL for sulfadiazine, sulfamethazine and streptomycin. The same amount of sporidesmin A was recovered by IAC when a column was loaded in slight excess of the matrix capacity with 1 000 ng of sporidesmin A, and when loaded with 20 times this amount. This would indicate that the capacity of 900 ng was a true value and not an underestimate resulting from the flow rate of 0.8 mL per minute being too high to allow complete capture.

In the present study the mean recovery of nine sporidesmin A standards (5-1 000 ng) using IAC was determined by cELISA and found to be 87%, ranging from 72-124%. These results were similar to those obtained also by cELISA by Azcona *et al.* (1990) who reported the mean recovery of 95% (range 76-120%) of zearalenone in distilled water from immunoaffinity columns. While it would have been useful to confirm the results by HPLC to ensure that the recovered material was intact sporidesmin A, some of these quantities were well below the limit of detection of the HPLC method routinely used for the quantification of sporidesmin A. Under optimal conditions the least amount of sporidesmin A measured by the HPLC method is 0.5 ng (A. D. Hawkes, AgResearch, Hamilton NZ; unpublished results) although the sensitivity may be much less depending on the matrix composition of the sample.

Previously, in cELISA, the polyclonal antibody used for IAC had been shown to bind to sporidesmin A and to cross-react to a lesser extent with sporidesmin D and urinary metabolites. Similarly the monoclonal antibody had been shown to bind to sporidesmin A, sporidesmin D and analogues where the antibody binding site remained sufficiently unmodified. There may, however, be a discrepancy between antibody reactivity in an immunoassay and the binding properties of antibody in IAC (Calton, 1984). This can occur when immobilisation of an antibody on a polymer matrix yields an antibody conformation that is different from that on a polystyrene ELISA plate. In either case the antibody structure may be deformed when coupled to the immunomatrix, active sites may be bound or sterically hindered and the formation

of an antibody-antigen complex is not possible (Calton, 1984). This might be expected to reduce analyte recovery by IAC. In the present study, however, when the polyclonal antibody was immobilised on Sepharose and used in IAC, good recoveries of 5-1 000 ng of sporidesmin A spiked into buffer were achieved. Recovery of sporidesmin A standards from milk using this matrix was greater than 80% only at low loadings, *i.e.* 25 ng per mL of gel, indicating that in the presence of milk the maximum binding capacity of the IAC was substantially reduced.

In this study the elution profile of sporidesmin A from the monoclonal matrix was seen to have a sharper trailing edge than that obtained from the polyclonal matrix. Goding (1986) suggested that elution curves from polyclonal immunoadsorbents have long trailing edges due to high-affinity interactions which are not easily disrupted. With monoclonal antibodies, however, peaks often emerge with less trailing because of the homogeneous antibody population binding a single analyte epitope, often giving lower-affinity interactions with the analyte. Elution of sporidesmin A from the polyclonal column was achieved by using relatively mild, non-denaturing conditions and recoveries were acceptable. Polyclonal columns have been reported to show better stability than the equivalent monoclonal columns and are able to be regenerated more times. Farjam *et al.* (1991) compared the performance of a polyclonal matrix with that of a monoclonal matrix for recovering aflatoxin M_1 and B_1 from milk. They found that the monoclonal matrix had a capacity drop of about 90% during the first six to ten analyses. The equivalent polyclonal column maintained its initial capacity during at least 50 analyses. They also found that both columns showed decreased recovery of analyte from spiked milk samples upon repeated use, although the loss was more significant with the monoclonal than with the polyclonal column. Therefore, in spite of the trailing of the sporidesmin A elution curve, the first matrix evaluated was the polyclonal one mainly because the greater availability of the purified antibody and also because some earlier workers (Farjam *et al.*, 1991) had found polyclonal matrix performance with milk better than that with monoclonal matrices.

6.7.3 Investigation of sporidesmin metabolites in sheep urine

An important advantage of affinity purification is the rapid separation of the target analyte from interfering materials in a diverse range of samples. When an antibody recognises a certain structural feature in an analyte which is maintained during metabolism of the analyte, the antibody can often be used in IAC for purification of the metabolites also. Groopman and Donahue (1988) reported such application of a

monoclonal antibody in preparative affinity columns for the isolation of aflatoxin metabolites, including DNA and protein adducts in serum and urine.

In the present study a maximum recovery of 40% of urinary metabolites was achieved initially using IAC columns with small dimensions but this dropped to 14% when the chromatography was scaled up. This loss of column efficiency may be explained by the exchange of the Wright column with a Mobicol which had different dimensions and therefore altered the chromatographic conditions. Preliminary purification of the urine before IAC could have been carried out using solid-phase extraction to remove impurities which may include material which binds non-specifically to the IAC matrix. This may have improved the efficiency of the IAC matrix as more binding sites could be available for the sporidesmin metabolite.

Although maximum recovery of 40% appears low, an important point to be considered is that it is highly likely that the polyclonal antibody used in the matrix does not bind some of the metabolites which are recognised by the monoclonal antibody used in cELISA. Therefore, because of the differing specificities of the antibodies used in the IAC matrix and in the cELISA, recovery of 100% may never be possible. Any further purification of sporidesmin metabolites from urine should be carried out using the matrix prepared with the monoclonal antibody (Section 6.1.2) which is the group B antibody used in the cELISA.

An attempt was made to determine whether purified conjugates not recognised by the antibodies in cELISA could be hydrolysed by enzymes to produce hydrolysates which could be recognised. The presence of these conjugates would be indicated when cELISA of the hydrolysed sample gave less colour development than that developed in cELISA of the untreated sample (analyte concentration is inversely proportional to absorbance). Hydrolysis was carried out on a sample purified by IAC from urine using β-glucuronidase, sulfatase and pronase. Slight reductions in cELISA absorbances suggested that sporidesmin glucuronides and protein conjugates may be present but this evidence was inconclusive. It is also possible that glucuronides, sulfates or protein conjugates could be present in sheep urine, but the derivatised regions of the sporidesmin molecule are not a part of the binding region for the antibodies used in cELISA. Therefore, enzymatic deconjugation would not significantly change the cross-reactivity of the metabolite measured and alter the immunoreactivity measured by cELISA.

As well as possible changes in immunoreactivity of purified metabolites after hydrolysis, enzyme hydrolysis should also lead to a decrease in polarity with the formation of deconjugated sporidesmin A. Therefore, clearer data might be obtained if the samples before and after hydrolysis were fractionated by HPLC and the fractions analysed by cELISA to locate and determine the retention time of the immunoreactive material in both cases. This was carried out earlier with unpurified hydrolysates (Section 5.3.3). A decrease in polarity after hydrolysis would demonstrate that polar metabolites were hydrolysed by a particular enzyme to form less polar compounds and further information concerning the conjugation of the metabolites would be obtained.

6.7.4 Investigation of sporidesmin residues in milk

It is indisputable that dairy cows graze pastures containing relatively high sporidesmin A loads. This makes it possible that sporidesmin metabolites occasionally occur in milk. The elimination of a toxin from the body via the milk depends on a number of factors: (1) the affinity of the toxin for constituents of the milk; (2) the ability of the toxin to diffuse across cell membranes; (3) competing routes of elimination, *i.e.* milk, urine and faeces; and (4) detoxifying mechanisms present in the animal (James *et al.*, 1994).

Scott *et al.* (1994) used IAC in a study of the transmission of fumonisin mycotoxins into milk after dosing animals orally with 1 or 5 mg of fumonisin B_1. Transmission of fumonisins into milk was not demonstrated despite demonstrated recoveries of fumonisin spikes (5-50 ng per mL) from milk using these methods. In contrast to this finding, aflatoxin M_1, a metabolite of the mycotoxin aflatoxin B_1, appeared in milk when feedstuffs containing aflatoxin B_1 was ingested by dairy cattle (Van Egmond and Paulsch, 1986). Other experiments with cows have shown that 1-4% of the ingested aflatoxin B_1 could be recovered in milk as aflatoxin M_1 (Van Egmond and Paulsch, 1986).

Milk is a complex substance containing an emulsion of fat in a protein and mineral solution which poses problems to the analyst when using IAC. Although some workers report the successful use of undiluted milk on IAC columns, many describe the necessity to pre-treat the milk in some way before chromatography, particularly if the column is to be used several times and column capacity and flow rates are to be retained. Pre-treatment may involve dilution (Zarba *et al.*, 1992), defatting by centrifugation (Mortimer *et al.*, 1987), deproteinisation (Zimmerli and Dick, 1995) or solvent extraction (Scott and Lawrence, 1988). In the present study solubilisation of

whole milk in buffer containing 0.5% Tween 20 was followed by protein denaturation induced by freezing and then centrifugation and filtration. Columns which were re-used 15 times did not block and the flow rate remained constant. The percentage recovery of four sporidesmin A standards (25-400 ng) by IAC from 5 mL of milk averaged 75.6 ± 9.8. The decrease in percentage recovery of sporidesmin A from spiked milk compared with spiked PBS containing 0.05% Tween 20, *i.e.* 75.6 *cf* 87, may be due to milk components binding non-specifically to the matrix and interfering with the binding of sporidesmin A. An additional purification step before IAC, such as solid-phase extraction, might remove these interferences. Alternatively the IAC matrix prepared using the group B monoclonal antibody may have bound less of the interfering material.

It is well known that circulating sporidesmin reaches the udder of lactating animals where it profoundly decreases milk yield (Towers, 1978) and alters milk composition. In earlier research, Mortimer and Stanbridge (1968), using a cytotoxicity bioassay sensitive to 4 ng per mL, could not detect sporidesmin A or toxic sporidesmin metabolites in milk from cows ingesting pasture contaminated with an unknown amount of toxin. The possibility that sporidesmin residues are secreted into milk was re-investigated using the more sensitive immunoassay and immunopurification techniques reported in this study. A milk sample, collected from a cow dosed with sporidesmin A, was purified using the IAC methods developed and the material eluted was analysed by cELISA using the group B antibody. The result confirmed the presence of immunoreactive substances in milk which were assumed to be sporidesmin metabolites. Immunoassay of the sample using the group A antibody indicated the presence of free sporidesmin A at picogram levels although the amount measured was near to the limit of detection for the assay. To conclusively determine if free sporidesmin A is transferred into milk it will be necessary to process a larger volume of milk so that the presence of sporidesmin A can be confirmed by independent analytical techniques such as HPLC and mass spectrometry.

In the present study a high dose of sporidesmin A was administered and the results may not be representative of the repeated low level exposure typical of field conditions. The rate and route of excretion from the body may differ with toxin dose rate and damage to detoxifying tissues in the liver or kidney may alter absorption, excretion, or metabolism of the toxin (James *et al.*, 1994). Because sporidesmin A is a potent hepatotoxin the excretion of a large dose such as that used in this trial would be expected to be somewhat different to excretion after prolonged exposure to small daily doses as would be expected to occur with natural exposure to facial eczema. For

this reason further research is necessary using dosing levels similar to those experienced during natural exposure to sporidesmin A, before it could be confirmed that sporidesmin A is transmitted into milk in dairy cattle exposed to contaminated pastures.

Because of its hydrophobic properties sporidesmin A would be expected to associate with the fat fraction in milk. Therefore, if free sporidesmin A was found in cream collected from cows after natural exposure to facial eczema, it would be found at concentrated levels in fat-rich dairy products such as cream, cheese and butter. Sample pre-treatment including solubilisation with detergent to evenly disperse sporidesmin A throughout the aqueous and fat phases, together with the immunopurification techniques developed in this study, could be applied to sample clean-up for determination of sporidesmin A in these dairy foods. Sharman *et al.* (1989) have reported the successful use of solvent extraction and IAC for sample clean-up to determine aflatoxin M_1 in cheese.

The procedures developed were used successfully for the preparative recovery of sporidesmin metabolites from urine and milk and they also show potential for use in the quantification of sporidesmin A in milk. It is highly probable that these methods could be applied to other areas of sporidesmin residue analysis in foods such as cheese, butter, cream and meat. For routine use the methods would have to be optimised to shorten the analysis time. This could be achieved by reducing the number of wash steps, increasing flow rates in the washing steps and by reducing the solvent volumes used. Further development and validation of IAC with the monoclonal matrix needs to be undertaken as this matrix could have greater binding capacities and be more suitable for use in the isolation and quantification of sporidesmin metabolites.

6.8 Summary

Two IAC matrices were prepared by coupling a polyclonal and monoclonal antibody to cyanogen bromide-activated Sepharose. Both antibodies were specific for sporidesmin A and also cross-reacted with sporidesmin D.

Since the amount of group B monoclonal antibody was limited and this was not the case with the polyclonal antibody, IAC method development was undertaken using the polyclonal matrix. The matrix prepared with the group B antibody was retained for later use in the quantitative recovery of sporidesmin metabolites.

Bound sporidesmin A was eluted from both matrices with 30 and 40% methanol in water. Urine was collected from sheep after dosing with sporidesmin A and from this immunoreactive material was extracted using the polyclonal IAC matrix. Elution of this fraction was also achieved with 30 and 40% methanol and the material recovered was hydrolysed with β-glucuronidase, sulfatase and pronase. Analysis of the hydrolysates by cELISA gave some indication that a glucuronide and/or a protein conjugate may be present.

The matrix capacity was determined to be 900 ng of sporidesmin A bound per mL of matrix. The percentage recovery of nine sporidesmin A standards, (5-1 000 ng), by IAC from buffer averaged 86.7 ± 15.9 and ranged from 72.0-124.4. Optimum recovery of sporidesmin metabolites from urine was achieved when the ratio of urine to gel volume was 1:2. The matrix capacity for sporidesmin metabolites extracted from urine was approximately 500 ng of sporidesmin A immunoreactive equivalents per mL of matrix.

A method for the preparation of milk for IAC was developed using detergent so that the analytes were evenly dispersed throughout aqueous and fat phases and any remaining lipids and particulate material were removed by centrifugation so that contamination and blocking of the column were avoided. The percentage recovery of four sporidesmin standards (25-400 ng) by IAC from 5 mL of milk averaged 75.6 ± 9.8 and ranged from 68.2-88.8. The affinity column lowered the limit of detection for the previously developed cELISA for sporidesmin A in milk (Section 4.4) by a factor of 100 .

A trial was carried out to determine whether sporidesmin residues appeared in milk following experimental ingestion of the toxin. Analysis of milk samples by cELISA showed that maximum levels of sporidesmin metabolites were found 8 h after the first dose of sporidesmin A. A milk sample collected from a cow at this time was purified by IAC. cELISA of the recovered material confirmed the presence of immunoreactive substances in milk which were assumed to be sporidesmin metabolites and indicated the presence of free sporidesmin A at picogram levels. Purification of a much larger sample and analysis by HPLC and mass spectrometry are needed to confirm this result.

Chapter 7:

GENERAL DISCUSSION

CHAPTER 7

GENERAL DISCUSSION

The work described in this thesis has provided high-affinity antibodies with the specificity against sporidesmin A, its metabolites and its analogues, required for use in ELISA and immunoaffinity chromatography. Their use in these techniques has enabled further research into facial eczema to be undertaken.

Facial eczema is of considerable economic significance to the agricultural industry, particularly in New Zealand, and for this reason further research is necessary to provide better control of the disease. There is also a need to understand the metabolism of sporidesmin so that selective breeding of animals resistant to sporidesmin A can be carried out with less cost to the farmer and less risk of injury to animals.

To facilitate studies of the metabolism of sporidesmin A, methods for the analysis of sporidesmins A and D and their metabolites in various body fluids were developed. There are existing HPLC methods only for sporidesmin A (Halder, 1980; Fairclough and Smith, 1983) although these involve extensive clean-up of samples before analysis, making the procedure time-consuming, and inconvenient when large numbers of samples are handled. Extraction of samples for analysis also introduces the likelihood of analyte recoveries being less than 100% and the added disadvantage of under-estimation of sporidesmin A.

When the production of anti-sporidesmin A antibodies for immunoassays was undertaken to provide a more suitable method for analysis of sporidesmin A, monoclonal as well as polyclonal anti-sporidesmin A antibodies were developed in mice, sheep and rabbits. Attempts by other researchers to produce anti-sporidesmin A antibodies resulted in either polyclonal antibodies with low titres (Jonas and Ronaldson, 1974; Jonas and Erasmuson, 1977) or antibodies which bound to the coating antigens but did not bind to free sporidesmin A in cELISA (Gallagher *et al.*, unpublished data, 1987). The ability to produce antibodies which bound free mycotoxin in immunoasssay as reported in this study may well be attributed to the lengthy immunisation protocols used. This maturation of the immune response (Section 3.9.1), appeared to be important for the production of suitable monoclonal

antibodies. When a mouse was immunised over 5 months (Section 3.4.1) the antibodies produced did not bind to free sporidesmin A, whereas those obtained when mice had been immunised over 10 months or more (Sections 3.4.2, 3.4.3), were suitable for use in cELISA.

The hybridomas secreting anti-sporidesmin antibodies are listed in Appendices II and III. Antibodies giving the most sensitive assay in buffer were selected from each group for use in cELISAs. It is possible, however, that some of the remaining hybridomas stored in liquid nitrogen may produce antibodies which give better assay performance in some matrices (*i.e.* are less affected by matrix interference) than the antibodies selected. Because of differences in cross-reactivities they may also give more sensitive assays for metabolites. These antibodies should be investigated for their suitability for specific applications.

The sporidesmin A binding sites for the group A and group B antibodies were investigated by cross-reactivity studies. As discussed in Chapter 3 these suggested that the epitope for the group A antibody includes the intact disulfide bridge and possibly the immunogen hemisuccinate bridge. Group B antibody, however, appeared to bind regions of the sporidesmin A molecule distal to the disulfide bridge, for example the chlorine, methoxy, adjacent nitrogen, 10b hydroxyl groups, and associated ring structures. The epitope described for the group B antibody appears similar to that proposed by Jonas and Ronaldson (1974), and Jonas and Erasmuson (1977), who also suggested that the chlorine group was important in the antigenic structure of sporidesmin A. This is in spite of the point of conjugation for immunogen preparation being different in our research (Figure 1.9.1) to that of Jonas and Ronaldson (1974) and to that of Jonas and Erasmuson (1977).

The production of anti-sporidesmin A antibodies and development of the immunoassay methods enabled a study of the metabolism of sporidesmin A in sheep to be undertaken and it was possible to monitor sporidesmin A and its metabolites in bile, and metabolites in urine, using the appropriate cELISA. The pattern of uptake and clearance of sporidesmin A was similar to that reported by Mortimer and Stanbridge (1968) who recorded peak concentrations between 2 and 8 h after sheep were dosed with sporidesmin A. Mortimer and Stanbridge (1968), however, administered a large dose of 1 mg per kg of body weight, and the maximum concentration measured, using a cytotoxicity test, was 20 μg of sporidesmin A per mL of bile. In the present study a sheep was dosed with 0.4 mg of sporidesmin A per kg of body weight and a maximum level of only 1.3 μg of sporidesmin A per mL

of bile was observed 2 h after dosing. Leaver (1968) also found that peak concentrations in sheep bile occurred 2 to 4 h after administering 0.5 mg of sporidesmin A per kg of body weight. A peak concentration of approximately 1 µg of sporidesmin A per mL of bile was measured using the rabbit eye test and a cytotoxicity test. This was similar to maximum levels measured in the present study where the slightly lower dose rate was used. When sheep were dosed with sporidesmin A by Fairclough and Smith (1983) trace amounts of ^{35}S-labelled sporidesmin A were included. After dosing with 0.08 mg of sporidesmin A per kg per day over 3 consecutive days, bile was collected and extracted with organic solvent and the extracts fractionated by HPLC. Sporidesmin A was determined by scintillation counting of HPLC fractions containing sporidesmin A. They also found maximum concentrations of sporidesmin A were detected in the bile 1 to 2 h after dosing. The peak concentrations of sporidesmin A measured reflected the lower doses given and ranged from 10 to 80 ng per mL of bile.

Although the amount of sporidesmin A dosed and the methods of analysis used in the various studies were different, the results in the present study indicated that the pattern of clearance of sporidesmin A in bile is similar to that observed by earlier researchers. The results also support the suggestion made by Fairclough and Smith (1983) that with an increase in dose rate there is a proportionately greater increase in the concentration of sporidesmin A in bile.

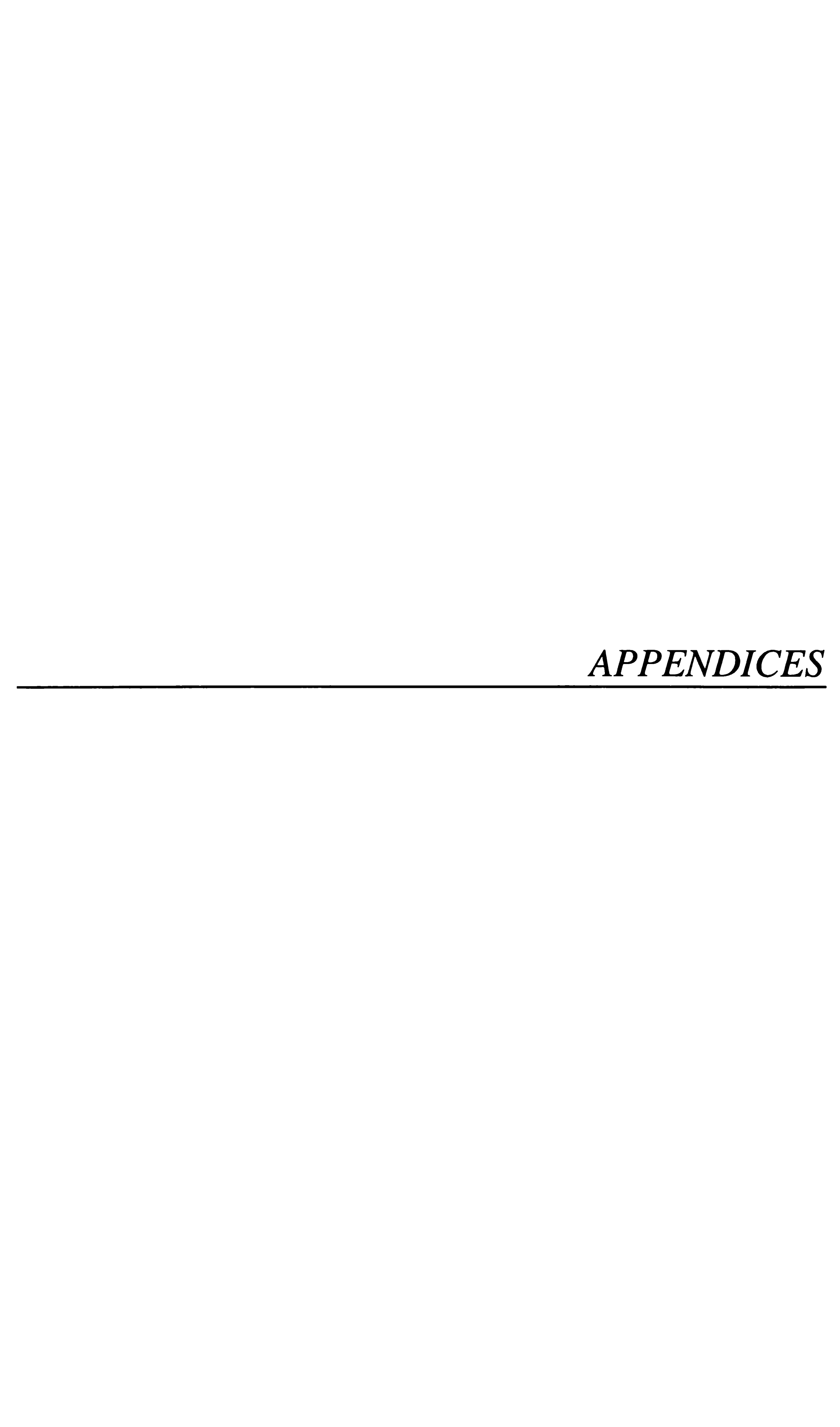
Unlike Mortimer and Stanbridge (1968) who were able to detect sporidesmin A by its cytotoxicity in urine samples 4 h after sheep were dosed with 1 mg of sporidesmin A per kg of body weight, in the present study it was not possible to detect by HPLC or cELISA sporidesmin A in the urine of sheep dosed with 0.2 mg of sporidesmin A per kg of body weight. Mortimer and Stanbridge (1968) found that maximum sporidesmin A cytotoxicity (equivalent to 4 µg of sporidesmin A per mL of urine) occurred in urine samples collected 12 h after dosing, and cytotoxic activity had disappeared in those collected after 24 and 48 h. The difference between our results and those of Mortimer and Stanbridge (1968) may be explained by the suggestion made for bile by Fairclough and Smith (1983), that with increasing dose rates there is a greater proportion of the dose excreted as sporidesmin A. It should also be recognised that the tissue culture cytotoxicity test used by Mortimer and Stanbridge (1968) to measure sporidesmin A is non-specific. It is possible that the cytotoxic material they found in urine may not have been unmetabolised sporidesmin A. The immunoreactive material excreted in urine and measured by

cELISA (Figure 5.1.2) may have been responsible for the cytotoxic activity measured.

The preparation of IAC matrices by coupling a polyclonal antibody to cyanogen bromide-activated Sepharose, allowed the extraction of immunoreactive material from urine and milk. The partial purification and sample concentration resulting from IAC prior to cELISA lowered the limit of detection of sporidesmin A in milk by a factor of 100 and provided confirmation of the transfer of sporidesmin residues into milk after experimental dosing with sporidesmin A. The methodology developed is being used to investigate an important animal health problem, but may also be useful for monitoring agricultural produce for possible contamination by sporidesmin residues.

This research has produced anti-sporidesmin antibodies which can now be used in the wide range of recently developed immunochemical technologies and formats. The new immunochemical methods have been developed to improve efficiency in monitoring for drugs and agricultural chemicals as well as mycotoxins. These methods have been used to reduce costs, reduce detection limits, improve reliability, simplify methods and carry out multianalyte testing. The development of technologies such as biosensors have provided assay simplification, while time-resolved fluorescent determination and label amplification have assisted in increasing assay sensitivity (Kricka, 1994). The use of labels for detection other than enzymes, such as fluorescent and chemiluminescent labels, has been another development giving increased sensitivity.

The anti-sporidesmin antibodies could be used with such technologies in the future to meet specific needs for improved assays. For example a simple test-kit could be developed for on-farm use to provide a more efficient way of detecting exposure to sporidesmin-contaminated pasture and to obtain more effective control of facial eczema. This would provide considerable savings to the agricultural industry.



APPENDICES

APPENDIX I

Iscoves Modified Dulbecco's Medium (IMDM)

The contents of one sachet (Gibco 430 2200) were emptied into 800 mL of milliQ water. The powder was dissolved by stirring at room temperature and the following added:

	per litre
NEAA	10 mL
penicillin-streptomycin stock	10 mL
sodium bicarbonate	3.024 g

The volume was made up to 1 L and the medium was filter-sterilised. It was stored for up to a month in the refrigerator but was usually used fresh.

Penicillin-streptomycin stock

	per 100 mL
Streptomycin sulphate (Sigma S 9137)	1 g
Benzylpenicillin sodium salt (Sigma P 3032)	10 000 units

The mixture was dissolved in milliQ water, dispensed into 5 mL aliquots and stored at - 20°C.

Complete IMDM-HAT or HT Medium (20% FCS)

	per 100 mL
IMDM	48 mL
HAT or HT(50x)	2 mL
FCS	20 mL
myeloma conditioned medium (see Section 2.5.1)	30 mL

The medium was filter-sterilised.

Storage Medium

	per 10 mL
IMDM	3.8 mL
FCS	2.2 mL
DMSO	1.0 mL
myeloma conditioned medium	3.0 mL

The medium was filter-sterilised.

Acetate buffer, *0.1 mol/L, pH 5.5*

	per 500 mL
anhydrous sodium acetate	3.3785 g
glacial acetic acid	360 µL in 4 mL

water

The sodium acetate solution was adjusted to pH 5.5 by addition of the diluted acetic acid.

Horseradish peroxidase substrate

Just before use, solutions 1 and 2 were prepared:

1.	30% hydrogen peroxide	37 µL
	milliQ water	2.5 mL
2.	acetate buffer, 0.1 mol/L, pH 5.5	11 mL/plate
	TMB (50 mg/5 mL of DMSO)	110 µL/plate

Solution 1 (110 µL) was then added to solution 2 (11.11 mL).

Acetate buffer, *0.2 mol/L, pH 5.5*
for use with β-glucuronidase and sulphatase

	per 100 mL
sodium acetate	1.64 g

The sodium acetate solution was adjusted to pH 5.5 by the addition of dilute acetic acid.

Tris/HCl buffer, *0.01 mol/L, pH 7.7, 0.015 mol/L $CaCl_2$,*
for use with pronase

	per 100 mL
Tris	121 mg
calcium chloride	15 mg

The Tris/calcium chloride solution was adjusted to pH 7.7 by the addition of hydrochloric acid. (0.1 mol/L).

APPENDIX II

HYBRIDOMA CELL LINES

Sixteen hybridoma cell lines produced in Fusion II and 30 in Fusion III, were shown to secrete antibodies which bound to the sporidesmin coating conjugates used in ELISA. Culture supernatants from these cell lines were analysed by cELISA to determine specificity for sporidesmin A and/or sporidesmin D.

Fusion II

cell line	subcloned	SN[a] dilution[b] in assay	% absorbance[c] without toxin	% absorbance[c] with spdm A (100 µg/mL)	% absorbance[c] with spdm D (100 µg/mL)	spdm A specific	spdm D specific
5E9		1:30	100	77	1	√	√√
2F4	√	1:4	97	37	2	√	√√
6B1	√	1:15	88	55	5	√	√√
2F1		1:2	96	76	32	√	√√
6C12		1:30	91	72	2	√	√√
3A12		1:30	80	85	5	√	√√
6H5		1	91	60	8	√	√√
8D7	√	1	98	55	1	√	√√
9C1		1	90	69	1	√	√√
12H10		1	53	42	35	√	√
8E12		1	27	22	15	√	√
5F12	√	1	98	73	10	√	√√
2G4	√	1	58	3	0.1	√	√√
5F4		1	55	3	0.2	√	√√
1G7		1	14	4	8	√√	√
12F11	√	1	88	40	1	√	√√

[a] SN represents hybridoma culture supernatant. [b] The supernatant dilutions used in cELISA were those which gave approximately 50% of the maximum absorbance recorded in ELISA when there was an excess of antibody. [c] Absorbances expressed as a percentage of the maximum value recorded on each plate.

APPENDIX III

Fusion III

cell line	subcloned	SN dilution in assay	% absorbance without toxin	% absorbance with spdm A (100 µg/mL)	% absorbance with spdm D (100 µg/mL)	spdm A specific	spdm D specific
1G6		1	35	3	35	√	
1B7		1	45	5	45	√	
2F2		1	38	4	38	√	
2C8		1	36	5	36	√	
4C2		1	37	5	37	√	
4F10		1	28	3	28	√	
5F2		1	29	3	29	√	
5A7		1	29	4	29	√	
5H10		1	27	6	26	√	
6A5	√	1:16	100	5	95	√√	
6D8		1:16	100	60	4	√	√√
7B5	√	1:32	77	22	77	√	
8G1		1	28	3	21	√√	√
7F11		1	27	4	27	√	
8F7		1	15	0.5	15	√	
9B7		1:4	84	15	2	√	√√
9D7		1	15	2	15	√	
11D4		1	21	3	21	√	
11B6		1:8	48	12	1	√	√√
11C11		1	22	3	22	√	

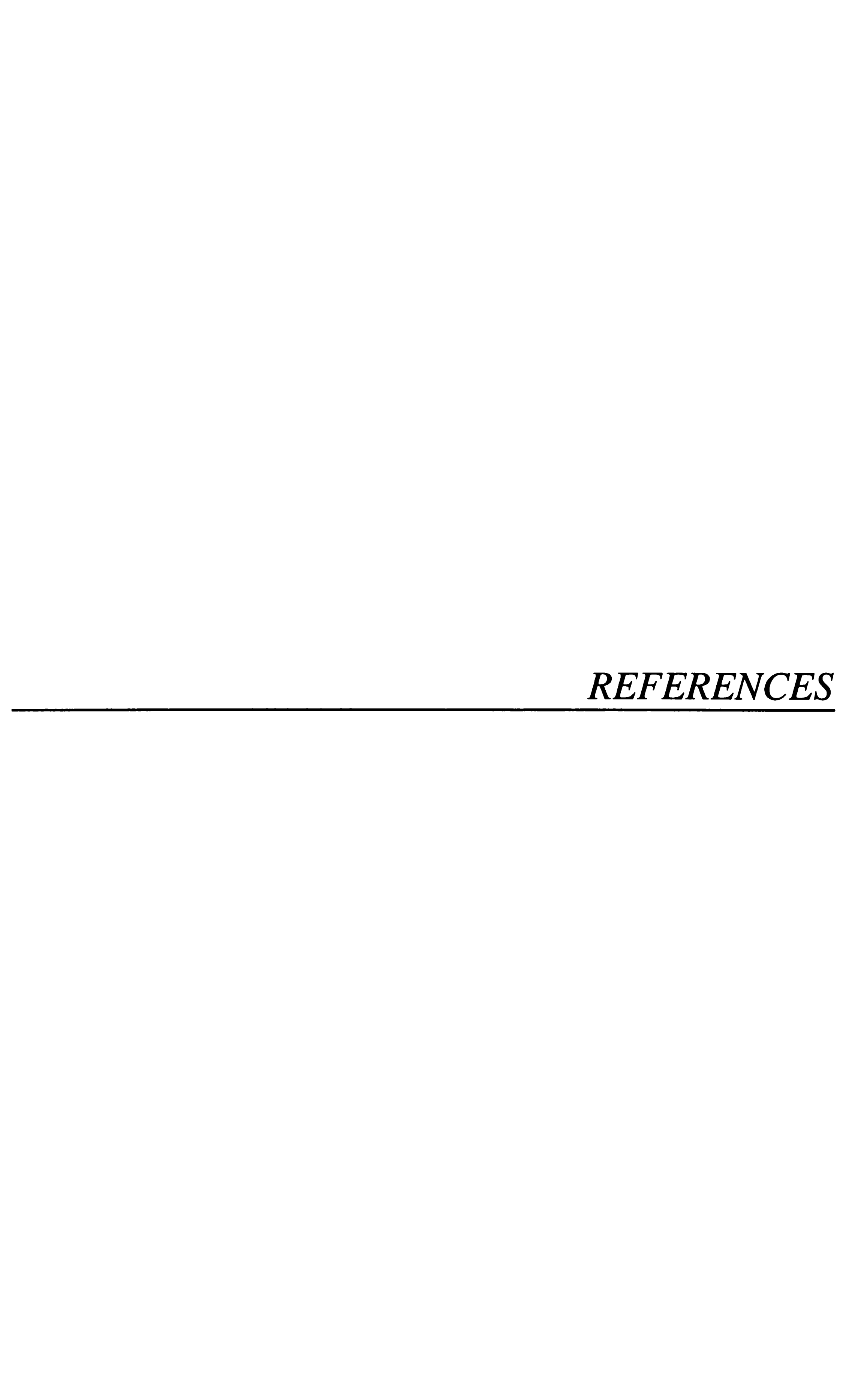
Fusion III (continued)

cell line	subcloned	SN dilution in assay	% absorbance without toxin	% absorbance with spdm A (100 μg/mL)	% absorbance with spdm D (100 μg/mL)	spdm A specific	spdm D specific
3G4		1	69	44	5	√	√√
3H4		1	44	22	42	√	
3A8		1	36	6	34	√	
12G12		1	59	10	7	√	√
4H9		1	85	40	7	√	√√
6D10		1	85	15	86	√	
7C4		1	99	30	93	√	
7E8		1	100	7	87	√√	√
8F11		1	77	46	10	√	√√
10E5		1	72	46	6	√	√√

APPENDIX IV

Publication:

Briggs, L. R.; Towers, N. R.; Molan, P. C. (1994) Development of an Enzyme-linked Immunosorbent Assay for Analysis of Sporidesmin A and Its Metabolites in Ovine Urine and Bile. *Journal of Agricultural and Food Chemistry 42,* 2769-2777.



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