DEVELOPMENT OF AN ELISA TO DETECT THE INCIPIENT STAGES OF *TRIBOLIUM CASTANEUM* IN FOOD COMMODITIES

Thesis

submitted to the

UNIVERSITY OF MYSORE

for the award of degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

by

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September 2006

DECLARATION

I hereby declare that the thesis entitled **"DEVELOPMENT OF AN ELISA TO DETECT THE INCIPIENT STAGES OF *TRIBOLIUM CASTANEUM* IN FOOD COMMODITIES"** submitted to the University of Mysore, for the award of the degree of Doctor of Philosophy in Biotechnology, is the result of the research work carried out by me in the Department of Food Protectants and Infestation Control, Central Food Technological Research Institute, Mysore, under the guidance of Dr. B. E. Amitha Rani, during the period June, 2003 to June, 2006.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

Roshni Srivastava
Senior Research Fellow

Date:
Place: Mysore

CERTIFICATE

I hereby certify that this thesis entitled **"DEVELOPMENT OF AN ELISA TO DETECT THE INCIPIENT STAGES OF *TRIBOLIUM CASTANEUM* IN FOOD COMMODITIES"** submitted by Mrs. Roshni Srivastava for the award of the degree of Doctor of Philosophy in Biotechnology, University of Mysore, is the result of the research work carried out by her in the Department of Food Protectants and Infestation Control, Central Food Technological Research Institute, Mysore, under my guidance and supervision during the period June, 2003 to June, 2006.

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Roshni Srivastava

CONTENTS

LIST OF ABBREVIATIONS

%	percentage
α	alpha
β	beta
γ	gamma
~	approximately
µg	microgram
µl	microlitre
µm	micrometer
AACC	American Association of Cereal Chemists
Ab	antibody
ACA	Antibody Capture Assay
Ag	antigen
BSA	bovine serum albumin
^{o}C	degree centrigrade
CH_3Br	methyl bromide
cm	centimeter
CO_2	carbon dioxide
EDTA	ethylene diamine tetraacetic acid
eg	example
ELISA	Enzyme Linked Immunosorbent Assay
FDA	Food and Drug Administration
Fig.	figure
g	grams
GC	Gas Chromatography
h	hour
HACCP	Hazard Critical Control Points
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HPLC	High Performance Liquid Chromatography
HRP	horse radish peroxidase
IgG	immunoglobulin G
IgY	immunoglobulin Y
IPM	integrated pest management
KCl	potassium chloride
kD	Kilo Dalton
kGy	Kilogray
M	molar
MDL	minimum detection limit
mg	milligram
mg/ml	milligram/milliliter
min	minutes
ml	milliliter
mm	millimeter
mM	millimolar
MW	molecular weight

N	noramality
NaCl	sodium chloride
NaOH	sodium hydroxide
NCM	nitro cellulose membrane
ng	nanogram
nm	nanometer
No.	number
N-PAGE	Native- Polyacrylamide gel electrophoresis
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid/ Schiff's reagent
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-Tween-20
PBS-FG	phosphate buffered saline-Fish gelatin
PEG	poly ethylene glycol
PH_3	phosphine
PMSF	phenyl methane sulphonyl flouride
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS- Polyacrylamide gel electrophoresis
SE	standard error
Sec.Ab-HRP	secondary antibody-HRP conjugate
SLAM	sanitation, loading, aeration and monitoring
SMP	skimmed milk powder
Std	standard
SSA	sulfosalicylic acid
TBS	Tris buffered saline
T. castaneum	*Tribolium castaneum*
TEMED	N, N, N', N'- tetramethyl ethylene diamine
TMB	3,3',5,5'-tetramethyl benzidine
tris	Tris (hydroxy methyl) aminomethane
Tris-Gly	Tris-glycine
V1	vitellin 1
V2	vitellin 2
Vm	vitellin mixture (mixture of V1 and V2)
VG	vitellogenin
Vt	vitellin
v/v	volume/volume
WSPF	water soluble protein fraction
w/v	weight/volume

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SYNOPSIS

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SYNOPSIS OF THE THESIS

DEVELOPMENT OF AN ELISA TO DETECT THE INCIPIENT STAGES OF *TRIBOLIUM CASTANEUM* IN FOOD COMMODITIES

In India, every year nearly 10 per cent of the food grains are lost during post-harvest processing and storage due to insect infestation. Increased production would hardly have any significance unless protected from post harvest loss. A recent estimate by the Ministry of Food and Civil supplies put the total preventable post-harvest losses of food grains due to insect infestation at about 20 million tons per year, which was nearly 10 per cent of the total production that could have fed upto 117 million people for a year.

Insect pest activity in agricultural produce may start at any stage from harvest to consumption. Insect infestation causes qualitative and quantitative losses of food commodities and changes the chemical composition affecting the nutritive value of the produce. Insect infestation in food commodities has health implications as well. Insects also play a significant role in the dissemination and proliferation of microorganisms including mycotoxigenic fungi in food commodities.

In national and international trade, cash value and marketability of different commodities are affected by insect infestation as are the processing and end-use qualities of food commodities. Quality maintenance by way of reduction in insect contaminants to meet the requirements of International Standards Organisation (ISO) and Hazard Analysis Critical Control Points (HACCP) is important for marketing the produce. Food and Drug Administration (FDA) has established Defect Action levels for live insects at two insects per

kilogram and insect damaged grains at 32 kernels/100g in food grains; in wheat flour, there is a limit of 75 insect fragments/50g; and in macaroni and noodle products it is 225 fragments in a 225g sample. In India, according to the Prevention of Food Adulteration Act, the uric acid level in food commodities should not exceed 100 mg/kg and the number of weevil-damaged grains should not exceed 10% by count. In countries like Canada and Australia, there is zero tolerance for insects in food grains and a similar standard is followed in international trade for grains.

Insect infestation detection methods, in samples and storage facilities, play a significant role as an indicator and also an effective infestation management tool in the food industry. The prominence of current methods used for detection of stored product insect pest such as fragment count, X-ray method, uric acid determination, carbon dioxide analysis, etc. are for the detection of adult or the visible life stages of the insect pest. Nevertheless, insect pest eggs have a key role in spread of infestation. Due to the small size of the eggs, they often go unnoticed and there are not many sufficiently sensitive methods to detect insect pest eggs. Currently, only few methods are available for detection of insect pest eggs like the egg staining techniques and breeding out method. These methods are not sufficiently sensitive; are exclusive in their application; or are time consuming. Sensitive insect pest egg detection technique would be advantageous especially for the milling industry, wherein the milled products of cereals get infested by eggs of insect pests such as *Tribolium castaneum*, *Oryzaephilus surinamensis* and *Corcyra cephalonica*, which gets transferred to the final product thereby reducing the quality of the product and also aiding in the spread of infestation. Therefore, development of sensitive, easy and quick infestation detection methods is imperative.

Currently, immunoassays due to their enormous specificity, resolution, rapidity, cost effectiveness and efficiency have gained importance in the field of insect pest detection systems. In view of this, the present work was aimed at the development of a Enzyme Linked Immunosorbent Assay (ELISA) for the detection of incipient stages of the incipient stages of the red flour beetle -*Tribolium castaneum*, with special reference to eggs.

Objectives of the study:

1. Development of ELISA for the detection of incipient and other developmental stages of *Tribolium castaneum.*

a. Purification of the antigen i.e. the major egg protein of *Tribolium castaneum* Herbst.

b. Production of antibodies against the purified antigen in rabbit and chicken.

c. Development of the standard ELISA based on the rabbit and chicken egg yolk antibodies.

2. Application of the ELISA developed to food commodities like whole wheat flour and rice flour and testing of market samples.

3. Comparison of the ELISA developed with the current insect pest egg detection methods i.e. AACC approved iodine method and bromocresol green staining method.

Chapter I comprising literature survey gives an overview of the stored product insect infestation and its effects; food quality control; methods of infestation control and its detection; and advantages of immunoassay as a detection tool. Literature on different insect infestation detection methods has also been surveyed.

Chapter II describes the purification and partial characterization of the antigen used in the present study i.e. the major egg protein–vitellin (phospholipoglycoprotein) of the red flour beetle *Tribolium castaneum.* Purification of the antigen was carried out using

preparative gel electrophoresis. Characterization of the antigen was carried out by determining the approximate molecular weight of the protein by PAGE and by determining the presence of phospho, lipo and glyco moieties using specific staining techniques in PAGE.

We found that the red flour beetle, *Tribolium castaneum*, showed the presence of two vitellins designated as V1 and V2, which were present throughout the embryonic development. This finding was also confirmed by western blot analysis using V1 antiserum. V1 was the major band and accounted to approximately 80% of the total extractable protein, hence chosen as the antigen in the present study.

The molecular weight of V1 was found to be ~440k. Both V1 and V2 stained positive for glycoprotein indicating the presence of glycoprotein moiety. However, the phosphate and lipid moiety were not detectable. SDS-PAGE analysis of V1 indicated that the protein comprised of three major subunits, the larger subunits of molecular weights ~150 kD each and the smaller subunit of molecular weight ~ 50 kD.

Further, Western blotting studies revealed that the V1 antiserum crossreacted with a protein band present in all other developmental stages of *T. castaneum*. Based on this finding, the reactive protein band could be vitellogenin (VG), the precursor of vitellin present in eggs, which are immunologically similar. These results affirm the choice of antigen for the present study and also help in relating the degree of applicability of the assay developed in detecting all the incipient stages and in addition the adult stages of the red flour beetle.

Chapter III describes the development of an Enzyme Linked Immunosorbent Assay (ELISA) based on rabbit (IgG) antibodies raised against the major egg protein of

T. castaneum and its application to whole wheat flour and rice flour. This involved production of antibodies in rabbit against V1 antigen, purification of IgG using immobilized Protein–A, ELISA formatting and development of a standard assay, determining the antibody titer and sensitivity of the different bleeds obtained. Further, crossreactivity studies of the antibodies against other stored product pests such as *Rhizopertha dominica, Corcyra cephalonica* and *Lasioderma serricorne* were done. Studies on the application of the ELISA developed in whole wheat flour and rice flour involved study of the matrix effect of the food extracts on the assay performance, development of a clean up protocol for the removal of matrix effect and spike recovery studies. The assay was also applied to detect infestation in market samples.

The antibodies raised against the major egg protein of *Tribolium castaneum* –vitellin were highly sensitive and specific. The ELISA developed using these antibodies proved to be highly sensitive with a minimum detection limit of 0.1ng of the protein which is equivalent to 1/73,000 parts of the extractable egg protein. In addition, the assay could also detect minimum of one number of each of the different developmental stages of *T. castaneum* such as larva, pupa and adult female and male insect. The *T. castaneum* vitellin antibodies showed low crossreactivity with egg protein from *Rhizopertha dominica* and *Corcyra cephalonica* and negligible crossreactivity with the egg protein from *Lasioderma serricorne*. Therefore, the ELISA developed is highly sensitive and specific for the detection of eggs and other developmental stages of *T. castaneum*.

The IgG based ELISA developed was applicable for both whole wheat flour and rice flour. The food extracts exhibited matrix effect, which was reduced by the dilution method. The spike recoveries in whole wheat flour and rice flour were 90-100% and 79-100%,

respectively. Analysis of market samples indicated that the assay developed could be applied successfully to unknown samples also.

Chapter IV describes the development of chicken egg yolk (IgY) antibody based Enzyme Linked Immunosorbent Assay (ELISA) and its application to whole wheat flour and rice flour. The antigen chosen for IgY production was Vm (mixture of V1 and V2). This was based on our studies which showed that V1 antiserum crossreacts with both V1 and V2. Further, the assay performance using V1, V2 and Vm antigen were comparable. Moreover, Vm can be easily extracted with a simple single step extraction. The study involved production of antibodies in chicken against Vm (mixture of V1 and V2) antigen, purification of IgY, ELISA formatting and development of a standard assay. The crossreactivity studies of the antibodies against other stored product pests such as *Rhizopertha dominica*, *Corcyra cephalonica* and *Lasioderma serricorne* were done. The application of the ELISA developed to whole-wheat flour and rice flour involved study of the matrix effect of the food extracts on the assay performance, development of a clean up protocol for the removal of matrix effect and spike recovery studies.

Highly sensitive and specific antibodies could be obtained from the egg yolk of the White Leghorn hen against the major egg protein of *Tribolium castaneum*–vitellin. The egg yolk antibodies could be easily purified with almost 90-95% purity by two methods using combinations of chloroform & PEG and carrageenan & PEG. The highest average yield of IgY obtained with good sensitivity was ~60mg/egg between weeks 3 to 7. The ELISA developed using these antibodies proved to be highly sensitive with a minimum detection limit of 0.1ng of the protein which is equivalent to 1/73,000 parts of the extractable egg protein. The method could also detect minimum of one number each of the different

developmental stages such as larva, pupa and adult female and male insect. The *T. castaneum* vitellin antibodies showed low crossreactivity with egg protein from *Rhizopertha dominica* and *Corcyra cephalonica* and negligible crossreactivity with the egg protein from *Lasioderma cephalonica*. Therefore, the ELISA developed is highly sensitive and specific for the detection of eggs and other developmental stages of *T. castaneum*.

The IgY based ELISA developed for the detection of eggs of *T. castaneum* was applicable for both whole wheat flour and rice flour. Simple dilution of the food extract could be used as an effective strategy for matrix effect reduction for both the food extracts and this was evidenced by good spike recoveries. The spike recoveries in whole wheat flour and rice flour were 90-100% and 75-85%, respectively.

The sensitivity, assay performance and applicability of the IgY based assay was comparable with that of the IgG based assay as described in chapter 3. Further, production of antibodies in chicken satisfies scientific and commercial interests as well as a concern for animal welfare thus making it a good alternate to IgG based assay.

Chapter V deals with the comparison of the ELISA developed with two methods of insect pest egg detection i.e. AACC (American Association of Cereal Chemists) approved iodine staining method and bromocresol green staining method. Development of new and novel methods of detection offers considerable opportunities. However, the key factor for acceptance of any method in the routine analysis is its comparative or better efficiency and sensitivity with that of the well established methods. The evaluation of the ELISA developed would enable its future use in the routine analysis of samples. The chosen food commodities for this study were refined flour, whole wheat flour and rice flour.

The ELISA developed for detection of incipient stages of *T. castaneum* was

comparatively more effective in detecting the eggs in both whole-wheat and rice flour as compared to that of the staining methods in terms of sensitivity, specificity, sample size, sample handling, throughput, data interpretation and applicability. The staining methods compared with the ELISA developed were applicable only for refined flour. Thus, ELISA proves to be a simple, quick and sensitive method for detection of eggs of *T. castaneum* in food commodities.

CONTRIBUTION OF THE THESIS

- This study reports for the first time development of an Enzyme Linked Immunosorbent Assay (ELISA) for the detection of incipient and other stages of the red flour beetle *Tribolium castaneum* Herbst- a stored product insect pest, based on rabbit (IgG) and chicken egg yolk (IgY) antibodies.
- The ELISA developed was highly sensitive. The minimum detection limit of the assay based on IgG and IgY antibodies was 0.1ng.
- The ELISA developed could detect all the developmental stages of *T. castaneum.*
- The ELISA developed was highly specific for *T. castaneum.*
- The ELISA developed was applicable for detection of *T. castaneum* eggs in wheat flour and rice flour and could be successfully used to detect *T. castaneum* infestation in market samples.
- The ELISA developed was comparatively more effective than the AACC approved iodine and bromocresol green staining methods in detection of *T. castaneum* eggs in terms of sensitivity, sample handling, data interpretation and throughput.
- The ELISA developed based on IgY antibodies, due to high quantity of sensitive antibodies obtained, puts forth the possibility of using IgY for the detection of *T. castaneum* infestation in the routine analysis of food commodities.

Research Guide

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CHAPTER I

INTRODUCTION

"Give a man a fish and he will eat for a day, teach a man to fish and he will eat for a lifetime," the old saying goes. Nevertheless, solutions to the problem of world hunger aren't that simple. Knowing how to fish or produce food successfully is only half the battle. For the past two decades, India has had the know-how and technology to produce high-yielding crops and, especially in fertile states such as Karnataka, this technology has been rather successful. However, the problem in India, where 60% of the economy is dependent on agriculture, is the inability to preserve agricultural surpluses. According to a 1999 World Bank Report, post-harvest losses amount to 12 to 16 million metric tons of food grains each year - an amount that the World Bank stipulates could feed one-third of India's poor. In India, post-harvest losses of fruits and vegetables range from 20 to 40%, while losses in pulses, oilseeds and cereals range from 10 to 30%. This represents a market value of approximately $15 billion U.S. per year, causing a serious dent in the economic wealth of the farm producers (Narang, 2002).

Food commodities are stored for varying periods in different types of storage structures for future consumption or trade purposes. During storage, pests such as birds, rodents, insects, mites, and microbes attack the food commodities (Rajendran, 2005). Adequate management of pests that attack and destroy post harvest food grains has always received less attention than pest management efforts on crops in the field. This is unfortunate, as losses of grain in storage are often equal to cereal grain losses in the field. Every year nearly 10 per cent food grains are lost during post-harvest handling and storage in India. Increased production would hardly have any significance unless protected from waste. A recent estimate by the Ministry of Food and Civil supplies put the total preventable post-harvest losses of food grains at about 20 million tons a year, which was nearly 10% of the total production, which could have fed upto 117 million

people for a year (Ref.: Website 1).

Insect pest activity in agricultural produce may start at any stage from harvest to consumption. In some cases, the infestation occurs in the standing crop itself. Insects such as *Sitotroga cerealla* (on paddy rice), *Sitophilus zeamais*, *Prostephanus truncates* (on maize), *Hypothenemus hampei* (on coffee), *Carpophilus* spp. (on dreid fruits), *Carydon serratus* (on peanuts) commence their activity on standing crop (Rossiter, 1970; Tigar *et al*., 1994). Similarly, post harvest insect appearance is not spontaneous. Infestations are the direct result of egg laying by insects that are already present in a godown or enter it along with the grain (Narang, 2002). Infested products unknowingly accepted into food-processing facilities presents a major sanitation problem. Storage insects, especially during incipient stages of infestation are difficult to detect. These insects are very small and often the same color as the food they infest. The beetle larvae are ~1-6 mm depending on the instar and species. The eggs of these insects are generally 500-600 μm in length and ~350 μm in width (Abels and Ludeshcher, 2003). These pests, thus, often go undetected until the population explodes and the insects begin to disburse.

Insect infestation causes qualitative and quantitative losses of food commodities and changes the chemical composition, affecting the nutritive value of the produce (Howe, 1965; Swaminathan, 1977). Insects like *Crytolestes* spp., *Trogoderma granarium* and *Plodia interpunctella* preferentially feed on the germ that is soft and highly nutritious. Because grains constitute a major part of the diet, the impact of post harvest insect pests on human nutrition is enormous. In addition, insects produce excrement and frass during their grain-boring and oviposition activities. Insect contaminants such as excreta (uric acid), exuviae (cast skins) and dead bodies, webbing and secretions in food commodities pose a quality-control problem for food industries (Gentry *et al*., 2001).

Insect infestation in food commodities has health implications as well. Exuviae from developing larvae and small insect fragments are inhalant allergens. Detached urticating hairs of dermestid beetle larvae cause mechanical injuries to skin, eyes and nasal membranes. Quinones excreted by *Tribolium castaneum* and *Tribolium confusum* impart an unpleasant odor and contaminate food. The excreted quinones can cause conjunctivitis and dermatitis and are associated with tumors in mice (Phillips and Burkholder, 1984; Hodges, 1996). Some of the effects of insect infestation on foods are summarized in Table 1.1. The processing, cooking quality and organoleptic properties may be affected in infested produce (Smith *et al*., 1971). Insects also play a significant role in the dissemination and proliferation of microorganisms including mycotoxigenic fungi in food commodities. In national and international trade, the channels of commodities that are infestation free are essential to avoid rejections. Therefore, processing and end-use qualities of food commodities are affected by insect infestation, as are cash value and marketability of different commodities.

Table 1.1. : Effects of insect infestation on stored food commodities*

Effect	*Insect*	*Commodity*
Chemical composition altered	*Rhizopertha dominica*	Wheat
	Sitophilus oryzae	Wheat
	Tribolium castaneum	Wheat
	Tribolium castaneum	Sorghum
	Callosobruchus maculatus	Cowpea, groundnut
	Corcyra cephalonica	Peanut
	Oryzaephilus surinamensis	Peanut
	Stegobium paniceum	Turmeric, coriander
Changes in nutritional quality	*Trogoderma granarium*	Wheat
	Sitophilus oryzae	Finger millet, wheat maize
	Callosobruchus maculatus	Cowpea
Off-odors	*Tribolium castaneum*	Sorghum
	Rhyzopertha dominica	Sorghum
Changes in end use quality	*Sitophilus granarius*	Wheat
	Rhyzopertha dominica	Wheat, maize
	Tribolium confusum, Tribolium castaneum	Wheat flour
	Tribolium castaneum	Peanut
Health implications		
1. Inhalant allergy 2. Ulcerative colitis 3. Carcinogenic quinines 4. Association with toxigenic fungi or pathogenic microbes	*Sitophilus oryzae, Trogoderma glabrum, Tribolium castaneum, Plodia interpunctella*	Wheat, maize
	Sitophilus oryzae, Tribolium castaneum	Maize, wheat, rice
	Ephestia kuehneilla, Stegobium paniceum, Tribolium castaneum	Peanut, cumin, wheat flour, copra

(* Adapted from Rajendran, 2005)

INSECT PESTS OF STORED FOODS:

Two major groups of economically important post-harvest insect pests are either beetles (Coleoptera) or moths and butterflies (Lepidoptera). Psocidae (psocids) are also found in most of the stored produce (Cotton and Wilbur, 1982). More than 600 species of beetles and 70 species of moths among insects, 355 species of mites, 40 species of rodent and 150 species of fungi have been reported to be associated with various stored products, including food commodities (Rajendran, 2002). Some of the principal stored grain insects are shown in Fig.1.1. Food commodities of agricultural origin are often infested by beetle pests belonging to the families Tenebrionidae, Cucujidae, Curculionidae, Cleridae, Ptinidae, Silvanidae, Bruchidae, Anobiidae, Anthridae and Bostrichidae and also moth pests belonging to Gelechiidae and Pyralidae. Crop damage by Lepidoptera is done only by the larvae. Several Lepidopteran larvae entangle the feeding media through silky secretion, which turns the products into entwined lumps. In the case of Coleoptera, both larvae and adults often feed on the crop and these two stages are responsible for the damage (Rees, 1996).

Post-harvest insect pests may be primary i.e. able to attack intact grains such as the genus *Sitophilus*, while others are secondary pests, attacking already damaged grains or grain products such as the genus *Tribolium* (Mound, 1989). Insects also have varied food habitats and can breed on foodstuffs containing less than 2% carbohydrate (e.g., *Lassioderma serricorne* and *Tribolium castaneum*), on dried fish with 20% salt (e.g., *Dermestes maculates*), on dry fruits having up to 60% sugar (e.g., *Carpophilus* spp.) and on tree nuts with 50-70% fat (e.g., *Ephestia cautella*). Thus, there is a need to control the insect infestation in food stuffs (Rajendran, 2005).

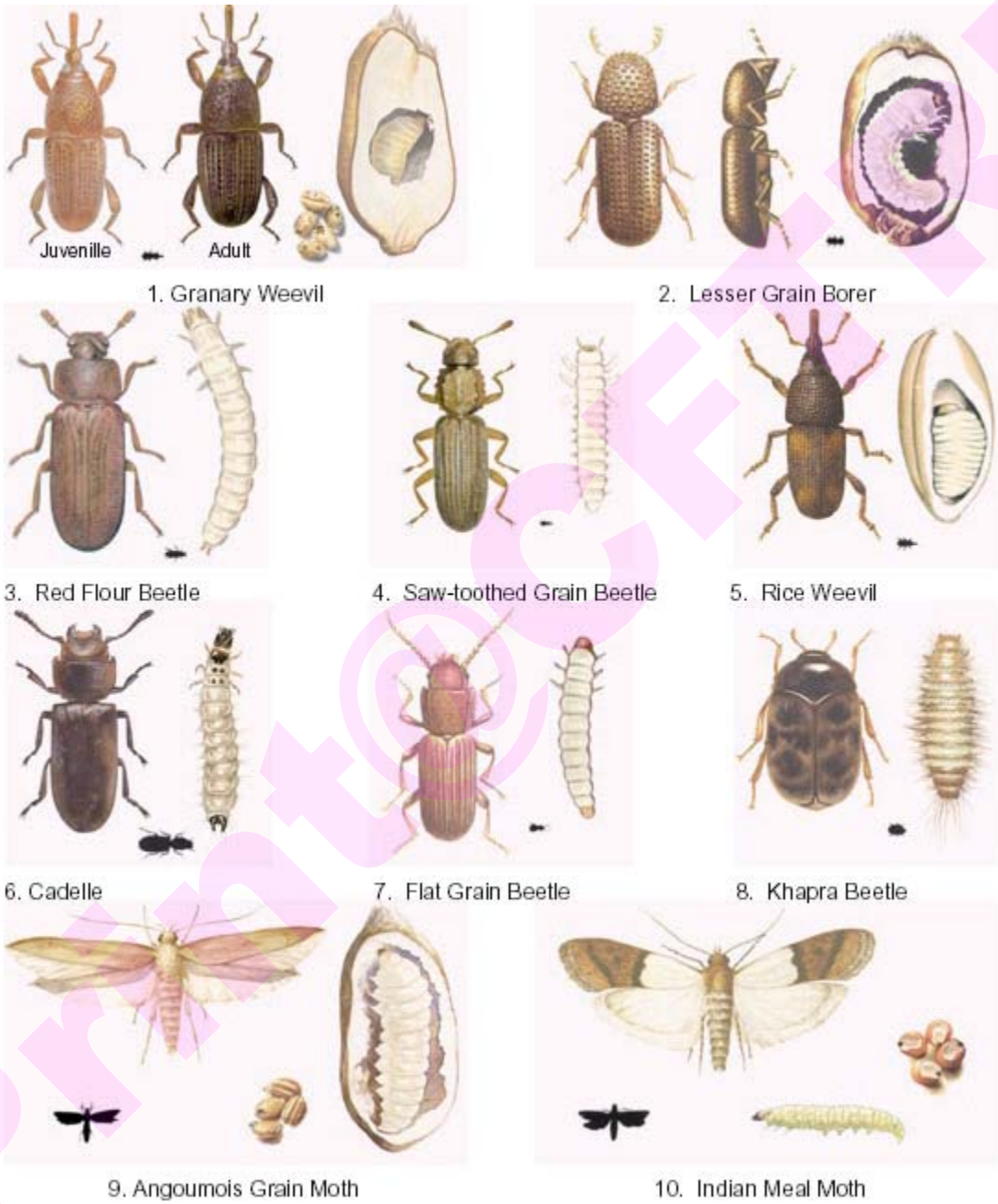


Figure 1.1. Principal Stored Grain Insects

CONTROL METHODS:

Several methods of insect control have been adapted from ages to protect the foods from insect infestation.

1. Traditional methods: Farmers, through a long history of battle against stored product pests, have learnt to exploit natural resources, or to implement accessible methods, that would lead to suppression of pest population. Traditional methods usually provide cheap and feasible ways of post-harvest handling of the crops. The following are the two commonly used methods.

(a) Exposure to sunlight: Exposure to sunlight or exposure followed by sieving of the grains, is a well known technique among farmers, especially against the pests of beans (Chinwada and Giga, 1996). In this method, grains are spread on a dark paper or a black polyethylene sheet and left exposed to sunlight for at least seven hours. After sunning, grains are sieved using a 5μm sieve. This method proved to be quite effective in reducing bruchid infestation with no or minimal effect on grain quality or germination (Songa and Rono, 1998; Chinwada and Giga, 1996; Lale and Sastawa, 1996).

(b) Drying: Drying of the food produce is done using bush dryers, solar dryers or light fire underneath the crop to reduce the water content and to deter or kill the different insect stages (Ntoukam *et al*., 1997).

2. Chemical methods: Chemical methods are effective and easy alternates to traditional methods wherein the insect pest are controlled/killed using chemical compounds.

(a) Pesticides: Due to the significant increase in the human population and the consequent increase in the amounts of food and grains produced, many small scale farmers adopted the use of pesticides as a means of pest control. Dusting and fumigation of grains are the most commonly used chemical methods among small-scale farmers (Rai

et al., 1987; Gwinner *et al.*, 1996). The most commonly used insecticide dusts among farmers belong to two main groups of chemicals: (1) organophosphorus compounds, such as chlorpyrifos-methyl, fenitrothion, malathion, methacrifos and pirimiphos-methyl, and (2) pyrethroids, such as cyfluthrin, deltamethrin, fenvalerate and permethrin. Health risks during handling of the pesticides, their residue in food commodities and environment pollution are the greatest concerns which, limit/minimize their use in infestation control.

(b) Fumigation: Fumigants are low molecular weight chemicals, highly toxic and volatile, that are used during storage to kill all insect stages residing in the produce. Fumigation is a widely used method all over the world on small as well as large storage scale. Fumigants are commercially available in a solid, liquid or gaseous state. The two commonly used compounds as fumigants are Phosphine (PH_3) and Methyl bromide (CH_3Br). However, the problem of human toxicity due to inadequate application of the method is considered a drawback, especially in the developing countries where inappropriate handling of such toxicants is widespread. Another recent problem with the use of fumigants is the development of resistance in insects against fumigants. Recently, fumigation is discouraged at a small-scale level; moreover the use of methyl bromide has been strongly restricted in industrialized countries because of its ozone-depleting potential. However, fumigation is still the most widely operated method as an essential large-scale post-harvest practice. Trials have been conducted on the use of alternative fumigants that are less toxic like carbon dioxide and 'biogas' with methane and carbon dioxide as its main components (Krishnamurthy *et al.*, 1993, Newton *et al*., 1993).

3. Temperature: Temperature is a crucial environmental factor that influences the development of insects. There is always a minimum, optimum and a maximum range of temperature at which insects can survive. The use of high temperature is a well-known

technique to control stored product pests. For example, temperatures of above 40^0C are lethal for most stored food pests (Gwinner *et al*., 1996). Adult emergence of *Sitotroga cerealella, Sitophilus oryzae* and *Rhyzopertha dominica* can be totally suppressed after exposing their pupae to 45^0C for 72 hours. In addition, low temperature treatment in combination with drying of grains is also effective in protecting grain from insect infestation (Sharma *et al*., 1997).

4. Biological control: Biological control may provide a useful and safe alternative for the control of crop pests. McGaughey *et al*.(1987) reviewed the use of the entomopathogenic bacterium *Bacillus thuringiensis* against pests of stored grain and seed. *B. thuringiensis* proved to be ideally suited for use on stored grain and seeds, being compatible with other protectants and available in different formulations for convenient application. In bulk stores, dressing a 10 cm deep surface layer with *B. thuringiensis* at 125 mg/kg controlled both *Plodia interpunctella* and *Ephestia cautella*. This method is gaining importance due to increasing health concerns; however, the use of biological control against stored product pests is still limited.

5. Irradiation: The use of varied short wave doses of about 0.2-0.5 (kGy) provides another alternative in the control of pests in store. Combined treatments of radiation and carbon dioxide produced a higher mortality in *T. confusum* than did either treatment alone (Omar *et al*., 1988). This method has the advantage of leaving no residues in the product, though it might not be feasible due to the high costs involved in application. Other experiments involved the use of microwave energy against stored product pests. A special microwave unit was developed with a variable speed conveyor belt and tested for insect control in stored milled rice. Results indicated that *T.confusum* and *Cryptolestes pusillus* could be killed economically with microwave energy (Langlinais, 1989).

6. Pheromones and trapping: The use of pheromones is one of the most promising techniques aimed at the control of stored product insects that may lead to a drastic reduction of chemical treatments against crop pests (Trematerra, 1997). Pheromone traps can be used to monitor the dynamics and occurrence of different stored product pests, such as *Phthorimaea operculella* (Trematerra *et al*., 1996). In addition, certain compounds extracted from insect bodies may serve as attractants, repellents or arrestants to other insects of the same species. For example, tobacco leaf disk stacks treated with hexane wash of *Lasioderma serricorne* females reduced egg laying by conspecific females; therefore it may have use as an oviposition deterrent (Howlader and Ambadkar, 1995). In another experiment on *Callosobruchus chinensis*, crude extracts of females trapped more than 60 percent of males in a laboratory culture using a pitfall trap, resulting in lower adult infestation levels in the following generation (Islam, 1994).

7. Stored Product Integrated Pest Management: Several studies have focused on developing post-harvest technologies as they play a key role in ensuring food security. Consumers are now aware of the danger in the use of chemical pesticides to protect stored products. Therefore, the world-wide trend to minimize the use of toxic substances applied on food products, has led scientists to develop Integrated Pest Management (IPM) techniques. IPM involves three basic sections - prevention, pest identification and treatment. The prevention section discusses the four basic principles - sanitation, loading, aeration and monitoring (SLAM), which can help maintain maximum grain quality during storage and discourage pest problems. The pest identification section includes a key to assist the user in identifying any of the most common stored grain pests. Finally, the treatment section includes information about biological control, cultural control, mechanical control, physical control, and where necessary, the judicious use of less-

hazardous pesticides (Krishna *et al.,* 1996, Lagnaoui *et al.,*1996).

FOOD QUALITY CONTROL:

Quality maintenance by way of reduction in insect contaminants to meet the requirements of International Standards Organisation (ISO) standards and Hazard Analysis Critical Control Points (HACCP) is important for marketing the produce. There are national tolerance limits such as the maximum quantity of substances including pesticides and natural or unavoidable defects (live or dead insects, insect fragments and related contaminants) allowable in a food. For instance, in the United states of America, the Food and Drug Administration (FDA) has established Defect Action levels (Table 2) for live insects at two insects per kilogram and insect- damaged grains at 32 kernels/100g in food grains; in wheat flour, there is a limit of 75 insect fragments/50g and in macaroni and noodle products it is 225 fragments in a 225g sample (Jeon, 2002). In India, according to the Prevention of Food Adulteration Act, the uric acid level in food commodities should not exceed 100 mg/kg and the number of weevil-damaged grains should not exceed 10% by count (Anon, 2001). In countries like Canada and Australia, there is zero tolerance for insects in food grains (White, 1995) and a similar standard is followed in international trade for grains (Fleurat-Lessard, 1997).

Table 1.2. : Defect Action levels by Food and Drug Administration

Food	Contaminant	Action Levels
Food Grains	Live insect	2 / kg
Food Grains	Insect-damaged kernels	32 / 100g
Wheat flour	Insect fragments	75/ 50g
Macroni	Insect fragments	225 / 225 g
Noodles	Insect fragments	225 / 225 g

There has been a growing concern throughout the world about contamination by pests and pesticides in food commodities. Detection of insect infestation is, therefore necessary (1) to ensure a supply of wholesome food to the consumers, (2) to assess effectiveness of fumigation and other pesticide treatment, and (3) to serve as an early warning for taking appropriate control measures. Any delay in detection may result in pest outbreaks, causing severe contamination of food materials and quantitative loss. In addition, the detection of insect infestation in stored food commodities or on storage premises is the foremost step in pest management in food industries (Mueller, 1998).

INSECT INFESTATION DETECTION METHODS:

There has been considerable progress in research in the area of insect detection for the past 65 years. Insect detection, in samples and storage facilities, continue to play a significant role as an effective management tool in the food industry. Various methods are used to detect insect infestation in commodity samples (Table 1.3). The choice of method depends on whether the infestation is inside or outside the food grains, in the surrounding premises or inside bulk grain, availability of equipment facilities and required sensitivity and the type of food commodity in diverse storage conditions. Most of the methods have the objective of detecting the presence of live insects either directly or indirectly. Free-living (external) insects are detected by visual inspection, sampling, sieving and heat-extraction methods, whereas hidden (internal) infesters are detected by radiography, staining techniques, near-infrared and fragment count methods. Infestation can also be detected indirectly by determining uric acid or carbon dioxide level. Methods for detecting both living and dead insects are rather limited (fragment count and ELISA methods) (Atui *et al.*, 2003).

Brief description of some of the commonly used techniques is given below:

1. Visual inspection: Several clues indicate the presence of insect infestation in stored foods. The presence of eggs of pulse beetles such as *Callosobruchus* spp. can be easily seen on infested pulses with naked eye. Similarly the exit holes of internal infesters such as *Sitophilus* spp., *R. dominica*, *Prostephanus truncates* are clearly visible in infested food grains (Nicholson *et al.,* 1953). In the case of khapra beetle (*T. granarium*) infestation, the exuviae of the larvae are indicators of the presence of the pest. Infestation by moth including *E. cautella*, *Plodia interpunctella*, and *Corcyra cephalonica* is marked by the presence of webbing or silken threads and large fecal particles in a sample of grain or grain product.

Disadvantages: Visual inspections are an indirect measure of detecting infestation and it is a qualitative test only (Rajendran, 2005).

***2. Sampling and sieving*:** In both developing and developed countries, for insect pest detection in food grains, the sampling and sieving method is commonly practiced. In this method representative samples are drawn from a stock of static bulk grain or grain transit, are sieved and are visually checked for the presence of insect pest. The method indicates the current infestation instantaneously.

Disadvantages: It is labor intensive and shows high degree of variation (Hagstrum, 1994).

3. Floatation method: Floatation methods are of two kinds: (1) The specific gravity method and (2) The crackling floatation technique. In the specific gravity method, a whole grain sample is directly tested with a suitable salt solution having specific gravity less than that of the grain (Dixon and Knowlton, 1994). In the crackling floatation technique, whole grain is coarsely ground and treated with a mixture of an alcohol

solution and light mineral oil to expose the insect particles, which are lighter than the grain and float on the top surface (AOAC, 1997).

Disadvantages: The specific gravity method is a qualitative test and does not indicate the species or the specific life stage present inside the grain; it is not suitable for hulled seeds such as barley, oats and paddy and for large- seeded grains like corn (Pederson, 1992). In case of crackling-flotation method, the results are least accurate in determining insect population in commodities and false negatives have been encountered.

4. Fragment count or acid hydrolysis method: Pest infestation in commodities can also be detected by the presence of fragments of insects such as elytra, head capsules, mandibles and other sclerotized parts. The fragment count method involves a floatation technique but in a modified way. In flotation, as applied for whole grains, simple salt solutions are used as the floating medium, whereas in the fragment count method, a mixture of oil and aqueous phase is used and the interfering food materials are digested with acid before allowing the insect fragments to float (Gentry *et al.*, 2001).

Disadvantages: This method requires a trained person to carry out the analysis, especially in flour, chocolate and powdered spices. In addition, it also shows wide variation in results and false positive results (Brader *et al.*, 2002).

5. Carbon dioxide (CO_2) analysis: Howe and Oxley (1944) proposed the use of CO_2 produced in food grains and grain products as an indicator of insect infestation, particularly hidden infestation. The CO_2 level can be measured by gasometric method or an infrared gas analyzer. The intergranular air in the normal grain, which is free from infestation, contains about 0.03% CO_2. A CO_2 concentration of about 0.5% or more is indicative of high-level pest activity in the sample (Fleurat-Lessard, 1988).

Disadvantages: CO_2 is an indirect method of detecting an existing insect infestation. The

respiratory rate of insect eggs or early larval stages is negligible, so this method is not applicable for grains having only those life stages. In addition, it cannot be used for grains with moisture content exceeding 15% because at higher moisture levels, grain alone evolves more CO_2. Furthermore, the CO_2 concentration inside the enclosure/container is also dependent on its air tightness (Semple, 1992).

6. Uric acid determination: The excreta of stored product insects composed primarily of uric acid is used as an indicator of insect infestation in cereals and cereal products since 1950s (Subramanya *et al.*, 1955). Some of the methods used for uric acid determination are colorimetric method using Benedict's reagent, enzymatic method, flourometry, HPLC, TLC, paper chromatography and liquid chromatography.

Disadvantages: The amount of uric acid excreted by an insect per day varies depending on the life stage, insect density and their nutritional status (Farn and Smith, 1963b).

Table 1.3. : Insect Detection Methods Applicable for Commodity samples[a]

Test method	*Applicability*	*Comments*
Physical Methods		
Visual inspection	Whole grains, milled products	Qualitative; only high-level infestation detected
Sampling and sieving	Whole grains, milled products	Hidden infestation not detected; commonly practiced
Heat extraction	Whole grains	Adults and larvae detected
Acoustics	Whole grains	Active stages are detected
Breeding out	Whole grains	Time consuming
Imaging techniques:		
X-ray method	Whole grains	Prohibitive capital cost
Near infrared spectroscopy	Whole grains	Rapid, expensive, can be automated
Nuclear magnetic resonance	Whole grains	Less sensitive
Chemical methods:		
Serological techniques	Whole grains, milled products	Highly sensitive, species specific; shows infestation from unknown past to till date
Uric acid determination	Whole grains, milled products	Shows infestation from unknown past to till date
CO_2 analysis	Whole grains	Simple, time consuming; indicates current level of infestation; not suitable for grains having >15% moisture
Specific gravity method	Whole grains	Simple and quick; suitable for oats and maize
Crackling and floatation method	Whole grains	Variable results noted
Fragment count	Whole grains, milled products	Highly variable results noted; shows infestation from unknown past to till date
Staining techniques:		
Egg-plugs	Whole grains	Specific for *Sitophilus* spp.
Ninhydrin method	Whole grains	Eggs and larvae not indicated

[a] Adapted from Rajendran (2005)

Methods for detection of insect pest eggs: Staining techniques are the commonly used method for detection of insect pest eggs. Some of the popularly used techniques are:

Egg plug staining: In this technique the mucilaginous secretions of weevils are stained with acid fuchsin, gentian violet or berberine sulfate. Weevils including *S. oryzae*, *S. granarius* and *S. zeamais* attached to stored cereals deposit their eggs inside the grains and plug the holes or egg cavities with saliva. These egg plugs are stained and the infestation is identified. The number of egg plugs observed estimates the extent of infestation in a grain sample (Potter and Shellenberger, 1952).

Drawbacks of the technique: The egg plugs are likely to be dislodged during grain handling, so there are chances of recording less than actual infestation level. Moreover this technique is not applicable for other internal infesters like *Rhizopertha dominica* and *S. cerealella* and the external infesters like *Tribolium*, *Cocyra* etc. that oviposit outside the grain (Xingwei *et al.,* 1999).

Breeding out or incubation method*:* In this method the infestation can be determined by incubating the grain under optimum temperature and humidity conditions and checking for adult emergence by sieving the sample at intervals of 3 or 4 days until no more insects emerge.

Drawbacks of the technique: It is a slow process because the time taken to complete the life cycle varies between species and depends on temperature and humidity. This method is accurate, but it takes a longer period of 4-6 weeks to arrive at the results (Xingwei *et al*., 1999).

Iodine method: This is the standard AACC (American Association of Cereal Chemists, (2000) method. In this method the flour is first sieved and the flour particles containing the eggs are boiled with dilute sulfuric acid and filtered through Whatman filter paper

No.1 and 0.1N solution of iodine is added and again washed with dilute sulfuric acid. The eggs appear yellow against brownish yellow flour particles.

Disadvantages of the technique: The contrast between the flour particles and the egg is not distinct (Leelaja *et al*., 2006). In addition, the eggs may be lost during sample processing. The method is applicable only for flour with low residual flour particles like refined wheat flour and only 0.1g of the flour particle can be analyzed at a time, hence not applicable for whole wheat flour, rice flour etc. which have higher (20%) residual flour particles (unpublished results).

The various methods developed have limitations in their application or interpretation of the results (Chambers, 2003). Currently, immunoassays due to their enormous specificity and resolution have gained importance in the field of insect pest detection systems (Ref.: Technical information, Washington,2000).

IMMUNOASSAYS AS DETECTION TOOLS:

Introduction to immunoassays: Immunoassay has a rather long history and has become a widely accepted technique, particularly in the clinical area. Its roots can be traced to Ehrlich and Landsteiner's work with blood typing in 1900, for which Landsteiner won the Nobel Prize in 1930. In the early 1960's radioimmunoassays were used to quantify insulin in plasma samples. Since their introduction, immunoassays have been used to detect and quantify various types of molecules which vary widely in size, chemical, physical and biological properties (Law, 1996).

The use of enzyme labels in an immunoassay was first published in 1971 by a Dutch group. Currently over a billion clinical tests are performed annually in the United States using immunoassay technology. The uses range from testing for drugs, both therapeutic and drugs of abuse, to infectious diseases such as hepatitis and AIDS, to

pregnancy tests, which can be purchased over the counter in any local drug store (Oellerich, 1984).

The use of immunoassays in the field of detection of food contaminants started first with its application to detect pesticide residues by 1970 (Gabaldon *et al.*, 1999). Although Ercegovich described early work on the detection of pesticides by immunochemical means and indicated the potential usefulness of this method for routine analyses in 1971, the level of interest has increased significantly only recently. This is apparent from the literature where the number of publications in this area has increased dramatically (Newsome, 1986).

The crux of an immunoassay is the natural response of a living organism to a foreign agent. When a foreign substance enters the body, it triggers the immune system to produce *"antibodies"* (Ab) which are immunoglobulin proteins that specifically binds to the foreign substance or the *"antigen"* (Ag). Once the antibody and antigen are bound together, the antigen's activity is reduced or eliminated and the cluster is removed from the body. It is estimated that the individual animal can produce antibodies specific for 10,000,000 to 100,000,000 antigens. The ability of an antibody to discriminate between the millions of naturally occurring molecules in a living organism is critical to the use of immunoassays in environmental analysis. Antibodies can be polyclonal if produced by antigen injection into experimental animal, or monoclonal if produced by cell fusion and cell culture techniques (Ref.: Website 2).

Antigen-Antibody Interactions:

Affinity: Affinity describes the strength of interaction between antibody and antigen at single antigenic sites. The specific association of antigens and antibodies is dependent on hydrogen bonds, hydrophobic interactions, electrostatic forces and van der Waals forces.

Within each antigenic site, the variable region of the antibody "arm" interacts through weak non-covalent forces with antigen at numerous sites; the more interactions, the stronger the affinity. Like antibodies, antigens can be multivalent, either through multiple copies of the same epitope or through the presence of multiple epitopes that are recognized by multiple antibodies. Interactions involving multivalency can produce more stabilized complexes, however multivalency can also result in steric difficulties, thus reducing the possibility for binding. All antigen-antibody binding is reversible and follows the basic thermodynamic principles of any reversible bimolecular interaction:

$$K_A = \frac{[Ab\text{-}Ag]}{[Ab][Ag]}$$

where K_A is the affinity constant, Ab and Ag are the molar concentrations of unoccupied binding sites on the antibody or antigen respectively and Ab-Ag is the molar concentration of the antibody-antigen complex.

The affinity constant for antibody-antigen binding can span a wide range, extending from below 10^5 mol^{-1} to above 10^{12} mol^{-1}. Affinity constants can be affected by temperature, pH and solvent. Affinity constants can be determined for monoclonal antibodies but not for polyclonal antibodies as multiple bondings take place between polyclonal antibodies and their antigens.

Avidity: Avidity is the measure of the overall stability or strength of the antibody-antigen complex. It is controlled by three major factors: antibody epitope affinity; the valence of both the antigen and antibody; and the structural arrangement of the interacting parts. Ultimately these factors define the specificity of the antibody, that is, the likelihood that the particular antibody is binding to a precise antigen epitope.

Cross-reactivity: Cross-reactivity refers to an antibody or population of antibodies binding to epitopes on other antigens. This can be caused either by low avidity or

specificity of the antibody or by multiple distinct antigens having identical or very similar epitopes. Crossreactivity is sometimes desirable when one wants general binding to a related group of antigens or when attempting cross-species labeling when the antigen epitope sequence is not highly conserved in evolution (Berzofsky and Berkower, 1984).

Immunochemical techniques capitalize upon the extreme specificity, at the molecular level, of each immunoglobulin for its antigen, even in the presence of high levels of contaminating molecules. The multivalency of most antigens and antibodies enables them to interact to form a precipitate. Examples of experimental applications that use antibodies are Western Blot, Immunohistochemistry and Immunocytochemistry, Enzyme-Linked Immunosorbent Assay (ELISA), Immunoprecipitation and Flow Cytometry.

Types of immunoassays:

Immunoassays are broadly classified into three classes:

Antibody capture assays: The antigen is attached to a solid support and labeled antibody is allowed to bind. After washing, the assay is quantitated by measuring the amount of antibody retained on the solid support.

Antigen capture assays: The antibody is attached to the solid support and labeled antigen is allowed to bind.

Two-antibody sandwich assays: One antibody is bound to the solid support, and the antigen is allowed to bind to this first antibody. The assay is quantitated by measuring the amount of a labeled second antibody that can bind to the antigen (Harlow and Lane, 1988).

Enzyme linked immunosorbent assays (ELISA): ELISAs are based on selective antibodies/antigen attached to solid supports in high affinity binding microtiter plates

combined with enzyme reactions to produce systems capable of detecting low levels of chemicals (Lai *et al.*, 2005).

Advantages of immunoassays:

1) Immunochemical methods provide a *rapid, sensitive and cost effective* analysis for a variety of environmental and food contaminants. These methods are highly sensitive to specific antibodies and provide analytical systems capable of detecting very low levels of chemicals. This high sensitivity is due to the powerful catalytic ability of enzymes. These methods are also highly selective due to the extraordinary discriminatory capabilities of antibodies.

2) Immunoassay methods are *fast* and *relatively easy*. This is because immunoassays involve fewer steps and less training to operate. The immunoassay kit is portable and can be used for testing the sample at the sampling site.

3) Immunoassays are *inexpensive*. Immunoassays also have the advantage of not requiring a multi-step cleanup process as do many traditional methods.

4) Immunoassays are very *efficient screening tools*. Detection limits are suitably low for environmental work and sample preprocessing is not required with its abundant losses. Recovery of analyte from spiked sample can be better with immunoassays than with conventional methods (Newsome, 1986).

IMMUNOASSAYS IN DETECTION OF INSECT INFESTATION:

Johnson *et al.* (1973) first proposed the feasibility of application of an immunoassay method for detection of insect pest contamination in food commodities. After a gap of 18 years, there is a renewed interest in the development of immunological tests for insect contamination in foods**.** Kitto (1991) and Quinn *et al.* (1992) reported immunoassays for insect detection in food commodities by an indirect Enzyme Linked Immunosorbent

Assay (ELISA) based on insect myosin, adopting the method of Browning *et al*. (1987). It is believed that there are very few differences in myosin structure between insect types, and hence, the ELISA technique is known to give reliable and reproducible results. However, a nonspecific background-level equivalent to 6.84± 1.45 µg of myosin/50 g of wheat was observed during the determination of *S.granarius* infestation in wheat by indirect ELISA (Schatzki *et al.,* 1993); the background level was still higher for milled products and some of the spices (Kitto *et al.,* 1994). Quinn *et al.* (1992) adopted a double sandwich ELISA (sELISA) procedure based on the method of Martin *et al.* (1988) for detection of infestation in wheat flour to overcome the non-specific binding. This technique has been reported to be more sensitive and is not much affected by the quantity of wheat flour sample taken for analysis.

The myosin or indirect ELISA uses only the polyclonal antisera, detects the total insect infestation and cannot quantify the contribution of individual species. However, in a practical situation, a grain or a milled product sample may carry more than a single insect species. In this context, monoclonal antibody technique was exploited to develop monoclonal antisera specific to *S. granarius* (Chen and Kitto, 1993) to conduct sELISA. Species – specific sELISA has also been developed to identify *T. granarium* (Stuart et al., 1994). Other immunological techniques such as double immunodiffusion and immuno-osmophoresis have been developed to detect hidden infestation of *S. granarius* (Germinara *et al.*, 2000; Rotundo *et al*., 2000). Udaya Kumari *et al.* (2004) have also reported a specific ELISA for *S. oryzae*. Immunoassay techniques for insect pest detection and quantification in food commodities require minimal laboratory facilities and proper training of the staff. The technique is applicable for detecting the presence of insects in various foodstuffs with appropriate modifications in extraction procedures

(Kitto *et al.*, 1994). Immunological assays are also found to be more reliable than X-ray and fragment count methods (Kitto *et al.*, 1994; Quinn *et al*., 1992).

Immunoassays for detection of eggs of insect pest: The immunoassays mentioned above based on muscle protein myosin can detect all the life stages of the insect except eggs since myosin is not present in eggs. Johnson *et al* (1973) however claimed that fruitfly eggs could be detected by serological/immunological methods. The authors reported that the fruit fly eggs in fruit juices are detected by solid-phase radioimmunoassay as described by Johnson *et al.* (1971). Sandwich ELISA for detection of *Anastrepha suspensa* eggs in fruits was reported by Kitto *et al.* 1996. The assay showed low cross reactivity to *Dacus* and *Ceratitis* flies. However, currently there is no report on development of and immunoassay for detection of eggs of stored product pest.

The current scenario of stored product insect pest detection methods is that most of the methods can detect the adult or the visible life stages of the insect pest. Only few methods are available for detection of insect pest eggs like the egg staining techniques and breeding out method. These methods are not sufficiently sensitive; are exclusive in their application or are time consuming. Due to their small size eggs often go unnoticed until the population explodes and by then the damage is already done. Insect infestation is one of the major problems faced by the milling industry wherein the milled products of cereals get infested by insect pest eggs which gets transferred to the final product. The red flour beetle, *Tribolium castaneum*, the saw-toothed grain beetle, *Oryzaephilus surinamensis*, and the rice moth, *Corcyra cephalonica* infestation is generally observed in plan-sifters, separators and conveyors (Leelaja *et al.*, 2006). From these sources, insect infestation is likely to be passed on to finished products like whole wheat flour, refined wheat flour and rice flour. Subsequently, the level of infestation in milled products

increases according to the storage period in the retail market. Detection of the insect pest eggs is therefore a crucial factor for millers and the processed food packaging industry.

In view of the above lacunae, the present study focuses on the development of an immunoassay for detection of incipient stages of *Tribolium castaneum* with special reference to eggs. This study is groundwork for the feasibility of the application of immunoassays in detection of stored product insect pest eggs.

TRIBOLIUM CASTANEUM

Common name: Red flour beetle

Scientific name: *Tribolium castaneum* (Herbst)

Phylum: Arthropoda

Class: Hexapoda

Order: Coleoptera

Family: Tenebroinidae (Sokoloff, 1972)

Fig. 1.2. Photograph of *Tribolium castaneum*

Red flour beetles attack stored grain products such as flour, cereals, crackers, beans, spices, pasta, cake mix, dried pet food, dried flowers, chocolate, nuts, seeds and even dried museum specimens (Via 1999, Weston and Rattlingourd, 2000). Adults are highly adapted to feed on a very wide range of commodities and perfect colonizers of new habitats. In tropical conditions, this species is dominant to *T. confusum* (Howe, 1965). They can be found in almost every store containing infested cereals or cereal products, specially in tropical and sub-tropical climates. These beetles have chewing mouthparts

but do not bite or sting. The red flour beetle may elicit an allergic response (Alanko *et al*. 2000), but is not known to spread disease and does not feed on or damage the structure of a home or furniture. These beetles are the most important pests of stored products in the home and grocery stores. *T. castaneum* adult females lay small, cylindrical, white eggs scattered in the product. Females are highly fecund and able to lay a maximum of 1000 eggs during a lifetime, with 40^0C and 22^0C as upper and lower limits for development. At an optimum temperature of 32.5^0C, females lay up to 11 eggs daily. This species is also highly tolerable to humidity as low as 11 percent.

Distribution : The red flour beetle is of Indo-Australian origin (Smith and Whitman, 1992) and is found in temperate areas, but will survive the winter in protected places, especially where there is central heat (Tripathi *et al*. 2001).

Description and Life Cycle: Although small beetles, about 1/4 of an inch long, the adults are long-lived and may live for more than three years (Walter, 1990). These beetles are reddish-brown in color and their antennae end in a three-segmented club. The head is visible from above, does not have a beak and the thorax has slightly curved sides. They may fly, especially before a storm. The eggs are white, microscopic and often have bits of flour stuck to their surface. The slender larvae are creamy yellow to light brown in color. They have two dark pointed projections on the last body segment. The pupae are lighter in color, being white to yellowish. These beetles can breed throughout the year in warm areas. The life cycle takes from 40 to 90 days and the adult can live for three years. At 25^0C and 70% RH, the total developmental period is about 40-45 days (egg- 6-8, larva-32, pupa-10 and adult-10 days). All forms of the life cycle may be found in infested grain products at the same time (Bousquet, 1990).

Habits: The red flour beetles may be present in large numbers in infested grain, but are unable to attack sound or undamaged grain. The adults are attracted to light but will move away from light when disturbed. Typically, these beetles can be found not only inside infested grain products but also in cracks and crevices where grain may have spilled. They are attracted to grain with high moisture content and can cause a grey tint to the grain they are infesting. The beetles give a displeasing odor and their presence encourages mold growth in grain (Walter, 1990 ; Sokoloff, 1972).

THE ANTIGEN: VITELLIN, THE MAJOR EGG PROTEIN

In insects, vitellins (Vt) are the major yolk proteins which, serve as the nutritive source for the developing embryo during embryogenesis. Vitellins usually comprise 60-90% of the soluble egg yolk protein (Hagedorn and Kunkel, 1979). Their precursors, vitellogenins (Vg), are synthesized extraovarially under the regulation of specific hormone in the female fat body (Socha *et al*., 1991; Wyatt, 1991; Venugopal and Kumar, 2000) and subsequently secreted into the haemolymph. These precursors are taken up by the maturing oocytes via receptor mediated endocytosis (Telfer *et al.*, 1982; Raikhel and Dhadialla, 1992) and deposited as vitellins (Giorgi *et al.*, 1999). Vitellin and its precursor-vitellogenin for majority of the insects appear to be largely identical molecules as evidenced by molecular weights, amino acid composition and immunological reactivity (Hagedorn and Kunkel, 1979). Insects may have single or multiple vitellins. Presence of multiple vitellin is known in cockroach families including *Periplaneta* spp., *Blatta orientalis*, *Blatella germanica*, the termite *Zootermopsis angusticollis*, the cricket *Acheta domesticus* (Storella *et al*., 1985) and *Leucophaea maderae* (Masler and Ofengand, 1982).

Insect vitellins are large oligomeric phosphoglycoproteins with molecular weight ranging from 210 to 652kD (Raikhel and Dhadialla, 1992) and containing subunits of the molecular weight ranging from 50-180kD (Giorgi *et al*., 1999). However, vitellins of the higher dipterans are known to compose of oligomers of a single polypeptide subunit (White and Bownes, 1997) a homologue of vertebrate lipases. Vitellin of *Derobrachus geminatus* (Coleoptera) is composed of three apoproteins of about 160, 140 and 50 kD (Osir and Law, 1986). Two large subunits of 199 and 162 kD have been reported for vitellogenin of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleopetera)

(Koopmanschap *et al*., 1992). Vitellogenin/vitellins of at least some Coleopterans do conform to the more typical pattern of one large and one small apoprotein e.g. Curculionidae (Heilmann *et al*., 1993) and Cerambycidae (Azuma *et al*., 1993). The soluble proteins of the eggs of the coleopteran insect *Anthonomus grandis* Boheman, the cotton boll weevil, consist almost entirely of two vitellin types with molecular weights of 160,000 and 47,000D (Heilmann *et al*., 2005).

Recent studies on *T. castaneum* have indicated that it consists of a single vitellin molecule with native molecular weight of 440kD and contains two larger subunits of 180kD, 160kD and a smaller subunit of 50kD (Kim *et al.*, 2001).

With the onset of embryonic development, the degradation of vitellin is initiated with a sequence of distinctive and limited proteolytic cleavages yielding intermediates of lower molecular weight. Also, about 50% of the reserved proteins are converted into non-proteinous components (de Chaffoy and Kondo, 1980). On degradation, the proteolytic products may or may not retain their antigenic properties. In *Periplaneta Americana* in the course of embryonic development Vt undergoes specific limited cleavages that result in the generation of new, smaller polypeptides with concomitant loss of several immunological determinants (Storella *et al*., 1985). On the contrary, in *Leucophaea maderae* and *Acheta domestica* it has been observed that the proteolytic products retain their antigenic properties. With embryonic development the vitellin titer also decreases giving rise to newer stage specific proteins. The rate of vitellin utilization varies among insects. In *Locusta migratoria,* vitellin is utilized rapidly during the early phase of embryogenesis (McGregor and Loughton, 1974). A longer persistence of vitellin is, however, observed during embryonic life in *Drosophila melanogaster* (Bownes and

Hames, 1977), *Blatella germanica* (Tanaka, 1977), *Bombyx mori* (Irie and Yamashita, 1980) and *Acheta domestica* (Handley *et al*., 1998).

Therefore, the presence of vitellins as a major egg protein constituting about 60-90% of the soluble egg yolk protein; their persistence up to the larval stage; and its immunological identity with its precursor vitellogenin that appears by the larval stage in most insects, has made it the obvious choice as the 'antigen' for the present study on the development of an ELISA for detection of incipient stages of *Tribolium castaneum* - a stored grain insect pest.

OBJECTIVES OF THE PRESENT STUDY

Insect infestation is one of the major problems faced by the milling industry wherein the milled products of cereals get infested by eggs of insect pests such as *Tribolium castaneum, Oryzaephilus surinamensis* and *Corcyra cephalonica,* which gets transferred to the final product thereby helping in the spread of infestation. The prominence of current methods used for detection of stored product insect pest is on the detection of adult or the visible life stages of the insect pest, especially in whole grains. Only few methods are available for detection of insect pest eggs like the egg staining techniques and breeding out method. These methods are not sufficiently sensitive, are exclusive in their application or are time consuming. Currently, immunoassays due to their enormous specificity, resolution, rapidity, cost effectiveness and efficiency have gained importance in the field of insect pest detection systems. The present study is aimed at the development of a sensitive Enzyme Linked Immunosorbent assay (ELISA) for detection of incipient stages of the red flour beetle - *Tribolium castaneum,* with special reference to eggs, in food commodities. The study involves the following objectives:

1. Development of ELISA for detection of incipient and other developmental stages of *Tribolium castaneum.*

a. Purification of the antigen i.e. the major egg protein of *Tribolium castaneum* Herbst.

b. Production of antibodies against the purified antigen in rabbit and chicken.

c. Development of the standard ELISA based on the rabbit and chicken egg yolk antibodies.

2. Application of the ELISA developed to food commodities like whole wheat flour and rice flour and testing of market samples.

3. Comparison of the ELISA developed with the insect pest egg staining methods i.e. AACC approved iodine method and bromocresol green staining method.

CHAPTER II

PURIFICATION AND PARTIAL CHARACTERIZATION OF THE ANTIGEN

INTRODUCTION

This chapter describes the purification and partial characterization of the antigen used in the present study i.e., the major egg protein – vitellin of the red flour beetle, *Tribolium castaneum.* The nutritive proteins present in the egg yolk are usually called vitellin and these proteins are phospholipoglycoproteins. Their precursors, vitellogenin, are synthesized extraovarially under the regulation of specific hormone in the female fat body (Socha *et al*., 1991; Wyatt, 1991; Venugopal and Kumar, 2000). Vitellins usually comprise 60-90% of the soluble egg yolk protein (Hagedorn, 1979). With the onset of the embryonic development, vitellin degradation starts with a sequence of distinctive and limited proteolytic cleavages yielding lower molecular weight polypeptides that provide the food supply to the developing embryo. In *Periplaneta Americana,* during embryonic development, vitellin undergoes specific limited cleavages that result in the generation of new, smaller polypeptides with concomitant loss of several immunological determinants (Storella *et al*., 1985). On the contrary, in *Leucophaea maderae* and *Acheta domestica*, it has been observed that the proteolytic products retain their antigenic properties. The rate of vitellin utilization varies among insects. In *Locusta migratoria,* vitellin is utilized rapidly during the early phase of embryogenesis (McGregor and Loughton, 1974). A longer persistence of vitellin is, however, observed during embryonic life in *Drosophila melanogaster* (Bownes and Hames, 1977), *Blatella germanica* (Tanaka, 1977), *Bombyx mori* (Irie and Yamashita, 1980) and *Acheta domestica* (Handley *et al*., 1998). The changes in vitellin titer and vitellin processing observed in insects during embryonic development are diverse. Therefore, this chapter deals with the study of the profile of vitellin at different embryonic stages of *T .castaneum* and use of vitellin as an antigen to develop a detection system for *T. castaneum* infestation in foods.

MATERIALS AND METHODS

1. Chemicals: Leupeptin, pepstatin, phenylmethanesulfonylflouride (PMSF), high molecular weight range markers, mercaptoethanol and N,N,N',N'- tetramethyl ethylene diamine (TEMED) were purchased from M/s Sigma Chemical Company, USA. Sodium chloride, potassium chloride, sodium phosphate, acrylamide, Bis-acrylamide, cupric chloride, Coomassie brilliant blue, Schiff's reagent were purchased from M/s Sisco Research Laboratories, Mumbai. All the other chemicals used were obtained locally and were of highest purity grade.

2. Insects: The red flour beetle *Tribolium castaneum* Herbst, adults were obtained from a five year old colony from the insect culture maintained in our departmental laboratory. Insects were reared in glass culture bottles with whole-wheat flour as the medium. The cultures were set at 30^{o}C with 60% relative humidity. Whole-wheat flour obtained locally was checked for infestation by incubating the flour for a period of 30 days and then supplied as diet, for insect culture.

3. Sexing of *Tribolium castaneum*: Sexing was carried out at the pupal stage since at this stage pupae move very little compared to the adults and do not need to be immobilized by cooling them on ice. The sexing was done as follows:

1. Some pupae were tapped onto a clean glass slide.
2. Using a small brush the pupae were arranged in a horizontal line about half way down the slide (all of them facing the same direction, i. e. all heads up or all heads down).
3. The slide was placed under a stereomicroscope and sexing was done based on the papillae present at the posterior end of the insect. The papillae in

females are much larger than those of the male and are two finger-like structures just anterior to the pointed urogomphi. The male papillae are small and look like fingertips (Plate 1). One sex was brushed into a new line above the original line and the other sex into a new line below the original line.

4. The pupae were double checked to verify the sex and placed in separately labeled containers filled with flour and allowed them to eclose to adulthood (Ref.: Website 3).

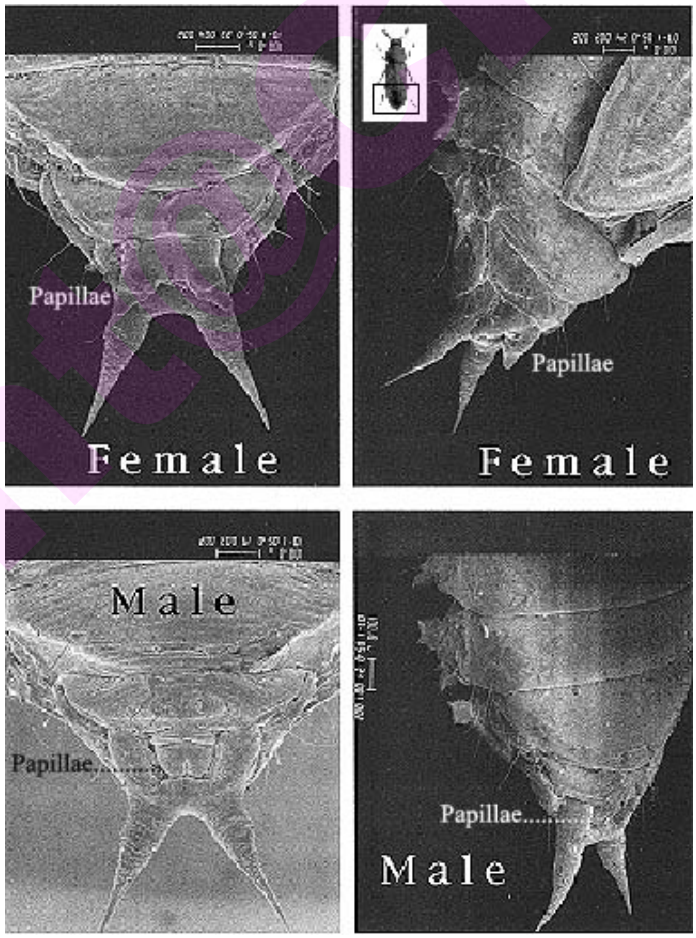


Plate 2.1. Photographs of *T. castaneum* pupal genitalia

(Reproduced from Ref.: Website 3)

4. Collection of eggs and other developmental stages: The whole wheat flour was sieved twice with 85mesh (pore size180 microns) standard sieve. *Tribolium castaneum* Herbst, adults with random male female ratio were released into the sieved whole wheat flour. The egg laying and yield of eggs are affected by quinones released by the insect and insect cannibalism (Sokoloff, 1972). In order to avoid or minimize such effects, the insect population was maintained around 500 insects per 250g of sieved flour and the flour was changed for every third batch of insect release i.e. after every 18h. For the collection of eggs, after 6 hours of insect release, adults were removed from the flour using 44 mesh (pore size 355 microns) standard sieve. The flour containing the eggs was subsequently sieved using 85mesh sieve to separate the eggs. Each batch of eggs retained in the sieve were left in a small quantity of the sieved flour for a further determined incubation period to obtain eggs with different embryonic stages. Therefore, each batch of eggs would contain eggs in embryonic development approximately between 0-6h, 12-18h, 24-30h, 42-48h, 66-72h, 86-92h, 114-120h and 138-144h (contained some freshly emerged larvae along with eggs which were separated). Mature larvae and pupae were collected by allowing the eggs to develop in the wheat flour till the desired stages were attained.

Eggs in the early embryonic development phase i.e. 0-6h as explained above were collected in higher quantities in order to isolate and purify the egg protein of interest for the present study.

5. Study of the protein profile in the embryonic and developmental stages: The criteria for selection of a protein as antigen in the present study was that it should be a major egg protein and be present in eggs throughout the embryonic development. Thus, protein profile was studied throughout the embryonic development.

The major egg proteins i.e. vitellins are derived from the extraovarian precursor vitellogenin. Therefore, as vitellogenin is present in the other developmental stages such as larvae, pupae and adult male and female insects, the protein profile of these stages were also looked into in order to study the precursor-product relationship and their crossreactivity. This knowledge would bring out the degree of usefulness of the assay in detecting the incipient and adult stages of the red flour beetle - *T. castaneum*.

The protein from the different stages i.e. egg, larvae, pupae and adult male and female insects was extracted by the method of Chinzei *et al.* (1981)**.** Batches of different stages of the insect was rinsed once with extraction buffer, (0.2 M sodium phosphate buffer containing 0.5M KCl, 5mM EDTA, PMSF, pepstatin and leupeptin, pH 7.5), and homogenized in 2 volumes of cold (4^0C) extraction buffer. The homogenate was centrifuged at 10,000 rpm for 30mins in a refrigerated centrifuge at 4^oC. The clear supernatant i.e. the water soluble protein fraction (WSPF) was recovered and subjected to Native PAGE (N-PAGE) to study the protein profile. Protein estimation was done by the Bradford method (1976).

6. Polyacrylamide gel electrophoresis: N- PAGE was performed at 4^0C on a 7% resolving gel with a 5% stacking gel according to the method of Laemelli (1970). α globulin (MW- 440 KD) was used as molecular weight marker. SDS-PAGE for the purified protein was carried out on an 8% resolving gel with 5% stacking gel according to the method of Laemelli. High molecular weight range markers were used for molecular weight determination. On termination of electrophoresis, the gels were fixed and stained with 0.1% Coomassie brilliant blue R-250 for total protein. As vitellin is a phospholipoglycoprotein, presence of carbohydrate (glycoprotein), phosphate and lipid moiety were detected by specific staining.

(a) Glycoprotein staining: The presence of carbohydrates was detected by staining the gel using periodic acid/Schiff's (PAS) reagent (Fairbanks *et al*., 1971). Briefly, gel was first incubated in 7.5% acetic acid for 1h at room temperature followed by 1h in 0.2% periodic acid at 4^0C. After 2-3 rinses in 15% acetic acid, the gel was stained with Schiff's reagent for 1h. The gel was then destained in 7% acetic acid at 4^0C. The carbohydrate moiety stains pink in color.

(b) Phosphoprotein staining: The presence of phosphate moiety was detected by methyl green staining (Cutting and Roth, 1973). Briefly, on termination of electrophoresis, the gels were placed in 10% sulfosalycylic acid (SSA) fixative for 10 min and then transferred and incubated for 1h in 10% SSA containing 0.5M calcium chloride. Subsequently, the gel was rapidly rinsed in distilled water to remove surface borne calcium chloride and placed in 0.5N sodium hydroxide at 60^0C for 30 min. Then the gel was rinsed at 10min interval in 1% aqueous ammonium molybdate. The gel was then placed in 1% solution of ammonium molybdate in 1N nitric acid for 30min and transferred to 0.5% solution of methyl green in 7% acetic acid for 30min. The gel was destained in 10% SSA and stored in 7% acetic acid. The phosphate moiety stains green in color.

(c) Lipid staining: Lipid moiety was detected using sudan black (De Vlamig *et al*., 1977). Briefly, on termination of electrophoresis, the gels were stained overnight in a saturated solution of Sudan black in 70% ethylene glycol and destained in 70% ethylene glycol for approximately 1h in order to visualize lipoprotein bands, which appear dark blue/black.

7. Isolation and purification of major egg protein (vitellin): One gram of eggs with embryonic stages between 0-6h was collected and processed as described above to obtain the WSPF. The isolation and purification of the major egg protein V1 from the WSPF

was performed by preparative N- PAGE as per the conditions above. The protein of interest was eluted from the gel adopting the method of Wen *et al.* (2001). Briefly, on termination of electrophoresis, the gel was stained with cupric chloride (0.3 M). After staining, the gel was rinsed with water for 2-3 minutes to remove the excess reagents. The major egg protein band was excised and destained with 25mM Tris-Gly (pH 8.3) followed by 12.5M Tris–Gly (pH 8.3) for 10 min in each. The protein band was then rinsed with water and immersed in 2 mL of extraction buffer (50mM Tris-HCl plus 50mM EDTA, pH8.8). The band was finely minced and left on a stirrer for 12-24h at room temperature. The supernatant was collected and dialyzed first against PBS containing 5mM EDTA and then against PBS. The supernatant containing vitellin was used as an immunogen for antibody production.

8. Western Blotting: Western blot was carried out as described by Towbin *et al*. (1979). Protein samples were eletrophoresed in the same gel in duplicates to correlate western blot patterns with total protein patterns. After electrophoresis, half of the gel with one set of sample was stained for total protein with Coomassie brilliant blue and the other was transferred to a 0.45μm nitrocellulose membrane (NCM) in 25mM Tris containing 19.2mM glycine buffer (pH 8.3) containing 20% methanol. The transfer of proteins on the NCM was visualized by staining with Ponceau S. The NCM was then blocked for 2h with 2% bovine serum albumin in 10mM Tris- HCl, 0.9% NaCl, pH 7.4 (TBS), again incubated for 2h at 37^0 C in a 1:50,000 dilution of polyclonal antiserum, washed in TBS-0.05% Tween 20, incubated for 90 min in 1:10,000 dilution of horse radish peroxidase labeled anti-rabbit IgG and washed again. The bands were visualized with 1:10 dilution of substrate-chromogen reagent. The method of obtaining the V1 antiserum is described in detail in Chapter III.

RESULTS

1. Collection of eggs: Insects collected within 6h of release into the stock culture medium layed more eggs amounting to 110mg of eggs as compared to those collected at later stage as can be seen from Fig. 2.1. Subsequent release of the insects into the old culture medium resulted in lower yield. For example eggs collected from 30h old culture medium yielded about 30mg eggs thereby registering a four-fold decrease. This decrease in the egg laying is attributed to the accumulation of quinones excreted by the growing adult *Tribolium castaneum* in the culture media. Based on these observations the culture media was changed after 18h of use in order to obtain good yield of eggs. The insect population was fixed at 500 insects per 250g of whole wheat flour.

2. Protein profile of eggs through embryonic development: Examination of protein profile of the embryonic stages by N-PAGE revealed two major protein bands (V1 and V2). V1 and V2 together obtained from the eggs between 0-6h of embryonic development is designated as Vm in this study and it represents the total antigen obtained from the eggs between 0-6h embryonic development. As evidenced from Fig-2.2, V1 is the major band and is present throughout the embryonic stage. V2 was a minor band in all the embryonic stages. From 72h upto immature larval stage, in addition to V1 and V2, another band of molecular weight lower than V2, was observed with embryonic development. Further, from 120h another new band of intermediate molecular weight between V1 and V2 was observed. From Fig. 2.2, it is observed that V2 and the other two new protein bands formed at the later embryonic stage were carried forward onto immature larval stage. The Western blot analysis of the egg proteins through embryonic development reaffirmed the PAGE findings that V1 and V2 are present throughout the embryonic development (Fig.2.3). Based on these observations V1, the

major band was selected as the antigen for this study.

3. Vitellin titer: Amount of extractable protein in the WSPF obtained per egg at 0-6h of embryonic development was found to be 7.3µg and V1 protein was approximately 80% and V2 approximately 20% in the young embryo. This data was obtained from the extraction of these proteins following electrophoresis. Protein pattern seen in Fig. 2.4 indicates slow decrease in the protein content with embryonic development. Approximately 40% of the initial level of total extractable protein remained unused at the end of embryonic life. However, there were other proteins, present as minor bands, which added to the protein content. Also it was clear from the N-PAGE that V2 continued to the larval stage.

4. Partial characterization of the antigen: The N-PAGE (Fig.2.2) indicates that the molecular weight of V1 was slightly more than 440kD. SDS-PAGE analysis of V1 protein showed three major subunits, the larger subunits V1a and V1b with molecular weights of about 150 kD and the smaller subunit V1c with molecular weight of about 50 kD (Fig.2.5). Both V1 and V2 stained positive with PAS reagent, indicating that they were glycoproteins (Fig.2.6). However, the phosphate and lipid moieties were not detectable by the methods adopted.

5. Protein profile in other developmental stages: The N-PAGE of the WSPF of immature larvae (Li), mature larvae (Lm), pupae (p), male (m) and female (f) adult insects revealed the presence of one major band designated as VG, which corresponded to V2 protein band found in egg (Fig.2.7). Immature larvae (Fig.2.2), mature larvae and pupae (Fig.2.7) revealed another protein band of a slightly lower molecular weight than VG. This finding is only of the WSPF obtained and does not reveal the total protein of the developmental stages. The amount of extractable protein obtained with VG as the

major protein is tabulated in Table 2.1.

Western blot analysis (Fig.2.8) revealed that VG protein band present in the developmental stages crossreacted with V1 antisera. This crossreactivity indicates that the VG protein may be the precursor for V1 and/or V2 protein present in the egg. This finding justifies the choice of antigen, as the antibodies raised against V1 protein from egg is able to crossreact with VG present in other developmental stages i.e. larvae, pupae and adult (male and female). There was no observed crossreactivity with the other protein band present in immature larvae, mature larvae and pupae (Fig.2.8).

The above findings indicate that the antigen of choice V1 protein is present throughout the embryonic development of *T. castaneum*. Further, the antibodies obtained against V1 protein from egg crossreacts with other developmental stages of *T. castaneum*, suggesting that in the present study V1 antibody based assay could be used to detect all the developmental stages of *T. castaneum*.

Table 2.1.: Total extractable protein from the developmental stages of *T. castaneum*

Developmental stage	Antigen (mg)/insect stage (1No.)
Egg	0.0073
Immature larva	1.5800
Mature larva	0.0291
Pupa	0.2710
Adult female	0.0920
Adult male	0.0820

(Each value represents mean ± SE of three different extractions)

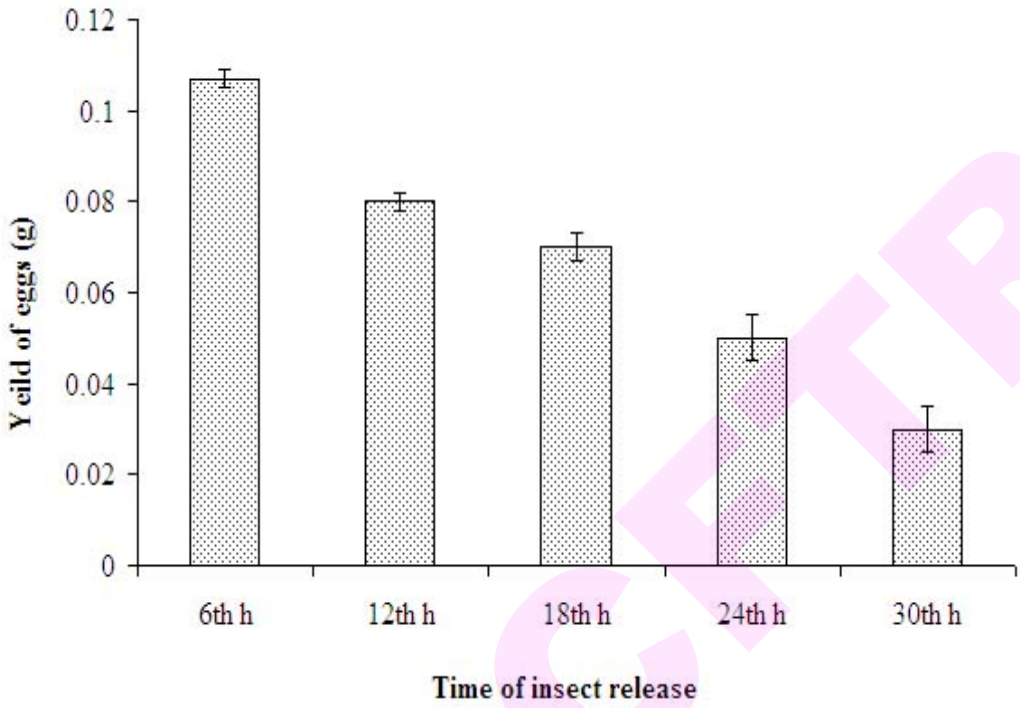


Fig.2.1. Effect of aging culture media on the yield of eggs of *T. castaneum*

(Each bar represents mean ± SE of three different experiments)

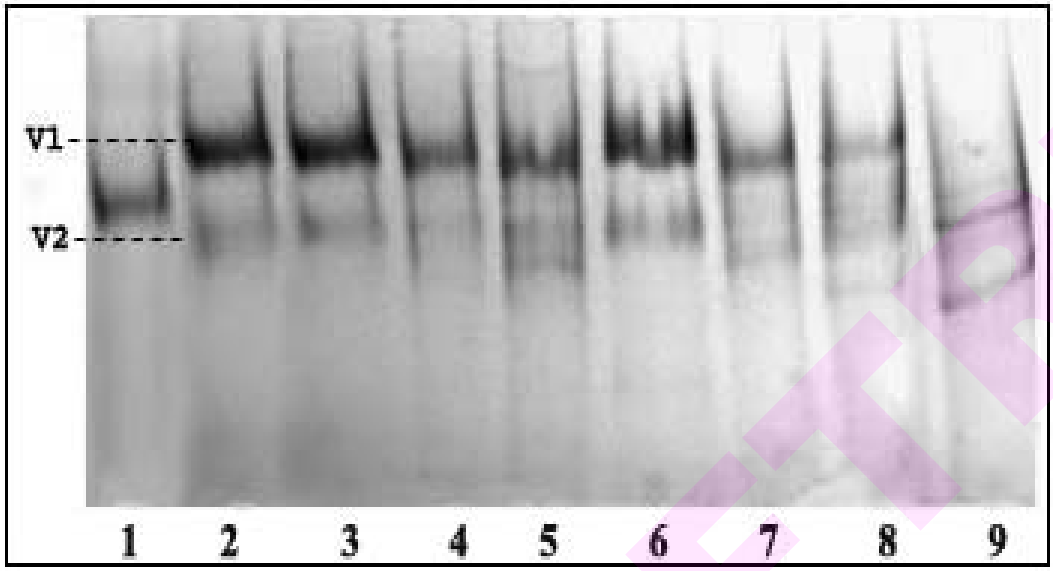


Fig.2.2. N-PAGE of eggs of *T. castaneum* during embryonic development

Lane: (1) MWM(440kD), (2) 6h, (3) 24h, (4) 48h, (5) 72h, (6) 96h, (7) 120h, (8) 144h, (9) Immature larvae

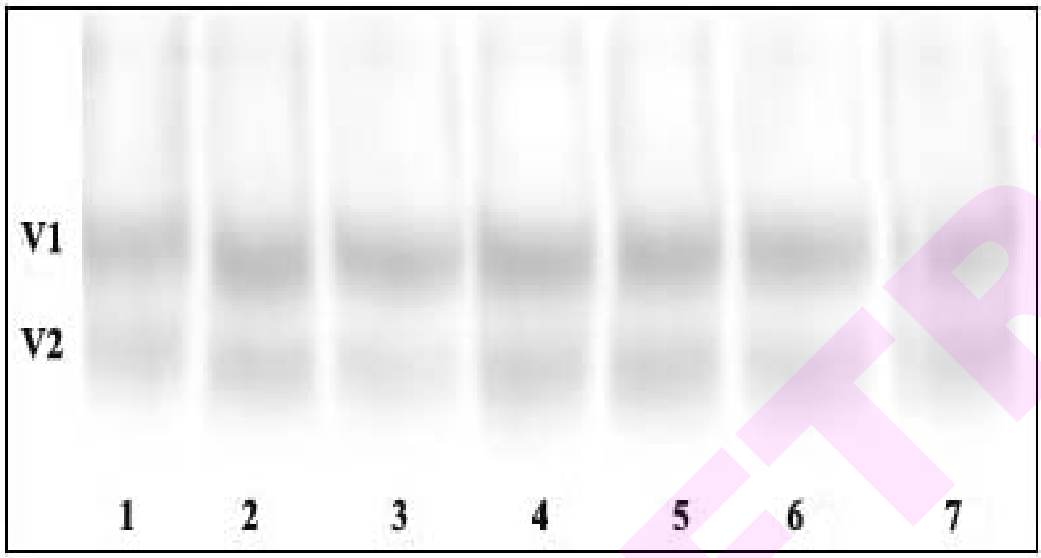


Fig.2.3. Western blot analysis showing reactivity/crossreactivity of embryonic stages of *T. castaneum* with anti V-I antiserum

Lane: Age of the embryo-(1) 6h , (2) 24h, (3) 48h , (4) 72h, (5) 96h, (6) 120h , (7) 144h

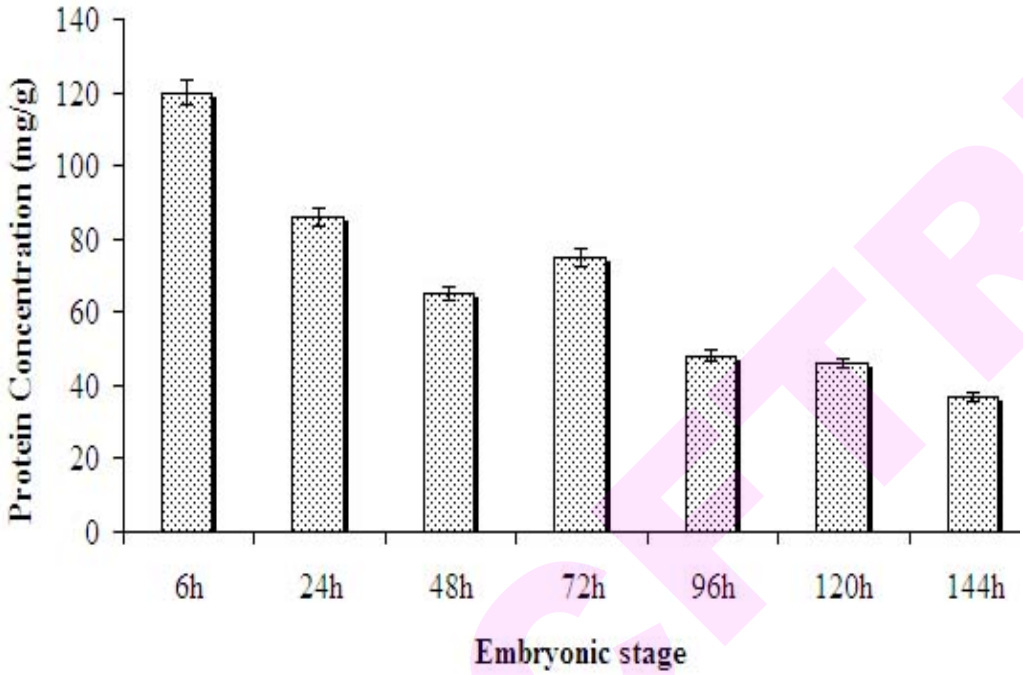


Fig.2.4. Total extractable protein concentration through embryonic development of *T. castaneum*

(Each bar represents mean ± SE of three different extractions)

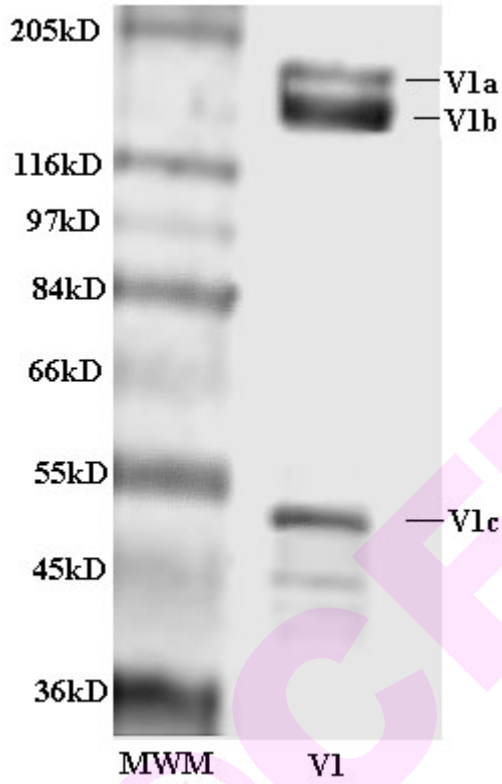


Fig.2.5. SDS –PAGE analysis of V1 protein of *T. castaneum* egg

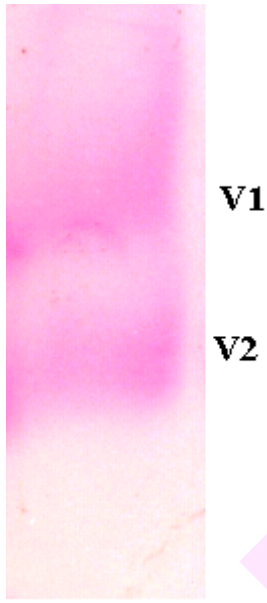


Fig.2.6. Glycoprotein staining of *T. castaneum* egg protein

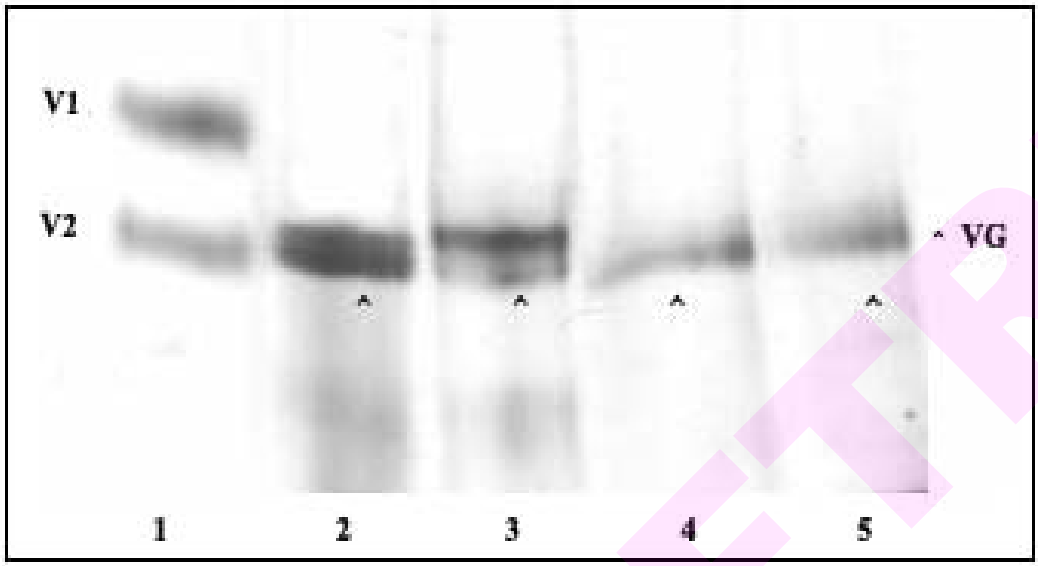


Fig.2.7. Native-PAGE of *T. castaneum* developmental stages

Lane: (1) 6h egg, (2) mature larvae, (3) Pupae, (4) adult female, (5) adult male

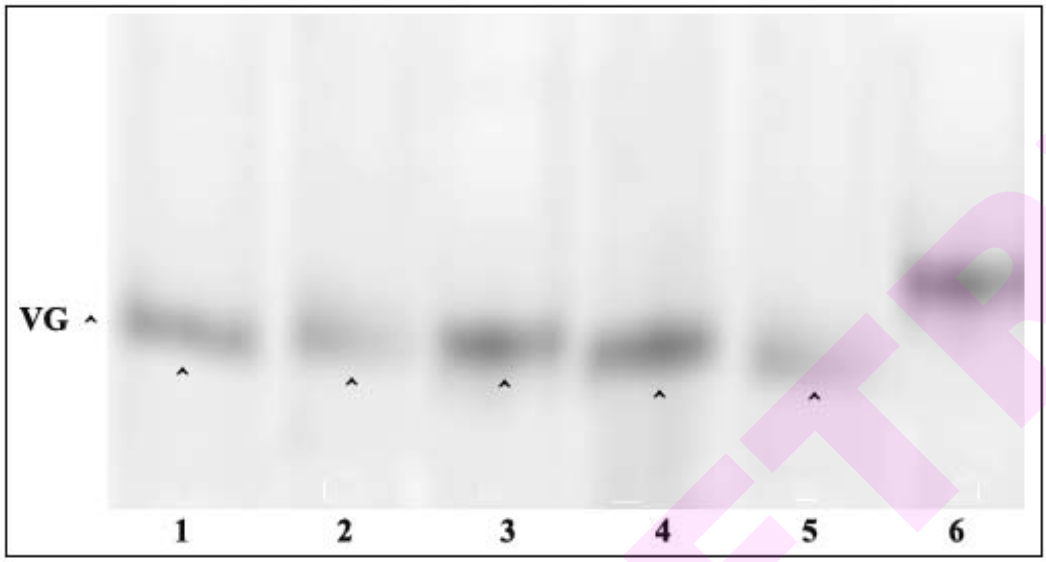


Fig.2.8. Western blot analysis showing crossreactivity of developmental stages of *T. castaneum* (VG protein band) with V1 antiserum

Lane: (1) adult female, (2) adult male, (3) pupae, (4) mature larvae, (5) immature emerged larvae, (6) purified V1 protein from egg

DISCUSSION

Vitellins are the major nutritive yolk proteins in the insect eggs. Insect eggs may contain single or multiple vitellins, which vary with species. The present study revealed the presence of two vitellins, V1 and V2. In a recently published study on *T. castaneum* presence of a single vitellin has been reported (Kim, *et al.,* 2001). This difference in the number of vitellins observed could be due to the two different approaches used in the extraction of egg protein in the present study and in the findings of Kim *et al.,* 2001. Further, the reactivity of V1 antiserum with V2 suggests that V1 and V2 share common antigenic properties (Fig.2.3). Therefore, V2 could probably be a proteolytic product formed during the utilization of vitellin that has retained the antigenic properties and this is supported by studies in *Leucophaea maderae*, and *Acheta domestica* (Dejmal and Brookes, 1972*;* Handley *et al*., 1998*).*

Presence of multiple vitellins have been reported in members of all three major cockroach families including *Periplaneta* spp., *Blatta orientalis*, *Blatella germanica*, the termite *Zootermopsis angusticollis*, the cricket *Acheta domesticus* (Storella *et al*., 1985) and *Leucophaea maderae* (Masler *et al*.,1982). Therefore, it is possible to have two vitellins in *T. castaneum* of which V2 appears to be carried forward to the larval stage cannot be ignored as seen in the present study. Furthermore, since vitellins are very large molecules, degradation of the proteins may occur relatively easily during sample preparation due to the proteolytic enzymes present in the eggs. Degradation can also occur under alkaline electrophoretic conditions and consequently one may identify a degradation product rather than the vitellin. For example, an artifactual breakdown product was identified in PAGE for the vitellogenin and vitellin of *Leucophaea.* In this case the purified proteins most often yielded two bands on the gels, only one of them

being the native molecule (Engelmann, 1976). In their study, the molecular size estimation and immunodiffusion of the eluted bands indicated that one of these bands is an artifact, which did, however, crossreact with antivitellogenin. Therefore all the above discussed reports suggest that more studies are required for *T. castaneum* vitellin/s to arrive at a conclusive statement.

In insects, vitellins are the major yolk proteins serving as nutritive reserve for the developing embryo and account for 40 to 88% of the total protein. Likewise, 88% of the yolk proteins of *Periplaneta* were reported to be vitellin (Bell, 1969), and in *Blatella* and *Leucophaea* (Oie *et al.,* 1975) it is about 93%. In the present study, V1 was found to be approximately 80% and V2 was about 20% of the total extractable protein.

With the onset of the embryonic development there is a slow decrease in the vitellin titer due to degradation initiated with a sequence of distinctive and limited proteolytic cleavages yielding polypeptides of lower molecular weight. These provide the primary food to the developing embryo. Also, about 50% of the reserved proteins are converted into non-proteinaceous components (de Chaffoy and Kondo, 1980). In insect embryos, presence of a special type of lysosome delays the vitellin degradation until bulk growth of the embryo is initiated (Fagotto, 1995). In the present study, the appearance of proteolytic products was observed beyond 72nd h. It was also observed that about 40% (with some minor contaminating proteins) of the initial level of the extractable protein remained till the end of the embryonic development and that V2 was carried over to larval stage. In support of this finding, in silkworm about 40% of the initial vitellin remained unused at the end of embryonic life and that considerable amount of vitellin was found in the first instar larvae (Irie, 1980). The presence of vitellin in fully differentiated larvae has been shown in *Drosophila melanogaster* by PAGE (Bownes and

Hames, 1977) and *Blatella germanica* by immunohistochemistry (Tanaka,1977). Electron microscope observations on the embryonic development in silkworms reveal that yolk cells are incorporated into the lumen of the midgut just after dorsal closure and somewhat degraded yolk globules are preserved therein to the hatched larvae (Miya, 1976). These results indicate that in some insects vitellin is not completely exhausted for embryonic development but is carried over to the hatched larvae until feeding takes place.

Vitellins contain appreciable amounts of carbohydrate and phospholipids and their molecular weight ranges from 210- 652 kD (Raikhel and Dhadialla, 1992; Dejmal and Brookes, 1972; Hagedorn and Judson, 1972; Englemann and Friedel, 1974). In *Tenebrio molitor*, a coleopteran insect, the molecular weight of vitellin was found to be 460kD, and its larger subunits with molecular weight ranging between 160-143kD and the smaller subunit of 56/45kD (Handley *et al*., 1998). Vitellin of *Derobrachus geminatus* (Coleoptera) has three apoproteins of about160, 140 and 50 kD (Osir and Law, 1989). The soluble proteins of the eggs of the coleopteran insect *Anthonomus grandis* Boheman, the cotton boll weevil, consist almost entirely of two vitellin types with MW of 160,000 and 47,000 (Heilmann *et al*., 2005). In the present study, the molecular weight of V1 was found to be ~440k. Both V1 and V2 stained positive for glycoprotein indicating the presence of glycoprotein moiety. However, the phosphate and lipid moiety were not detectable. SDS-PAGE analysis of V1 (Fig.2.5) indicated that the protein comprised of three major subunits, the larger subunits V1a and V1b with molecular weights of about 150 kD each and the smaller subunit V1c of ~ 50 kD. Similar findings are also reported in a recent study on vitellin of *T. castaneum* (Kim *et al*., 2001).

Vitellin is derived from its extraovarian precursor vitellogenin. It was first recognized by Telfer (1954) in the haemolymph of the female silkmoth *Hyalophora*

cecropia. It was found to be the predominant egg yolk protein (vitellin). The differences between vitellin and vitellogenin are subtle. In fact, vitellogenin and vitellin for the majority of the insect species appear to be largely identical molecules as evidenced by native molecular weights, electrophoretic mobility, amino acid composition and immunological reactivity (Hagedorn and Kunkel, 1979). In the present study, the observed crossreactivity through Western blotting of VG present in larvae, pupae, male and female insects with V1 antiserum indicates that VG band may probably be vitellogenin- the precursor of vitellin.

The crossreactivity of VG protein of male with V1 antiserum reveals the presence of small quantities of vitellogenin in adult male insects (Fig.8). In support with our findings, a study on the vitellogenin mRNA from olive fruitfly *Dacus oleae*, the translation of poly(A)+RNA isolated from male flies revealed of small quantities of vitellogenin in male haemolymph of *Dacus* (Levedakou *et al.,* 2005) It is also known that the haemolymph of the males of certain Lepidoptera, such as *Hyalophora* (Telfer, 1954) and *Bombyx* (Doira *et al.*, 1972) contain at least a low titer of vitellogenin. In *Rhodnius*, it was shown immunologically, that the male of this species has vitellogenin in high titers. For Drosophila it was demonstrated that vitellogenin is synthesized by the male and incorporated into the growing oocytes. Synthesis of vitellogenin in the male reportedly occurred only after implantation of ovaries (Kambysellis, 1977). Males of the orders Dictyoptera, Orthoptera, Hemiptera, and Coleoptera apparently will not mature eggs within implanted ovaries regardless of availability of vitellogenin. On the other hand, species of the order Lepidoptera can produce fully grown eggs within the male milieu even in the absence of vitellogenin. Further, for *Drosophila* and *Rhodniu*s the conclusion must be that the males contain the genetic information for vitellogenin (Engelmann,

1976) and hence, the same can be possible for *T. castaneum*, as observed in the present study.

CONCLUSION

The red flour beetle, *Tribolium castaneum* showed the presence of two vitellins designated as V1 and V2, which were present throughout the embryonic development. V1 was the major band and accounted to approximately 80% of the total extractable protein. Hence, V1 was chosen as the antigen to raise antibodies in the present study.

In the other developmental stages of the flour beetle, beginning from the larval stage, vitellogenin (VG), the precursor of vitellin was found. The crossreactivity of vitellogenin with the vitellin antiserum observed was in line with the earlier findings. These results help in relating the degree of relevance/significance of the assay developed in detecting all the incipient stages, and in addition the adult stages of the red flour beetle.

CHAPTER III

DEVELOPMENT AND APPLICATION OF ELISA BASED ON RABBIT ANTIBODIES (IgG)

INTRODUCTION

The major immunoglobulin in the serum is IgG which accounts for 70-75% of the total immunoglobulin pool. It is distributed evenly between the intravascular and extravascular pools and is the major class of antibody of the secondary immune response and the exclusive antitoxin. IgG consists of a single immunoglobulin molecule with a sedimentation coefficient of 7s and a molecular weight of 1,46,000D (Jeske and Capra, 1984). In 1962, Rodney Porter proposed a basic four-chain model of the immunoglobulin molecule based on two distinct types of polypeptide chains (Fig.3.1). In the IgG molecule, the smaller (light) chain has a molecular weight of 25kD, the larger (heavy) chain has a molecular weight of 55KD, and they conform to a 'Y' shaped structure. The polypeptide chains are linked together by covalent and non-covalent forces. One light chain associates with the amino terminal region of one heavy chain to form an antigen binding domain (Fab- fragment having the identical antigen binding site). The Fab is characterized by sequence of variability in both heavy and light chains, and they form the fork region of the 'Y' shaped molecule. The heterogeneity of variable region provides the structural basis for the large repertoire of binding sites used by an animal to mount an effective immune response. The rest of the molecule i.e. the stem of the 'Y' shape has a relatively constant structure. The carboxy terminal region of the two heavy chains fold together to make the Fc domain (Fc-fragment that crystallizes) which mediates the effector functions including binding of the immunoglobulin to host tissues. The hinge region is a segment of the heavy chain that provides flexibility in this area permitting the two antigen binding sites to operate independently (Silverton *et al.*, 1977).

The choice of animal for obtaining antibody is determined by four factors i.e. the amount of serum required, the source of antigen, whether monoclonal antibodies are

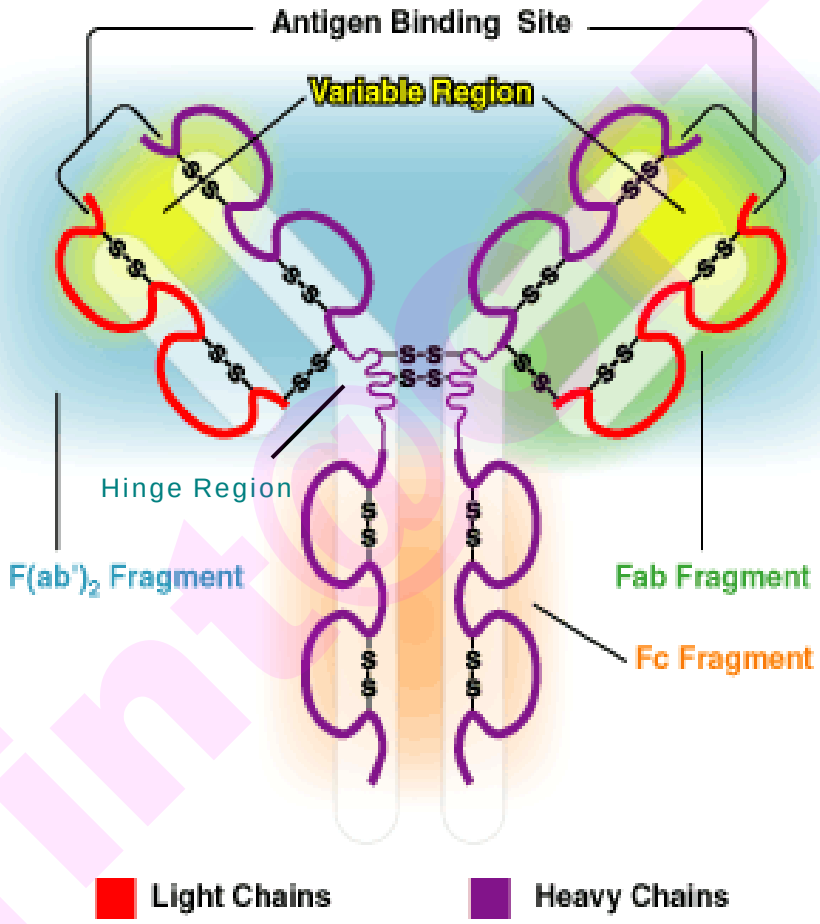


Fig. 3.1. The structure of IgG

needed, and the amount of antigen available. A wide range of vertebrate species are used for the production of antisera. The five most commonly used laboratory animals are rabbits, mice, rats, hamsters and guinea pigs. Pigs, horses, sheep and donkeys are used to produce antibodies for commercial purposes. In general, rats, mice, hamsters and guinea pigs are not used for polyclonal antibody production, because only small volumes of sera can be obtained. For practical reasons, rabbits represent an ideal choice for routine production of antisera. They are easy to maintain and handle, can be safely and repeatedly bled, the antibodies they produce are well characterized and easily purified. With careful management, at least 500mL of serum can be obtained from one rabbit through the course of an immunization regime (Harlow and Lane, 1988).

Enzyme Linked Immunosorbent Assay (ELISA) is based on immobilizing antibodies/antigen onto solid supports in a high affinity binding microtiter plates. These are then reacted with specific antigen/antibody conjugated to enzymes. The antigen-antibody reaction is visualized through enzyme reaction with specific substrate present along with a chromogen, which produces color that enables the visualization of the enzyme-substrate reaction. This system is capable of detecting low levels of contaminants present in a test sample (Lia *et al.*, 2005).

There are many variations in which ELISA or immunoassays can be performed, and are classified based on different criteria. They can be classified basically into three different classes i.e. (1) antibody capture assay format, (2) antigen capture assay format, and (3) the two-antibody sandwich assays. In an antibody capture assay, the antigen is attached to a solid support, and labeled antibody is allowed to bind. After washing, the assay is quantitated by measuring the amount of antibody retained on the solid support. This class of immunoassay is the most versatile and can be used to determine both

antigen and antibody levels and to compare antibody binding sites. In an antigen capture assay, the antibody is attached to the solid support and labeled antigen is allowed to bind. The unbound antibodies are removed by washing and the assay is quantitated by measuring the amount of antigen that is bound. This class can be used for many types of studies, but is most useful in determining the presence and quantitation of the antigen. In a two-antibody sandwich assay, the primary antibody is bound to a solid support and the antigen is allowed to bind to the primary antibody. The assay is quantitated by measuring the amount of labeled secondary antibody that can bind to the antigen. This class of immunoassay is one of the best techniques for determining the presence and quantity of antigens (Yalow and Berson, 1959).

Any immunoassay can be performed with four variations i.e. (1) in antibody excess, (2) in antigen excess, (3) as an antibody competition, or (4) as an antigen competition. Immunoassays, which use either of the first two variations, are termed non-competitive assays. Assays done in antibody excess or as antigen competitions are used to detect and quantitate antigens, while antigen excess or antibody competition assays are used to detect and quantitate antibodies (Miles and Hales, 1968b).

Based on the variation in the detection methods, immunoassays can be direct or indirect. In direct detection methods, the antibody or antigen is purified and labeled using either iodinated reagents or enzyme-labeled reagents. In indirect detection methods, a labeled secondary reagent that will bind specifically to an antibody is used. These labeled secondary reagents are easily available commercially (Van Weemen and Schuurs, 1971). In the present study, purified fraction of vitellin protein from eggs of *T. castaneum* was used as the antigen to produce specific antibodies using rabbit. The ELISA method developed in the present study was based on the non-competitive antibody capture assay

format wherein the assay was performed in antibody excess mode and used the indirect detection system.

The application of immunoassay to food samples requires consideration of several factors. When applying immunoassay to food samples one must characterize the robustness of the assay to a variety of sample conditions. Food samples can vary dramatically in their composition. Potential interferences include, sample pH, carbohydrates, lipids, pigments and protein. The extent to which any particular immunoassay is affected by interferences dictates the level of sample preparation required. In contrast to the traditional GC and HPLC methods, generally little or no sample preparation is required in an immunoassay, but this should be verified with each assay (Newsome, 1986).

As the common name suggests the red flour beetles are common pests in milled products in addition to being pest of oil seeds and their products. Most often the minute incipient stages especially the eggs are very difficult to detect and distinguish from the flour, hence these remain in the food causing further disbursal of infestation. Therefore, detection of the insect pest especially the incipient stages is a crucial factor for millers and the processed food packaging industry.

Application of ELISA to milled products poses greater challenge compared to the whole grains due to the elevated matrix interference levels attributable to the higher probability of extracting the food components in its milled form into the extraction system (Kitto *et al.,* 1994). 'Matrix interference'/ 'matrix effect' can be defined as any change in the assay performance that can be attributed to the effects of the components in the sample. In immunoassays, matrix effects are highly dependent on the nature of the food sample being tested i.e. the content of lipids, proteins, polyphenols, etc. that may be

coextracted during sample preparation (Skerritt and Rani, 1996). Good recovery and detection of the analyte from the fortified samples indicates the accuracy of the extraction method, the immunoassay and the applicability of the immunoassay. In spike recovery studies since the analyte is spiked to the food prior to extraction, it acts as a 'mimic' to real samples, the only difference being that the concentration of the analyte in the food sample is known in spiked food samples. Hence, failure to recover the added analyte may indicate the ineffectiveness of the clean up protocol when it comes to testing real food samples. Hence, spike recovery studies help in making the required changes for effective application of the assay developed to foods (Ahmed, 2001).

Application of IgG based assays in wheat for stored product insect pest detection have been described in myosin based ELISA (Kitto, 1991; Quinn *et al.* 1992; Schatzki *et al.*, 1993) and species-specific ELISA (Stuart *et al*., 1994, Germinara *et al*., 2000; Rotundo *et al*., 2000; Udaya Kumari *et al*., 2004). In addition, the application of ELISA to wheat flour and to grain and milled products including wheat, rice barley, oats and corn (Quinn *et al.*, 1992) have also been described. However, these ELISAs are applicable for almost all the life stages of the insect pest except egg. Until now there have been no reports on ELISA for detection of eggs of stored product pests and its application to grain and milled grain products. This chapter studies the development and application of rabbit IgG based ELISA for detection of incipient stages of the stored grain pest *T. castaneum* with special reference to egg, in whole wheat flour and rice flour.

MATERIALS AND METHODS

1. Chemicals: 3,3'5, 5'-Tetramethyl benzidine (TMB), urea-hydrogen peroxide, bovine serum albumin (BSA), anti-rabbit IgG, Freund's complete and incomplete adjuvant, β–cyclodextrin, leupeptin, pepstatin, phenylmethanesulfonylflouride (PMSF), mercaptoethanol, N, N, N', N'-tetramethylethylenediamine (TEMED), high molecular weight range native protein markers, 0.45μm nitrocellulose membrane were supplied by Sigma Chemical Company, USA. Protein A-Sepahrose was obtained from Pierce, U.S.A. Polystyrene 96-microwell maxisorp ELISA plates were purchased from Nunc (Roskilde, Denmark). Sodium chloride, potassium chloride, sodium phosphate, acrylamide, Bis-acrylamide, cupric chloride, Coomassie brilliant blue, Schiff's reagent were purchased locally. All the chemicals obtained were of analytical grade. Spectromax 340 microplate reader (Molecular Devices) was used to measure the absorbance in the micro titer plates.

2. Production of polyclonal antibodies: Polyclonal antibodies were raised against V1 antigen (i.e. purified major egg protein of *T. castaneum*).

2.1. Animals: Six-month-old New Zealand female rabbits with body weight of 3kg were used for the production of antibody. The rabbits were housed in large stainless steel cages in a well-aerated room having 12h light and darkness. The rabbits were maintained on commercial rabbit pellet diet and water *ad libitum.*

2.2. Immunization protocol: The immunization protocol followed was as described in Amitha rani *et al* (2004). Briefly, a primer dose of 1mg of the purified egg protein V1 mixed with equal volumes of Freund's complete adjuvant was injected into the rabbit at about 40 sites subepidermally at the nape region. We have observed in our laboratory, that multiple site injection increases the probability of the

immunogen stimulating the lymph nodes for antibody production rather than intramuscular injections. In fact, with multiple injections only a single animal is immunized to obtain the antibodies, making the experimentation more animal sparing (Unpublished results). Five successive booster injections of 0.5mg antigen each were administered into the thigh muscles at four-week interval, beginning four weeks after the primer dose. For the booster doses the antigen was mixed with equal volume of incomplete Freund's adjuvant. Bleeds were collected on the ninth day after each booster dose and allowed to clot at room temperature. The antisera thus obtained were collected, centrifuged at 2000rpm and the clear supernatant antisera was stored at -20^{o}C until further use.

3. Affinity purification of IgG: Affinity purification of IgG was performed using the protein A- sepharose affinity column.

Principle: Protein A is a cell wall protein of the bacterium *Staphylococcus aureus* with a molecular weight of 42kD. It contains little or no carbohydrate. Protein A binds specifically to the Fc portion of the antibody molecules especially those of the IgG class. The protein contains four high affinity-binding sites (approximate Ka=10^{8} M^{-1}) capable of interacting with the Fc region in the antibodies of several species. Protein A is typically immobilized through its amine groups.

The technique requires that the material to be isolated is capable of reversibly binding to a specific ligand. When a complex mixture containing the specific molecule to be purified is attached to the insolubilised ligand, generally contained in a conventional chromatography column, only the matrix under optimum experimental conditions bind to the ligand. All the other compounds can therefore be washed away and the molecule of interest can be subsequently recovered by displacement from the ligand. The binding and

displacement depend on the change in the pH.

The affinity purification using protein A column was carried out as follows:

(1) The affinity column of protein A-agarose was first rinsed with 10 volumes of binding buffer (10mM sodium hydrogen phosphate, 150mM sodium chloride and 10mM EDTA, pH7.0) at a flow rate of 1mL/min controlled using a peristaltic pump.

(2) The serum was diluted 1:1 with binding buffer pH 7.0 and loaded onto the column.

(3) The column was washed with 20X volumes of the binding buffer to remove the undesirable serum proteins such as albumins and to facilitate the binding of the IgG molecules to protein A.

(4) The bound IgG molecules were then eluted using elution buffer (0.5M acetic acid, pH 3.0).

(5) Fifteen 2mL fractions of the purified IgG were collected using a fraction collector.

(6) The protein concentration in these fractions was obtained by reading the absorbance at 280nm.

(7) The peak fractions were neutralized using 1M Tris HCl buffer pH 9.0 (1.4mL/2mL fraction) and dialyzed at 4^0C against 50mM phosphate buffered saline (PBS), pH 7.2, for 24h with three changes.

(8) The fractions were then concentrated using Amicon ultrafilteration unit using a cellulose membrane with 10kD cut-off.

(9) The column was rinsed using 20 volumes of regeneration buffer (1M acetic acid pH 2.5).

(10) The column was finally regenerated with 10 volumes of binding buffer (pH 7.2) containing 0.005% sodium azide and stored at 4^0C.

The affinity purified polyclonal antibodies (IgG) were used in the assay to check the

effect of antibody purification on the assay performance with that of the polyclonal antiserum.

4. ELISA formatting: ELISA based on non-competitive antibody capture assay (ACA) format (Fig.3.2) was used for the assay development. The assay was performed as described in Udaya Kumari *et al.* (2004). Polyclonal antiserum was used for assay development unless mentioned otherwise and the following parameters optimized:

(a) *Antigen concentration*: A linear working curve defines the portion of the curve that has the most reliable concentration dependence exhibiting a linear relationship between the signal and the analyte concentration (Pathak *et al*., 1997). The optimum linear working range for the present study was selected by testing the antigen (V1) concentration range between 0.1ng to 1000ng.

(b) *Antiserum dilution*: Antibody excess is one of the criteria in antigen detection assays. Hence, the antiserum dilutions should be appropriate in order to be able to detect traces of antigen, however excessive overload of the antibodies than required should be avoided in order to prevent the netting effect of antibodies. In the present study, the antiserum dilution range tested was between $1/1x10^1$ to $1/32x10^5$.

(c) *Blocking agent*: Choice of blocking agent is an important determining factor in assay performance as the blocking agents may crossreact with the assay components leading to a poor assay outcome. The background color is crucial, especially in antigen determining assays as negative samples can be wrongly reported as false positive samples leading to a serious error in the analysis. In the present study, four different blocking agents were tested for their blocking efficiency at zero antigen. Different concentrations of the blocking agents i.e. skimmed milk powder (SMP) at 1% and 2%, gelatin at 0.25 and 0.5%, bovine serum albumin (BSA) at 2% and 3%, and Tween 20 at 0.1 and 0.5% were

tested. The blocking agents were dissolved separately in 10mM PBS. Further, BSA solution prepared in carbonate buffer pH 9.6, is reported to have comparatively better blocking efficiency in carbonate buffer than in 10mM PBS (Nunc catalogue, 1995/96). Therefore 2% BSA prepared in carbonate buffer was also tested.

5. Standard assay: The immunoassay was standardized and performed as follows:

Step 1: Antigen i.e. the purified *T. castaneum* egg protein V1 was diluted in carbonate buffer (pH 9.6) to obtain a working range between 0.1ng / 100 µl to 15ng /100 µl. 100 µl of the buffer blank i.e. zero antigen was also included. 100µl of the antigen solution was coated onto Nunc Maxisorp ELISA plate and incubated overnight at room temperature.

Step 2: The antigen solution was tipped off the next day and the plate washed three times with 50mM PBS (pH 7.2) containing 0.05% Tween 20 (PBS-T). The plates were gently tapped dry.

Step 3: The plate was blocked with 150 µl of 2% BSA solution prepared in carbonate buffer (pH 9.6). After an incubation period of 1h, the blocking solution was tipped off and the plates gently tapped dry.

Step 4: The plate was loaded with 100 µl of the V1 antiserum solution diluted to $1/5x10^4$ in 50mM PBS containing 0.5% fish gelatin (PBS-FG) and allowed to bind to the antigen. Fish gelatin was used in the dilution buffer as a stabilizing agent for the protein molecules. After an incubation period of 1h, the antiserum solution was tipped off and washed 3X with PBS-T and gently tapped dry.

Step 5: Hundred µl of the secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Sec.Ab- HRP) diluted to 1:10,000 with PBS-FG was added to the plate. After an incubation period of 1h, the Sec.Ab- HRP solution was tipped off and washed (3X) with PBS-T to remove the excess unbound Sec.Ab- HRP and gently tapped dry.

Step 6: The bound antigen-antibody-HRP complex was visualized by the addition of 150 µl of substrate- chromogen mixture (substrate - 0.015% of H_2O_2 solution prepared in 0.1M sodium acetate buffer , containing 0.25% β-cyclodextrin, adjusted to pH 5.0 with citric acid mixed in the ratio of 97:3 with the chromogen - 3, 3', 5, 5'-tetramethyl benzidine (1%) in dimethyl sulphoxide). The reaction was stopped after 45 min with 50 µl of 2.5N sulphuric acid and plate was read at 450nm using ELISA plate reader.

Further, the ELISA performance using V2 and Vm antigen against V1 antiserum was also tested. This study was done based on the Western blotting results, which showed that V1 antiserum crossreacted with both V1 and V2 (Fig. 2.3). The findings would enable the use of Vm antigen for the standard assay, because Vm could be easily obtained through simple one step buffer extraction.

The ELISA data generated was plotted in log-log plot. The log-log plot provides the most precise estimate of true values in the unsaturated region of the curve. The region of receptor excess is completely linearized and ideally will have a slope of one. Deviations of the curve from the ideal are easy to discern and interpret (Maciel, 1985).

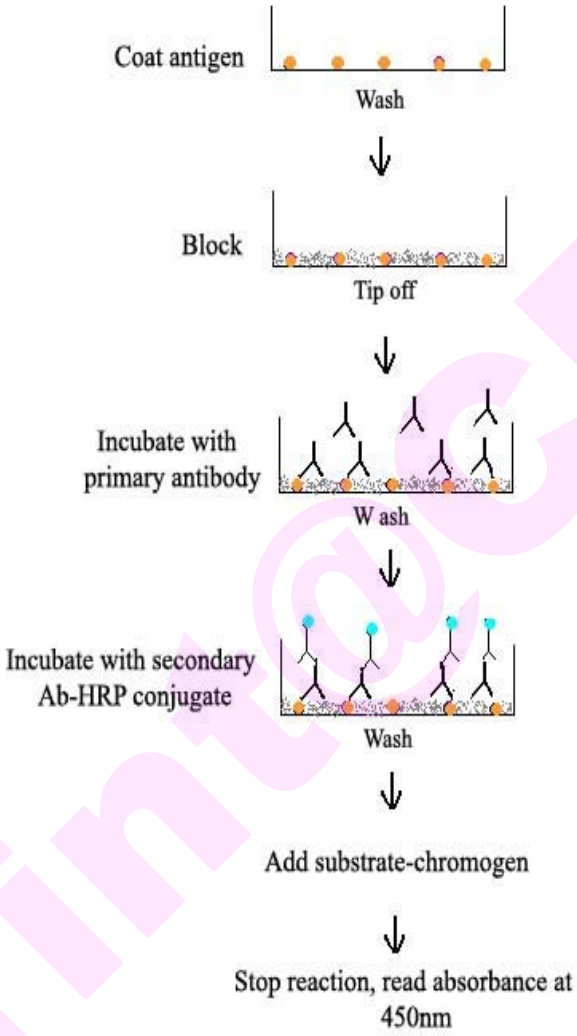


Fig. 3.2. Pictorial representation of ELISA in antibody capture assay (ACA) format

6. Detection of eggs throughout embryonic development and other incipient and adult stages of *T. castaneum*: For this study the eggs throughout embryonic development, larvae, pupae and adult male and female insects were collected and processed to obtain the antigen as described in Chapter II under the section 4 and 5, respectively. On obtaining the antigen, appropriate dilution of the antigen was coated onto ELISA plates. The antigen dilution ranged between 125 to 1.9ng. The assay was performed as described in this chapter under section 5. Comparative antigen (V1) dilutions were also coated, which was considered as the standard. The absorbance obtained for the different developmental stages was compared with the standard absorbance.

7. Crossreactivity studies with other stored product insect pest egg proteins: Vitellin is a highly evolving protein, hence vitellin antibodies of one insect exhibits low or no crossreactivity with the vitellin of other insects and thus generally species specific. In the present study, the crossreactivity of the *T. castaneum* antibodies with the egg proteins of the rice moth- *Corcyra cephalonica*, lesser grain borer- *Rhizopertha dominica* and the cigarette beetle - *Lasioderma serricorne* was studied, as these pests are commonly found with *T. castaneum* in the food.

7.1. Collection of eggs: *(a) Corcyra cephalonica* - The adult moths were released into a cylindrical cage with a standard 44 mesh at the bottom of the cage. The cage was placed on a filter paper spread over a petridish. The filter paper was checked the following day for eggs. The eggs were collected and processed to obtain the WSPF as described in Chapter II, section 5.

(b) Rhizopertha dominica: The adult insects were released into whole wheat flour sieved with 85 mesh (pore size180 microns) standard sieve. After 6h of insect release, the flour

was first sieved to remove the adult insect using 44 mesh (pore size 355 microns) standard sieve. The flour containing the eggs was subsequently sieved using 85mesh sieve to separate the eggs. The eggs were collected and processed to obtain the WSPF containing the antigen, as described in Chapter II, section 5.

(c) Lasioderma serricorne: The adult insects were released into refined starch powder containing 5% powdered yeast sieved with 85 mesh (pore size180 microns) standard sieve. The collection of eggs was carried out as explained under *Rhizopertha dominica.*

8. Application of the assay to whole-wheat flour and rice flour: Good quality whole-wheat flour and rice flour were obtained locally.

***8.1. Matrix effect studies*:** The first step in determining potential matrix effects is to examine the effect of the food extractants on the assay performance. In order to choose the most suitable extraction buffer, the food samples were extracted with three different buffers i.e. (i) 50mM PBS, pH 7.4, (ii) carbonate buffer and (iii) antigen extraction buffer (prepared as described in chapter II under section 5) and also with (iv) distilled water. 100g of the whole-wheat flour and rice flour were separately sieved in 85 mesh sieve. The flour was sieved in order to reduce the sample quantity and also because sieving does not lead to loss of eggs during sample testing as they are retained in the seive along with the residual flour particles. The residual flour particles was gently mixed 1:10 with the cold extractants, and centrifuged at 10,000 rpm at 4^0C. The supernatant was recovered and further diluted to 1:50 and 1:100 times with carbonate buffer. The antigen i.e. V1 was serially diluted in the above mentioned four different diluted extracts and coated onto the ELISA plate. This was compared with antigen dilutions prepared in extractant blank minus food extract and a standard curve of the V1 antigen prepared in carbonate buffer coated on the same plate. The extractant blanks were obtained by diluting the extractants

to 1:50 and 1:100 times with carbonate buffer as in the food extract. The standard and the carbonate buffer blank curve were common as standard dilutions of the antigen are prepared in carbonate buffer. The final dilutions were done in carbonate buffer because the antigen which is a glycoprotein binds well on to the Maxisorp ELISA plates in 50mM carbonate buffer. The assay was performed as described in chapter III under section 5.

Three different approaches were adapted for matrix clean up:

(a) Dilution: Dilution is one of the simplest and the easiest way of achieving matrix removal by either diluting or eliminating the matrix effect and is especially advantageous when the analyte is a protein which is susceptible to solvent treatment and heavy sample processing. However, during dilution the analyte concentration in the test volume should be kept well within the detection limits of the assay (Ahmed , 2001).

In the present study, 100g of whole wheat flour/rice flour was first sieved and the residual flour particles retained in the sieve was diluted 1:10 times with antigen extraction buffer, stirred well and centrifuged at 10,000 rpm. The clear supernatant was recovered and further diluted to 150, 300 and 600 times with carbonate buffer, keeping the dilutions within the detection limits of the assay. The antigen i.e. V1 was serially diluted in the above three different diluted extracts and coated onto the ELISA plate. This was compared with antigen dilutions prepared in extractant blank minus food extract and a standard curve of the antigen prepared in carbonate buffer coated on the same plate. The buffer blanks were obtained by diluting the antigen extraction buffer to 150, 300 and 600 times with carbonate buffer. The assay was performed as described in chapter III under section 5.

(b) Ultrafilteration: Ultrafilteration can be used as an effective method in filtering out matrices from that of the analyte. In extracts, especially of milled products numerous

components are coextracted during sample preparation, however, the contributing factor/s of matrix effect is difficult to discern. Ultrafilteration can be used to eliminate some of the components thereby reducing the matrix effect to a certain degree.

In the present study, 100g of whole wheat flour/rice flour was first sieved and the residual flour particles retained in the sieve was diluted 1:10 times with antigen extraction buffer, mixed gently and centrifuged at 10,000 rpm. The clear supernatant was recovered and ultrafiltered using cellulose membrane with 10kD cut-off. The retained fraction was spiked with the antigen and was used for the assay. This was compared with antigen dilutions prepared in buffer blank minus food extract and a standard curve of the antigen prepared in carbonate buffer coated on the same plate. The assay was performed as described in chapter III under section 5.

(c) 10% Chloroform treatment: Water-immiscible solvents can be used to precipitate the interfering components from that of the analyte thereby reducing/eliminating the matrix effect. This is advantageous in case of protein analyte wherein the protein remains in the aqueous phase without being effected by the solvent and in addition the extract does not require further evaporation to be made up into an immunoassay compatible solvent system. However, determining the solvent tolerance in an immunoassay is essential (Brun, 2004; Chang *et al.,* 1993). Since in the present study the analyte is a protein, the effect of chloroform on the assay performance is also studied.

In the present study, 100g of whole wheat flour/rice flour was first sieved and the residual flour particles retained in the sieve was diluted 1:10 times with antigen extraction buffer, gently mixed and centrifuged at 10,000 rpm. The supernatant was recovered and treated with 10% chloroform, mixed well and the aqueous layer was recovered. The aqueous layer was diluted further 1:150 times with carbonate buffer,

spiked with the antigen and the assay performed as described in chapter III under section 5.

(d) Solvent tolerance*:* The extractant blank was first spiked with known concentration of the antigen and then treated with 10% chloroform. The sample was mixed well and the aqueous layer was recovered. The aqueous layer was diluted further 1:150 times with carbonate buffer and the assay performed as described in chapter III under section 5. This was compared with antigen dilutions prepared in buffer blank and a standard curve of the antigen prepared in carbonate buffer coated on the same plate.

***8.2. Spike and recovery studies*:** In the present study dilution was found to effectively dilute/reduce the matrix interference. Hence, dilution was used as the strategy for matrix removal in spike recovery studies and in testing market samples. The whole-wheat flour/rice flour (100g) was spiked with eggs (1, 2, 4, 8 and 16 nos.) and free antigen equivalent of the eggs. The spiked whole wheat flour/rice flour was first sieved and the residual flour particles containing the spiked eggs retained in the sieve was crushed to break the eggs and then was gently mixed 1:10 times with cold antigen extraction buffer and centrifuged at 10,000 rpm. The clear supernatant obtained was further serially diluted to 1:600 with carbonate buffer. 100 µL of the diluted spikes were coated onto the ELISA plates. This was compared with the buffer blank spiked with antigen equivalent to 1,2,4,8 and 16 eggs. The buffer blank was prepared by diluting the antigen extraction buffer 1:600 with carbonate buffer. A comparative standard curve with the purified antigen only in carbonate buffer was also coated and the assay performed as described in chapter III under section 5.

9. Testing of market samples: Market samples were obtained locally. Both branded and unbranded samples were tested for infestation by the ELISA developed. Five samples

each of whole-wheat flour samples and rice flour samples were taken for testing.

100g of each sample was first sieved and the residual flour particles containing the spiked eggs retained in the sieve was crushed to break the eggs and then was gently mixed 1:10 times with cold antigen extraction buffer and centrifuged at 10,000 rpm. The clear supernatant was recovered and further diluted to 600 times with carbonate buffer. 100µL of the sample extract was coated on to ELISA plates. This was compared with antigen dilutions prepared in extractant blank minus food extract and a standard curve of the antigen prepared in carbonate buffer coated on the same plate. The buffer blank was prepared by diluting the antigen extraction buffer 1:600 with carbonate buffer. The assay performed as described in chapter III under section 5.

The ELISA results were validated by incubation/ breeding out method as any infestation that may be present can be visualized after the eggs develop into visible stages. For this, a part of the market samples were placed in clean glass jars covered at the mouth region with a clean white cloth in an incubator at 30^0C for 45 days. The flour was then sieved using a 44-mesh sieve. The sieve was checked for adult *T. castaneum* insects and the results compared with the ELISA findings. However, it may be noted that, in general, the results of the two methods need not be mutually complementary as the probability of finding infestation in each sample/sub-sample varies.

10. Statistical analysis: Data are expressed as mean $\pm$ standard error (SE) of three separate experiments. The minimum detection limit of the assay was defined as the lowest detectable analyte concentration that gives a response, which has a statistically significant difference ($p< 0.005$) from the response of the zero analyte concentration. Statistical analyses were done using Microsoft Excel and Origin software for obtaining the data plot, standard error and significance.

RESULTS

1. Optimization of ELISA:

1.1. Antigen concentration **-** The ELISA based on antibody capture assay format indicated that the workable linear antigen concentration for the antiserum and the affinity purified IgG was between 15.6 to 0.1ng (Fig.3.3). These optimized antigen concentrations produced a linear working curve with an 'r^2' value of 0.9.

1.2. Antisera dilution: For the present study, the workable serum dilution was fixed at $5x10^3$, which showed a minimum detection limit of 0.1ng (Fig3.4). At antiserum dilution lower than $5x10^3$ the assay sensitivity did not improve. The antiserum dilution for the assay was selected based on the assay performance that gave a comparable assay performance with that of the affinity purified antibody coated at 1μg/well. As seen from the Fig. 3.4, all the different antiserum dilutions tested ($5x10^3$ to $5x10^4$) showed the same sensitivity i.e. 0.1ng. However, the absorbance of the antiserum dilutions between $1x10^4$ and $4x10^4$ showed a gradual decrease in absorbance with dilution at higher antigen concentrations (1.9ng and higher) when compared to that of the purified antibody. This may be probably because of lower amount of available antibodies with increased dilution, resulting in lower absorbance. Hence, the workable antiserum dilution for the assay development was fixed at $5x10^3$ until mentioned otherwise.

1.3. Blocking agent: The effective blocking agent was chosen based on the minimal background color obtained at zero antigen concentration at different serum dilutions and minimum or no interference on the standard antigen curve**.** As seen in Fig.3.5, BSA at 2% was found to be the most suitable blocking agent with minimal background color at zero antigen in the present study. Even at lower serum dilutions i.e. antibody excess the background color was minimal indicating that 2 % BSA showed least non-specific

binding. BSA at 1 and 3% were not comparatively effective. Gelatin was the second best at higher dilutions of the serum, however, at lower antisera dilutions ($2x10^3$ and lower) it gave a high background color. SMP at 2% also exhibited a similar trend as that of gelatin, however, the minimum absorbance was more than that of gelatin at higher serum dilutions ($2x10^3$ to $4x10^4$). SMP at 1% though exhibited a trend of decreasing minimal background with serum dilution, but the absorbance was comparatively higher (0.17-0.32) and less effective as compared to 2% SMP. Tween 20 at the concentrations 0.1 and 0.5% resulted in the maximum background color (0.3 and higher) compared to all the blocking agents tested. Therefore, due to the high background color, Tween 20 and SMP were not effective blocking agents in the present study.

Thus, the IgG based assay developed based on the major egg protein vitellin of the red flour beetle worked best at an antigen concentration range of 15.6 to 0.1ng, with 2% BSA as the blocking agent and serum dilution of $5x10^3$. The standard assay developed showed a minimum detection limit of 0.1ng for both unpurified antiserum and the affinity purified IgG (Fig. 3.4). Thus, the unpurified antiserum at $5x10^3$ was used for all the further studies. Further, expressing the minimum detection limit of the assay in terms of parts of an egg, the assay can detect 1:73,000 part of an egg, which is equivalent to 0.1ng of the total extractable protein. This demonstrates that the assay developed is highly sensitive. The assay's linear response easily spanned a 150-fold range of the total protein concentrations (0.1–15ng), and demonstrated the possible potential for developing a quantitative assay for detection of incipient stages of *T. castaneum*.

Further, the assay performance of V2 and Vm antigens against V1 antiserum showed that there was no change in the assay performance as compared to that of V1 antigen curve (Fig 3.3.a). This comfirms that V1 antiserum crossreacts with V2 as

observed in Fig.2.3. (Chapter 2). Further, as the assay performance of Vm antigen was comparable with that of V1 antigen curve, Vm could be used as the antigen for the standard assay, and also Vm could be easily obtained with a simple single step extraction.

2. Sensitivity of antibody and antibody titer through bleeds I to V: The bleeds/antibodies obtained in the present study showed good sensitivity. As seen from Fig.3.6 , at $5x10^3$ serum dilution, the sensitivity of the antibodies, expressed as minimum detection limit, was 0.1ng for bleed II and I. The minimum detection limit of bleeds III, IV and V were 0.5, 5 and 10ng respectively. Although the sensitivity of the III, IV and V bleeds was almost 5-100 times lesser than that of I and II bleeds, it could still detect 73 - 15,000 parts of an egg, indicating that the polyclonal antibodies obtained were highly sensitive in all the bleeds tested.

Further, the results also demonstrated that the antibody titer obtained was good at all the bleeds tested (Fig.3.7). The minimum detection limit of the bleeds at $5x10^4$ was 0.1, 0.1, 0.5, 5 and 10ng for I, II, III, IV and V bleed, respectively. And at the highest serum dilution tested i.e. $320x10^4$ the minimum detection limit was 10, 10, 50, 50 and 100ng for I, II, III, IV and V bleed, respectively. These results indicate a good antibody titer and high sensitivity of the antibodies obtained.

3. Detection of eggs through embryonic development and other incipient and adult stages of *T. castaneum*: In the Western blotting (see Chapter II, Fig.2.3) the V1 antiserum reacted with the V1 protein band present at all the embryonic stages tested. This suggests that the antigen V1 present is similar throughout the embryonic development and that the ELISA developed is applicable for detecting eggs at any given stage of embryonic development. To further the findings, on testing the antigen (Vm) obtained from different embryonic stages against V1-antiserum by ELISA, the assay

performance was comparable with that of the antigen (V1) obtained from the egg at early embryonic development (0-6h) (Fig.3.8). There was no detectable change in the absorbance at any of the embryonic stage tested. This demonstrates that the immunoreactivity of the antigen remained unaltered through embryonic development and the assay developed could also be used to detect all the stages of the developing egg.

The crossreactivity studies of V1 antiserum with antigen-VG obtained from immature larvae, mature larvae, pupae and the adult males and females indicated that the assay could detect these stages also (Fig.3.9). The minimum detection limit for immature larvae was similar to that of the eggs i.e. 0.1ng, however, the signal intensity at higher antigen concentrations were lower as compared to that of the egg. The minimum detection limit of the other stages i.e. mature larvae, pupae, adult male and female insects was higher and was equivalent to 7.8, 7.8, 15.6 and 3.9ng respectively. However, this sensitivity was equivalent to one number of each stage. In line with the above results, in Western blotting (See Chapter II, Fig.2.8) the V1 antiserum crossreacted with VG of the developmental stages. This crossreactivity of the V1 antiserum with VG of the developmental stages indicates that the assay could detect all the developmental stages of *T. castaneum*, but with varied sensitivities.

4. Crossreactivity studies of the V1 antiserum with other insect pest egg proteins: V1-antisera exhibited low/negligible cross reactivity with the egg proteins of the other stored product insect pest/moth tested (Fig. 3.10). *Rhizopertha dominica* and *Corcyra cephalonica* exhibited low cross reactivity with V1-antiserum. Expressing the same in terms of percentage, the crossreactivity of V1-antiserum with that of *Rhizopertha dominica* and *Corcyra cephalonica* at the highest antigen concentration tested i.e. 500ng was 9.3 and 5.4% respectively. There was no observed crossreactivity at lower antigen

concentrations. *Lasioderma serricorne* egg protein did not show any crossreactivity with V1-antiserum even at 500ng of the antigen.

5. Application of the assay to food commodities:

(a) Matrix effect studies: Matrix effect studies revealed that the whole-wheat flour and rice flour extracts exhibited matrix effect/interference.

Evaluating effect of the extractants: The extractants alone spiked with antigen did not have any effect on the assay performance and was comparable with that of the standard curve (Fig.3.11). The poor assay performance when the food was extracted using different extractants was thus attributed to the matrix effect due to the food components and not due to the extractant. The matrix effect led to poor assay signals for both rice flour and wheat flour and among the three extractants used, the carbonate buffer extracted food extract gave slightly higher color when compared to PBS, antigen extraction buffer and distilled water extracted food. Based on the above results and also keeping in mind the final extraction of the antigen from the food sample, antigen extraction buffer was the obvious choice as the extraction buffer for further studies. Antigen extraction buffer as described in chapter II is a high salt buffer used for the extraction of the major egg protein. Only the carbonate buffer and the antigen extraction buffer curves are shown as they were finally used for the application of the assay to food commodities, the PBS and the distilled water curves are not shown for clarity.

Of the three different methods tested for the removal of the matrix interference, dilution was found to be effective compared to chloroform treatment and ultrafilteration. The efficiency of each of the method tested in matrix removal is discussed below.

(a) Ultrafilteration: As observed in Fig.3.12 wheat flour extract and rice flour extract affected the assay performance due to the presence of matrix interference. In both the

cases the curve shifted to the right as compared to the standard curve due to lowered absorbance. Rice flour extract showed comparatively lower color than that of whole-wheat flour extract. With ultrafilteration there was an observed betterment in matrix clean up, but it did not completely remove the matrix interference. Whole-wheat flour showed higher absorbance at higher antigen concentrations and with decrease in antigen concentration there was a shift in the curve to the right, as compared to that of the standard antigen curve and the extractant alone curve. This shift indicates the persistence of matrix interference. In the case of rice flour, there was betterment in the assay performance, but the absorbance was still lower than that of the standard curve indicating matrix effect. Therefore, ultrafilteration did not help much in matrix removal of both the foods.

(b) 10% Chloroform treatment: 10% chloroform treatment was comparatively more effective in reducing matrix interference in rice flour extract than for whole wheat flour extract (Fig.3.13). In case of rice flour, the matrix clean up was better at higher antigen concentration but the matrix interference affected the assay performance at lower antigen concentrations, hence, lower color compared to that of the standard curve was observed. The chloroform treatment was not very effective in matrix removal for wheat flour extract. Combination of this method with dilution would have probably helped in further matrix effect removal, but the solvent tolerance test showed the antigen was affected during chloroform extraction hence resulting in poor assay performance observed as lower absorbance as compared with that of the standard curve (Fig.3.14). Hence chloroform method was not found to be satisfactory in removal of matrix interference from both the foods.

(c) Dilution: Dilution was found to be the most effective method in diluting /eliminating

the matrix interference. As seen in Fig.3.15 the matrix effect did not dilute out until the food extract was diluted 1:600. However, there was a gradual improvement in the assay signal intensity with dilution for both rice and whole-wheat flour extracts. At a dilution of 1:600 the assay performance was comparable with that of the standard curve for both the food extracts. In this method, the high sensitivity of the assay allowed this dilution and the antigen concentration at the highest dilution tested was within the minimum detection limit of the assay i.e. 0.1ng.

6. Spike and recovery studies:

(a) Whole wheat flour: The percent spike recoveries ranged from 90-100% for the egg spikes 1,2,4 and 8, while at 16 eggs spike the recovery was only 43%. The results were comparable with the free antigen equivalent spikes (Fig.3.16). It was observed that at higher antigen concentrations (16 eggs and more) the recoveries were <50%. However, with increased dilution, this effect could be negated and good recovery of 85% could be achieved. Hence, in real unknown sample analysis such samples with high antigen concentrations could be diluted further and tested.

(b) Rice flour: The percent spike recoveries ranged from 79-100% for the egg spikes 1, 2, 4 and 8, while at 16 eggs spike the recovery was only 56%, and the results were comparable with the free antigen equivalent spikes (Fig.3.17). It was observed that with increased dilution (1:1200) at 16 eggs spike the recovery improved to 78%.

3. Testing of market samples: Of the five different samples each of wheat flour and rice flour tested by the ELISA developed one sample of wheat flour and two samples of rice flour tested positive for *T. castaneum* infestation (Fig.3.18). The rate of infestation from the standard graph was equivalent to one egg for wheat flour sample #3, and for rice flour it was equivalent to two eggs and one egg in sample #2 and #3, respectively. These

results were compared with that of the breeding out method after incubation of the samples for 45 days. The wheat flour sample #2 and rice flour samples #2 and #3 showed infestation. The other samples did not indicate any infestation until the observed time period.

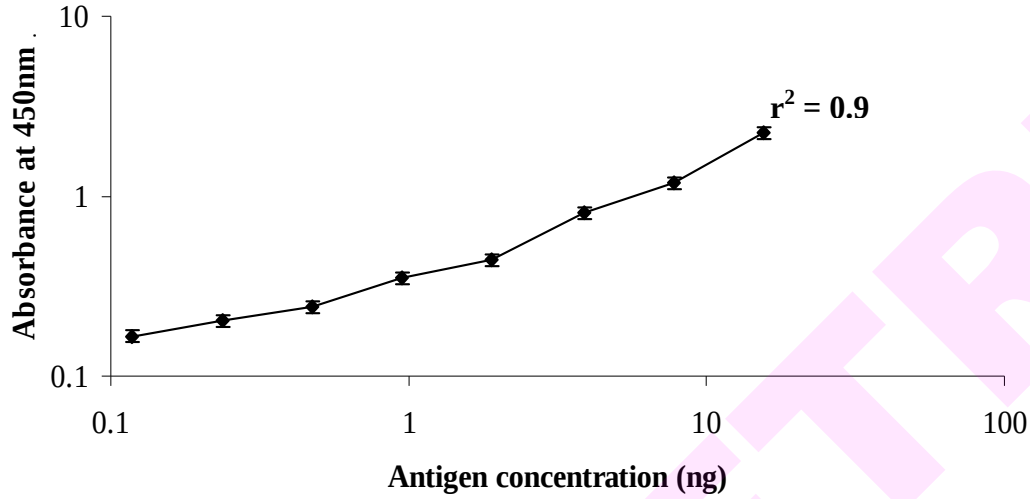


Fig. 3.3. IgG based standard linear working curve

(Data is expressed as mean ± SE of three separate experiments)

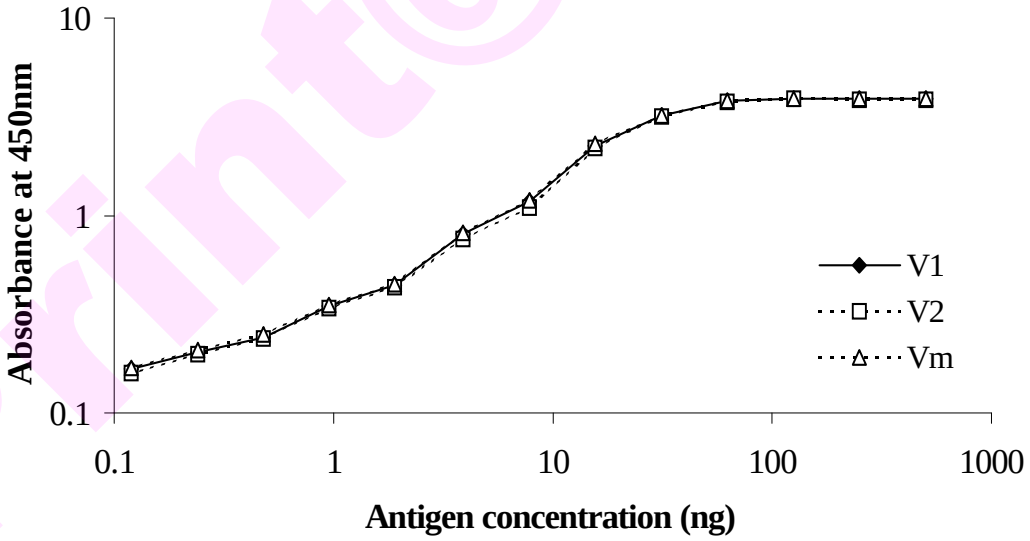


Fig. 3.3a. Standard assay performance using V2 and Vm as the antigen against V1-antiserum

((Data is expressed as mean ± SE of three separate experiments; error bars are not integrated for clarity)

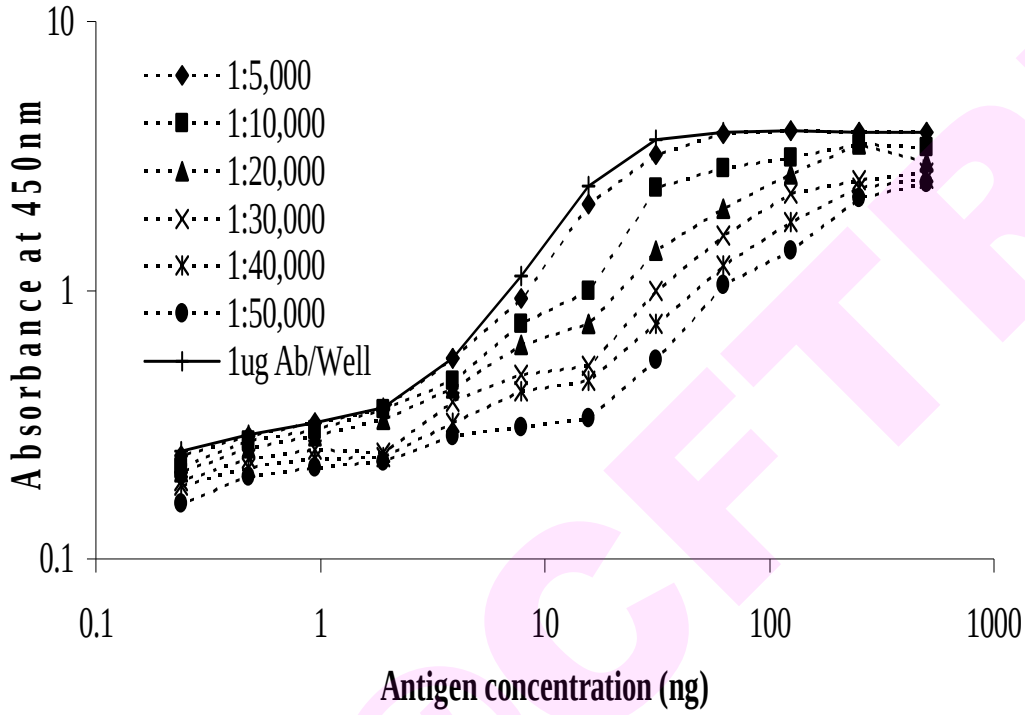


Fig. 3.4. Effect of serum dilution on the assay performance

(Data is expressed as mean ± SE of three separate experiments; error bars are not integrated for clarity)

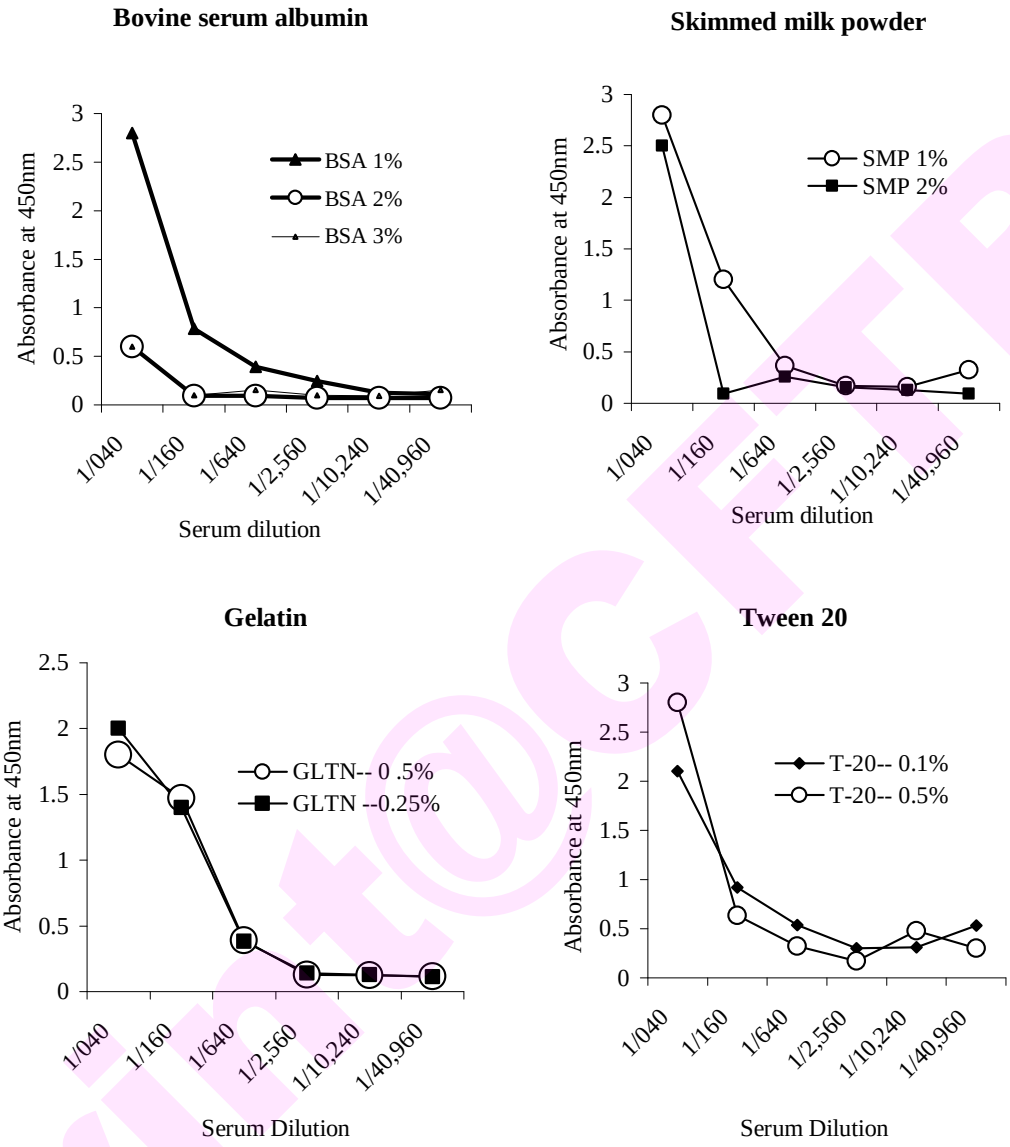


Fig. 3.5. Effect of different blocking agents on the assay at zero antigen

(Data is expressed as mean ± SE of three separate experiments; error bars are not integrated for clarity)

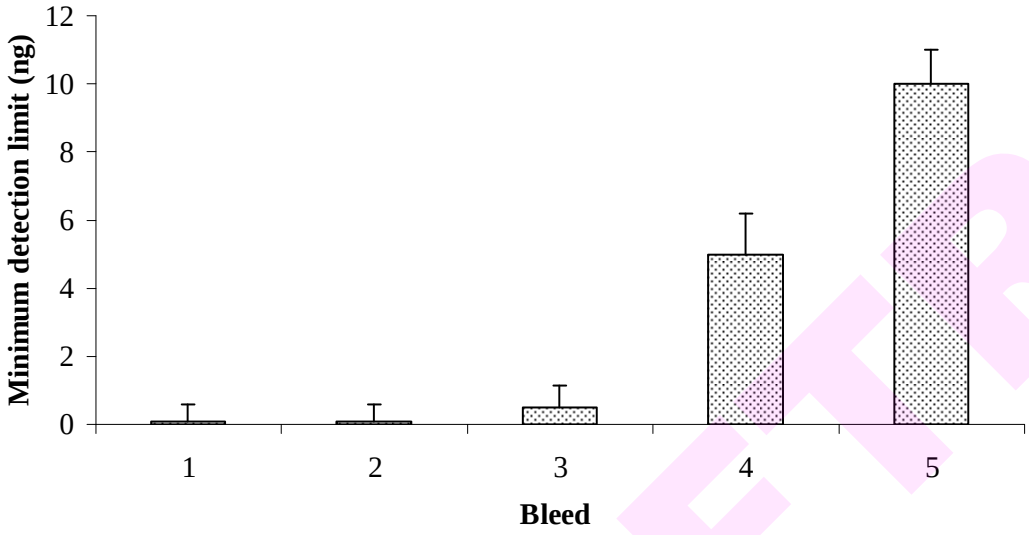


Fig. 3.6. Antibody sensitivity of different bleeds

(Data is expressed as mean ± SE of three separate experiments)

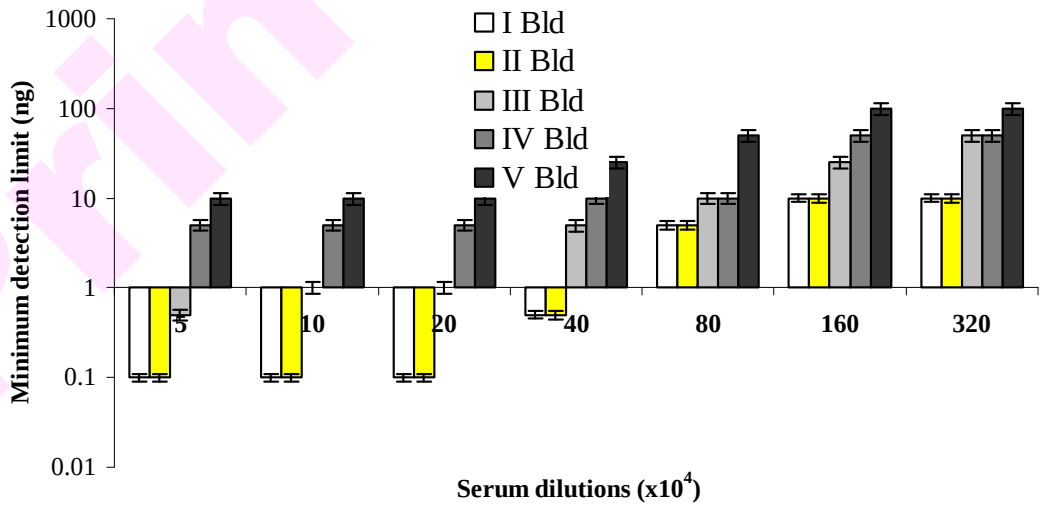


Fig. 3.7. Antibody titer of different bleeds

(Data is expressed as mean ± SE of three separate experiments)

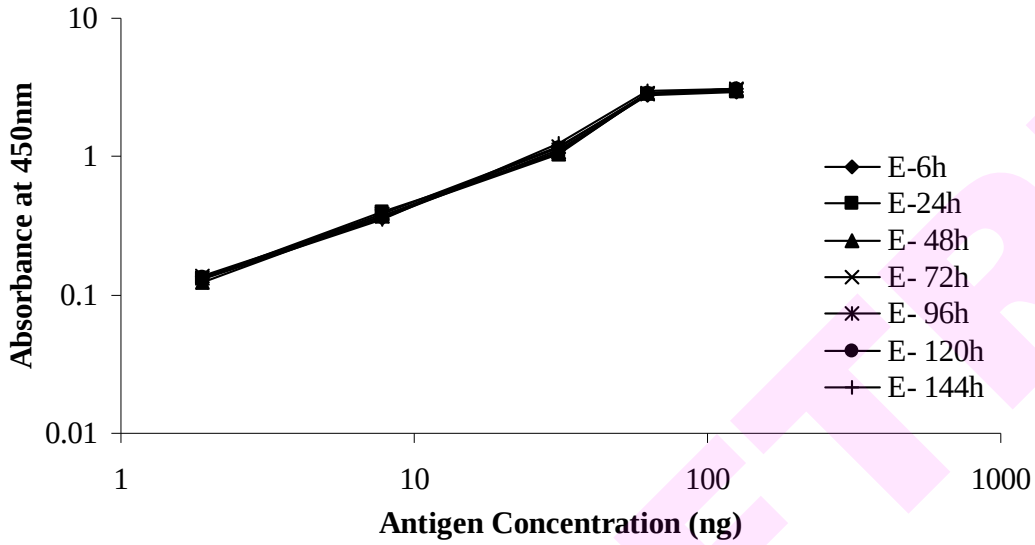


Fig. 3.8. IgG based ELISA showing reactivity of embryonic stages of *T. castaneum* with V1 antiserum

(Data is expressed as mean ± SE of three separate experiments; for clarity the error bars are not integrated)

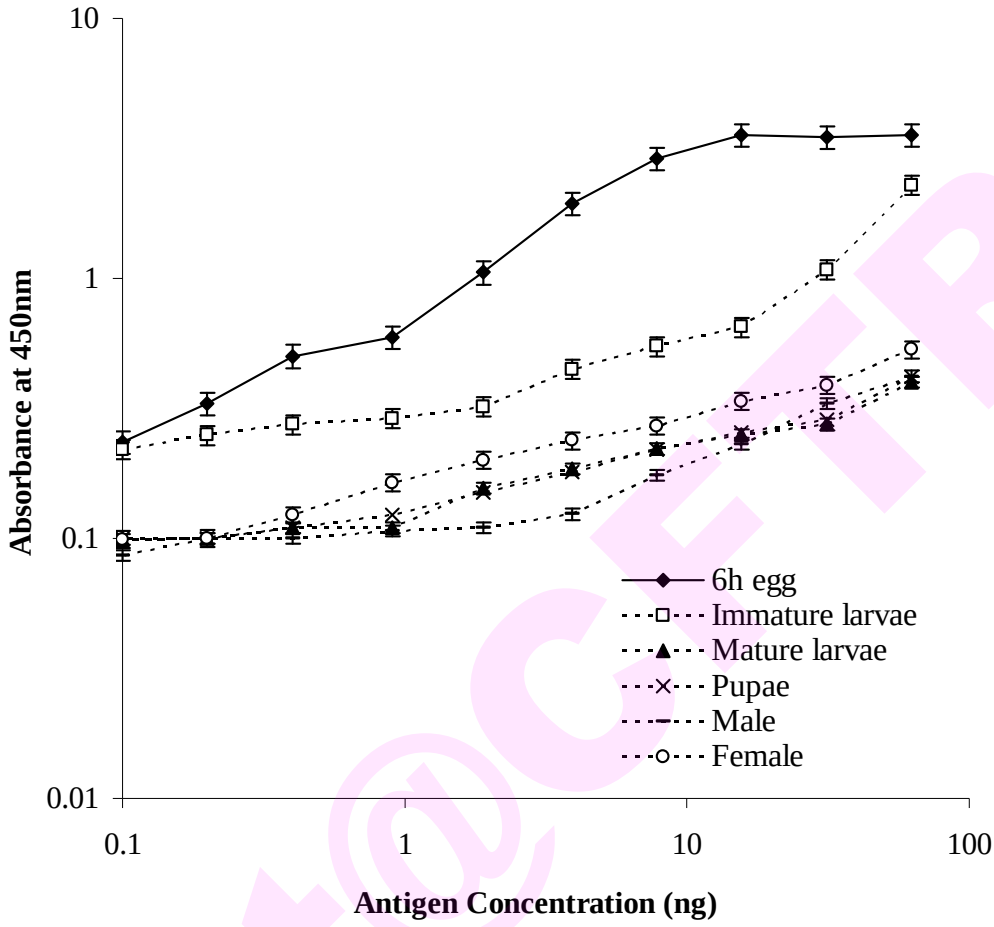


Fig. 3.9. IgG based ELISA showing crossreactivity of developmental stages of *T. castaneum* with V1 antiserum

(Data is expressed as mean ± SE of three separate experiments)

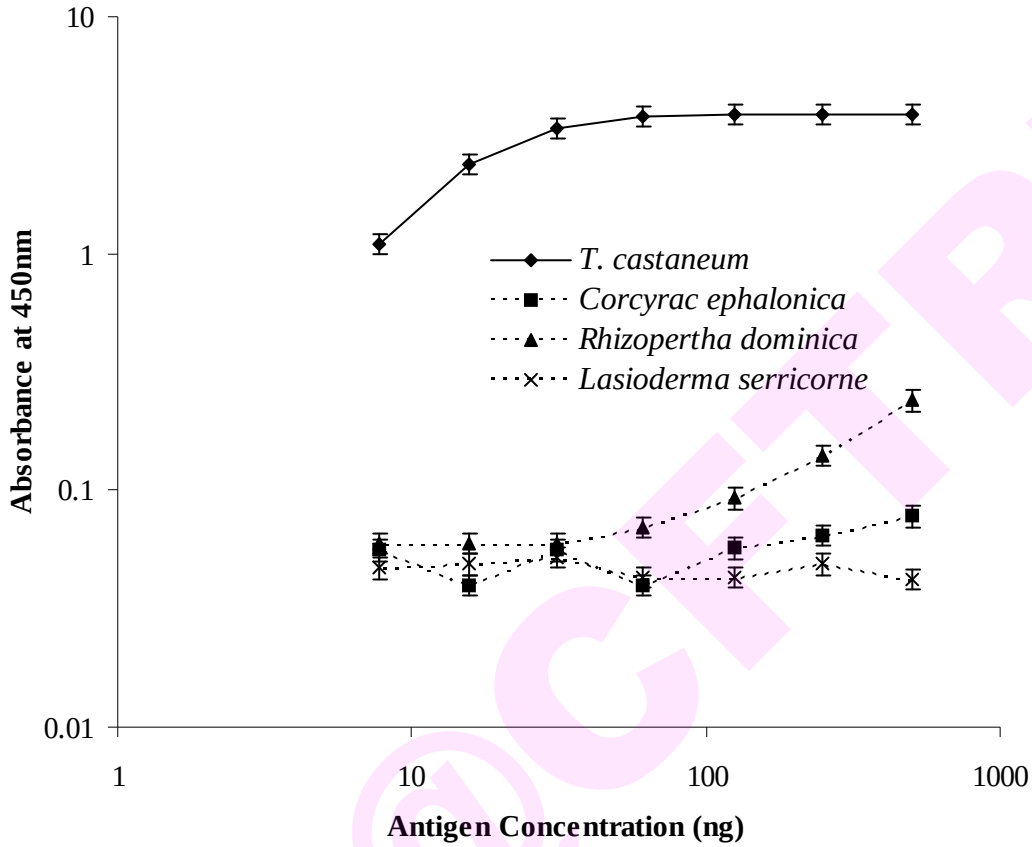


Fig. 3.10. IgG based ELISA showing crossreactivity of the other stored product insect egg protein against *T. castaneum* egg V1 antiserum

(Data is expressed as mean ± SE of three separate experiments)

(The antigen concentrations tested were at a higher range to determine even the least crossreactivity if present)

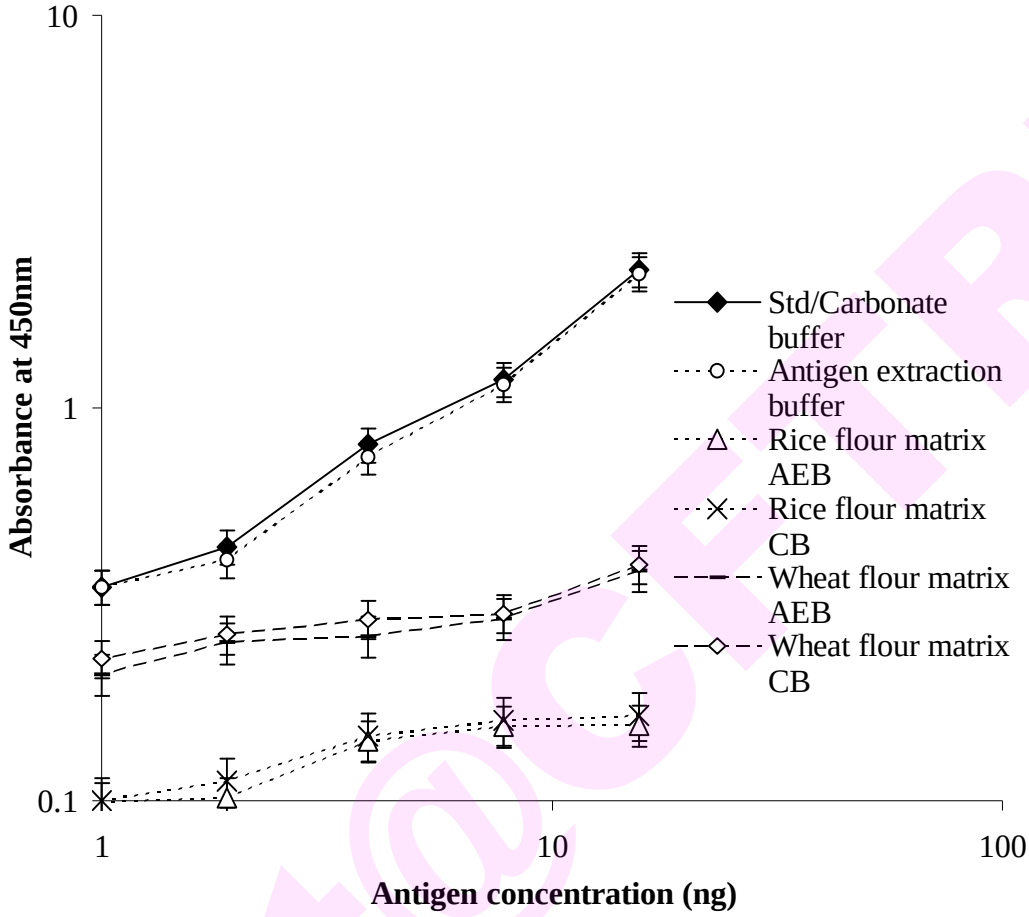


Fig. 3.11. Effect of extraction buffers on the assay performance

(Data are expressed as mean ± SE of three separate experiments; PBS and distilled water curves are not shown for clarity)

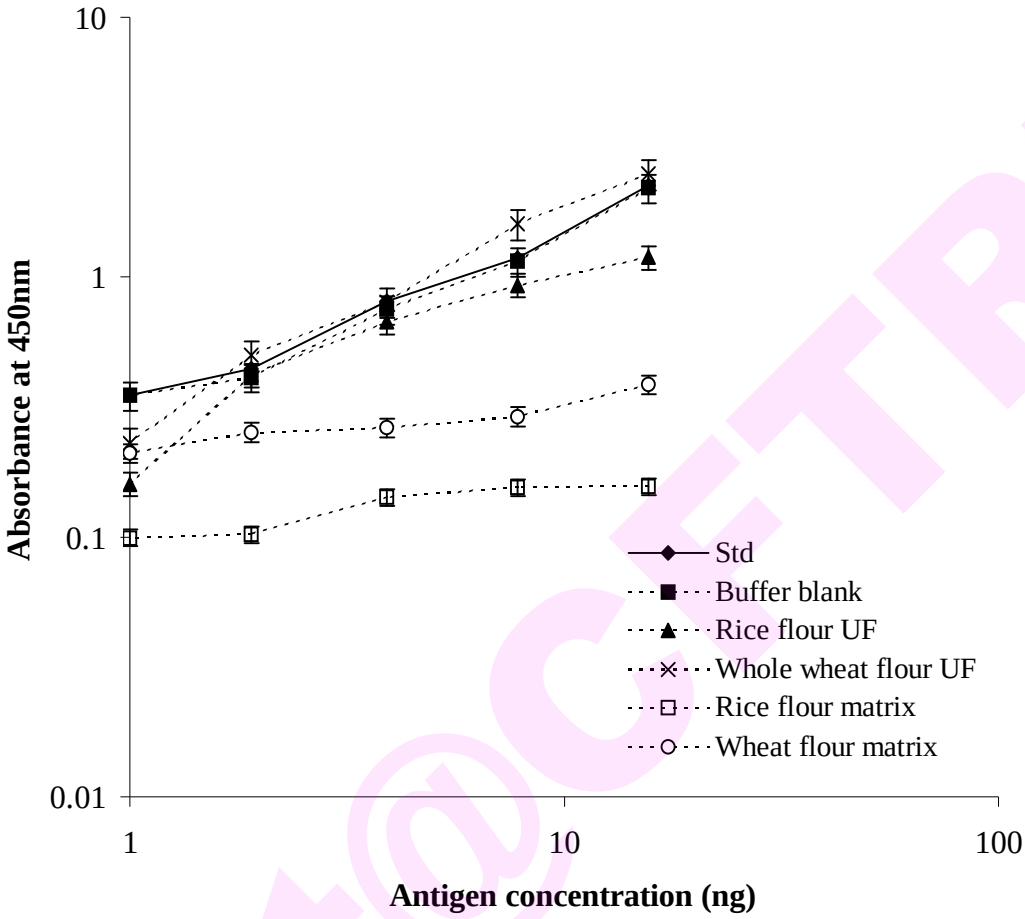


Fig. 3.12. Effect of ultrafilteration (UF) on matrix interference

(Data are expressed as mean ± SE of three separate experiments)

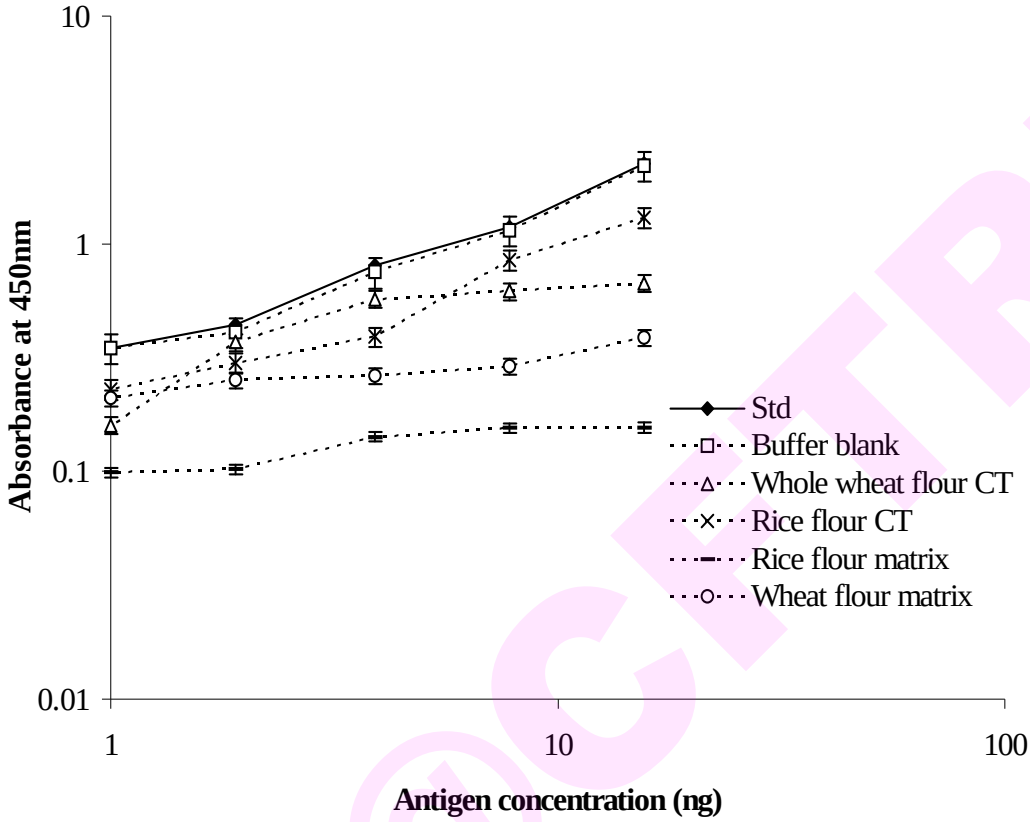


Fig. 3.13. Effect of 10% chloroform treatment (CT) on matrix interference

(Data are expressed as mean ± SE of three separate experiments)

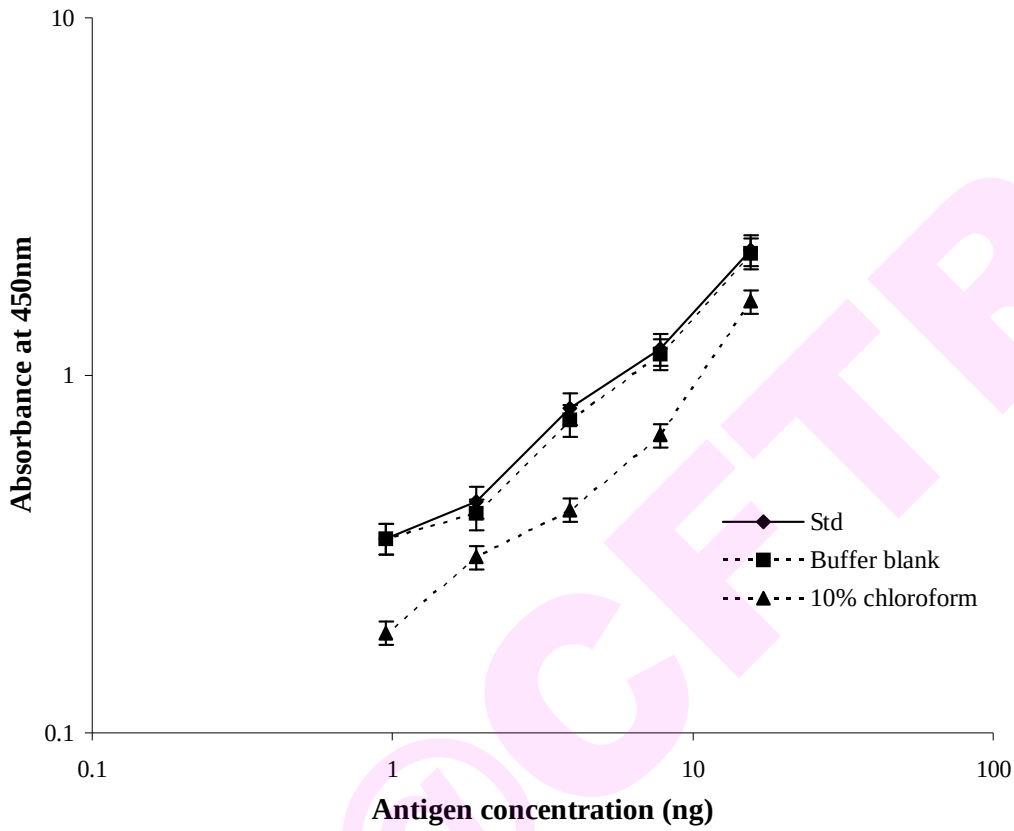


Fig. 3.14. Solvent tolerance of the assay to 10% chloroform

(Data are expressed as mean ± SE of three separate experiments)

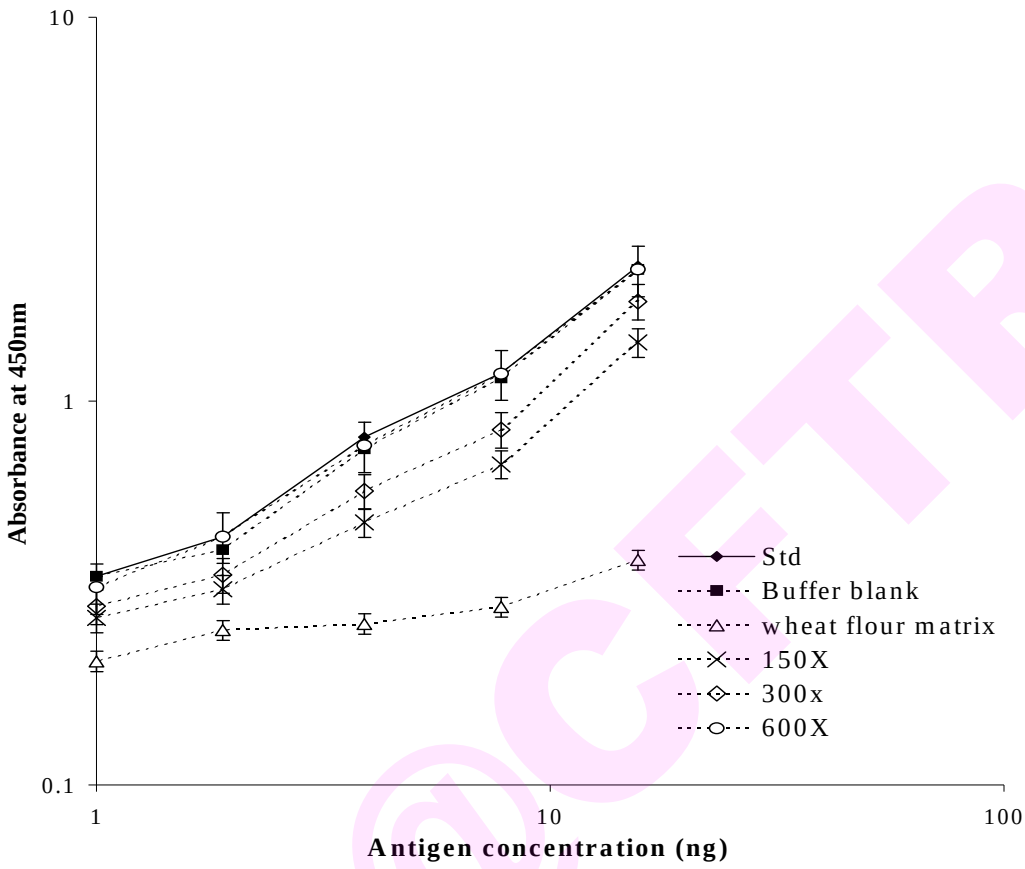


Fig. 3.15. Effect of dilution on matrix interference of whole-wheat flour extract

(Data are expressed as mean ± SE of three separate experiments)

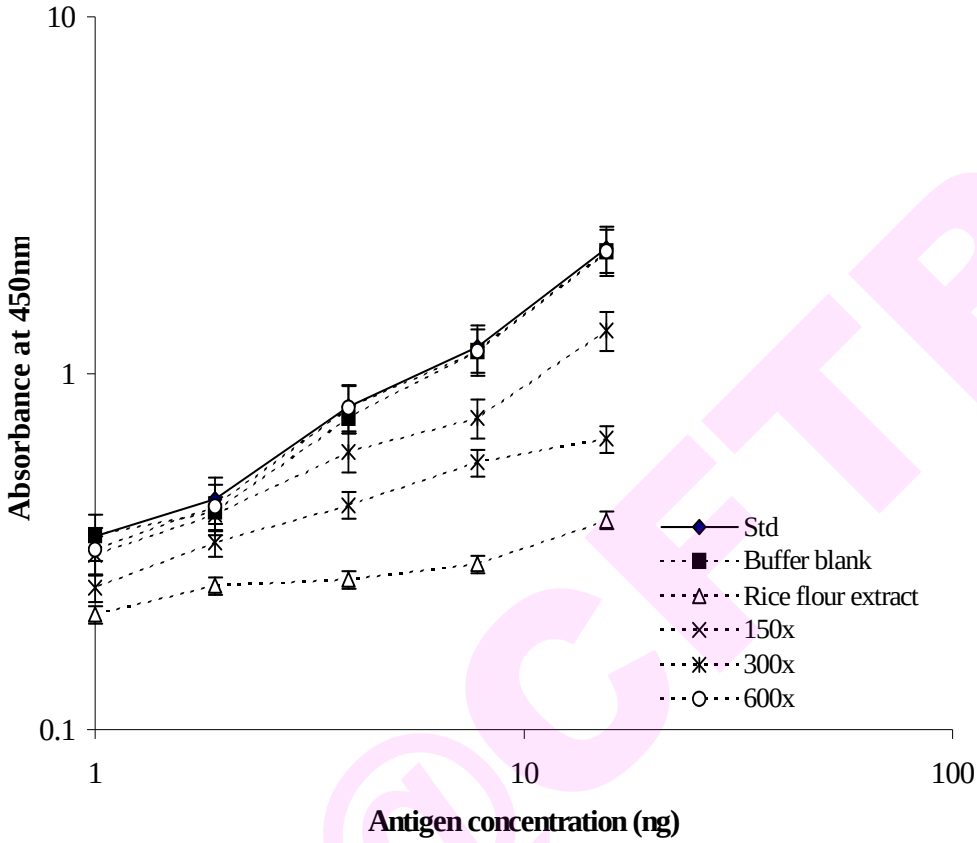


Fig. 3.16. Effect of dilution on matrix interference of rice flour extract

(Data are expressed as mean ± SE of three separate experiments)

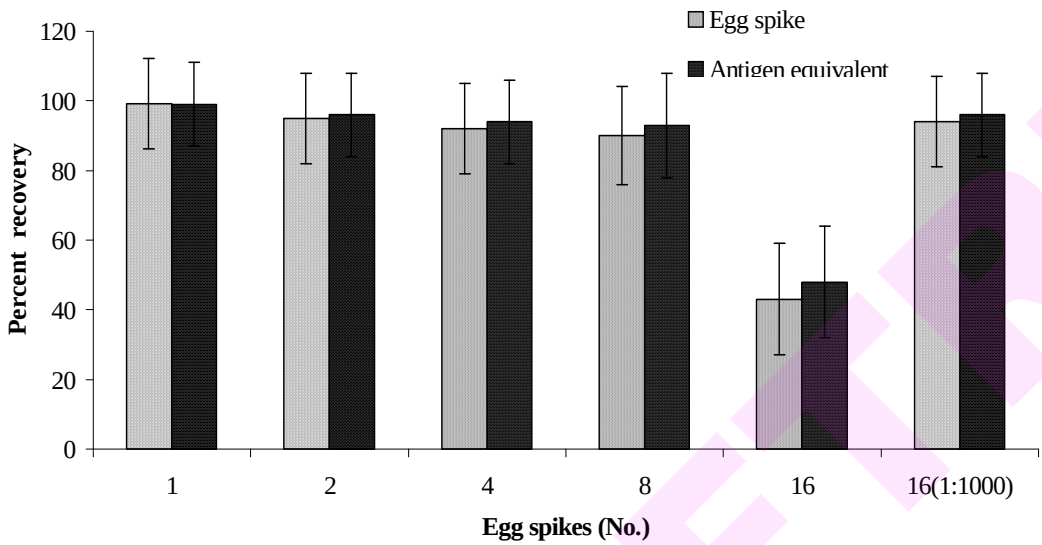


Fig. 3.17. Spike and recoveries in whole wheat flour

(Data are expressed as mean ± SE of three separate experiments)

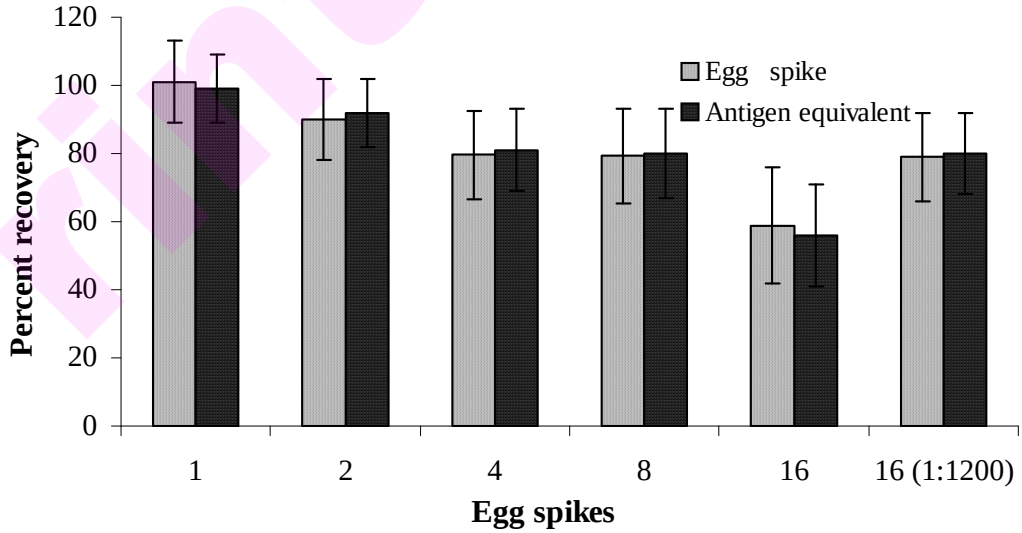


Fig. 3.18. Spike and recoveries in rice flour

(Data are expressed as mean ± SE of three separate experiments)

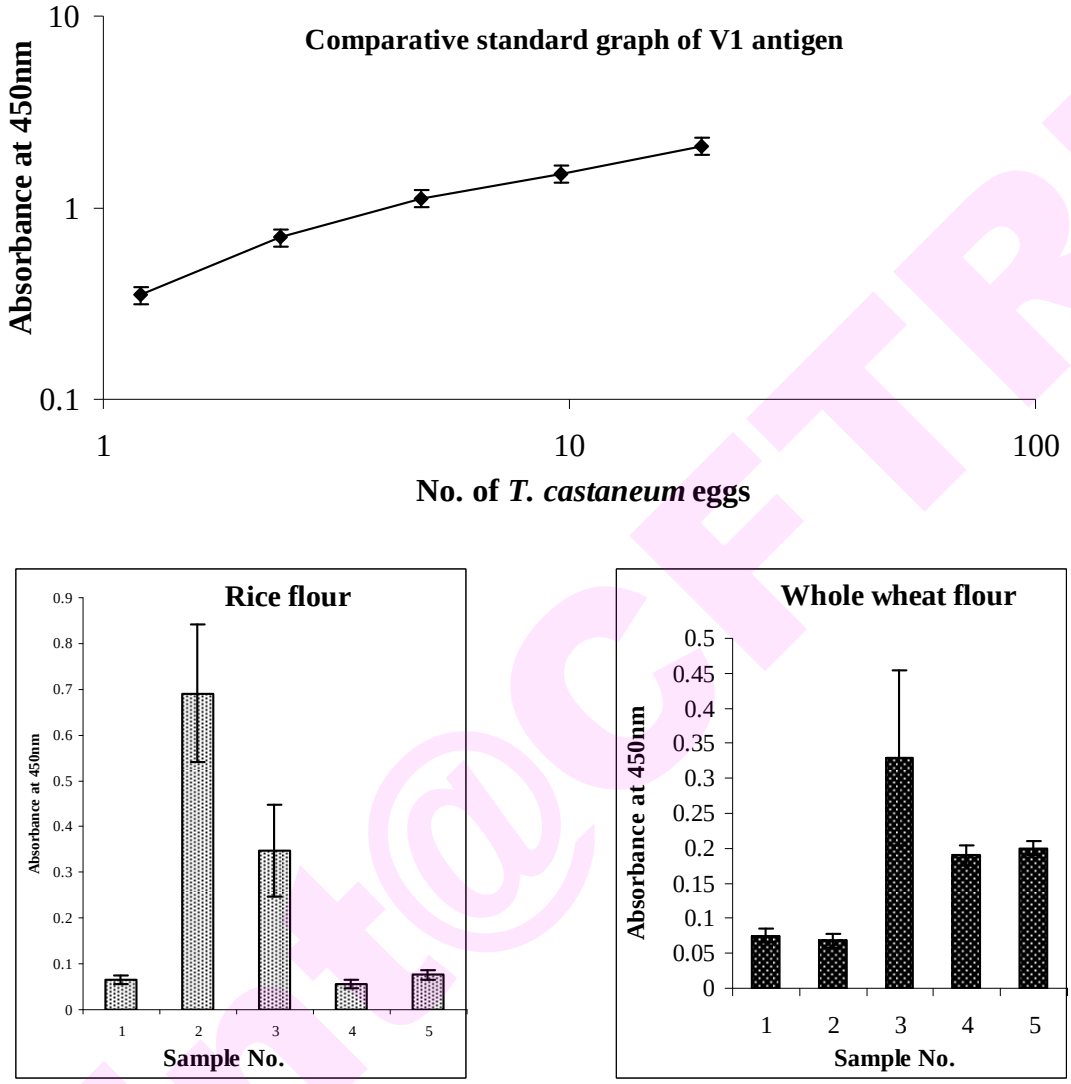


Fig. 3.19. Testing of market samples of whole-wheat flour and rice flour

(Data are expressed as mean ± SE of three separate experiments)

DISCUSSION

The immune system is challenged constantly by an enormous number of antigens. 'Antibodies' are the result of the natural response of a living organism's defense mechanism to fight against the invasion of a foreign molecule termed 'antigen'. One of the key features of the immune system is that it can synthesize a vast repertoire of antibodies and cell-surface receptors, each with different antigen binding site. The binding of the antibodies and T-cell receptors to foreign molecules provides the molecular basis for the specificity of the immune response (Roitt *et al.,* 1993). Immunoassays are based on this specific reaction between the antigen and the antibody.

Immunogenicity, the ability of a molecule to induce an immune response, is determined both by the intrinsic chemical structure of the injected molecule and by whether or not the host animal can recognize the compound. Proteins, peptides, carbohydrates, nucleic acids and many other naturally occurring or synthetic compounds can act as successful immunogens. The minimum size limit on an immunogen is not less than 3000-5000Da (Harlow and Lane, 1988).

In the present study the antigen is a phospholipoglycoprotein (major egg protein-vitellin) with a molecular weight of approximately 440kDa, thus making it an ideal choice as an antigen. The minimum detection limit of the assay was 0.1ng at an antibody titer of 1:200,000. A good antibody titer was obtained starting from the first bleed. The sensitivity was maintained till the second bleed obtained after the second booster dose. Although there was a decline in the sensitivity and the titer of the antibody with subsequent boosters, the minimum detection limit was still good at 0.5, 5 and 10ng for II, IV and V bleed equivalent to 1;14,000, 1:1400, and 1:730 parts of the extractable egg protein, respectively.

ELISA based on myosin for the detection of stored product insect infestation has been reported by Kitto (1991) and Quinn *et al.* (1992) and (Schatzki *et al.*, 1993). It is believed that there are very few differences in myosin structure between insect types, and hence, the myosin based ELISA technique can detect any insect pest in the given sample. Species–specific sELISA (sandwich ELISA) has also been developed to identify *T. granarium* (Stuart *et al*., 1994), *S. granarius* (Germinara *et al*., 2000; Rotundo *et al*., 2000). Udaya Kumari *et al.* (2004) have reported a sensitive myosin based ELISA for the detection of *Sitophilus oryzae*, with a minimum detection limit of 60pg. The assay showed low crossreactivity with *Rhizopertha, Tribolium, Lasioderma* and *Stegobium* up to an antigen concentration of 2ng. Chen and Kitto (1993) developed a species-specific ELISA to detect and quantify total and specific infestation levels in wheat. Monoclonal and polyclonal antibodies were developed against a *W* protein with 59,500Da and isoelectric point of 6.0 obtained from *S. granarius*. However, these ELISAs based on muscle protein myosin can detect all the life stages of the insect except eggs since myosin is not present in the egg stages.

There are a few routine methods used for the detection of eggs such as staining method and breeding out method, which are either time consuming or are not very sensitive. Currently there have been no reports on ELISA for detection of eggs of stored product pests. Eggs of insect pests are one of the major problems faced by the milling and packaging industry. Therefore, development of a sensitive method such as ELISA for their detection offers considerable opportunities. This is the first report on the ELISA for the detection of eggs and other developmental stages of *T. castaneum*. In the present study, the ELISA developed for the detection of eggs and other developmental stages of *T. castaneum* is highly sensitive and can detect 0.1ng of the antigen which is equivalent

to 1:73,000 parts of extractable protein of one egg. Further, unlike the myosin based assay this vitellin based assay can detect all the life stages of the insect, but with varied sensitivity. However, there has been a report on the detection of insect eggs in fruits i.e. a sandwich ELISA has been reported for detection of insect eggs of the fruit fly *Anastrepha suspensa* in fruits (Kitto *et al*, 1996). The assay was based on antibodies raised against vitellin from *Anastrepha suspensa* eggs. Johnson *et al* (1973**)** claimed that fruitfly eggs could be detected by serological/immunological methods. The authors reported that the fruit fly eggs in fruit juices are detected by solid-phase radioimmunoassay as described by Johnson *et al.* (1971).

The specificity of an antiserum is the result of the summation of the actions of the various antibodies in the total population each reacting with a different part of the antigen molecule and even different parts of the same antigenic determinant. However, when some of the determinants of the antigen, 'A', are shared by another antigen, 'B', the proportion of the antibodies directed to 'A' will also crossreact with 'B'. This phenomenon is termed 'crossreactivity' (Roitt *et al*, 1993). ELISA for detection of eggs of insect pests is based on vitellin, the major egg protein. Vitellin is a rapidly evolving protein (Kunkel and Pan, 1976); hence the antibodies against one insect vitellin may not cross react/show low crossreactivity with other insect vitellins. There are strong *a priori* reasons that yolk proteins, and especially vitellins and vitellogenins might have an evolutionary continuity back to annelids. There is, however, evidence, which suggests the possibility that the vitellins of insects do not form a homologous group. The reasons include (1) apparent rapid change in the structure of vitellin due to its largely nutritive function *vs* the conservation of certain compositional and structural features; (2) multiplicity of vitellin genes and tissues in which they are expressed; (3) differences in

the primary translation products and their subsequent processing (Hagedorn and Kunkel, 1979).

In the present study, the *T. castaneum* vitellin antiserum showed low crossreactivity with *Rhizopertha dominica* and *Corcyra cephalonica* egg protein and there was no observed crossreactivity with *Lasioderma serricorne* egg protein. In a study on the development of a sELISA for detection of eggs of the fruit fly *Anastrepha suspensa*, the antibodies obtained against the insect's vitellin proved highly specific for eggs from *Anastrepha* species, with little crossreactivity shown to *Dacus* and *Ceratitis* flies (Kitto *et al*, 1996). In another study on the properties of vitellin from the silkworm, *Bombyx mori*, the vitellin antibodies did not cross-react with the vitellin from *Locusta migratoria*, however, it showed crossreactivity with the vitellin from another silkworm, *Philosamia cynthia*. The low/no crossreactivity of vitellin antibodies of one insect with vitellin of other insects confers two advantages to the assay (a) the assay developed will be species specific, hence the contribution of individual species will be known (b) the assay unlike myosin based ELISA, may not detect beneficial insects like *Trichogramma pretiasum*, *Xylochoris flavipes*, *Bracon hebetor* and *Laelis pedatus* and unnecessarily add up to the increased pest numbers in a sample (Rajendran, 2005). Therefore, by adapting a specific method (species specific ELISA) such as the ELISA developed in the present study, one can determine the presence of a particular insect pest.

Application of the vitellin based ELISA/immunoassay is extensive in other fields such as study of the effect of environmental stress on the reproduction of the insect/animal, for determining the estrogenic activity of endocrine disrupting chemicals (EDCs), to study the role of juvenile hormone in vitellogenin expression, effect of insect control agents and in the study of the vitellin processing during the embryonic growth of

the insect/animal. A universal ELISA of vitellogenin as a biomarker for environmental estrogens was developed by Scott *et al.* (1995). However, the usefulness of a vitellin based ELISA in the detection of stored product insect infestation is reported for the first time in the present study.

Immunoassays are powerful tools, which augment monitoring and measurement capabilities in testing of food samples for contamination. Immunoassays can minimize cleanup in analysis and provide a rapid screen for the analysis of large sample loads where conventional analytical methods are too costly or cumbersome. Many immunoassay test kits are sensitive enough to directly analyze crude extracts with few or no steps involved in extract clean up. Adapting immunoassays to analyze food samples is the key to the development of a successful immunoassay. To adapt an immunoassay to food samples, potential problems can be systematically investigated.

The most important factors when developing a method are: 1) the required detection levels, 2) the choice of an extraction technique, and 3) matrix effects.

When applying an immunoassay to a food matrix, detection levels required for the testing and achieved by the immunoassay must be determined. A number of sources may indicate the concentration required for detection. They include tolerance published by the Codex Commission of the World health Organization, US EPA in the Federal Register, or internal organizational requirements (Jourdan *et al.*, 1996). Currently there are no quality standards for insect eggs in grain and flour. For insect infestation, the Food and Drug Administration (FDA) has established Defect Action Levels for live insects at two insects per kilogram. In countries like Australia and Canada, there is zero tolerance for insects in food grains (White, 1995) and a similar standard is followed in international trade for food grains (Fleurat-Lessard, 1997). The assay should be capable of detecting

the stipulated amount of contaminant or lower. This is based on the immunoassay sensitivity. In the present study, the assay developed can detect 0.1ng of the egg protein, which is equivalent to 1/73,000 parts of the extractable egg protein. Therefore, clearly the ELISA developed can detect minimum of one egg present in the food sample.

Once the detection level required is understood, an extraction technique can be developed. There is an obvious need to extract the residue from the foodstuff since the antigen–antibody reaction is typically optimum in an aqueous environment. In the present study since the egg is present in the flour, it becomes necessary to homogenize the food and extract the egg protein into the extracting buffer. Once an extraction method is chosen, it is important to evaluate the effect of the extracted sample on the immunoassay. All immunoassays rely on antigen and antibodies as the critical analytical reagents. Anything in the sample presented to the immunoassay that affects the antigen- antibody binding event can have a negative effect on the accuracy of the immunoassay (Jourdan *et al.*, 1996).

In a study on application of myosin based ELISA it was noted that there was a nonspecific background-level equivalent to 6.84$\pm$ 1.45 μg of myosin/50 g of wheat was observed during the determination of *S. granarius* infestation in wheat by indirect ELISA (Schatzki *et al.*, 1993); the background level was still higher for milled products and some of the spices (Kitto *et al.*, 1994). Although the standard assay results were very promising, the indirect ELISA procedure proved unsatisfactory for analyzing insect contamination in grain and flour. Background color formation and a decreasing response of the ELISA were noted as the concentration of the flour was increased to high levels. In milled products such as wheat flour, the huge background level is due to the formation of background color or nonspecific binding of plant materials (e.g., lectins) to the

carbohydrate moieties in the antibodies bound to the walls of the microwells. To overcome the problem, Quinn *et al.* (1992) adopted a double sandwich ELISA (sELISA) procedure based on the method of Martin *et al.* (1988).

In the present study it was noted that both whole wheat and rice flour extracts exhibited matrix effect leading to lower absorbance indicating false negative results. This problem was attributed to the possible nonspecific binding of the flour components such as lectins to the carbohydrate moieties in the antibodies and also to the walls of the microwells.

There are a number of ways, which help in effectively resolving matrix effect once it has been identified. They can be divided into two groups, those that attempt to remove the matrix effect e.g. cleanup of the extract, and those that attempt to live with the effect, e.g. standardization in the presence of the matrix. The preferred approach is to remove the effect, which results in a more robust method. In the present study, since the analyte is a protein, it is susceptible to lengthy sample processing methods and to solvent extractions. Also, for on-field application the sample cleanup method should be minimal and easy. Dilution, ultrafilteration and solvent treatment (within tolerance limit of the assay) are some of the methods for simple sample extractions. In the present study it was found that dilution of the food extract successfully removed the matrix effect. Based on the detection requirements and the sensitivity of the immunoassay the degree to which the food extract can be diluted should be calculated. It should be recognized that, while dilution steps will dilute the potential interferences, it would also dilute the analyte concentration. Therefore, it is advantageous to begin with an immunoassay with greater sensitivity than required (Jourdan *et al.*, 1996; Stanker and Beier, 1996). In the present study the food extract could be diluted up to 1:600 times to negate the matrix

interference. This was possible because of the high sensitivity of the immunoassay, which has a minimum detection limit of 0.1ng. This allowed higher dilution, yet keeping the analyte concentration in the diluted food extract well within the detection limit of the assay.

Currently, most of the extraction methods used for matrix clean up is for the pesticide residue in food products (Hall *et al.*1990; Jourdan, 1992; Mumma, 1987). There are very few studies and literature available on the extraction methods used in insect infestation detection assays. In a study by Quinn (1992) the matrix effect of wheat flour was alleviated by adoption of a double a sandwich ELISA procedure developed by Martin *et al* (1988). In this technique the microtiter plates are precoated with a "capture" antibody and any unoccupied sites are blocked with serum albumin. Upon incubation with a test sample, any myosin present is bound to the antibody and the matrix is washed away. This technique has been reported to be more sensitive and is not much affected by the quantity of wheat flour samples taken for analysis. They also described that the same method was applicable to various other grain and milled grain products like rice and rice flour.

To evaluate the proposed application for food, spike recovery experiments were performed using the matrix clean up protocol. As in development of methods using traditional chromatographic techniques like gas chromatography and high performance liquid chromatography, spike recovery studies are useful in assessing the performance of an immunoassay application. Spikes can be prepared by adding the analyte to the sample material prior to extraction or by adding analyte directly to the extract sample prior to immunoassay analysis. Samples should be analyzed without the spike (i.e. at zero analyte) and at several spike levels with the immunoassay. Good recovery of the analyte

from fortified samples indicates accuracy in the extraction method and the immunoassay. Failure to accurately recover added analyte may indicate the presence of interfering substances in the sample matrix (Stanker and Beier, 1996; Jourdan *et al*., 1996; Skeritt and Rani, 1996). As discussed earlier there are very few studies on the immunoassay development for insect infestation and in addition the literature available does not provide information regarding the sensitivity of the assay, matrix effect and spike recoveries. Udaya Kumari *et al* (2003) have reported 90% spike recovery studies in wheat for *Sitophilus oryzae* infestation. Burkholder *et* al (1996) have demonstrated that grain samples spiked with insect showed a good correlation with that of the standard antigen curve, indicating good spike recovery from the food sample. Similar results were observed in a study by Kitto *et al* (1992) wherein clean hard red winter wheat samples were spiked with known numbers of *Sitophilus granarius*. The results showed that the ELISA provides a signal that increases proportionately to the number of weevils present. The sensitivity of the assay was sufficient to detect the presence of a single insect in a 50g sample. In the present study the samples were spiked to the food prior to extraction and it was observed that good recovery (75-100%) was achieved for both whole wheat flour and rice flour. This demonstrates that the immunoassay developed could successfully detect *T. castaneum* infestation in whole wheat flour and rice flour.

CONCLUSION

The antibodies raised against the major egg protein of *Tribolium castaneum* – vitellin were highly sensitive and specific. The ELISA developed using these antibodies proved to be highly sensitive with a minimum detection limit of 0.1ng of the protein which is equivalent to 1/73,000 parts of the extractable egg protein. This is the first report on the development of an ELISA to detect the eggs of *T. castaneum* eggs. The method could also detect minimum of one number of the various stages of development such as larva, pupa and female and male adult insect. The *T. castaneum* vitellin antibodies showed low crossreactivity with egg proteins from *Rhizopertha dominica* and *Corcyra cephalonica* and there was no observed crossreactivity with the egg protein from *Lasioderma serricorne*. Therefore the ELISA developed is not only highly sensitive but also specific for the detection of eggs and other developmental stages of *Tribolium castaneum*.

The IgG based ELISA developed for the detection of eggs of *T. castaneum* was applicable for both whole wheat flour and rice flour. The food extracts exhibited matrix effect, which was negated by the dilution method. The spike recoveries for whole wheat flour and rice flour were 90-100% and 79-100%, respectively. Therefore, the IgG based ELISA developed was successful in its application for whole wheat flour and rice flour, with easy matrix clean up procedure and good spike recoveries. The assay was also applied to detect infestation in unknown market samples. The studies indicated that the assay developed could be applied successfully to unknown samples also to detect *T.castaneum* infestation as low as one egg in 100g of the food sample.

CHAPTER IV

DEVELOPMENT AND APPLICATION OF ELISA BASED ON CHICKEN EGG YOLK ANTIBODIES (IgY)

INTRODUCTION

This chapter describes the production of IgY (Immunoglobulin G from yolk) obtained from egg yolk of chicken and development and application of the IgY based ELISA for the detection of incipient stages of *T. castaneum* in whole wheat flour and rice flour.

The application of specific antibodies for the detection, quantification and isolation of various molecules in food and biological fluids is revolutionizing nutritional, chemical and biological research. At present, the major source of antibodies is from serum of different immunized mammals (Hartmann and Wilhelmson, 2001) and generally belongs to one of the two main categories: mammalian monoclonal antibodies and mammalian polyclonal antibodies. In addition to their therapeutic importance in medicine, monoclonal and polyclonal antibodies are of great value in biological research, where they serve as essential components in a variety of diagnostic systems used for the qualitative and quantitative determination of a wide range of substances. Therefore, there is a growing interest in alternative methods which focuses not only on the quality control of antibodies but also on the methods used for the production of antibodies (Tini, 2002).

Hen egg yolk represents an alternative source of antibodies. It has been known for more than a hundred years that chickens are able to transfer their immunoglobulins into the egg yolk in order to provide their descendants with an initial protection against infections (Cova, 2005). Beginning in the 1980's this option has been increasingly utilized due to certain advantages of the yolk antibodies in comparison to those from mammalian serum. Especially in cases where mammals do not respond to immunogens, chickens often provide sufficient antibody titers. Besides, antibody production using chickens is ethical, thus gaining more importance. Recently, in medical approaches remarkable efforts have been made to disseminate the chances and advantages of using

egg yolk antibodies. As the report of recommendations of the ECVAM (European Centre for the Validation of Alternative Methods) points out, this method satisfies scientific and commercial interests as well as a concern for animal welfare and should, therefore, be improved and gained to become widespread and common (Schade, 1991).

Egg yolk can be viewed as being an oil-in-water emulsion with a watery portion containing proteins and a dispersal portion of so-called yolk-granules and lipid drops (Fisher, 1996). The proteins in the yolk can be divided roughly into four fractions:

1. The vitellin or lipovitellin fraction (47.5%).
2. The vitellenin fraction (38.6%), with less phosphorous and more lipids than the vitellin fraction.
3. The phosvitin fraction (4.3%), very rich in phosphorus and without lipids
4. The livetin fraction (9.6%), with soluble proteins like globulin. This fraction is heterogeneous and differentiated into alpha-, beta-, and gamma-livetin. The gamma-livetin is same as the serum gamma globulins and IgY is identified with this fraction (Staak *et al.,* 1995).

The major serum antibody in chicken is IgG, but the antibody is also actively transported to the egg in a manner similar to the placental transfer of IgG in mammals. The protection against pathogens in the immuno-incompetent newly hatched chick is through transmission of antibodies from the mother via the egg. In the egg, chicken IgG is found mainly in the egg yolk, whereas the concentration in egg white is very low. While the egg is still in the ovary, hens transfer their serum immune globulins into the yolk. IgM and IgA are transferred together with other proteins in the oviduct into the egg white. IgG (or IgY as suggested in chickens) in the egg follicle is passed by receptors selectively in large amounts into the yolk. Recent genetic research suggests that the IgY

molecule is phylogenetically a progenitor of mammal's IgG and IgE. Avian sera contain the three principal classes of immuno globulins, namely IgA, IgM and IgY (IgG). However, structurally there is a big difference between hens IgY and mammal's IgG as demonstrated in Fig. 4.1. The molecular weight of the IgY is about 190kD vs. 150kD of IgG. Shimizu *et al*. (1992) suggested the structure of IgY in comparison to mammalian IgG. As can be seen from the Fig. 4.1, the heavy chain of IgY consists of a further domain (CH4) and a further carbohydrate chain (CHO), which is very flexible in the case of IgG, was estimated to be less flexible in IgY molecules.

IgY antibodies have been stored for over 10 years at 4^0C without any significant loss in antibody activity. Chicken antibodies have also retained their activity after 6 months room temperature or 1 month at 37^0C. Chicken antibodies are also useful in immunoprecipitation assays in agar. Chicken antibodies offer many advantages to mammalian antibodies and a change from mammalian to chicken antibodies may in many cases improve an immunological assay (Larsson *et al*., 1993).

The benefits of egg yolk antibodies (IgY) compared with mammalian IgG - which include its continuous production, cheap maintenance costs of hens, the lower susceptibility of hens to diseases and the lack of any need for bleeding are receiving growing attention. Also with regard to animal protection regulations the immunization of chickens offers two advantages: 1) the immunization with complete Freund's adjuvant is well tolerated and produces no local inflammatory reaction as seen in rabbits or other mammals, and 2) collecting eggs, in contrast to bleeding is non-invasive (Gassmann *et al*., 1990) The hen's higher production of antibodies is demonstrated by the results of Gottstein and Hemmler (1985). Each egg yolk contains upto 200mg of immunoglobulin

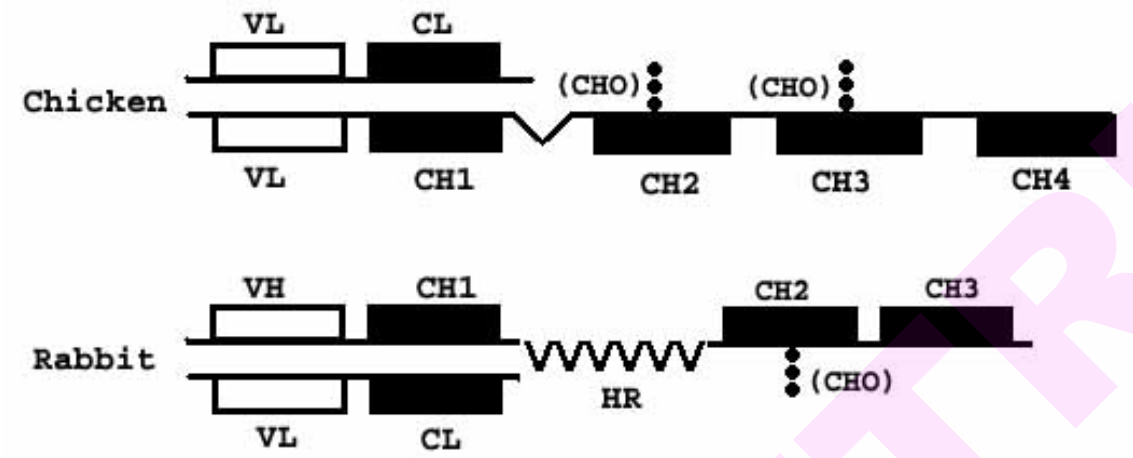


Fig. 4.1. Differences between the chicken IgY and the rabbit IgG

Table. 4.1. Comparison of the characteristics of mammalian IgG and avian IgY[a]

	Mammalian IgG	**Avian IgY**
Antibody sampling	invasive	non-invasive
Antibody amount	200mg IgG per bleed (40 ml blood)	50-100mg IgY per egg (5-7 eggs per week)
Amount of antibody per month	200 mg	~ 1500 mg
Amount of specific antibody	~ 5%	2-10%
Protein-A/G binding	yes	no
Interference with mammalian IgG	yes	no
Interference with rheumatoid factor	yes	no
Activation of mammalian complement	yes	no

([a] Adapted from Schade *et al.,* 1991)

(Jensenius *et al*., 1981), referred to as IgY (Leslie and Clem, 1969). A comparison of the characteristics of IgG and IgY is listed in Table 4.1 (Schade *et al.,* 1991)

Egg yolk antibodies have been effectively used for application in human and veterinary medicine, including strategies for the treatment of *Helicobacter pylori* infection or fatal intestinal diseases in children, particularly in poor countries, for reducing the use of antibiotics and in Asia and South America for producing antibodies against snake, spider and scorpion venoms (Schade *et al*., 2005).

Among numerous applications of immunoassays in human medical research, there are some emphasizing the advantages of antibodies isolated from chicken egg yolk. Carroll and Stollar (1983) presented an ELISA based on avian antibodies for the determination of the enzyme RNA polymerase II from calf thymus. Song *et al*. (1985) examined yolk antibodies against the α-subunit of an insulin receptor from rat and received specific antibodies from each of the five immunized hens. Earlier studies on immunization experiments employing the same immunogen to produce antibodies in rabbits were not successful. A greater antigenicity of the rat insulin receptor for chicken than for rabbits could be postulated as to be the reason for this phenomenon. Chicken egg yolk antibodies against high molecular weight mucin-like glycoprotein-A (HMGP-A) could be successfully applied in the breast cancer research.

Although, the yolk antibody technology has been mostly for medical approaches, the application in other fields of chemical/pesticide residue analysis in food and environment and in the analysis of food samples for biological contaminants like fungal and bacterial toxins and insect infestation seems promising. Amitha rani *et al.* (2006, in press) have reported the various applications of egg yolk antibodies in the field of pesticide residue analysis, insect infestation detection and detection of cell death markers.

This study for the first time reports the development and application of the egg yolk antibody based ELISA in detection of incipient stages of *T. castaneum* in whole-wheat flour and rice flour.

MATERIALS AND METHODS

1. Chemicals: 3,3'5, 5'-Tetramethyl benzidine (TMB), urea-hydrogen peroxide, bovine serum albumin (BSA), anti-rabbit IgG, Freund's complete and incomplete adjuvant, β-cyclodextrin, leupeptin, pepstatin, phenylmethanesulfonylflouride (PMSF), mercaptoethanol, N, N, N', N'-tetramethylethylenediamine (TEMED), high molecular weight range native markers and 0.45μm nitrocellulose membrane were purchased from Sigma Chemical Company, USA. ProteinA-Sepahrose was obtained from Pierce, U.S.A. Polystyrene 96-microwell maxisorp ELISA plates were purchased from Nunc (Roskilde, Denmark). Spectromax 340 microplate reader (Molecular Devices) was used to measure the absorbance in the micro titer plates. Sodium chloride, potassium chloride, sodium phosphate, acrylamide, Bis-acrylamide, cupric chloride, Coomassie brilliant blue and Schiff's reagent were purchased from Sisco Research Laboratories, Mumbai. All the other chemicals used were obtained locally and were of highest purity grade. Whole wheat and rice flour were purchased locally.

2. Production of egg yolk antibodies: Egg yolk antibodies were raised against the antigen Vm (mixture of V1 and V2). Vm was chosen as the antigen for raising antibodies in chicken based on the Western blotting results which showed that the V1 antiserum crossreacted with both V1 and V2 (Fig2.3, Chapter 2) and also the ELISA performance of Vm antigen against V1 antisera which was comparable with that of V1 antigen curve (Fig.3.3.a, chapter 3). Furthermore, as Vm can be easily obtained with a simple single step extraction, it was chosen as the antigen for IgY production in chicken. Vm was obtained from 0-6h eggs as described in chapter II (5).

2.1. Birds: Twenty-two weeks old White Leghorn hens/chickens were used for the antibody production. The birds were housed in large stainless steel cages placed in well-

aerated room having 12h light and 12h darkness. The hens were maintained on commercial poultry diet and water *ad libitum*.

2.2. Immunization protocol: The immunization protocol followed was as described in Udaya Kumari *et al.* (2004). Briefly, one milligram of the antigen Vm was mixed well with equal volume of Freund's complete adjuvant. The antigen mixture was injected subepidermally at about 40 sites in the breast region of the white leghorn hen. Five booster doses with 0.5 mg of the antigen were administered intramuscularly into the breast muscle at an interval of 4 weeks each, after 4 weeks of administration of the initial primer dose. Eggs were collected daily from the 7^{th} day onwards after each booster dose and stored at 4^0C until use.

3. Purification of egg yolk antibodies: Purification of IgY was carried out by employing three different methods to choose the best method of purification based on the efficiency of extraction of IgY and the assay performance of the purified IgY. One egg each from the first five weeks after the first booster dose was taken for initial screening of purification methods. Yolk of one egg was divided into three equal parts and purification of IgY was carried out using the following three different IgY purification protocols:

3.1. Method 1 (M1): Purification of IgY using CFTRI patented method (US patent No. US 2003068786, WO03033537-2003-04-24): Egg yolk was diluted 1:1 with distilled water and homogenized for 30 sec, filtered through cheese cloth. A known quantity (w/v) of carrageenan was added, mixed well and incubated at room temperature for 30 min. The solution was then centrifuged at 10,000 rpm for 15min at 4^0C, the lipid precipitate was discarded and the supernatant was filtered through Whatmann filter paper No. 2. IgY precipitation was carried by addition of PEG 8000 and centrifuged at 10,000rpm for 20 minutes. The supernatant was discarded; the IgY precipitate was reconstituted in PBS and

dialyzed against PBS for 24h at 4^0C with three changes. After dialysis, 0.005% sodium azide and 5% glycerol was added and aliquots of the IgY solution were stored at -20^0C until use.

3.2. Method 2 (M2): Purification of IgY using PEG *(Deignan et al., 2000 ; Polson et al., 1985):* One volume of yolk was diluted with 5 volumes of 0.1M phosphate buffer pH 7.6 Added 3.5% w/v PEG, centrifuged at 5000 rpm for 30minutes. The lipid precipitate was discarded and the supernatant was filtered through cheese cloth. IgY precipitation was done by adding 12% w/v PEG and incubated at for 10 minutes at room temperature, centrifuged at 7500 rpm for 25 min. The IgY precipitate was dissolved in 5mL of phosphate buffer and cooled to 0^0C. Then 5mL of precooled ethanol was added, centrifuged at 10,000 rpm for 25 min at 4^0C. The supernatant was discarded, the IgY precipitate was reconstituted in 10mM PBS, pH 7.2 and dialyzed against 10mM PBS for 24h at 4^0C with three changes. After dialysis, 0.005% sodium azide and 5% glycerol was added and aliquots of the IgY solution were stored at -20^0C until use.

3.3: Method 3: Purification of IgY using chloroform and carrageenan *(Clarke et al., 1990* ; *Polson, 1990):* 1 volume of yolk was diluted with 4 volumes of 0.1M PBS (pH 7.2) and 1 volume of chloroform. The yolk was mixed well and centrifuged for 30 min at 10,000g. The lipid precipitate was discarded and the supernatant was filtered through Whatmann filter paper No.2. IgY precipitation was carried out by adding 14% w/w PEG 8000. IgY precipitate was reconstituted in PBS and 25% v/v of precooled ethanol to remove the residual PEG. The solution was centrifuged and the IgY pellet was reconstituted in 10mM PBS, pH 7.2 and dialyzed against 10mM PBS for 24h at 4^0C with three changes. After dialysis, 0.005% sodium azide and 5% glycerol was added and aliquots of the IgY solution were stored at -20^0C until use.

4. Purification of weekly eggs over four month's period: Weekly eggs over four month's period were processed to check the IgY yield and sensitivity with booster doses. The purification of the egg yolk antibodies was carried out using method 1 as explained above. The sensitivity of the IgY was tested by ELISA.

5. Estimation of protein (antibody concentration) by Bradford's method: The protein (antibody) concentration in each of the purified IgY sample was estimated by Bradford's method (Bradford, 1976) as follows: BSA was used for obtaining the standard working curve. 10, 20, 30, 40 and 50µL of 1mg/mL BSA standard stock were pipetted into respective test tubes. 3mL of Bradford's reagent was added, and the total volume was made up to 3.05 mL with distilled water. The tubes were incubated at room temperature for 30 minutes and the absorbance was read at 595nm. The concentration of protein in the sample was obtained from the standard curve.

6. Polyacrylamide gel electrophoresis: The samples i.e. precipitate and supernatant obtained during the steps involved in IgY purification were subjected to N-PAGE to study the purity and efficiency of the extraction of three different purification methods employed. N-PAGE was performed on a 7% resolving gel with a 5% stacking gel according to the method of Laemelli (1970) at 4^0C. Rabbit IgG (146kD) was used as molecular weight marker. After electrophoresis, gels were fixed and stained with 0.1% Coomassie brilliant blue R-250 for total protein.

7. ELISA formatting: ELISA based on antibody capture assay (ACA) format was performed to test the sensitivity and specificity of the purified egg yolk antibodies and for the assay development. The assay was performed as described in Udaya Kumari *et al.* (2004). Assay formatting was done for optimizing the following parameters:

***(a) Antigen concentration*:** The optimum linear working range for the present study was

(b) selected by testing the antigen concentrations between 0.1ng to 1000ng.

(b) Antibody concentration: Different concentrations of the antibody i.e. 1, 0.5, 0.25, 0.125, and 0.0625 µg were tested to determine the optimum antibody concentration that could detect the least antigen concentration and a linear working curve.

(c) Blocking agent: BSA dissolved in carbonate buffer at four different concentrations i.e. 0.5, 1, 2 and 3% was tested for their blocking efficiency. In addition, effect of diluants i.e. PBS containing 0.5% Tween and PBS-FG, in the betterment of assay performance was studied in combination with the different concentration of BSA tested. Tween 20 reduces non-specific binding that is usually observed with the IgY, therefore 0.5% Tween was added to PBS in order to study the assay performance in presence of tween.

8. Standard assay: The standardized immunoassay was performed as follows:

Step 1: Antigen Vm obtained from *T.castaneum* egg protein was diluted in carbonate buffer (pH 9.6) to obtain a working range between 0.1ng / 100 µl to 15ng /100 µl. 100 µl of the buffer blank i.e. zero antigen was also added. 100µl of the antigen solution was coated onto Nunc Maxisorp ELISA plates and incubated overnight at room temperature.

Step 2: The solution was tipped off, and the plate was washed 3X with 50mM PBS (pH 7.2) containing 0.05% Tween 20 (PBS-T). The plates were gently tapped dry.

Step 3: The plate was blocked with 150□µl of 2% BSA solution prepared in carbonate buffer (pH 9.6). After an incubation period of 1h, the blocking solution was tipped off and the plate was gently tapped dry.

Step 4: The plate was loaded with 1µg of IgY/100 µl of 50mM PBS containing 0.5% fish gelatin (PBS-FG). After an incubation period of 1h, the antibody solution was tipped off , washed 3X with PBS-T and gently tapped dry.

Step 5: The plate was loaded with 100 µl of the secondary antibody conjugated to horseradish peroxidase (rabbit anti chicken-HRP conjugate) diluted to 1:5000 with PBS-FG. After an incubation period of 1h, the secondary Ab- HRP solution was tipped off and washed 3X with PBS-T and gently tapped dry.

Step 6: The bound antigen-antibody-HRP complex was visualized by addition of 150 µl of substrate- chromogen mixture (substrate i.e. 0.015% of H_2O_2 solution prepared in 0.1M sodium acetate buffer , containing 0.25% β-cyclodextrin, adjusted to pH 5.0 with citric acid mixed 97:3 with chromogen i.e. 1% solution of 3, 3', 5, 5'-tetramethyl benzidine in dimethyl sulphoxide). The reaction was stopped after 45 min with 50 µl of 2.5N sulphuric acid and plate was read at 450nm using ELISA plate reader.

9. Detection of eggs through embryonic development and other incipient and adult stages of *T. castaneum*: For this study, the eggs through embryonic development, larvae, pupae, adult male and female insects of *T. castaneum* were collected and processed to obtain the antigen as described in Chapter II under the section '4' and '5' respectively. On obtaining the antigen, appropriate dilutions of the antigen were coated onto ELISA plates and the assay performed as described under section '8' in this chapter. A comparative standard antigen (Vm) curve was also coated.

10. Crossreactivity studies with other insect eggs: The crossreactivity of the *T. castaneum* egg protein IgY was tested against the egg proteins of the cigarette beetle - *Lasioderma serricorne*, rice weevil-*Rhizopertha dominica* and rice moth - *Corcyra cephalonica*. The eggs of these insects were collected as described in Chapter III (7).

11. Application of the assay to whole-wheat flour and rice flour:

***11.1. Matrix effect studies*:** Based on the results of the application of IgG based assay on whole wheat flour and rice flour**,** dilution was used as the clean up method to overcome

matrix interferences in the application of IgY based ELISA.

In the present study, 100g of whole wheat flour/rice flour was first sieved and the residual flour particles retained in the sieve were diluted 1:10 times with antigen extraction buffer, gently mixed and centrifuged at 10,000 rpm. The supernatant was recovered and further diluted to 150, 300 and 600 times with carbonate buffer, keeping the dilutions within the detection limits of the assay, as described for IgG based assay. In addition, the whole wheat flour extract and rice flour extract was further diluted to 1000 and 1200 times, respectively, with carbonate buffer. The antigen i.e. Vm was serially diluted in each of the above diluted extracts and coated onto the ELISA plate. Comparative antigen dilutions prepared in the extractant (antigen extraction buffer diluted to 150, 300, 600, 1000 and 1200 times with carbonate buffer) minus food and also a standard antigen dilution prepared in carbonate buffer coated on the same plate. The assay was performed as described in chapter III under section 5.

***11.2. Spike and recovery studies*:** The whole-wheat flour/rice flour (100g) was spiked with eggs (1, 2, 4, 8 and 16 nos.) and free antigen equivalent of the eggs. The spiked whole wheat flour/rice flour was first sieved and the residual flour particles containing the spiked eggs retained in the sieve was crushed to break the eggs and then was gently mixed 1:10 times with cold antigen extraction buffer and centrifuged at 10,000 rpm. The clear supernatant obtained was further serially diluted to 1:1000 and 1200 times for wheat flour and rice flour, respectively, with carbonate buffer. 100 µL of the diluted spikes were coated onto the ELISA plates. This was compared to the extractant (i.e. antigen extraction buffer diluted 1:1000 and 1200 times with carbonate buffer for wheat flour and rice flour, respectively) spiked with antigen equivalent to 1,2,4,8 and 16 eggs. A comparative standard curve with the purified antigen only in carbonate buffer was also coated and the

assay performed as described in chapter III-A (5).

12. Testing of market samples: Whole-wheat flour and rice flour were obtained locally. Both high quality brands and local samples were tested for infestation by the ELISA developed.

100g of each sample was first sieved and the residual flour particles containing the spiked eggs retained in the sieve was crushed to break the eggs and then was gently mixed 1:10 times with cold antigen extraction buffer and centrifuged at 10,000 rpm. The clear supernatant was recovered and further diluted to 1000 and 1200 times for wheat flour and rice flour, respectively, with carbonate buffer. 100µL of the sample extract was coated on to ELISA plates. This was compared with antigen dilutions prepared in buffer blank minus food extract and a standard curve of the antigen prepared in carbonate buffer coated on the same plate. The buffer blank was prepared by diluting the antigen extraction buffer 1:600 with carbonate buffer. The assay was performed as described in chapter III under section 5.

The ELISA results were validated by incubation/ breeding out method as described in Chapter III (7).

13. Statistical analysis: Data are expressed as mean $\pm$ standard error (SE) of three separate experiments. The minimum detection limit of the assay was determined as the lowest detectable analyte concentration that gives a response, which has a statistically significant difference ($p< 0.005$) from the response of the zero analyte concentration. Statistical analyses were done using Microsoft Excel and Origin software.

RESULTS

1. Comparative analysis of the three different methods used in purification of egg yolk antibodies:

1.1. Purification of IgY: Based on the N-PAGE analysis of the different fractions obtained during IgY purification process**,** of the three methods used for the purification of IgY method 1 and 3 were found to be good as compared to method 2 in terms of IgY purity (Fig. 4.2).

The N-PAGE of diluted egg yolk revealed five bands corresponding to the lipovitellin, γ -livetins (IgY), β-livetins, α-livetins and the phosvitin (Fig.4.2). The IgY band was of slightly higher molecular weight compared to that of rabbit IgG band (Fig.4.2.a). The first step in IgY purification involved the lipid/lipoprotein precipitation using carrageenan, PEG and chloroform in method 1, 2 and 3 respectively. Method 1 (Fig. 4.2.a) and 3 (Fig. 4.2.c) were able to extract the IgY fraction along with other water-soluble proteins into the water-soluble protein fraction (WSPF) without lipoprotein contamination. However, in method 2 (Fig. 4.2.b), the WSPF contained some lipoproteins along with the other water-soluble proteins, probably as the PEG concentration (3.5%) did not precipitate all the lipoproteins.

In the second step which involved the IgY precipitation, methods 1 and 3 yielded almost 90-95% pure IgY fraction (Fig 4.2., a & c) with some contaminating proteins. The IgY fraction obtained from method 2 did not appear to be pure as seen in Figure 4.2.b. Contaminating proteins in a relatively high concentration were seen along with the IgY fraction.

1.2. IgY yield: The average comparative IgY yield obtained with the three different methods was of 15mg/5mL yolk for method 1 and 3, and 65mg/5mL yolk for method 2

(Fig. 4.3). These values are an average of the protein (IgY) obtained for the weeks 3, 4, 5, 6 and 7 tested.

1.3. Assay performance: The IgY purified using method 1 and 3 showed good assay performance as compared to that of the IgY purified using method 2 which showed comparatively low signal intensity. The minimum detection limit of the assay using IgY purified using method 1 and 3 was 0.1ng for both, while that for method 2 it was 4.5ng (Fig.4.4).

Therefore, based on the above results, it can be concluded that method 1 and 3 were efficient in purification of IgY. However, method 1 was chosen for further studies over method 3 as method 3 involved the use of chloroform and waste disposal of organic solvents is often considered a problem as they pose environmental hazard.

2. Weekly IgY yields over four-month period: The IgY yield as observed from Fig.4.5 was highest on week 2 with ~140mg of IgY yield/egg followed by week 8 with ~100mg IgY yield/egg. Between the weeks 2 and week 8 there was a decrease and then an increase in the IgY yield by the eighth week. Between week 2 and week 8 the IgY yields were approximately 61, 47, 21, 29 and 60mg/egg for the 3rd, 4th, 5th 6th and 7th week, respectively. At week 9 the IgY yield obtained was the lowest at 6.75mg/egg. Then there was a gain in the IgY yield by week 11 at 32mg IgY/egg and this was maintained until the last week tested (week 18). Overall, up to two booster doses, there was a rise in the titer and the titer fell by the end of the each booster. The titer fell to the minimum during the early phase of third booster but it increased with time, however, it was lower compared to that obtained with the first and second booster doses, at an average of 32mg IgY/egg.

3. Optimization of ELISA:

(a) Antigen concentration- The ELISA based on antibody capture assay format indicated that the workable linear antigen concentration for the purified IgY was between 15.6 to 0.1ng (Fig.4.6). These optimized antigen concentrations produced a linear working curve with an 'r^2' value of 0.9.

(b) Antibody (IgY) dilution: For the present study, the workable antibody concentration was fixed at 1µg/well which showed a minimum detection limit of 0.1ng (Fig.4.6). Of the different concentrations of the antibody i.e. 1, 0.5, 0.25, 0.125 and 0.0625 µg tested, 1 and 0.5µg of antibody showed almost comparable antibody performance with minimum detection limit of 0.1ng. Since antigen-determining assays are performed with antibody excess, IgY at 1µg/well was chosen for developing the standard assay. With the decrease in the IgY concentration i.e. IgY at 0.25, 0.125 and 0.0625 µg/well, there was a decrease in the number of antigen-antibody complex on plate, thereby leading to the lower absorbance with the decrease in the antibody concentration. This led to the lower sensitivity of the assay and the minimum detection limit of the assay for IgY concentration at 0.25, 0.125 and 0.0625 µg/well was 0.4, 0.9 and 0.9ng respectively.

(c) Blocking agent and antibody dilution buffer: Of the two different antibody dilution buffers tested, it was found that PBS-FG did not interfere with the assay performance. PBS-T-0.5% effected the assay performance resulting in lowered signal intensity evident at higher antigen concentrations (Fig.4.7). The different BSA concentrations tested did not show any significant change in their blocking efficiency. Therefore, 2% BSA was used for developing the standard assay, as in case of the IgG based assay.

Thus, the IgY based assay developed based on the major egg protein vitellin of the red flour beetle worked best at an antigen concentration range of 15.6 to 0.1ng, with

2% BSA as the blocking agent and at an antibody (IgY) concentration of 1µg/well. The standard assay developed showed a minimum detection limit of 0.1ng. Further, expressing the minimum detection limit of the assay in terms of parts of an egg, the assay can detect 1:73,000 part of an egg, which is equivalent to 0.1ng of the total extractable protein. This demonstrates that the assay developed is highly sensitive. The assay's linear response easily spanned a 150-fold range of the total protein concentrations (0.1–15ng) which demonstrated the possible potential for developing a quantitative assay for detection of incipient stages of *T. castaneum*. The good IgY yield demonstrated the possible potential of using the IgY based ELISA in the routine analysis/detection of incipient stages of *T. castaneum* in food commodities. The assay performance was comparable with that of IgG based assay. However, IgY based ELISA gave a slightly higher absorbance as compared to that of IgG assay (Fig.4.8).

4. Sensitivity of antibody obtained over four-month period: The antibodies obtained in the present study were highly sensitive and the maximum sensitivity obtained over four month period, expressed as minimum detection limit was 0.1ng. Maximum sensitivity of the antibodies was obtained on week 4, 6 and 7 (Fig.4.9). The sensitivity of the IgY obtained on week 1 after the first booster dose was 1.4ng. Gradually, there was an increase in the sensitivity of the antibodies. By week 4 the IgY reached the maximum sensitivity i.e. 0.1ng. Week 5 i.e. one week after the second booster, showed a slight decrease in the antibody sensitivity (MDL 0.3ng). However, the maximum sensitivity was retained by week 6 and the same trend was observed until week 7. By week 8, there was a four-fold decrease in the antibody sensitivity (MDL 0.4ng). However, the following weeks showed an improvement in the antibody sensitivity (0.2ng) and almost the same sensitivity of the antibodies was observed until the last week tested i.e. week 18.

On the whole, there was an increase in the sensitivity of the antibodies with booster doses. Though the IgY yield decreased to an average of 32mg/egg after the third booster, the antibody sensitivity was good hence could make up for the decrease in the IgY yield.

5. Detection of eggs through embryonic development and other incipient and adult stages of *T. castaneum*: The assay performed using the antigen Vm obtained from different embryonic stages of *T. castaneum* did not show any change in terms of assay performance and sensitivity (Fig.4.10). The assay performance of the different embryonic stages was comparable with that of the earliest embryonic stage (0-6h) tested. In addition, the minimum detection limit of the assay remained unaltered at 0.1ng. This shows that the assay developed can detect the eggs at any given embryonic stage in a food sample. These results are in line with that observed for the IgG based assay for *T. castaneum* described in Chapter III.

The crossreactivity studies of Vm antibodies with antigen VG obtained from immature larvae, mature larvae, pupae and the adult males and females indicated that the assay could detect these stages also (Fig.4.11). The assay performance and the sensitivity of the assay were in comparison with that of the IgG based assay. The minimum detection limit for immature larvae was similar to that of the eggs i.e. 0.1ng, while, the minimum detection limit of the other stages i.e. mature larvae, pupae, adult male and female insects was higher and equivalent to 7.8, 7.8, 15.6 and 3.9ng, respectively. However, due to the high sensitivity of the antibodies, the assay could still detect a minimum of one number of each stage, except male insects. This crossreactivity of the Vm IgY with VG of the developmental stages indicates that the IgY based assay could detect all the developmental stages of *T. castaneum*, with varied sensitivities but could however detect a minimum of one number of each developmental stage.

6**. Crossreactivity studies of the Vm antibodies with other insect pest egg proteins:** Vm-antibodies (IgY) exhibited low/negligible cross reactivity with the egg proteins of the other stored product insect pest/moth tested (Fig.4.12). Low crossreactivity of IgY with the egg protein of *Corcyra cephalonica* and *Rhizopertha dominica* was observed. The percent crossreactivity observed was 5.4 and 9.3 for *Corcyra cephalonica* and *Rhizopertha dominica,* respectively at 500ng of the respective antigen coated. There was no observed crossreactivity at lower antigen concentrations. *Lasioderma serricorne* egg protein did not show any crossreactivity with Vm-antibody even at 500ng of the antigen. This indicates that the antibodies obtained are highly specific for *T. castaneum* egg protein. The same results were observed even for the IgG based assay.

7. Matrix effect studies: Matrix effect studies revealed that the whole-wheat flour and rice flour extracts exhibited matrix effect/interference. Dilution was found to be effective in diluting/eliminating the matrix effect in case of both whole-wheat flour and rice flour.

The whole wheat flour matrix interference resulted in high background color (Fig.4.13). There was a noticeable improvement in the assay performance with dilution and the matrix interferences could be reduced/eliminated with 1:1000 times dilution of the whole-wheat flour extract and was comparable with that of the standard curve (Fig.4.13).

In the case of rice flour, the matrix interferences resulted in a poor assay performance with lower absorbance as compared to the standard curve (Fig.4.14). There was a gradual improvement in the assay performance with dilution for both rice and whole-wheat flour extracts. At dilution of 1:1200, the assay performance was comparable with that of the standard curve. The high sensitivity of the assay allowed this dilution, which was within the detection limit of the assay i.e. 0.1ng.

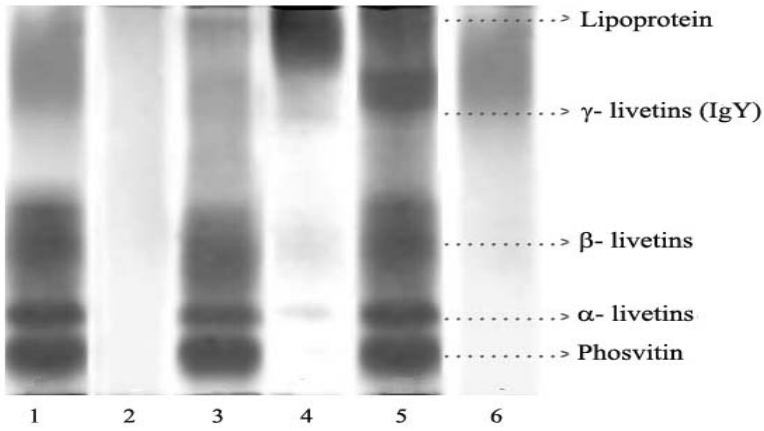
8. Spike and recovery studies:

8.1. Whole-wheat flour: The percent spike recoveries in whole-wheat flour ranged from 85-100%. Good recoveries between 95 -100% was observed for 4,8 and 16 egg spikes, while at 1 and 2 eggs spike the recovery was 85% (Fig.4.15).

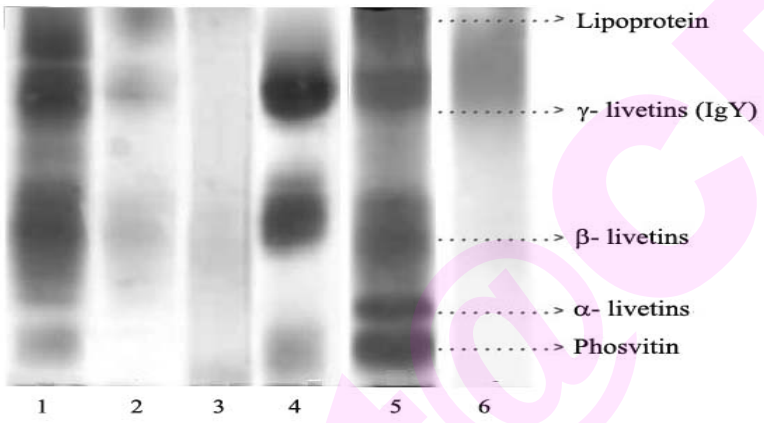
8.2. Rice flour: The percent spike recoveries in rice flour ranged from 75-85%. The highest recoveries of about 80-85% were achieved at lower egg spikes (Fig.4.16). There was slight decrease in the spike recovery with the increase in the number of egg spikes. However, the difference in the spike recoveries between the lowest egg spike (1No.) and the highest egg spike (16No.) was only 10%.

9. Testing of market samples: Of the five different samples each of wheat flour and rice flour tested by the ELISA developed one sample of wheat flour and 2 samples of rice flour tested positive for *T. castaneum* infestation (Fig.4.17). The rate of infestation from the standard graph was equivalent to one egg for wheat flour sample 3 and for rice flour it was equivalent to two eggs and one egg in sample 2 and 3 respectively. These results were compared with that of the breeding out method after incubation of the samples for 45 days. The wheat flour sample 2 and rice flour samples 2 and 3 showed infestation. The other samples did not indicate any infestation until the observed time period.

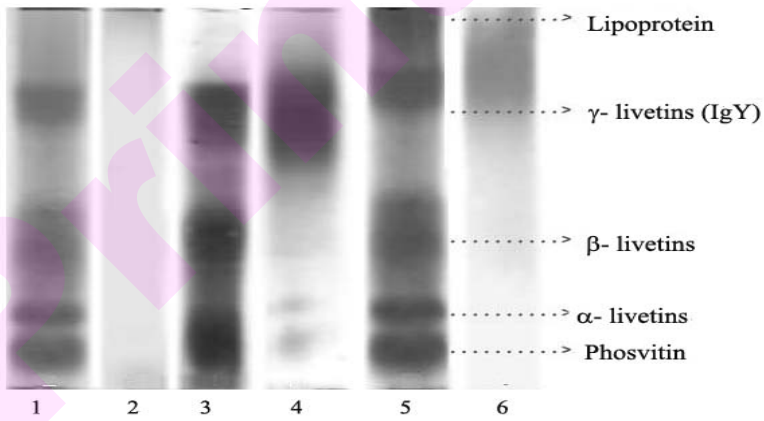
A summary of the comparison between the IgG and IgY based ELISA developed in the present study is given in Table 4.1



(a) Method 1: Gum + PEG



(b) Method 2 : PEG + PEG



(c) Method 3: Chloroform + PEG

Lane 1: Supernatant after lipoprotein precipitation

Lane2: Precipitate after lipoprotein precipitation

Lane 3: Supernatant after IgY precipitation,

Lane 4: Precipitate after IgY precipitation,

Lane 5: Diluted egg yolk

Lane 6: Rabbit IgG

Fig. 4.2. N-PAGE of IgY purified using three different methods

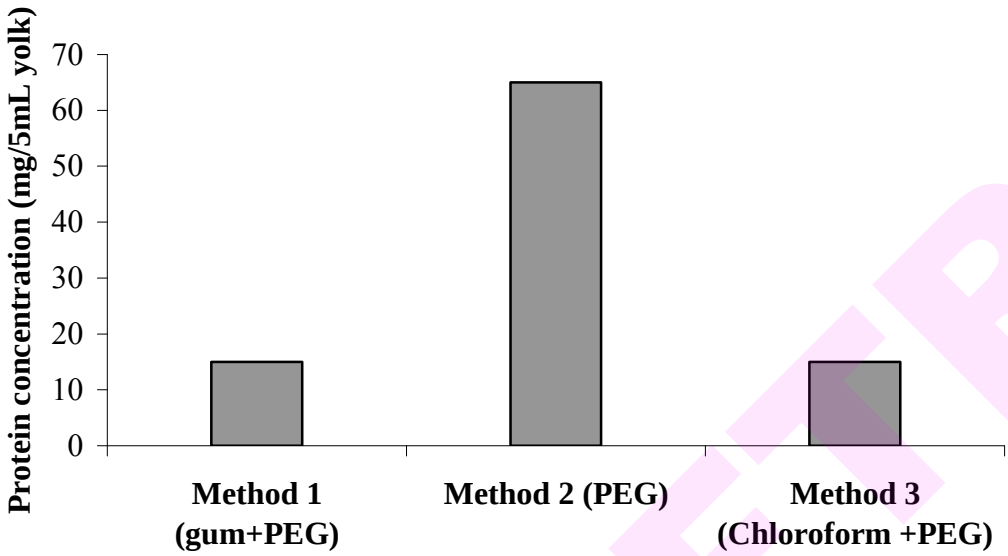


Fig. 4.3. IgY yield obtained with the different purification protocols

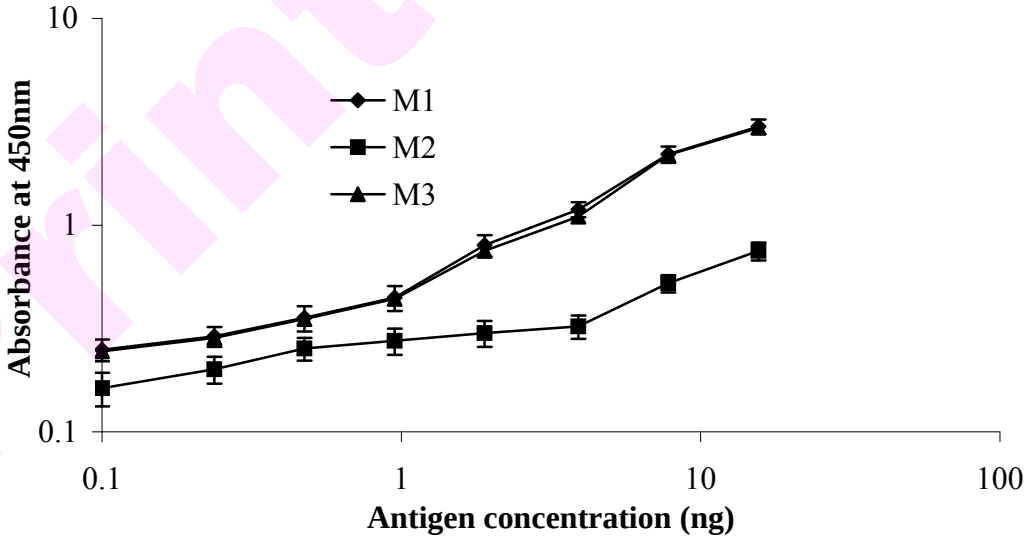


Fig. 4.4. Assay performance of IgY purified using three different protocols (M1-Gum+PEG, M2-PEG+PEG, M3-Chloroform+PEG)

(Data is expressed as mean ± SE of three separate experiments)

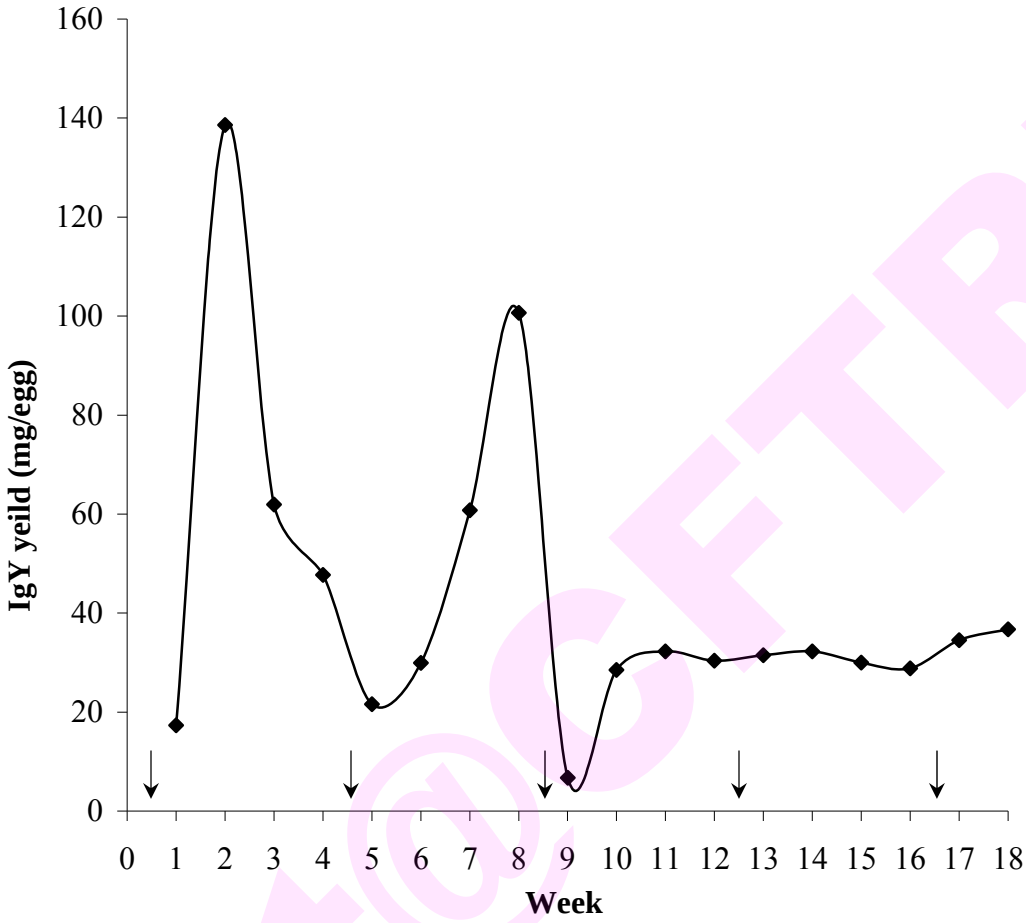


Fig. 4.5. Weekly IgY yields over four-month period

(Arrows indicates time of administration of booster doses)

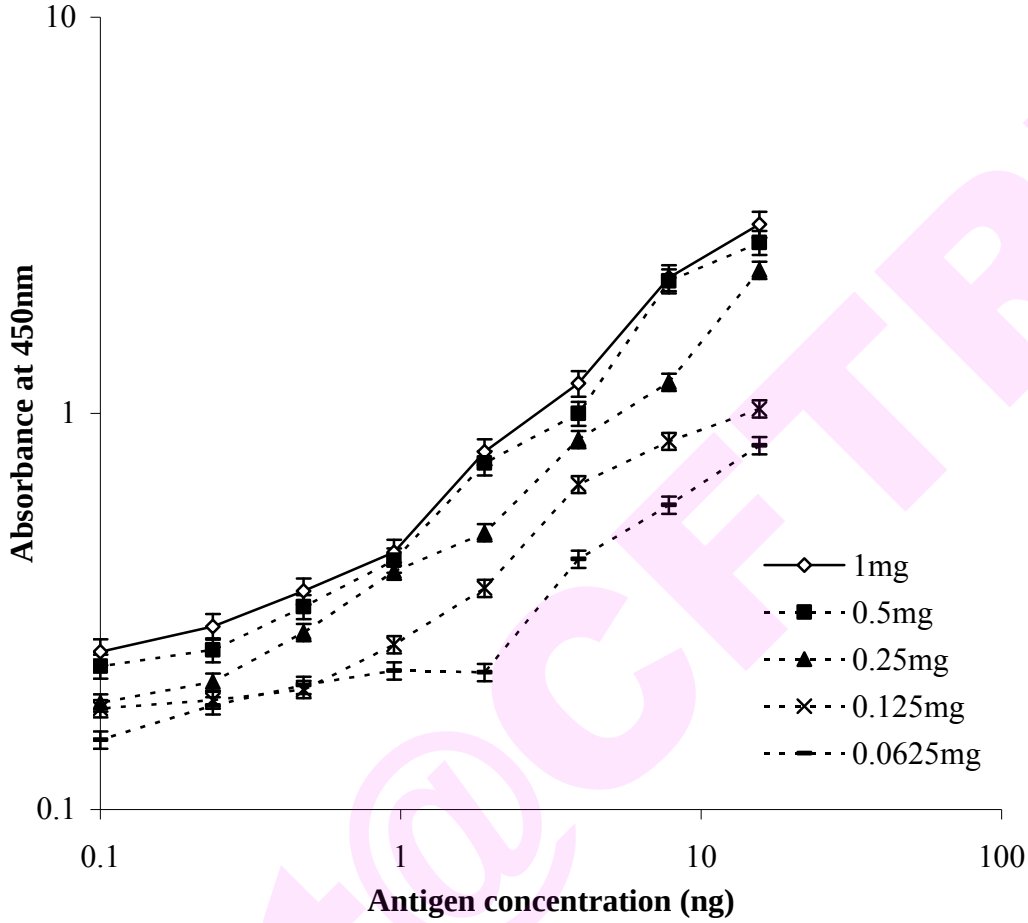


Fig. 4.6. Effect of different IgY concentration on the assay performance

(Data is expressed as mean ± SE of three separate experiments)

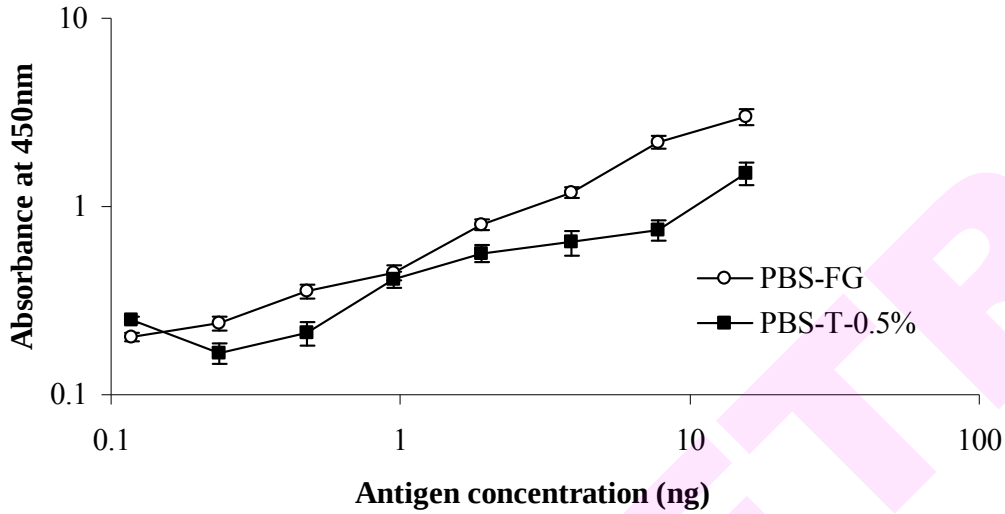


Fig.4.7. Effect of antibody diluants on the assay performance with 2% BSA (blocking agent)

(Data is expressed as mean ± SE of three separate experiments)

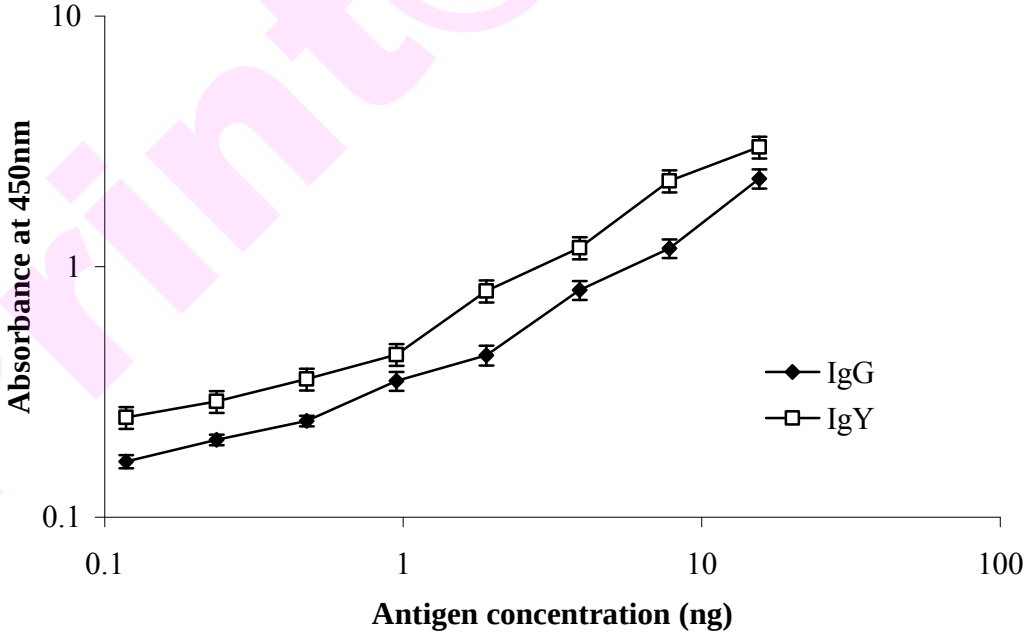


Fig. 4.8. Comparison of IgG and IgY based ELISA performance

(Data is expressed as mean ± SE of three separate experiments)

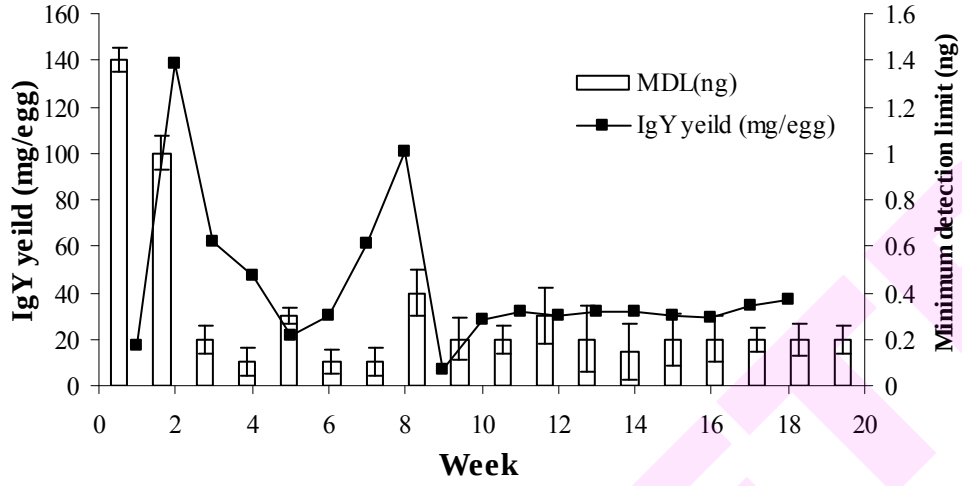


Fig. 4.9. Sensitivity of IgY antibody obtained over four-month period compared with the yield

(Data is expressed as mean ± SE of three separate experiments)

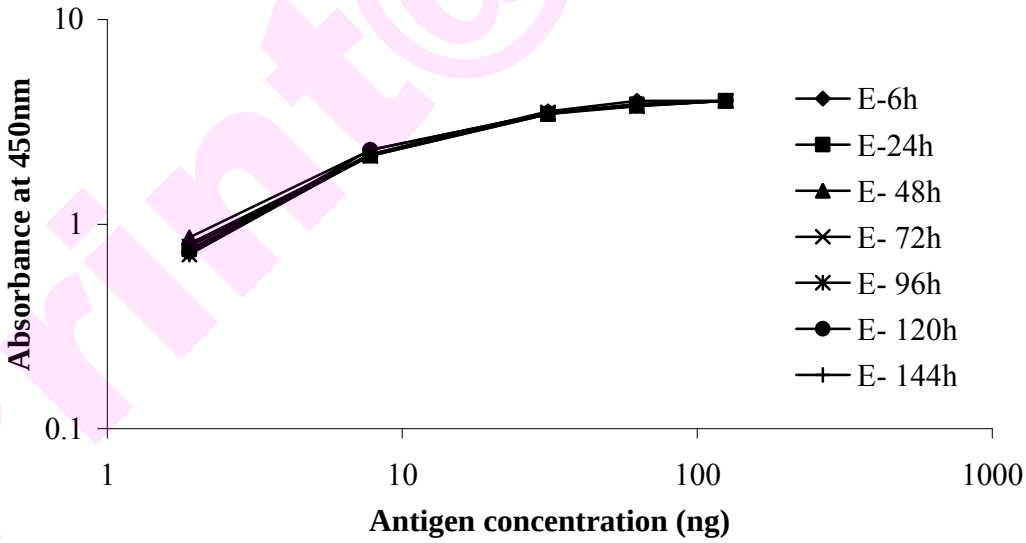


Fig.4.10. IgY based ELISA showing reactivity of embryonic stages of *T. castaneum* with Vm antibodies

(Data is expressed as mean ± SE of three separate experiments; for clarity the error bars are not integrated)

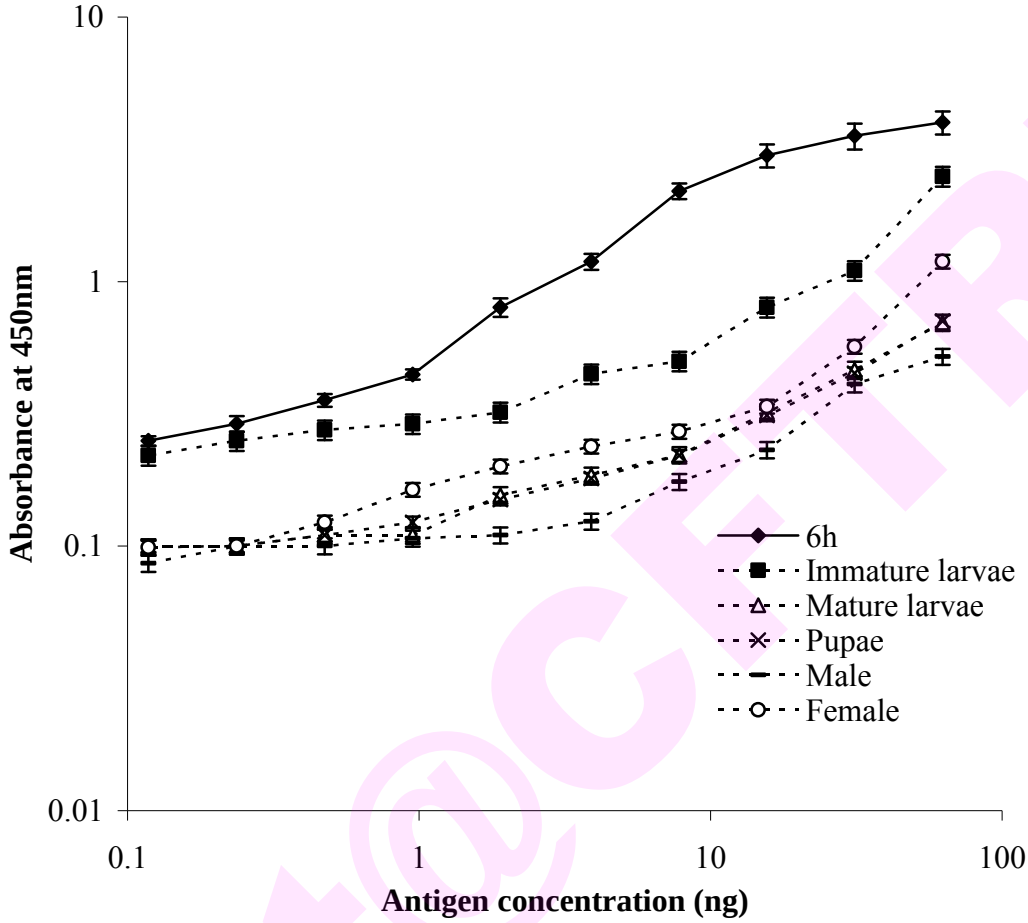


Fig.4.11. IgY based ELISA showing crossreactivity of developmental stages of *T. castaneum* with Vm antibodies

(Data is expressed as mean ± SE of three separate experiments)

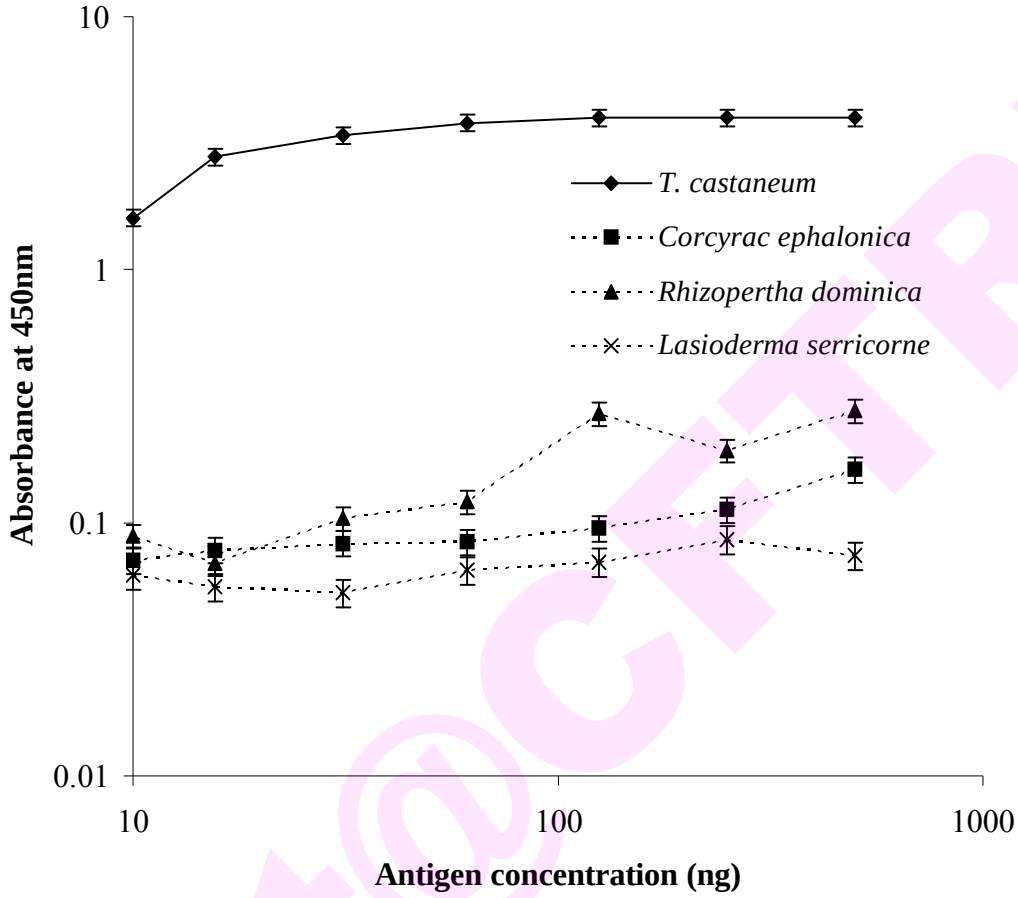


Fig.4.12. IgY based ELISA showing crossreactivity of the other stored product insect egg protein against *T. castaneum* egg Vm antibodies

(Data is expressed as mean ± SE of three separate experiments; the antigen concentrations tested were at a higher range to determine even the least crossreactivity if present)

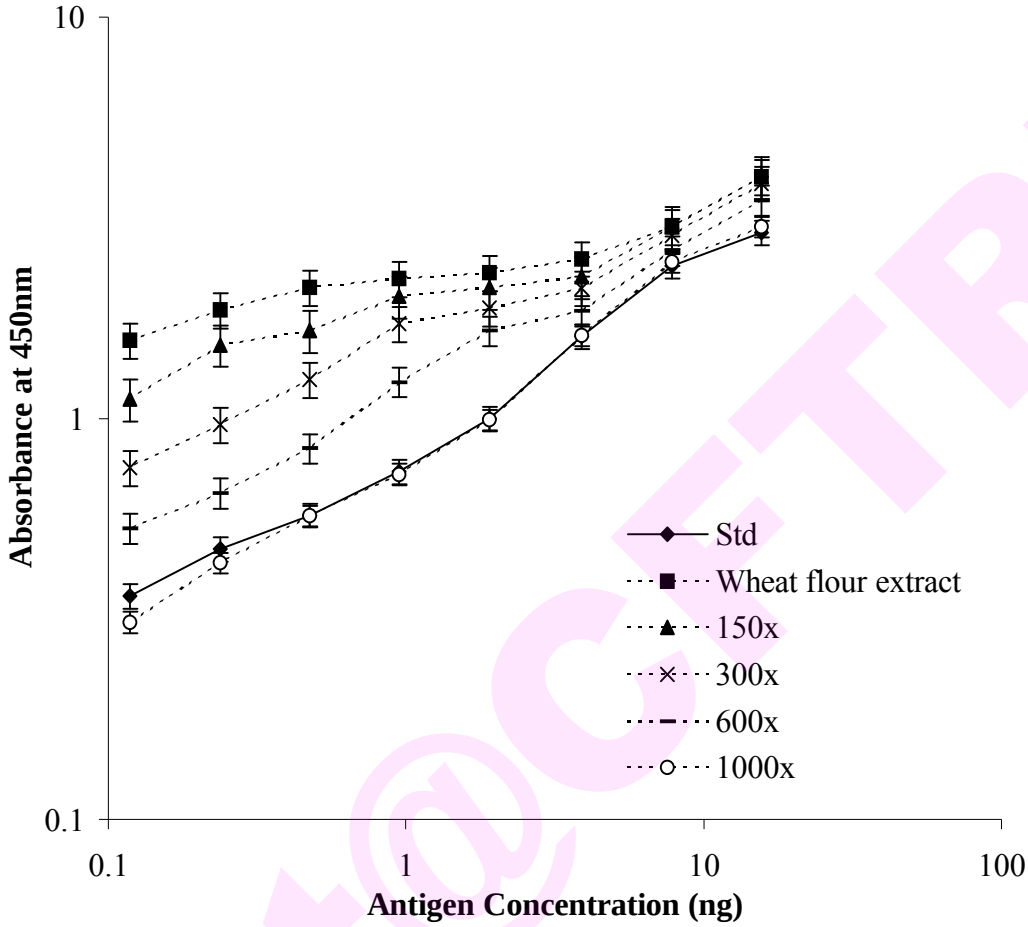


Fig.4.13. Effect of dilution on matrix interference of whole-wheat flour extract

(Data are expressed as mean ± SE of three separate experiments)

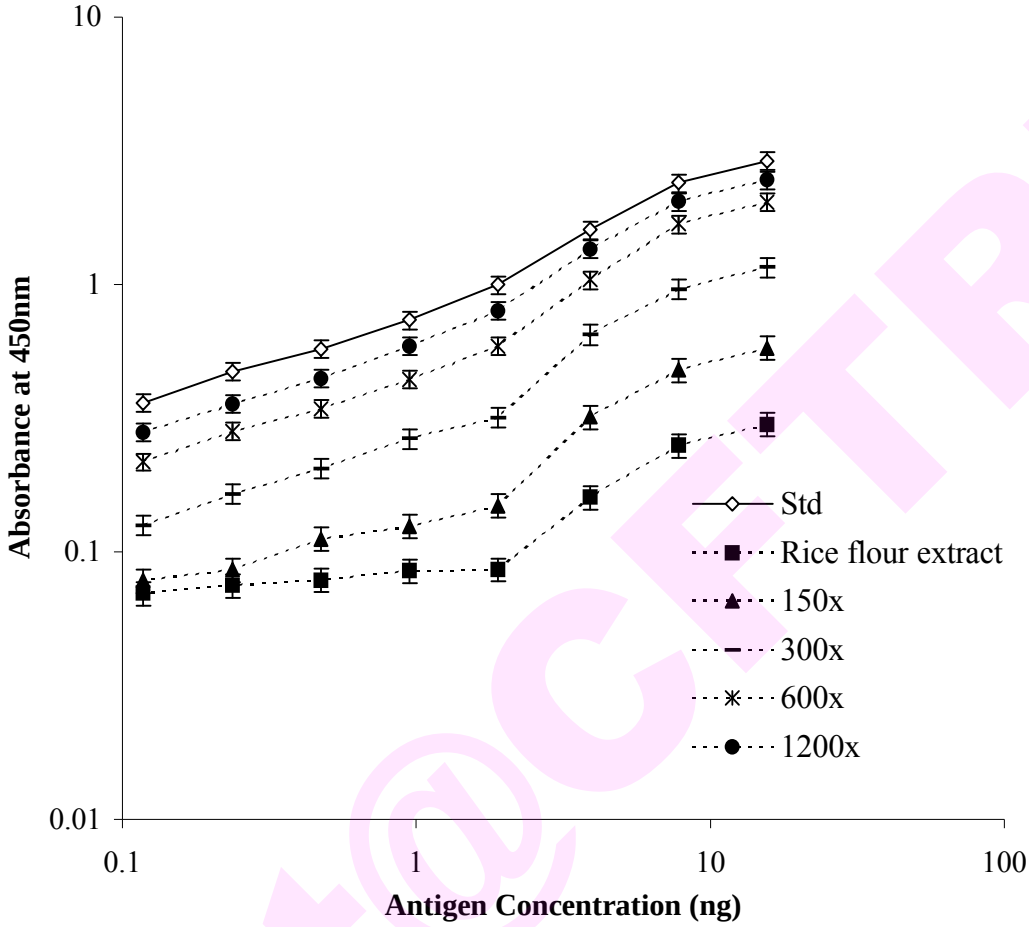


Fig.4.14. Effect of dilution on matrix interference of rice flour extract

(Data are expressed as mean ± SE of three separate experiments)

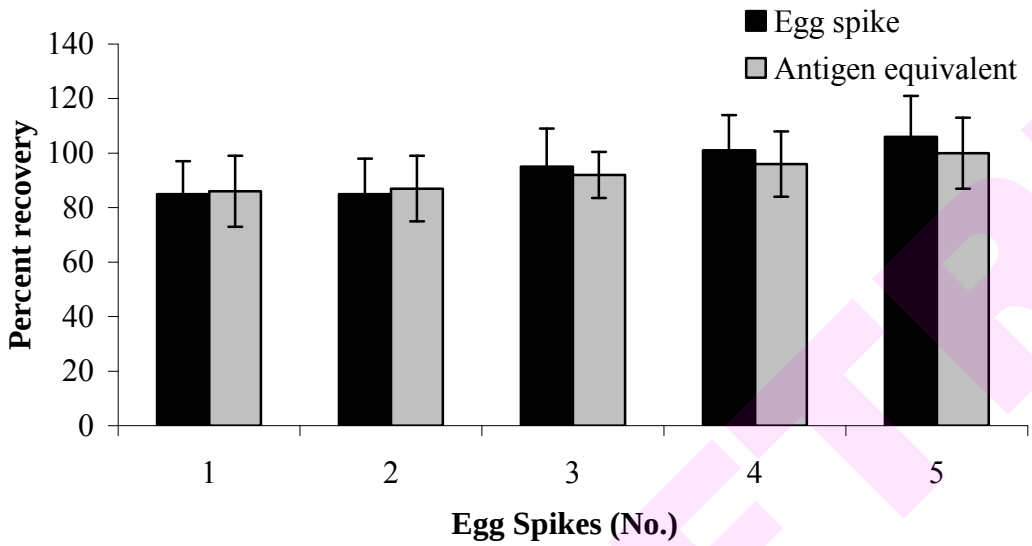


Fig.4.15. Spike and recoveries in whole wheat flour

(Data are expressed as mean ± SE of three separate experiments)

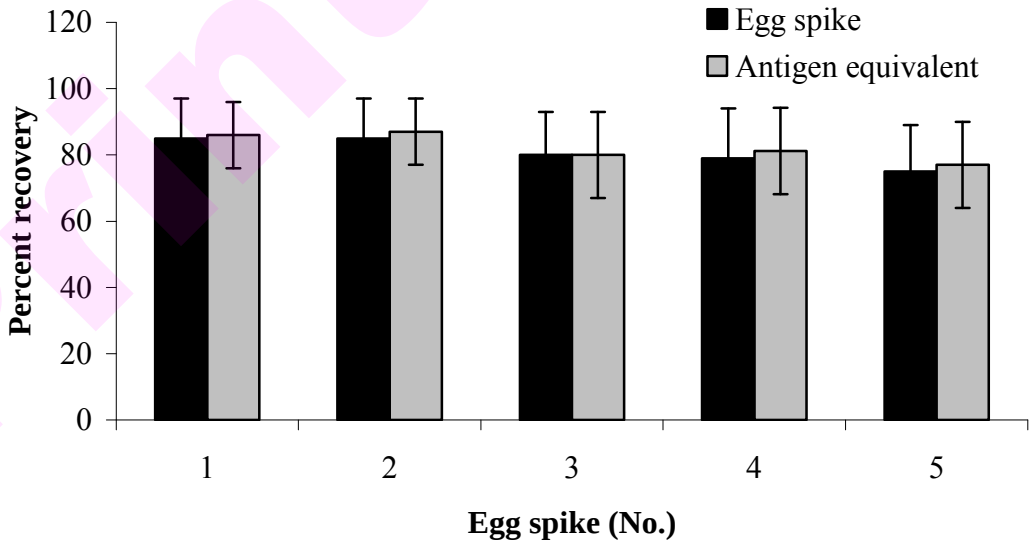
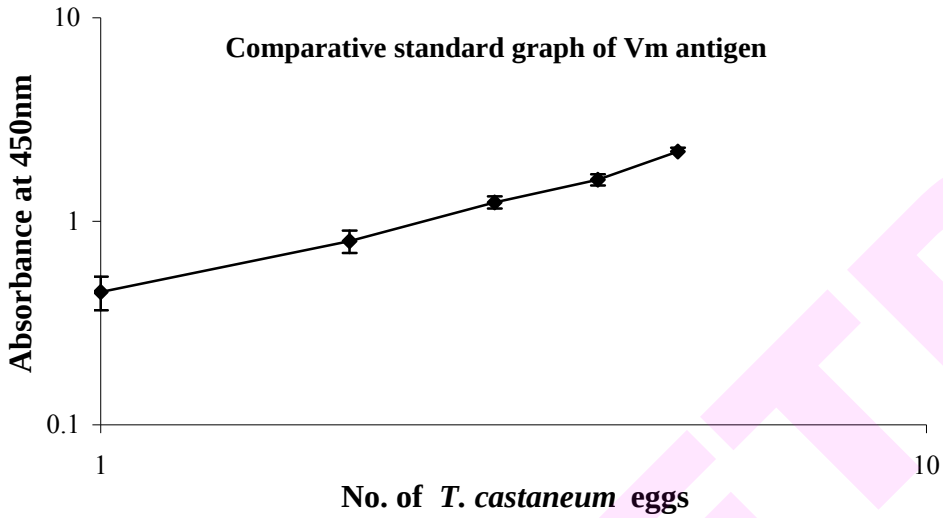


Fig.4.16. Spike and recoveries in rice flour

(Data are expressed as mean ± SE of three separate experiments)



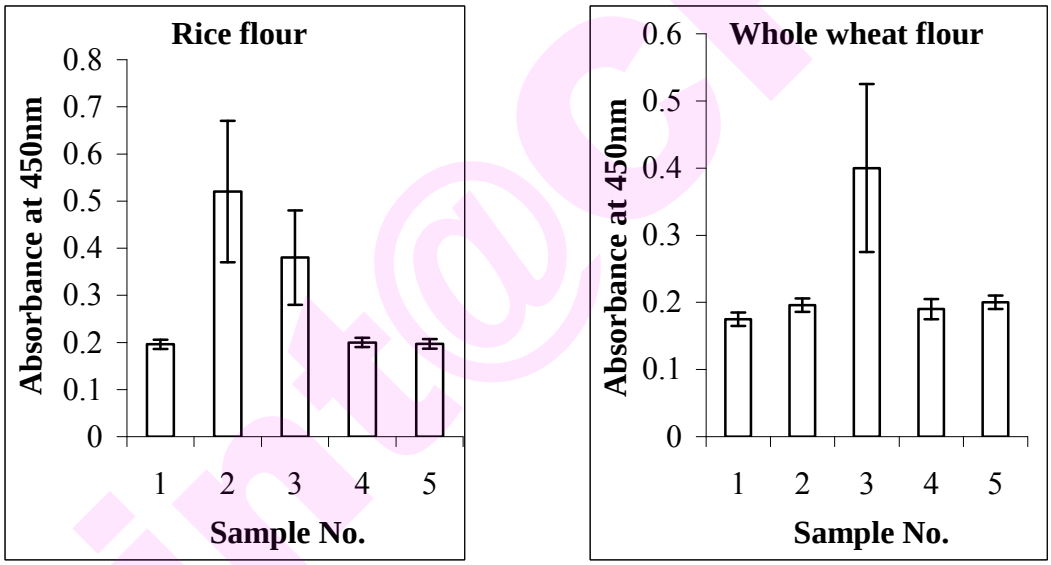


Fig.4.17. Testing of market samples of whole wheat flour and rice flour

(Data are expressed as mean ± SE of three separate experiments)

DISCUSSION

Polyclonal antibodies, widely used in research and diagnostics, are conventionally isolated from the blood of immunized mammals, especially rabbits. The fact that antibodies can also be detected in the yolk of the eggs laid by immunized hens, led to the development of the yolk antibody technology as an alternative method, which is less stressful to animals. Since hens can be kept under nearly natural conditions and antibodies can be isolated from the eggs, this technology has become an interesting alternative to the blood-collecting techniques (Schade *et al.,* 2005).

As with mammals, the immune response in hen is affected by three variables: antigen, adjuvant and the immunized animal. The properties of antigen that influences the development of the specific antibody are the quality and quantity of the antigen used. In 1945, Landsteiner pointed out that there exists a relation between the molecular weight and immunogenicity. The minimum molecular weight necessary to obtain a sufficient immune response is similar in both mammals and chickens and ranges from 5 to 10kD. The immunization of hens with proteins results normally in a good antibody production (Schwarzkopf *et al*., 2000). The amount of antigen used is relevant to the immune response. Optimal doses of antigens range between 10µg and 1mg (Mahn, 1998). Often in case of booster-immunizations, a smaller amount of the antigen is used than for the primary immunization. This way the highly affine B-cell receptors bind the antigen and mainly these B-cells clones are stimulated. This is to obtain antibodies with high affinity (Hudson and Hay, 1991). Adjuvants i.e. B-cell stimulating immune modulators are used to support the production of antibodies. Although this stimulation takes place independent of the kind of antigen and is thus non-specific, it improves the quantity and specificity of an immune response (Freund, 1951). Fruend's complete adjuvant (FCA),

which contains inactivated mycobacteria in paraffin oil, is considered as the 'gold standard' and is the most often used adjuvant for immunization. It is an extraordinary potent immune stimulant and owing to the good immune response to even weak antigens; its often-undesirable side effects are tolerated (Leenaars *et al.,* 1995).

In the present study, as mentioned earlier the antigen used is a phospholipoglycoprotein -major egg proteins of *T. castaneum* with a molecular weight of approximately 440kD, administered at 1mg for primary immunization in FCA followed by 0.5mg in the booster immunizations, all parameters in the optimal conditions for immunization to obtain good quantity and quality of polyclonal antibodies. This is reflected in the highly sensitive antibodies obtained in good quantity with a minimum detection limit of 0.1ng (Fig 4.5 and 4.9).

The antibody titer development in hens is same as observed in case of mammals, which depends on the immunogenicity of the antigen. The development takes one of the two forms. In the first type, after the booster dose, the development of antibodies rises for about 10 days then reaches a constant level lasting for about another 10 days before falling. After the second booster, the same trend is again repeated. In the second type after the booster, the antibody titer rises then remains for a longer time at the level reached (Schade, 2000). In the present study, the titer development followed the first type, with rise and fall in the antibody titer with booster doses. This result is supported by reports on IgY production against antigens of *Cryptosporidium* sps. bovine gamma globulin (Schade, 2000), ZON-BSA conjugate (Pichler, 1999), *Mycoplasma gallisepticum* (Tosil *et al*., 2004) etc. to list a few.

Further, when an animal is given booster doses of the same antigen, a much faster, more potent and more persistent response occurs. The booster doses i.e. the

secondary response yields higher affinity antibodies than those obtained in the primary dose. This can be attributed to the presence of higher numbers of helper T-cells and memory B-cells in the immune system of the animal (Harlow and Lane,1988). Similarly, in our study, we have observed that the there was an increase in the antibody sensitivity with subsequent injections of the same antigen as compared to that of the first booster dose. Further, sensitivity of the antibodies remained almost unaffected (MDL ranging between 0.1-0.3ng) from the second booster dose till the fifth booster dose.

Egg yolk is a fluid emulsion with a continuous phase of protein and a dispersed phase of lipoprotein particles. Yolk lipid thus exists associating with protein as lipoprotein. N-PAGE of diluted egg yolk showed five protein bands in the separation gel which corresponded to lipovitellin, γ-livetins, β-livetins, α-livetins and phosvitins. IgY is one of the livetins (γ-livetin) (Burley *et al.,* 1993). Therefore, removal of the lipoprotein (or lipid) is an important step for the purification of IgY. Many purification methods for Igy based on this strategy have been reported, e.g., lipoprotein separation by ultracentrifugation, delipidation by organic solvents, and lipoprotein precipitation by PEG or sodium dextran sulfate or natural gums such as carrageenan and xanthan gum.

In the present study lipid/lipoprotein, precipitation was carried out using chloroform, carrageenan and PEG. In a critical comparison of reported methods for the purification of egg yolk antibodies by Deignan *et al*. (2000), in the lipid removal studies using PEG (3.5%), the mean protein yield was 19.8 mg/mL and the IgY purity was low (57.1%) and hence the IgY yield was only 11mg/mL. In line with these findings, in the present studies of lipid removal using 3.5% PEG, it was observed that there was residual lipoprotein contamination in the WSPF (Fig. 4.2.b). In a report on lipoprotein precipitation by carrageenan (Hatta *et al*. 1990), it was observed that the number of

proteins obtained in the supernatant, after treating the egg yolk with carrageenan, were significantly decreased and the purity of IgY thus obtained was about 19.4% and there was almost negligible contamination of lipoprotein in the WSPF. Delipidation of egg yolk using chloroform yielded IgY that was 57% pure (Polson, 1990). In the present study after chloroform and carrageenan treatment for lipid removal, it was found that the WSPF obtained did not have any significant lipoprotein contamination and the IgY was found in a mixture of other water soluble proteins (Fig. 4.2.a&c).

Lipid removal using chloroform and gum removes significant quantities of protein as well as lipid, that the subsequent IgY precipitations yield very low levels of protein. Lipid precipitation using 3.5% PEG recovered higher protein yields (Deignan *et al.,* 2000). In the present study, following each of the three different methods used for lipid removal, the immunoglobulin precipitation from the WSPF was done using PEG at 12% for method 2 and at 14% for method 1 and 3. It was found that the protein yield in case of method 2 was 4.3 folds higher as compared to method 1 and 3 (Fig. 4.3). The higher protein yield may be because of the other contaminating protein present along with IgY, nonetheless, the 4.3 fold increase in the protein yield cannot be attributed solely to the contaminating protein. However, the lowered sensitivity observed with M2 purified IgY could be attributed to the contaminating protein present along with the IgY fraction.

The activity of the purified IgY is checked to know the effect of the chemicals and physical conditions such as freeze and thaw used in purification on the antibody activity and is generally studied by ELISA (Akita and Nakai, 1992). Residual PEG in the IgY has undesirable effect in biological systems such as excessive osmotic pressure (Polson *et al.,* 1985). PEG may also interfere with the assay performance. Excessive use of solvents effects the IgY and thereby the assay performance (Brun *et al.,* 2004). In the

present study it was found that both carrageenan and chloroform at 20% did not affect the assay performance. The antibodies thus purified were sensitive and was able to detect 0.1ng of the antigen. The IgY purified using PEG (M2) were not sensitive as compared to that purified by carrageenan and chloroform and the minimum detection limit of the assay was 4.5ng. The lower sensitivity may not be due to residual PEG as all the three methods used PEG for IgY precipitation and thus lower sensitivity should have been observed for all. Further all the IgY samples were dialyzed in 10kD cutoff cellulose membrane, thus, all the PEG would be removed from the IgY solution. The lowered sensitivity observed in case of PEG purified IgY may be because of the low purity of the IgY sample due to comparatively higher contaminating protein as compared to that in chloroform and carrageenan purified IgY samples.

Egg yolk antibodies are used extensively in human and veterinary medicine. Schade (2000) gives an overview of over 200 publications on diagnostics and therapeutic applications of specific egg yolk antibodies including egg yolk antibodies against virus antigens (both plant and animal viruses), bacterial antigens, antigens of parasites and other pathogens, proteins and peptides, enzymes, hormones and venoms.

Specific ELISA based on egg yolk antibodies have been developed for varied applications such as detection of fungal toxins like Ochratoxin A, zearalenone (ZON) (Pichler, 1999), direct sandwich ELISA for detecting bovine IgG, for detecting *Ascaris suum* antigens (Schniering,1996), for detection of antibody from horses against dourine (*Trypanosoma equiperdum*), ELISA to determine the content of mouse monoclonal antibodies by means of a mouse Ig-specific capture antibody and a labeled detecting antibody from chicken etc.

Currently IgY based ELISA is also being reported in the field of pesticide residue

analysis, in detection of cell death markers and insect infestation detection in stored products, and that the IgG based assay was comparable to the IgY based assay (Amitha rani *et al*., 2006, in press). The methods that have been reported for insect infestation detection are mainly IgG based myosin detection assays (Kitto, 1991; Quinn *et al.,* 1992; Schatzki *et al.,* 1993). The assays developed for insect infestation detection and different vitellin based assays reported for various purposes are discussed in detail in chapter 3, 'Discussion'. There has been one report on the IgY based assay for insect infestation detection by Amitha rani *et al.,* (2006, in press). However, these methods based on IgG and IgY for insect infestation detection can detect almost all life stages except eggs as it is based on the muscle protein-myosin, which is absent in the eggs. The present study, for the first time reports a species-specific vitellin based IgY assay that can detect all the stages of *T. castaneum* with varied sensitivities. Further, vitellin based immunoassays are usually species specific as antibodies against one insect vitellin does not cross react with that of the other as these are rapidly evolving proteins (Kunkel *et al.* 1976). In the present study, as observed for IgG based assay, the *T. castaneum* vitellin antiserum showed low crossreactivity with *Rhizopertha dominica* and *Corcyra cephalonica* egg protein, and there was no observed crossreactivity with *Lasioderma serricorne* egg protein. Therefore the assay could be highly specific for detection of *T. castaneum* infestations.

Application of immunoassays to food commodities especially milled products poses challenge as the food components affect the assay performance (Kitto, 1991). The potential problems and the effective ways in resolving these interferences on the assay are discussed in detail in chapter 3, 'Discussion'. The IgY based assay developed was tested for its application in whole-wheat flour and rice flour. It was observed that whole-

wheat flour components resulted in false positive results. This could be probably because of the flour components such as lectins binding to the carbohydrate moiety of the antibody (Kitto, 1996). It is known that IgY as compared to that of IgG has two carbohydrate chains, hence, the probability of more non-specific binding to the carbohydrate chains. However, in case of IgG based assay there was reduced assay signal intensity. In case of rice flour, the flour components reduced the assay signal both in IgY and IgG based assay. Various parameters influence the assay performance such as type of food, steric hindrances, non-specific binding of the food components to the antibody or antigen, blocking of antigen binding sites on the plate or antibody binding sites on the antigen, etc. which in turn may lead to either reduced or increased assay signals.

Dilution of food matrix is a simple and non-denaturing method especially for protein assays to negate the matrix effect on the assay (Van Emon *et al*., 1992). Immunoassays are highly sensitive, hence even traces of antigen present in the diluted extract can be detected. In the present study, the antigen used is a protein and as the assay developed is based on the highly sensitive antibodies with a minimum detection limit of 0.1 ng, which is equivalent to 1:73,000 parts of the total extractable egg protein, dilution could be used as an effective strategy to eliminate the matrix. The wheat flour extract diluted to 1:1000 and rice flour extract diluted to 1:1200 gave comparable results as that of the standard assay.

Good recovery of the analyte from fortified samples indicates accuracy in the extraction method and the immunoassay. Failure to accurately recover added analyte may indicate the presence of interfering substances in the sample matrix. In the present study, the spike recoveries in wheat flour was 90-100% and in rice flour 75-85. Spike recovery studies indicated that unlike IgG based ELISA, in IgY based ELISA there was no

observed decrease in the spike recovery with increase in the number of egg spikes. In case of rice flour there was slight decrease in the spike recovery with the increase in the number of egg spikes. However, the difference in the spike recoveries between the lowest egg spike (1No.) and the highest egg spike (16No.) was only 10% unlike in case of IgG based assay the difference was almost 50% between the lowest and the highest egg spikes.

Good spike recoveries in the present study indicated that matrix interferences have been effectively reduced and that the extraction method for the antigen from the sample was accurate. Therefore, the method developed could be used in the analysis of rice flour and whole wheat flour samples for *T. castaneum* infestation.

Table 4.2. : Comparison of the IgG and IgY based ELISA developed for detection of incipient stages of *T. castaneum*

	IgG based assay	IgY based assay
Host	New Zealand white female rabbit	White leghorn hen
Amount of antibody/animal or bird	~250mg (~7mg/1mL sera, total 5 bleed, sera obtained 5-7mL)	~5000mg (~40mg/egg, 18 weeks x 7 eggs)
Sensitivity of the antibody (expressed as minimum detection limit)	0.1ng	0.1ng
Amount of sensitive antibodies obtained	~150mg (I, II & III bleed)	~4000mg (from week 3 to week 18)
Minimum antibody required for ELISA	1µg/well	1µg/well
Detection of developmental stages of *T. castaneum*	All stages detected with varied sensitivity	All stages detected with varied sensitivity
Crossreactivity of the antibodies with other insect egg proteins	Low/negligible with other insect egg proteins	Low/negligible with other insect egg proteins
Application:		
(a) Whole wheat flour	Applicable	Applicable
o **Matrix interferences on the assay**	Yes, false negative results	Yes, false positive results
o **Removal of matrix interferences**	Dilution	Dilution
o **Spike recoveries**	85-100% False negative at higher egg spikes, negated with dilution	90-100% No such effect
(b) Rice flour	Applicable	Applicable
o **Matrix interferences on the assay**	Yes, false negative results	Yes, false negative results
o **Removal of matrix interferences**	Dilution	Dilution
o **Spike recoveries**	79-100% False negative at higher egg spikes, negated with dilution	75-85% No such effect
Total no. Ab coated ELISA plates /animal (at 1µg Ab/well)	~1500 assay plates	~4100 assay plates
Cost of one lab kit contanining one Ab coated plate*	Rs. 47/-	25 paise

*** Economics as quoted in Amitha Rani *et al., 2006***

CONCLUSION

Highly sensitive and specific antibodies could be obtained from the egg yolk of the White Leghorn hen against the major egg protein of *Tribolium castaneum*–vitellin. The egg yolk antibodies could be easily purified with almost 90-95% purity by two methods using combinations of chloroform & PEG, and carrageenan & PEG. The highest average yield of IgY/egg was approximately 60mg between weeks 3 to 7. The ELISA developed using these antibodies proved to be highly sensitive with a minimum detection limit of 0.1ng of the protein which is equivalent to 1/73,000 parts of the extractable egg protein. The method could also detect minimum of one number of the various stages of development such as larva, pupa and female and male adult insect. The *T. castaneum* vitellin antibodies showed low crossreactivity with egg protein from *Rhizopertha dominica* and that of *Corcyra cephalonica*, and there was no observed crossreactivity with the egg protein from *Lasioderma cephalonica*. Therefore, the ELISA developed is not only highly sensitive but also specific for the detection of eggs and other developmental stages of *Tribolium castaneum*.

The IgY based ELISA developed for the detection of eggs of *T. castaneum* was applicable for both whole wheat flour and rice flour. Simple dilution of the food extract could be used as an effective strategy for matrix removal for both the food extracts. The spike recoveries for whole wheat flour and rice flour were 90-100% and 75-85% respectively. Therefore, the IgY based ELISA developed was successful in its application for whole-wheat flour and rice flour, with easy matrix clean up procedure and good spike recoveries. The sensitivity, assay performance and applicability of the IgY based assay was comparable with that of the IgG based assay as described in chapter 3. Conclusively, the various advantages of IgY antibodies makes it a good alternate to IgG based assay.

CHAPTER V

COMPARISION OF THE ELISA DEVELOPED WITH OTHER METHODS FOR INSECT EGG DETECTION

INTRODUCTION

Insect infestation is one of the major problems faced by the milling industry wherein the milled products of cereals get infested by insect pest eggs which gets transferred to the final product. The red flour beetle, *Tribolium castaneum*, the saw-toothed grain beetle, *Oryzaephilus surinamensis*, and the rice moth, *Corcyra cephalonica* infestation are generally observed in plan-sifters, separators and conveyors (Leelaja *et al.*, 2006). From these sources, insect infestation is likely to be passed on to finished products like whole wheat flour, refined wheat flour and rice flour. Subsequently, the level of infestation in milled products increases according to the storage period in the retail market. Stored product insect pest egg detection methods thus play an important role in the milling and packaging industry.

In infested flour, the larvae, pupae and adults are visible due to their larger size as compared to that of the eggs. However, eggs of insects are very small in size hence difficult to distinguish by the naked eye from that of the flour particles. Eggs of *T. castaneum* and *T. confusum* are of 0.61mm x 0.35mm in size (Sokoloff, 1972), *O. surinamensis* of 0.72mm x 0.242mm (Kucerova and Stejskal, 2002) and *C. cephalonica* 0.5mm x 0.3mm in size (Hodges, 1979). In addition, adherence of flour particles to the eggs makes their identification even more difficult. There are a few reports on the detection methods for the eggs of stored product insect pests in food commodities. Abels and Ludeshcher (2003) reported that eggs of several stored product insects including *T. castaneum*, *T. confusum*, *O. surinamensis*, *C. cephalonica*, *Lasioderma serricorne*, *Rhyzopertha dominica*, *Ephestia kuehniella* and *Plodia interpunctella* exhibited fluorescence under long wave (365nm) UV excitation. However, autoflourescence of some of the food commodities interfered with the utility of this

technique. For example, blue fluorescence from wheat flour severely interfered with detection of eggs.

There are staining methods to detect insect eggs like egg plug staining in whole grains using acid fuchsin, gentian violet and berberine sulfate. However, these are used for *Sitophilus* spp.. Limited studies have been carried out on the detection of eggs of stored product insects by staining method in milled products. Some of the methods used for milled products are the American Association Cereal Chemists (AACC) iodine stain method and the acid-alkali method (Blumberg and Ballard, 1941). These methods are used for white flour or refined wheat flour. In the iodine method, the eggs stained yellow against brownish yellow flour particles. In the acid-alkali method, the eggs were yellow-orange even without the addition of any stain and the flour particles were yellow or orange red. They also reported that the contrast between eggs and flour particles can be improved by staining with either 1% aqueous iodine solutions or with Ehrlich's triacid stain and the flour particles appeared blue and green, respectively. Leelaja *et al.* (2006) has reported a new staining method using 0.1% bromocresol green for detection of insect pest eggs in refined wheat flour. The studies showed that bromocresol green differentially stained the insect eggs and the flour particles distinct orange and green, respectively.

Development of new and novel methods of detection offers considerable opportunities. However, the key factor for acceptance of any method in the routine analysis is its comparative or better efficiency and sensitivity with that of the well established methods. This evaluation of the method developed would enable its future use in the routine analysis of samples. This chapter deals with the comparison of the ELISA developed for the detection of the incipient stages of *T. castaneum* with special reference to eggs in food commodities with two different staining methods i.e. AACC iodine

staining method and bromocresol green staining method. The chosen food commodities are same as that used for application of the ELISA developed i.e. whole wheat flour and rice flour.

MATERIALS AND METHODS

1. Chemicals: Iodine, sulfuric acid, bromocresol green, ethanol, sodium hydroxide, filter paper were purchased locally. All the chemicals obtained were of analytical grade. Good quality whole-wheat flour and rice flour were obtained locally.

2. Egg staining methods: The sensitivity and efficiency of the ELISA developed for detecting *T. castaneum* eggs was compared with two different staining methods. The eggs of *T. castaneum* for this study were collected as described in chapter II under section 4.

(1) Iodine method: Iodine method is an AACC approved egg staining method to detect insect pest eggs in refined wheat flour. The iodine staining of the eggs was performed as follows:

(a) 100g of whole-wheat flour/rice flour/refined wheat flour was spiked with known number of eggs.

(b) The flour was then sieved using 80 mesh sieve and the flour particles retained in the sieve containing eggs was wetted with 2-3mL alcohol in a beaker.

(c) Then 40mL of 10% sulfuric acid was added to digest the flour particles, covered with petridish and kept in a steam bath for 10min.

(d) It was then filtered through Whatman No. 1 filter paper on Buchner funnel under low suction.

(e) Then 15mL of 0.1N iodine solution was added and filtered after 2-3mins under mild suction.

(f) It was then washed with 10% sulfuric acid and the filter paper transferred onto a petridish and examined under binocular microscope. Eggs stained yellow against brownish yellow flour particle

(2) **Bromocresol green method:** This method was reported by Leelaja *et al*. (2006) to detect insect pest eggs in refined wheat flour. The staining of eggs was performed as follows:

(a) 100g of whole-wheat flour / rice flour/refined wheat flour was spiked with known number of eggs.

(b) The flour was then sieved using 80 mesh sieve and the flour particles retained in the sieve containing eggs was wetted with 2-3mL alcohol in a beaker.

(c) 40mL of 10% sulfuric acid was added to digest the flour particles, covered with petridish and kept in a steam bath for 10min.

(d) It was then filtered through Whatman No. 1 filter paper on Buchner funnel under low suction.

(e) Then 15mL of 0.1% Bromocresol green stain prepared in 100mL of distilled water contaning 1.45mL of 0.1N sodium hydroxide was added and filtered after 2-3mins under mild suction.

(f) It was then washed with 10% sulfuric acid and the filter paper transferred onto a petridish and examined under binocular microscope. Eggs stained yellow-orange against green flour particle.

RESULTS

1. Detection of eggs of *T. castaneum* by bromocresol green staining method: The bromocresol green egg-staining method was not applicable for whole-wheat flour as the eggs were masked by the wheat flour particles. Fig. 5.1.b shows a portion of the filter paper containing sparsely spread whole-wheat flour particles with egg, for clarity of staining results. In rice flour, eggs were difficult to detect as they were masked by the swollen starch particles (Fig.5.1.c). However, in refined wheat flour (Fig.5.1.a) the eggs were clearly distinguishable from that of the flour particles.

(a) Whole wheat flour: In whole-wheat flour, the eggs stained yellow against green stained flour particles (Fig. 5.1.b). However, all the eggs were not visible under binocular microscope. The eggs were masked by the whole-wheat flour particles even at the minimum sample size taken for staining i.e. 0.1g due to the larger residual flour particles and also attributable to the higher the volume of the flour particles attributable to the thin flaky nature of the flour residues as compared to that of refined flour. Further, the flaky flour particles did not adsorb to the filter paper unlike that observed in case of refined flour, therefore, during addition of stains and acid/water washes they floated in the solution and settled on the filter paper as lumps when mild suction was applied, thus masking the eggs. The flour particles were not easily digestible even with 10% sulfuric acid as compared with that of refined wheat flour. Moreover, the amount of residual flour particles was 20% as compared to that of refined wheat flour (1.6%), which makes it difficult to stain these flour particles in a single step, thus requiring that the sample be divided into small quantities in order to facilitate the sample handling.

(b) Rice flour: In rice flour, the flour residue particles stained green, some particles stained light blue and the eggs appeared green (Fig. 5.1.c). However, the eggs were

difficult to detect due to the starchy flour particles masking them. The rice flour particles swelled up into starchy particles when placed in steam bath covering the whole surface of the filter paper, this made the filtration in the following steps slower also masking the eggs. Further, the starchy particles required high volumes of water washes (approximately 500mL for 0.1g of flour particles) to make the flour particles alkaline in order to regain the green staining from orange after acid wash. The volume of water wash required increased with the increased time of exposure of the flour particles with diluted sulfuric acid due to slow filtration. Moreover, the residual rice flour particles was about 15%, as compared to that of refined wheat flour (1.6%), which makes it difficult to stain these flour particles in a single step, thus, requiring that the sample be divided into small quantities in order to facilitate the sample handling.

2. Detection of eggs of *T. castaneum* by iodine staining method:

The iodine egg staining method was not applicable for whole-wheat flour (Fig. 5.2.b) as the eggs were masked by the wheat flour particles and in rice flour eggs were difficult to detect as they were masked by the swollen starch particles and in addition the flour particles including eggs stained deep blue (Fig.5.2.c). However, in refined wheat flour (Fig.5.2.a) the eggs which stained yellow were clearly distinguishable from that of the flour particles which stained yellow-orange.

(a) Whole-wheat flour: In whole-wheat flour, the eggs stained yellow and flour particles stained brown with few flour particles stained blue. The staining results using iodine in wheat flour was same as that observed for bromocresol green staining method. The eggs were masked by the flour particles hence not easily detectable.

(b) Rice flour: Iodine staining method in rice flour resulted in deep blue-stained eggs and also the flour particles with some particles stained brown. The drawbacks observed in

iodine staining was same as that discussed under bromocresol green staining for rice flour. Further, as iodine stains starch blue, both the flour particles and the starch-coated eggs stained blue thus making it difficult to distinguish the flour from that of the eggs. Moreover, all the spiked eggs were not visible due to masking by the flour particles. Therefore, iodine method was not applicable for rice flour. A comparison of the ELISA developed and the staining methods are given in Table 5.1.

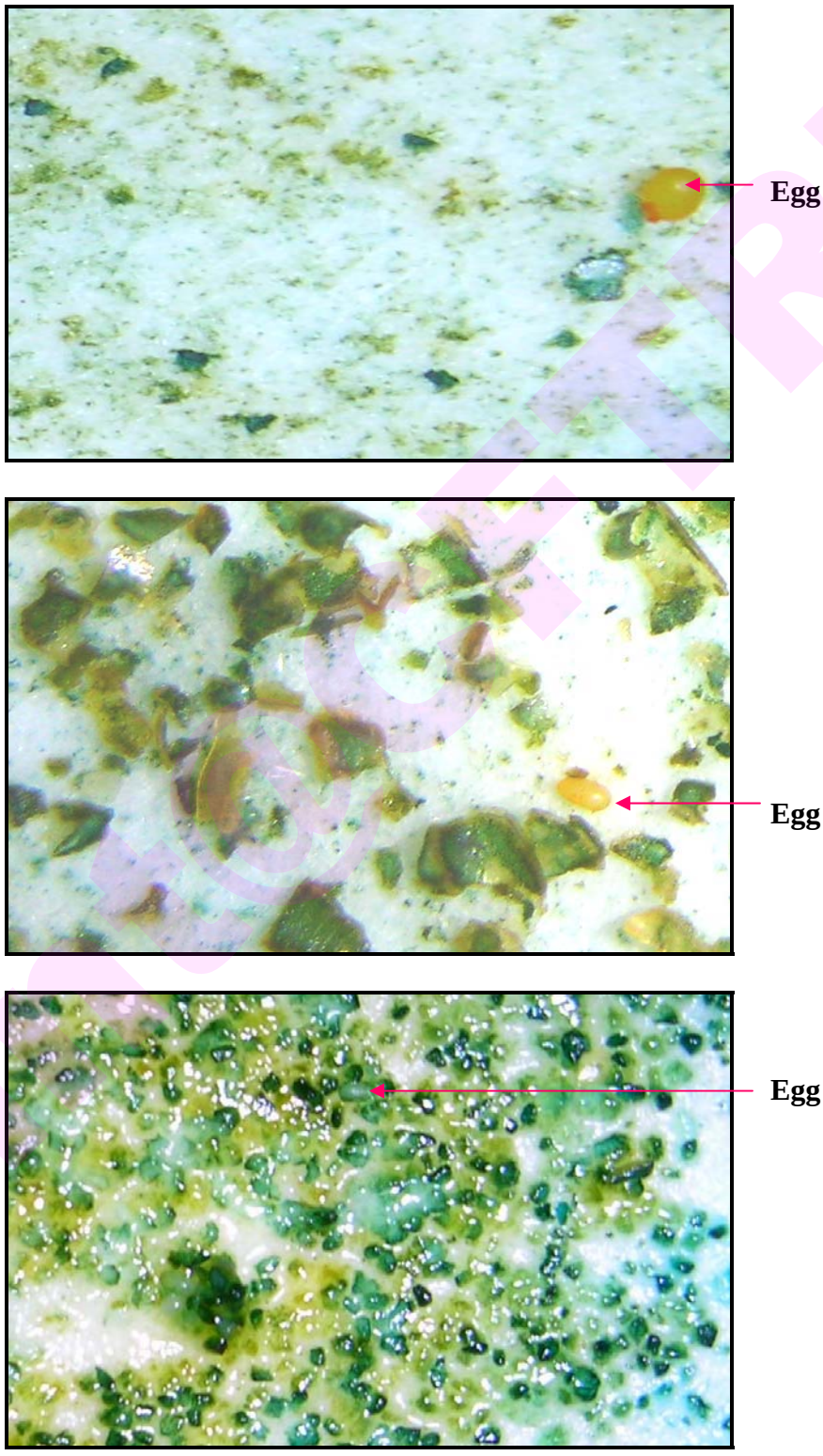


Figure 5.1. Comparison of bromocresol green staining method for egg in (a) refined flour, (b) whole wheat flour, (c) rice flour

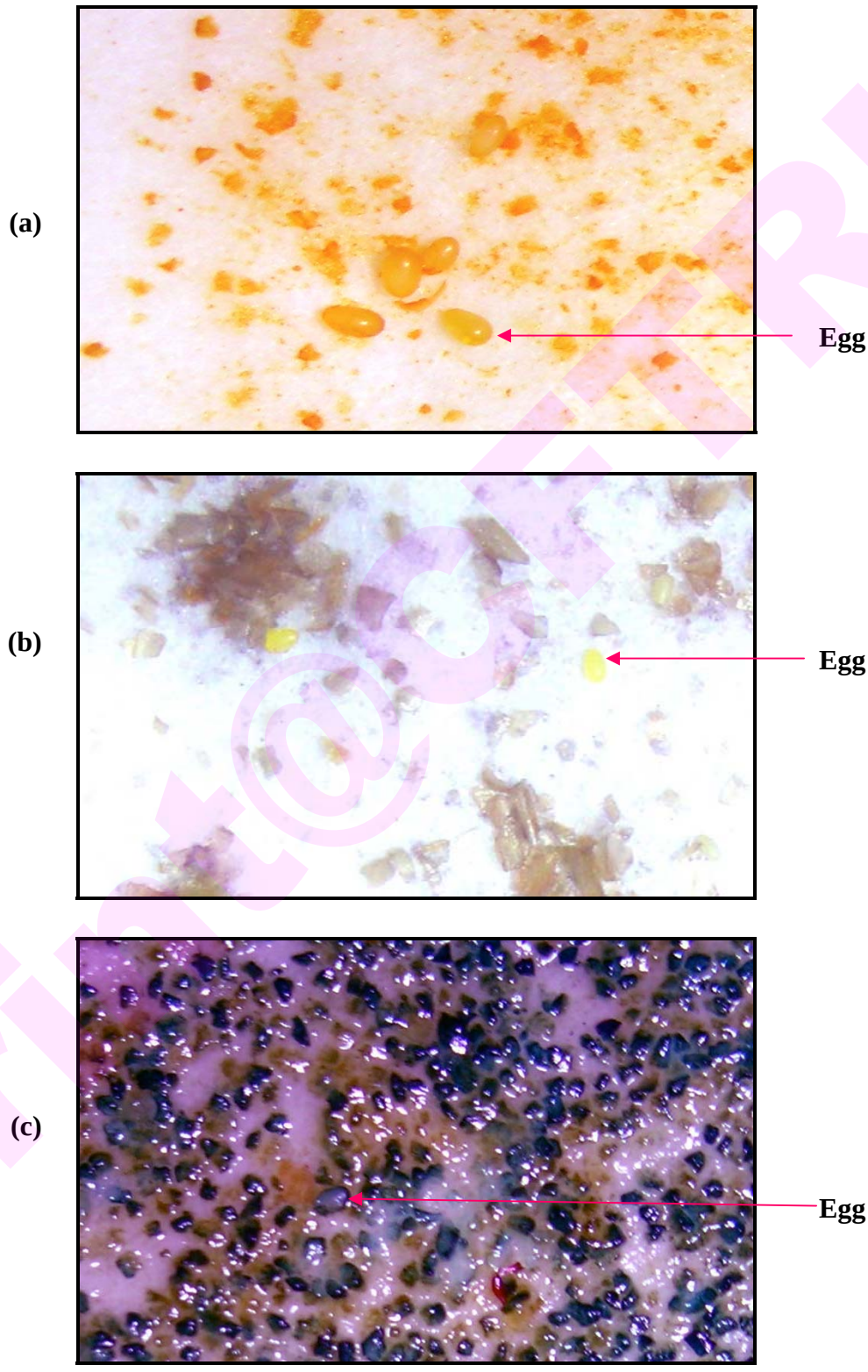


Figure 5.2. Comparison of iodine staining method for egg in (a) refined flour, (b) whole wheat flour, (c) rice flour

DISCUSSION

Staining secretions (egg plugs) or body fluids of insects (hemolymph) and entry holes as a means of detecting hidden insect infestation in food commodities were considered as early as in the 1950's. The staining method is a direct method of establishing hidden living infestation in a commodity.

Weevils including *S. oryzae*, *S. granarius* and *S. zeamais* attacking stored cereals deposit their eggs inside the grains and plug the holes or egg cavities with saliva. These mucilaginous secretions of weevils are stained with acid fuchsin, gentian violet or berberine. These methods are not applicable for other internal infesters like *R. dominica* and *S. cerealella* that oviposit outside the grain. Ninhydrin has been used to react with the body fluids of insects developing inside grains. However, these staining techniques are specific for internal infesters. There are staining methods for detection of external infesters *T. castaneum*, *O. surinamensis* and *Corcyra cephalonica*. In the AACC method (2000) using iodine as staining agent the eggs stain yellow against orange-yellow refined flour particles. In the Blumberg and Ballard acid alkali method, which involves treatment with 5% nitric acid, followed by 10% sodium hydroxide, the eggs stain orange even without the addition of stain. In a recently published method for egg staining using bromocresol green (Leelaja *et al.,* 2006), the eggs stain orange against green refined flour particles, which gives a good contrast thus aiding in easy distinction of the eggs from the flour.

The AACC method is an accepted method, which is used for the routine detection of insect pest eggs in flour. The bromocresol green staining method is a recently reported method for detection of insect pest eggs in refined flour (Leelaja *et al.,* 2006). This method was developed to overcome the drawbacks of other staining methods like AACC

iodine method, which showed low contrast between the flour particles and the insect pest egg on staining. In the present study, the AACC iodine stain method and the bromocresol green methods were tested for their sensitivity and applicability in whole wheat flour and rice flour as against the refined flour for which they were originally developed. The feasibility of application of these two staining techniques is good in refined flour because of the low residual flour particles obtained after sieving (~1.6%), hence the sample handling becomes easier and quicker. Further as the flour particles do not interfere with the staining or mask the eggs as the flour particles are small and are sparse as they are digested using acid, the detection of eggs is easier. However, in case of whole wheat flour and rice the feasibility of application of the two staining techniques is low because of the large amount of residual flour particles obtained (20% and 15%, respectively), hence, the sample handling becomes elaborate and laborious as only small sample sizes can be taken at a time for staining. Nevertheless, in ELISA the minimum sample size that can be taken per sample is 100g and many samples can be tested at a time (at least 88 samples/plate).

Due to the high amount of residue flour particles in both rice flour and wheat flour, the sensitivity of the staining techniques at minimal infestations (one egg/100g) is low due to the drawbacks observed in application of the iodine and bromocresol green staining techniques such has masking of eggs by the flour particles, low contrast between the rice flour particles and the egg, etc. Hence, minimal infestations may not be detectable or clearly visible. Further, there can be loss of the egg during sample processing thus indicating false negative results. However, the ELISA developed in the present study could detect a minimum of one egg and the sensitivity of the assay expressed as parts of an egg is equivalent to 1:73,000. Further, when the sample is processed the egg protein remains in the extract and added to it the sensitivity of the

assay which can detect even traces of the egg protein (0.1ng) makes the ELISA developed applicable even at minimal infestations.

The staining techniques in refined flour gave good contrast and hence easy detectability of eggs. However, in case of rice flour, the eggs stained blue as that of the flour particles in iodine method and green in bromocresol green method. Hence, due to the similar stain picked up by both the flour and the egg, they were not easily differentiated from each other, unless and otherwise carefully observed. The staining of eggs same as the color of the flour could be because of the starchy rice flour particles coated onto the eggs during boiling. In ELISA, such interferences of food components can be removed by specific clean up methods like dilutions as used in the present study and the effect can be negated. Since ELISA is based on the highly specific reaction between the antigen and antibody, it gives the direct measure of the infestation present in the food sample.

Table 5.1. : Comparison of the efficiency of ELISA and staining methods (AACC iodine method and bromocresol green method)

	ELISA	Staining method
Sensitivity	Can detect 1/73,000 parts of an egg	*WWF & RCF:* Not sensitive, cannot detect *RF:* Sensitive
Specificity	Specific	*WWF & RCF:* The eggs can be distinguished from the whole-wheat flour when detected. In rice flour, the eggs are not easily distinguishable. *RF:* Specific, the eggs can be distinguished from the flour
Sample size/test	100g of sample/test	*WWF & RCF:* 0.1g/15-20g of residual flour particles /100g sample/test *RF:* 100g of sample/test
Sample handling	Easy, can test 88 samples (100g each sample) per assay plate	*WWF & RCF:* Laborious, smaller quantities of 15-20g of residual flour particles/one sample (100g)/test *RF:* Easy
Throughput	High	*WWF & RCF:* Low *RF:* Low
Data interpretation	Easy, can be discerned from the standard curve	*WWF & RCF:* Ambiguous *RF:* Easy
Applicability	Applicable to both whole wheat flour and rice flour	*WWF & RCF:* Not applicable *RF:* Applicable

WWF- whole wheat flour, RCF - rice flour, RF- refined flour

CONCLUSION

The ELISA developed for detection of incipient stages of *T. castaneum,* with special reference to eggs, in the present study was comparatively more effective in detecting eggs in both whole-wheat flour and rice flour as compared to that of the staining methods in terms of sensitivity, specificity, sample size, sample handling, throughput, data interpretation and applicability. Thus, in the present study, ELISA proves to be a simple, quick, and sensitive method for detection of eggs of *T. castaneum* in whole wheat flour and rice flour.

CONTRIBUTION OF THE THESIS

- This is the first report on the Enzyme Linked Immunosorbent Assay (ELISA) developed for the detection of incipient and developmental stages of the red flour beetle *Tribolium castaneum* Herbst- a stored product pest, based on rabbit (IgG) and chicken egg yolk (IgY) antibodies.
- The ELISA developed based on both IgG and IgY antibodies was highly sensitive. The minimum detection limit of the assay for both the antibodies was 0.1ng.
- The ELISA developed could detect all the developmental stages of *T.castaneum*.
- The ELISA developed was highly specific for *T.castaneum*. The antibodies raised against *T.castaneum* egg protein showed low/negligible crossreactivity with other stored product insect pest tested i.e. *Lasioderma serricorne, Rhizopertha dominica* and *Corcyra cephalonica*
- The ELISA developed was applicable for detection of *T. castaneum* eggs in wheat flour and rice flour. Matrix interference of the food extracts could be easily reduced/negated by dilution of the food extract. Also the assay showed good spike recoveries ranging from 75-100%.
- The ELISA developed could be successfully used to detect *T. castaneum* infestation in unknown market samples.
- The ELISA developed was comparatively more effective than the AACC approved iodine method and bromocresol green staining method in detection of *T. castaneum* eggs in both wheat flour and rice flour.
- The ELISA developed based on IgY antibodies, due to high quantity of sensitive antibodies obtained, puts forth the possibility of using the ELISA for routine analysis of food commodities for detection of *T. castaneum* infestation.
- The ELISA developed based on both IgG and IgY antibodies thus is sensitive, specific, with easy sample handling, data interpretation and high through put and hence could be used for the analysis of wheat flour and rice flour for detection *T.castaneum* infestation at different levels of stored grain processing like milling and packaging industries and godowns.

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