Immunological Techniques For The Serum Determination Of Specific-IgE Levels Among Workers Exposed To Seafood Allergens

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DEDICATED TO MY PARENTS DANIE AND LIVIA

I declare that this thesis is my own work (unless otherwise stated). It is being submitted for the degree Magister Technologiae, to the Cape Technikon and has not been submitted before, for any examination, to any other tertiary institution. The work was carried out in the Allergology unit, Department of Immunology, Groote Schuur Hospital, University of Cape Town. The opinions and conclusions drawn are not necessarily those of the Cape Technikon.

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ABBREVIATIONS

AMPS	Amonium persulphate
CIE	Crossed immunoelectrophoresis
CLIE	Crossed-line immunoelectrophoresis
CRIE	Crossed radioimmunoelectrophoresis
DBPCFC	Double Blind Placebo-controlled oral Food Challenge
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
cDNA	complementary DNA
DTT	Dithiothreitol
EAST	Enzyme Allergosorbent-test
EDTA	ethylene diamine tetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
IEF	Isoelectric focussing
kDa	kilo Dalton
kU/l	kilo-Units per litre
LHR	Leukocyte histamine release
ml	millilitre
mg	milligram
mm	millimetre
M	Molar
Mm	millimolar
MoAB	Monoclonal antibody
mRNA	messenger Ribonucleic acid
PBS	Phosphate Buffered Saline
RAST	Radio Allergosorbent Test
RIA	Radio immunoassay
RNA	Ribonucleic acid
rpm	revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SPRIA	Solid-phase radioimmunoassay
SPT	Skin Prick Test
TBS	TRIS-buffered saline
µg	microgram

µl	microlitre

SUMMARY

Allergic conditions among workers processing seafood are most often related to inhalation of the seafood antigens or via direct unprotected handling of the seafood and its products. This can cause sensitised individuals to suffer from asthma, rhino-conjunctivitis, urticaria and protein contact dermatitis, which are IgE mediated. Food intolerance may also occur which is a non-IgE mediated reaction, however the exact mechanism is yet to be determined. There is therefore a need to develop reliable tests to identify sensitised workers processing seafood.

The objective of this study was to prepare specific seafood extracts from raw and cooked lobster; raw and cooked saltwater bony fish species (mackerel, red eye, maasbanker, pilchard and anchovy) and fishmeal dust obtained from a fish-processing factory. These extracts were tested by SDS-Polyacrylamide Gel Electrophoresis to characterise the seafood proteins, and the allergenicity was confirmed by the Western blot technique. Polyclonal IgG antibodies were also successfully generated in rabbits, using the specific seafood extracts isolated from the various species.

The second objective was to optimise and standardize an Enzyme Allergosorbent Test (EAST) method to quantify specific IgE antibodies in the sera of factory workers. This EAST was optimised and validated to detect allergen-specific IgE to each of the different fish species and also one crustacean species (rock lobster). Sera from a group of workers were selected and analysed for specific IgE antibodies by the optimised EAST (S) (South African laboratory), and commercial RAST techniques. Analysis was performed for the three most important extracts, pilchard (canned), anchovy, and lobster. The same samples were analysed by EAST (R) in the reference laboratory (Dr Gerald Reese; Paul-Ehrlich-Institute, Germany). The different techniques, and the EAST (R) and the EAST (S) results were compared by using a statistical software package.

An EAST method was successfully developed, however, compared to the results obtained by the reference laboratory the sensitivity and specificity was below 80%. The main reason for the low agreement between the two laboratories was the fact that the South African laboratory used a modified EAST method, and different data calculation methods, for categorising positive results. The South African laboratory did not use a kit-based assay and a serum dilution of 1:4 and not 1:2 were used when compared to the reference laboratory. When the EAST results were compared to the RAST results, poor agreement was found due to the fact

that canned pilchard was used in the EAST while raw pilchard in the commercial RAST assay. For pilchard, anchovy and lobster EAST, different species were utilized compared to the RAST, and this can also explain the poor level of agreement.

Future directions would be to further standardise the EAST method and to introduce reference sera and a standard curve to determine positive results, thereby ensuring more reproducible results between laboratories.

CHAPTER 1
INTRODUCTION

Seafood is an important food for humans particularly as a source of animal protein (Lopata and Potter, 2000). During the last decade, as concerns about dietary fat and cholesterol increased in the United States and Europe (Musmand *et al,* 1993) and rapid transportation and improved distribution methods became available, seafoods gained more widespread popularity (O'Neil and Lehrer, 1995).

Approximately 72% of fish and shellfish harvested worldwide are utilized for human food. In South Africa adverse food reactions to ingested seafoods are frequently reported but the prevalence of seafood allergy has not been determined (Zinn *et al,* 1997). Increased levels of production and processing of seafood have led, and continue to lead, to more frequent reporting of occupational health problems among workers in the industry (Jeebhay, *et al,* 2000).

The seafood industry is an important contributor to the regional economy of the West Coast in the Western Cape province of South Africa. The fishing and seafood industry in South Africa employs approximately 28,000 workers in more than 100 workplaces. These workers are directly dependent on the industry, supplying food for the whole of the southern African sub-region (Jeebhay *et al,* 2000).

Labour tends to be divided along gender lines with men almost exclusively going out to sea to catch the fish and women doing the majority of on-land seafood processing. A considerable proportion of the workforce is seasonal. The degree of exposure to seafood is likely to be highest during the harvest season, which varies according to the seafood type, when most of the processing occurs (Jeebhay *et al,* 2000).

Zinn and co-workers investigated the range of allergy due to seafood ingestion among a group of 105 volunteers from the general public in the Western Cape (Zinn *et al,* 1997).

These symptoms as seen by the study of Zinn, were usually due to domestic rather than occupational exposures. The likelihood that similar workplace exposures would be more intense and prolonged, confirmed the importance of investigating the health effects of these occupational exposures among workers in this province of South Africa.

This study focussed on developing and optimising a sensitive and specific EAST method in order to quantify the specific IgE in the serum of seafood processing workers working with saltwater bony fish (mackerel, red eye, maasbanker, pilchard, anchovy and fish meal dust) and rock lobster. Furthermore the cross-reactivity status of the different extracts and allergens responsible for sensitisation was also identified.

To quantify the specific IgE in the workers sera an Enzyme Allergosorbent Test (EAST) was optimised where the extracts (antigens) were coated onto a paper disc, and not onto a solid phase support. This EAST method is assumed to be more sensitive than the normal ELISA, especially when detecting specific IgE, because the levels in human serum are usually very low.

A group of workers were analysed by the EAST and the RAST method, and the same group of sera was tested with the EAST, by a reference laboratory (Dr Gerald Reese of the Paul-Ehrlich-Institute in Germany); agreement between these methods were then determined.

The specific IgE levels obtained from analysis of workers sera were correlated to the environmental exposure (department where worker works e.g. canning, bagging, degutting, etc.), presence of atopy and WRS (work-related symptoms).

In this way the clinical significance of the elevated IgE levels to specific seafood allergens among exposed workers could be established.

CHAPTER 2
LITERATURE REVIEW

2.1 The allergic response and the role of IgE

The adaptive immune response provides specific protection against infection with bacteria, viruses, parasites and fungi. It is able to provide rapid protection against a repeated challenge with the same or similar foreign organism or toxin. By contrast, some immune responses can give rise to an excessive or inappropriate reaction, this is usually referred to as hypersensitivity (Roitt, Brostoff and Male, 2001). Hypersensitivity may occur as an exaggerated form of an appropriate response, to a virus for example, or a response to an antigen that has no toxic potential, such as with asthma due to inhaled cat dander (Roitt, Brostoff and Male, 2001).

The most common forms of hypersensitivity are allergic responses, characterized by wheal and flare skin responses to the relevant antigen. IgE antibodies binding to mast cells mediate this response. Coombs and Gell classified hypersensitivity reactions into four forms (in Roitt, Brostoff and Male, 2001). Type 1 or immediate hypersensitivity is characterised by the production of IgE antibodies against foreign proteins commonly present in the environment. These antibodies bind specifically to a high-affinity receptor on mast cells and basophils, which are the only human cells that contain histamine. Subsequent exposure to the same antigens will lead to rapid release of histamine, and more gradual release of other mediators including leukotrines and cytokines (See figure 2.1).

The degranulation process is initiated in most cases by cross-linking of two specific IgE molecules by their relevant allergen. When two IgE receptors are cross-linked, signal transduction occurring through the gamma chains of the receptor leads to an influx of calcium; this initiates degranulation and the synthesis of newly formed mediators (Roitt, Brostoff and Male, 2001).

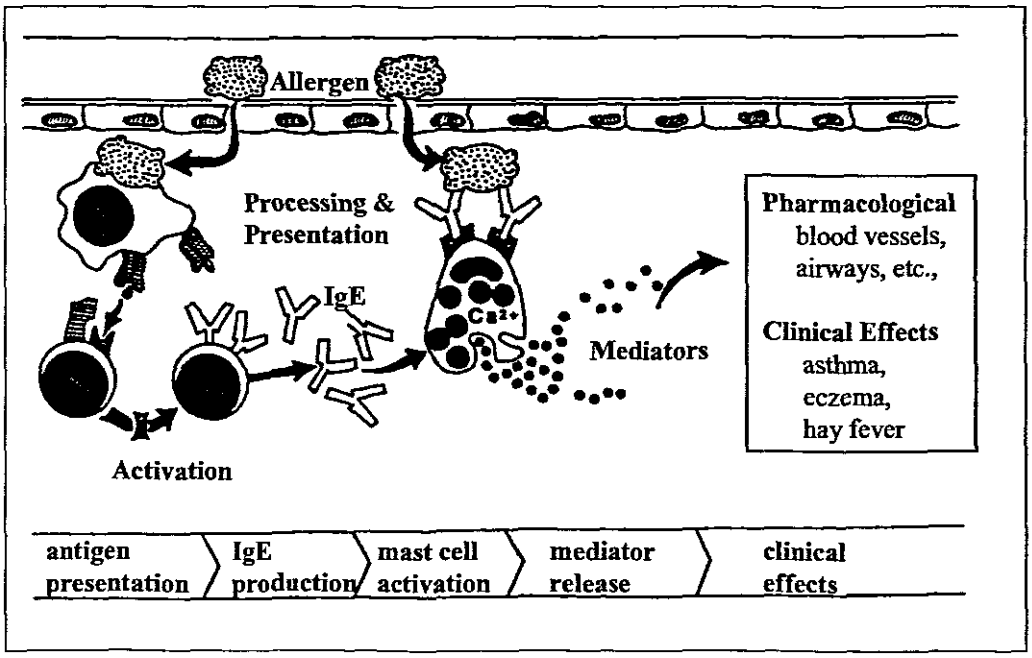

Figure 2.1: Induction and effector mechanisms in IgE-mediated hypersensitivity. (Source: Lehrer SB *et al.;* Critical Reviews in Food Science and Nutrition, 1996).

The effects of these mediators lead to the allergic symptoms that will be discussed later.

The IgE molecule

IgE is distinct from other dimeric immunoglobulins because it has an extra constant region domain, a different structure to the hinge region, and binding sites for both high and-low affinity IgE receptors.

The concentration of IgE in the serum of normal individuals is very low compared to all the other immunoglobulin isotypes. Most sera contain less than 1μg of IgE/ml. The reasons for this are as follows:

i. Serum IgE is known to have a very short half-life, less than two days compared to 21-23 days for IgG (Roitt, Brostoff and Male, 2001).

ii. IgE is produced in small quantities and only in response to a select group of antigens (Roitt, Brostoff and Male, 2001).

iii. IgE antibodies are sequestered on the high affinity receptor on mast cells and basophils (Roitt, Brostoff and Male, 2001).

2.2 Symptoms caused by the allergic reaction

Allergy to seafood can elicit almost any allergic symptom; some are more common than others. Some patients may have a single symptom, but more often there is a multi organ involvement (Lopata and Potter, 2000). Ingestion of fish or inhalation of aerosols

generated during the cooking of fish is common causes of both IgE-mediated and non-immunologic reactions. Such reactions following exposure to fish can be due to the fish itself, or due to contaminants (O'Neil, Helbling and Lehrer, 1993).

The symptoms of seafood allergy are as follows:

2.2.1 General Reactions

This includes anaphylaxis and exercise-induced anaphylaxis (Lopata and Potter, 2000). Anaphylaxis can be defined as an antigen-specific immune reaction mediated primarily by IgE, which results in vasodilation and constriction of smooth muscle, including those of the bronchus, which may result in death (Roitt, Brostoff and Male, 2001).

2.2.2 Respiratory Reactions.

The major localized anaphylactic categories that affect the respiratory tract include conjunctivitis, rhinitis, and asthma.

After the interaction of antigen with cell-bound reagin, there is a release of certain mediators that cause vasodilation, hypersecretion, oedema and swelling of the respiratory mucosa. Since there is a continuity of the mucous lining membranes of all components of the respiratory tract, all components are adversely affected. The degree to which each target organ is affected will determine the clinical manifestation of the disease (Bellanti, 1985).

2.2.3 Cutaneous/Skin Reactions

The skin is another frequently involved shock tissue. Reactions are classified under two broad categories.

1 Immediate e.g. urticaria
2 Delayed e.g. contact dermatitis

Ingested food, drugs or contactants may induce these conditions. There may also be secondary involvement including the respiratory tract and gastrointestinal tract (Bellanti, 1985).

2.2.4 Gastrointestinal Allergy

This type of allergy may be defined as hypersensitivity to certain exogenous substances, usually foods, which gain access to the body via the gastrointestinal tract. This hypersensitivity usually manifests as vomiting, diarrhoea or abdominal pain. It may also be severe and result in malabsorption or protein-losing enteropathy. It can also be accompanied by skin or respiratory manifestations (Bellanti, 1985).

2.3 Techniques used to diagnose allergy

2.3.1 *In-vivo*

I Skin Prick Test (SPT)

Skin Prick testing is a cheap, rapid and accurate way of identifying causative allergens in an atopic individual (The ALLSA Handbook of Practical Allergy, 1994). It is often used to screen patients with suspected IgE –mediated food allergies (Burks and Sampson, 1993). SPT's are uncomplicated and with practice and adherence to a few simple guidelines, it is possible to get highly reproducible results. The test is performed on the volar or inner aspect of the forearms, avoiding the flextures and wrists. The skin must be clear and free of active eczema. A drop of relevant allergen is placed on the arm and a lancet with a 1mm point is used to prick the skin through the drop. A positive and negative control must always be included in each series of tests. The negative control is the diluent used to preserve the allergen extract. The positive control is a 1 mg/ml histamine hydrochloride solution used to detect suppression by medication and to assess a positive skin reaction. Reactions should occur within 10-15 minutes (The ALLSA Handbook of Practical Allergy, 1994), and the skin test should be "read" after 15 minutes. The characteristic response is a wheal and flare reaction. The wheal is caused by extravasation of serum from the capillaries in the skin, which results from a direct effect of histamine. This is accompanied by pruritis (also a direct effect of histamine) and a larger erythematous flare mediated by an axon reflex. Skin tests are evaluated by the size of the wheal compared to a positive and negative control. A 4X4 wheal in adults can be considered positive. A positive skin test indicates that the patient has specific IgE antibodies on the mast cells in their skin (Roitt, Brostoff and Male, 2001).

II Double Blind Placebo Controlled Oral Food Challenge Test (DBPCFC)

This is considered to be the "gold standard" for diagnosis of food allergies. The foods to be tested are based on history and/or SPT or RAST results. Before undertaking this test the following conditions are necessary:

- Suspect foods should be eliminated 7-14 days before the challenge.
- Anti-histamine should be discontinued long enough to establish a normal histamine skin test.
- Other medications should be reduced to levels sufficient to prevent breakthrough of acute symptoms.
- In some patients with asthma short bursts of corticosteroids may be necessary to ensure adequate pulmonary reserve for testing (FEV_1 >70%)

The patient should be in a fasting state and the challenge is started with a dose of food unlikely to provoke symptoms. Generally it is started with 125-500 mg of lyophilised food, the dose is then doubled every 15-60 min depending on the type of reaction suspected to occur. Clinically, reactivity is generally ruled out when the patient has tolerated 10gm of lyophilised food blinded in capsules or liquid. If the blinded portion of the challenge is negative, however, it must be confirmed by an open feeding under observation to rule out rare false negative challenge. The test should generally be conducted in a hospital or clinic setting especially if IgE-mediated reactions are suspected. Trained personnel and equipment for treating systemic anaphylaxis should be present (Burks and Sampson, 1993).

2.3.2 *In-vitro*

I Radioallergosorbent Test (RAST)

The RAST and similar *in-vitro* assays including the Enzyme Linked Immunosorbent Assay (ELISA) are used for the identification of food specific IgE antibodies. These tests are often used to screen for IgE-mediated food allergies. In general, *in-vitro* measurements of serum food-specific IgE performed in high quality laboratories provide information similar to SPT (Burks and Sampson, 1993). A positive SPT indicate that the patient has specific IgE antibodies on the mast cells in their skin (Roitt, Brostoff and Male, 2001), whereas the RAST indicates specific IgE in the patient's serum. The principle of the RAST is as follows:

The allergen of interest, covalently coupled to an ImmunoCAP, reacts with the specific IgE in the patient's serum. After washing away non-specific IgE, enzyme labelled antibodies against IgE are added to form a complex. Unbound enzyme-anti-

IgE is washed away after incubation, and the bound complex is incubated with a developing agent. The fluorescence of the eluate is measured after the reaction is stopped. The higher the response value the more specific IgE is present in the specimen (Pharmacia and Upjohn UniCAP specific IgE Fluoroenzymeimmunoassay Information Booklet, 2000).

In a study done by Hansen *et al*, (1996) they aimed to evaluate the sensitivity and specificity of *in-vitro* tests in clinically allergic adult patients by comparing the diagnostic value of four specific IgE tests, and as a true diagnosis DBPCFC (Double Blind Placebo Controlled Oral Food Challenge Test) was employed. To ensure the quality of the allergen material SDS-PAGE and immunoblotting were applied to the fresh codfish extract and it was also used in an in-house RAST. They selected eight clinically codfish allergic patients, their food hypersensitivity was diagnosed by DBPCFC, and they all had positive SPT to the same batch of commercially available codfish extract. Thirty codfish tolerant adults served as the control group. They measured total IgE in all patients and controls with the PRIST kit (Pharmacia, Uppsala, Sweden). The codfish specific IgE were measured by Phadebas RAST (PHA) (Pharmacia Diagnostics, Uppsala, Sweden), Pharmacia Cap System RAST (CAP), and Magic Lite (ML) using the chemiluminescent assay from ALK (Horsholm, Denmark). A Maxisorp RAST (MAXI) was also done.

SDS-PAGE was also performed on the extract and the gels were stained by Coomassie Blue for protein determination or electroblotted to determine IgE binding.

They found the codfish specific serum IgE to be significantly higher in the challenge positive group compared to the codfish tolerant control group. All three the tests (PHA, CAP, and ML) correctly identified the eight codfish allergic patients as positive. However, false positives were seen in the tolerant control group using PHA and CAP, but no false positives using ML. The in-house MAXI specific IgE showed no advantage compared to the commercial tests. They found the rank of diagnostic accuracy of the specific IgE tests to be as follows: ML>PHA/MAXI>CAP. The SDS-PAGE showed numerous bands ranging between <14 kDa to 200 kDa, a band corresponding to the major allergen *Gad c 1* was also seen. The immunoblot was probed with all the patients and the controls, and all eight patients showed strong binding at 12-13 kDa, and no significant binding was observed for the codfish tolerant control subjects. Binding to other allergens was also seen in all the DBPCFC subjects and in 33% of all control subjects. The authors conclude by saying that their study suggests that *in-vitro* assays can identify patients who are clinically allergic to codfish,

and that overall the tests are acceptable for screening for codfish allergy, but only ML or specific and strong binding of IgE to 12-13 kDa protein are sufficient for establishing the diagnosis, when using DBPCFC as the "true diagnosis" (Hansen *et al*, 1996).

II Enzyme Linked Immunosorbent Assay (ELISA)

The basic indirect ELISA protocol is useful for screening anti-sera supernatants for specific antibodies, when milligram quantities of purified or semi-purified antigen are available. The wells of the microtiter pates are coated with antigen, the coated plates are incubated with test solutions containing specific antibodies, and unbound antibodies are washed away. A solution containing a developing reagent e.g. alkaline phosphatase conjugated to protein A, protein G, or antibodies against the test solution antibodies is then added to the plate. After incubation the unbound conjugate is washed away and a substrate solution is added. After a second incubation, the amount of substrate hydrolysed, is assessed with a spectrophotometer or spectrofluorometer. The measured amount is proportional to the amount of specific antibody in the test solution (Ausubel, *et al* (ed.) 1995).

In a study done by Doekes *at al*, (1996) they evaluated the use of relatively simple and inexpensive enzyme immunoassays (EIAs) in population studies in order to assess total IgE and specific IgE with various common or occupational allergens. They felt that extensive IgE serology in occupational or environmental health studies were often hampered by a lack of technical facilities and financial resources; this prompted their study.

Total IgE was measured with a sandwich EIA and calibrated with commercially available IgE standards. Reproducibility was studied by testing pooled normal human serum samples in each of a large series of test plates. A panel of 156 children's serum samples with known IgE values was used to compare the assay with other total IgE assays. A previously developed EIA for anti-yeast IgE was adapted for the measurement of IgE reacting with various common and occupational allergens. They also used panels of serum samples from school children, bakery workers, and laboratory animal workers to study sensitivity and specificity with reference to SPT's as the standard, and also to compare the EIAs with commercially available test kits.

The results they obtained with the 156 children's serum were strongly correlated with IgE concentrations measured previously by radioimmunoassay. The results of the EIA

for various occupational allergens correlated very well qualitatively and quantitatively with the results obtained by the commercial test kits. The sensitivity and specificity of the EIA results as a predictor of SPT reactivity to common allergens were remarkably high in the panel of school children. In the panel of bakery workers the specificity of the EIA was also very high but the sensitivity was notably lower in this adult panel. The authors feel however that it is in agreement with results reported for conventional IgE tests. The authors conclude by saying that as the costs were estimated to be 10-fold lower than commercial kits, these EIAs may be very useful to determine total and specific IgE's in epidemiological studies of atopic, respiratory or other disorders (Doekes *et al*, 1996).

III Enzyme Allergosorbent-Test (EAST)

The basic principle of the EAST and ELISA methods is the same, the only difference however is that the antigen is coupled to an activated paper disc in the EAST method and not to the wells of the ELISA plate. Ceska and Lundkvist (1972) previously described this paper-disc method in 1972, they used Munktell's Swedish filter paper and the discs were activated with BrCN (cyanogen bromide). In a study by Merget *et al* (1993) evaluating diagnostic tests in enzyme allergy, they evaluated the sensitivity and specificity of the SPT and the EAST. They found the SPT to yield a sensitivity of 100% and a specificity of 93%. For the EAST they used bromide-activated cellulose discs provided by Allergopharma and test yielded a sensitivity of 62% and a specificity of 96%. Baur *et al* (1996) evaluated EAST and CAP specificity for IgE and IgG antibodies to diisocyanate-HSA conjugates. They found the EAST to be 92% specific and the CAP to be 89% specific in IgE-binding, but they could however not confirm IgG-binding specificity.

The EAST method has been widely used in many other studies e.g. to determine the allergenicity of processed celery (Ballmer *et al*, 2002), in the identification of hazelnut major allergens (Pastorello *et al*, 2002), to determine allergy to Aspergillus-derived enzymes in the baking industry (Sander *et al*, 1998), and also various other studies. To our knowledge however this method has not been employed before in studying seafood specific IgE antibodies in sensitised subjects.

2.4 Seafood Allergens

Substances that give rise to wheal and flare responses in the skin and to the symptoms of allergic disease are derived from many sources. Any allergen can be described or classified by its source, route of exposure and nature of the specific protein. Extracts used for skin testing or *in vitro* IgE measurement are made from whole material, which can be an allergen. Individuals can react selectively to one or more different proteins within an extract.
Estimates of exposure can be made either by visual identification of particles or by immunoassay of the major allergens (e.g. *Gad c 1*).
While a very large number of food proteins can occasionally give rise to IgE responses, only a small number are common causes of allergic responses. These include egg, milk, peanut, soy, chicken and shellfish. In general, only a small fraction of the food proteins are absorbed. Small peptides can however be freely absorbed and may be recognised by T-cells and even by IgE antibodies in a minority of individuals. The bulk of the allergic and anaphylactic responses to foods are thought to be related to food proteins that have not been digested. They either trigger mast cells in the intestine or enter the circulation (Roitt, Brostoff and Male, 2001). Mast cell and basophil activation is most characteristically initiated when specific antigen triggers the response by binding to and cross-linking preattatched surface IgE molecules. The typical sequence is as follows: (Abbas. Lichtman and Pober, 1991)

- Production of IgE by B cells in response to the first exposure to an antigen, then
- binding of the IgE to specific Fc receptors on the surfaces of mast cells and basophils and,
- interaction of re-introduced antigen with the bound IgE, leading to,
- activation of the cells and release of mediators (Abbas. Lichtman and Pober, 1991).

The clinical and pathological manifestations of immediate hypersensitivity are due to the actions of the released mediators (Abbas. Lichtman and Pober, 1991)

For seafood allergens in particular, most of them are stable molecules that resist the effects of cooking, processing or digestive processes. They are generally glycoproteins with molecular weights between 10 and 70 kDa with an acidic isoelectric point (Lopata and Potter, 2000).

2.4.1 Groups of seafood

The term "fish" refers to a variety of cold-blooded aquatic vertebrates. It describes a life form rather than a single taxonomic group (O'Neil, Helbling and Lehrer, 1993).

The major edible seafoods that induce allergic reactions belong to 3 phyla, the first phylum is the Mollusca and other large groups are the classes Crustacea (figure 2.2), and Osteichthyes (bony fish) (figures 2.3 to 2.7). The two invertebrate phyla Arthropoda and Mollusca are generally referred to as "shellfish" (see table 2.1) (Lopata and Potter, 2000).

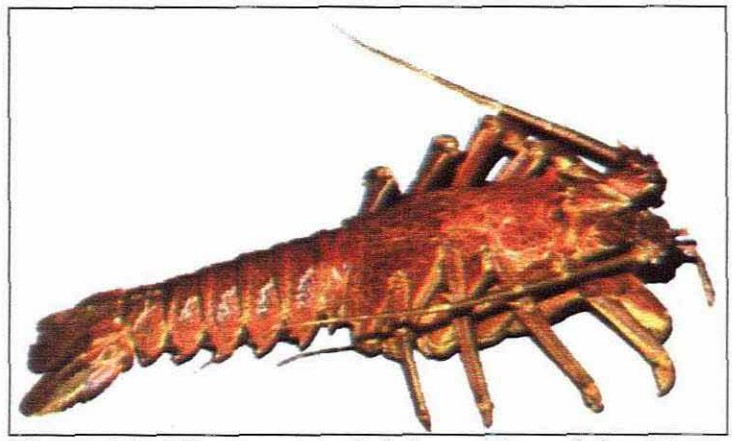

Figure 2.2: West coast rock lobster (*Jasus lalandii*) (class Crustacea).

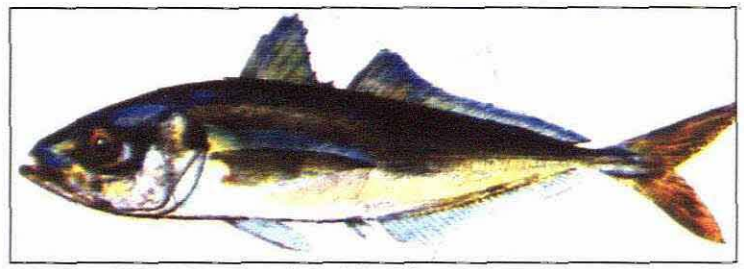

Figure 2.3: Maasbanker (*Trachurus trachurus capensis*) (class Osteichthyes (bony fish)).

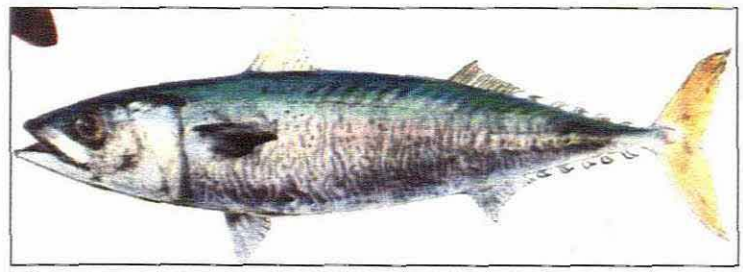

Figure 2.4: Mackerel (*Scomber japonicus*) (class Osteichthyes (bony fish))

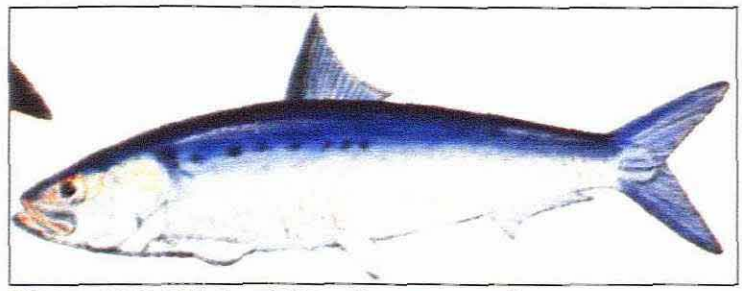

Figure 2.5: Pilchard (*Sardinops sagax*) (class Osteichthyes (bony fish)).

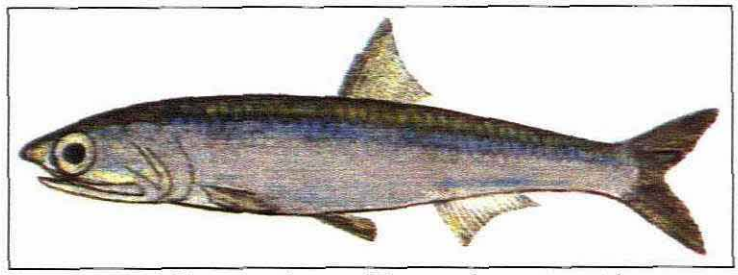

Figure 2.6: Cape anchovy (*Engraulis capensis*) (class Osteichthyes (bony fish)).

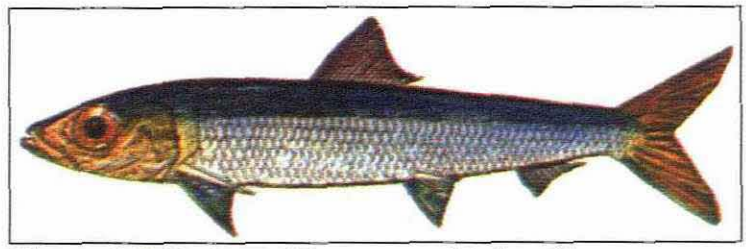

Figure 2.7: Red eye (*Etrumeus whiteheadi*) (class Osteichthyes (bony fish)).

Source: Figures 2.2-2.7 were all taken from the Fishing Industry Handbook, South Africa, Namibia and Mocambique. 25th edition 1997

Table 2.1: Groups of seafood

PHYLUM	**CLASS**	**ORDER**
Mollusca	Gastropoda	Abalone, Snail, Limpet
	Bivalvia	Clam, Mussel, Oyster, Scallop
	Cephalopoda	Octopus, Squid
Arthropoda	Crustacea	Lobster, Crayfish, Shrimp, Rock lobster
Chordata	Osteichthyes	Mackerel, Yellowtail, Tuna, Cod, Hake, Salmon, Sardine, Anchovy, Pilchard

(Source: Lopata and Potter, 2000)

Patients usually refer to offending seafood by its common name. There is widespread substitution of seafood products of high commercial value with closely related congeners. Patients may not be reacting to the seafood they perceive when consuming processed seafood, thus very careful *in vitro* and *in vivo* testing is essential to prove specific sensitivity (Lopata and Potter, 2000).

2.4.2 Classes of fish

The world of fish is divided in 50 orders containing 445 families representing > 4000 genera or > 20000 species. Their degree of relation can be judged from the distance indicated by the numbers of orders and families. (See Figure 2.8)

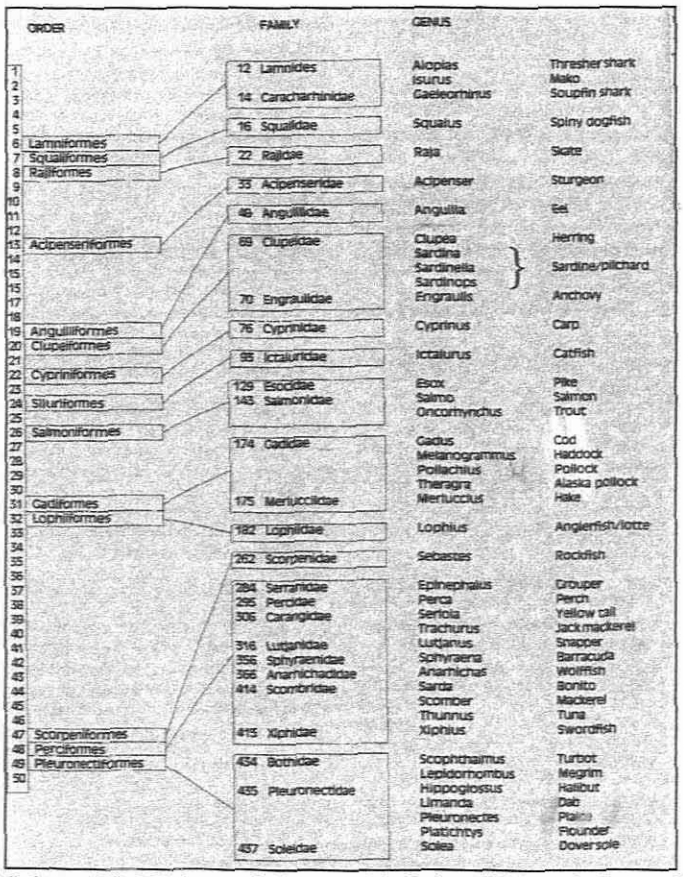

Figure 2.8: Source: Nelson JS: Fishes of the world, John Wiley & Sons, New York 1984

2.4.3 Fish Allergens

2.4.3.1 The model allergen *Gad c 1*

The major Cod allergen *Gad c 1* (allergen M) serves as a model of how allergens in general and particularly those in foods can be identified, purified and characterized.

The first studies assessing the antigenicity and allergenicity of cod proteins were done in the late 1960's and early 1970's.

Gad c 1 belongs to the parvalbumins, a group of vertebrate muscle calcium-chelating proteins. They mediate the concentration of calcium in the muscles and it is a very stable allergen; extremes in pH, temperature or random folding of the molecule following exposure to dissociating agents do not significantly alter the allergenicity. These findings suggest that allergenic activity is dependant on the amino-acid sequence and not necessarily on the steric conformation.

Gad c 1 contains 113 amino acid residues and one glucose molecule. It has a molecular weight of 12,328 kDa and an isoelectric point of 4,75. Specific tryptic cleavage of *Gad c 1* yields two polypeptide fragments (TM 1 and TM 2) that are allergenic. However, the allergenicity of these fragments are reduced when compared with the intact *Gad c1*. Three dimensionally *Gad c 1*, in common with other parvalbumins, is arranged in three domains. TM 1 (residues 1-75) corresponding roughly to domains AB and CD and TM 2 (residues 76-113) corresponding to the third domain, EF, exhibit extensive cross-reactivity in both the human IgE antibody system and the rabbit IgG system. This immunologic cross-reactivity is evidence for the three-domain arrangement of *Gad c 1*. Moreover, the relative allergenic reactivities of the fragments to *Gad c 1* are compatible with this structure. RAST inhibition shows an approximate ratio of 3:2:1 of the numbers of domains in *Gad c 1*, TM 1, and TM 2, respectively.

The *Gad c 1* molecule contains at least 5 IgE binding sites distributed along its polypeptide chain. Cross-reactivity among some fish species may be the result of common structures within related proteins (O'Neil, Helbling and Lehrer, 1993).

2.4.3.2 Other fish allergens

I Molecular level

Mata *et al* (1994) conducted a study comparing the allergenicity of codfish and surimi by skin testing, specific IgE Radioimmunoassay (RIA) and leukocyte histamine release (LHR) in six fish-allergenic patients.

Surimi is a Japanese food product. It is a mixture of small fish of many species lacking most soluble sarcoplasmic proteins and is mainly composed of myofibrillar proteins. Surimi may also have added starch, egg white and other ingredients forming an elastic gel after cooking. Fresh codfish and surimi was used as extracts.

The skin tests were positive for codfish (commercial and natural codfish) and to a lesser extent for surimi in the allergic patients. The controls were all negative and they could also show reactivity for both codfish and surimi proteins in the inhibition tests

of the IgE-RIA. As discussed before the major allergen in codfish is a parvalbumin belonging to the sarcoplasmic proteins. The preparation of surimi includes an extensive washing process of myofibrillar proteins which may eliminate the sarcoplasmic proteins.

Electrophoresis of the codfish filtration eluate identified a 13 kDa protein that could correspond to *Gad c 1* but this protein was not found in the surimi extract. The protein band found in the SDS-PAGE of both gel filtration eluates from surimi and codfish had a molecular weight of 63 kDa. They suggested that the band may represent an additional allergenic protein, different from *Gad c 1*, present in myofibrillar proteins from codfish, or that *Gad c 1* may derive from this 63 kDa protein and could be produced by proteolysis or reductive denaturation (Mata *et al*, 1994).

Codfish is one of the foods most frequently involved in allergy. The only cod allergen that has been purified and characterised is the 12.3 kDa parvalbumin *Gad c 1*. Galland *et al* (1998) have detected allergen bands, which have not previously been described, in particular, a 41 kDa protein. SDS-PAGE showed 18 proteins in the crude cod extract with their molecular weights ranging from 13 kDa to 130 kDa.

The crude extract was also brought to 80% ammonium sulfate saturation and centrifuged. The 41 kDa protein was purified and was still allergenic as shown by Western blotting with cod allergenic sera. Following the Western blot analysis *Gad c 1*, the 41 kDa allergen and two other proteins of 28, and 49 kDa respectively were detected with a pool of sera from cod allergenic patients, and also with anti-parvalbumin monoclonal antibody. The monoclonal antibody stained specifically the calcium bound form of parvalbumin and was detected against an epitope located in the calcium-binding site. This epitope corresponded to the same region than an IgE epitope of *Gad c 1*,located in the first calcium-binding loop (sequence 49-64). They concluded that this purified protein is definitely an allergen as it was detected in Western blot by a pool of allergenic sera, but it remains to determine the frequency of anti-p41-IgE in allergic patients (Galland *et al*, 1998).

II **<u>Influenced by fish processing</u>**

A study done by Bernhisel-Broadbent, Strause and Sampson (1992) found a 12 fold higher false negative rate in Double-Blind Placebo-controlled oral Food Challenges (DBPCFC) with fish.

They compared cooked lyophilized fish extracts (flounder, perch, cod and salmon) used for DBPCFC with cooked non-lyophilized fish extracts (flounder, perch, cod and salmon) used for the open challenges, by SDS-PAGE, immunoblot and ELISA-Inhibition assay. Altered fish allergenicity as a result of food processing with canned tuna and salmon was also investigated.

They found a 21% false-negative reaction rate that occurred with the fish DBPCFC's and this suggested an unexpected lability of major fish allergens to lyophilization. Their investigation supports the conclusion that the false-negative DBPCFC's to fish were due to lyophilization and not dose related, since the positive open challenges often occurred with the first bite of the cooked fish fillet. With the exception of the flounder extracts, they were unable to demonstrate this change in allergenicity of the fish extracts as a result of lyophilization by SDS-PAGE, immunoblotting or inhibition-ELISA. They found minor differences between cooked non-lyophilized and cooked lyophilized flounder extracts. Bands at 23 kDa and 15 kDa were prominent in the non-lyophilized lane but absent in the lyophilized lane. The authors were of the opinion that the *in vitro* methods used, lacked the sensitivity to detect the subtle, yet clinically relevant alteration in allergenicity.

They also evaluated 10 canned tuna extracts with SDS-PAGE and it appeared as nearly homogenous smears on the gel. The likelihood of allergenic epitopes that could still be present even though distinct bands were not obvious is unlikely because IgE binding to the immunoblots was minimal to absent with sera from seven patients with positive Skin Prick Tests (SPT) to raw tuna extract. In addition, canned tuna by ELISA-Inhibition revealed decreased allergenicity compared to cooked tuna. They concluded that canned tuna is not non-allergenic, but it appears to be significantly less allergenic than cooked fish. They also concluded that the process of canning tuna is responsible for its decreased allergenicity rather than inherent hypoallergenic property of the tuna itself. They support this conclusion by demonstrating decreased allergenicity of canned salmon as well (Bernhisel-Broadbent, Strause and Sampson, 1992).

III **Influenced by heat treatment**

In a study done by Crespo *et al*, (1995) they diagnosed 197 patients with IgE-mediated fish hypersensitivity. After diagnosis, all the patients were placed on a strict fish avoidance diet. After this they evaluated 21 of the 197 patients who reported symptoms after exposure to fish odours or vapours. During this period of avoidance

these patients reported allergic reactions after accidental exposure to airborne fish odours or vapours. Clinical manifestations associated with inhalation were respiratory (12 patients), and cutaneous (9 patients), with 19 of the 21 patients reported 3 or more episodes upon exposure to fish aerosols. In most cases these episodes occurred at home when other people were eating fish.

This demonstrates that the inhalation route could play an important role in accidental and unknown contacts with fish allergens. This could elicit clinical symptoms and could have some effect in delaying the development of tolerance (Crespo *et al*, 1995).

Pascual *et al* (1996) conducted a further study to evaluate the allergenicity of vapours from boiling fish, using boiling salmon as a model for generating aerosols. Extracts of raw salmon (*S. Salar)* and boiled salmon were prepared and salmon steam was collected through a refrigerated still from the boiling salmon.

CAP inhibition procedures showed the raw salmon extract to have lower fluid-phase inhibitory capacity (57%) while salmon steam and boiled salmon extract showed close inhibitory capacity, 85% and 88% respectively.

To identify the IgE-binding components, boiled, raw and salmon steam were analysed by SDS-PAGE, transferred to an Immobilon-P membrane and probed with a serum pool from patients with serum-specific IgE and positive prick tests to salmon.

The IgE immunoblot analysis of salmon steam showed multiple allergenic components with a double band between 12 and 14 kDa having the strongest allergenic activity, and also a single band around 30 kDa. With the boiled and raw extracts they found IgE-binding components of 12-30 kDa but also bands of >30 kDa exhibiting significant activity. On immunoblotting inhibition the boiled extract and the salmon steam inhibited each other completely but both only inhibited the raw extract partially.

The group concluded that IgE-binding components can be detected in steam from boiling salmon and the findings could be associated with other fish species. Therefore, fish avoidance as therapy, in patients properly diagnosed with fish hypersensitivity, should be based on the fish elimination diet and on avoiding the exposure to aerosol particles through inhalation (Pascual *et al*, 1996). This also supports the conclusion of Crespo *et al* (1995).

IV Influenced by aerosolisation

Taylor *et al* (2000) studied the possibility of passive aerosolisation of fish allergens in an open-air fish market, through air sampling and competitive IgE immunoassay. This was brought on by the fact that patients in Madrid, Spain, who suffer from fish allergy, exhibited respiratory symptoms after visits to an open-air fish market. Their aims were to demonstrate that fish protein is aerosolised through passive evaporation of raw fish in an open-air fish market, and to quantify the aerosolised fish allergen.

Air samples were collected from an open-air fish market at a single location and also from a residential area not located near a fish market. The allergens were directly extracted from the air samples. Serum samples from 19 Spanish individuals allergic to fish, with a history of symptoms after ingestion of cooked fish, exposure to raw fish or both, were tested for specific IgE. Extracts of raw whiff (*Lepidorhombus whiffiagonis)* and raw hake (*Merluccius merluccius)* were prepared as well as extracts of boiled whiff and hake. Snow crab (*Chinoecetes opilis)* was used as a control extract antigen. Fish allergens were detected in 39 different air samples from the fish market and no fish allergens were detected in the 8 residential samples. The amount of airborne allergens detected, appeared to be independent of duration of sampling time.

Concentrated fish market samples demonstrated dose-dependant reactivity similar to that of the raw whiff and hake standard. The fish aeroallergen concentration, although at the lower end of the scale, are of the same order of magnitude (10-100 ng/m^3) as many other occupational aeroallergens e.g. latex and snow crab. How the allergen becomes airborne is still unclear. The authors however, demonstrated that fish allergens are detectable in the air of an open-air fish market. They concluded that avoidance of a food allergen such as fish should extend to prevention of exposure to aerosolised particles as well (Taylor *et al*, 2000).

V Influenced by storage conditions

A study done by Dory *et al* (1998) aimed to characterize cod IgE-reactive protein bands by Western blotting of crude extract, in the pre-rigor mortis state, and also to evaluate the effect of post mortem degradation on the allergenic proteins.

The extracts were prepared as follows:

Pre-rigor mortis cod: A fillet was cut before the post mortem rigidity and immediately placed in liquid nitrogen.

Post-rigor mortis: The fillet was cut just after post mortem rigidity and then placed in liquid nitrogen.

Commercial cod: A fresh fillet was obtained from a fish shop that was kept at 4°C and the proteins extracted less than one hour after the fillet was collected. Results showed IgE-reactive protein bands over a wide molecular weight range. Freshness also played a part in IgE-reactive protein band distribution and in IgE binding. The relative content in IgE-reactive protein bands was greater after storing the fish for several days (Dory *et al*, 1998).

2.4.4 Crustacean allergens

Crustaceans are among one of the common seafood classes to cause adverse allergic reactions. Sera from patients who experienced several episodes of anaphylaxis after eating shrimp, was used to monitor the isolation of a major allergen from shrimp. The allergen was isolated from a fresh extract of boiled shrimp by gel filtration and from raw shrimp by gel filtration and electrophoresis. A 38 kDa band was identified on SDS-PAGE, the isoelectric point was about 4.5 and the allergen contained 4% carbohydrate and only 7 tyrosine and 9 phenalalanine out of 341 amino acid residues. The allergen was also a potent inhibitor of RAST to both cooked and raw shrimp. A second weaker allergen of 21 kDa was isolated from raw shrimp in a highly purified form (Hoffman *et al*, 1981).

In 1985 Lehrer *et al* undertook a study to analyse crustacean allergens/antigens by crossed immunoelectrophoresis (CIE), crossed radioimmunoelectrophoresis (CRIE) and also crossed-line immunoelectrophoresis (CLIE). CIE detected 18 precipitating antigens in extracts of shrimp. Of these antigens CLIE of shrimp extract demonstrated that 5 of them cross-reacted with crawfish, 3 with lobster and 1 with crab extract. Allergens present were identified by CRIE analysis using sera from 6 subjects with positive RAST's to shrimp extract.
Seven different precipitins were present in the shrimp extract that bound the patients' IgE antibodies. Three of these seven precipitins were detected in at least four of the six patient sera tested. None of the controls detected allergens. Their study also demonstrated that several allergens cross-react and several are unique. The one allergen, shrimp precipitin 1, was present in all 4 crustaceans tested, the other, shrimp precipitin 6, was present only in shrimp, crawfish and lobster extracts. Shrimp precipitin 3 appeared to be a unique allergen for shrimp (Lehrer *et al*, 1985).

In 1987 Halmepuro *et al* took it a bit further and their aim was to identify the antigenic and allergenic components of crawfish and lobster and also to study the structural similarities in crustacean antigens/allergens. Boiled extracts were made of crawfish (*Procambarus clarki*),

spiny lobster (*Panulirus argus*), white shrimp (*Penaeus setifecus*) and blue crab (*Callinectus sapidus*). Timothy pollen extract (Allergopharma, FRG) and coffee bean extracts were used as controls.

CIE was used to analyse the antigenic composition of crawfish and lobster; twenty-three different antigens in crawfish and seventeen in lobster were identified. A close relationship was found between the identified crawfish, lobster and other crustacean antigens. They found that most of the antigen present in crawfish or lobster was fully or partially identical within the crustaceans studied, and the predominant antigens were also among the common antigens. They demonstrated similar structural entities in crawfish and lobster extracts and also in the concentrated crawfish boiling water, including two crawfish and one lobster allergen. This indicates that the crustacean antigens are heat stable, water-soluble and can enter the atmosphere in steam aerosols from the cooking process.

The IgE binding capacity of 6 crawfish and 4 lobster antigens were also demonstrated. They suggested that crawfish extract antigen 11 and 12 and lobster antigen 8 and 13 are the main allergenic components, and that other crustaceans also share them. The group suggests further studies to isolate and characterise the major allergens present in crustacea (Halmepuro *et al*, 1987).

In 1989 Nagpal *et al* undertook a study in order to isolate and characterise heat-stable allergens from shrimp (*Penaeus indicus*). They made an extract from fresh whole shrimp, which they obtained from a local market. Two heat stable allergenic proteins, designated Sa-I and Sa-II, were isolated from the cooked whole shrimp extracts and its allergenicity was confirmed with sera from shrimp sensitive subjects. Both allergens were found to be homogeneous on PAGE and CIE. Sa-I had a molecular weight of 8.2. kDa and Sa-II, 34 kDa.

A comparison of Sa-II with antigen-II (Hoffman *et al*, reported the isolation of an allergen from cooked shrimp that appears to have properties in common with SA II, this heat stable protein was designated as antigen II) revealed that the former had a molecular weight of 34 kDa in contrast to a molecular weight of 38 kDa reported for antigen-II. Antigen-II was reported to contain 341 amino acid residues (excluding Tryptophan); Sa-II contained 301 amino acid residues (including Tryptophan). Antigen-II contained 4% carbohydrate but Sa-II did not reveal any detectable sugar residues. The authors suggest that Sa-II and antigen-II are the same molecules. They argue that the 4% carbohydrate associated with antigen-II could have been the result of contaminating material removed during additional isolation procedures used to purify Sa-II. The authors also suggest that Sa-I and Sa-II are different proteins having 54% of common allergenic determinants (epitopes). Alternately they suggest that Sa-I could

be a fragment of Sa-II generated by thermal denaturation during the process of cooking the shrimp (Nagpal *et al*, 1989)

In 1987 Halmepuro *et al* demonstrated marked similarities of the antigens in crawfish boil fluid and crawfish meat. In this study Lehrer *et al* (1990) aimed to analyse the shrimp boiling water for the presence of allergens and to compare the allergenicity of this fluid with the fluid of meat extracts. Water-soluble shrimp allergens released during boiling (shrimp water) were characterised and compared to allergen extracts from boiled shrimp (Shrimp meat). White shrimp (*Penaeus sefticus)* was boiled for 15 minutes in ion-depleted water and both the water and the shrimp meat were used as sources of shrimp extracts.
They demonstrated the extracts to be remarkably similar, containing many proteins with an acidic isoelectric point. Immunoprint results demonstrated that 86% of the shrimp sensitive individuals contained detectable shrimp-specific antibodies. The authors concluded that shrimp boiling fluid is an excellent source of shrimp allergens and that shrimp allergens also appear to migrate primarily in an acidic pH range. They also identified other shrimp allergens, which is in agreement with their earlier findings that demonstrated a number of precipitin bands that bind IgE antibodies (Lehrer *et al*, 1990)

Following on the study by Nagpal *et al* in 1989, Shanti *et al* (1993) reported that a comparison of amino acid sequences of different peptides, generated by proteolysis of Sa-II, revealed an 86% homology with tropomyosin from *Drosophila melangaster*, suggesting that Sa-II could be the shrimp muscle tropomyosin. Tropomyosin was isolated from uncooked shrimp (*Penaeus indicus*), and its physiochemical and immunochemical properties were compared with those of Sa-II. Both Sa-II and tropomyosin had the same molecular mass and focused in the isoelectric pH range of 4.8-5.4. Shrimp tropomyosin bound to specific IgE antibodies in the sera of shrimp-sensitive patients as was assessed by competitive ELISA and Western blotting. They also compared the amino acid sequence of shrimp tropomyosin in the region of IgE binding epitopes (residues 50-66 and 153-161) with the corresponding regions of tropomyosins from different vertebrates; this confirmed the lack of allergenic cross-reactivity between tropomyosins from phylogenic distinct species (Shanti *et al*, 1993).

In a study done by Run-Yee Lin *et al* (1993) a 39 kDa major allergen from shrimp was identified and characterised, according to the suggestions of the International Union of Immunological Societies; and this allergen was designated as *Par f 1*. Crude extracts were made from shrimp (*Parapenaeus fissures*), Crab (*Portunus* (*portunus*) *trituberculotus*),

cuttlefish (*Sepia (sepia) esculenta*) oyster (*Crassostrea gigas*) and pomfret (*Pampus argenteus)*. The protein components of the crude extracts were separated by SDS-PAGE and allergenic components were identified with IgE antibodies in immunoblotting. A total of six allergenic components ranging from 86 to 39 kDa were recognised by the IgE antibodies.
The component with a molecular weight of 39 kDa were considered a major allergen of shrimp, it was recognised by 70% of the sera tested. The component with a molecular weight of 74 kDa was also considered to be significant, it showed a frequency of IgE binding of 40%. Other components showed IgE binding frequencies equal to or less than 20%. This major allergen of shrimp with pI values from 5.1-5.6 was designated as *Par f 1*. The authors suggested that *Par f 1*, antigen-II and even Sa-II may represent the same or a very similar major allergen of shrimp (see table 2.2) (Lin *et al*, 1993).

Table 2.2: Characteristics of the major allergens of shrimp reported by different authors

Allergen	Shrimp	kDa	pI	Authors
Par f 1	*Parapenaeus fissurus*	39	5.1-5.6	Lin *et al* (1993)
Antigen-II	-	38	5.4-5.8	Hoffman *et al* (1981)
Sa-II	*Penaeus indicus*	34	-	Nagpal *et al* (1989)

Source Lin *et al*, 1993

Advances in molecular biology have allowed the rapid identification of antigens by molecular cloning of cDNA in an expression library and Leung *et al* (1994) isolated *Met e 1* from a *Metapenaeus ensis* expression library. M. *ensis* is a commercially important shrimp in the South China Sea. It is among the major commercial shrimp harvests in the waters of Malaysia, Singapore and Indonesia. As seen in previous studies, major IgE-reactive proteins have been characterised with respect to their molecular weight, isoelectric point and amino acid composition. However, the identity of shrimp allergens has only recently been identified as tropomyosin (Shanti *et al*, 1993). Tropomyosins are a diverse group of protein with distinct isoforms found in muscle (skeletal, cardiac and smooth), brain and non-muscle cells. The nucleotide sequence and amino acid sequence analysis of *Met e 1* showed significant homology to the fruit fly tropomyosin. In Musmand, *et al* (1993) it was reported that a 36 kDa allergen was isolated and termed *Pen a 1* which is similar to antigen-II (Hoffman *et al*, 1981) and also to Sa-II (Nagpal *et al*, 1989). The most important though was the study done by Shanti *et al* (1993) identifying this protein Sa-II as tropomyosin. Leung *et al* (1994) have compared the amino acid composition of their cloned shrimp allergen *Met e 1* with that of *Pen a 1*, antigen-II, and Sa-II and found they were extremely similar. They suggest that the major

allergen of shrimp is likely to include tropomyosin isoforms among various shrimp species. This has been reported in a variety of species including fruit flies, chickens, and mammals (Leung *et al*, 1994).

Daul *et al* (1994) set out to demonstrate and isolate the major shrimp allergen, *Pen a 1*. Raw Brown shrimp (*Penaeus aztecus*) meat and boiling water were used as extracts. Proteins from the shrimp meat and boiling water were separated by SDS-Page, and both extracts showed similar band patterns when stained with Coomassie blue stain. A major band was present in both extracts at 36 kDa. The proteins were transferred to nitrocellulose membrane and probed with positive patient sera. The most reactive band to which 82% of sera bound, was the 36 kDa band, *Pen a 1*. This allergen has an isoelectric point of 5.2 and a carbohydrate content of 2.9%. The amino acid composition indicated that it is rich in acidic amino acids, aspartic and glutamic acid, and as mentioned before, it indicates some degree of similarity to antigen-II and Sa-II. The amino acid sequence shows substantial homology with tropomyosin. The most significant homology (72-87%) was observed with fruit fly tropomyosin, and tropomyosins from mammalian species showed 60-62% homology. This then identifies the major shrimp allergen *Pen a 1* as shrimp tropomyosin. The authors have demonstrated that more than 80% of shrimp sensitive subjects tested had substantial IgE antibody reactivity to allergen *Pen a 1*. They suggested that *Pen a 1* is the only major shrimp allergen, and that the allergens identified by Hoffman *et al* (1981) and Nagpal *et al* (1989) are probably the same molecule. They have also shown the probability of tropomyosin being a major crustacean allergen in crab, shrimp, and crawfish (Daul *et al*, 1994).

Tropomyosin (*Pen a 1*) from brown shrimp *Penaeus aztecus* has been identified as the only major shrimp allergen. Beef, pork and chicken are other tropomyosin containing food that is not very allergenic. Compared to crustaceans, beef, pork and chicken, which also contains tropomyosin, seems to be less allergenic than crustaceans. Although people have reacted to beef, pork and chicken, the offending allergen seems not be tropomyosin. Tropomyosin can serve to investigate the contribution of the structural properties of a protein to its allergenicity. The main aim of the study by Reese *et al* (1997) was to determine the primary structure of *Pen a 1* and to identify IgE-binding epitopes. They sequenced four IgE-reactive, recombinant peptides of the major allergen *Pen a 1* from brown shrimp. An IgE-reactive peptide previously identified in Indian shrimp *P indicus* partially overlaps with E6, (one of four IgE-reactive peptides sequenced and aligned with the sequence of *Pen a 1* and *Met e 1*), indicating that this sequence of shrimp tropomyosins is a major IgE-binding site. No IgE-reactive *Pen a*

1 peptide shows complete sequence identity with vertebrate tropomyosins. They conclude by saying that since tropomyosins are a family of highly homologous proteins that contain both allergens and non-allergens, and the sequences of tropomyosins from a large variety of origins have been determined, tropomyosin is a good model to study the contribution of the primary structure to the allergenicity of proteins (Reese *et al*, 1997).

Currently tropomyosins are considered to be the major allergen in shrimp. However support have been lacking in other crustaceans. In a study by Leung *et al* (1998), lobster allergens were identified and characterised. The authors investigated the IgE-reactivity of crustacean sensitive individuals with lobsters *Panulirus stimpsoni* and *Homarus americanus*, at molecular level. They demonstrated that sera from subjects with clinical history of crustacea allergy have a high IgE-reactivity against the lobster tropomyosin. The IgE antibodies from the crustacean sensitive subjects show reactivity to a 34 kDa native lobster muscle protein, as well as the recombinant lobster tropomyosin *Pan s 1* and *Hom a 1*. Inhibition studies suggest that the epitopes between lobster and shrimp tropomyosins are highly similar if not identical. Results from this study demonstrate a muscle protein tropomyosin as a lobster allergen, as well as the possibility of tropomyosin as a major common allergen. Comparison of amino acid sequences of *Pan s 1* and *Hom a 1* with shrimp tropomyosin *Met e 1*, indicate these proteins are very similar with lower degree of identity when compared to tropomyosin of the fruit fly and chicken. The significant amino acid sequence identity of these shellfish proteins can be accounted for by their close phylogenic relationship, as well as the physiologic, biochemical and functional similarity (Leung *et al*, 1998).

Leung and Gershwin *et al* (1998) undertook a further study to clone, identify and determine the primary structure of a major IgE-reactive molecule in crab (*Charybdis feriatus*).
They produced a recombinant protein, biologically active in expressing its IgE determinants and immunoblotting suggested *Cha f 1* to contain all the IgE epitopes recognised by sera from patients with crustacea allergy. Nucleotide sequence analysis of *Cha f 1* also showed that the major IgE-reactive protein in crab is tropomyosin.
Cha f 1 showed extensive similarity in amino acid composition and peptide sequence identity with *Homarus americanus* slow muscle tropomyosin (95.8%) and to a lesser extent *Drosophila* tropomyosin (60.3%). *Cha f 1* is also significantly homologous with *Hom a 1* (92.4%), and *Pan s 1* (91.4%) respectively. Altogether their immunologic data and sequence analysis show that *Cha f 1* is the crab tropomyosin (Leung and Gershwin *et al*, 1998).

2.4.5 Cross reactivity studies

2.4.5.1 Cross-reactivity of fish species: *in vivo* and *in vitro* studies

de Martino *et al* (1990) ascertained whether cod-allergic children have increased frequency of positive skin tests to other fish species in order to determine which species might be included in their diet.

Sixty children were selected for the study. Twenty children had positive skin tests to cod and the positivity was closely related to their clinical history. Forty children had positive skin tests and RAST to one or more foods different from cod. The following allergen crude extracts were obtained from a commercial supplier (Lofarma, Milan, Italy): anchovy (*Engraulis enchrausicholus*), bass (*Dicentrarchus labrax*), carp (*Cyprinus carpio*), dogfish (*Mustelus plebeius*), eel (*Anguilla anguilla*), gilthead (*Chrysophrys aurata*), mackerel (*Scomber scomber*), mullet (*Mugil cephalus*), perch (*Perca fluviatilis*), red mullet (*Mullus barbatus*), salmon (*Salmo solar*), sardine (*Sardina pilchhardus*), tench (*Tinca tinca*), toothed gilthead (*Dentex dentex*), trout (*Salmo trutta*), tuna (*Thunnus thynnus*), and sole (*Solea vulgaris*).The children were Skin Prick Tested and they found that the number of subjects with positive SPT to one or more fish species was significantly higher in children with cod allergy (85%) than in children negative to cod (10%). The SPT positivities to the single fish species are presented in Table 2.3

The test was repeated one year later and in no subject was any change in the SPT results observed. As seen in table 2.3 all cod-positive children were not uniformly positive to all species tested, thus to counsel cod-allergic patients, a diet avoiding all fish may be incorrect without testing other fish species that could perhaps be eaten without any side effects. Total and specific IgE (RAST) levels to fish species, (cod, bass, dentex, dogfish, eel sole, and tuna), were studied with commercially available kits (Sferi kit, Lofarma). RAST to cod, bass, dentex. dogfish, eel, sole and tuna confirmed the SPT results. Total IgE results correlated with levels of specific IgE to cod and eel. No other correlations in IgE levels to other fish species studied by RAST was observed.

The presence of cross-reacting antigens among cod, bass, dentex, eel, sole, and tuna was suggested by the results of the RAST-inhibition assays. The authors propose that skin testing for cod allergy is a good approach to the diagnosis of allergy to fish, however, cod-allergic children may according to them, not be sensitive to, and may tolerate other fish species, particularly dogfish (de Martino *et al*, 1990).

Table 2.3 Prevalence of sensitivity to various fish species among Cod-positive and Cod-negative children.

Fish species	Cod-positive children (n = 20) (%)	Cod-negative children (n = 40)
Eel	17 (85)	0
Bass	11 (55)	0
Dentex	11 (55)	1 (2.5%)
Sole	11 (55)	3 (7.5%)
Tuna	11 (55)	2 (5%)
Perch	8 (40)	0
Tench	8 (40)	0
Anchovy	7 (35)	0
Red mullet	7 (35)	0
Trout	7 (35)	0
Mullet	6 (30)	0
Carp	5 (25)	0
Gilthead	4 (20)	0
Mackerel	4 (20)	0
Salmon	4 (20)	0
Sardine	4 (20)	0
Dogfish	2 (10)	0

Source de Martino *et al*, 1990

Bernhisel-Broadbent *et al* (1992) studied 11 patients with histories of fish hypersensitivity.

They underwent SPT, Double-blind, placebo-controlled oral food challenge (DBPCFC) and open oral challenges, each patient consumed four to six different fish species from at least four different fish families. SPT were done with raw fish extracts of catfish, bass, perch, mackerel, tuna, salmon, trout, codfish, flounder, and sardine. The fish species used represented 7 families and 6 orders of bony fish (Osteichthyes). The SPT positivities were as follows: see Table 2.4.

Table 2.4: Fish SPT results in 11 patients with a history of fish hypersensitivity.

Fish species	No of patients positive
Catfish	9
Bass	9
Perch	9
Mackerel	8
Tuna	9
Salmon	9
Trout	9
Codfish	9
Flounder	9
Sardine	8

Source Bernhisel-Broadbent *et al*, 1992

Forty-one DBPCFC's and nine primary open fish challenges were performed. All nine open challenges were negative. Eight of the 41-blinded challenges were positive and 33 negative. These 33 negatives were followed up by open challenges and 7 were positive. The false negatives did not appear to be secondary to insufficient quantity of fish antigen being consumed, since symptoms often appeared after the first or second bite in the open challenges. Compared to the oral challenges, the overall sensitivity of the fish SPT was 100% and the specificity 26%. The positive predictive accuracy was 37% and the negative predictive accuracy was 100%.

Immunochemical analysis of the fish proteins was evaluated by ELISA-inhibition assays. The assays were performed with extracts from a fish that provoked an allergic reaction, a fish from the same order, and a fish from a different order to which the patient had previously demonstrated tolerance. The concentration of the different fish proteins required to obtain 50% inhibition were similar, and the assays had comparable inhibition curves. In addition, clinically non-allergenic fish extracts were able to achieve 100% inhibition in these assays. No inhibition was observed with an unrelated protein (whey). It appears in some patients that fish allergy is species specific and the history of a reaction to one fish species should not automatically result in a restriction from eating all fish. The patients in this study were all able to consume one or more other fish species without adverse reactions (Bernhisel-Broadbent *et al*, 1992)

Hansen *et al* (1997) set out to demonstrate the immunologic reactivity of clinically codfish allergic adults to four species of fish: cod, mackerel, herring, and plaice.

Thirty-eight adults were investigated and Skin Prick Tested. Of the 38 subjects 8 were clinically codfish-allergic. Thirty subjects were considered to be controls, 3 of the 30 showed codfish allergy upon ingestion but were found to be clinically tolerant to codfish by DBPCFC, the remaining 27 subjects reported frequent intake of fish with no symptoms.

Of the 8 clinically codfish-allergic, all were SPT positive to the commercial extract of codfish, 7 were also positive to mackerel and all 8 were positive to herring and plaice. One challenge-negative patient to codfish with a positive SPT to codfish also had a positive SPT to herring and plaice. There were no reactions among the other control subjects.

In vitro testing involved determination of specific IgE to codfish, mackerel, herring, and plaice. Two commercially available kits, Phadebas RAST (PHA), and the Pharmacia cap system (CAP) measured this. Both systems were from Pharmacia diagnostics, Uppsala, Sweden. A Maxisorp RAST (MAXI) was also used and RAST-inhibition was also done on all subjects with elevated specific IgE antibodies to codfish (in MAXI). In the PHA test, all 8 clinically codfish allergic patients were positive to mackerel, herring, and plaice. In the CAP procedure all 8 were positive to mackerel and herring and 7 of the 8 were positive to plaice.

Inhibition studies were done in order to confirm whether mackerel, herring and plaice shared allergenic determinants with codfish. Cross-reactivity, in varying, degrees with these extracts were seen in all 8 adults, except with herring in one and plaice in another patient. Those with a lower degree of cross reactivity had a positive outcome in all other tests performed. The three other fish species were capable of inhibiting reactivity to codfish. It confirmed the hypothesis of cross-reacting antigens between codfish, mackerel, herring and plaice.

This study demonstrated that clinically, codfish-allergic patients do react to other fish species, and general caution is advisable for codfish-allergic adults ingesting other species of fish or fish based products. As only a small number of subjects were tested in this study the authors suggested that more extensive studies were needed (Hansen *et al*, 1997).

2.4.5.2 Cross-reactivity of crustacean species: *in vivo* and *in vitro* studies

Allergic reactions to oysters are also of interest because a number of crustacean-sensitive patients reported reactions upon oyster ingestion. Lehrer and McCants (1987) investigated IgE antibody reactivity to oyster extracts in oyster tolerant and oyster sensitive subjects and also the relationship to crustacean-specific responses.

The subject groups studied were chosen on the basis of sensitivity to oysters or crustaceans. Group 1 comprised 6 adults sensitive only to oysters, group 2 comprised seven adults sensitive to both oysters and crustaceans, and group 3 comprised twelve adults who reported a lack of prior exposure or hypersensitivity to oysters, but reported symptoms upon ingestion of crustaceans.

In-vivo testing involved SPT's with 10 common inhalant allergens and also with extracts of crustacea, white shrimp (*P. sefticus*), blue crab (*Callinectes sapidus*), spiny lobster (*Panulirus argus*), crayfish (*Procambarus clarkii*), and oyster (*Crassostrea virginica*) prepared in-house.

No individual skin test results were given.

In-vitro studies included specific IgE determination by RAST and RAST inhibition to determine cross-reactivity. One of the 6 subjects that were oyster-sensitive had an elevated RAST ratio of 6 to raw oyster, whereas all the others had essentially no reactivity. Of the oyster and crustacean sensitive subjects 3 of the 7 had elevated IgE antibodies (RAST ratios 7-41) to both boiled and raw oyster extract. Of the crustacean sensitive subjects, 6 of the 12 had elevated IgE antibody to boiled and raw oyster extract (RAST ratios of 6-34). Individual sera appeared to react similarly to both raw and boiled oyster extract, suggesting similar allergenic content, but essentially there was no correlation of RAST reactivity with total serum IgE levels. Comparison of skin and RAST reactivity suggested specificity; the results indicated that skin test negative individuals, in general, had little or no RAST reactivity to the oyster RAST discs. Sera from a number of skin test positive individuals had elevated IgE antibodies to the oyster extracts. The results suggest that positive oyster RAST's are specific immunological reactions rather than non-specific binding of the IgE to the oyster discs. Further studies compared reactivity of IgE antibodies in individual sera to oyster and crustacean extracts. The results indicated that IgE antibodies in individual sera appeared to react similarly to oyster and crustacean allergens. This supports the hypothesis of common antigenic epitopes in oyster and crustacean antigens. Antigenic relationships of oyster and crustaceans were further studied by RAST inhibition. Different crustacean preparations were tested and showed to significantly inhibit the

oyster RAST. RAST inhibition to either raw or boiled oyster was equally as effective with the crustacean extracts. Further tests were done which suggested oyster and crustacean allergens share common allergenic epitopes.

Although common allergenic epitopes have been reported for other foods, in particular crustaceans, they were unexpected in this study, because oyster and crustacea are not closely related in that they belong to different phyla (oysters, Mollusca; crustacea, Arthrapoda). The authors suggested it could be due to common antigenic structures, which are related to primitive molecular entities conserved in both phyla. Another possibility could be based on the fact that oysters are filter feeders, and it is possible that crustacean larva may be part of their diet. Thus the ingestion of small crustacea by oysters might lead to the incorporation of their allergens (Lehrer and McCants, 1987).

The presence of shared antigenic and allergenic determinants, on a molecular level among crustaceans have been demonstrated.

Desjardins *et al* (1995) assessed the prevalence of occupational IgE mediated sensitisation and asthma in processors who handled lyophilised clam and shrimp, (lyophilised powder extracts of various foods were put into bags).

In-vivo studies involved Skin Prick Tests (SPT) on 57 workers. Commercial preparations of clam, shrimp, lobster and crab were used, and extracts of clam, shrimp, and guar gum were prepared in-house. The results of the positive SPT's were as follows: clam 4/57, shrimp 9/57, lobster 5/57, and crab 5/57. Three of the 4 positive to clam were also positive to shrimp. Five of the 9 positive to shrimp were also positive to commercial lobster and 4 to crab.

In-vitro studies involved immunological testing of sera from 55 workers. Specific IgE measurements to clam, shrimp or both, and 5 negative controls were further tested for IgE antibodies to lobster, crawfish, and crab extracts. The results were as follows: 4 positive to clam, and 8 positive to shrimp. The 4 positive to clam were also positive to shrimp. Of the 8 sera positive to clam or shrimp, or both, 5 were positive to lobster, 7 positive to crawfish and 6 positive to crab. The authors demonstrated evidence of immunologic cross-reactivity between various seafoods used in their study, with regard to skin reactivity and specific IgE levels (Desjardins *et al*, 1995).

2.5 Studies of occupational seafood allergy due to crustaceans

Literature was reviewed on immunological studies that were done on occupationally exposed workers in the seafood industry. The review included workers that were exposed to fish as well as crustaceans in cross-sectional studies and case reports.

Gaddie *et al* (1980) investigated respiratory symptoms among a group of workers at a factory processing prawns. The factory employed 123 workers and 50 worked in the prawn-blowing section. The meat of the prawn was blown out of the tails by compressed-air jets, and small fragments of meat and droplets tended to spray in all directions. The 50 workers were interviewed and 18 of them showed significant respiratory symptoms. 15 were prawn blowers and the remainder worked within 1 metre of the prawn-blowing area. The symptoms started 6 weeks after the process of the factory had changed from hand peeling to air blowing.

The following immunological studies were done: Skin Prick Tests (SPT) with *D. pteronyssinus*, grass pollens, and prawn extract and serum was taken for total and specific IgE determination. The SPT results were as follows: Seven (39%) of the affected 18 workers were positive to the prawn extract and 6 (86%) of the 7 were atopic (positive to *D. pteronyssinus* and grass pollens). Six (19%) of the 32 unaffected workers were also positive to the prawn extract and 3 (50%) of them were atopic. The controls were all negative.
The serum investigations were as follows: 9 (50%) of the affected workers had elevated total IgE as measured by radioimmunoassay (Pharmacia), 7 (39%) of this group had increased specific IgE as measured by Radioallergosorbent test (RAST, Pharmacia). All of these were positive on SPT. Among the thirty-two unaffected workers, 11(34%) had elevated total IgE and 1 (3%) had elevated specific IgE. In most subjects an asthma-like reaction developed within 15 minutes of exposure and in others the illness occurred later, suggesting a delayed type of reaction. The authors suggested that the symptoms were serious enough to warrant prohibition of air blowing and mandatory surveillance of workers using water-blowing techniques (Gaddie *et al*, 1980).

Cartier *et al* (1984) investigated 303 crab-processing workers in two factories situated in Canada. They used 3 different commercial crab extracts (Alaska king crab, Dungeness crab, stone crab) and a crab boiling water extract for SPT.
During the pre seasonal study they found 298 workers had interpretable skin reactions and 65 (21.8%) of the 298 workers reacted to crab. During the seasonal survey, they found of the 298

subjects that 29 (10%) were positive to the commercial and the boiling water extracts, 25 (8%) were positive to only the commercial extracts and 11 (4%) were positive to only the boiling water extract. There was a strong statistical association between reactivity to the commercial extracts and the crab boiling water extract and the diagnosis of asthma. Twenty-seven (59%) of the 46 confirmed cases of occupational asthma had positive skin prick tests to the crab extracts and/or the crab boiling water, this suggested that at least one antigen, derived from crab aerosolised during boiling, was inhaled by the workers (Cartier *et al*, 1984).

Orford and Wilson (1985) determined the frequency of respiratory disease in 3 Bellingham Crab-processing plants and fish packers in another plant as control, to examine the possible causes of asthma, hypersensitivity, pneumonitis etc. Extracts of king crab meat, shell and processing dust from beneath the bandsaws were prepared for skin prick testing. Six (40%) of the 15 workers were positive to all 3-crab antigens and to one or two of the antigens in another three. Six (40%) of the workers were negative to the three antigens. The crabmeat was found to be more potent than shell and dust but the differences were small. As all three extracts provoked reactions in sensitive individuals it seems that antigens are not confined to the chitinous exoskeleton or the flesh (Orford and Wilson, 1985).

Carino *et al* (1985) reported a case study of a 37-year-old male with occupational asthma who was employed as a technician in a biological institute for nearly 9 years. His job included operations related to preparing and using both commercial and trial mixtures of shrimp meal for experimental intensive aquaculture of fish and shellfish. He underwent SPT with extracts of shrimp meal *(Artemia salina, Penaeus shrimp)* and shrimp constituents (shell, chitin) that he handled at work. Immediate reactivity to *Artemia salina* was documented on SPT. RAST showed 1+ to *Artemia salina* (Lofarma) and commercial shrimp 1+ and crab 2+ (Pharmacia, Uppsala, S.). The immunological findings indicate that specific IgE antibodies can mediate occupational asthma due to inhaled shrimpmeal, and antigens can be present both in the flesh and exoskeleton but the chitin itself induced no reactions. Nevertheless the pattern of bronchial responses may suggest that other mechanisms could be involved (Carino *et al*, 1985).

Cartier *et al* (1986) restudied a group of workers with different snow crab extracts for skin testing and specific IgE measurements. Their aim was to determine the prevalence of IgE sensitisation to snow crab extracts in these snow crab-processing workers with and without occupational asthma. One hundred and nineteen (39%) of the 303 workers had skin prick tests

with snow crab meat extracts and new extract of snow crab cooking water collected in 1984 (water-84). In this study they used a more specific and active water extract. RAST's with the latter extracts were done on 115 (38%) of the 303 workers with the 1982 sera that was stored at –20° Celsius, 58 (19%) of the workers had both tests done. A total of 54 confirmed cases of occupational asthma were skin tested and 48 (89%) of the 54 had RAST's done.
The RAST test was used to quantitate circulating IgE in the test sera. In-house RAST with activated filter paper discs were used and analysed using radioactivity The study concluded that there was better agreement between RAST and skin prick tests obtained with the water-84 than with the RAST and skin tests obtained with meat extracts. One possible explanation for the water that was more sensitive and potent than the meat is that the constituents, probably heat stable or denatured protein responsible for the reaction, were water soluble and present in higher concentrations once solubilized in water. (Cartier *et al,* 1986).

In a study done by Malo *et al* (1988), they focussed on pattern of improvement in spirometry, bronchial hyper-responsiveness and specific IgE antibody levels after cessation of exposure, in occupational asthma caused by snow-crab processing. They included 31 workers with occupational asthma caused by snow crab processing. Workers were tested on three occasions after leaving their work, at minimum intervals of 6, 24 and 57 months respectively. IgE levels were assessed on their last two visits and snow crab meat extracts and snow crab cooking water were used in the skin tests and the in-house RAST's. Initially 12 (48%) of the 25 serum samples that were assessed for specific IgE to crab meat and or boiling water showed significant increased values (% binding > 3). These 12 workers also showed immediate skin reactions to meat and or boiling water. Ten (40%) other subjects also showed immediate skin reactivity but did not have significantly increased specific IgE. A significant fall in the levels of specific IgE to crab meat was detected only at the time of the 3rd follow up. There was a significant and progressive fall in specific IgE levels to crab boiling water at the time of the 2nd and 3rd follow-ups and only 3 subjects showed a plateau of improvement (<30% change) in the levels of specific IgE antibodies between the 2nd and 3rd follow-ups (Malo *et al*, 1988).

In a case report by Patel and Cockroft (1992) a 25-year-old male chef was referred with symptoms of wheezing, dyspnoea and cough following ingestion of lobster, shrimp and scaly fish (pickerel) and while handling lobster. Skin prick tests were positive to mixed shellfish. There was a large response to lobster, and a 4+ to haddock, cod, oysters, and clam and 3+ to scallops (Patel and Cockroft, 1992).

Desjardins *et al* (1995) assessed the prevalence of occupational IgE mediated sensitisation and asthma in processors who handled lyophilised clam and shrimp. Skin prick tests were done on 57(95%) of the 60 workers and sera from 55 (92%) were available for immunologic testing. Commercial preparations of clam, shrimp, lobster and crab were used and extracts of clam, shrimp and guar gum were also prepared in-house. Specific IgE measurements to clam and shrimp were performed for all 55 (92%)of workers using an in-house RAST. Those sera with positive RAST to clam, shrimp or both and 5 negative control sera were further tested for IgE antibodies to lobster, crawfish and crab extracts. Four (7%) subjects had immediate skin reactivity to clam. Nine (16%) reacted to shrimp extract and none to guar gum. Five (9%) had positive reactions to lobster and five (9%) to crab, 3 (75%) of the 4 who reacted to clam also reacted to shrimp, 5 (55%) of the 9 that reacted to the shrimp also reacted to commercial extract of lobster and four of them reacted to commercial extract of crab. Four (7%) had significant increase in specific IgE levels to clam. Seventy five percent of them were positive on SPT, eight (14%) had increased specific IgE levels to one or another of the two extracts of shrimp and 63% of them had positive SPT. The four (7%) with increased specific IgE to clam also had high levels of specific IgE to shrimp. Of the eight sera that had increased specific IgE to clam, shrimp or both, five (63%) had increased levels to lobster, seven (88%) to crawfish and six (75%) to crab. In 5 negative control sera with normal specific-IgE to clam and shrimp, 1 (20%) had increased specific IgE to crawfish. Three (5%) subjects had increased specific IgE levels to guar gum. This study showed that clam and shrimp are causes of IgE- dependant sensitisation and occupational asthma in workers processing these agents. They also showed evidence of immunologic cross-reactivity between the various seafoods used in their study, in regard to both skin reactivity and specific IgE levels. Immunologic cross-reactivity between crustaceans like shrimp, crab and lobster has been documented (Desjardins *et al*, 1995).

Lemiere *et al* (1996) reported a 34-year-old woman working in a fishmonger shop who developed urticaria, rhinitis and asthma when handling lobster and shrimp. SPT to seafood; commercial extracts of clam, oyster, scallop and mussel were negative. Skin testing to lobster, shrimp and crab showed immediate whealing. RAST results were expressed as percentage binding of the amount of radiolabeled anti-IgE added to the disk indicated: 5.8% to lobster, 16.7% to shrimp, 3.6% to crab and 4.5% to crawfish. Occupational asthma and immunological sensitisation caused by both lobster and shrimp can be explained by the fact that she was exposed to both kinds of shellfish and or cross-reactivity between the two species (Lemiere *et al*, 1996).

Smith and Sechena (1998) investigated 51 individuals who worked on Alaskan offshore crab-processing ships. They analysed the sera using the Pharmacia diagnostic disc method. Diagnostic Products Corporation in Los Angeles, California quantified specific IgE. They used a AlaSTAT liquid phase EIA (Enzyme-labelled immunometric assay based on liquid allergens, monoclonal antibodies and ligand-coated tube separation). The IgE antibodies specific to crab components opilio crab (*Chinoecetes opilis)* were: meat (raw and cooked), blood, dinoflagellate (single-cell algae), blood+dinoflagellate, and crushed shell legs (raw and boiled). The degree of reactivity was classed as 0 to 4, 0 being no reactivity and 4 having highest reactivity. EIA testing for IgE specific to crab components were generally unreactive except to crab blood and crab blood + dinoflagellate (CB+DF), where 43% and 59% of subjects showed > class 2 reactivity respectively. Thirteen (25%) subjects had no reactivity to CB+DF and were also unreactive to all other antigens, forty seven percent of the fifty nine percent that were positive to CB+DF were also skin test positive. In this case the SPT seems less specific than the EIA. A specific IgE antibody is present in many patients but does not always correlate with the severity of symptoms or type and severity of abnormality of lung function tests. The presence of IgE antibody indicates sensitisation but does not prove that it is the mechanism of the systemic symptoms or physiologic abnormalities (Smith and Sechena, 1998).

Cartier *et al* (1999) reported that less than one year after starting their work with Atlantic rock crab (*Cancer irroratus*); several of the workers complained of respiratory symptoms suggestive of asthma. Twenty-nine subjects were referred for investigation of occupational asthma (OA), workers were interviewed, underwent SPT's to common allergens and rock crab extract, and had lung function tests. Only one (3%) worker had a previous history of asthma. Sixteen (57%) of the 28 subjects had at least one positive SPT to a common allergen and 7 (25%) of the 28 had positive SPT's to the rock crab extract. Taking into account these results and the lung function (not mentioned here), the authors concluded that Atlantic rock crab processors are at risk of developing OA but the exposure is also associated with high prevalence of asthma-like symptoms without any evidence of asthma (Cartier *et al*, 1999)

Ortega *et al* (2001) reported workers in a crab processing facility done both at the beginning and at the end of the crab processing season. The survey included questionnaires, spirometry, serological testing, and measurement of airborne crab allergens and microscopy of aerosolised materials. The aim of this study was to investigate factors, including workers' exposures, associated with the development of respiratory symptoms in crab processing workers.

For the serological studies, blood samples were collected for total and specific IgE antibodies and also for eosinophil cationic protein. IgE antibodies to crab were measured by using the Pharmacia CAP for *Cancer pagurus* crab. RAST assays were also developed in order to detect specific IgE to snow crab extracts of the species processed in the plant. Allergen extracts were prepared from cooking water, uncooked crabmeat, cooked crabmeat, and kanimiso. Of the 122 that completed both early and late season surveys, 15 (13%) were excluded because they did not spend at least 4 weeks in crab processing, thus a final number of 107 subjects completed the full survey. Serum levels of total and crab-specific IgE were determined in both early and late season surveys for 90% (96/107) of the crab processors. Levels of specific IgE were higher among "naïve" workers (they had 4 weeks or less lifetime experience in the crab industry at the early season survey), 12% of these workers had positive levels of IgE to kanimiso and crabmeat at the end of the season. In contrast, only 2% of experienced workers had specific IgE to kanimiso and none to crabmeat at the end of the season. The percentage specific IgE measured using the CAP for *C. pagurus* was similar among naïve and experienced workers (10% and 11% respectively). No significant changes were observed in the percentage IgE reactivity over the crab-processing season. In this study it is clear that the level of specific IgE to crab was higher in naïve than in experienced workers, suggesting that past exposure to crab processing work may result in the development of IgE antibodies and respiratory symptoms. These workers may be less likely to return to work and it is consistent with the "healthy worker effect". The authors feel that despite a few limitations in their study, an increased cross-seasonal development of respiratory symptoms in particular jobs such as, butchering and degilling were observed. This suggests that aerosols generated in these operations are able to trigger respiratory symptoms even when allergic sensitisation is absent (Ortega *et al*, 2001).

Table 2.5 a: Studies of occupational seafood allergy due to crustaceans

Study/Date	Agent	Number of subjects	SPT Positive	Other Immunological Methods	Results
Gaddie *et al.* 1980	Prawn *Nephrops norwegicus*	50 (18 with symptoms) (32 no symptoms)	7/18 to prawn extract (39%) 6/7 were atopic (86%) 6/32 to prawn extract (19%) 3/6 were atopic (50%)	Pharmacia RAST	7/18 +ve (39%) 1/32 +ve (3%)
Cartier *et al.* 1984	Snow crab *Chinoecetes opilis*	303 298 with interpretable skin results	29 (9%) to comm + bw 25 (8%) to comm only 11 (4%)to bw only	ND	
Orford and Wilson. 1985	King crab	15	6 (40%)to all 1 (7%)to meat 1 (7%)to shell 1 (7%)to meat + shell	ND	
Carino *et al.* 1985	Shrimpmeal *Penaeus shrimp* *Artemia salina*	1	*Artemia salina*	Pharmacia, Uppsala RAST	1+ to *Artemia salina* 1+ to comm shrimp 2+ to crab
Cartier *et al.* 1986	Snow crab	303 119 (39%) had SPT 115(38%) had RAST 58 (19%)had both	No SPT values given	Activated paper disc RAST	No Values given
Malo *et al.* 1988	Snow crab	31	22 (71%) to crab meat and or boiling water.	In-house RAST	Initial 12 (48%) of 25 +ve 1st follow-up ND 2nd follow-up 9 (29%) of 31 positive 3rd follow-up 5 (17%) of 29 positive

comm-Commercial (Alaska king crab, Dungeness crab, stone crab), bw-Boiling water, + ve-Positive, ND-Not done, SPT-Skin prick test, RAST-Radioallergosorbent test

Table 2.5 b: Studies of occupational seafood allergy due to crustaceans

Study/Date	Agent	Number of subjects	SPT Positive	Other Immunological methods	Results
Patel and Cockroft. 1992	Lobster	1	Large response to lobster, 4+ haddock, cod, oysters, clams 3+ scallops	ND	
Desjardins *et al.* 1995	Clam and Shrimp	60 57 (95%) for SPT 55 (92%) donated sera	Clam: 4 (7%) Shrimp: 9 (16%) Lobster: 5 (9%) Crab: 5 (9%) 3 (75%) + ve to clam also to shrimp. 5 (55%) + ve to shrimp also to comm lobster and 4 (44%) to crab.	Filter paper disc RAST	4 (7%) + ve to clam, 8 (14%) + ve to shrimp, The 4 + ve to clam also + ve to shrimp. 5 (63%) + ve to clam and/ or shrimp + ve to lobster, 7(88%)+ ve to crawfish and 6 (75%) + ve to crab.
Lemiere *et al.* 1996	Lobster and Shrimp	1	Lobster shrimp and crab.	Filter paper disc RAST	Lobster 5.8% binding Shrimp 16.7% binding Crab 3.6% binding Crawfish 4.5% binding
Smith and Sechena. 1998	Crab	51 49 (96%) SPT 46 (90%) RAST	47% of 59% POS EIA to CB+DF	EIA	Both >2 reactivity CB 43% CB+DF 59%

comm-commercial, bw-boiling water, + ve-positive, ND-not done, SPT-skin prick test, RAST-radioallergosorbent test, CB-crab blood, CB+DF-crab blood+dinoflagellate, EIA-enzyme labelled immunometric assay.

Table 2.5 c: Studies of occupational seafood allergy due to crustaceans

Study/Date	**Agent**	**Number of subjects**	**SPT Positive**	**Other Immunological methods**	**Results**
Cartier *et al.* 1999	Atlantic rock crab *Cancer irroratus*	28	16(57%) to at least 1 common allergen. 7(25%) to rock crab extract.	ND	
Ortega *et al.* 2001	Crab (cw, ucm, ccm, kanimiso from plant) *Cancer pagurus* CAP RAST.	107 96 (90%) IgE tested 45 (47%) naïve (n) and 51 (53%) experienced (e) workers.	ND	Pharmacia CAP RAST for *Cancer pagurus.* In house RAST for cw, ucm, ccm, and kanimiso from plant.	12% (n) +ve to kanimiso and cm. 2% (e) +ve to kanimiso none to cm. *Cancer pagurus.* 10% (n) +ve 11% (e) +ve

+ ve-positive, ND-not done, SPT-skin prick test, RAST-radioallergosorbent test, cw-cooking water, ucm-uncooked crab meat, ccm-cooked crab meat, cm-crab meat

2.6 Studies of occupational seafood allergy due to fish

Sherson *et al* (1989), measured antibodies in the sera of eight workers against trout intestines, trout skin scrapings, contaminated water from the gutting machine and to salmon. An aluminium radio-allergosorbent-test (RAST) was used (see Table 2.6 for results). Techniques were not discussed in detail. This study focussed mainly on the lung function results and the endotoxin content of the contaminated water from the gutting machine (Sherson *et al*, 1989).

Douglas *et al* (1995) screened 291 salmon factory workers and IgE and IgG antibodies against salmon serum were measured by radioimmunoassay and enzyme-immunoassay (no details provided of these assays). The seropositivity in the post modification group (new workers) was 0% (IgE) and 8.1% (IgG) respectively compared to the exposed group which was 8.6% and 33% respectively. It was concluded that IgE antibody production and disease was associated with working close to the aerosol source, but not with length of exposure (Douglas *et al*, 1995).

In a case report by Rodriguez *et al* (1997), two workers underwent skin prick tests with cooked and raw fish extracts (plaice, salmon, tuna, hake, anchovy, sardine, trout, sole and atlantic pomfret). Seafood specific serum IgE levels were determined by CAP-FEIA (Pharmacia Diagnostic, Sweden), in one worker and by Phadezym RAST (Pharmacia Diagnostic, Sweden), in the second worker. In the first worker SPT was positive to raw and cooked plaice, salmon, hake and tuna fish. Specific IgE antibodies were detected to salmon only but not to plaice, tuna and hake. In the second worker SPT was positive to anchovy, sardine, trout, salmon, atlantic pomfret and sole. Specific IgE was detected to trout, anchovy and salmon. Since the authors used different RAST methods for evaluating the two patients it is very difficult to compare the results they obtained (Rodrigues *et al*, 1997).

Because of the lack of sensitivity and specificity of assays used by the different authors in the studies included in this literature review, the main aim of this thesis was to develop a diagnostic assay which is sensitive and specific to screen factory workers for fish and lobster specific IgE to obtain good reproducible results.

Table 2.6: Studies of occupational seafood allergy due to fish

Study/Date	Agent	No of Subjects	SPT positive to fish	Immunological technique	Results
Sherson *et al.* 1989	Rainbow trout	8	ND	Aluminium RAST method	Contaminated water • 7 positive • 1 doubtful Trout skin • 4 positive • 3 doubtful Salmon • 7 positive • 1 doubtful
Douglas *et al.* 1995	Salmon (*Salmo salar)*	291	ND	Pharmacia RAST method	8.6% positive of exposed group
Rodrigues *et al.* 1997	Plaice, salmon, hake, sardine, anchovy, tuna, trout, sole, pomfret.	2	Patient 1 Raw and cooked plaice, salmon, hake, tuna. Patient 2 Anchovy, sardine, trout, salmon, atlantic pomfret, sole.	Patient 1 CAP-FEIA RAST method Patient 2 Phadezym RAST method	Patient 1 RAST +ve to salmon but -ve to plaice, tuna and hake. Patient 2 RAST +ve to trout, anchovy and salmon.

ND-not done, SPT-skin prick test, RAST-radioallergosorbent test, +ve-positive, -ve-negative

CHAPTER 3

AIMS AND OBJECTIVES

The main aims and objectives were as follows:

To prepare specific seafood extracts from the following species: Mackerel (*Scomber japonicus*), Red eye (*Etrumeus whitehead*), Maasbanker (*Trachurus trachurus capensis*), fish meal, Pilchard (canned) (*Sardinops sagax*), Pilchard (salted) (*Sardinops sagax*), Pilchard (gut) (*Sardinops sagax*), Pilchard (cooked) (*Sardinops sagax*), Pilchard (raw) (*Sardinops sagax*), Cape Anchovy (*Engraulis capensis*) and Rock Lobster (*Jasus lalandii*), and to confirm its antigen/allergen content.

To generate polyclonal IgG antibodies in rabbits using the specific seafood extracts isolated from the various species.

To determine IgE-mediated sensitivity to seafood allergens among seafood processing workers.

To develop a sensitive and specific EAST (Enzyme Allergosorbent Test) technique for detecting specific IgE-antibody levels in workers' sera: the reason for developing an EAST method rather that a RAST method is because the specific IgE in the workers' sera were determined against seafood species they were exposed to in the factory. These species were not available on commercial CAP RAST.

To quantify specific-IgE in the serum of the workers exposed to seafood allergens using the EAST (S) South African laboratory, EAST (R) Reference laboratory, and RAST methods, and to determine the level of agreement between these methods, in order to determine the best method to diagnose occupational seafood allergy.

CHAPTER 4
MATERIALS AND METHODS

4.1 Study Outline

The study population consisted of 650 workers from two seafood-processing factories in St. Helena Bay on the West Coast of South Africa. The population consisted of all workers including those working on the jetty and in the administration offices. Workers completed a comprehensive questionnaire and underwent skin prick tests (SPT) with the following extracts: commercial: Aspergillus (*Aspergillus fumigatus*), Bermuda grass (*Cynodon dactylon*), House dust mite (*D. pteronyssinus*), Cockroach (*Blatella germanica*), Rye grass (*Lolium perenne*), Cat (*Felis domesticus*), Mouldmix (*Cladosporium herbarum, Altenaria alternata, Fusarium*), Dog (*Canis familiaris*), Anisakis (*Anisakis simplex*) and Mussel, and in-house: Mackerel (*Scomber japonicus*), Red eye (*Etrumeus whitehead*), Maasbanker (*Trachurus trachurus capensis*), fish meal, Pilchard (canned) (*Sardinops sagax*), Pilchard (salted) (*Sardinops sagax*), Pilchard (gut) (*Sardinops sagax*), Pilchard (cooked) (*Sardinops sagax*), Pilchard (raw) (*Sardinops sagax*), Cape Anchovy (*Engraulis capensis*) and Rock Lobster (*Jasus lalandii*), and methacholine challenge tests to determine lung function.

A blood sample was also taken from each worker and those who could not undergo skin prick tests. Workers with a history of severe reactions to seafood and pregnant women were excluded from skin prick testing for safety reasons. A group of workers were selected for the purpose of this study. The group consisted of all skin prick test positive workers and a control group. The Enzyme Allergosorbent Test (EAST) (S) South African laboratory was optimised and together with the EAST (R) Reference laboratory, and RAST used to quantify the specific IgE in these workers sera. The level of agreement between the different methods was determined, and the specific IgE results compared to the specific working area of the person in the factory.

4.2 Antigen/Allergen analysis

I <u>Isolation of seafood extracts</u>

Protein extracts were prepared from ten fish species and one crustacean species;

Mackerel (*Scomber japonicus*), Red eye (*Etrumeus whitehead*), Maasbanker (*Trachurus trachurus capensis*), fish meal, Pilchard (canned) (*Sardinops sagax*), Pilchard (salted) (*Sardinops sagax*), Pilchard (gut) (*Sardinops sagax*), Pilchard (cooked) (*Sardinops sagax*), Pilchard (raw) (*Sardinops sagax*), Cape Anchovy (*Engraulis capensis*) and Rock Lobster (*Jasus lalandii*). The fishmeal consisted of about 80% of Anchovy and the rest of Red eye and cut-offs of Mackerel and Maasbanker. The tissue was cut into small pieces and extracted into phosphate buffered saline (PBS) (Appendix 1.5.2) overnight at 4° C. The extracts were sterile filtered (0.45µm, Millipore), the protein concentrations determined (BCA-protein assay, Pierce) and aliquots stored at -80°C until further use.

These different extracts were separated by SDS-PAGE to determine the antigen content and also the molecular weights of the different antigens.

SDS-PAGE

Glass plates were cleaned with 70% ETOH and assembled with spacers and clamps. A 1% agarose solution (Appendix 1.2.10) was melted in a microwave oven and the bottom and sides of the plates sealed. The running gel was made up as follows: (See Table 4.1)

After the 5% and 16 % solutions of the running gel was made up, the 16% solution was poured into the mixing chamber and the 5% solution poured into the reservoir chamber (the interconnecting and outlet valves remained closed). When the solutions were in place the magnetic stirrer was switched on and the interconnecting valve opened, the two solutions were allowed to mix for a few seconds after which the outlet valve was opened and the gel poured into the vertical gel unit, a space of about 3 cm was left at the top for the stacking gel. (See figure 4.1)

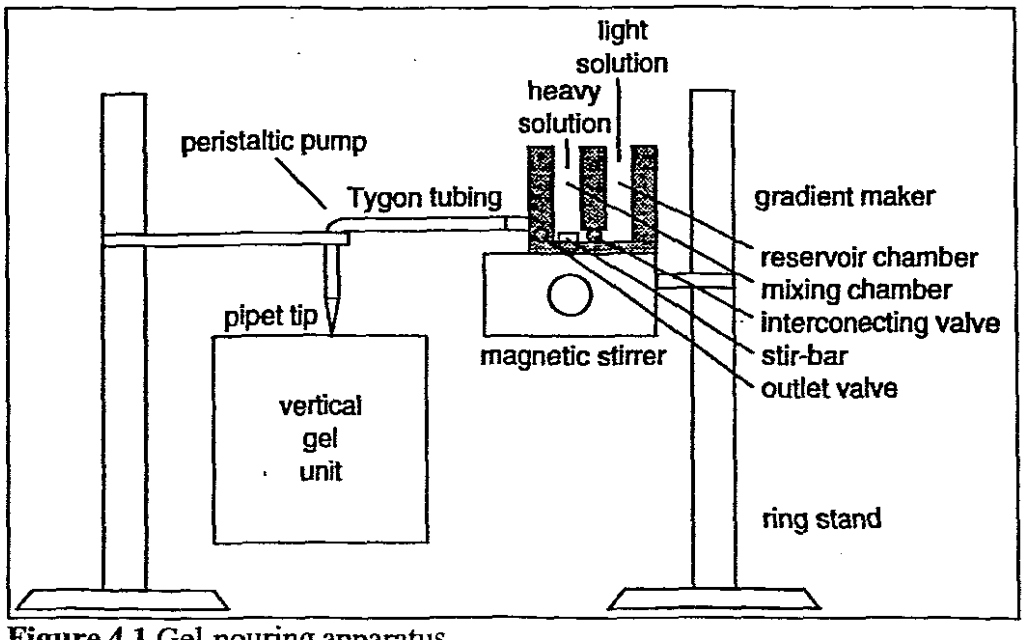

Figure 4.1 Gel-pouring apparatus

Table 4.1: Reagents used for the running gel.

REAGENT	Running Gel	
	5% GEL	16% GEL
30% Acrylamide 30%w/v Acrylamide Bis solution (Life Technologies Gibco BRL)	**0.833 ml**	**2.67 ml**
5% Running Gel Buffer (Appendix 1.2.1)	**1.6 ml**	-
16% Running Gel Buffer (Appendix 1.2.2)	-	**1.6 ml**
0.1% SDS (Appendix 1.2.3)	**2.51 ml**	**0.676 ml**
10%AMPS (Appendix 1.2.9) Amonium Peroxodisulphate, (BDH electran)	**50 µl**	**50 µl**
TEMED N, N, N', N', -Tetramethyl-ethylenediamine, (Sigma)	**2 µl**	**2 µl**

A layer of iso-propanol was poured on top and the gel left for 45 min to polymerise. The iso-propanol was rinsed off with distilled H_2O and a layer of dilute (1:4) running gel buffer poured on the gel and covered overnight.

The following day the stacking gel buffer was made up as follows: (See Table 4.2)

Table 4.2: Reagents used for the stacking gel.

Reagent	Stacking Gel
30% Acrylamide 30%w/v Acrylamide Bis solution (Life Technologies Gibco BRL)	**0.55 ml**
Stacking Gel Buffer (Appendix 1.2.4)	**1.0 ml**
Distilled H_2O	**2.45 ml**
10%AMPS (Appendix 1.2.9) Amonium Peroxodisulphate, (BDH electran)	**12.5 µl**
TEMED N, N, N', N', -Tetramethyl-ethylenediamine, (Sigma)	**12.5 µl**

The stacking gel was poured on the running gel, the comb inserted and left for 45 minutes to polymerise. After polymerisation the gel was assembled to the reservoir. The stock solution reservoir buffer (Appendix 1.2.5) was diluted five times and the top of the reservoir was filled with the buffer. If there were no leaks, the bottom of the reservoir was also filled (approximately 200ml of 1X reservoir buffer was needed).

Samples were prepared for loading:

- 2-5 µg of protein need to be loaded per lane for blotting purposes and 20-30 µg of protein for staining purposes.
- Samples were diluted with PBS (Appendix 1.5.2) to desired concentration

- 200 µl of Sample buffer (Appendix 1.2.7) and 10µl DTT (Appendix 1.2.8) were mixed and added to the diluted sample to make up 30µl for loading (sample buffer/DTT should make up half of the 30µl to allow the sample to sink to the bottom of the well when loaded).
- Rainbow molecular marker (Amersham) was boiled with the samples for 3 minutes and quickly centrifuged (3000 rpm for 3 minutes) before loading.

The comb was now taken out of the stacking gel and the buffer allowed to run into the wells, the samples and marker were loaded into the wells. Leads were attached to the power source and run on constant current, 15*m*A through the stacking gel then 20*m*A through the running gel.

II Protein concentration of the seafood extracts

The protein standards were prepared as specified by the BCA assay standard protocol (BCA Protein Assay kit, Pierce) and phosphate buffered saline (PBS) (Appendix 1.5.2) was used as a diluent. Samples were diluted as required and using Greiner F micro plates, 10µl of standard or sample were pipetted into the wells in duplicate. PBS was used as a blank. BCA working solution was prepared according to manufacturers instructions and 200µl of working solution were added to each well. The plate was then incubated at 37°C for 30 minutes. Absorbance was read (with a Versa Max Micro plate reader from Labotech) at 540 nm after the plate was allowed to cool to room temperature.

III Generation of polyclonal IgG antibodies in rabbits

For the production of the polyclonal antibodies in rabbits, 500µg (in 500µl of TBS) (Appendix 1.1.1) of each protein extract were used to immunise each of two New Zealand rabbits. The extracted proteins were mixed with 500µl of Complete Freund's Adjuvant (Difco), and it was injected subcutaneously at several sites of each rabbit. The rabbits were also immunized subsequently at three and six weeks after the initial immunization, with the same protein concentration in Incomplete Freund's Adjuvant. Blood samples were taken for antibody titer analysis before immunisation (used as a negative control), at week 3 and 6, and the final bleed was at week 8.

IV Confirmation of antigen/allergen content by Western blot and ELISA

The basic protocol of Western blot will first be described. This will be followed by a description of methods used to confirm antigen/allergen content using:

A) Human IgE

B) Rabbit IgG

C) Monoclonal anti-parvalbumin and anti-tropomyosin

Western blot

A piece of PVDF membrane (Hybond-p Amersham Pharmacia Biotech) was cut to size and placed in MeOH for two seconds. The MeOH was poured off immediately, the membrane placed in distilled H_2O for about 5 minutes and then placed in transfer buffer (Appendix 1.3.1) until needed. Two Scotch-Bright pads were also soaked in transfer buffer and placed together with the membrane at 4°C until needed (NB. When working with the PVDF membrane always wear gloves to prevent proteins from sticking to the membrane which is highly sensitive to protein binding). Two pieces of filter paper were cut to size (a bit smaller than the pads) and soaked in transfer buffer just before use.

Once the running of the gel (SDS-PAGE as previously described) was completed it was dissembled and the gel placed in transfer buffer. The sandwich for the blot was assembled as follows: (See figure 4.2)

The anode (red) base was placed at the bottom then yellow pad, filter paper, membrane and then the gel on the membrane. The layers were repeated in reverse and when all air bubbles were removed by rolling a glass tube over it, the cathode (black) base was placed on top. The sandwich was placed in a plastic container and connected to the power supply. The current was set at 0.3 Ampere and allowed to blot for 45 minutes. When the blotting was completed the power was switched off and the sandwich disconnected and dissembled. The gel was placed in stain to confirm blotting, Coomassie Brilliant Blue stain was used (Appendix 1.2.12), after 10 minutes the gel was placed in destain (Appendix 1.2.11) and left overnight. The membrane was allowed to dry. Before the membrane was used for immunoblotting it was placed in MeOH again for two seconds and immediately rinsed with distilled H_2O for 5 minutes. The membrane was blocked for 45 minutes with blocking buffer (WB) (Appendix 1.3.2).

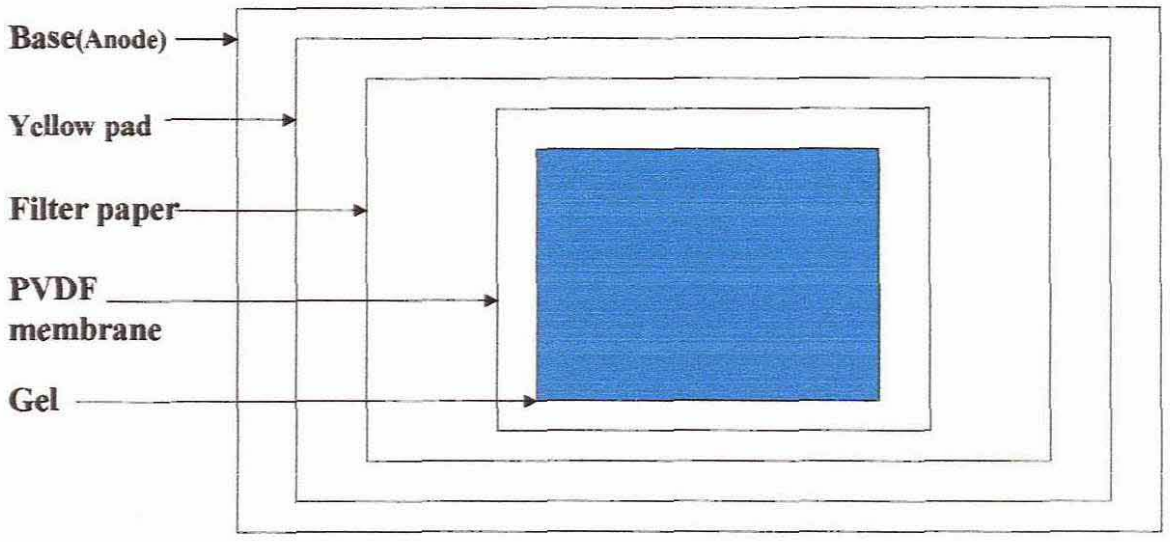

Figure 4.2: Assembling of sandwich blot.

A Human IgE

Western blot:

The following extracts were separated by SDS-PAGE: Raw mackerel, red eye, lobster, fish meal, canned pilchard, pilchard gut, cooked pilchard, raw pilchard, and raw anchovy. The proteins were transferred to a PVDF membrane by Western blot and the membrane was probed with a pool of sera from positive allergic patients. Four patients were used and 100µl of each serum was mixed and made up to 10ml with TBS (Appendix 1.1.1). The serum pool was left on the blot overnight. The next day the blot was washed 2X with TBS wash buffer (Appendix 1.3.3) and 1X with TBS. The secondary antibody mouse-anti-human IgE-AP (Southern Biotechnology) was diluted in TBS (1/1000), and left on the blot for 2 hours. The blot was then washed as before, substrate (Appendix 1.3.4) added and allowed to develop until purple bands became visible. Before the background developed, the blot was rinsed off with distilled water and left to dry.

B Rabbit IgG

Western blot:

Three extracts, lobster, anchovy, and pilchard (canned) were separated by SDS-PAGE (each extract was run separately using a solid comb, and 200µg of protein was loaded). Each gel was transferred onto a PVDF membrane respectively by Western blot, and each blot was now probed with its corresponding anti-rabbit antiserum. Each anti-serum was diluted 1/8000 in TBS and placed on its corresponding blot for 90 minutes. It was washed three times as described before and the secondary antibody goat-anti-rabbit IgG-AP (Southern Biotechnology) was diluted 1/2000 and left on each blot for 2 hours. The blots were washed

again and substrate (Appendix 1.3.4) was added and the blots were allowed to develop until purple bands became visible. Before the background developed, the blots were rinsed with distilled water and allowed to dry.

ELISA:

The plates were coated with lobster (raw), pilchard (canned), and anchovy (raw) protein extracts at two concentrations, 1µg/ml and 5µg/ml respectively; 100µl were added per well and left overnight at room temperature in the dark to coat. The next day the excess coating antigens were removed and the reaction was blocked with 2% blocking buffer (Appendix 1.4.1), 200µl per well for 1 hour at 37°C. Excess blocking solution was removed and the rabbit anti-sera was diluted serially from 1/1000 to 1/512000; 100µl per well were added and left for 90 minutes at room temperature. A control well was included where the anti-sera were replaced with TBS to ensure that the secondary antibody does not bind non-specifically. The plate was washed 3X with TBS wash buffer (Appendix 1.4.2), and the secondary antibody goat-anti-rabbit IgG-AP (Southern Biotechnology) was diluted (1/5000) and 100µl per well added. It was left to incubate for one hour, after which the plate was washed again, as before, and the substrate added (Appendix 1.4.3), 100µl per well, and read at 410 nm after 45 minutes.

C Monoclonal anti-parvalbumin and anti-tropomyosin

Western Blot:

The following seafood extracts were separated by SDS-PAGE: mackerel, red eye, maasbanker, lobster, fish meal, canned pilchard, salt pilchard, pilchard gut, cooked pilchard, raw pilchard, anchovy and anisakis. Two gels were run and the protein bands were transferred to two PVDF membranes respectively by Western blot. The one blot was probed with monoclonal anti-parvalbumin antibody (Sigma), and the other one with monoclonal anti-tropomyosin antibody (Sigma). The antibodies were diluted 1/2000 and left on their separate blots for 2 hours. After the 2 hours the blots were washed as described before and the anti-parvalbumin and anti-tropomyosin antibodies both detected by goat anti-mouse IgG1-AP (Southern Biotechnology), respectively. The antibody was diluted 1/1000 and left on each blot for 2 hours. The blots were washed again and substrate (Appendix 1.3.4) was added and the blots allowed to develop. The blots were rinsed with distilled water and allowed to dry.

An extra blot was done the same way as described above but the following extracts were used to include tropomyosin-containing controls: lobster, striped prawn, langoustine, cockroach body, locust body, and chicken extract.

4.3 Detection of IgE antibody by the Enzyme Allergosorbent method.

I Coating of paper discs

The paper discs were coated with the following extracts: Mackerel (*Scomber japonicus*), Red eye (*Etrumeus whitehead*), Maasbanker (*Trachurus trachurus capensis*), fish meal, Pilchard (canned) (*Sardinops sagax*), Pilchard (salted) (*Sardinops sagax*), Pilchard (gut) (*Sardinops sagax*), Pilchard (cooked) (*Sardinops sagax*), Pilchard (raw) (*Sardinops sagax*), Cape Anchovy (*Engraulis capensis*), and Rock Lobster (*Jasus lalandii*), lyophilized and stored at -80°C. Coating was done at the Paul-Ehrlich-Institute in Germany courtesy of Dr Gerald Reese.

II Evaluation of the EAST method

This method was evaluated and optimised by slightly changing the different conditions of the test in order to find the optimal working conditions for the best results. The following conditions were evaluated: serum concentration, antibody dilution, different antibody manufacturer (Southern Biotechnology, Pharmingen, Allergopharma), and also different blocking procedures. The different conditions and results were tabulated (see table 5.1 a, b and c)

III Final optimised EAST method

One disc was carefully placed in each well of the ELISA plate (Greiner) using a pair of forceps. The discs were washed once with working solution wash buffer (Appendix 1.1.3). Sera were diluted ¼ in TBS (appendix 1.1.1) and 50µl of diluted sera were pipetted onto the disc and the plate left to incubate in the dark at room temperature overnight. The next day the plate was washed with working solution wash buffer (Appendix 1.1.3) 3X with a multi channel pipette. After washing, the mouse-anti-human IgE Alkaline Phosphatase AP (Allergopharma) antibody, was diluted 1/1000 in TBS, 50µl pipetted into each well and left for 4 hours at room temperature. The plate was washed again with the working solution wash buffer with the multi channel pipette. The substrate (Appendix 1.1.4) was now added, 200µl per well, and left in the dark to incubate for one and a half hours. After incubation 100µl stop buffer (Appendix 1.1.5) was added to each well and mixed. Now 250µl of each well was transferred to a clean plate and the optical density was read with a Versa Max Micro plate reader (Labotech) at 405 and 650nm.

4.4 Quantification of IgE antibodies in workers sera.

I Quantification by RAST

A group of workers were selected for RAST and EAST testing. The specific IgE levels of the workers sera were determined by the Pharmacia CAP RAST (Pharmacia and Upjohn, Sweden) and the following caps were used: Langust (spiny lobster) (*Palinurus spp.*), Sardine, Pilchard (*Sardinops melanostica*) and Anchovy (*Engraulis encrasicolus*). A result was considered positive when the value was ≥0.35 kU/l.

II Quantification by EAST

Specific IgE levels in this group were also determined by the optimised EAST method and by the reference laboratory (Dr Gerald Reese, Paul-Ehrlich-Institute, Germany).
This was done by using the paper discs coated by the following extracts: Mackerel (*Scomber japonicus*), Red eye (*Etrumeus whitehead*), Maasbanker (*Trachurus trachurus capensis*), fish meal, Pilchard (canned) (*Sardinops sagax*), Pilchard (salted) (*Sardinops sagax*), Pilchard (gut) (*Sardinops sagax*), Pilchard (cooked) (*Sardinops sagax*), Pilchard (raw) (*Sardinops sagax*), Cape Anchovy (*Engraulis capensis*), and Rock Lobster (*Jasus lalandii*).
The reference laboratory used reference sera and a standard curve to determine which worker had a positive or negative EAST result. They calculated the specific-IgE content and the resulting EAST classes, using linear regression analysis of log-log-transformed reference data. In our laboratory however, no reference sera were available and no standard curve could be drawn up to determine positive and negative results. The values in the South African laboratory were treated as follows: the mean of the blank control were subtracted from the raw OD values of the workers and also from the negative control sera. A positive cut-off value was calculated by using the mean of the negative controls (after the blank was subtracted) plus two standard deviations. The following was considered to be positive: pilchard (canned) ≥0.034, anchovy (raw) ≥0.032, and lobster ≥0.029.

The level of agreement between the different methods was determined; using the three most important extracts namely Pilchard (canned), Anchovy (raw) and Lobster (raw).

4.5 Data Analysis

All the results were obtained, positive EAST, and RAST, determined as described (Appendix 2) and the following data analyses were done.

Descriptive data were obtained for each extract (Pilchard canned, Anchovy raw, and Lobster raw), and for each test (EAST, RAST). These included the number of workers or controls studied, the mean, standard deviation, range, median, number and percentage of positive workers. Descriptive data were also obtained for the negative controls used to calculate the cut-off value, (see Table 4.3). A pool of two negative control sera were used and the mean, standard deviation, range, and median were calculated using the values obtained in six experiments.

Table 4.3 Descriptive data for the negative control.

Negative Control (EAST)	Number of experiments	Mean	Std. Dev.	Range	Median
Pilchard (canned)	6	0.105	0.008	0.095-0.118	0.106
Anchovy	6	0.098	0.008	0.088-0.106	0.1
Lobster	6	0.102	0.008	0.094-0.112	0.099

2X2 Tables were drawn up for each extract (see Appendix 3) and the following statistical calculations made: Sensitivity (SS), Specificity (SP), % Agreement, and Kappa (k) value. The following formula referring to the 2X2 table (see Table 4.4) were used to calculate these parameters.

Formula

$SS = a/m_1 \times 100$

$SP = d/m_2 \times 100$

$$\% \text{ Agreement} = \frac{a + d}{n} \times 100$$

$$k = \frac{2(ad - bc)}{(n_1 m_2) + (n_2 m_1)}$$

Table 4.4 an example of a 2X2 table.

	Test 1 (+)	**Test 1 (-)**	**Total**
Test 2 (+)	a	b	n_1
Test 2 (–)	c	d	n_2
Total	m_1	m_2	n

CHAPTER 5

RESULTS

5.1 Results from the antigen/allergen analysis

I SDS-PAGE of the seafood extracts isolated

The protein extracts were successfully isolated from the different seafood species, and after the protein concentration was determined they could be separated by SDS-PAGE. In Figure 5.1 one can clearly see a range of proteins isolated between 10 and 250 kDa in molecular weight. Fish meal (no 5) only showed a smear of protein at low molecular weight. One can also clearly see that heat and other processes affect antigenicity, there are extra bands present in the cooked pilchard (no 8) extract that are not visible in the raw pilchard extract (no 9). Lane no 6 (canned pilchard) also show significant bands.

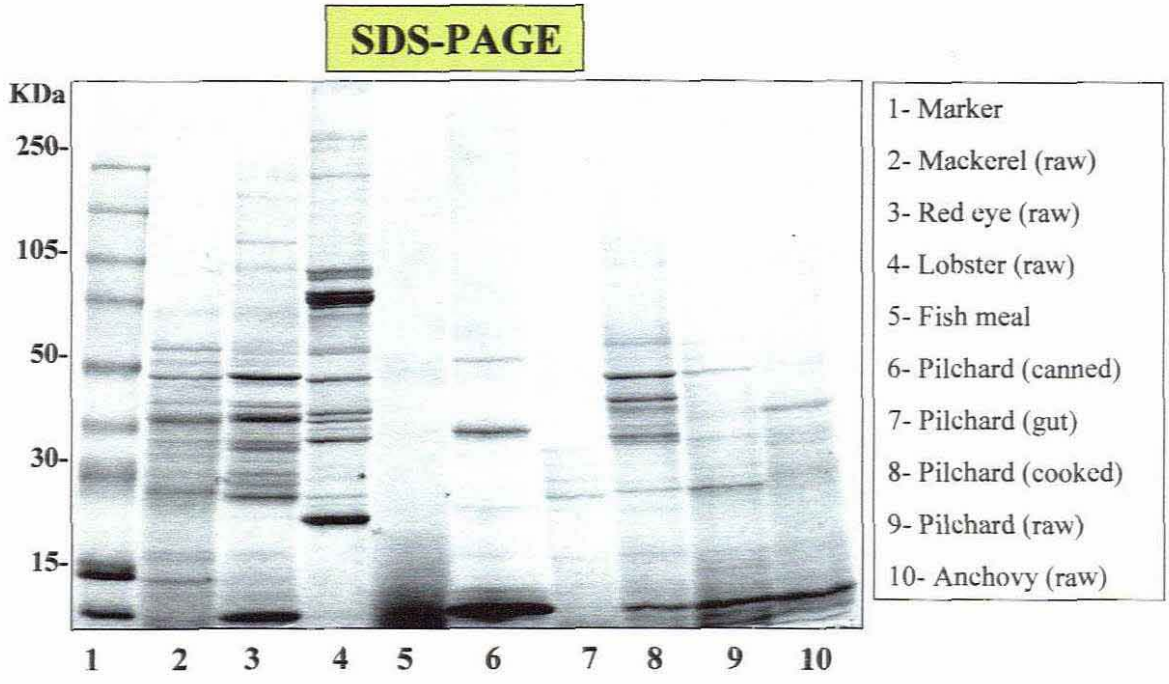

Figure: 5.1 A SDS gel of 9 of the protein extracts. The extract numbers are indicated on the right hand side and the molecular weights in kilo-Dalton are indicated on the left hand side.

II Polyclonal antibody titers in rabbits

Figure 5.2 shows the polyclonal antibody titers 3 and 6 weeks after the rabbit was immunized with the pilchard (canned) extract. It is clear that the rabbit responded well and 6 weeks after immunization a significant antibody titer was present.

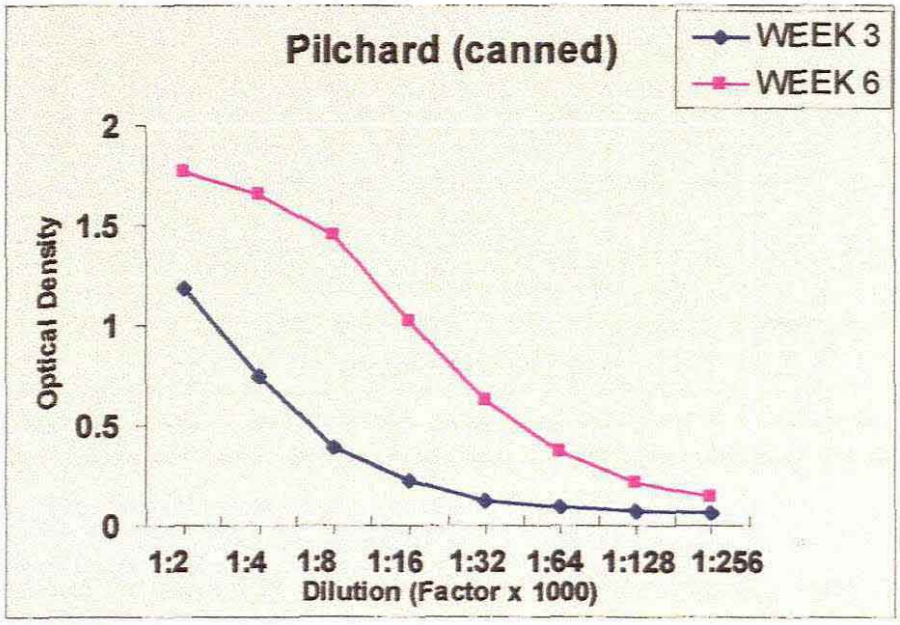

Figure 5.2: Antibody titers 3 and 6 weeks after immunization with canned pilchard extract. The optical density (OD) is indicated on the y-axis and the different antibody dilutions on the x-axis.

Figure 5.3 shows the polyclonal antibody titers 3 and 6 weeks after the rabbit was immunized with the anchovy extract. The response was similar to that of canned pilchard. This was also the case for all the other extracts except for fish meal, raw pilchard, and pilchard gut.

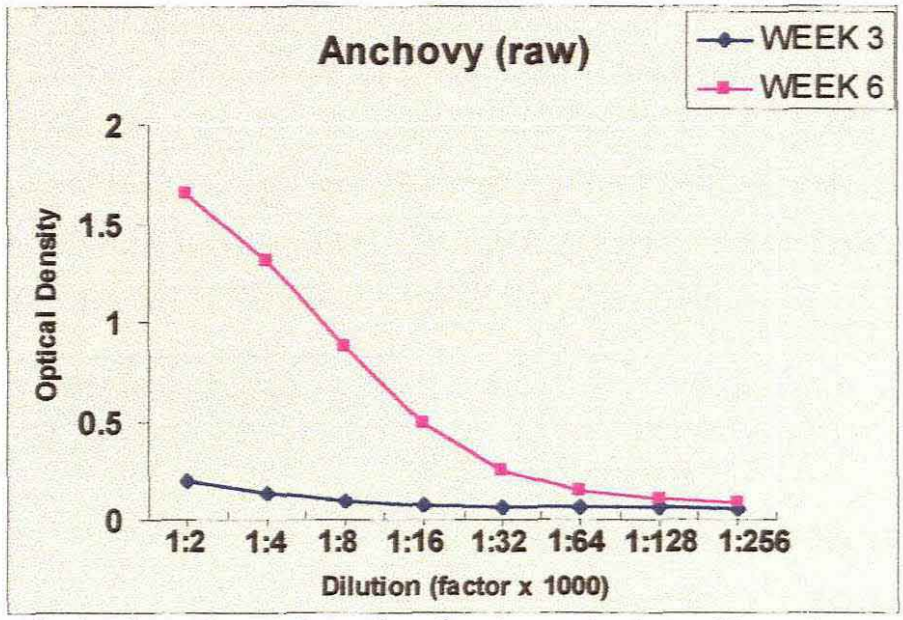

Figure 5.3: Antibody titers 3 and 6 weeks after immunization with anchovy extract. The optical density (OD) is indicated on the y-axis and the different antibody dilutions on the x-axis.

In the case of fish meal however (Figure 5.4) the antibody titer 6 weeks after immunization was very low compared to the other extracts. The rabbit was given a boost at week 8 and blood was again taken at week 12. Clearly at week 12 a significant antibody titer was reached. In the case of raw pilchard and pilchard gut a similar response was observed (data not shown).

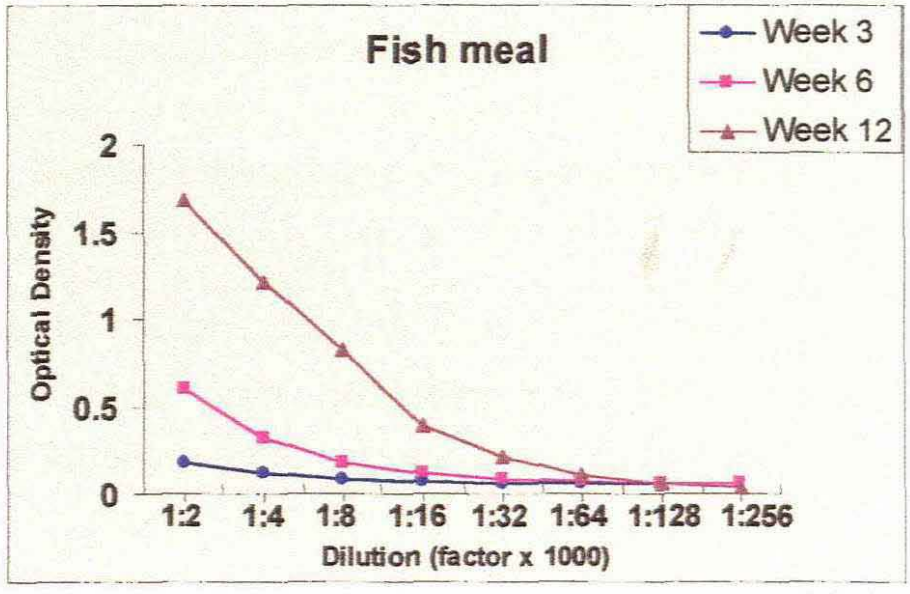

Figure 5.4: Antibody titers 3 and 6 and 12 weeks after immunization with fish meal extract. The optical density (OD) is indicated on the y-axis and the different antibody dilutions on the x-axis.

III Results of the Western blot and ELISA analysis of:

A Human IgE against the seafood extracts

The following extracts Mackerel (raw), Redeye (raw), Lobster (raw), Fish meal, Pilchard (canned), Pilchard (gut), Pilchard (cooked), Pilchard (raw) and anchovy (raw) were separated by SDS-PAGE and blotted onto a PVDF membrane as described previously. The blot was probed with a pool of positive patient's sera and various bands ranging from 30-75 kDa were visible. (See Figure 5.5)

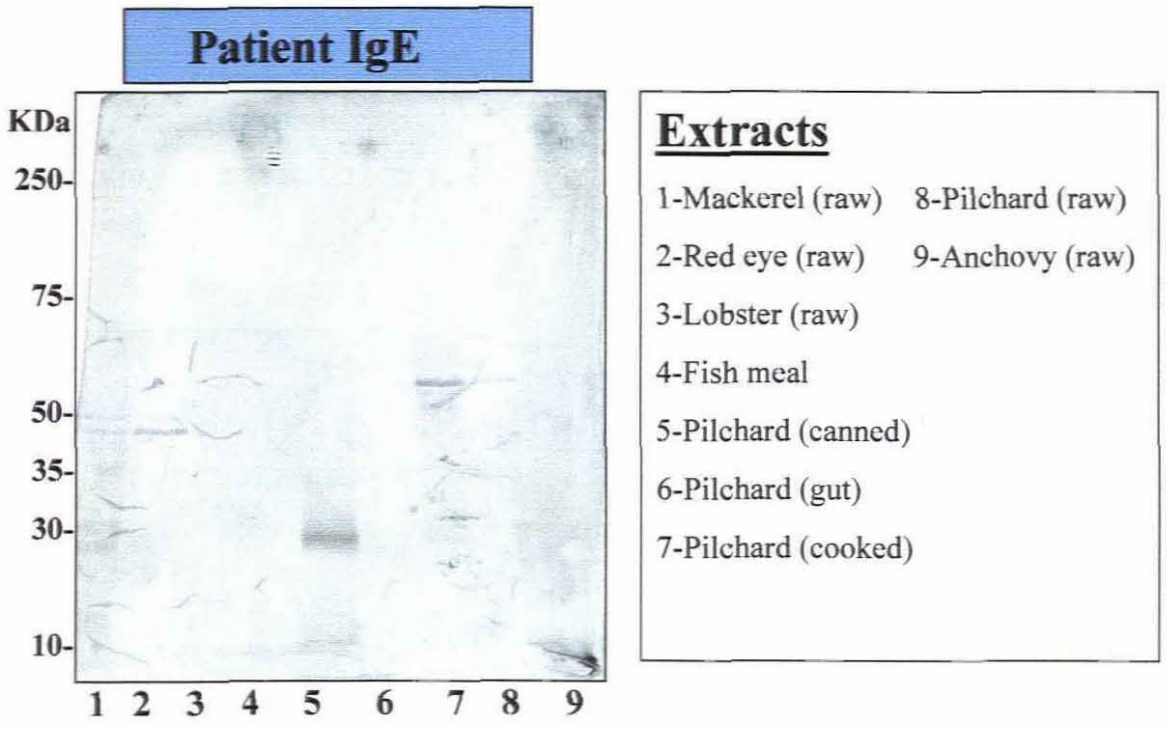

Figure 5.5: Western blot of a pool of positive patient sera binding to various extracts (numbers 1-9). The molecular weights in kilo Dalton is indicated on the left hand side and the seafood extracts no's 1-9 are indicated on the right hand side.

B Rabbit IgG against the seafood extracts (Western blots)

Pilchard (canned), Anchovy (raw) and Lobster (raw) extracts were separated by SDS-PAGE respectively and also blotted onto separate PVDF membranes. Each blot was probed with the rabbit antisera as indicated in no's 1-11 (See Figure 5.6). A range of bands is visible in all three blots. Each blot's self-sera (as indicated in Figure 5.6) showed the strongest bands but several cross-reactive bands were also clearly visible.

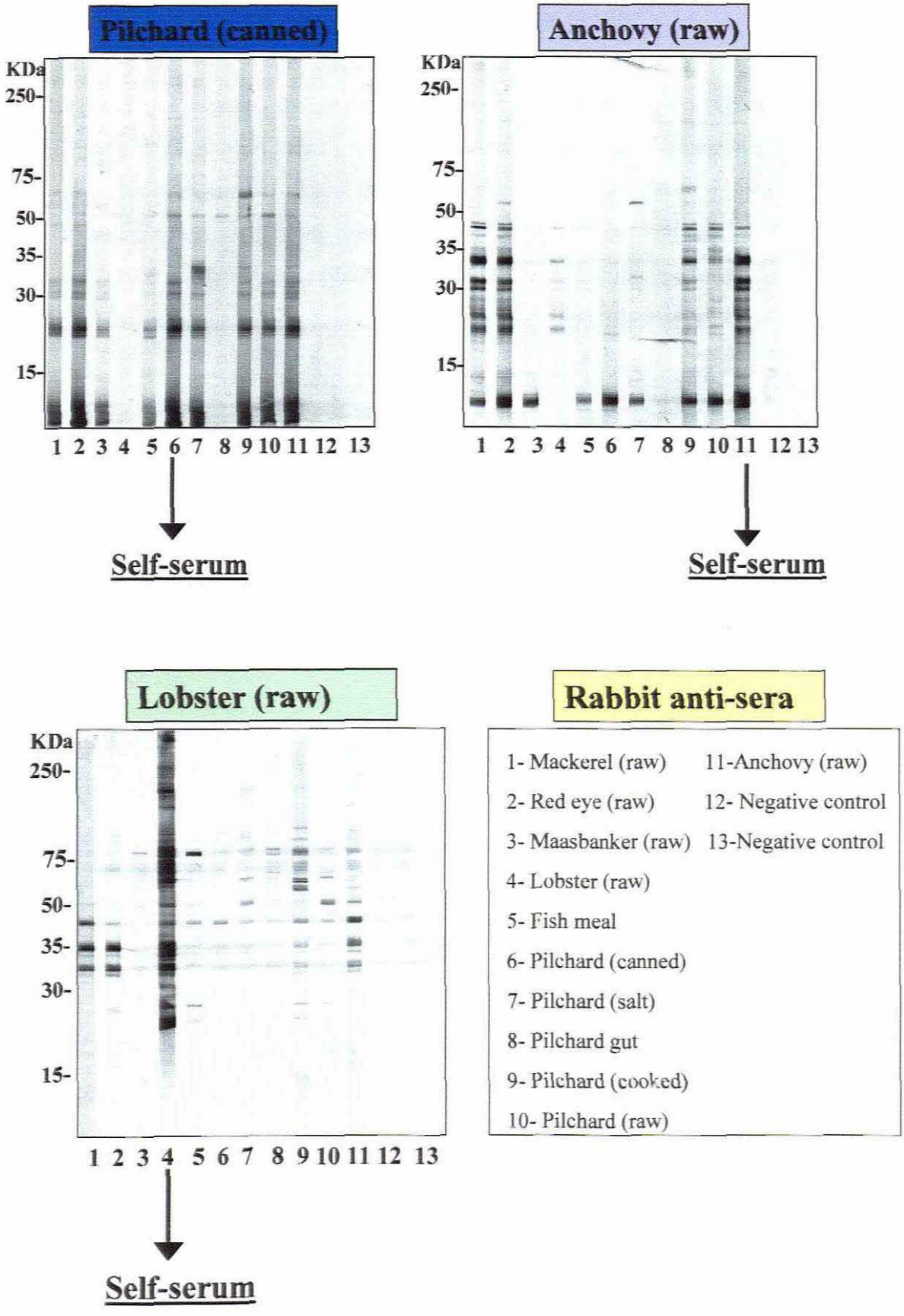

Figure 5.6: Western blots of rabbit anti-sera (numbers 1-11), binding to the protein extracts as shown here, pilchard (canned), raw anchovy and lobster. The molecular weights in kilo Dalton is indicated on the left hand side and the rabbit anti-sera no's 1-11 are indicated on the right hand side.

B **Rabbit IgG against the seafood extracts (ELISA)**

An ELISA assay was developed to confirm the antigen content of the pilchard (canned) Anchovy (raw), and the Lobster (raw) extracts. Two coating concentrations 1µg/ml and 5µg/ml were used and the antibody was diluted serially from 1/1000 to 1/512000. Figure 5.7 shows the results of the Pilchard (canned) extract. The different coating concentrations of the extract 1 and 5µg/ml give similar OD results. One can clearly see, even at an antibody dilution of 1/16000, that the anti-serum can still detect extract, giving an OD value of 0.5.

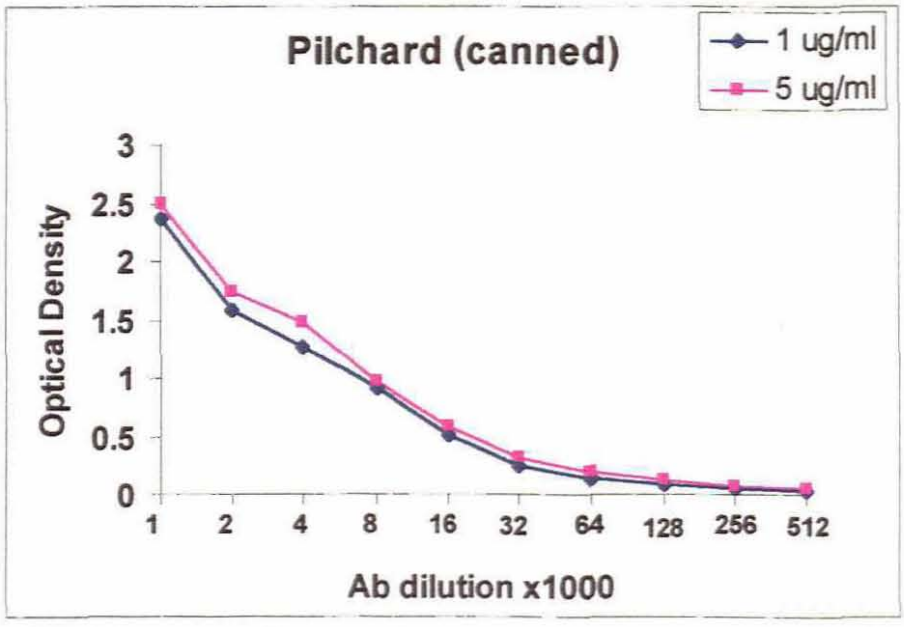

Figure 5.7: An ELISA assay confirming antigen content of the pilchard (canned) extract. The Optical Density (OD) is indicated on the y-axis and the antibody dilution is indicated on the x-axis.

Figure 5.8 shows the results of the Anchovy (raw) extract. The different coating concentrations of the extract 1 and 5µg/ml give similar OD results. At an antibody dilution of 1/32000 the anti-serum can still detect extract giving an OD value of 0.5.

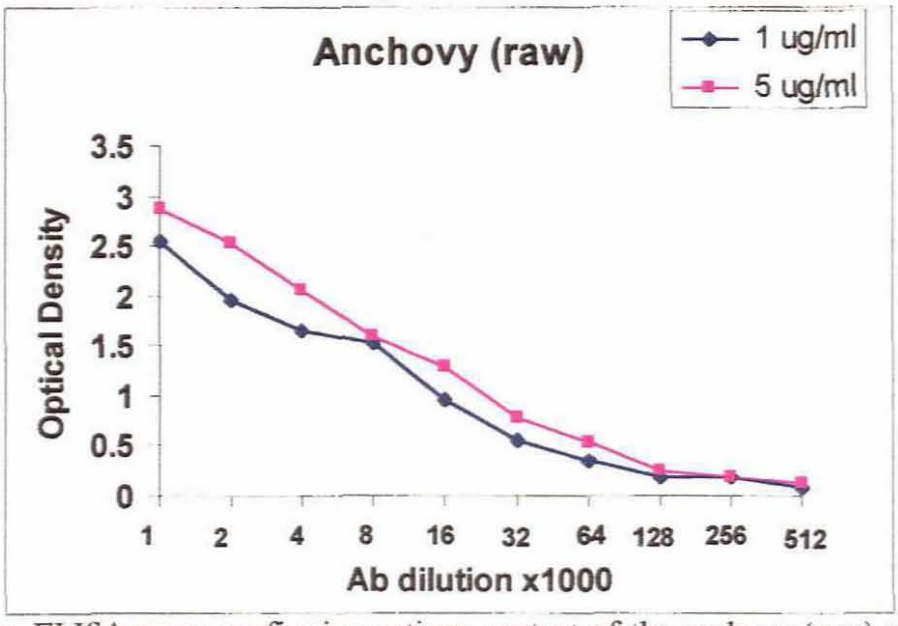

Figure 5.8: An ELISA assay confirming antigen content of the anchovy (raw) extract. The Optical Density (OD) is indicated on the y-axis and the antibody dilution is indicated on the x-axis.

Figure 5.9 shows the results of the Lobster (raw) extract. The different coating concentrations of the extract 1 and 5µg/ml give different results, with the 1µg/ml concentration giving lower OD values than the 5µg/ml concentration. Even at the lower coating concentration an antibody dilution of 1/16000 can detect extract giving an OD value of 0.5.

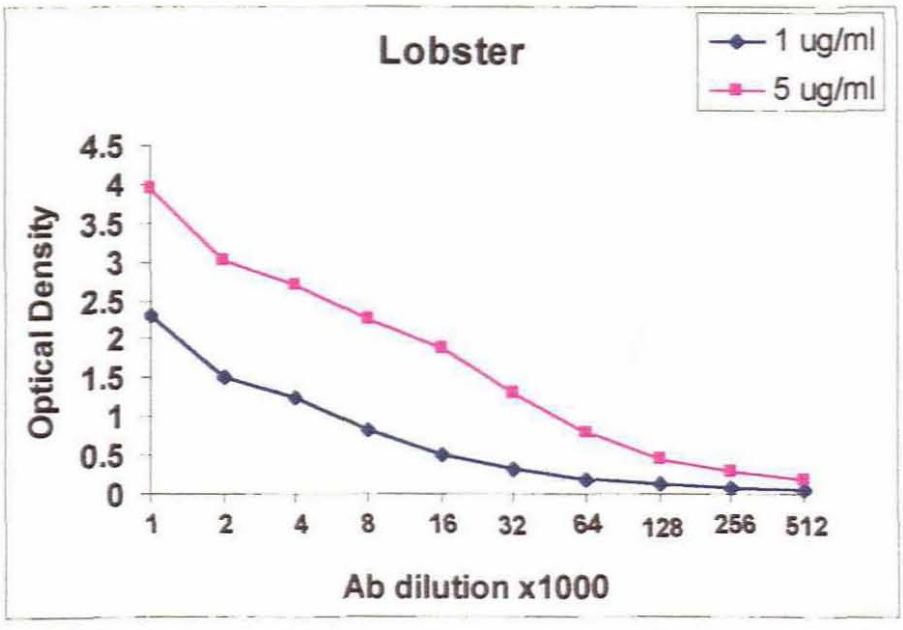

Figure 5.9: An ELISA assay confirming antigen content of the lobster (raw) extract. The Optical Density (OD) is indicated on the y-axis and the antibody dilution is indicated on the x-axis.

C Monoclonal antibodies against the seafood extracts

Extracts 1-12 (Figure 5.10) were separated by SDS-PAGE and blotted onto a PVDF membrane as described before. The blot was then probed with a monoclonal anti-parvalbumin antibody. Parvalbumin has a molecular weight of 12 KDa and as expected all the fish extracts show a band below 15 KDa but the crustacean (lobster lane 4), which contain no parvalbumin show no bands.

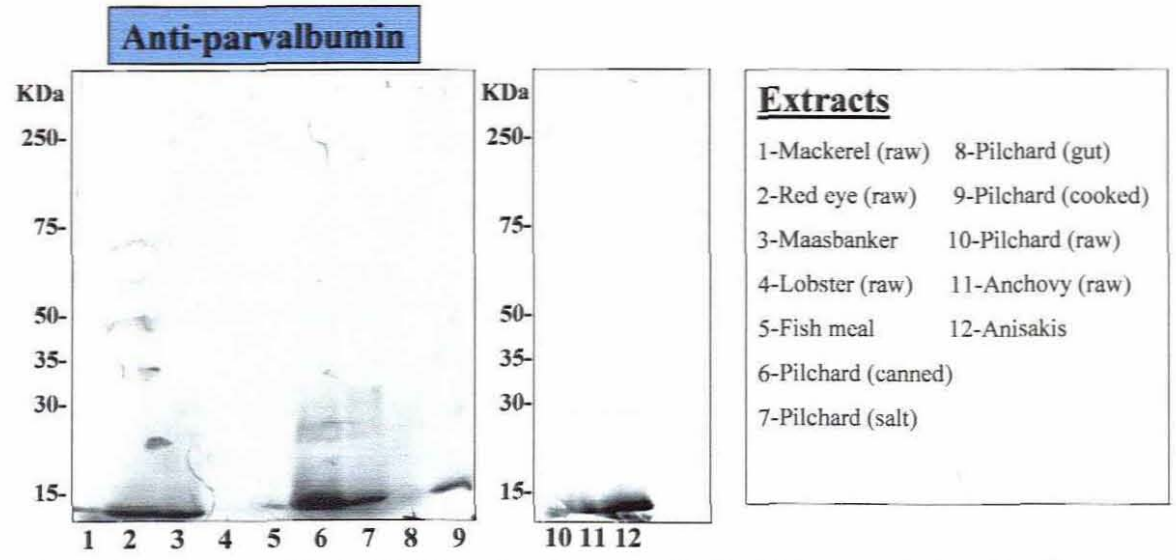

Figure 5.10: Western blots of different extracts, probed with the monoclonal anti-parvalbumin antibody. The molecular weight in kilo Dalton is indicated on the left hand side and the extracts 1-12 are indicated on the right hand side.

Figure 5.11 shows the same extracts 1-12 being separated by SDS-PAGE and blotted onto a PVDF membrane as described before. The blot was then probed with a monoclonal anti-tropomyosin antibody. Tropomyosins are a diverse group of protein with distinct isoforms found in muscle (skeletal, cardiac and smooth), brain and non-muscle cells. Tropomyosin has a molecular weight of 36 KDa and is found in crustaceans and a variety of other species including fruit flies, chickens, and mammals. In this blot, bands around 35 and 36 KDa can be seen but only in the fish species and not in the crustacean species (lobster lane 4), as we expected. (See Figure 5.11)

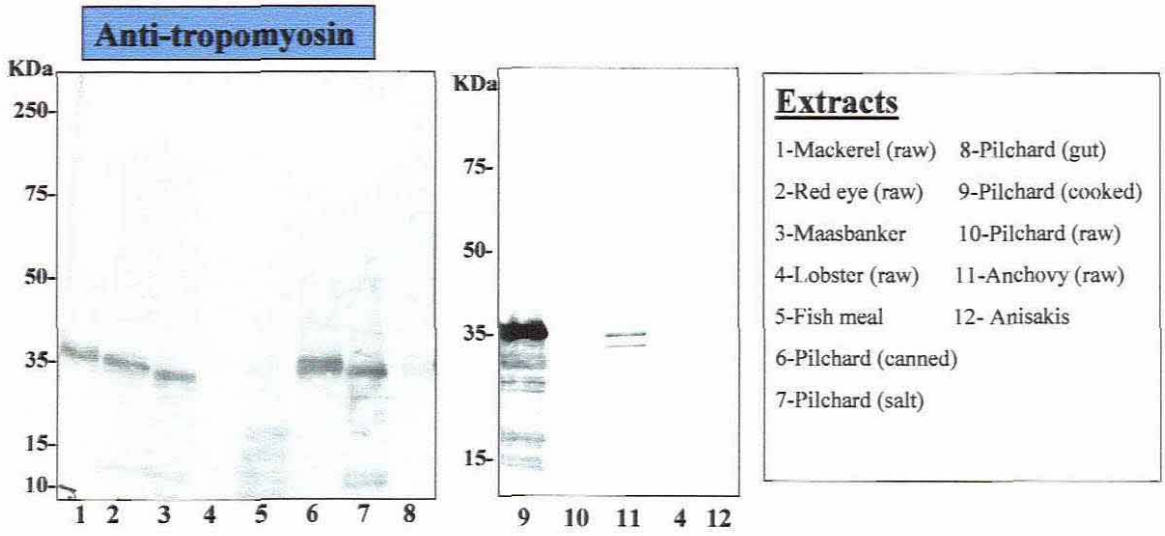

Figure 5.11: Western blots of different extracts, probed with the anti-tropomyosin antibody. The molecular weight in kilo Dalton is indicated on the left hand side and the extracts 1-12 are indicated on the right hand side.

The anti-tropomyosin blot (Figure 5.11) gave different results as expected (as described on page 62 paragraph 2) and another blot containing different crustacean extracts, some cockroach extract and chicken extract, known to contain tropomyosin, were probed with the same anti-tropomyosin antibody. The chicken positive control (lane 7 and 8) shows clear bands just above 35 KDa. The crustaceans (lane 1-4) however show no bands at 36 KDa but do show bands above 75 KDa, which may be aggregates of the 36 KDa band. (Figure 5.12) In SDS two molecules can bind and run at a molecular weight double of that of a single protein. This is also frequently seen with fish allergens (personal communication, Dr Ines Swoboda, University of Vienna Austria)

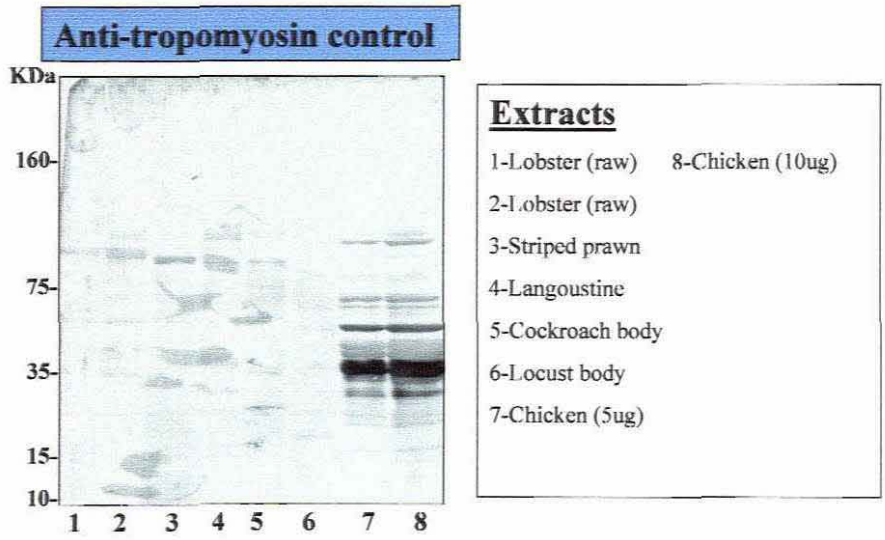

Figure 5.12: Western blots of different extracts, probed with the anti-tropomyosin antibody. The molecular weight in kilo Dalton is indicated on the left hand side and the extracts 1-8 are indicated on the right hand side.

5.2 Results from the EAST method used to detect IgE antibodies

I Coating of paper discs

The discs were tested with sera from patients known to have specific-IgE antibodies to seafood, and it was demonstrated that the coating of the paper discs was successful.

II Results using different conditions and anti-human antibodies

The experiments done using different conditions to optimise the method and to get the best results are found in Table 5.1 (a, b, and c)

Table 5.1a: Results using different conditions and anti-human antibodies.

Serum conc	Antibody dilution	Blocking	Far. +ve pt	Sho. +ve pt	Dia. -ve control	And. -ve control	Mag. -ve control
1/4	1/1000	None	1.256 mean OD	0.759 mean OD			
1/4	1/1000	Milk before disc 90 min 37°C	2.174 mean OD				0.987 mean OD
1/4	1/1000	Milk with disc 90 min 37°C	2.646 mean OD				1.178 mean OD
1/4	1/1000	1% BSA 30 min RT°	1.921 mean OD			0.654 1 well OD	0.807 mean OD
1/4	1/1000	Milk 30 min RT°	1.813 mean OD			0.992 mean OD	0.818 mean OD
1/4	1/4000	3%BSA 5hrs RT°	0.571mean OD 0.441 s-b		0.136 mean OD 0.005 s-b		
1/4	1/1000	3%BSA 5hrs RT°	1.243 1 well OD		0.402 1 well OD		
1/4	1/1000	Milk 5hrs RT°	1.492 mean OD 1.01 s-b		0.418 mean OD -0.0665 s-b		

Conc-concentration, +ve pt-positive patient, -ve-negative, RT°-room temperature, s-b – sample mean minus blank mean, OD-optical density. Far, and Sho, are patients known to be positive to seafood. Dia, And, and Mag are subjects used as negative controls.

Table 5.1b: Results using different conditions and anti-human antibodies.

Serum conc	Antibody dilution	Blocking	Far. +ve pt	Sho. +ve pt	Dia. -ve control	And. -ve control	Mag. -ve control
1/8	1/1000	Milk 5hrs RT°	0.819 1 well OD 0.605 s-b		0.210 1 well OD -0.004 s-b		0.237 1 well OD 0.023 s-b
1/4	1/4000	Milk 5hrs RT°	0.435 1 well OD 0.351 s-b		0.081 1 well OD -0.003 s-b		0.084 1 well OD 0 s-b
1/8	1/4000	Milk 5hrs RT°	0.295 1 well OD 0.211 s-b		0.078 1 well OD -0.006 s-b		0.080 1 well OD -0.004 s-b
1/4	1/2000	Milk 5hrs RT°	1.120(substr 1.5 hrs) 1 OD 1.022 s-b		0.093(substr 1.5 hrs) 1 OD -0.005 s-b		
1/4	1/2000	Milk 5hrs RT°	1.112(substr 2 hrs) 1 OD 1.005 s-b		0.133(substr 2 hrs) 1 OD 0.026 s-b		
1/4	1/1000 Pharmingen	Milk 5hrs RT°	1.739 s-b	0.461 s-b	0.5245 s-b		
1/4	1/1000 New SB	Milk 5hrs RT°	0.667 s-b	-0.002 s-b	0.1 s-b		

Conc-concentration, +ve pt-positive patient, -ve-negative, RT°-room temperature, SB-Southern Biotechnology,
s-b – sample mean minus blank mean, OD-optical density. Far, and Sho, are patients known to be positive to seafood. Dia, And, and Mag are subjects used as negative controls.

Table 5.1c: Results using different conditions and anti-human antibodies.

Serum conc	Antibody dilution	Blocking	Far. +ve pt	Sho. +ve pt	Dia. -ve control	And. -ve control	Mag. -ve control
1/3	1/1000 Pharmingen	Milk ON RT°		0.353 s-b	0.1725 s-b		
1/3	1/1000 New SB	Milk ON RT°		0.019 s-b	0.062 s-b		
1/3	1/1000 Pharmingen	3%BSA ON RT°		1.223 s-b	0.938 s-b		
1/3	1/1000 New SB	3%BSA ON RT°		0.1005 s-b	0.066 s-b		
1/4	1/2000 Allergo-pharma	Milk 2hrs	0.226 s-b		0.004 s-b		
1/3	1/1000 Allergo-pharma	NONE	0.623 s-b		0.046 s-b		
1/4	1/1000 Allergo-pharma	NONE	0.554 s-b		0.04 s-b		

Conc-concentration, +ve pt-positive patient, -ve-negative, ON-overnight, RT°-room temperature, SB-Southern Biotechnology, s-b – sample mean minus blank mean, Far, and Sho, are patients known to be positive to seafood. Dia, And, and Mag are subjects used as negative controls.

5.3 Results obtained from analysis of workers sera.

I Descriptive Data

A total of 128 samples of serum, obtained from workers with positive skin prick tests and a comparable number of controls were analysed, using the commercial CAP RAST and the EAST assays, in the reference laboratory (R) and in the South African Laboratory (S). The descriptive data are presented in Tables 5.2-5.4. A much lower proportion of samples were detected as positive on the commercial CAP RAST (8% of the Pilchard (canned), 8% of the Anchovy (raw) and 23% of the Lobster (raw)), with the lobster performing better than the fish CAP RASTS.

Table: 5.2 Descriptive data for Pilchard (canned) extract.

	Number	**Mean**	**Std. Dev.**	**Range**	**Median**	**Number positive** (using cut-off value) **(%)**
EAST (S)	128	0.135	0.061	0.074-0.406	0.111	49 (38%)
RAST	128	0.118	0.853	0-9.47	0	10 (8%)

EAST positive when ≥0.034 (using cut-off value) [O.D.]
RAST positive when ≥0.35 [kU/l]

Table: 5.3 Descriptive data for Anchovy (raw) extract.

	Number	**Mean**	**Std. Dev.**	**Range**	**Median**	**Number positive** (using cut-off value) **(%)**
EAST (S)	128	0.136	0.057	0.075-0.402	0.116	66 (52%)
RAST	128	0.076	0.295	0-2.13	0	10 (8%)

EAST positive when ≥0.032 (using cut-off value) [O.D.]
RAST positive when ≥0.35 [kU/l]

Table: 5.4 Descriptive data for Lobster (raw) extract.

	Number	**Mean**	**Std. Dev.**	**Range**	**Median**	**Number positive** (using cut-off value) **(%)**
EAST (S)	128	0.148	0.079	0.069-0.549	0.119	65 (51%)
RAST	128	1.043	4.784	0-48.9	0	30 (23%)

EAST positive when ≥0.029 (using cut-off value) [O.D.]
RAST positive when ≥0.35 [kU/l]

II Results obtained by comparison of different methods

All three methods (RAST, EAST (R), and EAST (S)) were compared with each other and the sensitivity (the proportion of true positives that are correctly identified as such, and is one minus the false negative rate) (Kirkwood, 1998), specificity (the proportion of true negatives that are correctly identified as such and is one minus the false positive rate) (Kirkwood, 1998), % agreement and kappa values calculated as per formula described previously. The results are presented in Tables 5.5, 5.6, and 5.7. When the results of the different analytical methods were compared, the commercial RAST lacked sensitivity but had good specificity. The sensitivity of the commercial lobster CAP RAST performed the best (having the best agreement), when compared to the reference laboratory EAST assay. The EAST assay of the South African laboratory was more sensitive but lacked specificity, and again the lobster assay performed the best and showed the highest kappa values.

Table: 5.5: Comparison of analytical assay methods for Pilchard (canned) extract.

	Sensitivity (SS)	**Specificity (SP)**	**%Agreement**	**Kappa (K)**
EAST (R) vs RAST (S)	8%	95%	84%	-0.001
EAST (S) vs RAST (S)	6%	91%	59%	-0.03
EAST (R) vs EAST (S)	69%	65%	66%	0.15

R=Reference laboratory
S=South African laboratory

Table: 5.6: Comparison of analytical assay methods for Anchovy (raw) extract.

	Sensitivity (SS)	**Specificity (SP)**	**%Agreement**	**Kappa (K)**
EAST (R) vs RAST (S)	12%	93%	77%	0.06
EAST (S) vs RAST (S)	6%	90%	47%	-0.04
EAST (R) vs EAST (S)	58%	50%	52%	0.05

R=Reference laboratory
S=South African laboratory

Table: 5.7: Comparison of analytical assay methods for Lobster (raw) extract

	Sensitivity (SS)	**Specificity (SP)**	**%Agreement**	**Kappa (K)**
EAST (R) vs RAST (S)	50%	83%	77%	0.3
EAST (S) vs RAST (S)	35%	89%	62%	0.24
EAST (R) vs EAST (S)	83%	57%	62%	0.24

R=Reference laboratory
S=South African laboratory

CHAPTER 6
DISCUSSION

6.1 Antigen/Allergen analysis

SDS-PAGE

The results demonstrated that the seafood extracts were all successfully separated by SDS-PAGE and a range of protein bands between 250 and 10 kDa in molecular weight could be demonstrated. However fish meal only showed a smear of protein at a low molecular weight, which can be explained by the extreme conditions the products are exposed to when fish meal is produced.

Bernhisel-Broadbent, Strause and Sampson (1992) evaluated fish allergens influenced by processing and found that canned tuna extract appeared as nearly homogenous smears on SDS-PAGE, they also found it to be less allergenic than cooked tuna. In our study however clear and distinct bands in the region of 10-50 kDa were found in the canned pilchard extract, and allergenic activity could also be demonstrated using a pool of sera from positive patients on immunoblot.

The SDS-PAGE also clearly demonstrated that heating generates extra proteins. Extra bands were present in the cooked pilchard extract, which were not present in the raw pilchard extract. These added proteins in the cooked extract might be volatile and cause allergic reactions after inhalation of the cooking vapors, which has previously been demonstrated. Findings by Crespo *et al* (1995) and Pascual *et al* (1996) reported fish hypersensitive patients to be allergic to vapours from boiling fish. However the exact nature of volatile seafood allergens has not been established yet.

Generation of polyclonal IgG antibodies in rabbits

Rabbits were immunized with the selected seafood extracts respectively in order to produce polyclonal antibodies to confirm antigen content of the extracts. Significant antibody titers were reached, demonstrating that all the extracts contain antigen and that they were suitable to use in the study.

Confirmation of antigen/allergen content

Human IgE

The allergen content of the extracts were also confirmed when the Western blot was probed with a pool of positive patient sera. Various bands could be observed, the serum pool reacted to the following: a double band around 50 kDa in mackerel and red eye, a 30kDa band in pilchard (canned), and a band just above 50 kDa in pilchard (cooked) and pilchard (raw). The bands in mackerel and red eye and in pilchard (cooked) and pilchard (raw) were cross-reactive bands. Cross reactivity in fish species has been previously established. In a group of children studied by de Martino *et al* (1990) the number of children with positive SPT to one or more fish species was significantly higher in children with cod allergy than children negative to cod. All children however were not uniformly positive to all species tested. They could also demonstrate cross-reacting antigens among cod, bass, dentex, eel, sole and tuna in RAST-inhibition assays (de Martino et al, 1990). Hansen *et al* (1997) also demonstrated cross-reacting antigens among Codfish, Mackerel, Herring and Plaice; the extracts indicated the existence of both general fish allergens and species-specific allergens. Bernhisel-Broadbent *et al* (1992) investigated 11 patients with a history of fish hypersensitivity; SDS-PAGE and immunoblot techniques were performed on cooked and raw extracts from catfish, bass, perch, mackerel, tuna, salmon, trout, codfish, and flounder. They demonstrated that all the patients possessed IgE antibodies specific to various fish-protein fractions, the most prominent band being that of 13 kDa.

Rabbit IgG

Antigen content of the extracts could also be demonstrated by probing the pilchard (canned), anchovy (raw) and lobster (raw) Western blots with the rabbit anti-sera produced. The "self-sera" reacted strongest to the corresponding extract. Various cross-reactive bands could also be observed which indicates that antigens in different seafoods extracts could be similar. The antigen content of the extracts could further be confirmed by an ELISA assay. In the case of pilchard (canned) an antibody dilution of 1/16000 still detected antigen giving an O.D. of 0.5. With the anchovy (raw) extract even an antibody dilution of 1/32000 gave an O.D. of 0.5. Coating concentrations of 1µg/ml and 5 µg/ml of extract gave similar results. With the lobster (raw) extract the results differed slightly with the two coating concentrations, but even at the lower concentration of 1 µg/ml an antibody dilution of 1/16000 could detect antigen giving an O.D. of 0.5. The Western blot and ELISA both confirmed that the extracts used, contained protein antigens, detected by rabbit polyclonal antibodies, and allergens as detected by IgE antibodies in patients' sera.

Monoclonal anti-parvalbumin and anti-tropomyosin.

Anti-Parvalbumin

A Western blot of different seafood extracts screened with an anti-parvalbumin antibody demonstrated a band below 15 kDa in all bony-fish extracts but not in the lobster (crustacean) extract. This band is considered to be the 12 kDa parvalbumin, major fish allergen *Gad c 1.* Parvalbumins represent low molecular weight (~ 12 kDa), acidic, water-soluble-calcium-binding proteins with remarkable stability regarding thermal and enzymatic degradation (Bugajska-Schretter *et al* 1999). The major cod allergen *Gad c 1* belongs to the parvalbumins and serves as a model for how allergens in general and particularly those in food can be identified, purified and characterized (O'Neil, Helbling and Lehrer, 1993). Parvalbumin is considered a major allergen in bony-fish (class Osteichthyes). James *et al* (1997) demonstrated IgE-binding to a 12 kDa protein in catfish, cod, and snapper and they found this protein to be similar to *Gad c 1.* Lindstrom *et al* (1996) reported that white muscle parvalbumin was similarly found to be a major allergen in Atlantic Salmon (*Salmo salar, Sal s 1*). Bugajska-Schretter (1999) and co-workers have also characterized carp parvalbumin as a major cross-reactive fish allergen, which contains most of the fish-specific IgE epitopes. The parvalbumin protein was however absent in lobster extract, as expected, because crustacean species do not contain parvalbumin but rather tropomyosin as a major allergen.

Anti-Tropomyosin

Tropomyosin belongs to a family of highly conserved proteins with multiple isoforms found in muscle and non-muscle cells of all species of vertebrates and invertebrates. Tropomyosin has been demonstrated as a major allergen in shrimp, dust mite, cockroach, lobster, squid and other mollusks (Reese, Ayuso and Lehrer, 1999). According to the literature tropomyosin can be identified in the region of 34-38 kDa in molecular weight. Western blot of the different seafood extracts, probed with an anti-tropomyosin antibody demonstrates bands around 34-36 kDa in some fish species (mackerel, red eye, maasbanker, pilchard (canned), pilchard (salt), pilchard (gut), pilchard (cooked), and anchovy), but not in the lobster (crustacean) species, which was expected. A second blot with other crustacean extracts (prawn and langoustine) and cockroach known to contain tropomyosin, as well as chicken (which served as a positive control), was probed with the same anti-tropomyosin antibody.

The chicken positive control demonstrated bands in a range between 20-80 kDa, with a very strong band at 36 kDa indicating a strong presence of tropomyosin as expected. The other extracts from lobster, prawn, langoustine, and cockroach only demonstrated a band above 75

kDa. Here again the crustacean species and the cockroach, known to contain tropomyosin, did not demonstrate bands in the region of 34-38 kDa. A possible explanation is that the commercial anti-tropomyosin antibody used was generated against chicken gizzard, hence the strong band with the chicken extract.

In a study by Reese, Tracey *et al* (1996) they found that *Pen a 1*-specific (major allergen of brown shrimp) monoclonal antibodies and *Pen a 1*-specific IgE of shrimp allergic subjects did not bind to any vertebrate (beef, pork, chicken and rabbit) tropomyosin, both by dot blot and immunoblot analysis (Reese, Tracey *et al*, 1996). However, Leung, Chow *et al* (1996) demonstrated IgE reactivities of shrimp sensitive sera to the muscle extracts from grasshopper, cockroach and fruit fly, but the IgE reactivity was not detected in muscle proteins from either chicken or mouse. They suggested the presence of unique IgE-reactive epitopes on invertebrate tropomyosins (Leung, Chow *et al*, 1996).

It could be that the tropomyosin in these extracts was not recognized by the anti-tropomyosin antibody produced from chicken (vertebrate source), or that the 75 kDa bands present aggregates of the tropomyosin protein. Some fish extracts show bands around 34-38 kDa, which has previously not been demonstrated. To our knowledge no studies have been done to determine if tropomyosin is also present in bony-fish (Osteichthyes).

6.2 Optimisation of the EAST method

The EAST method was evaluated and optimised by slightly changing the different conditions of the test in order to find the optimal working conditions for the best results. Throughout this process different blocking times and solutions were used, as well as different antibody and serum concentrations. None of these showed a remarkable difference in results. The only difference in results, and positive change made to optimize this method, was demonstrated by using a secondary antibody from a different manufacturer. The mouse-anti-human IgE Alkaline Phosphatase AP, antibody from Allergopharma, was used instead of the one manufactured by Pharmingen, or Southern Biotechnology. This demonstrates that during a process of optimizing a method all aspects should be considered in order to obtain the best results.

6.3 Analysis of results

EAST vs. RAST

Pilchard (canned)

The results of the EAST (R) (reference laboratory), and the EAST (S) (South African laboratory) respectively, were compared to the RAST results for each extract. For the pilchard (canned) extract the sensitivity was very low (below 10%), and the specificity high (above 90%). The percentage agreement ranged from 59%-84% for EAST (S) vs. RAST and EAST (R) vs. RAST respectively. The kappa value, which is also a measure of agreement, was poor (< 0). The fact that the agreement between these tests is so poor can be explained by the following: For the EAST, the discs were coated with Pilchard (canned) (*Sardinops sagax*) and for the RAST the Sardine; Pilchard (raw) (*Sardinops melanostica*) Cap was used. It has been shown previously that processing alters the allergens present in fish extracts. Mata *et al* (1994) compared the allergenicity of codfish and surimi. Surimi is a Japanese food product, which is extensively processed. They demonstrated reactivity to both codfish and surimi using allergic patient's sera, and found a 13 kDa protein in the codfish extract, that could correspond to *Gad c 1*, and a 63 kDa protein in both the codfish and surimi extracts, on SDS-PAGE. Bernhisel-Broadbent, Strause and Sampson (1992) found that clinically, patients reacted differently to lyophilized and non-lyophilized fish extracts. They found a 21% false-negative reaction rate that occurred with the DBPCFC's using lyophilized extract. With the flounder extract they could also demonstrate prominent bands in the non-lyophilized extract, not present in the lyophilized extract. They also investigated canned tuna extracts and found only homogenous smears on the gel, and not distinct bands. They demonstrated decreased allergenicity compared to raw tuna on SPT, and to cooked tuna on ELISA-inhibition. They also demonstrated decreased allergenicity of canned salmon (Bernhisel-Broadbent, Strause and Sampson 1992). In this current study however, we could demonstrate distinct bands on SDS-PAGE using the pilchard (canned) extract, some bands were also much stronger in the pilchard (canned) extract compared to the pilchard (cooked), and pilchard (raw) extract. The allergenicity of the pilchard (canned) extract could also be demonstrated by probing a Western blot with a serum pool of positive patients. Dory *et al* (1998) evaluated fish allergenicity influenced by storage conditions, and found the relative content in IgE-reactive protein bands to be greater after storing the fish for several days. Thus the fact that the one extract was pilchard (canned) and the other pilchard (raw) could explain this low correlation because the canned extract might have different allergens when compared to the raw extract.

The low correlation between the two methods can further be explained by the fact that different species of pilchard were used. It is known that parvalbumin is not the only antigen present in fish (Lopata, 1999); Mata *et al* (1994) identified a 63 kDa protein in codfish and surimi. Galland *et al* (1998) purified a 41 kDa-cod allergenic protein, and demonstrated it to be allergenic following a Western blot probed with a pool of sera from cod-allergic patients, and anti-parvalbumin monoclonal antibody. On this same Western blot they could also identify *Gad c 1*, and two other proteins at 28 and 49 kDa respectively.

Anchovy (raw)

With the anchovy extract the EAST results were as follows: the sensitivity was very low (6%-12%), however, the specificity was high (90%-93%), and the percentage agreement between 47% and 77% respectively for the EAST (S) vs. RAST, and the EAST (R) vs. RAST, the kappa value was also poor. A possible explanation for the poor correlation between the two methods could be the fact that different species of anchovy were used, for the EAST, Cape Anchovy (*Engraulis capensis*) was used and for the RAST Anchovy (*Engraulis encrasicolus*) was used. As described, it is known that other antigens other that parvalbumin are present in fish, Mata *et al* (1994) described a 63 kDa protein in codfish and surimi, and Galland *et al* (1998) described 41, 28 and 49 kDa allergens in codfish.

Pascual *et al* (1996) evaluated the allergenicity of boiled, raw and salmon steam. Immunoblot analysis of salmon steam showed a strong allergenic double band between 12 and 14 kDa, and also a single band around 30 kDa. With the boiled and raw extracts they found IgE-binding components of 12-30 kDa and bands >30 kDa that exhibited significant activity.

Lobster (raw)

In the case of the lobster extract the measures of agreement were much better compared to that of the two bony-fish species. The sensitivity range from 35%-50%, the specificity is > 80%, and the percentage agreement > 60%. The kappa values were also better although not perfect. The fact that the correlation was not perfect can be explained by the different species of extract used in the two different methods. For the EAST, Rock Lobster (*Jasus lalandii*) was used and for the RAST Langoustine (spiny lobster) (*Palinurus spp.*). However the correlation was still better compared to that of pilchard and anchovy, where the species also differed. This could be explained because tropomyosin seems to be a more homologous antigen, when a comparison is drawn between the different crustacean species. Halmepuro *et al* (1987) studied different crustacean species and found a close relationship between crawfish, lobster and other crustacean antigens. They also found that most of the antigens present in crawfish

or lobster were fully or partially identical within the crustaceans studied. Lin *et al* (1993) demonstrated a 39 kDa major allergen in shrimp (*Parapenaeus fissures*), and raised two monoclonal antibodies against this major allergen. MoAb 2-7-1 E showed immunoblot activity to both 39 kDa components of shrimp and crab (*Portunus (portunus) trituberculotis*). Leung *et al* (1998) could also demonstrate muscle protein tropomyosin as a lobster allergen. Comparison of the amino acid sequence of *Pan s 1* and *Hom a 1* (lobster tropomyosin) with *Met e 1* (shrimp tropomyosin) indicates these proteins to be very similar. Leung and Gershwin *et al* (1998) undertook further studies and aimed to clone, identify and determine the primary structure of a major IgE-reactive molecule in crab (*Charybdis feriatus*). They found expression of the *Cha f 1* cDNA produces a recombinant protein recognized by serum IgE from patients with crustacean allergy. The sera from patients with crustacean allergy also reacted to *Met e 1,* (shrimp) *Pan s 1,* and *Hom a 1.* They could also demonstrate that *Cha f 1* shares the common IgE-epitope with other shellfish tropomyosin, and that *Cha f 1* is the major crab allergen. They also found *Cha f 1* to be significantly homologous with *Hom a 1* (92.4%), and *Pan s 1* (91.4%) respectively. And *Cha f 1* shows extensive similarity in amino acid composition and peptide sequence identity with *Homarus americanus* slow muscle tropomyosin (95.8%) (Leung and Gershwin *et al* 1998). This homology between the different crustacean allergens could explain why the lobster showed better correlation even though the species differed.

Even though the level of agreement between the RAST and the EAST methods was low it was better for the EAST method of the reference laboratory compared to the South African laboratory. This could be explained by the fact that the reference laboratory and the South African laboratory used modified methods. The reference laboratory used an Allergopharma, kit-based, assay with reference sera and a standard curve for the specific-IgE content and the resulting EAST classes, using linear regression analysis of log-log-transformed reference data. The South African laboratory did not use a kit-based assay, and positive results were determined using a cut-off value calculated by utilizing negative patient sera, and no standard curve was calculated.

EAST (R) vs. EAST (S)

Comparing the same EAST method from two different laboratories the overall agreement for all three extracts was very similar. The sensitivity, specificity and percentage agreement for all three extracts were below 80%, except for lobster, which showed a high sensitivity of

83%. The kappa values were also relatively poor. Overall the EAST (S) picked up more positive results than the EAST (R). This could be because the two laboratories used different assays, and different calculations to determine positive results. A possible explanation for the difference in results found in the reference laboratory and the South African laboratory was the following: the reference laboratory used a kit-based assay and a serum dilution of 1:2. They used a standard curve to calculate the specific IgE content in kU/l, and the resulting 4 EAST classes, using linear regression analysis of log-log-transformed reference data. The South African laboratory did not use a kit-based assay, and the serum was diluted 1:4. No standard curve was used and a modified method was employed to determine the cut-off value. Negative control serum was used to determine the cut-off and not the blank control, in order to avoid false positive results.

In order to obtain better agreement it would be advisable to further standardize this EAST method by also using reference sera and a standard curve to categorize positive results.

CHAPTER 7
CONCLUSION

In conclusion this study demonstrates specific seafood extracts were isolated from the bony fish and crustacean species, and their antigen/allergen content established. Polyclonal antibodies were successfully raised in New Zealand white rabbits using these isolated extracts, and significant antibody titers were demonstrated.

A fairly sensitive and specific EAST method was developed, and the specific-IgE levels in the fish processing workers' sera established. Compared to the EAST (R) method employed by the reference laboratory the EAST (S) method was not as sensitive and specific as was to be expected. The reason for this is that modified methods were used by the two laboratories, resulting in different methods of calculating positive results, leading to fair but not excellent level of agreement.

It would be advisable, in future to look at both these modified methods and calculations, for categorising positive results and to take the good qualities of both to standardise and optimise this method further to obtain agreement of these methods.

The level of agreement between the EAST and RAST methods were relatively low in all three extracts. The fact that pilchard (canned) was used in the EAST and pilchard (raw) in the RAST, and in addition different species of pilchard, anchovy and lobster employed in the two methods is a possible explanation. It will however always be difficult to compare the EAST with the RAST because only certain species are available for commercial RAST. This however could support the use of the EAST where the paper disc can be coated with a specific species of seafood, and the assay is not limited to what is commercially available on the CAP RAST.

Since the EAST (S) was much more sensitive than the RAST which was more specific, the two tests can be used together, with the former used as a screening test and the latter as a diagnostic test in patients and workers reporting seafood allergy, while the EAST is still being optimised.

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APPENDIX 1

Buffers and solutions

1.1 Enzyme Allergosorbent Test (EAST)

1.1.1 Tris-buffered saline (TBS)

100 Mm Tris CL, pH 7.5

0.9% NaCL

Store at 4°C

Tris· CL [tris (hydroxymethyl) aminomethane], 1 M

1 M Tris

Adjust to pH 7.4 with concentrated HCL

Mix and make up to 1 litre with distilled H_2O.

(Approximately 70 ml of HCL is needed to achieve a pH of 7.4)

1.1.2 Stock Solution Wash Buffer PH 7.4

1M Tris

1M NaCL

25 mM $MgCL_2$

Make up to 1 Litre with distilled water

Sterilize

1.1.3 Working Solution Wash Buffer

Dillute100ml of the stock wash buffer into 900ml of distilled water

Add 500 µl of Tween-20 (0,05%)

Mix well and store at 4°C

1.1.4 Substrate Buffer PH 9.5

100 mM Tris

100 mM NaCL

5 mM $MgCL_2$

Make up to 1 Litre with distilled water

Sterilize

Dilute the substrate (4-Nitro phenol Phosphate di-Sodium salt $6H_20$) in the substrate buffer (final concentration 1mg/ml)

1.1.5 Stop Buffer

1 N NAOH

Make up with distilled water

1.2 SDS-Polyacrylamide Gel Electrophoresis

1.2.1 Running Gel Buffer (5%)

150 mM Tris

Dissolve in 60 ml distilled H_2O

Adjust the pH to 8.8

Make up to 100 ml

Sterile filter

1.2.2 Running Gel Buffer (16%)

150 mM Tris

Dissolve in 60 ml distilled H_2O

Adjust the pH to 8.8

Sterile filter

Add 30 ml glycerol

Make up to 100 ml

1.2.3 (0.1%) SDS solution

Dissolve 100 g of SDS in 1 litre of distilled H_2O

Store at 4°C

1.2.4 Stacking Gel Buffer

50 mM Tris

Dissolve in 60 ml distilled H_2O

Adjust the pH to 6.8

Add 1.39 mM of SDS

Make up to 100ml

1.2.5 Stock solution Reservoir Buffer

125 mM Tris

960 mM Glycine

17.3 mM SDS

Make up to 1 Litre with distilled water

The pH must be 8.5 +/- 0.2 do not adjust

1.2.6 Working Solution Reservoir Buffer

Dilute the stock solution five times.

1.2.7 Sample Buffer (non-reduced)

Stacking Gel Buffer	2.5 ml
Glycerol	2.0 ml
SDS	0.4 g
Bromophenol Blue	0.1 g

Dilute the SDS and Bromophenol Blue in the Stacking Gel Buffer first before adding the glycerol. If need be make up to 5 ml with distilled H_2O

1.2.8 DTT

Dissolve 600 mg of Dithiothreitol (DTT) (Boehringer Mannheim) in 1 ml of distilled H_2O

Aliquot and freeze.

1.2.9 (10 %) Amonium persulphate (AMPS)

Dilute 10µg of AMPS (BDH electran) into100 µl of distilled H_2O

Store in fridge

1.2.10 Sealing Agar

Make up a 1% agarose solution with distilled H_2O

Melt in the microwave until dissolved

Store at room temperature and use as needed

1.2.11 Coomassie Destain

30% Methanol

10% Acetic Acid

60% Distilled H_2O

1.2.12 Coomassie Brilliant Blue Stain

0.1% Brilliant Blue in destain solution

1.3 Western Blot

1.3.1 Transfer Buffer (pH 8.3 – 8.5)

41.6 mM Tris

320 mM Glycine

Add 400 ml Methanol and make up to 2 litres with distilled H_2O

1.3.2 Blocking Buffer (WB)

2% milk, 0.05% Tween-20 in TBS

1.3.3 TBS Wash Buffer

0.05% Tween-20 in TBS

Mix well and store at 4°C

1.3.4 Substrate for Western Blot

1 NBT/BCIP (Nitro blue tetrazolium chloride 5-Bromo-4-Chloro-3-indolyl-phosphate, toluidine-salt) tablet (Roche) dissolved in 15 ml distilled H_2O.

Cover substrate dilution with foil.

10 ml can be used at a time and the rest stored at 4°C covered with foil. If substrate dilution turns purple it cannot be used anymore.

1.4 Enzyme Linked Immunosorbent Assay (ELISA)

1.4.1 Blocking Buffer

2% Milk powder

0.1% NaN_3

100 ml stock solution PBS (Phosphate buffered saline)

Make up to 1 litre with distilled water

1.4.2 TBS Wash Buffer

0.05% Tween-20 in TBS

Mix well and store at 4°C

1.4.3 ELISA substrate

Dilute the substrate (4-Nitro phenol Phosphate di-Sodium salt $6H_20$) in the following substrate buffer (final concentration 1mg/ml)

Substrate buffer:

0.02% NaN_3

97 ml di-ethanolamine

800 mg $MgCL_2.6H_2O$

Add 700 ml H_2O and adjust the pH to 9.8 with 10M HCL then make up to 1 litre and store at 4°C.

1.5 General

1.5.1 Stock Solution Phosphate Buffered Saline (PBS)

25 mM Sodium di-hydrogen Phosphate 1-hydrate

74.7 mM di-Sodium Hydrogen Phosphate di-hydrate

1.45 M NaCL

Add 800 ml distilled water and adjust pH to 6.8

Make up to 1 litre

1.5.2 Working solution Phosphate Buffered Saline (PBS)

Dilute the stock solution ten times

The pH of the working solution must be 7.4

APPENDIX 2

EAST and RAST results obtained upon analysis of workers sera.

ID NO	Lobster EAST	Pilchard (canned) EAST	Anchovy EAST	Lobster RAST	Pilchard (canned) RAST	Anchovy RAST
1	0	0	0	0	0	0
2	0.178	0.119	0.152	0	0	0
4	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0.032	0	0	0.51	0	0.42
11	0	0	0	0	0	0
13	0.083	0.1	0.085	0	0	0
14	0	0	0	0	0	0
17	0	0	0	0	0	0
20	0	0	0	0	0	0
25	0.204	0	0	14.6	0	0
26	0.095	0.066	0.077	1.9	0	0
31	0.081	0	0	2.24	0	0
35	0.242	0	0	8.95	1.31	1.4
54	0	0	0	0	0	0
55	0.134	0.156	0.153	0	0	0
57	0	0	0	0	0	0
61	0.098	0.145	0.119	0	0	0
66	0	0	0	0	0	0
73	0	0	0	0	0	0
75	0	0	0	0	0	0
76	0.128	0.111	0.124	0	0	0
78	0	0.037	0	0	0	0
85	0	0	0	0.48	0	0
89	0.075	0.069	0.112	0.71	0	0
94	0	0	0.032	0	0	0
95	0	0	0	0	0	0
96	0.145	0.178	0.136	0	0	0
98	0.081	0.076	0.104	0	0	0
99	0.205	0	0	13.1	0	0
101	0	0	0	0	0	0
102	0.115	0.159	0.155	0	0	0
108	0.361	0.274	0.232	0	0	0
114	0	0	0	0	0	0
118	0	0	0	0	0	0
128	0.052	0.045	0.051	0.53	0.49	0
133	0	0	0	0	0	0
135	0	0	0	0	0	0
141	0.03	0	0.043	0	0	0
147	0.139	0.11	0.125	2.12	0	0
148	0	0	0.041	0	0	0
153	0.103	0.085	0.071	0	0	0
162	0	0	0.038	0	0	0
165	0.075	0	0.069	0	0	0

ID NO	Lobster EAST	Pilchard (canned) EAST	Anchovy EAST	Lobster RAST	Pilchard (canned) RAST	Anchovy RAST
166	0	0	0	0	0	0
171	0	0	0	0.89	0	0
172	0	0	0	0	0	0
177	0.039	0	0.032	0	0	0
180	0	0	0	0	0	0
185	0	0	0	0	0	0
188	0.032	0	0.039	0	0	0
190	0	0	0	0	0	0
195	0.461	0.318	0.32	0	0	0
196	0	0	0	0	0	0
198	0	0	0	0	0	0
203	0.113	0.093	0.113	0	0	0
207	0.062	0.06	0.064	0	0	0
209	0.049	0.047	0.047	0	0	0
214	0.098	0.052	0.069	0.71	0	0
220	0.131	0.148	0.125	0	0	0
228	0	0	0.036	0	0	0
230	0.108	0.132	0.111	0.81	0	0
233	0	0	0	0.58	0	0
242	0	0	0.035	0	0	0
244	0.089	0.113	0.13	0	0	0
246	0.193	0.276	0.152	0	0.39	0.38
247	0	0	0	0	0	0
256	0.165	0.113	0.118	0.87	0	0
261	0	0	0.037	0	0	0
262	0.036	0.043	0.046	0	0	0
266	0	0	0.041	0	0	0
271	0	0	0	0	0	0
276	0.033	0.042	0.046	0	0	0
282	0.101	0.115	0.124	0	0	0
294	0.135	0	0.067	5.23	0.49	0.81
306	0	0	0	0	0	0
308	0	0	0	0	0	0
309	0	0	0	0.38	0	0
313	0.052	0.075	0.041	0	0	0
322	0.091	0	0.044	2.23	0.48	0.66
338	0.044	0.054	0.073	0	0	0
339	0.032	0	0.05	0	0	0
342	0	0	0	0	0	0
343	0.109	0.088	0.085	0	0	0
344	0.058	0.055	0.082	0	0	0
367	0	0	0.066	0	0	0
387	0	0	0	0	0	0
388	0.042	0	0.046	0	0	0
389	0.048	0.059	0.099	0	0	0
396	0	0	0	0.67	0	0
404	0.135	0.099	0.111	0.91	0.47	0.99
411	0.034	0.039	0.044	0	0	0
438	0.077	0.101	0.071	0	0	0
443	0	0	0	1.78	0	0
446	0	0	0	0	0	0
457	0.36	0.082	0.129	48.9	0	0

ID NO	Lobster EAST	Pilchard (canned) EAST	Anchovy EAST	Lobster RAST	Pilchard (canned) RAST	Anchovy RAST
466	0	0	0	0	0	0
473	0.122	0.067	0.072	0	0	0
485	0	0	0.036	0.51	0	0
489	0.145	0.141	0.17	0	0	0
494	0.047	0.048	0.067	1.55	0	0
499	0	0	0	0	0	0
503	0.101	0.062	0.061	0	0	0
524	0.078	0	0	5.14	1.12	1.23
532	0	0	0.043	0	0	0
535	0.185	0.095	0.124	5.14	0	0
537	0	0	0	0	0	0
542	0	0	0	0	0	0
547	0	0	0	0	0	0
551	0	0.038	0	0	0	0
563	0.149	0	0	8.58	0.52	0.73
571	0	0	0	0	0	0
579	0.043	0	0	2.52	0.48	0.93
581	0	0	0	0	0	0
590	0	0	0	0	0	0
592	0.102	0.097	0.127	0	0	0
604	0	0	0	0	0	0
614	0.06	0.05	0.054	0.39	0	0
615	0.079	0.171	0.123	0.55	0	0
617	0.032	0	0.042	0	0	0
618	0	0	0	0	0	0
621	0.189	0.181	0.179	0	0	0
626	0	0	0	0	0	0
629	0.036	0	0	0	0	0
632	0	0	0	0	9.47	2.13
641	0.074	0.092	0.096	0	0	0
642	0.205	0.198	0.25	0	0	0

APPENDIX 3

2X2 Tables used to calculate sensitivity, specificity, % agreement, and Kappa values

Canned Pilchard

	EAST (R) Positive	EAST (R) Negative	Total
RAST (S) Positive	1	9	10
RAST (S) Negative	12	106	118
Total	13	115	128

R-Reference laboratory
S-South African laboratory

	EAST (S) Positive	EAST (S) Negative	Total
RAST (S) Positive	3	7	10
RAST (S) Negative	46	72	118
Total	49	79	128

R-Reference laboratory
S-South African laboratory

	EAST (R) Positive	EAST (R) Negative	Total
EAST (S) Positive	9	40	49
EAST (S) Negative	4	75	79
Total	13	115	128

R-Reference laboratory
S-South African laboratory

Anchovy

	EAST (R) Positive	EAST (R) Negative	Total
RAST (S) Positive	3	7	10
RAST (S) Negative	23	95	118
Total	26	102	128

R-Reference laboratory
S-South African laboratory

	EAST (S) Positive	EAST (S) Negative	Total
RAST (S) Positive	4	6	10
RAST (S) Negative	62	56	118
Total	66	62	128

R-Reference laboratory
S-South African laboratory

	EAST (R) Positive	EAST (R) Negative	Total
EAST (S) Positive	15	51	66
EAST (S) Negative	11	51	62
Total	26	102	128

R-Reference laboratory
S-South African laboratory

Lobster

	EAST (R) Positive	EAST (R) Negative	Total
RAST (S) Positive	12	18	30
RAST (S) Negative	12	86	98
Total	24	104	128

R-Reference laboratory
S-South African laboratory

	EAST (S) Positive	EAST (S) Negative	Total
RAST (S) Positive	23	7	30
RAST (S) Negative	42	56	98
Total	65	63	128

R-Reference laboratory
S-South African laboratory

	EAST (R) Positive	EAST (R) Negative	Total
EAST (S) Positive	20	45	65
EAST (S) Negative	4	59	63
Total	24	104	128

R-Reference laboratory
S-South African laboratory