Validation and application of the ELISA technique for the detection of fish aero-antigens

By

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DECLARATION

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I, Dashwill Anton George, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise), and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other Technikon or University.

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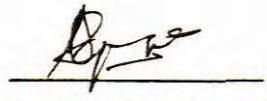

Dashwill Anton George

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ABSTRACT

Background:

Increased seafood consumption due to its nutrition and promotion of a healthy diet has lead to more frequent reports of allergic reactions. In the seafood industry, workers are exposed to the antigens through inhalation of the vapours created during the seafood processing and cooking. Most seafood allergens are stable molecules, which are resistant to the effect of cooking and processing. The prevalence of occupational asthma varies from 7-36% among different groups of workers including seafood processing and fishmeal workers, fishermen and restaurant cooks (Jeebhay et al 2001).

Purpose of Study:

The purpose of the study is to determine total protein and the specific fish antigen concentrations in the environment by means of personal air sampling filters obtained from various categories of workers in the seafood processing industry.

Objectives:

- To determine the correlation between total protein concentrations and specific fish (pilchard and anchovy) antigen concentrations on personal air sampling filters using the linear response model of the standard curve.

- To determine the correlation between total protein concentrations and specific fish (pilchard and anchovy) antigen concentrations on personal air sampling filters using the sigmoidal response model with a variable slope of the standard curve.

- To identify the most efficient standard curve response model for fish antigen detection by comparing the percentage recovery of the linear standard curve response model and the sigmoidal standard curve response model.

Methodology:

A sample population of 195 samples was taken from workers in the seafood industry at the St. Helena Bay Fisheries and West Point Processors using personal air sampling pumps. The filters were analysed to determine specific fish (pilchard and anchovy) antigen concentrations using a specialized assay called the Enzyme-Linked Immunosorbent Assay (ELISA). This study focused on total protein, pilchard and anchovy antigen concentrations. This entailed preparing extracts from personal air sampling filters to monitor various exposure zones of workers in the seafood processing factory. The investigation adhered to the prescribed antigen-antibody concentration combinations conducted by Tulane University for the detection of these fish extracts.

Results:

The reproducibility in the ELISA inhibition assays, coefficient variation (CV) percentages were calculated on the percentage inhibition of each antigen standard over three consecutive days and showed good CV of less than 15%. The study revealed that cross-reactivity between anchovy antigens with pilchard polyclonal antisera, does exist, as expected due to the major fish antigen (*Gad c1*), which is present in most fish species. The comparison of the assays performance showed good agreement between Tulane and Groote Schuur laboratories for the standard curves of both pilchard and anchovy antigen detection assays. The agreement test between the two laboratories for both pilchard and anchovy antigen detection on personal air sampling filters was not as good as the standards. This could be explained on the basis that Tulane laboratories diluted their elutes and the Groote Schuur Laboratories performed the assays on undiluted elutes, yielding more sensitive and accurate results. The Shapiro-Wilk test was performed to test whether the distribution of the environmental sample concentrations were normal. The correlation analysis was performed between the total protein concentrations, pilchard and anchovy antigen concentration derived from a linear standard curve and a sigmoidal standard curve of environmental sample filters. As expected, a significant correlation was found between ambient pilchard and anchovy antigen concentrations as they share a major fish antigen (*Gad c1*). No significant

difference was evident amongst the correlation analysis of the sample concentrations derived from the linear standard model and from the sigmoidal standard model. However, the sigmoidal standard curve yielded a better average percentage recovery than the linear standard curve. The linear standard curve model was significantly different to the known standard concentration. The study demonstrated the difference between the limit of detection of the linear standard curve and the limit of detection of the sigmoidal standard curve, as it is of importance to assess and monitor the performance of a method. The detection limit of the linear standard curve recovered only 0.14µg/ml pilchard antigen instead of 0.20µg/ml. The detection limit of linear anchovy standard was 0.28µg/ml anchovy antigen instead of 0.20µg/ml. This demonstrated that there is significantly difference ($p<0.001$) between the linear standard curve and the known standard concentration in both pilchard and anchovy antigen standards. The results therefore proved that the sigmoidal standard curve was more precise and accurate in recovering its detection limit than the linear standard curve.

Conclusion:

This study illustrated that it is certainly possible to detect fish antigens derived from fish species such as pilchard and anchovy on personal air sampling filters using the ELISA inhibition assay. However, special precaution needs to be taken with the behaviour of the antigen standard curve, as traditionally linear standard curves were used to obtain results for environmental sampling filters. This study demonstrated the variation between two standard curve response models in order to identify the most efficient standard curve model for fish antigen detection on personal air sampling filters. The analysis of the standard concentrations revealed that the sigmoidal standard curve model was better than the linear standard curve model. The limit of detection is considered to be an important part of assessing the performance of the ELISA inhibition assay, and based on this the sigmoidal standard curve brings more confidence and certainty with the reporting of low antigen concentrations than the linear standard curve.

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CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Recent years have seen increased levels of production and consumption of processed seafood, leading to more frequent reporting of allergic reactions in occupational and domestic settings. Workers involved in either manual or automated processing of crabs, prawns, mussels, fish and fishmeal production are commonly exposed to various seafood constituents. Occupational dermal exposure occurs as a result of unprotected handling of seafood and its by-products. In addition, aerosolisation of seafood and cooking fluid during processing, are potential occupational hazards resulting in sensitisation through inhalation. There is great variability of aerosol exposure within and amongst various jobs with reported allergen concentrations ranging from 0.001 - 5.061($\mu g/m^3$) (Jeebhay M. et al 2001). Airborne antigens associated with asthmatic reactions have not yet been fully described as only a few seafood antigens have been isolated and characterized in detail. Previous studies isolated additional antigens from cod and salmon (Malo JL et al 1993). Antigens with amino acid homology similar to the major crustacean antigen, shrimp muscle protein tropomyosin, have been isolated from several shrimp species, lobster and crab. A high degree of IgE cross reactivity has been shown between shrimp, crab, lobster and crawfish antigens. (Lopata A et al 2000).

Studies assessing the antigenicity and allergenicity of cod proteins showed the major Cod allergen *Gad c 1* (allergen M) serves as a model as to how allergens in general and particularly those in foods can be identified, purified and characterized. *Gad c 1* belongs to the parvalbumins, a group of vertebrate muscle calcium-chelating proteins. It mediates the concentration of calcium in the muscles and is a very stable allergen. The allergenicity of the Gad c 1

allergen is not significantly altered even in extreme pH and temperature conditions or random folding of the molecule following exposure to dissociating agents. These findings suggest that allergenic activity is dependant on the amino-acid sequence and not necessarily on the steric conformation. 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).

Until recently, few exposure assessment techniques existed to measure high molecular weight protein antigens of plant or animal origin directly (Heederik D. et al 1999). Traditional methods relied on total thoracic particulate mass, the protein concentrations or functional assays measuring enzyme activity. These quantification methods are not sensitive and specific enough to investigate exposure-response relationships among sensitized workers. With the introduction of highly sensitive immunoassays for the quantification of seafood antigens in environmental air samples and their efficiency has contributed towards a better understanding of these relationships in population studies, including occupational settings (Doukes G. et al 1996). These studies provide clear evidence for the existence of exposure-response relationships for occupational antigens (Baur X. et al 1998). Most previous attempts to measure airborne seafood antigens in the occupational context utilized human serum in a radioimmunoassay procedure. This technique however has significant drawbacks such as lack of specificity, since each human serum aliquot may demonstrate varying degrees of avidity to the different antigens. Only a sensitive immunological techniques such as utilizing rabbit antiserum and containing polyclonal antibodies for detecting antigens in fish processing workplaces will enable the assessment of exposure-response relationships among workers in jobs with differential exposure to fish antigens (Jeebhay MF et al 2001).

1.2 PROBLEM STATEMENT:

The purpose of this study was to detect total protein and the specific fish antigen concentrations in the environment by means of personal air sampling filters obtained from various categories of workers in the seafood processing industry. The study investigated the relationship between total protein and the specific fish antigen concentrations with reference to linear and sigmoidal response models in order to determine the most efficient recovery percentage. The ELISA technique was used to isolate and quantify the fish antigens present in aerosols generated during the processing of seafood.

1.3 SUBPROBLEMS:

Subproblem 1:

To determine the correlation between total protein concentrations and specific fish (pilchard and anchovy) antigen concentrations on personal air sampling filters using the linear response model of the standard curve.

Subproblem 2:

To determine the correlation between total protein concentrations and specific fish (pilchard and anchovy) antigen concentrations on personal air sampling filters using the sigmoidal response model with a variable slope of the standard curve.

Subproblem 3:

To identify the most efficient standard curve response model for fish antigen detection by comparing the percentage recovery of the linear standard curve response model and the sigmoidal standard curve response model.

1.4 HYPOTHESES:

The first hypothesis is that a correlation exists between total protein concentrations and specific fish (pilchard and anchovy) antigen concentrations on personal air sampling filters using the linear response model of the standard curve.

The second hypothesis is that a correlation exists between total protein concentrations and specific fish (pilchard and anchovy) antigen concentrations on personal air sampling filters using the sigmoidal response model with a variable slope of the standard curve.

The third hypothesis is that the percentage recovery of the linear standard curve response model and the sigmoidal standard curve response model is significantly different.

1.5 ASSUMPTIONS:

- Any particulate collected on the personal air sampling filters represents the ambient air concentrations within the breathing zone of the workers.
- The protein content on the air filters consists mainly of fish antigens originating from meat, exoskeleton, blood and endolymph.
- The protein and fish antigens obtained by means of personal air sampling filters, is representative of the working conditions in the seafood processing industry.
- All procedures used to obtain the samples are reliable and valid indicators of protein and fish antigens in the working environment of the seafood processing workers.

1.6 DELIMITATIONS:

- The study was conducted on personal air sampling filters of two seafood-processing factories in St Helena Bay along the West Coast of South Africa.
- The study only concentrated on two seafood antigen species e.g. Pilchard (*Sardinops sagax*) and Cape anchovy (*Engraulis* capensis) detected in air samples.
- Only the ELISA technique was used to identify and quantify fish antigens present on the personal air sampling filters and excludes any other immunoassays.

1.7 DEFINITIONS:

ELISA – Enzyme-linked immunosorbent assay refers to a technique used to detect antigens in a sample. One of the reaction components is nonspecifically adsorbed to the surface of a solid phase (the wall of a microtiter well). After washing, enzyme-labeled antibody (different from the bound antibody) is added and forms a complex of solid phase antibody-antigen-antibody-enzyme. Excess (unbound) antibody is washed away and enzyme substrate is added. The enzyme catalytically converts the substrate to products, the amount of which is proportional to the quantity of antigen in the sample.

ELISA Inhibition assay – The microplate wells are firstly coated with a known concentration of antigen. This is incubated overnight at 4^{0}C and then blocked, followed by the normal sequence of the ELISA. The percentage inhibition is directly proportional to the concentration of antigen in the sample.

Linear response model – This is a statistical model, called linear regression, which is used to find the line that comes closest to the data. More precisely, the linear regression finds values for the slope and intercept that define the line

that minimizes the sum of the square of the vertical distances between the points and the line.

Sigmoidal response model – This is a statistical model, called sigmoidal dose-response with a variable slope, which is used to find the line similar to an S-shape through the data. This model has variable slopes, one at the bottom, one in the middle and one at top of the S-shape curve.

Limit of Detection – is the lowest level of the antigen that can be measured with acceptable certainty above the background noise.

Percentage Recovery – is a percentage calculated to express the ability of an analytical method to accurately measure an analyte when a known amount of it is added to authentic samples.

Allergen – is any substance that can trigger an inappropriate immune response, or allergy, in susceptible people.

Sensitisation - *is repeated or single exposure to an allergen that results in the exposed individual becoming hypersensitive to the allergen.*

IgE (Immunoglobulin E) - is formed as an antibody against allergens which attaches to cell membranes causing the release of histamine and other substances responsible for the local inflammation characteristic of an allergy

1.8 MOTIVATION FOR THE STUDY:

Allergy to fish is common among fish-eating populations and in fish processing communities. High-risk occupational exposure to seafood allergens occurs mainly in the food and fishing industry. In the fishing industry, immunologically mediated reactions have been documented to occur primarily through the respiratory route by inhalation of aerosols generated during cutting, scrubbing,

cleaning, cooking and drying. Analysis of the occupational environment and exposure will reveal which workers are at high risk of developing allergic reactions. The ELISA technique was developed and evaluated by Tulane University Laboratories to detect antigens on air sampling filters. The same technique and protocol was used in this study to isolate and quantify the seafood antigens present in aerosols generated during the processing of seafood. The study sought to determine whether total protein could be used as a proxy for fish antigen concentrations. The study also sought to identify the most efficient standard curve response model for fish antigen analysis, so as to determine a reliable and valid detection limit for the assay.

CHAPTER TWO

2. THE REVIEW OF THE RELATED LITERATURE

This review will focus primarily on the methodological issues employed in the collection and analysis of ambient air environmental samples collected during the processing of proteins in general and seafood proteins on particular.

2.1 Introduction:

"Seafood" refers to any aquatic organism that is intended for human or animal consumption. Recent years have seen a growing demand for seafood, which has led to increased production and consumption (Lehrer 1993). Approximately 72% of harvested fish and shellfish are utilized as human food worldwide. It is estimated that between 1985 to 1989 the world harvests of all seafood species increased by 15% (shellfish increased as a rate of 22% and finfish at a rate of 14%) (Moody et al 1993). Increased levels of production and consumption of seafood have led and continue to lead to the more frequent reporting of adverse reactions, including immunologically-mediated reactions. Allergy to fish is common among fish-eating populations and in fish processing communities (O'Neil et al 1993). The prevalence of immediate-type fish allergy is higher when the intake of fish constitutes a greater part in the diet of the community (Aas 1987). Despite these reactions being a common occurrence in the general population, their prevalence in the occupational setting has until recently largely been understudied (Durborow 1999).

During the processing of seafood, aerosols are generated when cutting, scrubbing, cleaning, cooking and boiling, which is then inhaled (through the respiratory route). This can then lead to allergic sensitization and occupational asthma among workers in the seafood processing industry (Malo and Cartier 1993). The Food and Agriculture Organisation of the United Nations (FAO) estimates that between 1970 and 1990, the number of people engaged in fishing, aquaculture and related activities doubled from 13 million to 28.5

million worldwide (ILO 1999). Among these workers 52% worked aboard fishing trawlers, 32% were involved in aquaculture production (marine and freshwater) and 16% worked inland as capture fishers or other land-based activities such as processing. In 1990, 95% of the world fishers and fish farmers were from developing countries, producing 58% of the 98 million tons of world fish. In many countries, labour in the fishing industry 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 processing (Jeebhay, Lopata and Robins 2000). Most of these workers are seasonal workers. The degree of exposure is likely to be highest during the harvest season when most of the processing occurs. Detailed detection and quantification of fish antigens in the occupational setting has not been documented previously, despite being a potential cause of occupational allergy and asthma (Jeebhay et al 2001).

2.2 Environmental Air Sampling:

The general principle underlying assays for detecting environmental allergens is the collection of airborne particles onto suitable filters or the collection of settled dust samples and subsequent extraction and quantification of individual allergens and total allergens by an inhibition immunoassay. High volume samplers (Quan-Tec Air, Inc., Rochester, Min) operate at flow rates of up to 3 litres per minute and provide samples large enough to measure clinically significant concentrations of aeroallergens (Yungringer JW. et al 1997).

Personal air sampling is useful for defining the particular tasks in the job that are associated with elevated exposures. Proper filter media are essential and should offer low resistance to the air being sampled, but yet offer efficient retention of respirable (less than 3 microns) particles. These filter media should not denature the protein, nor adsorb the allergen, but permit high yields of recovery. Polytetrafluoroethylene (PTFE or Teflon) has proven to be the most satisfactory filter medium (Quan-Tec Air, Inc., Rochester, MN) (Reed et al. 1999).

There have been other studies that have used other filter media with limited success. Laitinen evaluated four different sampling filters for the quantification of endotoxin, which are generally used in occupational environments. In this evaluation study performed by Laitinen (1999) replicate samples were collected as close to each other as possible with sterile 37 mm diameter filters in plastic filter holders (Millipore Corp., USA) using calibrated suction pumps (SKC, Model 222-3) at a flow rate of 2 litres/min. Sampling times varied from 0.5–2 hours, but for replicate samples they were the same. Before collection, the plastic filter holders were cleaned by sonication for 30 min in 1.0% triethylamine (Fluka Chemie AG, Buchs, Switzerland) and dried at 70°C in an oven. All glassware and pipette tips were autoclaved at 121°C for 20 min or heat-sterilised at 180°C for 4 hours before use. A blank sample was used as a control to check for endotoxin (pyrogen) contamination during analysis. Four different filters were evaluated. All samples were controlled for bacterial growth at the same time as endotoxin concentrations of the extracts were analysed. After collection, samples were extracted and stored at -20°C. The concentrations of endotoxin were the highest (28 µg/m^3) when the glass fibre filters were used as the gold standard, for air sampling. Comparison of the various filter media showed that the endotoxin concentrations determined from air samples collected on the cellulose esters filters were, on average, 53% and on the polycarbonate membrane filters 26% of endotoxin concentrations collected on the glass fibre filters, while the lowest concentrations of endotoxin were detected on the polyvinyl chloride filters.

Houba R et al extracted wheat antigens from the filters with 2.5ml 0.15M Phosphate Buffered Saline (PBS, pH 7.4) in a 10ml centrifuge tube. Each tube was vortexed for 2 minutes, successively. The extract was centrifuged at 5.000g for 15 minutes. The supernatant was collected and stored at –20^{0}C for up to 6 months (Houba R. et al 1996). Malo JL et al detected snow-crab antigens in environmental samples. In this study type A/E glass fiber filters were cut into approximately 2 x 3mm pieces and sonicated in 1.5ml phosphate buffered saline (0.01M, pH 7.2) on an ice pack for 30s/100watts. The sonicate

was centrifuged (20 min, 100 000 x g) and the supernatant stored at $-4^{0}C$ (Malo JL. et al 1997).

2.3 Analytical techniques:

With the introduction of highly sensitive and efficient immunoassays for the quantification of allergens in environmental air samples has contributed towards a better understanding of exposure-response relationships in population studies, including occupational settings (Doekes. et al 1996). Most seafood antigens, like many other food allergens, are very stable proteins that resist the effects of cooking, processing or even digestive processes (Lopata A and Potter P. 2000). This has been demonstrated for crustacean allergens and recently also for fish allergens (Pascual CY et al 1996). Most previous attempts to measure airborne seafood allergens utilized human serum in radioimmunoassay procedures (Jeebhay et al 2001). With the introduction of the ELISA inhibition technique, it has become possible to measure airborne crab antigens in seafood processing factories (Malo JL. et al 1997).

2.4 Protein analysis

A comparative study done by Sapan CV et al (1999) using several assays with the same sample population suggests that the most critical issue in the use of a chromogenic protein assay is the selection of a standard for the calibration of the assay. It is crucial that the "standard" be representative of the sample. If it is not possible to match the "standard" with the sample from the perspective of protein composition, then it is preferable to use an assay that is not sensitive to the composition of the protein such as a micro-Kjeldahl technique, quantitative amino acid analysis or the biuret assay. In a complex mixture it might be inappropriate to focus on a general method of protein determination and much more informative to use specific methods relating to the protein(s) of particular interest, using either specific assays or antibody-based methods. In a study characterizing the metabolites in fresh trout, the tissue was homogenized, centrifuged and the supernatant protein concentration was measured using the BCA method with bovine serum albumin as standard (Zhengrong Y. et al 1999).

The results of this study suggested that the tolerance of the BCA method to many commonly encountered detergents and buffers offers a definite advantage over general methods for protein determinations. The study done by Griffin P. et al (1994), demonstrated that total protein is a less accurate measure of exposure to the sensitizing agent, due to the protein assay detecting non-crab meat proteins and non-specific reducing compounds.

2.5 Enzyme Linked Immunosorbent Assay (ELISA)

The basic indirect ELISA protocol is useful for screening anti-sera supernatants for specific antibodies, when microgram 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 FM. et al 1995). This assay can be modified into an inhibition assay to measure antigens by introducing fluid-phase extracts as inhibitors. The dose-response curves produced by various test extracts (air filter samples) can then be compared with the dose-response curve produced by the standard extract (Yungringer JW. et al 1997).

A recent study by Taylor AV. et al, detected aerosolized fish allergen in an open-air fish market through area sampling and a competitive IgE immunoassay. Ambient air samples were collected on polytetrafluoroethylene filters using air samplers. Fish allergens were specifically quantified by competitive IgE immunoassay using pooled sera from fish-sensitive individuals. Allergen was quantified in all 39 fish market air samples (2-25 ng/m^3). The study concluded that by using air sampling and an immunochemical analytic

technique, fish allergen was detectable in the ambient air of an open-air fish market (Taylor AV. et al 2000).

Beaudet N. et al recently performed a study among processing workers aboard crab-processing vessels (Beaudet N. et al 2002). Crab allergen concentrations were quantified during specific work activities with 25 personal air samples collected on polytetrafluoroethylene filters and analyzed by a competitive IgE immunoassay technique. Aerosolized crab allergen concentrations ranged from 79 ng/m3 to 21,093 ng/m3 (mean = 2797 ng/m3, SD = 4576 ng/m3). The highest concentrations were measured at butchering/degilling workstations, which were combined on the smallest vessel. Substantial concentrations of crab allergen exposure were measured, confirming the wide variability in exposure during crab processing aboard fishing vessels.

2.6 Calibration curves of ELISA:

Enzyme-linked immunosorbent assays are rapid and sensitive methods for quantifying environmental analytes in trace amounts. (Hall JC. 1990, Van Emon et al 1989 and Vanderlaan M. et al 1988). The choice of expressing the best-fitting curve for simple calculatory purposes can reflect irrelevant calibration values (Bunch DS. et al 1990).

Sasaki D. et al (2002) indicate that sandwich ELISA assays and competitive ELISA assays differ fundamentally with respect to the standard curves obtained and the methods used for data analysis. To obtain reliable results it is recommended that the sample dilutions provide signals that fall in the vicinity of 50% signal. Also, to report values that have statistical significance, biological samples require 3 samplings or dilutions (n=3) regardless of the number of replicate wells. Typically coefficients of variance (CV, standard deviation/mean) will be less than 15%. If statistical significance or precision is not a major concern (e.g. for well established assay procedures designed to determine the presence or absence of an analyte), single dilutions of a sample can be used to obtain reproducible results as long as the values fall within the linear range of

the standard curve. Standard curve for a typical sandwich ELISA assay showing a linear relation between the signal and the analyte concentration is defined as $y = mx + b$, where y = signal, x = concentration, m = slope, b = y-intercept. The standard curve of the sandwich ELISA is represented with a straight line and a positive slope. A linear regression curve can be used to obtain sample concentration estimates. For a competitive ELISA, the standard curve can be mathematically represented as a 4-parameter logistic fit and subjected to nonlinear regression analysis (Diamandis EP. et al, 1996 and Maciel RJ. 1985). Competitive ELISA data can be linearized and analyzed using a log-logit transformation. The resulting straight line can be used to evaluate samples by linear regression. However, only the linear portion of the standard curve can be used (Diamandis EP. et al, 1996)

2.7 Assay Comparisons using statistical techniques:

Renström A. et al (1997) performed a comparison study between radioallergosorbent inhibition and an ELISA for aeroallergen measurement. The statistical tools used for the comparison, were regression analysis and the Bland-Altman plot to test agreement between the two methods. The regression analysis of log values of aeroallergen concentrations from 37 air filter elutes showed that the value of the two methods were highly correlated, $r^2 = 0.72$ ($p<0.001$) (three samples below the lowest standard point were excluded). However, according to the Bland-Altman plot, the values differed by several orders of magnitude: the median (range) values of all 40 samples in both methods. When the difference in sample between the two assays was plotted against the sample mean (using log transformed data), a systematic difference was shown between the assays ($p<0.001$); moreover the difference in the measured value increased as the quantity of protein increased. If there had been a close agreement between the values measured for the same sample in each assay, the data points would be distributed around zero.

The underlying theoretical framework used by Renström A. et al (1997) is described by Bland and Altman (Bland JM and Altman DG 1986). It is most unlikely that different methods will agree exactly, by giving the identical results for all samples. The most common approach is to perform regression analysis; however all the data points will be clustered near the regression line and it will be difficult to assess between-method differences. A plot of the difference against the standard measurement is sometimes suggested, but this will always appear to show a relationship between difference and magnitude when there is none. A plot of the difference between the methods against their mean may be more informative. The mean difference would be the estimated bias, the systematic difference between methods, and the standard deviation (SD) of the differences would measure random fluctuations around this mean. Bland and Altman recommended 95% confidence limits of agreement, mean difference plus or minus 2 (more precisely, 1.96) SD's, which would indicate how far apart measurements by the two methods were likely to be for most samples.

2.8 Limit of Detection issues:

At low analyte concentrations the relative uncertainty increases to the point where it is no longer pertinent to refer to as a quantitative result (limit of detection). At low concentrations the uncertainty for the net signal is of increasing importance in the overall estimation process. One can take the conservative view that no concentration can be estimated below the limit of detection or the EURACHEM view that the "limit of detection is the lowest concentration with stated trueness and precision". The limit of detection is that concentration (amount of substance) value where the measurement uncertainty amounts to 1/3 the value of the measured and uses the same procedures for its estimation as laid down in the EURACHEM Guide First Edition 1995 (http://www.vtt.fi/ket/eurachem).

It is important to remember that in environmental measurements, the coefficient variation at the limit of detection should not exceed 10% (Wegscheider W. 1997). Limitations of the analytical methodology used to measure the

concentration of substances in the environment have had an important role in environmental modeling and regulation. The limit of detection is the true concentration of an analyte that will, with high confidence, produce a measured value above the critical level. An important use of limit of detection is to assess and monitor the performance of a laboratory (Rocke, DM et al, 2003).

In summary it is evident that detection of seafood antigens on air sampling filters are certainly possible by using the ELISA inhibition technique. Previous studies have concentrated mainly on crustacean antigens and very seldom on fish antigens detection on air samples. Fish allergen was detectable in the ambient air of an open-air fish market by competitive IgE immunoassay using pooled sera from fish-sensitive individuals. There is therefore a need to evaluate the ELISA inhibition assay using polyclonal antibodies for fish antigen detection on air sampling filters of occupational exposed individuals as they are likely to be exposed to higher concentrations of fish antigen. Polytetrafluoroethylene filters were the most common and satisfactory filter medium used in previous crab and fish antigen detection studies. No previous studies investigated the relationship between total protein concentrations and specific fish antigen concentrations on air sampling filters. The Bicinchoninic acid (BCA) method has proven to be the most efficient total protein determination method on air sample filters when compared to the micro-Kjeldahl technique and the biuret assay. Various statistical methods have been described to determine analyte concentrations on air sampling filters. The sandwich ELISA is generally represented with a linear standard curve and a competitive ELISA is represented with a sigmoidal standard curve. The evaluation of the linear and sigmoidal standard curve responses should theoretically bring certainty reporting the limit of detection for each antigen assay.

CHAPTER THREE

3. METHODOLOGY

3.1 Aerosol sampling instrumentation: Personal sampling equipment was used to collect the fraction of aerosols containing protein antigens with Polytetrafluroethylene (PTFE or Teflon) filters as the filter medium. Full shift time weighted average samples was obtained from each participating worker using SKC Aircheck Samplers (Model 224-PCCXR), Gill Air and Du-Pont Alpha-1 battery operated air-sampling pumps running at an average flow rate of 2 liters per minute with a Personal Environment Monitor (PEM_{10}) (manufactured by MSP Corp., Minneapolis, MN). The PEM is a two-stage sampler consisting of a single impaction stage (preselector) and a backup filter. The preselector has an upper median cut diameter of 10 micrometer, resulting in collection of approximately the "thoracic" fraction particulate on the filter. The PEM_{10} was fixed to the lapel of the overall/apron, near the breathing zone of the worker. Filters were weighed before and after sampling in a humidity and temperature controlled environment using a microbalance. After weighing, the samples were prepared for immunological quantification.

3.1.1 Preparation of environmental sample filters:

For elution, filters were cut into 4 equal pieces, the membrane backing removed and all 4 pieces placed into 0.5ml PBS (Phosphate buffered saline) containing 0.05% Tween 20 (detergent). The filter pieces were mixed overnight on a shaker at 4 degrees Celsius. The mixture was centrifuged at 2000g for 2 minutes, and the supernatant removed and stored at –80 degrees Celsius until further use.

3.2 Protein Analysis of Samples: The technique used for protein analysis is called bicinchoninic acid (BCA) Protein Assay (Pierce Biotechnology products). A protein standard was prepared by diluting BSA (Bovine Serum Albumin) in the same diluent (Distilled Water) as the protein sample to be determined. The protein standard series should cover the range of concentrations determined by the assay protocol, which is 0.5 µg/ml to 2.50 µg/ml of total protein standard. The BCA working reagent was prepared by mixing 50 parts of solution A to 1 part of solution B. The assay was conducted in 96-well Greiner F plates, using 10µl sample within 200µl BCA working reagent. Mixed well and incubated at 60°C for 30 minutes. After incubation, the plate was cooled to room temperature. The absorbance was read vs. the blank (distilled water) at 540nm.

3.3 Production of polyclonal antibodies in rabbits: For the production of polyclonal antibody, 500 µg (in 0.5 ml TBS) of each protein extract (antigens) was used to immunize two New Zealand rabbits each. The Ethics Committee for Animal Experiments of the University of Cape Town approved this study. Standard guidelines for laboratory animal care were followed. The rabbits received subcutaneous injections at several sites with the extracted proteins in 0.5 ml of Complete Freund's Adjuvant (Difco). Subsequent immunizations were performed with the same protein concentration in Incomplete Freund's adjuvant at three and six weeks after the first immunization. Blood samples were taken for specific antibody titer analysis before the immunization and at week 3 and 6 with the final bleed performed at week 8.

3.4 Analysis of samples for Antigen detection: Relevant seafood extracts (1:10 w/v) of Pilchard (*Sardinops sagax*) and Cape anchovy (*Engraulis* capensis) were prepared from fresh specimens obtained from the two factories. These extracts were used as internal standards for the

Enzyme-linked immunosorbent assay (ELISA) techniques, to identify antigens present on the sampling filters.

ELISA-inhibition assay for antigen determinations of samples:

3.4.1 Pilchard antigen determination:

- To determine the Pilchard antigen concentration of each environmental sample, each polystyrene 96-well microtitre plate (Dynex flat bottom 2HB plates) was coated with 100µl of 1.56µg/ml Pilchard extract, using 100mM Sodium carbonate buffer.
- The microplates were incubated at 4 degrees Celsius overnight, flicked out and blotted.
- The microplates were blocked using 200µl of blocking buffer (Powder skim milk in TBS with 0.05% Tween 20) and incubated for 60 minutes at 37 degrees Celsius. The microplates were flicked out and blotted.
- One standard inhibition curve was used for each plate assay by adding 50µl per well in duplicate of pilchard extract ranging from 0.2µg/ml to 200µg/ml.
- The same volumes were added to each well in duplicate for the eluant of each environmental sample.
- The rabbit polyclonal pilchard antiserum was diluted to 1:8 000 of which 50µl was added to each well.

  Provision was made to demonstrate "Total Specific Binding" (TSB) and "Non Specific Binding" (NSB).
- Total Specific Binding consists of 50µl of blocking buffer (Powder skim milk in TBS with 0.05% Tween 20) and 50µl of rabbit polyclonal pilchard antiserum (1:8000) to yield 100% binding, which means complete binding of antibodies of the antiserum to the antigens coated on the microplate; no inhibition.
- Non Specific Binding consists of 100µl of blocking buffer (Powder skim milk in TBS with 0.05% Tween 20) to yield 0% binding because of no antigen-antibody complexes being formed, i.e. it is a background (blank) reading of the microplate.

- The microplates were incubated at room temperature for 90 minutes, then flicked out and blotted.
- The microplates were rinsed once with washing buffer (TBS-0.05%-Tween 20), then flicked out and blotted.
- The goat anti-rabbit IgG antibody (Southern Biotechnology Associates, Inc) was diluted to 1:10 000 of which 100µl was added to each well and incubated at room temperature for 90 minutes.
- The microplates were flicked out, washed thrice with washing buffer (TBS-0.05%-Tween 20) and once with TBS-AP buffer (diluted 1:5) and blotted.
- Substrate (PNPP; Sigma) with a volume of 100µl was added and incubated at room temperature for 45 minutes to develop colour.
- The Optical densities were read at 410nm using the NSB as blanks and the TSB as 100% -binding.
- The standard inhibition curves were expressed by a simple linear regression response model and a sigmoidal dose-response with a variable slope, to calculate the concentration of allergens per filter.

3.4.2 Anchovy antigen determination:

The same protocol (see 3.3.1) was followed for the Anchovy antigen determination except for the coating concentration which was 3.13µg of Anchovy extract and the rabbit polyclonal anchovy antiserum which was diluted to 1:16 000.

3.5 THE DATA, THEIR ANALYSIS, AND THEIR INTERPRETATION:

3.5.1 Primary data:

- The total protein concentrations were obtained by the Bicinchoninic acid Assay (BCA) technique for each air-sampling filter.

- The antigen (Pilchard, Cape anchovy) concentrations were obtained by the ELISA Inhibition technique for each air-sampling filter.

3.5.2 Secondary Data:

The data and current documentation regarding the ELISA Inhibition technique was developed by the Tulane University Laboratories for fish antigen detection. Quality control of the analytical methods was assured by sending a 10% subset of samples to a second laboratory for cross validation (Tulane Medical Centre). Similar analytical protocols were used in both collaborating laboratories (Groote Schuur Hospital in South Africa and Tulane Medical Centre). The Bland-Altman plot was used to test the level of agreement of the pilchard standard curve and the anchovy standard curve between Tulane and Groote Schuur laboratories.

3.6 The criteria governing the admissibility of the data:

Only data linked with a Standard Curve per 96-well microplate was accepted.

Only the data where all the exposure metrics were established was accepted, i.e. (Total protein, Pilchard and Cape anchovy antigen concentrations per sample must be available).

3.7 The proposed research process:

Since the objective of the study was to investigate the relationship between total protein and specific antigen concentrations using two standard curve response models it was important to determine the outcome of each subproblem. This involved determining the Total protein, Pilchard and Cape anchovy antigen concentrations of all the air sampling filters on a microplate reader. Only the Optical Density readings were obtained from the instrument, which were manipulated statistically to express the concentrations in microgram per cubic meter.

3.7.1 Instruments: For this type of investigation a sophisticated clinical microplate reader was used viz. Biotek automated microplate reader Model EL311s. This technique also required an incubator whose temperature was adjusted to 37^{0}C (Memmert Incubator by Lasec), a waterbath whose temperature was adjusted to 60^{0}C (Memmert Waterbath by Lasec), a graduated 12-channel multipipette (Labopette by Hirschmann Laborgerate) and a fridge whose temperature was set at 2-8^{0}C.

3.7.2 Sample: This study was limited to 195 samples obtained by personal air sampling from two factories in St Helena Bay viz. on the West Coast of South Africa. All filters were treated with the same extraction procedure for elution of antigens. Approximately 10% of the samples were collected as field blanks, in order to exclude or correct for possible background contamination during analysis.

3.8 The specific treatment of each subproblem:

3.8.1 Subproblem 1:

To determine the correlation between the total protein concentrations and specific fish (pilchard and anchovy) antigen concentrations on personal air sampling filters using the linear response model of the standard curve.

The data needed:

It is required to measure the Total protein, Pilchard and Cape anchovy antigen concentrations of each filter, using the linear response model of the standard curve.

Locality of the data:

Only samples with Total protein, Pilchard and Cape anchovy antigen concentrations per filter were used. The samples were taken from two seafood processing factories on the West Coast of South Africa, namely West Point Processors and St. Helena Bay Fishing.

The means of obtaining the data:

The Total protein concentration of each air-sampling filter was determined by using the Bicinchoninic acid Assay (BCA) technique. The Pilchard and Cape anchovy antigen concentrations were determined using the ELISA inhibition technique. All the Optical Density readings were obtained from the Biotek microplate reader. The researcher as a qualified medical technologist processed the samples and operated within the Standard Operating Procedure protocol prescribed for the microplate reader.

The analysis of the data:

The optical density readings of total protein and specific antigen concentrations were subjected to a linear response model of the standard curve to express the concentrations in µg/ml. All the concentrations reported were corrected by the volume of air flowing through the sampling pump to express the total protein and specific antigen concentrations in $\mu g/m^3$. These concentrations were manipulated statistically using Analyse-it® Version 1.6 for the linear standard curve model. Stata® version 7 was used for the basic statistical calculations and to identify the correlation amongst the determinants.

Interpretation of the data:

The data were analysed using linear regression analysis and depending on the p-value a correlation can be identified, proving the hypothesis of the first subproblem to be positive or null.

3.8.2 Subproblem 2:

To determine the correlation between the total protein concentrations and specific fish (pilchard and anchovy) antigen concentrations on personal air sampling filters using the sigmoidal response model with a variable slope of the standard curve.

The data needed:

It is required to measure the Total protein, Pilchard and Cape anchovy antigen concentrations of each filter, using the sigmoidal response model of the standard curve.

The locality, the means of obtaining, the treatment and interpretation of data:

The same research methods as in subproblem 1 were used to test the hypothesis of subproblem 2, except antigen concentrations, which were manipulated statistically using Prism® Version 4 for the sigmoidal standard curve model.

3.8.3 Subproblem 3:

To identify the most efficient standard curve response model for fish antigen detection by comparing the percentage recovery of the linear standard curve response model and the sigmoidal standard curve response model.

The data needed:

It is required to measure pilchard antigen and Cape anchovy antigen concentrations of each specific standard.

Locality of the data:

The standards of each microplate were used.

The means of obtaining the data:

The Total protein concentration of each standard was determined using the Bicinchoninic acid Assay (BCA) technique. The Pilchard and Cape anchovy antigen concentrations were determined by using the ELISA inhibition technique. All the Optical Density readings were obtained from the Biotek microplate reader.

The treatment of the data:

The optical density readings of total protein standard and each specific antigen standards were subjected to a linear response model and then to a sigmoidal response model to express the concentrations in µg/ml. The percentage recovery of each standard concentration was calculated and evaluated for each response model.

Interpretation of the data:

The two response models were compared based on the percentage recovery of the known standard concentration, the r^2-value derived from the expression equation and depending on the level of significance the model that best fitted all the data points was obtained.

3.9 ETHICS:

This study concentrated on the immunological analysis of seafood aero-antigens in the occupational environment, which forms part of a collaborative project between the Environmental Health Research Unit at the University of Cape Town and the Department of Health Sciences at Peninsula Technikon. The principal researcher, Dr M. Jeebhay at the University of Cape Town, provided permission to perform and use the report of the analysis as the basis of an MTech degree in Biomedical Technology (Appendix 1). The principal researcher obtained all ethics approval from the Research Ethics Committee of the University of Cape Town (Appendix 2). Letters of both seafood processing factories confirming to participate in this project are included as (Appendix 3) and (Appendix 4).

CHAPTER FOUR

4. RESULTS:

A researcher Fernandes JK developed the ELISA inhibition assay for fish aero-antigen detection at Tulane University and sourced the checkerboard titration experiments, sensitivity and cross-reactivity of the rabbit antiserum to the Groote Schuur Laboratories.

4.1 Checker-board titration experiments:

To determine the optimal antigen and antiserum concentrations for the ELISA inhibition assays, checkerboard titrations were performed of the Pilchard extracts with rabbit anti-pilchard serum and anchovy extracts with rabbit anti-anchovy serum. The optical density readings of each fish antigen coating concentration versus fish antiserum serial dilution were plotted and represented as curves in (Figures 1 and 2).

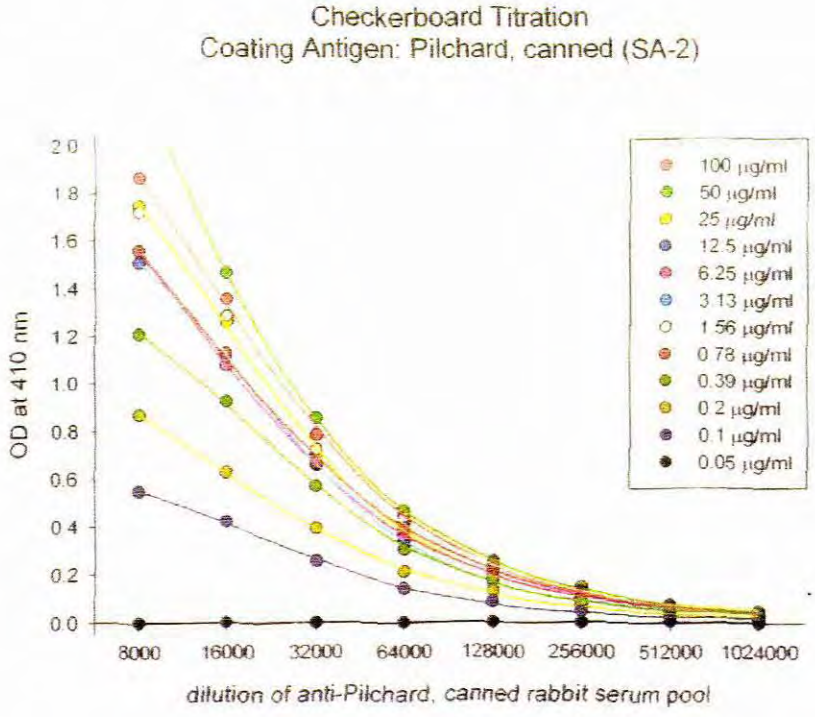

Figure 1: Checkerboard titration for Pilchard coating antigen and antibody dilutions

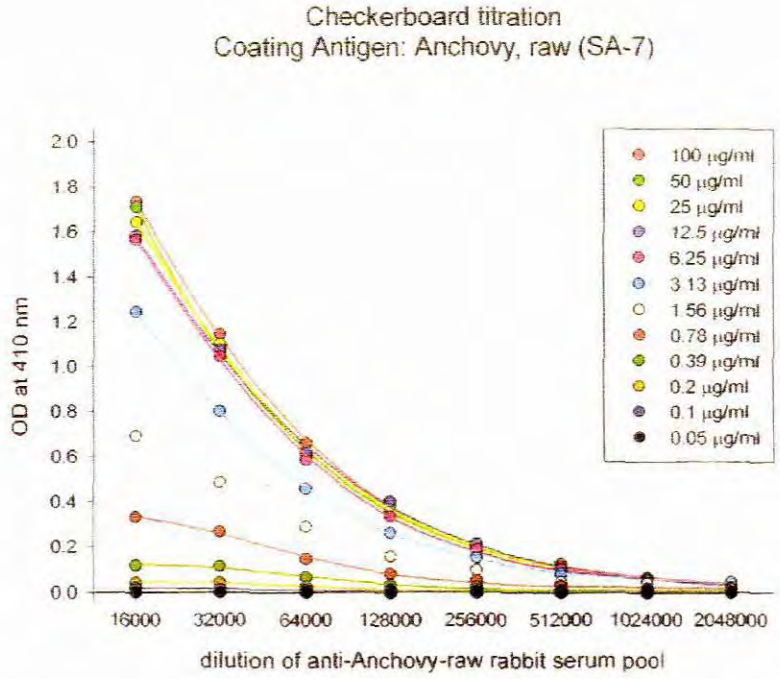

Figure 2: Checkerboard titration for Anchovy coating antigen and antibody dilutions

The optimal coating concentration and antiserum dilution for the ELISA inhibition assay were chosen from that point on the curve corresponding to the lowest antigen coating concentration and highest antiserum dilution. The optimal pilchard antigen coating concentration was 1.56µg/ml versus 1:8000 pilchard antiserum dilutions. The optimal anchovy antigen coating concentration was 3.13µg/ml versus 1:16000 anchovy antiserum dilution.

4.2 Sensitivity of rabbit antiserum and coating antigen: The sensitivity of the rabbit antibodies was assessed by ELISA inhibition assays with fish antigens and two antiserum dilutions. The inhibition curves of pilchard and anchovy were very similar (Figure 3) for all coating concentration-antiserum combinations, implying that the previously chosen concentration were appropriate.

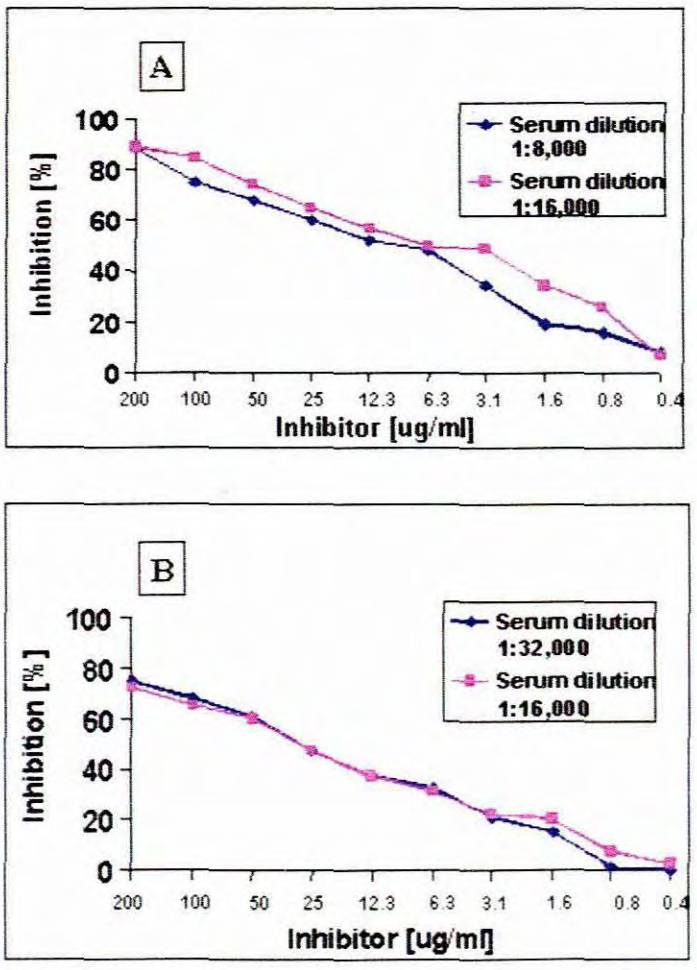

Figure 3: ELISA inhibition assays for Pilchard antigen detection (A) and Anchovy antigen detection (B)

4.3 The assessment of the Pilchard and Anchovy inhibition assays:

The experiments were conducted over three days using the prescribed coating concentration and antibody dilutions as described in chapter three and are illustrated in (Figures 4 and 5)

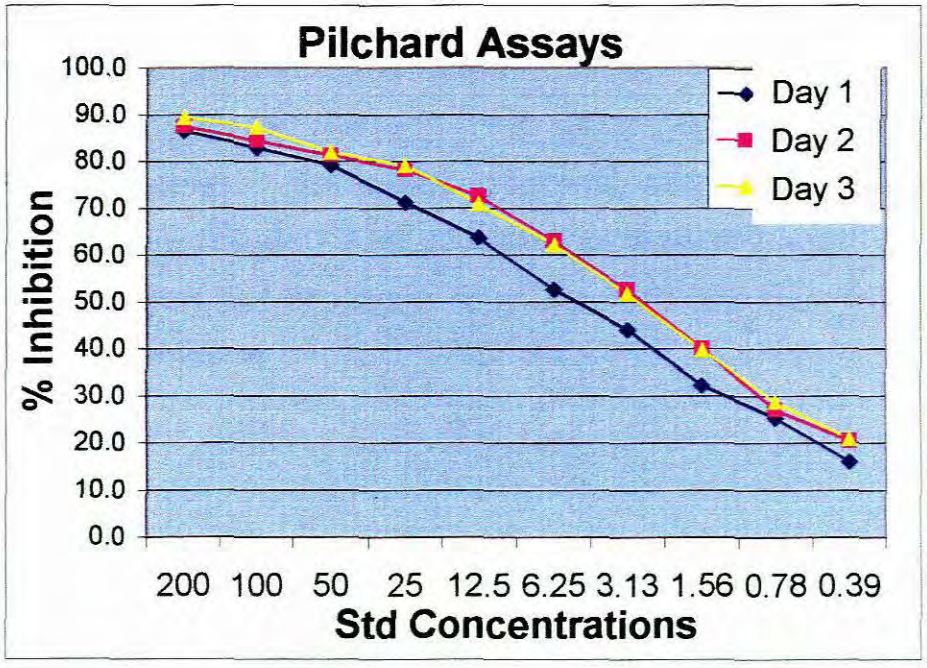

Figure 4: Pilchard inhibition assay assessment over three days

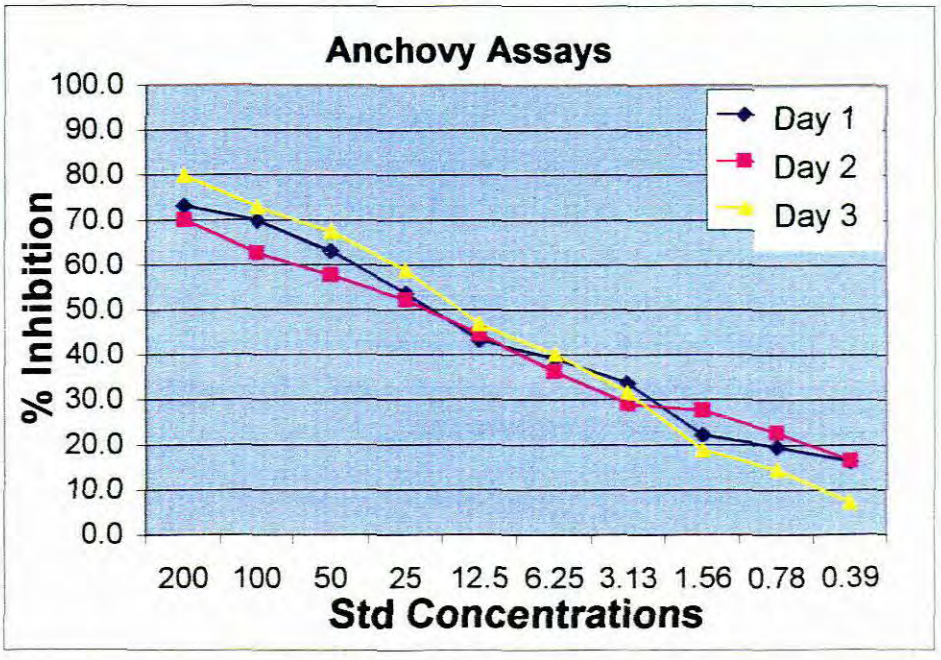

Figure 5: Anchovy inhibition assay assessment over three days

The standard curves of the Pilchard inhibition assays demonstrated good consistency, as the coefficient variation percentage was 7% over three consecutive days. The coefficient variation percentage for the Anchovy inhibition assays over three consecutive days was 12.7%.

4.4 Cross-reactivity between Pilchard and Anchovy antigens:

To establish whether cross-reactivity existed between Pilchard and Anchovy antigen concentrations, a microplate was coated with 1.56µg/ml pilchard extract. Thereafter Pilchard and Anchovy antigen concentrations ranging from 0.4µg/ml to 200µg/ml were added as standards. This was allowed to react with 1:16 000 dilution of pilchard polyclonal antiserum.

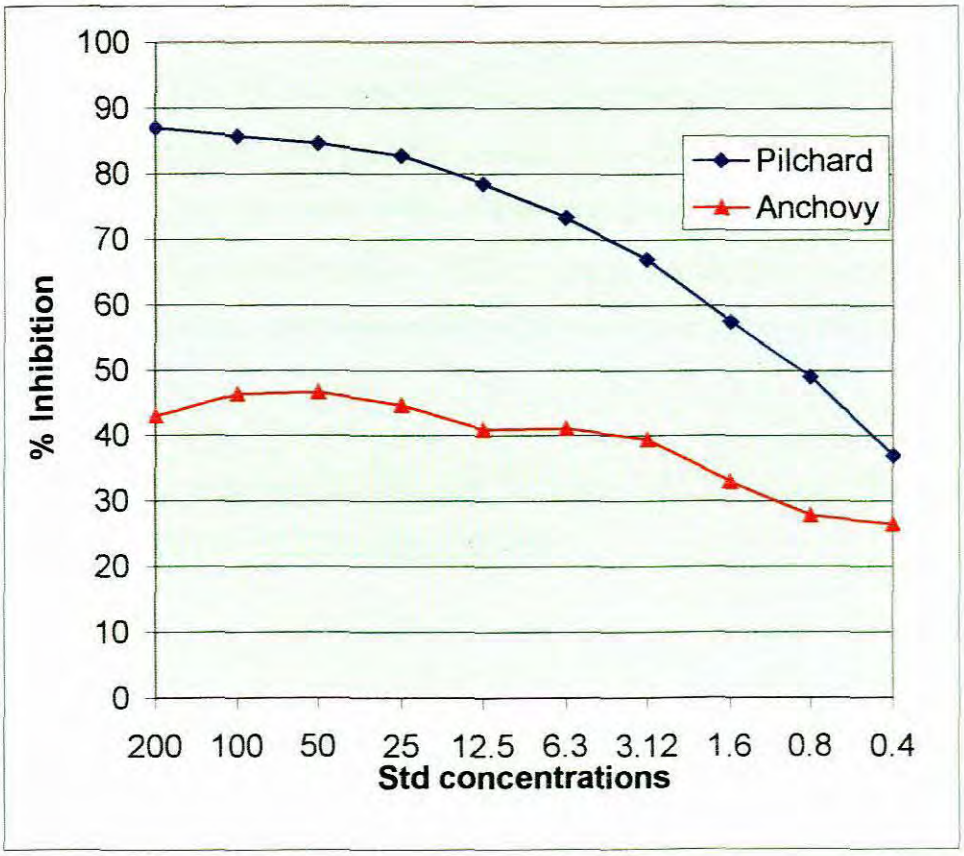

Figure 6: Cross-reactivity testing of Anchovy antigen on Pilchard antigen coated plate

As illustrated with in (Figure 6) cross-reactivity exists between anchovy antigen and pilchard antiserum of 25.7% inhibition in the low antigen concentration to 45.2% inhibition in the high antigen concentration. These results were similar to the cross-reactivity analysis performed at Tulane laboratories. The homologous combinations of coating antigens i.e. pilchard antigens and homologous rabbit anti-pilchard serum were set to 100% and other reactivities were calculated accordingly. Anti-

anchovy serum demonstrated cross-reactivity to pilchard coating antigen with 59% binding and anti-pilchard serum showed cross-reactivity to anchovy coating antigen with 45% binding.

4.5 Comparison of ELISA inhibition assays between Tulane and Groote Schuur laboratories:

The Pilchard Standard Inhibition curves showed good agreement between the Tulane and Groote Schuur laboratories (Table 1). The Log_{10} *mean value for the two standard curve measurements* (X-axis) was plotted against the difference between the standard curve values (Y-axis) (Figure 7). The mean for the difference between values is indicated by a solid line, which equals 0.055. The two standard curves agree closely, as most the differences were distributed around 0 and within the 1.96 SD zones.

Table 1: Comparison of the Pilchard Standard Inhibition Curve between Tulane and Groote Schuur Laboratories

Pilchard Standard Concentration (µg/ml)	% Inhibition	
	Tulane	Groote Schuur
200	96.9	90.6
100	93.3	89.1
50	88.1	85.2
25	82.3	78.9
12.5	74.7	70.6
6.25	65.5	63.4
3.13	54.1	50.9
1.56	44.5	36.0
0.78	30.1	25.2
0.39	28.0	16.5

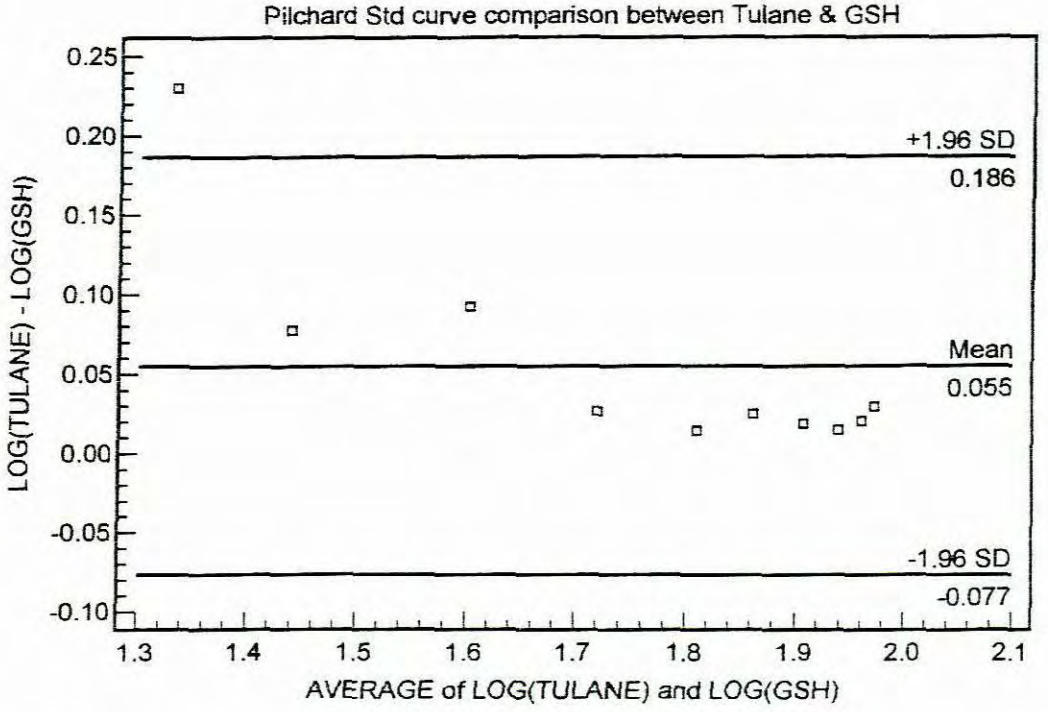

Figure 7: Bland-Altman plot of the Pilchard standard curve between Tulane and Groote Schuur Laboratories

The Anchovy Standard curves showed good agreement between the Tulane and Groote Schuur laboratories (Table 2). The Log_{10} mean value for the two standard curve measurements (X-axis) was plotted against the difference between the standard curve values (Y-axis) (Figure 8). The mean for the difference between values is indicated by a solid line, which equals 0.04. The two standard curves agree closely, as most of the differences were distributed around 0 and within the 1.96 SD zones.

Table 2: Comparison of the Anchovy Standard Inhibition Curve between Tulane and Groote Schuur Laboratories

Anchovy Standard Concentration (µg/ml)	% Inhibition	
	Tulane	*Groote Schuur*
200	79.5	79.9
100	65.0	72.9
50	56.2	67.4
25	47.6	58.5
12.5	39.6	46.8
6.25	35.3	40.2
3.13	28.9	31.6
1.56	23.7	18.8
0.78	23.1	14.4
0.39	23.4	7.2

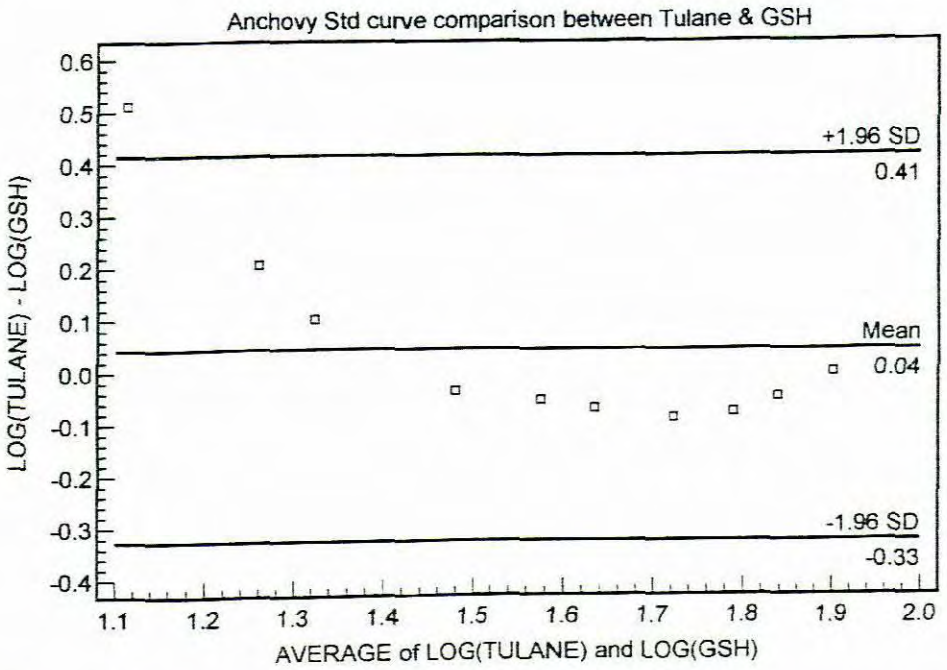

Figure 8: Bland-Altman plot of the Anchovy standard curve between Tulane and Groote Schuur Laboratories

One field blank was collected for each batch having a maximum of 10 samples. Replicate analyses were conducted on 20 environmental sample filters to test for agreement between Tulane and Groote Schuur laboratories. The assay comparisons are shown below: (Figure 9 and 10).

The Bland-Altman plot test showed not good agreement between Tulane & Groote Schuur as most differences were distributed within 1.96 SD, except in one case where antigen concentrations of both Pilchard and Anchovy were high and were diluted in order to be measurable. The Log_{10} mean value for the Tulane & Groote Schuur Laboratory Pilchard measurements (X-axis) was plotted against the difference between two laboratories values (Y-axis) (Figure 9). The mean for the difference between values is indicated by a solid line, which equals -0.47. The Log_{10} mean value for the Tulane & Groote Schuur Laboratory Anchovy measurements (X-axis) was plotted against the difference between two laboratories values (Y-axis) (Figure 10). The mean for the difference between values is indicated by a solid line, which equals -0.62. In both Pilchard and Anchovy antigen assay comparisons the mean for the difference between the two laboratory values were not close to zero.

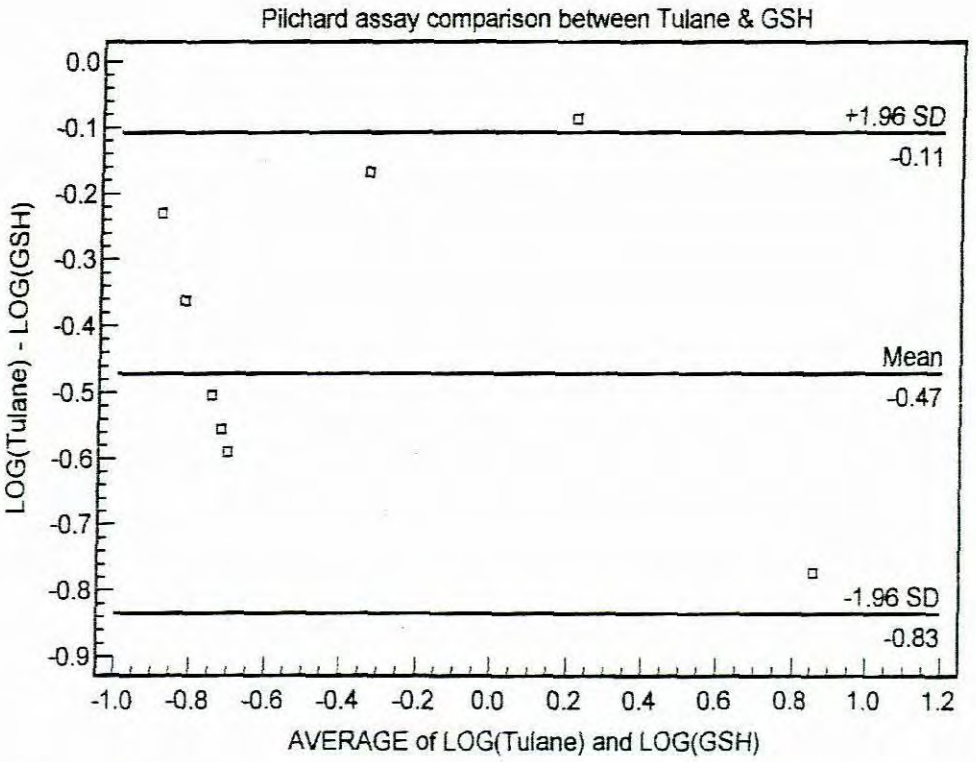

Figure 9: Bland-Altman plot of the samples for Pilchard antigen determination between Tulane and Groote Schuur Laboratories

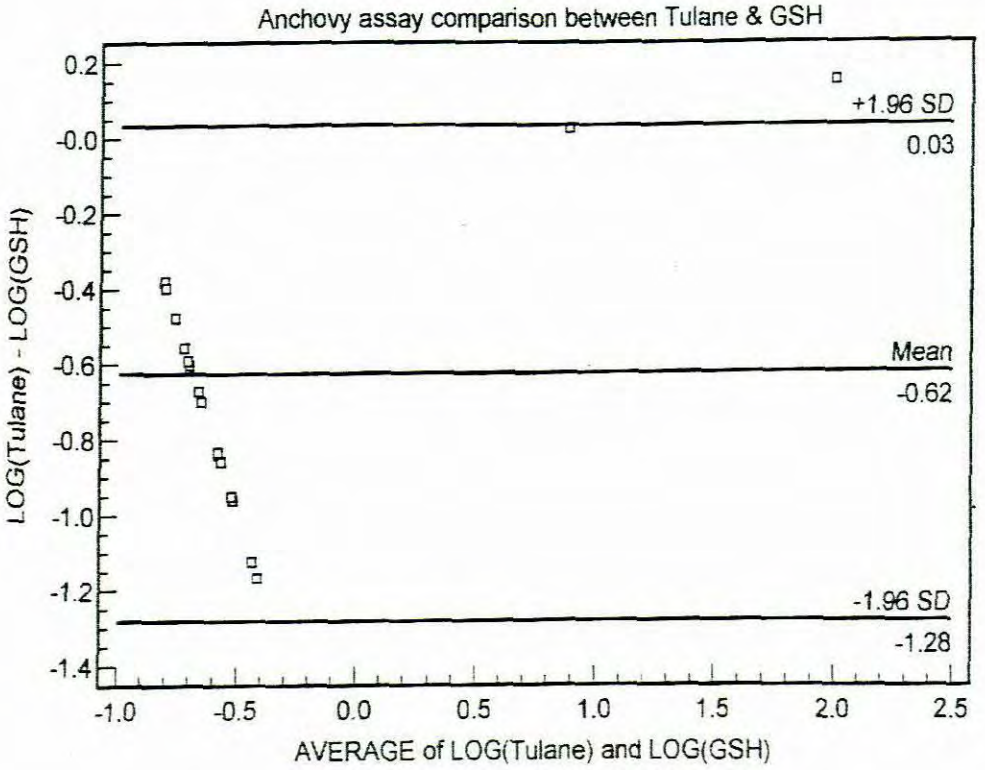

Figure 10: Bland-Altman plot of the samples for Anchovy antigen determination between Tulane and Groote Schuur Laboratories

4.6 Analysis of environmental samples:

Each sample filter was analysed for total protein, pilchard and anchovy antigen concentrations. For total protein determinations only the linear standard curve response model was used. The percentage inhibition results from the pilchard and anchovy antigen determinations of all the samples were subjected to both the linear standard curve response model and the sigmoidal standard curve response model. The results of all the samples are shown in (appendix 5).

Table 3: Summarised results of samples for total protein, pilchard- and anchovy antigen determinations (appendix 5)

	N	AM	GM	GSD	Range
Protein	180	0.870	0.693	0.601	(0 – 10.865 µg/ml)
PilcLin	195	0.160	0.123	0.381	(0 – 17.538 µg/ml)
AncLin	195	0.593	0.289	0.454	(0 – 83.171 µg/ml)
PilcSig	195	0.159	0.219	0.267	(0 – 10.657 µg/ml)
AncSig	195	0.697	0.319	0.451	(0 – 99.500 µg/ml)

PilcLin – Samples with pilchard antigen concentrations derived from the linear standard curve response model; AncLin - Samples with pilchard antigen concentrations derived from the linear standard curve response model; PilcSig - Samples with pilchard antigen concentrations derived from the sigmoidal standard curve response model; AncSig - Samples with pilchard antigen concentrations derived from the linear standard curve response model; N=no of samples; AM=Arithmetic mean; GM=Geometric mean; GSD=Geometric standard deviation.

4.7 Explanation of the linear standard curve response model:

The optical density (OD) readings of each sample were calculated to express the percentage inhibition of each sample based on the OD reading of the Total Specific Binding, which equaled 100% inhibition. The percentage inhibitions of the standard concentrations were plotted against the Log transformed standard concentrations ranging from 0.20 - 200µg/ml. The Linearity of all the percentage inhibition points were expressed with a straight line as in $y=bx+a$, where a is equal to the

intercept of the straight line, b is equal to the slope of the straight line, x is equal to the concentration of the standard and y is equal to the percentage inhibition. The Log concentrations were transformed to anti-log to express it as µg of antigen per ml. The antigen concentration was corrected by multiplying the volume of air flowing through the personal air sampling pump to obtain a final concentration µg of antigen per m^3.

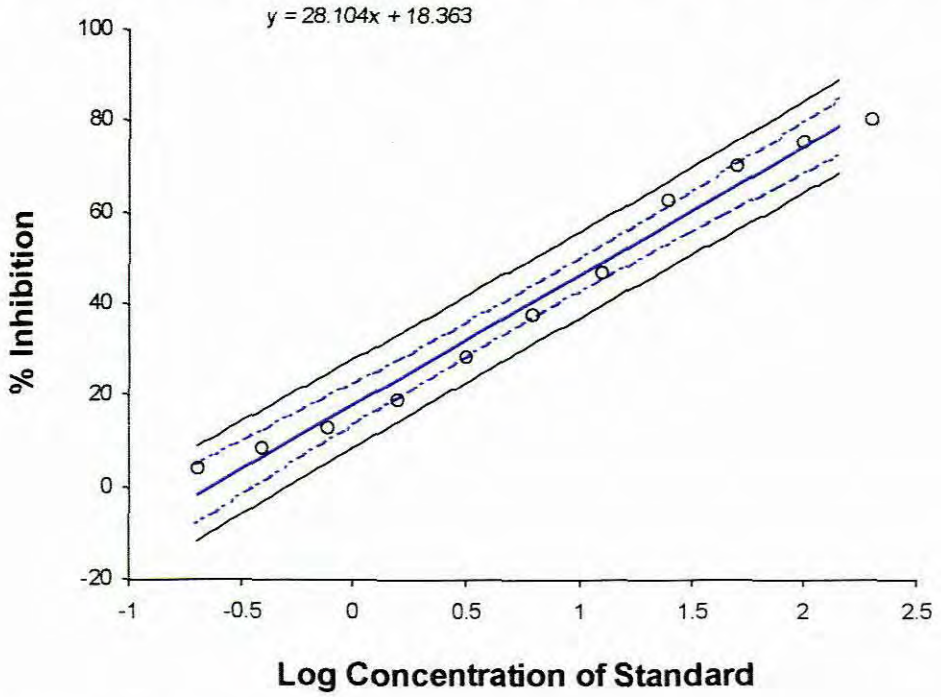

Figure 11: Example of a linear standard curve

4.8 Explanation of sigmoidal standard curve response model:

The percentage inhibition of the standard concentrations were plotted against the Log transformed standard concentrations ranging from 0.20 - 200µg/ml. All the percentage inhibition points were expressed with a variable slope sigmoidal equation:

$$Y = Bottom + (Top - Bottom) / (1+10^{(LogEC50 - X)*HillSlope})$$

The model parameters were *Bottom*, which denoted the value of Y for minimal percentage inhibition standard curve response, *Top*, which denoted the value Y for the maximal percentage inhibition standard curve response, $LogEC_{50}$, which denoted the logarithm of standard concentration halfway between the *Bottom* and *Top* standard curve

response, and the *Hill Slope*, which denoted the steepness of the standard inhibition curve. The independent variable, X, was the logarithm of the standard concentration. The variable slope sigmoidal equation was alternatively referred to as the Hill equation or the four-parameter logistic equation.

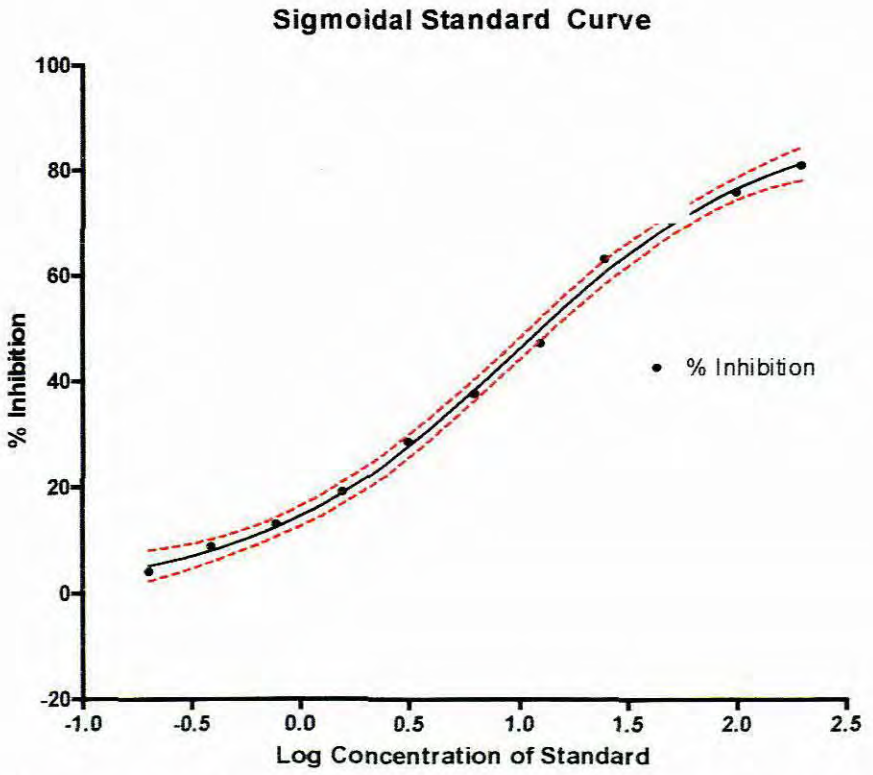

Figure 12: Example of a sigmoidal standard curve with a variable slope

4.9 Correlation analysis:

The Shapiro-Wilk tests were performed to determine whether the distribution of the data were normal. The data for total protein concentrations pilchard antigen concentrations and anchovy antigen concentrations with both standard curve response models were all skewed. Based on these findings, the Spearman correlation analyses were performed.

Correlation between total protein concentrations, pilchard antigen concentrations and anchovy antigen concentrations derived from the linear standard curve response are summarised in (Table 4) and illustrated in (Figures 13-15)

Table 4: Correlation between various exposure metrics derived from the linear standard curve response

	N	R-value	p-value
Total protein concentration & Pilchard antigen concentration	180	0.26	<0.0001
Total Protein concentration & Anchovy antigen concentration	180	0.25	<0.0001
Pilchard antigen concentration & Anchovy antigen concentration	195	0.74	<0.0001

N= no of samples; R-value=Spearman's rho; p-value=probability test

There was a significant ($p < 0.0001$) correlation (Spearman's rho = 0.26, CI = 0.122 – 0.395) between total protein concentrations and pilchard antigen concentration. The correlation (Spearman's rho = 0.25, CI = 0.106– 0.381) between total protein concentrations and anchovy antigen concentration was also significant ($p < 0.0001$). The strongest correlation (Spearman's rho = 0.74, CI = 0.670 – 0.798) was between pilchard and anchovy antigen concentrations, with high significance of ($p < 0.0001$).

The correlation between total protein concentrations, pilchard antigen concentrations and anchovy antigen concentrations derived from the sigmoidal standard curve response are summarised in (Table 5) and illustrated in Appendix (Figures 16-18)

Table 5: Correlation between various exposure metrics derived from the sigmoidal standard curve response

	N	R-value	p-value
Total protein concentration & Pilchard antigen concentration	180	0.302	<0.0001
Total Protein concentration & Anchovy antigen concentration	180	0.253	<0.0001
Pilchard antigen concentration & Anchovy antigen concentration	195	0.742	<0.0001

N= no of samples; R-value=Spearman's rho; p-value=probability test

The correlation (Spearman's rho = 0.302, CI = 0.163 – 0.429) improved slightly between the total protein and the pilchard antigen concentrations derived from the sigmoidal standard curve response. The correlations between total protein concentrations and anchovy antigen concentrations, and between pilchard and anchovy antigen concentrations were similar to that of the linear standard curve model.

4.10 Intra-assay Variation:

Obtaining reproducible results with the ELISA method requires minimizing intra-assay variation (replicate values within a microplate), which can be used to assess and monitor the performance of the laboratory. The Mean, Standard Deviation and the Coefficient Variation were calculated on the Optical Density Readings of each Standard Concentration ranging from 0,02µg/ml to 200µg/ml for each antigen. These findings are plotted on the graphs below (Figure 19 & 20).

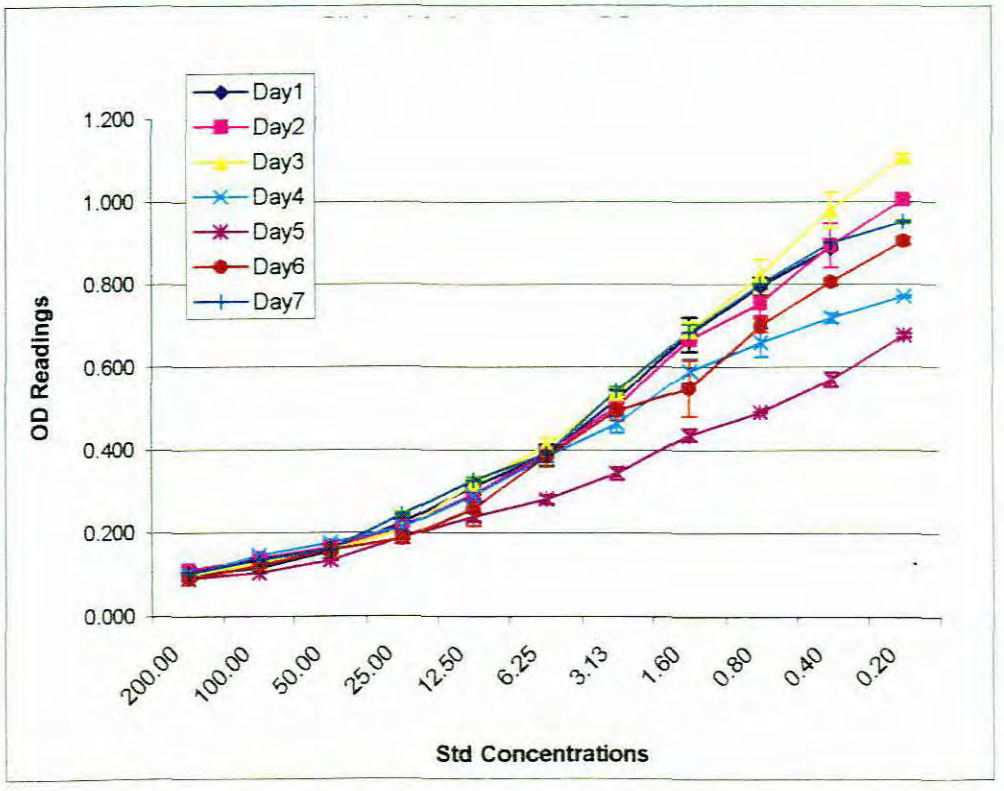

Figure 19: Variation of Pilchard Optical Density Readings

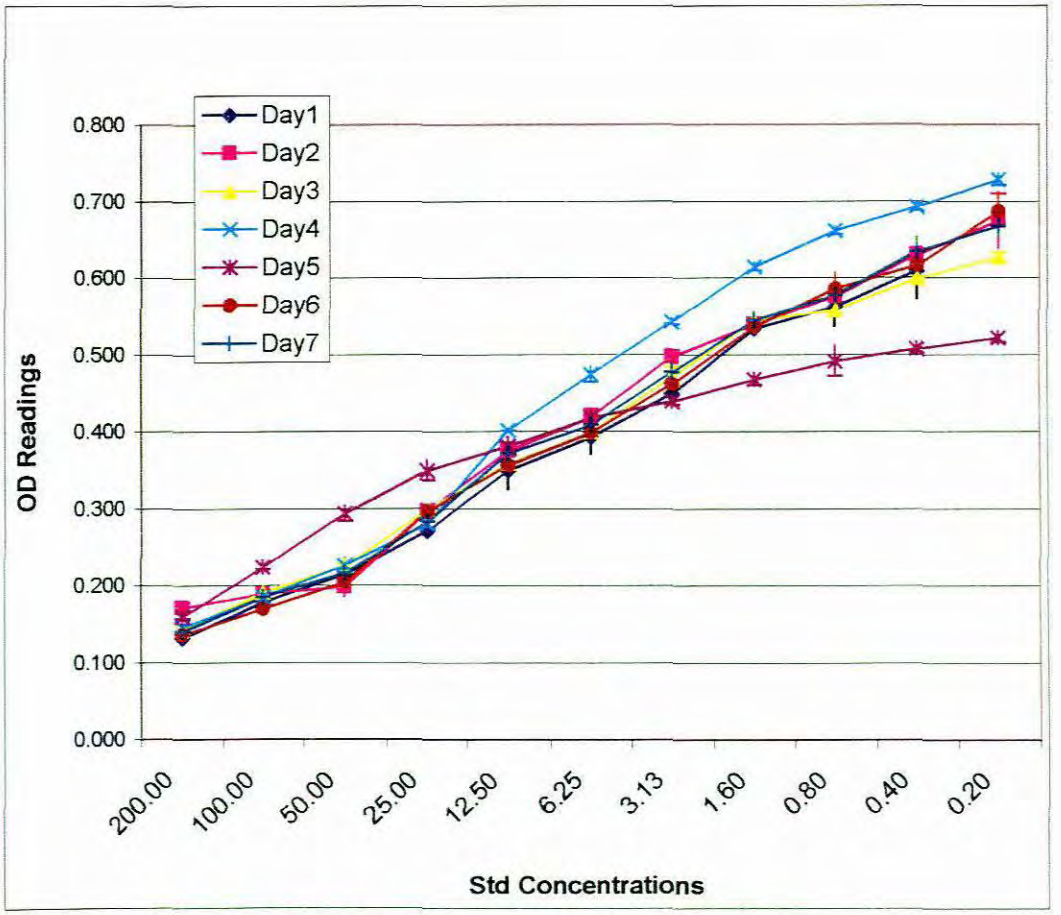

Figure 20: Variation of Anchovy Optical Density Readings

The variations are indicated with the Y-error bars for each standard concentration. Averages Coefficient Variation was calculated for all concentrations:

Table 6: Average coefficient variation percentage for each day

Day	Pilchard	Anchovy
One	5,1%	3,7%
Two	3,1%	2,8%
Three	3,2%	1,6%
Four	4,4%	1,7%
Five	2,2%	2,1%
Six	5,4%	2,8%
Seven	2,5%	2,5%

A desirable coefficient variation (CV) percentage is <5%, which was observed in most plates except on day one and day six of the pilchard antigen plates where the CV was >5%.

4.11 Inter-assay Variation:

The inter-assay variation refers to "day to day" variation between microplates in order to determine the reproducibility of results within the ELISA method. The variation was calculated using the mean, standard deviation and coefficient variation of all the Optical Density Readings for each standard concentration ranging from 0,02µg/ml to 200µg/ml for each antigen. An average coefficient variation was calculated for all concentrations, which revealed a higher CV than the CV for the intra-assay variations.

Table 7: Average coefficient variation percentage for 7days per concentration

Std [µg/ml]	Pilchard	Anchovy
200	8,5%	9,5%
100	11,1%	8,8%
50	8,5%	14,0%
25	10,2%	8,5%
12,5	11,7%	5,2%
6,25	11,4%	6,7%
3,13	13,7%	7,3%
1,56	15,3%	7,6%
0,79	15,6%	8,6%
0,40	16,3%	9,0%
0,20	16,6%	10,7%
Average	12,6%	8,7%

4.12 Percentage Recovery and its significance:

Table 8: Average percentage recovery for all concentrations per day

	Pilchard		Anchovy	
	Linear	Sigmoidal	Linear	Sigmoidal
Day 1	108%	101%	101%	100%
Day 2	110%	100%	104%	105%
Day 3	110%	100%	103%	101%
Day 4	105%	98%	105%	102%
Day 5	107%	101%	109%	101%
Day 6	109%	101%	101%	103%
Day 7	107%	100%	102%	101%

The average percentage recovery from day one to day seven for pilchard antigen concentration in linear standard curve equals 108% which is higher than the sigmoidal standard curve of 100%. The anchovy antigen concentration in the linear standard curve equals 104%, which is higher than the sigmoidal standard curve of 102%. In both cases the sigmoidal standard curve appears to reveal better results than the linear standard curve.

Table 9: Average percentage recovery and significance per standard concentration

	Pilchard				Anchovy			
Std	Lin-Recov	Linear P-value	Sig-Recov	Sig-P-value	Lin-Recov	Linear P-value	Sig-Recov	Sig-P-value
200	48%	<0.001	92%	<0.001	101%	0.74	92%	0.56
100	75%	<0.001	92%	0.18	116%	0.18	99%	0.69
50	116%	<0.001	105%	0.38	138%	<0.001	124%	0.09
25	154%	<0.001	108%	0.28	116%	0.31	97%	0.61
12,5	163%	<0.001	98%	0.72	80%	<0.001	88%	0.05
6,25	161%	<0.001	101%	0.86	87%	0.05	105%	0.19
3,13	136%	<0.001	101%	0.88	80%	<0.001	104%	0.33
1,56	102%	0.84	96%	0.48	74%	<0.001	96%	0.45
0,79	89%	0.12	103%	0.27	96%	0.05	112%	0.11
0,40	76%	<0.001	103%	0.24	117%	<0.001	107%	0.22
0,20	71%	<0.001	101%	0.36	140%	<0.001	100%	1.00

Lin-Recov = percentage recovery of the linear standard response model; Linear P-value = significance level of linear standard response model; Sig-Recov = percentage recovery of the sigmoidal standard response model; Sig P-value = significance level of sigmoidal standard response model. (One-sided t-test were performed against the known standard concentrations to generate a p-value)

The pilchard average percentage recovery for the complete linear standard concentration range was 108% and for the complete sigmoidal concentration range 100% (Table 9). The anchovy average percentage recovery for the complete linear standard concentration range was 104% and for the complete sigmoidal concentration range 102%. Two one-sided t-tests were performed for each standard concentration to indicate whether there were significant differences between the antigen concentrations derived from linear standard curve and the known standard concentrations. The same procedure was followed for the antigen concentrations derived from the sigmoidal standard curve. The P-value of both the pilchard and anchovy standard concentrations derived from the linear model indicates a significant difference to the

original (known) standard concentrations. Based on the P-value of the pilchard and anchovy standard concentrations derived from the sigmoidal response model, yielded better recovery of the original standard concentrations.

The r^2-value of each standard curve demonstrated how good all the percentage inhibitions best expressed the standard equation and are summarised in the (Table 10). The r^2-values of the Sigmoidal standard curves for all the seven plates, pilchard and anchovy antigen standards, were higher than the r^2-values of the linear standard curves.

Table 10: The r^2-value of each standard curve

	PilcLin	PilcSig	AncLin	AncSig
Plate one	0.958	0.999	0.994	0.997
Plate two	0.958	0.999	0.985	0.992
Plate three	0.957	0.999	0.988	0.997
Plate four	0.979	0.998	0.982	0.998
Plate five	0.969	0.998	0.934	0.999
Plate six	0.964	0.996	0.994	0.996
Plate seven	0.970	0.998	0.993	0.997

PilcLin – pilchard antigen concentration of the linear standard curve; PilSig - pilchard antigen concentration of the sigmoidal standard curve; AncLin - anchovy antigen concentration of the linear standard curve; AncSig – anchovy antigen concentration of the sigmoidal standard curve.

The r^2-value differences between the two models in the lower standard concentration yielded more certainty in the detection limit of the sigmoidal standard curve model, than the linear standard curve model. The detection limits are summarised in (Table 11).

Table 11: Detection limits in µg/ml of linear standard curve & sigmoidal standard curve

	PilcLin	PilcSig	AncLin	AncSig
Plate one	0.14	0.20	0.22	0.20
Plate two	0.12	0.20	0.23	0.20
Plate three	0.11	0.20	0.30	0.20
Plate four	0.20	0.20	0.31	0.20
Plate five	0.10	0.20	0.43	0.20
Plate six	0.13	0.20	0.21	0.20
Plate seven	0.19	0.22	0.26	0.20

PilcLin – pilchard antigen concentration of the linear standard curve; PilSig - pilchard antigen concentration of the sigmoidal standard curve; AncLin - anchovy antigen concentration of the linear standard curve; AncSig – anchovy antigen concentration of the sigmoidal standard curve.

The detection limit of the linear standard curve yielded 71% recovery of 0.20µg/ml pilchard antigen concentration (i.e. the average of measured pilchard antigen concentration equals 0.14µg/ml instead of the expected 0.20µg/ml) and 40% higher than 0.20µg/ml of anchovy antigen concentration (i.e. the average of measured anchovy antigen concentration equals 0.28µg/ml instead of the expected 0.20µg/ml) in (Table 11). The detection limit of the sigmoidal response curve yielded 101% recovery at 0.20µg/ml pilchard antigen concentration and 100% recovery at 0.20µg/ml anchovy antigen concentration.

CHAPTER FIVE

5.1 DISCUSSION:

Aerosolisation of seafood antigens during processing has been identified as a potential route for allergic sensitization and occupational asthma in the crustacean processing industry (Malo JL et al 1993). The recent introduction of highly sensitive immunoassays for the quantification of allergens in environmental air samples and their efficiency has contributed towards a better understanding of these relationships in population studies, including occupational settings (Doekes G. et al 1996). Most studies that addressed occupational exposure to seafood allergens concentrated mainly on crustacean (crab and shrimp) and very scantily on fish allergens (Cartier A et al 1986). This study utilised polyclonal antibodies from rabbits to detect fish antigens on personal air sampling filters with a sensitive immunological assay and investigated the relationship between total protein and the specific fish antigen concentrations with reference to linear and sigmoidal response models.

To assess the reproducibility in the ELISA inhibition assays, coefficient variation (CV) percentages were calculated on the percentage inhibition of each antigen standard over three consecutive days (Figures 4 and 5). The pilchard antigen inhibition assay showed a CV of 7%. Day one pilchard inhibition percentages were slightly lower than day two and day three. This could be explained by the fact that day one pilchard inhibition assay was performed on the last day of the first trial week. Diluents were prepared in smaller volumes and fresh diluents were prepared on day two, the second week. To exclude this type of variation, sufficient diluent volumes were prepared to perform all assays on actual air sampling filters. The anchovy inhibition assays showed a CV of 12.7% over three consecutive days. Typically coefficients of variance of 3 consecutive values for biological samples should be less than 15% (Sasaki D. et al 2002). This confirmed that the reproducibility of the standard inhibition curves in both antigen detection assays were acceptable.

Based on these findings the study tested for cross-reactivity of anchovy antigens with pilchard polyclonal antisera, which revealed that cross-reactivity does exist, 25.7% inhibition in the low antigen concentration to 45.2% inhibition in the high antigen concentrations (Figure 6). The cross-reactivity between anchovy antigen and pilchard antisera was expected as both anchovy and pilchard have the major fish antigen (*Gad c1*), which is present in most fish species (O'Neil et al 1993).

To compare the assays performance between Tulane and Groote Schuur laboratories replicate analyses were performed on 20 environmental sample filters. Renström A. et al used the Bland-Altman plot to test agreement between radioallergosorbent inhibition and an ELISA inhibition for aeroallergen measurement. If there were close agreement between the methods of the two laboratories, the data points would be distributed around zero (Renström A. et al 1997). This was indeed the case for the standards of the anchovy and the pilchard inhibition assays (Figures 7 and 8). The mean for the difference between the values of Tulane and Groote Schuur laboratories was 0.055 for the pilchard inhibition standard and 0.04 for the anchovy inhibition standard (close to zero). The agreement test between the two laboratories for both pilchard and anchovy antigen detection on environmental sample filters was not as good as the standards (Figures 9 and 10). The mean for the difference between the values of Tulane and Groote Schuur laboratories was -0.47 for the pilchard and -0.67 for the anchovy antigen detection. The reason for this could be explained on the basis that Tulane laboratories diluted their elutes to 1:2, 1:4 and 1:8 dilutions. When elutes are diluted to these factors, the antigen concentration is decreased by the same factor, which is well below the limit of detection for the assay. The Groote Schuur Laboratories performed the assays on undiluted elutes, yielding more sensitive and accurate results.

Sandwich ELISA assays and competitive ELISA assays differ fundamentally with respect to the standard curves obtained and the methods used for data analysis. Standard curves for a typical sandwich ELISA assay show a linear relation between the signal and the analyte concentration (Sasaki D. et al

2002). For a competitive ELISA, the standard curve can be mathematically represented as a 4-parameter logistic fit and subjected to nonlinear regression analysis (Diamandis EP. et al, 1996; Maciel RJ. 1985). It was for this reason that correlation analysis was performed between the total protein concentrations, pilchard and anchovy antigen concentration derived from a linear standard curve and a sigmoidal standard curve of environmental sample filters. The total protein concentrations of the environmental samples were calculated from a linear standard curve as the reaction principle based on optical density of each sample is directly proportional to the total protein concentration of each sample (i.e. a linear reaction). Before correlation analysis was performed, the Shapiro-Wilk test was performed to test whether the distribution of the environmental sample concentrations were normal. Based on these findings the Spearman correlation analysis was performed, which revealed similar correlation results between total protein and pilchard antigen concentrations; and total protein and anchovy antigen concentrations. As expected, a significant correlation was found between ambient pilchard and anchovy antigen concentrations as they share a major fish antigen (*Gad c1*) commonly found in fish species (O'Neil et al 1993). No significant difference was evident amongst the correlation analysis of the sample concentrations derived from the linear standard model and from the sigmoidal standard model.

To obtain reproducible results with the ELISA method requires minimizing intra-assay variation (replicate values within a microplate) and inter-assay variation ("day to day" variation between the microplates). The intra-assay variation for the pilchard and anchovy standard curve is consistent with most studies and ranged from (2.2% to 5.4%) and (1.6% to 3.7%) respectively over seven days. The average inter-assay variation for the pilchard and anchovy standard curve equals 12.6% and 8.7%, respectively over seven days. The calculations were performed on optical density readings. These results are considered to be good repetition since the coefficients of variance was less than 15% (Sasaki D. et al 2002). The pilchard standard curve demonstrated higher variation of 15.3% to 16.6% between concentrations 1.56µg/ml and 0.20µg/ml, respectively.

To assess how well the standard concentrations were represented by the two standard curve response models, the percentage recovery was calculated for each model per day. The recovery refers the ability of an analytical method to accurately measure an analyte when a known amount of it is added to authentic samples. A good percentage recovery would be close to 100%. If percentage recovery is less than 100%, it indicates that the method under estimated the known standard concentration; alternatively if the percentage recovery is greater than 100%, the method over estimated the known standard concentration (Koch D. et al 1996). The average percentage recovery of the linear standard curve was 108% for the pilchard standard and 104% for the anchovy standard concentrations. The sigmoidal standard curve yielded a better average percentage recovery of 100% for the pilchard standard and 102% for the anchovy standard.

These findings were calculated in detail for each standard concentration ranging from 0.20µg/ml to 200µg/ml and to distinguish between the two standard curve models, the P-value was generated per known standard concentration by a one-sided t-test. The linear standard curve model was significantly different to the known standard concentration and the sigmoidal standard curve model was the best to express the known standard concentration as demonstrated in (Table 9.)

The r^2-values of the sigmoidal standard curve were higher than the linear standard curve in both pilchard and anchovy antigen standards. This demonstrated how good the equation expressed each standard curve model for the complete concentration range 0.20µg/ml to 200µg/ml (Table 10). This suggested that antigen concentrations derived from any point in the sigmoidal standard curve revealed yielded more accurate results than antigen concentrations derived from the linear standard curve.

An important use of detection limit is to assess and monitor the performance of a method (Rocke, DM et al, 2003). The study demonstrated the difference between the limit of detection of the linear standard curve and the limit of

detection of the sigmoidal standard curve. The detection limit of the linear standard curve yielded 71% recovery of 0.20µg/ml pilchard antigen concentration (i.e. only 0.14µg/ml pilchard antigen was recovered instead of 0.20µg/ml, under estimated). The detection limit of linear anchovy standard curve was 40% higher than 0.20µg/ml anchovy antigen (i.e. 0.28µg/ml anchovy antigen was recovered instead of 0.20µg/ml, over estimated). This was also demonstrated in (Table 9) to be significantly different ($p<0.001$) to the known standard concentration in both pilchard and anchovy antigen standards. The detection limit of the sigmoidal standard curve for both pilchard and anchovy antigen detection was 0.20µg/ml. This proved that the sigmoidal standard curve was more precise and accurately in recovering its detection limit than the linear standard curve.

5.2 LIMITATIONS OF THE STUDY:

Most of the sample filters collected had no fish antigens and were below detection limit. This could be explained by the fact that this ELISA inhibition assay utilized polyclonal rabbit antiserum, which comprises the sensitivity and specificity of the assay to detect fish antigens. Monoclonal rabbit antibodies are more sensitive and specific, which also influence the cross-reactivity analysis of different fish antigens. The standard concentration range was 0.20µg/ml to 200µg/ml and most of the positive samples were in the lower range of the standard curve. A lower standard concentration should have been considered. A larger positive sample population would definitely improve the correlation between total protein and fish antigen concentration.

5.3 CONCLUSION:

This study illustrated that it is certainly possible to detect fish antigens derived from fish species such as pilchard and anchovy on air sampling filters using the ELISA inhibition assay developed by the Tulane laboratories. Special precautions need to be taken with behaviour of the antigen standard curve, as

traditionally linear standard curves were used to obtain results for environmental sample filters. However, reproducibility of these results is only possible if the sample concentrations fall within the linear range, but in this study most of the results reported were below the linear range. The sigmoidal standard curve model brings confidence and certainty with the reporting of low antigen concentration on air sampling filters. The limit of detection is considered to be an important part of assessing the performance of the ELISA inhibition assay, and based on this the sigmoidal standard curve yielded better results than the linear standard curve.

5.4 RECOMMENDATIONS:

This is the first study of its kind conducted in South Africa, detecting fish antigens on personal air sampling filter using the ELISA inhibition technique. This technique needs some attention but it shows relevance and promise. As for future studies comparing results between laboratories and to measure performance of the ELISA inhibitions any researcher should minimize all possibilities of variation, one of which is the standard curve expression. The analyst should not manipulate data of the ELISA standard curve to behave in a linear format, rather than using the appropriate standard curve model to obtain antigen concentrations for environmental air samples. Consideration of larger microplate formats with 384 wells to accommodate the complete sample population would exclude inter-assay variations.

6. QUALIFICATIONS:

Masters Candidate:

Mr. D.A. George

National Diploma: Biomedical Technology

B. Tech. Degree: Biomedical Technology

Laboratory Technician: Peninsula Technikon

Supervisors (External):

Dr. A Lopata (Ph. D)

Specialist Scientist (Infectious Immunology / Allergology)

Division of Infectious Immunology

Groote Schuur Hospital / National Health Laboratory Services

University of Cape Town

Dr Mohamed F Jeebhay (MBChB DOH MPhil (Epi) MPH (Occ. Med))

Associate Director - Occupational Health

Occupational and Environmental Health Research Unit

School of Public Health and Family Medicine

Faculty of Health Sciences

University of Cape Town

Supervisors (Internal):

Mr. Mogamat Shafick Hassan (M. Pharm) & (MA Health Management)

Head: Department of Health Sciences

Peninsula Technikon

Mr Emmanuel Rusford MPH (Epidemiology & Biostatistics)

Senior Lecturer

Environmental and Occupational Health

Faculty of Science

Peninsula Technikon

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8. APPENDICES

Appendix 1: Letter of approved candidate to participate and conduct analysis of environmental samples of the study.

UNIVERSITY OF CAPE TOWN

OCCUPATIONAL AND ENVIRONMENTAL HEALTH RESEARCH UNIT

School of Public Health & Family Medicine
Faculty of Health Sciences
Anzio Road
Observatory 7925
SOUTH AFRICA
Telephone: (27 21) 406 6306/6300
Fax: (27 21) 406 6163
e-mail: mjeebhay@commed.uct.ac.za

Dear Mr Dashwill George,

RE: RESEARCH INTO OCCUPATIONAL SEAFOOD ALLERGY AMONG WORKERS IN THE WESTERN CAPE PROVINCE OF SOUTH AFRICA

I would like to confirm that you have been selected to conduct the *immunological analysis of environmental samples collected for the* abovementioned study. I also understand that the report on this analysis will form the basis of your MTech degree for which you have registered with Peninsula Technikon. This collaboration between the Occupational and Environmental Health Research Unit at the University of Cape Town with the Department of Health Sciences at Peninsula Technikon is part of an ongoing broader initiative to build capacity in research and training in occupational and environmental health in the region.

Please find attached letters from the various shareholders participating in the study as well as the ethics approval from the Research Ethics Committee of the University of Cape Town.

Sincerely,

Dr Mohamed F Jeebhay, MBChB DOH MPhil (Epi) MPH (Occ. Med)
Principal investigator - Occupational Seafood Allergy Research project

Appendix 2: Letter of approval for the research study by the University of Cape Town Research Ethic Committee

Research Ethics Committee
Faculty of Medicine
Anzio Road, Observatory, 7925
Queries : Martha Jacobs
Tel : (021) 406-6492 Fax: (021) 406-6390
E-mail : Martha@medicine.uct.ac.za

REC REF: 109/99

Dr M F Jeebhay
Community Health

Dear Dr Jeebhay

OCCUPATIONAL ALLERGY ASSOCIATED WITH ROCK-LOBSTER AND SALTWATER BONY FISH PROCESSING IN THE WESTERN CAPE PROVINCE OF SOUTH AFRICA

I have pleasure in informing you that the above study has been formally approved by the Research ethics Committee on 30 April 1999.

Included is a list of Research Ethics Committee Members who have formally approved your protocol.

Please quote the above Reference number in all correspondence.

Yours sincerely,

PROFESSOR FOR
CHAIR: RESEARCH ETHICS COMMITTEE

Queries: Martin Jacobs
Research Ethics Committee
Room 212 Werner and Beit
UCT Medical School
Anzio Road, Observatory, 7925
Tel: (021) 406-6492 Fax (021) 406-6390
E-mail: martha@medicine.uct.ac.za

Appendix 3: Letter of St. Helena Bay Fishing approving research project to be conducted at their facilities.

ST. HELENA BAY FISHING

P O Box [illegible] South Africa
Tel: (022) 742 1911 Fax: (022) 742 1847 E-Mail: [illegible]

Dr M Jeebhay
University of Cape Town
Department of Public Health

Seafood Allergy Research Project

Dear Doctor Jeebhay

This serves to confirm our willingness to participate in the research project you intend to undertake. We understand that your actions will be guided by the protocol established for the project and agree to make our facilities available to you when required.

For the purpose of communication, as discussed at the introductory meeting you can liaise with our Clinic sister directly regarding the logistics and patient flow.
Any other information and/or queries must be directed to me directly.

We look forward to have you in our presence.

Regards

D Marin
Manager: Occupational Health, safety & Environmental Protection

Appendix 4: Letter of West Point Processors approving research project to be conducted at their facilities.

WEST POINT PROCESSORS

Mnr G. Wiese
P.O.Box. 15
St Helena Bay
7390

Dr. Jeebhay
Department of Public Health
Faculty of Health Science
Anzio Road
Observatory
7925

Dear Dr. Jeebhay

Re: FOOD ALLERGY PROJECT

I would like to inform you about the outcome of our discussion that was held on 31 January 2001.
For the research project, our facilities and workers are available to you. All parties involved have been informed.

We are looking forward to start with the project.

Appendix 5: Environmental air sampling filters results for Total Protein, Pilchard & Anchovy Antigen determinations expressed in µg/ml

Job Title	Filter	Vol	PilcLin	PilcSig	AncLin	AncSig	Protein
		litre	ug/ml	ug/ml	ug/ml	ug/ml	ug/ml
Fish Packer	100	624	<0.137	<0.20	0.466	0.514	N/A
Quality Controler	101	582	<0.137	<0.20	0.396	0.425	N/A
Line inspector	102	621	<0.06	<0.20	<0.12	<0.20	0.435
Tank operator	103	600	<0.137	<0.20	0.363	0.381	N/A
Seamer operator	104	585	0.085	0.130	0.190	0.150	0.000
Drainer & Saurcer	106	1206	0.075	0.125	0.180	0.150	1.245
Floor foreman	108	1137	0.075	0.120	<0.12	<0.20	0.775
Cooker & press operator	112	555	0.000	0.000	0.000	0.000	0.000
Dryer operator	113	864	0.110	0.170	0.000	0.000	0.000
Separator operator	114	873	0.070	0.120	0.000	0.000	0.000
Fitter	115	666	0.095	0.155	0.170	0.130	0.735
Stuk Water Plant operator	116	840	0.000	0.000	0.000	0.000	0.000
Drainer & Saurcer operator	118	666	0.100	0.160	0.140	0.100	0.000
Floor foreman	119	738	0.000	0.000	0.000	0.000	0.000
Retort operator	120	672	0.000	0.000	0.000	0.000	0.000
Spice Mixer	121	690	<0.06	<0.20	0.150	0.100	2.845
Catcher	122	762	0.000	0.000	0.000	0.000	0.000
Retort operator	124	1170	0.231	0.332	0.687	0.780	N/A
Floor foreman	126	1077	<0.137	<0.20	0.297	0.297	N/A
Catcher	127	1161	0.233	0.336	0.721	0.820	N/A
Spice Mixer	128	1149	0.234	0.350	0.914	1.040	N/A
Tank operator	130	894	0.000	0.000	0.000	0.000	0.000
Quality Controler	131	684	<0.06	<0.20	0.000	0.000	2.410
Fish packer	132	1005	0.000	0.000	0.000	0.000	0.050
Seamer operator	133	720	0.070	0.115	0.000	0.000	1.000
Line inspector	134	654	0.000	0.000	0.000	0.000	0.000
Vacuum operator	136	894	0.000	0.000	0.000	0.000	0.000
Admin clerk	137	942	0.000	0.000	0.000	0.000	1.165
Cleaner	138	909	0.000	0.000	0.000	0.000	0.605
Sorting Table Supervisor	139	906	0.100	0.160	<0.12	<0.20	0.800
Mechanic	140	888	0.000	0.000	0.000	0.000	2.150
Storeman	142	1047	0.000	0.000	0.000	0.000	2.525
Store manager	143	1047	<0.06	<0.20	0.140	0.110	0.370
Store buyer	144	930	0.000	0.000	0.000	0.000	0.000
Team leader (F.M.B)	145	897	0.335	0.390	0.305	0.350	1.875
Scale operator (F.M.B)	146	987	1.811	1.515	7.361	6.769	N/A
Ass. Scale oprt.(F.M.B)	147	981	0.185	0.250	0.355	0.430	1.255

Job Title	Filter	Vol	PilcLin	PilcSig	AncLin	AncSig	Protein
		litre	ug/ml	ug/ml	ug/ml	ug/ml	ug/ml
Bag leveler (F.M.B)	148	990	0.090	0.140	<0.12	<0.20	0.945
Forklift driver (F.M.B)	149	978	0.200	0.270	0.335	0.395	1.650
S.Table supervisor	151	876	0.060	0.110	<0.12	<0.20	0.295
Pump operator	152	885	0.060	0.105	0.000	0.000	0.105
Empty can operator	153	861	0.000	0.000	0.000	0.000	2.520
Drainer operator	154	834	0.120	0.190	0.000	0.000	0.000
Soucer	155	816	0.075	0.120	<0.12	<0.20	0.095
Spice mixer	156	777	0.070	0.100	0.180	0.170	0.625
Hoist operator	157	768	0.690	0.680	0.455	0.575	1.170
Supervisor	159	876	0.000	0.000	0.000	0.000	0.000
Label maschine operator	160	921	0.000	0.000	0.000	0.000	0.000
Tea lady	161	1215	<0.20	<0.20	0.000	0.000	0.235
Label checker	162	921	0.000	0.000	0.000	0.000	0.000
Palet packer (label)	163	921	0.000	0.000	0.000	0.000	0.000
Forklift driver (label)	165	882	0.000	0.000	0.000	0.000	0.000
Clerk (full can store)	166	909	0.000	0.000	0.000	0.000	0.000
Cleaner (full can store)	167	987	<0.137	<0.20	<0.22	<0.20	0.000
Forklift driver (full can store)	168	873	0.000	0.000	0.000	0.000	0.000
Autopacker operator	170	942	0.230	0.295	0.420	0.520	2.225
Kitchen Supervisor	171	828	0.000	0.000	0.000	0.000	0.035
Trolley Pusher (cannery)	172	894	0.115	0.170	<0.20	<0.20	0.035
Retort operator	173	891	0.215	0.280	0.310	0.380	2.570
Computer operator (Retorts)	174	813	0.245	0.315	0.460	0.580	0.495
Cleaner (Cannery)	175	1020	0.175	0.230	0.000	0.000	0.000
Clerk (Pick-ups)	176	855	0.000	0.000	0.000	0.000	0.000
Packer (Pick-ups)	177	825	0.000	0.000	0.000	0.000	0.000
Pick-up operator	178	810	0.000	0.000	0.000	0.000	0.000
Trolley Pusher (Pick-ups)	179	912	0.000	0.000	0.000	0.000	0.000
Forklift Driver (Pick-ups)	180	813	0.000	0.000	0.000	0.000	0.025
Foreman (Boiler)	182	918	0.000	0.000	0.000	0.000	0.000
Boiler operator	183	1155	<0.10	<0.20	0.000	0.000	0.900
Fireman (Boiler)	184	1050	<0.10	<0.20	0.000	0.000	0.870
Coalshoval operator	185	1149	0.000	0.000	0.000	0.000	0.340
Cleaner (Boiler)	186	1158	<0.10	<0.20	0.000	0.000	0.000
Storeman (Cannery Store)	187	975	0.000	0.000	0.000	0.000	0.365
Cooker & Press operator	188	1044	0.324	0.442	0.893	1.016	0.000
Pit worker (Fishmeal)	189	1020	0.000	0.000	0.000	0.000	0.000
Cash Book Clerk	191	981	0.000	0.000	<0.16	<0.20	0.000
Receptionist	192	984	<0.10	<0.20	<0.16	<0.20	0.025
Creditors Clerk	193	1002	0.000	0.000	0.000	0.000	0.000
Debtors Clerk	194	987	0.000	0.000	0.000	0.000	0.000

Job Title	Filter	Vol	PilcLin	PilcSig	AncLin	AncSig	Protein
		litre	ug/ml	ug/ml	ug/ml	ug/ml	ug/ml
Fleet Accountant	195	906	0.000	0.000	0.000	0.000	0.000
Pit Worker	196	990	0.359	0.515	1.470	1.632	N/A
Pit Worker	197	990	0.171	0.255	0.497	0.549	0.685
Pit Worker	198	990	0.330	0.440	0.440	0.630	1.240
Pit Worker	199	1047	<0.10	<0.20	<0.16	<0.20	0.000
Supervisor (Jetty)	200	954	<0.137	<0.20	0.240	0.222	N/A
Pipe/Switch operator (Jetty)	201	954	<0.137	<0.20	0.240	0.222	N/A
Supervisor (Jetty)	203	1233	0.000	0.000	0.000	0.000	0.035
Hole Worker	204	1233	<0.10	<0.20	0.000	0.000	0.035
Pipe/Switch operator	205	1233	0.000	0.000	0.000	0.000	0.000
Supervisor (Workshop)	206	1011	0.000	0.000	0.000	0.000	0.000
Canning Manager (Cannery)	207	933	0.000	0.000	<0.16	<0.20	0.395
Fitter & Turner (workshop)	208	1137	0.000	0.000	0.000	0.000	0.000
Handyman (workshop)	209	1113	0.000	0.000	0.000	0.000	0.000
Mechanic (workshop)	210	945	<0.10	<0.20	0.000	0.000	10.865
Cleaner (Full Can Store)	211	1068	0.000	0.000	0.000	0.000	0.050
Supervisor (Full Can Store)	212	1068	0.000	0.000	0.000	0.000	0.000
Hygiene Inspector (Cannery)	213	1092	0.000	0.000	0.000	0.000	0.060
Handyman (Workshop)	215	1020	0.000	0.000	0.000	0.000	2.440
Fitter (Fishmeal Plant)	216	915	0.166	0.247	0.678	0.767	N/A
Supervisor (Fishmeal)	218	828	0.000	0.000	0.000	0.000	2.140
Cooker & Press operator	219	972	0.210	0.270	0.000	0.000	1.785
Dryer operator	220	1008	0.350	0.460	0.000	0.000	0.845
Separator operator	221	1005	<0.10	<0.20	0.190	0.150	0.280
Stuk Water Plant operator	222	1008	<0.05	<0.20	0.000	0.000	0.000
Pit Worker	223	1002	<0.05	<0.20	0.000	<0.20	1.465
Pit Worker	224	1002	0.564	0.670	1.330	1.490	N/A
Front end loader driver	225	975	<0.05	<0.20	<0.21	<0.20	0.000
Coalshoval operator	226	945	0.000	0.000	0.000	0.000	0.530
Fireman	227	960	<0.05	<0.20	<0.21	<0.20	1.250
Assistant	228	933	<0.05	<0.20	<0.21	<0.20	2.920
Pit Worker	230	963	<0.137	<0.20	0.249	0.235	0.935
Pit Worker	231	963	0.000	0.000	0.000	0.000	0.290
Pump operator	233	1080	<0.05	<0.20	<0.21	<0.20	2.070
Fish packer	234	1083	0.050	0.100	<0.21	<0.20	3.340
Quality Controler	235	1101	0.090	0.140	<0.21	<0.20	2.450
Autopacker operator	236	981	0.050	0.100	<0.21	<0.20	1.005
Supervisor (Boiler)	237	975	0.000	0.000	0.000	0.000	1.895
Head of Dept. (Boiler)	238	1215	0.000	0.000	0.000	0.000	2.070
Pit Worker (fishmeal)	239	1215	0.000	0.000	0.000	0.000	1.800
Scale operator	240	1104	0.100	0.150	<0.21	<0.20	2.490

Job Title	Filter	Vol	PilcLin	PilcSig	AncLin	AncSig	Protein
		litre	ug/ml	ug/ml	ug/ml	ug/ml	ug/ml
Ass. Scale operator	241	1098	17.538	10.657	83.171	99.500	N/A
Labourer (FMB)	242	1098	0.380	0.370	1.250	2.190	4.820
Forklift Driver (FMB)	243	1098	0.000	0.000	0.000	0.000	0.830
Supervisor (workshop)	245	1131	0.000	0.000	0.000	0.000	1.705
Handyman (workshop)	246	1131	0.000	0.000	<0.21	<0.20	1.780
Fitter & Turner (workshop)	247	1005	<0.05	<0.20	0.000	0.000	1.465
Fitter (Cannery workshop)	248	1215	0.000	0.000	0.000	0.000	1.625
Washer (Laundry)	249	1176	<0.137	<0.20	<0.22	<0.20	N/A
Washer (Laundry)	250	1074	0.000	0.000	0.000	0.000	2.855
Supervisor (Jetty)	252	1059	<0.05	<0.20	<0.21	<0.20	1.515
Pipe/Switch operator (Jetty)	253	897	<0.05	<0.20	0.000	0.000	1.085
Hole worker (Jetty)	254	1056	0.000	0.000	0.000	0.000	0.425
Tank operator (Jetty)	255	984	0.000	0.000	0.000	0.000	5.840
Line Inspector (Cannery)	256	951	<0.05	<0.20	<0.21	<0.20	2.270
Seamer operator (Cannery)	257	948	<0.05	<0.20	0.000	<0.20	1.345
Trolley Pusher (Cannery)	258	933	0.000	0.000	0.000	0.000	2.250
Handyman (Jetty)	259	1050	0.000	0.000	0.000	0.000	1.235
Buyer (Store)	261	1095	0.000	0.000	0.000	0.000	1.640
Storeman (Store)	262	1095	<0.06	<0.20	0.000	<0.20	0.480
Receptionist (Admin)	263	1029	0.070	0.100	<0.10	<0.20	0.000
Wage Clerk (Admin)	264	1017	0.000	0.000	0.000	0.000	0.000
Tea Lady (Admin)	265	1011	0.000	0.000	0.000	0.000	0.250
Creditors Clerk (Admin)	266	1023	0.000	0.000	0.000	0.000	0.000
Invoice Clerk (Admin)	267	1047	<0.06	<0.20	<0.10	<0.20	1.420
Washer (Laundry)	268	1038	<0.137	<0.20	<0.22	<0.20	0.000
Washer (Laundry)	269	1038	0.110	0.170	0.190	0.190	2.005
Pick-up operator	270	990	<0.06	<0.20	<0.10	<0.20	0.010
Packer (Pick-ups)	271	975	0.140	0.200	0.350	0.380	1.105
Washer (Laundry)	273	888	0.000	0.000	0.000	0.000	1.125
Label machine operator	274	1266	0.000	0.000	0.000	0.000	1.095
Palet Packer	275	1260	0.000	0.000	0.000	0.000	0.615
Label Checker	276	1140	0.000	0.000	0.000	0.000	0.335
Ice plant operator	277	741	0.000	0.000	0.000	0.000	0.000
Ice Plant operator	278	585	0.000	0.000	0.000	0.000	0.000
Breakdown Controler	279	933	0.230	0.200	0.200	0.200	1.840
Empty can operator	281	981	0.210	0.270	0.290	0.310	2.125
Weigher (Cannery Store)	282	981	0.110	0.160	0.160	0.160	0.660
Teller	283	1080	0.000	0.000	0.000	0.000	0.195
Supervisor	284	1089	0.000	0.000	0.000	0.000	0.000
Scale operator	285	1059	0.000	0.000	0.000	0.000	0.000
Stamper	286	1050	0.000	0.000	0.000	0.000	1.710

Job Title	Filter	Vol	PilcLin	PilcSig	AncLin	AncSig	Protein
		litre	ug/ml	ug/ml	ug/ml	ug/ml	ug/ml
Packer	287	1230	0.000	0.000	0.000	0.000	0.345
Packer	289	498	0.000	0.000	0.000	0.000	0.605
Packer	290	501	0.000	0.000	0.000	0.000	0.040
Packer	291	498	0.000	0.000	0.000	0.000	0.090
Packer	292	789	0.000	0.000	0.000	0.000	0.405
Packer	293	819	0.000	0.000	0.000	0.000	0.565
Labourer	296	1290	0.000	0.000	0.000	0.000	0.000
Labourer	297	1242	0.000	0.000	0.000	0.000	0.130
Labourer	298	1263	0.000	0.000	0.000	0.000	0.405
Labourer	299	1209	0.000	0.000	0.000	0.000	0.185
Sorter	300	1197	0.000	0.000	0.000	0.000	0.515
Sorter	301	1200	0.000	0.000	0.000	0.000	1.475
Sorter	302	1200	0.000	0.000	0.000	0.000	0.010
Sorter	303	969	0.000	0.000	0.000	0.000	0.000
Sorter	304	1209	0.000	0.000	0.000	0.000	1.225
Mastering	306	1164	0.000	0.000	0.000	0.000	0.585
Mastering	307	1164	0.000	0.000	<0.13	<0.20	0.000
Mastering	308	1164	0.000	0.000	1.760	2.020	0.000
Mastering	309	1164	0.000	0.000	0.000	0.000	0.930
Mastering	310	1164	0.000	0.000	0.000	0.000	0.940
Stacker	311	855	0.000	0.000	0.000	0.000	0.815
Forklift driver	312	1185	<0.09	<0.22	<0.13	<0.20	2.540
Supervisor	313	1260	0.000	0.000	0.000	0.000	0.815
Storeman	314	1095	0.000	0.000	0.000	0.000	0.060
Cleaner	315	1227	0.120	0.150	<0.13	<0.20	1.755
Packer	317	1077	0.000	0.000	0.000	0.000	0.540
Packer	318	1080	0.000	0.000	<0.13	<0.20	0.000
Packer	319	1098	0.000	0.000	0.000	0.000	0.000
Packer	320	1092	0.000	0.000	1.620	1.880	0.000
Quality controler	321	1071	0.000	0.000	0.000	0.000	0.865
Quality controler	322	840	0.000	0.000	0.000	0.000	1.335
Clerk	323	1248	0.000	0.000	0.000	0.000	0.000
Accountant	324	1233	0.000	0.000	0.000	0.000	0.900
Tea lady	325	1014	0.000	0.000	0.130	0.100	5.330
Tea lady	327	996	0.000	0.000	0.000	0.000	4.565

Vol- Volume of air flowing through sampling filter ; N/A – insufficient, sample not analysed;
PilcLin – pilchard antigen concentration of samples derived from the linear standard curve;
PilSig - pilchard antigen concentration of samples derived from the sigmoidal standard curve;
AncLin - anchovy antigen concentration of samples derived the linear standard curve;
AncSig – anchovy antigen concentration of samples derived the sigmoidal standard curve.

Appendix 6: Environmental air sampling filters results for Total Protein, Pilchard & Anchovy Antigen determinations expressed in µg/m³:

Job Title	Filter	Vol	PilcLinM	PilcSigM	AncLinM	AncSigM	Protein
		litre	ug/m³	ug/m³	ug/m³	ug/m³	ug/m³
Fish Packer	100	624	<0.220	<0.321	0.747	0.824	N/A
Quality Controler	101	582	<0.235	<0.344	0.680	0.730	N/A
Line inspector	102	621	<0.097	<0.322	<0.193	<0.322	0.700
Tank operator	103	600	<0.228	<0.333	0.605	0.635	N/A
Seamer operator	104	585	0.145	0.222	0.325	0.256	0.000
Drainer & Saurcer	106	1206	0.062	0.104	0.149	0.124	1.032
Floor foreman	108	1137	0.066	0.106	<0.106	<0.176	0.682
Cooker & press operator	112	555	0.000	0.000	0.000	0.000	0.000
Dryer operator	113	864	0.127	0.197	0.000	0.000	0.000
Separator operator	114	873	0.080	0.137	0.000	0.000	0.000
Fitter	115	666	0.143	0.233	0.255	0.195	1.104
Stuk Water Plant operator	116	840	0.000	0.000	0.000	0.000	0.000
Drainer & Saurcer operator	118	666	0.150	0.240	0.210	0.150	0.000
Floor foreman	119	738	0.000	0.000	0.000	0.000	0.000
Retort operator	120	672	0.000	0.000	0.000	0.000	0.000
Spice Mixer	121	690	<0.086	<0.290	0.217	0.145	4.123
Catcher	122	762	0.000	0.000	0.000	0.000	0.000
Retort operator	124	1170	0.197	0.283	0.587	0.666	N/A
Floor foreman	126	1077	<0.127	<0.186	0.276	0.276	N/A
Catcher	127	1161	0.201	0.289	0.621	0.706	N/A
Spice Mixer	128	1149	0.204	0.305	0.795	0.905	N/A
Tank operator	130	894	0.000	0.000	0.000	0.000	0.000
Quality Controler	131	684	<0.088	<0.292	0.000	0.000	3.523
Fish packer	132	1005	0.000	0.000	0.000	0.000	0.050
Seamer operator	133	720	0.097	0.160	0.000	0.000	1.389
Line inspector	134	654	0.000	0.000	0.000	0.000	0.000
Vacuum operator	136	894	0.000	0.000	0.000	0.000	0.000
Admin clerk	137	942	0.000	0.000	0.000	0.000	1.237
Cleaner	138	909	0.000	0.000	0.000	0.000	0.666
Sorting Table Supervisor	139	906	0.110	0.177	<0.132	<0.221	0.883
Mechanic	140	888	0.000	0.000	0.000	0.000	2.421
Storeman	142	1047	0.000	0.000	0.000	0.000	2.412
Store manager	143	1047	<0.057	<0.191	0.134	0.105	0.353
Store buyer	144	930	0.000	0.000	0.000	0.000	0.000
Team leader (F.M.B)	145	897	0.373	0.435	0.340	0.390	2.090
Scale operator (F.M.B)	146	987	1.835	1.534	7.458	6.858	N/A
Ass. Scale oprt.(F.M.B)	147	981	0.189	0.255	0.362	0.438	1.279

Job Title	Filter	Vol	PilcLinM	PilcSigM	AncLinM	AncSigM	Protein
		litre	ug/m^3	ug/m^3	ug/m^3	ug/m^3	ug/m^3
Bag leveler (F.M.B)	148	990	0.091	0.141	<0.121	<0.202	0.955
Forklift driver (F.M.B)	149	978	0.204	0.276	0.343	0.404	1.687
S.Table supervisor	151	876	0.068	0.126	<0.137	<0.228	0.337
Pump operator	152	885	0.068	0.119	0.000	0.000	0.119
Empty can operator	153	861	0.000	0.000	0.000	0.000	2.927
Drainer operator	154	834	0.144	0.228	0.000	0.000	0.000
Soucer	155	816	0.092	0.147	<0.147	<0.245	0.116
Spice mixer	156	777	0.090	0.129	0.232	0.219	0.804
Hoist operator	157	768	0.898	0.885	0.592	0.749	1.523
Supervisor	159	876	0.000	0.000	0.000	0.000	0.000
Label maschine operator	160	921	0.000	0.000	0.000	0.000	0.000
Tea lady	161	1215	<0.165	<0.165	0.000	0.000	0.193
Label checker	162	921	0.000	0.000	0.000	0.000	0.000
Palet packer (label)	163	921	0.000	0.000	0.000	0.000	0.000
Forklift driver (label)	165	882	0.000	0.000	0.000	0.000	0.000
Clerk (full can store)	166	909	0.000	0.000	0.000	0.000	0.000
Cleaner (full can store)	167	987	<0.139	<0.203	<0.223	<0.203	0.000
Forklift driver (full can store)	168	873	0.000	0.000	0.000	0.000	0.000
Autopacker operator	170	942	0.244	0.313	0.446	0.552	2.362
Kitchen Supervisor	171	828	0.000	0.000	0.000	0.000	0.042
Trolley Pusher (cannery)	172	894	0.129	0.190	<0.223	<0.22	0.039
Retort operator	173	891	0.241	0.314	0.348	0.426	2.884
Computer operator (Retorts)	174	813	0.301	0.387	0.566	0.713	0.609
Cleaner (Cannery)	175	1020	0.172	0.225	0.000	0.000	0.000
Clerk (Pick-ups)	176	855	0.000	0.000	0.000	0.000	0.000
Packer (Pick-ups)	177	825	0.000	0.000	0.000	0.000	0.000
Pick-up operator	178	810	0.000	0.000	0.000	0.000	0.000
Trolley Pusher (Pick-ups)	179	912	0.000	0.000	0.000	0.000	0.000
Forklift Driver (Pick-ups)	180	813	0.000	0.000	0.000	0.000	0.031
Foreman (Boiler)	182	918	0.000	0.000	0.000	0.000	0.000
Boiler operator	183	1155	<0.087	<0.173	0.000	0.000	0.779
Fireman (Boiler)	184	1050	<0.095	<0.190	0.000	0.000	0.829
Coalshoval operator	185	1149	0.000	0.000	0.000	0.000	0.296
Cleaner (Boiler)	186	1158	<0.086	<0.173	0.000	0.000	0.000
Storeman (Cannery Store)	187	975	0.000	0.000	0.000	0.000	0.374
Cooker & Press operator	188	1044	0.310	0.423	0.855	0.973	0.000
Pit worker (Fishmeal)	189	1020	0.000	0.000	0.000	0.000	0.000
Cash Book Clerk	191	981	0.000	0.000	<0.163	<0.204	0.000
Receptionist	192	984	<0.102	<0.203	<0.163	<0.203	0.025
Creditors Clerk	193	1002	0.000	0.000	0.000	0.000	0.000

Job Title	Filter	Vol	PilcLinM	PilcSigM	AncLinM	AncSigM	Protein
		litre	ug/m^3	ug/m^3	ug/m^3	ug/m^3	ug/m^3
Debtors Clerk	194	987	0.000	0.000	0.000	0.000	0.000
Fleet Accountant	195	906	0.000	0.000	0.000	0.000	0.000
Pit Worker	196	990	0.363	0.520	1.485	1.648	N/A
Pit Worker	197	990	0.173	0.257	0.502	0.554	0.692
Pit Worker	198	990	0.333	0.444	0.444	0.636	1.253
Pit Worker	199	1047	<0.096	<0.191	<0.168	<0.191	0.000
Supervisor (Jetty)	200	954	<0.144	<0.210	0.252	0.232	N/A
Pipe/Switch operator (Jetty)	201	954	<0.144	<0.210	0.252	0.233	N/A
Supervisor (Jetty)	203	1233	0.000	0.000	0.000	0.000	0.028
Hole Worker	204	1233	<0.081	<0.162	0.000	0.000	0.028
Pipe/Switch operator	205	1233	0.000	0.000	0.000	0.000	0.000
Supervisor (Workshop)	206	1011	0.000	0.000	0.000	0.000	0.000
Canning Manager (Cannery)	207	933	0.000	0.000	<0.171	<0.214	0.423
Fitter & Turner (workshop)	208	1137	0.000	0.000	0.000	0.000	0.000
Handyman (workshop)	209	1113	0.000	0.000	0.000	0.000	0.000
Mechanic (workshop)	210	945	<0.106	<0.212	0.000	0.000	11.497
Cleaner (Full Can Store)	211	1068	0.000	0.000	0.000	0.000	0.047
Supervisor (Full Can Store)	212	1068	0.000	0.000	0.000	0.000	0.000
Hygiene Inspector (Cannery)	213	1092	0.000	0.000	0.000	0.000	0.055
Handyman (Workshop)	215	1020	0.000	0.000	0.000	0.000	2.392
Fitter (Fishmeal Plant)	216	915	0.181	0.270	0.741	0.838	N/A
Supervisor (Fishmeal)	218	828	0.000	0.000	0.000	0.000	2.585
Cooker & Press operator	219	972	0.216	0.278	0.000	0.000	1.836
Dryer operator	220	1008	0.347	0.456	0.000	0.000	0.838
Separator operator	221	1005	<0.010	<0.199	0.189	0.149	0.279
Stuk Water Plant operator	222	1008	<0.050	<0.198	0.000	0.000	0.000
Pit Worker	223	1002	<0.050	<0.200	0.000	<0.200	1.462
Pit Worker	224	1002	0.563	0.668	1.327	1.487	N/A
Front end loader driver	225	975	<0.051	<0.205	<0.215	<0.205	0.000
Coalshoval operator	226	945	0.000	0.000	0.000	0.000	0.561
Fireman	227	960	<0.052	<0.208	<0.219	<0.208	1.302
Assistant	228	933	<0.054	<0.214	<0.225	<0.214	3.130
Pit Worker	230	963	<0.142	<0.208	0.259	0.244	0.971
Pit Worker	231	963	0.000	0.000	0.000	0.000	0.301
Pump operator	233	1080	<0.046	<0.185	<0.194	<0.185	1.917
Fish packer	234	1083	0.046	0.092	<0.194	<0.185	3.084
Quality Controler	235	1101	0.082	0.127	<0.191	<0.182	2.225
Autopacker operator	236	981	0.051	0.102	<0.214	<0.204	1.024
Supervisor (Boiler)	237	975	0.000	0.000	0.000	0.000	1.944
Head of Dept. (Boiler)	238	1215	0.000	0.000	0.000	0.000	1.704
Pit Worker (fishmeal)	239	1215	0.000	0.000	0.000	0.000	1.481

Job Title	Filter	Vol	PilcLinM	PilcSigM	AncLinM	AncSigM	Protein
		litre	ug/m^3	ug/m^3	ug/m^3	ug/m^3	ug/m^3
Scale operator	240	1104	0.091	0.136	<0.190	<0.181	2.255
Ass. Scale operator	241	1098	15.973	9.706	75.748	90.619	N/A
Labourer (FMB)	242	1098	0.346	0.337	1.138	1.995	4.390
Forklift Driver (FMB)	243	1098	0.000	0.000	0.000	0.000	0.756
Supervisor (workshop)	245	1131	0.000	0.000	0.000	0.000	1.508
Handyman (workshop)	246	1131	0.000	0.000	<0.186	<0.177	1.574
Fitter & Turner (workshop)	247	1005	<0.050	<0.199	0.000	0.000	1.458
Fitter (Cannery workshop)	248	1215	0.000	0.000	0.000	0.000	1.337
Washer (Laundry)	249	1176	<0.116	<0.170	<0.187	<0.170	N/A
Washer (Laundry)	250	1074	0.000	0.000	0.000	0.000	2.658
Supervisor (Jetty)	252	1059	<0.047	<0.189	<0.198	<0.189	1.431
Pipe/Switch operator (Jetty)	253	897	<0.056	<0.223	0.000	0.000	1.210
Hole worker (Jetty)	254	1056	0.000	0.000	0.000	0.000	0.402
Tank operator (Jetty)	255	984	0.000	0.000	0.000	0.000	5.935
Line Inspector (Cannery)	256	951	<0.053	<0.210	<0.221	<0.210	2.387
Seamer operator (Cannery)	257	948	<0.053	<0.211	0.000	<0.211	1.419
Trolley Pusher (Cannery)	258	933	0.000	0.000	0.000	0.000	2.412
Handyman (Jetty)	259	1050	0.000	0.000	0.000	0.000	1.176
Buyer (Store)	261	1095	0.000	0.000	0.000	0.000	1.498
Storeman (Store)	262	1095	<0.055	<0.183	0.000	<0.183	0.438
Receptionist (Admin)	263	1029	0.068	0.097	<0.097	<0.194	0.000
Wage Clerk (Admin)	264	1017	0.000	0.000	0.000	0.000	0.000
Tea Lady (Admin)	265	1011	0.000	0.000	0.000	0.000	0.247
Creditors Clerk (Admin)	266	1023	0.000	0.000	0.000	0.000	0.000
Invoice Clerk (Admin)	267	1047	<0.057	<0.192	<0.096	<0.191	1.356
Washer (Laundry)	268	1038	<0.132	<0.193	<0.21	<0.193	0.000
Washer (Laundry)	269	1038	0.106	0.164	0.183	0.183	1.932
Pick-up operator	270	990	<0.060	<0.202	<0.101	<0.202	0.010
Packer (Pick-ups)	271	975	0.144	0.205	0.359	0.390	1.133
Washer (Laundry)	273	888	0.000	0.000	0.000	0.000	1.267
Label machine operator	274	1266	0.000	0.000	0.000	0.000	0.865
Palet Packer	275	1260	0.000	0.000	0.000	0.000	0.488
Label Checker	276	1140	0.000	0.000	0.000	0.000	0.294
Ice plant operator	277	741	0.000	0.000	0.000	0.000	0.000
Ice Plant operator	278	585	0.000	0.000	0.000	0.000	0.000
Breakdown Controler	279	933	0.247	0.214	0.214	0.214	1.972
Empty can operator	281	981	0.214	0.275	0.296	0.316	2.166
Weigher (Cannery Store)	282	981	0.112	0.163	0.163	0.163	0.673
Teller	283	1080	0.000	0.000	0.000	0.000	0.181
Supervisor	284	1089	0.000	0.000	0.000	0.000	0.000
Scale operator	285	1059	0.000	0.000	0.000	0.000	0.000

Job Title	Filter	Vol	PilcLinM	PilcSigM	AncLinM	AncSigM	Protein
		litre	ug/m^3	ug/m^3	ug/m^3	ug/m^3	ug/m^3
Stamper	286	1050	0.000	0.000	0.000	0.000	1.629
Packer	287	1230	0.000	0.000	0.000	0.000	0.280
Packer	289	498	0.000	0.000	0.000	0.000	1.215
Packer	290	501	0.000	0.000	0.000	0.000	0.080
Packer	291	498	0.000	0.000	0.000	0.000	0.181
Packer	292	789	0.000	0.000	0.000	0.000	0.513
Packer	293	819	0.000	0.000	0.000	0.000	0.690
Labourer	296	1290	0.000	0.000	0.000	0.000	0.000
Labourer	297	1242	0.000	0.000	0.000	0.000	0.105
Labourer	298	1263	0.000	0.000	0.000	0.000	0.321
Labourer	299	1209	0.000	0.000	0.000	0.000	0.153
Sorter	300	1197	0.000	0.000	0.000	0.000	0.430
Sorter	301	1200	0.000	0.000	0.000	0.000	1.229
Sorter	302	1200	0.000	0.000	0.000	0.000	0.008
Sorter	303	969	0.000	0.000	0.000	0.000	0.000
Sorter	304	1209	0.000	0.000	0.000	0.000	1.013
Mastering	306	1164	0.000	0.000	0.000	0.000	0.503
Mastering	307	1164	0.000	0.000	<0.112	<0.172	0.000
Mastering	308	1164	0.000	0.000	1.512	1.735	0.000
Mastering	309	1164	0.000	0.000	0.000	0.000	0.799
Mastering	310	1164	0.000	0.000	0.000	0.000	0.808
Stacker	311	855	0.000	0.000	0.000	0.000	0.953
Forklift driver	312	1185	<0.076	<0.186	<0.110	<0.169	2.143
Supervisor	313	1260	0.000	0.000	0.000	0.000	0.647
Storeman	314	1095	0.000	0.000	0.000	0.000	0.055
Cleaner	315	1227	0.098	0.122	<0.106	<0.163	1.430
Packer	317	1077	0.000	0.000	0.000	0.000	0.501
Packer	318	1080	0.000	0.000	<0.120	<0.185	0.000
Packer	319	1098	0.000	0.000	0.000	0.000	0.000
Packer	320	1092	0.000	0.000	1.484	1.722	0.000
Quality controler	321	1071	0.000	0.000	0.000	0.000	0.808
Quality controler	322	840	0.000	0.000	0.000	0.000	1.589
Clerk	323	1248	0.000	0.000	0.000	0.000	0.000
Accountant	324	1233	0.000	0.000	0.000	0.000	0.730
Tea lady	325	1014	0.000	0.000	0.128	0.099	5.256
Tea lady	327	996	0.000	0.000	0.000	0.000	4.583

Vol- Volume of air flowing through sampling filter ; N/A – insufficient, sample not analysed;
PilcLin – pilchard antigen concentration of samples derived from the linear standard curve;
PilSig - pilchard antigen concentration of samples derived from the sigmoidal standard curve;
AncLin - anchovy antigen concentration of samples derived the linear standard curve;
AncSig – anchovy antigen concentration of samples derived the sigmoidal standard curve.

Appendix 7: **Spearman correlation analysis of antigen *concentration derived from the linear standard curve.***

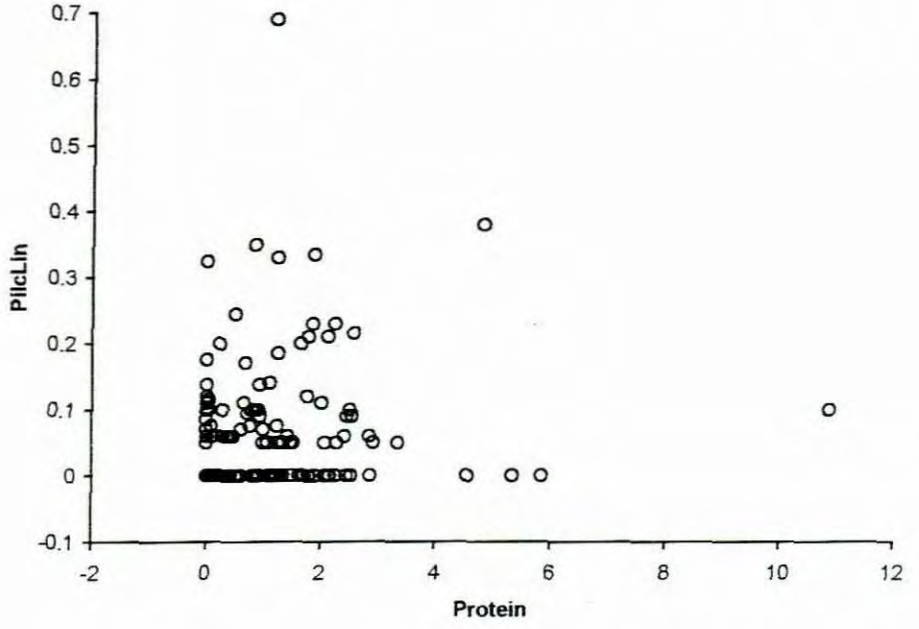

Figure 13: Correlation analysis between (Protein) total protein concentration [ug/ml] and (PilcLin) pilchard antigen concentrations derived from the linear standard curve

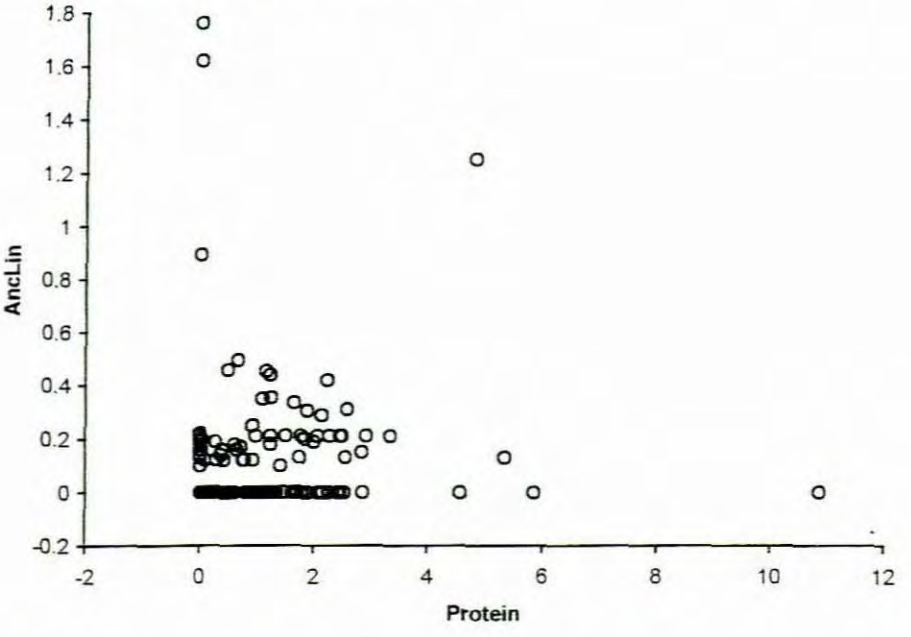

Figure 14: Correlation analysis between (Protein) total protein concentration [ug/ml] and (AncLin) anchovy antigen concentrations derived from the linear standard curve

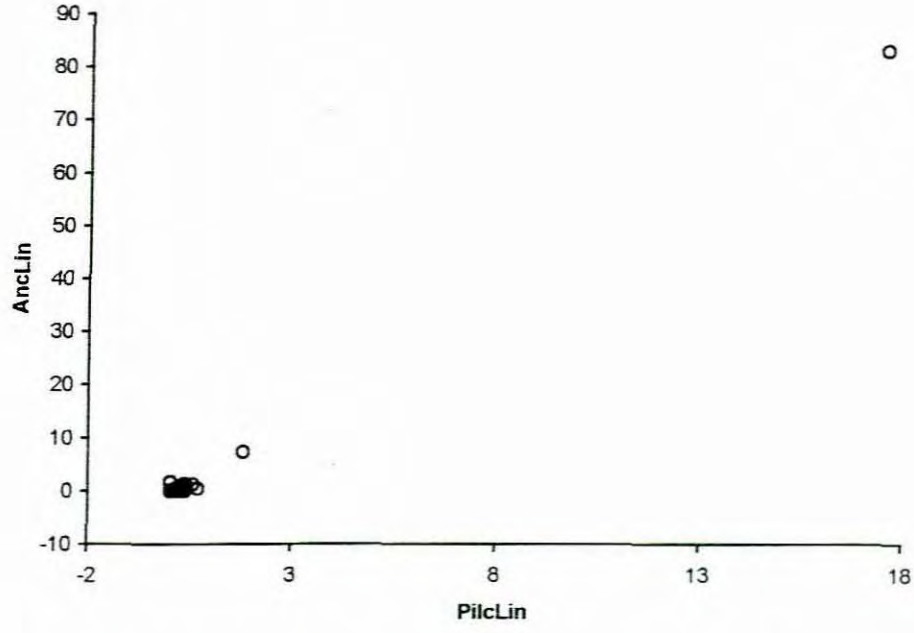

Figure 15: Correlation analysis between (PilcLin) pilchard antigen concentration [ug/ml] and (AncLin) anchovy antigen concentrations derived from the linear standard curve

Appendix 8: Spearman correlation analysis of antigen concentration derived from the sigmoidal standard curve.

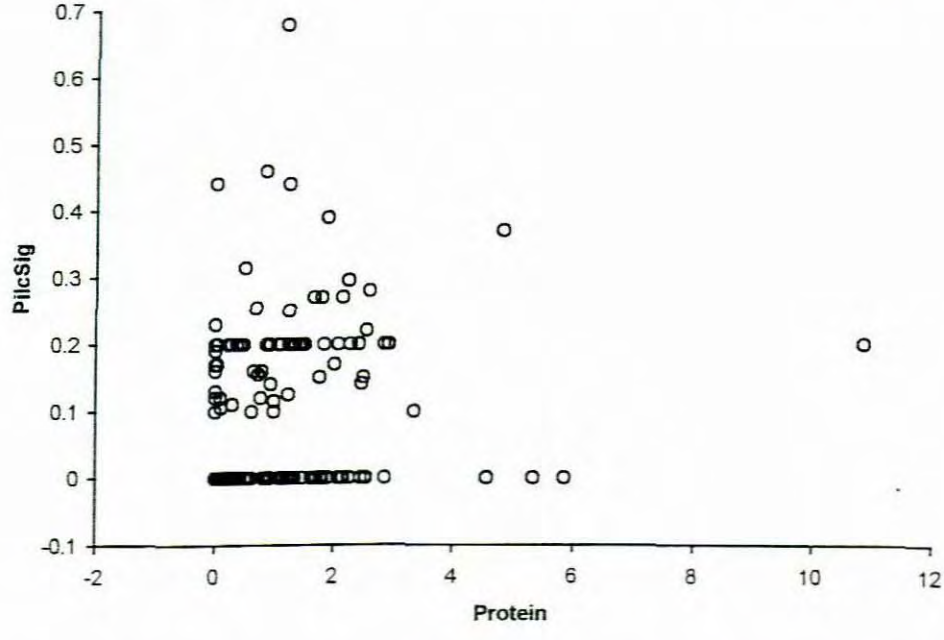

Figure 16: Correlation analysis between (Protein) total protein concentration [ug/ml] and (PilcSig) pilchard antigen concentrations derived from the sigmoidal standard curve

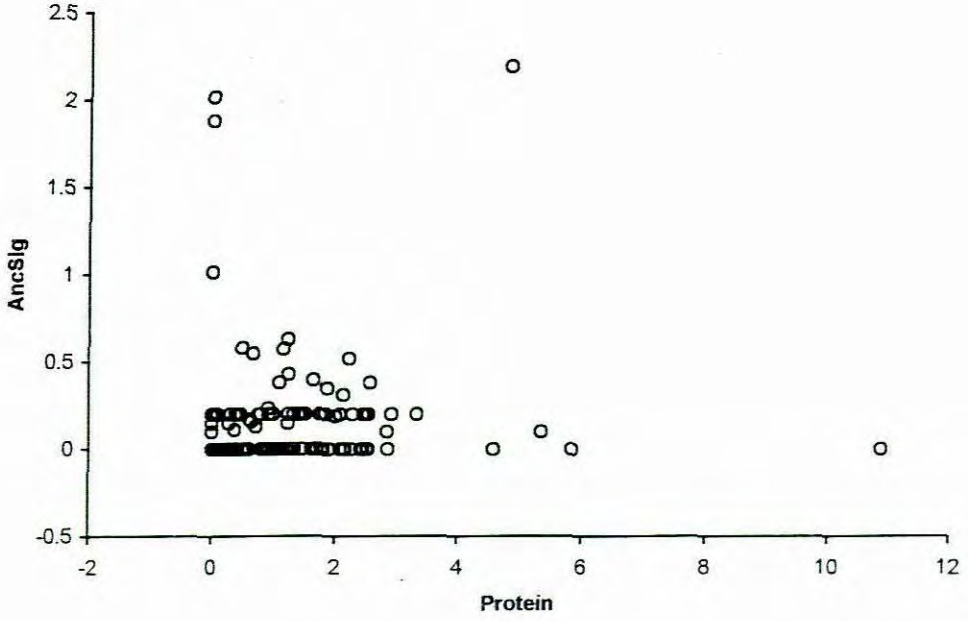

Figure 17: Correlation analysis between (Protein) total protein concentration [ug/ml] and (AncSig) anchovy antigen concentrations derived from the sigmoidal standard curve

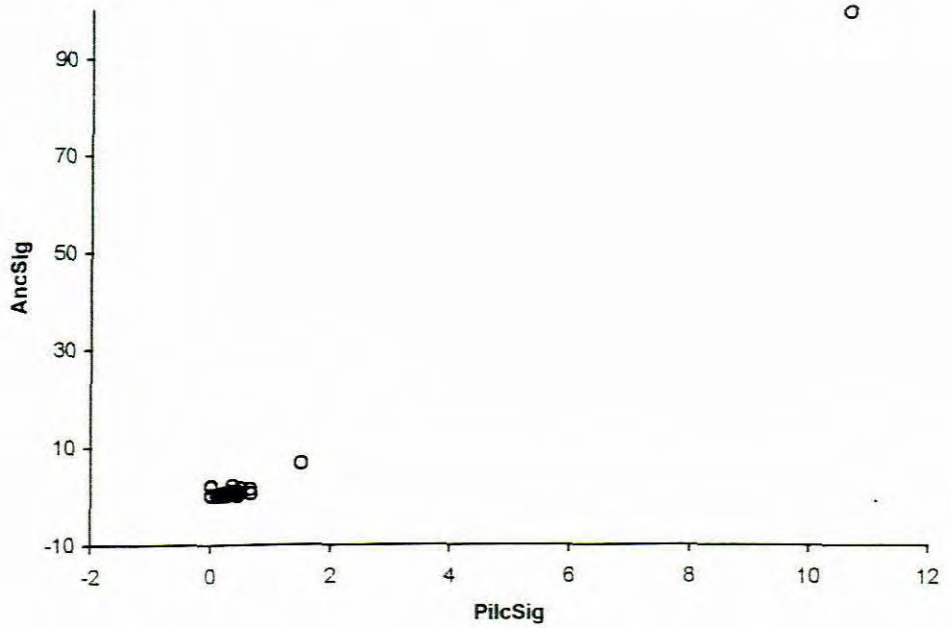

Figure 18: Correlation analysis between (PilcSig) pilchard antigen concentration [ug/ml] and (AncSig) anchovy antigen concentrations derived from the sigmoidal standard curve