THE IDENTIFICATION OF AEROSOLIZED SNOW CRAB PROTEIN BY ELECTROPHORESIS AND MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME OF FLIGHT MASS SPECTROMETRY

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The Identification of Aerosolized Snow Crab Protein by Electrophoresis and Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry

By

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ABSTRACT

Modern proteomics tools allow for quick identification of proteins that may be present in aerosolized samples collected from snow crab processing. The coupling of electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has allowed for a rapid, 2-3 day, technique for the identification of these proteins. The protein bands are excised from gel slices, the protein present digested using trypsin and then analyzed using a MALDI-TOF MS instrument to produce a peptide mass fingerprint.

On-line database searching of the peptide mass fingerprints through Swiss-Prot or other database engines provide an efficient and effective means for protein identification, provided the protein has been sequenced. As well, unmatched peptide peaks can be analyzed through a post-translational modification database to help understand any chemical modifications that have occurred.

This combinational approach has produced a rapid technique for the screening of possible proteins aerosolized during commercial snow crab processing, some of which are allergens. Using the Swiss-Prot database, tropomyosin was identified in both lab-produced condensate and an air filter sample collected from a commercial snow crab processing plant.

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I would like to express my gratitude to my supervisor, Dr. R. Helleur, for his patience, and advice during the course of this research project. I would like to thank Ms. Linda Winsor for her time and cooperation, the Marine Institute and Memorial University of Newfoundland for the use of their facilities. I would like to express my gratitude to Mr. Dave Bonnell of the Marine Institute for support and patience during this undertaking.

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TABLE OF CONTENTS

LIST OF APPENDICES

LIST OF FIGURES

LIST OF TABLES

Page No.

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CIHR	Canadian Institute of Health Research
Da	Daltons
DBPCFC	Double-blind placebo-controlled food challenge
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
IgE	Immunoglobulin E
kDa	Kilodaltons
MALDI-TOF MS	Matrix assisted laser desorption ionization time of flight mass spectrometry
$[M+2H]^{2+}$	Diprotonated molecular ion
$[M+3H]^{3+}$	Triprotonated molecular ion
$[M+H]^{+}$	Monoprotonated molecular ion
PAGE	Polyacrylamide gel electrophoresis
RAST	Radioallergosorbent test
R_f	Relative mobility
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
TRIS	Tris(hydroxy methyl) aminomethane

CHAPTER 1 INTRODUCTION

Combining modern electrophoresis techniques and highly sensitive mass spectrometry with on-line protein databases allows for rapid and accurate identification of proteins. This analytical proteomics combination offers potential benefit to the snow crab industry which is a quintessential part of many rural communities in Newfoundland and Labrador. The impact of protein-induced allergic reactions on individuals working in the snow crab industry is only now becoming understood. These allergic reactions, affecting about 16% of all workers, not only affect the ability of individuals to perform their assigned tasks but may also lead to life threatening anaphylactic reactions (1).

The objective of this study was to isolate and identify aerosolized proteins produced during snow crab processing by employing modern proteomics techniques: one-dimensional electrophoresis coupled with in-gel digestion, matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), and on-line computerized protein database searching. This combination of tools provides a quick approach requiring only 2-3 days to determine which proteins are present in the air of seafood processing plants.

1.1 Snow Crab Processing

Since the collapse of the groundfish fishery in 1992, the importance of the snow crab (*Chinoecetes opilis*) fishery has increased greatly in Newfoundland and Labrador. Today, some 35 processing plants participate in the snow crab industry employing about 17,000 people (2).

Of the 35 snow crab production plants in operation, all undertake crab processing similar to the process illustrated in figure 1.1. Slight variations between plants occur depending on each individual operation. Nevertheless, the vast majority of product manufactured in Newfoundland is in the form of cooked frozen sections, with limited amounts being further processed into finished meat products.

As illustrated in Figure 1.1, the crabs are butchered to remove the carapace and viscera. The two resulting sections are cleaned of any remaining gut material prior to sorting. After sorting, the sections (shoulder and leg) may be cleaned with high-pressure washers to remove foreign objects, such as leach eggs, prior to cooking. Cooking normally takes 9-14 minutes depending on the size of the section. The sections are cooled using a chilling bath set at 1-2°C for 15 minutes. At this stage some sections may be sent for meat extraction while others may be cleaned, graded and frozen as sections. Meat extraction from the shell is accomplished by one of 5 methods: vacuum sucking, removal by water-jet, centrifuge system, leg splitting machine or roll extrusion. The meat is then packaged and frozen (3).

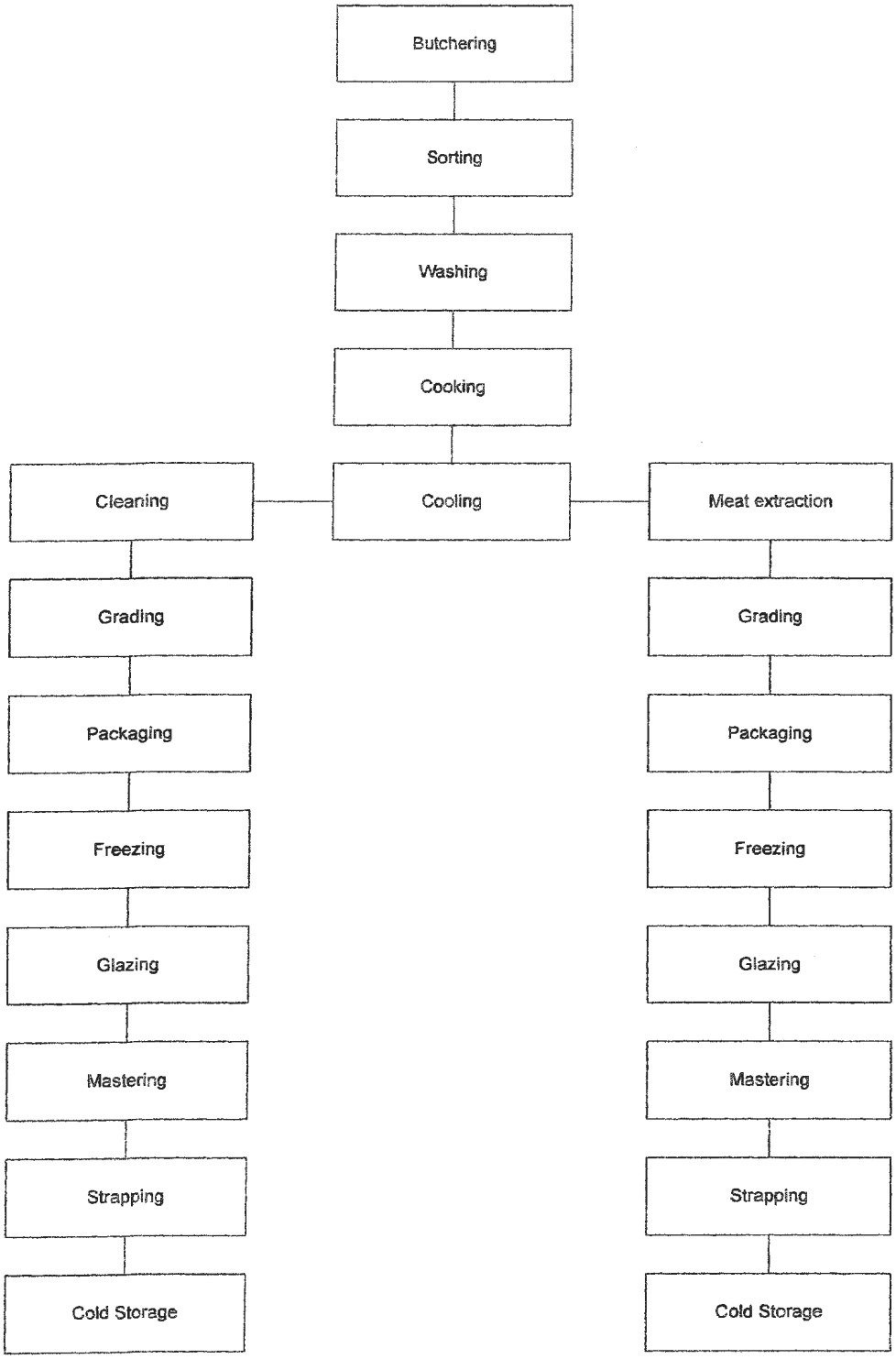

Figure 1.1 **Flow diagram of snow crab processing for in-shell and meat production.**

1.2 Occupational Asthma in Snow Crab Processing Operations

Adverse reactions of shellfish workers to their work environment is a global issue. Allergenic and other adverse reactions have been reported in crab, shrimp, and lobster operations (1, 4-6). These adverse reactions include itching, swelling, nausea, vomiting, eczema, asthma, headache, anxiety, diarrhea and dizziness (4, 6). While the number of workers affected varies, Cartier *et al.* (1) estimated that 15.6% of the employees within snow crab operations show symptoms of occupational asthma because of the processing plant environment. As well, Weytjens and Cartier (7) were able to identify areas (job/location) of concern within snow crab processing operations. These included crab cracking (butchering), cooking, sorting, and cleaning. The concentration of the allergens for different job/locations was determined using air sample filters and is given in table 1.1. These findings have lead Weytjens and Cartier (7) to concluded that different areas within a snow crab operation pose different levels of risk to the workers.

Malo and his co-workers (8) studied the presence of proteins and allergens in snow crab facilities utilizing air sampling in a manner similar to Weytjens and Cartier (7). From their study they were able to detect both proteins and allergens on the air sampling filters, leading Malo *et al.* (8) to conclude that the mechanism of sensitization to snow-crab in plant workers occurs through an airborne dispersion of snow-crab antigen during the boiling process. The estimated level of allergens on the filter was 1.657 μg while the total protein level reached about 8.6 μg (8).

Shellfish allergens are stable molecules and resist the effects of cooking, processing, or digestive processes. They are generally glycoproteins with molecular weights ranging from 10 to 39 kDa and an acidic isoelectric point of 4.7 (5). All, with the exception of shrimp antigens I and II, fall into the family of proteins known as tropomyosin. Tropomyosin may be part of either muscle or non-muscle cells; in striated muscle it controls muscle contraction by mediating the interaction between troponin complexes and actin. Its function in non-muscle cells is unclear however (6).

The tropomyosin from various shellfish contains about 264 amino acid residues with the number of amino acids varying depending on the species. Snow crab tropomyosin has not yet been sequenced, thus the exact number of amino acids in the tropomyosin is unknown (5).

Table 1.1 Airborne snow crab allergen estimates (7)

Filter no. Type	Job/location	Estimated concentration(ng/m^3)	ng for total filter
Area			
1*	Crab cracking	547	11806
2*	Crab cracking	84	1156
3	Outlet of boiler	53	1927
4	Outlet of cooling basin	100	2502
5	Near final section	63	1568
Personnel			
A	Crab cracker	5061	3664
B	Crab cracker	4961	4862
C	Crab sorter-outlet of cooling basin	604	568
D	Crab sorter-outlet of cooling basin	196	174
E	Crab sorter-underwater jet	220	169
F	Crab sorter-underwater jet	204	194
G	Crab sorter-cleaner	197	191
H	Crab sorter-cleaner	179	154

Note: * - Crab cracking area 1 and 2 are at different locations with in the processing plant.

1.3 The SafetyNet Project

It has long been recognized that a comprehensive research program studying occupational health and safety of marine and coastal workers was needed in Newfoundland and Labrador. SafetyNet, a Community Alliance for Health Research, is undertaking this research with funding from the Canadian Institutes of Health Research (CIHR). SafetyNet has created a community alliance that includes researchers in medicine, nursing, social science, engineering and marine sciences, and involves partners in the public sector, private sector and in the coastal communities in which the research is taking place (9).

One project in which SafetyNet is actively engaged is a study of "Occupational Asthma in Snow Crab Processing Workers" at four different snow crab processing plants in Newfoundland and Labrador. The study has numerous components including a two-phase air-sampling component to establish allergens and chemical levels in different areas of the snow crab plants. The outcome of the air-sampling will help in determining future plant design and changes to work processes. Also, questionnaires, skin tests, RAST assays and Peak Expiratory Flow monitoring have been used to try to ascertain the prevalence of occupational asthma within current and former plant workers (9).

1.4 Current Practice for Identification and Quantification of Shellfish Allergens

Several methods are currently used for measuring protein allergens from seafood. These include, the radioallergosorbent test (RAST), the western blot, the inhalation challenge and the double-blind placebo-controlled food challenge (DBPCFC). The RAST test is the most common approach (5).

The RAST test, which is currently the most commonly used method, is an *in vitro* diagnostic test used to demonstrate food-allergen-specific immunoglobulin E (IgE) antibodies. This method allows for the amount of specific IgE antibodies for a given allergen to be semi-quantified. The methodology involves the sample being separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then reacted with IgE. The IgE is subsequently reacted with anti-IgE that is radioactively labeled with ^{125}I. The amount of reactivity is measured by the development of an X-ray film. The time

required for the completion of the test is approximately 4 weeks and the concentration of the protein allergen is based on the level of radioactivity (5, 10).

1.5 Instrumental Techniques for Protein Identification Used in This Study

The following sections describe the theory and practice of each step involved in protein identification used in this study. 1) one-dimensional polyacrylamide gel electrophoresis to separate proteins followed by 2) in gel enzymatic digestion to produce peptides and finally 3) peptide or intact proteins analyzed by MALDI-TOF MS. Figure 1.2 illustrates the steps required to separate and identify the snow crab proteins.

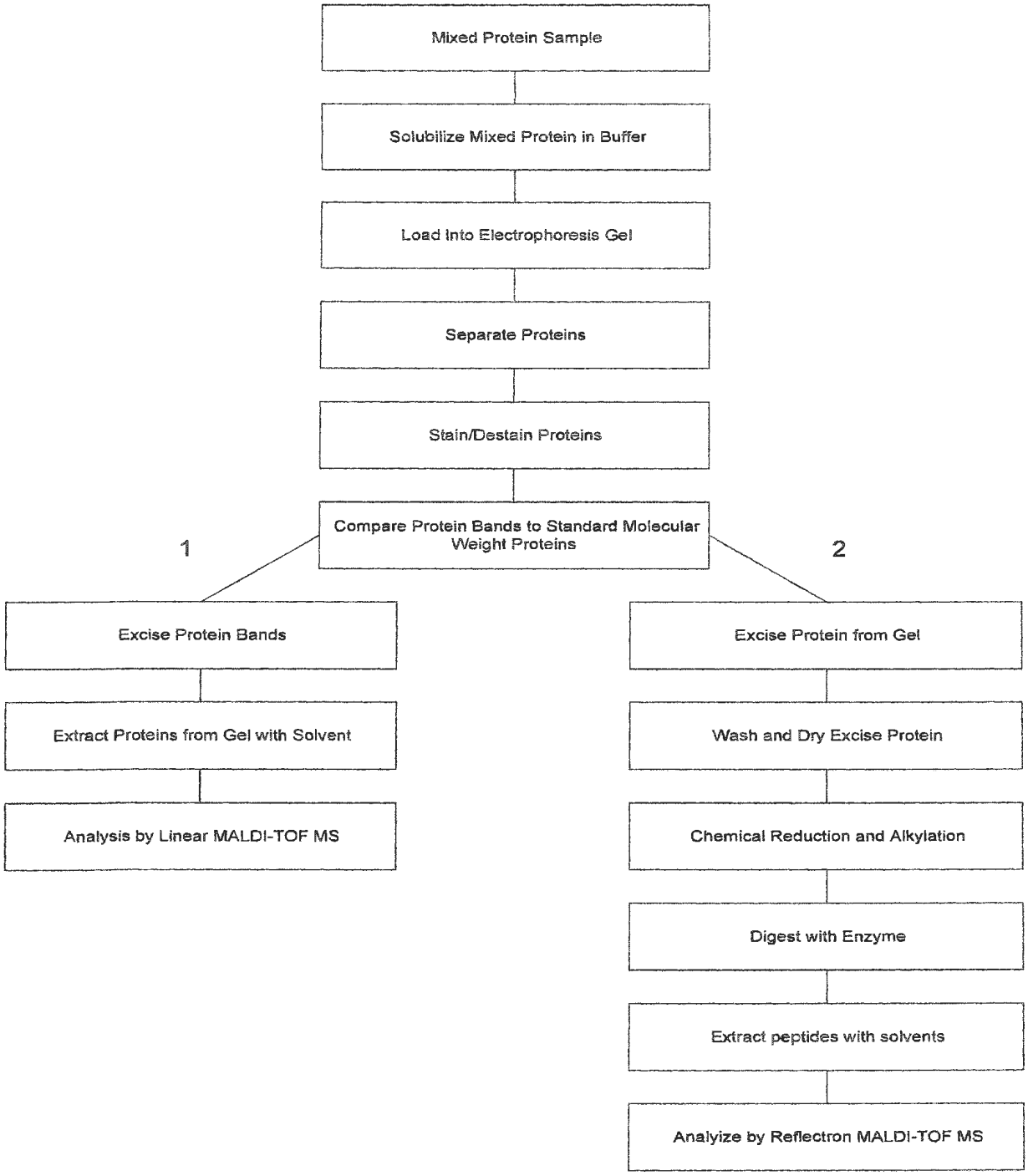

Figure 1.2 **The steps involved in the separation and identification of Snow crab proteins using electrophoresis and MALDI-TOF MS. 1) Analysis of intact protein and 2) enzymatic digestion to produce peptides.**

1.5.1 Electrophoresis

Under the influence of an electrical field, charged molecules migrate in the direction of the electrode bearing the opposite charge to the molecules. During this process, the substances are in an aqueous solution. Because of their varying charges and masses, different molecules of a mixture will migrate at different speeds and will be separated into different fractions (11).

The electrophoretic mobility, which is the speed of migration, is a major and characteristic parameter of a charged molecule and is dependent on the pK_a value of the charged molecule. It is affected by the type, concentration and pH of the buffer, by the temperature and field strength as well as by the nature of the support material which typically may be gels, films or thin-layer plates (11).

Discontinuous gel electrophoresis, which uses a gel with a distinct stacking and resolving section in buffers containing sodium dodecyl sulfate (SDS) is very widely used for the determination of molecular weight of protein subunits, as well as purity estimations. The functionality of the electrophoresis gel is defined by the matrix material, polyacrylamide. The polyacrylamide used in gel electrophoresis provides a wide continuum of pore sizes for exerting molecular sieving effects on a charged molecular species. In the case of crossed linked polyacrylamide, the pore sizes allow for effective molecular sieving of species ranging from a few hundred to several hundred thousand Daltons (12). The polyacrylamide gel is formed by reaction between acrylamide and bis-acrylamide (Figure

1.3) which proceeds via a free radical mechanism and therefore depends on; 1) the concentration of available free radical, 2) temperature and 3) reagent choice and purity (11, 13).

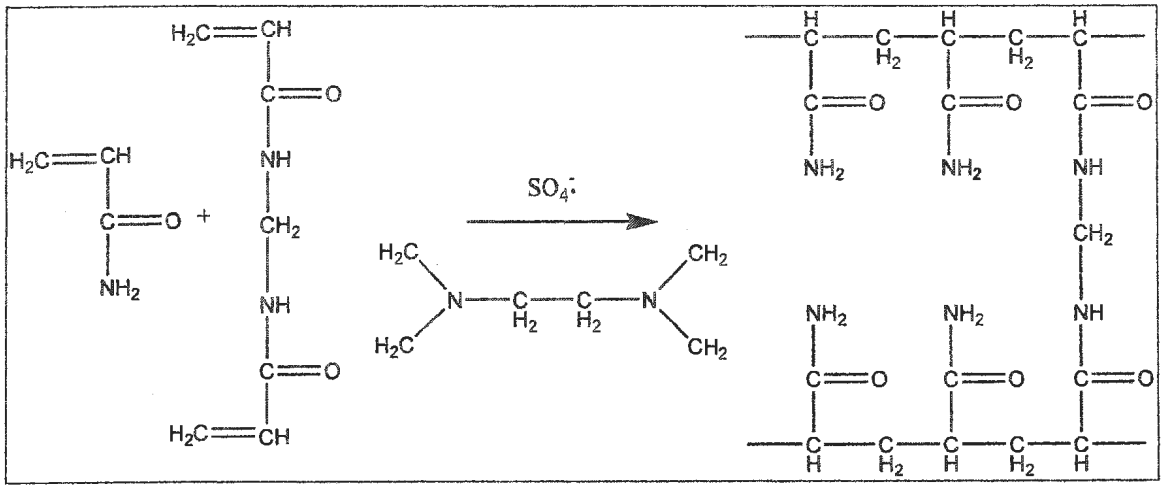

Figure 1.3 The polymerization of acrylamide and bis-acrylamide via persulfate (initiator) and N,N,N',N' tetramethylelthylendiamine (catalyst).

The pore characteristics of the polymer is expressed through two factors, %T and %C. %T is the total monomer concentration:

%T = acrylamide (g) + crosslinking agent (g) / 100mL

The % crosslinking (%C) is the ratio of the weight of the crosslinking agent to the total monomer weight.

%C = crosslinking weight (g) X 100 / %T

Common %T and %C for low molecular weight proteins, less than 100 kDa, are in the range of 12-30%T with crosslinking ranging between 2-5%C. The higher the %T and %C the lower the electrophoretic mobility, thus longer separation times result (11, 12).

Protein location in an electrophoretic gel is determined by means of staining or radioactive tagging. The staining may be direct staining of the protein molecule by Coomassie blue or silver type stains, or conversely by staining the gel with metal solutions like copper and zinc chloride, which leaves the protein molecules unstained. The detection limits for the different staining methods vary greatly; Coomassie blue detection limit is 40 ng protein/mm^2, silver is 0.4 ng protein/mm^2, while copper and zinc can be used to measure protein concentrations in the range of 10 ng protein/mm^2 (14, 15). Both direct and negative (in-direct) staining approaches not only allow for molecular weight determination by comparison with known standards, but also aid excising of the protein bands by identifying their location. These protein bands can then be used for further study through other proteomics techniques.

Polyacrylamide gel electrophoresis (PAGE) or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), polyacrylamide gel with SDS detergent added, have traditionally contained a separate stacking and resolving section. The stacking and resolving sections have differing pHs and pore sizes to permit protein separation. Modern PAGE and SDS-PAGE allow for gradient gels that have a continuously changing pore size throughout the gel. The gel starts with a %T of 4% and then changes through a continuum until 20% is reached at the opposite end of the gel. This approach allows for better resolution and greater separation compared to the standard stacking and resolving gel (11).

1.5.2 Trypsin Digest

Biochemists exploit the ability of enzymes to digest proteins to aid in protein identification and sequence determination. With the advent of modern electrospray and MALDI-TOF mass spectrometry, the application of enzymes for protein sequencing has expanded greatly (17). The most commonly used enzyme is trypsin, because of its specificity. Trypsin only cleaves peptide bonds at the carboxyl end of basic amino acids, that is, lysine and arginine. However, if the lysine or arginine is followed by proline in the amino acid sequence the cleavage is missed. The cleave patterns produced by the digest - often call a peptide fingerprint - have unique molecular masses that can be determined by mass spectrometry, allowing for rapid and simple comparisons with known peptide mapping of protein standards (17).

With the new developments of SDS-polyacrylamide gel, trypsin digest techniques were adapted to allow for in-gel digestion. This technique provides a simple way for complex protein mixtures to be separated, and the purified proteins to be digested before extraction from the gel. Furthermore, the small peptides are more easily extracted from the gel than larger intact protein molecules. Typically, 10 μg of protein or greater would be required to allow a sufficient quantity of protein to be extract from a gel for analysis, using electroelution techniques. However, by doing in-gel digestion, the concentration required was reduced to about 1 μg, a ten-fold improvement in sensitivity (16, 18, 19).

To further enhance the tryptic digest, biochemists have taken to carrying out reduction and alkylation prior to the digestion stage (Figures 1.4 and 1.5). The reduction is carried out to reduce the cystine disulfide bridge to two cysteine residues. With the reduction of the cysteine, any potential rings in the protein molecule are opened, allowing for more efficient peptide bond cleavage. To prevent the disulfide bridge from reforming, iodoacetamide is often used as an alkylating agent as seen in Figure 1.5 (17).

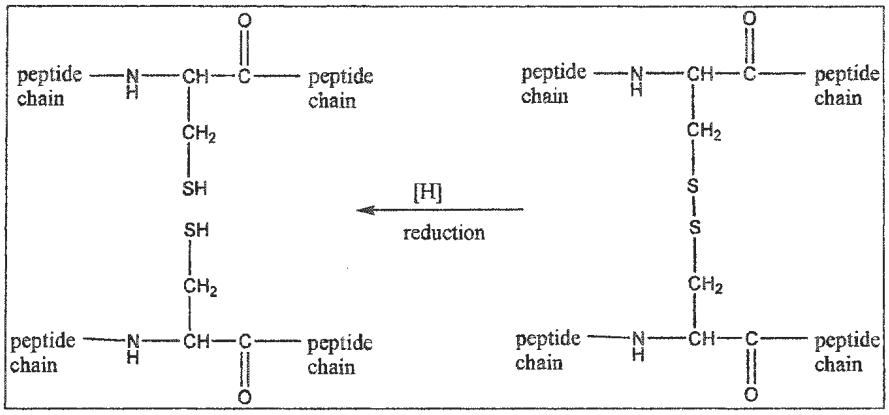

Figure 1.4 Reduction of cystine to cysteine by cleavage of the disulfide bridge

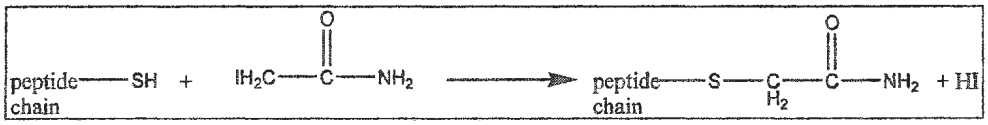

Figure 1.5 Alkylation of cysteine by iodoacetamide

It should also be noted that trypsin digests are not without problems. Trypsin does not distinguish one protein source from another. It will digest any protein contaminants present, for example keratin (from skin flaking), and if given time will perform autolysis

(16). These "background" peptide products can greatly interfere with the ability to identify the peptides of the targeted protein. Regardless, there is a positive side to having small amounts of trypsin autolysis peaks present. Because these peaks have precise molecular masses, and if the exact molecular mass is known, the autolysis peptide peaks can be used as an internal standard, greatly increasing the mass accuracy of unknown peaks versus external standards (20).

1.5.3 MALDI-TOF MS and Proteins

The advent of MALDI-TOF MS has had a profound affect on protein analysis. Through this soft ionization technique, intact molecular ions are produced from parent molecules having molecular masses between 100 and 1,000,000 Daltons. The MALDI-TOF MS combines the use of a laser and a sample matrix to create molecular ions. The mechanism of the MALDI ionization is illustrated in Figure 1.6. The current understanding of the mechanism is that strong absorption by the matrix at the wavelength of the incident laser beam makes it possible to transfer the energy of the laser to the sample in a controlled way. Typically one photon per matrix molecule in the uppermost layer is absorbed. This amount of energy is enough to induce ablation of a sample volume, freeing intact matrix and analyte neutrals and ions. The analyte is diluted in a high molar excess of the matrix-forming solid solvent, thus preventing analyte molecules from aggregation or fragmentation, and reducing strong intermolecular interaction. Analyte ionization occurs by proton transfer between excited matrix molecules and analyte molecules, possibly in

the solid phase, and also by collisions in the expanding plume. These ions are stable protonated ions (21).

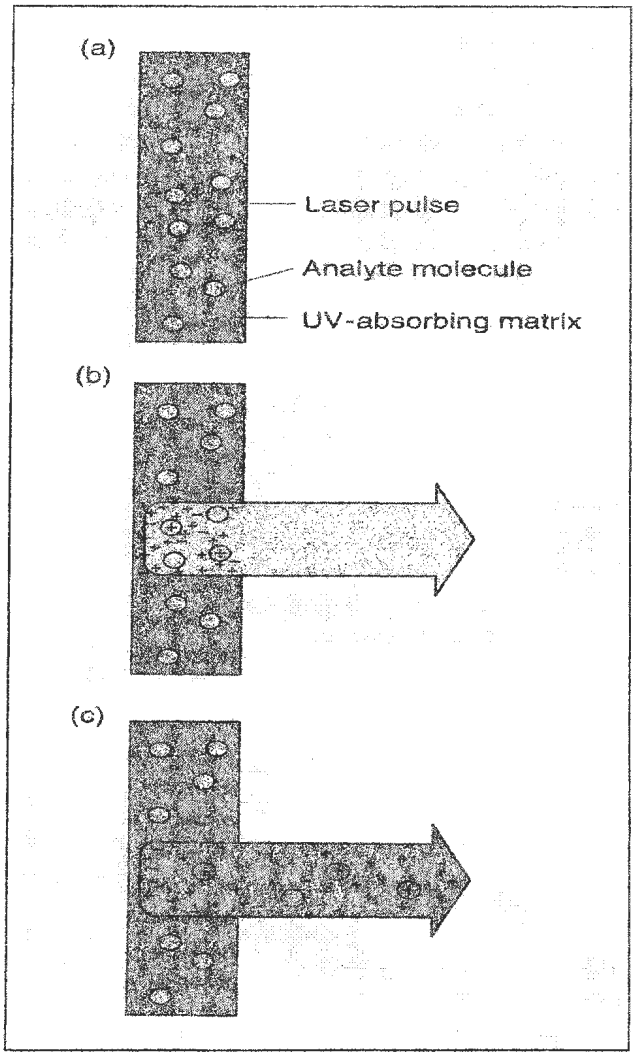

Figure 1.6 **Mechanism for matrix ionization in MALDI using an ultra-violet laser (a) Absorption of UV radiation by the matrix and ionization of the matrix. (b) Dissociation of the matrix, phase change to supercompressed gas, and transfer of charges to analyte molecules. (c) Expansion of matrix at supersonic velocity, entrainment of analyte in expanding matrix plume, and transfer of charges to analyte molecules (22).**

The most commonly used matrix materials include α-cyano-4-hydroxycinnamic acid and sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid); the appropriate choice depends on whether peptides or proteins are to be analyzed (Figure 1.7). Not only do these matrices allow for proton transfer but they are also chemically non-reactive, stable under a vacuum and are easy to prepare (21).

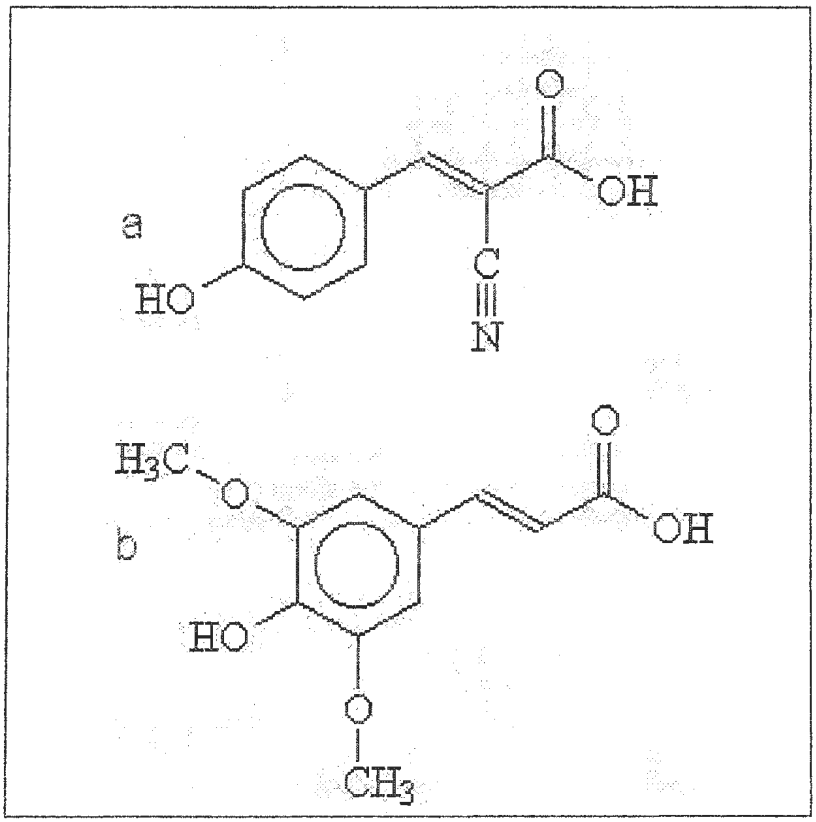

Figure 1.7 **Common matrix materials, (a) α-cyano-4-hydroxycinnamic acid and (b) sinapinic acid.**

The unique combination of ionization and solid solution shows no limitations in the analyses of proteins regardless of their primary, secondary or tertiary structures. Proteins with different solubility properties, including proteins that may or may not be soluble in

aqueous solutions or glycoproteins with large percentage of carbohydrates, can be analyzed. As well, MALDI is minimally impacted by the presence of non-proteinaceous impurities, including salts and buffers that are commonly used in biochemical methodologies. Detergents like SDS, widely used in biochemistry, are tolerable up to 1% for most matrices, and with 2,5-Dihydroxybenzoic acid the tolerance is increased to 10%. This tolerance for SDS allows for analysis of proteins from SDS-PAGE gels through enzymatic or chemical digests (21).

The MALDI-TOF MS (Figure 1.8) instrument allows for the analysis of intact proteins and peptides. The intact proteins are measured in linear mode while the peptides are measured in reflectron mode. The reflection, located at one end of the flight tube, compensates for the differences in flight times of the same m/z ions of slightly different kinetic energies (21). Thus resulting in the focusing of ions in time and space at the detector. With both linear and reflectron modes being present in a single instrument, two detectors are required for detection of the molecular masses.

To improve analyte detection in linear and reflectron mode, adjustment can be made to the laser energy, voltage grid, and guide wire between each test. As well, in reflectron mode, the reflection (electrostatic mirror) is employed to increase analyte resolution. The ease of instrument adjustment between each test is one of the great strengths of the instrument.

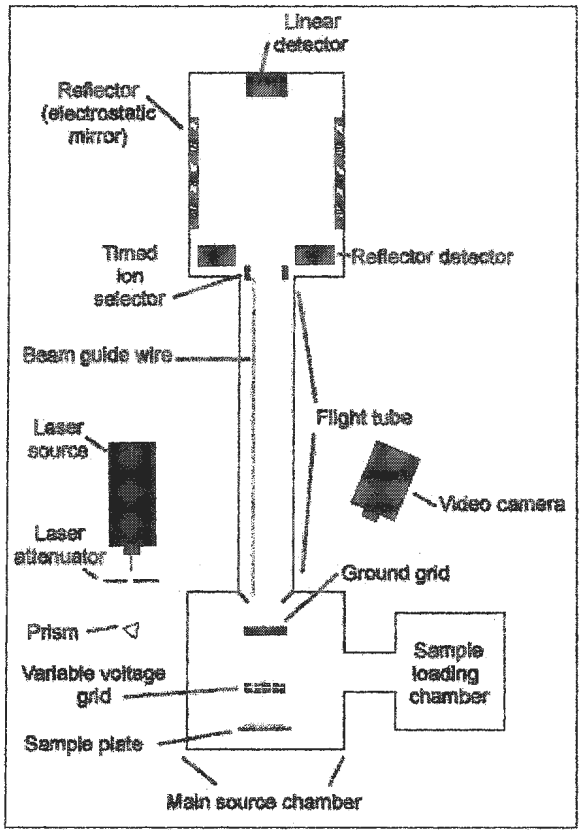

Figure 1.8 MALDI-TOF MS instrument capable of linear and reflectron operating modes.

High accuracy mass determination for proteins and peptides, (0.10% for proteins below 30,000 Da and 0.05% for peptide below 5,000 Da) can be achieved with the reflectron MALDI-TOF MS described above. These mass accuracies can be further increased with internal standards, as opposed to external standards, to within 0.01% for peptides. For trypsin digested peptides, porcine and bovine trypsin autolysis peaks have been determined and either can be used as internal reference peaks (16, 20).

When using the MALDI-TOF MS for peptide analysis, it is expected that the number of peptide peaks detected will vary from sample to sample, and at times, a very dominant peak may reduce the apparent intensity of other peaks, therefore producing spectrum variations. Protein modifications during the electrophoresis process may also affect observed peaks; oxidation of methionine (+16Da) and trypophan (+32Da), and alkylation of cysteine to cysteinyl-S-β-propionamide (+71Da) have all been documented (16). As well, peaks arising from post-translational modifications, changes after the DNA codes for the protein, may not be integrated in any peptide mass search database. As a result, some peptide peaks will be unaccounted for. Furthermore, genetic variation of single individuals can result in peptides of differing masses and render the peptide mass search more difficult (16). Nonetheless, the identity of the protein can be confirmed especially when the approximate protein molecular weight and biological origin of the protein are known.

CHAPTER 2 MATERIALS AND METHODS

2.1 Aerosolized Protein Samples

For the purpose of this study, two aerosolized crab protein samples were studied. One sample was generated in a controlled laboratory setting the other collected from a snow crab processing operation.

2.1.1 Snow Crab Condensate Collection

Two snow crab sections along with 750 mL of distilled water were placed in an aluminum cooker and the resultant condensate was collected using a glass condenser with collection flask attached, Figure 2.1. The sections were cooked in boiling water until 150 mL of condensate was produced, taking about 30 minutes. The cooker was cleaned between each sample with distilled water and fresh water was used for each cooking. A total of 20 crab section were processed in this way to provide 10 replicate samples which, in turn, were subdivided into aliquots of 15 mL before freeze drying in an Edwards Freeze Dryer at –18°C. The freeze-dried samples were stored at –18°C until used. These samples represented the controlled isolation of aerosolized proteinous material from a small scale crab cooker.

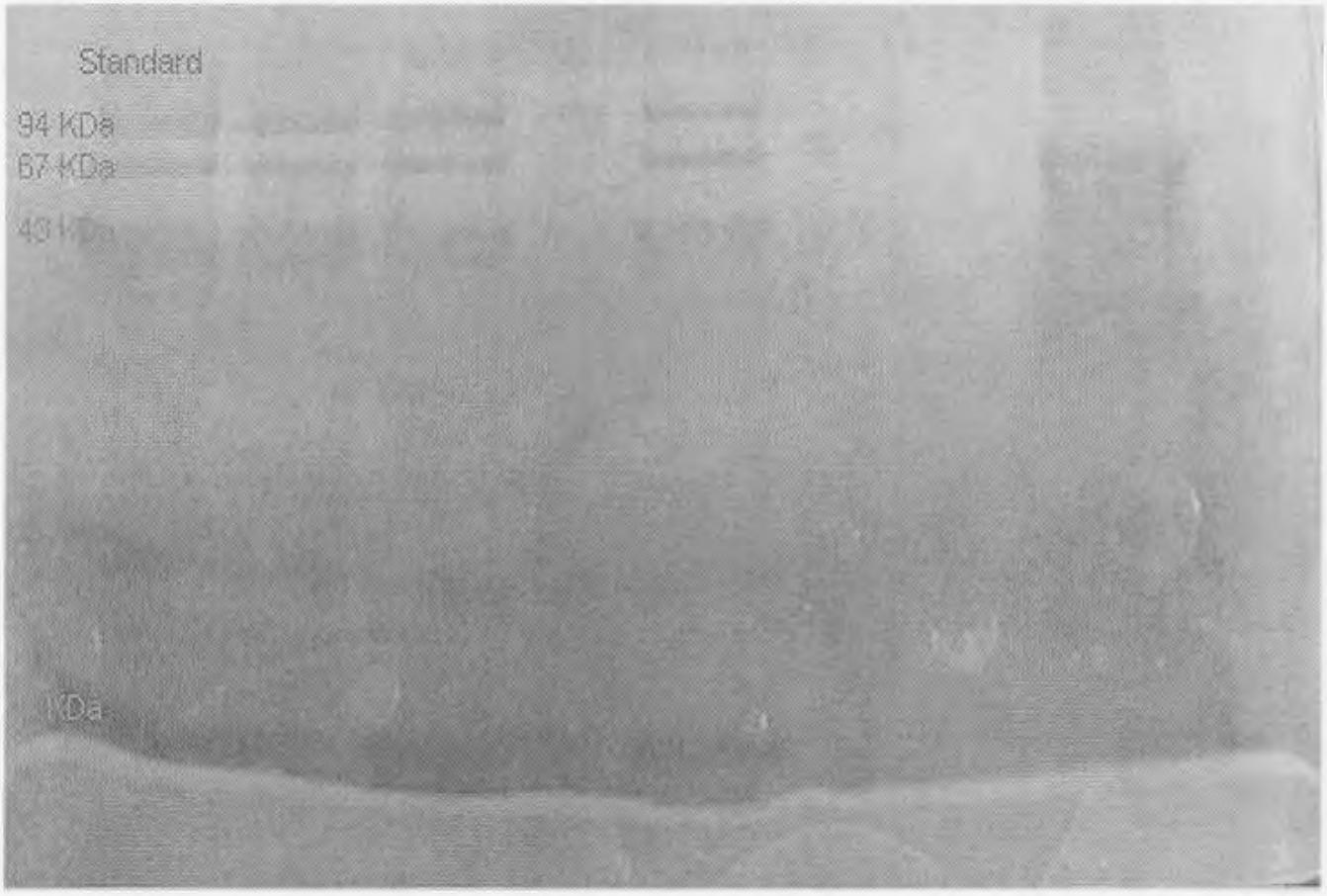

Figure A1: Copper stained electrophoresis gel used for passive elution and Linear MALDI analysis of protein molecular weight determination.

The following chemicals were obtained from Sigma-Aldrich Canada Ltd.: TRIS, glycine, sodium dodecyl sulfate, dithiotheritol, bromophenol blue, trifluoroacetic acid, ammonium bicarbonate, bovine trypsin (sequencing grade), iodoactamide, α-cyano-4-hydroxycinnamic acid, sinapinic acid and 2-mercaptoethanol. The following chemicals were obtained from Bio-rad Laboratories Canada Ltd.; Ready gel (4-20%T), Coomassie blue R-250, and glycerol. The following chemicals were obtained from Fisher Scientific Limited: ethanol, methanol, acetonitrile, formic acid, disodium ethylenediaminetetraacetic acid and acetic acid. An electrophoresis calibration kit was obtained from Amersham Biosciences Corp.

A tropomyosin protein sample isolated from salmon was kindly provided by Dr. D. Healey of the Biochemistry Department of Memorial University.

2.4 Electrophoresis

2.4.1 Electrophoresis Standards

The electrophoresis calibration standard kit, purchased from Amersham Biosciences Corp, contained the proteins listed in Table 2.1.

Table 2.1 Electrophoresis molecular weight calibration protein standards.

Standard	Molecular weight	Micrograms / vial
Phosphorylase b	94,000	64
Bovine Serum Albumin	67,000	83
Ovalbumin	43,000	147
Carbonic Anhydrase	30,000	83
Soybean Trypsin Inhibitor	20,100	80
α-Lactabumin	14,400	121

The electrophoresis standards were suspended in 500 μL of sample buffer and frozen at −18°C in 100 μL aliquots.

2.4.2 Sample Buffer

A Sample buffer solution is used to solubilize sample proteins. The resulting solution is loaded into the sample wells of the electrophoresis gel. Twenty millilitres of sample buffer was prepared containing 0.08 *M* tris(hydroxymethyl) aminomethane-HCL (TRIS-HCl), pH 6.8. The sample buffer was made by combining 0.5 mL of 2-mercaptoethanol, 4 mL of 10% sodium dodecyl sulfate, 1.6 mL of stacking gel buffer (section 2.4.3), 2.5 g of 87% glycerol, and 0.5 mg of bromophenol blue and was made up to 20 mL with distilled deionized water (14).

2.4.3 Stacking Gel Buffer

The stacking gel buffer is a pH buffer, used in the preparation of the sample buffer and the stacking gel to fix the pH. Two hundred and fifty millilitres of stacking gel buffer was prepared to contain 1 *M* TRIS-HCl, pH 6.8, by dissolving, 30.3 g of TRIS in 200 mL of distilled deionized water, and adjusting pH to 6.8 with HCl, before dilution to 250 mL (12).

2.4.4 Electrophoresis Stock Buffer

The electrophoresis stock buffer is an electrode buffer that provides for electrical conduction through the gel when the external power is applied. One liter of electrophoresis stock buffer solution containing 0.25 *M* TRIS, 2 *M* glycine, and 1% sodium dodecyl sulfate, pH 8.3, was prepared by dissolving 30,0 g TRIS, 144 g glycine, and 100 mL of sodium dodecyl sulfate in distilled deionized water. The pH was adjusted to 8.3 with HCl and diluted to one liter. Prior to use, the electrophoresis buffer was diluted 1:10 with distilled water (12).

2.4.5 Staining Solution

The Coomassie blue R-250 (azo dye) is used to permanently stain the proteins in the gel. Coomassie blue R-250, 1.25 g, was dissolved in a solution of 230 mL of methanol, 230 mL of distilled deionized water and 40 mL of acetic acid (12).

2.4.6 Destaining Solution

The destaining solution was used to remove any Coomassie blue R-250 from the gel that is not bound to protein. This allows for differentiation between the gel containing proteins and the remainder of the gel. This destaining solution was made by mixing five hundred millilitres of acetic acid with 1500 mL of ethanol, followed by dilution to 5 liters with distilled deionized water (12).

2.4.7 Electrophoresis Gel

Commercial gels were purchased from Bio-rad Laboratories Canada Ltd., Mississauga, Ontario. All gels were gradient gels (a gel with a continuum of pore sizes, large to small

– 4 to 20%T), thus allowing the resolving of proteins with molecular weights from 10-200 kDa.

2.4.8 Sample Loading

All samples were loaded using a variable volume Eppendorf pipette, 10-100 μL, equipped with gel loading tip. Fifteen microlitres of each of the standard and the sample solution were loaded into wells at the very top of the gel.

2.4.9 Electrophoresis Unit and Running Conditions

A Bio-Rad Mini Protein II electrophoresis chamber (Figure 2.2) was loaded with the gradient gel, and electrophoresis buffer was added to both the inner and outer chambers, requiring about 1000 mL of buffer. Samples and protein standards (between 6-10 in total) were loaded on the top of the gel, the chamber sealed, and the electrodes connected to a constant voltage power supply. The power supply was set to a constant DC voltage of 125 V. The samples ran until the bromophenol blue dye front (representing a low molecular weight marker) was about 1 cm from the bottom of the gel, with an approximate running time of 45 minutes to 1 hour.

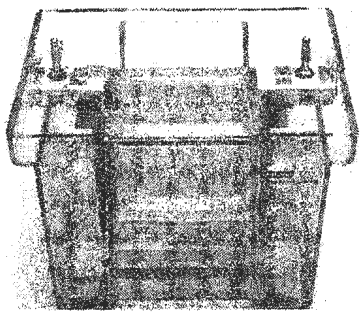

Figure 2.2 Boi-Rad mini gel electrophoresis unit.

2.4.10 Staining and Destaining

After running, the gel was stained by soaking it in Coomassie blue for one hour; then destained by soaking in destaining solution for at least 4 hours or until the gel was clear of the Coomassie blue. To shorten the destaining time, the destaining solution was changed once after 20 minutes (12).

2.5 Molecular Weight Determination by Electrophoresis

Protein mobilities were calculated as R_f values (distance of protein migration divided by the distance of migration of the dye front). Proteins of known molecular weight were run under the same conditions as the samples, and the slope generated defined the linear relationship between R_f and the molecular weight. The molecular weights of the unknown proteins were extrapolated from the curve of the molecular weight standards (14).

2.6 Molecular Weight Determination by MALDI-TOF MS

Two approaches were used to try to calculate the accurate molecular mass of the intact proteins present in the snow crab condensate sample. The first method involved protein separation and extraction from an electrophoresis gel followed by MALDI-TOF MS, while the second method involved re-solubilization of the lypholized condensate and analysis of the protein mixture by MALDI-TOF MS.

2.6.1 Protein Mass Determination from Electrophoresis Gel by MALDI-TOF MS

Proteins separated on a 4-20%T ready–gel were stained for 20 minutes with 0.3 *M* copper (II) chloride (Coomassie blue cannot be used because it binds to the proteins while copper (II) chloride only stains the gel and not the proteins). Once stained, the protein bands were excised and stored at 4°C in sealed micro-centrifuge tubes until needed.

The bands were destained by washing, with shaking, in 0.25 *M* EDTA / 0.25 *M* TRIS for 10 minutes. Then the bands were washed 3 times, with shaking, in 20 *mM* TRIS / 150 *mM* glycine / 0.01% SDS for 10 minutes each time. Each destained band was crushed and then incubated in 40 µL of an extract solvent, consisting of formic acid / acetonitrile / 2-isopropanol / water (50:25:15:10, v/v/v/v). The incubation was performed in an ultrasonic bath for 60 minutes at room temperature (24).

2.6.2 Protein Mass Determination Directly by MALDI-TOF MS

The freeze-dried protein condensate and the protein sample from the air filter, were solubilized in 100 µL of 0.1% trifluoroacetic acid (TFA). The samples were then analyzed by MALDI-TOF MS as described in section 2.9.

2.7 Tryptic Digest Protocol

Protein bands stained with Coomassie blue (~1 mm x 1 mm) were excised using a clean scalpel and placed into 1.5 mL micro-centrifuge tubes. A protein-free section of gel was cut as a digestion control. All gel pieces were frozen at –18°C until analyzed.

All pieces of gel were washed, to remove Coomassie blue, in 100 µL of 25 *mM* ammonium bicarbonate in 50% acetonitrile, agitated with a vortex for 10 minutes. This was repeated 2-3 times. The samples were then dried for 15 minutes in a vacuum desiccator. Once dried, reduction and alkylation were carried out. Reduction was accomplished by adding 10 *mM* dithiotreitol (DTT) (reducing agent) in 25 *mM* ammonium bicarbonate to a 1.5 millilitre centrifuge tube in sufficient quantity to cover the gel slices followed by incubation at 56°C for 60 minutes. After 60 minutes the samples were cooled, the DTT was removed and replaced with an equal volume of 55 *mM* iodoacetamide (alkylating agent) in 25 *mM* ammonium bicarbonate. The samples were incubated a second time for 45 minutes at room temperature. The samples were again removed from the incubator, the solution removed, and replaced with 100 µL of 25 *mM* ammonium bicarbonate and vortexed for 10 minutes. The ammonium bicarbonate was removed and replaced with 100 µL of 25 *mM* ammonium bicarbonate in 50% acetonitrile and vortexed for 10 minutes. The washing and dehydration steps were repeated 2 times and then the samples were dried in a vacuum desiccator for 20 minutes.

The gel particles were rehydrated in 25 *mM* ammonium bicarbonate, pH 8.0, containing 0.02 µg/µL trypsin (sequencing grade) and placed on ice for 7-10 minutes. Any excess trypsin solution was removed and 25 µL of 25 *mM* ammonium bicarbonate was added. The samples were incubated at 37°C for 12-16 hours (over night) to enhance digestion (18, 19, 25).

The peptides were recovered from the gel particles by three extractions. The first extraction was carried out with 30 μL of 25 *mM* ammonium bicarbonate accompanied by vortexing for 10 minutes. The resulting solution was transferred to a 1.5 mL collection micro-centrifuge tube. The two subsequent extractions were performed with 30-μL aliquots of 5% formic acid in 50% acetonitrile with 10 minutes of vortexing and combined with the first of the extract in the 1.5 mL collection centrifuge tube. The resulting peptide solution was completely dried in a vacuum desiccator and then resuspended in 10 μL of 0.1% trifluoroacetic acid and stored at 4°C until analyzed (26, 27).

2.8 Ziptip (C_{18})

The Ziptip is a 10μL pipette tip containing immobilized C-18 resin chromatography media that allows for the desalting and concentrating of peptide and protein samples. Some tryptic digest samples were concentrated and purified using these Millipore Ziptips. An Eppendorf pipette was set to 10 μL, a ZipTip attached, wetted at least twice using a 50% acetonitrile. The tip was equilibrated using 0.1% TFA and repeated. The peptides were then bound to the Ziptip by aspirating and dispensing 3-10 times from the peptide solution (about 10 μL each time). The tip was then washed at least twice with 0.1% TFA. The peptides were eluted off the tip with a 5 μL mixture of 50% acetonitrile 0.1% TFA (28).

2.9 MALDI-TOF Mass Spectrometry

2.9.1 Instrument

All mass spectra were recorded using an Applied Biosystem Voyager DEPRO MALDI-TOF MS operated in either linear or reflectron mode. Sample/matrix ionization was initiated by means of a nitrogen laser operated at 337 nm with repetition rate of 3 Hz.

For linear mode TOF MS the acceleration voltage was set at 25 kV, a grid voltage of 94%, a guide wire of 0.2%, the low mass gate set at 2000, and an extraction delayed time of 750 nsec. One hundred laser shots were used to generate the spectrum. The mass acquisition range was from 2,000 to 100,000 Daltons.

For reflectron mode TOF MS the acceleration voltage was set to 20 kV, the grid voltage set at 73.4%, the mirror voltage ratio set to 1.12, the guide wire at 0.004%, the extraction delayed time set to 200 nsec, the mass acquisition range between 1000-6000 Da, and the low mass gate at 500 Da. The number of laser shots used to generate the spectrum varied from 50-100.

2.9.2 MALDI Sample Preparation

Stock solutions of matrices were prepared fresh each day. For work in linear mode TOF MS, 10 mg of sinapinic acid was dissolved in 1 mL of 0.1% TFA / acetonitrile(70:30). The matrix was mixed with the samples at volume ratios ranging from 1:1 to 20:1, matrix to sample, depending on sample concentration. One microlitre of matrix/sample solution

was applied to the MALDI spotting plate by the dried-droplet method and allowed to dry in a desiccator for about 30 minutes.

For the reflection mode TOF MS, 10 mg of α-cyano-4-hydroxycinnamic acid was dissolved in 1 mL of 50:50 0.1% TFA / acetonitrile. The matrix was mixed with the sample at a volume ratio of 1:1. One microlitre of sample/matrix solution was spotted on the MALDI plate using the dried-droplet method and allowed to dry in the desiccator for about 30 minutes (29).

2.9.3 MALDI-TOF MS Standardization

External standards for mass measurement were used for both intact protein molecular weight and peptide mass fingerprint analysis. For the intact protein molecular weight determination, Perseptive Biosystems Sequazyme bovine serum albumin (BSA) test standard kit, Calmix 3, was used. For the peptide mass fingerprint analysis, Perseptive Biosystems Sequazyme peptide mass standard kit, Calmix 2, was employed. In the intact protein molecular weight determination, linear mode TOF MS, one part of Calmix 3 was mixed with twenty-nine parts of matrix. For the peptide mass fingerprint analysis, reflectron mode TOF MS, one part of Calmix 2 was mixed with twenty-four parts of matrix (29).

2.10 On-line Database Searching

The mascot search engine (www.matrixscience.com)was used to search the SwissProt database for mass spectrums that matched those generated from the MS analysis of the peptide from the tryptic digest During all searches the fixed modification was set to carbamidomethyl (C) indicating that reduction and alkylation had been carried out on the samples. The missed cleavages were set at one to start, however, for some searches the missed cleavages were adjusted to two or three (this accounts for errors in the enzymatic breakage of the amino acid linkages within the protein). The peptide tolerance was adjusted to 0.05% and the mass values were expressed as monoisotopic (MH^+). No results were considered if protein coverage (the percentage of peptides matching known peptides from a known protein) was less than 15%, and for all results, genera and species factors were assessed. The peptide masses matching trypsin autolysis peaks, and human contamination peaks (Tables 3.3 and 3.4) were not included in the searches. In all initial searches the mass peptide values between ~1000 and 2500 Da were analyzed. Any values below or above the 1000 – 2500 Da range were only added to the search after a possible identification was ascertained.

2.11 On-line Post-translational Modification (PTM) Searches

Post-translational modifications, were investigated for the proteins that provided a match giving a SwissProt database number and whose genus and species were in the SwissProt database. The potential protein post-translational modifications were carried out using the "FindMod" tool at http://us.expasy.org/tools/findmod. For each search, the SwissPort ID and, the peptide masses was entered, the maximum post-translational modification was

set at 2, the missed cleavages at 2, the cysteine amino acids were iodoacetamide treated, and a mass tolerance of 1.5 Dalton was established.

CHAPTER 3 RESULTS AND DISCUSSION

3.1 Protein Molecular Weight Determination

Attempts to determine the molecular weights of the intact aerosolized snow crab proteins met with mixed results. Two approaches were employed. The first was electrophoretic separation followed by passive elution and MALDI-TOF MS analysis. The second method involved the resolubilization of the protein mixture followed by MALDI-TOF MS analysis.

3.1.1 Molecular Weight Determination by SDS-PAGE Electrophoresis Only

Molecular masses of salmon tropomyosin, and of proteins in the crab condensate and air Filter 56 based on SDS-PAGE electrophoresis are presented in Table 3.1. These molecular masses are based on the masses for the electrophoresis standards (Table 2.1). The snow crab condensate had protein bands at approximately 70.1, 36.0, 20.1, 17.8 and 14.0 kDa. Air Filter 56 gave protein bands at approximately 74.6, 36.8, and 15.3 kDa with the bands varying greatly in intensity from one replicate to the next (Appendix C). The salmon tropomyosin was employed to help determine if the band observed in the 36 kDa region would be snow crab tropomyosin. Information from the literature shows the crab tropomyosin band is about 34.0 kDa (30), however, the difference of 2 kDa appeared to be inconsequential when viewing the gels (Appendix C). The slight differences between the values in this study and those published elsewhere may be occurring because of the use of a gradient gel rather than the traditional stacking and resolving gel.

It was hoped that the gradient gel would provide more resolving power than the standard stacking and resolving gel. This however did not occur. The band at about 14 kDa showed marked diffusion with a high probability that more than one protein band is mixed in the diffused region (Appendix C). This was unexpected because of the manufacture's claim that the gradient gel could resolve proteins in the 6 kDa range, that is, proteins measuring between 6 and 7 kDa should be distinguishable. In spite of these problems, all visible bands were excised for protein identification by peptide mass fingerprinting (section 3.2).

3.1.2 Electrophoresis / Passive Elution followed by Linear MALDI-TOF MS

Electrophoretic separated proteins are typically eluted from gels by electroelution. However, when electroelution is not available passive elution is an alternative. Cohen's (24) promulgation that his approach to passive elution (section 2.6.1) allowed for the recovery of proteins in the low microgram range could not be substantiated. Appendix A shows the separated proteins standards on the electrophoresis gel stained using copper (II) chloride, though all attempts to recover the proteins were unsuccessful. No mass signals were ever obtained when the standard extracts were analyzed by MALDI-TOF MS. The reasons for the failure to observe these proteins by mass spectrometry are unclear but a number of factors may have contributed to unsuccessful outcome. Some of these factors include issues regarding the sodium dodecyl sulfate (SDS) detergent and the nature of the proteins being analyzed.

The presence of SDS is known to suppress the MALDI-TOF MS signals even when proteins are present in appropriate concentrations (31). During sample preparation for electrophoresis, the ratio of SDS to protein is about 1.4 mg SDS per 1 mg of protein (11). Mass spectrometry detection may not be possible if some of the SDS remained attached to the protein during the mass spectrometry ionization process. Puchades *et al* (31) states that concentrations of SDS above 1% in a sample is enough to greatly reduce the sensitivity of the MALDI-TOF MS analysis. In this study the only indicator as to the level of SDS in the samples occurred when the matrix/sample mixture was spotted on the stainless steel sample plate. Large amounts of spreading would indicate the presence of SDS detergent. However, no excess spreading was observed. In addition, for some samples, C_{18} ZipTips were utilized to remove the SDS detergent and to concentrate the proteins present. This did not improve the results.

In this study, concentration of the protein standard in the copper stained gels was in the range of 2-5 μg (or 10-25 pmol). Other researchers have used protein concentrations of 300 pmol or greater (31,32). Deutscher (33), likewise, indicates that it is important to have a high ratio of protein to gel. Another factor with respect to the proteins that may have prevented passive elution from working could be the hydrophobicity of the proteins. Galvani (32) has shown that hydrophobic proteins do not elute well from electrophoresis gels.

3.1.3 Direct Analysis of Protein Mixture by Linear MALDI-TOF MS

Instead of separating the sample proteins first by electrophoresis, mass spectrometry experiments were performed directly on the protein mixture in order to obtain molecular weight information.

The resolubilized snow crab proteins (condensate and filter 56) and the resolubilized salmon tropomyosin were analyzed by MALDI-TOF MS. Salmon tropomyosin, used as a standard, yielded mass peaks at 31.9 kDa, 16.2 kDa, 11.1 kDa, (Table 3.2 and Appendix B). The 31.9 kDa mass peak is the $[M+H]^+$ for tropomyosin. The 16.2 kDa mass peak is likely the $[M+2H]^{2+}$, while the 11.1 kDa the $[M+3H]^{3+}$ ion. Doubly and triply protonated protein signals are commonly observed in MALDI experiments.

When the snow crab condensate samples were analyzed by MALDI-TOF MS, the detectable masses varied from sample to sample with some mass peaks not detected in other condensate samples. This variability may be the result of variable amounts of aersololyed proteins isolated from replicate runs. Table 3.1 lists the average molecular masses for the major high mass peaks detected and Appendix figure B3 through B5 shows each individual MALDI-TOF MS spectrums. The peaks observed include masses at approximately 32.6 kDa, 12.1 kDa, and 11.1 kDa, with other peaks being present in some condensate samples at 65.1 kDa, 16.4 kDa and 14.0 kDa ranges. The 32.6 kDa mass peak is believed to correspond to the band at about 36.0 kDa found on the Coomassie stained electrophoresis gels (Table 3.2). The 14.0 kDa and the 12.1 kDa mass

peaks are likely represented by the 14 kDa electrophoresis band (Figure C1). What is less clear is the source of the 65.1 kDa and 16.4 kDa mass peaks. The 65.1 kDa peak may be a $[2M+H]^+$ ion of the 32.6 kDa peak or it may be the protein that is responsible for the protein electrophoresis band at about 70.1 kDa on the Coomassie stained electrophoresis gels (Appendix C). The 16.4 kDa mass peak may be a $[M+2H]^{2+}$ of the 32.6 kDa or it could be the protein visible at 17.8 kDa on some of the Coomassie stained electrophoresis gels (Appendix C). The 11.1 kDa mass peak is most probably the $[M+3H]^{3+}$, the same as one sees with the salmon tropomyosin.

The Filter 56 sample yield similar results to the snow crab condensate. Mass peaks were detected at 32.6 kDa, 16.3 kDa, 14.0 kDa, 12.7 kDa, and 11.2 kDa, and a mixture of smaller ion peaks were seen as well (Table 3.1). As with the mass spectrometry results of the condensate, it is believed that the 32.6 kDa, 14.0 kDa and the 12.7 kDa mass peaks are separate proteins while the 16.3 kDa and 11.2 kDa are the $[M+2H]^{2+}$ and the $[M+3H]^{3+}$ ions of the 32.6 kDa peak respectively.

The difference in the molecular weight values obtained from the MALDI-TOF MS (Table 3.2) and for the electrophoresis gels (Table 3.1) can be attributed to the fact that SDS-PAGE has an error of about 10 % (30). Additionally, the presence of large quantities of SDS (about 1.4 mg per 1 mg of protein) and the ability of proteins to form adducts with acrylamide monomers could help account for perceived differences in molecular weight measurements (34).

This approach did provide useful information regarding the molecular weight for some of the proteins present in the samples. However, because of the difference between the MALDI results and the electrophoresis gels, some questions still remain about what proteins are actually present around the 14 kDa range.

3.2 Peptide Mass Fingerprinting by MALDI-TOF MS

Peptide mass fingerprinting proved to be an effective and efficient means of identifying some of the proteins aerosolized during snow crab processing. The on-line databases searches yielded positive identification for those proteins that have been sequenced and stored in the databases. Proteins from the snow crab condensate and Filter 56 were analyzed along with BSA and salmon tropomyosin as reference standards. The BSA served as a separate reference standard with every tryptic digest undertaken to ensure the suitability of the trypsin activity.

3.2.1 On-line Database Searches

As of January 26, 2003 Matrixscience has updated their Mascot protein search engine (35). These changes have resulted in adjustments to their search engine. All results reported here-in (Table 3.5-3.11) reflect the latest mowse scores and percentage protein coverage. The major change appears to be related to the mowse score and the peptide mass tolerance. Every 0.01% increase in the peptide tolerance brings about a 7-point change in the mowse score. Changes in the mowse score, while important, are not critical because the genus and species information of the proteins studied was known.

3.2.2 Trypsin Autolysis Mass Peaks and Human Contamination Mass Peaks

Both trypsin autolysis mass peaks and human contamination mass peaks occur regularly in trypsin digested proteins samples. Table 3.3 shows some of the trypsin autolysis mass peaks (background peaks) observed in this study. Table 3.4 gives the most common trypsin autolysis mass peaks and human contamination mass peaks sited in the literature (20). Some of the peaks, notability 1066, 2163 and 2273, were observed often while others were not. The main problem encountered with these types of contamination peaks is suppression of other mass peaks and therefore reducing the probability of peptide matching during the database searches.

3.2.3 Peptide Mass Fingerprint for Bovine Serum Albumin Reference Standard

The peptide mass fingerprint of the bovine serum albumin (BSA) showed strong matches through the SwissProt database. In all samples the mowse score was 66 or higher. A mowse score of 66 or greater means that the protein match is significant at the 95% confidence level. The BSA peptide mass fingerprinting gave mowse scores ranging from 66 to 124 (Table 3.5). The protein coverage ranged from 18-24%. A protein coverage of 15% is considered acceptable, while coverage greater than 20% is considered very good (35).

Even though the bovine serum albumin was a reference, peptide mass fingerprint variations were observed in the mowse score and protein coverage. These variations can be attributed to suppression of some peptide mass peaks, post-translational modifications

and unspecific cleavages. The suppression of peptide mass peaks is not uncommon because the SDS detergent used during the electrophoresis separation is extracted along with the protein for the tryptic digest step (28). Instrument settings may also play a role in the suppression of some peaks. Based on the MALDI-TOF MS spectrum (Appendix E), it appears that peaks below 2600 Daltons show greater peak intensity. Therefore, in some samples, mass peaks at higher masses may be suppressed.

Post-translational modifications of proteins, that is, physiological adaptation of the protein after DNA coding are expected occurrences. These modifications can occur because of age, disease of the organism, physiological function of the protein and/or the chemical treatment of the protein during the peptide mass fingerprint analysis (36). Potential post-translational modifications can be observed with the BSA standard as some of the peaks present (Appendix E) were not matchable through the SwissProt database for BSA. The most probable reason for the unmatched peaks is due to post-translational modifications. When FindMod software (http://us.expasy.org/tools/findmod) was utilized to study the unmatched peaks, a number of peaks were attributable to modifications, namely, changes to the amino acids cysteine and methionine. Cysteine changes may be due to the iodoacetamide used for acetylation and acrylamide adduct formations arising from the electrophoresis gel. The modification of methionine is due to oxidation that may occur in the separation or digestion stages of the peptide mass fingerprint (36). Also, other modifications including phosphorylation and trimethylation were indicated by the FindMod software as potential reasons for the unmatched peaks. Unspecific cleavages

consist of peptides whose termini do not correspond to common cleavage patterns of the protease used to digest the sample (36). These unspecific cleavages can indicate the presence of other proteases, for example, chymotrypsin. Chymotrypsin is often found in trace amounts in commercial sequencing grade trypsin. Other factors including reagents, temperature and, more importantly, incubation time, can impact the occurrence of unspecific cleavages (36).

Despite the fact that some peaks did not match during the SwissProt database search, the results did give conclusive matches for BSA every time bovine serum albumin was analyzed by SDS-PAGE, in-gel trypsin digest and peptide mass fingerprinting by MALDI-TOF MS.

3.2.4 Peptide Mass Fingerprint for Salmon Tropomyosin

The peptide mass fingerprint for the salmon tropomyosin (Table 3.6 and Appendix F) was analyzed using the NCBInr database. The SwissProt database used for the other peptide mass fingerprints could not be used because salmon tropomyosin is not part of the SwissProt database. Mowse scores ranging from 68 to 90 were obtained for the 4 peptide mass fingerprint analyses carried out. With the NCBInr database, a mowse score of 74 is required to have a significant match at the 95% confidence level. Three of the four peptide mass fingerprints undertaken gave matches at the 95% confidence level. The reason for the fourth sample not meeting the 95% confidence level may be attributed to post-translational modifications.

Post-translational modifications were not determined for the salmon tropomyosin because the FindMod software does not support the NCBInr protein codes. Without conducting post-translational modification searches, it is impossible to determine what changes may have occurred. Also, the electrophoresis analysis may have had an effect on this protein. The protein mass listed in the NCBInr database is 32,530 Daltons while the linear MALDI-TOF MS result showed the molecular weight to be closer to 31,900 Daltons (Table 3.1).

3.2.5 Peptide Mass Fingerprint for 70.1, 20.1, 17.8, and 14.0 kDa Electrophoresis Bands from Snow Crab Condensate

The 70.1 kDa, 20.1 kDa, 17.8 kDa and 14.0 kDa protein bands (Table 3.2) could not be identified by the peptide mass fingerprinting. The five snow crab replicates used from the 70.1 kDa protein bands showed that many of the peptide masses were consistent throughout all replicates (Table 3.7). Peptide masses at 1207, 1286, 1578, 1828 and 2497 kDa were detected in all the replicates. This consistent occurrence in peptide masses indicates that one protein is present. The most likely reason for the inability to match the protein through the on-line databases is the fact that the protein has not been sequenced and added to the on-line databases.

Only one of the SDS-PAGE separated condensate replicates showed a protein band at 20.1 kDa while two showed protein bands at 17.8 kDa (Table 3.9). None of these proteins could be matched to any proteins in the on-lines databases. The apparent low

concentrations (Appendix I) of these protein bands made it difficult to produce good quality peptide mass fingerprints.

The 14.0 kDa snow crab condensate protein was problematic. Of the five replicates, only 3 yielded results and of the 3 there was a significant difference between replicates 1, 2, and replicate 5 (Table 3.10 and Appendix J). The 14 kDa electrophoresis band was very diffuse, making it possible that more than one protein was mixed within the band. The greatly differing results between the replicates for the peptide mass fingerprint indicates that there is a high degree of probability that more than one protein is present in the band.

3.2.6 Peptide Mass Fingerprint for 36.0 kDa Bands from Snow Crab Condensate and Filter 56 (Air Sampling Filter)

The 36.0 kDa protein band from the snow crab condensate showed homology to tropomyosin from other shellfish when the peptide mass fingerprints were analyzed. The five snow crab condensate replicates had mowse scores that ranged from 57 to 91 (Table 3.8) when the peptide mass fingerprints (Appendix H) were database searched. The 36.0 kDa protein bands from filter 56 also gave homology to tropomyosin from other shellfish with mowse scores from 62 to 100 (Table 3.11). A mowse score of 66 is required for a for a positive protein match at the 95% confidence level. The protein coverage for both the snow crab condensate and filter 56 was between 33 and 46%, far beyond the minimum15% recommended (35).

The mowse score was not the only factor used to aid in the correct protein identification. Since the organism from which the protein originated was known, when studying the likely proteins matches derived from the on-line database, the genus and species for the organisms were assessed. Since snow crab is a shellfish, only proteins from shellfish were considered. All the tropomyosin matches were from other genus and species of shellfish. The strongest homology was to Cha f I tropomyosin from *Charybdis feriatus*, rock crab. Other shellfish that showed homology included the greasyback shrimp, spiny lobster and the american lobster. No homology was evident to tropomyosin from other organisms.

The low mowse scores from some of the peptide mass fingerprints is not surprising because snow crab tropomyosin is not in the SwissProt database. Some of the mass peaks that did not match with the other tropomyosins may indeed be valid amino acid sequences that are not found in the other shellfish.

3.3 Radioallergosorbent Test (RAST) of Snow Crab Condensate and Filter 56

A sample of snow crab condensate and of Filter 56 were sent to Dr. Samuel Lehrer, Tulane University, New Orleans, for RAST analysis (see section 1.4). While the condensate did not show any allergenic reactivity, filter 56 did (Appendix M). The RAST test showed activity in the 34.0 kDa and 14.0 kDa area. Both of these bands were also are visible on the Coomassie blue stain electrophoresis gels (Appendix C). The reason why

the snow crab condensate did not produce allergenic reactivity is unclear. It may be due to a concentration factor which may have been below the detection limit.

These results, along with positive protein identification of 34.0 kDa by peptide mass fingerprinting, confirm that the tropomyosin present in the crab plant air filter (filter 56) is responsible for allergenic reactivity in seafood plants. Based on the intensity of the x-ray film, tropomyosin constitutes a large part of the airborne allergen. Further studies should be undertaken to identify the protein at 14.0 kDa.

Table 3.1 **The average molecular weights in daltons for the crab condensate, filter 56 and salmon tropomyosin determined by Coomassie blue stained electrophoresis gels.**

Crab Condensate (n=6)	Filter 56 (Air Sample Filter) (n=6)	Salmon Tropomyosin (n=4)
70100 ± 4500	74600 ± 2400	34600 ± 900
36000 ± 1700	36800 ± 3300	
20100 ± 850	15300 ± 1200	
17800 ± 450		
14000 ± 800		

Note: See appendix C for electrophoresis gels.

Table 3.2 **The molecular masses in daltons from linear MALDI for solubilized proteins.**

Snow Crab Condensate (n=3)	Filter 56 (Air Sample Filter) (n=2)	Salmon Tropomyosin (n=3)
65077 ± 471	32595 ± 18	31943 ± 44$[M+H]^{+}$
32580 ± 15	16330 ± 25	16186 ± 10$[M+2H]^{2+}$
16357 ± 74	13983 ± 45	11149 ± 58$[M+3H]^{3+}$
13980 ± 248	12681 ± 9	
12142 ± 31	11267 ± 139	
11141 ± 2	10557 ± 170	
	8728 ± 9	

Note: See appendix B for individual spectrums.

Table 3.3 **Peptide mass peaks evident from trypsin digest of gel slice; background peptides.**

Gel slice 1	Gel slice 2
1005.4515	1042.3954
1111.4537	1064.4495
1165.5886	1026.3942
2163.0297	1539.7293
2273.0173	1838.9264
2193.9519	2162.6636
2288.9788	2273.7060
3153.4585	3904.6239
3210.4048	

Note: See appendix D for individual spectrums.

Table 3.4 **Bovine Trypsin Autolysis peaks (MH^+) and human contamination peaks listed in the literature (19).**

Bovine Autolysis Peaks (MH^+)	Human Contamination peaks (MH^+)
659.3839	1016.842
805.4167	1030.850
906.5048	1031.847
1020.5034	1032.845
1153.5740	1036.861
1175.5228	1037.860
1433.7209	1038.857
1493.5999	1046.806
1676.7766	1047.812
1774.8506	1050.849
2163.0566	1054.841
2193.9946	1060.823
2273.1599	1066.822
2289.1548	1072.841
2305.1497	1082.815
2514.3389	1225.939
2550.2329	1268.919
2552.2485	1346.550
2612.1812	1556.591
2613.3499	1582.608
3211.4746	1688.746
3227.4695	1774.830
	1859.818
	2285.191

Table 3.5 **Peptide mass fingerprint peaks evident from trypsin digest of the Bovine Serum Albumin (standard) electrophoresis bands – replicate analysis.**

BSA standard (replicate 1)	BSA standard (replicate 2)	BSA standard (replicate 3)	BSA standard (replicate 4)	BSA standard (replicate 5)
1040.1294	1083.5363	1063.6709	1147.7825	1083.6702
1056.0866	1163.5441	1279.7154	1163.6709	1163.6596
1249.2408	1305.5532	1306.6753	1279.7154	1305.6492
1439.2681	1439.5663	1439.6504	1306.6753	1439.6591
1479.2330	1479.5267	1448.7620	1439.6504	1441.6599
1568.0876	1567.4145	1479.6880	1448.762	1568.5127
1640.2399	1640.5598	1567.5136	1479.6088	1640.6468
1724.0373	1724.4007	1639.6381	1567.5136	1724.5059
1946.9691	1748.2377	1724.4867	1639.6381	1881.4445
2044.9500	1881.3616	1731.4655	1724.4867	2044.4150
2295.8195	2045.3510	1880.4278	1731.4655	2300.2236
2497.7587	2163.3058	2045.390	1880.4278	2529.2327
2528.6865	2273.3898	2163.3616	2045.3900	
2868.3370	2529.1912	2273.3174	2301.2565	
3589.9858	2612.1583	2301.2565	2499.2667	
3279.0227		2512.2569	2512.2569	
		2529.1654	2529.1654	
		2612.0229	2612.0229	
Mowse score = 94	Mowse score = 124	Mowse score = 66	Mowse score = 86	Mowse score = 115
% protein coverage = 20%	% protein coverage = 24%	% protein coverage = 18%	% protein coverage = 23%	% protein coverage = 23%

Note: See appendix E for individual spectrums; only replicates 1-3 included.
Mowse score >66 means that the protein has been identified at the 95% confidence level based on peptide matching.
Protein coverage is the percentage of peptide sequences matching the peptide sequences in a known protein.

Table 3.6 Peptide mass fingerprint peaks from salmon tropomyosin electrophoresis bands – replicate analysis.

Salmon tropomyosin	Salmon tropomyosin	Salmon tropomyosin	Salmon tropomyosin
1060.2368	1308.9950	1309.5758	1308.5733
1271.1128	1711.6827	1589.5242	1433.5328
1727.5903	1728.0683	1642.4042	1490.5414
1815.5105	1816.0176	1728.4542	1589.5150
1854.4204	1884.0949	1815.3703	1728.4517
1883.5412	1944.0706	1884.3928	1794.3995
1944.5054	2175.0366	2175.2243	1815.3675
2174.4241	2274.1174	2491.0711	1866.9390
2491.2792	2417.1197	2544.0181	1884.3881
2544.3823	2492.0160	2853.7955	2174.2117
	2545.1234	3153.5097	2416.1100
			2490.0288
			2544.0388
Mowse score = 78	Mowse score = 79	Mowse score = 68	Mowse score = 90
Percent protein coverage = 35%	Percent protein coverage = 37%	Percent protein coverage = 40%	Percent protein coverage = 40%

Note: See appendix F for individual spectrums.
See table 3.5 for meaning of mowse score and percent protein coverage.

Table 3.7 **Peptide mass fingerprint peaks for the 70 kDa snow crab condensate electrophoresis bands.**

70 kDa (replicate 1)	70 kDa (replicate 2)	70 kDa (replicate 3)	70 kDa (replicate 4)	70 kDa (replicate 5)
1016.7689	1286.6403	1070.7211	1207.5495	1207.7253
1207.7321	1403.6958	1207.6945	1268.5819	1217.7849
1268.7216	1465.5766	1286.6188	1380.5069	1268.7347
1286.6901	1550.6527	1562.6067	1578.5079	1287.7070
1337.7351	1562.5815	1578.6112	1620.5006	1320.6295
1352.6829	1578.6310	1621.6623	1818.4488	1336.7254
1414.7648	1626.5639	1714.4109	1828.3956	1352.6948
1428.7776	1839.4989	1776.5234	1870.4344	1385.7314
1576.6624	1866.5067	1828.4935	2288.2323	1414.7771
1714.4892	1964.5459	2195.3430	2443.0532	1541.6764
1743.5948	2028.4146	2266.2159	2497.0413	1578.6733
1770.5911	2110.4860	2454.0552	2561.1132	1599.6318
1828.5723	2157.4066	2496.0789		1714.4938
1870.5821	2259.3663	2511.0965		1770.5811
1898.5766	2412.2601	2531.1439		1827.5762
1915.5132	2453.2361	2547.1491		1871.5751
1923.5674	2497.2404	2561.1168		1898.5832
2060.4372	2502.2351	2775.9276		1914.4822
2359.3645	2549.2297	2807.8975		1923.5620
2497.2761	2808.2073	2829.9318		2359.3582
2549.3322	2830.2104	3230.5832		2497.2698
2809.2160		3264.6597		2561.3822
		3279.6880		2643.3001
		3314.8371		2809.2025
		3732.3613		3152.9473
		3727.0146		
Mowse score = ND	Mowse score = ND	Mowse score = ND	Mowse score = ND	Mowse score = ND
Percent protein coverage = ND	Percent protein coverage = ND	Percent protein coverage = ND	Percent protein coverage = ND	Percent protein coverage = ND

ND – Protein could not be identified from database search.

Note: See appendix G for individual spectrums; only replicates 1-3 included.

Table 3.8 **Peptide mass fingerprint peaks for the 36kDa snow crab condensateelectrophoresis bands.(The protein was identified as tropomyosin from rock crab.)**

36 kDa (replicate 1)	36 kDa (replicate 2)	36 kDa (replicate 3)	36 kDa (replicate 4)	36 kDa (replicate 5)
1220.6752	1035.0800	1129.4656	1220.4264	1129.9324
1432.6793	1051.5215	1220.4370	1432.3531	1433.8900
1518.6711	1131.4149	1432.4898	1588.3141	1588.9106
1589.6864	1148.5170	1589.5289	1663.1947	1663.8153
1664.5449	1165.5150	1663.4912	1812.1562	1812.8164
1812.5591	1220.3400	1811.5220	1940.1249	1850.7499
1854.6496	1376.2644	1854.6234	2159.0673	1940.8359
2234.3917	1588.2701	1940.5826	2232.9249	2136.8096
2342.4427	1620.2863	2135.6096	2315.0049	2234.7180
2409.3190	1663.1821	2233.6166	2347.8941	2347.7238
2449.3552	1759.2070	2315.7023	2488.8733	2488.7021
2488.4180	1794.9005	2488.7400	2537.7969	2538.6825
2538.3236	1811.1524	2538.6643		
2644.3870	1827.1750			
2753.2332	1940.2346			
	2216.4948			
	2233.9783			
	2438.9944			
	2488.0237			
	2537.9124			
	2733.9462			
	3047.5526			
	3306.7243			
Mowse score = 73	Mowse score = 77	Mowse score = 91	Mowse score = 57	Mowse score = 71
Percent protein coverage = 46%	Percent protein coverage = 43%	Percent protein coverage = 42%	Percent protein coverage = 33%	Percent protein coverage = 35%

Note: See appendix H for individual spectrums; only replicates 1-3 included.
See table 3.5 for the meaning of mowse score and percent protein coverage.

Table 3.9 Peptide mass fingerprint peaks for 18 and 20 kDa snow crab condensate electrophoresis bands.

18 kDa (replicate1)	18 kDa (replicate 2)	22 kDa (replicate 1)
1165.7763	1165.8081	1058.7347
1279.7208	1449.7600	1079.5279
1478.5333	1634.2191	1207.5437
1674.4788	1674.5170	1287.5704
1830.4172	1831.4814	1295.6595
1901.4175	1883.5144	1352.5587
1919.4997	1902.4712	1477.5509
2172.3801	2052.3979	1542.5889
2272.3175	2172.4399	1599.5591
2301.2130	2272.3185	1742.6104
2335.2688	2302.3179	1757.5557
2721.1259	2721.2496	1770.5865
		1812.5915
		1827.6077
		1871.6339
		1950.7214
		2238.6230
		2274.6297
		2563.7192
Mowse score = ND	Mowse score = ND	Mowse score = ND
Percent protein coverage = ND	Percent protein coverage = ND	Percent protein coverage = ND

ND – Protein could not be identified from database search.

Note: See appendix I for individual spectrums.

Table 3.10 Peptide mass fingerprint peak for the 14 kDa snow crab electrophoresis bands.

14 kDa (replicate 1)	14 kDa (replicate 2)	14 kDa (replicate 5)
1010.1142	1037.1852	1005.6585
1037.2222	1080.2502	1077.6535
1080.2629	1202.6081	1192.5805
1259.6417	1259.6128	1233.6281
1267.6684	1267.6398	1264.5915
1282.6580	1348.8330	1502.5059
1348.8788	1507.1574	1740.3332
1425.0214	1631.3842	1902.2630
1506.1759	1922.9410	2053.1698
1722.6119	1943.0194	2073.1648
1922.9642	2058.1795	
1944.9939	2079.2148	
2058.2054	2190.4627	
2070.2295	2306.7553	
2077.2445	2492.1313	
2190.4257		
2493.1269		
Mowse score = ND	Mowse score = ND	Mowse score = ND
Percent protein coverage = ND	Percent protein coverage = ND	Percent protein coverage = ND

ND – Protein could not be identified from database search.

Note: See appendix J for individual spectrums.

Table 3.11 **Peptide mass fingerprint peaks for the 36kDa protein (isolated from crab plant air filter) (filter 56) electrophoresis bands. (The protein was identified as tropomyosin from rock crab.)**

36 kDa (replicate 1)	36 kDa (replicate 2)	36 kDa (replicate 3)	36 kDa (replicate4)	36 kDa (replicate 5)
1220.5674	1130.1185	1111.6201	1220.4205	1220.4197
1432.5612	1377.0739	1433.5546	1433.4630	1432.3537
1589.5648	1433.1218	1588.5482	1588.5243	1588.3119
1573.1261	1590.1740	1631.5415	1663.4391	1664.1790
1795.1031	1664.1035	1759.4868	1759.5268	1794.7519
1811.4869	1760.1808	1811.4284	1795.0555	1812.1559
1853.5794	1812.1217	1877.3491	1812.4846	1853.2367
1940.5544	1846.1451	1940.4476	1854.6077	1939.1388
2234.4522	1940.1966	2136.3541	1940.5631	2159.0633
2315.5932	2290.1911	2348.2143	2159.6071	2233.9315
2347.4373	2350.1555	2488.2346	2233.5594	2315.0323
2488.5373	2489.1762	2538.1555	2315.6562	2348.8652
2538.4652	2539.2199		2348.6110	2487.8663
			2470.6702	2537.7728
			2488.6406	
Mowse score = 62	Mowse score = 75	Mowse score = 100	Mowse score = 83	Mowse score = 78
Percent protein coverage = 33	Percent protein coverage = 37	Percent protein coverage = 33	Percent protein coverage = 41	Percent protein coverage = 43

Note: See appendix k for individual spectrums; only replicates 1-3 included.
See table 3.5 for the meaning of mowse score and percent protein coverage.
See appendix L for sample database search results.

CHAPTER 4 CONCLUSION

Combining one dimensional electrophoresis and MALDI-TOF MS allowed for the identification of aerosolized snow crab proteins. The in-gel digestion with trypsin is a quick and effective method when combined with on-line database searching, shortening the testing time of traditional methods by as much as 3 weeks (RAST method). In addition, the peptide mass fingerprinting facilitated the identification of any post-translational modifications for some samples.

The positive results of this study include:

1. Tropomyosin was identified as a major aerosolized protein.
2. RAST study showed that tropomyosin is a major aerosolized allergen.
3. Rapid MALDI-TOF MS can be used for direct conformation for the presence of specific proteins on air filters.

The SDS-PAGE technique used in this study could be further improved by the use of two-dimensional electrophoresis to allow for enhanced separation of the proteins present and the sequencing and adding of snow crab proteins to the on-line databases. The disadvantage of direct analysis by MALDI-TOF MS is the lack of quantitation, and the inability to tell if the protein mass causes an allergic reaction. The current RAST test allows for both quantitation and determination of allergenic reactivity.

While the issue of allergic reactivity cannot be addressed by the direct MALDI-TOF MS analysis, the problem of quantification can be addressed. Quantitative proteomic studies involving isotope labeling and cysteine alkylation offer promise as reliable quantitative MALDI techniques (26) when conducting peptide mass fingerprinting. Even without quantitation the peptide mass fingerprinting allows for the identification of some of the aerosolized snow crab proteins, which was the major objective of the study.

With the trend towards shorter snow crab fishing seasons the amount of time required to complete the RAST analysis may become a major disadvantage. Peptide mass fingerprinting on the other hand may afford the snow crab industry a rapid method that can help predict potential occurrences of occupational snow crab asthma from aerosolized proteins before it becomes a problem within the processing plant environment.

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Appendix A

Electrophoresis Gel for Linear MALDI-TOF MS Analysis

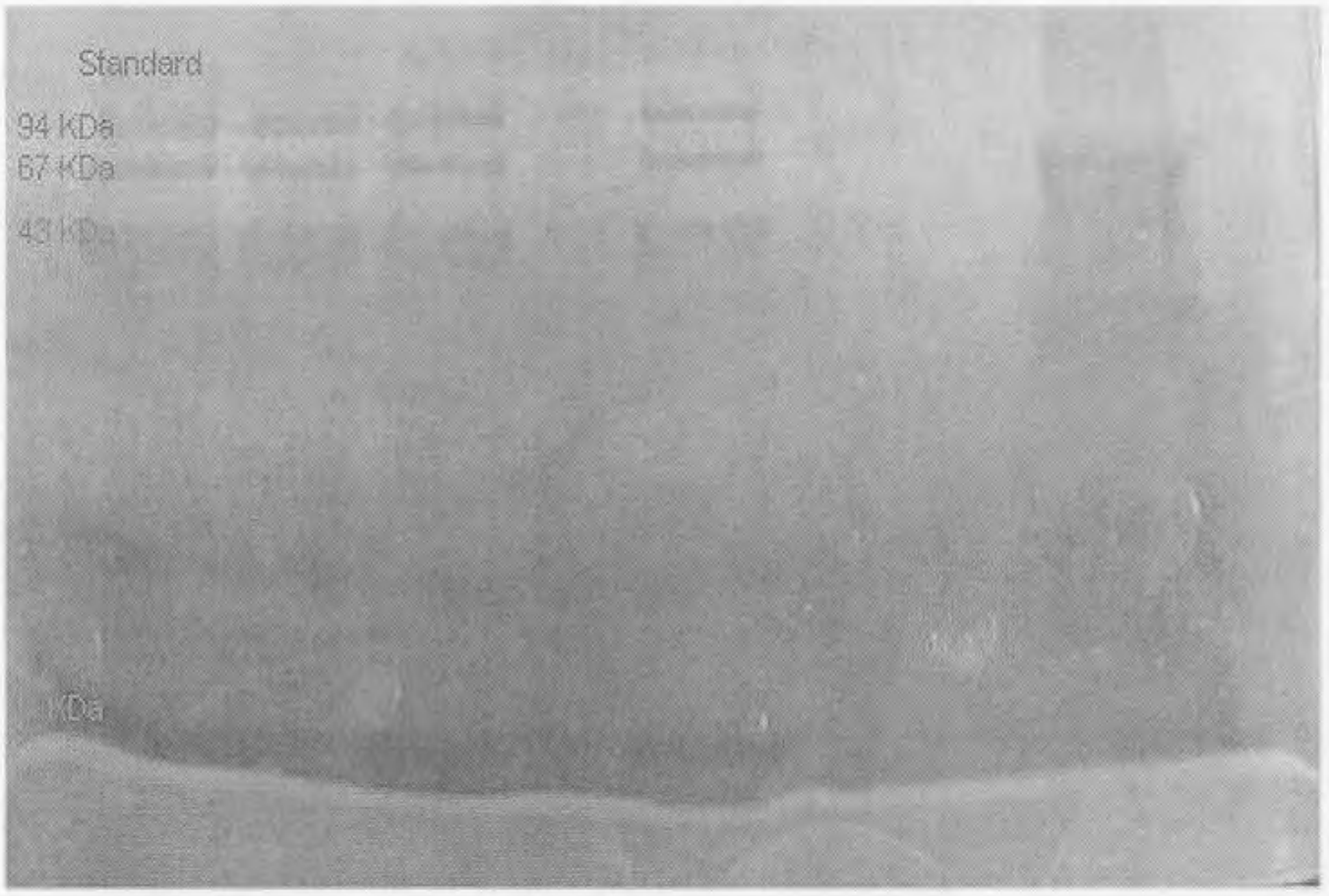


Figure A1: Copper stained electrophoresis gel used for passive elution and Linear MALDI analysis of protein molecular weight determination.

Appendix B

Protein Molecular Weight Spectrum from Linear MALDI Analysis

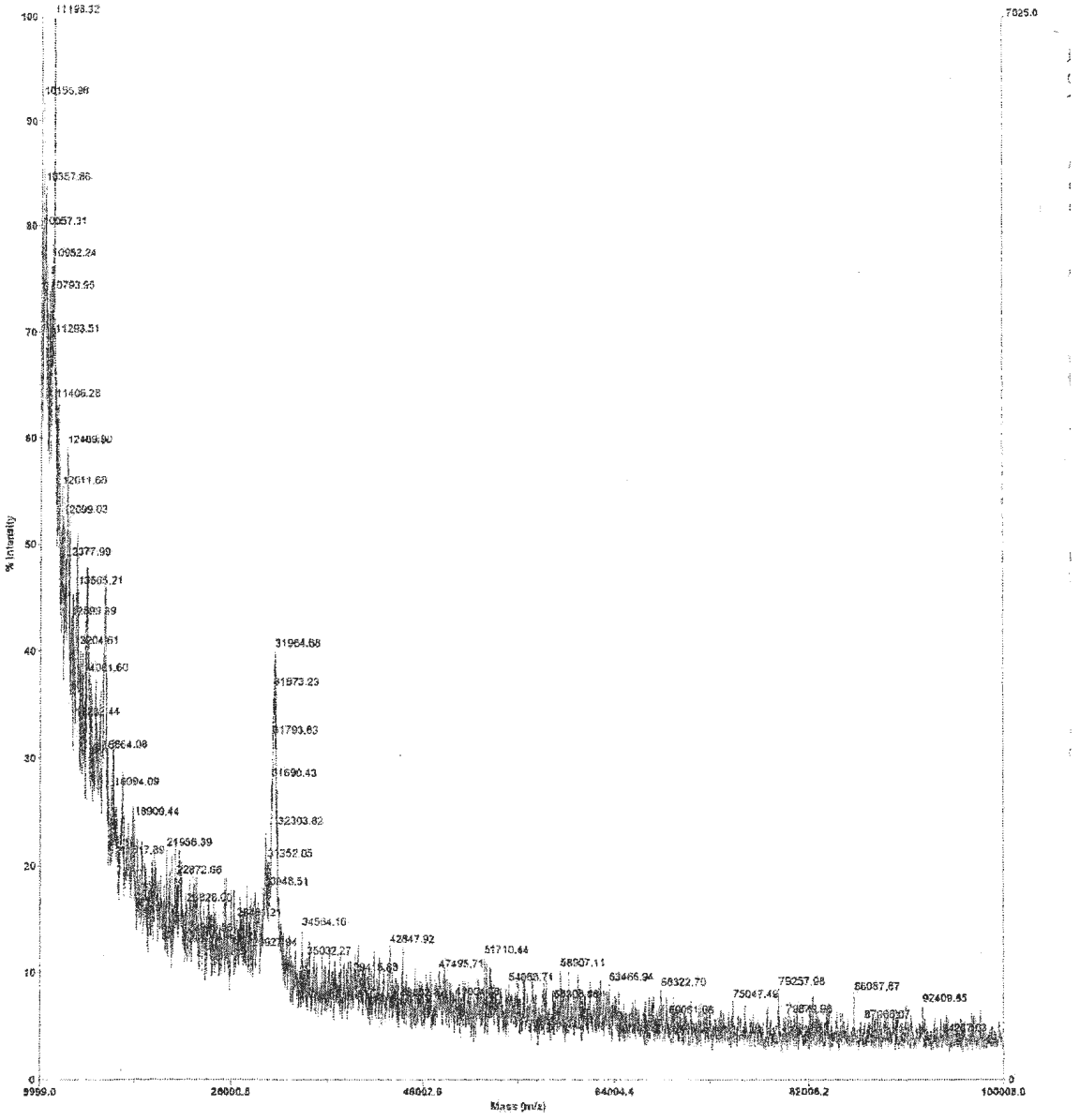


Figure B1: Linear MALDI spectrum for salmon tropomyosin protein (replicate 1).

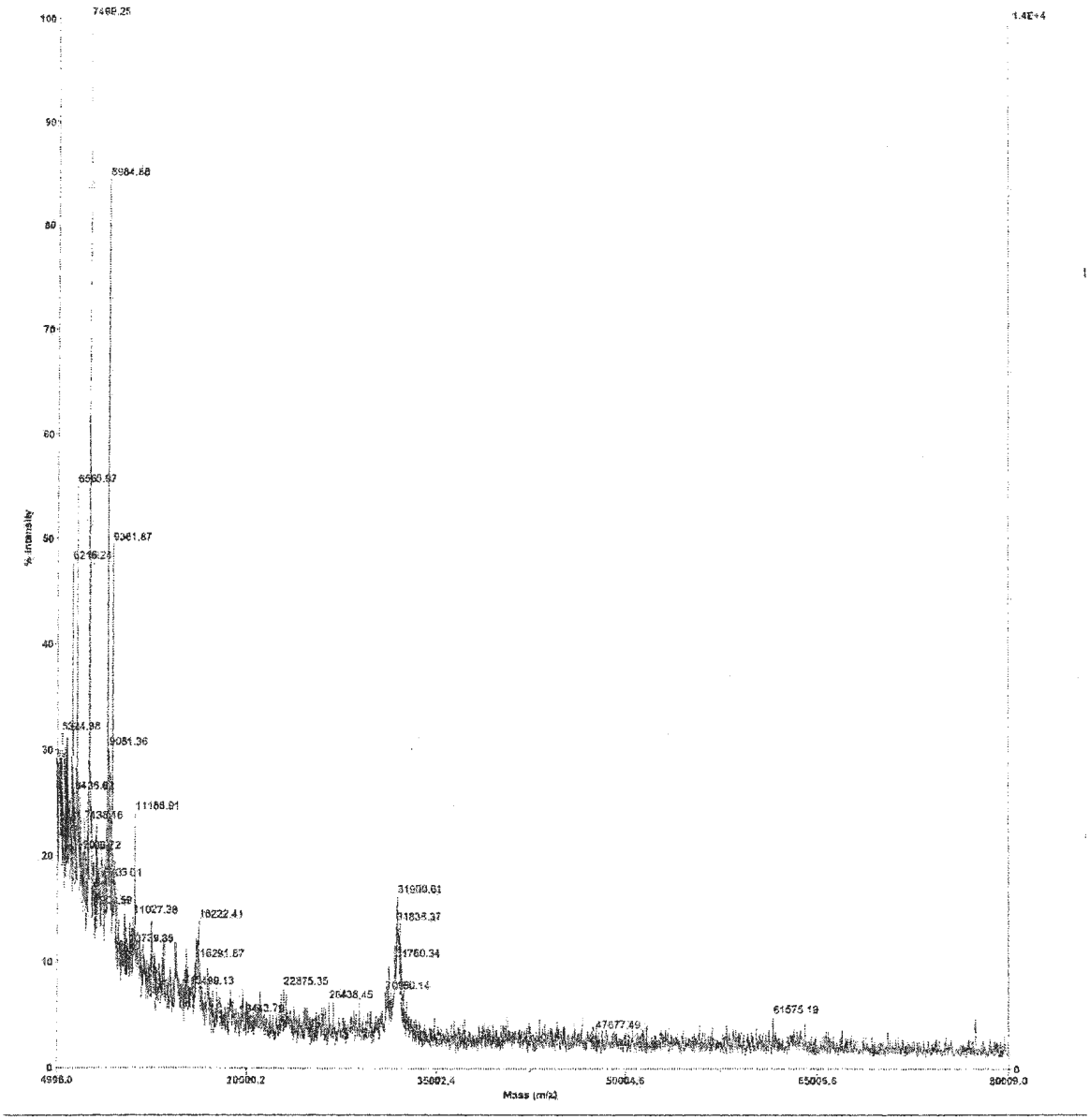


Figure B2: Linear MALDI spectrum for salmon tropomyosin protein (replicate 2).

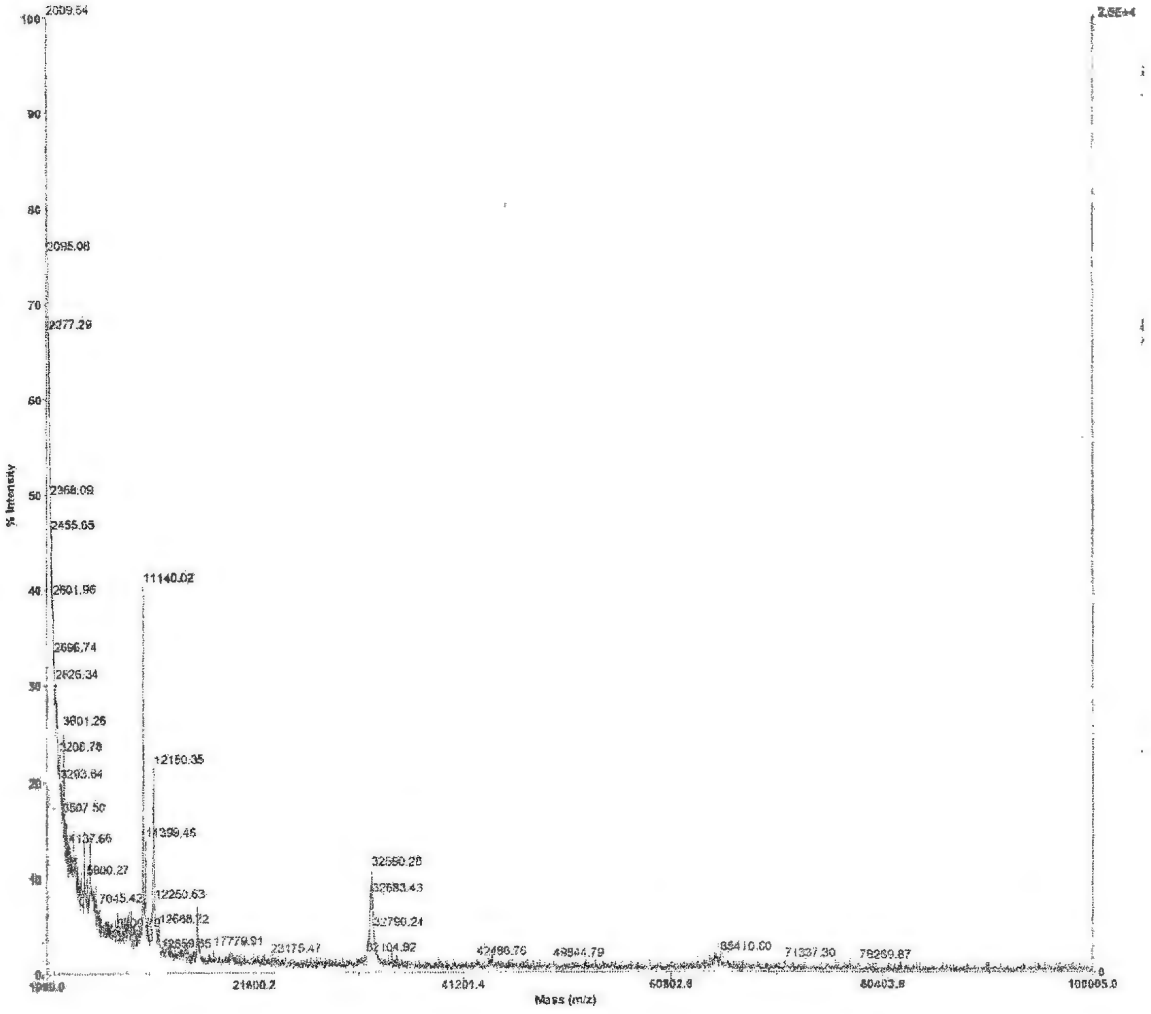


Figure B3: Linear MALDI spectrum of resolubilized snow crab condensate (replicate1).

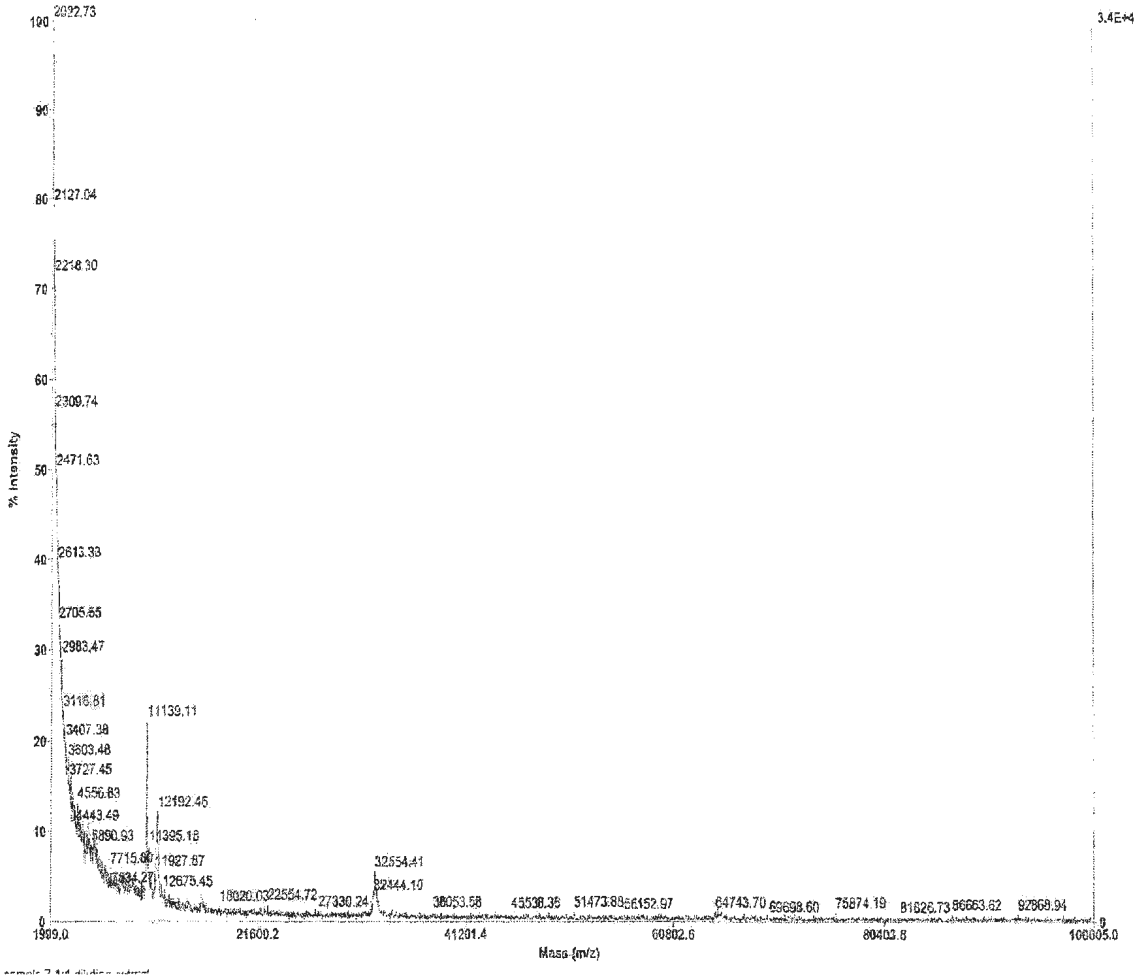


Figure B4: Linear MALDI spectrum of resolubilized snow crab condensate (replicate 2).

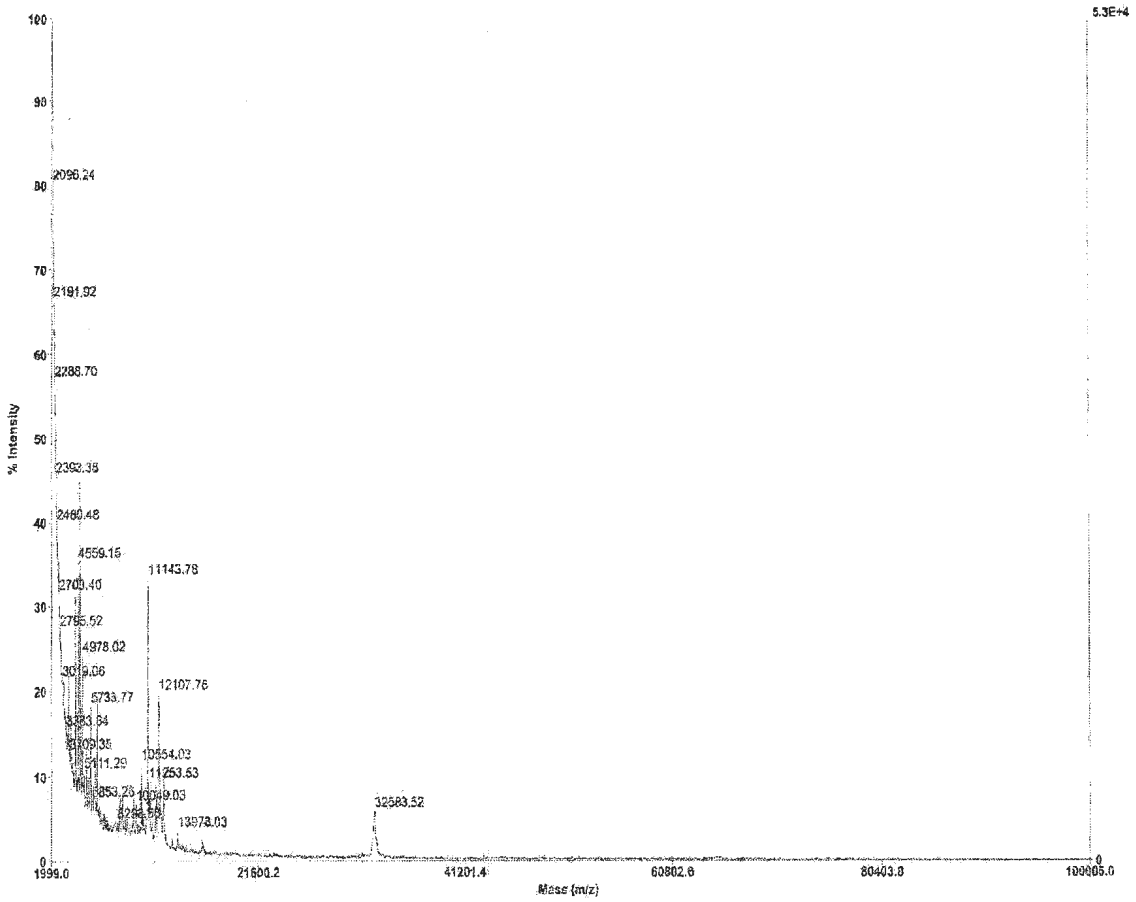


Figure B5: Linear MALDI spectrum of resolubilized snow crab condensate (replicate 3).

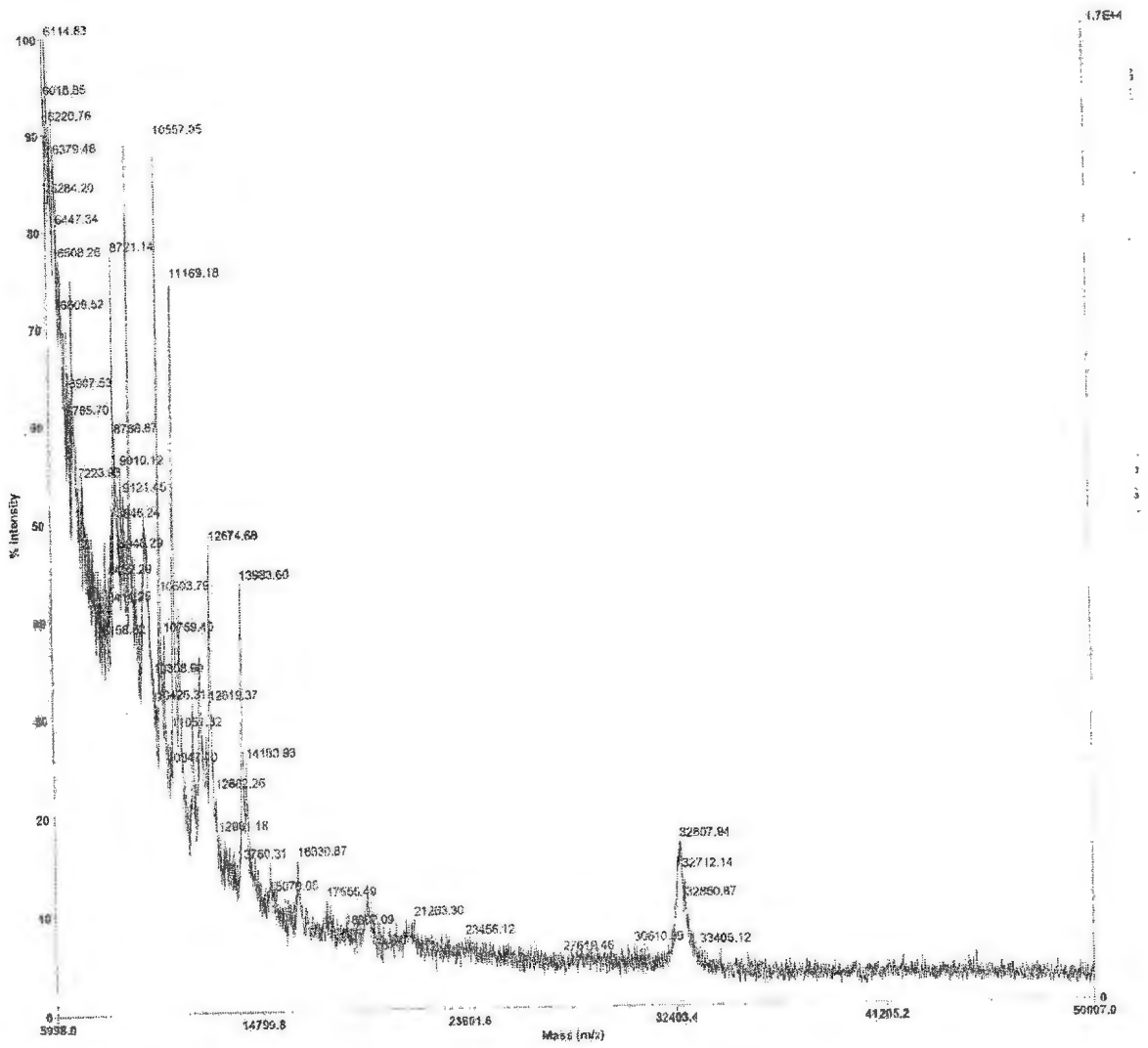


Figure B6: Linear MALDI spectrum of filter 56 (air filter sample replicate 1).

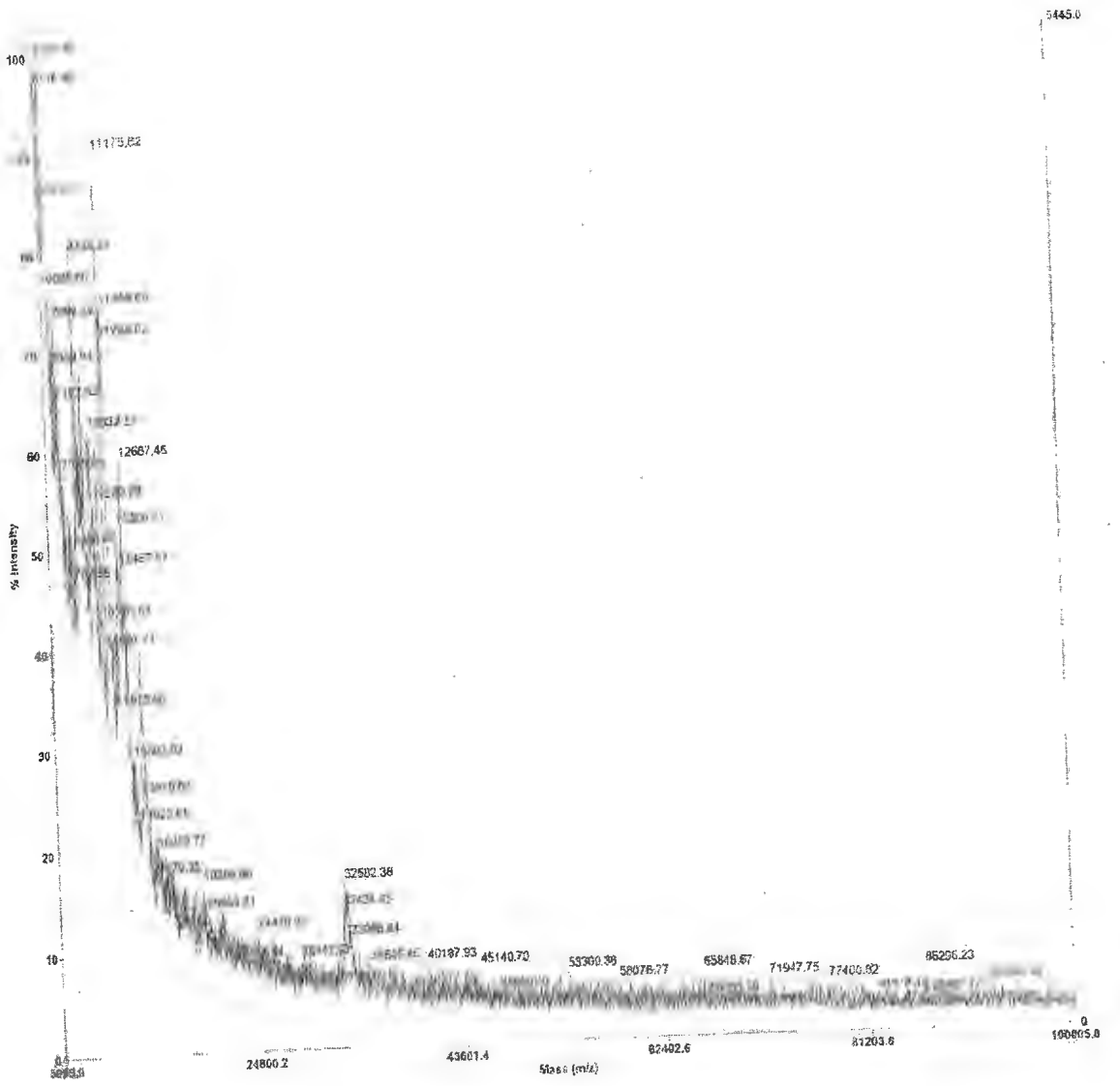


Figure B7: Linear MALDI spectrum of filter 56 (air filter sample replicate 2).

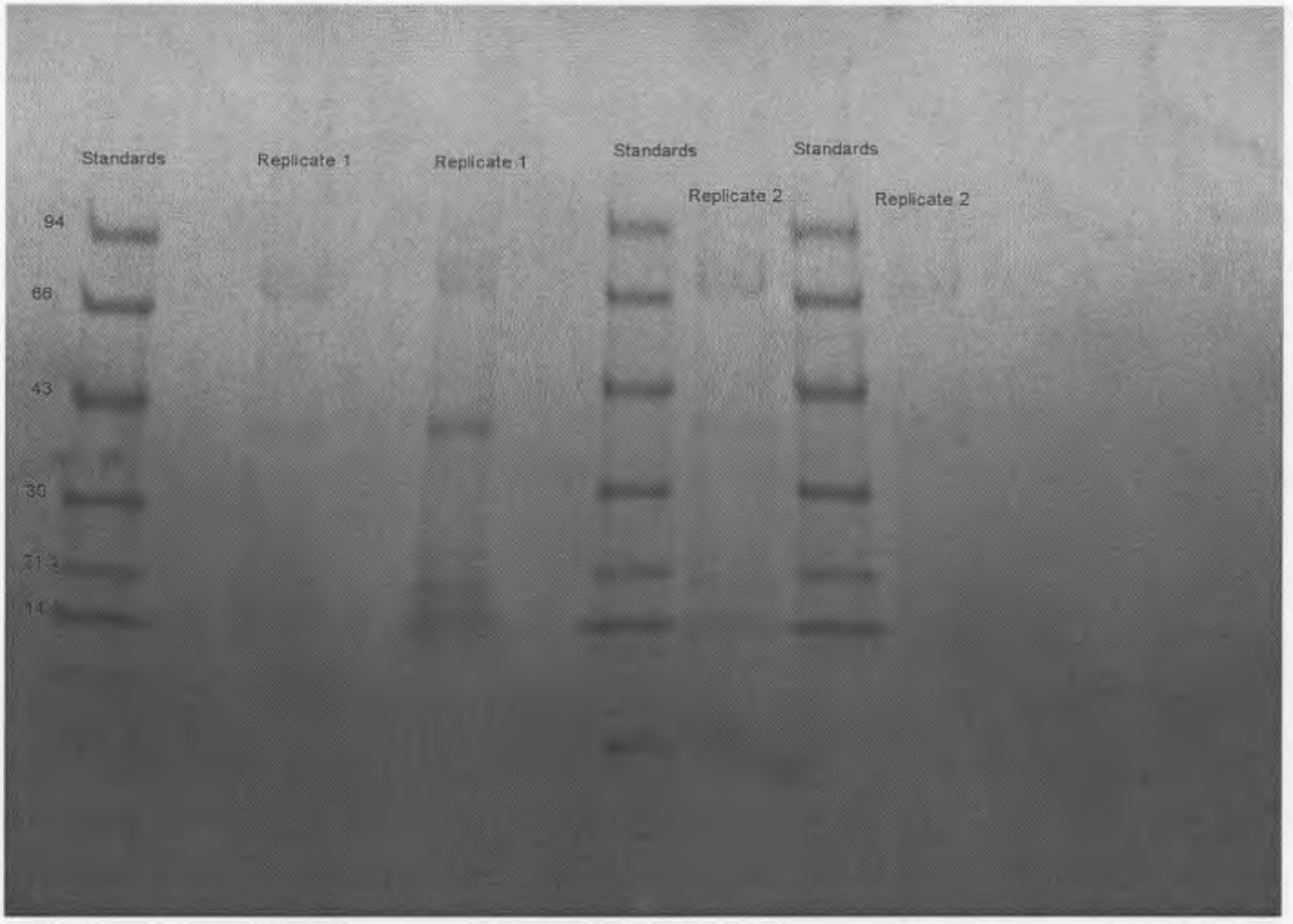


Figure C1: Coomassie blue stained electrophoresis gel used for peptide mass fingerprinting (replicate 1 and 2 are crab condensate samples).

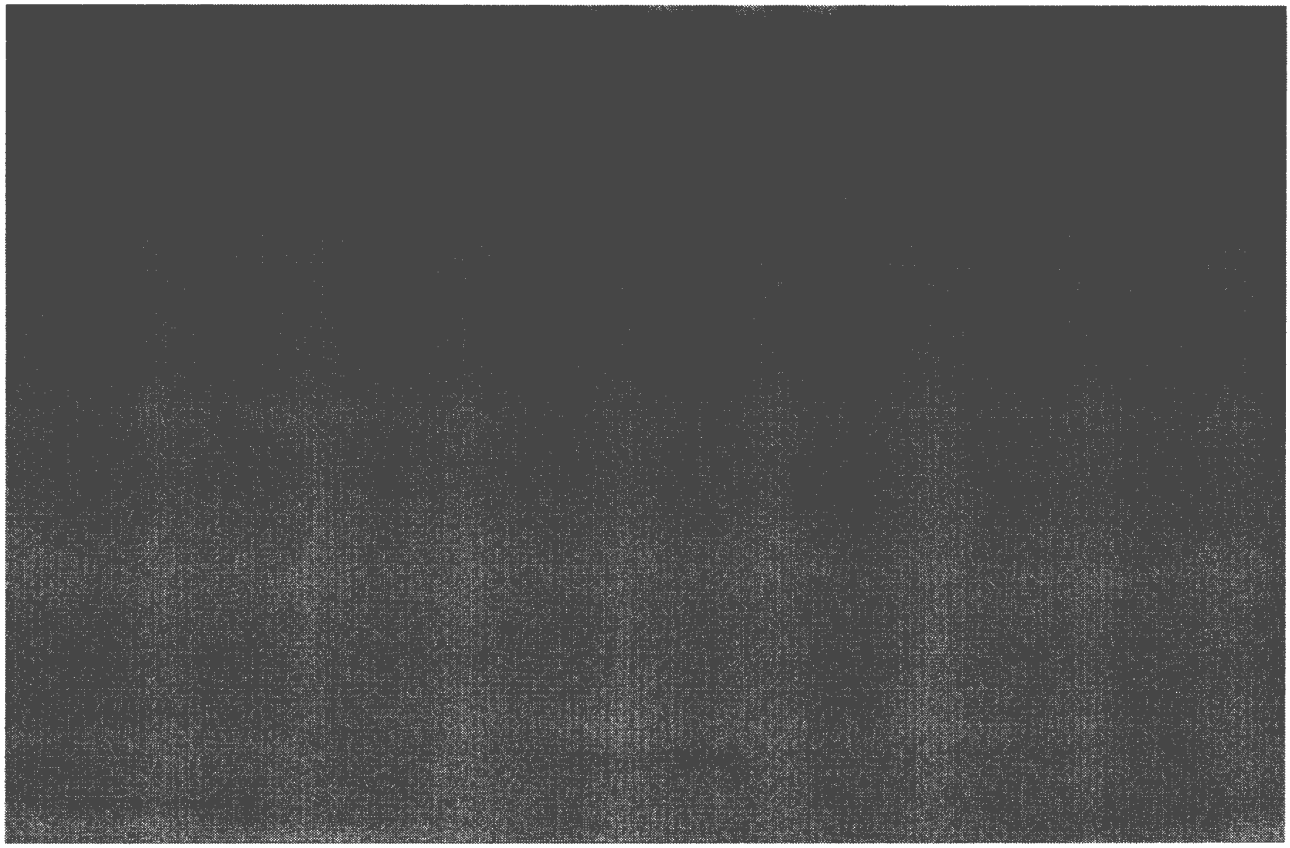


Figure C2: Coomassie blue stained electrophoresis gel used for peptide mass fingerprinting (filter 56 is the air filter sample and replicate 3 is a snow crab condensate sample).

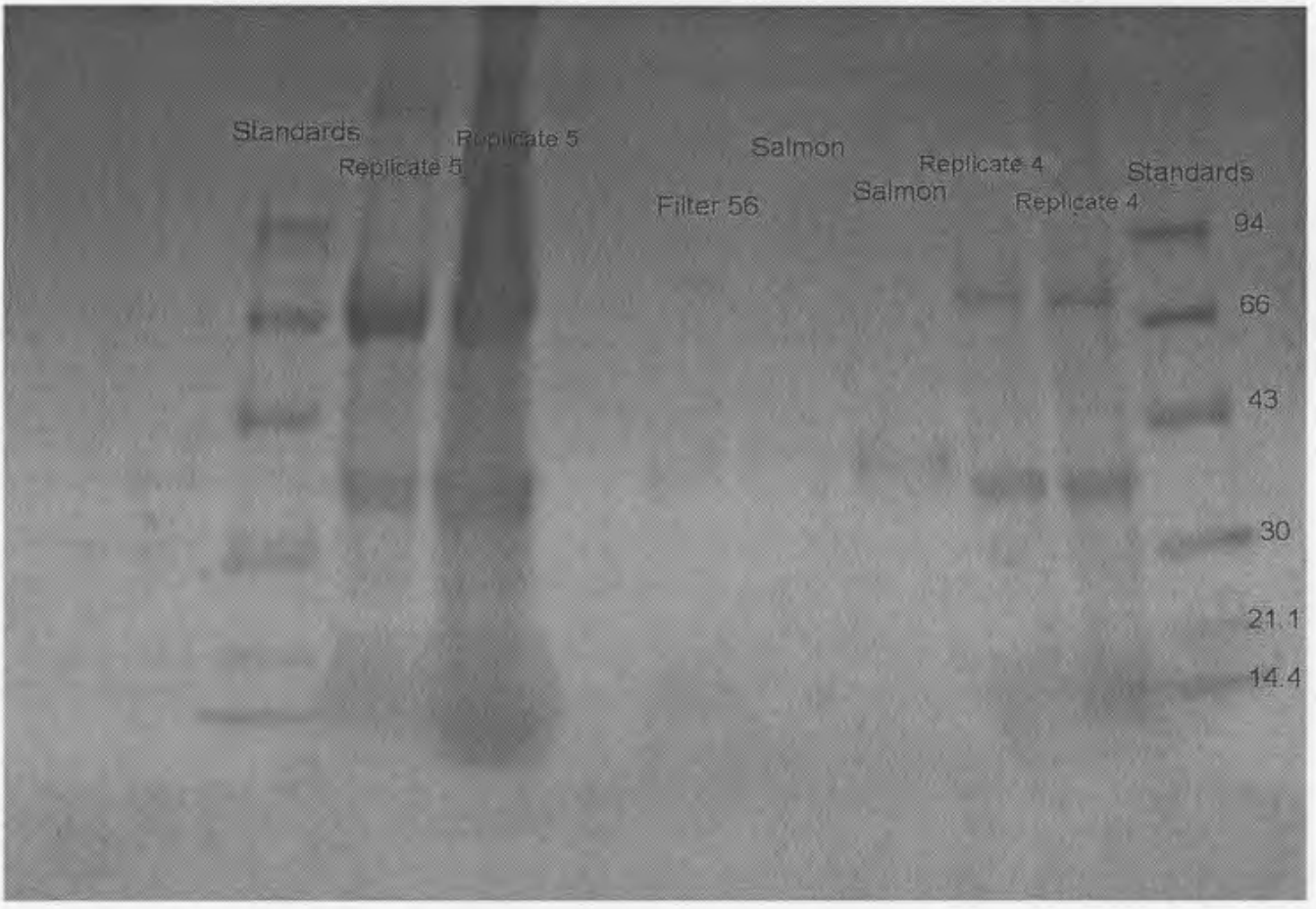


Figure C3: Coomassie blue stained electrophoresis gel used for peptide mass fingerprinting (Replicate 4 and 5 are snow crab condensate samples; filter 56 is an air filter replicate and salmon is the standard salmon tropomyosin).

Figure C4: Coomassie blue stained electrophoresis gel used for peptide mass fingerprinting (Replicate 5 and 6 are snow crab condensate samples; filter 56 is an air filter replicate and salmon is the standard salmon tropomyosin).

Appendix D

Background Gel Slice Spectrums from Tryptic Digest for Peptide Mass Fingerprinting

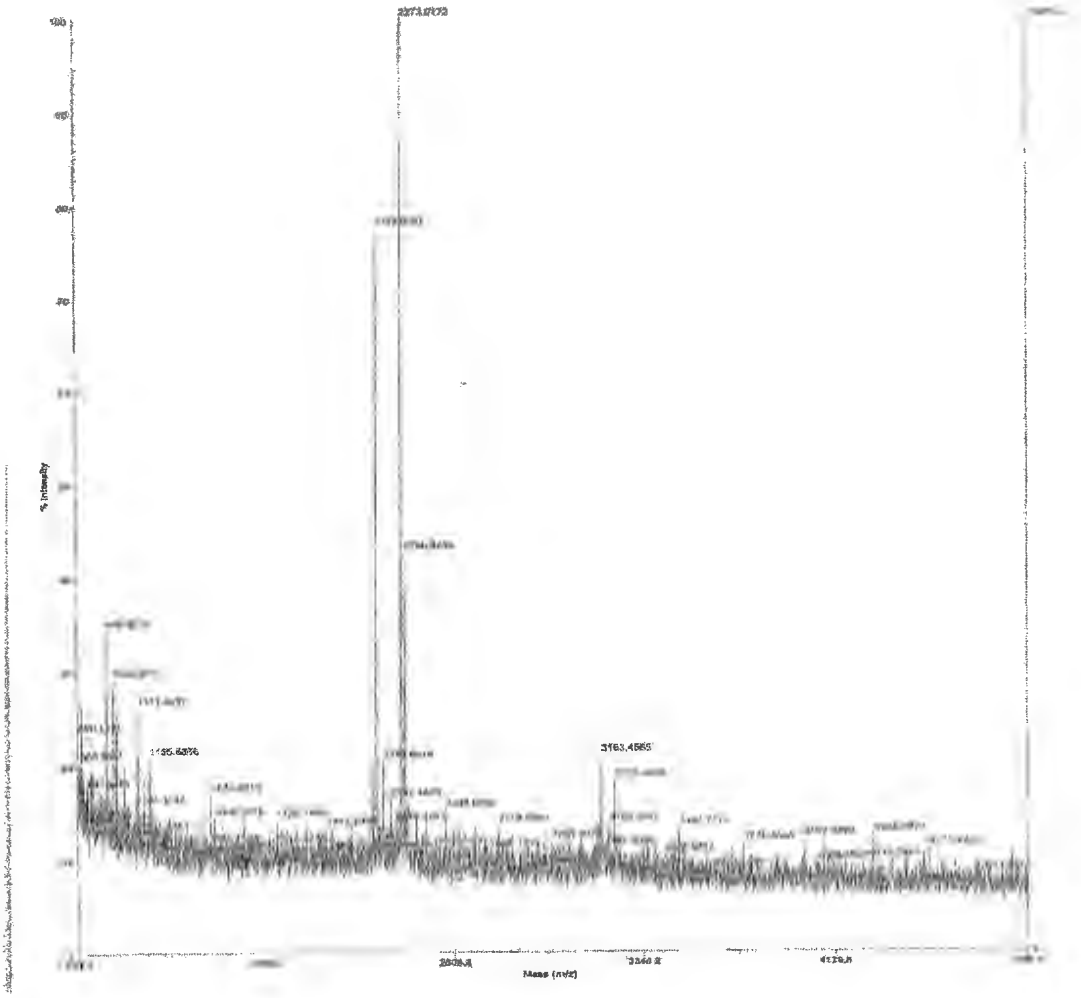


Figure D1: Peptide mass fingerprint spectrum produced from tryptic digest autolysis of gel slice.

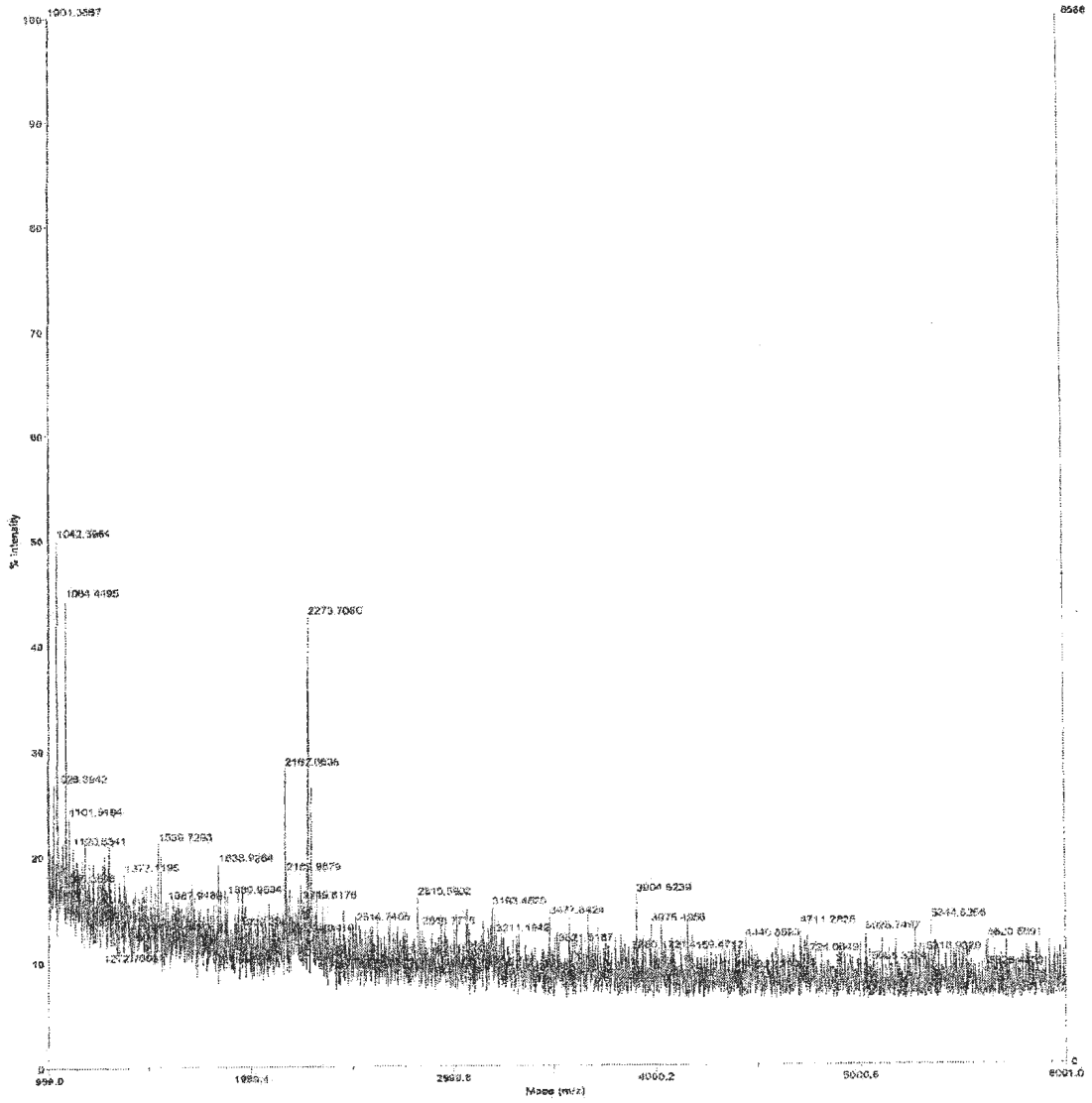


Figure D2: Peptide mass fingerprint spectrum produced from tryptic digest autolysis of gel slice.

Appendix E

Spectrums of BSA Standards for Peptide Mass Fingerprinting

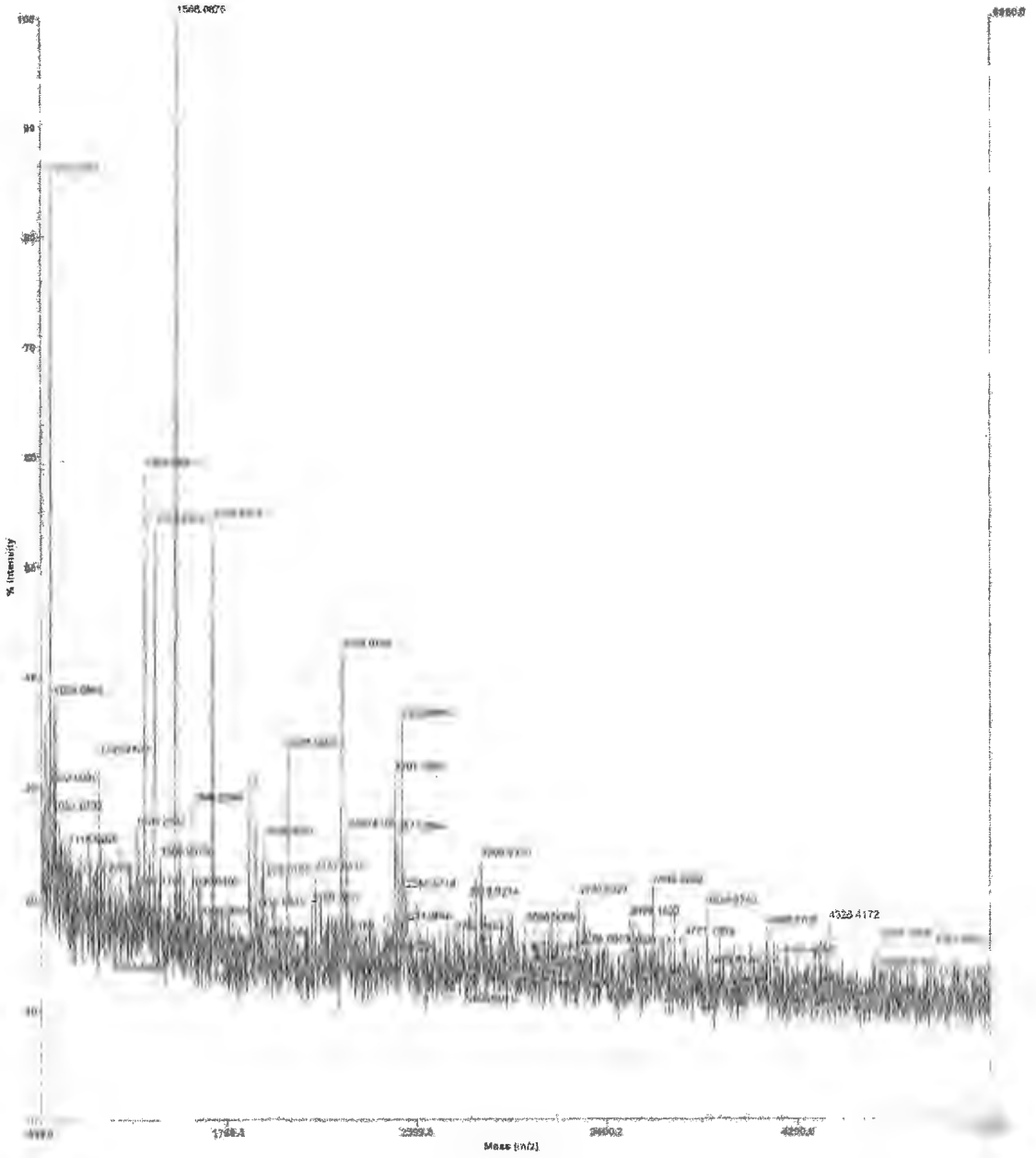


Figure E1: Peptide mass fingerprint spectrum produced from tryptic digest of BSA electrophoresis band (replicate 1).

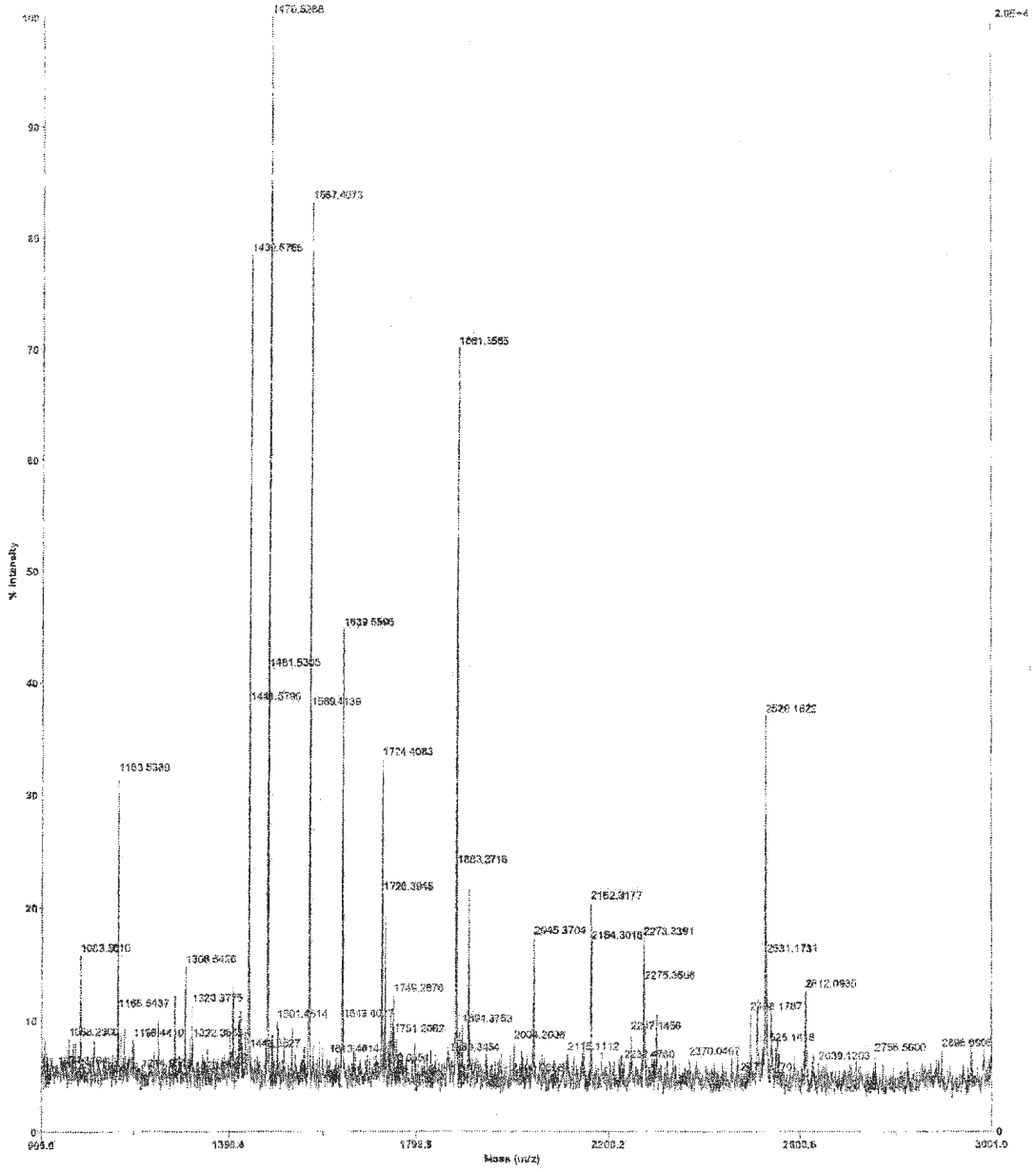


Figure E2: Peptide mass fingerprint spectrum produced from tryptic digest of BSA electrophoresis band (replicate 2).

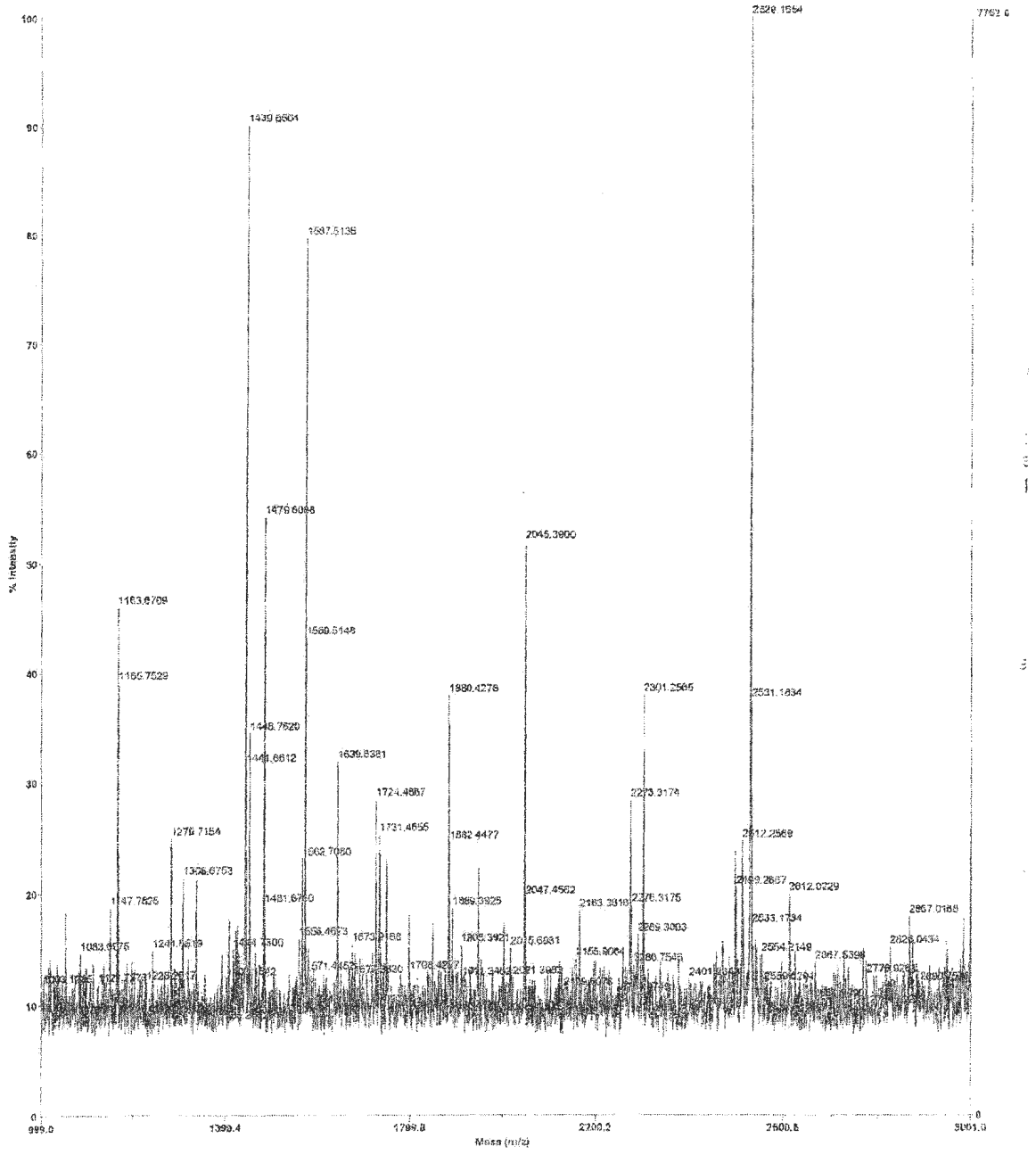


Figure E3: Peptide mass fingerprint spectrum produced from tryptic digest of BSA electrophoresis band (replicate 3).

Appendix F

Spectrums of Salmon Tropomyosin from Peptide Mass Fingerprinting

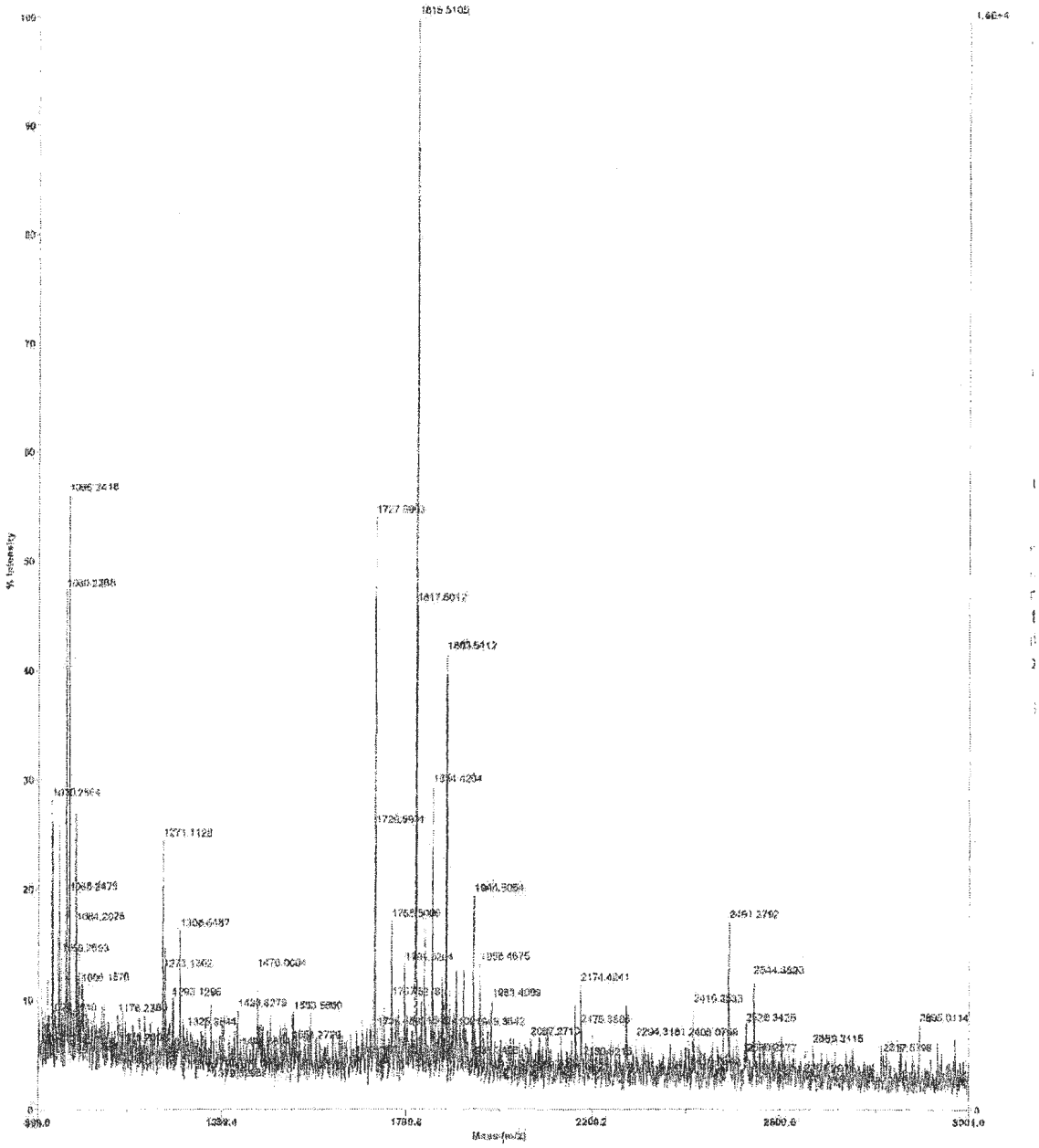


Figure F1: Peptide mass fingerprint spectrum produced from tryptic digest of salmon tropomyosin electrophoresis band (replicate 1).

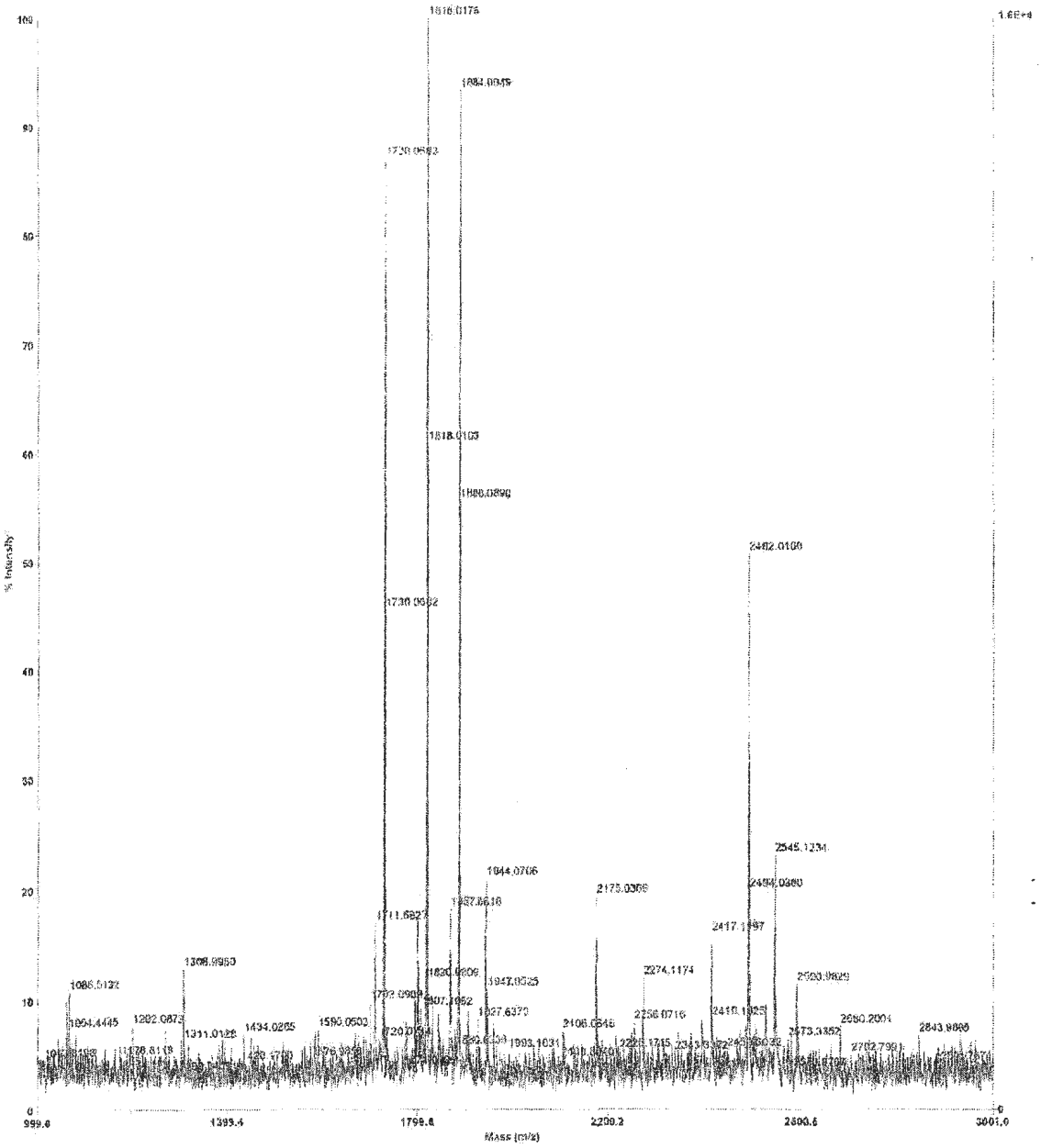


Figure F2: Peptide mass fingerprint spectrum produced from tryptic digest of salmon tropomyosin electrophoresis band (replicate 2).

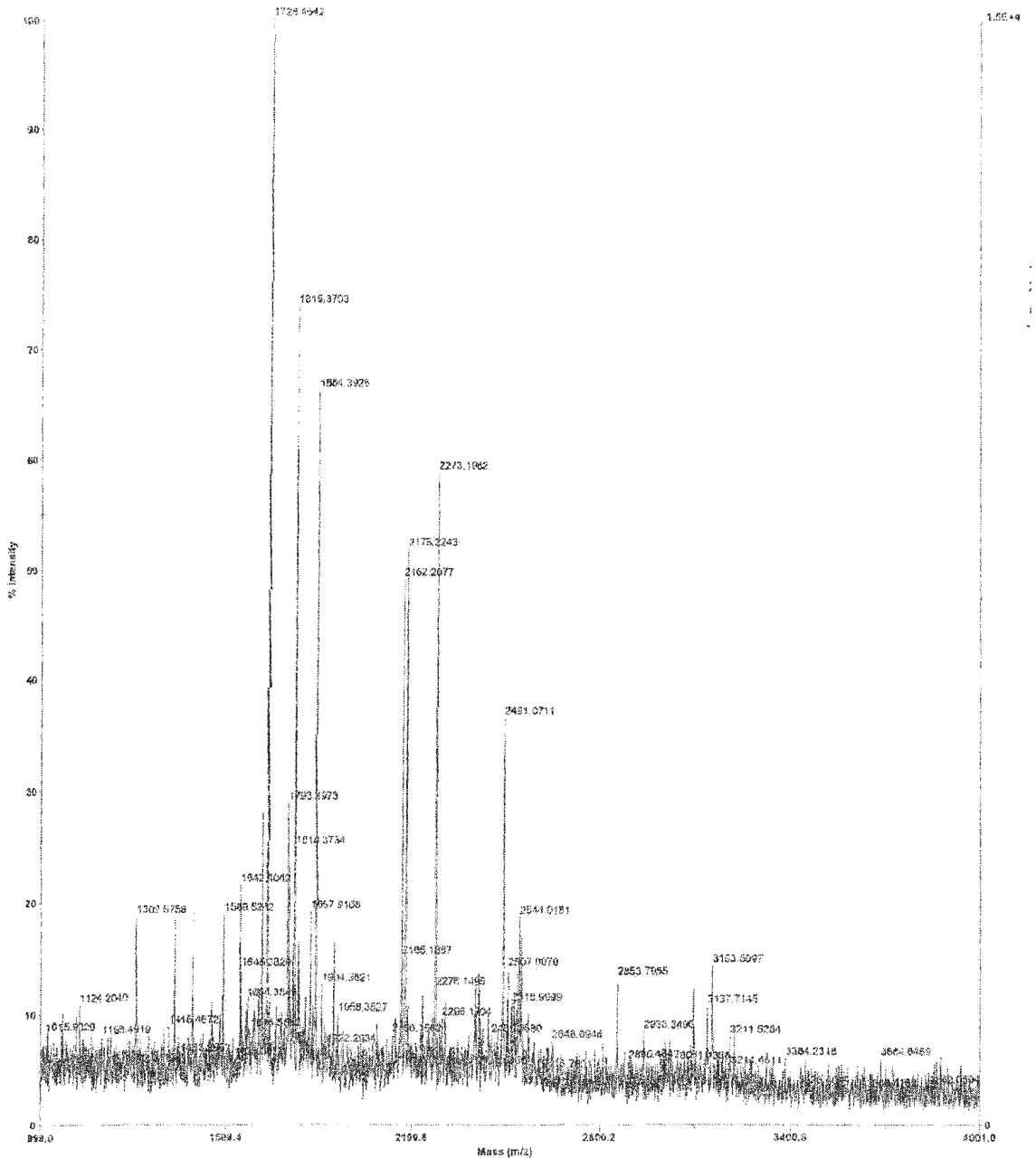


Figure F3: Peptide mass fingerprint spectrum produced from tryptic digest of salmon tropomyosin electrophoresis band (replicate 3).

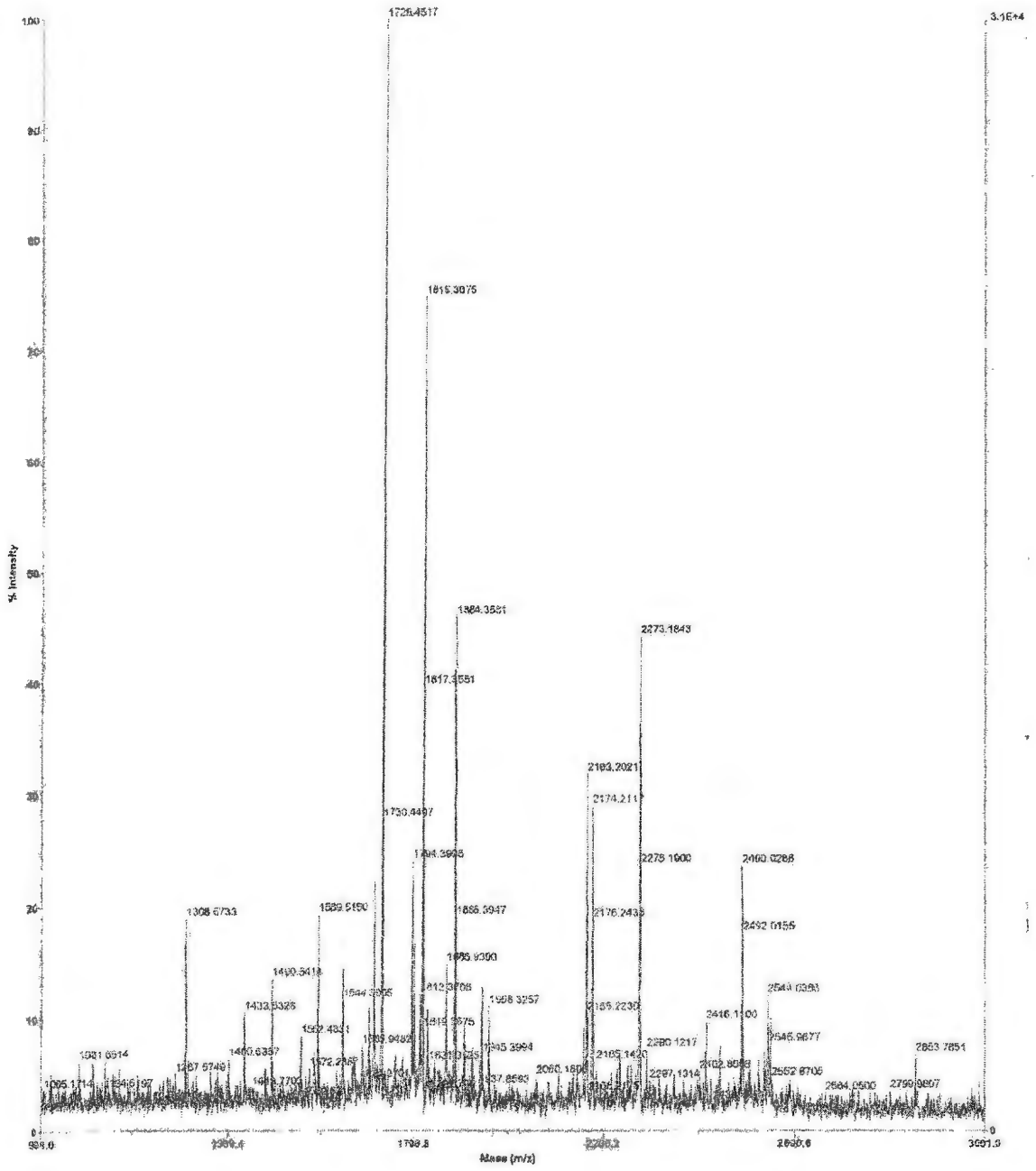


Figure F4: Peptide mass fingerprint spectrum produced from tryptic digest of salmon tropomyosin electrophoresis band (replicate 4).

Appendix G

Spectrums for 70 kDa band (Snow Crab Condensate) from Peptide Mass Fingerprinting

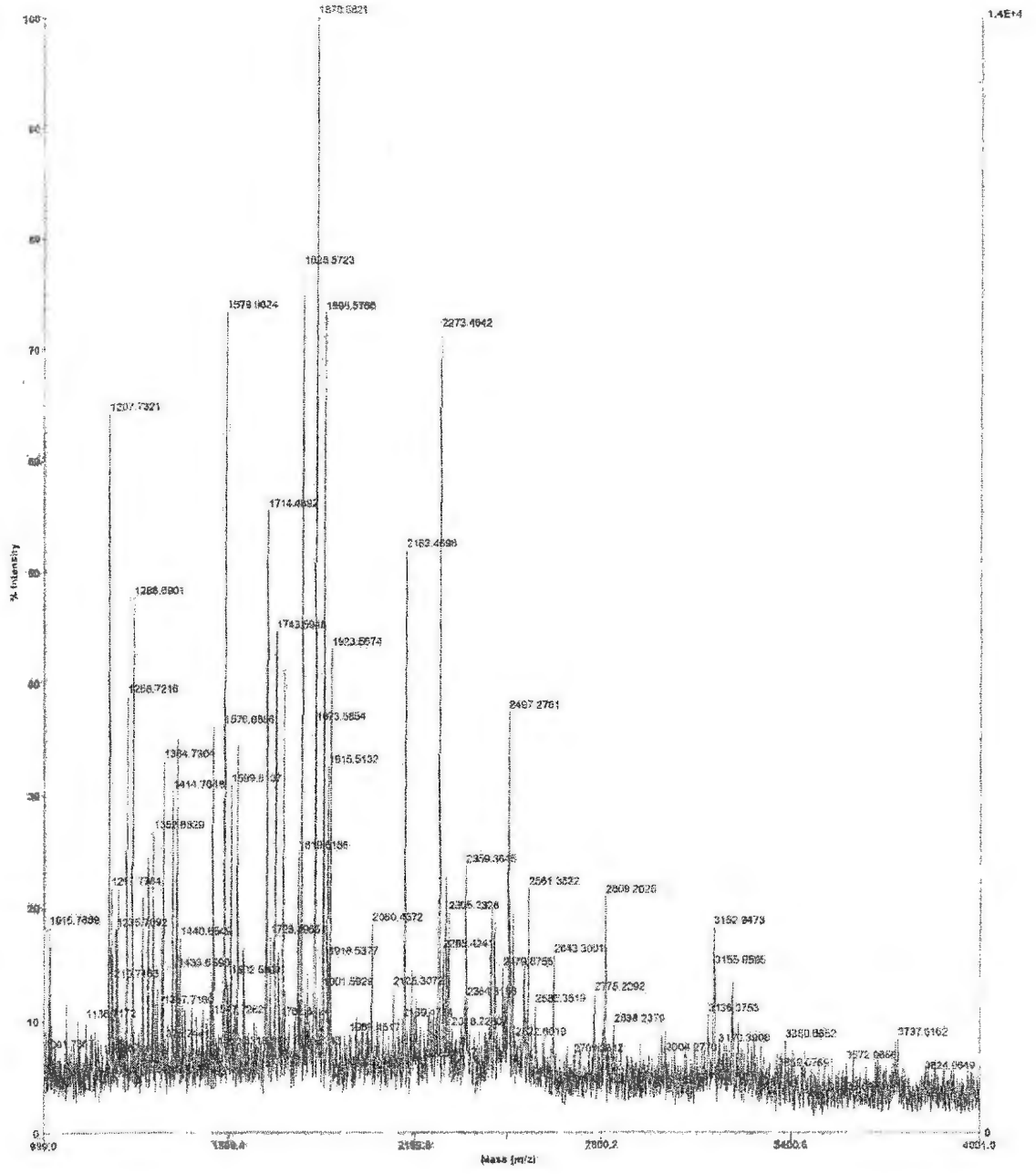


Figure G1: Peptide mass fingerprint spectrum produced from tryptic digest of 70kDa electrophoresis band (replicate 1).

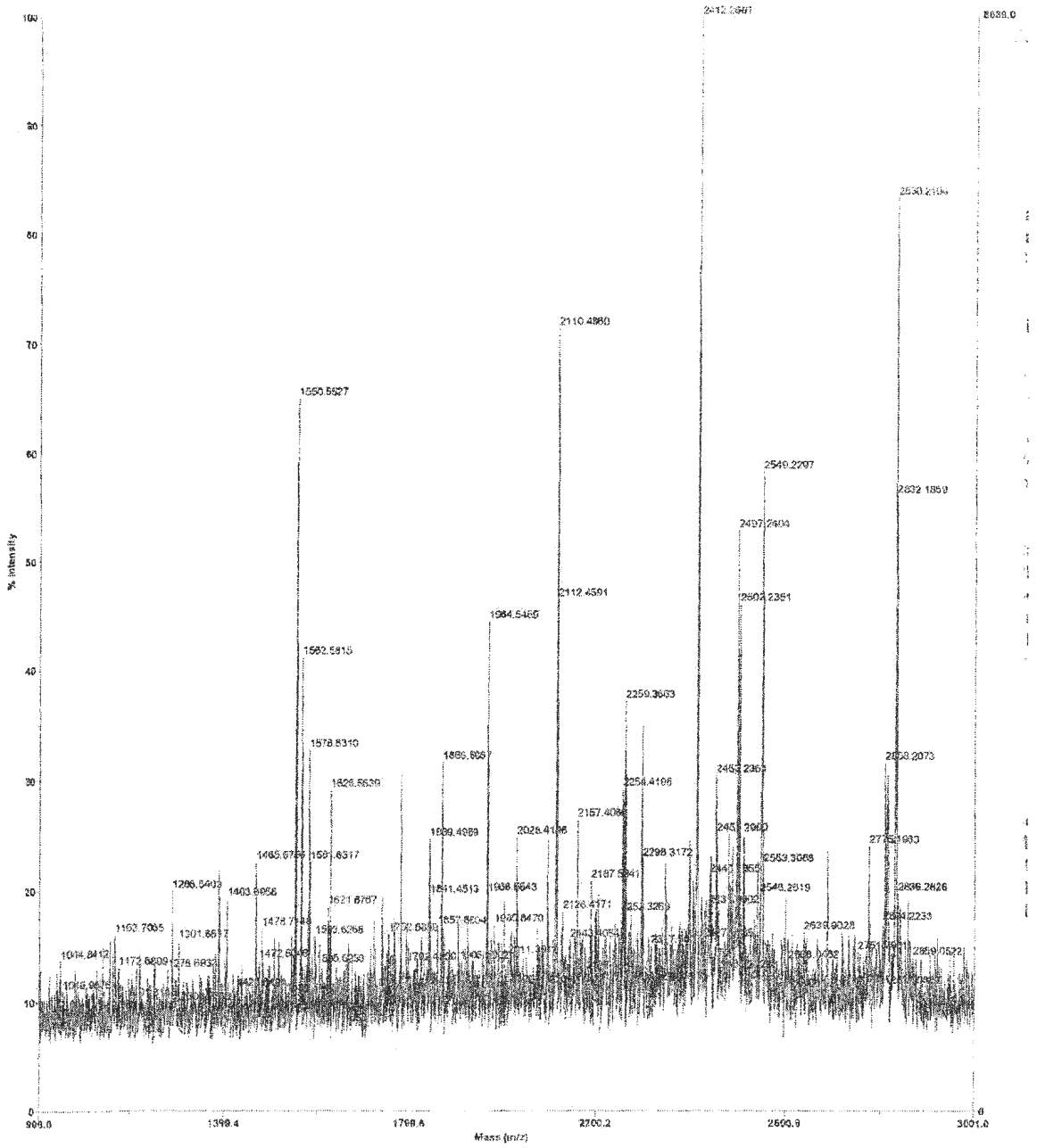


Figure G2: Peptide mass fingerprint spectrum produced from tryptic digest of 70kDa electrophoresis band (replicate 2).

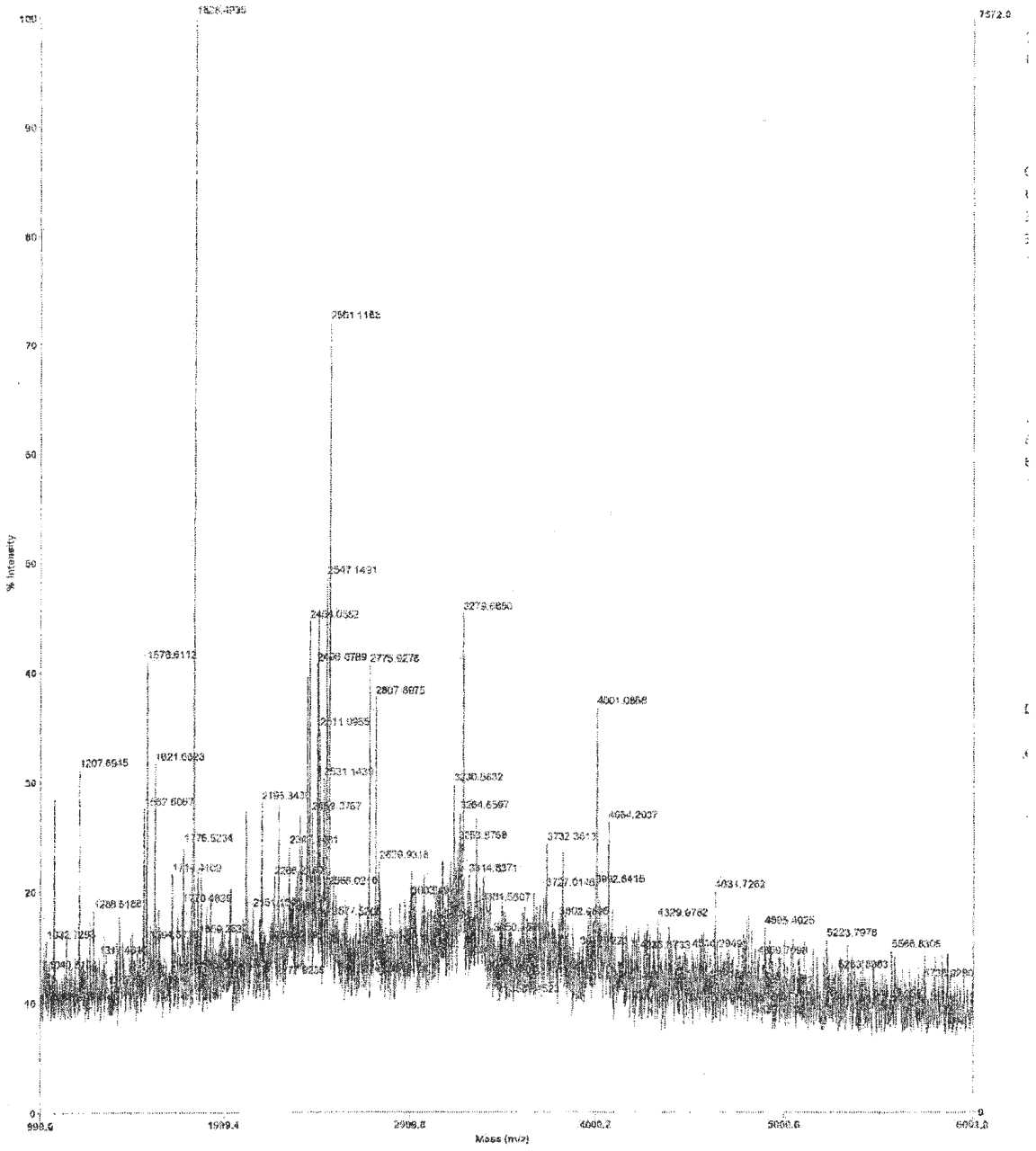


Figure G3: Peptide mass fingerprint spectrum produced from tryptic digest of 70kDa electrophoresis band (replicate 3).

Appendix H

Spectrums for 36 kDa band (Snow Crab Condensate) From Peptide Mass Fingerprinting

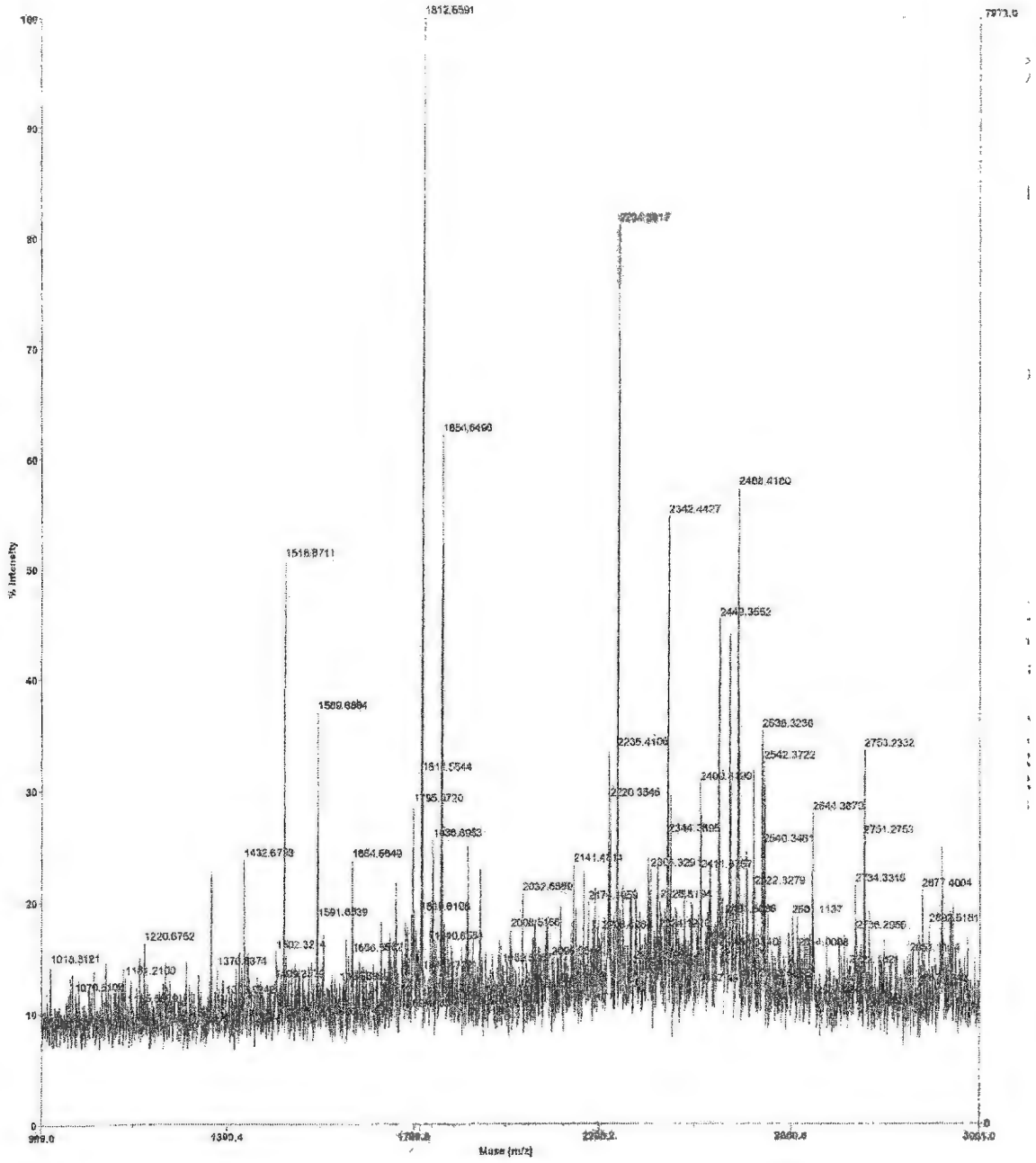


Figure H1: Peptide mass fingerprint spectrum produced from tryptic digest of 36kDa electrophoresis band (replicate 1).

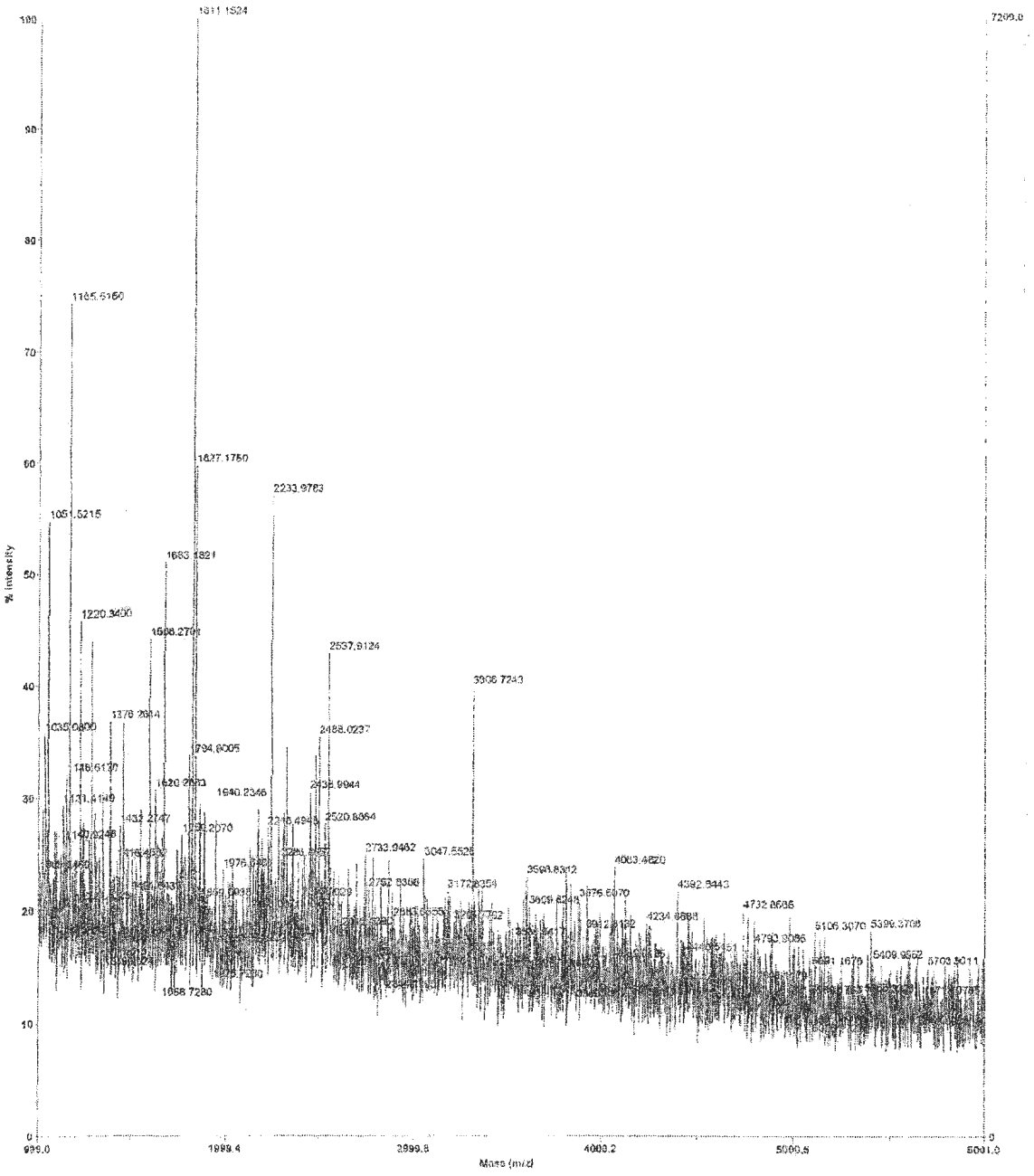


Figure H2: Peptide mass fingerprint spectrum produced from tryptic digest of 36kDa electrophoresis band (replicate 2).

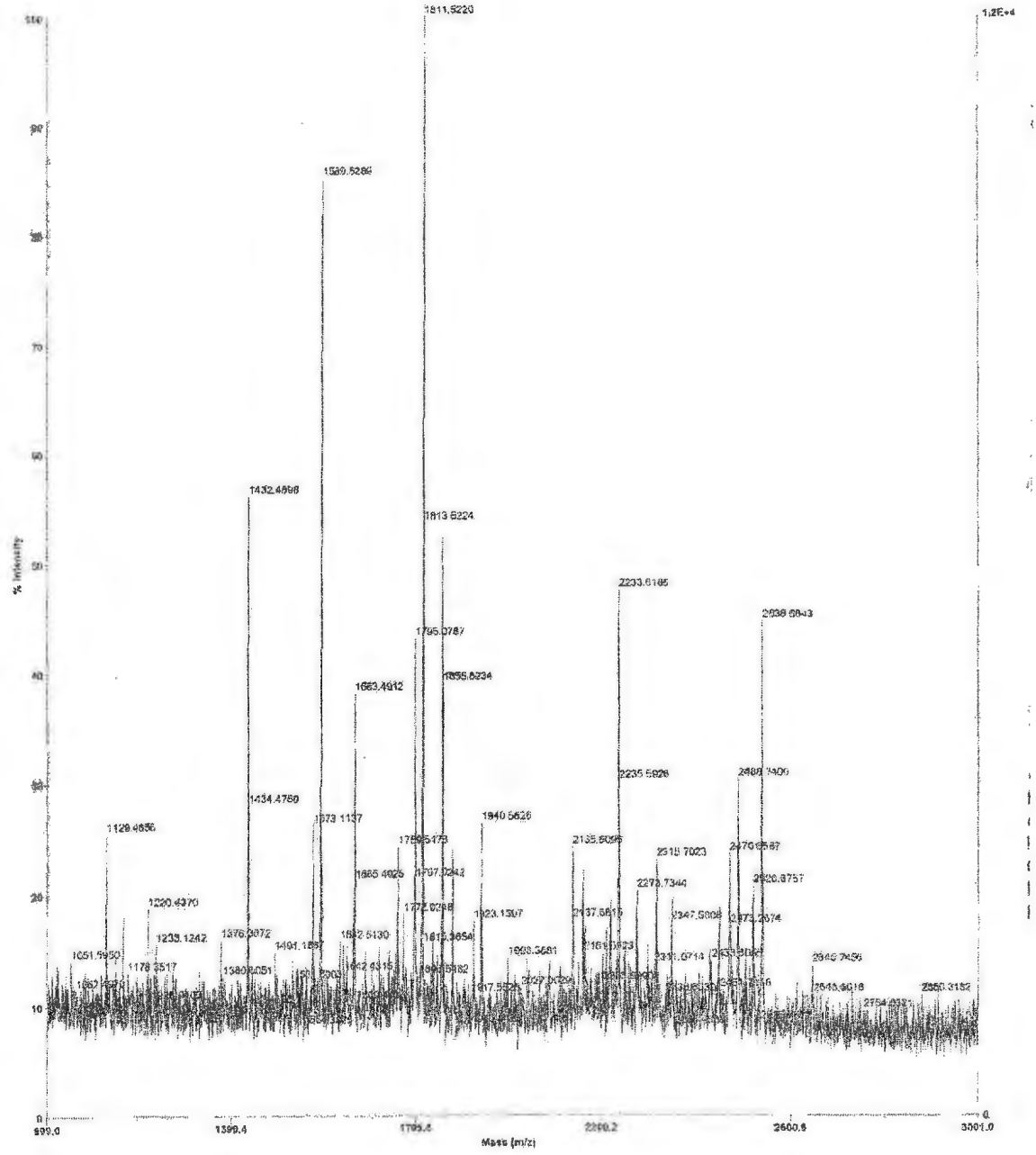


Figure H3: Peptide mass fingerprint spectrum produced from tryptic digest of 36kDa electrophoresis band (replicate 3).

Appendix I

Spectrums for the 18 and 20 kDa bands (Snow Crab Condensate) from Peptide Mass Fingerprinting

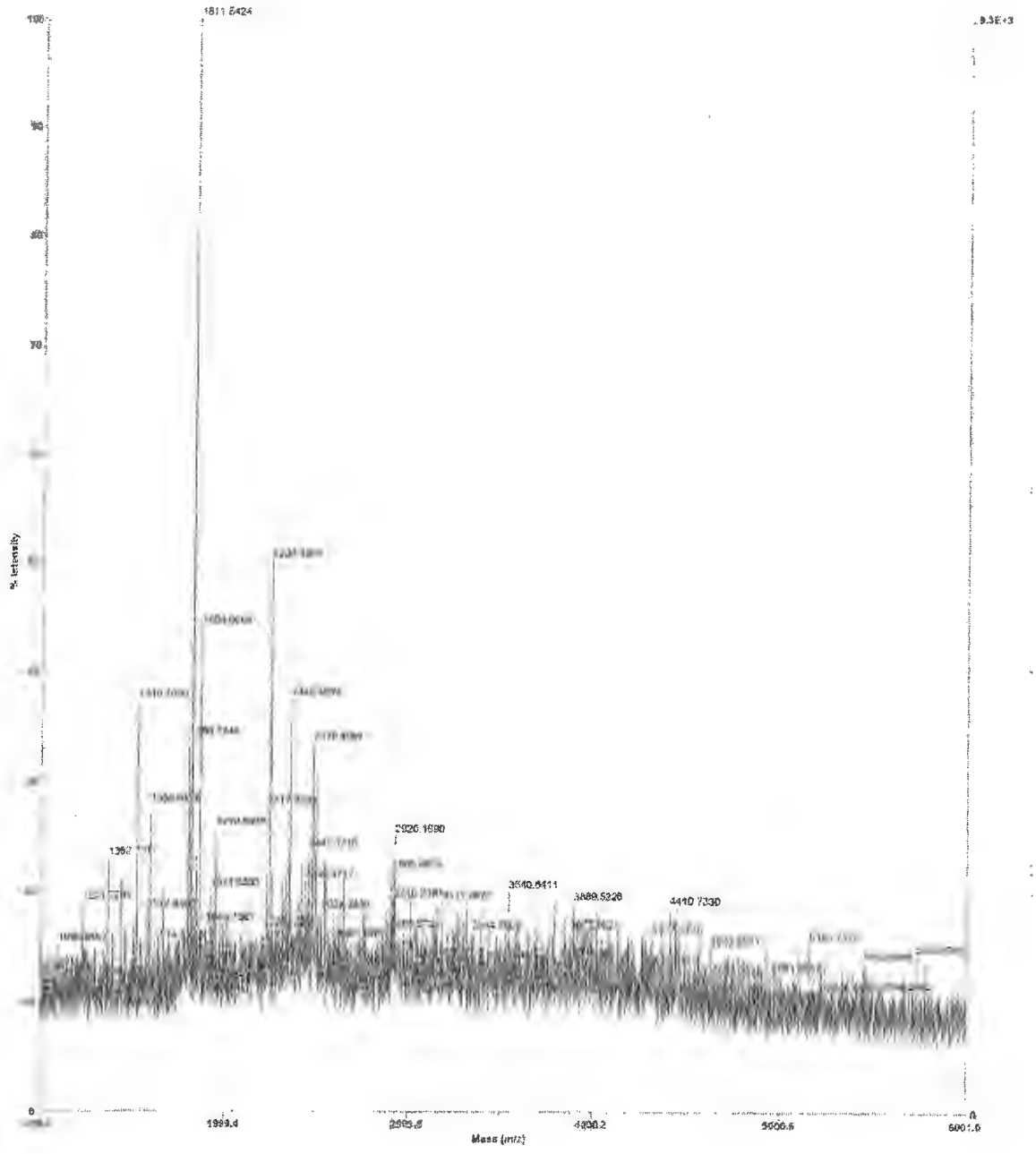


Figure I1: Peptide mass fingerprint spectrum produced from tryptic digest of 18kDa electrophoresis band (replicate 1).

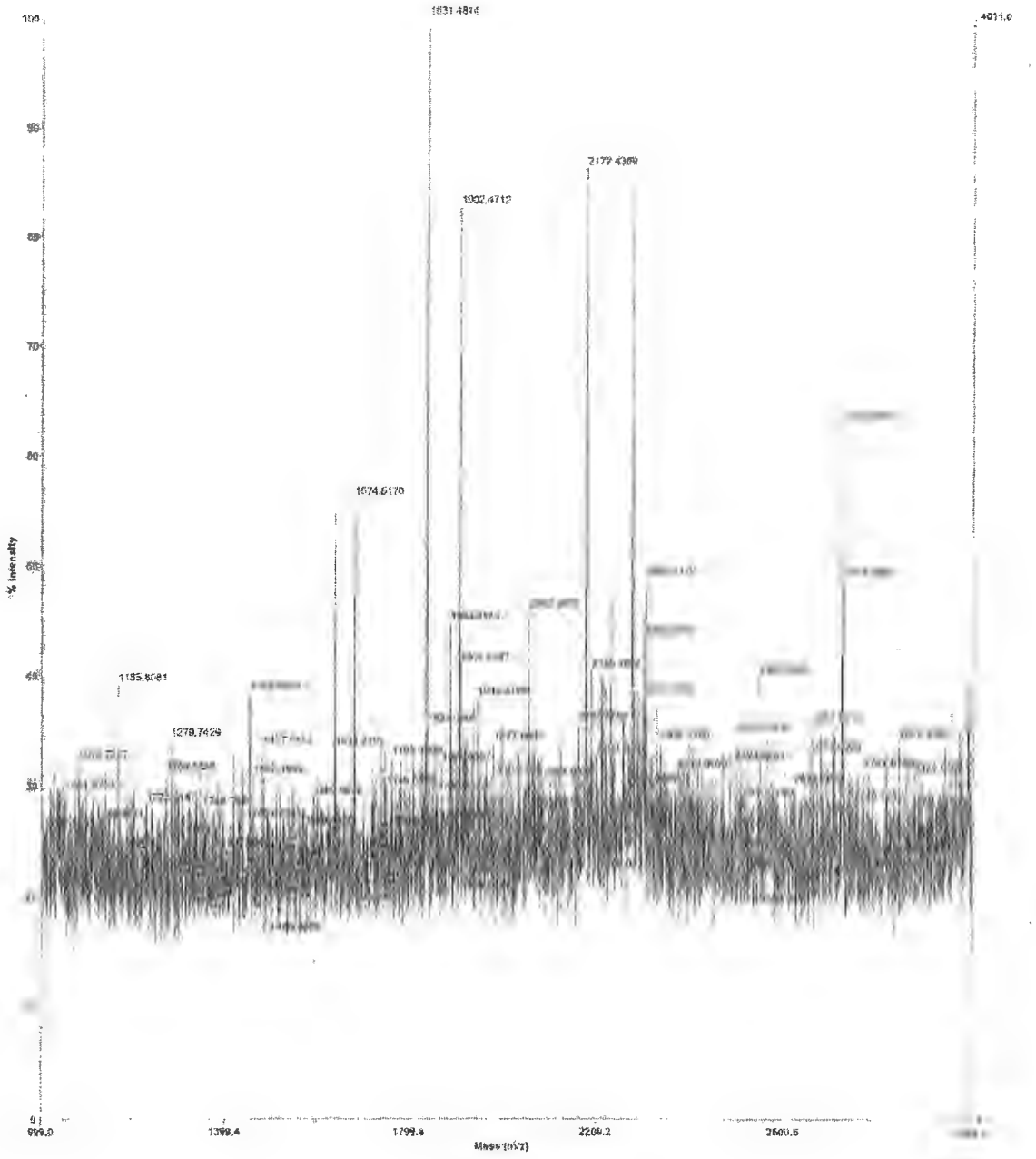


Figure I2: Peptide mass fingerprint spectrum produced from tryptic digest of 18kDa electrophoresis band (replicate 2).

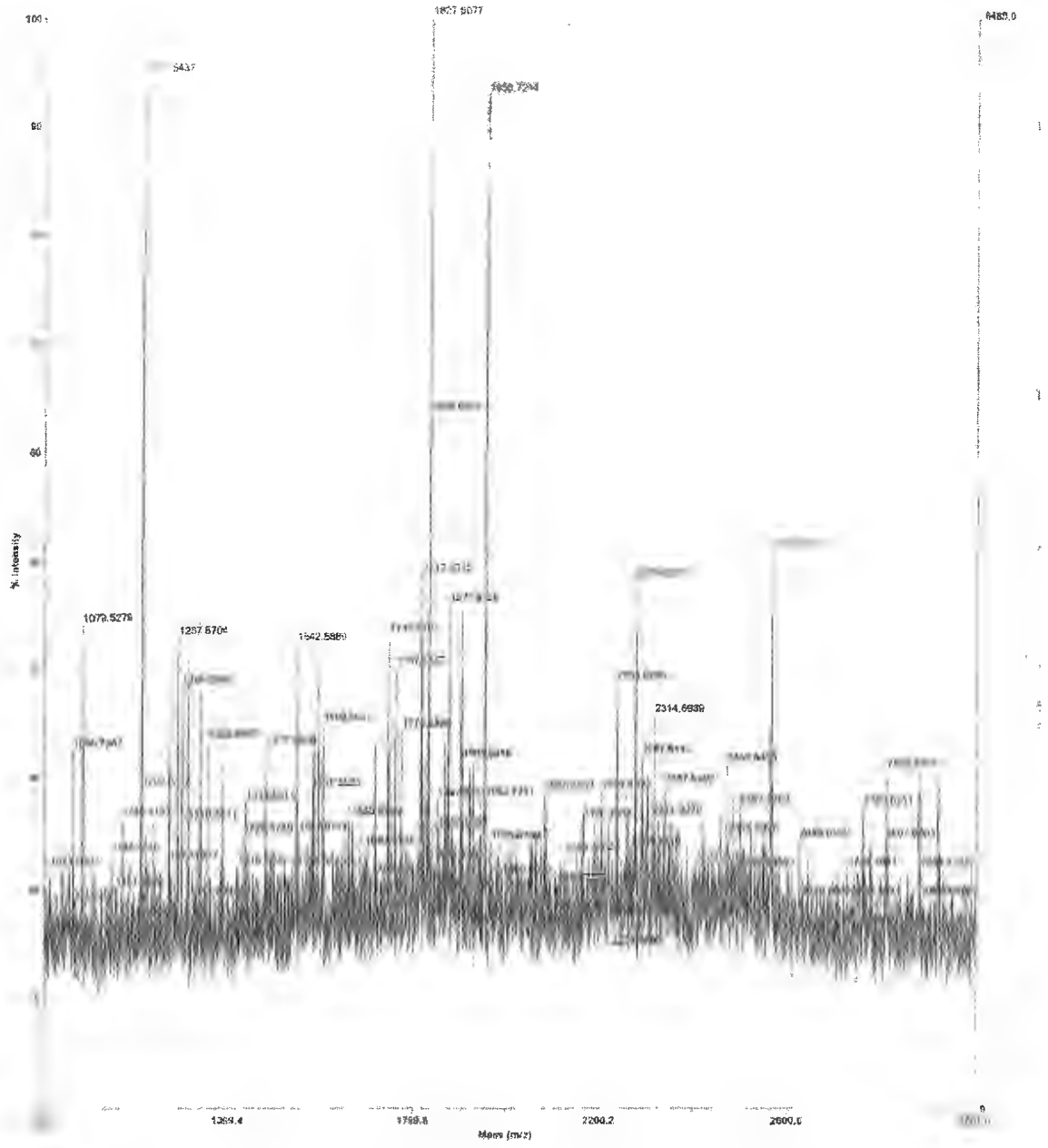


Figure I3: Peptide mass fingerprint spectrum produced from tryptic digest of 20kDa electrophoresis band (replicate 1).

Appendix J

Spectrums for the 14 kDa band (Snow Crab Condensate) from Peptide Mass Fingerprinting

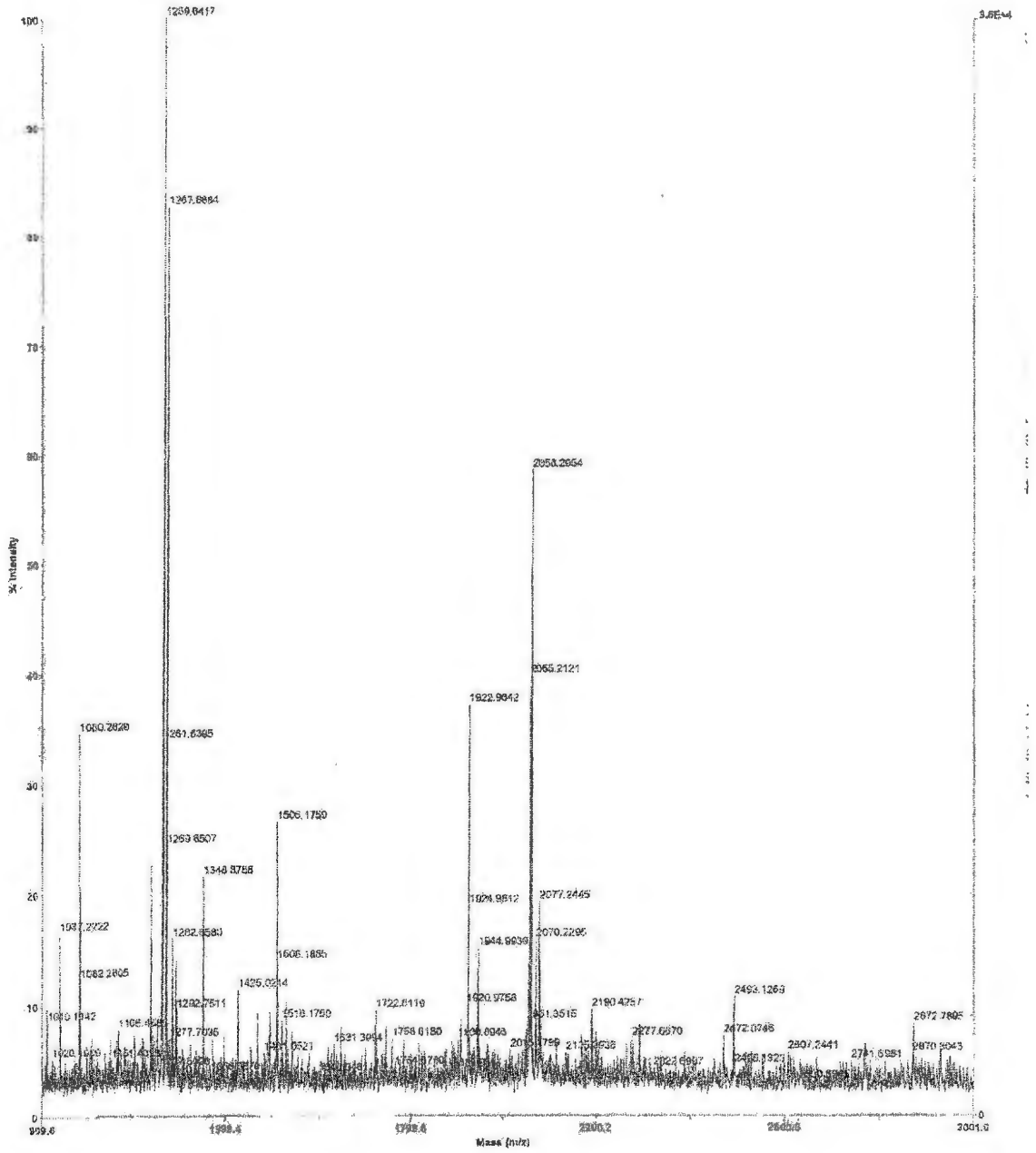


Figure J1: Peptide mass fingerprint spectrum produced from tryptic digest of 14kDa electrophoresis band (replicate 1).

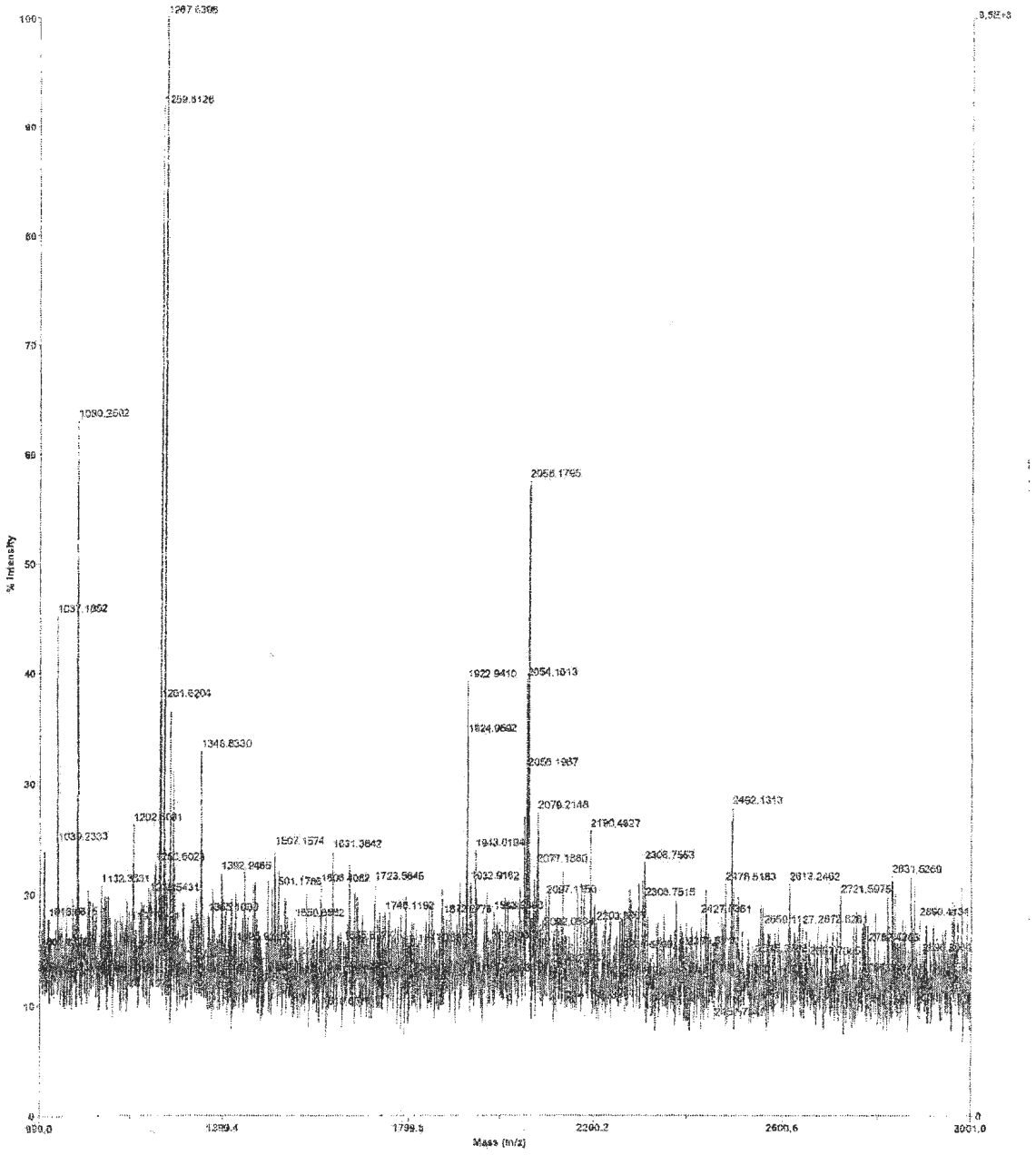


Figure J2: Peptide mass fingerprint spectrum produced from tryptic digest of 14kDa electrophoresis band (replicate 2).

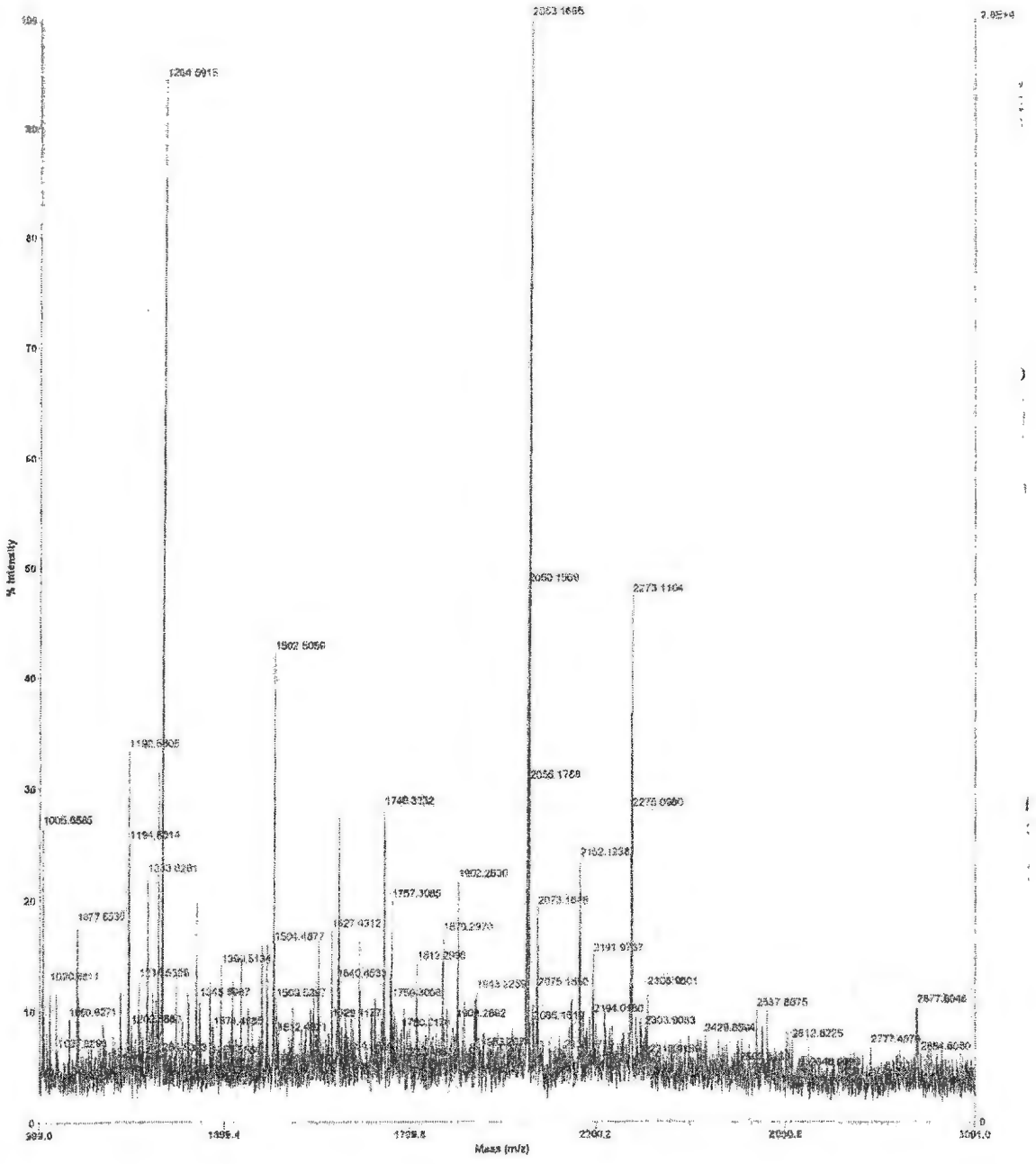


Figure J3: Peptide mass fingerprint spectrum produced from tryptic digest of 14kDa electrophoresis band (replicate 5).

Appendix K

Spectrums for the 36 kDa band (Filter 56) from Peptide Mass Fingerprinting

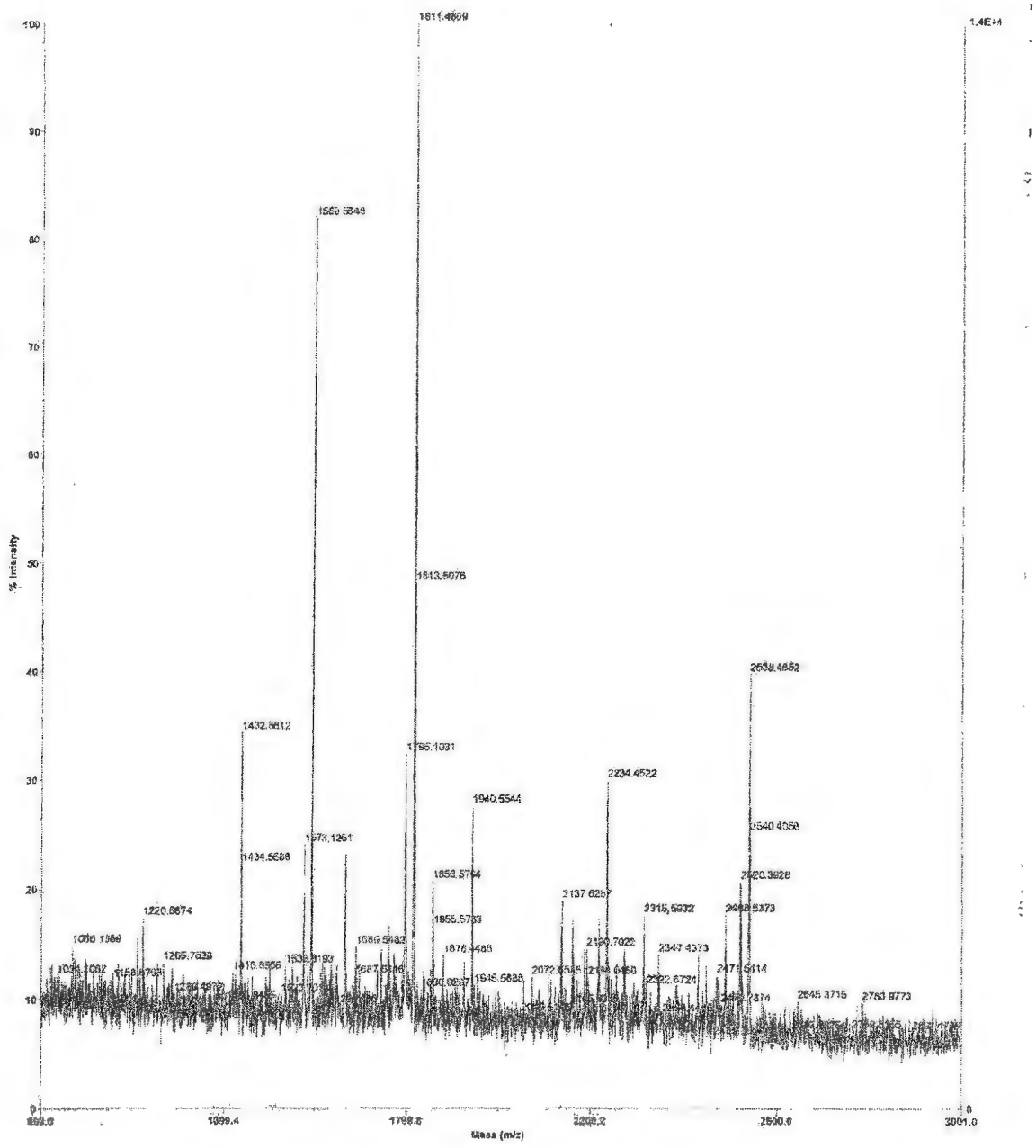


Figure K1: Peptide mass fingerprint spectrum produced from tryptic digest of 36kDa (filter 56) electrophoresis band (replicate 1).

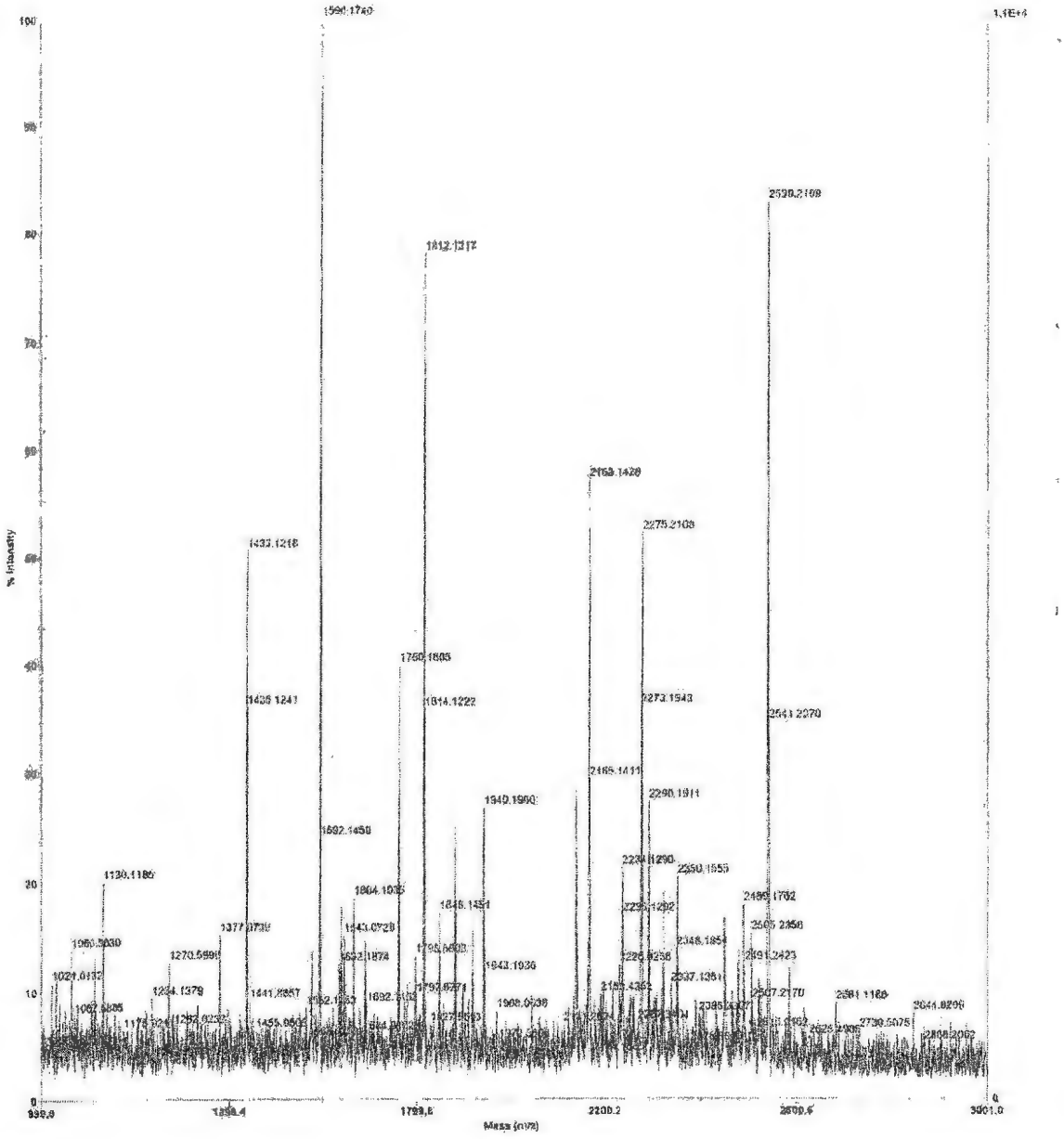


Figure K2: Peptide mass fingerprint spectrum produced from tryptic digest of 36kDa (filter 56) electrophoresis band (replicate 2).

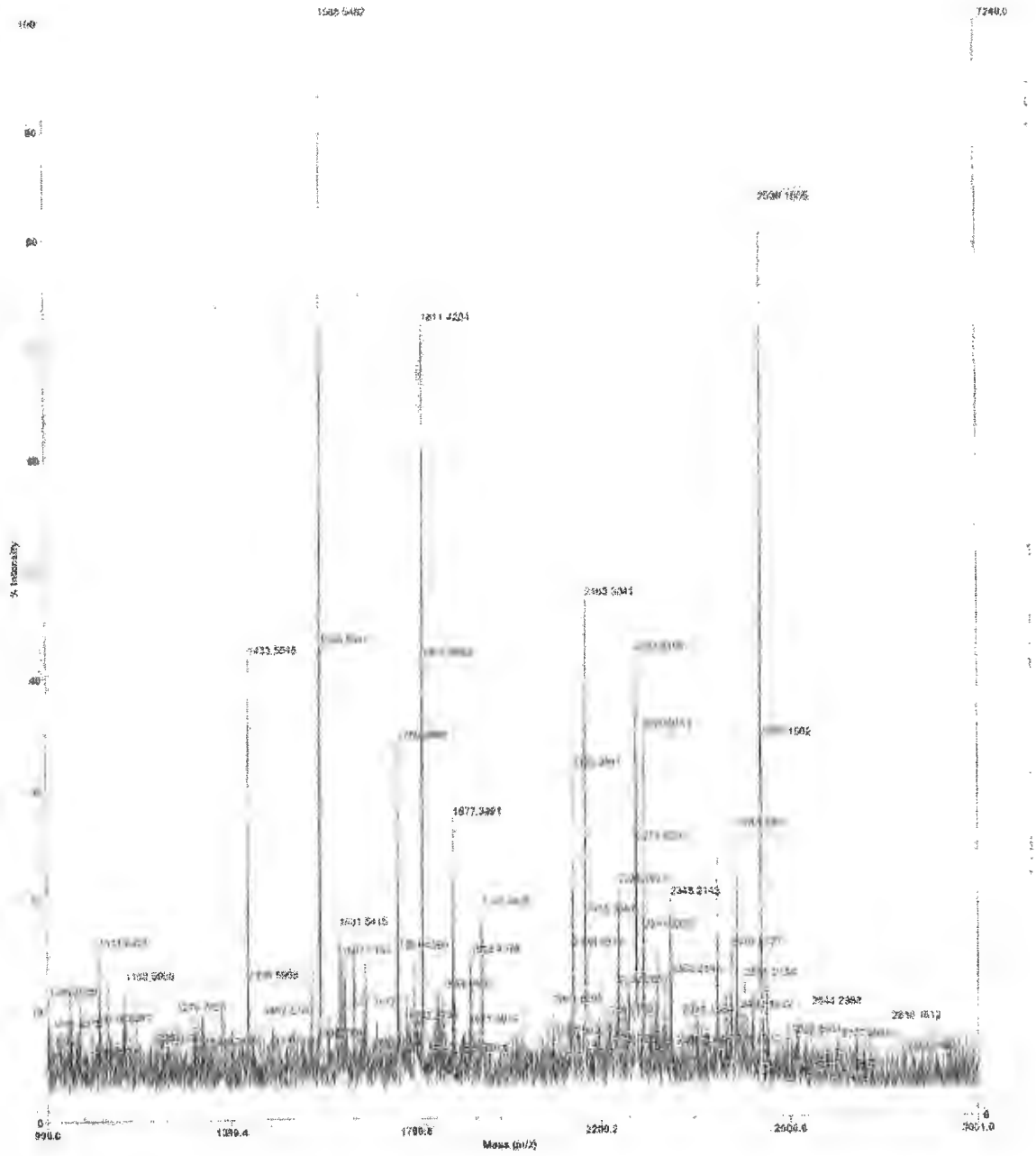


Figure K3: Peptide mass fingerprint spectrum produced from tryptic digest of 36kDa (filter 56) electrophoresis band (replicate 3).

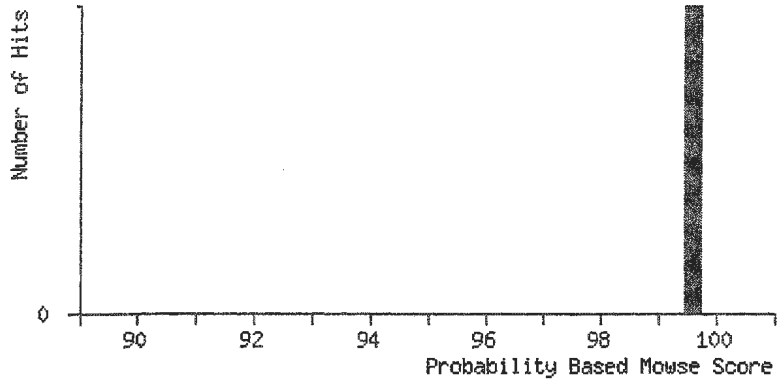
Appendix L

Sample Database Search Results for Filter 56, Replicate 3

{MATRIX}
{SCIENCE} Mascot Search Results

User : gwhiteway
Email : geoff.whiteway@mi.mun.ca
Search title :
Database : SwissProt 41.3 (184902 sequences; 92763446 residues)
Timestamp : 15 Apr 2003 at 15:14:38 GMT
Top Score : 100 for Q9N2R3, Tropomyosin (Allergen Cha f 1) (Cha f I) (Fragment)

Probability Based Mowse Score
Score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 65 are significant (p<0.05).



Concise Protein Summary Report
Switch to full Protein Summary Report
To create a bookmark for this report, right click this link: Concise Summary Report (../data/20030415/Fteumzan.dat)

1. Q9N2R3 **Mass:** 30417 **Total score: 100 Peptides matched:** 10
Tropomyosin (Allergen Cha f 1) (Cha f I) (Fragment)
Q25456 **Mass:** 31686 **Total score: 78 Peptides matched:** 9
Tropomyosin (Allergen Met e 1) (Met e I)
O61379 **Mass:** 31720 **Total score: 78 Peptides matched:** 9
Tropomyosin (Allergen Pan s 1) (Pan s I)
O44119 **Mass:** 32887 **Total score: 77 Peptides matched:** 9
Tropomyosin (Allergen Hom a 1)
Q8RGX1 **Mass:** 18641 **Total score:** 55 **Peptides matched:** 5
Phosphopantetheine adenylyltransferase (EC 2.7.7.3) (Pantetheine-phosphate adenylyltransferase) (PPAT) (Dephospho-CoA pyrophosphorylase)
P77829 **Mass:** 57384 **Total score:** 50 **Peptides matched:** 7
60 kDa chaperonin 1 (Protein Cpn60 1) (groEL protein 1)
P13927-00-00-01 **Mass:** 43208 **Total score:** 48 **Peptides matched:** 6

Elongation factor Tu (EF-Tu) STANDARD VARSPLIC; STANDARD VARIANT; REF. 3 FROM P13927

P13927 **Mass:** 43195 **Total score:** 48 **Peptides matched:** 6

Elongation factor Tu (EF-Tu) STANDARD VARSPLIC; STANDARD VARIANT; STANDARD CONFLICT FROM P13927

P25712 **Mass:** 11849 **Total score:** 45 **Peptides matched:** 4

NADH-ubiquinone oxidoreductase 9 kDa subunit, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-9KD) (CI-9KD)

P20277 **Mass:** 13611 **Total score:** 44 **Peptides matched:** 4

50S ribosomal protein L17 (BL15) (BL21)

P23394 **Mass:** 66774 **Total score:** 44 **Peptides matched:** 7

Pre-mRNA splicing factor RNA helicase PRP28 (Helicase CA8) STANDARD VARSPLIC; STANDARD VARIANT; STANDARD CONFLICT FROM P23394

P00284 **Mass:** 13964 **Total score:** 43 **Peptides matched:** 4

Azurin

Q9UXU7 **Mass:** 114514 **Total score:** 43 **Peptides matched:** 8

V-type ATP synthase alpha chain (EC 3.6.3.14) (V-type ATPase subunit A) [Contains: Pab atpA intein (Pab VMA intein)]

P23394-00-00-01 **Mass:** 66859 **Total score:** 42 **Peptides matched:** 7

Pre-mRNA splicing factor RNA helicase PRP28 (Helicase CA8) STANDARD VARSPLIC; STANDARD VARIANT; REF. 3 FROM P23394

P11943-00-00-01 **Mass:** 14449 **Total score:** 42 **Peptides matched:** 4

Acyl carrier protein, mitochondrial precursor (ACP) (NADH-ubiquinone oxidoreductase 9.6 kDa subunit) STANDARD VARSPLIC; STANDARD VARIANT; REF. 3 FROM P11943

P44243 **Mass:** 34696 **Total score:** 42 **Peptides matched:** 5

Hypothetical protein HI1523

P53218 **Mass:** 18315 **Total score:** 41 **Peptides matched:** 4

Ribonucleases P/MRP protein subunit POP6 (EC 3.1.26.5) (RNases P/MRP 18.2 kDa subunit) (RNA processing protein POP6)

Q9Y619-00-04-00 **Mass:** 33236 **Total score:** 41 **Peptides matched:** 5

Mitochondrial ornithine transporter 1 (Solute carrier family 25, member 15) STANDARD VARSPLIC; HHH SYNDROME-VAR_012760; STANDARD CONFLICT FROM Q9Y619

P41369 **Mass:** 17707 **Total score:** 41 **Peptides matched:** 4

Hypothetical protein in ileS 5'region (ORF B) (Fragment)

P51782 **Mass:** 17350 **Total score:** 41 **Peptides matched:** 4

Lysozyme C precursor (EC 3.2.1.17) (1,4-beta-N-acetylmuramidase C)

P30614 **Mass:** 60068 **Total score:** 40 **Peptides matched:** 6

Pyruvate kinase (EC 2.7.1.40)

Q01367 **Mass:** 34449 **Total score:** 40 **Peptides matched:** 5

Stage III sporulation protein AA

Q9MTN7 **Mass:** 82378 **Total score:** 40 **Peptides matched:** 4

Photosystem I P700 chlorophyll A apoprotein A2 (PsaB) (PSI-B)

P41134-00-00-02 **Mass:** 16498 **Total score:** 40 **Peptides matched:** 4
DNA-binding protein inhibitor ID-1 (ID) STANDARD VARSPLIC; STANDARD VARIANT; REF. 2 FROM P41134
P41134 **Mass:** 16470 **Total score:** 40 **Peptides matched:** 4
DNA-binding protein inhibitor ID-1 (ID) STANDARD VARSPLIC; STANDARD VARIANT; STANDARD CONFLICT FROM P41134
O50003 **Mass:** 17988 **Total score:** 38 **Peptides matched:** 4
60S ribosomal protein L12
P15846-00-00-01 **Mass:** 33086 **Total score:** 38 **Peptides matched:** 6
Tropomyosin, muscle STANDARD VARSPLIC; STANDARD VARIANT; REF. 2 FROM P15846
P15846 **Mass:** 33031 **Total score:** 38 **Peptides matched:** 6
Tropomyosin, muscle STANDARD VARSPLIC; STANDARD VARIANT; STANDARD CONFLICT FROM P15846
P35663 **Mass:** 68283 **Total score:** 38 **Peptides matched:** 6
Cylicin I (Multiple-band polypeptide I) (Fragment)
P39110 **Mass:** 22459 **Total score:** 38 **Peptides matched:** 4
GTP-binding protein CIN4
Q9XT73 **Mass:** 21266 **Total score:** 38 **Peptides matched:** 4
Ferritin heavy chain (Ferritin H subunit)
O52394 **Mass:** 37999 **Total score:** 37 **Peptides matched:** 5
RecA protein (Recombinase A)
Q9ZDA4 **Mass:** 22737 **Total score:** 37 **Peptides matched:** 4
Recombination protein recR
Q95031 **Mass:** 57970 **Total score:** 37 **Peptides matched:** 6
Cytochrome P450 6B6 (EC 1.14.14.1) (CYPVIB6)
Q43138 **Mass:** 39990 **Total score:** 37 **Peptides matched:** 5
Probable mannitol dehydrogenase 3 (EC 1.1.1.255) (NAD-dependent mannitol dehydrogenase 3)
O33964 **Mass:** 57575 **Total score:** 37 **Peptides matched:** 6
60 kDa chaperonin (Protein Cpn60) (groEL protein) (Heat shock protein 60)
P31834 **Mass:** 8668 **Total score:** 36 **Peptides matched:** 3
VPU protein (ORF-X protein) (UPX protein)
P23568 **Mass:** 43354 **Total score:** 36 **Peptides matched:** 5
Elongation factor Tu (EF-Tu) STANDARD VARSPLIC; STANDARD VARIANT; STANDARD CONFLICT FROM P23568
P39498 **Mass:** 8677 **Total score:** 36 **Peptides matched:** 3
Hypothetical 8.7 kDa protein in cd-pseT intergenic region
Q38841-00-00-01 **Mass:** 24063 **Total score:** 36 **Peptides matched:** 4
Agamous-like MADS box protein AGL12 STANDARD VARSPLIC; STANDARD VARIANT; REF. 1 FROM Q38841
P27488 **Mass:** 24397 **Total score:** 35 **Peptides matched:** 4
Colanic acid capsular biosynthesis activation protein A
Q980K7 **Mass:** 9622 **Total score:** 35 **Peptides matched:** 3

30S ribosomal protein S17e
Q05668 **Mass:** 28212 **Total score:** 35 **Peptides matched:** 5
Hypothetical protein ML1370
P42311 **Mass:** 9045 **Total score:** 35 **Peptides matched:** 3
Hypothetical protein yxiT precursor
P24219 **Mass:** 49844 **Total score:** 35 **Peptides matched:** 5
RNA polymerase sigma-54 factor
P96692 **Mass:** 23602 **Total score:** 35 **Peptides matched:** 4
Putative NAD(P)H nitroreductase ydfN (EC 1.-.-.-)
P75969 **Mass:** 42831 **Total score:** 35 **Peptides matched:** 6
Prophage lambda integrase (Int(Lambda)) (Prophage e14 integrase)
Q9ZDH7 **Mass:** 25025 **Total score:** 35 **Peptides matched:** 4
Putative 3-methyladenine DNA glycosylase (EC 3.2.2.-)
P13645-00-08-02 **Mass:** 59518 **Total score:** 35 **Peptides matched:** 5
Keratin, type I cytoskeletal 10 (Cytokeratin 10) (K10) (CK 10) STANDARD VARSPLIC; EHK-VAR_003830; REF. 2 FROM P13645
P43613 **Mass:** 34286 **Total score:** 35 **Peptides matched:** 5
Hypothetical 34.2 kDa protein in SAP155-YMR31 intergenic region precursor

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Bottom of Form
Search Parameters
Type of search : Peptide Mass Fingerprint
Enzyme : Trypsin
Fixed modifications : Carboxymethyl (C)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 0.05 %
Peptide Charge State : 1+
Max Missed Cleavages : 3
Number of queries : 10

Mascot: http://www.matrixscience.com/index.html

Appendix M

Scans of the RAST Analysis X-ray Films

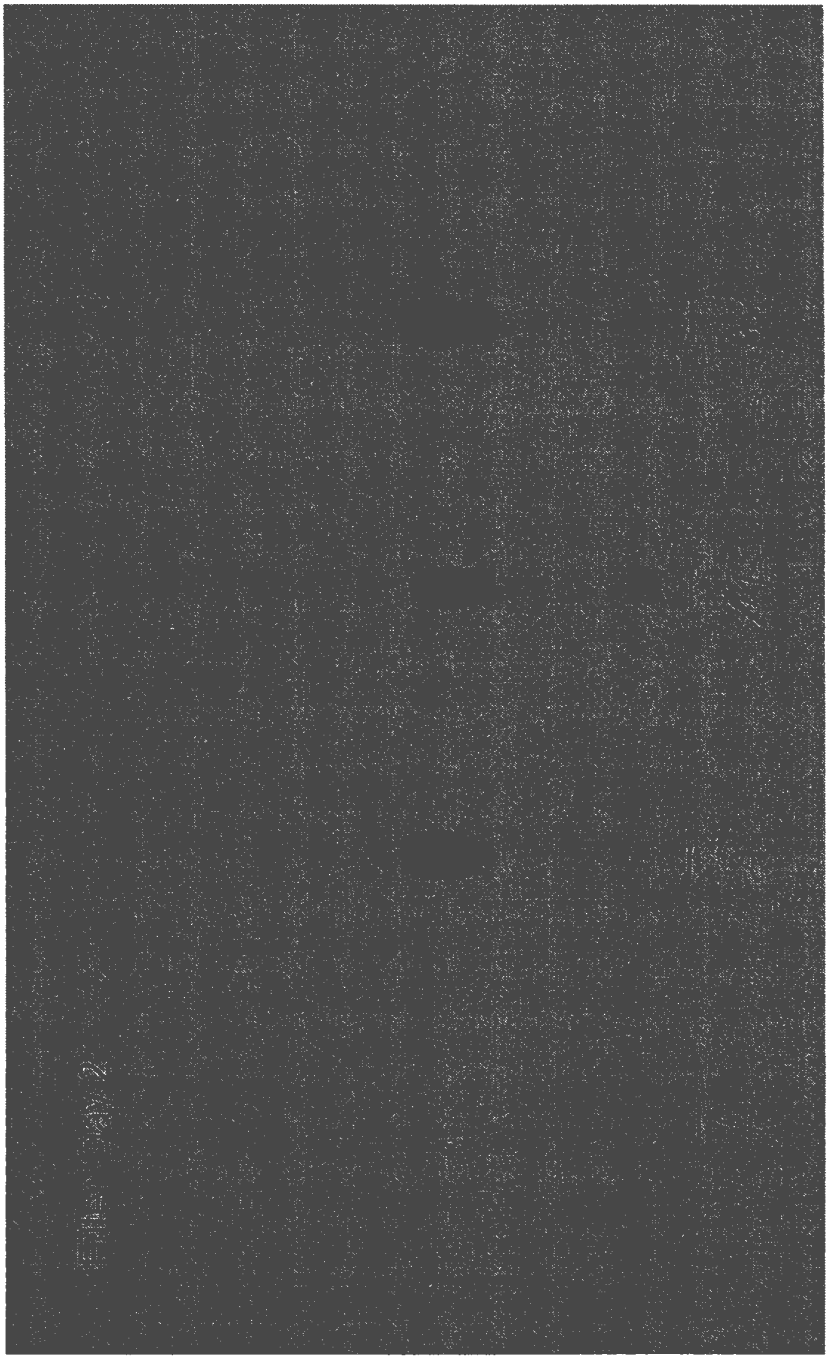

Figure L1: X-ray film demonstrating the reactivity of (1) snow crab condensate, (2) filter 56, (M) crab meat extract and (W) crab water extract after 2 days of development.

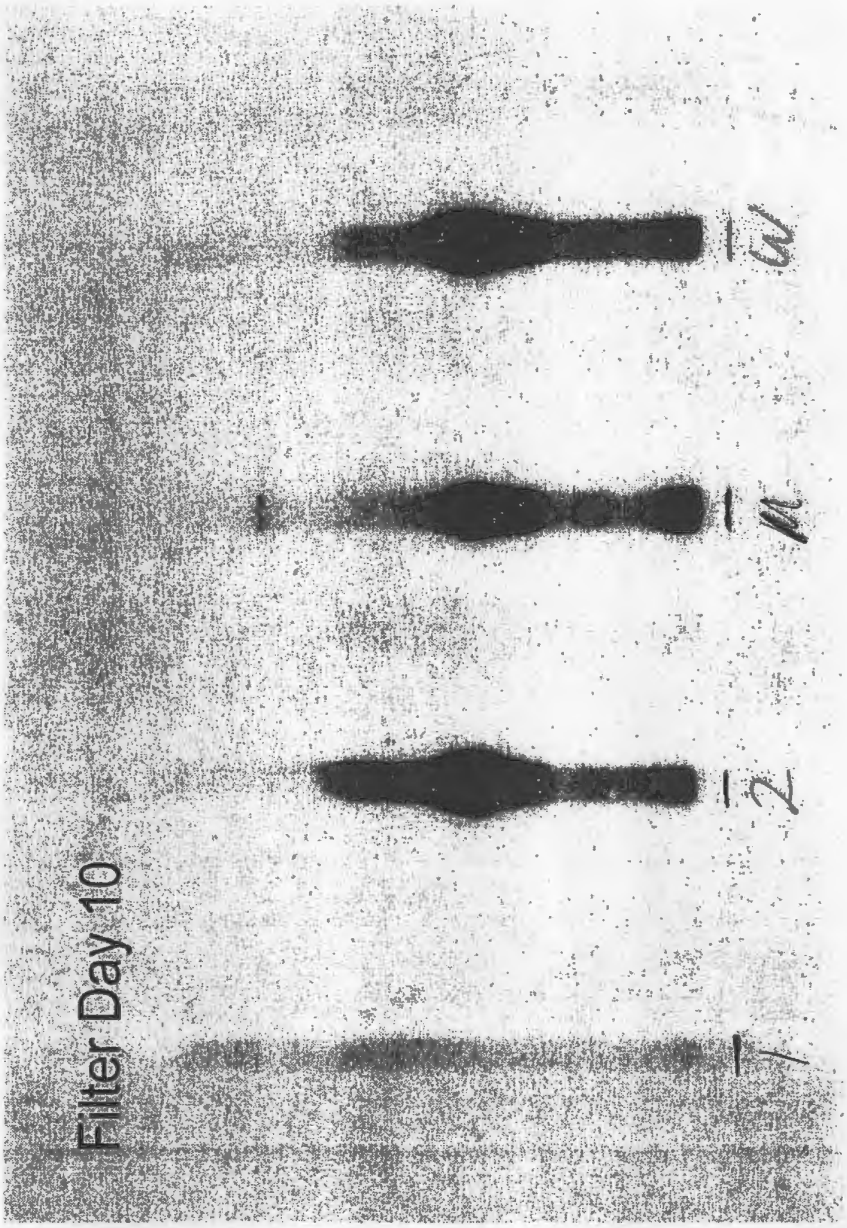

Figure L2: X-ray film demonstrating the reactivity of (1) snow crab condensate, (2) filter 56, (M) meat extract and (W) water extract after 10 days of development.