TOXICITY EFFECTS OF δ-ENDOTOXINS OBTAINED FROM NATIVE *Bacillus thuringiensis* ISOLATES AGAINST MAIZE STALK BORER (*Busseola fusca* FULLER)

MUIGANO, MARTIN NG'ANG'A

B.SC. (BIOLOGY)

Thesis submitted to the School of Biological Sciences of the University of Nairobi in partial fulfillment of the requirements of the degree of Master of Science in Microbiology

November 2014

DECLARATION

This research project is my original work and has not been presented for award of a degree in any other University.

Martin Ng'ang'a Muigano

Sign …… Date ……

This Thesis has been submitted for examination with our approval as University supervisors

Dr. Maina Wagacha

School of Biological Sciences

University of Nairobi

Sign …… Date ……

Prof. F. B. Mwaura

School of Biological Sciences

University of Nairobi

Sign …… Date ……

Dr. Muo Kasina

National Agricultural Research Laboratories

Kenya Agricultural and Livestock Research Organization

Sign …… Date ……

DEDICATION

This Thesis is dedicated to wife Grace Wathithi for her support and understanding throughout the work, and to my loving mum Margaret Wambui and dad Paul Muigano who have gone out of their way to support my education. I also dedicate this work to my son Mark Ng'ang'a who was born in the period of the project work.

ACKNOWLEDGEMENTS

I am heavily indebted to my supervisors Dr. Maina Wagacha, Prof. F. B. Mwaura, and Dr. Muo Kasina for their invaluable support, training, mentorship, and advice throughout this work. Without your support, this work would not have achieved anything significant. I am also grateful to Jane Wamaitha for the supply of the local Bt isolates and the Genetic Stock Center, USA, for providing the reference strain *Bacillus thuringiensis aizawai* and to Peter Kimani for his assistance in the maintenance of the isolates.

This work would not have been successful without the financial assistance provided by the Kenya Agricultural Productivity Agribusiness Programme (KAPAP) through the Kenya Agricultural Research Institute (KARI), to whom I am very grateful. I am also grateful to Dr. Kasina for his coordination that ensured financial support was available when needed.

I am grateful to Dr. Z. M. Kinyua and Dr. S. Gichuki for allowing me to use the facilities at the Plant Pathology and Biotechnology centers respectively during this work. My gratitude also goes to Mutisya of KARI, Katumani Center for supplying the neonates.

The technical personnel at the KARI, NARL Center have greatly contributed to this work. I am grateful to Janet Njeri, Pamela Kipyab, and Joseph Kinoti for their technical assistance in the bioassay work and to Boniface Mbevi for his assistance in handling the neonates. To Evans Mwasawe, Dominic Nzeve, and Bramwel Wanjala, I am grateful for your technical assistance, especially in protein analysis work.

Special thanks to John Agira of ChromAfrica LLC for his technical assistance in conducting the HPLC work and to Esther Kaduma for her assistance in Gel electrophoresis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

ABBREVIATIONS

ANOVA	Analysis of variance
Bt	*Bacillus thuringiensis*
Bta	*Bacillus thuringiensis aizawai*
Cry	Crystal
Cyt	Cytolytic
ddH_2O	Double distilled water
ICIPE	International Centre of Insect Physiology and Ecology
KARI	Kenya Agricultural Research Institute
NARL	National Agricultural Research Laboratories
rpm	Revolutions per minute
Subsp.	Subspecies
SDS PAGE	Sodium dedocyl sulfate polyacrylamide gel electrophoresis
KDa	KiloDalton
RP-HPLC	Reverse phase high performance chromatography
v/v%	Volume-volume percent
MPa	Megapascal
US	United States
USA	United States of America
USD	United States Dollar
TEMED	Tetramethylethylenediamine
BSA	Bovine Serum Albumin
DAP	Diammonium Phosphate
CARD	Caspase activation and recruitment domains
LSD	Least significance difference

mAU	Microabsorbance unit
LC50	Lethal concentration 50
mV.s	Milli volts.seconds

ABSTRACT

Decrease in maize crop yield due to infestation by the maize stem borer (*Busseola fusca*) presents a major food security threat in Kenya and many sub-Saharan countries. Farmers in Kenya have relied on the use of insecticides to control *B. fusca*. However, this approach presents several challenges including negative effects on human health, killing of non-target organisms, widespread pest resistance, and harm to the environment. *Bacillus thuringiensis* (Bt) produces δ–endotoxins during the sporulation phase, and these proteins have specific toxicity against a wide range of pests including lepidopteran insects. This study was carried out to evaluate the efficacy of δ-endotoxins from local Bt isolates in control of *B. fusca*, determine the effective dosage, and characterize the proteins from the most effective isolates. The pest neonates were fed on maize leaf discs contaminated with broth of various Bt isolates. A total of 43 isolates, including the standard *Bacillus thuringiensis aizawai*, were included in the study. The first study involved mortality tests to determine the most effective isolates and a bioassay was used to determine the LD50. Proteins from the most effective isolates were harvested using the centrifugation method and used in a bioassay in which five concentrations of the proteins were applied to the maize leaf discs and fed to the neonates. The effective isolates were characterized to determine their differences using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and reverse phase high performance chromatography (RP-HPLC) methods. The gel electrophoresis involved running the prepared samples through a gel of matrix concentration of 0.25% w/v and recording the results on a UV transilluminator. RP-HPLC enabled the elucidation of the protein profile and involved running the centrifuged proteins on a HPLC system whose solid phase comprised of Jupiter C18 column and mobile phase comprising of methanol and water at ratio of 70:30 (v/v%). The flow rate was maintained at 1ml/min, 10 MPa and the separation monitored using UV detector. There were temporal differences in the mortalities recorded at different

sampling regimes. The average mortalities increased with increase in time, with the lowest mortality being observed after 24 h (6.6%) and the highest after 120 h (86.4%). *Bacillus thuringiensis* isolates 25, 30 and 52 showed the highest overall mean mortalities at 73%, 69.5% and 67.5% and 90%, 90% and 87.5%, respectively when sampling was done after 72 h. The 24-hr, 48-hr, 72-hr, 96-hr, and 120-hr sampling regimes were used in this study. The bioassay conducted for 12-hr, 24-hr, 36-hr, and 48-hr sampling times showed that toxicity increased with increase in concentrations. The LC50 for the most effective isolates at 36-hour sampling were 0.003201 mg/ml, 0.00367 mg/ml, 0.000942 mg/ml, and 0.002395 mg/ml for isolates Bta, Bt 25, Bt 30, and Bt 52, respectively. The LD50 values of Bta and Bt 25 were not significantly different ($p \geq 0.05$). At these concentrations, more than half of the neonates had succumbed to toxic effects of the toxins. The gel electrophoresis results showed that proteins with a size of 130 KDa existed in the Bt samples together with others of between 70 KDa and 100 KDa. The HPLC results showed that the characteristic peak was attained at 190 nm for all isolates at which the highest peaks had a height of 1.64×10^6 mAU, 6.36×10^6 mAU, 1.66×10^6 mAU, and 1.63×10^6 mAU for Bta, Bt 25, Bt 30, and Bt 52, respectively. Peaks with similar patterns were observed in all the isolates, suggesting that the isolates had similar Cry proteins. The findings of this study suggest that local Bt isolates have great potential for use in managing *Busseola fusca* and other lepidopteran insects.

Key words*: Bacillus thuringiensis, Busseola fusca,* δ–endotoxins, Cry protein.

CHAPTER ONE

INTRODUCTION

1.1. Background Information

Maize is one of the most important staple foods in Sub-Saharan Africa. In Kenya, it accounts for at least 36% of total food calorific intake and 65% of total calorific intake in staple foods. The per capita consumption of maize in Kenya is 98 kilograms annually (Munyua *et al*., 2010). According to Mugo *et al*. (2011), about 400,000 metric tons of maize is lost annually to stem borers in Kenya, costing the economy about 72 million USD. Reduction of stem borer damage to maize in Kenya is therefore a priority among stakeholders in the agricultural sector. Effective control of maize stem borers would contribute to food security, improved maize yields, and improved standards of living by increasing the farmers' income (Mulaa *et al*., 2011).

Traditionally, farmers have relied on conventional methods of pest control that have solely relied on the use of synthetic pesticides in control of maize stem borers in Kenya (Mulaa *et al.*, 2011). However, the use of chemical pesticides presents serious adverse effects on human health such as immune system disorders and cancers as well as contributing to environmental pollution (Kumar *et al.*, 2008). In addition, the continued use of synthetic pesticides has led to widespread pest resistance and subsequent outbreak of secondary pests (Bravo *et al*., 2011). Resistance to chemical insecticides is particularly prevalent in developing countries and this result in high cost due to high application rates required (Raybould and Quemada, 2010). For instance, widespread resistance to *Helicoverpa armigera* has been reported in West Africa with subsequent high application rates by farmers (Martin et al., 2002). Additionally, chemical pesticides do not discriminate while killing pests thus resulting in killing non-target organisms (Raybould and

Quemada, 2010). A need therefore arises for the development of methods of controlling stem borer that are safe to humans and animals, environmental friendly, and have a narrow spectrum of activity. Biotechnology offers a possible solution to problem of stem borer infestation of maize in Kenya and other developing countries. It is currently possible to develop transgenic crops that can produce insecticidal proteins, which protect the plant against pest infestation (Mulaa *et al*., 2011). According to Bravo *et al*. (2011), microbial pesticides are a good replacement to chemical insecticides due to their narrow spectrum activity and, therefore, only kill specific insect pests. Moreover, microbial insecticides are environmentally friendly since they are biodegradable. One of the most widely used bioinsecticide is *Bacillus thuringiensis*, which is completely innocuous in humans, animals, and other invertebrates and therefore safe to use (Soberón *et al.*, 2009).

Bacillus thuringiensis (Bt) is a soil bacterium that contains proteins that have insecticidal activity against various pests and has been used as a bio-pesticide for several decades (Raybould and Quemada, 2010). The genes encoding δ-endotoxin from *B. thuringiensis* have been cloned in plants to kill the pests when they ingest parts of the plants. Within the *B. thuringiensis* species, there is a wide diversity of strains, which are grouped according to the toxin protein that they release. The toxins may be described according to the crystal (cry) protein structures, amino acid sequences, and modes of activity (Sanchis and Bourguet, 2008). The toxins are in form of parasporal crystals produced during the sporulation phase. The crystals consist of two groups of proteins (Cry and Cyt toxins). Cry proteins refer to Bt parasporal inclusion proteins showing a toxic effect against a target organism or exhibiting considerable similarity to known Cry protein sequences. On the other hand, Cyt proteins refer to parasporal inclusion proteins obtained from

B. thuringiensis with cytolytic activity or exhibiting sequence similarity to a known Cyt protein (Bravo *et al.,* 2007). The different Cry proteins that have been characterized using X-ray crystallography include Cry 1 Aa, Cry 2Aa, Cry 3Aa, Cry 3Bb, Cry 4Aa and Cry 4Ba (Sanahuja *et al.*, 2011).

The Bt toxins have been used to control wide range of pests such as mosquitoes and blackflies where it has been applied in water bodies (Go´mez *et al*., 2006). Other pests such as the tomato pinworm (*Keiferia lycopersicella*), corn earworm (*Helicoverpa zea*), Colorado potato beetle (*Leptinotarsa decemlineata*), tuber moth (*Phthorimaea operculella*), pink bollworm (*Pectinophora gossypiella*), European corn borer (*Ostrinia nubilalis*), and stem borers have been controlled using Bt toxins (Sanahuja *et al*., 2011). There is need to extend the research in the investigation of the potential for use of native isolates of *B. thuringiensis* collected in Kenya to control *B. fusca*.

1.2. Problem statement and justification

Maize is the staple food in Kenya and an important economic crop in many countries in the world. According to Mugo *et al.* (2011), the average maize output in the world was 5.1 tons per hectare in 2008. At the same period, the average maize yield in sub-Saharan countries such as Kenya was 1.3 t/ha. This is in contrast to the industrialized countries which produced an average yield of 8 t/ha. Africa contributes about 7% of the world maize yield from 29 million hectares (Mugo *et al*., 2011). Pests contribute a large share of this low yield of maize in Africa (Mulaa *et al*, 2011). The African stem borer is one of the major causes of maize yield loss in Kenya.

The reduction in crop yield due to infestation by *B. fusca* is a major threat to food security in Kenya. In addition, farmers incur heavy costs associated with management and control of these

pests since the cost of chemical pesticides is relatively high. Furthermore, the pests lower the quality of food consumed and thus present a potential public health concern. Control of *B. fusca* in Kenya has mainly relied on the use of chemical pesticides. However, the use of conventional pesticides in control of major crop pests has major limitations despite their success in history. Chemical pesticides have a broad spectrum of activity and, therefore, have greater risks of killing non-target insects. They are also expensive and harmful to humans, animals, and the environment (Bravo *et al*., 2011). A need, therefore, arises for the development of a sustainable, highly specific, non-expensive, and safe method of managing crop pest damage. The toxicity of *B. thuringiensis* endotoxins to various insect orders presents a potential for biological control of insect pests.

Even with the current international success in utilization of Bt within the genetic modification (GM) technology for pest management, there have been limited research efforts in the control of *B. fusca* in Kenya using *B. thuringiensis* (Mugo *et al*., 2011). This shows the need to continue with investigations aimed at identifying the best isolates that can be used to manage these pests. *B. fusca* is also more challenging to control compared to caterpillars. The current study sought to establish the insecticidal activity of Bt toxins against *B. fusca*. Subsequently, the efficacy of Bt toxins as potential insecticides and their utilization in control of this important crop pest was investigated.

1.3. Objectives of the study

1.3.1. Broad objective

The broad objective of this study was to determine the efficacy of delta endotoxins from native *Bacillus thuringiensis* isolates against *Busseola fusca* (Fuller).

1.3.2. Specific objectives

The specific objectives of the study were:

i. To determine the mortality rates caused by native *B. thuringiensis* isolates against neonates of *B. fusca.*

ii. To determine the mean lethal concentration (LC50) of Bt toxins causing 50% larval mortality of *B. fusca* neonates.

iii. To characterize *B. thuringiensis* δ-endotoxin proteins present in the three most effective native isolates against *B. fusca* neonates.

1.4. Hypotheses

The hypotheses of the study are:

i. There is no variation in the insecticidal activity of different *Bacillus thuringiensis* isolates against *B. fusca*.

ii. The toxicity of different Bt isolates, measured through LC 50, is not significantly different.

iii. There is no difference in the protein structure of endotoxins of the effective Bt toxins.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical background of Bt technology

The year 1996 was a landmark in agricultural technology as Monsanto Company commercially grew and harvested the first transgenic crops. Following the regulatory approval of transgenic crop technology, scientists have conducted intensive research on the use and safety of transgenic crops (Manjunath, 2005). Numerous studies have been conducted on the control of maize pests in Africa using biological methods while many more have focused on the use of *Bacillus thuringiensi*s as a potential biopesticide against susceptible lepidopteran pests. However, there are limited studies on the potential for use of *Bacillus thuringiensis'* delta endotoxins in the control of *Busseola fusca* in Kenya.

2.2 Stem borers in Kenya

The two most important stem borers in Kenya are *Chilo partellus* (Swinhoe) and *Busseola fusca* (Fuller) found in warm, low areas and cooler, higher areas respectively (De Groote, 2002). *Sesamia calamistis* (Hampson) is also found in Kenya but is of less economic importance (DeGroote, 2002). Maize stem borers cause an estimated 13.5% loss in annual maize production in Kenya (Mugo *et al*., 2011). It is estimated that for every 10% of maize plants infested by *B. fusca*, a corresponding loss of 12% of the maize grain is occasioned. *C. partellus* and *C. orichalcociliellus*, on the other hand, cause a loss of 18% on maize yields (Kfir *et al*., 2002). Stem borers damage maize by feeding on leaf tissues followed by tunneling and feeding of the stem (DeGroote, 2002).

2.3 Distribution and biology of *Busseola fusca*

Busseola fusca is a pest of maize that is indigenous to Africa and occurs throughout the sub-Saharan Africa region. In Southern and Eastern Africa, its distribution is limited to higher altitudes (above 600m above sea level) while its distribution varies from sea level to high altitudes (over 200m above sea level) in Central Africa (Tefera *et al*., 2010). The eggs of *B. fusca* are round, flattened and measure about 1 mm in diameter. The larvae measure about 40 mm in length when they are fully-grown. Although their color varies, it is usually creamy white with a characteristic grey tinge. The crotchets of the larvae of *B. fusca* are arranged in a semicircle thus distinguishing it from other stem borers such as *C. partellus*. The pupas are shiny yellow blown with the female measuring about 25 mm in length and the male being slightly smaller. The adult moths have a wingspan of about 20-40 mm with males being generally smaller than females (Harris and Nwanze, 1992).

Female moths lay eggs in batches of about 150 eggs in between the maize stalk and leaf sheath (Figure 1). Freshly laid eggs are white but the color darkens, as they get older. The female moth lays eggs in a long column in the stem below the leaf sheath, which hatches after 10 days (Tefera *et al*., 2010). The larvae initially dispatch into the leaf whorls where they feed before boring into the stem tissues where they form tunnels in maize stems and cobs (Harris and Nwanze, 1992). If the attack is severe, the plant turns yellow and die, and the larvae moves to another plant. The larvae period lasts 35 days or more after which the fully-grown caterpillars cuts a hole in the stem and pupates within the tunnel. The larval period is dependent on environmental factors such as temperature and humidity as well as the availability of food (Tefera *et al*., 2010). In some cases, the caterpillar may undergo a long diapause before pupating until the next rains appear.

The pupa stage of the first generation takes about two weeks after which the adult moth emerges from the hole made by the caterpillar on the stem (Tefera *et al*., 2010).

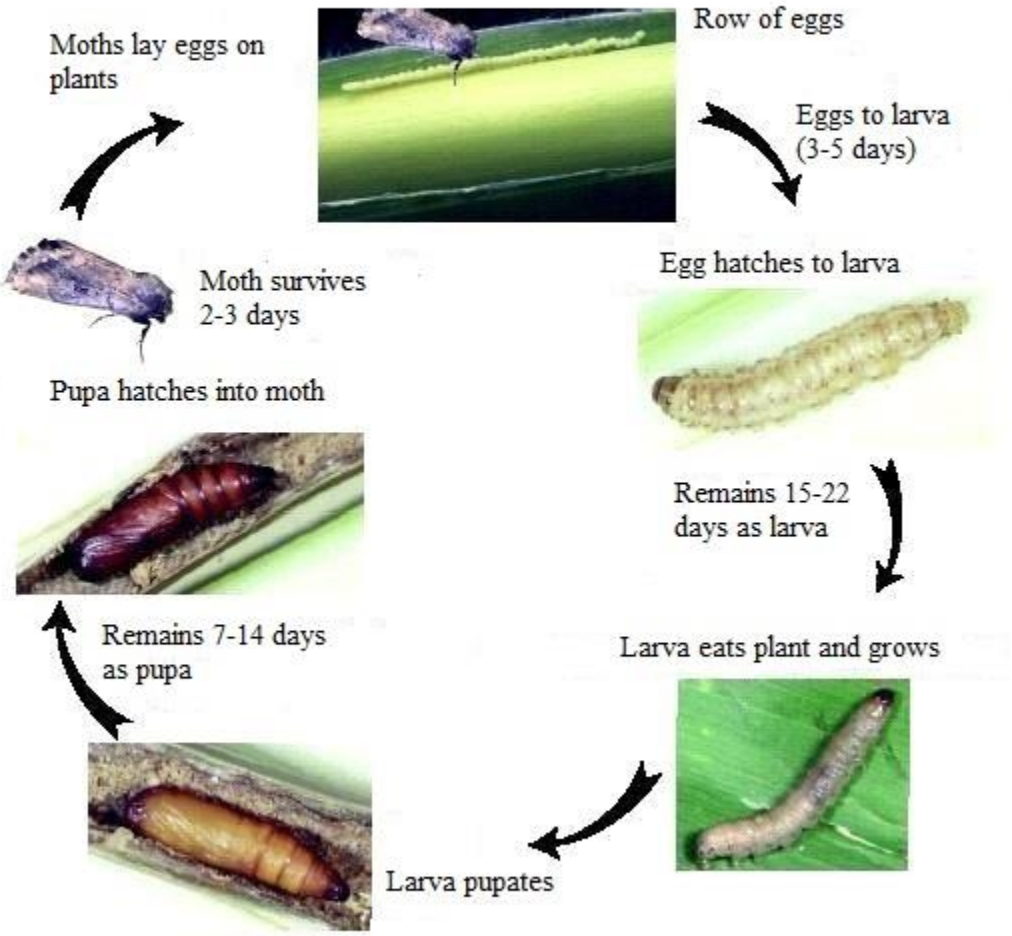


Figure 1: Schematic presentation of the life cycle of *Busseola fusca*
(Adapted with modification from http://www.push-pull.net/stemborers.html)

2.4 Isolation and biology of *Bacillus thuringiensis*

Bacillus thuringiensis was first isolated from infected silk worms (*Bombyx mori* (L.) in 1901 by Shigetane Ishiwata, a Japanese bacteriologist. Berliner, a German biologist, later isolated the bacterium from the Mediterranean flour moth, *Ephestia kuehniella* (Zell.) infected chyrsalids and named it *Bacillus thuringiensis*. The bacterium has continued to attract the interest of many scientists due to its entomopathogenic activity (Sachis and Bourguet, 2008). *Bacillus thuringiensis* is a spore-forming bacterium that is naturally found in the soil. The bacterium is also found in aquatic environments, in plants, in live and dead insects, in plants foliage, and in animal feces (Porcar and Caballero, 2000). The bacterial spores are formed during the stationary phase of the growth cycle (Sanahuja *et al*., 2010). *Bacillus thuringiensis* is a Gram positive, rod-shaped bacterium that measures about 1-1.2 cm in width and 3-5 cm in length. The spores of *B. thuringiensis* are ellipsoidal to cylindrical and have two envelopes, endo- and exosporium enclosing a thin sporewall. The spores are located in the central to terminal position and have no distension of the mother cell (Sharma and Manandhar, 1997).

During sporulation, the bacterium produces crystalline proteins (δ-endotoxins) that have insecticidal activity against many leptidopterans. About 25% of the bacterium's dry weight may consist of the crystalline inclusion (Sachis and Bourguet, 2008). *Bacillus thuringiensis* ferments carbohydrates such as mannose, sucrose, salicin, esculin, glucose among others, producing acid, but not gas. The bacterium may grow in both aerobic and anaerobic conditions. However, *B. thuringiensis* cannot produce acid from sugars such as xylose, arabinose, and mannitol. Moreover, *Bacillus thuringiensis* cannot reduce nitrite or free nitrogen gas from nitrate compounds. It may utilize lipids and acetone. It may also utilize citrate as a sole carbon source

resulting in alkalinity. *Bacillus thuringiensis* has a growth temperature range of 30-40°C although some strains may grow at 10°C. The bacterium survives in a pH range of 5.7 to 6.8 although a more neutral pH is favourable for protein crystals production. The bacterium may be cultured in a liquid medium such as nutrient broth or in a solid medium such as nutrient agar. The addition of antibiotics is recommended to make the medium more selective in isolation of *B. thuringiensis* (Sharma and Manandhar, 1997).

2.5 Bacillus thuringiensis as a potential biological control agent

Bacillus thuringiensis has the potential for use as a biological control agent against many crop pests since it has been proved to be a potent insect pathogen. *Bacillus thuringiensis* has toxicity effects against adult insects as well as the larval stages of various insect orders. During sporulation, *B. thuringiensis* synthesizes crystalline inclusions that are composed of proteins that are toxic to certain groups of insects (Deilamy and Abbasipour, 2013). The Bt toxins cause a disruption of the midgut tissue of the insect resulting in septicemia. Although the insecticidal toxins contribute to the *Bt* action against the insects, the bacterium produce an array of virulence factors that enhance the killing of the insect (Bravo *et al*., 2011).

The need to look for alternative ways of pest control instead of chemical pesticides has been motivated by the desire to alleviate the challenges associated with these pesticides. According to Pardo-Lo´pez *et al*. (2013), some chemical pesticides are toxic to non-target organisms while some are deleterious to human and animal health. *Bacillus thuringiensis* is an appropriate biological control agent of susceptible pests because it is not only non-toxic to humans but also to many non-target fauna. Moreover, Bt formulations are easy to use thus may be popular among

farmers as alternatives to chemical pesticide (Sanchis and Bourguet, 2008). The narrow spectra of activity of Cry proteins from *B. thuringiensis* mean that they result in less adverse environmental effects of the insecticides (Raybould and Quemada, 2010). *Bacillus thuringiensis* is also widespread and may be isolated from natural habitats including soils, insect larvae, insect habitats, and grain dusts (Al-Momani and Obeidat, 2013), thus may be a reliable and sustainable source of biological pesticide.

Formulations of Bt have been developed to control some of the common caterpillars that destroy crops. For instance, formulations have been developed to control the larvae of the European corn borer, *Ostrinia nubilalis* (Hbn.), in corn fields as well as the gypsy moth, *Lymantria dispar* (L.), in forests. Some common trade names for commercial Bt insecticides utilizing *B. thuringiensis aizawai* and *B. thuringiensis kurstaki* include Thuricide®, Caterpillar Killer®, Dipel®, Bactospeine®, Worm Killer®, and Javelin®. These commercial products have been made into sprayable products for use in control of lepidopteran insects. Other products using *Bacillus thuringiensis* include Skeetal®, Bactimos®, and Teknar® (Sanchis and Bourguet, 2008). The major threat to the adoption of *Bt* technology in management of pests has been the development of resistance by pests towards the *Bt Cry* toxins. When the *Bt* technology was introduced, scientists assumed that insects would not develop resistance to *Bt* and its insecticidal proteins due to their co-existence and co-evolutions with the insects for many years. However, resistance to *Bt* and its insecticidal proteins have frequently been reported in various parts of the world (van Rensburg, 2007). This has been blamed on the extensive and widespread use of the *Bt* and its proteins. In addition, the strong evolutionary pressure on insects by the *Bt* results in development

by the insects of defense mechanisms to enable them circumvent the bacterial and toxin attack (Ibrahim *et al*., 2010).

Recent cases of insect resistance to *Bt* toxins have been documented in the United States in which *Bt*-cotton expressing *Cry*1Ac showed resistance to *Helicoverpa zea* (Tabashnik *et al*., 2008) while in South Africa, *Busseola fusca* was shown to have resistance to Bt-corn expressing *Cry*1Ab (van Rensburg, 2007). Tabashnik *et al*. (2013) has reviewed the resistance of insects to *Bt* crops by analyzing the results of 77 studies from five continents. The review showed that although most of the pest populations were susceptible, there was reduced efficacy due to field-evolved resistance for populations of 5 out of the 13 major insect pests examined in these studies.

In order to overcome the challenge of resistance by insect pests to *Bt* Cry toxins there is need to conduct continuous screening of new isolates in order to identify effective and highly potent strains whose resistance has not been developed by insects. Screening the environment for highly potent and effective strains has become one of the key strategies for managing insect resistance to Bt products (Ammouneh et al., 2011).

2.6 Diversity of *Bacillus thuringiensis* δ-endotoxins

The *Bt* δ-endotoxins consist of two multigenic protein families, *Cry* and *Cyt*. *Cry* proteins are toxic to various insect orders such as Leptidoptera, Hymenoptera, Coleoptera, Diptera, or nematodes. *Cyt* proteins are toxic against the *Diptera* insects. Therefore, *Cry* toxins are the most popular in the control of major crop pests and mosquitoes (Gómez *et al.*, 2006). Based on the similarities in the amino acid sequence, the various Bt δ-endotoxins are classified into classes, *Cry* 1, 2, 3, 4, and so on. These classes are further classified into subclasses (for example *Cry*1A,

*Cry*1B, *Cry*1C, and so on) which are further composed of variants or sub-families such as *Cry*1Aa, *Cry*1Ab, *Cry*1Ac and so on. The *Cry* proteins nomenclature proposed by Crickmore *et al* (2011) consists of 51 classes (*Cry* 1 to *Cry* 51) (Sanchis and Bourguet, 2008). The protein product encoded by each cry gene has a restricted spectrum of activity since they act against certain larval stages of a limited number of insects (Sanchis and Bourguet, 2008). The *Cry* proteins are therefore classified into six groups based on host insect specificities. Group 1 consists of *Cry* 1, *Cry* 9, and *Cry* 15 and these are active against leptidopterans. Group 2 (*Cry* 2) is active against leptidopteran and dipteran insects while group 3 (*Cry* 3, *Cry*7, and *Cry* 8) is toxic to coleopterans. Group 4 (*Cry* 4, *Cry*10, *Cry*11, *Cry*16, *Cry*17, *Cry*19 and *Cry*20) is toxic against dipterans, group 5 (*Cry*1I) against coleopteran and leptidopteran and group 6 (*Cry* 6) is active against the nematodes (Ibrahim *et al*., 2010).

2.7 Structure of *Cry* toxins

The delta endotoxins produced by Bt bacteria during sporulation are two multigenic groups, cyt and cry. *Cry* toxins are specifically toxic to various insect orders including Coleoptera, Hymenoptera, Lepidoptera, and Diptera. *Cyt* toxins, on the other hand, are specifically toxic against *Diptera* insects although Guerchicoff *et al*. (2001) have documented various *Cyt* proteins that are toxic against Coleopteran larvae. Crickmore *et al.* (2011) have defined a cry protein as a parasporal inclusion protein exhibiting a toxic effect to a target organism or any protein with an obvious sequence similarity to a known Cry protein. There are at least 70 groups of Cry toxins that have been isolated and described (Pardo-Lo´pez *et al*., 2013). Among these toxins, the 3-d-Cry toxins are the most important and most abundant representing more than 53 groups (Crickmore *et al*. (2011). The key feature of the 3d-Cry toxins is the presence of protoxins with

two different lengths, one 65 KDa and the other one 130 KDa. The 65- and 130-KDa Cry toxins differ in the C-terminal extension that is present in the 130-KDa protoxin and is useful in toxicity since it is cleaved by the insect proteases (de Maagd *et al.,* 2001). The N-terminal region 3d-Cry genes codes for the N-terminal fragment of the protoxin that is composed of 20 to 60 residues and an active toxin that is composed of about 600 amino acid residues (Pardo-Lo´pez *et al*., 2013).

The 3d-Cry toxins are globular molecules with three different domains that are connected by single linkers. Of specific interest is *Cry*1Aa which is active against lepidopteran larvae and whose crystal structure has been determined by Li *et al*. (1991). The three-dimensional structures of several *Cry* toxins have been determined and published. *Cry* toxins have three-dimensional domains and are similar in topology to a high degree (Ibrahim *et al*., 2010). The three domain toxins comprise of proteins that have toxicity effects against the insect orders *Diptera*, *Lepidoptera*, *Coleoptera*, and *Hymenoptera* as well as nematodes (de Maagd *et al*., 2003). *Cry* protoxins are comprised of two different lengths (about 130 and 70 KDa). The long protoxin has a C-terminal extension that is dispensable for toxicity but may be involved in the bacterial crystal formation (de Maagd *et al*., 2003).

2.7.1 Domain I structure

Domain I is made of 7 hydrophobic ∝ helices with at least five of them having structural features that enable them to be inserted into the cytoplasmic membrane (Sachis and Bourguet, 2008). Six amphipathic outer helices surround the central helix ∝5. The domain is implicated in pore formation and exhibit structural similarities with other bacterial toxins that cause pore formation

such as colicin Ia and N (Icii, 1a87), haemolysis E, and the diphtheria toxin domain involved in translocation (de Maagd *et al*., 2003).

2.7.2 Domain II structure

Domain II comprise of three antiparallel β-sheets that have a similar topology and form a β–prism. The three antiparallel β-sheets terminate in loops at the domain apex. Smedley and Ellar (1996) have shown that the amino acids present in this domain are involved in the interaction between the insect receptors and the toxin (Sachis and Bourguet, 2008). Domain II has significant topological similarities with various carbohydrate-binding proteins such as lectin jacalin, lectin Mpa, and vitelline (de Maagd *et al*., 2003).

2.7.3 Domain III structure

The third domain comprise of two antiparallel β-sheets in a jellyroll formation that form a β-sandwich. Domain III is involved in pore function and receptor binding. The structure of Domain III is similar to that of the domains of carbohydrate-binding proteins such as cellulose-binding domain of galactose oxidase, 1,4-β-glucanase CenC, β-glucuronidase, sialidase; carbohydrate-binding domain of xylase U and β-galactosidase (de Maagd *et al*., 2003).

2.8 The mechanism of *Cry* toxin action in lepidopteran insects

The mode of action of *Cry* toxins has been elucidated by careful analysis of guts of caterpillars fed on contaminated food. Such analysis has shown that, shortly after crystal ingestion, the cilia on the brush border of midgut epithelia cells become disrupted followed by swelling and cell lysis. The contents of the caterpillar's guts are released in the body cavity thus enabling the

bacteria to breed. After nutrients exhaustion the spores formed by the bacteria spread to a new host through contaminated vegetation or a cadaver. The activation of the Bt toxins in the gut requires the presence of specific proteases that cleaves the innocuous pro-toxin into active form and an alkaline environment (Sanahuja *et al*., 2011).

The exact mechanism of action of Cry toxins remains controversial with several models being described in literature. The first model hypothesizes that Cry toxins bind to the midgut receptors where they oligomerize and inserts into the membrane forming lytic pores. The detection of ion fluxing in brush border membrane and artificial lipid bilayers treated with Cry toxins is the basis of the postulation that lytic pores are assembled by Cry toxins in the plasma membrane through formation of oligomers. The model has, however, not been proved and no evidence has been provided in living cells or in an insect (Ibrahim *et al*., 2010).

Zhang *et al*. (2005) proposed a second model that led to the insect killing as one involving the binding of the Cry toxin monomer to the cadherin receptor BT-R_1. This leads to the activation of a Mg^{2+} -dependent signal-transduction pathway that leads to cell death (Ibrahim *et al*., 2010). According to this model, the action of Cry toxin is a complex and dynamic process involving the binding of the toxin to the structural motif in the cadherin receptor BT-R_1. This triggers a cascade of events that results in programmed cell death (Oncosis). Once Cry1Ab toxin binds to BT-R_1 receptor, a molecular signal is induced which results in stimulation of adenylyl cyclase and heterotrimeric G protein with accompanying rise in cAMP production. Protein kinase A is activated by cAMP causing several cellular alterations including ion fluxing and cytoskeletal

rearrangement. This second pathway results in cell chemistry alteration and subsequent cell death (Ibrahim *et al*., 2010).

The Cry toxins binds to the surface proteins located in the microvilli of the midgut cells of the larvae. The Cry proteins are produced as protoxins, which are dissolved and processed through a proteolytic process by insect proteases. The protoxins then release the toxin fragment in an active form. According to Bravo *et al*. (2011), there are two groups of prototoxins: the large protoxins such as the 130 kDa Cry1Aa and the short protoxins such as the Cry2Aa of 70 kDa. When large protoxins go through proteolytical cleavage, they lose half of the C-terminal end and about 20 to 50 amino acids of the N-terminal end. The short protoxins, on the other hand, are processed at the N-terminal end.

Once the protoxin is activated by the insect proteases, it goes through complex sequential binding events with the various insect gut Cry-binding proteins resulting in membrane insertion and pore formation. Binding interactions of the activated Cry protein occurs through exposed amino acid regions of domain II and III. The result of these binding interactions is a concentration of the activated toxin in the microvilli membrane of the midgut cells. The toxin then binds with high affinity through the exposed domain II loops to the cadherin receptors. The interaction with cadherin receptors facilitates further proteolytic cleavage of domain I's N-terminal leading to formation of a toxin pre-pore oligomer. Apart from the cadherin receptors, the other Cry proteins involved in lepidopteran insects' toxicity as reviewed by Pigott and Ellar (2007) include glycosylphophatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), P252 (a 250 kDa protein), and GPI-anchored alkaline phosphatase (ALP) (a 270 kDa glycoconjugate).

The pre-pore oligomer binds to the GPI-anchored proteins leading to final insertion into the membrane. This results in formation of pores in the membrane and subsequent cell lysis (Bravo *et al*., 2011).

Much of the knowledge on the mechanism of Cry proteins in lepidopteran insects have been elucidated through experiments conducted with *Manduca sexta*. Cry1Ab toxins' binding regions of monomeric and oligomeric forms in *M. sexta* receptors have been mapped. Mapping of these regions involve use of various programs and algorithms. Pardo-Lopez *et al*. (2013) mapped the structural alignments of Cry1Aa using FATCAT algorithm and dynamic programming. The binding regions of Cry 1Ab receptors in lepidopteran insects are cadherin, alkaline phosphatase (ALP), and aminopeptidase-N (APN). The three receptors in Cry1Ab are shown in Figure 2.

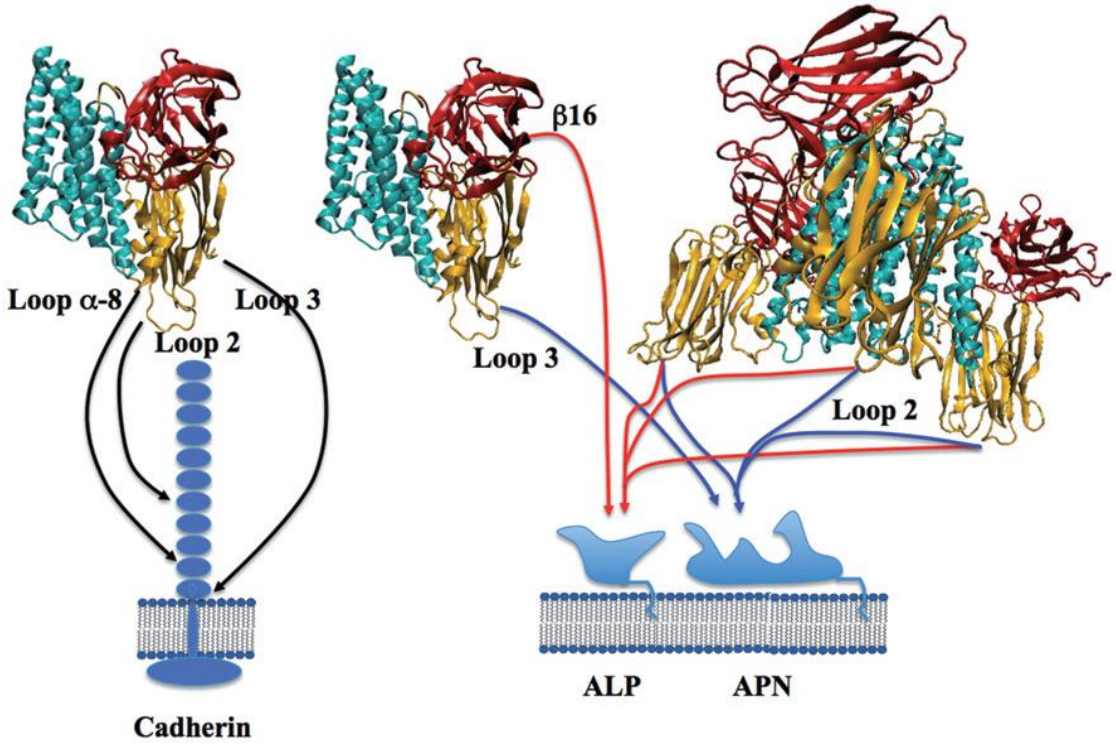


Figure 2: The receptors in Cry toxins.
Adapted from Bravo *et al*. (2013)

Go´mez *et al*. (2006) have demonstrated the role of the pre-pore oligomers in membrane insertion and subsequent cell lysis in insect gut walls. In the study by Go´mez *et al*. further demonstrated the role of APN in mediating Cry toxin toxicity. In their study, the pre-pore oligomer was shown to have a higher affinity of 200-fold (0.75 nMK_d) to APN receptor compared with that seen towards the monomeric structure of the toxin (100 nMK_d). Go´mez *et al.* (2006) have proposed a model of the mode of action of the Cry toxins in *Manduca sexta*. The model involves sequential interaction of the various toxin structures with CADR, a cadherin-like protein and APN. Figure 3 illustrates the model of Cry toxin activity in lepidopteran insects.

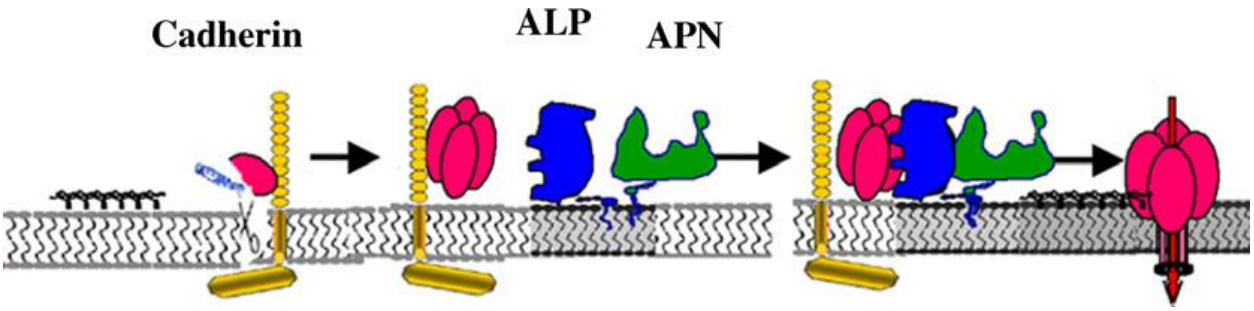


Figure 3: A model for the mode of action of Cry toxins in lepidopteran larvae
Adopted from Go´mez et al. (2006)

2.9. Isolates used in the study

The isolates used in this study were isolated by Wamaitha J. M. as part of her MSc thesis. The isolation involved the collection of samples of soil, grain, and dead insects from various maize growing areas in Kenya. The isolates were prepared using the sodium acetate selection method to obtain pure colonies if the bacteria. The isolates were then stored in glycerine under refrigeration at 0°C (Wamaitha, 2006). The isolates have been subjected to several tests against various insect pests. Wamaitha (2006) tested 68 isolates against *Prostephanus truncates* and found high

potency of between 50% and 80% with seven isolates Bt 41, Bt 61, Bt 51, Bt 60, Bt 46, Bt 45, and Bt 44 against the pest. In a later study, Kimani (2012) randomly selected 20 isolates from the 68 isolates and tested them against *Chilo partelus* and the results showed that Bt 44 and Bt 48 were most potent against the tested pest.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental insects and rearing of *Busseola fusca*

Busseola fusca neonates at the blackhead stage were purchased from the Kenya Agricultural Research Institute, Katumani. The neonates were reared on artificial diets at the KARI Katumani insectaries using the method described by Songa *et al*. (2001) (Appendix 1). The neonates were reared up to the first instar stage and brought to NARL for testing.

3.2 Retrieval of *Bacillus thuringiensis*

Bacillus thuringiensis isolates were obtained from the Biotechnology Laboratory at KARI, Biotechnology Center. A total of 44 isolates including the standard *Bacillus thuringiensis aizawai* were screened. The isolates had been collected from different parts of the country and have been stored in the laboratory for more than 2 years in glycerine, under refrigeration at 0°C. The isolates were tested against different stem borer species. The isolates were streaked on nutrient agar for growth and multiplication. The agar provided a substrate for the growth of the bacteria. Nutrient agar was prepared according to the manufacturer's instructions by suspending 28 g of nutrient agar in 1 liter of distilled water and boiled to dissolve completely (Oxoid Limited, Hampshire, United Kingdom). The agar was then sterilized in an autoclave at 121°C for 15 minutes, and about 10 ml of the agar poured on sterile plates. The media was then allowed to cool for about 5 minutes. Isolates of *Bacillus thuringiensis* were streaked using a sterile loop on to agar plates and then incubated for 7 days at 30°C for the *B. thuringiensis* cultures to grow. Nutrient broth was then prepared by dissolving 13.0g of Nutrient broth in 1 liter of distilled water in a 1-liter jar. The broth was sterilized by autoclaving at a temperature of 121°C and

pressure of 15 psi for 15 minutes. The nutrient broth was used in this study to encourage further growth and multiplication of the bacteria. Five colonies each of the sporulated cultures of each isolate were then inoculated in 250 ml fluted Erlenmeyer flasks containing nutrient broth and placed in a rotary shaker for 120 h at 30°C at 100 rpm to allow the Bt cultures to multiply. The broth was then used for the bioassay.

3.3 Insect bioassay

3.3.1 Growth of maize seedlings

Maize variety H 516 was planted under greenhouse conditions in plastic pots measuring 164 cm^3. The maize variety was used because it does well in highland areas, where the site of the study was located in Kabete. About 25 g of Diammonium Phosphate (DAP) fertilizer was added to each pot. Growth was allowed for two weeks after germination and leafs of the healthy plants then cut out from the seedlings. The young-soft leaves were cut into small leaf discs of 5 cm in diameter using a sterile scapel.

3.3.2 Testing the mortality of *B. fusca* neonates based on crude Bt protein

The test was conducted between November 2012 and April 2013. Maize leaf discs measuring 5 cm in length were cut out and perforated using a mounting needle. The maize pieces were then dipped in crude Bt broth cultures for five minutes. The discs were dried in the air for a further five minutes before being placed on a sterile Petri dish covered with paper towels from inside. Five neonates were placed on each leaf disc and 10 neonates were put in each Petri dish. For each isolate tested, four replicates were included. Nine controls were also included in the study in which untreated maize leaf discs were fed to the neonates. The controls corresponded to different trials since all the 44 isolates were tested in 9 different trials. The inclusion of the

controls in each trial was necessary to ensure that observations were due to treatments and not due to confounding factors. The bioassay setup was placed in a chamber and maintained at 50% relative humidity (RH), 28°C, and a 16:8 (L: D) h photoperiod. Each isolate had four replicates and a control giving a total of 40 neonates for each trial. In addition, a positive control involving *B. thuringiensis aizawai* was included in the study. Data on the mortality of the neonates was collected at intervals of 24 hours for five days. Observation was also made on their feeding behaviour and data collected by observing how the neonates behaved at each sampling. Feeding was observed from beneath the leaves a day after introduction of the neonates and daily for the next four days. A total of 7 controls were also included in the trial with each control corresponding to an individual trial. In the control samples, neonates were allowed to feed on untreated maize. The standard error of means was used to show significance in mean mortalities. The first screening involved the determination of the mortalities caused by different Bt isolates on *B. fusca* neonates using crude Bt broth. This enabled the determination of the most effective isolates that were subjected to further screening using extracted Bt toxins.

3.3.3 Bioassay of the most effective Bt isolates based on Bt endotoxins

The isolates with the highest mortality rates at 72 hours sampling and the overall mortality for all sampling times were selected as the most effective. Toxins from the broths of the three most effective Bt isolates were harvested and used for the second bioassay. In each 50 ml falcon tube, 40 ml of the liquid nutrient broth was put and placed in a centrifuge. The δ-endotoxin crystals were harvested by centrifugation at 4,000 rpm at 4°C (Eppendorf 5810, Eppendorf-Nether-Hinz, Hamburg, Germany). The supernatant obtained was discarded to remove the lysed cell debris and spores. The crystals were then centrifuged three times at low speed of 1300 rpm and 4°C in

0.85% saline. The washed crystals were then air dried and weighed, and then stored by suspending in 5 ml 0.85 saline at -20°C to reduce exoprotease activity (Kimani, 2012). The storage medium comprised of distilled water with 0.85% sodium chloride.

The first bioassay involved the standard Bta. Toxins extracted from the Bta were prepared through serial dilution to obtain various concentrations of the toxins (0.12 mg/ml, 0.012 mg/ml, 0.0012 mg/ml, 0.00012 mg/ml, and 0.000012 mg/ml), which were then used in the bioassay for testing against *Busseolla fusca*. Based on the LC50 determined from the Bta, the three most effective isolates against the Bt were subjected to further screening using the Bt proteins. Serial dilutions of the toxins (0.3 mg/ml, 0.03 mg/ml, 0.003 mg/ml, 0.0003 mg/ml, and 0.00003 mg/ml) were prepared and used to feed the first instar larvae of the maize stalk borer. Ten neonates per Petri dish were used for each concentration. Three Bta isolates Bta1, Bta2, and Bta7 were included in the study corresponding to trials 1, 2, and 7. The bioassay was conducted in a chamber at 50% RH, 28°C, and a 16:8 (L: D) h photoperiod. The number of larvae that survived and those that died were recorded every 24 hours for 5 days after inoculation (Ning *et al.*, 2010). The LC_{50} values were determined and used to rank the toxicity nature of the isolates for selection to carry out the Bt strain and Cry protein characterization. The LC50 of the three isolates were compared to determine the most effective Bt isolate against *B. fusca*.

Figure 4: Introducing the neonates to the Petri dishes containing different treatments at NARL Plant Pathology laboratory (February 10, 2013)

Figure 5: The experimental set-up showing Petri dishes with neonates introduced to the different treatments at KARI Pathology laboratory (February 13, 2013)

3.4 Determination of total protein concentration

The concentration of the total protein in the crystals was determined by Bradford protein determination method using a UV spectrophotometer (Bradford, 1976). Preparation of the Bradford reagent involved dissolving 10 mg of coomassie brilliant blue in 5 ml of 95% ethanol and then adding 10 ml of 85% phosphoric acid. The mixture was diluted to 100 ml and the solution filtered through Whatman no. 1 prior to the use. The standard, Bovine Serum Albumin (BSA) was used at stock solution concentration of 2 mg/l. In each of labelled test tubes, 0.1 ml each of the Bta and the three effective *Bt* samples were separately pipetted, and 5.0 ml of Bradford reagent added to each sample, thoroughly mixed and incubated at room temperature for

5 minutes. Absorbance was then measured at 595 nm and a calibration curve obtained for the standard protein. The curve was used to determine the total protein concentration for the native *Bt* isolates (Bradford, 1976).

3.5 Protein profiling using high performance liquid chromatography

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) was conducted at ChromAfrica's Bunyala road laboratories, Nairobi in order to elucidate the protein profile of the endotoxin. The Bt endotoxin crystals from the standard and the three most effective isolates were solubilized by suspending the crystals in 50 mM $Na_2CO3.NaHCO_3$ buffer and then digested with porcine trypsin solution to give trypsin-resistant fragments. The resulting solution was then subjected to centrifugation at 15000 rpm for 15 minutes before introduction into the HPLC system. The purification of the protein was achieved using reverse-phase high-performance liquid chromatography (RP-HPLC). The solid phase comprised of a system utilizing a Jupiter C_{18} column (5µm, 250 × 4.6mm, Phenomenex, USA) (Leetachewa *et al*., 2006). An aliquot of 100µl trypsin-digested δ-endotoxins was mixed with 25µl acetic acid and 20µl of the previously centrifuged sample was introduced into the HPLC system. Isolation was achieved by isocratic elution using a mobile phase that contained methanol and water at a ratio of 70:30 (v/v) at room temperature. The flow rate was maintained at 1 ml/min, 10 MPa and UV detector (absorption at 190 nm, 220 nm, 254 nm, 270 nm, 300 nm, and 360 nm in a photodiode array detector) used to monitor the separation. The 190-360 nm absorbance range was used because most biological substances have UV wavelengths in this range (Lundblad and Macdonald, 2010).

3.6 Characterization of the isolates' proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was conducted according to the method of Laemmli and Favre (1973) in order to determine the sizes of the cry proteins extracted from the bacteria isolates. The protein samples from the three most effective isolates along with the standard Bta were subjected to SDS PAGE. A separating gel comprising of double-distilled water 2.5 ml (ddH_2O), 3 ml Acrylamide mix (30%), 75 µl 10% SDS, 62.5 µl 10% ammonium per sulphate, and 2.5 µl tetramethylenediamine (TEDEM) were prepared and added to five clean tubes to make five gels. To each of the gels, 150 µl ddH_2O was added gently to make the volume to 300 µl and excess water drained off. A stacking gel was prepared and added to each universal tube. The gels were attached to the gel tank apparatus and running buffer added to the tank. The running buffer contained 72.0 g/l glycerine, 15.0 g/l Tris base, and 5.0 g/l of SDS. On the other hand, the stacking gel was prepared from a solution containing 1.125 ml of 0.5 M Tris (pH 6.8), 0.75 ml of acrylamide mix (30%), 45 µl of 10% SDS, 45 µl of 10% ammonium persulfate, 2.5 ml double distilled water, and 4.5 µl of TEMED. The combs were removed and the wells washed with the running buffer chamber using a 1 ml pipette tip. The samples were preheated at 96°C for 5 minutes. The samples were then microfuged for 5 minutes at room temperature and the samples loaded (30 µl of sample for each well comb). Power was then allowed to pass through the system for about 45 minutes. Staining was achieved through the use of Coomassie Brilliant Blue stain (0.25% (w/v). The digital image of the gel was recorded on a UV transilluminator (Laemmli and Favre, 1973).

3.7 Data analysis

The data analysed in this study included the LC50 values, the endotoxin content, the standard dosage effective against first instar larvae, as well as the larval mortalities. The GenStat Discovery Edition (VSN International Ltd, 2011) software was employed in analysing the means of mortalities of *B. fusca* caused by the *B. thuringiensis* isolates.

The LD50 calculation was done using probit analysis procedure (StatsDirect Statistical Software (StatsDirect Ltd., 2013)). The probit model employed in this study was as follows:

$$Y' = \Phi^{-1}(p)$$

where Y' is the probit transformed value, p is the proportion of subjects responding the treatment and $\Phi^{-1}(p)$ is 100*p% quantile from the standard normal distribution. The model uses logarithms of the responses to calculate LD50.

Dose-response curves were plotted based on the percent mortality versus the concentrations and the fitted model assessed by heterogeneity testing following a chi-square distribution. For all isolates, LD50 was calculated at 36 hours since this was the time at which normal distribution of mortalities was achieved. At 24 hours of observation and below, mortalities caused by all isolates was below 50% while those recorded at 48 hours and above had mortalities higher than 50%.

The mortalities of the neonates were plotted against the logarithms of the different concentrations to obtain dose-response curves. The LD50 was determined by taking the antilog of the concentration at which 50% of the neonates succumbed to the toxins.

The HPLC results were analysed by a comparison of the peaks at different band levels to determine the association among the Bt isolates. Similarly, the analysis of SDS-PAGE results involved a comparison of the unique bands obtained to determine the association among the Bt isolates.

CHAPTER FOUR

RESULTS

4.1 Efficacy of Bt isolates against *Buseola fusca*

4.1.1 Mortality of Bt isolates during initial screening

Generally, the neonates fed on the maize meal making numerous perforations on the maize discs after introduction. However, there was slowed feeding after 24 hours and the insects withdrew from the maize leaves. There was virtually no further feeding after 48 hours except for untreated trials. High mortality of *B. fusca* neonates was recorded from 48 hours and by 120 hours, most of the neonates had succumbed to the toxins. The surviving neonates in the treatments were sluggish and appeared weak. The dead larvae appeared shrunk, darkish, and small in size (Figure 6).

Figure 6: A dead larva of *Busseola fusca* after ingesting maize leaves contaminated with Bt toxins for 120 hours

In the controls where the neonates fed on untreated maize discs, normal feeding occurred up to the fifth day of observation with mortalities of less than 20% occurring in all controls. The mortality in the controls was significantly ($p \leq 0.05$) lower when compared to that in the treated samples. On the final day of observation, some neonates had grown to big sizes of up to 20 mm in length. The highest average mortality in the control samples was 19.5% (Table 1).

Table 1: Mean mortality *of B. fusca* recorded in the control samples between November 2012 and April 2013 at NARL, Pathology laboratory

Control sample	Mean mortality (%)
Control 1	15.0
Control 2	8.5
Control 3	13.0
Control 4	10.5
Control 5	15.0
Control 6	17.5
Control 7	19.5
Mean	14.1

4.1.2 Effect of duration of exposure of *Buseola fusca* to crude Bt broth

The toxicity of the different Bt isolates against the *B. fusca* neonates was significantly ($p < 0.05$) varied. Generally, mortality increased with the time of exposure (Figure 7). Mortality was lowest (66%) after 24 hours when few insects had died and highest (86.4%) after 120 hours when most of them had succumbed. More than 50% mortality was at 72 h exposure period.

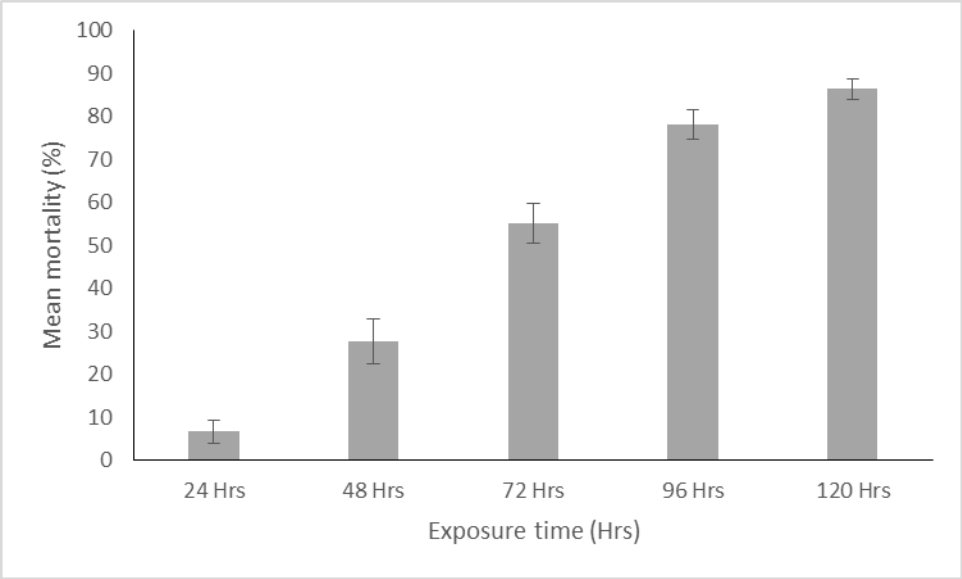


Figure 7: Mean mortality of *Buseola fusca* caused by Bt isolates at different sampling times between November 2012 and April 2013 at the NARL Plant Pathology laboratory

4.1.3 Mortality of *B. fusca* neonates after exposure to Bt broth at different exposure periods

Low mortalities of *B. fusca* were observed after 24 hours of exposure to broth from all the Bt isolates. Isolates 42 and 61 showed relatively higher mortalities at 20% each. No mortality was recorded for several isolates including Bt 34, Bt 49, Bt 51, and Bt 58 after 24 hours. Similarly, no deaths were observed in untreated controls 1, 2, 4, 6, and 7 after 24 hours.

After 48 hours, mortality rates of *B. fusca* neonates increased in comparison to that achieved after 24 hours. Isolates 25 and 30 resulted in the highest mortality rates at 67.5% and 60%, respectively. Isolates 52, 17, and 47 also resulted in high mortality at this sampling time at 52.5% each. Only five isolates caused mortality rates of 50% or higher including isolates 25, 30, 17, 52,

47, which had mean mortality rates of 67.5%, 60%, 52.5%, 52.5%, and 52.5%, respectively (Table 2).

Table 2: Mortality of *B. fusca* neonates recorded after 48-hour exposure to crude Bt broth from different *B. thuringiensis* isolates between November 2012 and April 2013 at KARI Pathology laboratory

Isolate	Mean mortality (%)	Isolate	Mean mortality (%)
25	67.5	12	25.0
30	60.0	19	25.0
17	52.5	11	22.5
47	52.5	3	22.5
52	52.5	61	22.5
56	47.5	65	22.5
26	45.0	69	22.5
59	45.0	8	22.5
62	45.0	49	20.0
42	42.5	66	20.0
43	42.5	48	17.5
63	42.5	Bta2	17.5
73	42.5	14	15.0
9	42.5	16	15.0
18	40.0	44	15.0
Bta7	40.0	41	10.0
46	37.5	45	10.0
5	37.5	7	10.0
51	35.0	*Control 3	10.0
74	32.5	34	7.5
20	30.0	55	7.5
22	30.0	*Control 5	7.5
29	30.0	*Control 1	5.0
60	30.0	*Control 2	5.0
Bta1	30.0	*Control 4	5.0
58	27.5	*Control7	2.5
		*Control 6	0.0

*The controls were run separately for different trials

After 72 hours, most of the *B. fusca* neonates had succumbed to the toxin and died. A 100% mortality was observed in some Petri dishes although others had lower mortalities. Isolates 30 and 52 caused the highest larval mortalities (90% each) after 72 hours. Similarly, isolates 25 and 73 caused high mortality rates of 87.5% after 72 hours. Effective mortality seemed to occur after 72 hours since more than half of the isolates had caused more than 50% death of the neonates (Table 3). At this sampling time, 34 isolates caused mortality rates of above 50%. High mortalities of *B. fusca* neonates were recorded after 96 hours. At this sampling, 85.2% of all neonates in the treated samples had died with some isolates causing 100% mortality. Isolates 63, 51, 49, 48, 46, 25, 18, and 14 caused 100% mortality. In addition, 25 isolates showed mortality rates of more than 90% at this sampling time.

Table 3: Mortality of *B. fusca* neonates recorded after 72-hour exposure maize discs treated with crude Bt broth from different *B. thuringiensis* isolates between November 2012 and April 2013 at KARI Pathology laboratory

Isolate	Mortality rate (%)	Isolate	Mortality rate (%)
30	90.0	5	65.0
52	90.0	56	62.5
25	87.5	60	60.0
73	87.5	65	55.0
51	85.0	12	55.0
46	82.5	9	52.5
49	80.0	7	52.5
58	80.0	Bta1	45.0
74	80.0	3	42.5
29	77.5	61	40.0
59	77.5	11	40.0
62	77.5	34	40.0
63	77.5	44	35.3
16	75.0	20	30.0
22	75.0	66	30.0
43	75.0	8	27.5
18	72.5	69	22.5
26	72.5	45	20.0
42	72.5	41	20.0
47	72.5	55	15.0
Bta7	72.5	*Control 5	12.5
14	70.0	*Control 1	12.5
48	70.0	*Control7	10.0
Bta2	70.0	*Control 4	7.5.0
17	65.0	*Control 2	7.5.0
19	65.0	*Control 3	2.5.0
		*Control 6	0.0

*The controls were run separately for different trials

The highest mortality of *B. fusca* was recorded after 120 hours exposure to crude Bt broth at which most isolates showed mortalities of 100%. The average mortality in the treated samples was 94.6%, which was the highest for all the sampling times recorded. Although most samples had almost no surviving neonates, a few samples including those infected with isolates 66, 11, and 44 had survival rates of 50%, 30%, and 27.5%, respectively. Similarly, the untreated controls had high survival rates of between 50% and 80%. Nevertheless, the mortalities recorded in the controls were significantly lower ($p < 0.05$) than that observed in the treatments.

Overall, thirty-one of the Bt isolates were found to be effective against *B. fusca*. Among the isolates, isolate 9 resulted in the lowest mortality (55.5%) while isolate 25 resulted in the highest (73%). The 31 isolates had significant toxicity effects as they resulted in death of more than half of the neonates. However, isolates 25, 30, 73, and 52 were the most effective with mean toxicity rates of 73%, 69.5%, 68.5%, and 67.5%, respectively. There was no significant difference ($p \leq 0.05$) in the mortality of *B. fusca* associated with isolates 6, 7, 11 and 66 compared to the untreated controls (Table 4).

Table 4: Mean mortality of *B. fusca* neonates caused by toxicity of crude Bt broth from 24 to 120 hours between November 2012 and April 2013 at KARI Pathology laboratory

Isolate	Mortality (%)	Isolate	Mortality (%)
25	73.0	48	58.0
30	69.5	14	57.5
73	68.5	Bta2	56.5
52	67.5	65	56.0
18	66.5	9	55.5
63	65.5	7	51.0
43	65.5	Bta1	51.0
46	65.0	12	50.5
59	65.0	3	48.0
74	64.5	34	48.0
22	64.0	69	47.0
47	64.0	8	46.0
51	64.0	20	45.0
17	63.5	61	42.5
62	62.5	45	42.5
26	62.0	44	41.5
42	61.5	41	41.0
29	61.0	11	38.5
5	61.0	55	37.0
58	61.0	66	29.5
56	60.5	*Control 7	19.5
49	60.0	*Control 6	17.5
60	60.0	*Control 1	15.0
Bta7	59.0	*Control 5	15.0
16	58.0	*Control 3	13.0
19	58.0	*Control 4	10.5
		*Control 2	8.5

*The controls were run separately for different trials

4.2 Bioassay of the most effective Bt isolates against *B. fusca*

The four isolates that were most effective were 25 (73%), 30 (69.5%), 73 (68.5%), and 52 (67.5%). Isolate 52, 25, and 30 were included among the three most effective as they had higher mortalities at lower sampling period (48-hour) as well the highest mortalities at 72-hour exposure, the period at which the effectiveness of the toxins occurred. The 72-hour period was selected because it was the period at which most isolates (33) caused over 50% mortality rates. The dry weights of proteins extracted from the three most effective *Bt* isolates as well as the standard Bta varied significantly (Figure 8). Isolate 30 had the highest protein yield at an average of 665 mg from 100 ml of broth. The lowest protein yield was obtained from isolate 25, which had an average yield of 110 mg from 100 ml of broth. The standard Bta had a yield of 245 mg per 100 ml of broth. The extracted Bt proteins were used in the subsequent bioassay.

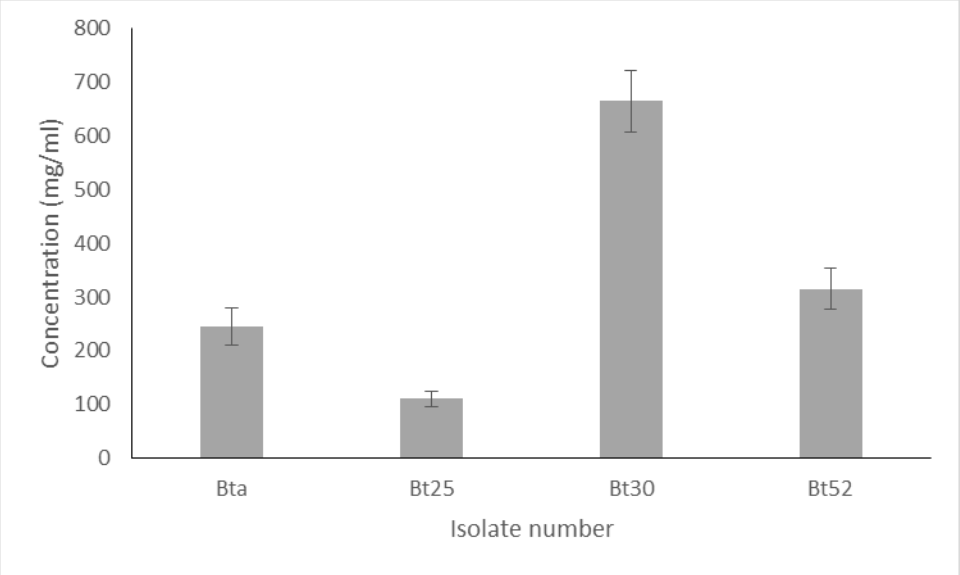


Figure 8: The weights (mg) of pellets extracted from various Bt isolates on May 17, 2013 at KARI Biotechnology center

The mortality of *B. fusca* neonates caused by the Bta was significantly different ($p \leq 0.05$) for the different sampling times (Table 8). There was increase in mortality with increase in exposure time with the highest mortality being observed after 120 hours and lowest after 24 hours as expected.

Table 5: Mean mortalities of *B. fusca* neonates caused by exposure to various doses of *Bacillus thuringiensis aizawai* toxins at different exposure times at KARI Plant Pathology laboratory, 2-7 October 2014

Concentration (mg/ml)	Mortality (%)					
	24 hours	48 hours	72 hours	96 hours	120 hours	Average
12	2.5	10.0	42.5	87.5	100.0	48.5
1.2	17.5	42.5	77.5	82.5	95.0	63.0
0.12	7.5	70.0	90.0	100.0	100.0	73.5
0.012	7.5	15.0	77.5	97.5	100.0	59.5
0.0012	0.0	20.0	70.0	95.0	100.0	57.0
0.00012	0.0	0.0	20.0	65.0	82.5	33.5
0.000012	0.0	2.5	7.5	45.0	65.0	24.0
Control	0.0	0.0	12.5	22.5	27.5	12.5
Mean	3.4	22.5	49.7	74.4	83.8	

Means represent mortality averages over concentration ranges

The mortality of *B. fusca caused by* standard Bt isolate dropped with reduction in concentration (Figure 9). The concentration of 0.12 mg/ml was the most effective against the *B. fusca* neonates causing 73.5% mortality while the lowest was 0.000012 mg/ml. The mortalities caused by the

different concentrations were significantly different from each other ($p \leq 0.05$) except for concentrations 0.012 mg/ml and 0.0012 mg/ml, which had no significant difference.

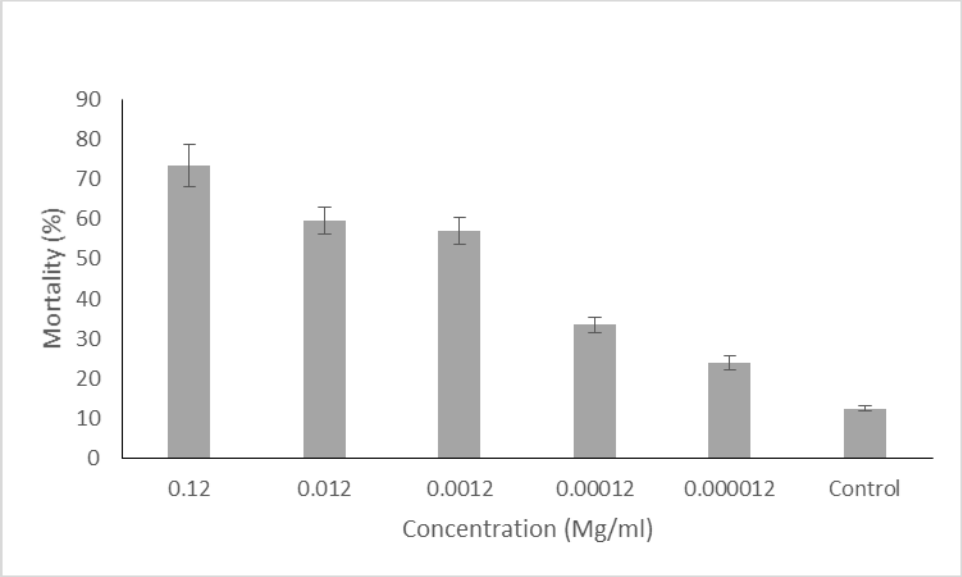


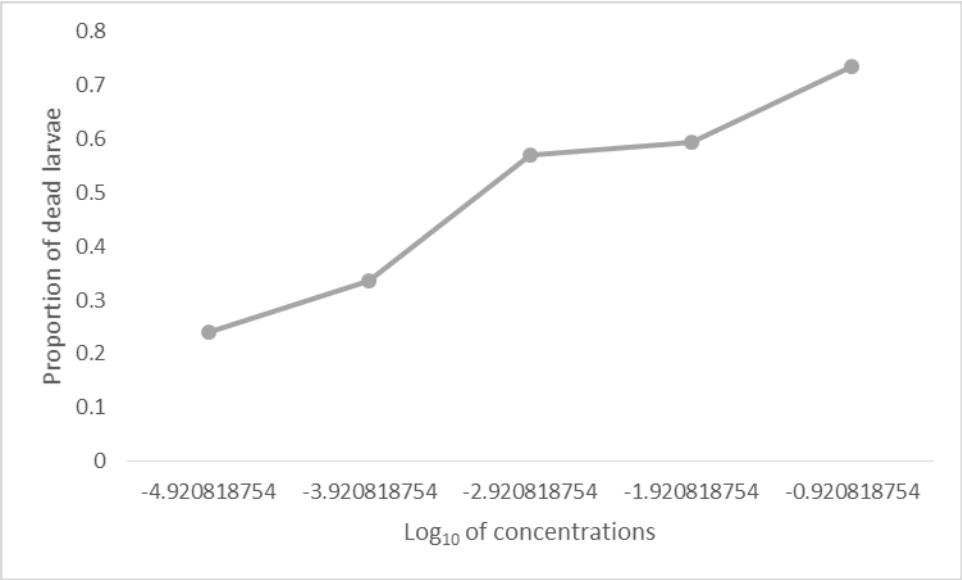
Figure 9: Larval mortalities from exposure of *B. fusca* neonates to various *Bacillus thuringiensis aizawai* protein concentrations on 24-28 September 2013 at the NARL Plant Pathology laboratory

Key: Error bars represent the standard error of the means.

4.2.1. LD50 for Bta

The mean lethal dose causing 50% mortality at 36-hour sampling was determined for the standard Bta. The 36-hour sampling was selected because it was the period at which a near sigmoid curve was obtained. Probit analysis conducted using StatsDirect Statistical Software (StatsDirect Ltd., 2013). A curve drawn for the proportion of dead neonates against different Bta concentrations. The mortalities of the neonates when subjected to different concentrations (0.12 mg/ml, 0.012 mg/ml, 0.0012 mg/ml, 0.00012 mg/ml, and 0.000012 mg/ml) of the standard *Bacillus thuringiensis aizawai* proteins ranged from 24% to 73.5%. Using the probit model, the

LD50 was found to be 0.003201 mg/ml (p=0.0199) (Figure 10). At this dosage, 50% of the neonates succumbed to the effects of the toxin.

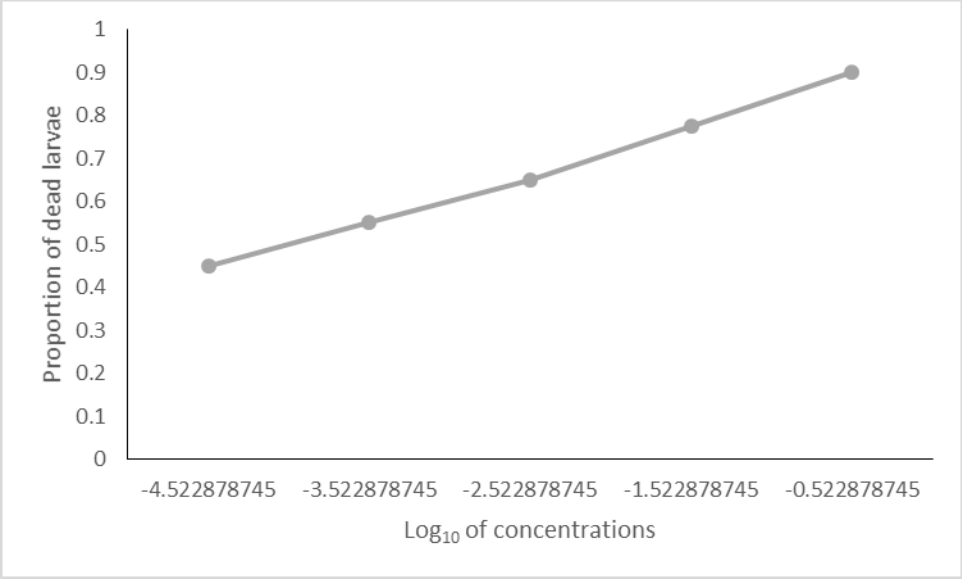


LD50 = Antilog of -2.4947 = 0.003201 mg/ml

Figure 10: The proportional response achieved from subjecting *B. fusca* neonates to different concentrations of Bta toxins at 36 hours at the NARL Plant Pathology laboratory between 18 and 19 October 2014

4.2.2. LD50 for Bt 25

The mortalities of the neonates when subjected to different concentrations of Bt 25 (0.3 mg/ml, 0.03 mg/ml, 0.003 mg/ml, 0.0003 mg/ml, and 0.00003 mg/ml) ranged from 45% to 90%. The LD50 for Bt 25 at 36 hours was calculated using the probit model and found to be 0.00367 mg/ml (p=0.0195). This was the concentration at which 50% mortality was achieved (Figure 11).

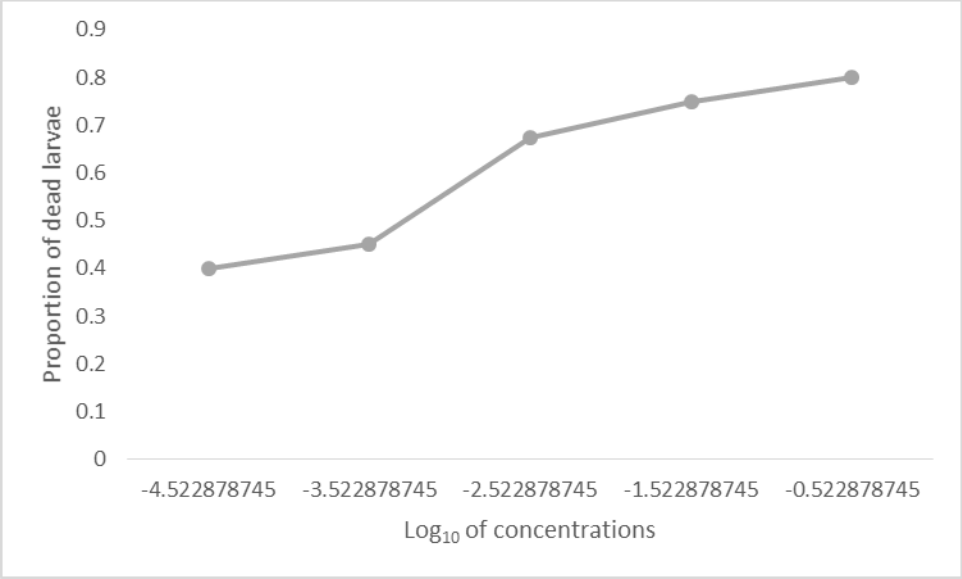


LD50 = Antilog of -3.43533 = 0.00367 mg/ml

Figure 11: The proportional response achieved from subjecting *B. fusca* neonates to different concentrations of Bt 25 toxins at 36 hours at the NARL Plant Pathology laboratory between 18 and 19 October 2014

4.2.3. LC50 for Bt 30

The mortalities of the neonates when subjected to different concentrations of Bt 30 (0.3 mg/ml, 0.03 mg/ml, 0.003 mg/ml, 0.0003 mg/ml, and 0.00003 mg/ml) ranged from 40% to 80%. Using the probit model, the LD50 of Bt 30 at 36 hours was determined to be 0.000942 mg/ml (p=0.0239). This was the concentration that caused 50% mortality in the neonates (Figure 13).

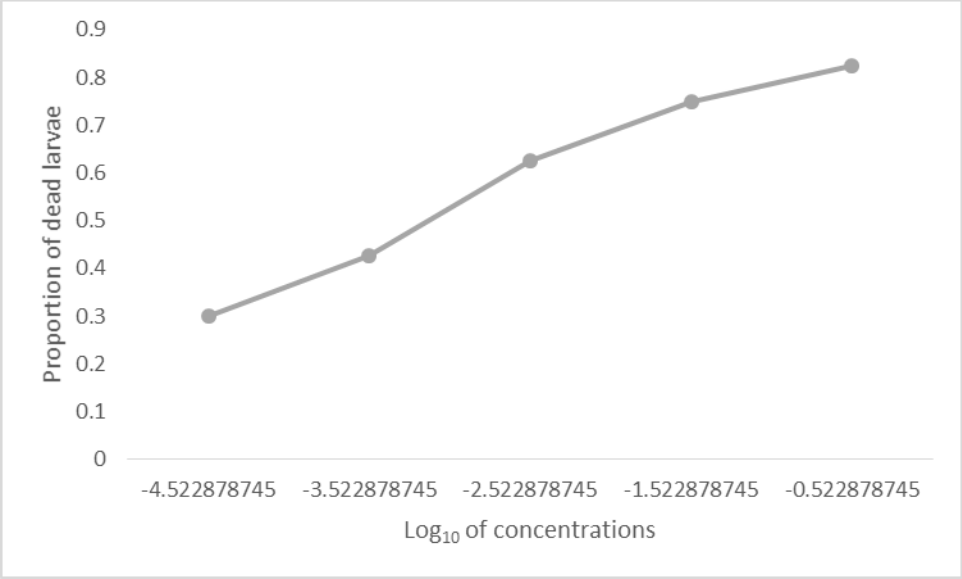


LD50 = Antilog of -3.025949 = 0.000942 mg/ml

Figure 12: The proportional response achieved from subjecting *B. fusca* neonates to different concentrations of Bt 30 toxins at 36 hours at the NARL Plant Pathology laboratory between 18 and 19 October 2014

4.2.4. LC50 for Bt 52

The mortalities of the neonates when subjected to different concentrations of Bt 52 (0.3 mg/ml, 0.03 mg/ml, 0.003 mg/ml, 0.0003 mg/ml, and 0.00003 mg/ml) ranged from 30% to 82.5%. The LD50 for Bt 52 at 36 hours was 0.002395 mg/ml (p=0.0195) when calculated using the probit model. This was the concentration that caused 50% mortality for Bt52.



LD50 = Antilog of -2.62069 = 0.002395 mg/ml

Figure 13: The proportional response achieved from subjecting *B. fusca* neonates to different concentrations of Bt 52 toxins at 36 hours at the NARL Plant Pathology laboratory between 18 and 19 October 2014

4.2.5. Summary of the dose-response effects

The dose-effect analysis was calculated at 36 hours since mortality rates at this sampling time was within the normal distribution range. Isolate Bt 30 had the lowest LD50 at 0.000942 mg/ml while Bt 25 had the highest LD50 at 0.00367 mg/ml. There was no significant difference between the LD50 of Bta and that of Bt 25 ($p \geq 0.05$).

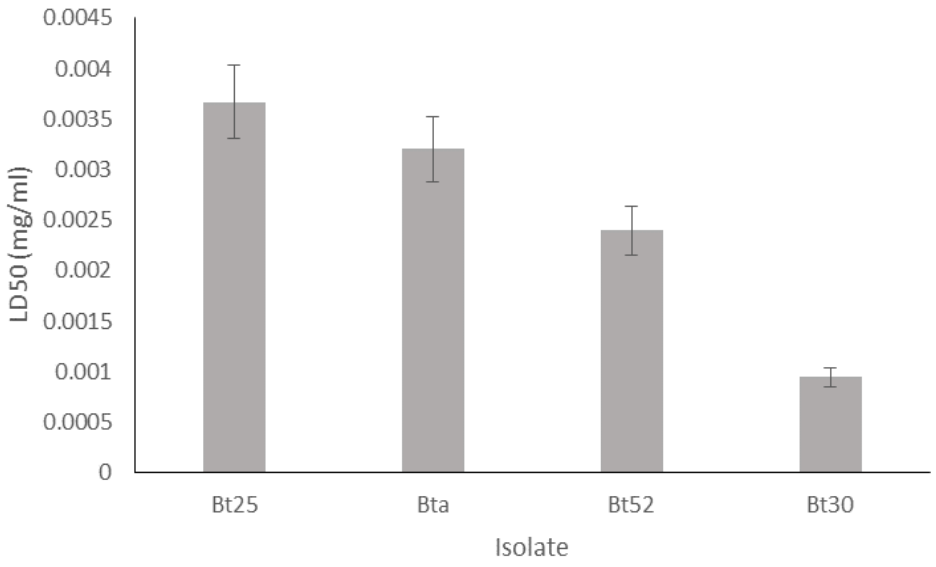


Figure 14: The LD50 of different isolates subjected to *B. fusca* neonates at 36 hours

4.3. Characterization of cry protein for Bta, Bt 25, 30, and 52

4.3.1 Characterization of cry protein by gel electrophoresis

Sodium dodecyl sulphate Polyacrylamide gel electrophoresis was conducted on the proteins extracted from the three isolates including *Bacillus thuringiensis aizawai*. An analysis of the separation of the proteins in the isolates showed that a protein of approximately 130 KDa existed in the isolates (Figure 17). This was in line with the expected molecular weight for Bt proteins. The four isolates Bta, Bt 25, Bt 30, and Bt 52 contained protein sub-units of 130 KDa molecular size. Further analysis showed that smaller protein molecules with sizes ranging from approximately 70 KDa to 100 KDa existed in all the isolates.

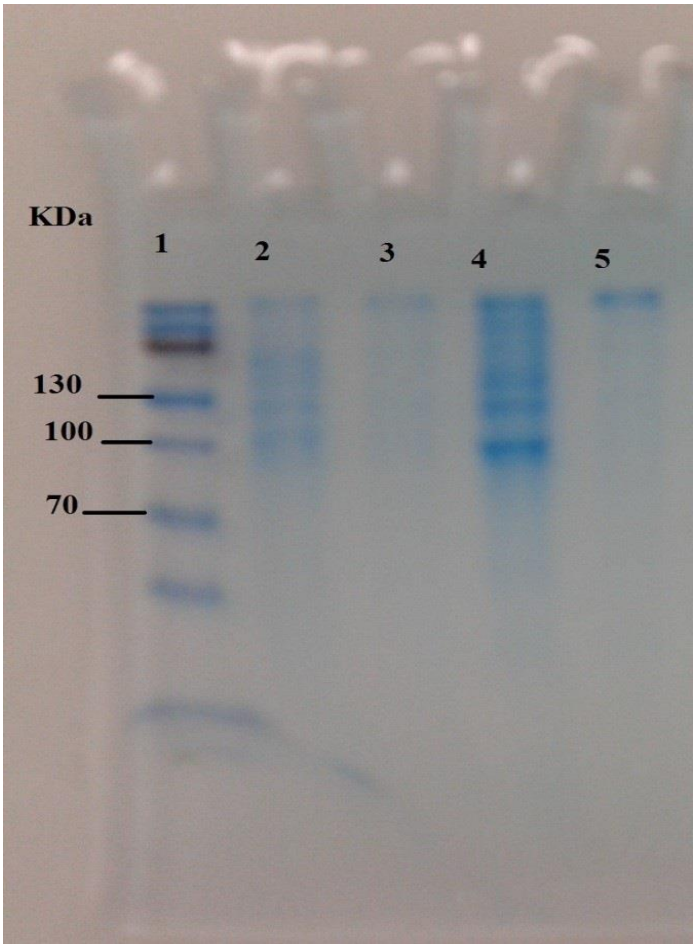


Figure 15: Band from SDS PAGE of *B. thuringiensis* isolates

Key: Lane 1: molecular weight markers (Appendix 2); lane 2: Bta; lane 3: Bt 25, lane 4: Bt 30, lane 5: Bt 52.

4.3.2 Protein profile of selected Bt isolates using HPLC

The proteins in different isolates had a different number of peaks at similar absorbances but the highest for all isolates was achieved at 190 nm. Isolate 52 had the highest number of peaks at 42 (220 nm) and 39 (360 nm) (Table 6). For all isolates, the optimum absorbance was achieved at 190 nm at which the highest peaks were obtained. At this absorbance, Bta endotoxins had a height of 1.64×10^6 mAU while the protein area percentage was 79.5%. A similar trend was

observed for isolates 25, 30, and 52, which achieved heights of 6.36× 10^6 mAU, 1.66 10^6 mAU, and 1.63× 10^6 mAU, respectively for an absorbance of 190 nm. The protein area percentages for isolates 25, 30, and 52 were 72.6%, 79.9%, and 34.6%, respectively. Isolates 30 and 52 were, therefore, very similar to Bta.

Table 6: Total number of peaks obtained for proteins from different Bt isolates at different spectral absorbance at ChromAfrica laboratories on December 6, 2013.

Isolate	Number of peaks					
	190 nm	220 nm	254 nm	270 nm	300 nm	360 nm
Bta	18	39	24	18	14	16
25	13	29	11	12	14	13
30	10	34	14	16	11	18
52	34	42	14	12	12	39

The first peaks were attained 1.97 minutes, 1.92 minutes, 2.01 minutes, and 4.20 minutes for Bta, Bt 25, Bt 30, and Bt 52 respectively. The time taken to achieve the highest peaks were 4.20 minutes, 4.21 minutes, 4.20 minutes, and 4.20 minutes for Bta, Bt 25, Bt 30, and Bt 52.

Isolates Bta, Bt 30, and Bt 52 shared similar bands at 190 nm. The three isolates Bta, Bt30, and Bt 52 had peaks of 1.64 × 10^6 mAU, 1.66 10^6 mAU, and 1.63 × 10^6 mAU, respectively. These peaks were all attained at a retention time of approximately 4.2 minutes (Table 7).

Table 7**:** Characteristics of the highest peaks achieved at 190 nm after running different Bt isolates on HPLC at ChromAfrica laboratories on December 6, 2013.

Isolate	Height of highest peak at 190 nm (mAU)	Time taken to achieve highest peak (Minutes)	Area of the highest peak	Percentage of the area
Bta	1.64×10^6	4.20	7.20×10^7	79.50
Bt 25	6.36×10^6	4.21	6.36×10^7	72.61
Bt 30	1.66×10^6	4.20	6.41×10^7	79.97
Bt 52	1.63×10^6	4.20	6.80×10^7	34.60
Average	2.82×10^6	4.20	6.69×10^7	66.67

4.3.2.1. Elution profile for Bta, Bt 25, Bt 30, and Bt 52 at 190 nm

For Bta, the highest peak at an absorbance of 190 nm (peak 2) was attained at a retention time of 4.201 minutes and had a height of 1.64×10^6 (mAU), an area of 7.20×10^7 mV.s and an area percentage of 79.5% (Appendix 3). This was the highest point reached in the entire elution profile for Bta. The peak characterized the Bta protein (Appendix 4). Peak 2 covered nearly the entire peak area at 79.5% and, therefore, represents the trypsin-digested endotoxins from Bta.

Bacillus thuringiensis isolate 25 (Bt 25) achieved the highest peak at an absorbance of 190 nm. The peak was recorded at a retention time of 4.221 and had an area of 6.36×10^7, height of 1.55×10^6 mAU and an area percentage of 72.6% (Appendix 10). The highest peaks recorded for the

other absorbances were also achieved after the fourth minute. The protein profiles were similar to those observed for Bta.

Similar to the observations in the other isolates, the tallest peak for Bt 30 was obtained at an absorbance of 190 nm. The peak was achieved at a retention time of 4.198 had a height of 1.66×10^6 mAU and an area and area percentage of 6.41×10^7 and 79.9%, respectively. The rest of the peaks had insignificant heights and could, therefore, represent the impurities in the sample (Appendix 15). The elution profile for Bt 52 was similar to that of the other three isolates in that the tallest peak was obtained at 190 nm (Appendix 21). The tallest peak at 190 nm was obtained at a retention time of 4.199 and had a height of 1.63×10^6 mAU and an area and area percentage of 6.80×10^6 and 34.6% respectively.

4.3.2.2. Elution profile for Bta, Bt 25, Bt 30, and Bt 52 at 220 nm

For Bta, the peak characterizing the *Cry* protein at absorbance of 220 nm (peak 3) was attained at a retention time of 4.203 min (Appendix 5). Peak 3 had a height of 346,347 mAU, an area of 1.89×10^6 mV.s and an area percentage of 30.63%. This was lower than the heights of the other characterizing peaks for the other absorbances. Apart from peak 3, peaks 4 and 7 also achieved relatively higher heights at 25,180 mAU and 10,088 mAU respectively. The other peaks recorded at this absorbance were low (Table 8). In the elution profile of Bt 25 at 220 nm, the highest peak was recorded at a retention time of 4.213. The peak height was 329,011 mAU while the area was 2.55×10^6 mV.s and the area percentage was 59.32% (Appendix 11). The highest peak for Bt 30 at an absorbance of 220 nm was attained at a retention time of 4.199 minutes with a height of 4.234×10^6 mAU. The peak had an area of 2.10×10^7 and an area percentage of

30.0%. At an absorbance of 220 nm, the highest numbers of peaks (34) were recorded for Bt 30 (Appendix 16).

At an absorbance of 220 nm, isolate Bt 30 attained its highest peak at a retention time of 4.2 minutes. The peak had a height of 3.807×10^6 mAU and an area and area percentage of 3,770,728 mV.s and 61.9% respectively (Appendix 22).

Table 8: Properties of the tallest peaks at an absorbance of 220 nm for Bta, Bt 25, Bt 30, and Bt 52 at ChromAfrica laboratories on December 6, 2013

Isolate	Peak#	Retention time (min)	Area (mV.s)	Height (mAU)	Area%
Bta	3	4.203	1,894,515	346,347	30.63
Bt 25	3	4.213	2,550,157	329,011	59.33
Bt 30	2	4.199	2,095,907	423,815	30.01
Bt 52	1	4.20	3,770,728	380,760	61.88

4.3.2.3. Elution profile for Bta, Bt 25, Bt 30, and Bt 52 at 254 nm

At 254 nm, the highest peak for Bta was attained at 4.202 minutes (Appendix 6). This peak had a height of 1.35×10^5 (mAU) while its area and area percentage figures were $8.16821 \times 10^5$and 39.8%, respectively. A total of 24 peaks were eluted for Bta at 254 nm. For Bt 25, the highest peak at 254 nm had a height of 130,853 mAU and was attained at a retention time of 4.2 minutes (Appendix 12). The peak's area and area percentage were 1,490,512 mV.s and 91.87%, respectively. For isolate 30, peak 3 was the characterizing peak for the protein (Appendix 17). There were 14 peaks at 254 nm for Bt 30, with the highest being observed at retention time of

4.198 minutes. This peak had a height of 286,107 mAU while its area and area percentage were 1,335,662 mV.s and 38.9%, respectively.

The highest peak at an absorbance of 254 nm for Bt 52 was peak 3. The peak had a height of 156825 mAU and was attained at a retention period of 4.2 minutes (Appendix 23). The peak had an area and area percentage of 1,031,634 mV.s and 61.24%, respectively (Table 9).

Table 9: Properties of the tallest peaks at an absorbance of 254 nm for Bta, Bt 25, Bt 30, and Bt 52 at ChromAfrica laboratories on December 6, 2013

Isolate	Peak#	Retention time (min)	Area (mV.s)	Height (mAU)	Area%
Bta	2	4.202	816,821	135,013	39.82
Bt 25	2	4.212	1,490,512	130,853	91.87
Bt 30	3	4.198	1,335,622	286,107	38.90
Bt 52	3	4.20	1,031,634	156,825	61.24

4.3.2.4. Elution profile for Bta, Bt 25, Bt 30, and Bt 52 at 270 nm

Generally, the heights of the tallest peak reduced from those observed in lower absorbances of 190 nm, 220 nm, and 254 nm. The protein profile of Bta showed that the highest peak 270 nm had a height of 1.123×10^5 nm. However, the area percentage for the peak was much higher than that attained at 254 nm at 66.7%. The highest elution peak at 270 nm for Bta was attained at a retention time of 4.201 (Appendix 7). The peak had an area of 1,117,128 m.Vs and an area percentage of 66.7%. For Bt 25, peak 2 was the highest at an absorbance of 270 nm with a height of 114,322 mAU. This peak was attained at 4.21 minutes and had an area of 787,653 mV.s

(Appendix 13). The area percentage for the highest peak at 270 nm for Bt 25 was 53.29%. The peak was slightly higher than that achieved by Bta at this absorbance.

For Bt 30, the highest peak at 270 nm was attained at a retention time of 4.2 minutes. The peak had a height of 252,244 mAU, an area of 1,179,607, and area percentage of 38.2%. The peak was taller than that of the other three isolates at 270 nm absorbance (Appendix 18). The tallest peak for isolate Bt 52 at 270 nm was attained at a retention time of 4.2 minutes (Appendix 24). The peak had a height of 147,130 mAU, an area of 978,971, and area percentage of 59.07%. At an absorbance of 270 nm, isolates Bta, Bt 25, and Bt 52 showed similar profiles as they attained comparable peak heights (Table 10).

Table 10: Properties of the tallest peaks at an absorbance of 270 nm for Bta, Bt 25, Bt 30, and Bt 52 at ChromAfrica laboratories on December 6, 2013

Isolate	Peak#	Retention time (min)	Area (mV.s)	Height (mAU)	Area%
Bta	2	4.201	1,117,128	112,300	66.67
Bt 25	2	4.212	787,653	114,322	53.29
Bt 30	4	4.198	1,179,607	252,244	38.20
Bt 52	3	4.2	978,971	147,130	59.07

4.3.2.5. Elution profile for Bta, Bt 25, Bt 30, and Bt 52 at 300 nm

A similar trend of decrease in the size of the tallest peak was observed at 300 nm. At this absorbance, the height of the tallest peak for Bta was 4.0534×10^5 mAU, which was attained at a retention time of 4.2 minutes (Appendix 8). The tallest peak for Bta at this absorbance had an

area of 312269 and area percentage of 78.04%. For Bt 25, the tallest peak at 300 nm was observed at 4.2 minutes and had a height of 35,875 mAU (Appendix 14). The area and area percentage for this peak were 260,766 mV.s and 76.2% respectively. Similarly, Bt 30 and Bt 52 attained their highest peaks at retention times of 4.2 minutes at 300 nm (Appendices 19 and 25). The heights of the peaks were comparable for Bt 30 (44,195 mAU) and Bt 52 (46,191 mAU) (Table 11).

Table 11: Properties of the tallest peaks at an absorbance of 300 nm for Bta, Bt 25, Bt 30, and Bt 52 at ChromAfrica laboratories on December 6, 2013

Isolate	Peak#	Retention time (min)	Area (mV.s)	Height (mAU)	Area%
Bta	3	4.203	312,269	40,534	78.04
Bt 25	3	4.213	260,766	35,875	76.18
Bt 30	2	4.20	265,625	44,195	70.80
Bt 52	3	4.20	334,539	46,191	73.70

4.3.2.6. Elution profile for Bta, Bt 30, and Bt 52 at 360 nm

At an absorbance of 360 nm, the proteins for all the isolates attained their highest point at 4.2 minutes (Table 12). For Bta, the tallest peak had a height of 27,776 mAU, an area of 148,915 mV.s and an area percentage of 53.05%. The heights of the tallest peaks for Bt 30 and Bt 52 were 16,868 mAU and 21,058 mAU, respectively (Appendices 9, 20, and 26, respectively). The retention time for the tallest peaks for all isolates was above 4 minutes for all absorbances. Despite the differences in heights and area of the peaks, all isolates demonstrated similar

profiles. However, the numbers of peaks were more distributed for Bt 52, with multiple tall peaks being observed.

Table 12: Properties of the tallest peaks at an absorbance of 360 nm for Bta, Bt 30, and Bt 52 at ChromAfrica laboratories on December 6, 2013

Isolate	Peak#	Retention time (min)	Area (mV.s)	Height (mAU)	Area%
Bta	2	4.204	148,915	27,776	53.05
Bt 30	2	4.20	110,916	16,868	54.03
Bt 52	4	4.20	195,536	21,058	22.73

CHAPTER FIVE

DISCUSSION

5.1. Toxicity effects of *Bacillus thuringiensis* isolates against *Busseola fusca*

Bacillus thuringiensis has been reported as the most effective commercial biocontrol agent for insect pests (Prabhakar *et al*., 2013). This study involved screening of the insecticidal activities of native *Bacillus thuringiensis* isolates against the maize stalk borer, *Busseola fusca*. An initial screening was conducted by feeding *B. fusca* neonates on maize leaf discs contaminated with crude Bt broth. A selection of the most effective isolates was conducted and subsequent quantitative bioassays using various toxins concentrations to determine the effective dosage. According to Jisha *et al.* (2013), crude Bt broth comprise of a mixture of protein crystals, spores, and cellular debris. The crude Bt broth is, therefore, highly toxic to specific lepidopteran insects. After feeding the insect neonates on two-week old maize leaf discs contaminated with crude Bt broth, the mortalities caused by the different isolates were recorded.

In the treated samples, feeding was observed to take place within the first 24 hours after which the neonates stopped feeding completely, became sluggish, and died. However, normal feeding continued in the controls up to the final sampling time at 120 hours. These findings corroborate the findings of previous studies (Ibrahim *et* al., 2010; Bravo *et* al., 2011) that have defined the mechanisms of action of the Cry toxin in lepidopteran insects. For instance, the most accepted model for explaining the mode of action of *Bacillus thuringiensis* endotoxins is the pore-formation model. According to this model, insects ingest crystalline inclusions, which are then solubilized in the guts to release Cry proptoxins. The cleavage of the Cry protoxins by the insect

midgut proteinases results in formation of active Cry toxins. The activated Cry toxins bind to specific receptors in the insect midgut epithelium resulting in pore formation in the membrane and subsequent cell lysis (Zhang *et al*., 2005; Bravo *et al*., 2011). The formation of pores in the insect midgut and the resulting cell lysis results in inability of the insects to feed.

In the current study, the neonates failed to feed after approximately 24 hours from exposure to the toxins. This may have resulted in the formation of pores in the insects' midgut and, therefore, the inability to feed. The insects, therefore, succumbed to starvation soon after. Tende *et al.* (2010) while studying the susceptibility of *Chilo partellus* and *Busseola fusca* reported that neonates feeding stopped within the 1-2 hours after ingestion of the Bt toxin. In this study, however, data collection was conducted on a 24-hour interval and, therefore, the phenomenon was identified after 24 hours. The treated samples showed higher mortalities than the controls. The highest average mortality recorded in the control samples was 19.5% while the highest mortality in the treated samples was 73.0% for isolate Bt 25. Insignificant mortalities of 8.5% were observed in some untreated controls and, therefore, the results were consistent with similar studies. Kimani (2012), for instance recorded 10% larval mortality after 72% in the control treatment in a study involving *C. partellus*. The significant difference between the controls and the treated samples demonstrate that the mortalities observed in the treated samples were due to the treatment and not due to extraneous factors. Within the first 48 hours, all controls except controls 3 and 5 had 100% survival rates while the treated samples showed mortalities ranging from 2.5% to 20% within 24 hours. These results were consistent with those reported in Syria on a study of the efficacy of *Bacillus thuringiensis* on various insect pests in which high survival

rates of 100% were observed in the controls within the first 24-48 hours (Ammouneh *et al.*, 2011).

The mortalities of *Busseola fusca* neonates increased with sampling time. The lowest average mortality was recorded after 24 hours at 6.6% while the highest average mortality was observed at 120 hours where 86.4% of the neonates died. The low mortality observed in the 24-hour sampling could be attributed to the short duration of exposure of the insects to the toxins. At this time, the amount of toxins ingested by the neonates could not have been sufficient to cause death. Additionally, the toxin exposure time was less and therefore the toxicity process could not have been complete. Previous studies have shown that organisms generally die at higher rates after a long exposure to a toxic substance than when exposed for a shorter duration. In a recent study on the insecticidal activity of *Bacillus thuringiensis*' Cry toxins against several Syrian insect pests, Ammouneh *et al*. (2011) showed that the effects of the toxins were more pronounced after a 48-hour exposure compared to a 24-hour exposure at which toxicity were lower. The difference in mortality of insects with time could be attributed to the occurrence of different toxicity effects of the toxins with time. For instance, electron micrographs obtained by Ogutcu *et al*. (2005) have demonstrated that *Bacillus thuringiensis kurstaki* has different effects on malphigian tubules of *Thaumetopoea pityocamba* larvae. The study by Ogutcu *et al*. (2005) has shown that swelling of the swelling of the mitochondria occurred within 12 hours while dissolution of the basal cytoplasm and dissolution of nucleoplasm occurred after 24 hours and 72 hours respectively. The current findings, were therefore, consistent with previous studies on dosage exposure to insects over time. There was low mortality in some samples. Twelve isolates caused average mortality that was lower than 50%. These included isolates 3 (48%), 34 (48%), 69 (47%), 8 (46%), 20 (45%), 61 (42.5%), 45 (42.5%), 44 (41.5%), 41 (41%), 11 (38.5%), 55

(37%), and 66 (29.5%). Nevertheless, all isolates had significantly higher mortalities than the untreated controls except isolate 66. Kimani (2012) while testing the insecticidal activity of *Bacillus thuringiensis* on *Chilo partellus* reported low mortalities in some isolates, with 11 isolates showing mortalities of less than 50%. In another study, Tabashnik *et al*. (1990) reported low mortalities caused by isolates of *Bacillus thuringiensis* subsp. *kutstaki* among the diamond back moth, *Plutella xylostella*. In this study, low mortalities of 34-35% were reported in the resistant populations while the effective isolates caused average mortalities of 90-100%. This is comparable to some of the observations in the current study, for instance, with isolate 29 having a mean mortality of 29.5%.

The most plausible explanation for the low mortalities observed in some isolates is the specificity of the *B. thuringiensis* strains to lepidopteran insects. According to Gómez et al. (2006), Cry proteins are specifically toxic to specific insect orders such as Celeoptera, Lepidoptera, Coleoptera, and Hymenoptera. Cry1A toxins are specifically toxic to lepidopteran insects. Therefore, the various isolates screened in this study may have contained different Cry toxins and, therefore, causing different toxicity effects due to lack of specificity in some isolates.

5.2. Effective dosage of *Bacillus thuringiensis* toxins against *Busseola fusca*

Effective dosage was also determined for the three most effective isolates by testing the different concentrations of *Bacillus thuringiensis* proteins against *Busseola fusca* neonates. The standard isolate *B. thuringiensis* subsp. *aizawai* had an LD_{50} of 0.003201 mg/ml. This was the dosage required to kill 50% of the neonates. Using serial dilutions of this concentration, the LD_{50} of the three most effective isolates were determined and found to be 0.00367 mg/ml, 0.000942 mg/ml,

and 0.00239 mg/ml for isolates Bt 25, Bt 30, and Bt, 52 respectively. For all isolates, mortality was seen to generally increase with increase in concentrations. This could be attributed to the fact that higher concentrations of the dosage meant that there was higher rate of ingestion of the toxin by the insects and, therefore, higher mortality. The results of this study were comparable with the findings of Avilla et al. (2009). While working on the toxicity of *Bacillus thuringiensis* against *Helicoperpa armigera* from Spain, Avila *et al.* (2009) found that the LC50 was in the range of 0.0035 mg/ml to 0.0063 mg/ml.

5.3. Protein characterization

Using the sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS PAGE), the size of the toxin proteins was determined. Proteins of approximately 130 KDa were found in the toxins in addition to smaller sized proteins of between 70 KDa and 100 KDa. The findings of this study corroborate previous findings that have demonstrated that Cry proteins present in *Bacillus thuringiensis* contain proteins of about 130 KDa. While working on the characterization of Mexican strains of *Bacillus thuringiensis*, Bravo *et al*. (1998) showed that most of the Cry proteins had approximate sizes of 120 KDa to 145 KDa. Proteins of sizes 130 KDa and 85 KDa were also identified in *B. thuringiensis* in the study by Bravo *et al.* (1998). In another study, Ning et al. (2010) characterized the Cry1Ac proteins obtained from the larvae of *Helicoverpa armigera* (Hubner) infected with *Bacillus thuringienesis*. The study found the molecular weight of the proteins was ~59 KDa to ~68 KDa. The Bt strain containing Cry1Ac was, therefore, present in samples of the current study.

The protein profiles of the three isolates including *B. thuringiensis aizawai* were determined using reverse phase high performance liquid chromatography. The highest peaks for all isolates

were found at an absorbance of 220 nm. The characterizing peaks at an absorbance of 220 nm showed maximum heights of 1.64×10^6 mAU, 6.36×10^6 mAU, 1.66×10^6 mAU, and 1.63×10^6 mAU for Bta, Bt 25, Bt 30, and Bt 52, respectively. Similar peak patterns were observed for all the isolates at absorbances of 254 nm, 270 nm, 300 nm, and 360 nm. The four isolates may therefore, contain similar Cry proteins. The similarity in the nature of these isolates explains their high efficacy in killing neonates of *Busseola fusca*. However, the peak pattern at absorbance of 190 nm differed significantly from those observed in the other absorbances. In his study, Kimani (2012) while working on *Chilo partellus* found that similar peaks existed in two isolates Bt 44 and Bt 48. The two isolates caused the highest average mortalities when fed to *C. partellus* neonates and were shown to have similar Cry proteins. The results of this study, therefore, corroborate those of Kimani (2012).

The control of stem borers in Kenya has been a major challenge in Kenya because strains that control one species of the stem borers are ineffective towards others. Therefore, a single strain that is effective against most of the stem borers is lacking. However, employing gene stacking strategy allows the conferment of two or more Bt genes in a crop. For instance, Yang et al. (2011) have developed transgenic rice expressing two *Bacillus thuringiensis* genes. A gene stacking strategy involving genes from isolates 25 and 48 could, therefore, be used in the control of the two most important pests of maize in Kenya, *Busseola fusca* and *Chilo partellus*. The two isolates have been shown to be most effective in Kenya against *Busseola fusca* and *Chilo partellus*, respectively in the current and previous study by Kimani (2012). Future studies should, therefore, evaluate the possibilities of developing maize carrying genes from the two strains.

5.4 Limitations of the study

While every effort was made to make the results of this study as novel and objective as possible, several limitations were encountered. First, the data collection on neonates' mortalities was done on a 24-hour interval. This may have limited the monitoring of the behaviour of the insects after ingesting the toxins or the crude Bt broth. For instance, our results show that the neonates stopped feeding after 24 hours upon the ingestion of the toxin. However, a recent study of the effect of *Bacillus thuringiensis* on *B. fusca* and *C. partellus* showed the neonates stopped feeding within the first two hours of feeding on the toxin (Tende *et al.,* 2010). Nevertheless, the duration period did not significantly influence the findings of the study, as the focus was not on the feeding behaviour. An ideal situation would have been the collection of data within shorter sampling times of say once every six hours. However, resource and other constraints make it impossible or too costly to do so.

This study involved the laboratory tests in which neonates reared in the lab were subjected to different concentrations of Bt toxins and to different strains of *B. thuringiensis*. The insects were confined in Petri dishes where mortality data was recorded. The confinement of the neonates to the restricted laboratory environment may have affected the insects' response to the toxins. The behaviour of the insects under their natural environment may have differed slightly from that observed in the laboratory. Therefore, conducting greenhouse tests would be appropriate to confirm the results of the laboratory tests in the field. However, this study relied entirely on the results of the laboratory tests with no field studies. This may have limited the interpretation of the results. However, this was overcame by use of controls, which helped to ensure that the findings in the laboratory reflected the true behaviour of the neonates.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The aim of this study was to identify possible Bt candidates for the development of *B. fusca* management strategy. A leaf-disc method was used for the toxicity tests while SDS PAGE and reverse phase HPLC were used for protein characterization. From the findings of the study, the following conclusions are drawn:

- Some isolates caused mortality while others did not. The study showed that the mortalities caused by the different isolates were significantly different.
- The high mortalities observed in the neonates were due to the effect of the treatment since there was significant difference in mortality rates between treated and untreated samples ($p = 0.05$). Based on the results of the initial screening tests, it is concluded that the mortalities observed on the neonates fed with food containing the Bt toxins died from the effects of the toxins. The neonates in untreated food samples had insignificant mortality levels. It is further concluded that the insignificant mortalities (of less than 50%) observed in the untreated control samples were due to normal deaths present in natural populations.
- Isolates 25, 30, and 52 had the highest mortalities at 72 hour sampling and were deemed the most effective. The high mortalities observed in samples containing these isolates have been attributed to the specificity of the isolates on *Busseola fusca* while the low mortalities observed in some isolates have been attributed to lack of specificity and possible reduction in viability as a result of long storage duration.

- Isolate 66 had insignificant effect on *Busseola fusca* and could be tested against other pests such as Diptera and Coleoptera. This isolate caused the lowest mortality of 29.5%, which had no significant difference with the mortality of 19.5% in the untreated control 7. It is therefore concluded that isolate 66 was not specific to lepidopteran insects.
- There was a significant correlation between concentration of the δ-endotoxin and the mortality of the *Busseola fusca* neonates. The findings showed that the extracted proteins were highly effective in killing the neonates compared to the crude broth. Since isolates 25 and 30 were the most effective after 48 hours, insecticides targeting to kill the pests within a short time should involve these isolates.
- Cry1 endotoxins existed predominantly in the screened samples since proteins of sizes 130 KDa were detected. Small quantities of Cry2 endotoxins were also present since smaller protein sizes of 70 KDa were observed.
- All the isolates were characterized by high peaks at 190 nm and 220 nm. Isolates 25, 30, Bta, and 52 had a similar characterizing peak at a retention time of 4 minutes. It is therefore conclude that isolates Bt 30, Bt 25, and Bt 52 had profound genetic similarities and possibly belonged to the same strain as *Bacillus thuringiensis* subsp. *aizawai*.

6.2 Recommendations

Research interest on the development of pest resistant crops in Kenya has increased immensely over the past two decades. The use of *Bacillus thuringiensis* in controlling common crop pests has been the center of this study. However, more still needs to be done in order to realize the benefits of *B. thuringiensis* as a biocontrol agent. Based on the findings of the current study, the following are recommended:

i) The effective *Bacillus thuringiensis* isolates 25, 30, and 52 should be used as biocontrol agents for *Busseola fusca* in Kenya. This study has shown that *B. thuringiensis* is highly toxic to *B. fusca* neonates and, therefore, a good candidate for controlling the maize stem borer. However, the levels of *B. thuringiensis* usage are still low in the country and greater adoption levels are needed to realize the benefits. Formulations of Bt proteins from the three isolates should, therefore, be made and packaged as commercial pesticides in order to encourage use among farmers.

ii) It is necessary to conduct field studies for isolates 25, 30, and 52 in order to determine the efficacy of the Bt δ-endotoxin under field conditions. The current study focused on laboratory tests. However, field experiments may provide useful information on the enhancement of the toxin delivery.

iii) There is need to conduct periodic isolation and screening of *Bacillus thuringiensis* from the soil. Such continuous screening could enable the exploration of possibilities for the discovery of novel and highly potent strains.

iv) Future studies should compare toxicity of strains towards multiple insects in order to evaluate the possibilities of discovering a strain that could be effective on two or more insect pests. Isolates 25, 30, and 52 should be tested against other insect pests in order to explore their potential use against multiple pests. Most of the Bt strains are effective to a single insect pest and largely ineffective to most other insects. The establishment of a strain that could control a group of insect pests would be highly desirable for the farmer.

v) There is also need to increase funding for research into the area of crop resistant pests in Africa. Greenhouse and field tests are particularly expensive to conduct and

inadequate funding in this area has been a major setback for researchers. It is, therefore, important for greater funding in order to enable researchers carry out more and greater tests on a variety of other biocontrol agents.

In summary, the current study has shown that local isolates of *Bacillus thuringiensis* can contribute to the control of the maize stem borers. The study supports the current scientific knowledge that the δ-endotoxins from *B. thuringiensis* have specific insecticidal activity against maize stem borers. It is, therefore, important that the opportunities presented by this technology be harnessed in order to deal with the food security challenges in Kenya, reduce environmental impacts of pesticides, and maintain biodiversity by avoiding non-discriminatory killing of organisms by chemical insecticides.

REFERENCES

Al-Momani, F., and Obeidat, M. 2013. Ecology, toxicity, and hydrolytic activities of *Bacillus thuringiensis* in forests. *Turkish Journal of Agriculture and Forestry*, 37(1), 76-82.

Ammouneh, H., Harba, M., Idris, E., and Makee, H. 2011. Isolation and characterization of native *Bacillus thuringiensis* isolates from Syrian soil and testing of their insecticidal activities against some insect pests, *Turkish Journal of Agriculture and Forestry* 35, 421-431.

Ariga, J., Jayne, T. S., and Njukia, S. 2010. Staple food prices in Kenya. In *Prepared for the COMESA policy seminar on "Variation in staple food prices: Causes, consequence, and policy options", Maputo, Mozambique* (pp. 25-26).

Armes N. J, Jadhav, D. R and DeSouza, K. R. 1996. A survey of insecticide resistance in *Helicoverpa armigera* in the Indian subcontinent. *Bulletin of Entomological Research* 86, 499-514.

Avilla C., Vargas-Osuna E., González-Cabrera J., Ferré J., and González-Zamora J. E. 2009. Toxicity of several delta-endotoxins of *Bacillus thuringiensis* against *Helicoverpa armigera* (Lepidoptera: Noctuidae) from Spain. *Journal of Invertebrate Pathology* 90(1), 51-54.

Babariya, P.M., Kabaria, B.B., Patel, V.N. and Joshi, M.D. 2010. Chemical control of gram pod borer, *Helicoverpa armigera* Hübner infesting pigeonpea, *Legume Research* 33, 224-226.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248 – 254.

Bravo A., Gill, S. S., and Soberon, M. 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control, *Toxicon* 49: 423-435.

Bravo A., Likitvivatanavong, S., Gill, S. S., and Soberon, M. 2011. *Bacillus thuringiensis*: A story of a successful bioinsecticide. *Insect Biochemistry and Molecular Biology* 41, 423-431.

Bravo, A., Gómez, I., Porta, H., García-Gómez, B. I., Rodriguez-Almazan, C., Pardo, L., and Soberón, M. 2013. Evolution of *Bacillus thuringiensis* Cry toxins insecticidal activity. *Microbial Biotechnology*, 6(1), 17-26.

Crickmore N, Zeigler D R, Schnepf E, Van Rie J, Lereclus D, Baum J, et al. 2011. '*Bacillus thuringiensis* toxin nomenclature' Available Online at: http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/

De Groote, H. 2002. Maize yield losses from stemborers in Kenya. *International Journal of Tropical Insect Science*, 22(02), 89-96.

de Maagd, R. A., Bravo A., Berry, C., Crickmore, N., and Schnepf, H. E. 2003. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria, *Annual Reviews of Genetics*, 37: 409-33.

Deilamy, A., and Abbasipour, H. 2013. Comparative bioassay of different isolates of *Bacillus thuringiensis* subsp. *kurstaki* on the third larval instars of diamondback moth, *Plutella xylostella* (L.)(Lep.: Plutellidae). *Archives of Phytopathology and Plant Protection*, 46 (12), 1480-1487.

Dulmage, H. T. 1993. Development of Isolates of *Bacillus thuringiensis* and Similar Aerobic Microbes for Use in Developing Countries, Cairo: National Research Center.

Fouad, A. L., and Obeidat, M. 2013. Ecology, toxicity, and hydrolytic activities of *Bacillus thuringiensis* in forests. *Turkish Journal of Agriculture and Forestry* 37, 76-82.

Ghaly, A.E. and Alkoaik, F.N. 2010. Nutritional value of the Maize Stalk Borer and American Bollworm as unconventional protein sources, *American Journal of Applied Sciences* 7(1), 1-12.

Gómez, I., Pardo-López, L., Muňoz-Garay, C., Fernandez, L. E., Pérez, C., Sánchez, J., Soberón, M., and Bravo, A. 2006. Role of receptor interaction in the mode of action of insecticidal Cry and Cyt toxins produced by *Bacillus thuringiensis*. *Peptides*, 28(1), 169-73.

Guerchicoff, A., Delécluse, A., and Rubinstein, C. P. 2001. The *Bacillus thuringiensis* cyt genes for hemolytic endotoxins constitute a gene family. *Applied and Environmental Microbiology*, 67(3), 1090-1096.

Hamed, M. and Nadeem, S. 2008. Rearing of *Helicoverpa armigera* (Hub.) on artificial diets in laboratory. *Pakistan Journal of Zoology*, 40, 447-450.

Harris, K. M., and Nwanze, K. F. 1992. *Busseola fusca* (Fuller), the African maize stalk borer: a handbook of information. Information Bulletin no. 33. Patancheru, A.P. 502 324, India: International Crops Research Institute for the Semi-Arid Tropics, and Wallingford, UK: CAB International.

Ibrahim, M., Griko, N., Junker, M., and Bulla, L.A. 2010. *Bacillus thuringiensis*: A genomics and proteomics perspective, *Bioengineered Bugs* 1, 31-50.

Jisha, V. N., Smitha, R. B., and Benjamin, S. 2013. An Overview on the Crystal Toxins from *Bacillus thuringiensis*. *Advances in Microbiology*, 3(05), 462.

Karim, S. 2000. Management of *Helicoverpa armigera*: A review and prospectus for Pakistan, *Pakistan Journal of Biological Sciences* 3, 1213-1222.

Kfir, R., Overholt, W.A., Khan, Z. R. and Polaszek. 2002. Biology and management of economically important lepidopteran cereal stem borers in Africa, *Annual Reviews of Entomology* 47, 701-731.

Kimani, G., Nyambaka, H., Gichuki, S., Amata, R., Okomo, M. and Kasina, M. 2012. Determination of insecticidal activity of Kenyan Bt isolates against the spotted stem borer, *Chilo partellus, Proceedings, KARI Biennial Scientific Conference*, 777-783.

Kumar, S., Chandra, A., and Pandey, K. C. 2008. *Bacillus thuringiensis (Bt)* transgenic crop: An environment friendly insect-pest management strategy. *Journal of Environmental Biology* 29(5), 641-653.

Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. I**.** DNA packaging agents. *Journal of Molecular Biology* 80, 575-599.

Leetachewa, S., Katzenmeier, G., and Angsuthanasombat, C. 2006. Novel preparation and characterization of the alpha4-loop-alpha5 membrane-perturbing peptide from the *Bacillus thuringiensis* Cry4Ba delta-endotoxin. *Journal of Biochemistry and Molecular Biology*, 39(3), 270.

Li J. D., Carroll J., and Ellar D. J. 1991. Crystal-structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5-A resolution. *Nature* 353(6347), 815–821.

Lundblad, R. L., and Macdonald, F. 2010. *Handbook of Biochemistry and Molecular Biology* (4th ed.). Boca Raton, FL: CRC Press.

Manjunath, T. M. 2005. A decade of commercialized transgenic crops-analyses of their global adoption, safety and benefits. *The 6th Dr. S. Pradhan Memorial Lecture*, 1-18.

Martin T, Ochou G. O., Djihinto A., Traore D., Togola M., Vassal J. M., Vaissayre M, Fournier D. 2002. Controlling an insecticide resistant bollworm in West Africa. *Agriculture, Ecosystems and Environment* 107, 409–411.

Mittal, V. and Ujagir, R. 2005. Evaluation of naturalyte spinosad against pod borer complex in early pigeon pea. *Indian Plant Protection*, 33, 211-215.

Mugo S., Mwimali M., Taracha C., Songa M J., Gichuki S T.,Tende R., Gichuki S T., Tende, R., Karaya H, Bergvinson, D. J., Pellegrinschi, A., and Hoisington, D. A. 2011. Testing public Bt maize events for control of stem borers in the first confined field trials in Kenya, *African Journal of Biotechnology* 10, 4713-4718.

Mugo S., Taracha, C., Bergvinson, D.,Odhiambo, B., Songa, J., Hoisington, D., Mclean, S., Ngatia, I., and Gethi, M. 2001. Screening Cry proteins produced by Bt maize leaves for activity against Kenyan maize stem borers. *Seventh Eastern and Southern Africa Regional Maize Conference*, pp. 102-105.

Mulaa, M. M., Bergvinson, D. J., Mugo, S. N., Wanyama, J. M., Tende, R. M., Groote, H. D., and Tefera, T. M. 2011. Evaluation of stem borer resistance management strategies for Bt maize in Kenya based on alternative host refugia, *African Journal of Biotechnology*, 10: 4732-4740.

Munyua, B., Hellin, J., Nyikal, R., and Mburu, J. 2010. Determinants for use of certified maize seed and the relative importance of transaction costs. *Joint 3rd African Association of Agricultural Economists (AAAE) and 48th Agricultural Economists Association of South Africa (AEASA) Conference, Cape Town, South Africa, September 19-23, 2010*.

Nasreen, A. and Mustafa, G. 2000. Biology of *Helicoverpa armigera* (Hubner) reared in laboratory on natural diet. *Pakistan Journal of Biological Sciences* 3, 1668-1669.

Ning C., Wu, K., Liu, C., Gao, Y., Jurat-Fuentes, J. L., and Gao., X. 2010. Characterization of a Cry1Ac toxin-binding alkaline phosphatase in the midgut from *Helicoverpa armigera* (Hübner) larvae, *Journal of Insect Physiology* 56, 666–672.

OFAB Africa. 2010. *Bt cotton Research Progress in Kenya*. Accessed July 28, 2012 from: http://www.ofabafrica.org/meeting_reports/OFABReport-Kenya-April10.pdf

Ogutcu, A., Suludere, Z., Uzunhisarcikli, M., and Kalender, Y. 2005. Effects of *Bacillus thuringiensis kurstaki* on Malpighian tubule cells of *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae) larvae. *Folia Biologica*, 53(1-2), 1-2.

Pardo-López, L., Soberon, M., and Bravo, A. 2013. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. *FEMS Microbiology Reviews*, 37(1), 3-22.

Pigott, C. R., and Ellar, D. J. 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiology and Molecular Biology Reviews*, 71(2), 255-281.

Porcar, M., and Caballero, P. 2000. Molecular and insecticidal characterization of a *Bacillus thuringiensis* strain isolated during a natural epizootic. *Journal of Applied Microbiology*, 89(2), 309-316.

Prabhakar, A., Hugar, P. S., Krishnaraj, P. U., Vastrad, A. S., and Nandihalli, B. S. 2013. Bio efficacy of crude protein extract of native *Bacillus thuringiensis* isolates against lepidopteran pests of cabbage. *Karnataka Journal of Agricultural Sciences*, *26* (3).

Raybould, A., and Quemada, H. 2010. Bt crops and food security in developing countries: realised benefits, sustainable use and lowering barriers to adoption. *Food Security*, 2(3), 247-259.

Sanahuja, G., Banakar, R., Twyman, R. M., Capell, T., and Christou, P. 2011. *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnology Journal* 9, 283-300.

Sanchis, V. and Bourguet, D. 2008. *Bacillus thuringiensis*: applications in agriculture and insect resistance management. A review. *Agronomy for Sustainable Development*. 28, 11–20.

Sezonlin, M., Dupas, S., Le Ru, B., Le Gall, P., Moyal, P., Calatayud, P. A., Giffard, I., Faure, N. and Silvain, J. F. 2006. Phylogeography and population genetics of the maize stalk borer *Busseola fusca* (Lepidoptera, Noctuidae) in sub-Saharan Africa, *Molecular Ecology* 15, 407-420.

Sharma, A. P. and Manandhar, S. P. 1997. *Bacillus thuringiensis*- potent and valuable alternative of conventional insecticides, *Tribhuvan University Journal*, XX (1), 13-24.

Smedley, D. P., and Ellar, D. J. 1996. Mutagenesis of three surface-exposed loops of a *Bacillus thuringiensis* insecticidal toxin reveals residues important for toxicity, receptor recognition and possibly membrane insertion. *Microbiology*, 142(7), 1617-1624.

Soberon, M., Gill, S. S., and Bravo, A. 2009. Signaling versus punching hole: How do *Bacillus thuringiensis* toxins kill insect midgut cells?. *Cellular and Molecular Life Sciences*, 66(8), 1337-1349.

Songa J.M., Bergvinson, D., and Mugo, S. 2001. Mass rearing of the maize stem borers *Chilo partellus, Busseola fusca, Sesamia calamistis, Chilo orchalcociliellus* and *Eldana saccharina* at KARI, Katumani. *Seventh Eastern and Southern Africa Regional Maize Conference*, pp. 120-124.

StatsDirect Ltd. 2013. *StatsDirect Statistical Software.* 9 Bonville Chase, Cheshire, United Kingdom.

Tabashnik, B. E., Brévault, T., and Carrière, Y. 2013. Insect resistance to Bt crops: lessons from the first billion acres. *Nature Biotechnology*, 31(6), 510-521.

Tabashnik, B. E., Cushing, N. L., Finson, N., and Johnson, M. W. 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology*, 83(5), 1671-1676.

Tabashnik, B. E., Gassmann, A. J., Crowder, D. W., and Carrière, Y. 2008. Insect resistance to Bt crops: evidence versus theory. *Nature Biotechnology*, 26(2), 199-202.

Tefera T, Mugo S, Tende R, Likhayo P 2010. *Methods of mass rearing of stem borers, maize weevils and larger grain borer insect pests of maize*. CIMMYT: Nairobi, Kenya.

Tende, R.M., Mugo, S.N., Nderitu, J.H., Olubayo, F.M., Songa, J.M. and Bervinson, D.J. 2010. Evaluation of *Chilo partellus* and *Busseola fusca* susceptibility to δ -endotoxins in Bt maize. *Crop Protection* 29: 115-120.

Van Rensburg, J. B. J. 2007. First report of field resistance by the stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. *South African Journal of Plant and Soil*, *24*(3), 147-151.

VSN International Ltd. 2011. *GenStat Discovery Edition 4*. Hemel Hempstead, United Kingdom.

Wamaitha J. M. 2006. Isolation, evaluation and molecular characterisation of *Bacillus thuringiensis* isolates against *Prostephanus truncatus*, a major storage pest in maize. Kenyatta University, M.Sc. Thesis.

Yang, Z., Chen, H., Tang, W., Hua, H., and Lin, Y. 2011. Development and characterisation of transgenic rice expressing two *Bacillus thuringiensis* genes. *Pest Management Science*, 67(4), 414-422.

Zhang, X., Candas M., Griko N. B., Rose-Young, L., and Bulla L. A. 2005. Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the

cadherin receptor BT-R-1 expressed in insect cells. *Cell Death and Differentiation* 12(11):1407–1416.

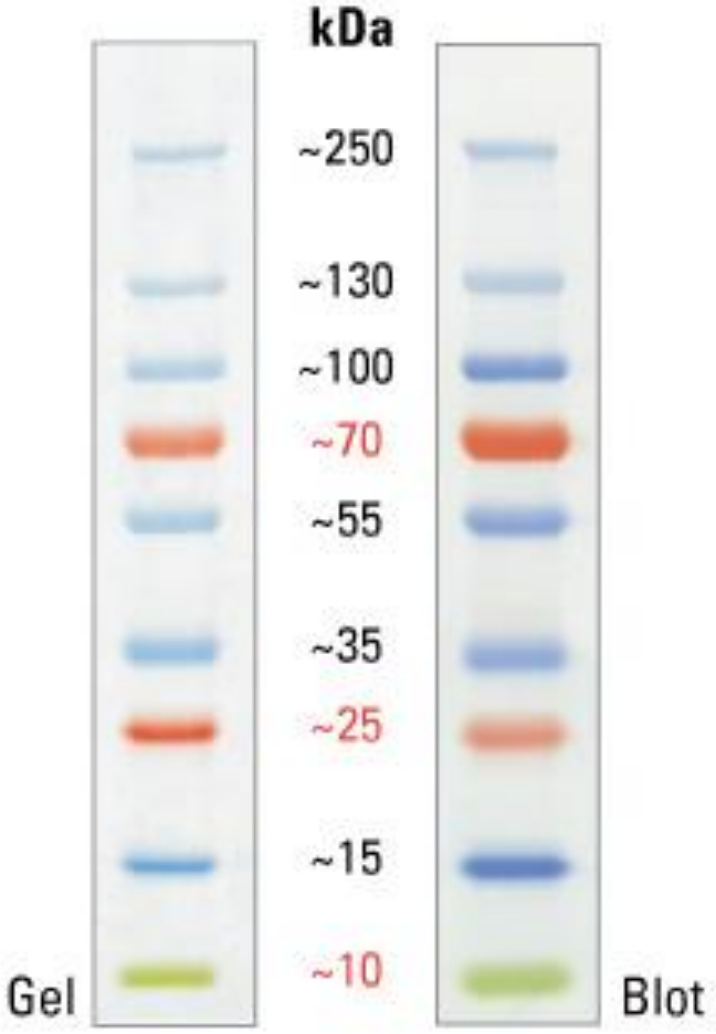
APPENDICES

Appendix 1: Ingredients of the artificial diet used for rearing *Busseola fusca*

Components for each fraction	Amount at different volumes			
FRACTION A	1.5 Litres	3 litres	6 litres	9 litres
Distilled water	604.65	1209.3	2418.6	3627.9
FRACTION B				
Beans (*Phaseolus vulgaris*) Powder	132.3	264.6	529.2	793.8
Maize leaf powder	37.8	75.6	151.2	226.8
Brewer's yeast	34.05	68.1	136.2	204.3
Ascorbic Acid	3.75	7.5	15	22.5
Vitamin E capsule (200i.u)	4 capsules	8 capsules	15 capsule	22 capsules
Sorbic Acid	1.95	3.9	7.8	11.7
Methyl p-hydrobenzoate (dissolved in 20ml Ethanol, i.e 3 litre diet)	3.0	6.0	12.0	18.0
Sucrose (Table sugar)	52.95	105.9	211.8	317.7
Formaldehyde 40%	3.0ml	6.0ml	12ml	18.0ml
FRACTION C				
Agar Technical No.3	18.9	37.8	75.8	113.4
Distilled water	604.65	1209.3	2418.6	3627.9
TOTALS	1500.15	3003.3	6000.8	9000.9

Note: Measurements in grams except where indicated otherwise

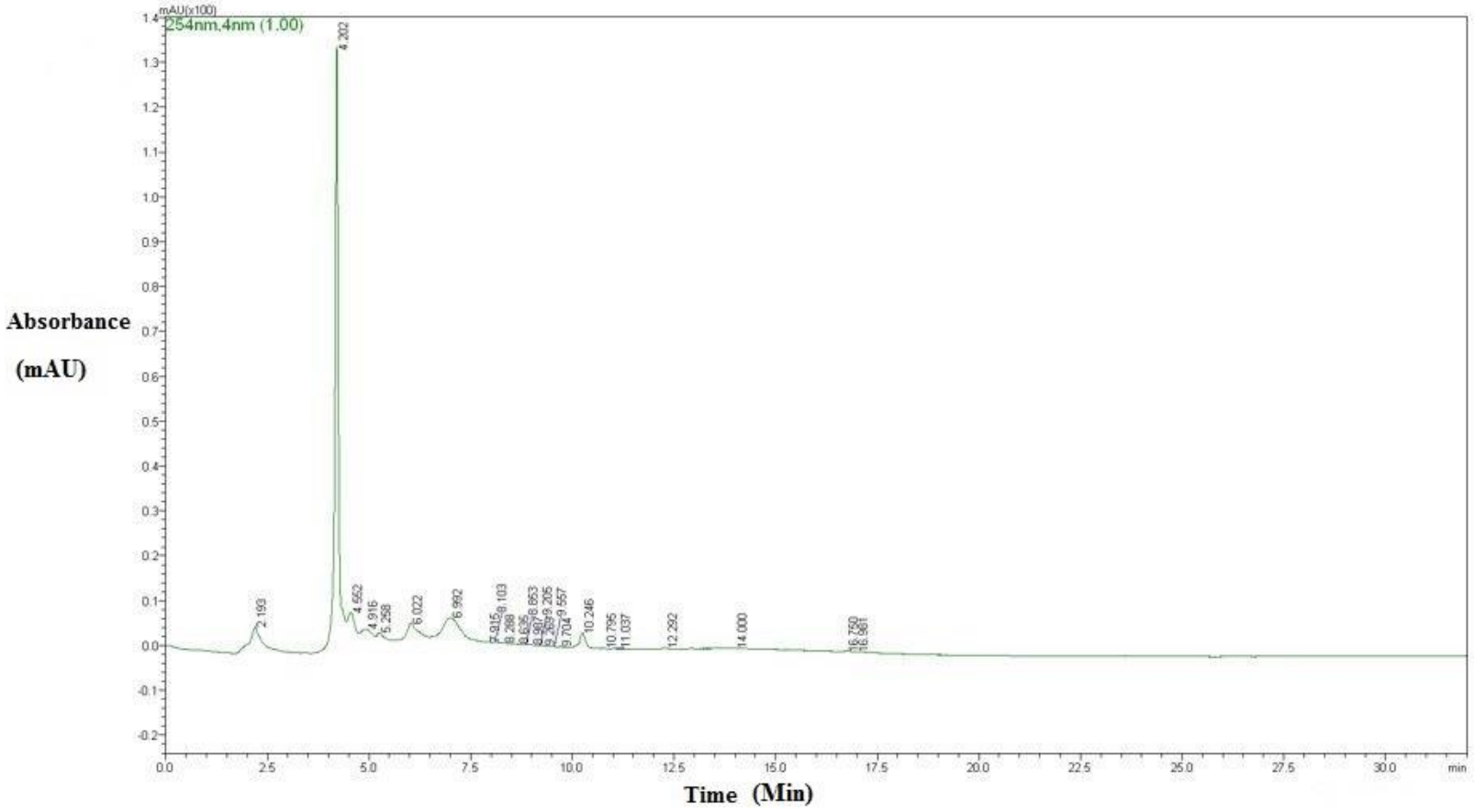
Appendix 2: Molecular weight markers used in interpretation of SDS PAGE results



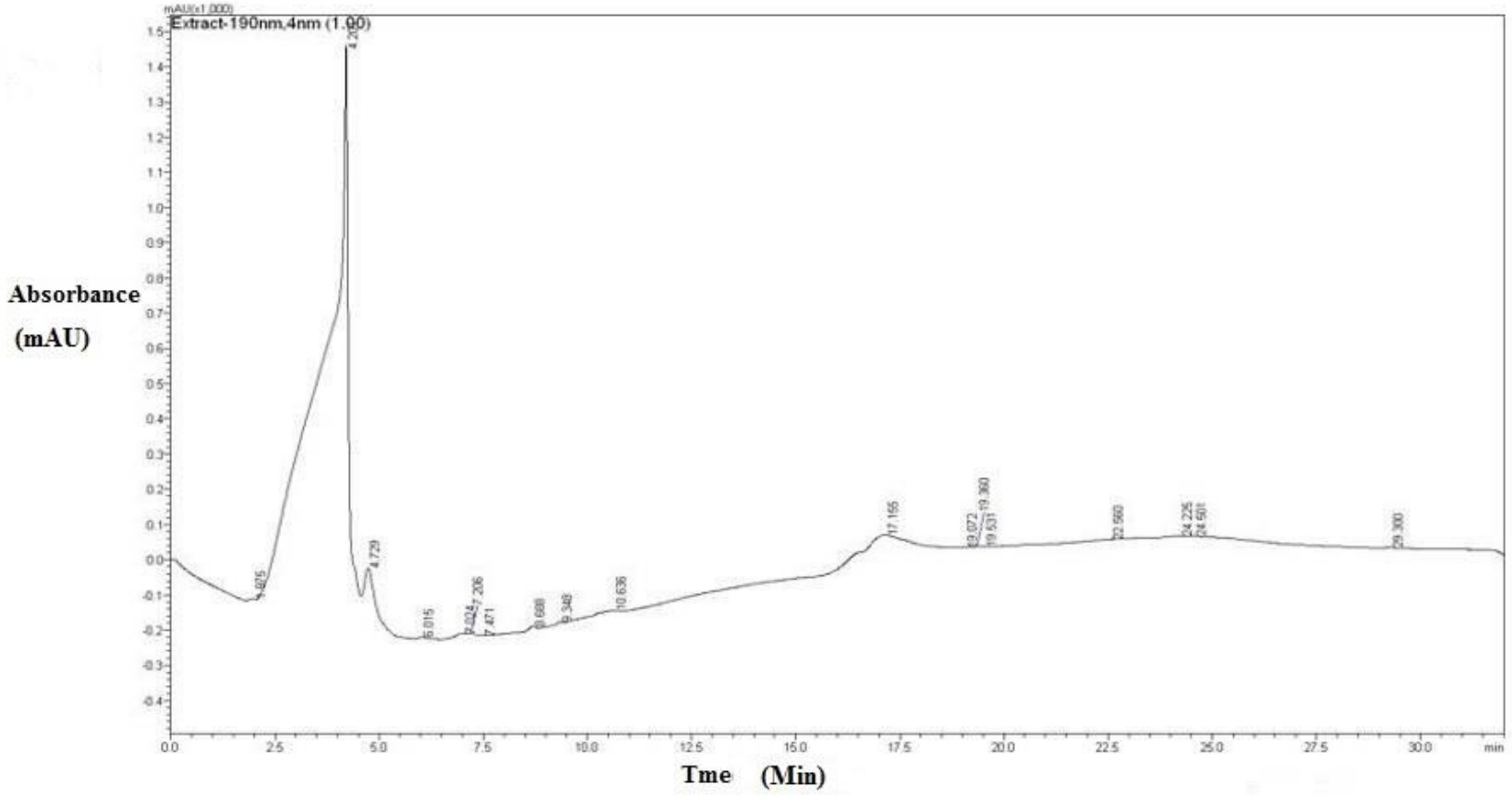
Appendix 3: Reverse Phase high performance chromatography (RP-HPLC) profile of Bta at absorbance of 190 nm

Peak#	Retention Time	Area	Height	Area%
1	1.975	96773	10242	0.1068
2	4.201	72014063	1644690	79.5029
3	4.729	3473704	174353	3.8349
4	6.015	63293	4827	0.0699
5	7.024	223743	11766	0.247
6	7.206	97544	9101	0.1077
7	7.471	2684	556	0.003
8	8.688	88563	9394	0.0978
9	9.348	58293	6877	0.0644
10	10.636	515065	8267	0.5686
11	17.155	5152514	67803	5.6883
12	19.072	7101	332	0.0078
13	19.36	5481	657	0.0061
14	19.531	4692	963	0.0052
15	22.56	1833568	23467	2.0242
16	24.225	3001900	32895	3.3141
17	24.501	3915534	32779	4.3227
18	29.3	25889	1289	0.0286

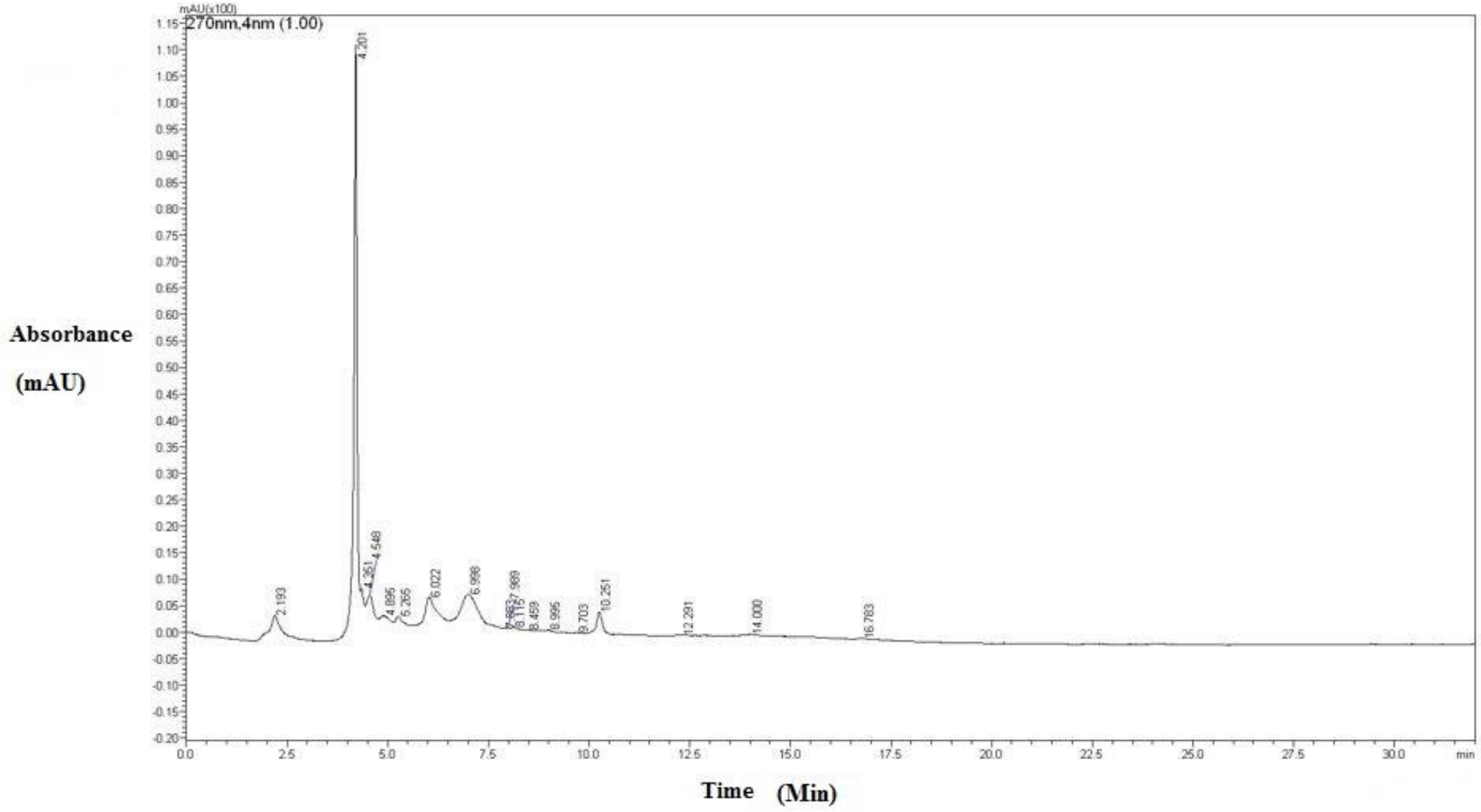
Appendix 4: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bta at 190 nm



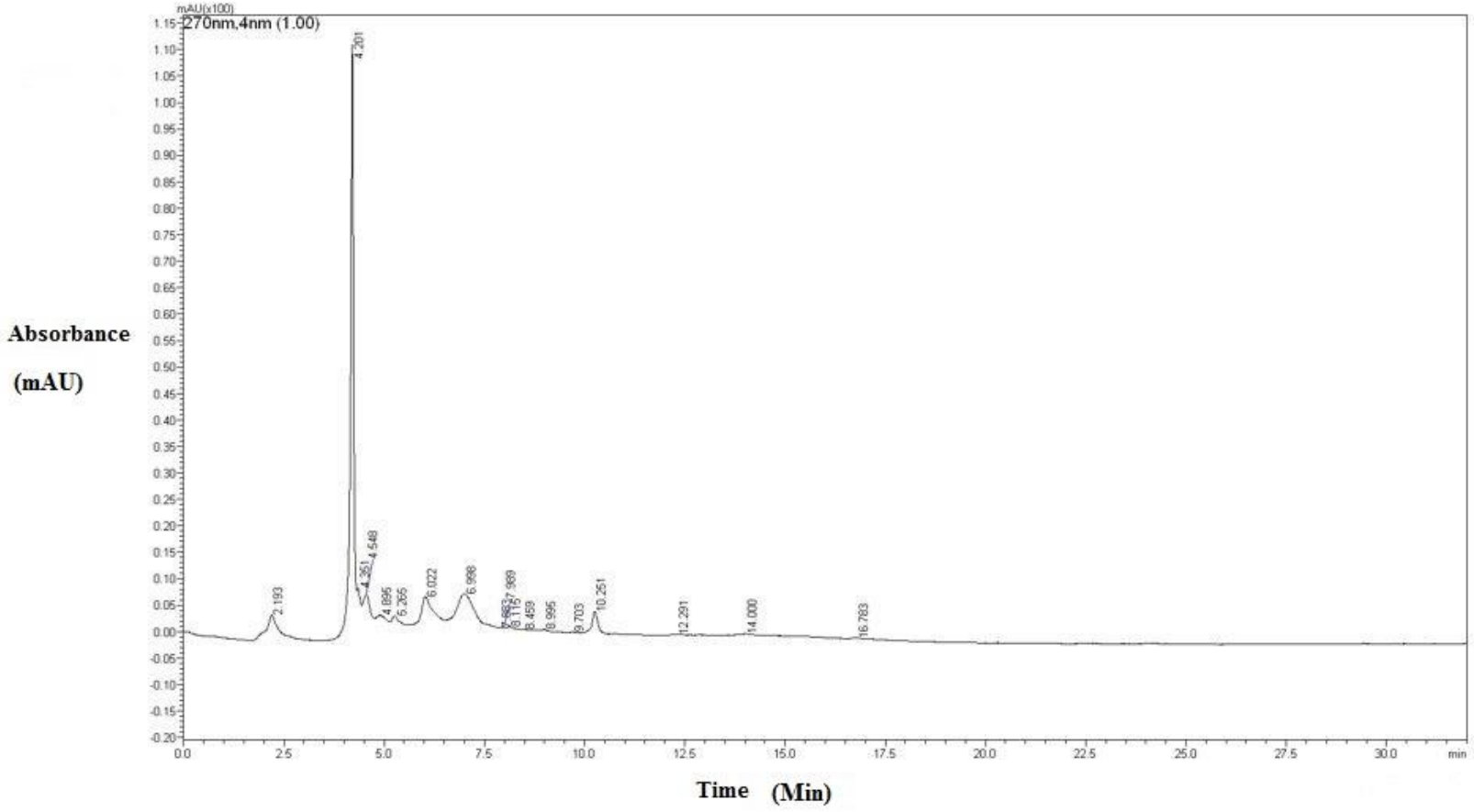
Appendix 5: Reverse Phase high performance chromatography (RP-HPLC) profile of Bta at absorbance of 220 nm



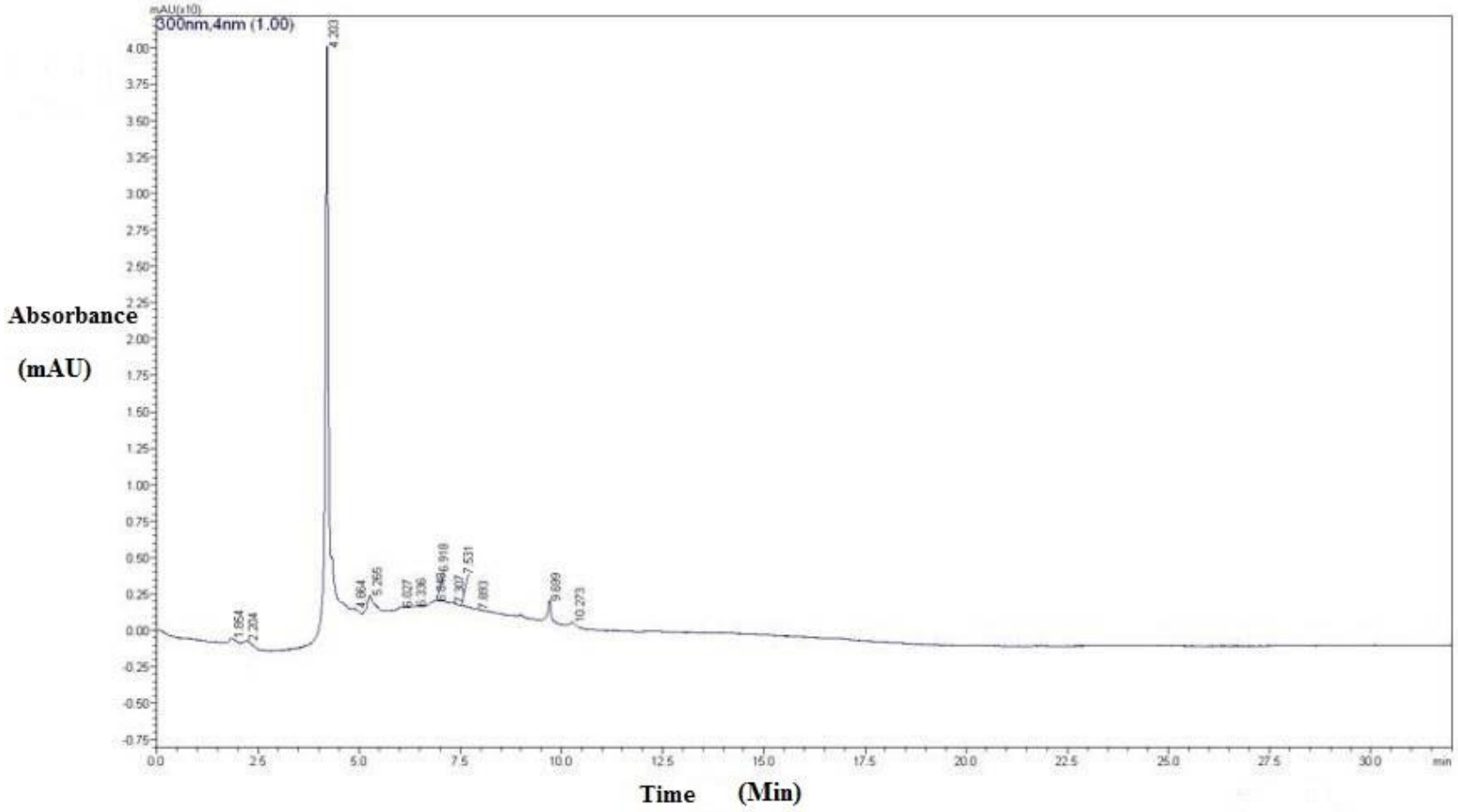
Appendix 6: Reverse Phase high performance chromatography (RP-HPLC) profile of Bta at absorbance of 254 nm



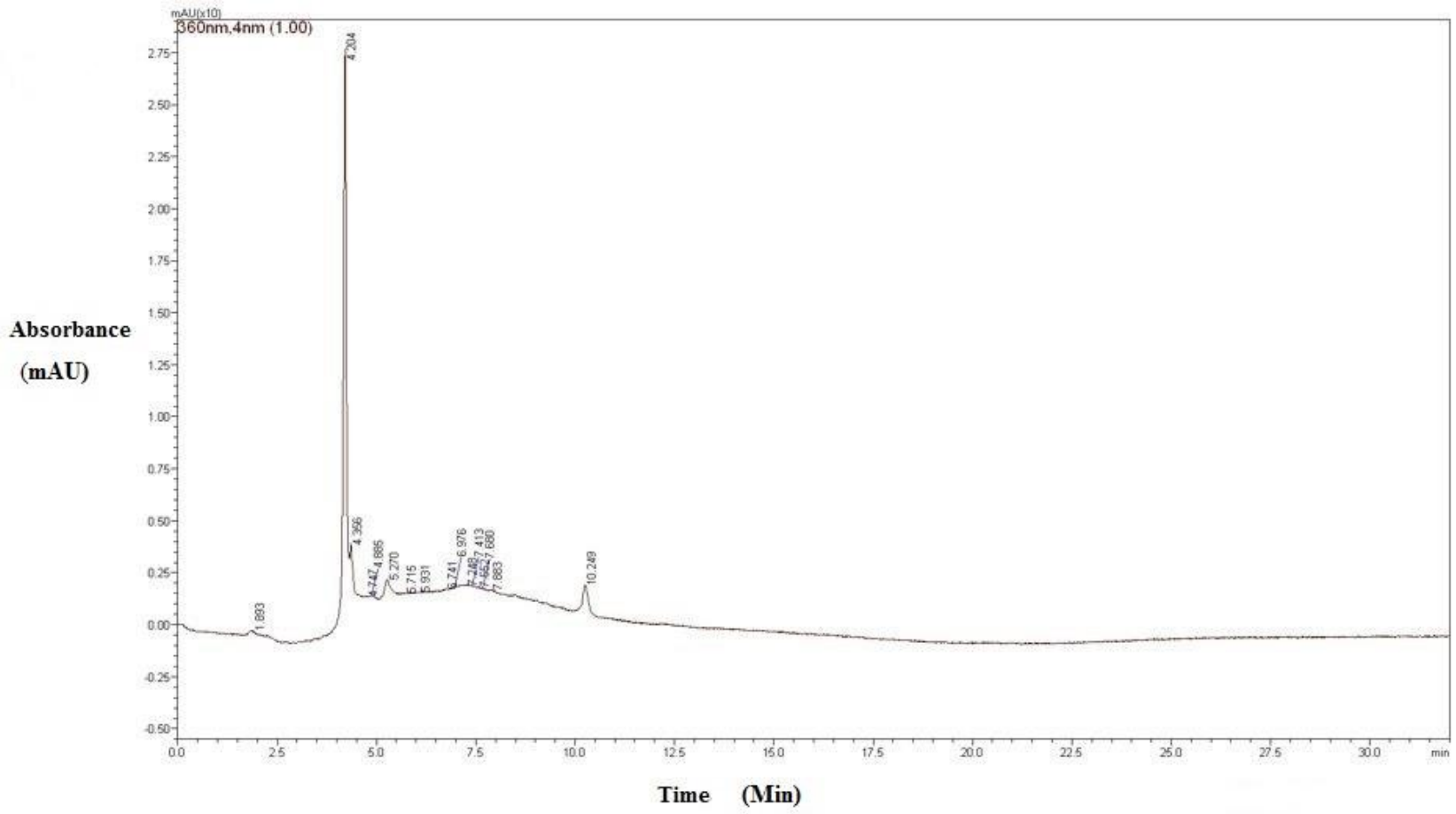
Appendix 7: Reverse Phase high performance chromatography (RP-HPLC) profile of Bta at absorbance of 270 nm



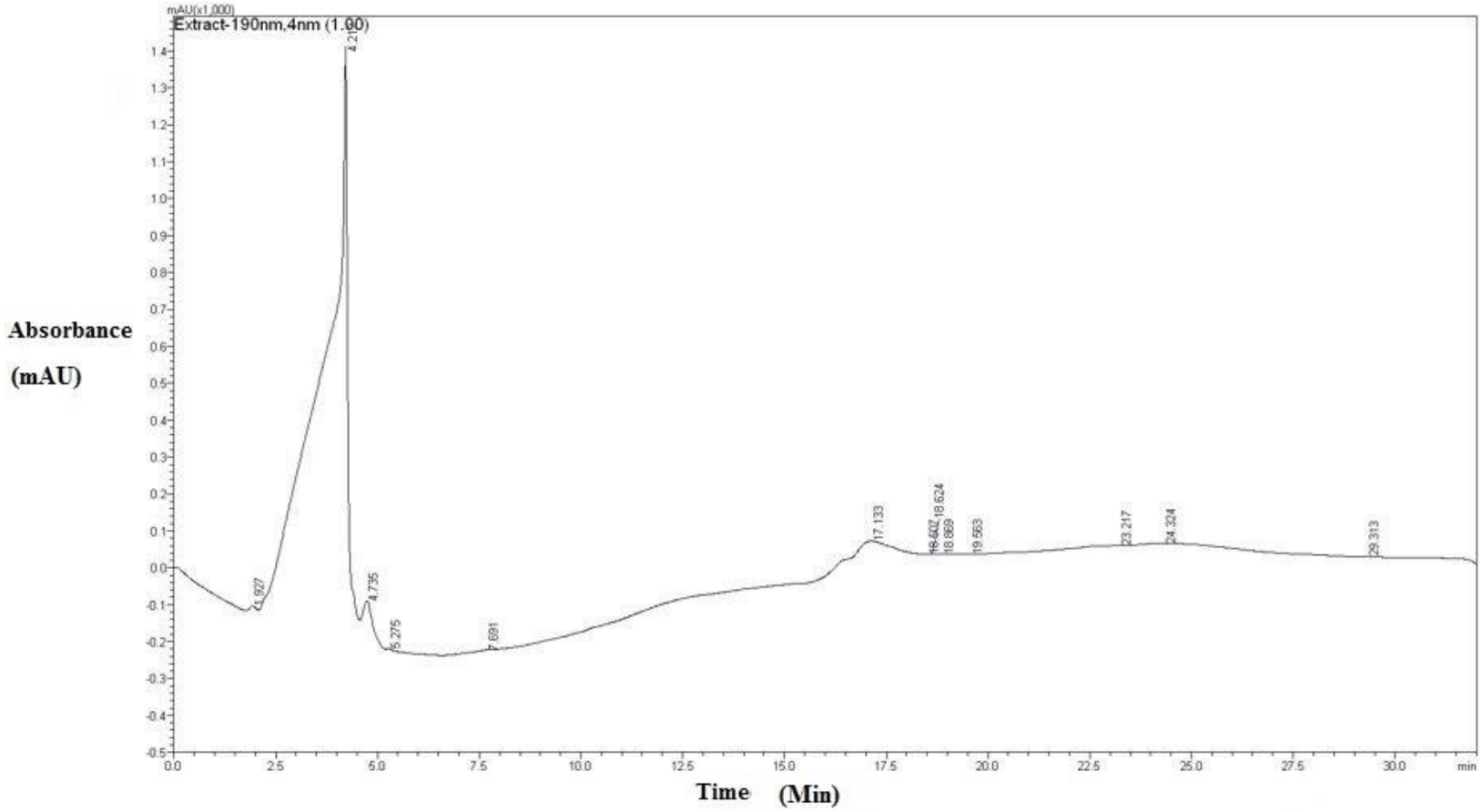
Appendix 8: Reverse Phase high performance chromatography (RP-HPLC) profile of Bta at absorbance of 300 nm



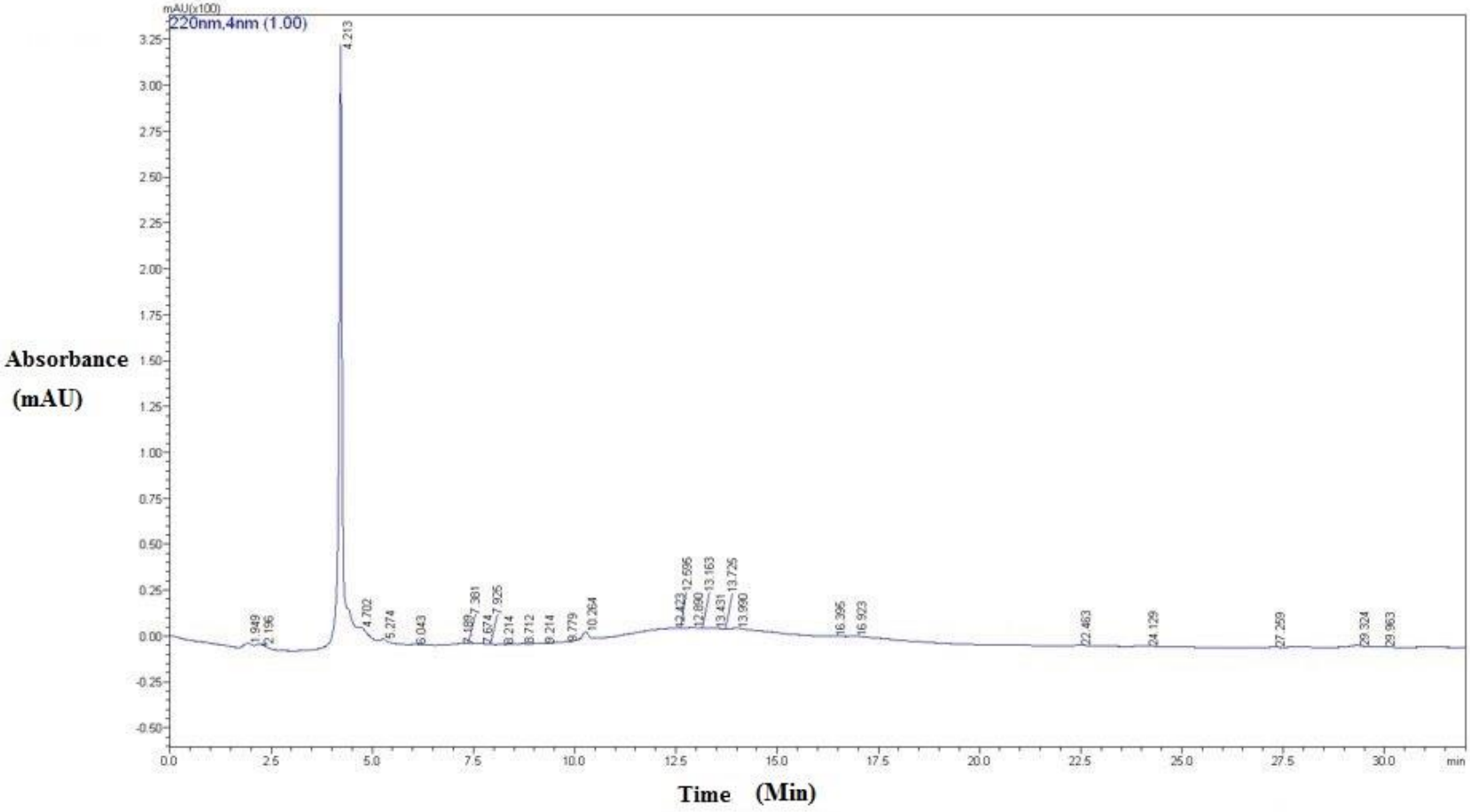
Appendix 9: Reverse phase high performance liquid chromatography (RP-HPLC) elution profiles of endotoxins from Bta at 360 nm



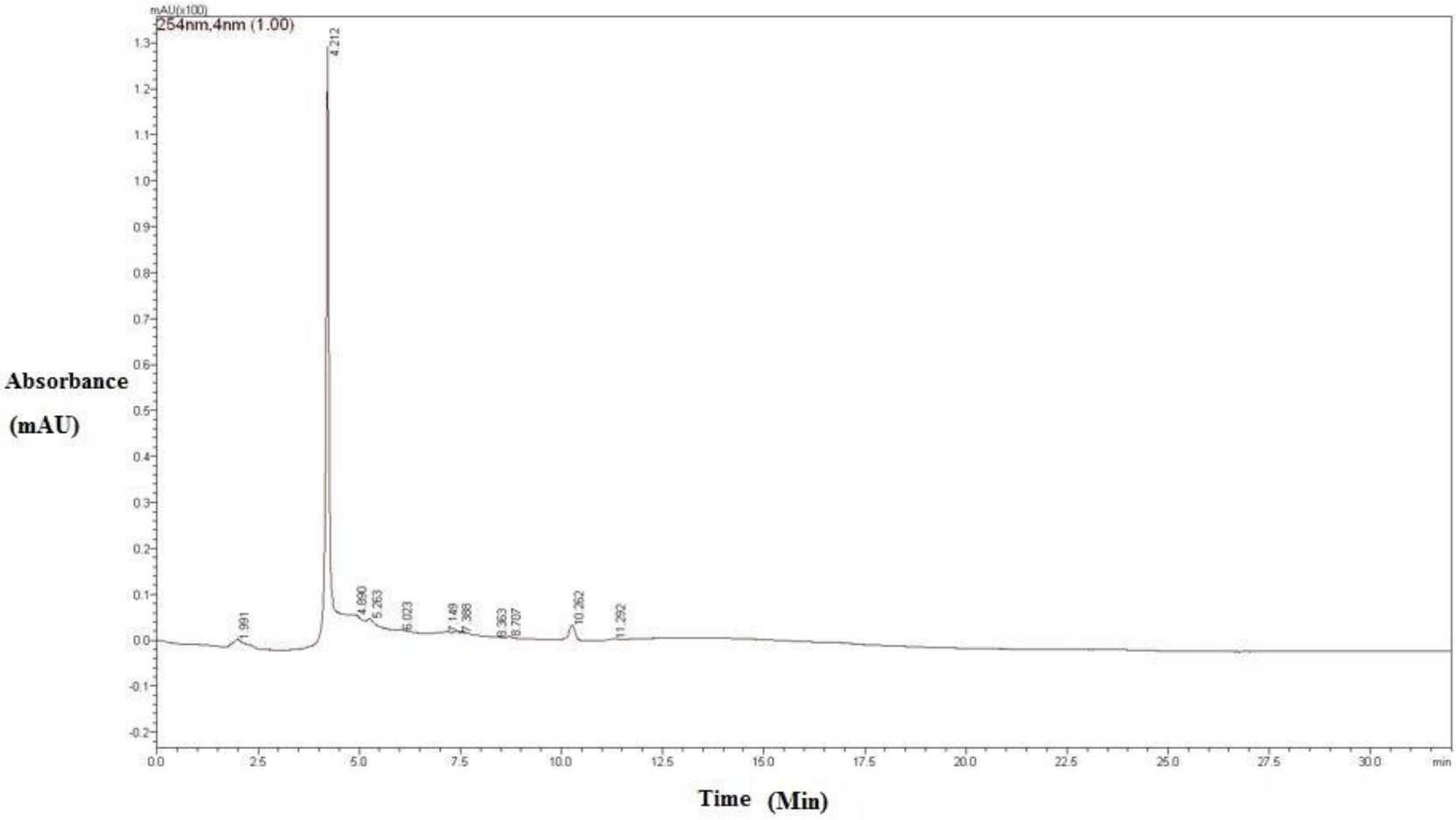
Appendix 10: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 25 at 190 nm



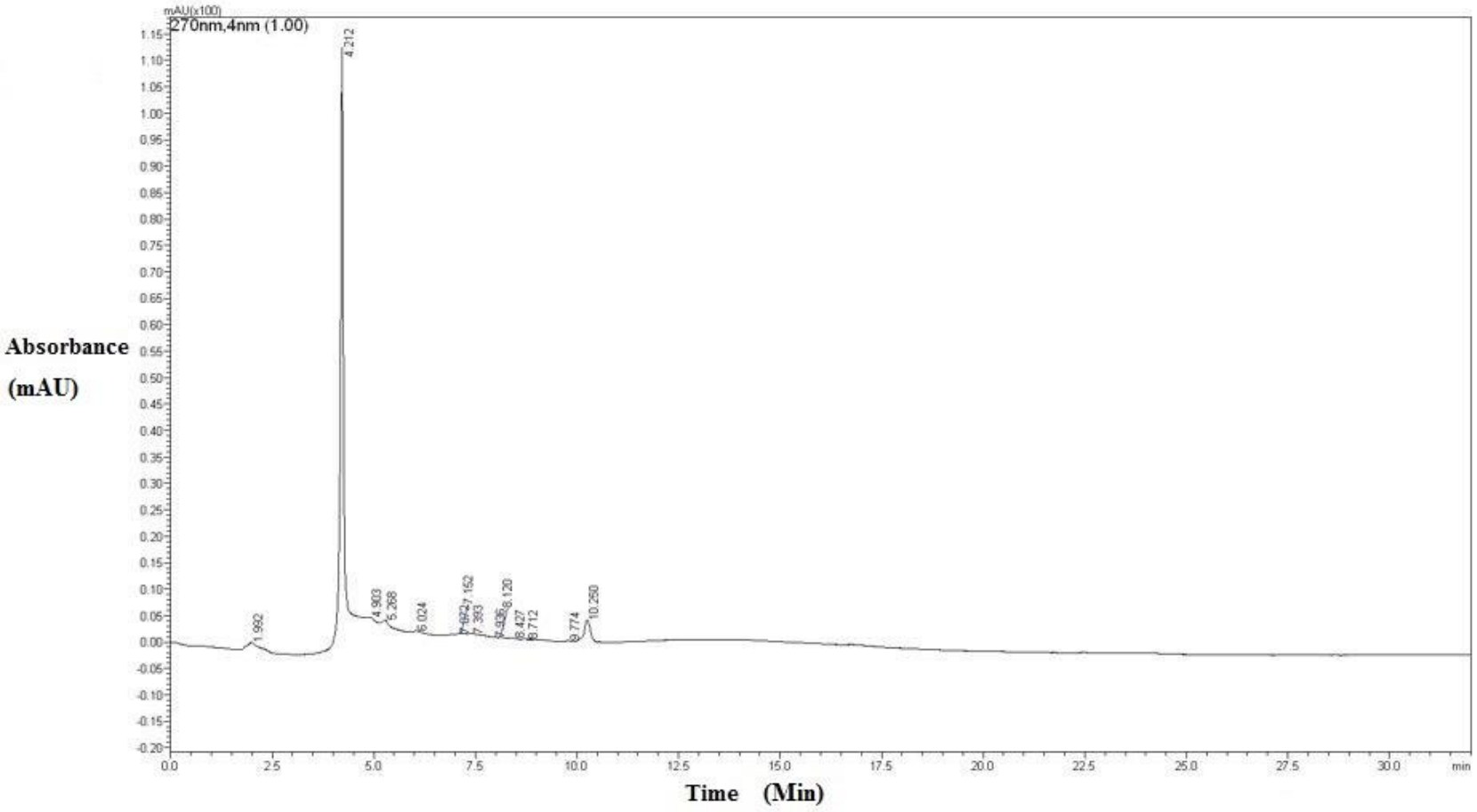
Appendix 11: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 25 at 220 nm



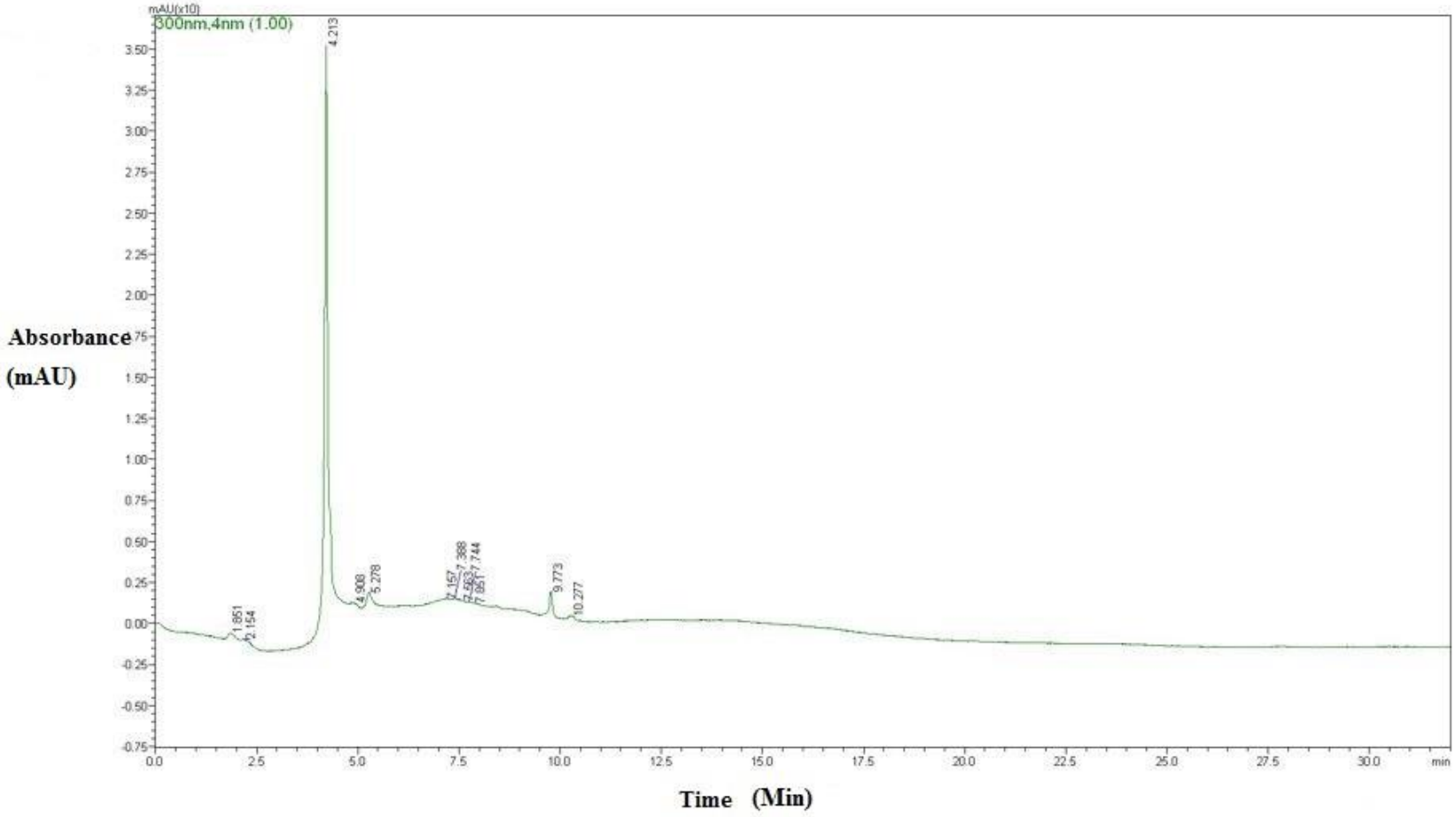
Appendix 12: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 25 at 254 nm



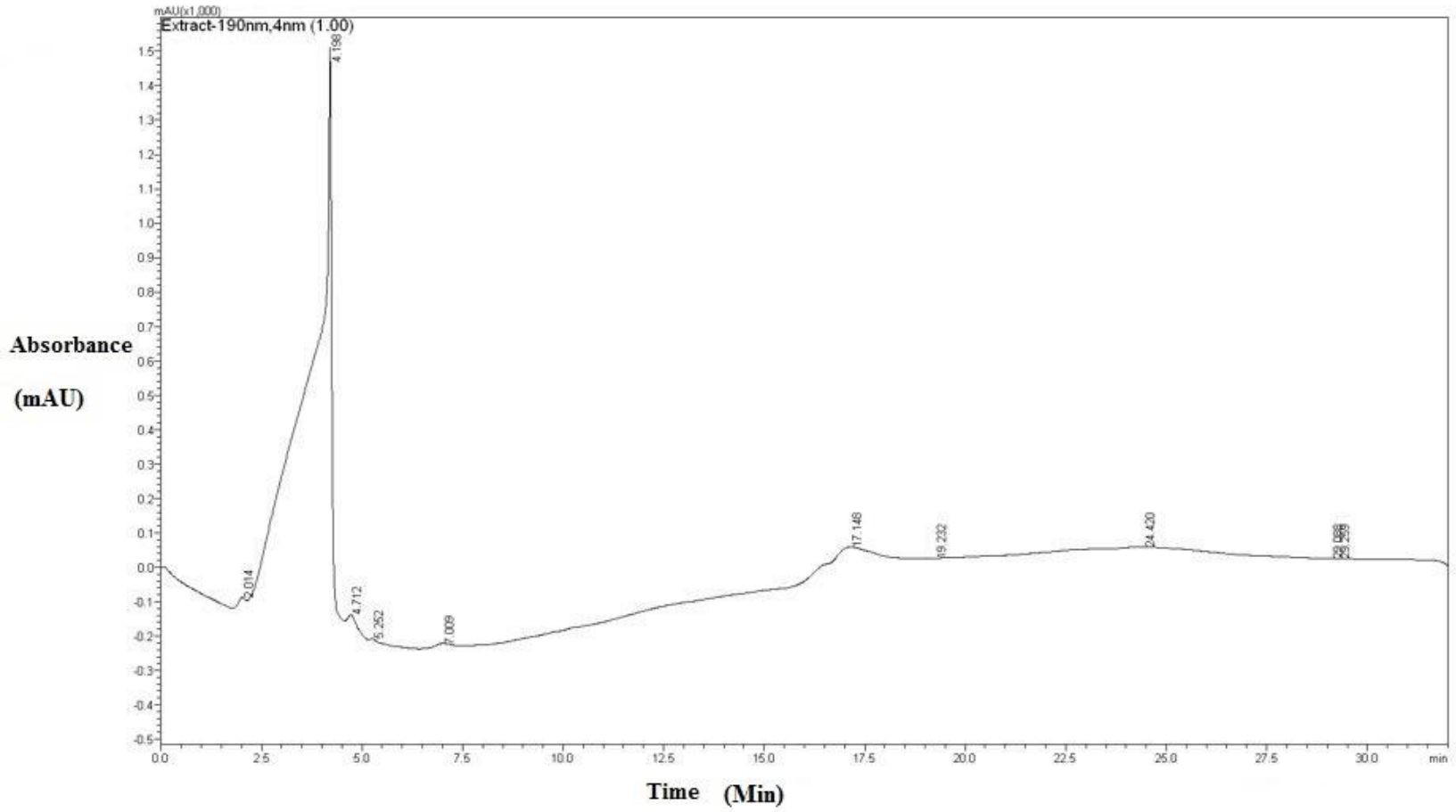
Appendix 13: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 25 at 270 nm



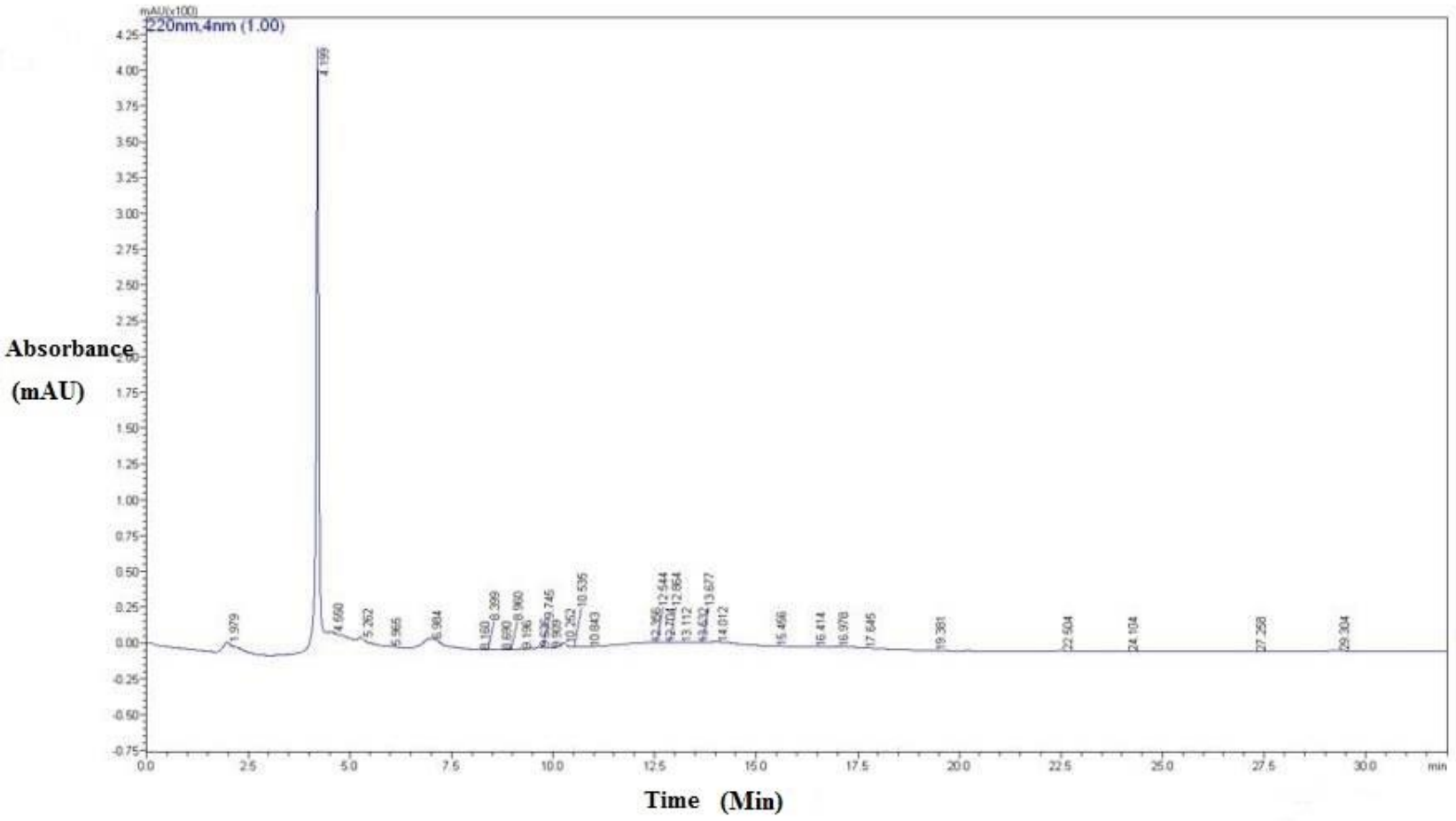
Appendix 14: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 25 at 300 nm



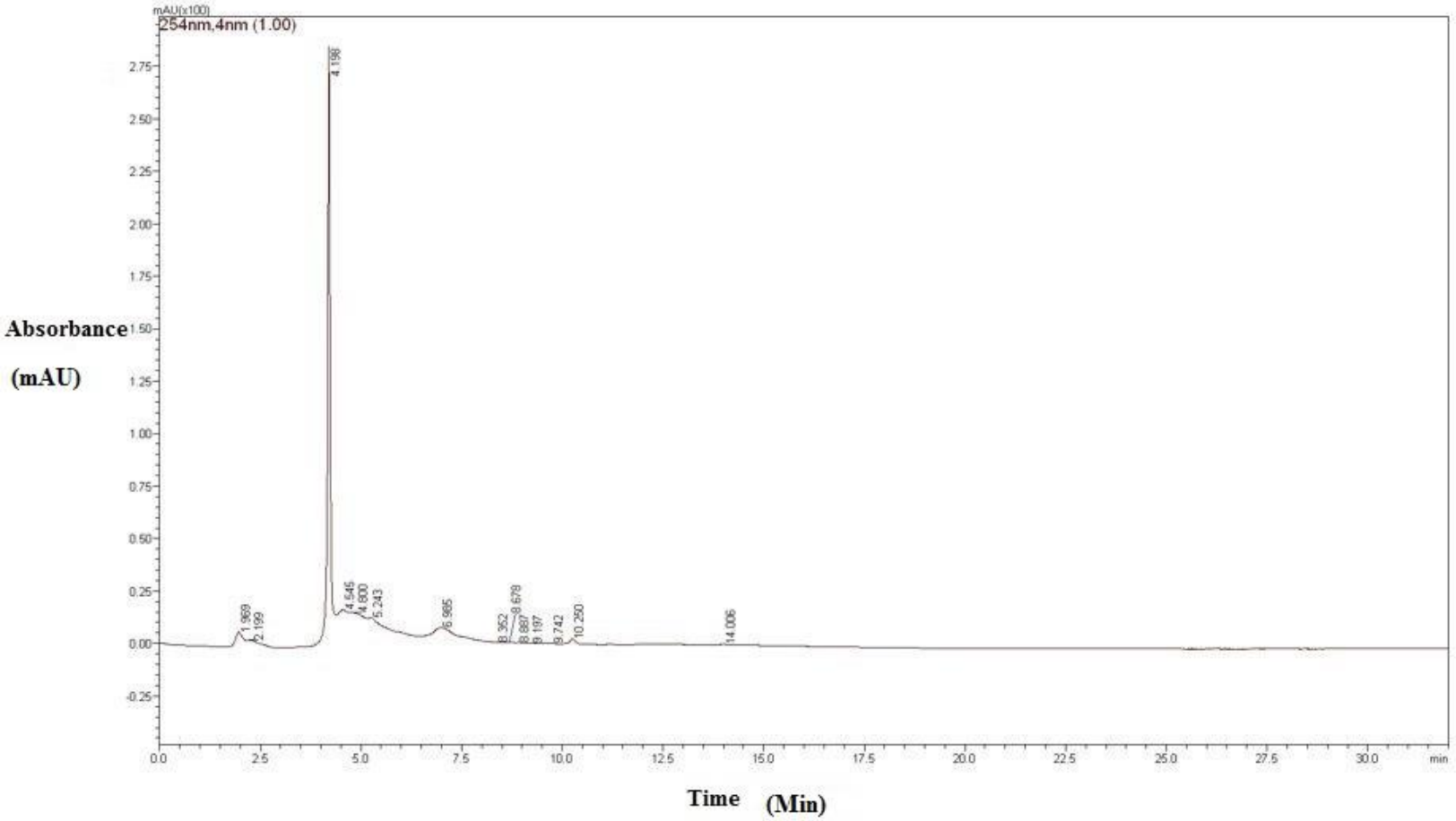
Appendix 15: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 30 at 190 nm



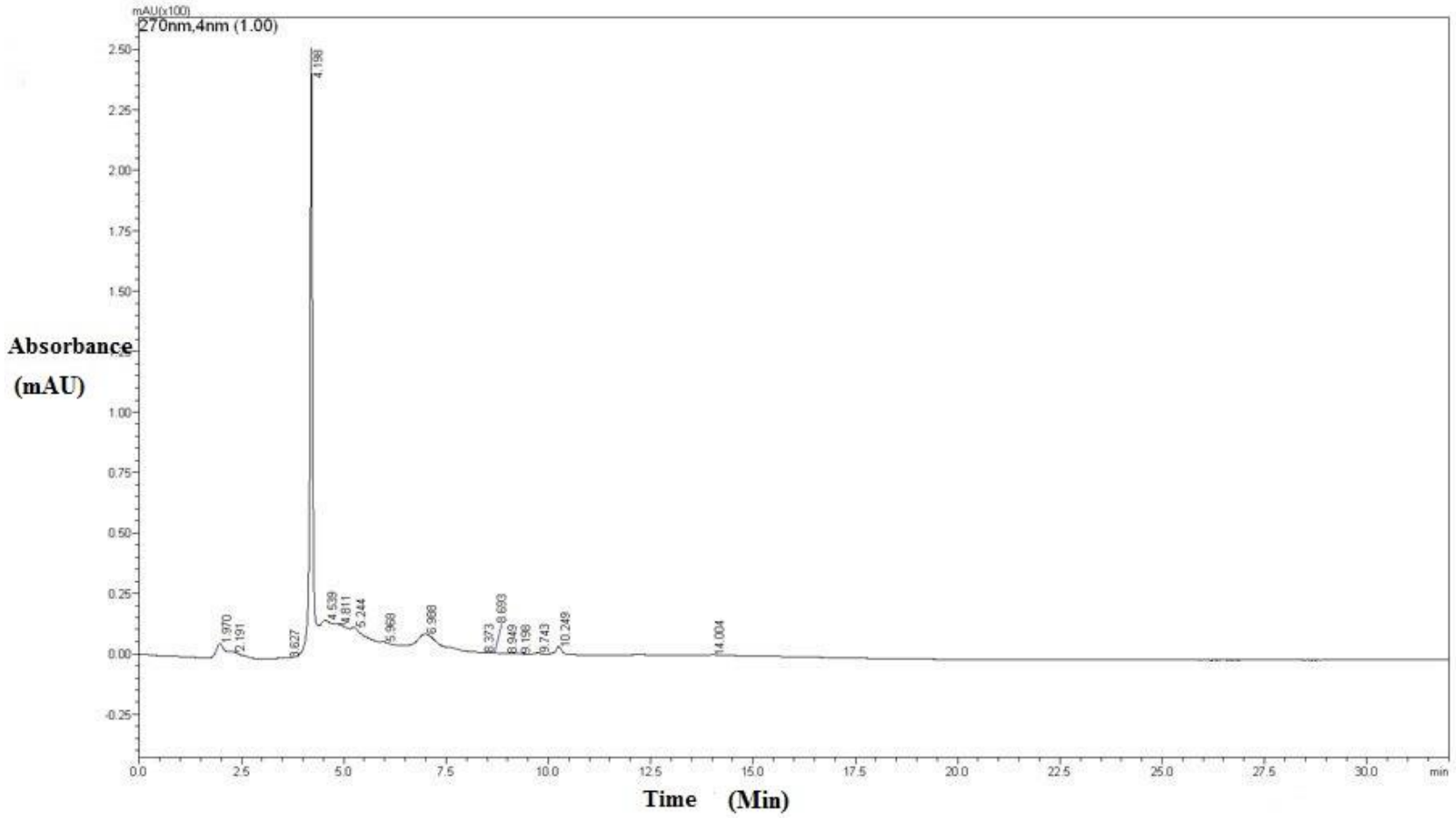
Appendix 16: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 30 at 220 nm



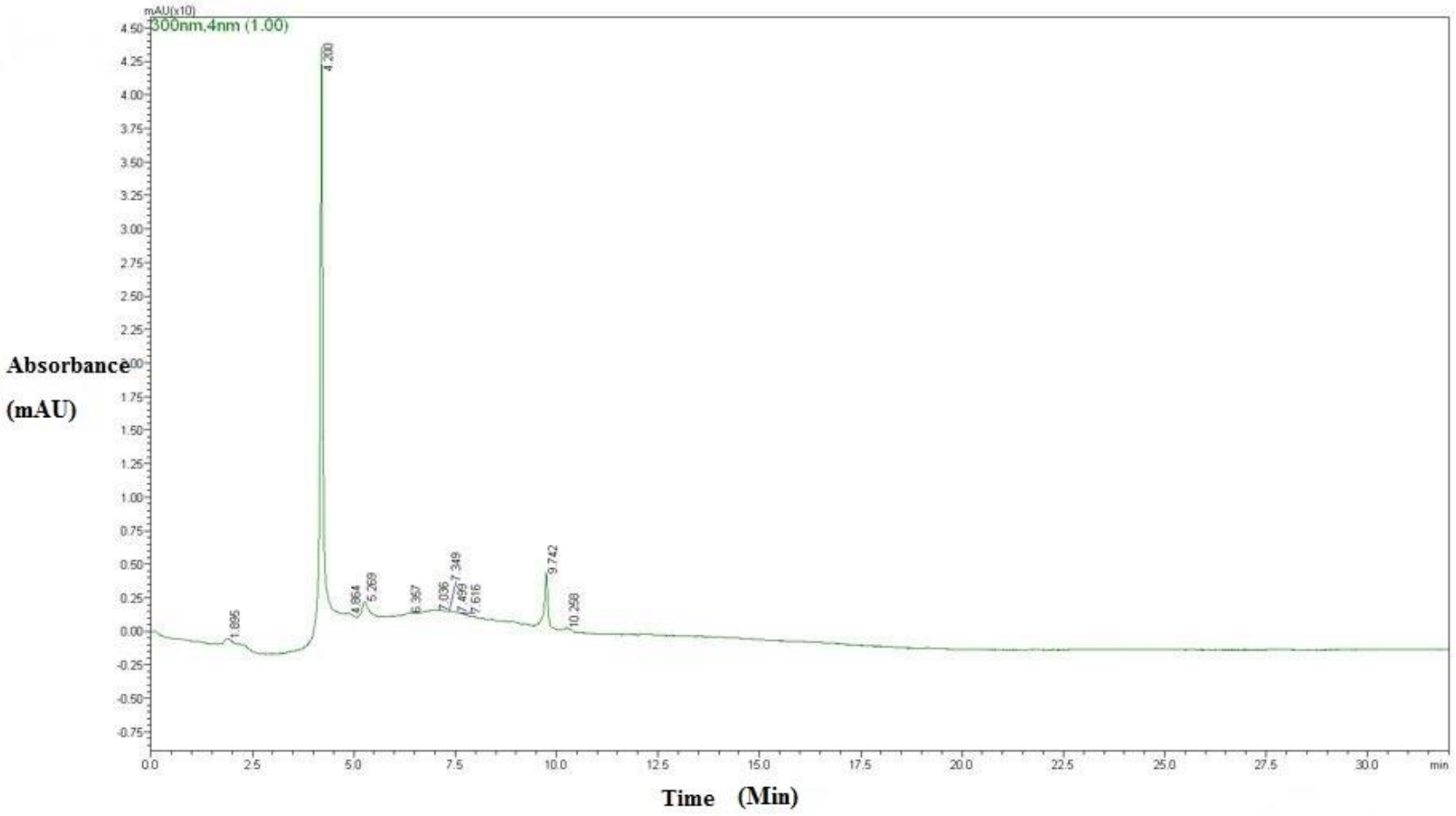
Appendix 17: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 30 at 254 nm



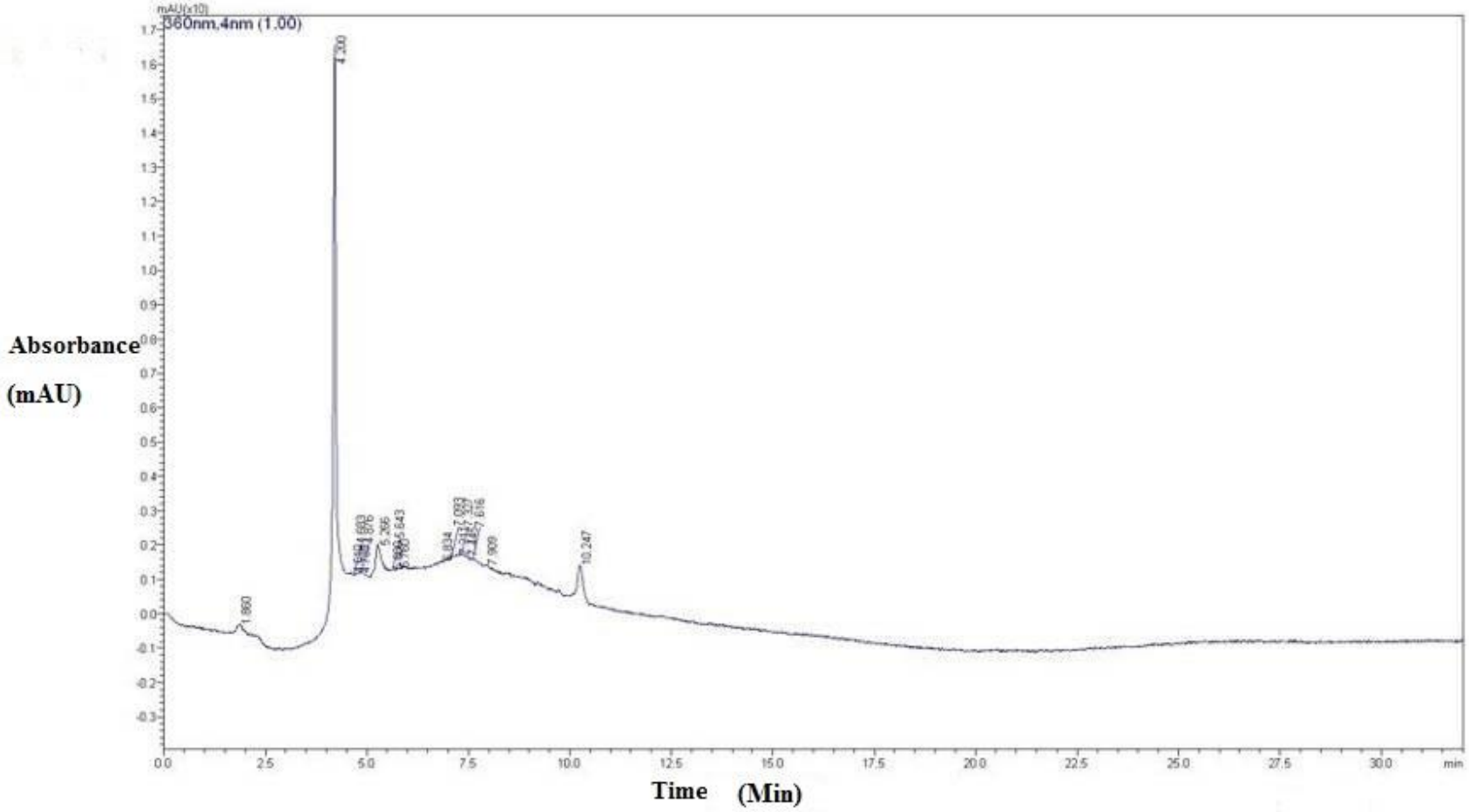
Appendix 18: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 30 at 270 nm



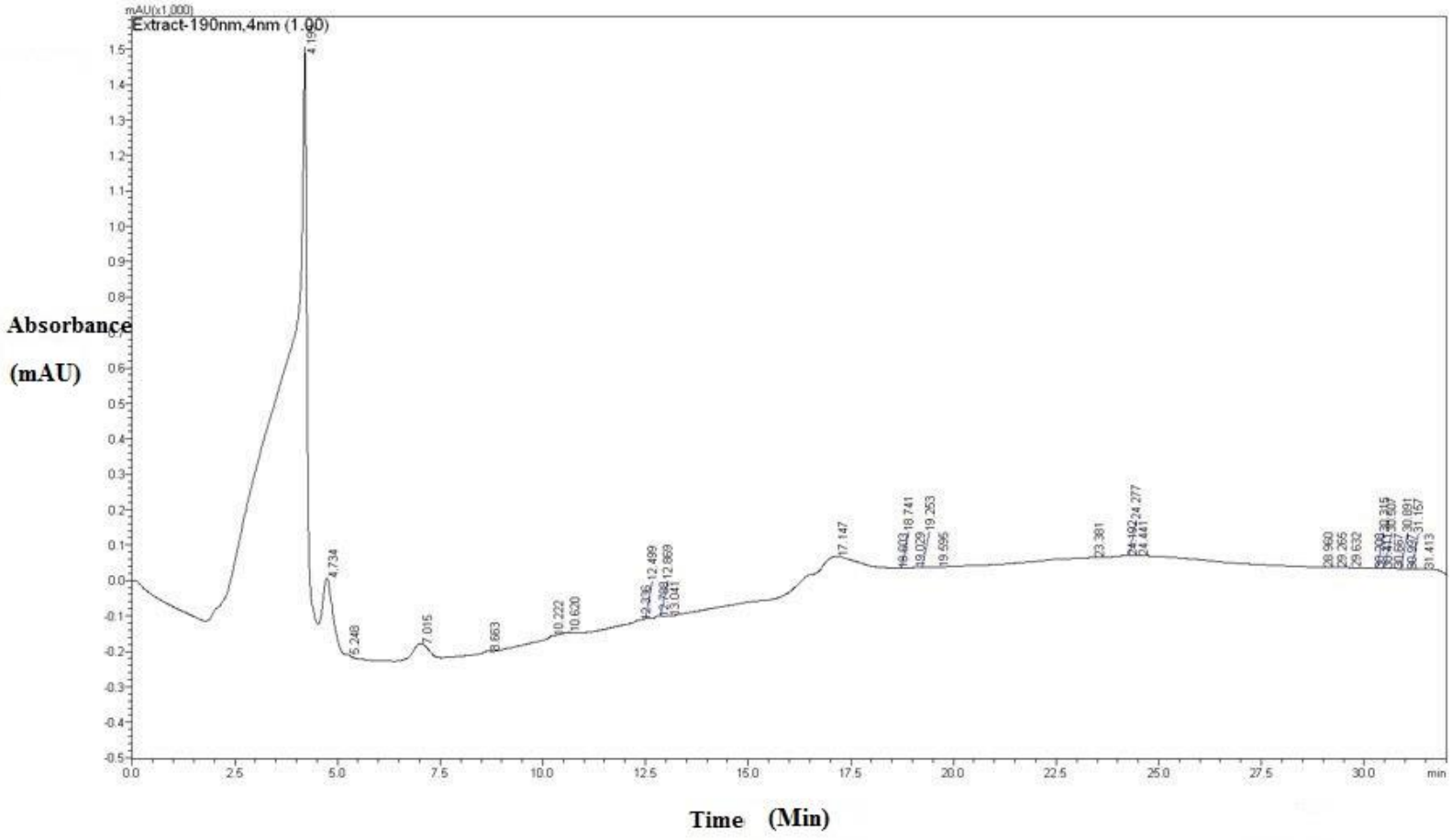
Appendix 19: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 30 at 300 nm



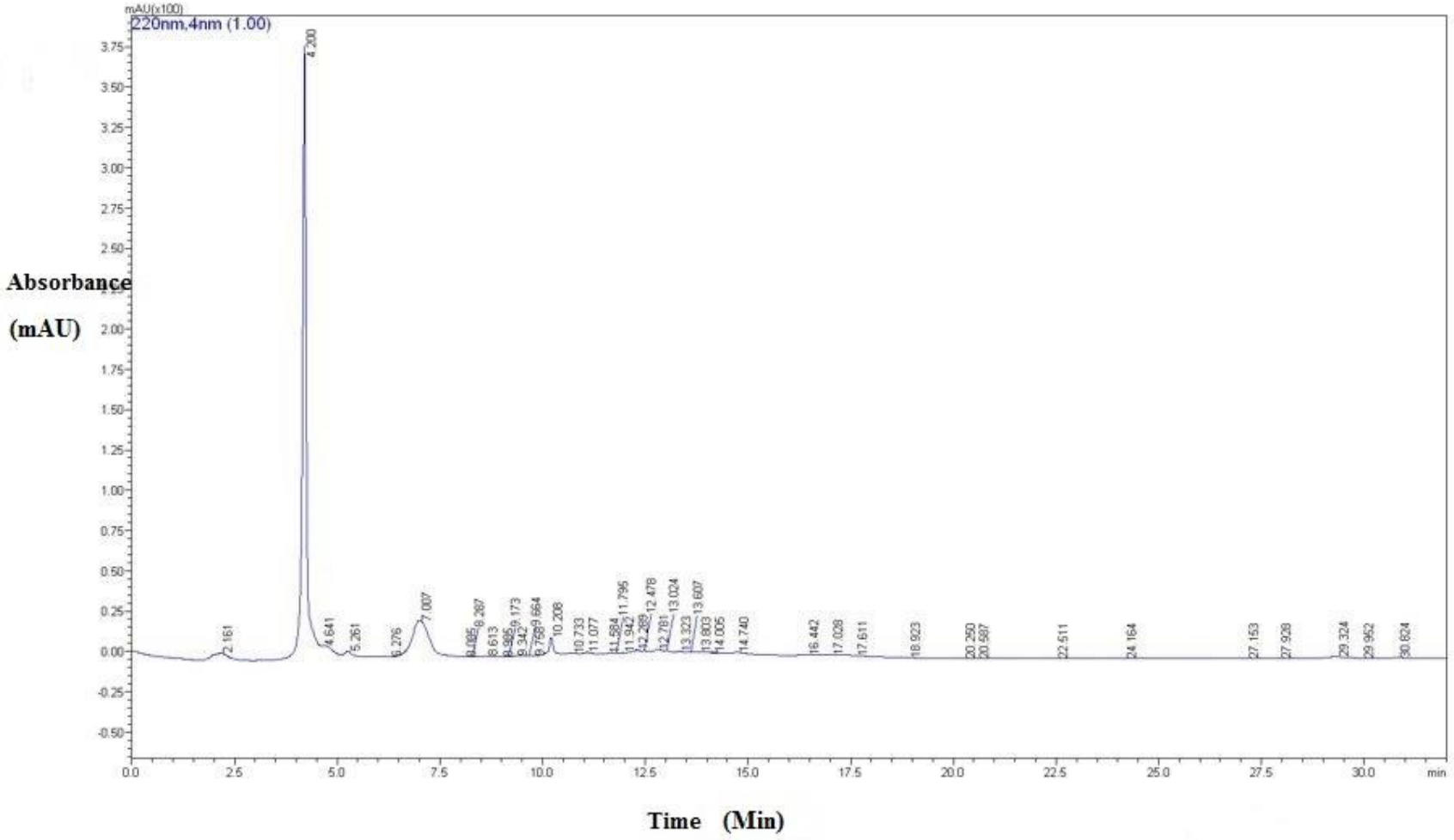
Appendix 20: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 30 at 360 nm



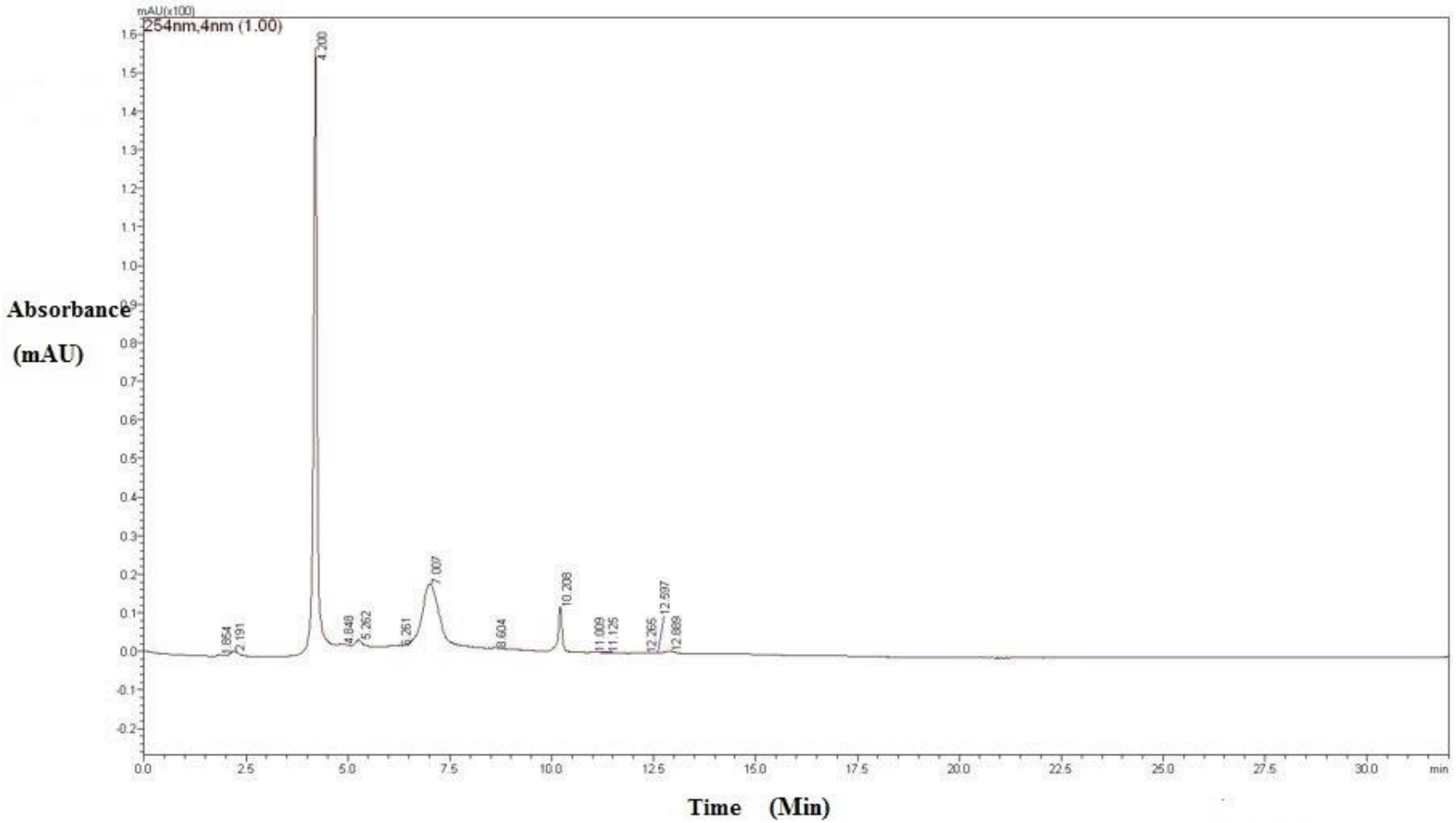
Appendix 21: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 52 at 190 nm



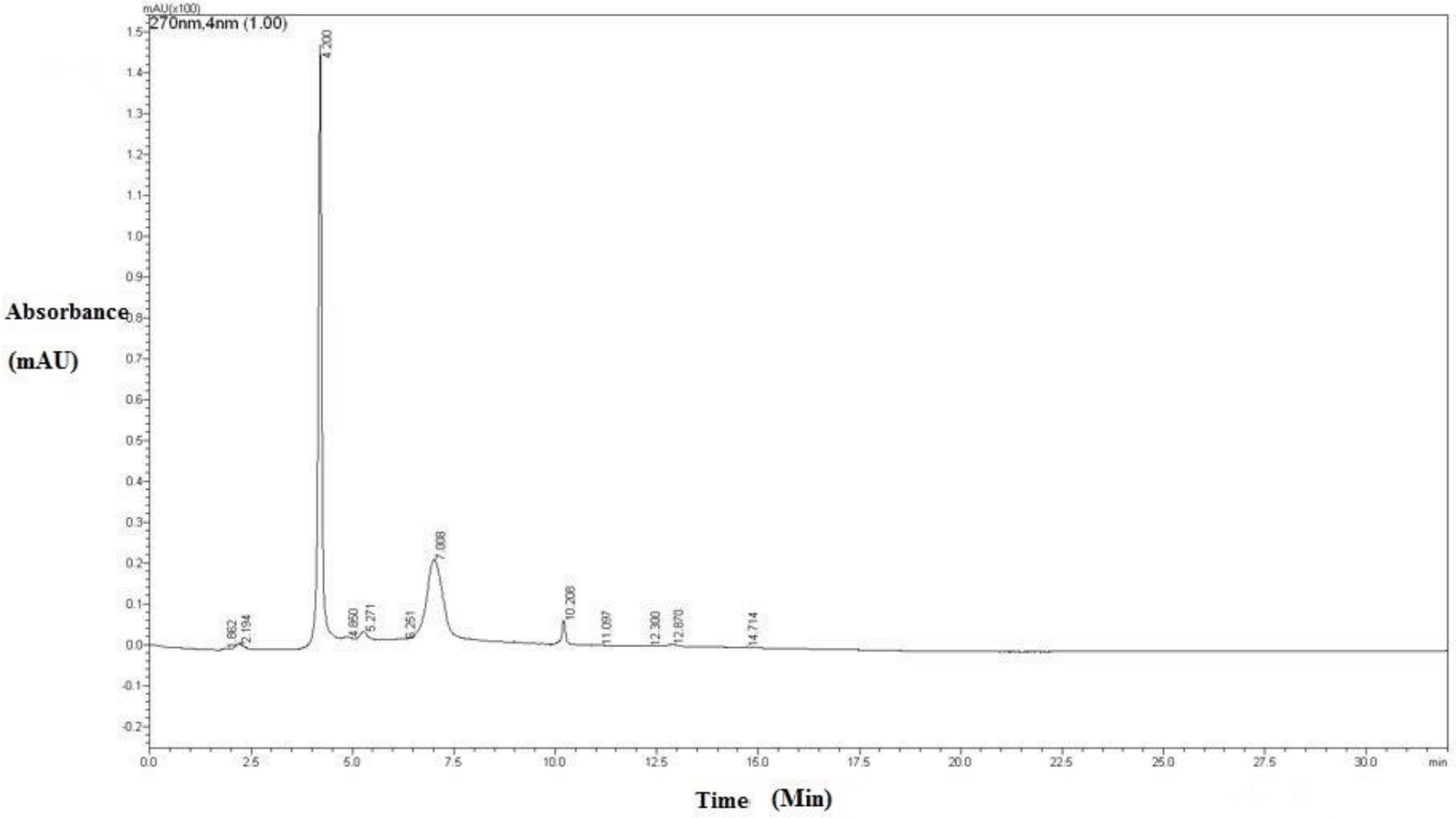
Appendix 22: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 52 at 220 nm



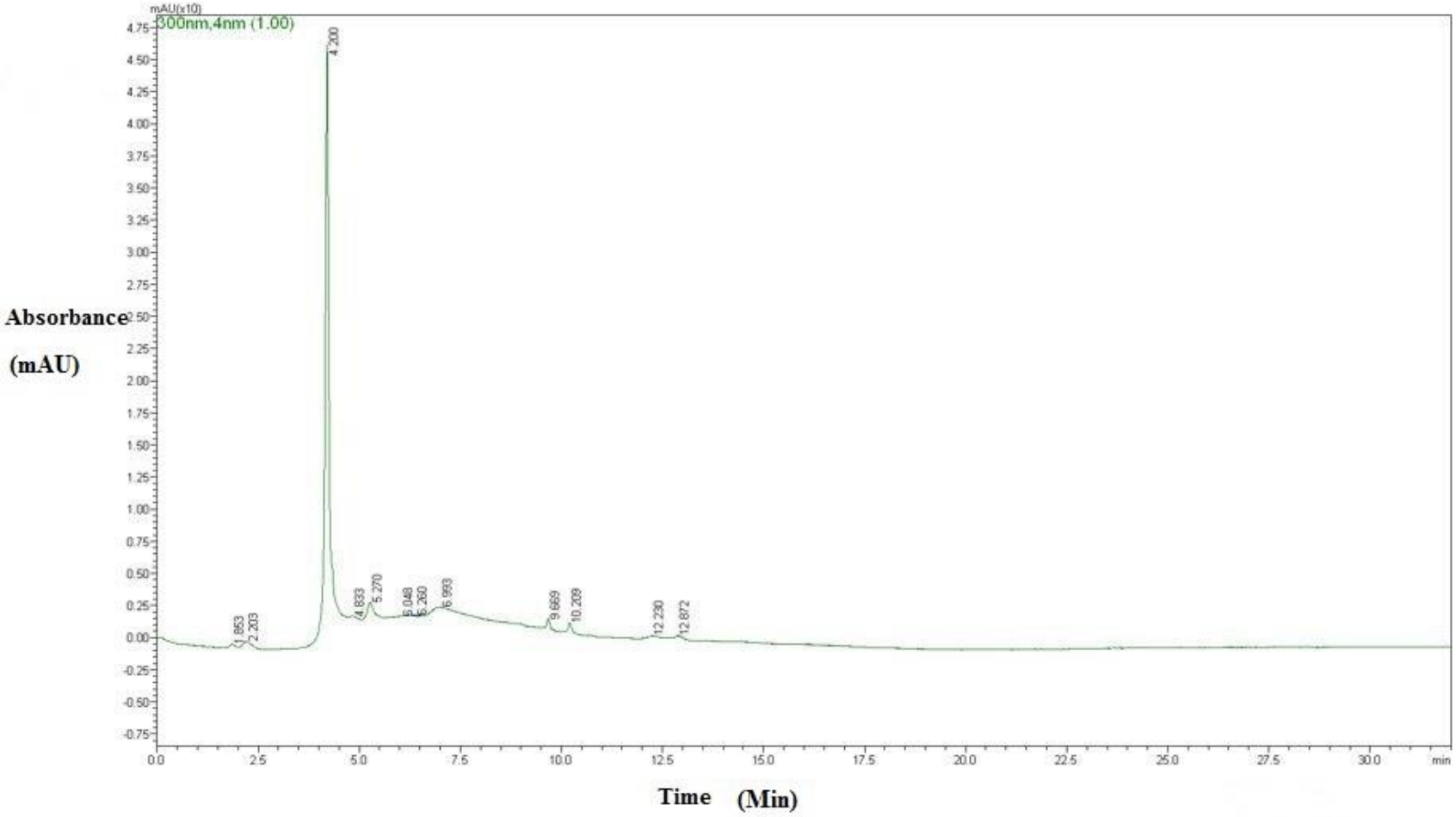
Appendix 23: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 52 at 254 nm



Appendix 24: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 52 at 270 nm



Appendix 25: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 52 at 300 nm



Appendix 26: Reverse phase high performance liquid chromatography (RP-HPLC) elution profile of endotoxins from Bt 52 at 360 nm