Biochemical Investigations on Genetically Modified Oil Crops

Dissertation zur Erlangung des Doktorgrades
Der Naturwissenschaften (Dr. rer. nat.)

Fakultät Naturwissenschaften
Universität Hohenheim

Institut für Lebensmittelchemie

Vorgelegt von

Enas Mahmoud Mohamed Ibrahim Mekawi

aus

Ägypten

Dekan:		Prof. Dr. Hanz Breer
1.	berichtende person (1. Püefer):	Prof. Dr. Wolfgang Schwack
2.	berichtende person (2. Püefer):	Prof. Dr. Heinz-Dieter Isengard
3.	Prüfer:	Prof. Dr. Lutz Graeve

Eingereicht am:	28.09.2009
Mündliche pruefung am:	08.12.2009

Die vorliegende Arbeit wurde am 19.11.2009 von der Fakultät Naturwissenschaften der Universität Hohenheim als "Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften" angenommen.

Acknowledgement

The work with this dissertation has been extensive and trying, but in the first place exciting, instructive, and fun. Praise and thanks be to Allah for providing me with health, strength, and guidance to pursue my endeavours in this life. Without help, support, and encouragement from several persons, I would never have been able to finish this work. Though it will not be enough to express my gratitude in words to all those people who helped me, I would still like to give my many thanks to all these people.

First of all, I would like to thank Prof. Dr. W. Schwack, for many years of confidence in my person and in my research. His valuable guidance and his reliability as my mentor made possible the completion of this work. He showed me different ways to approach a research problem and the need to be persistent to accomplish any goal. I learned a lot from him, without his help I could not have finished my dissertation successfully.

I would also like to acknowledge Prof. Dr. H. D. Isengard and Prof. Dr. L. Graeve for their constructive comments through the review of my dissertation and for asking me good and hard questions at my doctor exam.

A special thanks goes to everyone in Prof. Dr. W. Schwack working group: Dr. W. Armbruster for his valuable and continuous help, Dr. G. Morlock for her comments and time during my research, Miss K. Lehnert for spontaneous help and for technical assistance with protein purification.

I am also very grateful to everyone that has read parts of the manuscript, especially Dr. M. El Nagar and Dr. J. Pfannstiel.

Thanks also to all my colleagues and members of the Faculty and staff in the University of Hohenheim: Miss. A. Manns, Institute of Food Technology, for generous help in Capillary Electrophoresis analysis, Dr. M. Lacorn Institute of Animal Husbandry and Animal Breeding, for providing antiserum and Dr. J. Pfannstiel, Life Science Center, for his valuable and continuous help in Mass Spectrometry analysis.

My sincere thanks go to Dr. H. N. Thu Georg-August University Göttingen, Germany for providing Cry1Ab-toxin.

Gratitude is also extended to all staff members of the Agricultural Chemistry Department, Faculty of Agriculture at Moshtohor, Benha University, Egypt for getting me interested in research and coming to Germany.

I deeply cherish the unconditional support I got from my parents, my sister Doaa and brothers Mohamed and Mostafa throughout my studies and also during these years abroad.

I am greatly indebted to my husband Dr. Mahran, my son Mohamed and my daughter Sofia. They form the backbone and origin of my happiness.

I will be always indebted to "Wiedereinstiegsstipendium der Universität Hohenheim" for providing financial support to this work (05.2005-10.2006).

Finally, it was a great opportunity to study in Germany. I have had a valuable time and experience that I would never forget. Therefore, I would like to pay my tribute to German study system that provides students extensive facilities, opportunities and knowledge.

Abbreviations and symbols

A	Ampere, unit of electric current
Bt	*Bacillus thuringiensis*
CE	Capillary electrophoresis
cm	Centimeter
CR	Coleopteran-resistance
CRL-GMFF	Community Reference Laboratory for GM Food and Feed
DAD	Diode Array Detector
DEAE	Diethylaminoethyl cellulose
dw	Dry weight
ECB	European corn borer
EFSA	European Food and Safety Authority
ELISA	Enzyme-Linked ImmunoSorbent Assay
ENGL	European Network of GMO Laboratories
ESI	Electrospray ionization
EU	European Union
g	Gram
g	Relative centrifugation force
GMOs	Genetically modified organism
GPC	Gel permeation chromatography
h	Hour
HOSF	High-oleic sunflower
HPLC	High performance liquid chromatography
HPTLC	High Performance Thin Layer Chromatography
HRP	Horseradish peroxidase
IHCP	Institute of Health and Consumer Protection
JRC	European Commission's Joint Research Centre
kDa	Kilodalton (the unified atomic mass unit dalton Da)
L	Liter
LC	Liquid chromatography
LC-MS/MS	Electrospray-ion trap mass spectrometry
LR	Lepidopteran-resistance
MALDI-TOF	Matrix assisted laser desorption time of flight mass spectrometry
mAU	Milli-absorbance units
min	Minute
mm	Millimeter
MS	Mass spectrometry
MW	Molecular weight
m/z	Mass-to-charge ratio

nm	Nanometer
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
pI	Isoelectric point
PI	Phosphatidylinositol
PMF	Peptide mass fingerprint
RP	Reversed-phase
SAP	Scientific Advisory Panel
sec	Second
SEC	Size exclusion chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	Sunflower
Synpro	Synthetic protoxins
Unsap. matter	Unsaponifiable matter
USDA	United State of Agriculture Department
UV	Ultraviolet
V	Volte, the international system unit of electric potential and electromotive force
δ	Delta
α	Alpha
γ	Gamma
μ	Micro
∆	Delta

List of tables

List of figures

1 Introduction

1.1 Importance of oil crops

Oil seed crops are one of the most valuable and widely traded agricultural commodities because of their nutritional and economic importance. Oilseed crops have been grown since time immemorial and are grouped as conventional and non-conventional. The conventional oilseeds include rapeseed/mustard, groundnut, linseed, castor seed and sesame. The non-conventional oilseeds are canola, sunflower, soybean and safflower. Cotton, though a fibre crop is an important source of edible oil. Corn also yields edible oil as a by-product. Oil palm, olive, jojoba and salicornia are also edible oil crops.

Oil crops have many other uses beside food and feed purposes. According to the United State of Agriculture Department (USDA, 2007) the total world production of major vegetable oils in 2007 was over 400 million tonnes. A part of the total supply was used by the oleochemical industry as starting material for range of industrial products. Some was used also in the animal feed but the vast majority of vegetable oils were used for edible commodities, such as margarines, cooking oils, and processed foods. For many years it has been assumed that the produced oils and fats were used for food, feed, and oleochemical industry in the ratio 80:6:14 but this is changing, mainly through the rapidly growing demand for biodiesel. At the end of 2007 it was probably close to 74:6:20 and it has been suggested that by 2020, it could be 68:6:26. This ratio does not mean that less will be consumed as food but that this will represent a smaller portion of a larger total. In 2008/09 one half of the year's additional supply will be probably be used for non-food purposes (mainly biodiesel) and the other half for food purposes.

1.2 Genetically modified plants

Biotechnology has allowed the targeted modification of the genetic composition of plants resulting in organisms with novel traits. The new crop varieties produced by modern biotechnology are called genetically modified (GM) plants or transgenic plants (Gao 2004). The tools of modern biotechnology allow plant breeders to select single genes that produce desired traits and move genes from one plant or organism to another.

During the period 1996 to 2007, the proportion of the global area of GM crops grown by developing countries had increased consistently. In 2007, 43% of the global biotech crop area, (up from 40% in 2006), was in developing countries. The growth between 2006 and 2007 was substantially higher in developing countries (8.5 million hectares or 21% growth) than industrial countries (3.8 million hectares or 6% growth) (James 2008).

The previous information shows that the genetically engineered food and feed-grade varieties being currently cultivated at commercial scale. GM crops were conceived to show enhanced agronomic properties in term of herbicide tolerance and/or insect resistance. The introduction of these traits has resulted in a restrained use of herbicides and insecticides, simplifying agricultural practice and reducing the costs for production of agricultural commodities.

Subsequent genetic engineering applications have been additionally focused on enhancement of wide variety of quality traits in agricultural products and derivatives.

Biotechnological engineering in plants can be categorized in several groups including improvement of important characteristics, the enhancement of content, composition or bioavailability of nutrients, increasing the nutrition value of food and enhancing processing properties and modified structures composition (Christau and Klee 2004).

1.2.1 Engineering of crops for improving agronomic traits

Important properties of agricultural products have been improved by mean genetic engineering. The best known examples of herbicide resistance are soya, maize, cotton and canola. Commercial crop resistance has been achieved with four different classes of herbicides such as glyphosate, glufosinate, bromoxynil, and the group of herbicides that include sulfonylureas imidazolinones (Christou and Klee 2004). Even in industrial countries, a major percentage of crop harvests are lost because of pests and diseases. Within this context, transgenic crops have played an important role delivering new strategies for management of pest and disease control measures. The best known example is the production of transgenic plants that express the insecticidal δ-endotoxins

produced by different strains of *Bacillus thuringiensis.* Bt toxin has been shown to act highly on a wide range of target organisms. A number of genes encoding for δ-endotoxins have been successfully introduced into maize, making crops resistant to corn borers and corn root worms (Thomson, 2006).

1.2.2 Quality and yield traits

Genetic engineering to modify the contents composition or bioavailability of macro- and micronutrients in food-grade crops address specific metabolites such as lipids, carbohydrates, proteins, and amino acids. For most major oilseed crop species, methods for genetic transformation are available. This opens up many opportunities to selectively modify their lipid composition and nutritional value by genetic engineering. These modifications aim at the modulation of chain length and the degree of saturation of fatty acids, as well as the production of oils containing a particular fatty acid in high proportions, such as high-stearate oil, high–oleic oil or accumulation of very long-chain polyunsaturated fatty acids (Christau and Klee, 2004).

1.3 Genetically modified Bt plants

Bacillus thuringiensis is a naturally occurring soil bacterium that produces proteins, called Bt proteins that are toxic to certain insects (Schnepf *et al*., 1998). Over 40 varieties of these proteins have been identified. For instance some of Bt toxin target larval forms of lepidoterans (butterflies and moths), dipterans (flies and mosquitoes) and others cleopterans (beetles). Bt proteins cause little or no harm to most non-target organisms, including humans and wildlife. They have been used in sprays in conventional and organic agriculture for decades with a little or no ill effects on environment or human health. Thus, Bt toxins are considered environmentally friendly alternatives to broad spectrum insecticides. Also, these and other insecticide sprays are rather effective against pests that burrow into plant tissues (Thomson 2006).

1.4 Genetically modified Bt corn

The Bt crops contain a toxin derived from *Bacillus thuringiensis*. Corporations have genetically inserted the gene for this toxin into crops to function as an insecticide

against harmful insects, such as the European corn borer and the Colorado potato beetle (Mendelsohn *et al*., 2003). However, Bt crops also kill and adversely effect beneficial insects, like monarch butterflies, bumblebees, and lacewings.

One of the most popular types of genetically engineered corn is an insecticide producing corn called Bt corn MON810 maize with a trade name YieldGard, produced by Monsanto. It was genetically engineered to resist European corn borer by producing a truncated version of the insecticidal protein Cry1Ab, derived from *Bacillus thuringiensis* (Holck *et al.*, 2002). The Cry1Ab protein produced by MON810 is insecticidal only to Lepidopteran insects. This specificity of action is directly attributable to the presence of specific binding sites in the target insects.

The native Cry1Ab protein has molecular weight 131 kD while the plant expressed Cry1Ab codes a protein 91 kD (Brandt 2004). After activation of the protoxin by solubilization of the toxin by insect midgut proteases or by trypsin, the protoxin becomes the active toxin. Commercially available Bt- expressing maize is shown in Table 1 (EPA 2008).

1.5 Mode of action of Cry toxins

The toxin that *Bacillus thuringiensis* produces is called Cry protein. Different strains of the bacterium produce different versions of the toxin; these versions are classified into groups CryI, CryII, CryIII, and CryIV. Each group was subdivided further into subgroups A, B, C, etc

Bravo *et al.,* (2007) reported that, *Bacillus thuringiensis* crystals are first solubilized in the midgut of susceptible insects, followed by activation of the protoxin to active toxins by the midgut protease. The activated toxin then binds to insect midgut membrane receptors, insert into the apical membrane and form pores. Formation of the pores causes loss of osmotic regulation, and eventually leads to cell lyses, which is thought to be responsible for insect death.

Table 1: Registered Bt maize for Lepidopteran-resistance (LR) and coleopteran-resistance (CR).

Plant-Incorporated Protectant	Insecticidal proteins	Traits	Companies	Date Registered	Trade name
Bt corn Event 176 [a]	Cry1Ab	LR, PAT[b]	Mycogen	Aug.,1995	NtureGuard
Bt corn Event 176 [a]	Cry1Ab	LR, PAT[b]	Ciba Seeds	Aug.,1995	KnockOut
Bt corn Event BT11	Cry1Ab	LR, PAT	Northup King	Aug.,1996	Agrisure CB
Bt Corn Event MON810	Cry1Ab	LR	Monsanto	Dec.,1996	YieldGard
DBT418 [a]	Cry1Ac	LR, PAT	DeKalb Genetics	Mar.,1997	Bt-Extra
Bt corn Event BT11 (sweet corn)	Cry1Ab	LR, PAT	Novartis Seeds	Feb.,1998	Attribute
Bt CornCBH351[a,c]	Cry9C	LR, PAT	Plant Genetics systems	May, 1998	StarLink
Bt corn Event TC1507	Cry1F	LR, PAT	DowAgroSciences/Pioneer Hi-Bred	May, 2001	Herculex I
Bt corn Event MON863	Cry3Bb1	CR	Monsanto	Feb.,2003	YieldGard RW
Bt corn stalk Events MON863+MON81[d]	Cry3Bb1 + Cry1Ab	CR, LR	Monsanto	Oct., 2003	YieldGard Plus
Bt corn event DAS-59122-7	Cry34Ab1 + Cry35Ab1	CR, PAT	Dow Agro Sciences/ Pioneer Hi-Bred	Aug.,2005	Herculex RW
Bt corn Events DAS-59122-7 [d] + TC1507	Cry34Ab1 + Cry351Ab1 + Cry1F	LR, CR, PAT	Dow AgroSciences / Pioneer Hi-Bred	Oct., 2005	Herculex Xtra
Bt corn Event MON88017	Cry3Bb1	CR, EPSPS [e]	Monsanto	Dec.,2005	YieldGard VT RW
Bt corn Events MON88017 [d]+ MON810	Cry3Bb1 + Cry1Ab	LR, CR, EPSPS	Monsanto	Dec.,2005	YieldGard VT Triple
Bt Corn Events MIR 604	modified Cry3A	CR	Syngenta	Oct., 2006	Agrisure RW
Bt Corn Events MIR 604 [d] + BT11 (Agrisure CB/RW)	Modified Cry3A + Cry1Ab	LR, CR, PAT	Syngenta	Jan., 2007	Agrisure CB/RW

[a] No longer registered
[b]PAT, phosphinothricin-N-Acetyltransferase, which allows use of herbicide glufosinate ammonium (e.g., Liberty®)
[c] Registered for animal feed and non-food use
[d]Stacks formed from conventional crossed
[e]Epsps, 5-enolpyruvylshikimate-3-phosphate synthase, which allows use of herbicide glyphosate (e.g. Roundup®)

1.6 Activation of Cry protoxins

A hallmark of Bt crystal proteins is their extreme insolubility. Endotoxins typically are protoxins, which need a proteolytic processing event to aid solubility and activate the

toxic component (Andrews *et al*., 1985). Crystal solubilization is facilitated by an alkaline pH of susceptible insects; the typical midgut pH is between pH 9-11 in insect midgut (Milne and Kaplan 1993). Cry1A toxins are fully soluble at pH 9.5 (Bietlot *et al*., 1989). Activation of Cry1 protoxins by protease according to Choma and Kaplan (1990) are shown in figure 1. As shown in the process of activation, it appears to resemble that mammalian gut proteases such as trypsinogen pepsinogen in that 25-30 amino acids from N-terminal peptide is removed and approximately half of the remaining protein from the C-terminus. The role of the C-terminal extension to the active toxin is believed to be in the formation of crystalline inclusion bodies within the bacterium and is dispensable for toxicity (Choma and Kaplan, 1990 and 1991, Bravo *et al*., 2002).

However, in case of protoxin activation, there are no internal cleavages generated with the toxic moiety during cleavage. It appears that conformation changes occurring during activation are rather subtle, affecting the tertiary structure but not the secondary structure of proteins.

1.7 Potential alternation in toxicity of plants after genetic modification

The insertion of a new gene results in either the production of either one or two of new substances in the plant or change in synthesis of existing substances. The effect can be direct and intentional. The introduced genes directly produce proteins or enzymes, which in turn results in the change of other substances in the plants. The introduction of new genes may increase or decrease the expression of existing protein or enzyme. Hence, GM products should be tested for levels of natural toxins and any change of other substances (Parekh 2004). As the expression of wild-type Bt genes in plants is suboptimal due to differences between bacterial and plant genomes (De Maagd *et al*., 1999), these genes were modified to enhance the quality and toxicity of Cry toxins (Perlak *et al*., 1991).

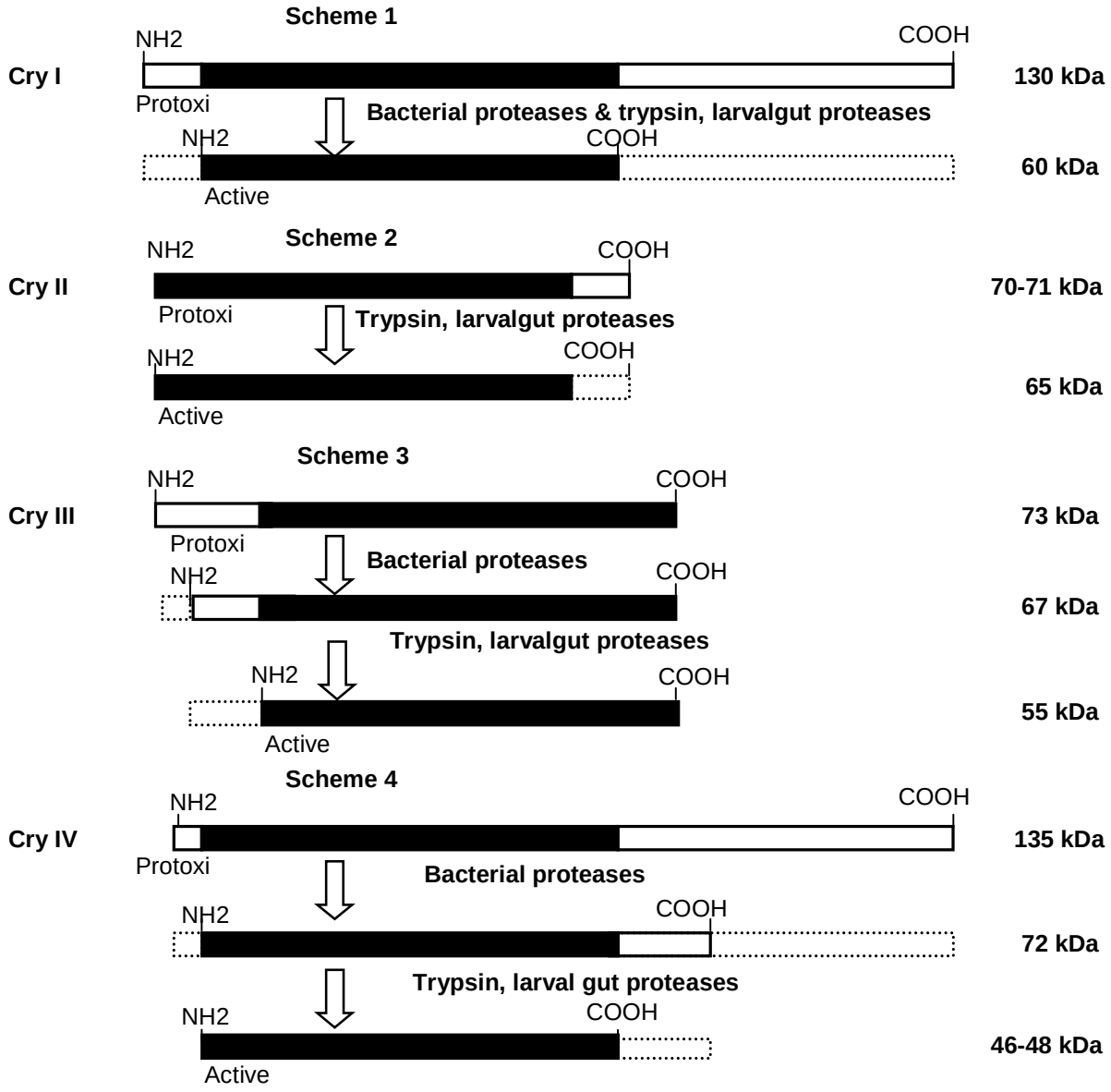

Figure 1: Scheme for activation of Cry protoxins.
Stippled region, removed by cutting with protease.

1.8 Economic and human health impacts of Bt crops

The proportion of the economic benefits that accrue to the farmer, the consumer and the technology company vary among countries depending on the degree of the protection provided for intellectual property rights and the degree of government control over commodity price. Direct health benefits accrue from the reduction in insecticide use on Bt crop, as a result of lower pesticide residues in food and water, and the reduced

exposure of farm workers during pesticide applications. These benefits are especially great in developing countries, where pesticide regulation is weak, the education level of farmers is generally low and pesticides are applied manually (Romeis *et al.,* 2008).

1.9 Methods for the detection of GMOs

GMOs research includes the development of transgenic plants as well as the characterization and detection of GMOs in plants and plant-derived materials. Last years, GMO research was focused mainly on development and optimization of strategies and methodologies for the control and analysis of GMOs.

The use of GMOs -their release into the environment, cultivation, importation and particularly, their utilization as food or food ingredients - is regulated in the European Union (EU) by a broad legislative framework. EU Regulations require critical measures such as access to validated methods and technical guidance documents for the detection of GMO. The European Commission's Joint Research Centre (JRC), acting via the Institute of Health and Consumer Protection (IHCP), and more particularly the "Biotechnology and GMOs Unit" is legally mandated under Article 32 and the Annex of Regulation No 1829/2003 on genetically modified food and feed to operate as the Community Reference Laboratory for GM Food and Feed (CRL-GMFF). The role of the CRL-GMFF in the process of authorization for placing on the market a GMO for food or feed is to validate the method for the detection and identification of the transformation event. Results of the validation are transmitted to the European Food and Safety Authority (EFSA) and are included in the Authority's overall opinion. The activities of the CRL-GMFF are carried out in collaboration with the European Network of GMO Laboratories (ENGL). ENGL is a unique platform of EU experts that play an eminent role in the development, assessment and standardization of means and methods for sampling, detection, identification and quantification of GMOs or derived products in a wide variety of seeds, grains, food, feed and environmental samples (Mazzara *et al*., 2007).

1.9.1 DNA based methods

The DNA that has been engineered into a crop consists of several elements that govern its functioning. Although several DNA based techniques are available, two are commonly used: Southern blot and particularly PCR analyses (Ahmed 1995; Sambrook and Russel 2000). The sensitivity of these DNA methods is extremely high for genetically modified crops and processed foodstuffs being able to detect traces of DNA (Schreiber, 1999).

1.9.2 Protein based methods

Immunoassay technologies with antibodies are ideal for qualitative and quantitative detection of many types of proteins in complex matrices when the target analyte is known (Brett *et al*., 1999). Both monoclonal (highly specific) and polyclonal (often more sensitive) antibodies can be used depending on the amounts needed and the specificity of the detection system (e.g. antibodies to whole protein or specific peptide sequences), depending on the particular application, time allotted for testing and cost. On the basis of typical concentrations of transgenic material in plant tissue (>10 µg per tissue), the detection limits of protein immunoassays can predict the presence of modified proteins in the range of 1% GMOs (Stave, 1999). Various methods based on the recognition of recombinant proteins have been developed. The most common test formats were sandwich ELISA and sandwich-type immunochromatographic (lateral flow) strip tests (Stave 2002). They are suitable for detection of bivalent or polyvalent antigens on the basis of direct double antibody binding strategy (sandwich). The analyte is trapped between a solid phase antibody and a labeled secondary antibody. Such methods are available for the protein expression in the most important GM crops, including insect–resistant maize, potato, cotton or herbicide-resistant maize, potatoes, cotton, soybean and canola (Lipp *et al*., 2000).

1.10 Protein Identification by Mass Spectrometry

Mass spectrometry is entirely different from the other sequencing techniques and offers several advantages such as the analysis of peptides within mixtures or of peptides that

are blocked on the N-terminal side or post translational modifications. Mass spectrometry is thus complementary to other existing methods.

Recent examples from the literature indicate that MS might also be viably applied for the analysis of protoxins produced by *B. thuringiensis* strains (Ranasinghe and Akhurst 2002; Lee *et al.*, 2006). There are two major methods that are widely used for protein identification by mass spectrometry: MALDI-TOF based protein fingerprinting and LC-MS/MS based peptide sequencing. Traditionally, proteomic analysis of complex protein samples involves the resolution of proteins using gel electrophoresis, followed by the identification of resolved proteins by mass spectrometry.

1.11 MALDI-TOF based protein fingerprinting

MALDI provides a high sensitivity and a broad mass range. MALDI-TOF MS has been widely used to study different classes of biomolecules such as proteins, oligonucleotides, polysaccharides and polymers (Feistner *et al*., 1995; Karas *et al*., 1995; Papac *et al.*, 1996). Compared to the traditional techniques, such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), MALDI-TOF MS has several advantages in the determination of protein molecular weight (Mr), peptide mapping, and purity (Guo-Hua *et al*., 1999).

In the MALDI-TOF based protein fingerprinting method, a sample is digested with certain proteolytic enzyme (usually trypsin) and an MS spectrum is acquired which generates the masses of all peptides. These masses are used as a fingerprint to search proteins in a database. The hits are ranked by a scoring method. A candidate protein that contains more proteolytic peptides, which can match measured masses, has a high score. Protein identification based on MALDI-TOF MS is a rapid method for protein mass fingerprinting.

1.12 LC-MS/MS peptide based sequencing

Liquid chromatography combined with tandem mass spectrometry has become a widely used technique for protein analysis. Reversed-phase (RP) LC is a preferred mode of

separation for LC−MS/MS due to its high separation power and compatibility of the mobile phases with ionization techniques such as electrospray ionization (ESI) (Fournier *et al*., 2007; Motoyama and Yates, 2008).

In protein identification by LC-MS/MS peptide sequencing, each MS/MS spectrum (corresponding a specific peptide sequence) is used to search protein database for matched peptides. A protein hit is often identified by multiple independently sequenced peptides from the same protein. However, there are two major factors that make the LC-MS/MS a superior platform for protein identification: sensitivity and reliability.

Since mass spectrometry detection is concentration-dependent, such concentration increase is effectively translated into the increase of MS signal. Consequently, it can dramatically increase real sensitivity in identifying proteins. On the other hand protein identification by LC-MS/MS is based on independent sequencing of peptides. Theoretically, an LC-MS/MS can identify a thousand protein mixture with the same reliability as that in identifying one protein.

1.13 Genetically modified Oilseeds

Because of the difficulties in domesticating wild plants greater effort has been directed to the modifying of plants that are already grown and harvested on commercial scale and where good agronomic products are already well developed. The oilseeds have economic and nutrition values, because of that the genetic modification of oilseeds was demonstrated to enhance the properties of oilseed crops. The changes to be sought are partly agronomic change in fatty acid composition, triacylglycerol composition, and in levels of minor components. These changes must be achieved without sacrifice of yield and must be biologically stable from season to season. Such changes may be brought about by conventional seed breeding or by newer procedures of genetic engineering (Christau and Klee, 2004).

Change of fatty acid composition which have been sought include: reducing levels of saturated acids for nutritional reasons, reducing levels of linolenic acid and/or higher levels of saturated acids to avoid hydrogenation (with consequent production of undesirable trans acids), and higher levels of oleic acid. One important and exciting

possibility is to develop plant systems that will produce long-chain polyunsaturated fatty acids such as arachidonic acid (12:4), EPA (20:5), and DHA (22:6) (Frank, 2008).

In view of the high demand for oils and fats for food and non-food purposes it is important to increase supplies. This may be achieved by seed breeding to give higher oil yields, or higher seed yield which are more drought resistant and will so give better yields under adverse conditions, and seeds which will grow under harsher conditions of climate (lower temperature or shorter growing seasons) or of soil (high salinity) (Christau and Klee, 2004).

1.14 The effect of genetic modification on chemical composition of plants

The use of genetic engineering in agriculture and food production has effects not only on the environment and for biodiversity, but also on human health, because genetic engineering introduces new genes, genetic information and constituents into the cell of food-producing organisms. These new proteins could cause allergies or be toxic (Taylor 1997). Alternatively they could alter the cellular metabolism of the food-producing organisms in unintended and unanticipated ways, and in turn, these alternations in metabolism could cause allergens or toxins to be produced in food.

Another possibility is that, as a result of these alterations in metabolism, the food-producing organism might fail to make some important vitamins or nutrients. Consequently, the genetically engineered food would lack important nutrients that are normally present in corresponding non-genetically engineered food. These unexpected and unintended adverse changes in composition or character of foods, food ingredients and food additives may cause foods to be unsafe and damaging to health (Stave, 2002). Therefore, how different genetic modifications can impact on the chemical composition of these products is of great interest. In this regard, studies which assess the safety equivalence between transgenic and parental non- transgenic organisms are important, including field investigations, animal nutrition, and basic chemical composition (National Research Council 2004). Moreover, the mentioned strategies devised to study the nutritional, safety assessment, and chemical composition of the first GMOs generation

will be much more difficult to apply to the coming new generation of GMOs, in which significant changes in other constituents have been deliberately introduced (e.g., increased fatty acids or amino acid content, polyphenols, vitamins, and reduced undesirable constituents), requiring the development of more powerful and informative analytical procedures (Flachowsky *et al*., 2005; Simo *et al*., 2005).

1.15 Oil content and oil quality

Oils have important physico-chemical and nutritional properties, and these have to be brought into appropriate balance. This is not always an easy task. Nutritionists may indicate a recommended quantity and quality of fat. Seed producers, farmers and those in the agricultural and food businesses may strive to produce material to meet these targets. It remains only for the consumer to fellow the advice. This is often the major difficulty (Gunstone 2008). Analysis of oil composition is an essential step which specifies the nature of occurring constituents and their relative quantities (Karleskind 1996). The characterization of oil composition of newly produced or uncharacterized genetically modified oil is still having a great interest. Among this oil composition are fatty acids, sterols, tocopherols and phospholipids.

1.15.1 Fatty acids

The composition of oleic, linoleic and linolenic acids in oil affect the oxidative stability (Min and Boff 2001; Nawar 1996). Sunflower oil has approximately 70% linoleic acid (Meydani *et al*. 1991) and is highly susceptible to lipid oxidation (Jeleń *et al*., 2000). Heating speeds up the oxidative reactions, which is a major concern for deep fat-frying operations (Muik *et al*., 2005). Ashton *et al*., 2001 reported that high-oleic sunflower oil may decrease the risk of coronary heart disease by decreasing susceptibility of low density lipoprotein (LDL) cholesterol to oxidation.

1.15.2 Phytosterols

Phytosterols have received attention recently due to their ability to block cholesterol levels (Gylling and Miettinen 2005). In addition to their cholesterol-lowering properties, some phytosterols have also been investigated for antioxidant or anti-polymerization activity during the high temperature heating of oils (Sims *et al*., 1971; Gordon and Magos, 1983; White and Armstrong, 1986; Lampi *et al*., 1999). It has

been observed that sterols with an ethylidene group in their side-chain, such as Δ5- and Δ7-avenasterol, and citrostadienol, seem to have antioxidant or anti-polymerization activity, while others, such as sitosterol, stigmasterol, and campesterol had either no effect or a slightly prooxidant effect (Sims *et al*., 1971; Gordon and Magos 1983; White and Armstrong 1986; Lampi *et al*., 1999).

The US Food and Drug Administration recently approved a health claim for certain foods containing at least 0.65 g or 1.7 g/serving of plant steryl or plant stanyl esters, respectively (United States Food and Drug Administration 2006). Phytosteryl and phytostanyl esters have been added to vegetable oil-based spreads, margarines, and salad oils, and new products are continually being developed (Ohr 2006).

1.15.3 Tocopherols

Tocopherols are well recognized as effective antioxidants both endogenously and as additives. Jung reported that optimum concentrations of tocopherol homologues were 100, 250, and 500 ppm for α-, γ-, and δ-tocopherols, respectively (Jung and Min 1990). However, if these compounds are in too high concentrations, they can act as pro-oxidants. Satue *et al*., 1995 found that if α-tocopherol was more than 250 ppm in olive oil, it acted as a pro-oxidant as measured by peroxide values. In addition, Jung found that 500 ppm of added α-tocopherol in soybean oil was a pro-oxidant by peroxide formation (Jung and Min 1990). The difference between these two findings might be due to differences in naturally occurring antioxidants and/or in fatty acid compositions of the oils used. Other researchers have also reported that fatty acid composition may not be the only determinant of oil quality (Kamal-Eldin and Andersson 1997; Yanishlieva and Marinova 2001).

1.15.4 Phospholipids

The stability and quality of vegetable oils are influenced by the presence of minor constituents, such as phosphatides. Phospholipids may act as antioxidants, the antioxidative activity being attributed to their synergistic action, their metal scavenging activity, and their catalytic activity to decompose hydroperoxides (Pokorny *et al*., 1982; Koga and Terao 1994).

Measurement of phospholipid components also can be used to evaluate the quality of crude oil from oilseeds that have been damaged due to environmental conditions in the field and/or improper handling, storage, and transportation (Mounts and Nash 1990). The major phospholipids in sunflower seed oil are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA), with a total concentration lower than 1.2% (Padley *et al*., 1994). Most of these phospholipids are hydratable and can be removed from the crude oil by using a water-degumming process. (Holló *et al.,* 1993).

1.16 Sunflower oil

Sunflower oil is good in taste, light in appearance and supplies more vitamin E than any other vegetable oil. It is a combination of monounsaturated and polyunsaturated fats with low saturated fat levels. Sunflower oil is valued for its light taste, frying performance and health benefits. Sunflower oil with high-oleic acid content is coming more into the focus of interest since the fatty acid composition is more comparable to rapeseed and olive oil. The linoleic acid content is negatively correlated with the oleic acid content (Marquard 1977), resulting in seeds that are either rich in linoleic acid or oleic acid. In regular sunflower oil linoleic acid is the predominant fatty acid comprising about 60%, while high-oleic sorts with a high content of oleic acid (80%) and only low amounts of linoleic acid (less than 10%) (Rass *et al*., 2008) In addition sunflower oil is gaining more and more importance. Sunflower oil also contains about 8–15% saturated fatty acids, mainly palmitic and stearic acid.

Another important aspect is that the high content of oleic acid results in a high oxidative stability, making this oil interesting for a wide range of applications (Rass *et al*., 2008).

1.17 Corn oil

Corn is the second crop after soybean in global GM modified crops area (James, 2008). The first GM maize was planted in USA in 1996 and accounted for 0.3 million hectares. There are several types of maize planted by farmers around the world (Tomson 2006). Corn oil is widely used as an all-purpose cooking oil and for margarine because of its unique flavour attributes and because it is more stable to

oxidation than linolenate containing oils, such as soybean or canola (Warner and Nelsen 1996).

1.18 The future of the genetically modified oil crops

The question of vegetable oils derived from genetically modified seeds is still an issue in Europe and in some other countries. Over one half (and increasing) of soybean oil comes from genetically modified seeds in both North and South America and also increasing proportions of canola. Non-GM material must be sourced from appropriate areas and be identity preserved. The first GM oilseeds were modified to be resistance to certain herbicides and to pests leading to more efficient farming. The second generation of GM oilseeds have been modified to have a different fatty acid composition and/or enhanced levels of minor components. There may be pressure for EU to accept oil from GM plants for industrial (biodiesel) purposes (Denis and Murphy 1999).

The challenge for researchers in the coming years will be to produce oil crops with higher yields to satisfy increased demands and also to increase the spectrum of useful products, whether for edible or industrial use, that can be derived from these crops. To date, the vast majority of research and development activities have focused on improving existing crops. More recently, there has been considerable interest in using recombinant DNA technology to transfer genes from other oil producing plants (Christau and Klee 2004).

1.19 Objectives of the study

Despite MON810 being one of the major genetically modified maize crops, no studies have characterized Cry1Ab, the toxic protein which is produced in MON810. Several methods of purification of bacterial Bt toxin had been described using HPLC and GPC. The Bt is much more difficult to separate from transgenic plants because of the low concentration of toxic protein (Wu *et al*., 2001). In additional, plants contain a large number of proteins that make the separation of the toxin by traditional chromatographic methods more difficult. Therefore the purification of the target Bt protein needs a highly specific purification method.

Since safety assessment studies should be achieved on the product that the people actually consume (EPA 2003), our study focussed on the characterization of the Bt toxic protein that is purified from GM corn and characterized by specific sensitive MS chromatographic methods, which only could be achieved by using a highly specific purified toxic protein.

The characterization of the oil content is of great interest for researchers because of the nutritional and economical values of oils. The study of the effect of genetic modification on oil composition is a target of this investigation. The GM corn and genetically modified sunflower oils are among the most important genetically modified crops to be investigated compared with conventional traits.

The main purpose of this study was to develop a method of purification and characterization of Cry1Ab purified from MON810 genetically modified maize. The second object was to study the effect of the genetic modification of MON810 and high-oleic sunflower on the oil composition. Therefore, the objectives of the thesis were as follows:

(1) Quantification of Cry1Ab toxin in different corn plant parts.

(2) Development of a suitable method for purification of Cry1Ab from genetically modified corn.

(3) Establishment of characterization method of Cry1Ab by MS with regard to high recovery.

(4) Evaluation of the effect of genetic modification on the oil composition compared with the conventional traits.

To achieve these goals, two genetically modified crops were used MON810 genetically modified corn and high-oleic sunflower. Different purification and characterization methods for obtaining highly purified characterized toxin were used, as well as the oil composition (fatty acid, sterols, tocopherols and phospholipids) of genetically modified corn and sunflower were evaluated comparing with the conventional plants.

2 Materials and Methods

2.1 Materials

2.1.1 Plant Material

MON810 maize samples were kindly provided by Bayerische Landesanstalt für Landwirtschaft (LFL) Schwarzbau-Bayern, Germany. As a control maize KYS was taken from Institute of Plant Breeding, Seed Science and Population Genetics, University of Hohenheim, Germany. High-oleic sunflower (DEKALB MH4310) was kindly provided by Monsanto GmbH, Germany. Conventional (Regular) variety was purchased from Ministry of Agriculture, Agricultural Research Centre, Giza, Egypt.

2.1.2 Bacterial toxin

Cry1Ab-protoxin was kindly provided by Dr. Hang Nguyen Thu, Georg-August University, Göttingen, Germany.

2.1.3 Insecticides

XenTari 10.3% *Bacillus thuringiensis* sub sp. Aizawai was purchased from Bayer, North Chicago, USA.

2.1.4 Antibodies

Secondary antibody horseradish peroxidase was purchased from Dianova (Hamburg, Germany). The δ-endotoxin antiserum was provided by Dr. Markus Lacorn, Institute of Animal Husbandry and Animal Breeding, University of Hohenheim, Germany.

2.1.5 Chemicals

Substance	**Manufacturer**
Acetic acid	VWR International GmbH. Darmstadt
Acetone	Technical grade, distilled before use
Acetonitrile	Technical grade, distilled before use

Substance	Manufacturer
Acrylamide	VWR International GmbH. Darmstadt
Ammonium bicarbonate	Fluka, Steinheim,Germany
Ammonium hydroxide solution 28%	Fluka, Steinheim,Germany
Ammonium persulfate	Fluka, Steinheim,Germany
Ammonium sulfate	VWR International GmbH. Darmstadt
Bovine serum albumin	Sigma, Steinheim,Germany
Butanol	Technical grade, distilled before use
Citric acid	Roth, Karlsruhe, Germany
Coomassie Blue G-250	Serva, Heidelberg, Germany
3-Cyclohexylamino-1-propanesulfonic acid (CAPS)	Sigma,Steinheim, Germany
Cytochrom	Sigma, Steinheim,Germany
Diethyl ether	Technical grade, distilled before use
Dithiothreitol (DTT)	Sigma,Steinheim, Germany
Ethylenediaminetetraaceticb acid (EDTA)	VWR International GmbH. Darmstadt
Fluorescamine	Sigma, Steinheim, Germany
Glycine	Sigma, Steinheim, Germany
Magnesium chloride	VWR International GmbH. Darmstadt
Methanol	Technical grade, distilled before use
Mercaptoethanol	Sigma, Steinheim,Germany
Milk powder	Roth, Karlsruhe, Germany
n- Hexane	Technical grade, distilled before use
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Fluka, Steinheim,Germany
Ovalbumin	Sigma, Steinheim,Germany
Phosphoric acid	VWR International GmbH. Darmstadt
Premulin	Sigma, Steinheim,Germany
2-propanol	Technical grade, distilled before use
Pyridine	Sigma, Steinheim,Germany
Sodium azide	VWR International GmbH. Darmstadt
Sodium carbonate	VWR International GmbH. Darmstadt
Sodium chloride	VWR International GmbH. Darmstadt
Sodium phosphate	VWR International GmbH. Darmstadt
SDS	Roth, Karlsruhe, Germany
Tetrahydrofuran (THF)	Fluka, Steinheim, Germany
Triethylamine	VWR International GmbH. Darmstadt
Trifluoroacetic acid (TFA)99%	Sigma, Steinheim, Germany
Tris (hydroxymethyl) aminomethane hydrochloride (Tris–Cl)	Roth, Karlsruhe, Germany
Trypsine	Sigma, Steinheim, Germany
Tween® 20	Sigma, Steinheim, Germany

All other standard chemicals were purchased as analytical grade from Sigma-Aldrich, Steinheim, or otherwise mentioned. Ultra pure water was obtained by a Synergy System (Millipore, Schwalbach, Germany) was used for the preparation of solutions.

2.1.6 Consumables

Material	Manufacturer
Bottles	Schott Duran ®, Germany
Films	AGFA, Cologne, Germany
Nitrocellulose membrane	Pharmacia Biotech, Freiburg, Germany
Parafilm	American National Can, Chicago, IL 60631, USA
Pipette tips	Eppendorf, Hamburg, Germany
Tubes	
0.5, 1.5, 2.0 ml	Eppendorf, Hamburg, Germany
Filteration devices	
Steriflip_Gp filters, 0.22µm, Centrifugal	Millipore, Eschborn, Germany
filter devices with a cut off 30 and 50 kDa	Millipore, Eschborn, Germany
Sterilize filters, 0.2	Macherey-Nagel, Düren, Germany
Filter paper	Schleicher&Schuell, Dassel, Germany

Remaining expendable materials were purchased from Eppendorf and Roth.

2.1.7 Apparatus

Equipment and supplies	Manufacturer
Balances	Scaltec Instruments GmbH, Göttingen, Germany
Centrifuges	
Biofuge Primo R	Heraeus, Osterode, Germany
Biofuge Pico	Heraeus, Osterode, Germany
Electrophoresis	Owl Separation Systems, Portsmouth, NH, USA.
Capillary electrophoresis	Agilent CE system (Model HP3D CE; Agilent Technologies)

Equipment and supplies	Manufacturer
Freezer mill 6800 SPEX CertiPrep	SPEX Industries GmbH, Grasbrunn, Germany
Gas chromatography GC PE 8600	PerkinElmer, Überlingen, Germany
Gel permeation chromatography GPC	GILSON, Germany
High performance Liquid Chromatography HPLC Series HP 1100 (Hewlett-Packard, Waldbronn) with diode array detector software version Rev.A.04.02	Agilent Technologies, Germany
Heater magnetic stirrer	Heidolph, Schwabach, Germany
Horizontal shaker Model M-1000	MedTec Inc., Chapel Hill, NC, USA
Incubator	WTB Binder Labortechnik GmbH, Germany
pH Meter pH 526 with temperature sensors TFK 150 (WTW, Weilheim) and electrode CAT® Inode	Gamma analysen-Technik, Bremerhaven, Germany
Pipettes	Eppendorf, Hamburg, Germany
Soxtherm (Extraction of oil)	Gerhardt, Germany
Mass Spectrometry	
Autoflex III MALDI-TOF-TOF mass spectrometer	Bruker Daltonics, Bremen, Germany
Acquity nano-UPLC system	Waters, Milford, USA
LTQ-Orbitrap XL hybrid mass spectrometer	Thermo Fisher, Bremen, Germany
High performance thin-layer chromatography (HPTLC)	
Application Automatic TLC sampler ATS 4	CAMAG, Muttenz, Switzerland.
Automatic developing chamber	CAMAG, Muttenz, Switzerland
Scanning TLC Scanner 3	CAMAG, Muttenz, Switzerland
Dipping Chromatogram immersion device	CAMAG, Muttenz, Switzerland
DigiStore2 documentation system	CAMAG, Muttenz, Switzerland
ProteoChrom HPTLC silica gel 60 F_{245s}	Merck KGaA Darmstadt, Germany

Equipment and supplies	Manufacturer
HPTLC Software	All instruments were controlled via the software platform winCats 1.4.1 Planar Chromatography Manager (CAMAG).
Ultra filtration	
Stir unit model 8050 with ultra filtration membrane XM 50 cutoff 50kDa	Amicon, Germany

2.1.8 Kits

Kits	Manufacturer
ELISA Cry1Ab/Cry1Ac Microtiter Plate Kit	Abraxis L.L.C., Warminste, PA, USA
Protease inhibitor cocktail complete	Roche, Germany
Protein concentration, BCA protein assay kit	Pierce, Germany
Western blot detection ECLplus detection reagent	GE Healthcare Bio-Sciences, Germany

2.1.9 Buffers and solutions

Buffer	Solutions
Antibody purification	Sodium azide 0.05% **Coupling buffer pH 8.3:** 200 mM sodium carbonate, and 500 mM sodium chloride
Preparation of antigen-bound column	50mM Tris buffer pH 7.4. 100 mM glycine pH 2.5 **neutralization buffer NB**: 1ml Tris-HCl pH 8, 1.5 M NaCl, 1mM EDTA, and 0.5% sodium azide
Immuno-Affinity purification Solutions	0.5M CAPS buffer, pH10.5. 20mM sodium phosphate buffer pH 7.2.

Buffer	Solutions
	loading buffer pH 7.0: 20mM sodium phosphate, 0.5 M NaCl, and 0.5% tween20. **MacIlvaine buffer pH 3.0:** 0.1M citric acid: 0.2 M disodium hydrogen phosphate. 20 mM citric acid.
Capillary electrophoresis (CE)	1M NaOH, 125 mM borate buffer pH 9.2 containing 25 mM SDS. Carbonate/NaCl buffer pH 10.7.
Extraction buffer for Cry1Ab from corn leaves	50 mM CAPS containing 100 mM NaCl, 2 mM EDTA, and 2 mM DTT pH 10.8
ELISA	**PBS-buffer:** 0.136 M NaCl, 8.1 mM Na_2HPO_4, 2.7mM KCL and 1.5mM KH_2PO_4. **Coating Buffer:** 0.05 M $NaHCO_3$. **Wash buffer:** 10% PBS-buffer and 0.05% Tween20. **Blocking buffer:** 0.12 M NaCl, 0.02 M Na_2HPO_4, 0.01 M EDTA, 0.1 % Gelatine, 0.05 % Tween 20, 0.002 % Phenol red, 0.005 % Chlorhexidindigluconat. **Substrate buffer:** 0.1 M Na-Acetate*$3H_2O$. **Substrate solution:** 35 ml substrate buffer

Buffer	Solutions
	571 µl TMB in DMSO (9 mg/1.5 ml DMSO) 143 µl 1% H_2O_2 in water.
HPLC	**Solvent A:** 10 mM Tris PH 9.8. **Solvent B:** 10 mM Tris containing 0.18 M NaCl PH 9.8.
Gel permeation chromatography (GPC)	50 mM Tris-HCl buffer pH 8.0 containing 1 mM-EDTA and 0.1 % 2-mercaptoethanol
SDS-PAGE	**Electrode buffer pH 8.3:** 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS **Sample buffer:** 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% Mercaptoethanol, and 0.001%(w/v) bromophenol blue **Separating gel buffer:** 1.5 M Tris-HCl, pH 8.8, and 0.1% (w/v) SDS **SDS-PAGE running buffer:** 50 mM Tris-HCl, pH 8.3, 384 mM Glycine, and 0.1% (w/v) SDS **Stacking gel buffer:** 1 M Tris-HCl, pH 6.8, and 0.1% (w/v) SDS **Blue silver micellar Dye in aqueous solution:** 0.12%(w/v) Coomassie Blue G-250, 10% (w/v) ammonium sulfate, 10% (v/v) phosphoric acid, and 20%(v/v) methanol

Buffer	Solutions
Western blot	**1x transfer buffer:** 25 mM Tris, 250 mM Glycine, and 15% (v/v) methanol **TBS-T buffer pH 7.6:** 150 mM NaCl, 50 mM Tris base, and 0.05% Tween 20

2.2 Methods

2.2.1 Characterization of Cry1Ab

2.2.1.1 Quantification of Bt endotoxin in plant tissue by ELISA

Bt endotoxin concentrations were determined in MON810 maize tissues. The expression of the Cry1Ab toxin was examined in the plant tissue using the commercially available ELISA Bt Cry1Ab/Cry1Ac Microtiter Plate kit, Abraxis. This rapid and simple method is based on recognization of the toxin by specific antibodies. For ELISA screening, maize tissues (Leaf, seeds and stalks) were ground under liquid nitrogen until they were completely pulverized. 100 mg of the grounded maize tissue sample was transferred to an eppendorf tube. Each sample was extracted by 5 ml of sample extraction buffer provided in the kit by incubation 30 min. Three extracts were prepared from each sample. The extracted samples were centrifuged at 15.000g for 5 min and diluted 10 fold (if necessary, the samples could be diluted 50 fold to have a final concentration of nearly 4 ng) with the extraction buffer before applying to the ELISA plate .The diluted extractions at 100 µl per well were added to Cry1Ab/Cry1Ac antibody coated ELISA plate wells. Five concentrations (0, 0.25, 1.0, 2.0, 4.0 ng/ml) of Bt standard were used for the calibration. The plate was moved in a rapid circular motion for 30 sec to mix the vial contents. The plate was covered and incubated at room temperature for 30 min allowing the antibody to bind to the Cry1Ab toxin. After incubation, the ELISA plate was washed three times by microplate washer using 300 µl of wash buffer, then 100 µl of horse radish peroxidase-labelled goat anti-rabbit enzyme conjugate was added, mixed and further incubated for 30 min. After incubation the plate was washed by the buffer, and 100 µl of color solution was added to the wells and incubated for 30 min.

The stop solution of diluted acid was added to the wells at 50 µl per well and read at 450 nm. Three replicates were measured for each sample.

2.2.1.2 Extraction of Bt toxin from insecticides

The Bt (Cry1C) toxin was extracted from the insecticide powder (from pesticide XenTari, *Bacillus thuringiensis aizawai*) at 2 mg/ml ratio in a buffer which consisted of 50 mM CAPS with constant stirring for 30 min. 1 mg/ml trypsin was added to the supernatant. The mixture was stirred for 60 min. and centrifuged in a centrifugal filter devices with a cut off 50 kDa (Millipore, Germany), according to the procedure reported by Masson *et. al*., 1998.

2.2.1.3 Gel permeation chromatography GPC

For purification of Cry toxin from both the insecticidal toxin extract (from pesticide XenTari, *Bacillus thuringiensis aizawai*) and from MON810 maize leaf extract, the extract was loaded to 29x260 mm Sephadex G-100 gel glass column. The elution was made with 50mM Tris-HCl buffer containing 1mM EDTA and 1% mercaptoethanol, pH 8 through a UV detector at 280 nm. The column was calibrated with bovine serum albumin (66 kDa), Ovalbumin (45 kDa), and Cytochrom (12.4 kDa). The flow rate of 1 ml/min was used for the fraction, 10-40 min for the pesticidal extract and 15-40 min for leaf extract. The purified fractions were collected and stored at –20^{o}C until further use.

2.2.1.4 Cry1Ab purification and HPLC analysis

Applied to an HPLC system equipped with ProSwift WAX-IS (Dionex) DEAE weak anion exchange column, the separation of the toxin was achieved using the mobile phase of solvent A, 10 mM Tris PH 9.8 and solvent B, 10 mM Tris containing 0.18 M NaCl pH 9.8. Gradient elution was used, 0-20 min 0-45%B, 20-60 min 45-55%B and 60-90% B, Flow rate 0.5 ml/min. The detection was at 215 nm. The peaks were collected and tested with ELISA Cry1Ab/ Cry1Ac kit to recognize the desired peak of Cry1Ab.

2.2.1.5 Development of Cry1C antisera

Antisera (blood serum containing polyclonal antibodies), was prepared by using 0.5 mg of the pre- purified insecticidal Bt toxin. The antibodies developed in rabbits.

2.2.1.6 Indirect ELISA test for the activity of Cry1C antisera against activated insecticidal Cry1Ab

The assay was performed in transparent 96-well microtiter plates as follows: For coating of microtiter plates, 100 µl activated Cry1Ab insecticidal toxin was diluted with coating buffer at a concentration of 5 ng/100 µl, then added to the wells and incubated overnight at 4ºC. The solution was then removed and the plate was blocked with blocking buffer 300 µl per well for 50 min at 25ºC. After the incubation the plate was washed three times with 300 µl washing buffer.

Primary incubation was carried out with 100 µl of different dilutions of the Bt-antiserum 1:1000 to 1:582000. The antiserum was diluted in blocking buffer (test buffer) for 3 h, and then the plate was washed twice with 300 µl washing buffer.

For second incubation, secondary antibody 100 µl (HRP) diluted 1:1000 in blocking buffer was added for 1 h. The wells were washed again three times with washing buffer.

For colorimetric detection 100 µl of substrate solution was added and after incubation for 40 min, the reaction was stopped by diluted by 100 µl 1M HCL to give yellow color and the intensities were measured at 450 nm. All the incubation steps performed at 25℃.

2.2.1.7 Purification of Cry1 antibodies

The Cry1 (Cry1C) antibody was purified by preparation of Antigen (Cry1Ab) bound column, then the bound column was used for capturing the Cry1 Ig Gs from antisera. The purified antibodies were eluted, concentrated and used for the purification of Cry1Ab from the MON810 maize leaf tissue.

2.2.1.7.1 Preparation of antigen-bound column

The protoxin Cry1Ab was activated by incubation with trypsin. The trypsin: protoxin ratio was 1:50 w/w for 2 h at room temperature. 4 mg of activated Cry1Ab was dissolved in the coupling buffer and applied on 1 ml HiTrap NHS-activated column, (GE Healthcare, Germany). Coupling of Cry1Ab protein was performed at 4ºC for 4 h with calculated efficiency of approximately 90 %. After deactivation of the excess amino groups, which did not couple to the ligand, column was ready for use. Coupling of Cry1Ab to the column was done following GE Healthcare recommendations.

2.2.1.7.2 Preparation of purified anti-Cry1Ab antibody

Preparation of the sera: Sera that exhibited a strong immunogenic response were tested for the antibody IgG concentration by BCA protein assay kit (Pierce). The frozen antiserum was thawed overnight at 4ºC. Sodium azide was added at a concentration of 0.05%, and centrifuged at 15000 g for 5 min. After that the purified antiserum was filtered by Steriflip_Gp filters (0.22µm).

Removing of the anti-Cry1Ab antibodies from the serum: Serum was diluted 1:1 with 50 mM Tris buffer, pH 7.4 and recirculated in the antigen-bound HiTrap NHS-activated column several times to allow efficient binding of Cry1Ab-specific antibodies. Specific, anti-Cry1Ab antibodies were eluted using 100 mM glycine pH 2.5.The elution was performed in 1.5 ml previously prepared tubes containing 100 µl neutralization buffer (NB) per 1 ml of the collected fraction. Each 1 ml fraction was mixed immediately and placed on ice before collecting the next fraction. The column was equilibrated and ready for the next run. Finally, the total IgGs concentration was determined in the combined fraction by BCA protein assay kit as Pierce recommended.

2.2.1.8 Extraction of Cry1Ab from corn leaf tissue

Corn leafs (MON810 and regular) were ground and extracted with constant stirring in a 1:3 (w:v) ratio in a buffer which consisted of 50 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid) pH 10.8, 100 mM NaCl, 2mM EDTA (ethylenediaminetetra-acetic acid), 2mM DTT (dithiothreitol), 20 µl/ml Protease inhibitor cocktail (complete,

Roche, 1tablet/ml). The mixture was incubated for 30 min on ice and filtered through two layers of cheese cloth and then centrifuged for 10 min at 15.000g at 5℃. The concentration of the protein was determined according BCA protein assay kit.

2.2.1.9 Immuno-Affinity purification of Cry1Ab from corn extract

The purification of Cry1Ab from MON810 corn leaf extract was carried out according to the method of Gao *et al.* (2006). The purification was performed by coupling of highly purified antibodies with CNBr 4 fast flow activated sepharose media (GE Healthcare, Germany). The antibody density was 8 mg/ml of the media, which backed in disposable syringe like plastic column. The lyophilized GM corn extract (2.5% of the column volume) was dissolved in 0.5 M CAPS buffer pH 10.5 and then centrifuged 5 min at 13.000 g. The pH of the supernatant was adjusted to 7.0 with 20 mM citric acid and mixed with 20 mM sodium phosphate buffer pH 7.2 in a 1:1(v/v). The column was equilibrated by three column volume of loading buffer pH 7.0. After that the sample was loaded and recirculated in the Vislprep (Supelco, North Harrison road, USA) system for three times, the column was washed by three column volume of loading buffer and the elution was done with three column volume MacIIvaine buffer pH 3.0. The fractions were collected in 1.5 ml tubes containing neutralization buffer as 100µl/1ml of the fraction. Finally, the fractions were concentrated and desalted using centrifugal filter devices with a cut off 30 kDa and use SDS-PAGE and western blot analysis.

2.2.1.10 Gel preparation

Gels were sandwiched between 1.0-mm-thick glass plates approximately 10 cm wide, 10 cm long and formed with thin (0.5 mm) spacers to facilitate cooling. Combs with eight 8-mm-wide teeth (2 mm between teeth) are typically used to form loading wells. These items can be prepared by a, or similar sized plates, spacers, and combs purchased from gel electrophoresis manufacturers. Gels should be cooled to approximately 12℃ during electrophoresis. Table 2 shows the components and volumes for preparing cast and stacking gels.

Table 2: Volume (ml) of components required to cast gel and stacking gel

Components	Cast gel	Sticking gel
	10%	5%
H2O	5.9	3.4
30% acrylamide mix	5.0	0.83
1.5 M Tris (pH8.8)	3.8	-
1 M Tris (pH6.8)	-	0.63
10%SDS	0.15	0.05
10% ammonium persulfate	0.15	0.05
TEMED	0.006	0.005
Total volume	15	5.0

2.2.1.10.1 SDS-PAGE, and in-gel digestion of proteins

SDS-PAGE was performed on 10% polyacrylamide gels using the system of Laemmli (1970) and protein bands were visualized by colloidal Coomassie blue (Candiano *et al.,* 2004). Proteins were in-gel-digested using trypsin (Roche, Penzberg, Germany) according to (Shevchenko *et al*., 1996). After the tryptic digestion the supernatant was removed and transferred to a new tube. The gel pieces were extracted with 50% acetonitrile (ACN)/50% 0.1% formic acid (FA) (v/v) for 15 min. After incubation, the supernatant was collected and the gel pieces were covered with 5% FA for 15 min before the same volume of ACN was added. After incubation for 10 min the supernatant was collected. The pooled supernatants were then lyophilized in a vacuum centrifuge and stored at -20ºC.

2.2.1.10.2 Preparation of blue silver micellar solution

The SDS-PAGE gel staining was achieved according to the method of Candiano *et al.* (2004). The final concentrations adopted in the working colloidal “blue silver“ solution were: 0.12% dye, 10% ammonium sulfate, 10% phosphoric acid, and 20% methanol. This produced a dark green dye solution, which turned to a deep blue when adsorbed onto the polypeptide chains fixed in the polyacrylamide gel, or blotted onto membranes. The dye solution was prepared as follows, by sequentially adding the various ingredients as here indicated: to a water solution (1/10 of the final volume) the desired amount of phosphoric acid was added, so that, in the final volume, its concentration would be 10%; to this, the required amount of ammonium sulfate (in powder) was added, calculated to obtain a final concentration of 10%. When the ammonium sulfate has dissolved, enough Coomassie Blue G-250 (in

powder) added to obtain a final concentration of 0.12%. When all solids have dissolved, the water added to 80% of the final volume. To this solution, under stirring, anhydrous methanol had been added to reach a 20% final concentration. This stock dye solution should be kept in a brown bottle and was stable at room temperature for 6 months.

2.2.1.11 Western blot analysis

2.2.1.11.1 Semi dry transfer of proteins onto an immobilon-p PVDF membrane

For the specific detection of particular proteins, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using Western Blot technique. At least twenty minutes prior to blotting, six pieces of electrode paper (Whatman, 3 mm), same size as the PAGE gel, were incubated in 1x transfer buffer for 10 min, the PVDF membrane was wetted in methanol for 15 seconds and then transferred to a container filled with ddH_2O for 5 min. The membrane was then soaked in 1x transfer buffer for 10 min and a sandwich of buffer papers and gel nitrocellulose membrane was made as shown in Figure 2, Transfer was performed at 8 Ampere per blot for 30 min.

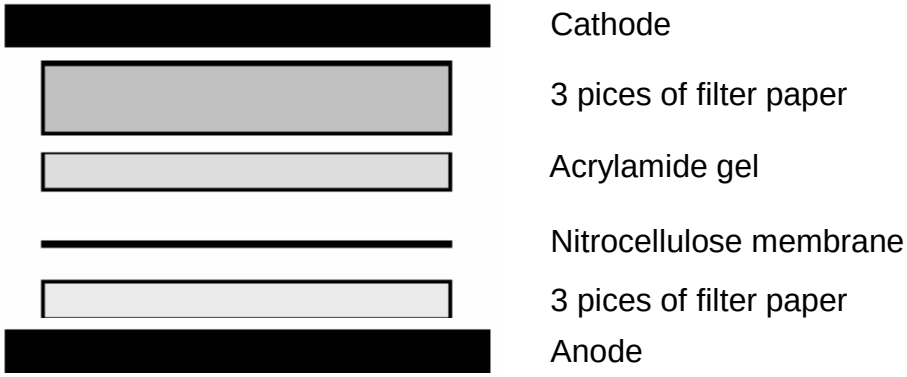

Figure 2: Schematic assembly of Western blot apparatus.

2.2.1.11.2 Immuno-detection of the proteins with specific antibodies

The efficiency of blotting was determined by visualization of the protein by 0.2% Ponceau-S prestaining solution on the nitrocellulose membrane. The membrane was washed three times for 5 minutes in 1xTBS-T buffer. The non-specific protein-binding capacity of the membrane was blocked by incubation with non-fat dried milk (5% w/v in 1x PBS-T) for 2 h with continuous shaking (100 rpm) at room temperature. The

blocked membrane was washed three times with 1x TBS-T buffer for 5 min. The membrane was incubated with the primary antibody (diluted 1:1000 a solution of 5% w/v non-fat dried milk dissolved in1x TBS-T) over-night at 4℃ under continuous gentle agitation. The membrane was washed three times with 1x TBS-T buffer for 5 min. The membrane was incubated for 2 h with the secondary antibody horseradish peroxidas (Dianova, Hamburg, Germany), diluted 1:5000 in a solution of 5% w/v non-fat dried milk dissolved in1x TBS-T. The membrane was washed three times with 1 x TBS-T for 5 min. Western blotting was developed by using ECLplus detection reagent, the western blot image developed in (Fuji film).

2.2.1.12 Characterization of Cry1Ab by mass spectrometry

Lyophilized peptides from tryptic digests were dissolved in 20 µl of 0.1% TFA and desalted by reversed phase chromatography on µC18 ZipTips (Millipore, Schwalbach, Germany) following the manufacturer's protocols. Peptides were eluted directly onto a stainless steel target using 1 µl of a CHCA matrix solution (5 mg/ml in 50% ACN/ 50% 0.1% TFA, v/v). Identification of the purified Cry1Ab protein by peptide mass fingerprint was performed on an Autoflex III MALDI-TOF-TOF mass spectrometer. Peptide mass fingerprint data were recorded in positive ion reflector mode using an accelerating voltage of 21 kV and 2000 laser shots per sample to ensure good S/N quality. Flex Analysis 3.0 and Bio-Tools 3.0 software (Bruker Daltonics, Bremen, Germany) were used for data processing.

Nano-LC-ESI-MS/MS experiments were performed on an Acquity nano-UPLC system (Waters, Milford, USA) directly coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher, Bremen, Germany). Tryptic digests of Cry1Ab were concentrated and desalted on a precolumn (2 cm x 180 µm, Symmetry C_{18}, 5 µm particle size, Waters, Milford, USA) and separated on a 20 cm x 75 µm BEH 130 C_{18} reversed phase column (1.7 µm particle size, Waters, Milford, USA). Gradient elution was performed from 1% ACN to 50% ACN in 0.1% FA within 1 hr. The LTQ-Orbitrap was operated under the control of XCalibur 2.0.7 software. Survey spectra (m/z = 250-1800) were detected in the Orbitrap at a resolution of 60.000 at m/z = 400. Data dependent tandem mass spectra were generated for the five most abundant peptide precursors in the linear ion trap. For all measurements using the Orbitrap Detector internal calibration was performed using lock-mass ions from ambient air as described in Olsen *et al*. (2005).

Mascot^{TM} 2.2 software (Matrix Science, London, UK) was used for protein identification. Spectra were searched against the NCBI protein sequence database ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz. Search parameters specified trypsin as cleaving enzyme allowing two missed cleavages, a 3 ppm mass tolerance for peptide precursors and a 0.6 Da tolerance for fragment ions. Carbamidomethylation of cysteine residues was set as fixed modification and S,T,Y phosphorylation, methionine oxidation and N-terminal acetylation of proteins were allowed as variable modifications.

2.2.1.13 Capillary zone electrophoresis (CZC)

Capillary electrophoresis has a very good sensitivity based on mass detection. This application is important when the sample size is very limited, thus a minute amount of sample is sufficient for analysis (Flachowsky *et al*., 2005).

Capillary zone electrophoresis was performed on Agilent CE system (Model HP3D CE; Agilent Technologies). Fused silica capillary, 57 cm long with effective length 47 cm, was conditioned for use by flushing the capillary with 1M NaOH, rinsing with de-ionized water followed by conditioning with buffer to equilibrate the capillary. The separation was carried out by 125 mM borate buffer pH 9.2 containing 25mM SDS. Purified desalted Cry1Ab was diluted to the concentrations of 0.3 mg/ml, 0.15 mg/ml, 0.07mg/ml and 0.035mg/ml in Carbonate/NaCl buffer, pH 10.7, at 25℃ and voltage of 20 kV (Simo *et al*., 2005).

2.2.1.14 HPTLC for Cry1Ab peptide identification

The HPTLC plates ProteoChrom HPTLC silica gel 60 F_{245s} (Merck KGaA Darmstadt, Germany) were washed (pre-chromatography) with methanol and dried for 15 min at 120^{o}C. Samples were applied with the Automatic TLC Sampler. The following settings were used: band length 6 mm, distance from left plate edge 10 mm and from the lower plate edge 10 mm, from each edge in the left corner of the HPTLC sheet, dosage velocity 120 nl/s, as sample application volumes 50 µL of Cry1Ab digest 0.3 µg /µl dissolved in 25 mM ammonium bicarbonate buffer. This gave a total of 15 µg protein per band. The samples plates were developed in automatic developing chamber 20 cm × 10 cm, up to a migration distance of 60 mm (from the lower plate edge) using a mixture of 2-butanol/pyridine/ammonia/water (39:34:10:26) as mobile

phase. The plate was dried for 10 min at room temperature. The detection of the peptides was carried out by two solutions: Solution A; 0.02%fluorscamin in acetone and Solution B; 10% triethylamine in acetone. The plates were dipped using chromatogram immersion device, in solution A for 1 s. and dried at room temperature for 10 min, and solution B for 1 sec and dried at room temperature for 10 min before UV detection at 366 nm by scanner. Wavelength detection was performed by TLC Scanner with a slit dimension of 4 mm × 0.2 mm and a scanning speed of 100 mm/s.

All instruments were controlled via the software platform winCats 1.4.1 Planar Chromatography Manager.

2.2.2 Oil analysis

2.2.2.1 Extraction of the seed oil

The seeds were finely ground, by freezer mill, the seed powder was extracted by *n*-Hexan by means of soxtherm solvent extractor equipped with 6 soxhelt posts. After the extraction process the extract was evaporated on a rotary evaporator at 40ºC. The residual lipids were weighed to determine the total yield and stored at 4ºC before further analysis.

2.2.2.2 GLC/FID analysis of fatty acids

Fatty acids were transesterified into, fatty acids methyl esters (FAME) by heating in boron trifluoride according to the procedure reported by Metcalfe *et al*., 1966. FAME was identified using flame ionization detector (FID). The flow rate of the carrier gas hydrogen was 45 ml/min. A sample of 1µL was injected on a 25 m x 0.53 mm x 0.5 µm film onto a J&W's DB 23-Megabore-capillary column (50% Cynopropyl, 50% Methylsilicon). The injector and FID temperature was set at 250ºC. The initial column temperature was 100ºC (2 min) programmed by15ºC/min until 180ºC and by7 ºC/min until 220 and then kept 8 min at 220ºC. A comparison between the retention times of the samples with those of authentic standards (C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1 and C22:0 methyl esters) purchased from Sigma (St. Louis, MO, USA), was made to facilitate identification.

2.2.2.3 GLC/FID analysis of phytosterols

2.2.2.3.1 The saponification process

Characterization of sterols was performed after saponification of the total lipids. The separation of sterols was done according to the method described by the Official Journal of European Communities (European union commission, 1991). The samples (250 mg) were saponified by refluxing with 5 ml ethanolic solution of 2 M potassium hydroxide solution for 60 min with bumbing granules. After cooling to room temperature, 100 ml water was added. The unsaponifiable matter was separated in separation funnel, and the aqueous phase was washed three times with10 ml diethyl ether. The diethyl ether fraction was collected, washed with water, dried with anhydrous sodium sulphate and filtered. The extract was evaporated in rotary evaporator at 25 ºC under reduced pressure, and the residual ether was evaporated under nitrogen. The sterols were analyzed as described below.

2.2.2.3.2 GLC/ FID analysis

Analyses were carried out using an Auto system XL, equipped with FID. The following parameters were used; GLC column: DB 5, packed with 5% phenylmethylpolysiloxan (J&W scientific, Falsom, CA, USA), 60 m length, 0.25 mm internal diameter, 1.0 µm film thickness; carrier-gas (hydrogen) flow rate 45 mL/min. Detector and injector were maintained at 270ºC. The oven temperature was kept constant at 300ºC and injection volume was 1 µL. Phytosterols were identified by comparison of their retention times (relative to 5 α-cholestane) with retention times of commercially available standards, stigmasterol (95%), β-sitosterol (95%), campesterol (98%) and sitostanol (96.7%) purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phytosterols without commercially available standard such as Δ5-Avenasterol and Δ7-Avenasterol were identified by their relative retention time as available in the literature (Winkler *et al.,* 2007) and calculated according to the method of Brufau *et al.* (2006).

2.2.2.4 HPLC analysis of tocopherols

Normal-phase liquid chromatography analysis of tocopherols was performed with a HP 1100 HPLC equipped with diode array detector. The column was stainless steel

with dimension of 250x3 mm, packed with silica 5 µm (YMC Europe GmbH, Germany). The solvent flow rate was maintained at 1 ml/min. The solvent system was hexane/THF/acetic acid (97/3/0.25), column temperature was 30ºC, and the detection wavelength was 295 nm. The seed oil was diluted 5-100 mg/ml in the selected mobile phase and 50 µl directly injected onto the HPLC column. In the case of highly concentrated or cloudy solution, it was filtered (25 mm syringe filter, 0.45 µm membrane Macherey-Nagel, Düren, Germany). Each sample was prepared three times and analysed twice.Tocopherols were separated isocratically within 20 min.

2.2.2.4.1 Preparation of standard curves

Standard solutions were prepared by serial dilution of approximately 0.1- 2 mg/l of tocopherols, 20 µl were injected onto the HPLC column, and peak areas were determined to generate standard curve data. Slope of standard curves (9 concentrations levels) was obtained by linear regression.

2.2.2.5 Solid phase extraction of phospholipids

Solid phase extraction was used for the fractionation of lipids. The procedure of Kaluzny *et al.* (1985) was modified to separate the neutral lipids (NL), fatty acids and phospholipids. Discovery DSC-NH_2 (Supelco, North Harrison road, USA), 6 mL tubes, 1 gram were used, instead of Bond Elut aminopropyl column (500mg), The oil extract 200- 500 mg was dissolved in chloroform to minimize volume. The column was placed on Vislprep (Supelco, North Harrison road, USA) and pre-washed by two volumes of 4 ml of hexane. The vacuum applied at 7.5 mm Hg after the column activation. The lipid classes were recovered by sequential elution as followes: 8 ml of chloroform:2-propanol 2:1 (w:w) was used for elution of neutral lipids, 8 ml of solution of 2% acetic acid in diethyl ether was used for elution of fatty acids, while phospholipids were eluted by 8 ml ethanol. Samples fractions were dried under nitrogen and stored at 4ºC for subsequent analysis.

2.2.2.6 HPTLC

For calibration, phospholipids standards, L-α phosphatidylcholine (PC) L-α phosphatidylethanolamine (PE), 3-sn-lysophosphatidylethanolamine (LPE), phosphatidic acid sodium salt (PA) (Sigma, Germany), and phosphatidylinositol sodium salt (PI) (Fluka, Germany) were dissolved in chloroform-methanol 2:1.

Standard phospholipids were oversprayed in five concentrations 0.03 to 4.8 µg/band range (Table 3). Samples fractions (regular maize oil, MON810 maize oil, high-oleic sunflower oil and regular sunflower oil) extracted by solid phase extraction were dissolved in 10 ml chloroform-methanol 2:1, 20 µl were applied of each sample.

HPTLC glass plates were washed (pre-chromatography) with methanol and dried for 15 min at 120℃. Standards and samples were applied with the automatic TLC Sampler 4 using the following settings: band length 6 mm, distance from left plate edge 10 mm and from the lower plate edge 10 mm, dosage velocity 120 nl/s.

Plates were developed in the Automatic Developing Chamber 2 (ADC2) with a mobile phase consisting of chloroform/methanol/2-propanol/triethylamine/0.25% KCl (30:9:25:18:6) to a migration distance of 60 mm (from the lower plate edge). Plate activity was adjusted to 33% relative humidity by saturated aqueous magnesium chloride ($MgCl_2$ x 6 H_2O). The plate was dried for 10 min at ambient temperature. The plate was dipped in premulin (100mg in 200 ml acetone/H_2O 1:4) for 1 sec using the TLC Immersion Device III at dipping speed of 1 cm/s, following by drying for 5 min. Wavelength detection was performed by TLC Scanner 3 at 366 nm with a slit dimension of 4 mm × 0.45 mm and a scanning speed of 10 mm/s via peak area. The data obtained were processed with winCATS software, version 1.3.2 (CAMAG).

Table 3: Standard phospholipids amount applied for determination of phospholipids by HPTLC

Phospholipids Standard	**Amount µg/spot**				
PC	0.15	0.30	0.60	1.20	2.40
LPE	0.03	0.06	0.12	0.24	0.48
PA	0.05	0.10	0.20	0.40	0.80
PI	0.05	0.10	0.20	0.40	0.80
PE	0.30	0.60	1.20	2.40	4.80

2.2.2.7 Statistical analysis

All experiments were performed in replicates. Data were estimated as the percentage as well as mean and its standard deviation of the different traits. The calculations were done using Microsoft Excel 2000 Program.

3. Results and Discussion

3.1 Characterization of Cry1Ab endotoxin expressed in transgenic corn plants MON810

Characterization of the newly introduced protein(s) in genetically modified plants is a critical component for the food, feed, and environmental safety assessment. Large quantities (tens to hundreds of grams) of the transgenic proteins are required to perform toxicological and other safety oriented studies (Gao *et al*., 2004). Ideally, the protein to be used in safety tests would be directly purified from the transgenic plants. However, in many cases, it is technically impossible to obtain sufficient quantities of the subject protein in high purity from the transgenic plants due to the low expression levels. The National Academy of Science, the Scientific Advisory Panel (SAP) Bt plant-Pesticides and other experts recommend use of plant product protein that people actual consume rather than bacterial product for safety testing, because bacterial and plant product protein can have significant differences (Freese 2001). Many Cry proteins were previously purified, and characterized. The SDS-PAGE and western blot analysis were the most used methods for characterization of Bt toxins like Cry1Ab (Miranda *et al.,* 2001, Díaz-Mendoza *et al*., 2007). Increasingly, Mass spectrometry is being used as a rapid and sensitive method for screening and identification of Cry proteins. Peptide mass fingerprint (PMF) and mass sequencing were employed for characterization of Cry proteins such as Cry1F, Cry34Ab1, and Cry35Ab1 (Gao *et al.,* 2004, Gao *et al.,* 2006) and Cry1D, Cry1H, Cry9A and Cry9B (Ranasinghe and Akhurst 2002). Although MON810 maize with the trade name YieldGard produced by Monsanto, is one of the major GM maize crops, the properties of truncated Cry1Ab expressed in MON810 remain uncharacterized (Freese, 2004).

In our knowledge, this investigation produced the first PMF and mass sequencing study of Cry1Ab characterize the Cry1Ab expressed in transgenic maize MON810.

In this investigation, the Cry1Ab expressed in transgenic corn plants was characterized by the Cry1Ab ELISA, Molecular mass by SDS-PAGE and western blot analysis, HPLC, peptide mass fingerprinting and peptide sequencing by matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF) and liquid

chromatography-electrospray-ion trap mass spectrometry (LC-MS/MS). Additionally the Cry1Ab was examined for glycosilation. For the successful characterization of Cry1Ab, high efficiency purification methods have been used. The specifically purified antibody against recombinant Cry1A, purified by antigen-bound column, proofed their usefulness in the purification process of Cry1Ab from leaf tissue extract of MON810. The Cry1Ab characterization data are presented in this part.

3.1.1 Quantification of Cry1Ab in MON810 by ELISA

ELISA technique is widely used for screening of Cry1Ab/Cry1Ac protein, to determine the express levels of Cry protein (Palm *et al.,* 1994, Zwahlen *et al.,* 2003). Since fresh weight measurements depends on the water concentration of the plants, local, seasonal concentration, and on irrigation of fields, it becomes difficult to compare the data from different studies. So in our study we determined the Bt concentration for the dry weight (dw) (Table 4).

Table 4: Concentration of Cry1Ab protein in tissues of transgenic MON810 Bt maize (dw)

Plant tissue	**Cry1Ab Concentration µg/g dw**
Leaf	26.82 ± 0.28
Stalk	1.52 ± 0.26
Grain	1.02 ± 0.09

Cry1Ab protein concentrations were determined by quantitative enzyme-linked immunosorbent assay (ELISA). Values are means of three sub samples from a homogenized powder of ten leaves each from a different plant. As well as three sub samples from ten grams of homogenized powder of grains and Stalks, each gram from a different plant.

The commercial ELISA kit was recommended to detect and quantify the Bt in corn and cotton leaf tissue, single seed and bulk grain. The concentration in general of corn plant was 26.82 µg/g dw for leaves, and 1.52, and 1.02 µg/g for stalks and grains respectively (Table 4). The Bt content in dry weight of leaf tissue in our samples was on average about 2.7 to 5.5 times higher than that in fresh weight determined by Monsanto product safety description (2002), and for dried grains was higher on average about 1.1-3.4 times.

To compare the results from different studies, the authors related the Bt concentration of fresh weight to the dry weight of the samples. The Bt concentration

for dry weight was only given in the study of Lorch and Then (2007). The Bt content in leaves of in our study was about six times higher than in the authors study. Additionally, there are no available data for the dry weight content of Bt in stalk and grain plant parts that we could compare with our results.

The considerable variability and differences of up to hundred-fold in Bt concentration (0.1 and 10µg Bt/g fw) challenge the significance of trends and average Bt levels published in different studies (Lorch and Then, 2007).

ELISA is a method widely used for screening purposes with a high throughput capacity that allows rapid, preliminary testing and is easy to handle. However, it is not a suitable method for drawing definitive conclusions, as it does not provide information on the chemical structure. To comply with legal requirements according to the Commission of the European Communities (93/256/EEC), an identifying analytical procedure, which consists of a suitable combination of cleanup, chromatographic separation, and spectrometric or immunochemical detection is recommended.

3.1.2 Extraction and purification of Cry1C from Bt pesticide

The results from SDS-PAGE indicate that the purity of protein obtained by gel permeation chromatography of Cry1C pesticide was high. There was only one protein band visible (Figure 3) indicating a purity of Cry1C greater than 90% and this was considered sufficient to be used directly as an immunogen for producing antibodies.

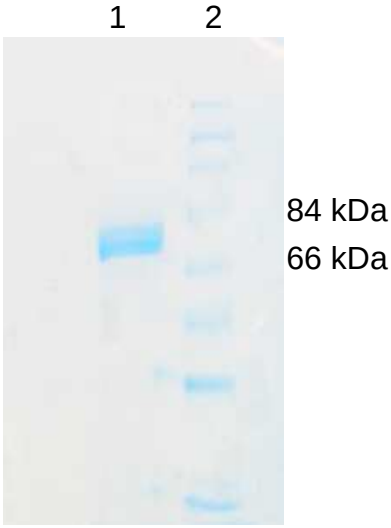

Figure 3: SDS-PAGE for partially purified pesticide by GPC.
Lane 1: partially purified Cry1C from pesticide extract Lane 2: protein molecular weight marker.

3.1.3 Preparation of antisera

Antibodies from rabbits immunized by Cry1C protein were applied for ELISA test for studying the cross reactivity of Cry1C antibodies against Cry1Ab protein.

3.1.4 Indirect ELISA test for the cross reactivity of Bt Cry1C antisera against activated insecticidal Cry1Ab

To evaluate the reactivity of Cry1C antisera against Cry1Ab endotoxin, indirect ELISA was employed. The purpose of studying the reactivity of Cry1C antibodies against Cry1Ab was for evaluating the suitability of Cry1C antibodies for isolation of Cry1Ab protein from transgenic plants.

Though many ELISAs described in the literature have been developed for the determination of Bt Cry toxins, the proteins of Bt Cry1A or Bt Cry1Ab toxins were obtained from bacterial Bt HD-1. (Tapp and Stotzky1995; Sims and Berberich 1996; Takahashi *et al.,* 1998; Hori *et al*., 2000) However, there are no reports on the usage of protein of Bt Cry1C which was isolated from pesticides for detection of Cry1Ab.

Cry1Ab Insecticidal toxin was diluted in coating buffer to the concentrations of 5 ng/100 µl, while Bt-antisera was diluted to the ratio of 1:5000 to 1:3200000. The antisera showed a high reactivity against Cry1Ab up to a concentration of 1:80000.

Wang *et al.,* (2007) studied the cross reactivity of Cry1Ac antisera with five different Cry proteins (Cry1C, Cry2A, Cry3A, Cry3Bb1, Cry9C) by sandwich ELISA. They found that there was no cross reactivity for the tested protein except the cross reactivity of Cry3A.The cry genes are classified cry1 to cryV1 based on amino acid sequence homology and insecticidal activity (Hoefte and Whiteley 1989; Feitelson *et al*., 1992) The cross reactivity of different kinds of Cry proteins was not unexpected, since the amino acid sequence in the same class are resembled.

3.1.5 Separation and purification of Cry1Ab from transgenic maize by liquid chromatography

Several methods of purifying Bt toxin from extracts of various strains of Bt or Bacillus *thuringiensis* genetically engineered bacteria by HPLC have been reported (Yamamoto 1983, Masson *et al*., 1998). However, compared with Bt strains or Bt engineering bacteria, the Bt transgenic plants have a very low concentration of toxic proteins in their tissues. For this reason, it is much more difficult to separate the Bt toxic proteins from Bt transgenic plants than from Bt strains and engineered bacteria (Wu *et al.*, 2001).

In the present investigation, a high amount of corn leaf extract was used. Size exclusion chromatography was employed for partial purification of Cry1Ab from transgenic maize extract. The column was calibrated with bovine serum albumin (66kDa), ovalbumin (45kDa), and cytochrom (12.4kDa). The peaks were eluted at 24, 33 and 40 ml elution volume. The appearent molecular masses of the fractions were determined by comparing their elution volumes to those of known molecular masses. Since the SDS-PAGE and western blot analysis refered to MW 65-70 kDa Cry1Ab in corn leaf extract, the eluted fraction was collected 25-45 ml. The concentrated fraction was applied to HPLC.

Wu *et al.,* 2001 developed and evaluated a separation method for Bt toxic protein from Bt transgenic rice containing Cry1Ab. The separation achieved by HPLC, employed two separation steps, anion exchange chromatography and cation exchange chromatography for separation of Cry1Ab. The isolated peaks tested for production of Cry1Ab by engaging ELISA. The previous investigation did not use further test for the positive produced peak by another chromatographic analysis such as MS methods to characterize and test the Cry1Ab purity.

Separation by HPLC was achieved by DEAE weak anion exchange column HPLC under operating conditions described in the methods, gave a chromatogram as seen in Figure 4. To identify peaks, ELISA was engaged. The collected fractions of candidate peaks were checked by Cry1Ab/Cry1Ac plate kit.

The ELISA results showed that fractions of peak 5 contained Cry1Ab toxin. Chromatographic separations were employed for preparation and purification of Bt toxin from Bt transgenic plant two. Firstly, using the size exclusion chromatography (SEC) was used for partial purification of the fraction, secondly weak anion exchange chromatography was used to obtain more purified fraction. However, the separation was poor, that could be due to the fraction containing large number of maize proteins, additionally, the peak five was isolated, concentrated, and after trypsin digestion, applied to the MALDI-TOF to examine the purity of Cry1Ab.

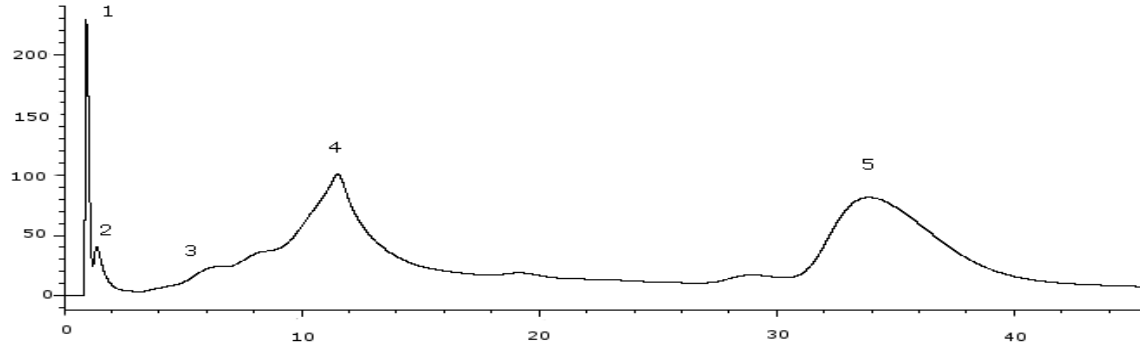

Figure 4: Chromatogram of anion-exchange separation of Cry1Ab toxic protein from transgenic maize.

3.1.6 Tryptic mass fingerprint of Cry1Ab separated by HPLC

Peptide mapping by MALDI-TOF following trypsin digestion is a powerful tool for protein identification. The Cry1Ab peak isolated by HPLC was concentrated and subjected to heat denaturation and digested by trypsin. The resulting peptide mixture was anayzed by MALDI-TOF and LC-MS/MS. The masses of detectable peptides were compared to those deduced from databases.

The result showed that, in the digest of HPLC peak there was a mixture of two proteins, β-D-glucosidase (*Zea mays*) and of Cry1Ab. Figure 5 shows the LC-MS/MS spectrum of β-D-glucosidase (*Zea mays*) precursur from transgenic MON810 maize, 22 β-D glucosidase peptides were identified and 13 peptide of Cry1Ab. The major component of the mixture was β-D-glucosidase, which may be due to inefficient purification and separation of Cry1Ab by GPC and HPLC.

The molecular mass of β-D-glucosidase was previously estimated to be 60 kDa by

SDS-PAGE (Esen and Cokmus1990). The PI of the Enzyme was 6.4 (Cuevas *et al*. 1992), while the active Cry1Ab mass was 60-70 kDa and the isoelectric point was 6.5 (Lung *et al.,* 2004).

In anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support. The binding on anion-exchangers is achieved mostly through interaction with functional groups on the surface of the beads. However, the glucosidase enzyme and Cry1Ab had isoelectric point (pI) of 6.4, and 6.5 respectively, therefore, when run at pH 9.2 the overall charges on the protein and the enzyme being negative, it was highly problematic to be separated by ion exchange. In fact, the pore separation of two substances by DEAE column could be related to the relative net charge in the enzyme and the protein.

In order to separate and characterize Cry1Ab from MON810 leaf extract, a high efficiency purification method should be followed. In our investigation we followed a specific immuno-affinity purification to get a highly purified Cry1Ab.

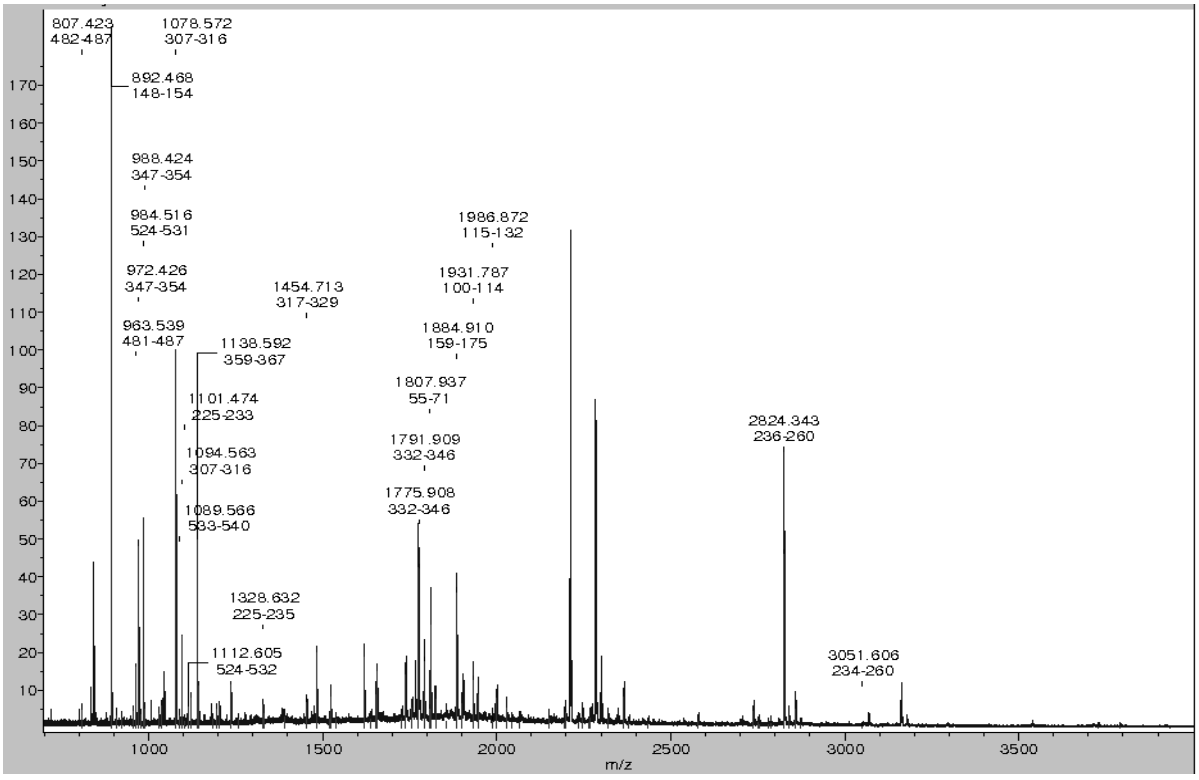

Figure 5: MS/MS Spectrum of β-D-glucosidase precursur from transgenic MON810 maize.

3.1.7 Immuno-Affinity purification of the protein Cry1Ab from plants

For the successful characterization of Cry1Ab, we followed a high efficiency purification method. The specifically purified antibodies against recombinant Cry1A proofed their usefulness in the purification process of Cry1Ab from leaf tissue extract of MON810. The eluted fraction (1ml) was collected from elution effluent and concentrated to 200 µl. The SDS-PAGE examination of the fraction showed that the fraction had a major band at approximately of 69 kDa while the protein profile of Cry1Ab crude leaf extract of transgenic and non transgenic were indistinguishable on SDS-PAGE. Further characterization by western blot confirmed that the Cry1Ab protein band was immunoreactive to the specific antibody against Cry1Ab, since the truncated core was detected in transgenic plant extract as well as in the purified Cry1Ab (Figure 6).

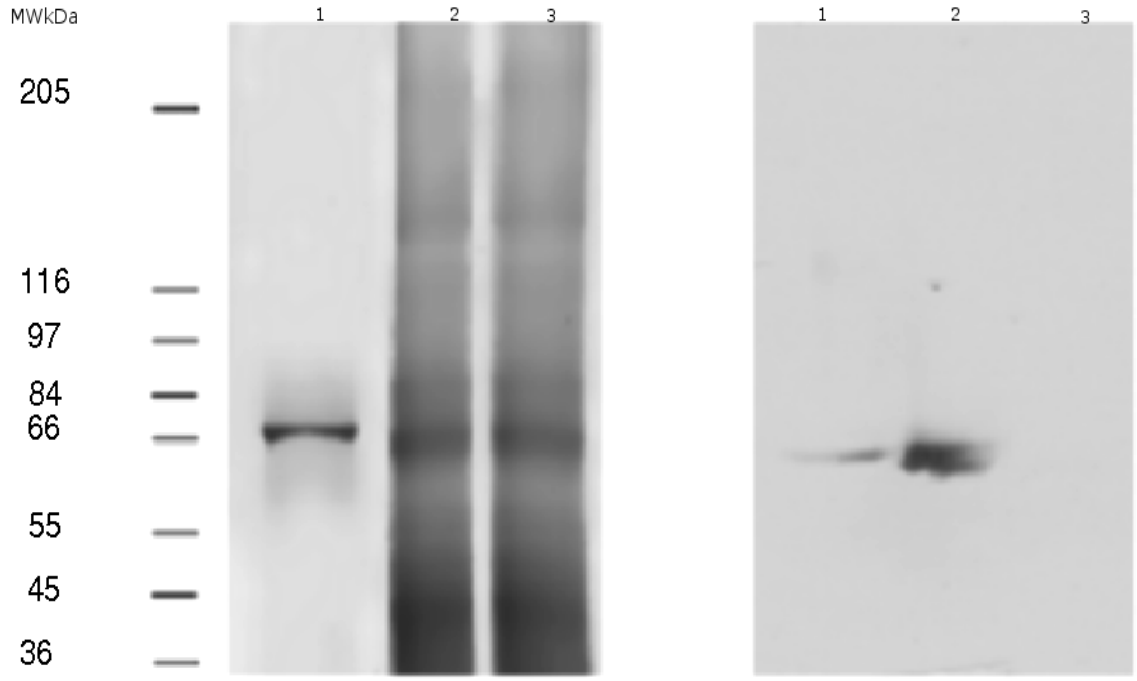

Figure 6: SDS-PAGE and Western blot of Cry1Ab protein.
Lane 1: Cry1Ab purified from transgenic maize leaf extract MON810. Lane 2: Transgenic Maize leaf extract. Lane 3: Non transgenic maize leaf extract.

3.1.8 Truncation of Cry1Ab

The expected molecular weight of Cry1Ab is 92 kDa, since MON810 contains a truncated Cry1Ab coding sequence, that introduce the N-terminal fragment of the full length Cry1Ab protein (130 kDa) of *B. thuringiensis* ssp. *kurstaki* strain HD1. Western blot analysis showed that the Cry1Ab which produced by MON810 had a molecular

weight of 69 kDa (Figure7, lane2). This Cry1Ab protein band was cut from SDS gel, digested with trypsin, and purified by zip-Tip column. Because of the limitation of SDS-PAGE in determining accurate MW of proteins, the purified Cry1Ab was digested and subjected to MALDI-TOF to measure the MW of the MON810 Cry1Ab band. On the basis of expected amino acid sequence of Cry1Ab, the MW was calculated to 68.9 kDa. These results refers that the Cry1Ab (92 kDa) is subjected to truncation by the plant cell proteases. Indeed, the truncated core toxin was detected in maize concentrated extract by western blot analysis which had the same MW of Cry1Ab of purified Cry1Ab protein. These results suggest that on exposing of Cry1Ab (92 kDa) to plant proteases, truncation occurs. It had been well documented that Cry1 toxins, such as Cry1Ab and Cry1Ac, are synthesisted as inactive toxins 130 kDa (Schnepf *et al*., 1998). The crystal protoxin is activated by proteases in the susceptible insect gut through removal of N-terminal peptide consisting of 25-30 amino acids. The removal involves domain I α1 of N-terminus, and cleavage of approximately half of the sequence from C-terminus (Gill and Cowles 1992, Schnepf *et al*., 1998, Bravo *et al*. 2002). The role of the C-terminus to the active toxin is believed to be in the crystalline inclusion bodies within Bt bacterium (Park *et al*., 2000). Bravo *et al*., (2002) reported that the removal of the N-terminal peptide is essential before the Cry1Ac toxin becomes fully active. Miranda *et al*. (2001) previously reported that the activation of Cry1A is commonly achieved by commercial enzymes such as trypsin, usually at low concentration or by cleavage by proteases in the susceptible insect guts producing 60-70 kDa toxin fragment. The removal of N-terminal and C-terminal peptides is the result of enzymatic cleavage by proteases in the susceptible insect guts. The major proteases in the insect midgut are trypsin-like or chymotrypsin-like proteases. On the basis of the results, the removal of N-terminal and C-terminal peptides could also happened in transgenic Bt cells, which was not unexpected since similar serine proteases are present in plants.

3.1.9 Tryptic peptide mass fingerprinting

The Cry proteins had been previously characterized by MALDI-TOF for many toxins, like Cry1F, Cry34Ab1, and Cry35Ab1 with sequence coverage of 40-53% (Gao *et al*., 2004, Gao *et al*., 2006). On the other hand, there are reports, which refer to MALDI-TOF as a tool of characterization of novel Bt toxins (Cry1D, Cry1H, Cry9A and Cry9B), but the coverage of the sequence was low relating the lack of data which

was available in databases (Ranasinghe and Akhurst 2002). The Cry1Ab endotoxin derived from transgenic maize was subjected to denaturation by heating, the band related to Cry1Ab protein (69 kDa) was cut out of the gel, and in gel digested before applying to the MALDI-TOF fingerprinting characterization. The masses of the digested protein peptides were performed and the m/z values between 750 and 3500 Da were compared to theoretically predicted values, based on protein sequence in databases (Figure 7). The results showed the digest of Cry1Ab has 26 peptides matching the theoretically mass of Cry1Ab synthetic protein (synpro) (Table 5). These peptides covered 41% of the Cry1Ab (synpro) protein masses. In general, a protein identification made by peptide mass fingerprint is considered to be reliable if the measured coverage is 15% or higher with minimum five peptide matches (Jensen *et al*., 1997).

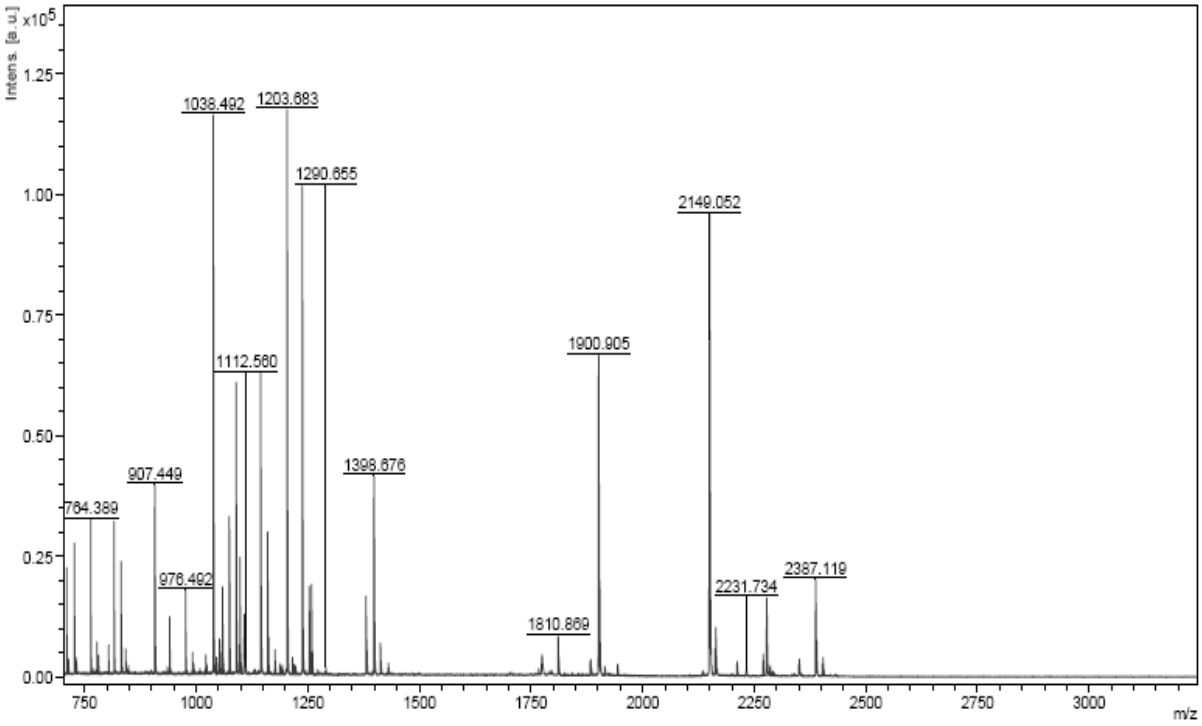

Figure 7: Tryptic peptide mass fingerprint spectrum of proteolytic fragments from transgenic maize MON810.

Table 5: Mass fingerprinting and sequences of a tryptic peptide of Cry1Ab isolated from MON810.

Theoretical mass m/z	Mass m/z observed	Identified peptide sequence	Amino acid range
763.38	764.38	YNDLTR	88 - 93
780.36	781.37	TVSQLTR	193 - 198
803.43	804.44	VWGPDSR	259 - 265
815.38	816.39	DVSVFGQR	218 - 224
906.44	907.44	TLSSTLYR	174 - 181
939.48	940.49	LSHVSMFR	361 - 368
975.48	976.49	LSHVSMFR	430 - 437
991.47	992.48	WYNTGLER	430 - 437
1037.48	1038.49	TSPGQISTLR	210 - 217
1058.56	1059.57	GSAQGIEGSIR	503 - 512
1073.53	1074.54	GPGFTGGDILR	282 - 292
1088.55	1089.56	VNITAPLSQR	491 - 501
1097.61	1098.61	VNITAPLSQR	513 - 522
1143.55	1144.56	APMFSWIHR	450 - 458
1159.55	1160.56	APMFSWIHR	450 - 458
1202.67	1203.68	IVAQLGQGVYR	350 - 360
1214.67	1215.67	RTSPGQISTLR	502 - 512
1236.59	1237.60	WGFDAATINSR	182 - 192
1252.64	1253.65	SGFSNSSVSIIR	438 - 449
1257.65	1258.66	LIGNYTDHAVR	199 - 209
1397.66	1398.67	EWEADPTNPALR	116 - 127
1899.89	1900.90	EIYTNPVLENFDGSFR	266 - 281
1942.88	1943.89	EWEADPTNPALREEMR	116 - 131
2148.04	2149.05	SGTVDSLDEIPPQNNNVPPR	404 - 423
2210.09	2211.10	LSHVSMFRSGFSNSSVSIIR	430 - 449
2276.1491	2277.15	SGTVDSLDEIPPQNNNVPPR	403 - 423

3.1.10 Liquid chromatography-electrospray-ion trap mass spectrometry

HPLC and LC coupled to ESI-MS/MS have become the methods of choice for the identification of proteins by tandem mass spectrometry and database searching. In reality, the identification of proteins by LC−ESI-MS/MS is based on the characteristic MS/MS spectra and not on the retention times of the peptides. Moreover, it provides extremely high degree of reproducibility.

ESI-MS/MS had been successfully used in peptide identification of Cry proteins for determination of selected peptide ion from a Cry protein digest. The use of this technique was to prove the equivalence of bacterial Cry proteins and genetically modified Cry protein from plant source (Gao *et al*., 2004, Gao *et al*., 2006). In the current study, the peptide mixture of trypsin-digested Cry1Ab from MON810 maize

was analyzed, full scan mass spectra of the sample were acquired and used to identify the protein.

The sequence coverage was 73% which is rather high compared with MALDI-TOF coverage. Due to many factors 100% sequence coverage with single enzyme digest is not feasible for most proteins. These factors include the frequency of specific cleavage sites in the sequence, conditions of digestion, peptide recovery from post digestion purification, and the response of mass spectrometer. The analyzed peptides by LC−ESI-MS/MS produced signals that differ by the masses of individual amino acid residues; therefore it has been successfully used in peptide sequencing. The high coverage of ESI-MS/MS is related to the use of a reversed phase C18 column which effectively concentrates the peptides 50-200 fold before MS detection, which can increase the sensitivity in identifying the target protein, since mass spectrometry detection is a concentration dependent method.

3.1.11 Lack of glycosylation

The purified Cry1Ab was examined for glycosylation by a glycoprotein staining kit. The separation of purified Cry1Ab was performed by SDS-PAGE. Horse radish peroxidise was used as a positive control as shown in Figure 8. The staining method is able to detect 0.16 µg of horseradish peroxidase (Pierce technical information of the test kit).

The 1 µg Cry1Ab was loaded on the gel. That means, the applied amount was 6 fold of the detection limit of the horseradish peroxidase. The results refered to that the Cry1Ab had no detectable glycosides.

The results demonstrate that the Cry1Ab protein produced by MON810 transgenic maize plants could be truncated by the plant host proteases into core toxin with approximately 69 kDa, which corresponds to core fragment of the Cry1Ab toxin generated by commercial enzymes such as trypsin, or by cleavage by proteases in the susceptible insect gut. The truncation occurred by removal of 25-30 amino acids in the N-terminal peptide and approximately half of the remaining protein from the C-terminus. MALDI-TOF analysis could be a useful component for screening of Cry1Ab. Using of LC-ESI MS/MS produced higher sequence coverage of Cry1Ab peptide digest compared to MALDI-TOF analysis. Since the LC-ESI MS/MS is highly efficient in the identification of Cry proteins, it can be used also as a final step to identify Bt

strains that produce Cry proteins which have not been characterized. In addition it can be used for the detection and identification of novel Cry proteins.

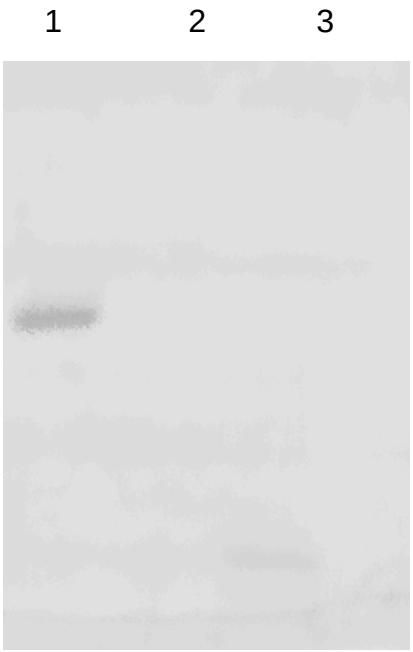

Figure 8: SDS-PAGE gel stained by pirce gelcode glycoprotein staining kit.
Lane1, HRP (positive control of glycoproteins); Lane2, Soybean trypsin inhibitor (negative control); Lane 3, immunoaffinity- purified corn Cry1Ab.

3.1.12 Capillary electrophoresis

Recent case studies screening Cry proteins and Cry1Ab content in GMOs and food stuffs were reported (Ezequiel *et al*., 2006, Lorch and Then 2007, Nguyen and Jehle 2007). These studies revealed the Cry proteins content with ELISA.

ELISA is a method widely used for screening purposes with a high throughput capacity that allows rapid, preliminary testing and is easy to handle. However, it is not a suitable method for drawing definitive conclusions, since it does not provide information on the chemical structure. Lutz *et al*., 2005 found that the antibody used in ELISA recognized fragmented yet immunoactive parts of Cry1Ab protein, while the immunoblotting assays with polyclonal and monoclonal antibodies against epitopes of the Cry1Ab protein showed that the full length protein active core was absent or at least below the detection limit of the immunoblotting assay in the bovine gastrointestinal tract samples. It means that ELISA technique not only recognizes the full length Cry proteins but also reacts with fragmented yet immunoactive epitopes of Cry1Ab.

However, to ensure adequate measurements and to confirm the presence of the full-length Cry1Ab protein, the use of an alternative method, for example capillary electrophoresis (CE) was advisable. Capillary electrophoresis was used for analysis of Bt endotoxin. Liu and Tzeng (2001) used the CE for quantitative determination of solubilised crystal δ-*endotoxins* from *Bacillus thuringiensis*. Lung *et al.* (2004) found that CE technique is able to identify three similar Bt toxins, Cry1Aa, Cry1Ab and Cry1Ac derived from bacteria. In our investigation, we determined Cry1Ab derived from MON810 transgenic maize by CE. The choice of CE for determination of Cry1Ab purified from MON810 for many factors include; its speed, efficiency, reproducibility, low consumption of solvent, easy removal of contaminants and ultra small sample volume, especially since the purification of a high amount of Cry1Ab from transgenic maize was difficult. For this reason we had to minimize the volume of analyzed sample. Samples obtained directly from the immuno-affinity columns were not sufficient; therefore it was necessary to concentrate the samples by centrifugal filter tubes. The samples were prepared in carbonate/NaCl buffer, pH 10.7. Proteins are polyelectrolytes and adsorption often occurs because of Coulombic attractions between the negatively charged capillary wall and the positive charges on the protein molecule. Therefore, separation was performed at high pH, above the isoelectric point of the toxins to minimize their adsorption onto the capillary surface, which were mainly due to electrostatic interaction. Above the isoelectric point of the protein, the protein molecule would be repelled from the negatively charged capillary surface due to its net charge.

The high pH borate buffer has been used for protein analysis with excellent resolution and repeatable results because it can provide the repulsion force between negatively charged proteins and fused silica surface of the capillary, and prevent the protein from adhering to the surface of capillary. At the same time SDS offered better resolution. Figure 9 shows that Cry1Ab had a migration time of nearly 26-27 min. The purified protein Cry1Ab was investigated by CE. Samples which containing 0.3, 0.15, 0.07, and 0.03 mg/ml were determined (Figure 10). Linearity observed in peak area mode (Figure 11a). The linearity also could be observed from peak height (Figure 11b). The correlation coefficient (R^2) for the peak width mode was 0.999 and the slope was 607.94, with intercept at 4.5618 of y axis (y =607.94x + 4.5618). Amount of the Cry1Ab toxin in the solution could be assayed by the linear regression equation. In other words, from the resultant electrophorograms, either peak area or peak height

could be used for determination of Cry1Ab by the equation. Detection limit was 0.03 mg/ml. According to the detection results using CE method, concentrations lower than, 0.03 mg/ml were not detectable. Therefore the calibration curve consisted of four points only.

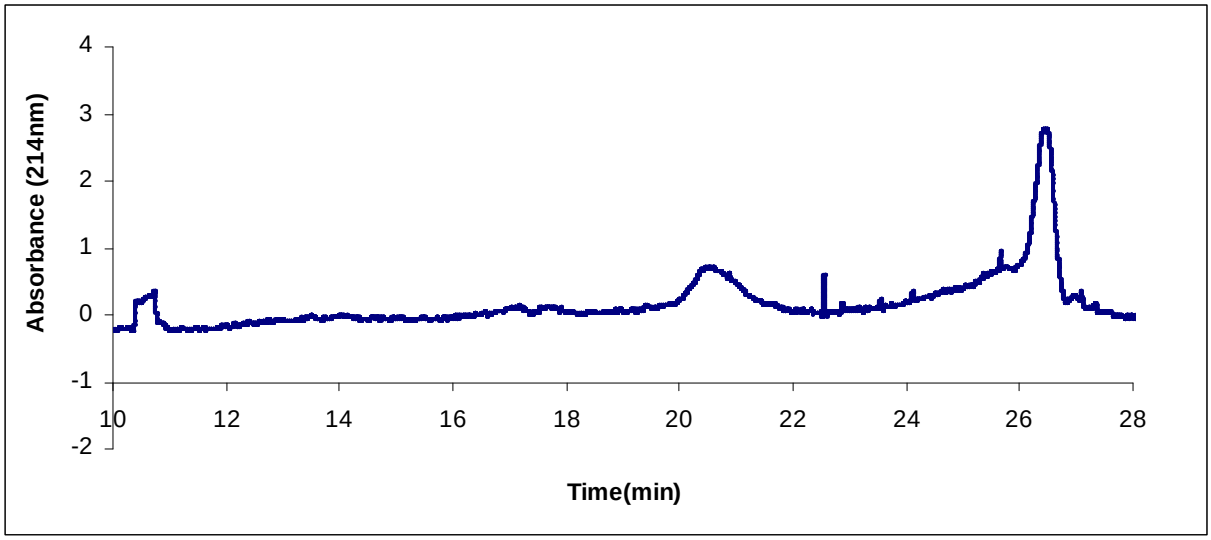

Figure 9: Electropherogram of purified Cry1Ab.
The 125mM borate buffer (pH 9.2) contained 25mM SDS.

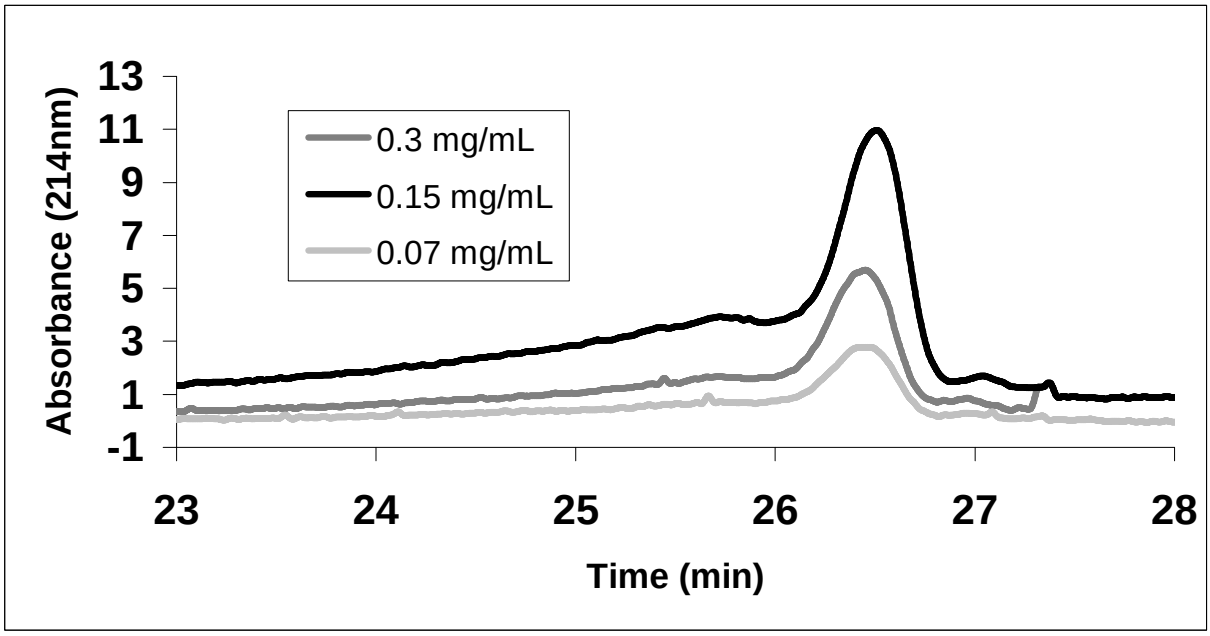

Figure 10: Standard electropherogram using different concentrations of purified Cry1Ab.
The 0.3, 0.15 and 0.07 mg/ml Cry1Ab which separated by 125mM borate buffer pH 9.2 containing 25mM SDS were used.

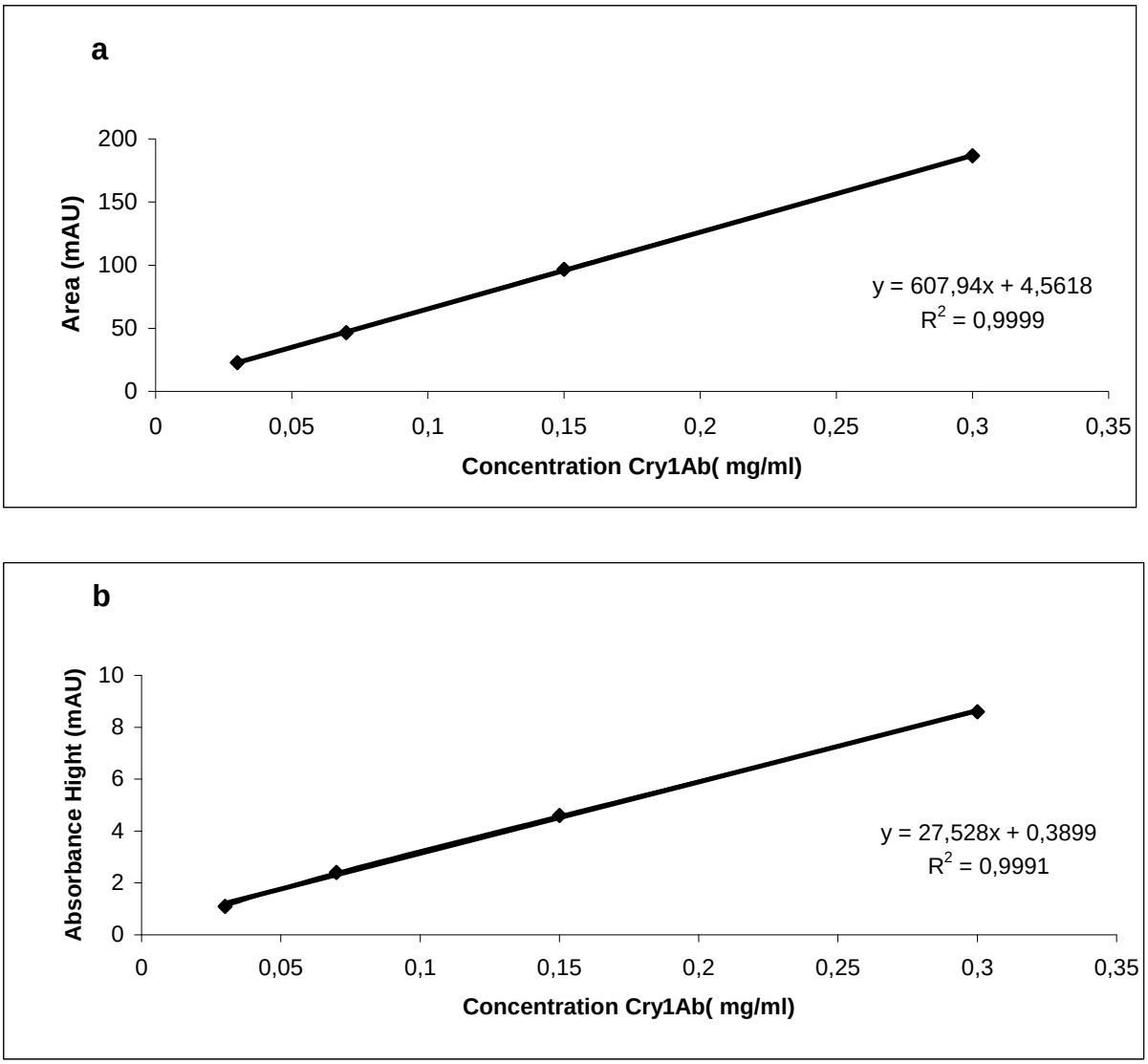

Figure 11: Plot of quantified Cry1Ab analyzed by capillary electrophoresis.
(a) Peak area (b) peak height.

In brief, capillary electrophoresis technique using SDS was a good tool for determination of Cry1Ab purified from Mon810 transgenic corn. The toxin complexed with SDS and migrated against the electroosmotic flow and this counter current electrophoretic mobility broadened the CE window and more effectively made it a good tool for determination of Cry1Ab. Capillary electrophoresis proved to be a suitable method for determination of the Cry1Ab isolated from MON810 genetically modified maize, but it did not meet the requirement of determination of very low quantities (lower than 30µg/ml was not detectable).

3.1.13 Peptide mapping

Using thin-layer chromatography (TLC) for peptide mapping is a commercial, efficient and rapid method. In this investigation we compared two methods of peptide mapping, capillary electrophoresis as an ideal method for peptide mapping of proteins digest and high-performance thin-layer chromatography (HPTLC) using relatively new plates (Proteo Chrom) for peptide mapping of Cry1Ab purified from transgenic corn.

3.1.13.1 Peptide mapping by capillary electrophoresis

Peptide mapping is one of the most powerful and successful tool for protein identification and characterization, it is a method for comparing protein primary structures. The similarity of proteins can be determined by evaluating the likeness of peptides generated by endopeptidic cleavage. Figure 12 shows the separation of Cry1Ab protein digests from trypsin. Since the peptides are amphoteric, they are ideally suited for the characterization by electrophoresis. The separation of peptides at intermediate pHs, partly ionized termini, and side chain residues allow optimization of the peptide separation. At pH<2, all ionizable groups of peptides would be protonated. The number of basic residues in the peptide chain would determine the overall charged state of the molecule. At pH>10, all ionizable groups will be deprotonated, resulting in a negatively charged peptide. At these extreme pH conditions, the separation of peptides could not be adjusted. The use of buffer of pH 9.2 for the separation of the peptides gave a good selectivity of peptide map of Cry1Ab by CE. Mapping by CE can done within 30 min, whereas up to 19 peptides were resolved.

In general, peptide mapping by capillary electrophoresis provided a powerful approach for detailed characterization of Cry1Ab purified from MON810 transgenic corn, and the use of capillary electrophoresis for a high-throughput, low-cost peptide mapping was demonstrated in the analysis of Cry1Ab.

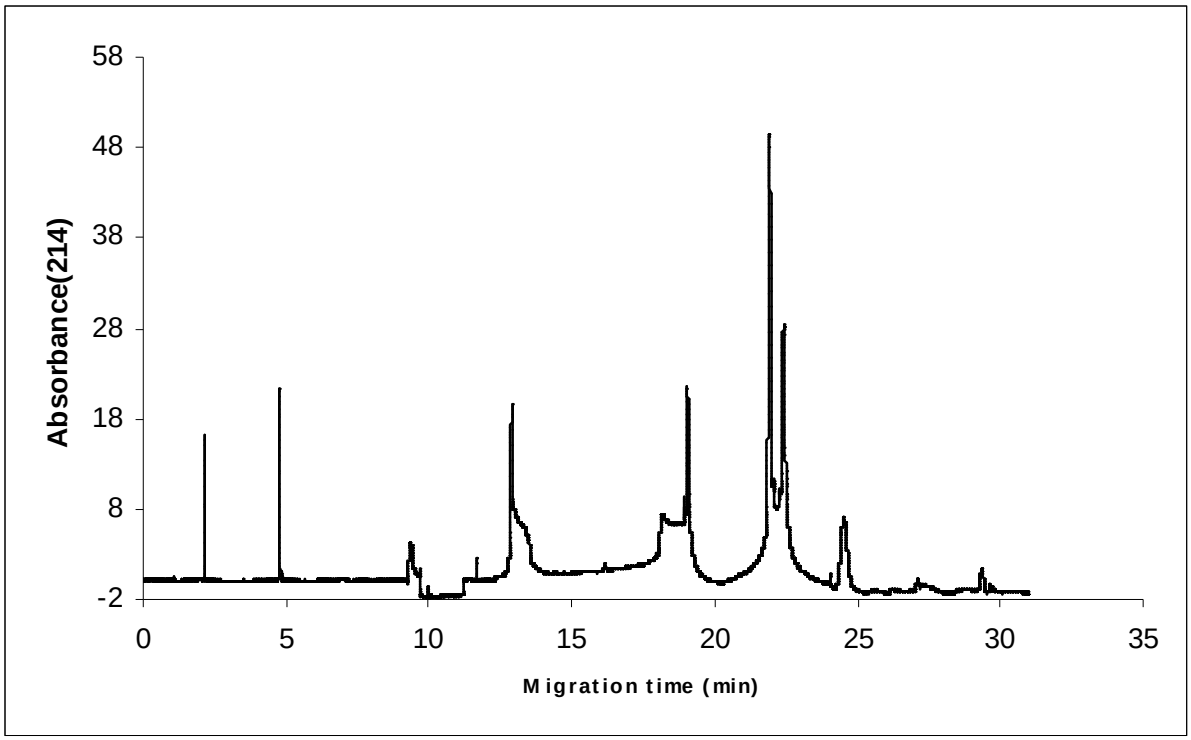

Figure 12: Electropherogram showing peptide maps of Cry1Ab digest.

3.1.13.2 Peptide mapping by HPTLC

Some advantages to the use of HPTLC include the ability to separate many samples in parallel, save/store the separation on the TLC plate, and to detect small and hydrophobic peptides. (Van Berkel and Kertesz, 2006)

HPTLC peptide mapping of proteins was successfully used (Van Berkel and Kertesz 2006, Pasilis *et al.,* 2008). In our investigation, the efficiency of HPTLC in the separation of purified digested Cry1Ab was studied.

The relatively new type of plates used in the experiment utilize an extra thin separation phase (100 µm) enabling highly efficient separation of peptides compared with commonly used HPTLC layers of 200 µm.

The developed HTLC plate was screened directly by TLC Scanner in the fluorescence mode (366 nm). HPTLC method was found to be simple, rapid, and could be routinely used to analyse a large number of samples. In fact, it requires a simple approach and a short analysis time. The protein digest was purified through Zip-Tip tips C18 for sample preparation. As shown in Figure13 a 1D separation of

Cry1Ab protein digest was achieved on ProteoChrom HPTLC silica gel 60 F 254s plates. After the separation, the resolved peptides were detected by dipping in fluorescamine and triethylamine. The scan of the plate showed that the HPTLC could resolve 13 peptides, according to manufacturers of ProteoChrom silica gel plates, the detection system by fluorescamine and triethylamine was very sensitive (Figure13 b). It was able to detect 0.1-5 ng. Based on the results presented, we can conclude that, although the capillary electrophoresis peptide mapping gave a relatively better resolution than HPTLC, the use of HPTLC in peptide mapping could be a convenient alternative if considerd that HPTLC is less costly than capillary electrophoresis or even HPLC.

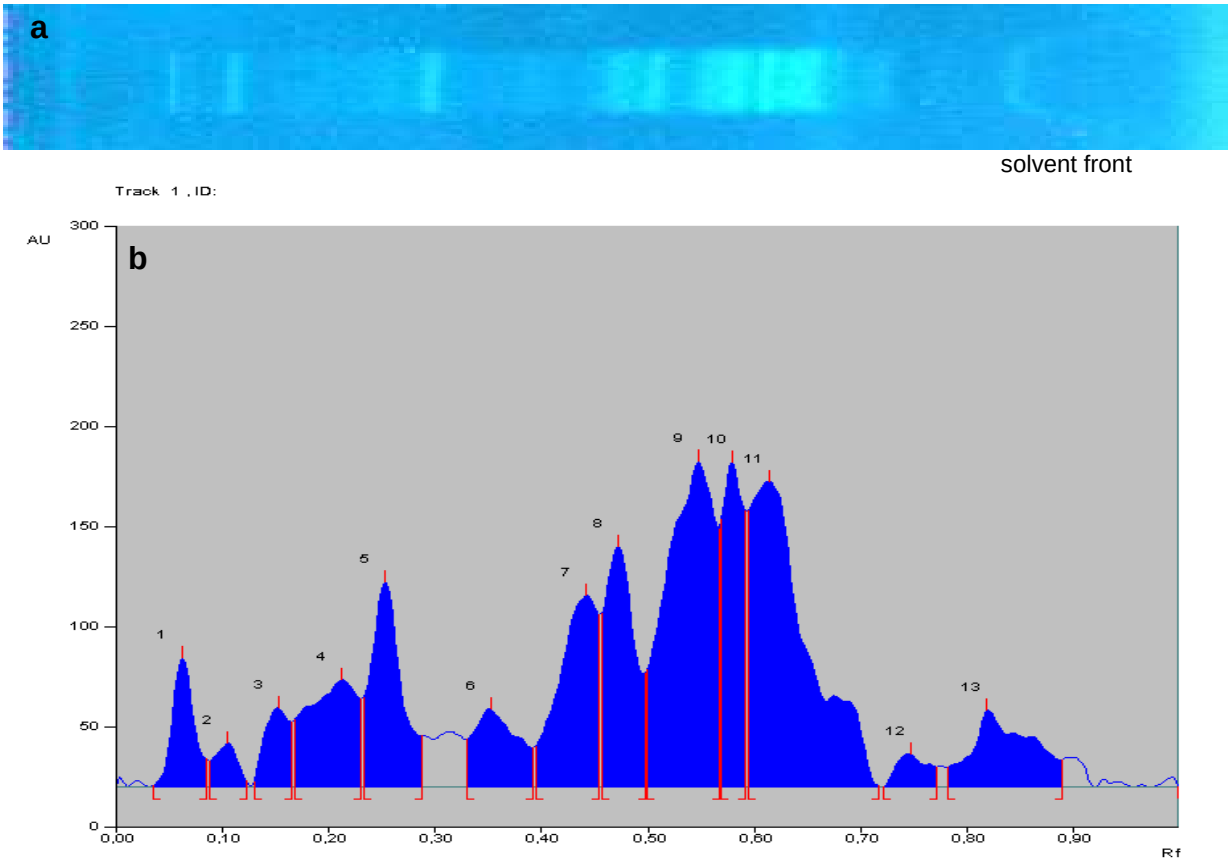

Figure 13: Tryptic digest of Cry1Ab.
The Cry1Ab was separated on ProteoChrom HPTLC silica gel 60 F_{245s} (a) and scan (at 366 nm) showing of the separated peptides (b).

3.2 Oil analysis

Over the past ten years, a number of biotechnology-derived products have been introduced into the market place (Ridley *et al.*, 2002, George and Ridley 2004). All plant breeding procedures can produce unexpected effect. Because of this, novel food research on how the different genetic modifications can effect on the oil composition have great interest. At the same time, evaluation of oil composition of GMOs has been reported previously (El-Sanhoty *et al*., 2006, Normand *et al*., 2006, Smith *et al*., 2007, Merrill *et al*., 2008). These studies were investigated on genetically modified maize Bt-176, sunflower, canola and soybean oil composition. Although genetically modified MON810 is one of the major GM maize crops, to our knowledge no data are available to compare or screen the sterol, phospholipid and tocopherol composition.

In general, this study evaluated whether the oil content extracted from genetically modified oilseeds are compositionally equivalent to conventional oil extracted from regular oilseeds. We determined and compared the fatty acid, sterol, tocopherol and phospholipid distribution as well as total unsaponifiable levels in two major of genetically modified oilseeds, GM maize to regular maize as well as GM high-oleic sunflower to regular sunflower .

3.2.1 Fatty acid composition of oils

The distributions of maize fatty acids are summarized in Table 6. The results showed that levels of lipid components in the grain of genetically modified maize (MON810) were relatively comparable to those in grain of the control maize. The lipids in maize grain were 3.1%-3.3% of dry mass. The genetic modification caused a slight change in total lipid content. The level of total lipid in modified maize was approximately 0.2% lower than the control sample. The levels of ten fatty acids in Bt MON810 were relatively comparable to those in control maize. Linoleic acid was the predominant fatty acid with a range 62.1%-62.3% of total fatty acids. These values were within the range of those determined from previous studies on maize.

The total lipids of high-oleic sunflower (HOSF) and regular sunflower (SF) were 39.1 and 25.5 respectively. The total lipids in high-oleic sunflower was higher than in conventional sunflower.

Table 6: Fatty acid compositions (%) of Bt MON810 maize and regular maize.

Variable	Bt-Mon810	regular Maize
Total lipids%	3.08	3.28
Fatty acid composition %		
C16:0	10.82 ± 0.28	11.89 ± 0.5
C16:1	0.14 ± 0.1	0.17 ± 0.01
C18:0	1.72 ± 0.04	1.29 ± 0.05
C18:1	23.44 ± 0.86	22.60 ± 0.64
C18:2	62.31 ± 1.23	62.11 ± 1.20
C18:3	1.08 ± 0.1	1.23 ± 0.03
C20:0	0.14 ± 0.01	0.31 ± 0.03
C20:1	0.21 ± 0.09	0.25 ± 0.01
C22:0	0.14 ± 0.01	0.15 ± 0.08

Values are expressed as mean ± standard deviation SD (n=3).

The fatty acid composition of HOSF and SF is listed in Table 7. Genetically modified high-oleic sunflower contained 84.8% oleic acid, about 62% more than conventional sunflower oil, and 3.9% linoleic acid which is about 58% less than conventional sunflower. These data were within the range of values determined from previous studies on sunflower (Normand *et al.,* 2006, Merril *et al*., 2008).

Table 7: Fatty acid compositions of high-oleic acid sunflower and regular sunflower.

Variable	High-oleic sunflower	regular Sunflower
Total lipids%	39,07	25,49
Fatty acid composition %		
C16:0	3.71 ± 0.1	6.96 ± 0.67
C16:1	0.02 ± 0.02	0.08 ± 0.04
C18:0	3.45 ± 0.05	3.39 ± 0.59
C18:1	84.78 ± 0.07	23.02 ± 0.29
C18:2	3.91 ± 0.5	62.35 ± 0.25
C18:3	1.51 ± 0.50	0.36 ± 0.47
C20:0	1.48 ± 0.72	2.59 ± 0.06
C20:1	1.09 ± 0.11	1.20 ± 0.02
C22:0	0.05 ± 0.02	0.05 ± 0.03

Values are expressed as mean ± standard deviation SD (n=3).

3.2.2 Phytosterol content of oils

Sterols comprise the bulk of unsaponifiable matter in many oils. They are of interest due to antioxidant activity and impact on health (Dutta *et al.,* 1994). Moreover, analysis of sterols provides a powerful tool for quality control of vegetable oils

otherwise not recognized by the fatty acid profile. The phytosterol content of genetically modified oils and regular oils is summarized in Tables 8 and 9**.** It showed that levels of unsaponifiables were relatively higher in modified maize (2.8%) than conventional maize (2.2%), while the unsaponifiables for HOSF and SF samples were (1.5 and 1.8%) respectively. When considering the sterol distribution, five components were detected (campsterol, stigmasterol, β-sitosterol, ∆5-avenasterol, ∆7-avenasterol). Since β-sitosterol was the most abundant sterol in corn oil and SF oil samples, our data was in agreement with the studies reported by Phillips *et al.* (2002) and Normén *et al.* (2007). β-sitosterol comprised 70 % of total sterols in MON810 maize while it was 74 % of the total sterols in conventional maize.

Table 8: Unsaponifiable matter and phytosterol content of Bt MON810 maize and regular maize.

Variable	**Bt-Mon810**	**Regular Maize**
Unsap. Matter(% total lipids)	2.83	2.21
Phytosterol content mg/100g oil		
Campsterol	349.12 ± 1.70	213.05 ± 1.68
Stigmasterol	87.59 ± 1.5	51.82 ± 1.48
β- sitosterol	1218.58 ± 2.8	925.14 ± 7.24
∆5Avenasterol	36.153 ± 5.12	26.00 ± 1.5
∆7Avenasterol	47.14 ± 14.5	32.07 ± 1.75

All values are mean ± standard deviation (n=3).

Table 9: Unsaponifiable matter and phytosterol content of high-oleic acid sunflower and regular sunflower.

Variable	**High-oleic Sunflower**	**Regular sunflower**
Unsap. matter (% total lipids)	1.51	1.83
Phytosterol content mg/100g oil		
Campsterol	49.13 ± 1.52	34.41 ± 1.52
Stigmasterol	20.23 ± 0.85	22.02 ± 0.27
β- sitosterol	260.12 ± 4.36	271.62 ± 8.70
∆5Avenasterol	15.50 ± 1.25	13.76 ± 0.17
∆7Avenasterol	10.11 ± 1.81	10.32 ± 1.23

Values are expressed as mean ± standard deviation SD (n=3)

On the other hand sitosterol consisted of 75.2 and 73.4 % in HOSF and SF, respectively. Winkler *et al.* (2007) reported that total sterols in corn oil are higher than in sunflower oil. In our study, regular maize had 3.5 times total phytosterols as compared to regular sunflower. Sterol content of corn oil was higher than that determined by Winkler *et al*. (2007) and in agreement with the results reported by Verleyen *et al*., 2002. The sterol distribution in the lipid fraction of modified maize was similar as in the unmodified samples. The values determined for HOSF and regular SF demonstrate that the phytosterol distributions were in the range of the study reported by Codex (2005).

3.2.3 Tocopherols content of oils

Tocopherols and tocotrienols, together abbreviated as tocols and also termed as vitamin E, are a group of fat soluble antioxidants with a chromane ring and hydrophobic side shain.

Many studies published have made direct analysis of tocopherols after diluting the oil in an organic solvent (Pocklington and Dieffenbacher, 1988, Warner and Mounts, 1990). Saponification causes pronounced losses of tocopherols even in protective conditions such as darkness and high nitrogen (Rupérez *et al.,* 1998). However, direct analysis after dilution, unlike saponification and extraction, simplifies the procedure and shortens the analysis. Moreover, many oil samples can be analyzed several times without altering the chromatographic efficiency or the column efficiency, which remains high. Normal phase HPLC has been found capable of separating isocratically all the tocopherols in seed oils (Brubacher *et al*., 1985) which involves dissolving the oils in hexane for injection on to a silica column. Muralidharan and Husain (1985) reported that both reversed and normal phase HPLC is used for tocopherol analysis, the latter is, however, more suitable for separating different tocopherols. The present study was, therefore, initiated to evaluate the quality of different seed oils for their tocopherol contents by an HPLC assay with a silica column and n-hexane/THF/acetic acid as eluent mixture.

The distributions of individual tocopherols in assessed corn oils are reported in Table 10. Consistent with published data (Syväoja *et al*., 1986, Bonvehi *et al*., 2000), which reported that α- and γ-tocopherols proved to be the major tocopherols in vegetable oils and fats. MON810 corn oil had a higher tocopherol content (886.7 mg/kg) than

regular maize (679.4 mg/kg). γ-Tocopherol consisted of 77.4% of total MON810 tocopherols, while it was 64.5% of total tocopherols in conventional maize. α-Tocopherol was the second most abundant tocopherol consisting of 12.6-26.5% of total tocopherols. Generally γ-and α-tocopherol were the major components in both types of maize. The results of the conventional maize were in the range of previous studies (El-Sanhoty *et al*., 2006, codex, 2005).

Table 10: Tocopherols in Bt MON810 maize and regular maize.

Tocopherols (mg/kg)	Bt-Mon810	Conventional maize
α-Tocopherol	111.76 ± 0.05	180.18 ± 1.18
β-Tocopherol	46.41 ± 0.85	32.80 ± 0.57
γ- Tocopherol	686.39 ± 0.77	438.55 ± 0.13
δ- Tocopherol	42.14 ± 0.36	27.87 ± 0.06

Values are expressed as mean ± standard deviation SD (n=3).

Table 11: Tocopherols in high-oleic acid sunflower and regular sunflower.

Tocopherols (mg/kg)	High-oleic Sunflower	regular sunflower
α-Tocopherol	641.77 ± 5.74	708.11 ± 0.06
β-Tocopherol	31.59 ± 0.88	40.95 ± 0.09
γ- Tocopherol	18.69 ± 0.56	11.42 ± 0.35
δ- Tocopherol	8.23 ± 0.21	6.88 ± 0.01

Values are expressed as mean ± standard deviation SD (n=3)

The sunflower samples were in agreement with the values reported for sunflower oils in the Codex (2005). SF had much higher tocopherol content (767.4 mg/kg) than in maize samples. The α-isomer was the predominant tocopherol present with 94% in HOSF and 92% in SF of total tocopherols. The δ-isomer only accounted 0.8-0.9% of total tocopherols. The results obtained referred that the tocopherol distribution of HOSF and SF had no significant differences between the two oils. The concentration of δ-Tocopherol in all oils was not higher than 43 mg/kg (Table 11).

3.2.4 Phospholipid content of oils

Phospholipids are components of foods and have antioxidant effects (Rathjent and Steinhart, 1997). Spectrophotometric methods have been employed to determine the concentration of phospholipids and phosphorus in vegetable and animal tissues (Keenan *et al*., 1968, Zhukov and Vereshchagin, 1976 and AOCS, 1994). However, many of these methods are tedious and inaccurate. There are a number of high-performance liquid chromatographic (HPLC) methods available for the analysis of

phospholipids in vegetable oils (Mounts and Nash, 1990, Singleton and Stikeleather, 1995, Helmerich and Koehler, 2003). Thin-layer chromatography (TLC) provides a quick separation and identification of a variety of compounds with differing polarities. A limitation is the accurate quantification of separated species, which could be done by a scanning method. An advantage of this technique is its ability to analyze the whole TLC plate at once, hence making it a rapid method for routine work.

In order to determine the phospholipid composition of oil samples, solid phase extraction was employed. Standard conditions were used for detection, immersing the HPTLC plate into the detection reagent premulin was achieved according to the method of Ramesha and Pickett (1987) with regular distribution of the reagent. With the premulin reagent, phospholipids emitted blue fluorescence if inspected under UV 366 nm. The detection limits were 0.06-0.43 µg/band (Table 12). Detection limits of the phospholipid classes were 2-5 times lower than in previous studies (Du Plessis and Pretorius 1983; Helmrich and Koehler, 2003). The low detection limit of the phospholipids fraction could be due to the different method of visualization (premulin reagent system).

Great linearity was achieved over the calibration range with correlation coefficients of 0.9880-0.9996. HPTLC was shown to give a good separation of phospholipids. Helmrich and Koehler (2003) compared the methods: TLC, HPLC and nuclear magnetic response spectroscopy (NMR) for the determination of phospholipids lecithins and flour improvers. They found that HPTLC gave the best separation of phospholipids.

Table 12: Determination coefficient $R^2$of calibration curves of major phospholipid classes and detection limits.

Phospholipid classes	R^2	Detection limit µg/band
PC	0.9978	0.19
PE	0.9948	0.43
PI	0.9880	0.09
PA	0.9958	0.11
LPE	0.9996	0.06

Determination coefficient $R^2$of calibration curves of major phospholipid classes and detection limits were determined with authentic mixture of phospholipid references.

In figure 14, the HPTLC separation of the phospholipids of regular maize and transgenic maize is shown. The fluorescence measurements in corn oil samples are shown in Figure 15. Typical Rf values were PC 0.12; LPE 0.23; PA 0.33; PI 0.39 and PE 0.44, the different Rf values refer to that phospholipid classes were well separated (Figure 16). Sunflower oil phospholipids in plate image (Figure 17) and fluorescence measurement (Figure 18) showed similar separation as in the maize oil phospholipids.

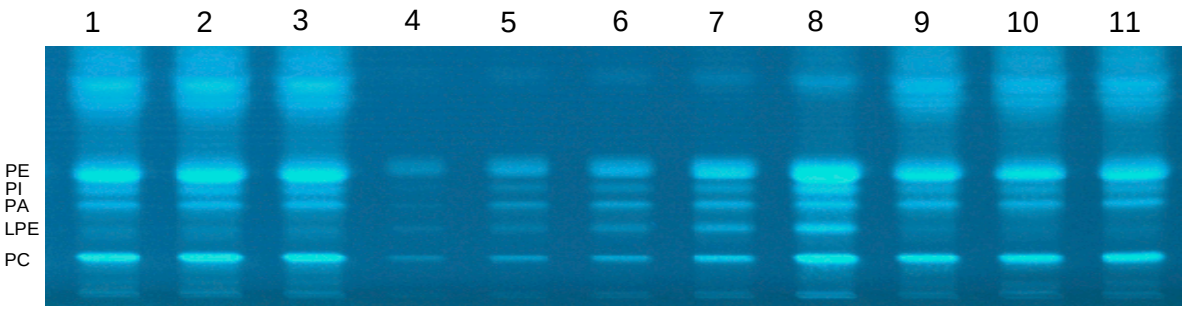

Figure 14: High-performance thin-layer chromatography plate image under UV (366 nm) obtained during determination of maize phospholipids.
Lanes1-3 regular maize phospholipids; Lanes 4-8 phospholid standards; 9-11Bt MON810 maize phospholipids.

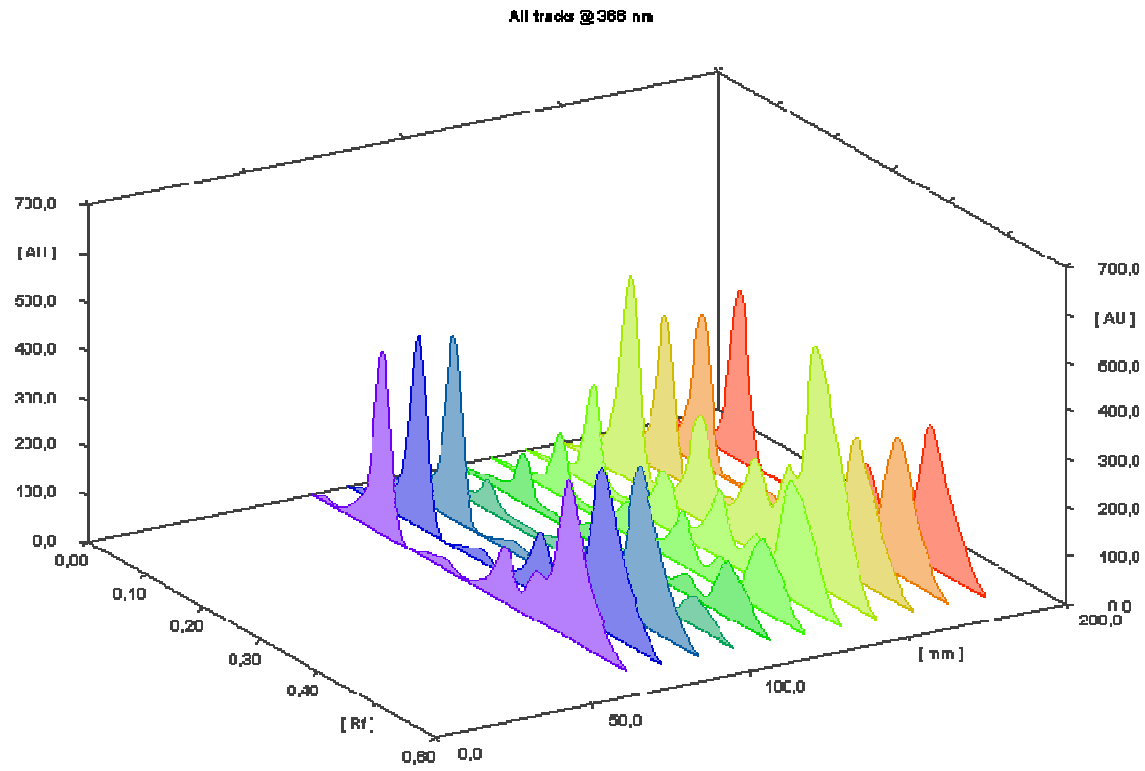

Figure 15: HPTLC-fluorescence measurement of maize phospholipids at UV (366 nm).
Tracks 1-3 conventional maize; Tracks 4-8 phospholid standards; 9-11Bt MON810 maize.

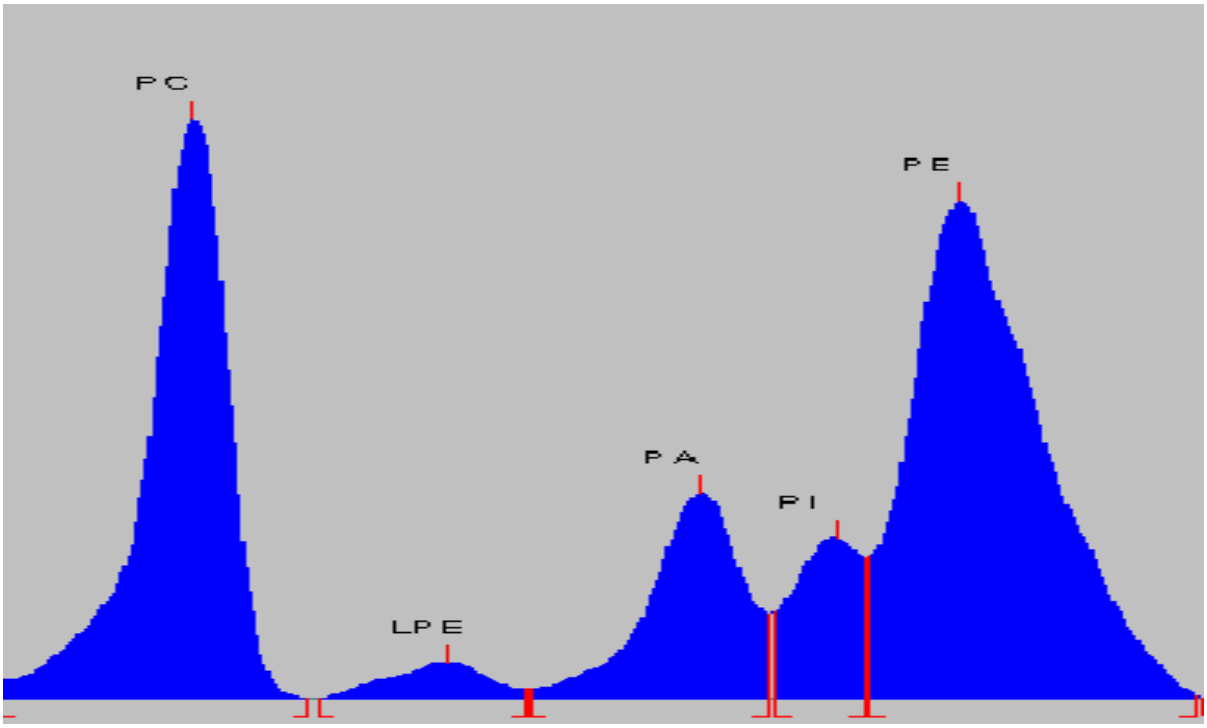

Figure 16: HPTLC-separation of phospholipids of conventional maize

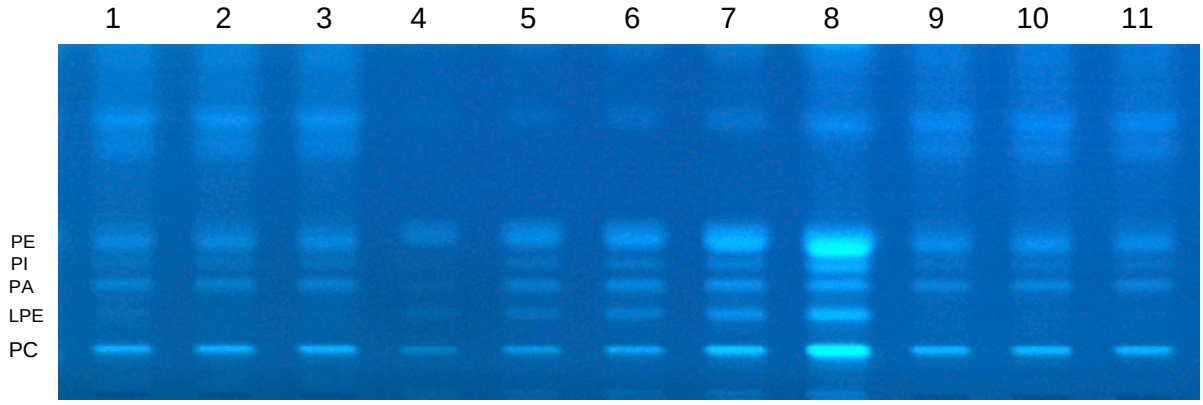

Figure 17: High-performance thin-layer chromatography plate image at UV (366 nm) obtained during determination of sunflower phospholipids.
Lanes1-3 conventional sunflower; Lanes 4-8 phospholid standards; 9-11high-oleic sunflower phospholipids.

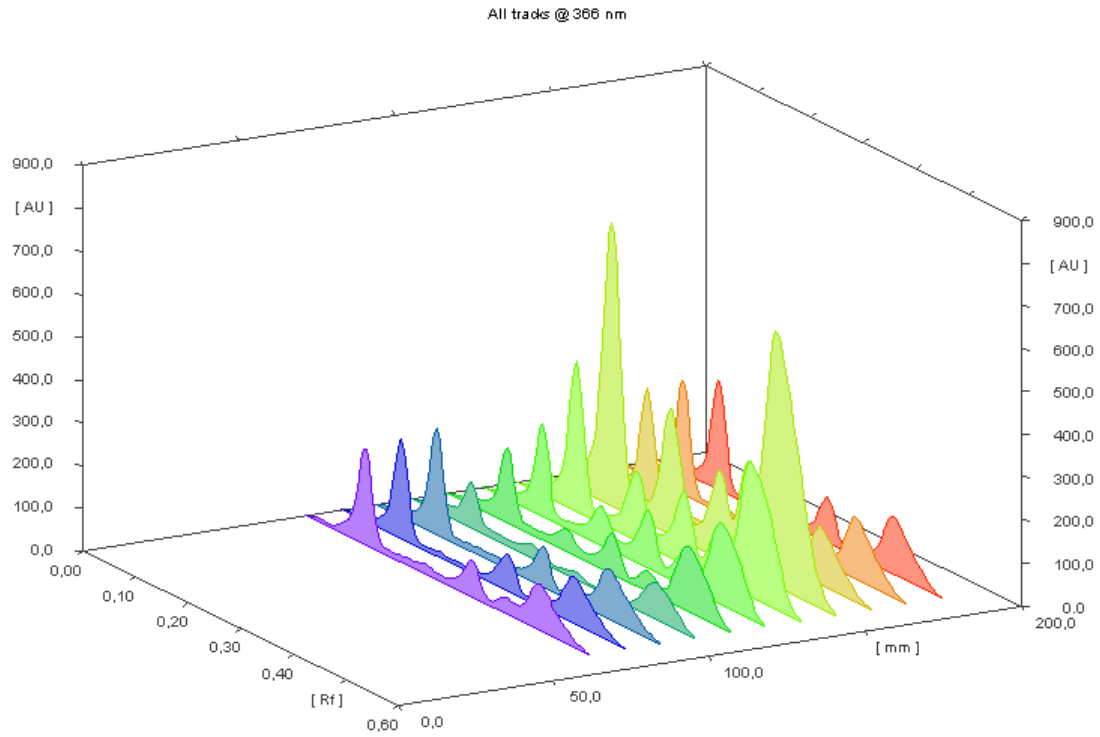

Figure 18: HPTLC-fluorescence measurement of sunflower phospholipids at UV (366 nm). Tracks 1-3 conventional sunflower. Tracks 4-8 phospholid standards; 9-11 high-oleic sunflower phospholipids.

major phospholipid was PC, (57.5-61.8%), followed by PI (19.8-14.5%) and PA (3.8-5.7%). According to the previous studies, the major phospholipid was PC (>50%). In our study, the second major PL was PE and the lowest amount of the phospholipids was PI, while, in previous investigations, the major PL was PA or PI. Bt MON810 had higher amount of total phospholipids (8.2 mg/g oil) while it was 5.1 mg/g oil in regular maize.

Table13: Phospholipid composition of Bt MON810 maize and regular maize.

	Oil					
	Bt-Mon810			**Regular Maize**		
Phospholipid	mg/g oil	RSD (% n=3)	% PL composition	mg/g oil	RSD (% n=3)	% PL composition
PE	1.76	5.5	21.4	1.02	6.2	20.1
PA	1.82	4.4	22.0	0.76	3.4	14.9
LPE	+	-	+	+	-	+
PI	0.92	2.9	11.2	0.72	2.8	14.1
PC	3.74	4.0	45.4	2.59	7.1	50.9
Total	8.24			5.09		

The phospholipids composition of sunflower showed that PC (50.5%) was the major phospholipids and PA (7.5%) was the lowest (Table 14). The results of regular sunflower PL were in agreement with Du Plesses and Pretorius (1983). LPE was the only minor component and the detected quantity was under the limit of detection in all oils. The total amount of phospholipids in both high-oleic sunflower and conventional sunflower were resembled (4.7 and 5.2 mg/g oil, respectively). The RSD ranged from 2-8.8% (n=3).

Table 14: Phospholipid composition of high-oleic acid sunflower and regular sunflower.

	Oil					
	High-oleic Sunflower			**Regular sunflower**		
Phospholipid	mg/g oil	RSD (% n=3)	% PL composition	mg/g oil	RSD (% n=3)	% PL composition
PE	1.49	2.0	32.1	1.64	2.6	31.3
PA	0.46	8.7	9.8	0.39	8.0	7.5
LPE	+	-	+	+	-	+
PI	0.50	5.8	10.7	0.56	4.2	10.7
PC	2.21	8.8	47.4	2.65	4.7	50.5
Total	4.66			5.24		

The results of the present investigation indicated that the HPTLC method was highly useful for analysis of phospholipids from vegetable oils. A great advantage is that the separation plate is only used once, and the matrix fixed at the starting region does not matter. In our study the determination of sixteen samples at the same time was achieved for comparing the phospholipid content of studied oils. The determination of these samples was performed in 65 min (10 min for application, 45 min for separation, and 10 min for detection). Thus the HPTLC method can be useful in the determination of phospholipids on a large scale in laboratories.

The results of oil composition analysis generate from corn oils demonstrate that the corn oil of MON810 is comparable with the regular corn hybrids. Along with the safety evaluations concluded on proteins of genetically modified crops (Harrison *et al*. 1996, Leach *et al*. 2001) and the studies produced for evaluation of products containing those proteins (Ridley *et al*., 2002, George *et al*. 2004, MacCann *et al*. 2007), this study further demonstrated MON810 oil is as safe as conventional hybrid oil on the market today. On the other hand the sunflower high-oleic oil modification did not produce unexpected effects on the oil composition.

4 Summary

The main purpose of this study was to develop a method of purification and characterization of Cry1Ab isolated from MON810 genetically modified maize. The second object was to study the effect of the genetic modification of MON810 and high-oleic sunflower on the oil composition. Therefore, the following investigations were performed:

(1) Quantification of Cry1Ab toxin in different corn plant parts.
(2) Development of a suitable method for purification of Cry1Ab from MON810.
(3) Establishment of characterization method for Cry1Ab by mass spectrometry with regard to high peptide sequence coverage.
(4) Evaluation of the effect of genetic modification on the oil composition compared with the conventional traits.

The following results were obtained:

Screening of Cry1Ab by ELISA is the most predominant technique for determination of Cry toxin content in plants. The determination of the toxin concentration resulted in highest levels for leaves (26.8 µg/g dry matter), while it was 1.5, and 1.0 µg/g for stalks and grains respectively. In our study, toxin content in leaves was about six times higher than in a previous study. There are no data available for the dry weight content of Bt toxins in stalk and grain, which could be compared to the obtained results.

Although MON810 maize is one of the major genetically modified crops, informations on the character of the Cry1Ab purified from the MON810 maize is still limited, although such data are important for safety assessment studies. To my best knowledge, this study is the first investigation charactizing Cry1Ab toxin isolated from MON810 maize.

The results of the present investigation indicated that the separation of the Cry1Ab protein from MON810 leaf extracts by HPLC techniques was not efficient. MALDI-TOF analyses showed that the major component separated with Cry1Ab was β-D glucosidase, which may be due to resembled isoelectric points.

However, immuno-affinity purification using self-prepared affinity columns was very efficient to isolate pure Cry1Ab from MON810. The characterization of purified Cry1Ab was successfully done by SDS-PAGE, Western blot analysis and MS techniques.

MALDI-TOF MS analyses were useful for component screening of Cry1Ab. Results showed that Cry1Ab is subjected to truncation by plant proteases into a core toxin with approximately 69 kDa. LC(ESI)-MS/MS gave a higher sequencing coverage of Cry1Ab (73 % of peptide sequence) compared to MALDI-TOF analysis (41% of peptide sequence). Further studies revealed that Cry1Ab had no detectable potential carbohydrates which might be covalently linked to the protein.

The capillary electrophoresis technique was used for determination of the Cry1Ab purified from MON810 maize and proved to be a suitable method for determination of the Cry1Ab, but it was not successful for the detection of very low quantities (less than 0.03 mg/ml).

Peptide mapping is one of the most powerful tools for protein identification and characterization. The use of HPTLC with the relatively new plates (ProteoChrom) was identified as a convenient tool for peptide mapping as compared to capillary electrophoresis, especially if put into consideration that HPTLC is less costly than capillary electrophoresis. The HPTLC method was able to resolve 13 peptides, while capillary electrophoresis resolved 19 peptides, obtained from the digested Cry1Ab toxin.

Concerning lipid analyses, fatty acids and sterols were determined by gas chromatography, tocopherols by HPLC. For the determination of phospholipids, an HPTLC method was developed, resulting in lower detection limits than reported in previous studies.

The present study proved that the genetic modification did not significantly affect the contents of fatty acids, sterols, tocopherols and phospholipids in transgenic maize oil. Apart from the increased amount of oleic acid in high-oleic sunflower oil, the genetic modification in sunflower did not produce unexpected effects on the oil composition. Therefore, with regard to the oil composition, both oils from genetically modified plants will be as safe as conventional oil types.

5 Zusammenfassung

Das Hauptziel dieser Arbeit war es, ein Verfahren zur Aufreinigung und Charakterisierung des Toxins Cry1Ab in dem gentechnisch modifiziertem Mais MON810 zu entwickeln. Weiterhin war es das Ziel, die Auswirkungen der genetischen Veränderung von MON810 und high-oleic Sonnenblumen auf die Lipid-Zusammensetzung zu studieren. Daher wurden folgenden Untersuchungen durchgeführt:

(1) Quantifizierung des Cry1Ab-Toxins in verschiedenen Teilen der Maispflanze.
(2) Entwicklung einer geeigneten Methode zur Reinigung von Cry1Ab aus MON810.
(3) Charakterisierung von Cry1Ab mit Hilfe der Massenspektrometrie.
(4) Chemische Analyse der Öl-Zusammensetzung von MON810 Maisöl und high-oleic Sonnenblumenöl im Vergleich zu konventionellen Ölen.

Folgende Resultate wurden erzielt:

Die Bestimmung von Cry1Ab in MON810 Mais mittels ELISA ergab die höchsten Konzentrationen in Blättern (26,8 µg/g Trockenmasse), während die Gehalte im Stengel und im Korn deutlich niedriger ausfielen (1,5 bzw. 1,0 µg/g Trockenmasse). Die in den Blättern bestimmten Gehalte waren etwa sechsfach höher als in einer früheren Literaturstudies. Für Stengel und Korn gibt es keine Vergleichsdaten in der verfügbaren Literatur.

Obwohl MON810 Mais eine der wichtigsten gentechnisch veränderten Nutzpflanzen ist, sind proteinchemische Informationen zu Cry1Ab begrenzt, eine wesentliche Voraussetzung für Sicherheitsbewertungen. Nach meinem besten Wissen liefert die vorliegende Arbeit die ersten Ergebnisse zur Charakterisierung des Cry1Ab-Toxins aus MON810 Mais.

Die durchgeführten Untersuchungen zeigten, dass die Aufreinigung des Toxins aus Blattextrakten mittels HPLC nicht effizient war. MALDI-TOF-Analysen ergaben, dass die isolierte Hauptkomponente insbesondere mit dem Enzym β-D-Glucosidase verunreinigt war, wahrscheinlich auf ähnliche isoelektrische Punkte zurückzuführen.

Dagegen erwies sich die Immuno-Affinitätsextraktion an selbst hergestellten Immuno-Affinitätssäulen als sehr effizient und erlaubte die Untersuchungen rein vorliegenden Cry1Ab mittels SDS-PAGE, Western-Blot-Analyse und MS-Techniken.

Die Charakterisierung durch MALDI-TOF-Analysen zeigte, dass Cry1Ab durch pflanzliche Proteasen auf ein Kernprotein von etwa 69 kDa zugeschnitten wird. Mit Hilfe der LC(ESI)-MS/MS konnte eine höhere Abdeckung von Cry1Ab-Peptiden (73% der Peptid-Sequenz) im Vergleich zur MALDI-TOF-Analyse (41% der Peptid-Sequenz) erreicht werden. Weitere Studien zeigten, dass Cry1Ab keine potenziell nachweisbaren Kohlenhydrate aufwies, die kovalent an das Toxin gebunden sind.

Die Capillarelektrophorese erwies sich als eine geeignete Methode zur Bestimmung von Cry1Ab, isoliert aus MON810, leider aber eignete sie sich nicht zur Bestimmung sehr geringer Mengen (weniger als 0,03 mg/ml).

Peptid-Mapping ist eine der besten Methoden zur Protein-Identifizierung und Charakterisierung. Im Vergleich zur Capillarelektrophorese oder HPLC stellte die HPTLC auf speziellen ProteoChrom-Schichten eine preisgünstige und schnelle Alternative zum Peptid-Mapping dar. Mit der HPTLC-Methode konnten 13 Peptide aufgetrennt werden, während die Capillarelektrophorese Signale für 19 Peptide nach tryptischem Verdau von Cry1Ab lieferte.

Im Rahmen der Lipidanalytik wurden die Fettsäure- und Sterinverteilung mittels Gaschromatographie, das Tocopherolmuster mittels HPLC untersucht. Zur Bestimmung der Phospholipide wurde eine HPTLC-Methode entwickelt, mit der geringere Nachweisgrenzen erreicht wurden als in den beschriebenen Methoden.

Die erhaltenen Ergebnisse zu MON810 Maisöl zeigten keine Unterschiede zu konventionellem Maisöl. Abgesehen vom höheren Ölsäuregehalt konnten auch beim high-oleic Sonnenblumenöl keine Differenzen zu konventionellem Sonnenblumenöl aufgedeckt werden. Insofern kann es hinsichtlich der Lipidzusammensetzung keine Sicherheitsbedenken bei den beiden Ölen aus getechnisch modifizierten Pflanzen geben.

6 References

Ahmed, F.E. (1995). Application of molecular biology to biomedicine and toxicology J. Environ. Sci. Health C11**,** 1–51.

Andrews, R. E., Bibilos, M. M., and bulla, L. A. (1985). Protease activation of the entomocidal protoxin of *Bacillus thuringiensis* subsp *kurstaki*. Appl. Environ. Microbiol. 50, 737-742.

AOCS (1994) Official methods and recommended practices of the American oil chemists' society, 4th Edition. J.AOCS Press, Illinois.

Ashton, E.L., Best, J.D., Ball, M.J. (2001). Effects of monosaturated enriched sunflower oil on the CHD risk factors including LDL size and copper-induced LDL oxidation. J. Am. College of Nut. 20, 320–326.

Bietlot, H., Carey, P. R., Choma, C., Kaplan, H., Lessard, T., and Pozsgay, M. (1989). Facile preparation and characterization of the toxin from *Bacillus thuringiensis* var. *kurstaki*. Biochem. J. 260 (1):87–91.

Bonvehi, J.S., Coll, F.V. and Rius, I.A. (2000). Liquid chromatographic determination of tocopherols and tocotrienols in vegetable oils, formulated preparations, and biscuits. J. AOCS.INT. 83, 627-634.

Brandt, P. (2004). Transgene Pflanzen. 2. Auflage. Birkhäuser Verlag, Basel pp 364.

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

Bravo, A., Sanchez, J., Kouskoura, T. and Crickmore, N. (2002). N-terminal activation is an essential early step in the mechanism of action of the *Bacillus thuringiensis* Cry1Ac insecticidal toxin. J. Biol. Chem. 277, 23985−23987.

Brett, G.M., Chambers, S.J., Huang, L. and Morgan, M.R.A. (1999). Design and development of immunoassays for detection of proteins. Food Control 10, 401–406.

Brubacher, G., Mulot, W. M. and Southgate, D. A. T. (1985) Vitamin E in foodstuffs : HPLC method. Methods for the determination of vitamins in food. Elsevier Applied Science. London and New York.

Brufau, G., Codony, R., Canela, M.A. and Rafecas, M. (2006). Rapid and quantitative determination of total sterols of plant and animal origin in liver samples by gas chromatography. Chromatographia, 64, 559–563.

Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G.M, Carnemolla, B., Orecchia, P., Zardi, L. and Righetti, P.G., (2004). Blue silver: A very sensitive colloidal coomassie G-250 staining for proteome analysis. Electrophoresis 25, 1327-1333.

Choma, C.J. and Kaplan, H. (1990). Folding and unfolding of protoxin from *Bacillus thuringiensis*: Evidence that the toxic moiety is present in an active confirmation, Biochemistry 29, 10971-10977.

Choma, C.J. and Kaplan, H. (1991). The toxic moiety of *Bacillus thuringiensis* protein undergoes a conformational change upon activation, Biochem. Biophys. Res. Commun. 179, 1934-1938.

Christau and Klee, P. and Klee, H. (2004). Handbook of plant biotechnology volume 1 John Wiley & Sons Ltd, England. pp. 738.

Codex (2005). Standard for named vegetable oils. Available at http://www.codexalimentarius.net/download/standards/336/CXS_210e.pdf, 5.02.2009.

Cuevas, L., Niemeyer, H.M. and Jonsson, L.M.V. (1992). Partial purification and characterization of a hydroxamic acid glucoside β-D-Glucosidase from maize. Phytochem. 31 (8), 2609-2612.

De Maagd, R.A., Bosch, D. and Stiekema, W. (1999). *Bacillus thuringiensis* toxin-mediated insect resistance in plants. Trends in Plant Science 4, 9-13.

Denis, J. and Denis and Murphy , D. J. (1999). Perspectives on new crops and new uses, ASHS Press, Alexandria, VA, 216-219.

Díaz-Mendoza, M., Farinós, G. P., Castañera, P., Hernández-Crespoand, P. and Ortego, F. (2007). Proteolytic processing of native Cry1Ab toxin by midgut extracts and purified trypsins from the Mediterranean corn borer Sesamia nonagrioides. J. Insect Physiol. 53, 428-435.

Du Plessis, L.M. and Pretorius, H.E. (1983). Phospholipid composition of some plant oils at different stages of refining, measured by the latroscan-chromarod method. J. AOCS.INT. 60, 1261-1265.

Dutta, P. C., Helmersson, S., Kebedu, E., Alemaw, G. and Appelqvist, L., (1994). Variation in lipid composition of Niger seed. J. Am. Oil. Chem. Soc. 71, 839-843.

El-Sanhoty, R., Shahwan, T. and Ramadan, M.F. (2006). Application of artificial neural networks to develop classification model between genetically modified maize by applying lipid analysis data. J. Food Comp. Anal. 19, 628-636.

EPA (2003). Environmental protection agency, Bt Plant-Pesticides Biopesticides Registration Action Document. Available at http://www.agbios.com/docroot/articles/2000264-A.pdf.

EPA (2008). Environmental protection agency, Current & Previously Registered Section 3 PIP. Registrations at http://www.epa.gov/oppbppd1/biopesticides/pips/pip_list.htm.

Esen, A. and Cokmus, C. (1990). Maize genotypes classified as null at the Glul locus have f3-glucosidase activity and immunoreactive protein. Biochem Genet 28, 319-336.

European Union Commission. (1991). Regulation EEC 2568/91 on the characteristics of olive oil and olive pomace and their analytical methods offecial Journal of European Communities L248.

Ezequiel, M., Reggiardo, M. I., Vallejos, R. H. and Permingeat, H. R. (2006). Detection of BT transgenic maize in foodstuffs**.** Food Res Inter. 39, 250–255.

Feistner, G. J., Faull, K. F., Barofsky D. F., Roepstorff, P. (1995). Mass spectrometric peptide and protein charting. J. Mass Spectr. 30, 519-530.

Feitelson, J.S., Payne, J. and Kim, L. (1992). *Bacillus thuringiensis:* insects and beyond. Bio/Technol. 10, 271-275.

Flachowsky, G., Chesson, A. and Aulrich, K. (2005). Animal nutrition with feeds from genetically modified plants. Arch. Anim. Nutr. 59 (1), 1-40.

Fournier, M., Gilmore, J., Martin-Brown, S. and Washburn, M. (2007). Multidimensional separations-based shotgun proteomics. Chem. Rev. 107, 3654- 3686.

Freese, B. (2001). Environmental protection agency EPA. Critiques of EPA's Decision to re- register Bt crops and an examination of the potential allergenicity of Bt proteins. http://www.foe.org/safefood/gapseval.pdf.

Freese, W. and Schubert, D, (2004). Safety testing and regulation of genetically engineered foods. Biotechnol. Genet. Eng. Rev. 21, 299-323.

Gao, Y. (2004). Biosafety issues, assessment, and regulation of genetically modified food plants. In The GMO Handbook: Genetically modified animals, microbes, and plants in biotechnology; Parekh, S. R., Ed.; Humana Press: Totowa, NJ, pp 297−344.

Gao, Y., Fancil, K.J., Xu ,X., Schwedler, D.A., Gilbert, J.R. and Herman, R.A. (2006). Purification and characterization of a chimeric Cry1F delta-endotoxin expressed in transgenic cotton plants. J. Agric Food Chem. 54, 829-35.

Gao, Y., Schafer, B.W., Collins, R.A., Herman, R.A., Xu, X., Gilbert, J.R., Ni, W., Langer, V.l. and Tagliani, L. A. (2004). Characterization of Cry 34Ab1 and Cry35Ab1 insectcidal crystal proteins expressed in transgenic corn plants and pseudomonas fluorescens. J. Agric Food Chem. 52, 8057-8065.

George, C., Ridley, W.P., Obert, J.C., Nemeth, M.A.; Breeze, M.L. and Astwood, J.D. (2004). Composition of grain and forage from corn rootworm-protected corn event MON863 is equivalent to that of conventional corn (*Zea mays* L.). J. Agric. Food Chem. 52, 4149−4158.

Gill, S. S., Cowles, E. A. and Pietrantonio, P. V. (1992). The mode of action of *Bacillus thuringiensis* endotoxins. Annu. Rev. Entomol. 37, 615−636.

Gordon, M.H., Magos, P. (1983). The effect of sterols on the oxidation of edible oils. Food Chem. 10:141–147.

Gunstone, F. D. (2008). Oils and fats in the food industry John Whiley & sons Ltd, United Kingdom, pp 146.

Gunstone, F.D. (2008). Oils and fats in the food industry John Whiley & sons Ltd, United Kingdom, pp 146.

Guo-Hua, Z., Guo-An, L., Guo-Qing, S., Ya-Cheng C., Mimg-Sheng, Z. (1999). Study on the quality of recombinant proteins using matrix-assisted laser desorption ionization time of flight mass spectrometry. WJG. 5 (3), 235-240.

Gylling, H. and Miettinen, T.A. (2005). The effect of plant stanol- and sterol-enriched foods on lipid metabolism, serum lipids and coronary heart disease. Ann. Clin. Biochem. 42 (4), 254–263.

Harrabi, S., Herchi, H., Kallel, W., Mayer, P. M. and Boukhchina, S. (2006) Liquid chromatographic–mass spectrometric analysis of glycerophospholipids in corn oil. Food Chem. 114 712-716.

Harrison, L., Bailey, M., Naylor, M., Ream, J., Hammond, B. G., Nida, D., Burnette, B. (1996). The expressed protein in glyphosate-tolerant soybean, 5-enolypyryvylshikimate-3-phosphate synthase from Agrobacterium sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. J. Nut. 126, 728−740.

Helmerich, G. and Koehler, P. (2003). Comparison of methods for the quantitative determination of phospholipids in lecithins and flour improvers. J. Agric. Food Chem. 51 (23), 6645–6651.

Hoefte, H. and Whiteley, H. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis.* Microbial. Rev. 53, 242-255.

Holck, A., Vaïtilingom M., Didierjean, L., Rudi, K. (2002). 5'-Nuclease PCR for quantitative event-specific detection of the genetically modified Mon810 MaisGard maize. European Food Research &Technol., 214, 449-454.

Holló, J., Perédi, J. Ruzics, A., Jeránek, M. and Erdélyi, A. (1993). Sunflower lecithin and possibilities of utilization. J. Am. Oil Chem. Soc. 70, 997–1001.

Hori, H., Takahashi, Y., Takahashi, M. and Wada, Y. (2000). Detection of the *Bacillu thuringiensis* serovar *japonensis* strain *Buibui protoxin* with enzyme-linked immunosorbent assay and its application to detection of the protoxin in soil. Appl. Entomol. Zool. 35, 401–411.

James, C. (2008). Global status of commercialized biotechn/GM crops. International Service for the Acquisition of Agri-Biotech Applications (ISAAA). Available at http://www.isaaa.org/resources/publications/briefs/37/executivesummary/default.html,2008 5.12.08.

Jeleń H.H., Obuchowska M., Zawirska-Wajtasiak R. and Wasowicz, E. (2000). Headspace solid-phase microextraction use for the characterization of volatile compounds in vegetable oils of different sensory quality. J. Agric Food Chem. 48, 2360–2367.

Jensen, O. N., Podtelejnikov, A. V. and Mann, M. (1997). Identification of the components of simple protein mixtures by high accuracy peptide mass mapping and database searching. Anal. Chem. 69, 4741-4750.

Jung, M.Y. and Min, D.B. (1990). Effects of α, γ and δ tocopherols on oxidative stability of soybean oil. J. Food Sci. 55, 1464−1465.

Kaluzny, M.A., Ducan, L.A., Merritt, M.V. and Epps, D.E. (1985). Rapid separation of lipid classes in high yield and purity using bonded phase column. J. Lipid Res. 26, 135–140.

Kamal-Eldin, A., Andersson, R.A (1997). Multivariate study of the correlation between tocopherol content and fatty acid composition in vegetable oils. J. Am. Oil Chem. Soc. 74, 375−380.

Karas, M., Bahr, U., Strupat, K., Hillenkamp, F. (1995). Matrix dependence of metastable fragmentation of glycoproteins in MALDI TOF mass spectrometry. Anal. Chem. 67, 675-679.

Karleskind, A. (1996). Oils & Fats manual. Vol. 2, Intercept Ltd, Andover, UK, pp. 1572.

Keenan, R. W., Schmidt, G. and Tanuka, T. (1968). Quantitative determination of phosphatidyl ethanolamine and other phosphatides in various tissues of the rat. Anal. Biochem. 23, pp. 555–566

Koga, T., Terao, J. (1994). Antioxidant activity of a novel phosphatidyl derivative of vitamin E in Lard and its model system. Am. Chem. Soc. 42, 1291–1294.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) 227, 680-685.

Lampi, A.M., Dimber, L.H. and Kamal-Eldin, A. (1999). A study on the influence of fucosterol on thermal polymerization of purified high-oleic sunflower triacylglycerols. J. Sci. Food Agr. 79, 573–579.

Leach, J., Pyla, P., Holleschak, G.; Hileman, R., Lee, T. C., Bechtel, C., Hammond, B. and Astwood, J. D. (**2001).** Safety assessment of insect control Bacillus thuringiensis Cry3Bb1 protein for use in transgenic crops, Abstract ID 1973. Toxicologist, 60.

Lee, K. Y., Kang, E. Y., Park, S., Ahn, S. K., Yoo, K. H., Kim, J. Y., Lee, H. H. (2006). Mass spectrometric sequencing of endotoxin proteins of *Bacillus thuringiensis* ssp. konkukian extracted from polyacrylamide gels. Proteomics 6, 1512–1517.

Lipp, M., Anklam, E., Stave, J.W. (2000). Validation of immunoassay for detection and quantitation of genetically modified soybean in food and food fractions using reference materials: interlaboratory setudy. J. AOAC INT. (4), 919-927.

Liu C.-M. and Tzeng, Y.M. (2001). Quantitative analysis of parasporal crastal protein from *Bacillus thuringiensis* by Capillary electrophoresis. J. Food Drug Anal. 9, 79-83.

Lorch, A. and Then, C. (2007). How much Bt toxin do genetically engineered MON810 maize plants actually produce? Bt concentration in field plants from Germany and Spain. Available at http://www.greenpeace.de/fileadmin/gpd/user_upload/themen/gentechnik/greenpeace_bt_maize_engl.pdf, 17.02.08.

Lung, J. H. T., Male, K. B., Mazza, A., Masson, L. and Broussau, R. (2004). Analysis of insecticidal proteins from *Bacillus thuringiensis* and recombinant *Escherichia coli* by capillary electrokinetic chromatography. Electrophoresis, 25, 3292-3299.

Lutz, B., Wiedemann, S., Einspanier, R., Mayer, J. and Albrecht, C. (2005). Degradation of modified maize in the bovine gastrointestinal tract Cry1Ab protein from genetically. J. Agric. Food. Chem. 53 (5), 1453-1456.

Marquard, R., Schuster, W. and Seibel, K.H. (1977). Fettsäuremuster und Tokopherolgehalte im Öl verschiedener Sonnenblumensorten aus weltweitem Anbau. Fette Seifen Anstrichmittel., 79, 137–142.

Masson, L., Erlandson, M., Puzstai-Carey, R., Brousseau, V., Juarez-Perez, and Frutos, R. (1998). A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. Appl. Environ. Microbiol. 64:4782-4788.

Mazzara, M., Charles-Delope, C., Savini, C., Van den Eede, G. (2007). Plant & Animal Genomes XV Conference**,** Method validation for the detection of GMOs. In the context of EU regulation January 13-17.

McCann, M.C., Trujillo, W.A., Riordan, S.G., Sorbet, R., Bogdanova, N.N. and Sidhu, R.S. (2007). Comparison of the forage and grain composition from insect-protected and glyphosate-tolerant MON88017 corn to conventional corn (*Zea mays* L.). J. Agric. Food Chem. 55, 4034-4042.

Mendelsohn, M., Kough, J., Vaituzis, Z. and Matthews, K. (2003). Are Bt crops safe?. Nat. Biotechnol. 21, 1003 – 1009

Merril, L. I., Pike, O. A., Ogden, L.V and. Dunn, M. L. (2008). Oxidative stability of conventional and high-oleic vegetable oils with added antioxidants. J. Am. Oil Chem. Soc. 85:771–776.

Metcalfe, L.C., Schmitz, A.A. and Pleca, I. R. (1966). Rapid preparation of acid esters from lipids for gas chromatographic analysis. Anal. Chem. 38, 514-515.

Meydani, S.N., Lichtenstein, A.H., White, P.J. Goodnight, S.H., Elson, C.E. and Woods M. (1991). Food use and health of soybean and sunflower oils, J. Am. College of Nut. 10, 406–428.

Milne, R. and Kaplan, H. (1993). Purification and characterization of a trypsin-like digestive enzyme from spruce budworm (Choristoneura fumiferana) responsible for the activation of δ-endotoxin from *Bacillus thuringiensis*. Insect Biochem. Mol. Biol. 23, 663-673.

Min, D.B., Boff, J.F. In: C. Akoh and D.B. Min, (2001). Lipid oxidation of edible oil, Food lipid, New York, pp. 335–363.

Miranda, R., Zamudio, F. Z. and Bravo, A. (2001). Processing of Cry1Ab delta-endotoxin from *Bacillus thuringiensis* by Manduca sexta and Spodoptera frugiperda midgut proteases: role in protoxin activation and toxin inactivation. Insect Biochem. Mol. Biol. 31(12), 1155-63.

Monsanto (2002). Safety assessment of YieldGard insect-protected event MON810. Published by agbios.com as Product Safety Description. http://agbios.com/docroot/decdocs/02-269-010.pdf 17.02.09

Motoyama, A. and Yates, J. (2008). Multidimensional LC Separations in Shotgun Proteomics. Anal. Chem. 80, 7187 -7193.

Mounts, T. L. and Nash, A. M., (1990). HPLC analysis of phospholipids in crude oil for evaluation of soybean deterioration. J.OACS 67, 757–760

Muik, B., Lendl, Molina-Díaz, B.A. and Ayora-Canada, M. J (2005). Direct monitoring of lipid oxidation in edible oils by Fourier transform and Raman spectroscopy, Chemistry and Physics of Lipids 134**,** 173–182.

Muralidharan, D and Husain, S (1985) HPLC methods for determination of fat soluble vitamins in foods. Oriental J. Chem. 1, 53-60.

National Research Council (2004). Safety of genetically engineered foods. The National Academies Press, Washington DC, pp236.

Nawar, W.W. (1996). Lipids. In: O.R. Fennema, Editor, Food chemistry (3rd ed.), Marcel Dekker, New York pp. 225–319.

Nguyen, H.T. and Jehle, J.A. (2007). Quantitative analysis of the seasonal and tissue-specific expression of Cry1Ab in transgenic maize MON810. J Plant Dis Prot. 114 (2), 820-87.

Normand, L. Eskin ,N.A.M., and Przybylski, R. (2006). Comparison of the frying stability of regular and high-oleic acid sunflower oils. J. AOCS. INT. 83, 331-334.

Normén, L., Ellegand, L., Brants, H., Dutta, P. and Andresson, H.A. (2007). A phytosterols databases: fatty foods consumed in Sweden and the Netherlands. J. Food Comp. Anal. 20, 193-201.

Ohr, L.M. (2006). The (heart) beat goes on. Food. Technol. 60, (6):87–92.

Olsen, J.V., de Godoy, L.M., Li, G., Macek, B., Mortensen, P., Pesch, R., Makarov, A., Lange, O., Horning, S.and Mann, M. (2005). Parts per million mass accuracy on an orbitrap mass spectrometer via lock-mass injection into a C-trap. Mol.Cell. Proteomics 4(12), 2010-2021.

Padley, F. B., Gunstone, F. D. and Harwood, J. L. (1994) Occurrence and characteristics of oils and fats. In *The Lipid Handbook*, eds F. D. Gunstone, J. L. Harwood and F. B. Padley, 2nd Edition, 65–223. Chapman and Hall, London.

Palm, C.J., Donegan, K.K., Harris, D. and Seidler, R.J. (1994). Quantification in soil of *Bacillus thuringiensis* var. *kurstaki* δ-endotoxin from transgenic plants. Mol. Ecol. 3, 145-151.

Papac, D.I., Wong, A., Jones, A.J.S. (1996). Analysis of acidic oligosaccharides and glycopeptides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Chem. 68, 3215-3223.

Parekh, S.R. (2004). The GMO handbook genetically modified animals, microbes, and plants in biotechnology. Humana press Inc. Totowa, New Jersey, pp 374.

Park, H.W., Bideshi, D.K. and Federici, B.A. (2000). Molecular genetic manipulation of truncated Cry1C protein synthesis in *Bacillus thuringiensis* to improve stability and yield. Appl. Environ. Microbiol. 66, 4449−4455.

Pasilis, S. P., Kertesz, V., Van Berkel, G. J., Schulz, M. and Schorcht, S. (2008). Using HPTLC/DESI-MS for peptide identification in 1D separations of tryptic protein digests. Anal Bioanal Chem 391, 317.

Perlak, F.J., Fuchs, R.L., Dean, D.A., McPherson, S.L. and Fischhoff, D.A. (1991). Modification of the coding sequence enhances plant expression of insect control protein genes. PNAS. 88 (8), 3324-3328.

Phillips, K.M., Ruggio, D.M., Tovio, J.I., Swank, M.A. and Simpkins, A.H. (2002). Free and esterified sterol composition of edible oils and fats. J. Food Comp. Anal. 15, 123-142.

Pocklington, W. D. and Dieffenbacher, A. (1988). Determination of tocopherols and tocotrienols in vegetable oils and fats by high performance liquid chromatography: results of a collaborative study and the standardised method Pure Appl. Chem. 60, 877-892.

Pokorny, J., Marcín, A. and Davídek, J. (1982). Stability of phospholipid-enriched sunflower seed oil against oxidative rancidity, Scientific papers of the Prague Institute of Chemical Technology, 53, 7–23.

Ramesha, C.S. and Pickett, W.C. (1987). Fatty acid composition of diacyl, alkylacyl, and alkenylacyl phospholipids of control and arac h idonate-depleted rat polymorphonuclear leukocytes. JLR. 28, 326-331.

Ranasinghe, C. and Akhurst, J. R. (2002). Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) for detecting novel Bt toxins. J. Invert. Pathol 79: 51-58.

Rass, M., Schein, C. and Matthäus, B. (2008). Virgin sunflower oil Eur. J. Lipid Sci. Technol., 110, 618–624.

Rathjent, T. and Steinhart, H. (1997). Natural antioxidants in lipids in: Richard, E. M., Mossoba M. M. (eds) New techniques and applications in lipid analysis. AOCS press, Champaign. USA, pp 341-355.

Ridley, W.P., Sidhu, R.S., Pyla, P.D., Nemeth, M.A., Breeze, M.L. and Astwood, J.D. (2002). Comparison of the nutritional profile of glyphosate-tolerant corn event NK603 with that of conventional corn (*Zea mays* L.). J. Agric. Food Chem. 50, 7235−7243.

Romeis, J., Shelton, M. M. and Kennedy, G.G. (2008). Integration of insect-resistant genetically modified crops within IPM programs. Springer Science and business media, USA, pp. 441.

Rupérez, F. J., Barbas, C., Castro, M., Martínez, S. and Herrera, E. (1998). Simplified method for vitamin E determination in rat adipose tissue and mammary glands by high-performance liquid chromatography. J. Chromatography A. 823**,** 483-487

Sambrook, J. and Russel, D. (2000). Molecular Cloning: A Laboratory Manual (3rd ed.), Cold Spring Harbor Laboratory Press, New York.

Satue, M.T., Huang, S.W. and Frankel, E.N. (1995). Effect of natural antioxidants in virgin olive oil on oxidative stability of refined, bleached, and deodorized olive oil. J. Am. Oil Chem. Soc. 72, 1131−1137.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R. and Dean, D. H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 775−806.

Schreiber G.A. (1999). Challenges for methods to detect genetically modified DNA in foods. food cont. 10, 351-352.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68, 850-858.

Simó, C., Barbas, C., Cifuentes, A. (2005). Capillary electrophoresis-mass spectrometry in food analysis. Electrophoresis 26, 1306-1318.

Sims, R. J., Fioriti, J. A. and Kanuk, M. J. (1971). Sterol additives as polymerization inhibitors for frying oils. J. Am. Oil Chem. Soc. 49:298–301.

Sims, S.R. and Berberich, S.A. (1996). *Bacillus thuringiensis* CryIA protein levels in raw and processed seed of transgenic cotton: determination using insect bioassay and ELISA J. Econ. Entomol. 89, 247–251.

Singleton, J. A. and Stikeleather, L. F.(1995). High-performance liquid chromatography analysis of peanut phospholipids. Injection system for simultaneous concentration and separation of phospholipids. J.OACS 72, 481–483.

Smith, S.A., King, A.R.E. and Min, D.B. (2007). Oxidative and thermal stability of genetically modified high-oleic sunflower oil. Food chem. 102, 1208-1213.

Stave, J.W. (1999). Detection of new or modified proteins in novel foods derived from GMO-future needs. Food Control 10**,** 367–374.

Stave, J.W. (2002). Protein immunoassay methods or detection of biotech crops: Applications, Limitations, and practical considerations. J. AOAC INT. 85 (3), 780-786.

Syväoja, E.L., Piironen, V., Varo, P., Kouvistoinen, P. and Salminen, K. (1986). Tocopherols and tocotrienols in finished foods:oils and fats. J. Am. Oil Chem. Soc. 63, 328-329.

Takahashi, Y., Hori, H., Furuno, H., Kawano, T., Takahashi, M. and Wada, Y. (1998). Enzyme-linked immunosorbent assays for rapid and quantitative detection of insecticidal crystal proteins of Bt pesticides*.* J. Pesticide Sci. 23*,* 386–391.

Tapp, G. and Stotzky, G. (1995). Dot blot enzyme-linked immunosorbent assay for monitoring the fate of insecticidal toxins from *Bacillus thuringiensis* in soil Appl. Environ. Microbiol. 61, 602–609.

Taylor, S.L. (1997). Food from genetically modified organisms and potential for food allergy. Environmental toxicology and pharmacology 4, 121-126.

Thomson, J.A. (2006). GM crops: the impact and the potential. Collingwood, Vic CSIRO, PP.158.

United States Food and Drug Administration, Department of Health and Human Services, (2006). Plant sterol/stanol esters and risk of coronary heart disease. In: code of Federal Regulations. http://www.access.gpo.gov/nara/cfr/waisidx_06/21cfr101_06.html.

USDA (United States Department of Agriculture) (2007). Oilseeds: World markets and trade. http://www.fas.usda.gov/oilseeds/circular/2007/September/oilseeds.pdf

Van Berkel, G. J. and Kertesz, V. (2006) Automated sampling and imaging of analytes separated on thin-layer chromatography plates using desorption electrospray ionization mass spectrometry. Analytical Chemistry; 78, 4938.

Verleyen, T., Forcades, M., Verhe, R., Dewettinck, K., Huyghebaert, A. and De Greyt, W. (2002). Analysis of free and esterified sterols in vegetable oils. J. Am. Oil Chem. Soc. 79, 117-122.

Wang, S., Guo, A.Y., Zheng, W.J., Zhang, Y., Qiao, H. and Kennedy, I.R. (2007). Development of ELISA for the determination of transgenic Bt-cottons using antibodies against Cry1Ac protein from *Bacillus thuringiensis* HD-73. Eng. Life Sci. 7,149–154.

Warner, K. and Mounts, T. L. (1990). Analysis of tocopherols and phytosterols in vegetable oils by HPLC with evaporative light-scattering detection. J. Am. Oil Chem. Soc. 67, 827-831.

Warner, K. and Nelsen, T. (1996). AOCS collaborative study on sensory and volatile compound analyses of vegetable oils. J. Lipid Sci. Technol. 73,157–166.

White, P.J., Armstrong, L.S. (1986). Effect of selected oat sterols on the deterioration of heated soybean oil. J. Am. Oil Chem. Soc. 63 (4), 525–529.

Winkler, J. K.., Warner, K. and Glynn. M. T (1997). Effect of deep-fat frying on phytosterol content in oils with differing fatty acid composition. J Am oil Chem Soc. 84, 1023-1030.

Winkler, J.K., Warner, K. and Martin, T.G. (2007). Effect of deep-fat frying on sterol content in oils with differing fatty acid composition. J. Am Oil Chem Soc. 84, 1023-1030.

WU, Z.,P., Xu, B.,J. and Chen, Z.,Y. (2001). Separation and purification of *Bacillus thuringiensis* (Bt) toxic protein Cry1Ab from transgenic rice by Ion-Exchange Chromatography. Chromatogarphia 53, 571-573.

Yamamoto, T. (1983). Identification of entomocidal toxins of *Bacillus thuringiensis* by high performance liquid chromatography, J. Gen. Microbiol. 129, pp. 2595–2603.

Yanishlieva, N. and Marinova, E. (2001). Stabilisation of edible oils with natural antioxidants. Eur. J. Lipid Sci. Technol., 103, 752−767.

Zhukov, A. V. and Vereshchagin, A. G. (1976). Quantitative content of total polar lipids in soybean seeds. JOACS, 53, pp. 1–7.

Zwahlen, C., Hilbeck, A., Gugerli P. and Nentwig, W. (2003). Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the fields. Mol. Ecol. 12, 765-775.