2 Review of Literature

2.1 Genetically modified organism (GMO)

As described by Holst-Jensen (2001) genetically modified organism (GMO) is a living organism, e.g. bacteria, plant, animal, whose genetic composition has been altered by means of gene technology. The genetic modification usually involves insertion of a piece of DNA and/or synthetic combination of several smaller pieces of DNA, into the genome of the organism to be modified. This process is called transformation. These DNA pieces are usually taken from other organisms such as bacteria or virus.
A typical insert (gene construct) in a GMO is composed of three elements: 1) The promoter element functions as an on/off switch for reading of the inserted gene(s); 2) The gene(s) that has been inserted, which coding for a specific selected feature; 3) The terminator element functions as a stop signal for reading of the inserted gene(s). In addition, several other elements can be present in a gene construct, and their function is usually to control and stabilize the function of the gene, demonstrate the presence of the construct in the GMO or facilitate combination of the various elements of the construct.

The area planted with genetically modified (GM) crops, including soybeans and maize has increased worldwide in recent years. Between 1996 and 2002, it rose from 1.6×10^6 to more than 58×10^6 hectares (James, 2002) (Figure 2). Recent report released by James (2003) mentioned that worldwide plantings of biotech crops increased 12 percent in 2002. Between 5.5 million and 6 million farmers in 16 countries planted biotech seeds in 2002, up from 5 million farmers in 13 countries in 2001. By the same way biotech plantings will likely increase.

Among the other interestingly findings in this report:

- The area planted with biotech crops increased 35-fold between 1996 and 2002.
- Four countries accounted for 99% of the global biotech acreage [Argentina (23%), Canada (6%), China (4%) and the United States (63%)]. The other 12 countries accounted for the remaining 1% [Australia, Bulgaria, Colombia,

Germany, Honduras, India, Indonesia, Mexico, Romania, South Africa, Spain and Uruguay].

- The three new countries to begin planting biotech crops in 2002 were Colombia, Honduras and India. Columbia and Honduras conducted "pre-commercial" biotech plantings in anticipation of full commercial approval in 2003.

- Four crops accounted for 99% of the global biotech plantings: Soybeans (63%), maize (19%), cotton (13%) and canola (5%). Biotech squash and papaya each accounted for less than 1%.

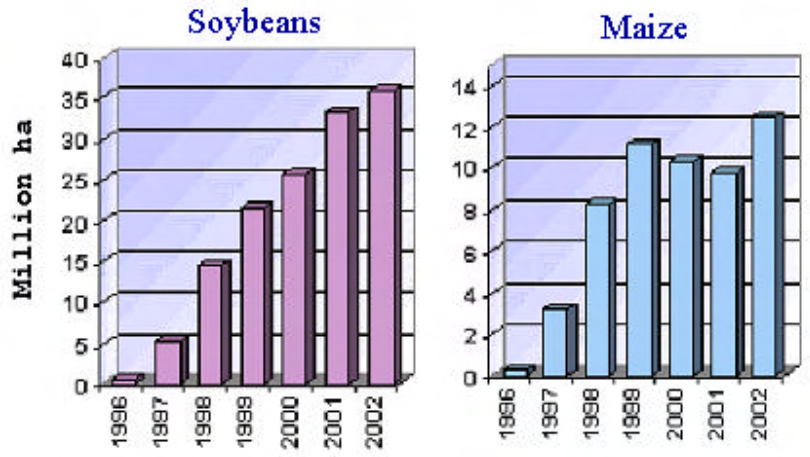

Figure 2. Worldwide production of GM soybeans and maize 1996-2002 (James, 2002)

This rapid increase has provoked an explosion of concern over the health and environmental impacts of these crops. Despite claims of safety and warnings against popular panic, public concern over GM crops has resulted in changes in their marketing, labelling, planting, and trade.

2.2 Actually present and future of genetically modified crops

Gaede (1997) and Flachowsky et al. (2002) classified edible crops developed by modern biotechnology from the nutritional point of view into two generations:

- 1st Generation: Feed plants are characterized by changed tolerance or resistance to insects, herbicides, pesticides or other influencing factors with minor changes in nutrient content (e.g. Bt-maize, Pat-maize, Roundup Ready soybeans™, etc.).

- 2nd Generation: Feeds are characterized by substantial changes in the content of valuable or undesirable major ingredients (e.g. protein, amino acids, fat, fatty acids, starch, sugars, lignin, etc.) or minor ingredients (e.g. vitamins, minerals, enzymes, antinutritive substances, etc.).

The following overview (Matissek, 1998) summarized the objects of molecular biological modifications in plants with respect to food and feed production. The objects of previous as well as prospective research work allow to distinguish 4 groups:

A) Direct increase in yield by creating high productive varieties of plants.
B) Enhancement of productivity by elimination or reduction of disturbing biotic and abiotic influences and limitations, for instance:
    - introduction of herbicide resistance.
    - control of pest and diseases.
    - reduced losses due to abiotic stresses.
C) Improvement of post harvest management and storage features.
D) Improvement of food and feed qualities by:
    - direct improvement of nutritive value through modulation of nutrient synthesis.
    - elimination of unfavorable ingredients and increase contents of health benefit substances.

2.3 Insect resistant Bt-maize

Shah et al. (1995) estimated the worldwide annual costs of chemical control of maize plant insect (European corn borer insecticides) at 3-5 billion US $. In addition, great losses in maize plant yield are still caused by insect pests. To overcome this economical as well as ecological impact of chemical insecticides and to reduce the permanent

danger of residue accumulation of insecticides, genes encoding for the insecticidal protein of *Bacillus thuringensis* (Bt) have been inserted into maize and other crop species by biotechnological methods to increase the resistant of the GM plants to insect pests (Ives, 1996).

Bt (*Bacillus thuringiensis*) is a naturally occurring soil bacterium that is found worldwide. A unique feature of this bacterium is its production of crystal-like proteins (Cry proteins) that selectively kill specific groups of insect larvae (Bt-delta-endotoxins). There are several strains of Bt, each with differing Cry proteins. Scientists have identified more than 60 Cry proteins (Gasser and Fraley, 1989). Most of the Bt maize hybrids, targeted against European corn borer (*Ostrinia nubilalis*), produce only the Cry1A(b) protein (Bt176 maize line), a few produce the Cry1A(c) protein or the Cry9C protein. Gasser and Fraley (1989) reported that all types of Cry proteins revealed no toxicity to beneficial insects, animals or human.

These crystal proteins (Cry proteins) are toxic to the European corn borer larvae. Once it ingested, the larvae own digestive enzymes activate the toxic form of the proteins in the gut. The Cry proteins bind to specific receptors on the intestinal lining epithelium and rupture the cells. Larvae stop feeding within two hours and die. Therefore, the Bt proteins are expected not to be toxic to other organisms, which have no receptors for Cry1A(b) in their guts (Hofmann et al., 1988).

Novartis (1999) has developed two different types of genetically modified maize plants (Bt176 and Bt11 maize lines). Both of these maize lines contain the truncated Cry1A(b) protein. Bt11 maize expresses the Bt protein in all areas of the modified plant, while Bt176 maize only express the Bt protein in the green tissues and pollen of the maize plant.

Frankenhuyzen (1993) reported that the *Cry* gene encoded Bt-delta-endotoxins (Cry proteins) that have been expressed in many transgenic Bt-crops to protect against Lepidopteran (Butterfly, Moth) or Coleopteran (beetle)-larvae pests are the same

proteins that have been used for over 30 years as commercial organic microbial insecticides.

Bt176 maize contains three new genes, *Cry1A(b)* from *Bacillus thuringiensis* [coding for Cry1A(b) protein], *bar* from *Streptomyces hygroscopicus* [coding for phosphinothricin-acetyl-transferase "PAT", this enzyme used during selection of successfully modified plant cells at the cultural level], and antibiotic marker gene *bla* (beta-lactamase) from ampicillin resistant bacteria (such as *E. Coli*). Although ampicillin resistant gene is also present in Bt176 maize, it is not functional in the plants, does not produce a new protein and cannot confer antibiotic resistance on the maize plant or the organisms that consume the plants as reported by Novartis (1999).

2.4 Methods for detection, screening and quantification of GMO

Commission of the European Communities (EC, 2000) recommended that food products and ingredients containing detectable amounts of DNA or proteins from genetically modified organisms have to be labelled, if their amount exceeds 1% per ingredients. In addition, the European Union has decided that additives and flavours containing GM material have to be declared on the product's label, regardless of the amount used. However, up to now no labelling is required for animal feeds. This will be considered in the near future as reported in the White Paper of Food Safety (EC, 2001). To fulfill the legislation requirements, reliable and accurate, sensitive methods for detection, quantification and monitoring of GMO are required (Buhk, 2002).

Numerous analytical methods (qualitative and quantitative) for detection and determination of GM maize and food-derived products were reviewed by Anklam et al. (2002). They reported that, at the time the new analytical issues and challenges cannot be addressed the integration of conventional and new molecular tools for plant varieties developed by genetic manipulation, which will give rise to an increasingly wide range of GMO with multiple gene constructs. It is therefore necessary to work towards the establishment of internationally accepted and harmonized detection protocols. Considering the potential economical impact of GMO on the market, it appears to be of

importance that analytical determinations of GMO presence/absence in food, feeds and agricultural products must be made by use of internationally validated and approved methodologies and standards.

Monitoring of genetically manipulated edible crops by polymerase chain reaction (PCR) was reported earlier by Meyer (1995). Pöpping and Broll (2001) mentioned that most laboratories analyze products for the presence of GM material by PCR. However, since several countries have already introduced a threshold level above which GM material requires labelling, quantitative methods are more and more in the focus of attention.

2.4.1 Detection and monitoring of GM maize

A simple, sensitive, economically and reliable protocol for extraction and purification of DNA from foods and feeds was described by Anonymous (2002). The method depends upon chemical isolation of DNA from tested material by using cetyltrimethyl ammonium bromide (CTAB). Purified DNA using CTAB-method can be amplified during different PCR techniques without inhibition and it is a good substrate for restriction endonucleases as well.

A method for the automated extraction of DNA from several maize tissues and various foods containing maize was studied by Hahnen et al. (2002). The results showed that the system provided for DNA isolation by Roche Applied Science® (MagNA Pure LC system) was capable of isolating DNA from any tested source. Furthermore, quantification of an endogenous gene by LightCycler real-time PCR revealed that the DNA was suitable in quality and quantity for multiple PCR analyses.

New varieties of Bt maize detected earlier by Hupfer et al. (1997), using polymerase chain reactions followed by southern hybridization for confirmation of the PCR products. Ehlers et al. (1997) mentioned a PCR based detection method for genetic modification of Bt176 maize line. The primers were designed to amplify the three genes present in Bt176 maize. The PCR products were sequenced to confirm their identity.

Detection methods of recombinant DNA segments introduced to genetically modified maize were demonstrated by Matsuoka et al. (2002). In this study recombinant DNA(s) introduced into the seven lines of GM maize (Bt176, Bt11, T25, MON810, GA21, DLL25 and MON802) were detected using 14 different primer pairs. They concluded that these methods are useful for fast and easy screening of GM maize crops including the unauthorized ones.

Pauli et al. (1999) and Höhne et al. (2002) carried out several PCR assays combined within one reaction tube (multiplex PCR) for the detection of GMO. The methods were tested for the detection and quantification of the four maize liens that are approved in Europe and contain the cauliflower mosaic virus promoter (35S-CaMV): Bt11, Bt176, MON810 and T25 maize.

Rudi et al. (2003) designed a novel multiplex quantitative DNA array based PCR (MQDA-PCR) for quantification of different transgenic maize in food and feed samples. The method based on a two-step PCR using bipartite primers containing a universal 5′ ‘HEAD' region and a 3′ region specific to each genetically modified construct.

Hupfer et al. (2000) described PCR based quantification methods for genetically modified Bt maize. In these approaches single and dual competitive PCR were developed. Zimmermann et al. (2000) described an event specific transgene detection in Bt11 maize by quantitative PCR at the integration site. This study reported the characterization of the genomic sequence at the 5′-site of the integrated transgenic sequence in the Bt11-maize genome using PCR. Finally, the quantitative competitive PCR (QC-PCR) system was calibrated to an equivalence point of 1% Bt11-DNA.

Holck et al. (2002) determined the DNA sequence of the 5′-flanking region of the MON810 maize line-using ligation mediated PCR. A primer probe set overlapping the junction was designed and used in a quantitative, event-specific TaqMan 5′-nuclease assay. Moreover, Hernández et al. (2003) reported a specific real-time quantitative PCR detection system for event MON810 maize (YieldGard®) based on the 3′-transgene integration sequences.

For detection of StarLink™ maize line, the currently available commercial kit as that used in this study is mainly used (GMO/Ident Kit StarLink™ Maize, GeneScan, Freiburg, Germany). Recently a qualitative and event-specific PCR real-time detection methods for event CBH-351 StarLink™ maize were developed by Windels et al. (2003). The methodology proposed envisages detection of an internal target site in the *Cry9c* gene-coding region in the mentioned maize line.

Detection of the genetic modification in heat-treated products of Bt-maize by PCR was described by Hupfer et al. (1998). Conclusion of this work confirms that the higher the processing level (heat, pressure, low pH, etc.), the more degraded the DNA. As a result the length of the PCR amplification to the detection of genetic modification in processed products is very important.

Validation of a method based on PCR for the detection of GMO in various processed feeds and foods was addressed by Lipp et al. (2001). Qualitative screening method was validated for the detection of genetically modified organisms in various processed food and feed containing cooked maize. Furthermore, Rønning et al. (2003) reported an event specific real-time quantitative PCR for genetically modified Bt11 maize line in processed food and feed materials. This study described a highly specific method for the detection of Bt11 maize in the processed food and feed.

2.4.2 Detection and monitoring of GM soybeans

Zimmermann et al. (1998) compared 9 different extraction methods for nucleic acids from soybean food samples. They concluded that with all of the extraction methods it has to be considered that they will have a certain saturation limit above which no more DNA will be extracted, independent of the amount of the starting material. Therefore, when large amounts of nucleic acids are expected, it would be advisable to break up the samples into small fractions and to extract them separately. Furthermore, these experiments have documented that extraction methods could lead to inhibitory effects in downstream processing and should therefore always be tested.

Characterization of the Roundup Ready soybean® inserts was described by Windels et al. (2001). This study dealt with the isolation and characterization of the junction between insert DNA and plant DNA in the transgenic Roundup Ready soybean® line event 40-3-2. The results established that during integration of the insert DNA several rearrangements occurred at the 3`-NOS junction and the genomic plant DNA at the pre-integration site may have been rearranged. These findings highlighted the utility of junction regions to fulfill the request for information regarding which DNA sequences have been incorporated in commercialized transgenic lines. Furthermore, the characterization of junction regions is the method of choice to support development of the methods for detection and identification of GMO.

Identification of transgenic Roundup Ready soybeans® (RRS) was discussed by Wurz and Willmund (1997). They developed a PCR in which a specific DNA sequence combination present in RRS can be detected. Zagon et al. (1998) described official methods for the detection of certain DNA sequences in Roundup Ready soybeans®. The object of these methods was to distinguish DNA sequences, which introduced by genetic engineering from the sequences, which can be introduced by other methods or can occur naturally.

Jankiewicz et al. (1999) published the official methods for the detection of genetically modified glyphosate-tolerance soybeans (Roundup Ready®) and insect-resistant Bt maize. This study aimed to settle a concept for the standardized description of the potency of a PCR setup with respect to the detection limit. The main terms used in this work were the theoretical detection limit (L_{theor}) and the practical detection limit (L_{prac}). These detection limits were determined for the example of two PCR applied for the detection of genetically modified soybeans and maize.

Brunnert et al. (2001) applied a PCR-enzyme linked immunosorbent assay (PCR-ELISA) for the CaMV-35S promoter as a screening method for genetically modified Roundup Ready soybeans®. PCR-ELISA technique is a more precise alternative to gel electrophoresis for detection of the two PCR products obtained in a quantitative competitive PCR. This method allows the detection of as little as 0.1 ng amplicon in

only 2 h. With this new technique the analyses of GM soybean as well as soybean flour with GMO contents ranging from 0.1% to 2%.

Quantitative event-specific multiplex PCR detection of Roundup Ready soybean using LabChip technology demonstrated more recently by Burns et. al. (2003). This study presented a scaling technique for the determination of GM content using multiplex PCR and quantitative analysis from data obtained using LabChip technology.

Studer et al. (1998) discussed quantitative competitive PCR for the detection of genetically modified soybean and maize. This study was developed quantitative competitive PCR (QC-PCR) systems for the detection and quantitation of Roundup Ready® soybean (RRS) and the Maximizer maize (MM). This study allowed the detection of as little as 0.1% GMO-DNA in total DNA isolated from food samples.

Vältilingom et al. (1999) demonstrated a real-time quantitative PCR for detection of genetically modified Maximizer maize and Roundup Ready soybean® in some representative foods depending up on ABI Prism 7700 sequence detector. Fluorescent dyes were chosen to coamplify total and transgenic DNA in the same tube. In addition, using of cloned DNA fragments for event-specific quantification of genetically modified organisms in pure and mixed food products was studied by Taverniers et al. (2001). An event specific PCR method for detection and quantification of genetically modified soybean was described in this article. A real-time PCR method was developed with Light-Cycler System. The Authors concluded that this approach overcome the risk of errors in the analytical results.

Terry and Harris (2001) reported a specific detection method of Roundup Ready® soybean using two different real time PCR detection chemistries. In this work development of two different quantitative event-specific assays that utilize the detection of an endogenous or species-specific gene and an event or GMO-specific sequence. One assay has been designed to use TaqMan technology for detection using the ABI Prism 7700 and the second to utilize scorpion primers for use on the Roche Diagnostics LightCycler detection system. In both cases, the Roundup Ready gene was quantified

relative to the endogenous soybean lectin gene and results expressed as the percentage of GM soybean relative to total soybean content using standards of known GM content.

A real-time PCR method to detect the genetic alteration in the Roundup Ready soybean® of Monsanto Co. was developed by Vollenhofer et al. (1999). PCR amplification of the 35S promoter/chloroplast transit peptide junction and hybridisation of the amplicon with a digoxigenin-labeled probe. Furthermore, quantitative detection of the 35S promoter and the Nos terminator using quantitative competitive PCR (QC-PCR) in GM soybeans were addressed by Hardegger et al. (1999). This study presented methods for the quantification of the GMO. The QC-PCR systems were calibrated either using Roundup Ready soybean® DNA diluted with conventional soybean DNA, or using certified commercial standards containing defined amounts of GM soybeans. Both systems presented allowed the detection of 0.1% GM soybean DNA in total DNA isolated from food samples.

Estimation of the practical detection and quantification limits in GMO analysis were studied by Berdal and Holst-Jensen (2001). An event specific real-time PCR method for detection and quantification of genetically modified Roundup Ready soybean® with TaqMan chemistry on the LightCycler, targeting the nopaline synthase terminator (3`) junction between recombinant and host plant DNA was described. This study distinguished between three types of detection and quantification limits: the absolute limits (referring to the initial number of template copies in the PCR), the relative limits (referring to the relative percentage of initial template copies of the recombinant sequence to copies of the haploid soybean genome that is detected), and the practical limits (referring to what is applicable in the PCR with the DNA that is being analyzed). The absolute detection limit was determined to be a single initial template copy, while the absolute quantification limit was determined to be approximately 30 initial template copies.

A simple procedure for quantification of genetically modified organisms using hybrid amplicon standards was recorded by Pardigol et al. (2003). More recently, recombinant plasmid standards have been used as an alternative to commercial certified reference

materials. Their production, however, necessitates cloning and microbiological facilities. This investigation described an easy and cost-effective procedure for construction of hybrid amplicon standards containing both transgene and reference gene targets in a tandem orientation on the same molecule. These standards can be rapidly produced in abundant amounts without any equipment for cloning and culturing of recombinant strains. This study presented a new validation study for quantitative analysis of Roundup Ready soybean® and the 35S-CaMV promoter in GM maize and soybean.

Block and Schwarz (2003) discussed the validation of different genomic and cloned DNA calibration standards for construct-specific quantification by real-time PCR. This study concluded that the highest accuracy was obtained by using standards of mixed genomic DNA and plasmids in genomic background. This indicates a significant influence of background DNA on the quantification of GM contamination. Since construct- specific plasmid can be produced on a large scale, stored for a long time without loss of quality and spiked in different DNA backgrounds, plasmid standards can be used in GM quantification as “gold standards”.

However, soybean oil contains traces of plant DNA due to the degradation of DNA during the processes of extraction and purification, Pauli et al. (1998) were amplified the traces of DNA in soybean oil samples using PCR technique to amplify small size DNA. Detection methods to identify modification of DNA in genetically modified soybean and maize in different processed foodstuffs were reviewed by Broll et al. (1998).

2.5 Nutritional safety assessment of GM soybeans and maize

Several international organizations have already addressed the issues associated with the safety assessment of novel foods, especially that produced by genetic modification (WHO, 1991 and 1993; OECD, 1993; EC, 1997; FAO, 1996; ILSI, 1996 and EC, 2003). It is generally agreed that such an assessment requires an integrated and stepwise, case-by-case approach and some authorities have developed decision trees (see appendix Figure 10.1) to assist in determining the extent of testing required in specific cases (UK-ACNFP, 1995 and Flachowsky et al., 2002 and 2004). Flachowsky et al. (2002) concluded that in addition to nutritional assessment of novel feeds, risk assessment for human, animal and environment should be one of the central points of the present and future studies.

2.5.1 Substantial equivalence

Substantial equivalence represents an important component in the safety assessment of foods and ingredients derived from genetically modified plants intended for nutritional consumption (WHO, 1995; FAO, 1996; OECD, 1996 and EC 2003). This concept embodies a science-based approach in which a genetically modified plant is compared to its existing, appropriate counterpart. The approach is not intended to establish absolute safety for any food or feed crops. Rather, the goal of this approach is to ensure that the transgenic plant and any substances that have been introduced into the food as a result of genetic engineering is as safe as its traditional counterpart.

FAO/WHO (2000) recorded that the concept of substantial equivalence was developed as a practical approach to the safety assessment of genetically modified foods. The Consultation agreed that substantial equivalence should be seen as a key step in the safety assessment process. The application of the concept is not a safety assessment in itself; it does not characterize the hazard, rather it is used to structure the safety of a genetically manipulated plant relative to its conventional counterpart. As a starting point, the genetically modified organism and/or foods derived from it, is compared with

its closest traditional counterpart in order to identify any intended and unintended differences, which then become the focus of the safety assessment.

The concept of substantial equivalence in safety assessment of foods derived from genetically modified organisms was discussed by Schauzu (2000). The principle of substantial equivalence is a reasonable approach to identifying differences between novel foods and their traditional counterparts. The concept of substantial equivalence was applied for the first time to a GMO in the safety assessment of the F_{lavr} S_{avr}™ tomato before it was placed on the USA market in 1994. Data collected from field trials and from analyses of the molecular and chemical composition showed that the GM tomato was equivalent to the non-modified parent plant. Furthermore, GMO containing foods have been on the market in many countries for more than five years without any reports of adverse effects on human health.

Investigations on the substantial equivalence of the transgenic Bt maize and corresponding non-transgenic line were done by Aulrich et al. (2001). The results of the analyzed maize samples as well as of the silage samples manufactured from both GM and non-transgenic maize lines illustrated substantial equivalence in all investigated ingredients, such as crude nutrients, amino acids, fatty acids, minerals and non-starch polysaccharides.

Sidhu et al. (2000) studied the composition of glyphosate tolerant (Roundup Ready) maize line GA21 in comparison with that of conventional non-modified line. Compositional analyses were conducted to measure proximate composition, amino acids, fatty acids and mineral contents of the grains. Also proximate composition, mineral contents of maize forage collected from 16 field sites of the GM maize over two growing seasons. The results demonstrated that the grains and forage of GA21 maize were comparable in their composition to that of the control maize line.

Böhme et al. (2001) analyzed substantial equivalence of GM modified sugar beets and maize, in which the glyphosate resistant (*PAT*) gene is inserted. Crude nutrients, amino acids and fatty acids profiles as well as NDF fraction of the maize grains were

determined and compared with those of corresponding non-transgenic cultivars. There were no differences detected in the crude nutrient contents including sugars and starch. Amino and fatty acids profiles as well as composition of cell wall constituents did not show any influences as well.

Comparative study on concentration of the fusarium mycotoxins deoxynivalenol and zearalenone in kernels of transgenic Bt maize hybrids and non-transgenic hybrids were performed by Valenta et al. (2001). The study demonstrated that the kernels of transgenic Bt maize hydrids were contaminated to a lesser extent with the fusarium mycotoxins (deoxynivalenol and zearalenone) than those of the standard maize hybrids.

Flachowsky et al. (2002) reviewed that apart from lower mycotoxin content in Bt-maize no significant differences in nutritional assessment and food quality between feeds from isogenic and transgenic plants and by-products from GM-crops of the first generation have been reported.

Sommer, et al. (2002) and Chrenková et al. (2002) determined the nutritional value of Roundup Ready maize and Bt-maize. They recorded that there is no marked changes could be observed in transgenic RR and Bt maize lines. Both varieties have the same nutritional value in comparison with the conventional non-transgenic maize. This was not only demonstrated for the main nutrient analyses but also for the amino acids, minerals and cell wall constituents.

2.5.2 Nutritional studies to assess the safety aspect of GM soybeans and maize

This section deals with specific issues that are frequently raised with regard to the safety of genetically modified crops. These issues include the potential for gene transfer from genetically modified plants to gut microflora and mammalian cells, degradability of DNA derived from GMO in animal gut and its metabolic fate, the safety of antibiotic resistance genes as markers for the selection of genetically modified plants, and the assessment of the potential allergenicity of genetically modified foods that may be caused by novel gene products (novel proteins) which present in these foods.

2.5.2.1 Allergenicity

Food allergies are adverse reaction to harmless food or food component that involves an abnormal response of the immune system to specific protein(s) in foods. As demonstrated by Sampson and Burks (1996), true food allergies may involve several types of immunological responses (humeral or cell-mediated reactions). The most common type of food allergies is mediated by allergen-specific immunoglobulin E (IgE). IgE-mediated reaction is known as immediate hypersensitivity reactions because symptoms occur within minutes to a few hours after ingestion of the offending food. IgE-mediated reactions can occur to other stimulants such as pollens, mould spores, animal danders, insect venoms and other environmental stimuli. Although food allergies represent a small fraction of all allergic diseases, it must be addressed during safety study of foods.

Metcalfe et al. (1996) developed a decision-tree approach to assess allergen derived from GM food or feed. This strategy focuses on the source of the gene, the sequence homology of the newly introduced protein to known allergens, the immunochemical binding of the newly introduced protein with IgE from the blood serum of individuals with known allergies to the source of the transferred genetic material and the physicochemical properties of the newly introduced protein. Taylor (1997) reported that the genetic modification of the food and feed crops results in the introduction of new proteins into the food and feed plant. Therefore, the safety of the newly introduced proteins must be assessed and the assessment of the allergenicity of the newly introduced protein should focus on the source of the gene.

To improve the nutritional quality of soybeans by increasing methionine content, methionine-rich *2S albumin* gene from the Brazil nut (*Bertholletia excelsa*) has been introduced into transgenic soybeans. Since the Brazil nut is a known allergenic food, Nordlee et al. (1996) aimed to assess the allergenicity of the new line of soybeans. This study demonstrated that the new transgenic soybean contains the allergenic protein and concluded that the allergenic protein(s) from a food known to be allergic can be transferred into another food by genetic engineering.

FDA/CDC (2001) initiated blood serum testing to investigate the allergenic potential of the Cry9c protein in StarLink ™ maize in human using Enzyme Linked Immunosorbent Assay (ELISA). Blood samples from 12 of the 48 individuals with self-reported symptoms were tested for IgE and IgG. These 12 persons had symptoms of typical allergic reaction. The blood tests were designed to determine if their symptoms were an allergic reaction to StarLink™ maize in taco shells or other maize containing foods. ELISA was developed to detect IgE and IgG antibodies to the Cry9c protein. The negative control group was people who had been selected randomly from the US population and whose blood samples were collected prior to 1998 (before approval of StarLink™ maize for feed). In addition, two other groups were also tested: a) samples from people who tended to be allergic to many different types of allergens and b) maize allergic individuals. Persons with a clinical food allergy to the Cry9c protein showed a significant increase in Cry9c-specific IgE antibody amount compared to the control populations. The range of reactions to IgE in the people who tended to be allergic to many different types of allergens and maize allergic individuals did not differ significantly from the control group. The authors concluded that these people were not allergic to the Cry9c protein in StarLink™ maize and that there is reasonable evidence to conclude that either the small quantities of Cry9c protein found in food is unlikely to lead to an allergic reaction, or the biochemical properties of the protein (Cry9c) is unlikely to act as a food allergen.

2.5.2.2 Feeding value and animal performance

The possible unexpected dietary effects of Bt176 maize on broiler performance and were studied by Aeschbacher et al. (2002a). Feeding experiments with Bt176 maize and its isogenic control were performed. Two kinds of feed with a portion of either 60% conventional or Bt176 maize were fed to broiler chickens for 39 d. Body weight and feed consumption were recorded weekly to calculate performance parameters. The results revealed that there was no significant difference between the two groups concerning the performance parameters measured.

The nutritional safety of maize line GA21 (Roundup Ready maize) was evaluated in a broiler feeding study conducted with rapidly growing broiler chickens, at a dietary concentration of 50-60%. Two-day-old broiler chickens were sexed and placed into pens (8 birds/pen; 5 pens/sex). Feed and water were provided *ad libitum*. Chickens were weighed by pen at study start (day 0) and study termination (day 38 for males and day 40 for females). The average body weight/pen and for each treatment group by sex was calculated. The average feed conversion was calculated for the entire duration of the study. At study termination, abdominal fats were collected from each chicken. The abdominal fats from all chickens within a pen were combined and weighed. The results showed that there were no significant differences in body weight, feed efficiency and abdominal fat weights between chickens fed with GA21 grains or with parental control grains (Sidhu et al., 2000).

Taylor et al. (2003) carried out 2 experiments to compare the nutritional values of GM maize with the non-transgenic or commercial maize in broiler nutrition. Roundup Ready maize fed to broiler chickens for 42 d (experiment 1) and the birds of the experiment 2 were fed diet contained insect protected maize event MON810 mixed with Roundup Ready maize. The broilers were weighed at day 0 and day 42 (experiments termination). The average feed conversion was calculated as well as carcass measurements for all birds were taken at the end of the experiments. Final live weights and feed conversion were not significantly different ($P>0.05$) across all treatments in both experiments in comparison with the groups fed non-transgenic or commercial maize. Thigh and wing weights were not affected by diets as well. The Authors concluded that broilers performed consistently and had similar carcass yields and compositions when fed diets containing Roundup Ready maize or Roundup Ready maize mixed with insect resistant MON810 maize.

A 38 d feeding study evaluated whether standard broiler diets prepared with transgenic Bt176 maize had any adverse effects on broiler chickens as compared to diets prepared with non-transgenic control maize. No statistically significant differences in survival or body weight were observed between birds reared on mash or pelleted diets prepared with transgenic and similar diets prepared using control maize. Broilers raised on diets prepared from the transgenic maize exhibited significantly better feed conversion ratio

and improved yield of the pectoralis minor breast muscle. Although it is not clear in this study whether this enhanced performance was attributed to the transgenic maize or due to possible slight differences in overall composition of the formulated diets as concluded in this study. It was clear that the transgenic maize had no deleterious effects on broiler performance (Brake and Vlachos, 1998).

Chesson and Flachowsky (2003) discussed and summarized the use of transgenic plants in poultry nutrition. They concluded that comparative feeding studies with broilers and layers in which conventional maize (50 to 78%) or soybeans (27%) were replaced in feeds by transgenic varieties, have failed to show differences of any significance in production parameters. This data indicates that feeding studies with target livestock species contribute very little to the safety assessment of crops engineered for input traits that have little or no detectable effects on chemical composition. However, comparative growth studies made with broiler chicks, particularly sensitive to any change in nutrient supply or the presence of toxic elements in their feed, can be used to screen for any unintended adverse consequence of the recombinant event, which cannot be detected by compositional analyses.

Recently Halle et al. (2004) fed GM-maize (Bt176) to breeder quails till the fourth generation compared with the isogenic maize line. The conclusion of this study revealed that there is no significant difference (from 1st to 4th generation) between the group fed diet contained Bt176 maize and the control group reared on conventional maize concerning feed intake, laying intensity and hatchability percent.

Feed conversion of two feeding mixtures with 11% either of Roundup Ready maize or conventional non-transgenic maize intended for fattening rabbits. Rabbits fed *ad libitum*, consumption of feed and live body weight were recorded. Rabbits were slaughtered at 70 days age when they achieved 2.5 kg live weight. The muscles longissimus dorsi was homogenized and analyzed for individual nutrients. The results demonstrated that all parameters tested did not differ significantly. Also the results showed the suitability of 11% RR maize in feed mixture fit for production of rabbit meat of good quality (Chrastinová et al., 2002).

Aulrich et al. (2001) in a balance study using Bt-maize studied the parameters of nutritional physiology such as digestibility and energy content of transgenic Bt maize in comparison with the control non-modified maize line for poultry, pigs and ruminants (fattening bulls). The results were not influenced by genetic modification of maize. The determined values for the digestibility and the energy contents for poultry, pigs and fattening bulls were not affected by the used maize variety.

Flachowsky and Aulrich (2001a and b) summarized some studies on the nutritional assessment of feeds from genetically modified organism. Digestion and feeding experiments were carried out with broilers (Bt-maize), layers (Bt-maize and Pat-maize), pigs (Bt-maize, Pat-maize, Pat-sugar beet, soybeans), sheep (Bt-maize silage, Pat-maize silage), growing bulls (Bt-maize silage) and fistulated cows (Bt-maize silage). They concluded that up to now, no significant differences in nutritional assessment between feeds from isogenic and transgenic plants of the first generation were found. Furthermore, recombinant plant DNA constructs were not detected in animal tissue samples.

Clark and Ipharraguerre (2001) reported that maize grains, whole plant green chop maize, maize silage, maize residue, soybeans and soybean meal from the current genetically enhanced plants have been fed to chickens, sheep, beef cattle and dairy cows, and compared with feeds produced from isolines of non genetically modified plants. Results from 23 research trials indicate that genetically manipulated maize and soybeans, which are currently available on the market, have similar feeding values for livestock and did not influence the animal growth rate or the production.

Böhme et al. (2001) studied the digestibility of PAT-sugar beets and maize grains for pigs. This study concluded that the digestibility of GM sugar beets and maize did not demonstrated meaningful differences when compared to the corresponding non-transgenic cultivars. Furthermore, digestibility of sugar beet roots and sugar beet top silage for ruminants proved to be also in the scope of natural variance. As the digestibility of the macronutrients remained unaffected, the *PAT* gene introduction into both crops did not showed an influence on the energetic feeding value.

Investigations on genetically modified Bt maize in pig nutrition in comparison with the non-modified parental maize line were carried out by Reuter et al. (2001 and 2002). The objective of this study was to determine the composition and the nutritional value of parental and transgenic maize used. Diets containing 70% maize to attain a high effect level were fed to pigs. A balance study with 12 pigs was designed and the collected faeces were analyzed for crude nutrients. All obtained results in this study were comparable for both maize lines.

Beever and Phipps (2001) reported that studies in which GM feeds have been compared with non-GM feeds in diets for ruminants and non-ruminants have reported no adverse effects on animal health and performance or product quality. To date, no significant deleterious effects have been confirmed.

2.5.2.3 Degradation and metabolic fate of feed ingested DNA

FAO/WHO (2000) discussed the transfer of plant DNA into microbial or mammalian cells under normal circumstances of dietary exposure.
They mentioned that such transfer would require all of the following events to occur:

- The relevant gene(s) in the plant DNA would have to be released, probably as linear fragments
- The gene(s) would have to survive nucleases in the plant and in the gastrointestinal tract of the host
- The gene(s) would have to compete for uptake with dietary DNA
- The recipient bacteria or mammalian cells would have to be competent for transformation and the gene(s) would have to survive their restriction enzymes
- The gene(s) would have to be inserted into the host DNA by rare repair or recombination events.

The report concluded that the plant DNA couldn't be transferred and stably maintained in mammalian cells. There is additionally no evidence that intact genes from plants can be transferred to and be expressed in mammalian cells.

It should be noted that the vast majority of known bacteria are not naturally transformable and there is as yet no evidence of transfer to and expression of plant genes in bacteria under natural conditions. Transfer has been observed under laboratory conditions, but only if homologous recombination is possible (Nielsen et al., 1998).

Horizontal transfer of DNA from GM crops to bacteria and mammalian cells was studied by Thomson (2001). He concluded that the chances of horizontal transfer of antibiotic resistance marker genes and increasing the fitness of any bacteria acquiring the genes from a GM plant are remote. There is also no known risk associated with the possibility that mammalian cells could be transformed with these genes or other fragments of recombinant DNA. However, the incidences of horizontal gene transfer are very rare, the genes introduced into a GM plant should be focus of biosafety considerations.

The fate of an antibiotic resistance marker gene (ampicillin resistance), incorporated into transgenic maize when fed to chicks was addressed by Chambers et al. (2002). Plant derived markers were found in the crop contents of five birds fed transgenic maize and in the proventriculus and gizzard contents of two birds out of the five. The plant-derived marker gene was not found in the intestines. The survival of the antibiotic resistance marker gene mirrored that it survives no better than other DNA and indicating that it is very unlikely that bacteria in the gut of chickens will be transformed to ampicillin resistance when the birds are fed transgenic maize.

Evaluation of the suitability of Bt176 maize as a GM feed in poultry nutrition was mentioned in another study by Aeschbacher et al. (2002b). This investigation aimed to study the way of inactivation of nucleic acids in the digestive tract and their possible transfer to the meat and organs of chickens. Feeding experiments with Bt176 maize and its isogenic control were performed. Two kinds of feed with a portion of either 60% conventional or Bt176 maize were fed to broiler chickens for 39 d. To follow up the degradation of the nucleic acids, three times two broilers of each treatment were slaughtered and tissues from organs, blood and muscle as well as samples from the digestive tract were collected. The maize specific gene *ivr* (226 bp) could be detected in

maize, feed, digesta samples until small intestine, spleen and liver. These maize fragments could not be detected in the caeca, meat or in the blood. The Bt-specific gene bla (479 bp) could only be detected until the crop. Bt specific gene was not detected in organs, meat or blood. In conclusion, a transfer of DNA fragments into the animal products is possible in tiny amounts, while the transfer of transgenic DNA fragments into organs and tissues is extremely improbable.

The fate of ingested recombinant plant DNA in farm animals (cattle and chickens) was studied by Einspanier et al. (2001). Cattle and chickens fed a diet containing conventional maize or recombinant Bt-maize. The data indicated that only short DNA fragments (<200 bp) derived from plant chloroplasts could be detected in the blood lymphocytes of cows. In all other cattle organs investigated (muscles, liver, spleen, kidneys) plant DNA was not found, except for faint signals in milk. Furthermore, Bt-gene fragments were not detected in any sample from cattle. However, in all chicken tissues investigated (muscle, liver, spleen, kidneys) the short plant chloroplast gene fragment was amplified. In contrast to this, no foreign plant DNA fragments were found in eggs. Bt gene specific were not detected in any of these poultry samples investigated.

Schubbert et al. (1997) studied the fate of food ingested foreign DNA in mice. Phage M13mp18 DNA as a test molecule devoid of homology to mouse DNA was pipette-fed or added to the food supply of mice. The fate of this foreign DNA in the animals was followed by several methods. In 84 animals, fragments of phage DNA were detected in the contents of the small intestine, the caecum (until 18h), the large intestine and in the faeces. In 254 animals, fragments of phage DNA of up to 976 bp were found in blood 2-8h after feeding. In buffer fed control animals (control) phage DNA could not be detected in any sample. Also the foreign DNA detected in the peripheral leukocytes between 2-8h, and in spleen or liver cells up to 24h after feeding but not later.

Aulrich et al. (2002) reported some studies intended to investigate the possible transfer of plant DNA from Bt maize and Roundup Ready soybeans (RRS) into animal tissues and the derived food products for human consumption as well. Poultry and pigs were fed diets containing Bt maize; cattle were fed Bt maize silage. Two other experiments

were carried out with pig fed either a diet containing RRS or pat maize. After the feeding trials all animals were slaughtered. Blood and tissue samples were collected. Samples of pig gastrointestinal tract contents were also collected. All collected samples from the different study were subjected to DNA investigation in trials to detect the plant DNA using specific primer systems and PCR techniques. In all investigated tissues and blood no fragments of recombinant DNA were detected. The same results were obtained for milk and eggs. In contrast, plant chloroplast DNA fragments (199 bp) were amplified in muscle, liver, spleen and kidney of poultry. Degradation of the recombinant DNA was observed in the gastrointestinal tract contents collected from pigs, and the degradation depends up on the time of the last GM feeding.

Degradation and possible carry over of feed DNA monitored in poultry and pigs by Klotz et al. (2002). After feeding pigs with conventional and recombinant Bt maize, blood and different body samples were investigated using PCR to detect chloroplast gene of different length (199 bp and 532 bp), a maize specific zein and a specific transgene present in Bt maize (Cry1A). A time dependent degradation of feed DNA in the gastrointestinal tract of pigs was analyzed within the juices from stomach, duodenum, jejunum and ileum. Short chloroplast DNA fragments (199 bp) could be successfully amplified from the intestinal juices up to 12h after the last feeding. In contrast, chloroplast specific DNA was not found in any pig organ investigated. Specific gene fragments from the transgenic Bt maize were never detected in any pig sample. Furthermore, a field study examining supermarket poultry samples (leg, breast and wing muscle, stomach as well) led to frequent detection of the short chloroplast DNA fragment (199 bp).

Safety issues associated with the DNA in animal feed derived from GM crops were reviewed by Beever and Kemp (2000). They concluded that there is a growing body of scientifically valid information available that indicates no significant risk associated with the consumption of DNA or the resulting proteins from GM crops. Furthermore, based on the safety analyses of each new crop, consumption of milk, meat and eggs produced from animals fed GM crops should be considered to be safe as traditional practices.

Reuter and Aulrich (2003) followed the passage and metabolic fate of feed ingested DNA in pig bodies during fattening period. Feed used in this trial containing transgenic Bt maize or non-transgenic maize. They concluded that feed ingested DNA is partially resistant to the mechanical and enzymatic activities of the gastrointestinal tract and is not completely degraded. Small DNA fragments derived from feedstuffs can pass the gut wall and enter organs and tissues of pigs. The transit of feed ingested DNA into organs and tissues beyond gut wall epithelia takes place independently of the feed source, whether transgenic or non-transgenic.

Different animal experimental trials fed on different GM crops or feed products were recently summarized by Flachowsky (2004). These studies reviewed that the using of Bt-maize, Pat-maize, pat-sugar beet and Gt-soybeans in feeding of pigs, sheep, dairy cows, beef cattle, broiler and laying hen and impact of these crops on the food quality for human consumption. The results obtained from all the trial up to date have no deleterious effect on animal health and production as well as the quality of animal origin food for human consumption.

Beever and Phipps (2001) reviewed that the impact of feed processing, including grinding, milling, heating and steam pressure on plant DNA integrity demonstrated that heat (>95°C) and high pressure substantially disrupt plant DNA but grinding has no effect (heated or pressure treated feeds minimize the exposure of animals to contact plant DNA). Occasional reports of the existence of foreign DNA or protein fragments in the tissues or products of animals receiving GM feeds remain some cause for concern, but there are also many studies in which the presence of novel DNA and proteins has not been established.

Detection of the genetic modification in silage obtained from insect resistant Bt maize was discussed by Hupfer et al. (1999). The detectability of the transgene was shown to be dependent on the length of the genomic target sequence chosen for amplification by PCR. The effect of maize DNA degradation in the course of ensilage on the detectability of target sequences was demonstrated in the model experiments applied in this study.

Berger et al. (2003) studied the influence of processing of isogenic and transgenic rapeseed on DNA-degradation. Both rapeseed varieties were treated by four different ways during manufacture process. Samples were collected at all steps of processing. DNA was extracted and PCR was applied using different specific primer pairs revealed different DNA size after PCR to detect degradation of DNA. This study demonstrated that the degradation of DNA depends on the processing conditions. Mechanical treatment has no influence on the degradability of DNA, while the processes of extraction and toasting were causing highly fragmented DNA. The results of this study confirm that feed or food processing minimize the exposure of animals and human to intact plant DNA.

Recently Aulrich et al. (2004) studied the influence of ensiling on the DNA-degradation in isogenic and transgenic corn. The author concluded that forage conservation by ensiling caused degradation of DNA to small fragments of about 200 bp.

2.6 Aims of the present investigations

2.6.1 Screening and monitoring of GM soybeans and maize in Egypt

Although the detection methods were developed on different levels qualitative, quantitative, national or international validated and non validated, the detection methods and monitoring programmes of GMO in Egypt and other developing countries are not established and there is no concern about GM crops in such countries.

However, Egypt depends on the imported soybeans and maize for both human and animal nutrition, these crops are only evaluated for nutrients content and mycotoxin contamination, but not for the presence of GM constructs. As a result, there is no information available on the presence or absence of GM crops on Egyptian market. The first objective of the present work aimed to answer the following question:

- Is the Egyptian market free from GM soybeans and/or maize crops?

2.6.2 Nutritional safety assessment of Bt-maize (NX 6262-Bt176)

As a result of high production and worldwide distribution of insect resistant maize (Bt-maize), different new hybrids are generated. Bt176 hybrid NX 6262 is a new Bt176 maize hybrid produced by Novartis.

2.6.2.1 Substantial equivalence

As recommended by many authorities (WHO, 1995; OECD, 1996 and FAO, 1996), nutritional safety assessment of any new GM crop requires an thoroughly investigations case-by-case approach and the substantial equivalence represent an important component in the safety assessment of GM plants intended for nutritional consumption. This concept recommended a scientific approach in which the newly produced GM plant must be compared to its conventional counterpart.

The second aim of the present study is to investigate the substantial equivalence of transgenic maize Bt176 hybrid NX 6262 in comparison with the non-transgenic counterpart.

2.6.2.2 Impact of feeding NX 6262-Bt176 maize on broiler health and performance

Piva et al. (2001) observed a higher significant live weight gain ($P<0.05$) in the broiler fed diet contained insect protected (MON810) compared to the control group fed diet contained the conventional maize line. They concluded that a lower mycotoxin content in the GM maize compared to the conventional maize used in the diet of the control group might resulted in better weight gain.

The third goal of the present study is to investigate the influence of transgenic maize (NX 6262-Bt176) on broiler health and performance concerning feed intake, body weight gain and feed conversion. An experimental diet containing NX 6262-Bt176 or a control diet containing the non-modified maize grains were fed to broiler chickens for 35 days. Performance parameters were recorded and compared. Blood parameters and some serum enzyme values were investigated to demonstrate broiler health in both groups.

2.6.2.3 Degradation of maize DNA (GM and conventional) in broiler GIT and metabolic fate of feed-ingested DNA in some tissues and organs

At the present time there is limitation in the literature which deal with the digestibility, persistence of DNA in gut and the metabolic fate of the ingested feed-DNA in tissues and organs of livestock. Generally farm animals ingested daily a considerable amount of foreign DNA via feed. The possibility of DNA transfer from feed crops (GM or conventional) into animal tissues, organs and animal derived foods can be expected, but the significance of this process is still unclear.

The following questions arise especially during rearing broiler on GMO containing diet:

- What about the degradability of the maize ingested DNA in broiler GIT? Is there difference between GM-maize and conventional maize DNA degradability?
- Can ingested feed DNA be detected in broiler GIT after feed withdrawal?
- Can ingested feed DNA pass broiler gut barrier and enter broiler blood, tissues and organs or is it not digested and shaded via faeces?
- If the feed-DNA absorbed from the broiler gut, is there any difference in the metabolic fate of ingested GM-DNA and conventional one in blood, tissues or organs?

Using different molecular biology methods and collection of different samples according to time programme after feed withdrawal in the broiler feeding experiment with GM maize (NX 6262-Bt176), the present study try to demonstrate and discuss the answers of these questions.