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Quality control for foods produced by genetic engineering

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ABSTRACT

This work aimed to study the following points: firstly to monitor the incidence of genetically modified soybean and maize especially used for human nutrition purposes in Egypt. The results clearly demonstrate the incidence of genetically modified maize and soybean on the Egyptian food market. Furthermore, the existence of StarLink™ maize in the food chain supplies evidence for uncontrolled arrival of even unauthorised GMOs for food use in Egypt.

The second point was the development of a construct-specific, qualitative detection method for two genetically modified potato *Spunta* lines, G2 and G3. First the DNA was extracted in sufficient amount and quality from raw as well as processed material. Secondly - based on information about the newly inserted sequence - primer systems were selected by employing computer software. Thirdly, the primer system was carefully tested for specificity and sensitivity with isolated DNA from potato (GM and non-GM) and other plants and the PCR conditions have been optimised. The results indicate that a high sensitivity 0.001% (w/w) GM-potato DNA mixed with non –GM potato DNA for raw potato respectively 1% for processed material was achieved. A highly specific method for the detection of raw and processed GM potato *Spunta* line G2 and G3 is now available.

Thirdly, studies on safety assessment (substantial equivalence) of GMO potato *Spunta* were conducted. The following main components were investigated: 1) chemical composition, natural occurring toxicants and anti-nutrient compounds of genetically modified versus conventional potato. 2) evaluation of the nutritional and biological equivalence of genetically modified potato *Spunta*. The results indicate that there were no significant differences in the levels of glycoalkaloids, phenol and protease inhibitor activity in physiological studies with rats. Health and metabolic processes of the rats were not affected by the added transgenic potatoes (whether line G2 or G3) to the diets.

Fourthly: study on the influence of GMO containing diets on health and performance of broiler (chicken) and the fate of DNA in the gut and tissues. The results indicated that, feed ingested DNA is partially resistant to the mechanical, chemical and enzymatic activities of the broiler gastrointestinal tract and is not completely degraded. Modified and non-modified potato *Spunta* DNA was comparable during feed passage in the broiler gastrointestinal tract. Small DNA fragments derived from plant feed (chloroplast DNA) can pass the gut epithelium and enter some organs of broiler chickens. Modified constructs from GM potato Spunta lines G2 and G3 fragments DNA could not detected in any investigated tissue samples.

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ABBREVIATIONS

ABSP	Agriculture Biotechnology support Project
ACNFP :	Advisory Committee on Novel Foods and Processes
AGRI	Agriculture Genetic Engineering Research Institute
APHIS	Animal and Plant Health Inspection Service
BBEP	Biotechnology, Biologics and Environmental Protection
CaMV 35S	Cauliflower Mosaic Virus
CFIA	Canadian Food Inspection Agency
CFR	Co-ordinated Framework for Regulation
CFSAN	Center for Food Safety and Applied Nutrition
CONABIA	Commission on Agricultural Biosafety
CP4-EPSPS	5-enolpyruvylshikimate-3-phosphate synthase from Agrobacterium sp. strainCP4)
CTAB	Cyltrimethylammonium bromid
CVM	Center for Veterinary Medicine
DNMA	National Directorate of Agri food Markets
EC	European Commission
EFTA	EU regulations in the framework of the European Free Trade Area
ELISA	Enzyme-linked immunosorbent assay
EP	European Parliament
EPA	Environmental Protection Agency
ERMA	Environmental Risk Management Authority
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
GEAC	Genetic Engineering Approval Committee
GEF	Global Environment Facility
GEFs	genetically engineered foods
GIT	gastrointestinal track
GMAC	Genetic Manipulation Advisory Committee
GMM	genetically modified maize
GMO	genetically modified organism

GMS	genetically modified: soya
GTA	Gene Technology Act
GTCCG	Gene Technology Ethics Committee
GTEC	Gene Technology Ethics Committee
GTTAC	Gene Technology Technical Advisory Committee
ICGEB	International Centre for Genetic Engineering Biotechnology
IICA	The Instituto Interamericano de Cooperacio´ n para la Agricultura
ILSI	International Life Sciences Institute
INASE	National Institute of Seeds
IRMM	Institute for Reference Materials and Measurements
ISNAR	International Service for National Agricultural Research
LDL	low density lioprotein
LMOs	Living modified organisms
LOD	Limit of detection
MAFF	Ministry of Agriculture, Forestry and Fisheries
ME	metabolisable energy
MHLW	MHLW :Ministry of Health, Labour and Welfare
MHW	Ministry of Health, Labour and Welfare
NAS	National Academy of Sciences report
NFE	nitrogen free extract
Nos-3 terminator	(nos) 3 /terminator :Agrobacterium tumefaciens nopaline synthase
NPTII	Neomycinphosphotransferase
NRC	National Research Council
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
PAT,	Phosphinotrycinacyltransferase
PBO	The Plant Biosafety Office
PCR	Polymerase Chain Reaction
QC-PCR	quantitative competitative PCR
RH	relative humidity
SAGENE	South Africa Committee for Genetic Modification
SCBD	Secretariat of the Convention on Biological Diversity

SENASA	National Agri food Health and Quality Service
STAFF	Society for Techno-Innovation Agriculture, Forestry and Fisheries
UNEP	United Nations Environment Program.
USDA	United States Department of Agriculture

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1. INTRODUCTION

1.1 Definitions

Genetic engineering is defined as the genetic modification of organisms by techniques by which the genetic material of plants, animals, micro-organisms, cells and other biological units are altered in ways or with results that could not be obtained by methods of natural breeding or natural recombination. Apart from "classical" recombinant DNA techniques based on vector systems, micro injection or particle gun bombardment is applied to transform genetic material.

A genetically modified organism (GMO) is defined as an organism produced from genetic engineering techniques that is capable to transport and transfer genetic material. In case the gene or a gene cassette is successfully inserted, the term transformation line or transformation event is frequently used. “Living modified organisms” (LMOs), “genetically engineered foods” (GEFs) and “transgenic crops” are other terms often used in place of GMOs. Bacteria, fungi, viruses, plants, insects, fish and mammals are examples of organisms that have been artificially changed or altered in order to modify physical properties or capabilities.

1.2 The increasing role of genetic engineering in agriculture

Genetic engineering is being used in medicine, fuel production, agriculture and food production as well as in criminal science. On the field of agricultural biotechnology, genetic engineering has opened new ways in the development of plants (e.g. food, feed, fibre, forest production) with novel traits but also in animal engineering for human health purposes (e.g. production of pharmaceuticals). By applying gene modification, potential gains for the world's agriculture are the production of higher yields needing less amounts of herbicides and pesticides **(Anklam et al., 2002; Kohnomurase et al.; 1995)**. Furthermore, it offers a tempting new tool to develop traits sustaining harsh environmental conditions (e.g. arid - or acid areas; heavy metal/salt contamination) and needing less fertilizers **(Reilly, 1996)**. Additional goals are to increase and improve the nutritional value and quality of crops **(Herbers et al., 1996; Spencer et al., 2000; Denbow et al., 1998; Nordelee et al., 1996; Yang et al., 2002; Wyatt et al., 2002)** making use of the most outstanding advantage of biotechnology: it provides an accurate and precise method to insert a desired trait into an organism by selecting single genes and their products. In contrast, traditional plant breeding, results in the exchange of numerous genes (**Taylor, 2002**) and hence less predictable effects. Apart from economic advantages for the farmer, it is discussed that "green" engineering provides a means for enhancing world's crop

production and thus might contribute to solve conflicts derived from an increasing world population, stagnant food production in certain areas and dependency on food imports.

1.3 Plants with novel traits

Up to now, all genetically modified agricultural products which are commercialised for nutrition purposes are of plant origin. Starting from 1994, since the FlavrSavr™ tomato entered the market in the USA, more than hundred transgenic plant lines (transformation events) have been approved for food and/or feed use by competent authorities world-wide. The global surface of transgenic crops has grown 35 fold from 1.7 million hectares in 1996 to 58.7 million hectares in 2002. It is estimated that in 2002 about 6.0 million farmers in 16 countries are cultivating genetically modified plants **(Clive, 2002),** above all oilseeds like cotton, maize, oilseed rape (canola) or soybean. But also perishable vegetables like chicory, cucumber, papaya, tomato and potato with resistance to virus and other diseases are of increasing interest especially for developing countries. Meals, fats/oil, proteins or starch produced from genetically modified maize or soybean can be found in thousands of products like bakeries, snacks and sweets, dietary and supplementary foods, infant formula, sauces, soups and livestock feed.

1.4 Types of modification

Dependent on their target, roughly three types of modifications in plants can be distinguished: a) resistance to insects (or virus or fungi), b) herbicide tolerance and c) altered "functional" properties to improve nutritional or processing related properties (e.g. altered carbohydrate or fatty acid pattern). All types may occur in combination. The functional basis for a genetic modification frequently is, that it leads to changes in the activity or introduces selected enzymes engaged in specific metabolic pathways (**Preach et al; 2002)**. By this way, for example, herbicide tolerance is achieved by the expression of specific enzymes which are degrading poisonous glufosinate in weed killers like Basta® (Phosphinotrycinacyltransferase, PAT) or compensating for the blocking effect of glyphosate on aromatic amino acid metabolism in herbicides like Roundup™ (CP4-EPSPS). On the other hand, modified genes from *Bacillus thuringiensis* strains which are coding for a toxic, crystalline protein are commonly introduced to express an endogenous repellent to specific insects of the *Lepidoptera* or *Coleoptera* class.

1.5 Outlook

Whereas the first generation of genetically modified crops for human nutrition is characterized mainly by pesticide or herbicide resistance, there is a second generation of genetically modified plants for food production coming up with the aim to improve health properties, food functionality or even taste. These include for example modified rice varieties, one of which expressing beta-carotene ("Golden Rice") which was specifically designed to target Vitamin A deficiency, a cause of blindness among people living in developing countries (**Ye et al., 2000).** Other examples are oil seeds in which the fatty acid profile was changed either by traditional mutational techniques or recombinant DNA techniques. Recently, canola (oilseed rape) and soybean oils with combined low levels of saturated fatty acids and increased oleic acid, have been produced with the objective of lowering total and low density lipoprotein (LDL) cholesterol levels, one of the risk factors for cardiovascular diseases, while at the same time enhancing the functionality of the oil. Biotechnology is also discussed as a potential way to eliminate allergens from known allergenic foods **(et Matsuda al., 1993).** Also in animal nutrition functional traits are under investigation. Forage with increased content of free carbohydrate and maize with low phytate content **(Herbers et al., 1996**) was developed to improve nutritional properties. Further issues which are tackled by bioengineering are for example: increasing the bioavailability of phosphate in feed for chickens and pigs **(Denbow et al., 1998; Spencer et al., 2000)**, increasing the level of ∝- lactoalbumin in maize kernels, enhancing the bioavailability of calcium stocks in plants **(Wyatt et al, 2002)** or increasing the level of essential amino acids (Lysine, Methionine, Tryptophan).

These examples highlight the potential of foods and feed with modified nutritional profiles to reduce the incidence of nutrition-related conditions or diseases. On the other hand, practical implications like the extension of shelf life, decrease of additives, reduced processing costs and energy consumption, so as lowering the environmental impact from food processing are focused on by plant biotechnology. An example for processing related properties altered by genetic engineering would be another GM rice variety revealing decreased gluten level which is an undesirable component for sake brewing.

1.6 Safety concerns

The use of genetic engineering in agriculture, food production and animal development might have impacts not only on the environment and biodiversity, but also on human health. Since a gene is the blueprint for a protein, the new genetic information causes the organism to

produce one or more new proteins. In turn, the food produced by that genetically engineered organism will contain these proteins which could cause in the worst case allergies or toxicity **(Taylor, 1997).** Alternatively, the cellular metabolism of the food-producing organism could be altered in unintended and unanticipated ways and in turn, these metabolic alterations could cause malfunctions **(Fuchs et al., 1996)** like failure to synthesize important vitamins or nutrients. Consequently, the genetically engineered food would lack important nutrients that are normally present in the corresponding non-genetically engineered food causing unexpected adverse changes in food composition. Such foods could be health damaging and thus would have to be regarded unsafe.

Thus, introducing any new technology, including gene technology, into the food or feed chain needs appropriate safeguards to protect human and animal health. Europe, USA, Japan, Canada and other countries around the world have developed regulatory controls related to GM foods intended for marketing. To date, in the European Union a wide regulatory framework controls multiple aspects from the deliberate release of GM crops to the final product for consumption. The principal legislation related to novel foods (any kind of food that has not been consumed in Europe before) is the European Regulation on Novel Foods and Novel Food ingredients (EC) No. 258/97 that entered into force in May 1997. Apart from a thorough safety evaluation before placing on the market, mandatory labelling of GM foods is required on basis of the presence of either "foreign" protein and/or DNA. Furthermore, the EC established a 1% threshold for unintended contamination of unmodified foods with approved GMOs above which labelling is afforded. However, up to now no labelling is required for animal feed. The inclusion of feed derived from GMOs actually is in preparation in the scope of a modification of the European "Novel Foods" Regulation (EC) No. 258/97. In future there will be a separate regulation dealing exlcusively with GMO contained in food and feed (Proposal on a New Regulation of the European Parliament and Council on Genetically Modified Food and Feed, Brussels, 25.7.2001 KOM(2001), final version, 2001/0173 (COD). A second regulation is under way on traceability and labelling of GMO derived from food and feed (KOM(2001) 182, final version, 2001/080 (COD)). The threshold for labelling GMO which are marketed in Europe will be 0,9 %. In contrast to the Novel Foods Regulation (EC) No. 258/97, foods and feed will have to be labelled irrespective of analytical evidence in future. These new regulations meanwhile have been adopted by the European Parliament in July 2003 and are expected to enter into force in spring 2004.

Thresholds have also been introduced by other countries. For example, in Japan, a threshold of 5% for frequently used GM crops like soybean and maize was implemented. Aside from labelling, the Ministry of Health and Welfare announced that health testing of GM crops is required. In the United States, GM plants or products must not be labelled. However, GMO varieties must pass a consulting procedure prior to marketing. The responsible competent authorities in the USA are the Center for Food Safety and Applied Nutrition (CFSAN), the Center for Veterinary Medicine (CVM) and the Food and Drug Association (FDA). Based on information provided by the applicant the safety of the new product is evaluated case by case.

1.7 Objectives of this study

Although Egypt mainly depends on imported soybeans and maize, the control and evaluation of these crops only depends on its nutrient content and the acceptable level of mycotoxins without paying any attention to genetic manipulation. Consequently, there is no idea about the presence or absence of GM crops for both human and/or animal consumption in Egypt. Furthermore concepts for safety evaluation are urgently needed. Therefore, this work was planned to study the following points:

Firstly: to monitor the incidence of genetically modified soybean and maize especially used for human nutrition purposes in Egypt. To achieve this purpose, forty soybean and soybean products samples and forty maize and maize product samples have been randomly collected from the Egyptian market. The samples were subjected to detection techniques based on Polymerase Chain Reaction (PCR) using the official detection methods according to Article 35 of the German Federal Foodstuffs Act **(Anonymus, 2002); (Anonymus, 2002).**

Secondly: development of a construct-specific, qualitative detection method for two genetically modified potato *Spunta* lines, G2 and G3. The GM potato lines under investigation are the result of a common research project of the Michigan State University and Egypt **(USAID/CAIRO/AGR/A, co-operative Agreement No. 263-0152 A-00-3036-00)**. Both lines contain the same gene construct, basically a gene derived from the bacteria *Bacillus thuringiensis* which codes for a crystalline protein (CryV) which is toxic (Bt-toxin) to *Coleoptera*. Since insect resistant GM potatoes are a promising GM crop for the Egyptian market and an application for marketing in Egypt in the near future is not unlikely, the material was chosen as a model system for further studies on safety issues. In addition, the detection method

developed here should be well suited for the control of labelling obligations. A qualitative method usually is a first and relatively low-cost step in analysis.

Thirdly: studies on the safety of genetically modified potato *Spunta*. Safety assessment studies were conducted on genetically modified potato *Spunta* based on the principle of substantial equivalence, which has been adopted by leading international food and regulatory bodies including the World Health Organisation (**WHO, (1991, 1995),** the United Nations Food and Agriculture Organisation (**FAO, 1996),** the Organisation for Economic Co-operation and Development (**OECD, 1993, 1996, 1997**), and the International Life Sciences Institute **(ILSI, (1997).** According to this principle, a food derived from a genetically modified crop is regarded as safe, if it does not differ from its conventional counterpart. Governmental authorities in Japan (MHW, 1996), Canada (Health protection Branch, 1994), the United States **(FDA, 1992),** the United Kingdom **(ACNFP, 1991),** the European Union **(EC, 1997),** and many other countries have adopted the principle of substantial equivalence as an integral part of the basis for safety assessment of foods derived from GM crops. The food safety assessment of GMO potato *Spunta* in this work consists of the following main components:

(1) Biochemical composition of genetically modified potato and conventional potato.

(2) Determination of natural occurring toxicants, anti nutrient compounds and nutritive value.

(3) Evaluation of the nutritional and biological equivalence of genetically modified potato *Spunta*. According to the recommendation by (**WHO, 2000)** as a guide for safety aspects of genetically modified food of plant origin, animal studies are performed with rats to investigate potential adverse efffects of GM potato.

Fourthly: study on the influence of GMO containing diets on health and performance of broiler (chicken) and the fate of DNA in the gut and tissues. This work tried to evaluate the digestibility of genetically modified potato DNA in comparison with the non-genetically modified control line depending on PCR techniques. Furthermore, this work aimed to evaluate the possibility of transformation of plant DNA to broiler blood and organs with a special emphasis on detecting the recombinant *Bacillus thuringiensis* (Bt) toxin-coding gene in broiler organs and in the meat derived from it.

2. REVIEW OF LITERATURE

2.1 Legislation and regulation of the release of GM crops around the world

North America and Europe have paved the way for the development and environmental release of GM crops. They have also defined the general framework for a regulatory system. The 1989 framework of the National Research Council (NRC) in USA was an early attempt to regulate the application of GM technology in the field and still offers a good overview of concerns and regulatory issues **(NRC,1989**). The 1993 OECD guidelines for industrial applications of GM organisms **(OECD, 1993a, b)** resulted in an extended framework for evaluating the environmental impact of GM organisms and safety assessments for application of GM in food and feed. More recently, the Cartagena protocol on Biosafety helps to provide a more general framework for implementation in individual countries **(SCBD, 2000)**. BINAS offers a database of regulatory issues, providing information on competent authorities, relevant laws, regulations and/or rules for individual countries.

Many countries are now faced with the challenge to put in place regulatory systems to ensure safe and effective evaluation of the impact of GM crops. Several organisations are instrumental in helping countries to generate the capacity to establish such systems. Among these are the International Service for National Agricultural Research (ISNAR) of the Consultative Group on International Agricultural Research (CGIAR), **(Cohen, 1999; McLean et al., 2002; Persley et al., 1993)**, the international Centre for Genetic Enineering Biotechnology (ICGEB) and the United Nations Environment Program. UNEP issued International Technical Guidelines for Safety in Biotechnology in 1995 **(UNEP, 1995)**. A UNEP Global Environment Facility (GEF) project on the development of National Biosafety Frameworks is designed to assist countries to develop their National Biosafety Frameworks so that they can comply with the Cartagena protocol on Biosafety. Currently 77 countries are enrolled. Here, the review will present an overview of the main characteristics of the prevailing regulatory systems in various parts of the world, beginning with the four countries growing the major areas of GM crops in the world. Systems and details for regulating GM crops are complex, often confusing and constantly evolving. In view of the potential changes in this field, the information presented might be quickly superseded. The broad and geographical overview of regulations aims to facilitate finding relevant information sources and updates, as well as making comparisons. The ISNAR initiative to prepare up-to-date country reports with overviews of the regulatory policies and

procedures of individual countries, such as now available for Egypt **(Madkour et al., 2000)** and Argentina **(Burachik and Traynor, 2002)**, is worth following for the future.

USA

In 1986 the Co-ordinated Framework for Regulation (CFR) of Biotechnology specified the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA), the Environmental Protection Agency (EPA) and the Food and Drug Agency (FDA) as the primary governmental agencies for regulating biotechnology in USA. The 1986 'Co-ordinated Framework for the Regulation of Biotechnology' is still in use today **(MacKenzie, 2000)**.

A useful oversight of the regulatory process for transgenic crops in USA is maintained by **(Byrne et al., 2002).** Regulatory assessments had to be science, risk and case based. A crucial decision in this CFR was that no new and specific biotechnology regulation system was necessary.

The at-that-time-current laws, the Federal Plant Pest Act, the Federal Plant Quarantine Act and the Federal Insecticide, Fungicide and Rodenticide Act, provided adequate statutory authority for biotechnology regulation **(MacKenzie, 2000)**. This decision implies that in USA the regulation focuses primarily on the characteristics of the product, rather than the way in which the product is produced. This product-based assessment is a major difference with the philosophy of regulation in, for example, the EU, which is process based. This process–product difference of philosophy has sparked considerable controversy over recent years. The Biotechnology, Biologics and Environmental Protection (BBEP) unit of USDA-APHIS focuses on the environmental impact of GM plants under (revised) regulation 7 CFR Part 340. The current procedure for field testing is relatively simple. A notification process can be used for most crops and many genes for field testing of GM plants. This is a simplified process, as compared to a permit, and takes only 30 days before the field trial can commence. An acknowledgement from USDA-APHIS is required prior to planting. When a formal permit is required, APHIS must come to a Finding of No Significant Impact (FONSI) **(MacKenzie, 2000)**.

For the release of GM plants with anti-pest proteins, such as insect-resistant plants, the EPA has joint responsibility for the regulatory oversight along with the USDA. In current EPA terminology, such plants contain, plant-incorporated protectants'(PIPs), formerly known as 'plant-pesticides' **(Deegan, 2001)**. For field tests of PIP-containing GM plants greater than 4 ha (10 US acres) in size, an Experimental Use Permit (EUP) from EPA is required. Currently, BBEP reviews about 1000 applications for field testing and deregulation each year **(NAS, 2002)**.

Such a review takes on average 10 months for applications not involving the notification process. EPA's review typically requires 18 months **(CAST, 2000**).

The FDA is responsible for determining human food and animal feed safety and wholesomeness of all plant products, including those produced via genetic modification. The FDA follows a decision tree safety assessment approach essentially based on the concept of 'substantial equivalence' **(FDA, 1992)**. This concept in relation to the regulation of GM food and feed is reviewed in considerable detail elsewhere **(Kuiper et al., 2001)**. Global developments prompted the FDA in January 2001 to sharpen its assessments, but the 1992 regulations are still used. The time frame for approval ranges from 6 to 12 months after data submission. The USDA-APHIS/EPA regulation of the environmental release is based on the concept of 'familiarity' **(OECD, 1993a)**. This concept can be considered the ecological counterpart of the concept of 'substantial equivalence', although in some publications these two concepts are also considered separately for environmental release. Familiarity considers whether the GM plant is comparable to its traditionally bred counterpart in environmental safety. Such comparison may assess the relevant issues in a GM crop without direct experience. Familiarity considers the biology of the plant species, the trait introduced, and the agricultural practices and environment used for crop production. In comparison with a suitable counterpart, often the parental non-GM crop, the aim is to establish if the GM change presents any new or greater risks relative to that counterpart. This allows a relative level of safety to be established for the GM crop. A related concept is that of the 'antecedent organism'. If an organism has already been evaluated (i.e. is familiar), future assessments of that organism can be less stringent. The precise meaning of 'familiarity' and the subsequent consequences for regulation have been discussed extensively without national (or worldwide) consensus.

The main points of discussion are to define 'comparable' and to decide when something is 'sufficiently comparable'. Applications for environmental release are evaluated on a case-by-case basis and concern weediness, gene transfer, effects on wildlife, altered disease susceptibility and several related aspects of the GM crop **(CAST, 2000, 2001)**.

In 1994, EPA proposed to regulate anti-pest GM plants as if they were pesticides, then in 1999 questioned whether GM seeds should be subject to pest control regulations.

A National Academy of Sciences report **(NAS, 2000)** recommended the formal adoption of the EPA's 1994 proposed regulations and to further strengthen its oversight in various ways. These developments could be interpreted as a move in the direction of a process based rather than a product-based regulation in USA. Most of the EPA regulations were issued in 2001, but

some issues such as the way in which plants with viral coat proteins should be regulated, are still being debated **(Deegan, 2001).**

A recent National Academy of Sciences evaluation of current US regulation **(NAS, 2002)** suggested tighter monitoring of the environmental release of all crops, including those resulting from 'traditional breeding' **(Gewin, 2002)**.

Argentina

Argentina was among the earliest countries to establish a system for regulatory oversight of GM crops. Since 1991, its system has evolved and expanded to meet the changing context of scientific and international developments.

The Agricultural Directorate of the Secretariat of Agriculture, Livestock, Fisheries and Food has several agencies involved in regulating the use of GM crops and their products. Major, agencies are the National Advisory Commission on Agricultural Biosafety (CONABIA), the National Institute of Seeds (INASE) and the National Agri food Health and Quality Service (SENASA), while the National Directorate of Agri food Markets (DNMA) is also involved in the commercialisation of GM crops. Recently, a National Advisory Commission on Policies for Agricultural Biotechnology was created to define guidelines concerning broader policy issues **(Burachik and Traynor, 2002)**. CONABIA is a multidisciplinary advisory group that is responsible for the regulation of products of agricultural biotechnology. It evaluates the scientific and technical issues of environmental release of GM crops and makes recommendations to the Secretary of Agriculture who makes the final decisions. The guidelines developed by CONABIA are legally based on Resolution 289/97, modifying Resolution 837/93 **(Huarte, 2000)**.

The guidelines are basically similar to those in North America and are based on the characteristics and risks of the products and not on the process **(Mackenzie, 2000**). They involve a combination of pre-existing and newly written laws and regulations. After at least one release into the environment has been approved and the safety of the GM crop has been demonstrated, the applicant can apply for a 'flexibilisation' permit which allows future releases by simply providing notification of the location, area, sowing date and intended harvest date. **(Burachik and Traynor, 2002)** give a useful overview of the organisation, current status and future trends of the biosafety regulatory framework in Argentina.

Canada

In 1990, the Canadian federal government published its regulatory framework for biotechnology to harmonise the benefits of biotechnology-derived products with the need for protection of the environment and human health and safety. Canada uses a product-based

approach for evaluation, placing emphasis on the novel traits or attributes introduced into a plant. All plants or products with new characteristics not previously used in agriculture and food production in Canada are monitored, irrespective of whether GM or more traditional plant breeding methods were used for development. Since 1994, Canada has approved a total of 43 novel food products, many of which are GM crop based **(MacKenzie, 2000)**.

The concept of familiarity is also the guiding principle in the Canadian system. Regulatory agencies responsible for products derived from plant biotechnology in Canada are the Canadian Food Inspection Agency (CFIA), Health Canada and Environment Canada. Food, feed and seed are regulated by CFIA, whereas Health Canada and Environment Canada establish criteria and monitor the inspections. Health Canada regulates drugs, vaccines, diagnostics and medical devices. Environment Canada, under the Canadian Environmental Protection Act, regulates other biotechnology products. The Plant Biosafety Office (PBO) of the CFIA monitors all (confined) field trials of novel crop varieties to ensure that the trials comply with the guidelines for the environmental release (Regulatory Directive 2000–07; amended February 2002). Unconfined release aimed at marketing (Regulatory Directive 94–08) requires a molecular characterisation, the requirements of which have been harmonised between Canada and the USA in 1998. In addition to these regulatory requirements, a novel GM crop must be registered through the variety registration of the CFIA the same way as all other new crop cultivars grown in Canada. For transparency, all decision documents describing any assessment and its results are available for the public on the PBO web pages **(PBO, 2002).**

China

China has implemented a very pragmatic approach to GM crop regulations. Regulations are basically product based and explicit attention is given to the economic interest of a given application. The State Science and Technology Commission, jointly with the Ministry of Public Health, the Ministry of Agriculture, and the Chinese Academy of Sciences, drafted a ‘Regulation on Biosafety Control of Genetic Engineering’ that established the legal framework for the release of GM crops. Following wide discussion, the final document was issued and implemented by the State Science and Technology Commission in late 1993 **(Ding, 1995)**.

Between 1996 and 2000, the Chinese Office of Genetic Engineering Safety Administration approved 251 of 353 GMO applications **(Huang et al., 2002)**. China is also in

the process of labelling imported GM crops (soybean and oilseed rape) and locally produced GM tomato and cotton

European Union

In the EU, the GM crop regulatory system is composed of several regulations, directives and amendments thereof, that are assembled in a time-consuming and highly complex interplay between the European Commission (EC), the European Parliament (EP), the relevant Council of Ministers and the individual Member States. In the EU's legal framework, a 'regulation' is a law that all Member States should eventually adopt in their local laws by passing through their individual parliaments. A 'directive' is a minimal set of demands that should be interpreted and implemented in the national legislation of the Member States. As a result, a 'directive' can have different implementations in different Member States. This obviously adds to the complexity of understanding GM crop regulation in the EU. The Belgian Biosafety Server (http://biosafety.ihe.be) compiles a regularly updated access to all European legislation and regulations. In 1990, the EU implemented two directives. Directive 90/ 219/EEC, with amendment 98/81/EC added in 1998, involved the contained use of GM (micro)organisms. Directive 90/220/EEC involved the deliberate release of GM organisms, including plants, into the environment. In February 2001, the EP adopted Directive 2001/18/EC, which defines new GM crop rules to come into force in October 2002. It presents a substantially revised version of the previous directives. Central in these regulations is that GM is considered something new and special for which existing legislation is not sufficient.

The EU regulatory system is therefore process based rather than product based: the way something is made determines the regulatory framework. This is thought to contribute to better acceptance of genetic modification, notably in the food sector. However, it seems more likely that it may have resulted in a heightened awareness and concern in Europe compared to the North American continent.

The major philosophical shift in Directive 2001/18/EC compared to its predecessors is the explicit adoption of the precautionary principle as a guide, rather than or in addition to the concepts of familiarity and substantial equivalence. This was motivated by, among other considerations, the Cartagena protocol on Biosafety. The precautionary principle is a difficult concept that originates from the discussions about maintaining biodiversity.

This principle requires the evaluation of indirect or delayed effects and changes in agricultural practices. Marketing consents will be time-limited and conditional upon post-

marketing surveillance (the precautionary principle is discussed in more detail in the accompanying paper (**Conner et al., 2003**). The EC realises that the precautionary principle may be difficult to apply. Therefore, it is stated that reliance on the precautionary principle is no excuse for detracting from the general principles of risk management such as proportionality, non-discrimination, consistency, examination of the bene- fits and costs of action or lack of action and examination of scientific developments (**CEC, 2000**). How this will be put in practice remains to be seen. Directive 2001/18/EC, as its predecessor 90/220/EEC, distinguishes two categories for environmental release. Releases for research and development are made under Part B of the Directive, which is generally used for conducting experimental field trials on GM crops. These releases are filed and granted at the national level by the individual Member State concerned. The time frame required for approval differs between Member States and runs from about 3 months to essentially indefinite periods. Releases for placing a GM product on the market require consent under Part C of the Directive. Such consents are given at the EC level and may take from 2 years to indefinite periods for approval, but once issued, apply across all Member States. In contrast to its predecessor, Directive 2001/18/EC includes provisions for the labelling and traceability of GM food, feed, seeds and pharmaceuticals. Unfortunately, such process-based labelling seems much more prone to fraud. Other provisions include a time-limited consent and phasing out of genes encoding resistance to antibiotics in use for medical or veterinary treatment by 2005 for commercial releases and 2009 for research purposes. A public registry of all approved products will allow consumers to trace GM products. Although the basic philosophy of the regulation is quite different, the data requirements for assessing safety of GM plants and plant products are similar in USA and the EU. The information required in the EU tends to be more extensive, mainly with respect to molecular characterisation, monitoring and traceability. Traceability is defined as a possibility to prove the origin of GM organisms or their products at any stage and at any time during their progression along all steps of the market chain. However, validated standardised test methods do not yet exist. Overall, it is currently impossible to give a reasonable estimation of a time frame for approval in the EU.

Novel Foods and cultivar registration procedures, make commercial release of any GM crop in the EU a lengthy, and therefore possibly unappealing, endeavour. Since June 1999, a de facto moratorium on commercial licensing of new GM products has been in place in the EU. Six EU Member States (Austria, Denmark, France, Greece, Italy and Luxembourg) decided that they would not accept any new GM approvals at least until a revision of Directive 90/220/EEC was in place. Such legislation is under development. In July 2001, the EC presented proposals for

legislation on traceability and labelling of GM organisms and products derived fromGM organisms **(CEC, 2001a)** and for GM food and feed (**CEC, 2001b**).

In addition, regulations dealing with the transboundary transport of GM material across the EU are being established in accordance with the international obligations in the Cartagena protocol on Biosafety.

Australia

The regulatory methodology in Australia has largely developed alongside the technology as the need arose. A Genetic Manipulation Advisory Committee (GMAC) was initially established as a non-statutory body to oversee the development and use of novel genetic manipulation techniques. From June 2001, Australia's new gene technology regulatory regime is governed by the Gene Technology Act (GTA) which regulates all dealings (e.g. research, manufacture, production and importation) with organisms that have been modified by gene technology **(MacKenzie, 2000)**.

A key aspect is that the GTA provides one central, enforceable scheme for regulating GM organisms through the Office of the Gene Technology Regulator **(OGTR, 2002)**. The Regulator assesses applications for release of GM organisms and prepares a risk assessment and risk management plan. This activity is supported by three key committees **(MacKenzie, 2000)**, the Gene Technology Technical Advisory Committee (GTTAC), the Gene Technology Ethics Committee (GTEC) and the Gene Technology Community Consultative Group (GTCCG) to provide scientific, ethical and policy advice. New Zealand has taken a very conservative approach to adopting GM technology. Field trials on GM crops were initially approved by the 'Interim Assessment Group' administered by the Ministry for the Environment. In 1998, this was superseded by the new Environmental Risk Management Authority (ERMA), which currently regulates the development, field testing and release of GM organisms under the Hazardous Substances and New Organisms Act. The assessment process is one of the most rigorous in the world. New regulations continue to be put in place to limit the commercial development of GM crops. In July 2000, a moratorium on further applications to field test GM organisms was imposed pending a Royal Commission on Genetic Modification on the risks and opportunities for GM in New Zealand. Although this Royal Commission basically endorsed the continuation of GM technology **(Eichelbaum et al., 2001)**, the New Zealand government is currently developing further legislation for additional controls on the release and use of GM crops.

Japan

In 1987, Japan formulated and issued its guidelines for application of organisms derived from recombinant DNA technology in agriculture, forestry, fisheries, the food industry and other

related industries **(MacKenzie, 2000)**. These guidelines were based on the OECD guidelines and revised in 1992 and 1995. In 1995, The Society for Techno-Innovation Agriculture, Forestry and Fisheries (STAFF) established an 'Information Desk for the Application of rDNA Organisms', which is the main source of information presented here **(STAFF, 2002).**

The Japanese system is largely product based and the concepts of familiarity and substantial equivalence form the basis of the Japanese guidelines. Two guidelines have been established for experimentation (one for experiments in university research facilities and one for all other research facilities) and six guidelines apply to industry applications. Three of the six industry guidelines refer to the safety assessment of the application of GM crop plants. These fall under the jurisdiction of the Ministry of Agriculture, Forestry and Fisheries (MAFF) or the Ministry of Health, Labour and Welfare (MHLW). Cultivation of GM crops, and the importation of GM plant material that can propagate in the natural environment, is regulated under the guideline for rDNA organisms, which is overseen by MAFF. In order to utilise a GM crop, it must be confirmed that the plant will not have a new impact on the agriculture and ecology of Japan **(MacKenzie, 2000)**.

Two separate stages of applications are distinguished: application in a simulated model environment, and application in an open system. Before applying to either system, the applicant must obtain the approval of MAFF confirming that the safety assessments satisfy the requirements. In April 2001, new legislation was introduced that set a zero tolerance for imports containing GM products unapproved by Japan **(RNS, 2002).** At the same time, a threshold of 5% for approved GM crops was introduced for food products to be labelled as GM.

Other countries

Various Asian countries are in the process of establishing their legislative framework for environmental and commercial release of GM crops. India has established a Genetic Engineering Approval Committee (GEAC) to oversee GM crop applications. In the Philippines, the National Committee on Biosafety mandates the guidelines and approvals. The approval permit stipulates that the performance of the GMcrop and its effect on the environment as well as human and animal health are assessed. Recently, the Philippines government released guidelines to take effect from 1 July 2003 that will regulate the importation and commercialisation of GM crops. Malaysia is in the process of drafting their Biosafety law and is developing their field testing regulations. Currently, 26 Asian and Pacific countries participate in the UNEP-GEF project **(UNEP, 2002)**.

European countries on the way to becoming members of the EU are expected to fully implement the EU regulatory system. Hungary and Slovenia are the closest to having this legislation in place. Other countries of Central and Eastern Europe, including many republics of the former Soviet Union, are in the process of developing appropriate rules and legislation. Romania, Bulgaria, Estonia and the Russian Federation have laws, whereas several other countries (Belarus, Moldova) are preparing legislation **(BioSafety meeting, 1999)**.

The MATRA programme of the Dutch Ministry of Foreign Affairs aims to support the establishment of national biosafety frameworks in conformity with the EU regulatory system and other international obligations such as the Cartagena protocol on Biosafety **(MATRA, 2002)**. In Ukraine, a biosafety committee has been installed and legislation is being considered. In 1999, Ukraine turned to Canada to help establish its regulatory system. The philosophical differences between the legislation in the EU and North America is complicating matters considerably **(Blume, 2000)**.

Currently, 13 Central and Eastern European Countries participate in the UNEP-GEF project **(UNEP, 2002)**. Norway has implemented the EU regulations in the framework of the European Free Trade Area (EFTA)-EU agreement in its 1993 Gene Technology Act. Switzerland has a Federal Co-ordination Centre for Biotechnology in place to oversee dedicated legislation. In South America, legislation with a wide scope of GM organisms exists in Brazil, Cuba, Mexico and Peru, whereas other countries limit the scope to GM plants, or have no legislation in place yet **(Artunduaga-Salas, 2000)**. Bolivia's regulations were confirmed by law in 1994.

Regulations for commercialisation are specific, except for Columbia and Uruguay. The Instituto Interamericano de Cooperacio´ n para la Agricultura (IICA) has provided guidance on the development and harmonisation of regulations **(Jaffe´ ,1994)**, with the CGIAR Research Centres providing assistance. Currently, 16 Latin American and Caribbean countries participate in the UNEP-GEF project **(UNEP, 2002)**.

In Africa, several African governments are facilitating the applications of agricultural biotechnology to help increase productivity. The need for increased productivity is probably nowhere greater than in Africa **(Mushita, 2001)**, which is currently experiencing the highest population growth rate and the highest levels of malnutrition of any region in the world. Various GM crop research activities towards this aim are reaching the field testing stage. In South Africa, the South Africa Committee for Genetic Modification (SAGENE) has based its environmental release considerations on guidelines developed in the UK, with a GMO act dating from 1997. Decisions regarding GM organisms consider more than safety issues. In this manner, the

decisionmaking process has acquired public credibility and support. In Egypt, existing legislation not tailored to GM crops was used to permit a few field trials, with the expectation that commercialisation will proceed and appropriate legislation will be put in place. An overview of the current developments in biosafety legislative system in Egypt is given by (**Madkour et al., 2000)**.

Kenya implemented regulations and guidelines in 1997, overseen by the National Biosafety Committee. Zimbabwe has also developed national guidelines, with its 2000 regulations being legally binding. The policy framework to regulate and monitor the import, manufacture, use and release of GM organisms is currently being developed in several other African countries such as Uganda, Namibia, Nigeria and Cameroon, often with assistance from the United Nations Environment Programme (UNEP). Namibia has based its guidelines on the South African model. Many other African countries, however, currently lack the financial support to develop appropriate guidelines, policies and/or legislation **(Kandawa- Schulz, 2000)**. Currently, 22 African countries participate in the UNEP-GEF project **(UNEP, 2002)**.

2.2 Detection method of genetically modified food

2.2.1 Screening and identification method.

In all regulations, in European union labelling of genetically engineering food or food ingredients are the European commission (EC) Novel Food Regulations 258/97 and 1139/98. These require labelling if any characteristic or food property such as composition, nutritional value or nutritional effects or intended use of food renders a novel food or food ingredient no longer equivalent to an existing food or food ingredient, “or if DNA or protein of Roundup Ready Soya and Bt-176 maize can be detected. On April 10, 2000, two new regulations become effective: the 49/2000/EC, which introduces the 1% threshold for the labelling of products derived from identity preserved sources (e.g. organic and non genetically modified organisms (GMO) grain) and the 50/2000/EC, which requires labelling of flavours and additives (the later were previously exempt from labelling). In Switzerland, a 1% threshold for the labeling of products containing GM material had been introduced in June 1999; Norway had 2% threshold for several years. In Mexico, the majority or members of parliament also voted to label foods containing transgenic material. Japan and Korea announced the introduction of 5% and 3% threshold for labelling in 2001, respectively.

The official labelling policy in the US is that of the FDA, which does not require a mandatory label for items produced using modern biotechnology. The policy does require that food labels

be truthful and nonmisleading. Products must be labelled if there is a material change in the food, if it is no longer equivalent to its conventional counterpart, or if an allergen is present.

The detection of GMO crops has become necessary to allow consumers to make an informed choice and to comply with labelling regulations. Methods for the identification of GM food can be divided into 3 categories. The first category includes nucleotide based amplification methods, such as polymerase chain reaction (PCR). The second category includes protein – based methods, such as enzyme-linked immunosorbent assay (ELISA). The third category is detection of enzymatic activities.

Every detection method has its own specificity and limitations **(Chiueh et al., 2001)**. For example, the detection of enzymatic activity method is not recommended for processed foods, where proteins may be denaturalised. The methods based on PCR are suitable for detection of some processed food, however DNA fragments in foods could be broken into pieces. Among the 3 categories, PCR is the most popular method used worldwide. This review focuses on detection method for genetically modified food through identification of recombined DNA. Generally, the different procedures to assay sampled food for the presence of GMOs could be divided into three distinct steps: detection, identification and quantification. 1) Detection (Screening of GMOs). The objective is to determine if a product contains a GMO or not. For this purpose, a screening method can be used. The results a positive/negative statement. The analytical method must be reliable enough to obtain results. 2) Identification: The purpose of identification is to reveal how many different GMOs are present and if they are authorised or not. Specific information (I.e. details on the molecular make-up of the GMOs has to available for the identification, 3) Quantification: If food product has been show to contain (one or more) authorised GMOs, then it become necessary to assess compliance with 1 % threshold regulation by the determination of the amount of each of the GMOs present in the individual ingredents from which it has been prepared (**Anklam et al., 2002**).

The analytical methods contain the following steps: 1) Extraction of DNA: It is necessary to extract the genetic material free from other impurities which might interfere in further steps of the analysis. 2) PCR reaction (Polymerase Chain Reaction): the PCR reactions are suited to multiply and amplify specific fragments of DNA that are alien genes to the food being analysed. The primer starter molecules used in the beginning of the reaction decide which sequence of DNA will by multiply. To avoid false negative results due to inhibit action of impurities during extraction of the DNA it is important to include a positive reaction. Making the PCR product visible: Through gelelectrophoresis (Agarose gel electrophoresis). The products of the PCR

reaction can be made visible together with the determination of the length of the base pair, the alien gen. 3) Confirmation of the results: The confirmation of results are being made by controlling the sequence of the base in the PCR product using specific sequence restriction, hybridisation with specific snode **(Hupfer et al., 1997, Pietsch et al., 1997)**.

The first method for GMO identification in foodstuffs was specifically developed to identify the Flavr Savr^{TM} tomato was described by (**Mayer, 1995**). This tomato contains in addition to the already mentioned PG-gene the kan^{r} gene, conferring to resistance to kanamycin and the cauliflower moasaic virus promoter CaMV35S. the detection of these three foreign sequences could be achieved by developing two PCR assays. One primer pair amplifies a 173 bp fragment from kan^{r} . The second primer pair consists of an oligonucleotide hybridising to the promoter sequence, the other being complementary to the Flavr Savr^{TM}-gene. The resulting amplicon is 427 bp long. Fresh tomatoes from different countries, a sample of canned tomato and a Flavr Savr^{TM} tomato were analysed. Only the genetically engineered tomato yielded the two specific amplicons. A protein-based analytical method is not applicable in the case of the Flavr Savr^{TM} tomato (or in general when antisense technique is used) because the genetic modification does not produce a new protein in the plant.

A further example for genetically modified product is the soybean “Roundup Ready ^{TM}” which belongs to the category “agronomic benefits” and is admitted in the US and the EC. This plant contains as a new element in the genome, the EPSPS-gene from *Agrobacterium tumefaciens* producing the enzyme 3-enolpyrufyl-shikimate-5-phosphate-synthase. This bacterial enzyme is not inhibited by the herbicide glyphosate “Roundup Ready ^{TM}” and makes the plant resistant to the herbicide. The genetically modified maize “Maximizer “ belongs to the third category, “pest resistance”, and contains the synthetic endo toxin cryIA(b) gene, which is 65% homologus to the natural bacterial cryIA(b) gene originating from *Bacillus thuringhiensis*. From both plants a set of specific and sensitive PCR-systems have been developed.

The rapid method for identification of *Streptococcus thermophilus* by primer-specific PCR-technique based on its lac7 gene sequence was demonstrated by (**Lick et al., 1996)**. The PCR-assay can be applied for rapid detection of *S. thermophilus* in fermented milk products like yoghurt. For subsequently confirmation of PCR-product (968 bp in length), the assay can additionally be combined with a hybridization step involving a *S. thermophilus* specific oligonucleotide proble hybridizing to be amplified sequence. Non specific amplification was not observed with DNA’s from a number of lactic acid bacteria which are used in combination with *S. thermophilus* for manufacture of dairy products. The authors shown that this assay is fast

species-specific identification of *S. thermophilus* and were also shown to be detectable by amplification assays. Furthermore, a recombinated strain of *Lactobacillus sake* carying an additional catalase (kat A) gene, which served as starter culture in raw sausage was also shown to be detectable by amplification assay

The method for the detection of genetically modified insect-resistant maize expressing a synthetic gene encoding a truncated version of the Cry IA(b) protein derived from *Bacillus thuringiensis* described by **Hupfer et al., (1997)**. The procedure includes: 1) extraction of genomic DNA, (2) amplification of the inserted synthetic gene by PCR method, and (3) characterization and confirmation of the PCR product by southern hybridization with a digoxigenin-labeled oligonuleotide probe and DNA sequency were also described that the method might srve as a basis for the detection of genetically modified insect-resistant Bt maize.

The PCR-test for the genetically modified maize “Event 176” of Ciba-Geigy was used by **(Ehlers et al., 1997)**. The maize contains genes conferring resistnce to the European corn borer (delta-endo-toxin gene from *Bacillus thuringiensis*) and tolerance to the herbicide Basta (phosphirinothricin resistance gene for *Streptomyces hygroscopicus*). The maize contains also an ampicillin resistance gene. Primers were designed and using “Event 176”-maize-DNA as template internal regions of the three genes were amplified with PCR. The PCR products were sequenced to confirm their identity. Using the delta-endotoxin primers in PCR down to haploid genomes of “Event 176”-DNA could be detected , even in the presence of a 10 fold excess of DNA from non-modified maize.

A screening method suitable for the detection of 26 out of the 128 transgenic plants of interest in 1997 was already published by **(Pietsch et al., 1997**) and has been already established as an official method in German and Swiss food Law. This method is based on the detection of two regulatory sequences exiting in these 26 trangenic plants. These sequences contain fragments of the CaMV 35S promoter originating from the cauliflower mosaic from the *Agrobacterium turmefacens* **(Hemmer, 1997)**. virus and the NOS terminator originating from the nopalin synthase gene originating **1996)**. This screening method is based on the detection o modified DNA by using the polymerase chain reaction (PCR) technique.

The nestd PCR-system for the “Roundup Ready was designed by **(Koppel et al., 1997)**, a nestd PCR-system for the “Roundup Ready ^{TM}” b setting the primers into three of the four new introduced genetic elements, he 5-end of CP4-EPSPS gene, the chloroplast transit peptide gene and the 35S promoter. The detection of this combination of genetic elements is highly specific

for the “Roundup Ready ™” soybean. The detection limit 20 pg DNA corresponding to 0.01-0.1% GMO soybean in conventional material.

The screening method for the identification of genetically modified food plant origin was demonstrated by **(Waiblinger et al., 1997)**, six different genetically modified plants were tested: Ripening delayed (Flavrs-Savr™) soybeans, PVY-virus-resistant potato, B33-invertase-potatoes and the products of conventional plant origin were used as controls. The primer pair 355-1/355-2 is complementary to DNA the CaMV 355 promoter, the primer pair NOS-1/NOS-3 is complementary to the DNA sequence of the NOS-terminator from Agrobacterium, the primer pair Tns-1/Tns-2 is complementary to the DNA sequence of the neomycinphosphotransferase 11-gene on the transposon Tns. The primer pair plant-1/plant-2 is complementary to conserved sewences of the non-coding region of the chloroplast genome. This study indicated that screening method able to make detection of transferred DNA in plant material (355 promoter, NOS promoter, npt11 gene by using PCR).

For the detection of the “Maximizer” mize (**Studer et al., 1997**) set the primers into the cryIA(b) gene. Therefore, this PCR allows the detection of all genetically modified plants containing the synthetic cryIA(b) gene. The detection limit was in the same order of magnitude as the system described before. To control the amplification potential of the extracted DNA, a second maize specific PCR system based on the zein storage protein gene was developed. This system allows to check whether a maize-derived product contains amplifiable DNA or not.

The identification method for two potato varieties, (Rustica and Desiree) was developed by **(Hassan-Hauser et al., 1998)**. These potato variety were tested for a genetic modification consisting of a granule bound starch sunthase (gbss) gene in antisense orientation combined with the endogenous B33 promoter and parts of the pBIN19 plasmid vector including the neomycin phosphotransferase II (nptII) gene as a marker. Various Polymerase Chain Reaction (PCR) primers were constructed for vector4 sequences, target inserts and the marker gene. At first no products with the predicated target-insert size were obtained. However, a long-template PCR combined with a nested PCR led to the expected amplification product in the Desiree variety. Results were confirmed by a restriction endonuclease digest, Southern blotting of fragments and by biochemical tests. The potato variety Rustica showed no target insert sequences on molecular analysis, nor did biochemical methods indicate modificationas of phenoltype (i.e. reduced amounts of gbss protein and amlylose). Only vector sequences and the marker gene were detected by the PCR method.

(Broll et al., 1999), used the DNA analytical method to detect genetically modified tomato and tomato products. 14 samples from tomato and tomato products were collected from the Berlin market. A combination of the classical CTAB protocol and a subsequent nucleic acid purification step with the QIA quick PCR kit (Qiagen) was applied to extract DNA from all samples. Three primer pairs were used, primer TO-F/TO-R, PG34-L/PG34-R and primer pair HB-7/t-NOS. The first primer pair was used as a control for amplification and isolation of DNA. The amplicon appears at 236 bp. Second primers pairs were used for screening and the amplicons appear only with genetically modified tomato at 180 bp. A third primer pair used for specific PCR yields only amplicons with genetically modified tomato at 193 bp. The DNA analytically method for the detection of genetically modified tomato had been sensitive and the limit of detection is 0.1 % 50 pg DNA (modified DNA).

The rapid DNA extraction/PCR-based method for detection of genetically modified soya (GMS) and maize (GMM) in mixed samples of trangenic and unmodified soybeans and maize kernels, and a variety of processed samples including soya flour, soya protein isolates, extruded defatted soya, acid-and alcohol-precipitated soya concentrates, soya lecithin, maize grit, seasoned corn puffs and salted corn chips was reported by (**Hurst et al., 1999)**. The presence of GMS DNA was determined with two pairs of primers directed towards different GMS target sequences and GMM by one primer pair. In addition, a multiplex pr reaction which utilises an internal positive control was developed for both genetically modified organisms (GMOs). Results indicated that the methods are sensitive and specific enough to detect GMS down to a level of 0.01% dry weight in single-product PCRs and 0.1% in multiplex PCRs and GMM down to 0.001% dry weight in single-product PCRs and 0.01% in multiplex PCR. The methods are considered to represent a viable route for the commercial detection o GMS and GMM in foodstuffs.

Two types of method to make detection of genetically modified RR Soya beans had been used by (**Duijan et al., 1999)**. The applied DNA methodology was making use of Polymerase Chain Reaction (PCR) using set of primers a long the gene the Agrobacterium CP4 synthase. DNA extraction and purification conditions were examined on a case-by case approach for a scala of soy products (Lecithin, oil, soybean meal, soy protein isolates etc.), half-products and final consumer products. Detection limits were found between 0.01% and 0.1%. The protein based were used to detect RR soy. And the limits of detection were found between 0.5% and 1% and the method were validated for half-and final products, this study were demonstrated that both DNA method and protein based method are applicable for the detection of transgenic RR

soy protein in raw material and soy protein fractions and the sensitivity were around 1% in protein based method but the specific and sensitivity for DNA based method was higher than protein based method (0.01 %). The DNA based method is applicable in a wide range of samples starting from beans to soy bean meal, protein, Lecithin and oil to half-and final products. Besides a method for the detection of transgenic DNA, also a specific method is available for the quantification of RR protein.

The overview of the development and application of DNA analytical methods for GMOs in food was presented by **(Meyer, 1999)**. Several food ingredients, such as wheat, soy, maize, celery, etc., have been successfully investigated by the DNA analytical method based on PCR. A critical point in this method is sample preparation. Currently, two DNA isolation methods are used, the cetyltrimethylammonium bromide (CTAB) method based on incubting a food sample in the presence of the detergent CTAB and using DNA-binding silica resins (e.g. Wizard^{R} Purification Systems, Promega Corp.) to extract and purify DNA from food samples. It was shown that a combination of the classical CTAB method and a subsequent DNA purification step with DNA-binding resins increased the yield of DNA. Various factors contribute to the degradation of DNA (e.g. heat treatment, nuclease activity, and low pH); data suggest that a critical minimum average DNA size for successful analysis is approximately 400 bp.

The results of an interlaboratory study of a PCR screening method for GMO in Roundup Ready^{TM} soy bean and Maximizer (Novartis-Agribusiness)^{TM} maize co-ordinated by the EC Joint Research Centre was demonstrated by **(Lipp et al., 1999)**. A screening method was applied for the detection of the CaMV 35S promoter and NOS terminator gene from *Agrobacterium tumefacins*. The method was applied to 26-28 transgenic plants in 1997. A total of 41 laboratories in 14 countries were invited to participate, and results from 29 laboratories were received.

The sensitivity and reproducibility of the PCR method depend on the reagents and apparatus used. Each participating laboratory was requested to perform its own optimisation of the PCR method using defined samples containing varying amounts of GMO that were prepared at the IRMM. For DNA extraction, no particular method was specific, but detailed descriptions of two DNA extraction methods were given. For PCR measurements, primer specified to the CaMV 35S promoter and the NOS terminator were prescribed. Vertification of the amplification products was done by restriction endonuclease digestion. The results of this validation study demonstrated that the PCR screening method is suitable for the detection of GMO in raw material derived from soy beans and maize. Samples containing 2% transgenic soy beans or

maize were unequivocally and correctly identified by all laboratories. Furthermore, correct classification was achieved by analyzing the CaMV 35S promoter in samples containing 0.5% GMO soy beans. Th method for the detection of the NOS terminator is less sensitive compared with that for the detection of the CaMV 35S promoter, and therefore a somewhat higher number of false-negative results was reported. Owing to the larger size of the genomic DNA of maize, this screening method is somewhat less sensitive for the detection of transgenic maize in raw material (i.e. there was a larger number of false-negative with respect to the false-positive rate for soy beans).

The screening and specific detection from transgenic tomato (Zeneca) by using the Polymerase Chain Reaction were made by **(Buch et al., 1999)**. These authors used primers in PG gene and the adjacent nos-3 terminator to generate a specific 350-bp amplicon. These authors used the primer pair PG-34L/PG34-R for the screening detection and the primer pair PG34L and t-NOS for the specific detection for tomato zeneca in the other hand the primer pair A_1 and A_2 used as a control DNA extraction. The results indicated that the screening and specific are suitable for detection for transgenic tomato and these results confirmed by restriction endonuclease and southern-Blot hybridsierung.

Several publications have described detection methods for genetic elements used in the germination of transgenic sugar beet, soybean (**Pietsch et al., 1997, Waiblinger et al., 1997, Wurz and Willmund, 1997**), tomato (**Waiblinger et al., 1996; Pietsch et al., 1997**), cotton (Dupont-petition), potato (**Pietsch et al., 1997; Waiblinger et al., 1997**). Almost all of these methods were PCR-based and were applied to approved genetically engineered products or to genetic elements that have been frequently used for the generation of the approved transgenic plants

Three different existing 35s screening systems were evaluated by (**Wolf et al., 2000)** and report the development of two new CaMV-specific PCR systems. These PCR systems based on CaMV-specific genes allow the identification of positively screened 35S food samples as naturally virus-infected products or plants. Seven food samples tested positive in routine 35S screening analysis and negative in GMO specific systems were investigated using the new virus-specific PCR systems. The results were shown that simple screening methods are not sufficient to differentate unambiguously between genetically modified and conventional food products or plants. In case of a positive screening result for a common genetic element, such as the 35S promoter, a CaMV-specific system should be used in parallel with specific PCR systems.

The PCR technique was used by **(Stran et al., 2000)** to make detection of residues of genetically modified soybeans in breaded fried turkey cutlets which produced in Israel depending on the presence of 35S promoter sequence and the detection of CaMV. The results clearly shown hat the grain used commercially in Israel were contaminated with soybean residues that were easily detected by nested PCR.

The method for detection of recombinant DNA's from four lines of genetically modified maize by using polymerase chain reaction (PCR) was described by **(Matsuoka et al., 2000)**. This method allows specific etection of each Bt 11, Event 176, MON810 and LiBERTY by using pairs of specific primers designed to amplify a segment including part of the exogenously introduced sequence and part of the intrinsic maize sequence. The detection sensitivity was 0.5% for Event 176, MON 810 and LiBERTY, and about 0.01% for Bt 11 and the method is effective as a basic technique for detecting the four lines of GM-maize, but it might not be applicable to processed foods.

In a recent extensive review of GMO detection methods applied to tobacco, the Task Force Genetically Modified Tobacco– Detection Methods of CORESTA (Cooperation Centre for Scientific Research relative to Tobacco, Paris, France) (**Bindler et al., 1999**) has reported on the successful application of the above-mentioned PCR strategies to different types of dry tobacco leaf, and has highlighted the advantages and drawbacks of confirmatory tests such as nested PCR, restriction enzyme digestion of amplicons, and the nucleotide sequencing of the detected genetic elements.

Two method to detect and identify four types of genetically modified (GM) maize were discussed by **(Chiueh et al., 2001)**, a polymerase chain reaction (PCR) assay and an immuno-assay kit were performed in this study. Primers specific to inserted genes in the Event 176 GM maize (Novartis company), Bt11 (Novartis company), MON810 (Monsanto company) and Liberty (AgrEvo company) were used to conduct the PCR assay. Four pairs of primers, namely, CDPK-cry (Event 176), IV01-cry (bt11), HS01-cry (MON 810)and CM03-PA01 (Liberty) were used to identify the GM-maize. Results showed that the limit of detection for GM-maize were 0.01%, 0.1%, and 1% (w/w) as using HS01-cry, CDPK-cry and IV01-cry, and CM03-PA01 primers, respectively. The G-maize references as well as 20 food-grade maize samples were also tested using commercial immuno-kit. Three GM-maize references, Event 176, Bt11 and MON 810, and 6 corn-middling pellet samples were tested as positives; while 14 popcorn samples were negative. To confirm these results, the 20 food-grade maize samples were further tested by the PCR method. Two types of GM-maize were detected in popcorn samples; while 3-4 types of

GM-maize were detected in the samples of corn middling pellets. The results of this study demonstrate that using PCR method is capable of differentaiting four types of GM-maize from non-GM products; while the commercial immuno-kit can only be used for screening purpose. All samples, with positive or negative results as tested by immuno-kit, need to be further confirmed by the PCR method. The results of this study also reveal that marketed maize products are usually mixed with different types of GM-maize.

2.2.2 Detection of processed food

Final products often present particular problems for identification methods due to DNA degradation. Various factors contribute to degradation of DNA in processed foodstuffs as a consequence of:

1-Prolonged heat treatment such as autoclaving used in canning process which may result in DNA hydrolysis, fragmention of the DNA, or modification of the chemistry of the DNA in such a way that the PCR process may not be work. For this reason canned products can give inconsistent results.

2-Increased chemical modification and hydrolysis of DNA at low pH (e.g., vinegar). For example, the factors make tomato puree (and relate tomato products such as katchup). Exposure to acidic pH will cause extensive hydrolysis because of depurination and cleavage of 3-5^{1}-phosphodiester linkages, and also prolonged heating will result in denaturation and subsequent break up of DNA chains.

3-Enzymatic degradation of DNA by nuclease may also occur on prolonged of fresh stuffs.

The detection of the genetic modification in heat-treated products of Bt maize by PCR have described by **(Hupfer et al., 1998)**. A model study on cooked maize powder (polenta) containing a portion of Bt-176 maize revealed that the extent of DNA degradation was most affected by low pH. While the 211-bp target sequence could still be detected after 105 min of boiling at pH 7 and 9, respectively, negative PCR results for samples boiled at pH 2-3 for 15 min indicated complete hydrolysis of DNA at these conditions. Investigations on ensiled Bt-176 maize showed that less harsh conditions of silage left the target DNA sequence intact for up to 7 months **(Hupfer et al., 1999**). As a general rule, the lower the pH at which the product was treated the lesser the chance to succeed with DNA-based analytical methods.

In certain products, however, there may be special circumstances preventing large-scale DNA degradation at heat exposure or long-term storage. In a model system of cooked sausage, **(Straub et al., 1999a)** demonstrated that DNA of GM starter culture microorganisms was well

preserved over a period of more than 9 weeks. This was explained by protective effects of the meat matrix, which apparently even prevented access of exogenous DNase to bacterial DNA released from lysed cells. In this system, detectability of GM DNA regions was not hampered, but favored ecological factors. The same group of workers examined the detectability in bread of glyphosphate-tolerant soya DNA present in a baking aid at a total content of 0.4% **(Straub et al., 1999b)**. Their data revealed a general degradation of DNA to fragment lengths between 10 and 100 kbp during the various stages of bread dough preparation. The baking process itself caused further fragmentation to an average size below 500 bp. Nevertheless, as the target sequence was sufficiently short (172 bp), these authors were able to detect the presence of GM soya flour at all stages of bread production. Interestingly, the original content of GM additive appeared to remain unchanged throughout the entire process.

In another study on a variety of bakery products containing Bt-176 maize or RR soya, **(Moser et al., 1999)** demonstrated the effect of technological parameters on the detectability of transgenic DNA sequence in final products. The extent of DNA degradation increased with the number of enzymatic, chemical, thermal and/or mechanical operations and their intensity during the baking process. Accordingly, they succeeded in detecting GM target sequences defined by primers Cry03/04 or p35s-f2/petu-r1, respectively, only in products not subjected to low pH or prolonged heat treatment.

The methods for detection of seven lines of GM maize, such as Event 176, Bt11, T251, MON 810, GA27, DLL25 and MON by using of PCR techniques were discussed by **(Matsuoka et al., 2002)**. 14 primer pairs for the detection of segments, such as promoter, terminator regions, and construct genes were designed. Their results indicated that these methods are useful for fast and easy screening and detection of GM crops.

2.3 Limit of detection (LOD)

The Limit of detection method for the PCR method for herbicide tolerant soybean and insect resistance maize were estimated by (**Jankiewicz et al., 1999**), they estimated for their PCR experiments the theoretical detection limit to be 0.005% GMO/non-GMO (w/w), corresponding to 30 copies of haploid genome copies or to 9 copies of haploid genome. The authors showed that the practical detection limit is significantly (20x) lower; i.e. 0.1% GMO/non-GMO (w/w) corresponding to 596 copies of RR soybean haploid genome of to 185 copies of Bt maize haploid genome. No claims concerning limit of detection have been supported by appropriate validation studies. In addition, the accuracy of the method is certainly dependent on the type of GMO, food matrix and processing involved and the analyses of samples containing a low concentration of GMs may show a great error.

The limit of detection of Bt-maize in mixtures of DNA from transgenic and conventional maize by using the primer pair cry03/03 was studied by (**Haupfer et al., 1999)** and the results demonstrated that Bt maize DNA proportions as low as 0.01 were still clearly detectable using the present PCR set up. Detection limits of ethidium-bromide-stained agrose gels and southern hybridization with DIG-labelled probe 2 were in the same range. Taking into account the number of PCR cycles.

The sensitivity of the method of detecting recombinant DNA's from four lines of genetically modified maize was represented by (**Matsuoka et al., 2000)**. Their results indicated that the detection sensitivity was about 0.05% for Event 176, MON 810 and the Liberty, and about 0.07% for Bt11 by using a multiplex PCR methods.

The qualitative detection and identification methods for StarLink maize (event CBH-351) was presented by **(Windels et al., (2003)**. The methodology proposed envisages detection of an internal target site in the cry9c coding region, as well as two event-specific target sites at the junction between the CBH-351 insert DNA and the genomic plant DNA. The cry9c-specific primer pair, generating a 180 bp amplicon, has been tested and optimised for conventional end-point PCR amplification. The event-specific primer pairs, generating amplicons of 138 bp and 100 bp respectively, give good performance in a conventional end-point PCR and in a real-time PCR assay. The results clearly demonstrate that the primer pairs proposed can be used in an unambiguous and specific PCR identification assay for StarLink maize.

2.4 Quantitative competitive PCR

Disadvantage of the conventional PCR is lack of accurate quantitative information due to the influence of the amplification efficiency (E). If the reaction efficiency for each amplification cycle remained constant, the concentration of DNA following PCR would be directly proportional to the amount of initial DNA target. Unfortunately, E is not a constant parameter but varies between different reactions, as well as within one reaction, particularly in the later cycles of the PCR, when products are formed with an unknown reaction rate and in a non-logarithmic fashion. In order to have a maximum sensitivity, product formation is indeed measured when the amplification reaches the maximum product yield (known as the plateau phase), i.e. when the correlation between the product concentration and the number of the initial target molecules is very poor (reviewed in: **; Raeymaekers, 1993; Rasmussen, 1994; Köhler, T.1996; Bindler et al., 1999; Gadani et al. 1999)**. Thus, conventional PCR relies on end-point measurements, when often the reaction has gone beyond the exponential phase because of limiting reagents. Other PCR-based techniques, such as quantitative competitive PCR (QC-PCR) and real-time PCR have been recently developed which address the problems of establishing a relationship between the concentration of target DNA and the amount of PCR product generated by the amplification.

For relative GMO concentrations in food mixtures, the quantification of a GM-marker has to be normalized to a plant-specific reference gene **(Wurz, et al., 1999; Hübner et al., 1999b; Hupfer et al. 2000)**. In practice, accurate relative quantification might be achieved by a combination of two absolute quantification reactions: one for the GMO-specific gene and a second for the plant reference gene. With the assumption that GMO material has been submitted to the same treatment as the non-GMO material, the measurement can be expressed as genome/genome % (g/g %) or weight/weight (w/w%). By reducing the influence of the varying amplification efficiency, the accuracy of the quantitative information obtained by PCR can be improved. The analytical strategies can be divided into two groups:
Co-amplification of a target analyse with an internal standard, which allows a correction for the decrease in reaction efficiency, such as in Quantitative competitive.

2.4.1 PCR (QC-PCR) and in double QC-PCR

Measurement of the PCR amplicon in an early stage of the reaction, when the efficiency is still constant and the product concentration still correlates well with the concentration of the

initial target molecules, such as in PCR-ELISA and real-time PCR. The PCR procedures belonging to these two analytical methods are discussed here in more detail.

The first attempts to introduced elements of quantitation into PCR were reported in the late eighties and early nineties. This competitive PCR was based on co-amplification of the target region and a second template (competitor, internal standard) carrying the same primer bending sites. The later can be a modified version of the target sequence, either shortened by a detection or enlarged by an insertion, or non-related sequence flanked by the same primer binding sites. Assuming identical kinetics of DNA amplification for both sample and competitor target sequences the ratio of brand intensities of the co-amplified products cand serve as a semi-quantitative measure. Calibration if performed using dilution series of standard sample and sompititor DNA to establish the point of equivalence, i.e. where equal amounts of the two amplicons are produced. For convenience, a given PCR system can be adjusted to a practically relevant threshold value, such as 1% of GMO, thus enabling the analyst to work out whether the GMO content of a sample is above or below the threshold. The evaluation can be done by the visual inspection of ethidium bromide-stained agarose gels or, more precisely, by densitometric scanning of gel bands or HPLC analysis of PCR products **(Diviacco et al., 1992; Studer et al., 1998)** used competitive PCR examine whether samples from nine commercially available food products had GM soya and maize contents higher or lower than 1%. The procedure included quantitative evaluation of electrophoretic bands by scanning densitometry and was recommended for routine trails.

(Hubner et al., 1999) have development and evaluated of a quantitative competitative PCR (QC-PCR) sytem for the detection and quantification of Roundup Ready^{TM} soy beans and Maximizer ^{TM} maize DNA in food samples. The principle of the method is the amplification o internal DNA standards together with target DNA and their subsequence quantification by photometric methods. DNA standards were constructed in a manner similar to the GMO by specific sequence insertions. The system was calibrated by co-amplification of mixtures of GMO DNA and corresponding amounts of conventional soy bean or maize DNA. Such standards are commercially available and contain known amounts of standard DNA. Deterioration of the so-called equivalence point is the basis for quantification. The system has been tested on commercial food samples (lecithin, flour, protein, and grist), with varying GMO contents ranging from less than 0.1% to more than 2% using certified Roundup Ready^{TM} flour mixtures for calibration. Detection of as little as 0.1% GMO DNA in samples is possible. Results of an interlaboratory study carried out among laboratories mainly from Switzerland showed that there

are no problems with matrices containing more than 2% GMO (i.e., there were no false positives or false-negatives) and that the semi-quantitative method is applicable for matrices containing more than 1% GMO. One of the advantages of QC-PCR methods relative to non-competitive PCR methods is that interference of PCR inhibitors will be ready evident because they will affect both internal standard and target DNA amplification. QC-PCR methods provide excellent possibilities for the simple and reliable detection and quantification of GMOs in food samples.

Another approach favored target sequences in the CaMV35s promoter and nos-3 terminator regions for semi-quantitative detection transgenic ingredients. The suitability of competitive PCR for monitoring of 1% threshold values of RR Soya was demonstrated in a Swiss interlaboratory trail where relative standard errors were 9% and 2% for samples containing 0.5% or 2.0% GMO, respectively. Interestingly, interlaboratory variations were found to be lower than in a comparable qualitative PCR trail. **(Hardegger et al., 1999; Hubner et al., 1999)**.

The findings of the assessment of the so-called “Double QC-PCR”, a technique in which the concentration of soybean DNA in different samples is first normalised using a QC-PCR quantification of the soybean-specific lectin le1 gene was reported by (**Wurz et al., 1999)**. When the same samples are submitted to a second QC-PCR for a GM marker, relative quantification can be established. However, since the generation of calibration curves is rather complex and the accuracy depends on various factors, Wurz et al. only used one competitor concentration, equivalent to 1% GM soybean (Roundup Ready™). Therefore, the method could only distinguish if a sample contained more or less GM material compared to the calibration concentration of 1%. Within this determination, some degree of uncertainty could not be avoided.

The Double QC-PCR method for the quantification of Bt maize was described by **(Hupfer et al. 2000)**, in which multiple competitor concentrations were used for the quantification of the amount of transgenic DNA, as well as for the quantification of the total amount of amplifiable maize DNA. This allowed obtaining a good correlation between the actual and measured GMO concentration, even when the amount of amplifiable DNA was reduced by a heat treatment to less then 20% of the initial amount. Furthermore, they showed that the reduction of amplifiable DNA could not be observed by UV measurement, which demonstrates the need for an accurate quantification of both GM and endogenous plant marker. The use of dual QC PCR might reduce the inter-laboratory differences observed in ring trial studies.

The limitations of the (single-) competitive PCR approach were shown in a study on heat-treated maize samples by **(Hupfer et al., 2000).** As the recovery rate of Bt-176 maize DNA decreased continuously in the course of heat treatment the calculated GMO contents in standard mixtures were significantly below the true values. The resultant systematic error was compensated by using a dual-competitive approach based on co-amplification of maize-specific and GM-specific traget sequences that were located in the *ivr* (invertase) gene and the CDPK pomoter/cryIA(b) gene region, respectively.

2.4.2 PCR –ELISA

PCR-ELISA uses the strategy of the second group and can be quantitative when the PCR is stopped before a significant decrease in amplification efficiency occurs. ELISA has been used to quantify the relatively low amounts of PCR products (**Landgraf et al., 1991; González et al., 1999**). Despite the fact that relative quantification using PCR-ELISA has been applied in different fields (e.g. (**Taoufik et al., 1998)** and that a GMO detection kit using PCR-ELISA has been commercialised (D-Genos, Angers, France), this technique has not been widely adopted for accurate GMO quantification purposes.

(Petit et al, 2003) discussed the screening method for genetically modified organsms and specific detection of Bt 176 maize in flours and starchs by PCE -enzyme linked immunosorbent assay. Polymerase Chain reaction (PCR)_Enzyme Linked Immunosorbent assay (ELISAs) targeting either the 35s promoter or the Bt 176 specific junction seqencese were developed to screen for the presence of genetically modified organisms (GMOs) and Specifically detect Bt 176 maize in flours and straches. Two Additional PCR-ELISA assay were developed to validate the results: one, based on the detection of the alchol dehydrogenase 1 promoter specifically detected the presence of maize, and the other, based on the detection of a conserved sequence of the plants (25S ribosomal RNA gene), validated the extracted DNA amplification. The PCR.ELISA assay developed were highly specific and found to be as sensititve as the reference Southern hydridisation assay. The PCR_ELISA tests were at least 6 time more than gel electrophoresis and allowed 0.1 % GMOs to be detected in Bt 176, Bt 11, Mon 810 maize and Round up soybean. The PCR: ELISA tests are a method of choice for GMO screaning and identification Bt 176 maize in flours and native Starches. They may offer GMO a cheaper Alternative to the expensive real time PCR assay and may be useful in laboratory GMO monitoring.

2.4.3 Real-time PCR

Another method of the second group that improves the accuracy, specificity and throughput of quantitative PCR is "Real-time PCR" **(Heid et al., 1996).** This technique was originally developed by **(Higuchi et al., 1992**). and is rapidly gaining popularity due to the introduction of several complete real-time PCR instruments and easy-to-use PCR assays. A unique feature of this PCR technique is that the amplification of the target DNA sequence can be followed during the whole reaction by indirect monitoring of the product formation. Therefore, the conventional PCR reaction has to be adapted in order to generate a constant measurable signal, whose intensity is directly related to the amount of amplified product. Real time detection strategies rely on continuous measurements of the increments in the fluorescence generated during the PCR. The number of PCR cycles necessary to generate a signal statistically significant above the noise is taken as a quantitative measure and is called cycle threshold (Ct). As long as the Ct value is measured at the stage of the PCR where the efficiency is still constant, the Ct value is inversely proportional to the log of the initial amount of target molecules. One of the most popular assays for real-time PCR is the Taqman® or 5'-exonuclease assay, which employs a fluorogenic probe consisting of an oligonucleotide with both a reporter and a quencher dye attached. When the probe is intact the reporter fluorescence is quenched by the proximity of the quencher dye. Due to its target-specific sequence, the probe anneals specifically to the amplification product (target DNA) between the forward and reverse primers. During amplification, the 5'-3' exonuclease activity of the Thermus aquaticus (Taq) DNA polymerase cleaves the internal probe during extension, if hybridisation has occurred. The cleavage reduces the quenching effect and the fluorescent signal of the reporter dye becomes a measure of the amount of amplification product generated. Because the development of the fluorogenic reporter signal takes place only if both the PCR primers and the TaqMan® probe anneal to the target DNA, the specificity of real time PCR detection is considerably higher than that of conventional PCR. The relative quantification of a target gene is possible by preparing a standard curve from known quantities of an additional endogenous gene and extrapolating from the linear regression.

(Wurz et al., 1999) described how the real-time PCR is amenable to relative quanitification of GM soybean. Two different quantification reactions were applied to calculate the w/w% of GM soybean as a fraction of the total amount of soybean in a sample: a first one for the absolute quantification of total soybean DNA and a second for the absolute quantification o GM soybean DNA.

The similar method for the quantification of Maximizer^{TM} maize and Roundup Ready^{TM} soybean was described by (**Vaitilingom et al.,1999**), and demonstrated the suitability of real-time PCR for the relative quantifiction of GM material in different food ingredients. The authors also reported how the combined quantification of the GM merker and an endogenous reference gene could be made in a single tube by using a multiplex PCR. Therefore, the quantitative PCR analysis of each sample was not affected by random differences in experimental factors such as pipetting error, while the internal standard is an improved control of false negative results. The results indicated that the quantification procedure was then easily and reliably applied to various food products even for samples with low DNA quantity (muesli and lecitin). The use of multiplex PCR in the quantification is made possible by the use of different reporter dyes, which can be detected separately in one reaction tube. Multiplex reactions are not only an economical way of doing PCR, but they also allow accurate relative quantification without previous estimation of DNA quantity or copy numbers. With a multiplex reaction, a direct relation between % GMO and the results of the real-time PCR can be established. This reduces the variation and permits accurate data interpreation by simple statistical evaluation of the quantification results (Pijnenburg, 1998). Due to the above-mentioned advantages, multiplex real-time PCR is increasingly applied in genetic analysis (**Johnson et al., 2000; Siler et al., 2000)** devloped an assay similar to that described by (**Vaitilingom et al., 1999)**, with an additional assay for 35S promoter and the nos terminator.

The multiplex real-time PCR assay was successfully applied to GM maize was used by (**Hohne et al., 2000 ; 2002)** that employed zein as the endogenous reference gene and the P-35S promoter as GMO marker of four different types of GM corn.

(Holck et al, 2002) discussed the method for quentitive event-specific detection of the genetically modified Mon810 maizGard. The sequence of the 5 flaking region of the Mon 810 insert was determined by using ligation medited PCR . Aprimer probe set over lapping the junction was desigend and used in a quentified, event -specific Taqman, 5 nuclease assay Mon 810 DNA was quentified relative to endogenous maize zein gene DNA. The results were expressed as the percentage of genetically modified Mon 810 maize DNA relative to the total content of maize DNA.

The qualitative detection and identification methods for StarLink maize (event CBH-351) was presented by **(Windels et al., 2003)**. The methodology proposed envisages detection of an internal target site in the cry9c coding region, as well as two event-specific target sites at the junction between the CBH-351 insert DNA and the genomic plant DNA. The cry9c-specific primer pair, generating a 180 bp amplicon, has been tested and optimised for conventional end-point PCR amplification. The event-specific primer pairs, generating amplicons of 138 bp and 100 bp respectively, give good performance in a conventional end-point PCR and in a real-time PCR assay. The results clearly demonstrate that the primer pairs proposed can be used in an unambiguous and specific PCR identification assay for StarLink maize.

2.5 Safety of food produced by genetic engineering

2.5.1 Compositional, nutritional and toxicological analysis

The Flaver Saver TM tomato is the frist and only safety evaluation of a GM crop. The FLAVR SAVRTM tomato, was commissioned by Calgene, as required by the FDA. This GM tomato was produced by inserting kanr genes into a tomato by an 'antisense' GM method. The test has not been peer-reviewed or published but is on the internet. The results claim there were no significant alterations in total protein, vitamins and mineral contents and in toxic glycoalkaloids (**Redenbaugh et al., 1992)**. Therefore, the GM and parent tomatoes were deemed to be "substantially equivalent." Some rats died within a few weeks after eating GM tomatoes. In acute toxicity studies with male/female rats, which were tube-fed homogenized GM tomatoes, toxic effects were claimed to be absent. In addition, it was concluded that mean body and organ weights, weight gains, food consumption and clinical chemistry or blood parameters were not significantly different between GM-fed and control groups.

Several lines of GM cotton plants have been developed using a gene from *Bacillus thuringiensis* sub sp. kurstaki providing increased protection against major lepidopteran pests. The lines were claimed to be "substantially equivalent" to parent lines (**Berberich et al., 1996),** in levels of macronutrients and gossypol, cyclopropenoid fatty acids aflatoxin levels were less than those in conventional seeds. However, because of the use of inappropriate statistics it is questionable whether the GM and non-GM lines were truly equivalent, particularly as environmental stresses could have unpredictable effects on antinutrient/toxin levels (**Novake and Haslberger 2000).**

The compositional equivalence between conventional soybeans and glyphosate tolerant soybeans was studied by **Padgette et al., (1996)**. The results demonstrated that the composition of glyphosate tolerant soybeans seed is equivalent to that of conventional soybeans.

The results from feeding trials comparing soybean meal derived from glyphosate-tolerant soybeans and soybean meal from the conventional counterpart in broilers, catfish and dairy cows were reported (**Hammond et al. 1996)**. No differences were measured in feed intake, body weight gain, feed efficiency, breast meat composition and fat pad thickness in broilers. Catfish fed diets comparing both soybean meals exhibited no differences in weight gain, feed efficiency or meat composition. In the dairy cow study comparing the two soybean meal sources, no differences were measured in feed intake, milk yield, milk composition, dry matter digestibility and rumen.

In a short feeding study to establish the safety of GM potatoes expressing the soybean glycinin gene, rats were daily force-fed with 2 g of GM or control potatoes/kg body weight **(Hashimoto et al., 1999).** Although no differences in growth, feed intake, blood cell count and composition and organ weights between the groups was found, the potato intake of the animals was too low and unclear, whether the potatoes were raw or boiled. Feeding mice with potatoes transformed with a *Bacillus thuringiensis* var. kurstaki Cry1 toxin gene or the toxin itself was shown **(Fares et al., 1998**) reported that mice fed for 14 days on fresh potato immersed in a suspension of delta-endotoxin of B. thuringiensis var. kurstaki strain HD1 developed an increase of hyperplastic cells in their ileum. Feeding with fresh genetically modified potato expressing the cry1 gene caused mild adverse changes in the various ileac compartments, as compared to the control group on fresh potato. The occurrence of these effects in mice fed either 'spiked' potato or genetically modified potato may have been due to the toxicity of the Cry1 protein; however, no details were given on the intake of Cry1 protein or on dietary composition, which limits interpretation of this study.

In another study, young, growing rats were pair-fed on iso-proteinic and iso-caloric balanced diets containing raw or boiled non-GM potatoes and GM potatoes with the snowdrop (Galanthus nivalis) bulb lectin (GNA) gene **(Ewen et al., 1996).** The results showed that the potatoes was significantly increased. Most of these effects were due to the insertion of the construct and not to GNA which had been been pre-selected as a non-mitotic lectin unable to induce hyperplastic intestinal growth (**Putzai et al., 1990**) and epithelial T lymphocyte infiltration.

Teshima, et al., (2000) fed Brown Norway rats and B10A mice with either heat-treated genetically modified soybeanmeal containing the cp4-epsps gene, or control non-genetically modified soybean meal. These experimental animals were employed based on their immunosensitivity to oral challenges. The semi-synthetic animal diet was supplemented with 30% (w/w) heat-treated soybean meal, and fed over 105 days. Both treatments failed to cause immunotoxic activity or to cause the IgE levels to rise in the serum of rats and mice. Moreover, no significant abnormalities were observed histopathologically in the mucosa of the small intestine of animals fed either genetically modified or non-genetically modified soybean.

Herbicide tolerant and non-herbicide tolerant corn was compared in swine metabolism studies by **(Böhme and Aulrich., 1999)**. The results showed that no differences were measured in protein digestibility, nitrogen free extract (NFE) digestibility or metabolizable energy(ME).

The safety assesment of transgenic rice with glycinin gene was studied by (Momma et al., 1999a). The results demonstrated that no differences in most of composition analysis, but the transgenic rice with glycinin gene differs from the non transgenic control in the a mounts of protein, vitamine B6 and a few fatty acid but the level of fatty acids were much higher that the normal rice.

The safety of GM rice incorporating soybean glycinin by feeding studied on rats were evaluated by (Momma et al., 2000) which monitored the food intake and body weight, as well as the detailed blood composition, organ weight and histophtological findings. Throughout these feeding studies, there were no significant differences between the rat fed on the control rice and non-GM rice, expect for the salivary gland weight. The group, which fed GM rice, is similar to the group fed on non-GM rice. There were no differences in body weight changes. Minor histophtological findings such as microgranuloma or necrosis in the liver and basophilic change in the kidney are through to have accidental science they are sporadically found in any group of rats and there was no relevance with any other parameter.

Bt and non-Bt corn grain were fed by **(Aulrich et al., 1998)** in a five-day study to laying hens and measured no differences in nutrient composition, body weight, digestible organic matter and protein as well as metabolisable energy.

The broiler study comparing Bt and non-Bt corn was conducted (**Brake and Vlachos., 1998)** for a 38-day and measured no differences in mortality, body weight, feed intake while measuring an improvement ($p<.05$) in feed conversion. Carcass data was not different between groups with the exception of breast meat yield that was improved ($p<.05$) in broilers fed Bt corn.

Slight differences in overall composition of the diets may have been the cause for these improvements with the Bt corn.

(Halle et al., 1998) fed Bt and non-Bt corn in a 35 day broiler trial. There were no differences in body weight gain, feed intake, feed conversion or protein digestibility.

(Faust and Miller., 1999) fed green chop from Bt or non-Bt corn to lactating cows for 14 days. No differences were measured in feed intake, milk yield, milk composition or udder health. Two diets containing Bt or non-Bt corn silage were fed with grass silage and supplement in a five-week. There were no differences measured between diets for intake, milk production and milk composition (fat, protein, lactose, urea).

(Faust 1999) compared in-vitro digestibility between corn silages derived from Bt and non-Bt corn which were ensiled at two different stages of maturity. No differences were measured in cell wall, true or dry matter digestibility regardless of stage of maturity.

(Daenicke et al., 1999) compared the digestibility and animal performance of sheep and growing bull calves that were fed Bt corn silage or non-Bt corn silage. There were no differences in the digestibility of organic matter, fat, fiber or nitrogen free extract. Likewise, there were no differences in intake, body weight gain, feed conversion, hot carcass weight, dressing percentage and abdominal fat. The feeding value of whole plant corn silage and crop residues over a two year period was compared between Bt and non-Bt corn by **(Hendrix et al. 2000)**. Three studies were conducted each year: 1) performance of steer calves fed corn silage, 2) performance of beef cows grazing corn residue and 3) grazing pattern of beef cows when given a choice between Bt and non-Bt residue. There were no differences between steers fed the two corns silage sources for average daily gain or dry mater intake. Feed/gain was greater ($P<.05$) for Bt vs. non-Bt corn silage. There was no difference in weight change between cows grazing the Bt and non-Bt residues. Over the entire observation period, no differences were measured in preference for one corn residue over the other between grazing cows.

(Sidhu et al., 2000) measured no difference in growth, feed efficiency and fat pad weight between broilers fed diets containing glyphosate-tolerant corn and diets containing conventional corn.

(Mireles et al., 2000) conducted two studies to compare nutrient composition and availability in Bt and non-Bt corn. The first study measured true metabolizable energy (TME) and amino acid digestibility. There were no differences for TME or amino acid digestibility between the two corn sources. The second study was designed to measure the performance of

broiler chickens fed starter feeds. No differences were seen for the parameters that were measured between Bt and non-Bt corn (weight gain and feed efficiency).

(Folmer et al., 2000a) conducted research designed to compare the ruminal fermentation parameters and lactational performance between four balanced diets containing Bt or non-Bt corn silage either from early maturity or late maturity hybrids in a 4 x 4 Latin square design. No differences were measured between Bt and non-Bt diets at either maturity for rumen fermentation characteristics (in-situ NDF digestibility, rumen VFA concentration, rumen pH), milk production or milk composition. The animals fed early maturity hybrids (Bt and non-Bt) did have improved (P<.005) total rumen VFA and efficiency of production than later maturity hybrids (Bt and non-Bt).

The effect of feeding glyphosate tolerant and non-glyphosate tolerant corn silage andcorn grain fed in identical mixed rations to lactating dairy cows was determined by **(Donkin et al., 2000)**. No differences were measured between groups for dry matter intake, milk production, milk protein yield, lactose yield or milk fat yield. Likewise, no differences were measured in milk composition (percentage of: fat, protein, lactose, solids not fat, somatic cell count or milk urea nitrogen.

Animal performance of beef cows grazing Bt or non-Bt corn crop residue has been compared over a two-year period **(Russel et al., 1999, 2000b**). There was no difference in animal performance in either year of the two-year study.

Two trials were conducted to evaluate the utilisation of corn silage and corn residue by **(Folmer et al., 2000).** An absence of significant European corn borer pressure resulted in similar grain yield and residue corn between Bt and non-Bt corn. In trial 1, twenty-three acres of Bt corn residue and twenty-one acres of non-Bt were divided into 3 pastures each and stocked with 8 or 9 steers per pasture to result in equal stocking rates. Average daily gain (avg. 0.28 kg/d) was similar between both corn sources. In addition, 16 steers were allowed access to either 7 acres of Bt or non-Bt corn residue. No preference was show for grazing either field. In trial 2, 128 steers were fed diets containing either Bt or non-Bt versions of two hybrids as corn silage at a 90% inclusion rate with 10% supplement. The steers were fed in a 2 x 2 factorial design and performance parameters were measured. Dry matter intake was higher (P>0.05) for steers fed Bt than non-Bt corn silage (8.61 vs. 8.32 kg/d respectively). An interaction (P<0.05) was observed between genotype and the Bt trait for daily gain and feed efficiency. For hybrid A, steers receiving the Bt version had improved daily gain over those receiving the non-Bt version. For hybrid B, there was no significant difference in daily gain between the steers fed the Bt vs. the

non-Bt version. Steers fed both versions of hybrid A had improved (P<0.05) feed efficiency when fed the Bt version than when fed the non-Bt version. No differences were measured in feed efficiency between steers fed the Bt and the non-Bt versions of hybrid B. Improved (P<0.01) daily gain and feed efficiency were measured for steers fed hybrid B compared to hybrid A, although an interaction was present. The authors concluded that while hybrid genotype appeared to affect performance, there was no consistent effect on performance of growing steers due to the presence of the Bt trait.

(Weber et al., 2000) compared grower-finisher performance and carcass characteristics from pigs fed Bt, the non-Bt isogenic counterpart or commodity-sourced (CS) corn. For animal performance, no differences were measured in average daily gain, average feed intake, or feed efficiency between pigs fed any of the 3 corn sources. Pigs fed Bt and the non-Bt corn were not different in carcass weight however pigs fed CS corn had heavier carcass weights and higher dressing percentages (P<0.05) than the other two groups. Pigs fed the isogenic control had less (P< 0.05) percent lean, greater back fat depth at the 10th rib and P2 location than pigs fed diets containing Bt or the CS corn. Pigs fed the non-Bt had greater (P<.05) back fat depth at the last lumbar vertebrae than pigs fed CS corn. Marbling scores were highest (P<.05) for pigs fed Bt and non-Bt corn. Weber et al concluded that Bt corn had no adverse effects on growth performance or carcass characteristics.

(Russell et al., 2000a) studied the nutritive value of the crop residues from Bt and non-Bt corn hybrids. No differences were measured between the residues from Bt and non-Bt residues for in-vitro digestible dry matter. The in-vitro organic matter digestibility in residues selected by steers after two weeks of grazing also did not differ.

(Russell et al., 2000b) studied the nutritive value of the crop residues from Bt and non-Bt corn hybrids and their effects on performance of grazing beef cows. No differences were measured between the residues from Bt and non-Bt residues for dry matter or organic matter composition. Over the grazing season, no differences were measured between residues for rates of change of residue composition. In further work.

(Russell et al. 2001) studied the effects of grazing crop residues from Bt-corn hybrids on the performance of pregnant beef cows. Four hybrids planted on duplicate fields were utilized in the study that was conducted over 2 consecutive years. One hybrid was non-Bt while three hybrids contained the Bt gene (two with Yieldguard ® and one with Knockout ®). Thiry Angus x Charolais x Simmental cows in midgestation were allotted between to two dry lots or the eight crop residue fields to strip-graze for 126 days. Biweekly visually estimated body scores were

taken with dry alfalfa hay supplemented to maintain a mean body condition score of 5 out of a 9-point scale. Crop residue yields were determined monthly from a 4 square-meter location in each grazed and ungrazed area paddock. On two consecutive days following 2 weeks of grazing, forage selected during a two-hour grazing period by one fistulated steer per field or dry lot was harvested from via the rumen cannulae. DMI was calculated from the digestibility of the forage and the fecal output in two cows per field or drylot during the same 2-day period. There were no effects on yields of harvested grain, dropped ears or grain, residue DM or OM over the 2 years. AT grazing initiation, IVOMD as well as ADF and ADL differed (P<.05) by base genetics but not by Bt vs. non-Bt hybrids. Rates of change in NDF, ADF, ADL, CP and IVOMD over winter did not differ between hybrids. There were also no differences between hybrids for intakes of forage digestible OM, NDF and ADF. No differences were seen in the amount of hay required to maintain body condition score between hybrids.

(Kerley et al. 2001) compared Bt and non-Bt corn fed to beef steers for the last 49 days of the finishing period. Thirty-six crossbred steers were allotted to six pens and fed a 75% corn diet. Growth performance and carcass parameters were measured. There were no differences in corn composition, average daily gain, feed efficiency , yield grade or quality grade between Bt and non-Bt corn hybrids.

(Petty et al. 2001) compared Bt vs. isogenic non-Bt corn fed as whole plant silage (WPS) and dry rolled grain over a two-year period. Each year corn was grown under isolation and harvested as grain and WPS. A feeding study was performed each year utilitsing 56 Angus and Simmental sired steers were blocked and randomly allotted by weight and breed type one month post weaning into eight pens of seven steers each. Growing diets comprised primarily of WPS were fed for 89 and 85 days in years 1 and 2 respectively were followed by finishing diets comprised of 75% dry rolled corn, 15% WPS and 10% supplement for 101 and 84 days in years 1 and 2 respectively. During the grower phase in year one, there were no differences (P>05) in average daily gain or dry matter intake but feed efficiency was improved (P<.05) for the steers fed non-Bt corn however this difference was not measured in year 2. There were no differences (P>.05) in average daily gain, dry matter intake or feed efficiency during the finishing phase for either year. Steers were harvested each year when they were estimated to be 75% USDA choice as a group. There were no differences (P>.05) in carcass characteristics in either year. The investigators summarised that there were no major differences in the feeding value of the Bt-corn compared to it’s isogenic counterpart.

(Reuter et al., 2002), investigated that the variability of crude nutrients, minerals, amino acids, fatty acids and selected ingredients from transgenic Bt-maize seeds and parental maize seeds in within the expected range and in the gaven range in table value. There were differences in the ingredients, especially the crude nutrients for both maize lines and are equals feedstuff for pig.

(Tony et al., 2003) concluded that there was no significant difference between the Bt maize 176 an d conventional maize in crude nutrients, minerals, amino acids, fatty acids and selected ingredients from transgenic Bt-maize seeds and partental maize seeds in within the expected range and in the given range in table value. There were no differences between the broiler fed Bt maize and conventional maize in broiler performers.

2.6 Metabolic fate and safety of ingested DNA, including genetically modified DNA

The (**FAO, 1991, WHO, 1991**), have concluded that there is no inherent risk in consuming DNA, including that derived from biotech crops. A key reason for their conclusion is the long history of safe consumption of significant quantities of DNA from a wide variety of sources including plants, animals and microbes by animals and people. DNA, a nucleic acid, encodes the fundamental genetic information by which the vast majority of organisms convey instructions for function and survival of self to subsequent generations. Consequently, DNA is an essential component of most living organisms and, as such, is present in nearly all foods and feedstuffs. In biotech crops, the introduced transgenic DNA molecules are made of exactly the same basic chemical components as the endogenous DNA (four ubiquitous nucleotides-adenosine,guanosine, thymidine, and cytosine). Therefore, the addition of transgenic DNA into aplant does not introduce any new chemical entities to foods or feeds. Generally, the total DNA in food contributes less than 0.02% to the total dry matter of the food **(Waston and Thompson 1990).**

The amount of transgenic DNA in plants improved through biotechnology represents an extremely small proportion of the total amount of DNA in a biotech plant (<0.0004% of the total plant DNA). To help put into context the level of transgenic and total DNA consumed by an animal, it has been estimated that approximately two thirds of a gram (608,000 μg) of DNA is consumed on a daily basis by a 600 kg animal such as a cow **(Beever et al.,2000**). If 60% of the feed were from a biotech crop such as BT176 maize, the daily intake of transgenic DNA would be approximately 1.5 μg, which is approximately 0.00025% of the total amount of DNA ingested per day. The gastrointestinal tract is constantly exposed to foreign DNA that is released from

partially or completely digested foods or feeds, ingested microbes, and DNA from intestinal microflora. Ingested food is mechanically disrupted and the released DNA is cleaved through acid hydrolysis and enzymatic digestion (especially by DNase I from salivary and pancreatic secretions) into small DNA fragments and eventually converted to single nucleotides (**Macallan , 1980**), The presence of various phosphatases and deaminases continue to destroy the structural integrity of any free DNA. One study with beef steers showed that plant DNA in feed is progressively degraded as it moves through their digestive tract, with over 50% being degraded in the first third of the intestine and 80% having disappeared by the time the digesta reaches the terminal ileum DNA given directly to steers was shown to be completely degraded into mononucleotides by the animal's digestive tract in about 4 hours (**Macallan , 1982).**
The generated nucleotides are readily abundant in food and feed and exceed nutritional requirements of the host (**Yu, 1998**) and gut bacteria (**Macallan, 1982).**

The breakdown products of DNA are absorbed for use in cellular synthetic processes as they can be found in blood and tissues (**Macallan , 1982).,** however, as intact nucleotides they are non-essential nutrients. The nucleotides are typically deaminated before being rapidly absorbed. Once absorbed, they are further catabolized into nitrogenous bases, free bases and other metabolites including sugars and phosphates that are used in cellular biosynthetic pathways (**Sonoda et al., 1978).** Interestingly, intestinal epithelial cells have unique salvage pathways for using free nucleotides, owing to their high rate of cell turnover (**He et al., 1994).** Any small polynucleotide DNA fragments that might enter the body would be phagocytized by mononuclear leukocytes and further degraded by cellular enzymes and nucleases **(Doerfler et al., 1997**).

The fact that the nucleotides of any gene (endogenous or transgenic) are in a precise sequential order to encode for production of a specific protein becomes essentially irrelevant to the digestive processes. The genetic sequence for a protein introduced in a plant is only functional when the DNA (gene) is activated in the plant. The presence of DNA in the diet is so common that it is of virtually no consequence to animals and people consuming plant-derived products. A recent publication describes experiments that directly tested whether extensive feeding of DNA to mice results in detectable expression of mRNA and protein in any of several organs of the animals (**Holweg et al., 2001**). Approximately 50 mg of DNA was fed to the mice per day. The DNA fed to the mice encoded the green fluorescent protein (GFP) under the control of one of three strong mammalian viral promoters [human cytomegalovirus (hCMV), Rous sarcoma virus (RSV) or simian virus 40 (SV-40)]. Separate experiments used a "gene

therapy”approach with intramuscular injection into mice of the GFP gene coupled with either the hCMV promoter (pEGFP-C1) or the RSV promoter. These gene therapy studies showed clearly detectable expression of the GFP protein and mRNA at the site of injection **(Holweg et al., 2001**). By comparison, no GFP protein or mRNA expression was detectable in liver, spleen, blood or intestinal epithelia of 21 animals fed the exact same DNA over a three week period. Also, fragments of the GFP gene were not detectable by PCR analysis of DNA isolated from spleen, liver or tail tip samples from either this three week feeding study or a separate experiment that involved feeding 50 mg of the pEGFP-C1 DNA per day to mice over eight generations **(Holweg et al., 2001)**. Therefore, it can be concluded from these studies that gene/promoter constructs clearly capable of functioning in vivo when administered via a gene therapy procedure (e.g. intramuscular injection) do not lead to gene expression in somatic cells or detectable integration into the germline of animals when provided orally. In addition to digestive processes that degrade DNA, feed processing procedures (and food preparation methods) significantly degrades DNA, especially those that involve heating to temperatures greater than 95°C (200°F) **(Gawienwski et al. 1999, Forbes et al. 2000**). The stability of transgenic DNA in maize preserved as silage has been studied (**Hupfer et al., 1999**).

The intact transgene was only detectable during the first five days of ensiling, with only small fragments (about 200 bp) of DNA being identifiable using sensitive PCR methods for longer stored silage. The rapid breakdown of DNA during ensiling was not unexpected since this process creates a harsh environment that involves plant tissue being chopped which leads to cell breakage, release of cell contents including the DNA and nucleases, and mild acidic conditions from natural fermentation. Thus, feed generated by ensilage reduces an animal’s dietary exposure to intact DNA, including any introduced transgenic DNA, even before ingestion and further degradation by its own digestive system. On a related issue of DNA uptake by intestinal flora, the concern as such could lead to foreign DNA persistence in the mammalian system. The possibility of plasmid DNA being incorporated via a normal biological process into endogenous gut bacteria is minimized due to the non-conjugative nature of typical plasmids used in recombinant DNA laboratories (**Hamer. 1997),** and the low frequency with which unaided transformation (uptake of naked DNA) occurs. Furthermore, beyond the difficulty of unaided transformation is the lack of stable incorporation **(Behr et al. 1989)** for DNA in general. Moreover, the probability of transferring such plasmids into natural bacteria in the gut environment has been calculated to be less than one in one million (**Maniatis et al, 1982**).

In fact, the DNA remaining after digestion is small random pieces of DNA regardless of the food material. Thus, the fundamental question is not related to any effects from the presence of transgenic-DNA, it is related to whether such DNA will be incorporated into the host cells in a functional way. There is no precedence for DNA being incorporated into host cells beyond the use of the basic nucleotide building blocks as nutrients. And, in fact, acid hydrolysis in the stomach is expected to depurate most adenosine and guanosine nucleotides rendering the DNA sequence of questionable value **(Klinedinst and Drinkwater, 1992**). A series of papers from Prof. Walter Doerfler's laboratory **(Shubbert et al, 1994, Shubbert et al , 1997, Doerfler et al 1997, Shubbert et al. 1998, Doerfler. 2000, Holweg et al., 2001)** have addressed questions on the fate of ingested DNA. In preliminary work, **(Shubbert et al, 1994)** the Doerfler team fed mice circular M13 bacteriophage DNA (~ 7.2 Kb) and were able to detect small DNA fragments in certain organs and tissues. These fragments were mostly 200 - 400 bp in size, although up to 1.7 Kb fragments were detected in the feces and up to 500 bp fragments were detectable in the blood. These DNA fragments were detectable within 2-7 hours after feeding. The sum of all of the DNA fragments recovered from all tissues and feces could account for 2-4% of the total M13 fed to the mice, with only 0.01% detectable in the blood. Therefore, 96-98% of the ingested DNA was presumably digested quickly and completely to very small and undetectable pieces. Furthermore, in vitro incubation of intact M13 DNA in blood demonstrated complete elimination within 6 hours. The conclusions from this pioneering work were consistent with the general understanding that DNA is a normal component in food and subjected to extensive degradation during digestion. The authors stated "The implication that a random mixture of DNA including gene fragments or intact genes of animal, plant or microbial origin should have been constantly excreted by innumerable organisms over millennia does not appear startling given the complexities of evolution. This barrage of linear DNA fragments, i.e. of recombinationally highly active DNA fragments in Nature should mitigate any concerns that one might have had in the past about biological consequences of experiments carried out with recombinant DNA over the course of the past two decennia." Earlier research (**McAllan, 1982**), complements these reports on studies on the fate of naked DNA demonstrated complete and quick digestion in the intestine.

In recent publication by (**Reuter and Aulrich., 2003)** concluded that feed-ingested DNA is partially resistant to the mechanical and enzymatic activities of the GIT and is not completely degraded. These results confirm that the absorption of functional large size DNA is low.

2.7 Detection of genetically modified DNA in farm animal products

(Klotz and Einspanier, 1998) published that the CP4 EPSPS gene of Roundup Ready® soybeans was not detectable by PCR followed by Southern blot in either the blood of the cow or the milk from these animals (**Klotz and Einspanier, 1998).** This publication showed that the highly sensitive method of PCR followed by a Southern was able to detect a small fragment of a highly abundant endogenous chloroplast gene in blood lymphocytes but not milk. Very recently, Einspanier's laboratory has published data from a study in which dairy cows, beef steers and broiler chickens were fed either conventional maize grain or grain from Novartis' BT176 event **(Flachowsky et al. 2000; Einspanier et al, 2001)**

The investigators evaluated two DNA detection technologies [standard Polymerase Chain Reaction (PCR) and Light Cycler "real time" PCR]. Although Light Cycler PCR showed advantages for detecting Btmaize in feed, this technique did not provide additional sensitivity beyond standard PCR methods for animal tissue samples. The presence of even a small portion of the coding region of the Bt gene (Cry1Ab) was not detectable by either standard PCR or Light Cycler PCR in any samples from the cows, steers or chickens fed BT176 maize. Similar to their previous report, using standard PCR technology, a small portion of the coding region of a highly abundant chloroplast gene (tRNAleu) was detectable in lymphocytes of dairy cows and in muscle, liver, spleen and kidneys of chicken, but not in dairy milk, or any tissue samples from steers. It is important to note that plastid genome copy number per cell varies depending on tissue-type, ranging from ~500 to 10,000 copies in roots and leaves, respectively **(Bendich, 1987)**. Therefore, the copy number of plastid genes is orders of magnitude higher than a transgene in a biotech product, which is typically has only one copy present per haploid genome. In addition, plastid gene sequences are also present in high numbers in the nuclear genome, with sometimes >100 copies of some sequences being observed **Ayliffe et al., (1998)**, such that the nuclear copies of plastid genes are an additional source of positive PCR signals. As a consequence, the high copy number of plastid genes and their subcellular localization within organelles could explain detection of these endogenous genes while transgenic DNA fragments areundetected to date.

(**Khumnirdpetch et al., 2001**) of Thailand, presented a poster at the 9th Plant and AnimalGenome Conference in San Diego, January 2001, in which results from their studies attempting to detect transgenic DNA in broiler chickens were shown. Broiler chickens were maintained by commercial standards and fed diets containing meal from either conventional or

Roundup Ready® soybeans from birth to seven weeks of age. Samples (meat, skin, duodenum and liver) were isolated from the birds at 1, 3, 5 and 7 weeks.

Real-time PCR was used to test for the transgenic DNA in the various samples. PCR results of the broiler samples taken over this entire seven-week feeding period were all negative. The authors speculated that the negative detection results suggest that the transgenic DNA in Roundup Ready® soybean meal has been fully degraded in the digestive tract of the broilers. Weber and Richert's poster at the 2001 Midwest meeting of the ASAS and ADSA also included data on PCR studies attempting to detect both the Bt gene and an endogenous corn gene in DNA extracted from 24 pork loin samples (12 fed YieldGard® corn and 12 fed a control conventional corn). PCR, followed by Southern blot analysis for ~200 bp fragments of the cry1Ab and shrunken-2 (sh-2) were uniformly negative (**Weber and Richert, 2001**). The sh-2 gene is an endogenous single-copy corn gene. By comparison, an endogenous swine gene (pre-prolactin) was readily detected in all pork loin samples, and spiking corn DNA into the extracted swine DNA also yielded positive results, indicating that the DNA quality and PCR conditions were both favorable for detection of DNA fragments, had they been present in the original samples. The PCR assay coupled with Southern blot was shown to have a limit of detection of approximately 1 to 2.5 pg of target DNA per 1 μg of input DNA, or approximately 1 genome equivalent of the target gene per PCR, the theoretical limit of assay sensitivity. DNA degradation during the digestive process has been documented from mouse feeding studies with M13 phage DNA and recently reviewed **(Doerfler, 2001).** From studies feeding purified M13 phage DNA to mice, it was observed that up to approximately 0.1% of that ingested DNA could be detected in their blood. **(Schubbert et al., 1997; Schubbert et al.,1998).** This extremely high level of DNA observed in the circulation is most likely owing to unique features of this circular, non-methylated phage DNA. Using the M13 data from mice, however, a calculation can be performed to predict the theoretical level of transgenic DNA that might be present in animal tissues, assuming uniform tissue distribution of that DNA in the farm animal. Basing uptake of transgenic DNA in farm animals on the mouseM13 phage data, it can be estimated that approximately 0.002 fg of transgenic DNA (1 femtogram equals one-trillionth of a mg, or 10-15 of a gram) might be present per mg of muscle tissue in the farm animal. No transgenic DNA has been detected in meat, milk or eggs from farm animals fed biotech products. These results are consistent with the knowledge that there are extremely small amounts of transgenic DNA in plants improved through biotechnology (<0.0004% of the total plant DNA). However, it is important to remember that even if transgenic DNA is detected by a future study, scientific

evidence and opinion concludes that ingested transgenic DNA would not be any different from ingestion of DNA already in foods, which is deemed safe. The safety of ingested DNA cannot only be derived from the long natural history of animal and human consumption of DNA, but it is also significant that, as would be expected because of digestive processes, no intact genes, only relatively small fragments, have yet been detected in animal tissues, regardless of the gene's abundance. Instead, in the published reports describing detection of DNA from ingested plants in animal tissues, only small portions of the entire coding region of these highly abundant chloroplast genes were found **(Klotz et al., 1998; Einspaner et al., 2001)**. Furthermore, only samples from a fraction of the total number of tested animals are yielding positive detections for these highly abundant gene fragments, suggesting that most of the individual animals are degrading ingested DNA to levels below the most sensitive PCR detection limits. Therefore, the likelihood that a transgenic gene or fragments is absorbed to any significant degree following digestion remains extremely low, especially when the relatively low levels of the transgenic DNA per cell is also considered when compared to the highly abundant endogenous plastid genes.

3. MATERIAL AND METHODS

3.1 Detection of a genetic modification in soybean and maize from the Egyptian food market

3.1.1 Sampling

3.1.1.1 Soya Samples

Forty samples of commercially available soybean and soybean products were collected randomly from markets in Cairo and Giza, or provided by the Food Technology Research Institute or the Central Laboratory for Food and Feed (Egypt) throughout the years 2000 / 2001. Samples included diverse processing steps from relatively mild treated ground soybeans to highly processed bakery products and snacks.

3.1.1.2 Maize samples

Forty samples of commercially available maize and maize products were collected randomly from markets in Cairo and Giza, or provided by the Food Technology Research Institute, or the Central Laboratory for Food and Feed (Egypt) throughout the years 2000 / 2001. Samples included ground maize and processed products from maize meal. (All collected samples of soya and maize are listed in Tables (10) and (11).

3.1.2 Reference Materials

Certified reference materials (CRMs), produced by the institute for reference material and measurements (Geel, Belgium) were used as negative and positive controls for soy and maize lines Bt176 and Bt11 CRMs were purchased from Fluka; Table (1).

Table (1): CRMs ordering numbers (Fluka)

CRM	0% GMO	0,5% GMO	1% GMO	2% GMO	5% GMO
Soya RRS	85474	85477	-	85478	-
Maize Bt176	63195	63197	-	-	17111
Maize Bt11	09754	-	17947	09757	-

Because there is no CRM available for maize lines MON 810 and T 25, samples containing 1% GMO were prepared in the laboratory from these lines and used as positive

controls, whereas, the negative control that was used was the normal non GMO maize. For the Star Link maize, the positive control as well as the negative control, were provided with the commercial detection kit used for detection of this maize line (Commercial GMO/dent Star Link™ kit produced by Europe Gene Scan, Bremen Germany. Cat. No.: 5221102810).

3.1.3 Extraction and purification of genomic DNA

Soybean seeds and meal, and maize samples were ground in an electric grinder. Frozen products were placed at room temperature till thawed. 200 mg of samples as well as from the CRMs were used for the extraction of the genomic DNA according to the official German methods for soybean **(Anonymus, 1998)** and maize **(Anonymus, 2002)** by the cetyltrimethyl ammonium bromide (CTAB) method (3.1.3.2).

DNA from CRMs as well as from all investigated samples was extracted twice in independent procedures. Furthermore, a blank sample consisting of 200 µl autoclaved bi-distilled water was used to control reagents used in the work.

3.1.3.1 Reagents

Suitable molecular biology grade reagents were used and all procedures were carried out under substantially sterile conditions. The water used was bi-distilled and autoclaved or of equivalent quality. This specified solution (aqueous solution), was filtrated through 0.2 mm filter paper and autoclaved before used.

3.1.3.2 DNA preparation

Samples were stored dry and cool before subjected to DNA extraction. All centrifugation procedures were carried out at 4 ºC. All plastic material and glassware were obtained sterile and free from DNA. Due to the high sensitivity of PCR analysis and to avoid false positive results, I used aerosol-proof filter tips for the DNA preparation and the succeeding PCR procedures. About 200 mg of homogenised powder was prepared from samples and weighed in 2 ml reaction vessels. 1000 µl of CTAB-extraction buffer [CTAB (20 g/L), NaCl (1.4 mol/L), TRIS-base/HCl (0.1 mol/L) and Na_2-EDTA (20 mmol/L) adjusted with HCl, pH=8] were added and mixed well, (20 µl proteinase K and/or 20 µl RNAse may be also added). The mixture was incubated at 65ºC under shaking for 30 min. Centrifugation was done at 14000 g for 10 minutes and the supernatant transferred to a new 1.5 ml reaction vessel. 400 µl Ready Red® were added to the sample and mixed 30 sec. The mixture was centrifuged at 14000 g for 10 min to separate the two

phases. Then the aqueous (upper) phase was transferred to a new 1.5 ml reaction vessel. Two volumes of CTAB-precipitation solution [CTAB (5 g/l), NaCl (0,04 mol/l)] were added and mixed well. Then the mixture was incubated at room temperature for 60 min under shaking and centrifuged at 14000 g for 10 min. The supernatant was discarded. The precipitate was redissolved in 350 µl NaCl solution (1,2 mol/L) and allowed to stand about 5 minutes at room temperature (RNAse solution may be also used with the sodium chloride solution). 350 µl Ready Red® were added and mixed for 30 sec. and the solution subjected to centrifugation at 12000 g for 10 min until the two phases separated. The upper (aqueous) phase was separated into a new 1.5 ml reaction vessel. 0.8 volume parts of isopropanol were added, mixed and stored over night in the refrigerator at 4 ºC. The extract was centrifuged 30 min at 14000 g. The supernatant was discarded carefully using a pipette and 500 µl ethanol 70% were added and mixed. Centrifugation for 15 min at 14000 g was applied to precipitate DNA. The supernatant was discarded carefully and the DNA pellet was air dried under vacuum and resuspended in 50 µl sterile bi-distilled and deionized water purchased from (Fluka, Germany).

3.1.4 Quality control and concentration of the extracted DNA

The concentration of the isolated DNA was controlled and measured fluorometrically using a Dynaquant 200 system fluorometer (Hoefer) according to the manufactures instructions using the following reagents:

10x TNE buffer :containing, 100 mM Tris, 10 mM EDTA and 2 M NaCl.

Dye solution: (bisBenzimide 1 mg/ml water, Sigma B-2883) in 1x TE buffer (10 mM Tris-HCl and 1 mM EDTA).

DNA standard stock solution (1 mg/ml): 250 µg calf thymus DNA (Sigma, D-0805) in 250 µl 1x TE buffer.

DNA 1: 10 standard solution (100 µg/ml): 50 µl DNA standard stock Solution, 50 µl 10 x TNE buffer and 400 µl water.

The working solution (freshly prepared), contained 10 ml 10x TNE and 10 µl dye solution is completed to 100 ml by using autoclaved bi-distilled water.

Before measuring the DNA concentration in the tested samples the fluorimeter was adjusted by using the standard DNA 1:10 solution (100 µg/ml).

The extracted DNA concentration was measured and adjusted by dilution to conc. 20 - 25 ng/µl prior to PCR, using bi-distilled, deionized, sterile water (Fluka, Germany).

3.1.5 Oligonucleotide primers

Primers used in this section of study together with their target specific part of the investigated DNA are listed in Table (2). All primers were synthesized by TIB MOLBIOL, Berlin, Germany and obtained in a lyophilized state. All primers were solved before use to obtain a final concentration of 20 pmol/µl of each.

For Star Link maize the primer pair as well as the complete master mix without the polymerase enzyme were provided with the commercial detection kit used.

3.1.6 DNA amplification and PCR condition

PCR was carried out on a Gene Amp PCR system 2400 (Perkin Elmer, Germany) and Biometra thermocycler (Trio-Thermo block, Germany). For each series, a master mix was prepared. Each PCR reaction mix had 25 µl total volume and contained 2.5 µl PCR buffer (10 x concentrate, Perkin Elmer), 2 µl $MgCl_2$ solution (25 mM), 1 µl dNTPs solution (0.2 mM each of dATP, dCTP, dGTP and dTTP), 0.5 µM of each primer, 1 Unit AmpliTaq Gold polymerase (Perkin Elmer), 2 µl of template extracted DNA and was completed to 25 µl with water. For Star Link detection 1 Unit AmpliTaq Gold polymerase (Perkin Elmer) was added to the master mix obtained with the commercial kit prior to PCR.

Table (3) explains the time/temperature profiles used in PCR for each primer pair including the conditions for the detection of Star Link. All amplicons were stored at 4 ºC until gel electrophoresis.

3.1.7 Gel electrophoresis

Agarose gel preparation as well as electrophoresis were carried out using Tris-base/borate (TBE) buffer solution (pH 8.0), containing 45 mmol/L Tris-base / boric acid and 1 mmol/L EDTA adjusted with hydrochloric acid.

To determine the size of the DNA fragments, DNA of known size (50, 100 bp DNA marker, Gibco BRL, USA) together with the different amplicons were separated on 2% w/v agarose gel (LE, Roche)/TBE buffer stained with 0.01% ethidium bromide solution (0.5 mg/L). 10 µl of all amplicons and DNA marker were stained before gel electrophoresis by 2 µl

xylenecyanol dye solution (1 mg xylenecyanol, 400 mg sucrose and completed to 1 ml with water), and then subjected to electrophoresis for 45 min.

The amplicons were made visible by ethidium bromide staining and documented using UV transillumination (254 nm) with a Phoretix workstation (Biostep,Germany).

3.2 Development of a construct-specific, qualitative method for the detection of genetically modified potato lines *Spunta* G2 and G3 in raw potato and potato-derived products

3.2.1 Material

Two genetically modified lines and the non-genetically modified control line from *Solanum tuberosum* L., *Spunta*, were obtained from the Department of Crop and Soil Science, Michigan State University, USA. Both modified lines, designated G2 and G3, have been transformed with the same vector (pSPUD5), including a gene cassette consisting of [CaMV35S promoter - Cry5-Bt gene - NOS terminator] **(Mohammed A. et al., 2000)**. For control purposes, in addition non-transgenic potato was obtained from the Berlin market to be compared with the negative control material *Spunta*. (Velox - Hans-Willi Böhmer, Speisekartoffeln). For storage the potato tubers were cut and freeze dried (Christ BETA 2-16, Christ company, Osterode / Harz Germany) according to the following conditions: freezing temperature -10 ºC, main drying (time 20 hrs at 20 ºC, vacuum 0.110 mbar, and final drying (time 4-6 hrs at 25ºC, vacuum 0.0010 mbar).

3.2.2 Preparation of potato by-products

Note: all the mixtures for GM and non-GM potato were homogenized by Grind mix GM 100 (Willy A. Bachofer AG. Maschenenfabrik, Basel, Switzerland

3.2.2.1 Preparation of potato chips

Potato tubers were manually peeled and also sliced into 2 mm slices in thickness. The slices were washed by dipping in water to remove starch from cut surface, inspected to discard any discoloured or irregular shaped slices, and deep fried in vegetable oil (100g/1kg oil) at a temperature of 190 °C for 2 min. The excess of oil adhering to the produced chips was removed using tissue paper, then the chips were cooled at room temperature, and the mixtures of GM

potato chips with non GM potato chips were made by mixing (w/w) 10 % or 1% GM / non GM potoato.

3.2.2.2 Preparation of cooked potatoes

Potato tubers (GM and non GM potato) were manually peeled and cut into pieces of 2 cm in thickness. These pieces were boiled in distilled water for 20 min. 2 % sodium chloride was added at a temperature of 120 ^{o}C. The boiled potato were removed from the water and cooled at room temperature and mixed by electronic mixer for one minute. Mixtures of GM potato with non GM potato were made by mixing (w/w) 10% or 1% GM / non GM potato.

3.2.2.3 Preparation of mashed potato

To make mashed potato, potato tubers (GM and non GM) each separately were manually peeled and cut into pieces of 2 cm in thickness. These pieces were boiled in distilled water for 30 min. 2 % sodium chloride was added at a temperature of 120 ^{o}C. The boiled potatos were removed from the water, cooled at room temperature and mixed with butter, salt and milk by electronic mixer for one minute. The mixtures of GM mashed potato and non-GM mashed potato were made by mixing (w/w) 10%, 1% and homogenized by Grind mix GM 100.

3.2.3 Extraction of genomic DNA from potato samples

DNA from potato samples was extracted according to the procedure described by **Tinker et al (1993).** DNA was extracted from 100 mg of each samples by a procedure that is based on DNA precipitation with cetytrimethyl amonium bromide (CTAB) as described before, (3.1.3.2).

Table (2) Oligonucleotide primer pairs sequence and their target element

Primer	Sequence	Fragment length	Target element	References
GM03 / GM04	5'- gCC CTC TAC TCC ACC CCC ATC C - 3' 5- gCC CAT CTg CAA gCC TTT TTg Tg - 3'	118 bp	Soy lectin gene.	Meyer et al., (1996)
P35s-f2/ Petu-r1	5' - TgA TgT gAT ATC TCC ACT gAC g -3' 5' -TgT ATC CCT TgA gCC ATg TTg T-3'	172 bp	Transition site from the CaMV35S promoter sequence to the petunia hybrida chloroplast-transit-signal sequence in **RRS**.	Wurz et al., (1997)
IVR1-F/ IVR1-R	5 - CCg CTg TAT CAC AAg ggC Tgg TAC C- 3' 5 ggA gCC CgT gTA gAg CAT gAC gAT C- 3'	226 bp	Maize invertase gene.	Ehlers et al., (1997)
Cry03 / Cry04	5' - CTC TCg CCg TTC ATg TCC gT - 3' 5' - ggT CAg gCT CAg gCT gAT gT - 3'	211 bp	Transition site from the CCDPK-promoter into the amino terminal sequence of synthetic Cry1A(b) gene in **Bt 176** maize.	Hupfer et al., (1998)
IVS2-2 / PAT-B	5' - CTg ggA ggC CAA ggT ATC TAA T - 3' 5' - gCT gCT gTA gCT ggC CTA ATC T - 3'	189 bp	Transition site from the intron IVS2 into the PAT-gene in **Bt11** maize.	Anonymus (2002)
T25-F7 / T25-R3	5' - ATg gTg gAT ggC ATg ATg TTg - 3' 5' - TgA gCg AAA CCC TAT AAg AAC CC- 3'	209 bp	Transition site from the CaMV-terminator into the PAT gene in **T25** maize.	Anonymus (2002)
VW01 / VW03	5'- TCg AAg gAC gAA ggA CTC TAA Cg - 3' 5' - TCC ATC TTT ggg ACC ACT gTC g - 3'	170 bp	Transition site from the genomic maize DNA into the CaMV-promoter in **MON810** maize.	Anonymus (2002)

Table (3) Time / temperature profiles for qualitative PCR with DNA extracted from maize and soybean samples using the primer pairs described in Table (2)

Primer pair	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final elongation
GM03 /GM04	10 min. at 95 °C	30 sec. at 95 °C	30 sec. at 60 °C	1 min. at 72 °C	35	3 min. at 72 °C
P35s-f2 /petu-r1	10 min. at 95 °C	30 sec. at 95 °C	30 sec. at 62°C	25 sec. at 72 °C	35 - 40	10 min.at 72 °C
IVR1-F /IVR1 - R	12 min. at 95 °C	30 sec. at 95 °C	30 sec. at 64 °C	30 sec. at 72 °C	42	10 min. at72 °C
Cry03 /Cry04	12 min. at 95 °C	30 sec. at 95 °C	30 sec. at 63 °C	30 sec. at 72 °C	38	10min. at 72 °C
IVS2-2 /PAT-B	12 min. at 95 °C	30 sec. at 95 °C	30 sec. at 64 °C	30 sec. at 72 °C	38	10min. at 72 °C
T25-F7 /T25-R3	12 min. at 95 °C	30 sec. at 95 °C	30 sec. at 64 °C	30 sec. at 72 °C	40	10min. at 72 °C
VW01 /VW03	12 min. at 95 °C	30 sec. at 95 °C	30 sec. at 64 °C	30 sec. at 72 °C	40	10 min. at 72 °C
Star Link Kit	10 min. at 94 °C	25 sec. at 94 °C	30 sec. at 62 °C	45 sec. at 72 °C	50	3 min. at 72 °C

DNA was air-dried and redissolved in 50 µl 1x PCR buffer (Perkin Elmer). The quality of nucleic acid extraction was controlled by agarose gel electrophoresis. The concentration of DNA in solution was measured by fluorometry according to the standard instructions of the manufacturer (Dynaquant system; Hoefer, distributed by Pharmacia, Germany).

3.2.4 DNA target sequences and olignucleotides used for the detection of genetically modified and non modified control potato

The Department of Crop and Soil Science, Michigan State University, USA, has provided the complete sequence of the synthetic version of the CryV gene introduced into potato. Figure (1), represents the sequence of the CryV gene in GM potato *Spunta* indicating the position of the primers that were designed and used to amplify the modified structure. Primer pair Spu-35S1-f/ Spu-cryVm1-r spans a region between the CaMV 35S promoter and the CryV gene. It attaches to the last 53 bp of the CaMV promoter and the first 69 bp of the CryV gene to reveal an amplicon of 122 bp. The primers were designed using the software primer designer ABI PRIS, primer express, TM version 1.0. The primer pair A1/A2 that has been described by (**Taberlet et al., 1991)** see Table (4) was used as a control for the ability of the DNA to be amplified because it specifically detects multicopy chloroplast DNA. Primers were synthesized by TIB MOLBIOL, Berlin, Germany and obtained in lyophilized state and solved before use to obtain a final concentration of 20 pmol/µl. Primers were stored at - 20°C.

Table (4) Sequence of the primers used for the detection of DNA from nonGMO and GM potato lines by qualitative PCR

Primer (Reference)	**Target**	**Sequence**
A1 (Taberlet et al., 1991)	Chloroplast gene	5' CGA AAT CGG TAG ACG CTA CG 3'
A2 (Taberlet et al., 1991	Chloroplast gene	5' GGG GAT AGA GGG ACT TGA AC 3'
Spu-35S1-f	CaMV promoter	5' CTT CGC AAG ACC CTT CCT C 3'
Spu-cryVm1-r	Cry V gene	5' GCT GGA GAA CGA TTG GTG C 3'

3.2.5 Polymerase chain reaction conditions for the primer pairs A1/A2 and Spu.-35S1/ Spu-Cry Vm1-r

All amplifications were carried out on a Gene Amp PCR system 2400 (Perkin Elmer, Germany) and Biometra thermocycler (Trio-Thermoblock, Germany). For each series, a master mix was prepared. A single reaction mix contained 2.5 µl of 10x PCR buffer (Perkin Elmer, Germany), 2 µl $MgCl_2$ [25 mM], 2 µl dNTP solution 0.2 mM of each dATP, dCTP, dGTP and dTTP, 0375 µl (20 µM of each primer), 0.4 µl AmpliTaq Gold, (5 Unit/µl) (Perkin Elmer-Germany) and 2 µl of template DNA [25 ng/µl], and was completed to 25 µl with purified distilled water. As a reagent control 200 µl of water was subjected to extraction and further treated in the same way as samples. Amplification was carried out in 0.2 ml reaction tubes with a final volume of 25 µl. Reaction mixtures were subjected to PCR with the following time / temperature program: initial DNA denaturation and enzyme activation 94 °C for 10 minutes, 30 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final extension step at 72°C for 5 min for the primer pair A1/A2. The size of PCR products was 550 bp **(Taberlet et al., 1991)**. After PCR amplification, PCR products (10 µl each) were separated by agarose gel electrophoresis (5V/cm) followed by ethidium bromide staining. A standard 10 bp, 50 bp, and 100 bp ladder (Gibco BRL, USA) was used to determine the molecular weight of amplifications. The GM potato *Spunta* G2 and G3 specific primer pair (Spu-35S1-f /Spu- CryVm1-r) was applied with the following temperature program: initial DNA denaturation and enzyme activation at 96 °C for 10 minutes, 40 cycles at 96°C for 30 second, 65°C for 30 second, 72°C for 30 second, and a final extension step at 72°C for 7 min. The size of these GM specific PCR products was 122 bp. The PCR products were separated together with 50 bp DNA marker on 2 % w/v agarose gel as described (3.1.7)

3.2.6 Confirmation of PCR products

The specificity of PCR products was confirmed by two methods. The first method uses restriction enzymes. The PCR product obtained with primer pair Spu35S-f/spu-cryVm1-r was digested with BamHI enzyme (restriction enzyme was supplied by (Boehringer, Mannheim, Germany). Digests were made by mixing 10 µl of PCR product with 2 µl of 10x restriction enzyme buffer, 1 µl of restriction enzyme (10 U/µl) and 10 µl of water. The mixture was incubated at 37 °C for 3 h and restriction was stopped by thermal inactivation of the enzyme at

80 °C for 20 min. Restriction enzyme digestion products were visualised on 2 % agarose gel. The second method to confirm the specificity of the PCR products is sequencing which was applied to characterise the PCR product, which was produced by using the primer pair Spu35S-f/spu-cryVm1-r. Amplicons were cut out from the agarose gel, DNA was extracted by using the QIA quick gel extraction (Commercial Kit produced by QIA GEN in Hilden, Germany, Cat 28706). The DNA was submitted to sequence analysis. Sequencing was done on an ABI PRISM 310 Genetic Analyzer, Perkin Elmer, using a commercial Kit with slight modification[1] from the manufacturers protocol.

3.3 Quality and safety evaluation of genetically modified potatoes Spunta: Compositional analysis, determination of some toxins, antinutrients and feeding study in rats

3.3.1 Tubers

Genetically modified potato *Spunta* lines G2 and G3 and its parental non-GMO control line used in this study were cultivated and harvested under the same conditions during the year 2001 in the Department of Crop and Soil Science, Michigan State University, USA and has been freeze dried prior to mixing to feed as described under Section (3.2.1). The authenticity of transgenic and non-transgenic potato was checked by DNA analysis as described above (Section 3.2).

3.3.2 Analysis of chemical composition and antinutrient compounds

3.3.2.1 Chemical composition

To determine the compositional equivalence, both, the transgenic potato *Spunta* and non-transgenic control line were analyzed for total solid, protein, ash, lipids, crude fibre, total sugar, reducing sugar, starch and ascorbic acid according to the methods of the **A.O.A.C. (1995**).

3.3.2.1.1 Non-reducing sugar

Non-reducing sugar was determined by the difference between total sugar and reducing sugar.

[1] ABI PRISM® BigDYe™ Terminator Cycle Sequencing Ready Reaction Kits 2000 with decreased amount of Terminator Ready Extraction mix of 6 µl

3.3.2.1.2 Total carbohydrate

Total carbohydrate was calculated according to **(Winton et al., 1958**) by the following equation; Carbohydrate g/100g =100 - (moisture + protein + lipid + fibre +ash).

3.3.2.1.3 Total phenol

Total phenol was determined according to **(Vinson et al., 1998**).

3.3.2.1.4 Protease inhibitor activity

Protease inhibitor activity was determined according to **(Lin et al., 1980**).

3.3.2.1.5 Potato alkaloids (α-Chaconine and α-solanine)

Potato alkaloids (α-Chaconine and α-solanine) were determined according to a procedure described by **(Bushway et al., 1986 and Carman et al., 1986**).

3.3.2.1.6 Amino acids

Amino acids were determined according to **(Anderson et al., 1977)** using an Amino Acid Analyzer (System 7300 Beckman, equipped with Beckman 7700 Data system, Detector Spectrophotometer, sodium high– performance column/25 cm).

3.3.2.1.7 Fatty acids.

The lipid was extracted according to the method of **(Bligh et al., 1959).** The profile of fatty acid was determined by GLC equipped with flame ionisation detector (FID). Trimethylsulfoniumhydrxide (TMSH) was used for preparation of fatty acid methyl esters according to the procedure of **(Arnes et al., 1994).** A Shimadzu system, model GC-14A fitted with an sp-2380 fused silica capillary column (30m X 0.25 mm x 0.2 μm; Supelco Inc., Bellefonte, PA, USA) was used. The temperature of injection port and detector was set up at 250 °C. The oven temperature was increased from 110 °C to 175°C at a rate of 5 °C/min.

3.3.3 Feeding study with rats

3.3.3.1 Experimental conditions

Forty-eight weaning male Albino rats (50 ±5 g) were obtained from the Faculty of Veterinary Medicine Moshtoher, Zagazig University, and were fed in the animal house of the Faculty of Agriculture Moshtoher, Zagazig university. All animals were kept under normal healthy conditions and fed on a basal diet for one week. The basal diet consisted of casein (80≥ % protein (20 %), soybean oil (5 %), cellulose (5 %), mineral mixture (3.5 %), vitamin mixture

(1 %), choline bitartrate (0.2 %), sucrose (10 %), DL methionine (0.3%) and cornstarch (55 %) according to **(Reeves et al., 1993).** The control and experimental diets are listed in Table (5). Water and diet was provided ad libitum. After feeding on basal diet for one week, rats were divided randomly into 4 groups (n =12) according to the following scheme:

Group1: The control group, fed on basal diet only

Group 2: Fed on basal diet with the replacement of 30 % from cornstarch by freeze-dried non-genetically modified potato *Spunta* potato

Group 3: Fed on basal diet with the replacement of 30 % from cornstarch by freeze dried genetically modified potato *Spunta* G2.

Group 4: Fed on basal diet with the replacement of 30 % from cornstarch by freeze dried genetically modified potato *Spunta* G3.

During the experimental period (30 days) rats were kept separately in well-aerated cages. The diet consumed and body weight was recorded every day of the experimental period. The individual body weight as well as the rest of feed was recorded to compute and compare some of performance parameters like body gain, food intake, final body weight, daily body weight gain and feed efficiency. After the end of the experimental period (30 days) all feed was removed. Blood samples were collected from all rats within different treatment groups from the orbital venous plexuses by a capillary tube. Blood serum was separated by centrifugation at 3000 rpm for 15 min. Serum was analyzed colorimetrically for total protein, albumin, glucose, total cholesterol, urea, and transaminase GOT (glutamate oxaloacetate amino transferase) and GPT (glutamate pyruvate amino transferase). The animals were slaughtered and the internal organs of each rat (liver, kidney, spleen, heart and testes) were removed and weighed.

3.3.3.2 Serum analysis

For the determination of glutamate pyruvate amino transferase (GPT), glutmate oxaloacetate amino transferase (GOT), total protein, albumin, glucose, urea, total cholesterol and creatinine

kits were used and obtained from Bio con®, Germany.

The principles of methodologies applied for serum analysis are listed below.

3.3.3.2.1 Blood glucose:

Blood glucose level was determined using the method of **(Trinder, 1969).**

3.3.3.2.2 Albumin:

Serum albumin was determined according to the method of **(Webster, 1974).**

3.3.3.2.3 Total protein:

Serum total protein was determined colorimetrically according to the method of **(Josephson et al., 1975)**

3.3.3.2.4 Determination of total cholesterol:

Serum total cholesterol was determined according to the method described by **(Thomas, 1992**). The produced color was measured at 500 nm using a spectrophotometer.

3.3.3.2.5 Determination of plasma transaminase activity:

Glutamate pyruvate amino transferase (GPT) and glutamate oxaloacetate amino transferase (GOT) activates were determined colorimetrically at 540 nm according to the method of (**Reitman and Frankel, 1957**).

3.3.3.2.6 Determination of serum urea:

Serum urea was determined using a spectrophotometer adjusted at 580 nm according to the method described by **(Patton and Crouch, 1977**).

3.3.4 Statistical analysis

All results were expressed as mean values. Statistical analysis of the chemical composition of potato and feeding study data, weight of organ, and serum analysis were performed by statistical comparison between each groups using the t-test method. Analysis of variance for the recorded data was performed according to the method described by (**Gomez and Gomez, 1984)**.

Figure (1) Sequence of the cryV gene and relative location of the primer binding sites.

Legend to figure (1) The relative sites for binding of the primers Spu-35S1-f and Spu-CryVm1-r are indicated by arrows. The first 53 bp of the sequence are from the CaMV promoter.

3.4 Effect of diets containing genetically modified potato Spunta on broiler health and performance and degradation and possible carry over of genetically modified DNA from Spunta potato DNA monitored in broiler

3.4.1 Potato tubers used for feeding studies with broiler

The genetically modified potato tubers *Spunta* G2 and G3 and the non-modified parental line used for this study is identical with the material described in section (3.2.1 Material). Tubers have been prepared and investigated for authenticity prior to use for feed mixtures as described in the following sections.

3.4.2 Compositional analysis of freeze-dried potatoes

To determine the compositional equivalence, both, the freeze-dried genetically modified potato *Spunta* lines G2 and G3 and its control were analyzed for dry matter (DM), total ash, total-phosphorous (P), crude protein (CP), ether extract (EE), crude fibre (CF), as well as starch and sugar according to the methods of **(Naumann et al., 1993).**

3.4.3 Experimental animal groups

Thirty-six one-day-old male broiler chicks (Lohmann meat) were obtained from (Geflugelhof Mokern-Brüterei-Germany), weighed, identified by wing number and caged (three birds in one cage) under standard conditions for fourteen days. All birds were fed during this period on control diet and subsequently divided randomly into 4 groups (n= 9) according to the following scheme:

Group 1: The control group which was fed on control diet only

Group 2: Fed on control diet with the replacement of 30 % from maize in the control diet by freeze-dried conventional control potato *Spunta* (non-GM).

Group 3: Fed on control diet with the replacement of 30 % from maize in the control diet by freeze-dried genetically modified potato *Spunta* G2.

Group 4: Fed on control diet with the replacement of 30 % from maize in the control diet by freeze-dried genetically modified potato *Spunta* G3.

Every bird was caged in a separate cage during feeding on experimental diets. All birds were fed during the experimental period as recommended by the German Society of Nutrition Physiology **(GfE, 1999 and NRC, 1995**).

3.4.4 Diet Formulation

The control and experimental diets are listed in Table (6). Both, control and experimental diets were designed to contain maximum amounts of potato to study its impact on broiler health and the fate of DNA in broiler body. Furthermore, the percentage of conventional and transgenic potato material used was equivalent in all experimental diets. DNA from control and experimental diets was extracted according to **(Tinker et al., 1993**) and PCR was applied using both, primer pairs, A1/A2 and (Spu-35S1-f /Spu-CryVm1-r), under the same conditions mentioned above (3.2.5) to exclude the possibility of cross contamination between the control and experimental diets during preparation and mixing. Diets were consumed in the form of mash without pelleting. Both diets were subjected to proximate chemical analyses before use to determine dry matter, total ash, sodium, phosphorous, potassium, crude protein, ether extract, crude fibre, starch and sugars, amino acids and fatty acids according to the methods of the (**VDLUFA Naumann and Bassler, 1993)**.

3.4.5 Housing and environmental conditions

From the first day of age all birds were identified by wing number, weighed and housed at the facilities of the FAL (Bundesforschungsanstalt für Landwirtschaft, Braunschweig). House temperature was initially 33-35°C during the first two days of age, and then decreased to 32-33 till the fourth day of age. From five days of age until the end of the first week the temperature was 30°C. In the second week, the temperature was 29°C and decreased gradually to 26°C in the third week of experiment. The house temperature was decreased in the fourth week of age to 22°C. House relative humidity (RH) ranged from 40-60% during the time of experimental period. In the first three days of age all birds received 24 hours of electric light program (35-40 watt), while from the fourth day of life until the end of the experimental period only 16 hours of light and eight hours of dark. House temperature, relative humidity and light program were automatically controlled.

Table (5) Composition of the basal and experimental diet for rats.

Ingredients (%)	Control diet *	Experimental diets		
	Group 1	Group 2	Group 3	Group 4
Casein (≥ 80%) protein	20	20	20	20
Soybean oil	5	5	5	5
Cellulose	5	5	5	5
Mineral mixture	3.5	3.5	3.5	3.5
Vitamin mixture	1	1	1	1
Choline bitartrate	0.2	0.2	0.2	0.2
Sucrose	10	10	10	10
Corn starch	55	25	25	25
DL-methionine	0.3	0.3	0.3	0.3
Sp control NON GM	-	30	-	-
Sp.GM G2	-	-	30	-
Sp.GM G3	-	-	-	30

* Reeves et 1993

3.4.6 Experimental design and sampling

Diets and water were provided ad-libitum during the experimental period. The groups of broiler were compared during the experimental period to demonstrate bird behavior, feed intake, pathogenic and general health conditions. Every day of the experimental period the individual body weight as well as the rest of feed were recorded to compute and compare selected performance parameters (body gain, final body weight and feed efficiency). At the end of the experimental period all feed was removed and excrete was collected from all groups, freeze-dried and ground in an electric mill. The samples were collected from all birds after one hour, four hours and eight hours from feed removal to investigate the fate of DNA in broiler. Blood samples were collected by heart puncture. Three independent blood samples were collected from each bird using heparin for physiological analyses; another sample was collected on sodium citrate for DNA investigation and the last sample without using anticoagulant for serum preparation. After blood collection birds were sacrificed and the carcasses were opened under sterile condition to avoid any contamination. The gut was tied off with sterile surgical silk at the

distal part of esophagus and at the end of rectum. Then the gut was dissected carefully with further tying off to prevent any exposure of digesta. The gut was removed intact, laid on a flat surface and the digesta from each section (Crop, Proventriculus, Gizzard, Duodenum, Jejunum, Ileum, Caecei and Rectum) was carefully squeezed to obtain the digest from different parts of the gut separately. Small parts of liver, kidney, spleen, heart, testes, bursa, thymus glands, thorax skin, abdominal fat, and small parts of thigh muscles were also collected. All collected samples from gut contents and organs are stored at -70 °C until DNA investigation.

Because the amounts of digesta in different segments of the gut varied appreciably and some sections were completely empty of digesta, pooling of the samples collected at the same time of slaughter program within the same group was carried out prior to DNA analyses. Pooling of the organs samples were also applied prior to Polymerase chain Reaction (PCR) to minimize the error of individual variation and the false results.

3.4.7 Extraction and purification of DNA from organs and gastrointestinal tract contents

3.4.7.1 DNA extraction and purification from organs and digesta and different parts of the GIT

100 mg wet weight of digesta of different parts of GIT were subjected to total DNA extraction using CTAB-method **(Anonymus 2002)** followed by purification of the isolated DNA using a commercial kit (QIAquick DNA Clean-up, QIAGEN GmbH, no.: 02103-892-230). The concentration of the isolated DNA was measured fluorometrically using Dynaquant 200 system fluorimeter (Hoefer) according to the manufactures instructions. The quality of extracted nucleic acid was controlled by agarose gel electrophoresis. The DNA concentration was adjusted between 25 - 30 ng/µl prior to PCR.

3.4.7.2 DNA extraction and purificaton from excreta

DNA from excreta was extracted using the QIAamp DNA Stool Mini Kit (Commercial kit produced in Hilden, Germany. Cat. No.: 02103-892-230).

All samples were lysed in specific buffer, proteins were digested using proteinase K and DNA was purified on QIA amp spin columns.

Table (6) Composition of the control and experimental diets for chicken

Ingredients (%)	Control diet*	Experimental diets		
	Group 1	Group 2	Group 3	Group 4
Maize	33.98	3.98	3.98	3.98
Soybean seed, soy pure	17.89	17.89	17.89	17.89
Wheat	21.2	21.2	21.2	21.2
Soybean oil	3	3	3	3
Soybean meal	19.38	19.38	19.38	19.38
Dicalcium phosphate	1.85	1.85	1.85	1.85
Calcium hydroxide	1.04	1.04	1.04	1.04
Methionine	0.25	0.25	0.25	0.25
Sodium chloride	0.25	0.25	0.25	0.25
L-Lysine HCl	0.16	0.16	0.16	0.16
Premix, Vilomix, N 47770	1	1	1	1
Freeze dried potato (Non-GMO)	-	30	-	-
Freeze dried potato (GMO G2)	-	-	30	-
Freeze dried potato (GMO G3)	-	-	-	30

* Naumann et al. 1993

3.4.8 Determination of the concentration and quality of DNA extracted from organs, excreta and gastrointestinal tract contents

The concentration of the isolated DNA was measured fluorometerically using a Dynaquant 200 system fluorimeter (Hoefer) according to the manufactures instructions. The quality of nucleic acid extracted was controlled by agarose gel electrophoresis as well. The extracted DNA was diluted prior to PCR to obtain 30 ng/µl, using bi-distilled, deionized, sterile water (Fluka, Germany)

3.4.9 DNA analysis of diets, digesta, tissue and excreta by PCR

Primer and probes were designed using the software primer designer ABI PRISM, Primer Express ™ version 1.0. using special software (Mac Vector™ V 6.5.3).

All primer pairs and probes used with their specific target are listed in Table (7). All primers and probes were synthesised by TIB MOLBIOL, Berlin, Germany and obtained in a lyophilised state. The primers and probes were dissolved in sterile distilled water (Fluka, Germany) to obtain a final concentration of 20 pmol/µl.

3.4.9.1 Oligonucleotides for the investigation of diets

Diets were analysed for the presence of potato and GM potato using primer and PCR systems as described in section 3.2.4 - 3.2.6.

3.4.9.2 Oigonucleotides for the investigation of digesta and tissue samples

3.4.9.2.1 Primer and probes for the detection of transgenic potato Spunta DNA

The presence of potato *Spunta* G2 and G3 in the gastrointestinal tract (GIT) and tissue samples was analysed by using the primer pair 35S1-f/Spu-cryVm1-r with a detection probe which was designed to be suited for analysis on a real time PCR machine. Two additional primer pairs (RR02/CryVm-Pr; Spu-35S-f/CRYVM-P-r3) (Table 7) have been designed. The position of these primer pairs within the CryV gene is indicated in Figure 4. Conditions for the amplification of DNA specific for GM potato *Spunta* lines G2 and G3 by real-time PCR were studied and optimised as described in detail under Section (4.2.4)

3.4.9.2.2 Primer and probes for the investigation of plant and potato specific chloroplast DNA in tissue samples.

To design a chloroplast specific primer pair for the detection of potato, DNA from potato, soybean, tomato and maize was extracted and PCR was performed by using the primer pair A1/A2 (Table 4). This primer pair has the code number (210/211) in my work. PCR with this primer pair was done under conditions as described in Section 3.2.5 in a concentration of (5 pmol/µ) each. Amplicons were cut from the agarose gel, DNA was extracted by using the QIA quick gel extraction (Commercial kit produced by QIAGEN in Hilden, Germany) cat. 28706) and subjected to sequencing-cycling. The reaction-mix and the sequencing-cycling program are listed in Table (8) and Table (9). The DNA resulting from sequencing-cycling was precipitated and cleaned from ddNTPs and subjected to sequencing as described in section 3.2.6. Chloroplast DNA alignment to identify differences between potato, and tomato, maize and soybean sequences was done in silica (**Figure 2).** Figure 3 indicates the potato chloroplast DNA sequence

obtained with primer pair A1/A2 and the selected primer pair position Cp-Po2-f/ Cp-Po2-r for potato chloroplast specific detection giving rise to a 111 bp fragment. The sequence of the corresponding newly designed probe is included in Table 7.

3.4.9.2.3 Primer for amplification control

Primer pair (MY-F/MY-R) (Table 7) was used as a control for DNA extration from tissue samples. The DNA fragment amplified using MY-f/MY-r/MY is of 97 bp size as described by **(Laube et al, 2003**).

3.4.10 PCR

3.4.10.1 PCR conditions for the analysis of DNA extracted from potatoes and diets using primer pairs 35S1-f/Spu-cryVm1-r and A1/A2

PCR was carried out on a Gene Amp. PCR system 2400 (Perkin Elmer, Germany). For the primer pair 35S1-f/Spu-cryVm1-r, the master mix contained: 2.5 µl PCR buffer (10x concentration Perkin Elmer), 2 µl $MgCl_2$ solution (25mM), 2 µl dNTPs solution (0.2 mM each of dATP, dCTP, dGTP and dTTP), 0.5 µM of each primer, 0.4 µl AmpliTaq Gold polymerase (Perkin Elmer), 2 µl of extracted template DNA [25-30 ng/ml] completed to 25 µl with water (Fluka, Germany). For the primer pairA1/A2 the master mix contained: 2.5µl of 10x PCR buffer (Perkin Elmer, Germany), 2 µl $MgCl_2$ (25 mM $MgCl_2$), 2 µl dNTP solution 0.2 mM of each dATP, dCTP, dGTP and dTTP. 0.5 µM of each primer, 1 Unit AmpliTaq Gold polymerase (Perkin Elmer) and 2 µl of template DNA (25 ng/µl) or water for control PCR.

The amplification conditions for primer pair 35S1-f/Spu-cryVm1-r were as described in (3.2.5) The PCR products were separeted on 2 % agrose gel as described in (3.1.7).

Table (7) Names, sequences and target elements of the primers and probes used for investigation of the DNA after feeding of chicken

Primer and probe	Sequence	Fragment length	Target element	Ref.
MY-f MY-r	5'-TTg TgC AAA TCC TgA gAC TCA T-3' 5'-ATA CCA gTg CCT ggg TTC AT-3'	97 bp	Mammals, Avian and reptile myostatin gene (Aminotes	Laube et al.,(2003)
Spu-35S1-f/Spu-cryVm1-r Spu-CryVm-Probe	5'- CTT CgC AAg ACC CTT CCT C 3' 5' -gCT ggA gAA CgA TTg gTg C 3' 5'-TCA TTT CAT TTg gAg AgA ACA Cgg g 3'	122bp	CaMV promoter and Cry V gene	This thesis
RRO2 CryVm-Pr	5-'TCC TTC gCA AgA CCC TTC CTC-3' 5'-ggA gTC gTg gTA gTC AgC CAG-3'	504 bp	CaMV promoter and Cry V gene	This thesis
Spu-35S1-f/ CRYVM-P-r3	5' CTT CgC AAg ACC CTT CCT C- 3' 5'-ggg TgT CgT AgC TCg ggA AC-3'	1000bp	CaMV promoter and Cry V gene	This thesis
Cp-po2-f CP-po2-r Cp-Po-Probe	5'CTC TTT ACA TCg AAA CTT CAG AAA g -3' 5' AAA CTA Cgg ATT Cgg gTC g -3' 5'AAA AgA ATg AAg TgA Agg ATA AAC gTA TAT A- 3'	111bp	Potato chloroplast DNA	This thesis

Table (8) Reaction mix for cycle sequencing

Substance	**Amount**[ml]
API pre-Mix	6
Primer [5 pmol/µ]	1
Samples DNA [30-100ng]	5
Dist-water	8
Total volume	20

Table (9) Reaction condition for cycle sequencing

Step	**Process**	**Time**[**min**]	**Temperatures**	**Cycles**
1	Start	2:00	96	-
2	Denaturation	0:10	96	
3	Annealing	0.05	54	25
4	Extension	4	60	
5	Cooling	∞	4	-

3.4.10.2 Digesta and tissue samples

3.4.10.2.1 Real time PCR conditions for the detection of GM potato Spunta DNA in digesta and tissue samples using primer system 35S1-f/Spu-cryVM1r / Spu-CryVm-Probe

Real time PCR with the primer pair 35S1-f/Spu-cryVm1-r with Spu-CryVm-Probe was performed on an ABI PRISM® 7700 Sequence Detector (Real-Time TaqMan™ PCR-Technology) in a final volume of 25 µl per reaction mix, including 5 µl of template DNA, 12.5 µl TaqMan Universal PCR master mix (Applied Biosystems), 0.25 µl of each primer, 0.125 µl of the corresponding probe and completed to the total volume with sterile bi-distilled water (Fluka, Germany). The PCR conditions consisted of 50°C 2 min holding temperature (to prevent contamination, Uracil-DNA glycosylase is used) then denaturation of DNA at 95°C for 10 min and 45 cycles of 95 °C for 30 sec followed by an annealing temperature of 60 °C for 1 min.

3.4.10.2.2 PCR conditions for the detection of GM potato Spunta DNA in digesta and tissue samples by using primer pairs RRO2/CryVm-Pr and Spu-35S1-f/CRYVM-P-r3

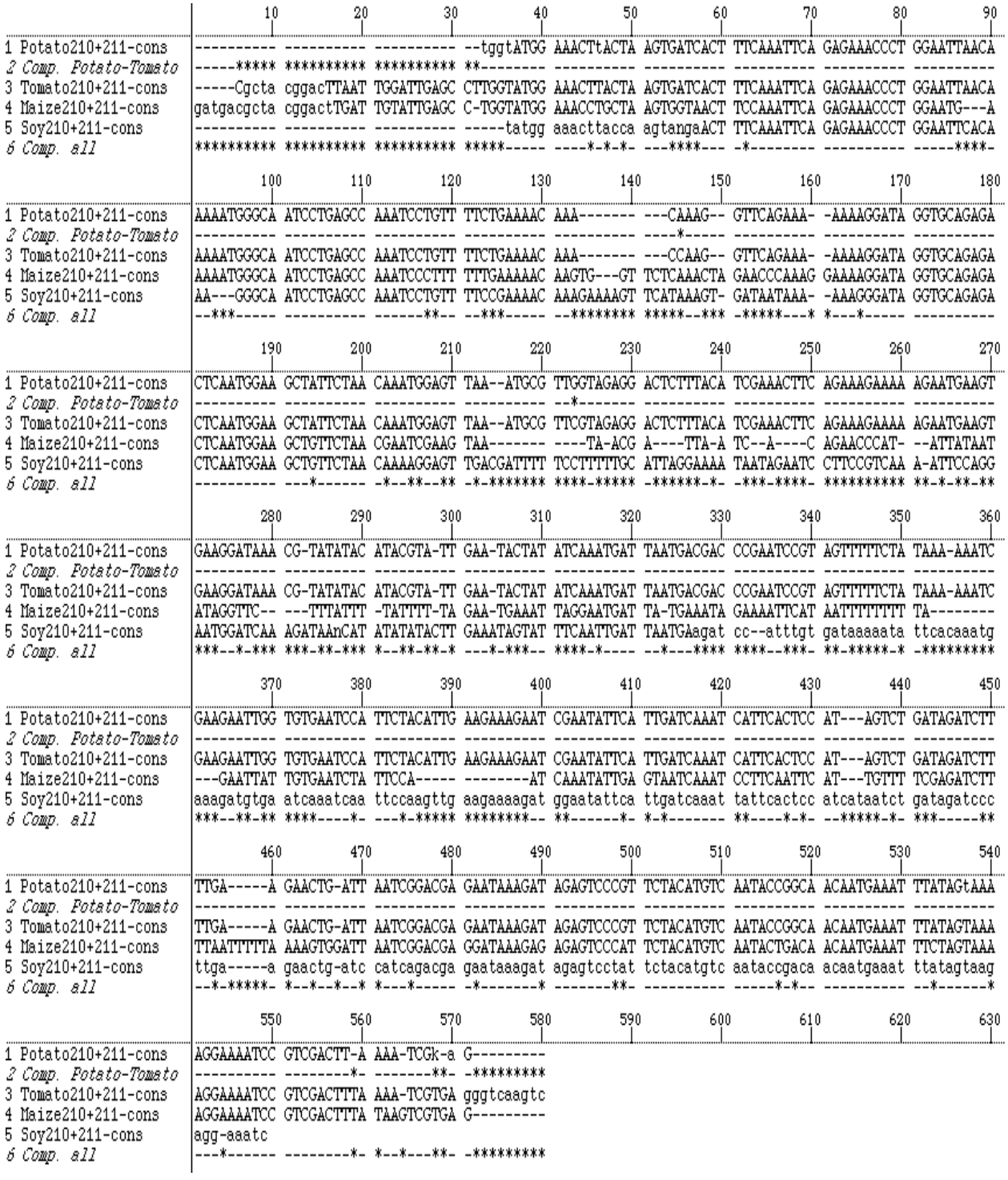
PCR was carried out on a Gene Amp. PCR system 2400 (Perkin Elmer, Germany). For each series, a master mix was prepared. Each PCR reaction mix of 25 µl total volume contained: 2.5 µl PCR buffer (10x concentration Perkin Elmer) 2 µl $MgCl_2$ solution (25mM), 2 µl dNTPs solution (0.2 mM each of dATP, dCTP, dGTP and dTTP), 0.5 µM of each primer, 0.4 µl AmpliTaq Gold polymerase (Perkin Elmer), 2 µl of extracted template DNA (25-30 ng/ml) and was completed to 25 µl with water. The amplification conditions for both, (RRO2/CryVm-Pr) and the (Spu-35S1-f/CRYVM-P-r3) system, the following conditions were chosen: initial DNA denaturation and enzyme activation at 96 °C for 10 minutes, 40 cycles at 96°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 7 minutes.

Real time PCR conditions for the detection of chloroplast DNA in digesta and tissue samples with primer system Cp-Po2-f/Cp-Po2-r / Cp-po-probe

The primer system Cp-Po2-f/Cp-Po2-r with the probe Cp-po-probe was used on an ABI PRISM® 7700 Sequence Detector (Real-Time TaqMan™ PCR-Technology) in a final volume per reaction mix of 25 µl, including 5 µl of template DNA, 12.5 µl TaqMan Universal PCR master mix (Applied Biosystems), 0.25 µl of each primer, 0.125 µl of the corresponding probe and completed to the total volume with sterile bi-distilled water (Fluka, Germany). The PCR conditions consisted of 50 °C for 2 min holding temperature (to prevent contamination, using Uracil-DNA glycosylase) then denaturation of DNA at 95 °C for 10 min and 45 cycles of 95 °C for 30 sec followed by annealing temperature at 60 °C for 1 min.

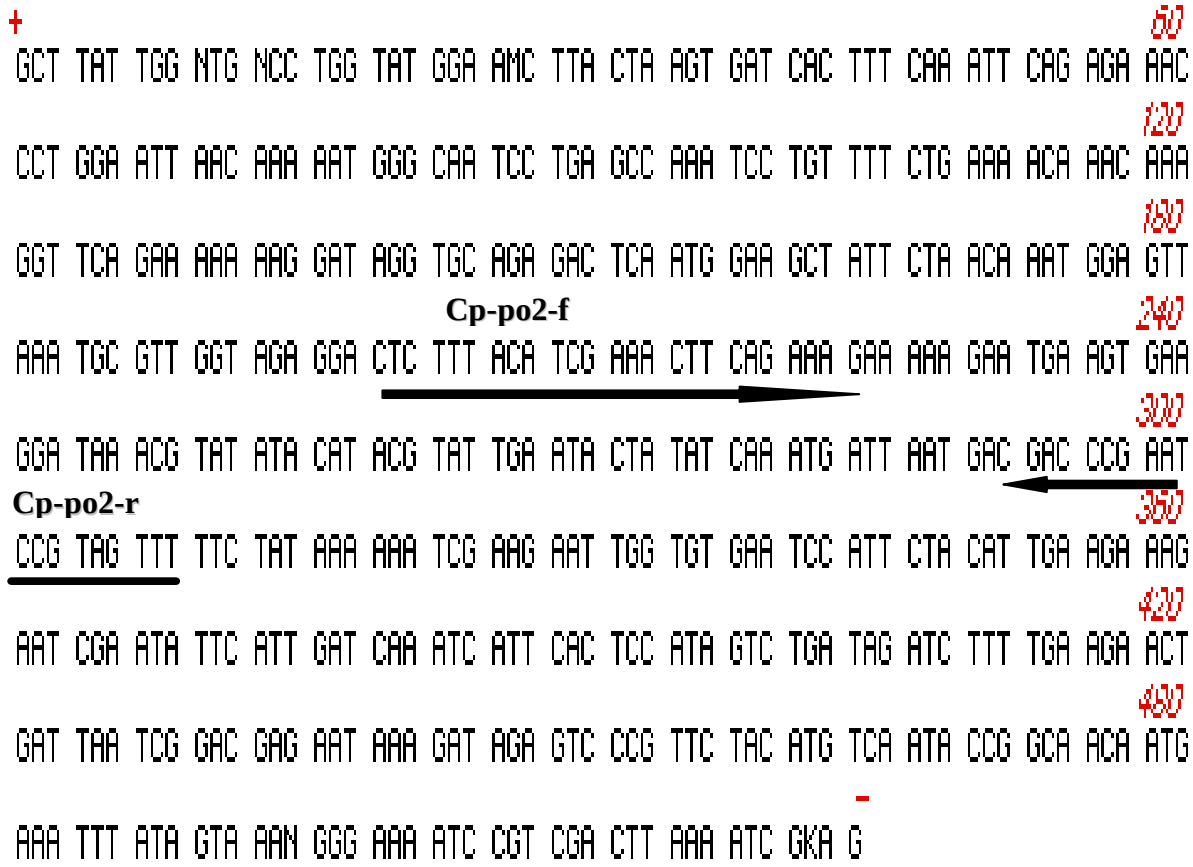
PCR products (Amplicons) were stained by 2 µl xylenecyanol dye solution (1 mg xylenecyanol, 400 mg sucrose and completed to 1 ml with water) and separated on 2% agarose gels containing 0.01% ethidium bromide together with 50 bp DNA marker.

Figure (2) Alignment of the introns of the tRNA Leucine genes encoded by the chloroplast-DNA from potato, tomato, maize, and soy bean amplified with a universal primer pair



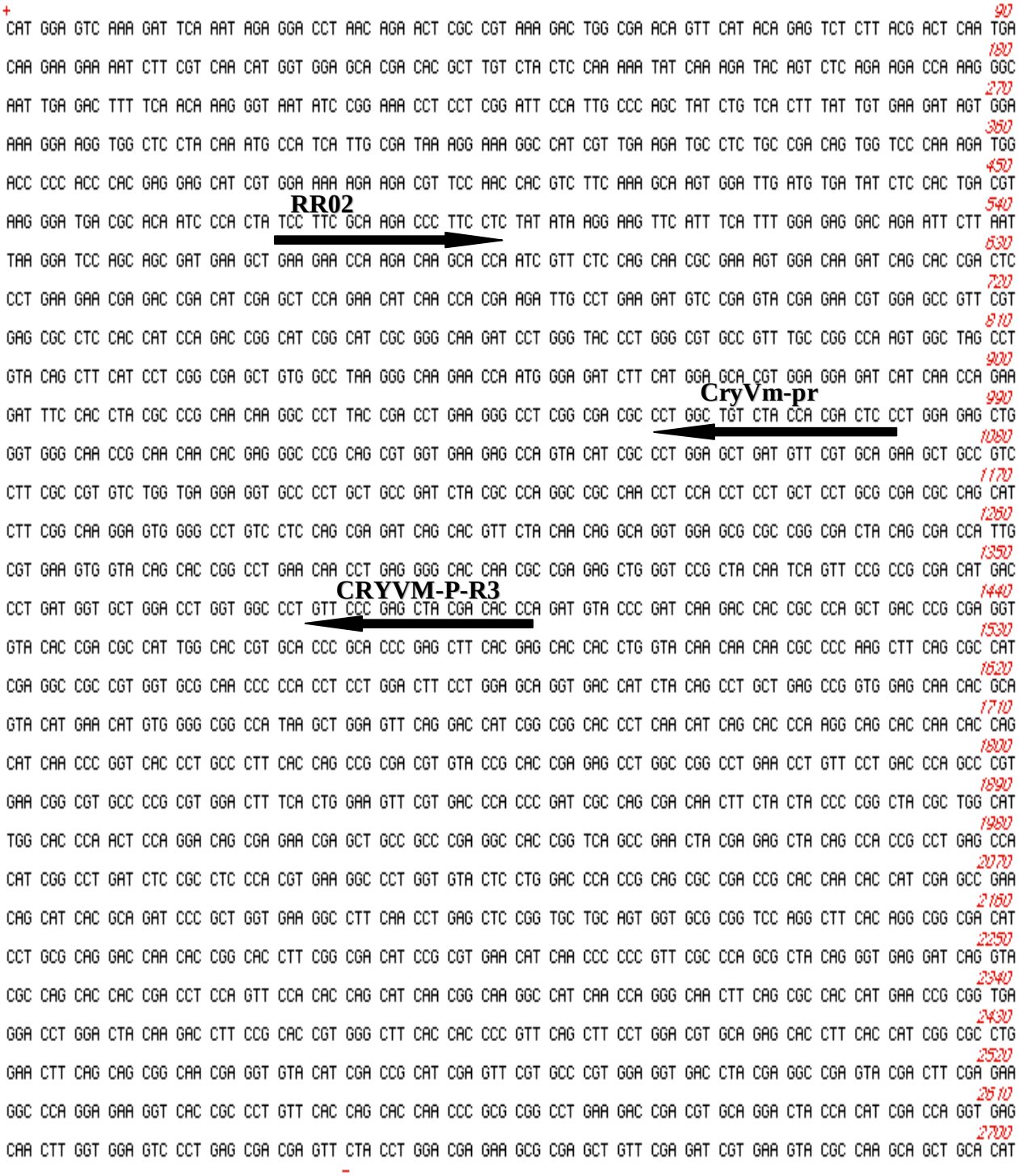
Legend to figure (2) The DNA was isolated, amplified, and sequenced as described in Materials and Methods 3.4.9.2.2. The universal primer used for amplification were A1 and A2 as described by Taberlet et al. 1991. For alignment the MacVector software was used.

Figure (3) Sequence of the intron of the tRNA Leucine gene from potato.



Legend to figure (3) The primers specific for the intron of the tRNA-Leucine gene of the potato chloroplast chromosome as indicated by arrows.

Figure (4) Sequence of the CryV gene in the GMO-potato.



Legend to figure (4) Binding sites of the primers RR02, cryVm-pr, and CRYVM-p-r3 are indicated.

4. RESULTS AND DISCUSSION

4.1 Part I: Detection of genetically modified soybean and maize from the egyptian food market

4.1.1 Introduction

Soybean and maize are widely used in human nutrition in Egypt. Furthermore, in 1995 a programme funded by the government was started to promote the use of soybean and maize as ingredients in traditional foods too like soybean milk, yoghurt, tofu, ice cream as well as bread made from soybean or maize meal (e.g. baladi, shami) (Research Capability and Programme, (1994). The aim of this study was to monitor the presence of products derived from GMOs on the Egyptian market. For this purpose about fourty food samples (Table 10 and 11) were collected randomly from markets in Cairo and Giza to detect those GMOs which are most abundant and detectable by the German official methods according to § 35 of the German Foodstuffs Act (Maize lines Bt176, Bt11, T25, MON810, Roundup Ready™ (RRS) soybean). In addition, samples were examined for the presence of StarLink™ corn (Aventis, CBH-351), a maize line exclusively approved by the Environmental Protection Agency (EPA) and the Animal and Plant Health Inspection Service (APHIS) of the U. S. Department of Agriculture for animal feed use in the USA (USDA/APHIS Petition 97-265-01p). Recently, this maize line entered the food chain unintentionally on a scale which is still unknown.

4.1.2 The presence of RRS soybean in egyptian food samples

4.1.2.1 The presence of soybean in foods (amplification control)

The presence of soybean and thus the functioning of the PCR system was checked by using the soybean lectin specific primer pair GMO3/GMO4 (Table 2 and 3). The primer pair GM03 /GM 04 is specific for the single copy lectin gene LE1 and yields a PCR product of 118 bp size **(Mayer et al, 1996).** It is detectable in transgenic as well as in conventional soy bean (soy specific primer pair). Specific primer pairs served as a control for the amplification of the isolated DNA and PCR procedure (PCR quality control). For all samples containing soya investigated here, an amplicon of the expected size have been obtained in PCR using primer pair GMO3/GMO4. The results are shown in figure (5) as an example for the PCR analysis. No amplification

were observed in neither with extraction control nor with the PCR control with out DNA.

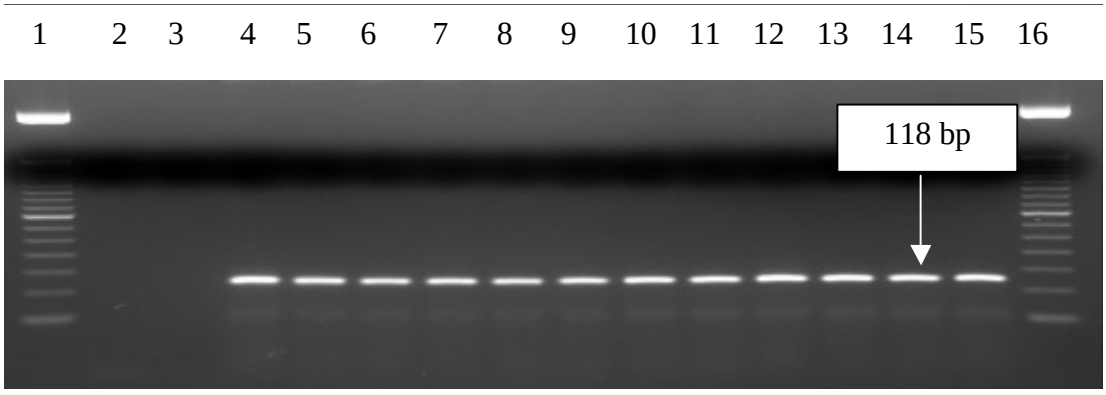


Figure (5) Detection of the soybean lectin gene in the DNA from different food samples. DNA was extracted from different samples and examined by PCR-analysis using primer pair GMO3/GMO4 as described in materials and methods (Section 3.1). The size and location of the expected amplification product is indicated. Lanes 1+16: 50 bp molecular weight ladder; lane 2: PCR control for DNA extraction; lane 3: PCR control without template-DNA; lanes 4+5: DNA from soybean granules; lanes 6+7: DNA from soybean cheese; 8+9: DNA from soybean milk; 10 +11: DNA from soybean granules; 12+13 DNA from soybean yoghourt; lane 14: DNA from non GMO soybean; lane 15: DNA from 2 % RRS.

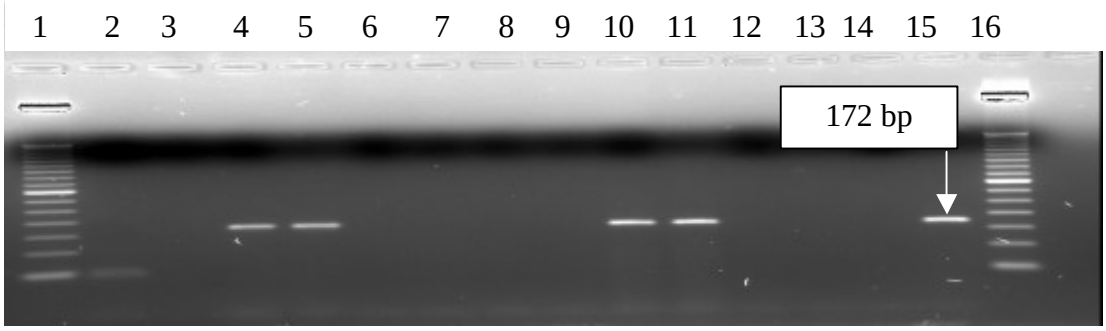


Figure (6) Analysis of different food samples for the presence of RRS specific DNA. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair p35S-f2/petu-r1was used for PCR-analysis. Lanes 1+16: molecular weight marker 50 bp ladder; lane 2: PCR control of the DNA extraction; lane 3: PCR control with out template DNA; lanes 4+5: DNA from soybean granules; lanes 6+7: DNA from soybean cheese; 8+9: DNA from soybean milk; 10+11: DNA from soybean granules; 12+13 DNA from soybean yoghourt; 14: DNA from non GMO soy bean; 15: DNA from 2% of RRS.

4.1.2.2 Specific detection of Roundup Ready™ (RRS) soybean

Forty soybean samples (Table 10) from different areas in Egypt were analysed for the presence of RRS soybean. PCR with RRS soybean-positive samples results in an amplicon of 172 bp in length using the primer pair P35s-f2/petu-r1 (Table 2 and 3). The primer pair p35s-f2/ petu-r1 is specific for the genetic modification in Roundup Ready soybean™ and amplifies a 172 bp segment. The primer pair attaches to the CaMV35S promoter sequence and the petunia hybrida chloroplast transit-signal sequence **(Wurz et al, 1997)**. The amplicon is only detected in transgenic samples.

Table (10): Soybean and soybean products analysed for the presence of Roundup Ready™ specific DNA

Tested raw materials and processed products	Number of samples	Number of RRS positive samples
Soybean granules	6	6
Tofu	3	—
Soybean flour	3	—
Soybean cheese	3	—
Natural soybean milk	2	—
Natural soybean yoghurt	3	—
Biscuit with soybean flour	1	1
Soybean mix for hamburger	3	—
Natural cheese supported with soybean milk	1	—
Cerelac with soybean protein	1	1
Soybean milk with chocolate flavour	2	—
Soybean milk with strawberry flavour	2	—
Soybean ground	3	1
Soybean ice cream with apricot flavour	1	—
Bread with soybean flour	1	—
Snack with soybean	1	—
Rice with soybean	1	—
Soybean yoghurt with strawberry flavour	2	—
Soybean ice cream with strawberry flavour	1	—
Total number of samples	40	9

Legend to table (10) DNA was extracted and analysed by PCR as discribed in Materials and Methods section (3.1). For PCR the primers p35s-f2 and petu-r1 were used to detect the transgene from RRS; the limit of detection for this PCR system is about 0.5 % of RRS; "-" indicates a negative RRS result (concentration below 0.5% RRS).

and GMO containing CRM. Nine out of 40 samples (22.5) tested are shown in table (10) and example for the PCR analysis is given in figure (6) lane 4, 5,10, and 11).

4.1.3 The presence of maize lines Bt176, Bt11, MON810, T25 and StarLink™ in egyptian food samples

4.1.3.1 The presence of maize in foods

The presence of maize and the functioning of the PCR systems was checked by using the maize invertase specific primers pair IVR1-F/IVR1-R (Table 2 and 3). The primer pair Ivr1-F/ Ivr1-R is specific for the invertase gene and flanks part of exon number 3 of this gene. It gives rise to a 226 bp amplicon **(Ehlers et al, 1997)**. This product is detectable in transgenic, as well as in conventional maize (maize specific primer pair). Maize specific primer pairs served as a control for the amplification of the isolated DNA and PCR procedure (PCR quality control). All tested samples gave positive results as shown in the example in Figure (7). with the amplification control primer pair. This resulted in an amplicon of the expected size in samples and CRM's. No amplification was observed neither with the extraction control nor with the PCR control without DNA.

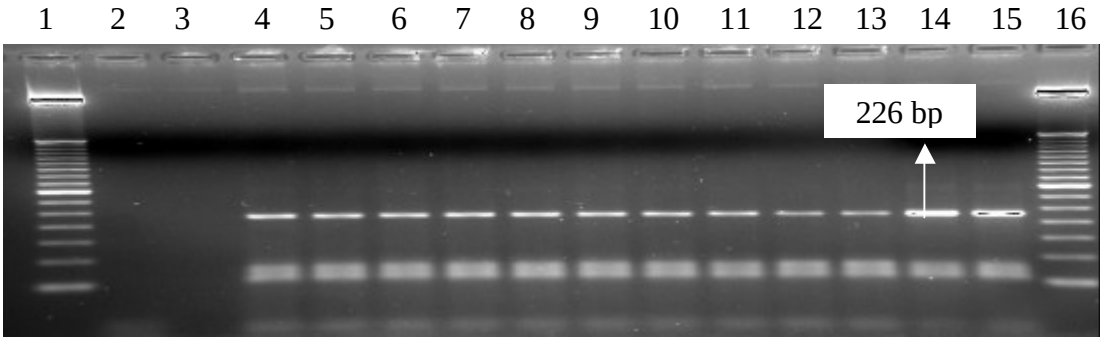


Figure (7) Example for the detection of the maize invertase gene in different food samples. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair ivr1-f/ivr1-r was used for PCR-analysis. Lanes 1+16: 50 bp marker DNA ladder, lane 2: blank sample (extraction control), 3: PCR control without DNA template; lanes 4 -13; DNA from different maize samples; lane 14: DNA from non GMO maize; lane 15: DNA from 0.5% genetically modified Bt 176 maize.

4.1.3.2 Specific detection of GM maize lines

Forty maize samples (Table 11) from different localities in Cairo and Giza were analysed using specific primer pairs to detect Bt176, Bt11, MON810, T25 and StarLink™ in PCR (Table 2 and 3). Bt176 was identified using the primer pair CRY03/CRY04. The primer pair Cry03 /Cry 04 is specific for the identification of transgenic maize Event BT 176. The resulting sequence of 211 bp size is amplified from a genomic region between two adjacent genetic elements, namely the CDPK promoter and the N-terminus of the synthetic cry IA (b) gene **(Hupfer et al, 1998)**.

The expected 211 bp amplicon only appeared with the transgenic samples and GMO containing CRM as shown in the example in Figure (8) (lanes 4, 5, 6 and 7). By using primers specific for Bt11 maize (IVS2-2/PAT-B) an amplified sequence of 189 bp length was obtained with maize grain samples imported from the US and the positive control (Figure (9) lanes 6-7, 10-13). The primer pairs IVS2-2 / PAT-B were used for the detection of the transition site from the intron IVS2 into the Pat-gene in BT11 maize The bacterial PAT gene codes for the enzyme phosphinotricine N-acetyl transferase giving rise to the resistance of Bt11 maize to the herbicide phosphinotricine**. (Anonymus, 2002**) Primer pair T25-F7/ T25-R3 is used for the detection of the transition site between the CaMV-terminator into the PAT gene in T25 maize and yields a PCR products of 209 bp. The amplicon only appear in GMO containing CRM as shown in the example in Figure (10). For the identification of maize MON 810 the primer pair VW01 / VW03 flanks the transition site from the genomic maize DNA into the CaMV- Promotor in MON810 maize **(Anonymus, 2002).** The expected 170 bp appeared with the GMO containing CRM as a positive control as shown in the example in Figure (11). The identification of StarLink™ maize was carried out with the GMOIdent StarLink™ test kit (GeneScan Europe AG). The expected 133 bp fragment only appeared with the maize grain samples from USA as shown in the example in Figure (12) (lane 5, 6).

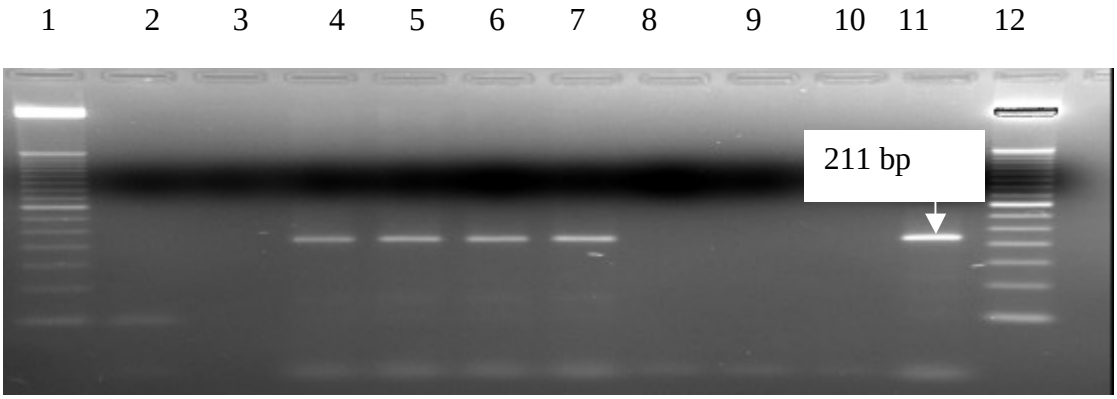


Figure (8) Detection of the transgene from Bt 176 maize in different food samples. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair CRY03/CRY04 was used for PCR-analysis. Lanes: 1+12: molecular weight marker 50 bp DNA ladder; 2: blank sample (extraction control), 3: PCR control without DNA; lanes 4 + 7: DNA from maize granules, USA; lanes 8 + 9: DNA from egyption maize granules, ; lane 10: DNA from non GMO maize; lane 11: DNA from 5 % Bt 176 maize.

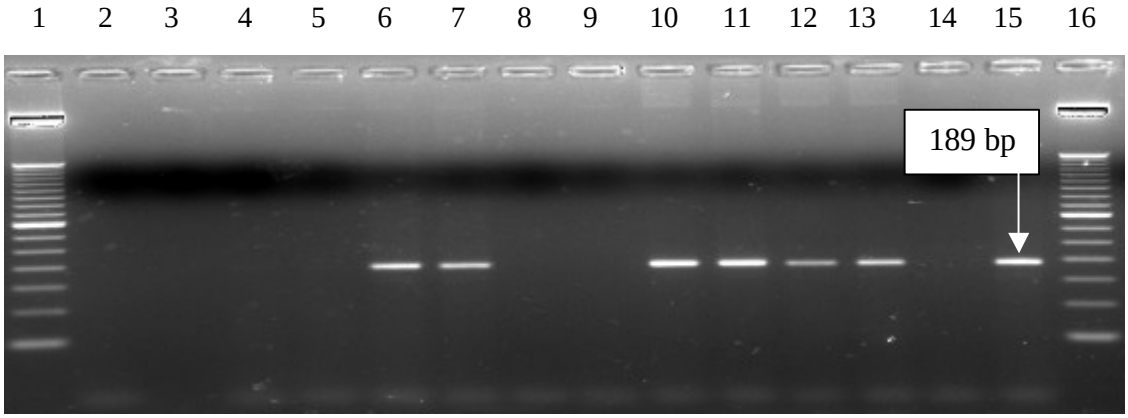


Figure (9) Detection of the transgene from Bt11 maize in different food samples. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair IVS2-2/PAT was used for PCR-analysis. Lanes: 1 + 16: 50 bp marker DNA ladder, lane 2: blank sample (extraction control), lane 3: PCR control without DNA template; lanes 4 + 5: DNA from egyptian maize samples; lanes 6 + 7: DNA from maize granules derived from the USA; lanes 8 + 9: DNA from maize flour from the USA; lanes 10 + 11: DNA from maize granules from the USA; lanes 12 + 13: DNA from maize granules from the USA; lanes 14: DNA from non GMO maize; lane 15: DNA from 2 % Bt11 maize.

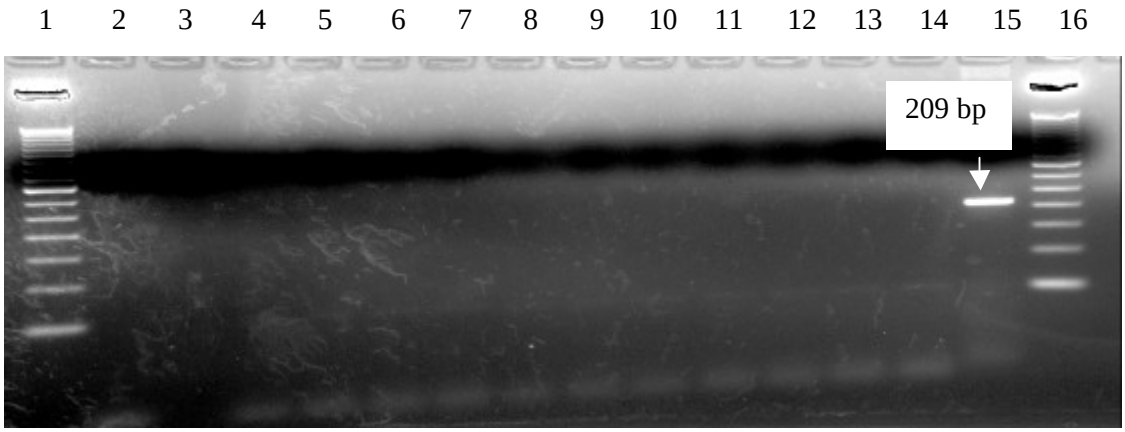


Figure (10) Example for the detection of the transgene from maize T25 in different food samples. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair T25-7/T25-R was used for PCR-analysis. Lanes 1+14: 50 bp marker DNA ladder, lane 2: blank sample (extraction control), lane 3: PCR control without DNA; lanes 4-11: DNA from raw and processed maize samples; lane 12: DNA from non GMO maize; lane 13: DNA from 1% GMO maize T25.

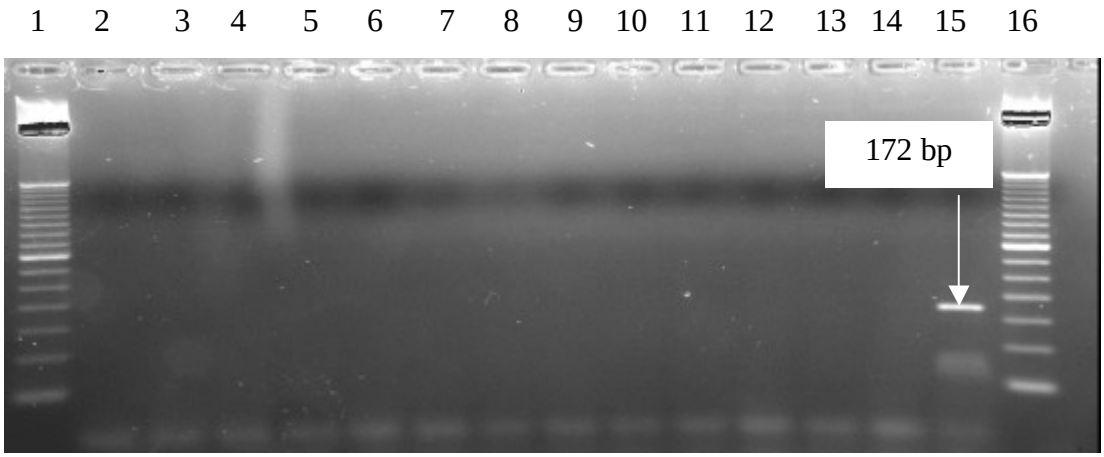


Figure (11) Example for the detection of the transgene from maize MON 810 in different food samples. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair vwo1/vwo3 was used for PCR-analysis. Lanes 1+16: 50 bp DNA ladder, lane 2 blank sample (extraction control), lane 3: PCR control without DNA; lanes 4-13: DNA from different raw and proceed maize samples, lane 14: DNA from non GMO maize, lane 15: DNA from 1% GMO maize MON 810.

The results for all 40 samples under investigation are compiled in Table (11). In particular maize granules imported from the USA and one sample of ground maize of unknown origin were tested positive for Bt176, Bt11 and/or StarLinkTM. 6 samples contained Bt176 maize (15 %) and 5 samples Bt11 maize (12.5 %). Four samples contained StarLink™ maize Table (11). The latter was found in combination with Bt176 and Bt11 in imported US maize. Quantitative analysis further confirmed the presence of StarLink™ maize and revealed a contamination of <1 % Table (11). Especially for this GMO event, health risks can not be fully excluded based on the investigations described in the authorisation by EPA for the purpose of feed production. No MON810 or T25 maize was detected. No GM maize was identified in maize flour and kernels or food samples of Egyptian origin.

4.1.4 Sensitvity of detection

By using specific primers (see material & Methods Table (3) p.(5). For the identification of Roundup Ready™ soybean DNA 2 % GM material (CRM) was detectable using the the present PCR set up as presented in Figure (5) (lane 15).
However, the method also functioned well with certified material containing 0.5 % GMO. This also applies to Bt176 maize using the respective primer pairs. For the transgenic maize lines Bt11, MON 810 and T25, PCR products were obtained when using sample material containing 1 % GMO. For StarLink™ a reference material the

transgenic maize lines Bt11, MON 810 and T25, PCR products were obtained when using sample material containing 1 % GMO. For StarLink™ a reference material provided with the detection kit was used. The purchaser indicates a detection limit of 0.1 % using the kit. The limit of quantification in the determinations of the StarLink™ content was 0.1%.

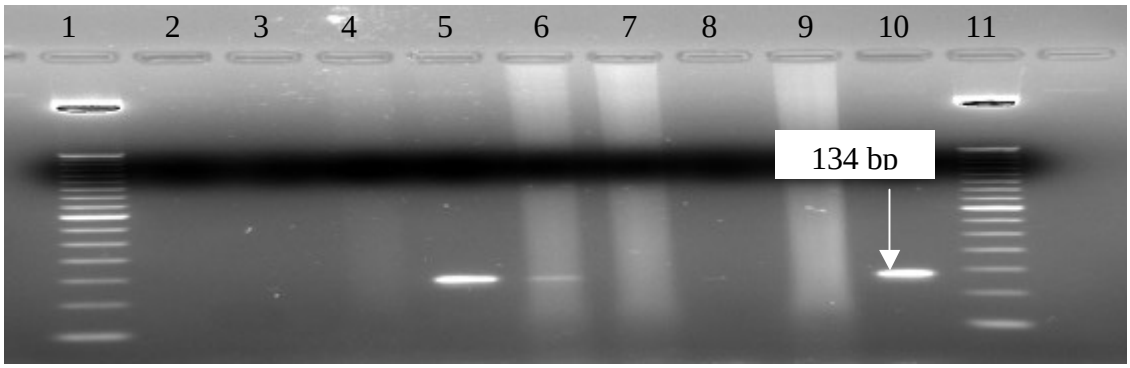


Figure 12. Detection of the transgene from StarLink™ maize in different food samples. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair from the GMOIdent StarLink test kit was used for PCR-analysis. Lanes: 1 + 11: 50 bp DNA ladder; lane 2: blank sample (extraction control), lane 3: PCR control without DNA; lane 4: DNA from corn flakes; lanes 5+6: DNA from maize granules; lane 7: DNA from maize flour for maize cake; lane 8: DNA from egyptian maize; lane 9: DNA from non GMO maize; lane 10: positive DNA from GMO*Ident* StarLink test kit.

4.1.5 Impact of processing

As a result of treatment during food production (e.g. heating, high pressure, etc.) the DNA content after extraction is much lower in processed than in raw samples. The degradation of especially high molecular weight DNA by heating and other physical processing steps is well known **(Ebbehøj et al, 1991; Mayer et al, 1993; Mayer et al, 1994)**. However, sufficient PCR nucleic acid was isolated from all samples using the described extraction procedure although the DNA concentration after extraction varied considerably among the processed samples.

Table 11. Presence of Bt176, Bt11, T25, Mon 810 and StarLink™ specific DNA in maize and maize products

Tested raw materials and processed products	Number of samples	Bt176	Bt11	T25	MON810	StarLink™
Maize flour	2	—	—	—	—	—
Maize starch	3	—	—	—	—	—
Biscuits with maize flour (20%)	1	—	—	—	—	—
Petit four with maize flour (5%)	1	—	—	—	—	—
Maize granules from USA	7	5*	5*	—	—	4*
Bread 100% maize flour	1	—	—	—	—	—
Baladi bread 20% maize	2	—	—	—	—	—
Maize cake	3	—	—	—	—	—
Maize flour for maize cake	1	—	—	—	—	—
Corn flakes	4	—	—	—	—	—
Maize ground	4	1*	—	—	—	—
Katcho-snacks with cheese and pepper flavour	3	—	—	—	—	—
Goulash with maize flour	2	—	—	—	—	—
Loopy nut-sweetened corn cereal coated	1	—	—	—	—	—
Local maize granules	2	—	—	—	—	—
Maize granules from Argentina	2	—	—	—	—	—
Katcho hot chilic and lemon flavoured	1	—	—	—	—	—
Total number of samples	40	6	5			4

*Number of positive samples for the respective GMO maize

Table (12) Presence of Bt176, Bt11 and relative quantitative amount of StarLink™ maize in seven samples of maize granules imported from the USA

Sample No.	Name of the sample	Maize Bt 176	Maize Bt 11	Starlink™ (%)
1	Maize granules	+	+	0.8 (±0.1)
2	Maize granules	-	+	0.3 (±0.1)
3	Maize granules	+	-	n.d.
4	Maize granules	+	+	0.1 ((±0.0)
5	Maize ground	+	+	n.d.
6	Maize granules	+	+	0.6 (±0.2)
7	Maize granules	+	-	n.d.

Legend to table 12: DNA was extracted and analysed by PCR as described in Materials and Methods section (3.1) with primer pairs specific for the transgenes introduced into Bt176, Bt11, and StarLink(TM) maize. Samples positive were tested by qualitative PCR for the presence of GM material from either Bt 176 and/or Bt 11 are indicate with +/-. The amount of StarLink maize was quantified by Genescan Analytics GMbH, Germany, and is shown in % ± standard deviation. The limit of detection was 0.1% of StarLink DNA.

4.1.6 Conclusion

Soybean and maize do play a relevant role in human nutrition in Egypt. Moreover, the consumption of soybean as a basic food component is promoted by the government.

The results clearly demonstrate the incidence of genetically modified maize and soybean on the Egyptian food market. Furthermore, the existence of StarLink™ maize in the food chain supplies evidence for uncontrolled arrival of even unauthorised GMOs for food use in Egypt.

The StarLink™-positive maize imported from the US was freely accessible on the local markets and the use of grains for food or feed purposes is not strictly defined nor monitored by the population. Although the amount of StarLinkTM in the samples was less than 1 % the allergenic potential is still a matter of discussion. Apart from StarLinkTM, Bt176 and Bt11 maize was detected in US imported raw or ground maize. The presence of further GMOs cannot be ruled out since the material was investigated only for the most abundant maize lines, which are approved for food use.

Because it was no longer possible to determine the origin of the maize and soybean material under investigation, it cannot be ruled out that, for instance, the grain samples could be homogenous batches which were imported and distributed all over the country. However, all the samples of the seven US maize grains were taken randomly from different places and at different time in Cairo and Giza. In particular, the percentage of StarLink™ contained in these samples is not equally distributed which strongly indicates different batches. In the case of soybean samples, RRS-positive material was also identified in processed products like biscuit and Cerelac, which is used as infant formula in Egypt. Taken together, GM material could mainly be detected in grains or meals representing the starting point for further processing but also in processed products. Thus, the question is whether the concentration in products is likely to be below the detection limits of the methods. The German official methods according to the § 35 of the German Foodstuffs Act, which were applied here, are reported to detect 0.1 % GM material. However, the positive controls within the framework of this study were carried out with materials between 0.5 – 5 % GMO. Therefore, it is possible that GM material contents below this range have not been detected.

4.2 Part II: Development of a construct-specific, qualitative detection method for genetically modified potato Spunta lines in raw potato and potato-derived products

4.2.1 Introduction

The genetic modification of Bt potato *Spunta* lines G2 and G3 consists of a CryV gene derived from a *Bacillus thuringiensis* strain which is coding for a crystalline toxine. The CryV gene is under the control of the 35S CaMV (Cauliflower Mosaic Virus) promoter. In addition, the inserted construct contains a gene coding for NPTII (Kanamycin resistance) as a selection marker under the expression of the NOS promoter **(Douches et al., 1998; Crikmore et al., 1998)**.

The development of a detection method based on the PCR technique usually comprises several steps. First the DNA is extracted in sufficient amount and quality from raw as well as processed material. Secondly - based on information about the newly inserted sequence - primer systems are selected usually by employing a computer software. Thirdly, the primer system is carefully tested for specificity and sensitivity with isolated DNA from potato (GM and non-GM) and other plants and the PCR conditions are optimised.

The following chapter describes and discusses the results of the different steps in method development including an evaluation of the limit of detection (LOD).

4.2.2 DNA extraction from potato and potato derived products was extracted by the CTAB protocol as described DNA extraction

DNA from potato and potato derived products was extracted by the CTAB protocol as described (Section 3.2.3) and the concentration of extracted DNA was measured fluorometrically (Section 3.1.4). Values obtained were in a range between a maximum of 75 ng/ mg extracted sample of raw material respective 38 ng/ mg extracted sample of chips. The quality of extracted DNA from all samples was tested by control PCR using primer pair A1/A2 **(Taberlet et al., 1991)** which amplifies part of the chloroplast genome and yields an amplicon of 550 bp size.

The CTAB extraction method applied here lead to satisfying results after PCR with DNA extracted from raw potato and cooked potato and hence is suited for the

investigation of this material. Figure (13) shows a typical example of 550 bp PCR products and the concentration of extracted DNA was measured fluorometrically (Section 3.1.4). Values obtained were in a range between a maximum of 75ng/ mg extracted sample of raw material respective 38 ng/ mg extracted sample of chips. The quality of extracted DNA from all samples was tested by control PCR using primer pair A1/A2 **(Taberlet et al., 1991)** which amplifies part of the chloroplast genome and yields an amplicon of 550 bp size. on the other hand, applying the CTAB method on higher processed products like potato chips and mashed potato gave no results in the control PCR. This seems not surprising if taking into account the relatively low amount of DNA which was extractable from these samples and the well known degradational effect of heat treatment and processing on DNA **(Mayer et al., 1993; Mayer et al., 1994)**. Therefore, to proceed the investigation, the NucleoSpin food kit (Macherey-Nagel, Germany) was tested for the extraction of DNA from mashed potatoes and chips. This kit finally gave good results after PCR with these samples. The amount of DNA after extraction was about 40 ng/mg.

4.2.3 Master mix and PCR conditions

The suitable PCR conditions and the master mix for the newly developed primer pair Spu-35S1-f/Spu-cryVm1-r (3.2.4 – 3.2.6) which was selected by sequence alignment to amplify the modified construct (transition site between CaMV promoter and the CryV gene; Figure 1) were studied. Optimum results were achieved with the primer pair Spu-35S1-f/Spu-cryVm1-r using the following conditions: denaturation at 96°C for 10 min, 40 cycles at 96°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec, and a final extension step at 72°C for 7 min. The master mix reagents for a PCR reaction mix of 25 µl total volume contained 2.5 µl PCR buffer [10x concentration Perkin Elmer], 2 µl $MgCl_2$ solution [25mM], 2 µl dNTPs solution (0.2 mM each of dATP, dCTP, dGTP and dTTP), 0.5 µM of each primer, 0.4 µl AmpliTaq Gold polymerase (Perkin Elmer), 5 µl of template extracted DNA [25-30 ng/ml] completed to 25 µl with water. Figure (14) demonstrates the fragments of 122 bp size resulting from PCR with the respective primer pair and the optimised PCR system using DNA extracted from raw material. In order to ascertain a robust limit of detection (LOD) and specific detection, an annealing temperature of 62°C and the conditions as mentioned above were chosen for further experiments. Both lines, G2 and G3, gave similar results.

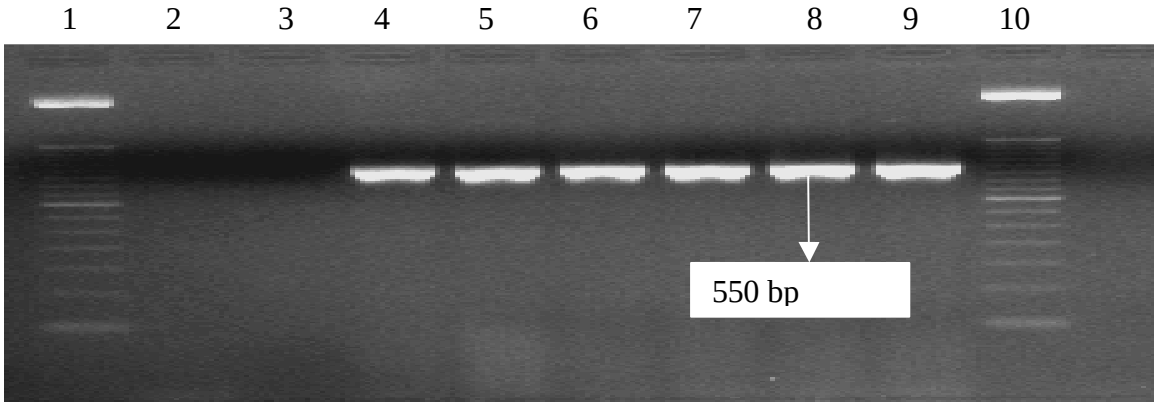


Figure 13; Detection of the tRNA-Leucin gene from chloroplast DNA in different GMO and nonGMO potatoe-lines. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair A1/A2 was used for PCR-analysis. Lanes1+10: 50bp DNA ladder, lanes 2: PCR control without DNA template, lane3: blank sample (extraction control), lanes 4, 5 and 6: amplification of DNA from non GM potato Spunta, lanes 7, 8 and 9: amplification of DNA from GM potatoes.

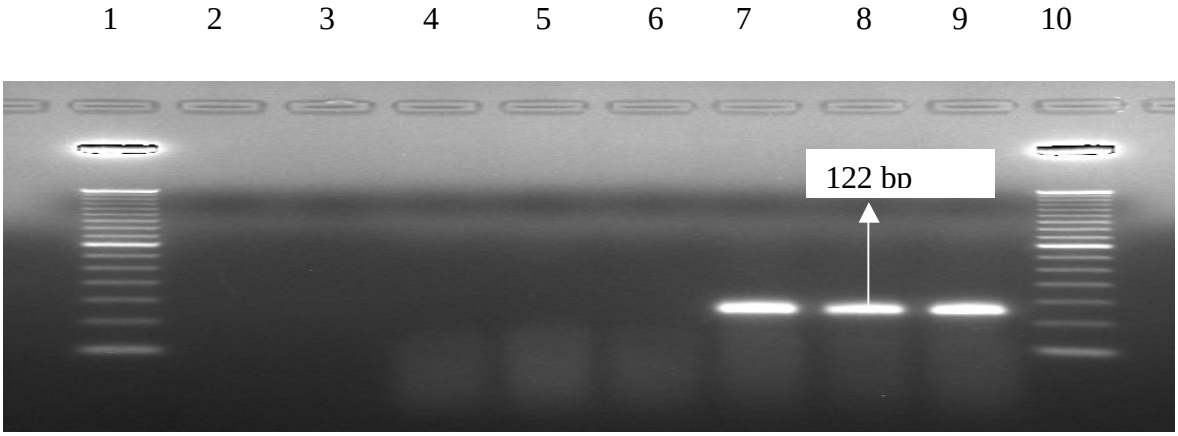


Figure 14: Detection of the transgene of the GMO potato Spunta. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair Spu-35S1-f/Spu-cryVm1-r was used for PCR-analysis. Lanes 1+10: 50bp DNA ladder, lanes 2: PCR control without DNA template, lane3: blank sample (extraction control), lanes 4, 5, 6: DNA from non GM potato Spunta lines, lanes 7, 8 and 9 DNA from GM potato Spunta.

4.2.4 Specificity of the potato Spunta PCR-system

The specificity of the construct specific PCR system was tested with DNA extracted from raw material of the non-modified, conventional *Spunta* line, as well as with commercially available non-GM potato from the market, wheat, the genetically modified maize lines Bt 176, Bt 11, Mon 810, T25, GM soybean (RRS) and a member of the *Solanaceae* family (tomato). DNA from each sample was extracted and then amplified by using the genetically modified potato *Spunta* PCR- system as described above. The PCR products were analysed by gelelectrophoresis on agarose. Only amplification of DNA obtained from genetically modified potato *Spunta*

resulted in a PCR product of the expected size of 122 bp (Figure 15; line 12) whereas DNA extracted from the conventional potato *Spunta* as well as from the commercially non-GMO potato from the market and other tested samples did not yield amplification products (Figure 15; line 4-9). Maize and soya DNA was checked prior to this investigation for PCR inhibitors by amplifying maize DNA samples with a maize specific primer pair (IVR1-F/IVR1-R; Table 2) targeting the invertase gene and soybean DNA with a soy-specific primer pair targeting the lectin gene (GM03/GM04; Table 2). All other plant DNA used in this study was checked for amplification capability by using the A1/A2 primer system as described above (Section 4.2.2). All controls (data not shown) were positive, so that negative results achieved with the GMO specific primer pair Spu-35S1-f/Spu-cryVm1-r in Figure (15) are definitely not based on inhibition but on the absence of the construct. The results clearly show, that the detection system developed here is highly specific, neither cross reacting with a closely related *Solanaceae* species like tomato nor with other GMO which are very common in foodstuffs (Bt176, T25, MON810, Bt11 and RRS) . There is no difference between line G2 and G3 in specificity of the method.

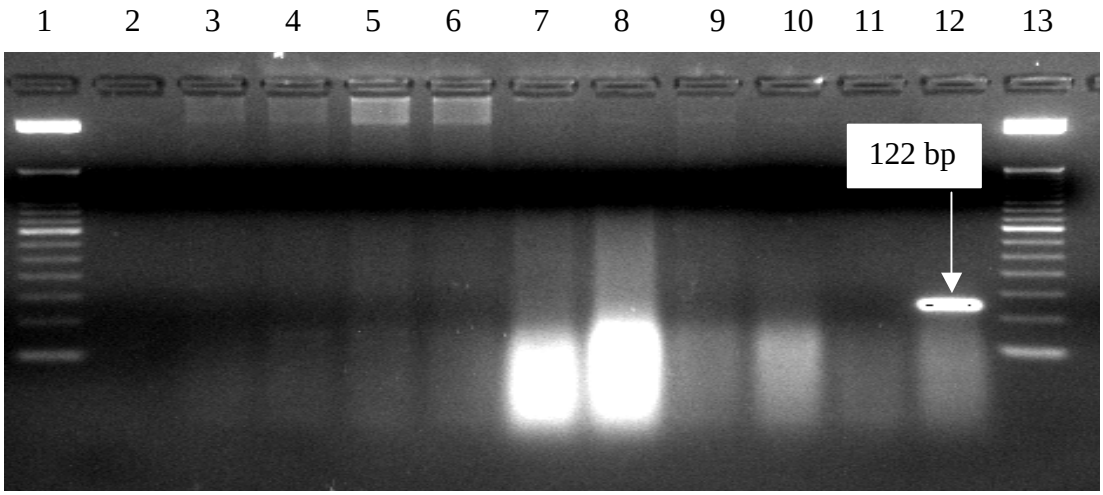


Figure 15: Test of the specificty of the primer pair Spu-35S1-f/Spu-cryVm1-r for the GMO potato Spunta. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair Spu-35S1-f/Spu-cryVm1-r was used for PCR-analysis. Lanes 1 + 13: 50 bp DNA ladder, lanes 2: PCR control with out DNA, lane 3: extraction control, lane 4: DNA from maize Bt176, lane 5: DNA from maize B11, lane 6: DNA from maize Mon 810, lane 7: DNA from maize T25, lane 8: DNA from RRS, lane 9: DNA from nonGMO wheat, lane 10: DNA from GMO (zeneca) tomato, lane 11: DNA from non-GM potato Spunta, lane 12: DNA from GMO potato Spunta.

4.2.5 Detection limit of the detection system for genetically modified Potato Spunta, lines G2 and G3, using raw material

To evaluate the detection limit of the GM potato *Spunta* PCR-system, DNA was extracted from the genetically modified potato (raw material) and stepwise diluted with DNA extracted from non genetically modified potato with proportions of 1 %, 0.1 %, 0.01% and 0.001% (w/w) (GMO/non-GMO DNA). PCR was performed by using the same conditions as described (4.2.4). The results show that a PCR product of 122 bp was generated using all dilutions down to 0.001 %. This amount of DNA corresponds to about 0.4 pg genomic potato DNA. Figure (16) demonstrates that the measured limits of detection (LOD) for the genetically modified potato *Spunta* reaches 0.001 % for raw potato. The sensitivity is similar to that reported for genetically modified soybean and maize **(Matsuoka et al., 1999); Hupfer et al., 1998).** Thus the present method is highly sensitive with raw material and well suited to detect the GM potato during transportation, storage and preprocessing, provided sampling is done properly. No difference of the sensitivity of the method using line G2 or G3 was observed.

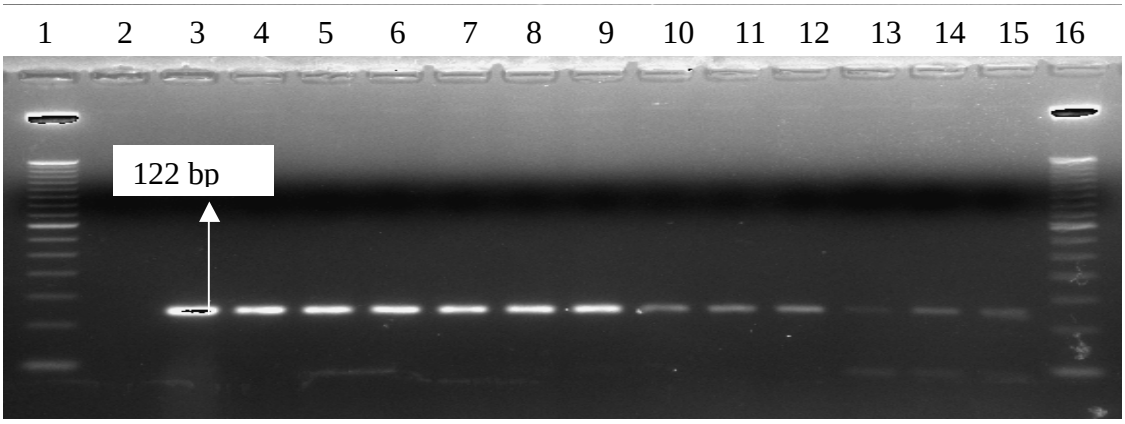


Figure 16: The limit of detection of the GMOpotato Spunta assay system. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair Spu-35S1-f/Spu-cryVm1-r was used for PCR-analysis. Lanes 1 + 16: 50 bp ladder DNA ladder, lane 2: PCR control without DNA template, lane 3: DNA from 100 % GMO potato Spunta, lanes 4, 5, 6: DNA from 1% GMO potato Spunta (w/w), lanes 7, 8, 9: DNA from 0.1% GMO potato Spunta (w/w), lanes 10 + 11: DNA from 0.01% GMO potato Spunta (w/w), lanes 13, 14, 15: DNA from 0.0001 % GMO potato Spunta (w/w).

4.2.6 Detection limit of the detection system for genetically modified potato Spunta, lines G2 and G3, after processing

Exposure to heat is known to cause fragmentation of high molecular weight DNA (**Mayer et al.,1993; Mayer 1994**). In order to prove the suitability of the

selected primer pair Spu-35s1-f/Spu-CryVm1-r for the detection of a genetic modification also in processed potato, products such as potato chips, cooked potato, and mashed potato were chosen as model systems. These examples of thermally treated potato products allow a fairly simple simulation of the influence of heating and processing under laboratory conditions. Serial dilution of DNA from potato chips, mashed potato and cooked potato resulted in a detection limit of 1% (w/w) (GMO / non-GMO DNA) for all processed samples. Figure (17) examplary shows the results obtained with DNA extracted from processed materials (cooked, mashed chips) consisting of 100% GMO and DNA extracted from realistic mixtures (3.2.2.1 – 3.2.2.3) containing 10% and 1% of GMO/non-GMO (w/w). A PCR product clearly was obtained down to 1 % (w/w) GMO/non-GMO for all processed sample types. No differences ocurred between line G2 and G3. This result indicates that the method is sensitive enough to fit in for example with GMO labelling regulation set by Europe (threshold for labelling: 1% (0.9%) GMO/non-GMO) or Japan (threshold for labelling 5 % GM/non-GM) **(Chiueh et al., 2001**).

4.2.7 Confirmation of PCR products

The specificity of the PCR products was further confirmed by appropriate restriction enzyme digestion or by sequencing of DNA (3.2.6). The amplified fragment derived from PCR with primer pair Spu-35S1-f/Spu-cryVm1-r was digested using the enzyme *Bam*HI into two fragments of 69 bp and 53 bp size (Figure 18). The result of sequencing is reflected in Figur (19). It can be concluded that there are no differences between the sequence of DNA produced by using the primer pair Spu-35S1/Spu-cryVm1-r and the sequence of amplified GM DNA of potato *Spunta* compared by alignment (see also Figure 1). These results underline the specificity of the developed detection system for the GM potato *Spunta*.

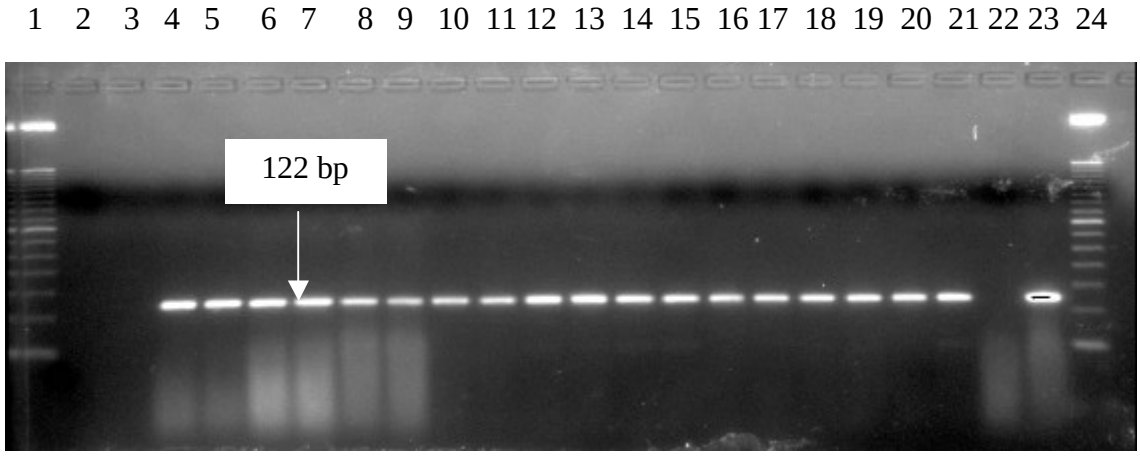


Figure (17) Application of the GMO potato Spunta assay system on processed potato products. Analysis was performed and is documented as described in legend to figure 5 except that the primer pair Spu-35S1-f/Spu-cryVm1-r was used for PCR-analysis. Lanes 1 + 24: 50 bp DNA ladder, lane 2: PCR control without DNA, lane 3: extraction control, lanes 4, 5: DNA from 100% GMO potato Spunta chips, lanes 6, 7: DNA from 10 % GMO potato Spunta chips (w/w), lanes 8, 9: DNA from 1% GMO potato Spunta chips (w/w), lanes 10, 11: DNA from 100% GMO potato Spunta cooked, lanes 12, 13: DNA 10% GMO potato Spunta cooked, lanes 14, 15: DNA from 1% GMO potato Spunta cooked (w/w), lanes 16, 17: DNA from 1% GMO potato Spunta mashed, lanes 18, 19: DNA from 10% GMO potato Spunta (w/w) mashed, lanes 20, 21: DNA from 100% GMO potato Spunta mashed, lane 22: DNA from of nonGM potato Spunta, lane 23: DNA from GM potato Spunta 100 %.

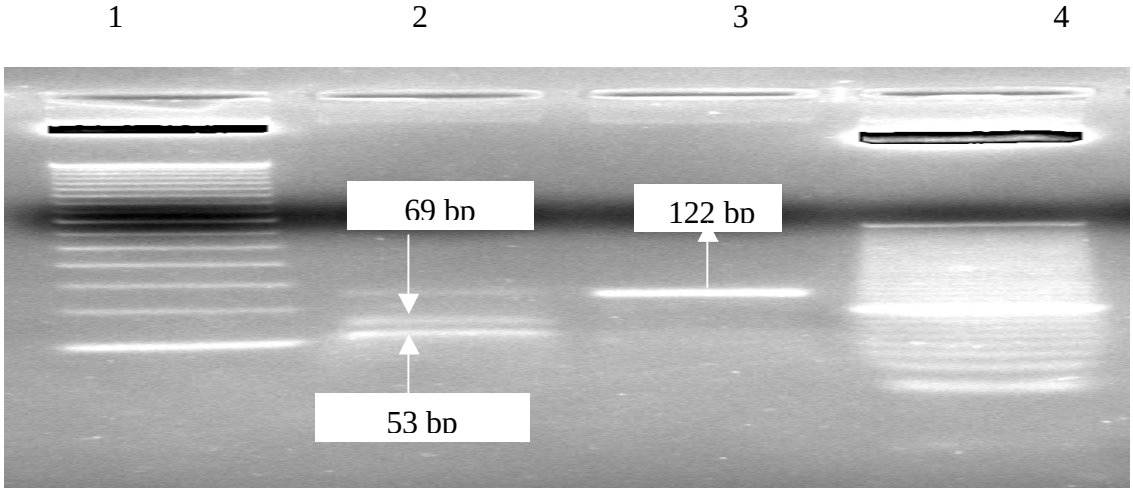
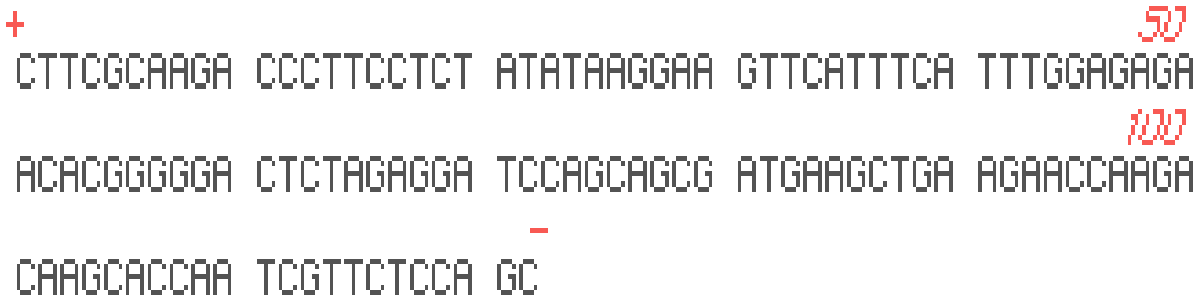


Figure (18) Restiktion analysis of the PCR products derived from the GMO potato Spunta assay system. Amplified products digested with BamHI should result in cleavage of the 122bp fragment in two of 53 and 69 bp. Lane 1: 50 bp DNA ladder, lane 2: PCR product digested with BamHI , Lane 3: not digested PCR product, Lane 4: 10 bp ladder

Figure (19) DNA sequence of the 122 bp fragment amplified using DNA from GMO potato Spunta with the primer pair Spu-35S1-f/SpucryVm1-r



Legend to figure (19) After PCR amplification the DNA fragment was isolated and sequenced as described in Material and Methods (3.2.6).

4.2.8 Conclusion

The following requirements were fulfilled to make available a method suited for the identification of GM potato *Spunta* lines G2 and G3:

- The primer binding sites were selected in a way that the amplified region represents a sequence which does not occur in nature to ensure highest specificity. The specificity was proven with plant DNA from non-GMO potato, tomato, wheat, GM maize lines and soybean. No cross-reaction was observed.
- The amplified sequence is short in size (122 bp) and therefore suited for the use in processed products which have been exposed to heat and/or acid conditions.

The sensitivity of the method goes down to 0.001% (w/w) GM-potato DNA mixed with non-GM potato DNA for raw potatoes respectively 1% for processed material. The detection method presented here is well suited to be used within a framework of statutory control activities with respect to labelling obligations.

4.3 Part III: Quality and safety evaluation of genetically modified potatoes Spunta: Compositional analysis, determination of some toxins, antinutrient compounds of diets and feeding study with rats

4.3.1 Introduction

In an exemplary investigation, the safety of genetically modified potato (*Spunta*) was studied. The potato was developed in the scope of an Agriculture Biotechnology project Support (ABSP) research project between the Department of Crop and Soil Science, Michigan State University, USA and the Agriculture Genetic Egineering Research Institute (AGERI), Egypt (**www.iia.msu.edu**) and is intended for the Egyptian market.

The food safety assessment of GM potato Spunta line G2 and G3 in this part of the study consists of two main elements:

(1) evaluation of the equivalence by determination of natural occurring toxicants, anti-nutrient compounds and components influencing the nutritive value.

(2) Study on potential effects on growth and performance of animals (rats) caused by GM potato *Spunta* lines G2 and G3.

4.3.2 Biochemical composition of Spunta lines G2, G3 and the parental control

4.3.2.1 Proximate analysis of potato tubers

The level of the major components of potato tubers (protein, fat, total solid, carbohydrate, ash, ascorbic acid, starch, crude fibre, total sugar, non-reducing sugar, reducing sugar, calcium, phosphorus, sodium, potassium) was determined for potato tuber from each of the genetically modified potato lines *Spunta* G2, G3 and the non genetically modified parental line (using fresh material). The results of compositional analysis are presented in Table (13). There were no statistically significant differences for any of the components ($P>0.05$) between the non-GM and GM potato G2 and G3 . These results are in agreement with the published literature data and ranges of the historical conventional control values determined in previous studies **(Hashimoto et al., 1999, Habiba et al., 2000; Preascha et al., 2002).**

Table (13) Fiber, mineral and proximate chemical composition of GMO and Non- GMO potato Spunta (g/100g dry weight matter)

Compoundes	**Variety**		
	Spunta Non-GMO	**Spunta GMO G2**	**Spunta GMO G3**
Total solid (%)	18.17 ± 0.105	19.75 ± 0.083	18.76 ± 0.148
Starch (%)	71.5 ± 0.63	71.20 ± 0.26	70.30 ± 0.65
Total protein N×6.25 (%)	11.25 ± 125	11.15 ± 0.228	11.20 ± 0.101
Total lipid (%)	1.2 ± 0.036	1.00 ± 0.060	1.1 ± 0.01
Ash (%)	2.31 ± 0.07	2.15 ± 0.05	2.26 ± 0.05
Crude fibre(%)	1.74 ± 0.01	1.73 ± 0.02	1.72 ± 0.02
Total carbohydrate(%)	65.33 ± 0.33	64.22 ± 0.19	64.96 ± 0.17
Reducing Sugar (%)	1.06 ± 0.02	1.20 ± 0.03	1.07 ± 0.02
Non-reducing sugar (%)	2.76 ± 0.076	2.74 ± 0.06	2.77 ± 0.06
Ascorbic acid mg/100g	31.87 ± 0.01	32.86 ± 0.3	33.66 ± 0.1
Sodium (mg/100 g)	1.35 ± 0.04	1.29 ± 0.01	1.37 ±0.03
Potassium (mg/ 100g)	2.88 ± 0.08	2.82 ± 0.01	2.7 ± 0.01
Phosphorous (mg/ 100g)	34.11 ± 0.09	33.49 ± 0.09	33.82 ± 0.02
Calcium (mg /100g)	8.2 ± 0.03	7.9 ± 0.01	8.4 ± 0.02

Data are expressed as means of triplicate +/- standard deviation (n=3). determinations. Ascorbic acid was determined as fresh weight basis

4.3.2.2 Amino acid composition

The contents of 12 amino acids in GM potatoes (G2 and G3) was comparable to those of the control line tuber, Table (14). In addition, these results were either within published literature ranges or within the range of historical conventional control values determined in previous studies. There were also no significant

differences (P>0.05) between GM potato G2, G3 and conventional non-GM potato Spunta. These results are in agreement with published data and in the range of historical control values determined in previous studies **(Hashimoto et al., 1999).**

4.3.2.3 Fatty acid composition

The results presented in Table (15) show the fatty acid profile in non-GM potato, GM G2 and GM G3 potato *Spunta*. Oleic acid is the predominant fatty acid followed by linoleic acid, these two fatty acids comprising together about 80% of total identified fatty acids. γ-linolenic acid was also estimated in appreciable amount. Palmatic acid constitutes the main saturated fatty acid (*ca*. 8%). No significant differences (P>0.05) between GM G2, G3 and conventional control line non-GM potato were found. These results are in agreement with the results reported by (**Hashimoto et al., 1999)**, and are also in agreement with published literature ranges and the range of historical control values.

4.3.3 Glycoalkaloids (α-chaconine and α-solanine)

Glycoalkaloids are toxic compounds and naturally found in members of *Solanaceae* family. Glycoalkaloids cause inhibition of cholinesterase, gastrointestinal symptoms, haemolysis and inflammation of kidney (**Novak et al, 2000).** Data in Table (16) show the levels of the glycoalkaloids in non-GM, GM G2 and G3 potato *Spunta*. The levels of α-chaconine were 1.47, 1.41 and 1.54 mg/100g, respectively. On the other hand, the level of α- solanine were 0.48, 0.44 and 0.41 mg/100g in non-GM, GM G2 and G3 potato *Spunta*, respectively. There were no significant differences (P>0.05) between GM and conventional control line non-GM potato *Spunta*. These results are in agreement with **(Bushway et al., 1986, Carman et al., 1986, and Friedman et al., 1992)** who found that the average levels of α- solanine in potato are 0.4 to 6 mg/100g and the average levels of α-chaconine are 0.8 to 3 mg/100 g. From these results, it can be concluded that the level of total glycoalkaloids in non-GM, GM G2 and G3 is 1.95, 1.85 and 1.95 mg/ 100g respectively and these findings are within the safety level recommended by FAO/WHO (200 mg/ kg) for acute toxicity (**Rodriguez et al., 1999).**

4.3.4 Total phenol and protease inhibitor

Phenol and protease inhibitor are defined as antinutrients. Usually antinutrients are understood as substances that inhibit or block important pathways in the metabolism, especially the digestion. Antinutrients reduce the maximum utilization of nutrients especially proteins, vitamins or minerals and as a consequence they obstruct optimal exploitation of the nutrients present in food decreasing its nutritive value (**Watzl et al., 1995).** Table (16) shows the total phenol compounds in potato lines GM and non-GM potato *Spunta.* Total phenol contents were 54.8, 55.6 and 70.5 mg/100g respectively. There is no significant differences (P>0.05) between GM and conventional control line and these findings are in agreement with **(Jah et al., 1991; Habiba et al., 2000).** The levels of protease inhibitor activity in non-GM, GM G2 and G3 potato Spunta, were 61.4, 65.6 and 70.5 mg/100g respectively. There are no significant differences (P>0.05) between GM G2, G3 and the conventional control line non-GM potato Spunta.

Table (14) Amino acid composition of potato tubers from transgenic and non-transgenic potaro potato Spunta (mg/100g protein).

Amino acid	**Variety**		
	Spunta non-GM	**Spunta GMO G2**	**Spunta GMO G3**
Alanine	226	238	241
Arginine	301	310	294
Aspartic	92.5	93.43	94.15
Cystine	77.69	82.15	83.09
Glutamic acid	73.38	77.90	76.19
Glycin	68.11	71.14	69.74
Histidine	121	125.03	119.76
Methionine	97.16	88.74	104.27
Troptophane	94.17	91.36	96.04
Tyrosine	23.6	24.3	22.9
Serin	25.4	23.9	24.4
Proline	24	23.3	24.2

Table (15) Fatty acid composition of transgenic and non-transgenic potato Spunta (mg/ 100g)

Fatty acids	**Variety**		
	Spunta non-GM	**Spunta GM G2**	**Spunta GM G3**
Palamitic	8.73	8.14	8.06
Stearic	2.21	2.05	2.13
Palmitoleic	1.39	1.41	1.31
Oleic	54.7	55.1	54.7
linoleic	23.7	23.00	23.2
linoolenic	9.17.	10.3	10.6

Table (16). Levels of glycoalkaloids, protease inhibitor activity and total phynol in GMO and non-GMO potato Spunta (mg /100g).

Variety	**Glycoalkaloids (a-Chaconine)**	**Glycoalkaloids (a-Chaconine)**	**Protease inhibitor activity**	**Total phynol**
Spunta non-GM	1.47 ± 0.03	0.48 ± 0.01	61.44 ± 0.85	54.77 ± 1.07
Spunta GM G2	1.41 ± 0.01	0.44 ± 0.02	65.58 ± 0.29	55.63 ± 0.95
Spunta GMG3	1.54 ± 0.02	0.41 ± 0.01	70.53 ± 0.4	70.53 ± 0.4

Data are expressed as means of triplicate determinations.

4.3.5 Feeding studies with rats (general performance and growth)

To investigate the nutritional impact of GM potato Spunta G2 and G3, a rat performance study was conducted to compare the wholesomeness of GM potato *Spunta* lines G2 and G3 to non-GM (using ground freeze dried material) and a basal diet. Forty-eight weanling male Albino rats (50 ±5 g) were fed with basal diet and housed as described (3.4.5). After feeding with basal diet (all experimental diets are listed in Table 5, Section 3.4.4) for one week, rats were divided randomly into 4 groups (n =12) according to the following scheme:

Group 1: The control group, fed on basal diet only

Group 2: Fed on basal diet with the replacement of 30 % from cornstarch by freeze-dried non-GMO potato

Group 3: Fed on basal diet with the replacement of 30 % from cornstarch by freeze dried genetically modified potato Spunta G2.

Group 4: Fed on basal diet with the replacement of 30 % from cornstarch by freeze dried genetically modified potato Spunta G3

4.3.5.1 General signs in the animals

During the study all test animals were in good condition and appeared healthy. No rat in any group died, and the rats in group 1, 2, 3, and 4 exhibited no abnormal signs throughout the test period.

4.3.5.2 Nutritional assessment in rats

Table (17) shows the effect of feeding diets containing 30 % GM or non-GM potato *Spunta* on the body weight gain, food intake and feed efficiency of the rats. The basal diet resulted ín a body weight gain (in average) of 57.99 g at the end of the experiment while the non-GM potato *Spunta* fed rats revealed a body weight gain of 58.50 g.

Table (17) Change in body weight, food intake and food efficiency of experimental animal groups at the end of experimental period (30 days).

Parameters	**Animal groups**			
	Group 1	**Group 2**	**Group 3**	**Group 4**
Initial body weight (g)	54.31 ± 1.02	53.62 ± 1.05	55.68 ± 0.46	55.96 ± 0.72
Final body weight (g)	112.55 ± 1.25	112.31 ± 1.30	114.23 ± 0.64	115.23 ± 0.65
Daily body weight gain (g)	2.07 ± 0.13	2.17 ± 0.15	2.28 ± 0.22	2.11 ± 0.01
Food intake (g)	6.51 ± 0.23	7.23 ± 0.13	7.27 ± 0.32	6.94 ± 0.22
Food efficiency (%)	0.340 ± 0.01	0.312 ± 0.01	0.363 ± 0.01	0.328 ± 0.003

Data are expressed as means +/- standard deviation (n=3).

Rats fed with the GM potato Spunta lines G2 and G3 showed a body weight gain of 58.55 g and 59.27 g respectively at the end of experimental period. The daily body weight gain for the aforementioned groups was 2.07, 2.17, 2.28 and 2.11 g in average. The daily food intake was almost identical in all groups ranging from 6.51 to 7.27 g, the feed efficiency ratio in basal diet was similar to that caused by diets containing 30 % non-GM potato and GM potato Spunta lines G2 and G3. It can be concluded that no statistical differences were observed in average daily body weight gain, food intake and feed efficiency between GM potato- and non-GM potato fed animals. The daily body weight gain was 2.07 (group I; basal diet), 2.17 (group II; Spunta control), 2.28 (group III/Spunta G2) and 2.11 g (group IV/Spunta G3) (average per animal) respectively for the aforementioned groups. No significant statistical difference (P>0.05) between all groups in final body weight gain was observed.

4.3.6 Effect of GM potatoes Spunta on weight of organs

Table (18) shows the effect of feeding on diets containing 30 % GM and non-GM potato *Spunta* on the relative ratio of selected organs, i. e. liver, heart, kidney, spleen and testicles to body weight of the rats compared with basal diet. The relative ratio of liver to body weight showed a value of 4.1 %, 3.99%, 3. 95 % and 3.98 % for feeding with the basal diet, non-GM or GM potato G2 and G3. The relative ratio for heart weight of the group fed on non-GM basal diet with or without non-GM potato (0.46-0.47 %) was in a similar range as the values resulting from feeding on GM G2, G3 (0.45, 046 %). Comparable results were obtained also for the relative weights of spleen, kidney and testicles, Table (18). From these results it is evident, that there is no significant statistical difference (P>0.05) between control groups and either (G2, G3) GM-fed experimental groups.

4.3.7 Effect of GM potatoes Spunta on selected serum constituents

The biochemical effects of feeding on non- GM and GM *Spunta* potato diets on selected biochemical serum parameters which are reflecting liver and kidney function are presented in Table (19). The data show no significant differences among the rats in all groups. The health condition of all experimental groups coincided with normal values of GOT, GPT, total protein, albumin, glucose and urea indicating that

the liver and kidney respectively were in a good functional state without suffering from any acute infection. Serum value of total cholesterol was also in the normal physiology level. These results showed that the general health and metabolic process of the rats were not affected by adding non-transgenic or transgenic potato to the diets used. These results are in agreement with those recorded by (**Hashimoto et al., 1999)** who studied the the safety assessment of transgenic potatoes with soybean glycin by feeding studies in rats.

Table (18) Effect of feeding of GMO and non-GMO potato Spunta on relative organ weight (percent) in rats.

Organ	**Animal groups**			
	Group 1	**Group 2**	**Group 3**	**Group 4**
Final body weight (g)	112.55 ± 1.25	112.31 ± 1.30	114.23 ± 0.64	115.23 ± 0.65
Liver (%)	4.1 ± 0.1	3.99 ± 0.01	3.95 ± 0.01	3.98 ± 0.01
Kidney (%)	0.63 ± 0.05	0.65 ± 0.03	0.60 ± 0.04	0.65 ± 0.001
Spleen (%)	0.40 ± 0.1	0.41 ± 0.1	0.41 ± 0.1	0.42 ± 0.2
Heart (%)	0.46 ± 0.01	0.47 ± 0.01	0.45 ± 0.001	0.46 ± 0.01
Testes (%)	2.13 ± 0.04	2.11 ± 0.04	2.10 ± 0.02	2.29 ± 0.04

Data are expressed as means +/- standard deviation (n=3).

Table (19) Effect of feeding on GMO and Non- GMO potato Spunta on some serum biochemical values in rats.

Parameters	**Animal groups**			
	Group 1	**Group 2**	**Group 3**	**Group 4**
Total protein (g/100ml)	3.36 ± 0.1	3.44 ± 0.2	3.54 ± 0.1	3.47 ± 0.1
Albumin (g/100M)	4.4 ± 0.3	5 ± 0.9	4.8 ± 0.6	5 ± 07
Glucose mg/100ml	79.35 ± 10.01	64.88 ± 4.51	63.63 ± 18.18	66.68 ± 18.91
T. cholesterol (mg/100ml)	73.51 ± 9.58	79.81 ± 3.85	67.43 ± 2.91	70.59 ± 9.45
Urea mg/100ml	66 ± 5.13	70 ± 2	69.3 ± 7.5	73 ± 1.15
GOT Umol/l	31.5 ± 5.19	33 ± 1.63	33.5 ± 2.88	33.5 ± 2.68
GPT Umol/l	11.2 ± 1.38	12.73 ± 0.66	13.30 0.98	12.36 ± 84

Componds were determined as described in Materials and Methods (3.3.3). Data are expressed as means +/- standard deviation (n=3).

4.3.8 Conclusion

One core instrument of safety assessment is the principle of “substantial equivalence”. Substantial equivalence may be classified into three different categories: (**I)** complete biochemical identity between the novel food and the existing food is expected for the properties conferred by the introduced gene; **(II)** substantial equivalence is expected for certain identifiable and defined differences, such as that the differing properties would require specific safety studies. **(III)** Lack of equivalence: the novel food differs from the traditional products in multiple undefined respects, such that the novel food would need to be thoroughly examined to assess its safety, before accepted or rejected **(Momma et al., 1999**).

The quality of genetically modified potato *Spunta* was assessed in this study by analysing about 36 parameters that are nutritionally and toxicologically important classes of components in potato. The values of proximate analysis obtained for the control potato were comparable with those previously reported for potato and thus are also reliable in terms of the compositional analyses made on transgenic potato. Moreover, there is no statistical difference between line G2 and G3. Against the background of these results, the genetically modified potato *Spunta* G2 and G3 would reveal complete "substantial equivalence" respecting the parameters investigated.

Taken together, the introduction of the CryV gene construct into *Spunta* potato, whether line G2 or G3, has no adverse effect on the whole chemical composition tested here which was not only demonstrated for the proximate protein, fat, total solid, starch, total carbohydrate, ash, crude fibre, total sugar, reducing sugar, non-reducing sugar, ascorbic acid content but also for the amino and fatty acids profiles.

On the other hand, there were no significant differences in the levels of glycoalkaloids, phenol and protease inhibitor activity in physiological studies with rats. Health and metabolic processes of the rats were not affected by the added transgenic potatoes (whether line G2 or G3) to the diets.

However, the safety assessment with laboratory animals is often influenced by many undefined factors apart from the property of the diet. First of all, it maybe problematical to feed the relevant dose of the tested material. In this study tentatively a dose higher than recommended by the guideline for the assessment of food additives was given.

Furthermore, the potential risks of unknown toxins (of the transgenic potato) and their capability to induce malformation, affecting reproductive function, mutagenicity or carcinogenicity are not confirmed by the short- term experiment done here. It should also be considered that studies with rodents do not allow a conclusion for the human situation. In order to construct experimental systems, that make it possible to be extrapolated to humans, long-term animals feeding experiments and the safety assessment by the use of cultured human cell system are now tackled with transgenic crops of potato **(Hashimoto et al. 1999)** and rice (**Momma et al., (1999**). However, such a study would have gone beyond the scope and time frame of this work.

4.4 Part IV: Effect of feeding diets containing genetically modified potato Spunta on broiler health and performance, degradation and possible carry over of genetically modified DNA to tissue

4.4.1 Introduction

Feeding studies that compare modified soybeans and modified maize with conventional products consistently showed no effects on the nutritional assessment of different animals such as rats, chickens, broiler chicken, catfish, dairy cattle, bulls or sheep (**Hammond et al., 1996., Brake and Vlachos., 1998; Flachowsky and Aulrich., 2001, Aulrich et al., 1998)**. Most of the DNA seemed to be inactivated and degraded by the low pH in the stomach or nucleases produced in the saliva and the small intestines **(Phillips and Beever., 2000; Duggan et al., 2000)**. Nevertheless in some cases small DNA fragments may pass through the gastrointestinal (GI) tract due to binding onto soil minerals or proteins **(Gallori et al., 1994; Steward and Carlson., 1986)**. which could act as a protection against degradation. On the other hand, the incorporation of foreign DNA fragments into the body is described for rodents, supposable through Peyer's patches in the GI tract containing M-cells **(Klotz and Einspanier., 1998; Einspanier et al., 2001; Doerfler et al., 1997; Doerfler et al., 1998., Schubbert et al., 1997; and Schubbert et al., 1998)**. In this regard for chicken the appearance of M-cells in the gut-associated lymph tissue has been recorded **(Jeurissen et al.,1999).** However, long term feeding of mice for eight generations did not indicate a germ line transfer of orally ingested foreign DNA **(Hohlweg and Doerfler., 2001).**

The objective of the present part of the study was to investigate the effect of genetically modified potato *Spunta* on broiler health and performance. Furthermore, the fate of modified (foreign) and non-modified DNA was studied to monitor the time-dependent degradation of feed DNA in the broiler GI tract as well as to trace a potential carry over of DNA from feed into broiler organs using DNA extraction and PCR methods.

4.4.2 Investigation of potato tubers and diets

The authenticity of genetically modified and non- genetically modified potato *Spunta* was verified by PCR analysis of the DNA from both, potato tubers and diets.

The primer pair A1/A2 was used as described before (3.2.4; 3.2.5) with freeze

dried potato material to be ground for feed mixtures. As expected, a 550 bp PCR product was detected in both, transgenic and conventional potato which indicates, that the DNA was successfully extracted and amplified, Figure (20). For the specific identification of genetically modified potato *Spunta*, the new developed primer pair Spu-35S1-f/Spu-cryVm1-r, resulting in an amplicon of 122 bp, was used under conditins as described previously (4.4.2). This fragment appeared only in genetically modified potato *Spunta* G2 and G3. Comparable results were observed in the investigation on diets used for feeding in this study, Figure (21). No cross-contamination of non-GM diets with GM material was observed, although the material yielded positive results with the amplification control primer pair A1/A2. The obtained results confirm the observation of (**Forbes et al., 2000**) that grinding a plant does not cause significant disruption of DNA.

4.4.3 Proximate chemical composition of freeze dried potato and diets

Compositional analysis from freeze dried potato. as used for preparation of GM and non-GM containing diets gave the results as presented in Table (20). These results show, that the levels of proximate components (dry matter, crude protein, Ether extract (fat), ash, sugar, crude fibre, phosphorus, starch, sodium and), of the freeze dried matter were comparable between GM-and conventional potato *Spunta* based diets. In addition, these values were either within published literature ranges, or within the range of historical conventional standard values determined from previous studies (**Hashimoto et al, 1999; Parkin et al, 1999; Habiba et al, 2000; and Prescha et al, 2002)**. Obviously the insertion of the construct of the CryV gene had not influence on the main chemical composition of the whole diet and did not affect its content of main nutrients. These results are in agreement with the results of previous studies (**Hashimoto et al, 1999;Habiba et al, 2000; Prescha et al, 2002;)**.

4.4.4 General signs in the birds

Birds were housed, raised, fed in four experimental groups as described (3.4.5). During the study all test birds were in good condition and appeared healthy. No birds in any group died, and the birds in group I, II, III, and IV (3.4.3) exhibited no abnormal signs throughout the test period.

4.4.5 Broiler performance

Table (21) shows the effect of feeding on diets containing genetically modified potato *Spunta* on broiler performance The results of this study show that there were significant differences (P>0.05) detectable concerning feed consumption, feed efficiency, final body weight and body weight gain between the control group fed diets without any potato and experimental groups fed on potato containing food. Obviously the chicken did not utilize the potato supplemented diets as well as the potato-free diet. But on the other hand, there is no significant difference (P>0.05) between either group fed on potato supplemented diet (group II – IV), whether containing non-GM control potato (group II) or GM potato G2 (group III) or G3 (group IV). The final body weight, feed consumption, body gain and feed efficiency among these groups revealed no differences. The results of the present study are in agreement with recently summarised studies by **(Chesson and Flachowsky, 2002)** in which it is concluded that feeds from isogenic and transgenic plants in poultry nutrition did not reveal any significant influence on bird performances.

4.4.6 Effect of feeding on diets contained GM potato Spunta on weight of organs

Table (22) shows the effect of feeding on diets containing 30 % GM and non-GM potato *Spunta* G2 and G3 on the relative ratio of some organs, i. e. liver, heart, spleen and testicles to body weight of the poultry compared with basal diet. These results show that there were no significant (P>0.05) differences detectable concerning the relative ratio of liver, splean, heart and testes to body weight between the group II which was fed on diet containing non-GM potato *Spunta* and groups III and IV which were fed on dietd containing GM potato *Spunta* G2 respectively G3. On the other hand, there were significant differences (P>0.05) in the relative ratio of (liver, Splean, and heart) between the group (I) which fed on the control diet without any potato and the other groups (II, III, IV). There was no significant difference (P>0.05) in the relative of testes to body weight between all groups.

Table (20) Proximate chemical composition of freeze dried nonGMO potato, GMO potato spunta and and diets.

Parameter	**Non-GM potato Spunta**	**GMO potato Spunta G2**	**GMO potato Spunta G3**	**Control diet**	**Control diet + 30% non-GM potato**	**Control diet + 30 % GM potato G2**	**Control diet + 30% GM potato G2**
DM	96.45	96.43	95.73	88.64	92.12	91.70	91.72
CP	15,06	15.20	14.85	22.22	23.24	23.74	24.41
EE	2,93	3,20	3,01	3,93	4,47	4,47	4,55
CF	0.46	0.47	0.43	8.44	8.29	8.52	8.63
Total ash	2.9	2.9	2.9	7	6.7	7.2	7.7
Total-P	2,88	2.82	2,7	7.6	8.04	7.99	7.79
Sodium	1,35	1.29	1.75	2.56	2.36	2.54	2.43
Starch	70.30	69,44	70.30	45,4	42,42	43,26	41,93
Sugars	5,4	5,2	5,61	5,16	4,31	5,27	4,41

Legend to table (20) Componds were determined as described in Materials and Methods (4. 3. 2). Amounts of crude proteine (CP), ether extract (EE) crude fiber (CF) are expressed as % of dry matter (DM).

4.4.7 Blood and Serum Enzymes Investigation

The biochemical effects of feeding on diets which contained non-GM, GM potato *Spunta* G2 and G3 and control group on biochemical parameters regarding liver, kidney and brain function are presented in Table (22). A change in the serum enzymes can even be observed if no other clinical abnormalities are detected so that the analysis of serum enzymes provides a simple test giving fast and general information about the state of the examined blood and the bird's health. The data showed that there are no significant differences (P>0.05) between the control group and experimental group's concerning selected biochemical parameters (GPT, GOT, glucose, cholinesterase, cholesterol, total protein, albumin and creatinine). The health conditions coincided with the values of GPT, GOT, Glucose, cholinesterase, cholesterol, total protein, albumin and creatinine which indicates that the liver, brain,

kidney were in a good functional state with out suffering from any acute infection. The results showed that the general health and metabolic process of the birds were not affected by the new variety of potato used, and the investigated parameters were in normal average as mentioned by (**Gylstorf and Grimm, 1998, Sahin et al., 2002**). From these results it can be concluded that the genetic modification in potato lines *Spunta* G2 and G3 have no significant influence on the general health and physiological process of broiler chicken.

Table (21) Effect of feeding of GMO and non-GMO potato Spunta on final body weight, body weight gain, feed consumption and food efficiency in chicken.

Parameters	**Animal groups**			
	Group 1	**Group 2**	**Group 3**	**Group 4**
Initial body weight (g)	340.45	328.10	335.22	339.99
Final body weight (g)	1270 ± 34	886 ± 110	1016 ± 21	1017 ± 21
body weight gain (g)	929.89 ± 40	663,85 ± 19	679.78 ± 1 8	816.57 ± 16
Feed consumption (g)	1455.22 ± 37	1268.67 ± 12	1314.8 ± 26	1304 ± 80
Feed efficiency (%)	0.64 ± 0.02	0.53 ± 0.01	0.51± 0.003	0.523 ±0 .01

Data are expressed as means +/- standard deviation (n=3).

Tables (22). Effect of feeding of GM and non-GM potato Spunta on relative organ weight (percent) in chicken

Organ	Animal groups			
	Group 1	Group 2	Group 3	Group 4
Final body weight (g)	1270±34	886.19±110	1016±21	1017.67±21
Liver (%)	9.22± 0.49	7.47±0.37	8.27± 0.96	7.71± 0.0,31
Spleen (%)	0.92± 0.01	0.86± 0.08	0.98± 0.07	0.79± 0.31
Heart (%)	8.31±0.25	6.33± 0.19	6.42± 0.34	6.43± 0.30
Testes (%)	0.39± 0.03	0.4± 0.03	0.35± 0.03	0.360.03

Data are expressed as means of triplicate determinations.

4.4.8 DNA Extraction

The quality of DNA from potato and diets which contained freeze dried potato which was extracted as described (3.1.3.2) was tested by PCR by using primer pair A1/A2 **(Taberlet et al, 1991)**. The amount of DNA from different samples was sufficient to give satisfying results after PCR. The results are shown in Figure (20). DNA from GIT- and digesta samples as well as from excreta were extracted as described (3.4.7.1 respectively 3.4.7.2). Subsequentely the quality of DNA extracted from gastrointestinal tract samples was tested by using the newly designed, potato chloroplast specific, primer pair Cp-Po2-f/ Cp-Po2-r. This primer pair was especially developed for the detection of potato DNA under degrading conditions (3.4.10.2.2 and Table 7) and yields a short 111 bp fragment. The results are shown in Figure (22). The quality of extracted DNA from broiler organs was tested by PCR using the primer pair My-f/My-r (3.4.9.2.3). This primer pair amplifies part of the mammalian and avian chromosomally encoded myostatin gene **(Laube et al., 2003)**. The results are shown in Figure (23). These results indicate that the DNA which was extracted from diets, samples collected from different sections of the gastrointestinal trakt (GIT) and organs of broiler chicken can be amplified by using PCR technique.

4.4.9 Investigation on the fate of potato DNA in digesta samples

To investigate the fate of potato DNA in digesta samples, a real time PCR system was newly developed, based on the potato chloroplast specific primer system CP-po2-f/CP-po2-r (3.4.7). An additional probe (3.4.9.2.2) allows a very sensitive on-line detection of the amplified target DNA and hence is best suited for the analysis of DNA in possibly higher degraded state.

The plots generated by real-time PCR represent the standardised DR_n value (normalized reporter dye fluorescence) as a function of the number of cycles. Cycle threshold (C_T) is inversely proportional to the number of template copies present in the reaction sample, therefore the higher the initial amount of genomic DNA tested, the sooner accumulated product is detected in the PCR process and the lower is the value of the C_T (Heid et al., 1996).

To investigate the passage and degradation of potato DNA in the different sections of the broiler GIT in all groups fed on diet containing potato (II – IV), birds were slaughtered at different time after feed withdrawal. The primer probe system CP-po2-f/CP-po2-r/ CP-po-probe gave a positive signal in all collected digesta samples from all groups described above Figure (24). The relative concentration of potato-specific DNA in crop, proventriculus and gizzard was high when the feed was offered continuously till slaughter. In the duodenum the concentration slightly decreases, because the duodenum is the shortest and narrowest part of the intestinal tract and the time of passage in duodenum is only about 10 min. In addition, digesting enzymes, including Dnase and Rnase, attack the digesta at the duodenum level. Furthermore, in poultry, there is a duodenum reflux, which transports the ingesta back to the stomach and this mechanism also affects the duodenum content **(Whittow, 2001**) of potato DNA fragments in this part of the GIT.

At the jejunum and ileum levels, which are the site of absorption, the relative concentration of the short potato DNA fragments (111 bp) is relatively increased, which indicates that DNA is on the way to degradation. The content in the caeca and rectum might have been affected by excretion activity of the birds, which frequently occurred and might have influenced the results obtained for this section of GIT. Even samples collected from GIT contents of birds slaughtered at 8 h after last feeding (group II, III and IV) revealed positive results in potato specific real-time PCR. However, the results obtained appear at high C_T which may be due to excretion or partially degradation of the target DNA during this time period.

From the obtained results it is clear that potato DNA can resist in the intestine without much degradation or absorption and is excreted via the faecal matter. The results are in line with a recent study by (**Reuter and Aulrich, (2003**) who concluded that feed-ingested DNA is partially resistant to the mechanical and enzymatic activities of the GIT and is not completely degraded.

In a second step the digesta samples collected from both, group III and group IV, fed on GM potato *Spunta* G2 and G3 DNA were also amplified by using the 35S1-f/ Spu-CryVm-r/ Spu-CryVm- probe system to see, whether – apart from potato DNA – also GM specific DNA is present. Figure (27) shows the results. The obtained results were similar to those using the potato specific primer CP-po2-f/CP-po2-r/ CP-po-probe system. In addition the primer pairs RR02/ CryVm-Pr and Spu-35S1-f/CRYVM-P-R3 were used (3.4.9.2.1 and Table 7) to detect GM potato *Spunta* DNA in samples collected from different sections of GIT. These primer pairs are construct specific, binding to the 35S promoter and the CryV gene, but in contrast to the primer pair 35S1-f/SpuCryVm-r they are giving rise to much larger PCR products of 504 respectively 1000 bp size. The aim of this additional investigation was to observe, if relatively large gene fragments are passing the different parts of the GIT. As a result, fragments up to 1000 bp size of the GM potato *Spunta* DNA were detected in the samples collected at 8 h after feeding withdrawal (Figure 25) thus resisting digestion and subsequent absorption in broiler gut. These results confirm that the absorption of functional large size DNA is low and these findings are in agreement with previous results published by (**Doerfler et al., 1997; Beever and Phipps 2001**). A Bt gene fragment was observed in pig GIT at various time up to 48 h. after the last feeding of a diet containing Bt maize **(Reuter and Aulrich, 2003)** detected a Bt gene fragment in broiler GIT at various time up to 24 h after feed withdrawal.

4.4.10 Fate of potato DNA in broiler organs

All investigated broiler blood and tissue samples revealed positive results in PCR with primer pair MY-F/MY-R (Figure 23). This primer pair amplifies the chromosomally encoded *myostatin* gene in mammals and poultry **(Laube et al., 2003).** The obtained results confirmed that the DNA was successfully isolated from blood and different tissues and is amplifyable during PCR. The same samples were examined for the presence of potato chloroplast gene by using the CP-po2-f/CP-po2-r/

CP-po-probe real-time primer/probe system, and for GM potato *Spunta* with the 35S1-f/Spu-CryVm-r/ Sp-Cryvm real-time primer/probe system. In contrast to the results obtained using the tissue specific primer pair MY-F/MY-R all investigated tissue samples gave negative results in GM potato-specific real-time PCR. However, in pectoral and thigh muscles, liver, spleen, kidney and thorax skin samples from group II group III and group IV, the plant potato DNA (chloroplast DNA) were successfully amplified in the samples collected at 1, 4, 8 h after feed withdrawal. Figure (26) and Table (24).

Table (23) Effect of feeding diets containing GMO potato Spunta G2 and G3 on serum in chicken by biochemical analysis

Parameters	Animals group			
	Group 1	Group 2	Group 3	Group 4
GOT *(U/L)	97.64 ± 4.47	95.26 ± 5.45	101.01 ± 4.09	98.28 ± 7.92
GPT** (U/L)	5.34 ± 1.33	5.51±57	5.65± 1.58	5.68 ± 0.77
Glucose (mmol/L)	18.36 ± 0.82	16.73 ± 1.03	16.76 ± 1.24	16. 42 ±0.68
Cholinesterase (U/L)	1.26 ± 0.06	1.19 ± 0.15	1.15 ±0.06	1.26 ± 0.11
Cholesterin (mmol/L)	4.31 ± 0.18	4.27 ± 0.18	4.040 ± 0.24	4.30 ± 0.31
Albumin (g/dl)	1.64 ± 0.04	1.67 ± 0.05	1.56 ±0.03	1.54 ±0.08
Total protein (g/dl)	3.27 ± 0.25	3.16 ± 0.13	3.48 ±0.22	3.02 ± 0. 09
Creatinine (µmol/l)	20.40 ±1.89	24.53 ± 1.30	22.62 ± 2.91	23.55 ± 1.03

Legend to table (21) Parameters were determined as described in the Ecoline ® 25 kits (Merck, Darmstadt, Germany). GOT* glutamic oxalic transaminase, (GPT)** glutamic pyruvic transaminase in units per liter (U/L). Data are expressed as means +/- standard deviation (n=3).

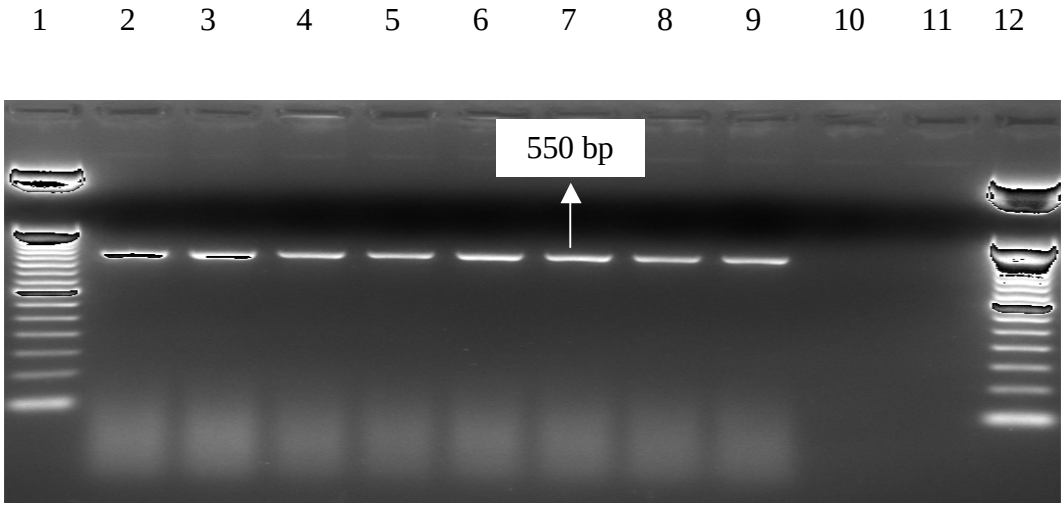


Figure (20) Detection of plant DNA using the universal primer pair A1/A2 in diets and potato samples. Analysis was performed and is documented as described in legend to figure 5. Lanes 1 + 12: 50 bp DNA ladder, lane 2: DNA from GMO potato Spunta G2, lane 3: DNA from nonGMO potato Spunta, lane 4: DNA from GMO potato Spunta line G2, lane 5: DNA from GMO potato Spunta line G3, lane 6: DNA from non-GM potato Spunta, lane 7: DNA from control diet with nonGMO potato Spunta, lane 8: DNA from diet with GMO potato Spunta G2, lane 9: DNA from diet with GMO potato Spunta G3, lane 10: PCR control without DNA, lane 11: extraction control.

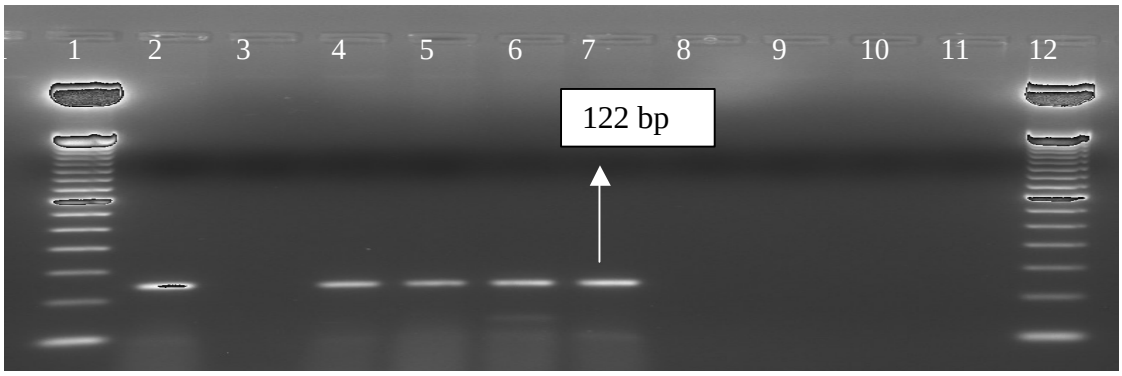


Figure (21) Detection of GMO potato Spunta DNA in diets. Analysis was performed and is documented as described in legend to figure 5 exept that the primer pair Spu-35S1-f/Spu-cryVm1-r was used. Lanes 1 + 12: 50 bp DNA ladder, lane 2: DNA from GMO potato Spunta G2, lane 3: DNA from nonGMO potato Spunta, lane 4: DNA from GMO potato Spunta line G2, lane 5: DNA from GMO potato Spunta line G3, lane 6: DNA from diet with GM potato Spunta line G2, lane 7: DNA from diet with GMO potato Spunta G3, lane 8: DNA from diet with nonGMO potato Spunta, lane 9: DNA from standard diet without potato, lane 10: PCR control without DNA, lane 11: extraction control.

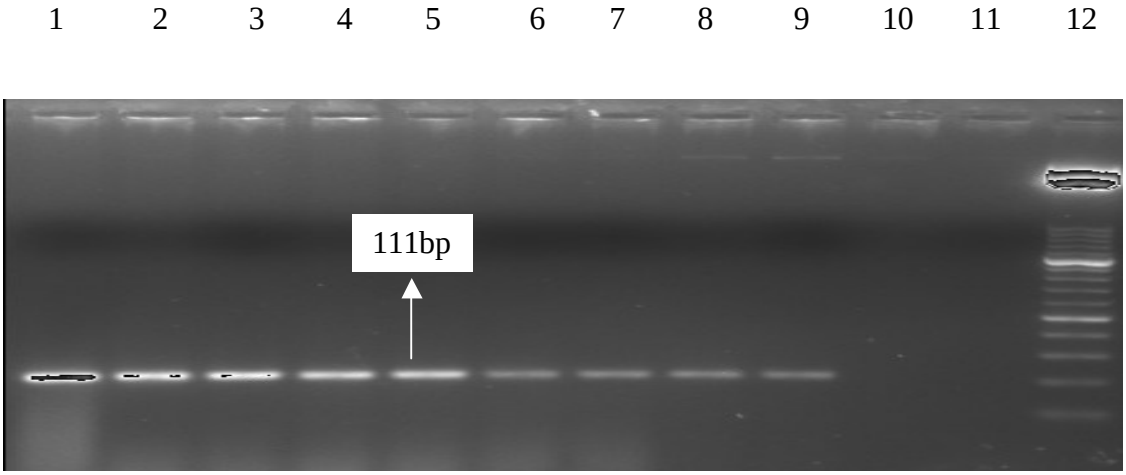


Figure (22) Detection of potato DNA in samples from the contents of the gastrointestinal tract and excreta. Analysis was performed and is documented as described in legend to figure 5 exept that the primer pair Cp.Po2-f/Cp-Po-r was used. Lane 1: DNA from excreta, lane 2: DNA from rectum, lane 3: DNA from ceacea, lane 4: DNA from ileum, lane 5: DNA from jejunum, lane 6: DNA from duodenum, lane 7: DNA from gizzard, lane 8: DNA from provetrucus, lane 9: DNA from crop; lane: 10: PCR control; lane: 11 extraction control lane 12: 50 bp DNA ladder.

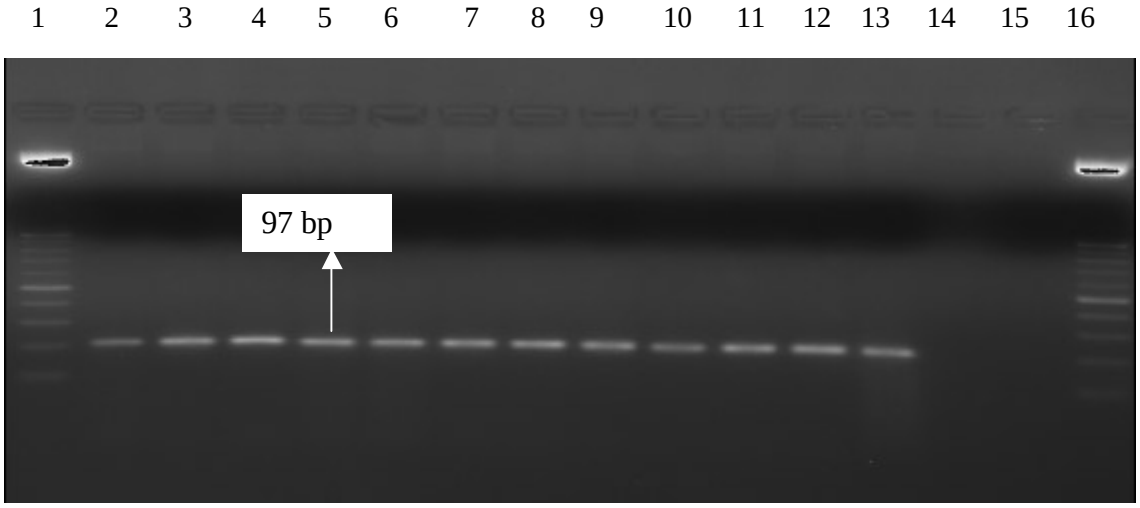
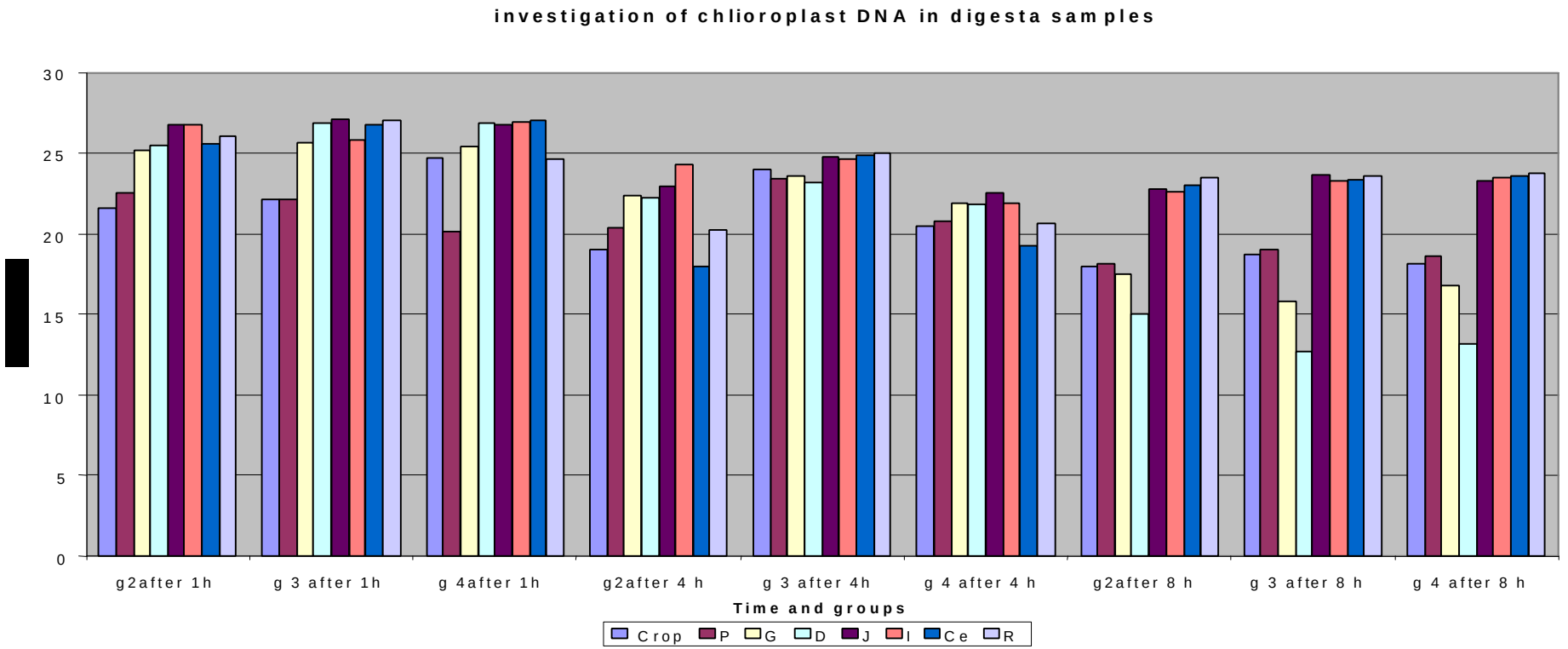


Figure (23) Detection of the myostatin gene in different tissue samples collected from broiler chicken using the primer pair MY-f/MY-r. Analysis was performed and is documented as described in legend to figure 5. Lanes 1 + 16: 50 bp DNA ladder, lane 2: DNA from musle tissue, lane 3: DNA from liver, lane 4:, DNA from kidney, lane 5: DNA from spleen, lane 6: DNA from testis, lane 7: DNA from abdominal fat, lane 8: DNA from thorax skin, lane 9: DNA from pectoral muscle, lane 10: DNA from thigh muscle, lane 11: DNA from bursa, lane 12: DNA from thymus gland, lane 13: DNA from heart, line 14: PCR control without DNA, lane 15: extraction control.

Figure (24) Releative quantification of the potato tRNA-leucine gene in the content of the gastrointestinal tract and excreta from broiler chicken fed with GMO and nonGMO potato Spunta by real time PCR



Legend to figure (24) The hours shown in the plot indicate the time of slautering after the last feeding. g2: group of animals fed with diet containing nonGMO potato Spunta, g3: group of animals fed with diet containing GMO potato Spunta G2, g4: group of animals fed with diet containing GMO potato Spunta G3. DNA was extracted and real time PCR was performed as described in Material and Methods (3.4.7). For PCR the primers Cp-po2-f/ Cp-po2-r and the probe Cp-po were used. The amount of potato DNA determined is shown as cycle threshold as mean of three samples. P: proventriculus; G: gizzard; D: duodenum; j: jejunum; I: ileum; Ce: caecei; R: rectum.

Table (24) Detection of the DNA from tRNA-Leucin gene in tissues samples (n=3) by real time PCR.

Organ	Group 2			Group 3			Group4		
	Slaughtering time			Slaughtering time			Slaughtering time		
	1 h	4 h	8 h	1 h	4 h	8 h	1 h	4 h	8 h
Liver	+	+	+	+	+	+	+	+	+
Kidney	+	+	+	+	+	+	+	+	+
Testes	-	-	-	-	-	-	-	-	-
Spleen	+	+	+	+	+	+	+	+	+
Heart	-	-	-	-	-	-	-	-	-
Abdomenal fat	-	-	-	-	-	-	-	-	-
Bursa	-	-	-	-	-	-	-	-	-
Thorax skin,	+	+	+	+	+	+	+	+	+
Burst muscle	+	+	+	+	+	+	+	+	+
Thigh muscle	+	+	+	+	+	+	+	+	+
Thymus glands	-	-	-	-	-	-	-	-	-
Excreata	+	+	+	+	+	+	+	+	+

Legend to table (24) The hours indicate the time difference between last feeding and slaughtering. DNA was extracted analysed by real time PCR as described in Material and Methods (3.4.7/3.4.10) with the primers Spu-35S1-f/Spu-cryVm1-r and the probe Spu-CryVm.

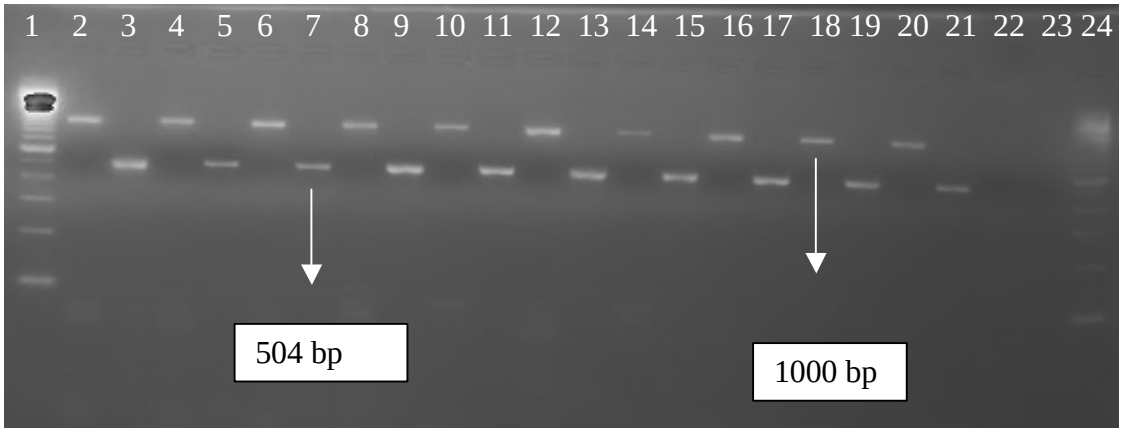


Figure (25) Detection of GMO potato spunta DNA in samples form the contents of the gastrointestinal tract and excreta with two different primer pairs. Analysis was performed and is documented as described in legend to figure 5 exept that the primer pairs RR02/CryVm-Pr (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22) and Spu-35S1-f/CRYVM-P-r3 (lanes 3,5,7,9,11,13,15,17,19,21,23) were used. Lanes 1+24: 100 bp DNA ladder, lane 2: DNA from GMO potato Spunta; lanes: 4+5: DNA from content of excreta; lanes 6+7: DNA from content of rectum; lanes 8+9: DNA from content of ceacea; lanes 10+11: DNA from content of ileum; lanes 12+13: DNA from content of jejunum; lanes 14+15: DNA from content of duodenum, lanes 16+17: DNA from content of gizzard; lanes 18+19: DNA from content of provetrucus; lanes 20+21: DNA from content of crop; lane 22: PCR control with out DNA, lane 23: extraction control.

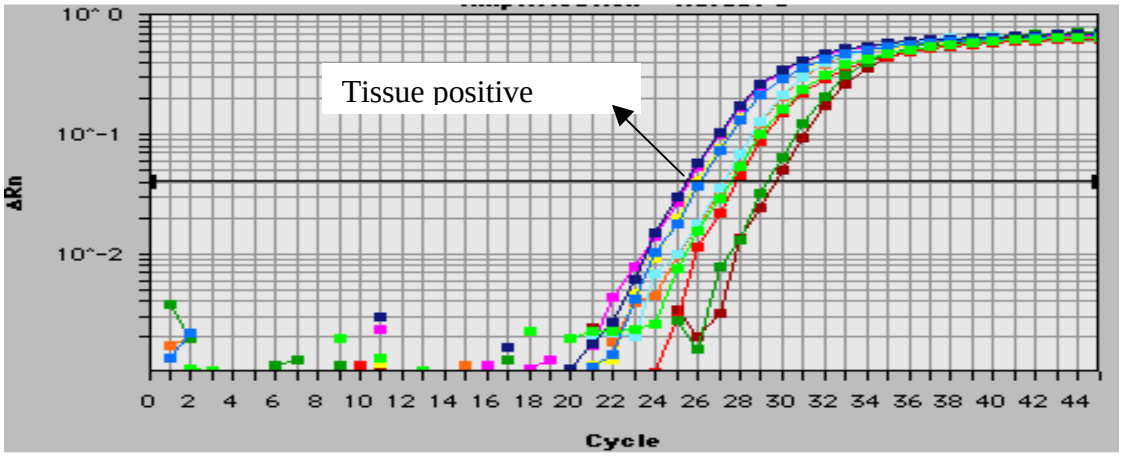


Figure 26. Example of an amplification plot for analysis of DNA from broiler tissue using the primer pair Cpu-Po2-f/ Cp-po2-r and the probe CP-po by real time PCR.

4.4.11 Conclusion

Sanderson-He-Y. Walker,(1994), reported that intestinal epithelial cells have unique salvage pathways for using free nucleotides, owing to their high rate of cell turnover. Any small poly nucleotide DNA fragments that might enter the body would be phagocytized by mononuclear leukocytes and further degraded by cellular enzymes and nucleases in different tissues, which was confirmed by the disappearance of chloroplast gene fragments detected in the tissues of broiler chickens after feed withdrawal. These results are also in agreement with the previous results found by (**Doerfler, 2000**).

The gastrointestinal tract is constantly exposed to DNA that is released from partially or completely digested food or feeds, ingested microbes, and DNA from intestinal micro flora. Ingested food is mechanically disrupted and the released DNA, although it is poorly digested, cleaved through acid hydrolysis and enzymatic digestion into small DNA fragments and eventually some of these fragments are converted to single nucleotides. Acid hydrolysis in the gastrointestinal. tract is expected to depurinate most adenine and guanine nucleotides of the food DNA **(Klinedinst and Drinkwater, 1992)**. The presence of various phosphatases and deaminases continue to destroy the structural integrity of any free DNA The breakdown products of DNA are absorbed being used at the cellular level for synthetic processes as they may be found in blood and tissues. The nucleotides are typically deaminated before being rapidly absorbed. Once absorbed, they are further catabolised into nitrogenous bases, free bases and other metabolites including sugars and phosphates that are used in cellular biosynthetic pathways (**Sonoda and Tatibana, 1978).**

DNA is an essential component of all living organisms and as such, is present in nearly all foods and feedstuffs. In biotech crops, the introduced transgenic DNA molecules are based on the same basic chemical components as the endogenous DNA (adenine, guanine, thymine, and cytosine). Therefore, the introduction of transgenic DNA into a plant does not introduce any new chemical entities to foods and the total DNA in food contributes less than 0.02% to the total dry matter of the food as reported by **(Watson and Thompson, 1988).** Similarly, the amount of transgenic DNA in plants manipulated through biotechnology represents a small proportion of the total amount of DNA in a biotech plant (<0.0004% of the total plant DNA) **(Beever and Kemp, 2000)**.

Furthermore, the genetic sequence coding protein introduced in a plant by biotechnology is only functional when the complete DNA sequence (gene) is activated in the plant as a complete gene without any degradation. A recent publication describes experiments

that directly tested whether extensive feeding of DNA to mice results in detectable expression of mRNA and protein in organs of the animals (**Hohlweg and Derfler, 2001).** Approximately 50 mg of DNA, which encoded the green fluorescent protein (GFP) were fed to 21 mice for three weeks and in a separate experiment that involved feeding 50 mg of the pEGFP-C1 DNA per day to mice over eight generations.. There is no GFP protein or mRNA expression was detectable in liver, spleen, blood or intestinal epithelia of animals. Also, fragments of the GFP gene were not detectable by PCR analysis of DNA isolated from spleen, liver or tail tip samples from either this three-week feeding study or that extended to eight generations.

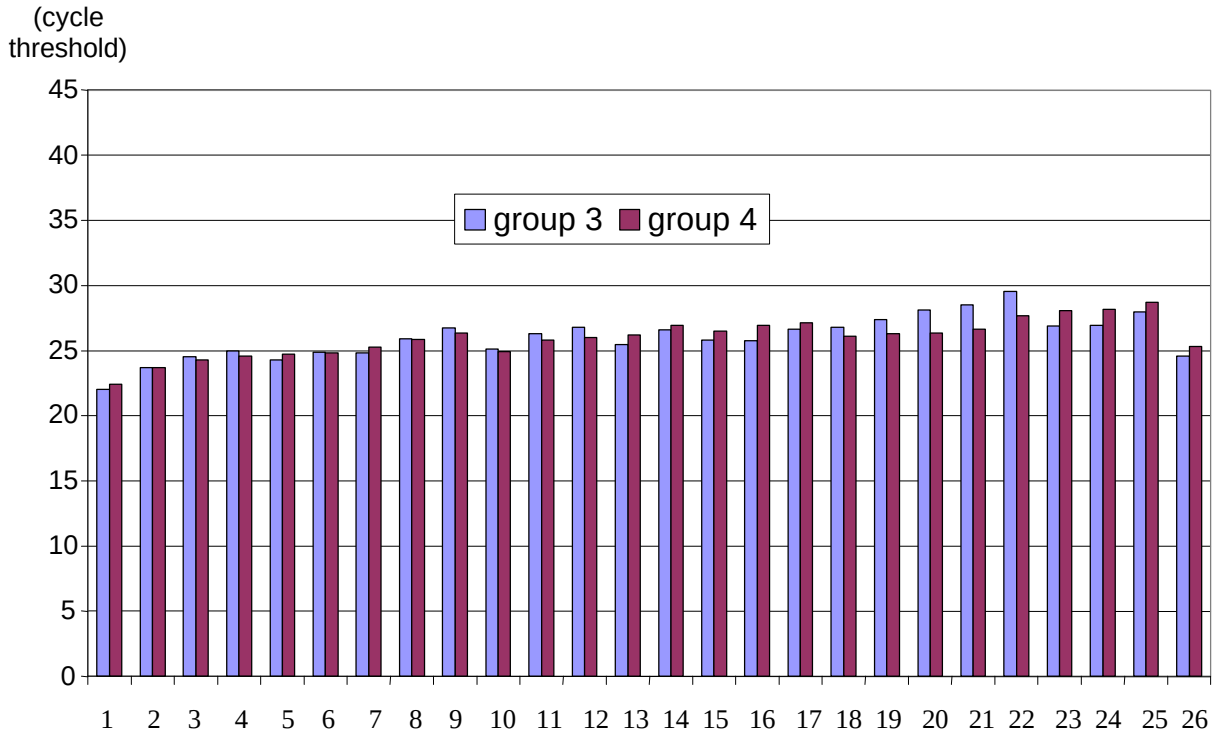
Another experiments used a gene therapy approach with intramuscular injection into mice of the GFP gene. These gene therapy studies showed clearly that detectable expression of the GFP protein and mRNA only at the site of injection **(Hohlweg and Derfler, 2001**).

Therefore, it can be concluded from these studies that gene constructs capable of functioning in vivo when administered via a gene therapy procedure (e.g. intramuscular injection) do not lead to gene expression in somatic cells or detectable integration into the germline of animals when provided orally. Feed processing procedures and food preparation methods resulted in significantly degraded DNA, especially techniques that involve heating at high temperatures or acid treatment **(Ebbehøj et al.,1991; Mayer et al., 1993; Mayer et al., 1994; Forbes, et al., 2000)** or extraction processes **(Berger et al., 2003).** For example, the stability of transgenic DNA in maize preserved as silage has been studied **(Hupfer, et al., 1999).** The intact transgene was only detectable during the first five days of ensiling; with small fragments (200 bp). The rapid breakdown of DNA during ensiling was expected as concluded by **Hupfer, et al., (1999)**. Thus, feed generated by treatments reduces dietary exposure to intact DNA, even before ingestion and further degradation by the digestive system.

From the results obtained in the present study and against the background of previous findings, it can be concluded that, feed ingested DNA is partially resistant to the mechanical, chemical and enzymatic activities of the broiler gastrointestinal tract and is not completely degraded. Modified and non-modified potato Spunta DNA was comparable during feed passage in the broiler gastrointestinal tract. Small DNA fragments derived from plant feeds (chloroplast DNA) can pass the gut epithelium and enter some organs of broiler chickens. Modified constructs from GM potato Spunta lines G2 and G3 fragments DNA could not detected in any investigated tissue samples, however the diets offered in the form of mash, without degradation of GM potato and chloroplast plant DNA. PCR and primer amplifying

plant specific (chloroplast DNA) or amplifying modified construct in GMO potato Spunta fragments could be a practical tool to determine the rate of feed passage and degradation.

Figure (27) Fate of the DNA from GMO potato Spunta in GIT in brioler chicken after feeding.



Legend to figure 27: DNA was extracted and analysed as described in Materials and Methods (3.4.7.2). For PCR the primer pair Spu-35S1-f/Spu-cryVm1-r and the probe Spu-CryVm. was used. The cycle theshold correspondes with the amount of DNA present in the samples 1: DNA from positive potato samples; DNA from crop after 1hour fasting; 3: DNA from Proventriculus Jejunum; 4: DNA from Gizzard V; 5: DNA from Duodenum after 1hour fasting ; 6:DNA from Duodenum after 1hour fasting; 7: DNA from Jejunum after 1hour fasting; 8: DNA from Ileum after 1hour fasting; 9: DNA from Caecei after one hour fasting; 9: DNA from Rectum after one hour fasting; 10: DNA from crop after 4 hour fasting; 11: DNA from Proventriculus after 4 hour fasting; 12: DNA from Gizzard after 4 hour fasting; 13: DNA from Duodenum after 4 hour fasting; 14: DNA from Jejunum after 4 hour fasting; 15: DNA from Ileum after 4 hour fasting; 16: DNA from Caecei after 4 hour fasting; 17: DNA from Rectum after 4 hour fasting; 18: DNA from crop after 8 hour fasting; 19: DNA from Proventriculus after 20 hour fasting; 21: DNA from Gizzard after 8 hour fasting; 22: DNA from Duodenum after 8 hour fasting; 23: DNA from Jejunum after 8 hour fasting; 24: DNA from Ileum after 8 hour fasting; 25: DNA from Caecei after 8 hour fasting; 26: DNA from Rectum after 8hour fasting; 26: DNA from excreat after 8 hour fasting

5. SUMMARY

This study contributes to control the presence and safety of GMO entering the Egyptian food and feed market in future. It includes four basic parts, which are summarised as follows.

PART I (Chapter 4.1)
Detection of genetically modified soybean and maize on the Egyptian food market

In this part, forty samples of commercially available soybean and soybean products and forty samples of commercially available maize and maize products were collected randomly from markets in Cairo and Giza or provided by the Food Technology Research Institute or the Central Laboratory for Food and Feed (Egypt) throughout the years 2000 / 2001. Samples included different processing steps from relatively mild treated ground soybeans to highly processed bakery products, snacks, ground maize and processed products from maize meal. The genetic modification was detected by polymerase chain reaction (PCR) using the official detection methods according to § 35 of the German Foodstuffs Act. Samples were investigated for the presence of material derived from the following genetically modified organisms (GMO's) all of which are approved for food use in Europe: Roundup Ready^{TM} soybean (RRS) and maize lines Bt176, Bt11, T25 and MON810. In addition, samples were examined in qualitative and quantitative analysis for the presence of material derived from the transgenic maize line StarLink^{TM} (Aventis) which was approved for animal feed use exclusively in the US.

<u>The results obtained can be summarised as follows:</u>

- 20% of fourty investigated soy samples contained Roundup ReadyTM soybean. 15% of fourty maize samples tested positive for Bt176 and 12.5% positive for Bt11 maize.
- Furthermore, the presence of StarLink^{TM} maize could clearly be demonstrated in four samples mixed with Bt176 and Bt11.
- The percentage of StarLink^{TM} was less than 1% in quantitative analysis.
- The maize lines T25 and MON810 were not detected.
- The conclusion drawn from the results obtained here is, that GM maize and soybean have already entered the Egyptian food market, including GMO's not approved for food use. To ensure that consumers have a choice and in order to build up an effective national food

control, Egypt needs a legal basis for the import, processing and labelling of GM foods in future.

PART II (Chapter 4.2)

Development of a construct-specific, qualitative detection method for genetically modified potato Spunta in raw potato and potato-derived products.

This part aimed to develop a construct-specific, qualitative detection method for the unambiguous identification of genetically modified potato *Spunta* lines G2 and G3. The detection method was based on the polymerase chain reaction (PCR). The PCR conditions and master mix were optimised empirically.

The results obtained can be summarised as follows:

- The DNA extraction method: the DNA extraction method based on the CTAB protocol leads to satisfying results after PCR with raw and cooked potatoes, but gives no positive results with higher processed material. This problem was solved using a commercial kit. Inhibition of PCR due to the extraction method (co-extraction of PCR inhibiting substances) was neither observed applying the CTAB method nor after using the kit as proven by control PCR with a plant chloroplast primer system.
- Primer design: a primer pair designated Spu-35S1-f/spu-CryVm1-r was newly designed based on sequence information obtained from the Michigan University. The amplified sequenced spanned from the constitutive 35S CaMV promoter region into the CryV gene. The amplified sequence is 122 bp of size and thus short enough to yield good results with higher processed samples.
- PCR conditions and master mix were optimised
- Specificity of the detection system for GM *Spunta* potato lines G2, G3: the specificity of the primer pair Spu-35S1-f/spu-cryVm1-r was tested with DNA from plant material including a closely related *Solanaceae* species (tomato), commercially available potato from the market, the non-modified *Spunta* negative control, wheat, genetically modified maize lines (MON810, Bt176, Bt11) and soybean (Roundup Ready^{TM}). Only with genetically modified potato *Spunta* the expected 122 bp fragment appeared after PCR with the respective primer pair. It was further shown, that the negative results obtained with

plant DNA was not based on inhibitory effects by performing control PCR with plant, maize or soybean specific primer pairs. G2 and G3 gave identical results.

- Detection limit of the detection system for GM *Spunta* potato lines G2, G3: The limit of detection (LOD) for this method was determined with serial diluted DNA from GM- and non-genetically modified potato which was extracted and mixed. The measured limits of detection are 0.001% (w/w) for raw material and 1% (w/w) for processed products.
- Confirmation of PCR products: the specificity of the PCR products was confirmed by appropriate restriction enzyme digestion. The amplified fragment of 122 bp size obtained with GM potato *Spunta* G2 or G3 was digested using the enzyme *Bam*HI into two fragments of 69 bp and 53 bp size. In addition, sequence analysis was performed to confirm further the PCR product.
- As a conclusion, a highly sensitive and specific method for the detection of raw or processed GM potato Spunta lines G2 and G3 has been made available. The method is suited for control purposes or further research on the fate of DNA after digestion.

Part III (Chapter 4.3): Quality and safety evaluation of genetically modified potatoes Spunta: Compositional analysis, determination of some toxins, antinutrients compounds and feeding study in rats

This part aimed to evaluate the safety of the composition, nutritional and toxicological of GM potato *Spunta* lines G2 and G3 compared with the parental non-GM *Spunta* line. Additionally, a feeding study was done with rat.

Compositional analyses was conducted to measure proximate chemical composition with reference to 36 components, total solid, protein, lipid, crude fibre, ash, carbohydrate, starch, reducing sugar, non reducing sugar, sodium, calcium, potassium, phosphorus and ascorbic acid. Additionally selected toxins and antinutrients compounds were determined.

The feeding study in rats was done for 30 days. Four groups of albino rats were used for studying the effect and the safety assessment of GM potatoes *Spunta* G2 and G3. Group (I) was fed on control basal diet, group (II) was fed on control diet plus 30 % freeze dried non-genetically modified potato *Spunta*, group (III) was fed on control diet plus 30 % freeze dried genetically modified potato *Spunta*, and group (IV) was fed on control diet plus 30 % freeze dried genetically modified potato *Spunta* GMO G3**.**

<u>The obtained results can be summarised as follows:</u>

a) Chemical composition

- There are no significant differences between GM potatoes G2, G3 and *Spunta* control potato line in the proximate chemical composition.
- There were no significant differences between the GM potatoes and the conventional potato line with respect to the levels of glycoalkaloids. Measured levels do not exceed a safety level which is recommended by FAO/WHO (200 mg/kg) for acute toxicity.
- No significant differences between the GM potatoes line and the conventional potato *Spunta* line were found with respect to protease inhibitor activity and total phenol.
- No significant differences in chemical composition between the GM potato lines G2 and G3 was observed.

b) Feeding study with rats

- During the period tested, rats in each group (I, II, III, IV) grew well without marked differences in appearance.
- No statistical differences were found in food intake, daily body weight gain and feed efficiency. But there is a significant difference in finally body weight between the control group (receiving basal diet without any potato) and experimental groups fed on potato. However, no statistical difference was observed between non-GMO or GMO potato fed animals.
- No significant differences were found in serum biochemical value and also between relative organs weight (liver, spleen, heart, kidney; testes) between each group.
- Based on these data, it can be concluded that the composition of GM potato *Spunta* G2 and G3 is substantially equivalent to that of the conventional control line regarding the substances measured, as well as the GM potato lines G2, G3 did not reveal any toxicological effects.

Part IV (Chapter 4.4) : Effect of diets containing genetically modified potato Spunta on broiler health and performance, degradation and possible carry over of genetically modified potatoes Spunta DNA monitored in broiler

In this part, the effects of feeding GM potato *Spunta* line G2 and G3 to broiler was studied including an evaluation of the degradation of potato *Spunta* DNA and its metabolic fate in broiler muscle and organs. One day old broilers were fed ad-libitum on either an experimental diet containing non-GM potato *Spunta* , GM potato *Spunta* G2 and G3 or a control diet without any potato. To confirm the nutritional equivalence of GM potato Spunta

and non-GM potato Spunta parameters of nutrition were evaluated (feed consumption, body weight gain, feed efficiency and final body weight). The biochemical parameters regarding liver, kidney and brain function were examined. Additionally, the effect of GM and non-GM potatoes *Spunta* on the relative weight of organs were compared. The degradation and fate of potato *Spunta* DNA and potato specific DNA (chloroplast DNA) in gastrointestinal tract and organs of broiler chicken were investigated.

The obtained results can be summarised as follows:

a) Growth and performance

- No birds in any group died, and the birds in group I, II, III, and IV exhibited no abnormal signs throughout the test period.
- There were significant differences (P>0.05) detectable concerning feed consumption, feed efficiency, final body weight and body weight gain between the control group which was feed on control diet without any potato and experimental groups fed on potato containing diets.
- But there is no significant difference (P>0.05) between group II, III and IV which were fed either on diets supplemented with non-GM potato or GM potato Spunta lines G2, G3 in the parameters of the previous point. Thus the effect described above was due to poorer utilization of potato containing diet in general, but not GM-specific.
- There were significant differences (P>0.05) detected concerning the relative ratio of liver to body weight between the control group which was fed on standard diet and experimental groups II-IV receiving potato with their diets. On the other hand, there is no significant difference (P>0.05) detected among these latter groups. Again, from these values a slightly decreased general utilization of potato containing diets versus non-potato doted basal diet is to conclude.
- There is no significant differences (P>0.05) between the control group I and experimental groups (II, III, IV) regarding biochemical parameters (GPT, GOT, glucose, cholinesterase, cholesterol, total protein, albumin and creatine).
- It can be concluded that the genetic modification potato Spunta with *Cry V* gene to resistance to potato tuber moth (PTM) has no significant influence on the general health and physiological processes of broiler chickens.

b) Fate of DNA in GIT, tissue and organs

- Fragments of feed-ingested potato chloroplast DNA could be demonstrated all over the GIT from crop to faeces.

- Genetically modified DNA fragments of 122, 504 and 1000 bp size could be demonstrated all over the GIT from crop to faeces.
- No amplification products derived from the genetic modification were detected in all tissue samples. However, pectoral and thigh muscles, liver, spleen, thorax skin and kidney samples from both trial groups, potato DNA fragments (chloroplast gene fragments) were successfully amplified in the samples collected at 1 h , 4 h, and 8h after the last feeding.

6. ZUSAMMENFASSUNG

Die vorliegende Studie ist ein Beitrag zur Kontrolle und Sicherheitsbewertung von gentechnisch modifizierten Pflanzen, welche zukünftig auf dem ägyptischen Lebens- und Futtermittelmarkt eine Rolle spielen könnten. Sie umfasst vier praktische Teile, welche sich wie folgt zusammenfassen lassen.

TEIL I (Kapitel 4.1)

Nachweis von gentechnisch verändertem Soja und Mais auf dem Ägyptischen Markt

- Vierzig kommerzielle Zufallsproben von Soja (Körner) und Sojaprodukten sowie vierzig Proben von Mais (Körner) und Maisprodukten wurden vom Einzelhandel in Kairo und Giseh sowie vom Food Technology Research Institute, Central Laboratory for Food and Feed (Ägypten) in den Jahren 2000/2001 bezogen und auf die Anwesenheit folgender transgener Pflanzen untersucht: Roundup Ready™ Sojabohne (RRS) sowie die Maislinien Bt176, Bt11, T25, MON810 und StarLink™. Im Probenumfang waren verschiedene Verarbeitungsstufen von einfachen Behandlungen wie gemahlenen Sojabohnen oder Mais bis zu stark verarbeiteten Produkten (Backwaren, Snacks) enthalten. Die gentechnische Modifikation wurde durch die Methode der Polymerase Kettenreaktion (PCR) nachgewiesen. Die Untersuchungen wurden gemäß der offiziellen deutschen Methoden nach § 35 LMBG durchgeführt.

Die Ergebnisse lassen sich wie folgt zusammenfassen:

- 20% der untersuchten Sojaproben enthielten RRS Soja, 15% der untersuchten Maisproben enthielten Bt176 und 12,5% Bt11 Mais.
- Weiterhin wurde in vier Maisproben die Anwesenheit von StarLink™, vermischt mit Bt176 und Bt11, festgestellt.
- Der Anteil von StarLink™ in den positiven Proben war geringer als 1%.
- Die Maislinien T25 und MON810 waren nicht nachweisbar.

Schlussfolgerung

Produkte aus gentechnisch verändertem Mais oder Soja sind bereits unkontrolliert auf den ägyptischen Lebensmittelmarkt vorgedrungen, inklusive Spuren einer Maislinie (StarLink™, Aventis), die nicht für den Gebrauch in Lebensmitteln autorisiert wurde. Soll den ägyptischen Konsumenten die Wahlfreiheit eingeräumt und eine effektive nationale Lebensmittelkontrolle aufgebaut werden, benötigt das Land zukünftig eine klare gesetzliche Grundlage für den Import, die Verarbeitung und Kennzeichnung von Produkten, die aus GVO hergestellt

wurden. Parallel sind der Aufbau speziell ausgestatteter Kontrolllaboratorien sowie Kenntnisse und Zugang zu standardisierten Nachweisverfahren notwendig.

TEIL II (Kapitel 4.2)

Entwicklung einer konstruktspezifischen, qualitativen Nachweismethode für gentechnisch veränderte rohe Kartoffeln oder Kartoffelprodukte der Linien *Spunta* G2 und G3

Dieser Teil verfolgte das Ziel, eine Konstrukt-spezifische, qualitative Methode für den unzweifelhaften Nachweis der gentechnisch veränderten Kartoffellinien *Spunta* G2 und G3 zu entwickeln. Die Methode basiert auf der Polymerase Kettenreaktion. PCR-Konditionen und Mastermix wurden empirisch optimiert. Im folgenden sind die Schritte der Methode kurz charakterisiert:

- Die standardmäßig eingesetzte DNA-Extraktion basierend auf dem CTAB Protokoll führt zu befriedigenden Ergebnissen in der PCR mit Material aus rohen und gekochten Kartoffeln, jedoch wurde kein positives Ergebnis mit höher prozessierten Proben erzielt. Dieses Problem konnte durch die Anwendung eines kommerziellen Kits zur DNA-Extraktion aus diesen Proben gelöst werden. Eine Inhibition der PCR z.B. durch Ko-Extraktion von PCR-inhibierenden Substanzen wurde weder mit der CTAB Methode noch mit dem Kit festgestellt, da ein multicopy Gen aus Chloroplasten in allen Proben in einer Kontrollreaktion amplifizierbar war.
- Aufgrund dieser Ergebnisse wurde DNA aus rohen Kartoffeln nach dem kostengünstigeren CTAB Verfahren, aus verarbeiteten Kartoffeln mittels eines kommerziellen Kits extrahiert.
- Anhand von Sequenzinformationen und einer speziellen Software (PrimerExpress) wurde ein auch in prozessierten Produkten einsetzbares Primerpaar Spu-35S1-f/Spu-CryVm1-r entwickelt. Die Primer binden im 35S-Promoter und im CryV-Gen und ergeben in der PCR ein Produkt von 122 bp Länge. Der Verdau dieses PCR-Amplifikats mit dem Restriktionsenzym *Bam*H1 ergibt spezifische Fragmente von 69 und 53 bp Länge.
- Für die amplifizierte Sequenz wurde eine geringe Größe von 122 bp gewählt, um die Einsetzbarkeit in höher prozessierten Proben zu ermöglichen.
- Die PCR-Konditionen wurden empirisch optimiert. Folgende PCR-Bedingung und Mastermixzusammensetzung wurden ermittelt: Denaturierung bei 96^{o}C für 10 min, 40 Zyklen bei 96^{o}C für 30 sec, 62^{o}C für 30 sec, 72^{o}C für 30 sec und ein abschließender Extensionsschritt bei 72^{o}C für 7 min. Der Mastermix für eine PCR Reaktion von 25 µl

Endvolumen enthielt: 2,5 µl PCR Puffer [10x konzentriert, Perkin Elmer], 2 µl $MgCl_2$ Lösung [25 mM], 2 µl dNTPs Lösung [0,2 mM jeweils dATP, dCTP, dGTP and dTTP], 0,5 µM von jedem Primer, 0,5 µl AmpliTaq Gold Polymerase (Perkin Elmer), 2 µl extrahierte Template-DNA [25-30 ng/ml] aufgefüllt auf 25 µl mit Wasser.

- Die Spezifität wurde anhand folgender Materialen getestet: unveränderte *Spunta* Kontroll-Linie, Kartoffel vom Markt, Tomate (*Solanaceae*), Weizen, gentechnisch veränderter Mais (MON180, Bt176, Bt11) und gentechnisch veränderter Soja (RRS). Nur mit der gentechnisch modifizierten (GM) Kartoffel *Spunta* erschien das erwartete 122 bp Fragment nach der PCR mit dem spezifischen Primerpaar. Die Linien G2 und G3 zeigten identische Ergebnisse. Es wurde weiterhin gezeigt, dass die negativen Ergebnisse mit pflanzlicher DNA nicht auf inhibitorischen Effekten beruhten. Eine Kontroll-PCR mit Pflanzen-, Mais- bzw. Soja-spezifischen Primer Paaren führten zu positiven Ergebnissen in allen Proben.
- Die Sensitivität (Limit of Detection) des Systems beträgt 0,001% (w/w) GM-Kartoffel-DNA in nicht-GM-Kartoffel-DNA für rohe, bzw. 1% (w/w) GM-Kartoffel-DNA in nicht-GM-Kartoffel-DNA in prozessierten Produkten.
- Es gibt keine Unterschiede in der Leistungsfähigkeit der Nachweismethode bei den *Spunta*-Linien G2 und G3.

<u>Schlussfolgerung</u>

Es wurde eine hoch sensitive und spezifische Methode für die Detektion von rohem oder prozessiertem Material der gentechnisch modifizierten Kartoffellinien *Spunta* G2 und G3 entwickelt. Die Methode ist potentiell geeignet für die Lebensmittelkontrolle sowie weiterer Forschungsarbeit zum Metabolismus der DNA nach Aufnahme und Verdauung in Fütterungsstudien.

TEIL III (Kapitel 4.3): Qualität und Sicherheitsbewertung gentechnisch modifizierter Kartoffeln (Spunta): Analyse der Zusammensetzung; Bestimmung von Toxinen und Anti-Nutrients sowie Fütterungsstudie mit Ratten.

Dieser Teil dient zur Bewertung der Sicherheit durch Vergleich der Zusammensetzung von ernährungsphysiologischen und toxischen Komponenten zwischen der gentechnisch veränderten Kartoffel *Spunta* der Linien G2 and G3 und den parentalen, nicht gentechnisch veränderten S*punta* Linien. Zusätzlich wurde eine Fütterungsstudie mit Ratten durchgeführt.

Für die Untersuchung der chemischen Zusammensetzung wurde der Gehalt von 36 Komponenten in der Kartoffel bestimmt, u.a.: Trockenmasse, Protein, Fett, Ballaststoffe, Asche, Kohlenhydrate, Stärke, reduzierende sowie nicht-reduzierende Zucker, Natrium, Calcium, Stickstoff, Phosphor und Ascorbinsäure. Zusätzlich wurde der Gehalt von ausgewählten Toxinen und Anti-Nutrient-Bestandteilen untersucht.

- Die Fütterungsstudie von Ratten wurde 30 Tage lang durchgeführt. Vier Gruppen von Albinoratten wurden verwendet, um den Effekt und die Sicherheitsbewertung der GM Kartoffeln *Spunta* G2 and G3 zu untersuchen. Gruppe (I) wurde mit einer Kontroll-Basisdiät gefüttert, Gruppe (II) mit der Kontroll-Diät plus 30 % gefriergetrockneten nicht-GM *Spunta* Kartoffeln, Gruppe (III) wurde mit einer Kontrolldiät plus 30 % gefriergetrockneten GM-Kartoffeln *Spunta* G2 gefüttert und Gruppe (IV) wurde mit der Kontrolldiät plus 30 % gefriergetrockneten GM-Kartoffeln *Spunta* G3 gefüttert.
- Es wurden keine signifikanten Unterschiede zwischen den Linien G2, G3 und der parentalen Ausgangslinie hinsichtlich der chemischen Summenparameter gefunden.
- Es wurden keine signifikanten Unterschiede zwischen den Linien G2, G3 und der parentalen Ausgangslinie hinsichtlich der biochemische Komponenten inklusive Glykoalkaloide gefunden. Hinsichtlich der untersuchten Merkmale wird die gentechnisch modifizierte Kartoffel mit der nicht-modifizierten als äquivalent eingestuft.
- Die gemessenen Werte überschreiten nicht die von der FAO/WHO empfohlene Sicherheitsgrenze für akute Toxizität (200 mg/kg).
- Hinsichtlich der Protease-Inhibitionsaktivität und des Gesamtphenolgehaltes konnten keine signifikanten Unterschiede zwischen den GM-Kartoffel Linien und den konventionellen Kartoffeln der *Spunta* Linien gefunden werden.
- Bei den Fütterungsversuchen mit Ratten wurden keine signifikanten Unterschiede zwischen den Tieren der Experimental-Gruppen (II, III, IV) gefunden (Nahrungsaufnahme, Gewichtszunahme). Somit sind keine signifikanten Unterschiede zwischen den Tieren, die mit GVO bzw. mit nicht-.GVO-Kartoffeln gefüttert wurden, vorhanden.
- Es wurden signifikante Unterschiede zwischen der Kontrollgruppe (I; mit Basis-Diät ohne Kartoffeln gefüttert) und der Experimental-Gruppe (II, III und IV; mit Kartoffeln gefüttert) gefunden (Nahrungsaufnahme, Gewichtszunahme).
- Es wurden keine signifikanten Unterschiede zwischen den Gruppen der Fütterungsversuche beim Endgewicht und Organgewicht gefunden.

- Es wurden keine signifikanten Unterschiede zwischen den Gruppen der Fütterungsstudie bei den Serumwerten festgestellt.
- Zusammenfassend kann gesagt werden, dass die Zusammensetzung der untersuchten Substanzen der GM-Kartoffeln *Spunta* G2 und G3 substantiell äquivalent zu denen der konventionellen Kontrolllinien ist. Weiterhin zeigten die GM Kartoffel Linien G2, G3 in den Untersuchungen keine toxikologischen Effekte.

TEIL IV (Kapitel 4.4): Effekt von Futter, das gentechnisch modifizierte Kartoffeln (Spunta) enthält auf Gesundheit und Wachstum von Broilern sowie Untersuchung möglicher Übergänge von gentechnisch veränderter DNA in den Broiler-Organismus.

In diesem Teil der Arbeit wurde der Effekt der Fütterung von GM-Kartoffeln (*Spunta* Linien G2 und G3) auf Broiler untersucht. Dies schließt die Bewertung der Degradation der DNA der *Spunta* Kartoffeln und ihres Metabolismus in Broilermuskeln und Organen ein. Einen Tag alte Broiler wurden *ad-libitum* entweder mit einer Experimental-Diät gefüttert, die nicht-GM-Kartoffeln *Spunta* , GM-Kartoffeln *Spunta* G2 und G3 oder eine Kontrolldiät ohne Kartoffeln enthielten. Um die Äquivalenz der GM-Kartoffeln *Spunta* und der nicht-GM Kartoffeln *Spunta* hinsichtlich der ernährungsphysiologischen Eigenschaften zu prüfen, wurden verschiedene Ernährungs-Parameter (Futterverbrauch, Gewichtszunahme, Futtereffizienz und finales Körpergewicht) bestimmt. Die biochemischen Parameter der Leber, Niere und Gehirnfunktion wurden untersucht. Zusätzlich wurden die Effekte der GM bzw. der nicht-GM Kartoffeln *Spunta* auf das relative Gewicht der Organe verglichen. Die Degradierung und Verstoffwechselung der DNA von GM-Kartoffeln und nicht-GM-Kartoffeln (anhand von Chloroplasten-DNA) im Gastrointestinaltrakt (GIT) und den Organen der Broiler wurde untersucht.

<u>Die Ergebnisse können wie folgt zusammengefasst werden:</u>

a) Wachstum und Leistung

- In keiner Gruppe sind Vögel gestorben. Die Vögel in den Gruppen I, II, III, und IV zeigten keine abnormalen Anzeichen während des Versuchszeitraums.
- Signifikante Unterschiede ($P>0,05$) beim Futterverzehr, bei der Futter-Effizienz, dem finalen Körpergewicht und der Gewichtszunahme wurden zwischen der Kontrollgruppe, die mit Kontrolldiät ohne Kartoffeln gefüttert wurde und den experimentellen Gruppen, die mit einer Diät gefüttert wurden, die Kartoffeln enthielt, festgestellt. Es gab jedoch

keine signifikanten Unterschiede zwischen den Gruppen II, III und IV, bei denen zur Basis-Diät GM- und nicht-GM-Kartoffeln zugesetzt wurden. Daher sind die Unterschiede zwischen Gruppe I und den übrigen Gruppen auf eine grundsätzlich schlechtere Verwertbarkeit der Kartoffel-haltigen Diät zurückzuführen und nicht auf den Zusatz von GVOs.

- Es wurden keine signifikanten Unterschiede (P>0,05) hinsichtlich des Verhältnisses von Leber zu Köpergewicht zwischen der Kontrollgruppe, die mit Standard Diät und der Experimentalgruppen II-IV, die eine Diät mit Kartoffeln enthalten hatten, festgestellt. Auch zwischen den Tieren der Experimentalgruppe wurden keine signifikanten Unterschiede entdeckt (P>0,05). Auch aus diesen Werten kannauf eine geringfügig schlechtere Verwertbarkeit der Kartoffel-enthaltenden Diät gegenüber der Kartoffel-freien Basis-Diät geschlossen werden.
- Es wurden keine signifikanten Unterschiede (P>0,05) zwischen der Kontrollgruppe I und den Experimentalgruppen (II, III, IV) hinsichtlich der biochemischen Parameter gefunden (Gehalt an Glutamat-Oxalacetat-Aminotransferase, Glutamat-Pyruvat-Aminotransferase, Glukose, Cholinesterase, Cholesterin, Gesamtprotein, Albumin and Kreatin).
- Aus den Untersuchungen kann geschlossen werden, dass die gentechnische Modifikation der *Spunta* Kartoffel mit dem *Cry V*-Gen für die Resistenz gegen die Kartoffel-Knollen-Motte (potato tuber moth = PTM) keinen signifikanten Einfluss auf die allgemeine Gesundheit und die physiologischen Prozesse der Broiler hat.

b) Metabolismus der DNA in GIT, Gewebe und Organen.

- Fragmente der mit dem Futter aufgenommenen Kartoffel-Chloroplasten-DNA können in allen Bereichen des GIT vom Kropf bis zum Faeces nachgewiesen werden.
- Fragmente des gentechnisch eingebrachten Konstruktes der Größe 122, 504 and 1000 bp konnten ebenfalls in allen Bereichen des GIT vom Kropf bis zum Faeces nachgewiesen werden.
- In allen untersuchten Geweben konnten keine Amplifikationsprodukte der gentechnischen Modifikation erhalten werden. Demgegenüber konnten Fragmente der Kartoffel-Chloroplasten-DNA in Brustmuskel and Oberschenkelmuskel, Leber, Milz, Brusthaut und Niere in allen Proben, die 1 h , 4 h und 8h nach der letzten Fütterung von beiden Versuchsgruppen genommen wurden, erfolgreich amplifiziert werden.
- Es wurden keine signifikanten Unterschiede zwischen den Linien G2, G3 und der parentalen Ausgangslinie hinsichtlich der Auswirkung auf Hühner (Nahrungsaufnahme, Gewichtszunahme, Endgewicht, Serumwerte) in einer Fütterungsstudie festgestellt.

- Es konnten sowohl Kartoffel-spezifische, als auch gentechnisch modifizierte DNA-Fragmente bis zu einer Länge von 1000 bp entlang des gesamten Verdauungstraktes von Kropf bis Faeces nachgewiesen werden.
- In keiner der untersuchten 11 Gewebetypen wurde gentechnisch veränderte Kartoffel-DNA gefunden, jedoch ließen sich in einigen dieser Proben nach 1, 4 und 8 h Fütterungsstop noch Fragmente von (multicopy) Kartoffel-Chloroplasten DNA nachweisen.

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