# 3 Materials and Methods

## 3.1 Materials

### 3.1.1 Chemicals

| | |
|---|---|
| Agarose LE | Roche, Mannheim, Germany |
| Bisbenzimide dye H33258 | Sigma, St. Louis, USA |
| Boric acid | Merck, Darmstadt, Germany |
| Cetyltrimethyl ammonium bromide (CTAB) | Merck, Darmstadt, Germany |
| Ethanol 96% | Merck, Darmstadt, Germany |
| Ether | Merck, Darmstadt, Germany |
| Ethidium bromide | Sigma, St. Louis, USA |
| Ethylene diamine tetraacetic acid (EDTA, Titriplex® III) | Merck, Darmstadt, Germany |
| HCOOH (formic acid) | Merck, Darmstadt, Germany |
| HCl (hydrochloric acid) | Merck, Darmstadt, Germany |
| $H_2SO_4$ (sulfuric acid) | Merck, Darmstadt, Germany |
| Hexan | Merck, Darmstadt, Germany |
| Isopropanol | Merck, Darmstadt, Germany |
| Magnesium chloride | Perkin Elmer, Germany |
| Norleucine solvent | Merck, Darmstadt, Germany |
| Proteinase K | Sigma, St. Louis, USA |
| Ready Red® (Chloroform / isoamyl alcohol mixture) | Qbiogene, USA |
| See sand | Merck, Darmstadt, Germany |
| $SnCl_2$-$H_2O$ (stannous hydrochloride) | Merck, Darmstadt, Germany |
| Sodium chloride | Merck, Darmstadt, Germany |
| Sodium citrate | Merck, Darmstadt, Germany |
| Sodium disulfide | Merck, Darmstadt, Germany |
| Sodium hydroxide | Merck, Darmstadt, Germany |
| Sucrose | Merck, Darmstadt, Germany |
| Tert-butyl methyl ether | Merck, Darmstadt, Germany |
| Trimethyl sulfonium hydroxide | Macherey-Nagel, Germany |
| Tris-(hydroxymethyl)-aminomethane | Merck, Darmstadt, Germany |
| Water (distilled-purified) | Fluka, Germany |
| Xylencyanol dye | Merck, Darmstadt, Germany |

### 3.1.2 Apparatus

| | |
|---|---|
| Amino acid analyzer (Beckman 6300) | GMI, Inc. Minnesota, USA |
| Autoclave (Varioklav 500 EV-Z) | H+P Labortechnik GmbH, Oberscheißheim, Germany |
| Beckman table centrifuge | GMI, Inc. Minnesota, USA |
| Digital camera | Biostep, Jahnsdorf, Germany |

| | |
|---|---|
| Electrophoreses Chamber (Mighty Small II, Se 250) | Amershan Bioscience, Freiburg, Germany |
| Fluorometer (Hoefer DyNA Quant™ 200) | Amershan Bioscience, Freiburg, Germany |
| Gas chromatography (Hewlett Packard 5880 A/FID) | GOW-MAC® Instrument Co., USA |
| Gel stand (Hoefer Mighty Small Multi Gel Caster) | Amershan Bioscience, Freiburg, Germany |
| Micro wave | Bosch, Stuttgart, Germany |
| pH-meter (766 Calimatic) | Knick, Berlin, Germany |
| Phoretix (photo-Documentation programme) | Biostep, Jahsdorf, Germany |
| Precision balance (PM 2000) | Mettler Toledo, Columbus,USA |
| Real-time PCR System (TaqMan™ technology 7700 sequence deterctor) | Applied Biosystems, Foster City, USA |
| Rotatory evaporator | Chemical Instrumentation Laboratories, USA |
| Sensitive balance (Bp 210 S) | Sartorius, Göttingen |
| Table cooling centrifuge (Centrifuge 5417 R) | Eppendorf, Hamburg, Germany |
| Thermocycler (GeneAmp 2400) | Perkin Elmer, Foster City, USA |
| Thermocycler (Trio-Thermoblock) | Biometra, Göttingen, Germany |
| Thermomixer (comfort) | Eppendorf, Hamburg, Germany |
| UV-Kappen | Biometra, Göttingen, Germany |
| Vacuum concentrator (Concentrator 5301) | Eppendorf, Hamburg, Germany |
| Vortex (MS1 Minishaker IKA®) | ROTH (Chemikalien Laborbedarf), Karlsruhe, Germany |

### 3.1.3 Other materials

| | |
|---|---|
| Calf thymus DNA (DNA standard stock solution, "1 mg/ml") | Sigma, St. Louis, USA |
| Filter papers (595.5 Ø 270 mm and 589.2 Ø 150 mm, white ribbon ) | Schleicher & Schuell, Dassel, Germany |
| Filter papers (0.22 and 0.45 µm Type VS) | Millipore, Bedford, USA |
| Filter Tipps, 2.5, 10, 100, and 1000 µl (sterile) | Eppendorf, Hamburg, Germany |
| Heparin containing tubes | Sarstedt, Germany |
| Micro-pipits (2.5, 10, 100, 200, 1000 µl) | Eppendorf, Hamburg, Germany |
| PCR optical caps (8 caps/strip) | Applied Biosystems, Foster City, Calif., USA |
| PCR optical vessels MicroAmp® (96-well reaction plate) | Applied Biosystems, Foster City, Calif., USA |
| PCR reaction vessels 0.5 ml | Eppendorf, Hamburg, Germany |
| Reaction vessels 0.5, 1.5, and 2.0 ml | Eppendorf, Hamburg, Germany |

### 3.1.4 Buffers, solutions and Kits

- <u>Cetyltrimethyl ammonium bromide extraction buffer (CTAB-extraction buffer)</u>

  20 g Cetyltrimethyl ammonium bromide
  81.82 g NaCl
  100 ml 1 M Tric-hydrochloride solution
  40 ml 0.5 M $Na_2$-EDTA
  Add 1000 ml dist.-$H_2O$ and pH adjusted with HCl to pH 8.0 then filtrated through 0.22 µm filter paper and autoclaved.

- <u>Cetyltrimethyl ammonium bromide precipitation buffer (CTAB-precipitation buffer)</u>

  5 g Cetyltrimethyl ammonium bromide
  2.34 g NaCl
  Add 1000 ml dist.-$H_2O$ then filtrated through 0.22 µm filter paper and autoclaved.

- <u>Sodium chloride solution (1.2 mol/l)</u>

  70.128 g NaCl
  Add 1000 ml dist.-$H_2O$ then filtrated through 0.22 µm filter paper and autoclaved.

- <u>Sodium-EDTA 0.5 M</u>

  93.05 g EDTA (Titriplex III)
  400 ml dist.-$H_2O$
  11 g NaOH
  Add 500 ml dist.-$H_2O$ and pH adjusted to 8.0 then filtrated through 0.22 µm filter paper and autoclaved.

- <u>Tris-borate-EDTA buffer (TBE)</u>

  54 g Tris-(hydroxymethyl)-aminomethane
  27.5 g Boric acid
  20 ml 0.5 M EDTA (pH 8.0)
  Add 1000 ml dist.-$H_2O$

- <u>Tris-hydrochloride solution (1M)</u>

  12.11 g Tris-(hydroxymethyl)-aminomethane solved in 80 ml dist.-$H_2O$
  8 ml HCl 25%
  Add 100 ml dist.-$H_2O$ and pH adjusted with HCl to pH 8.0 then filtrated through 0.22 µm filter paper and autoclaved.

- Tris-sodium chloride-EDTA buffer (TNE)

  12.11 g Tris-(hydroxymethyl)-aminomethane
  3.722 g EDTA
  116.89 g NaCl
  Dissolved in 800 ml dist.-$H_2O$ and adjusted pH to 7.4 then 200 ml dist.-$H_2O$ added. Filtrated through 0.45 µm filter paper and autoclaved

- Commercial Kits

| | |
|---|---|
| Alkaline Phosphatase Kit | Merck, Darmstadt, Germany |
| Gamma glutamyl transferase (γ-GT) Kit | Merck, Darmstadt, Germany |
| GMO / Ident Kit StarLink™Maize | GeneScan Eur., Freiburg, Germany |
| GOT (ASAT) Kit | Merck, Darmstadt, Germany |
| GPT (ALAT) Kit | Merck, Darmstadt, Germany |
| NucleoSpin Tissue kit | Macherey-Nagel, Germany |
| QIAamp DNA Blood Mini Kit | QIAGEN, Hilden, Germany |
| QIAamp DNA Stool Mini Kit | QIAGEN, Hilden, Germany |
| QIAGEN-DNA purification kit | QIAGEN, Hilden, Germany |
| Uric acid Kit | Merck, Darmstadt, Germany |

### 3.1.5 Reference Materials

Certified reference materials (CRM) in the form of fine powder contained known amount of GMO (Roundup Ready soybeans 0%, 0.1% and 5%) and maize (Bt176 and Bt11: 0%, 0.1% and 5% of each line). CRM produced by Fluka, Germany, and all information provided and submitted by the Institute for Reference Material and Measurements (Geel, Belgium), according to Pauwels et al. (1999). CRM were used as negative and positive controls.

Because of there is no CRM available for maize lines MON810 and T25, samples containing 1% GMO were prepared in the laboratory from these lines and used as positive control, while the non-transgenic conventional maize was used as a negative control.

For StarLink™ maize the positive and negative controls were provided with the commercial detection kit used (GMO / Ident Kit StarLink™ maize, GeneScan Eur., Freiburg, Germany).

## 3.2 Detection of genetically modified soybeans and maize in Egypt

### 3.2.1 Sampling

Fifty-one soybean samples and 61 maize samples were randomly collected from 22 different localities in Egypt during the years 2000 / 2001. The soybean samples contained 28 samples of soybean seeds, of which 27 were local Egyptian varieties and one was of USA origin. Twenty-two samples of soybean meal, which were imported from either USA or Argentina. Additionally one sample of full fat soybean was obtained from the Egyptian market (Table 7).

The maize samples consisted mainly of whole grains. Thirty-three samples were from different Egyptian varieties, 24 samples were imported from USA or Argentina and 4 additional samples consisted of maize gluten. One of the gluten samples was produced in Egypt whereas the other 3 samples were imported from USA (Table 8).

### 3.2.2 DNA extraction procedures

All procedures were carried out under substantially sterile conditions. The water used was bi-distilled and autoclaved. Soybean and maize samples were ground in an electric grinder. Tow-hundred mg of the resulting flour as well as 200 mg from the CRM were used for the extraction of the genomic DNA according to the method described in the German guide line No. L-15.05-1 (Anonymous, 2002). From each samples two independent extractions were performed. In addition, deionized distilled water was used as a blank sample and subjected to extraction and further treated in the same way as samples to control the reagents used and procedures of the work. The extracted DNA pellet was air dried under vacuum and was resuspended in 50 µl purified water (Fluka, Germany) as the following:

1- 200 mg of homogenized prepared examined samples powder weighed in 2 ml reaction vessel

2- 1000 µl of the CTAB-extracted buffer were added and mixed well (during DNA extraction from soybean and maize samples proteinase K and RNase were not used)
3- The mixture incubated at 65ºC under shaking for 30 min
4- Centrifugation at 14000 g for 10 min and the supernatant transferred to a new reaction vessel
5- 400 µl Ready Red® (Qbiogene, USA) were added to the sample and mixed 30 second
6- Centrifugation at 14000 g for 10 min (to separate the two phases). Then the aqueous (upper) phase transferred to a new reaction vessel
7- Tow volumes of CTAB-precipitation solution were added and mixed well, then incubated at room temp. for 60 min under shaking
8- Centrifugation at 14000 g for 10 min were applied and the supernatant were discarded. The precipitate redissolved in 350 µl NaCl solution and allowed to stand about 5 min at room temperature
9- 350 µl Ready Red® were added and mixed for 30 second and the solution subjected to centrifugation at 12000 g for 10 min
10- The upper (aqueous) phase separated into a new reaction vessel
11- 0.8 volume parts of isopropanol added, mixed and stored over night in refrigerator at 4ºC
12- Centrifugation 30 min at 14000 g, the supernatant were discarded carefully using pipette, then 500 µl ethanol 70% were mixed with the sediment and centrifugation for 15 min at 14000 g were applied to precipitate the DNA
13- The supernatant were discarded carefully and the DNA pellets were air dried for 2 min under vacuum
14- The DNA pellets were resuspended in 50 µl TE buffer or sterile bi-distilled, deionised water (Fluka, Germany)

All centrifugation procedures were carried out at 4ºC. All materials and glassware were obtained sterile and free from DNA. Aerosol-proof filter tips were used for the DNA preparation and the succeeding PCR procedures.

### 3.2.3 Concentration measurements and control of extracted DNA

The concentration of the isolated DNA was measured fluorometerically using Dynaquant 200 system fluorimeter at 365 $\pm$ 7 nm, according to the manufacturers instructions:

- The fluorimeter was adjusted by using the standard calf thymus-DNA 1:10 solution (100 µg/ml).
- The working solution (fresh prepared), containing 10 ml 10x TNE, 10 µl bisbenzimid dye solution and complete to 100 ml by using autoclaved bi-distilled water.
- 2µl of the sample DNA added to 2 ml of the working solution, mixing well and measured (Reading recorded as ng DNA/µl).
- The DNA concentration prior to PCR was adjusted by dilution using purified distilled water to 25 ng/µl.

### 3.2.4 Oligonucleotides primers used

Primers used in this study are listed in Table 1. All primers were synthesized by TIB MOLBIOL (Berlin, Germany) and obtained in a lyophilized state. All primers were solved in purified water (Fluka, Germany) before using to obtain final concentration of 20 pmol/µl.

For detection of StarLink$^{TM}$ maize (Aventis) a commercial kit purchased from GeneScan Europe, Freiburg, Germany (GMO/Ident Kit StarLink$^{TM}$ maize) was used, which amplified a fragment of 133 bp.

**Table 1.** Oligonucleotides primer pairs sequence and their target element to investigate Egyptian samples

| Primer | Sequence (5´ to 3´) | Target element and amplification length | References |
|---|---|---|---|
| GM03<br>GM04 | GCC CTC TAC TCC ACC CCC ATC C<br>GCC CAT CTG CAA GCC TTT TTG TG | Soybean lectin gene (118 bp) | Meyer et al. (1996) |
| P35s-f2<br>petu-r1 | TGA TGT GAT ATC TCC ACT GAC G<br>TGT ATC CCT TGA GCC ATG TTG T | Transition site from the CaMV35S promoter sequence to the petunia hybrid chloroplast-transit-signal sequence in RRS (172 bp) | Wurz and Willmund (1997) |
| IVR1-F<br>IVR1–R | CCG CTG TAT CAC AAG GGC TGG TAC C<br>GGA GCC CGT GTA GAG CAT GAC GAT C | Maize invertase gene (226 bp) | Ehlers et al. (1997) |
| Cry03<br>Cry04 | CTC TCG CCG TTC ATG TCC GT<br>GGT CAG GCT CAG GCT GAT GT | Transition site from the CDPK-promoter into the amino terminal sequence of synthetic cry1A(b) gene in Bt176 maize (211 bp) | Hupfer et al. (1998) |
| IVS2-2<br>PAT-B | CTG GGA GGC CAA GGT ATC TAA T<br>GCT GCT GTA GCT GGC CTA ATC T | Transition site from the intron IVS2 into the PAT-gene in Bt11 maize (189 bp) | Anonymous (2002) |
| T25-F7<br>T25-R3 | ATG GTG GAT GGC ATG ATG TTG<br>TGA GCG AAA CCC TAT AAG AAC CC | Transition site from the CaMV-terminator into the PAT gene in T25 maize (209 bp) | Anonymous (2002) |
| VW01<br>VW03 | TCG AAG GAC GAA GGA CTC TAA CG<br>TCC ATC TTT GGG ACC ACT GTC G | Transition site from the genomic maize DNA into the CaMV-Promotor in MON810 maize (170 bp) | Anonymous (2002) |

### 3.2.5 DNA amplification and PCR conditions

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 (25 µl total volume) contained 2.5 µl PCR buffer (10x concentrate, Perkin Elmer), 2 µl $MgCl_2$ solution (25 mM $MgCl_2$), 1µl dNTP solution 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.5 µl of each primer, 1 Unit AmpliTaq Gold polymerase (Perkin Elmer), 2 µl of template DNA and completed to 25 µl with purified water.

Table 2 explains the time/temperature profiles and the number of cycles used in PCR. All amplicons were stored at 4°C until gel electrophoresis.

### 3.2.6 Gel Electrophoresis

#### 3.2.6.1 Agarose gel preparation

Agarose gel preparation as well as electrophoresis buffer were prepared using Tris-base/borate (TBE) buffer solution (pH 8.0), 3 g agarose (LE, Roche) were added to 150 ml TBE to obtain 2% w/v agrose gel.

#### 3.2.6.2 Electrophoresis

The PCR products were stained by 2 µl xylenecyanol dye solution (1 mg xylenecyanol, 400 mg sucrose and completed to 1 ml with water). Amplicons together with 50 bp DNA marker (Gibco BRL, USA) were separated on 2% w/v agarose gels (LE, Roche) using 150 Volt and 100 mA for 45 min. The amplicons were made visible by ethidium bromide staining (0.01%) and documented using UV transillumination (254 nm) with the help of Phoretix workstation software (Biostep, Germany).

**Table 2.** Time / temperature profiles and number of PCR cycles

| **Process** | **GM03/ GM04** | **P35s-f2/ petu-r1** | **IVR1-F/ IVR1-R** | **Cry03 / Cry04** | **IVS2-2 / PAT-B** | **T25-F7 / T25-R3** | **VW01 / VW03** | **StarLink Kit** |
|---|---|---|---|---|---|---|---|---|
| Initial denaturation[1] | 10 min at 95 °C | 10 min at 95 °C | 12 min at 95 °C | 12 min at 95 °C | 12 min at 95 °C | 12 min at 95 °C | 12 min at 95 °C | 10 min at 94 °C |
| Denaturation | 30 s at 95 °C | 30 s at 95 °C | 30 s at 95 °C | 30 s at 95 °C | 30 s at 95 °C | 30 s at 95 °C | 30 s at 95 °C | 25 s at 94 °C |
| Annealing | 30 s at 60 °C | 30 s at 62 °C | 30 s at 64 °C | 30 s at 63 °C | 30 s at 64 °C | 30 s at 64 °C | 30 s at 64 °C | 30 s at 62 °C |
| Extension | 1 min at 72 °C | 25 s at 72 °C | 30 s at 72 °C | 30 s at 72 °C | 30 s at 72 °C | 30 s at 72 °C | 30 s at 72 °C | 45 s at 72 °C |
| Cycles | 35 | 35 - 40 | 42 | 38 | 38 | 40 | 40 | 50 |
| Final elongation | 3 min at 72 °C | 10 min at 72 °C | 10 min at 72 °C | 10 min at 72 °C | 10 min at 72 °C | 10 min at 72 °C | 10 min at 72 °C | 3 min at 72 °C |

1) time for the denaturation step is adapted to the use of Ampli Taq Gold DNA Polymerase from Perkin Elmer (enzyme activation)

## 3.3 Nutritional value assessment of Bt176 maize and derived feed

### 3.3.1 Maize grains and diets

GM Valmont maize hybrid NX 6262-Bt176 (Novartis) and its parent non-GM control line used in this study were cultivated and harvested under the same conditions during the year 2000 in Germany. The control and experimental diets for broiler chickens were formulated according to the recommendation of the German Society of Nutrition Physiology (GfE, 1999) and were designed to contain equivalent and maximum amount of maize as possible (Table 3). Moreover, Table 4 shows the calculated ingredients of both, control and experimental diets.

**Table 3.** Composition of the control and experimental diets (%)

| Ingredients | Control diet | Experimental diet |
|---|---|---|
| Conventional maize | 73.58 | - |
| Bt176 maize | - | 73.58 |
| Soybean oil | 2.00 | 2.00 |
| Wheat gluten | 14.81 | 14.81 |
| Fish meal (60% CP) | 3.00 | 3.00 |
| Dicalcium phosphate | 2.92 | 2.92 |
| Calcium carbonate | 0.88 | 0.88 |
| Common salt | 0.51 | 0.51 |
| DL-Methionine | 0.10 | 0.10 |
| L-Lysine | 0.83 | 0.83 |
| L-Threonine | 0.29 | 0.29 |
| L-Tryptophane | 0.08 | 0.08 |
| Premix[1] | 1.00 | 1.00 |

1) Per kg premix: 1 200 000 I.U. vit. A, 350 000 I.U. vit. $D_3$, 4 000 mg vit. E, 250 mg vit. $B_1$, 800 mg vit. $B_2$, 600 mg vit. $B_6$, 3.2 mg vit. $B_{12}$, 450 mg vit. $K_3$, 4.5 mg nicotinic acid, 1.5 mg Ca-pantothenate, 120 mg folic acid, 5 mg biotin, 55 g cholinchlorid, 3 g Fe, 2 g Cu, 10g Mn, 8 g Zn, 120 mg I, 40 mg Se, 40 mg Co (Deutsche Vilomix GmbH, Germany).

**Table 4.** Calculated ingredients of the control and experimental diets

| Parameter | Control diet | Experimental diet |
|---|---|---|
| Protein (%) | 21.50 | 21.50 |
| Ether extract (%) | 5.32 | 5.32 |
| $AME_N$ MJ/kg[1] | 13.38 | 13.38 |
| Lysine (%) | 1.20 | 1.20 |
| Methionine (%) | 0.50 | 0.50 |
| Threonine (%) | 0.90 | 0.90 |
| Tryptophane (%) | 0.25 | 0.25 |
| Ca (%) | 1.20 | 1.20 |
| P (%) | 0.80 | 0.80 |
| Na (%) | 0.25 | 0.25 |

1) Calculated accordino to Vogt (1986)

### 3.3.2 Proximate composition

Both maize grains and diets were analyzed using methods of the VDLUFA (Naumann and Bassler, 1993) for dry matter (DM), organic matter (OM), total ash, calcium (Ca), total-phosphorus (P), potassium (K), crude protein (CP), ether extract (EE), crude fibre (CF), acid detergent fibre (ADF), neutral detergent fibre (NDF) as well as for starch and sugars. Nitrogen free extracts (NFE) were calculated from the data of proximate analyses.

### 3.3.3 Analyses of amino and fatty acids

For amino acids and fatty acids analyses the corresponding procedures recommended by Naumann and Bassler (1993) were applied for maize grains and diets, by the help of amino acid analyzer and gas chromatography respectively.

### 3.3.3.1 Amino acids

Two independent samples of both maize grains and diets were used. For all investigated amino acids except methionine and cystine unoxidative hydrolyses of the samples was applied:

- 8 mg finely ground powder sample weighed in 2 l calibrated flask
- 60 mg $SnCl_2.H_2O$ was added as catalyst and antioxidant
- 500 ml 6n HCl added to the mixture with some glass beads and boiled for 24h using reflux condenser to remain the volume of the mixture and the acid concentration
- Cooling under tap water at room temperature, then 5 ml norleucine solvent solution (0.008 M) (internal standard) was added.
- Filtrate (589.2 Ø 150 mm) then evaporating the solvent solution in rotatory evaporator
- Washing of the precipitate 7 times by using distilled water then re-evaporation in rotatory evaporator
- The residue of the sample collected using see sand (Merck, Germany) and sodium citrate buffer (pH 2.2)
- Filtrate then add 1.2 ml 1n sodium hydroxide to the filtrate solution to adjust the pH

For methionine and cystine determination oxidative hydrolyses process of the samples was applied:

- Oxidative mixture composed of 31.5 ml HCOOH and 3.5 ml $H_2O_2$ incubated under shaking in water bath at 30 ºC for 1h. Then incubated in ice bath for 15 min. This mixture enough for 7 samples.
- 5 ml oxidative mixture added to 8 mg finely ground powder sample
- Incubation in ice bath (0 ºC) for 24h
- 0.9 g $Na_2S_2O_5$ (Sodium disulfide) and 500 ml 6n HCl added to the mixture with some glass beads and boiled for 24h using reflux condenser to remain the volume of the mixture and the acid concentration
- Cooling under tap water at room temperature, then 5 ml norleucine solvent solution (0.008 M) (internal standard) was added.

- Filtrate (589.2 Ø 150 mm) then evaporating the solvent solution in rotatory evaporator
- Washing of the precipitate 7 times by using distilled water then re-evaporation in rotatory evaporator
- The residue of the sample collected using see sand (Merck, Germany) and sodium citrate buffer (pH 2.2)
- Filtrate then add 1.2 ml 1n sodium hydroxide to the filtrate solution to adjust the pH

All hydrolyzed samples centrifuged in BECKMAN-table centrifuge (air fugue) 60 min then transferred to amino acid analyzer (BECKMAN 6300 amino acid analyzer).

**3.3.3.2 Fatty acids**

Two independent analyses from each sample were carried out. For fatty acids analyses gas chromatography was used. The preparation of the samples was performed as follow:

- 20 g finely ground powder sample weighed in the preparation flask (250 ml)
- 100 ml n-Hexan was added and shaking for about 2h (240 U/min)
- Incubated over night in refrigerator
- Filtration using fold filter (595.5 Ø 270 mm), the residue collected using nitrogen and rotatory evaporator
- For 100 mg added 5 ml ether (tert-Butyl methylether 99.8%, Sigma) and mixing
- 100µl from the prepared sample with 50 µl TMSH (Trimethyl sulfonium hydroxide, Macherey-Nagel) injected in GC (Gas Chromatography).

## 3.4 Broiler performance, degradation and metabolic fate of Bt176 maize in comparison with conventional maize

### 3.4.1 Birds

Thirty-five one day old male broiler chicks (Lohmann meat, Lohmann Tierzucht-Cuxhaven, Germany) identified by wing number were used. weighed and caged individually for 35 days. Twenty-six birds were reared from the first day on a diet containing the Bt176 maize and served as experimental group, while nine birds in the control group consumed a diet containing the non-transgenic conventional maize.

### 3.4.2 Housing, feeding and experimental design

Two groups of broiler chicks, control group (9 birds) consumed diet containing non-GM maize while experimental group (26 birds) reared on diet containing Bt176 maize used. The composition of diets listed in Table 3 (see 3.3.1). From the first day of age all birds housed individually in cages and consumed the investigated diets. House temperature was initially 34 - 35 °C during the first two days, then decreased to 32 - 33 by the fourth day of age. From the fifth day till the end of the first week, the temperature was 30°C. In the second week the temperature was 29°C, decreased gradually to 26°C in the third week. Temperature was decreased in the fourth week of age to 22°C and in the fifth week to 20°C. House relative humidity (RH) was ranged from 40 - 60% during the time of the experimental period. In the first three days of age all birds received 24h electric light program, while from fourth day till the end of the experimental period only 16h light and 8h dark. House temperature, relative humidity and light program were automatically controlled.

The diets, in the form of mash, and water were provided *ad libitum* during the entire experimental period. The individual body weight as well as the rest of feed was recorded weekly. Body weight gains and feed conversion ratio (FCR) were calculated.

Between days 20 to 25 a digestion experiment was carried out. During this period the faeces were collected individually from all birds and dried to obtain the dry matter

content. Apparent digestibility of dry matter were calculated from the differences between dry matter intake (feed intake) and dry matter excreated (faecal dry matter) according to McDonald et al. (1995). The dried faecal matter stored at -70°C until DNA investigations.

At the end of the experiment (day 35). The birds were slaughtered at several intervals after feed withdrawal (Table 5) and different samples were collected (digesta, blood and tissue samples) to investigate the metabolic fate of maize DNA.

### 3.4.3 Sampling

At the end of the experiment (day 35). The birds were slaughtered at several intervals after feed withdrawal and samples were collected (Table 5). To avoid cross contamination of DNA, slaughtering of control birds followed by experimental ones.

**Table 5.** Time program of sampling

| Time[1] | Control group | Experimental group |
|---|---|---|
| 0 h | Three birds | Five birds |
| 2 h | n.d[2] | Four birds |
| 4 h | Three bids | Four birds |
| 8 h | n.d[2] | Four birds |
| 12 h | n.d[2] | Four birds |
| 24 h | Three birds | Five birds |

1) Time after feed withdrawal before slaughtering

2) Not done

#### 3.4.3.1 Blood samples

Blood samples were collected immediately before slaughter by heart puncture. Three blood samples were collected from each bird, one sample collected in heparin to determine packed cell volume (PCV). Another sample was collected in sodium citrate for the detection of maize DNA in whole blood using polymerase chain reaction (PCR)

technique, and the last sample was collected without anticoagulant for serum separation to estimate serum glutamic oxalic transaminase (GOT), serum glutamic pyruvic transaminase (GPT), uric acid, alkaline phosphatase and γ-glutamyl transferase (γ-GT). Serum analyses were performed photometrically using commercial kits according to the manufacturers instructions (Merck, Germany).

#### 3.4.3.2 Gut-contents and tissue samples

After blood collection birds were slaughtered and the carcasses were opened under sterile condition to avoid DNA contamination. Gastrointestinal tract was ligated at the distal part of the esophagus and at the end of the rectum. The gut was removed intact, laid on a flat surface and ligated after different sections. Digesta from each section (crop, proventriculus, gizzard, duodenum, jejunum, ileum, caeca and rectum) was carefully squeezed separately. Tissue samples of pectoral and thigh muscles, liver, heart, spleen, kidney, bursa and thymus glands were collected as well.

Citrate blood, tissue samples as well as gut contents were stored at -70°C until DNA investigations using PCR. Samples from each group were pooled just prior the examination.

### 3.4.4 DNA extraction and purification

#### 3.4.4.1 From maize grains, diets and CRM

DNA from both maize lines and diets were extracted according to the German procedures-No. L-15.05-1 (Anonymous, 2002). The same procedure was also used for extraction of DNA from certified reference materials (CRM) containing 0% GMO (negative control) and another containing 0.1% transgenic Bt176 maize, which used as a positive control (see 3.2.2).

### 3.4.4.2 DNA extraction and purification from digesta, excreta, blood and tissue samples

**A) Digesta samples**

Digesta samples (100 mg wet weight) of pooled digesta of different sections of the GIT were subjected to total DNA extraction using the method described by Anonymus (2002) with some modifications. In digesta and faecal samples there are a lot of enzymes, proteins and other substances, which may inhibit the amplification of the isolated DNA from these samples (Rossen et al.,1992; De Broer et al., 1995).
To prevent PCR inhibitors substances, the extracted DNA was purified on silica gel columns (DNA commercial purification kit, Qiagen, Germany). The following procedures were used:

1- 100 mg wet weight of homogenized pooled digesta (GIT-contents) weighed in 2 ml reaction vessel
2- 500 µl of the CTAB-extracted buffer were added and mixed well
3- 20 µl proteinase-K added and mixed with the mixture
4- The mixture incubated at 65ºC under shaking for 90 min
5- Centrifugation at 14500 g for 10 min and the supernatant transferred to a new reaction vessel
6- 200 µl Ready Red® were added to each 350 µl of the supernatant and mixed 30 s
7- Centrifugation at 21000 g for 15 min (to separate the two phases)
8- 300µl of the aqueous (upper) phase transferred to a new reaction vessel containing 300µl isopropanol mixed and stored at room temperature for 20 min
9- Centrifugation 15 min at 21000 g and the supernatant were discarded carefully using pipette
10- 500 µl ethanol 70% were added and mixed
11- Centrifugation for 5 min at 21000 g were applied to precipitate the DNA
12- The supernatant were discarded carefully and the DNA pellets were air dried under vacuum for 3 min

13- The DNA pellets were resuspended in 50 µl sterile bi-distilled, deionised water (Fluka, Germany)

**B) Faecal DNA extraction**

DNA from excreta samples were isolated using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) following the manufacture's recommendation.

**C) Blood and tissue DNA**

DNA from blood and tissue samples was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Germany) and NucleoSpin Tissue kit (Macherey-Nagel, Germany) respectively.

### 3.4.5 Concentration measurements and control of extracted DNA

The concentrations of the extracted DNA were measured fluorometerically (see 3.2.3). DNA concentration was adjusted to 30 ng/µl prior to PCR.

### 3.4.6 Oligonucleotides primers and probes

Primer pairs and probes used with their target specific part listed in Table 6. All primers and probes were synthesized by TIB MOLBIOL, Berlin, Germany and obtained in a lyophilized state. The primers and probes were dissolved in sterile distilled water (Fluka, Germany) to obtain a final concentration of 20 pmol/µl.

#### 3.4.6.1 Maize grains and diets

The primer pair Ivr1-F/Ivr1-R used as a control for detection and amplification of maize DNA (*invertase* gene) from both maize grains and diets, revealed amplicons at 226 bp as described by Ehlers et al. (1997).

For detection of transgenic maize DNA in the grains and diets the primer pair Cry03/Cry04 was used, amplified a fragments of 211 bp (Hupfer et al., 1998).

#### 3.4.6.2 Digesta, excreta, blood and tissue samples

**a) Control primer-probe system for DNA isolated from blood and tissue samples**

Primer pair (MY-F/MY-R) together with MY-probe was used as a control for DNA in blood and tissue samples. The DNA amplification size is 97 bp as described by Laube et al. (2003).

**b) Maize DNA**

For Real time PCR as a target for detection of maize DNA in digesta, excreta, blood and tissue samples, the *high mobility group* gene was selected using a specific primer-probe-system (ZM1-F/ZM1-R/ZM1), which produces a 79 bp amplicons.

c) **Transgenic maize DNA**

The presence of Bt176 DNA in GIT contents, excreta, blood and tissue samples was analysed using a primer-probe-system (Cry2-F/Cry2-R/BTSYN) specific for the genetic modification constructs and amplified at 129 bp DNA size.

Primer-probe-systems ZM1-F/ZM1-R/ZM1 and Cry2-F/Cry2-R/BTSYN were newly designed for this project by GenScan, GmbH, Germany. The methods used to perform amplification and detection of an endogenous maize gene (*high mobility group protein* gene in the maize genome) and of the synthetic *Cry1A(b)* gene introduced into the genome of maize event Bt176 (Novartis, Inc.) respectively. The methods were based on fluorescence coupled DNA amplification and is optimized for real-time PCR. The evaluation was conducted by the German Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV).
Both detection systems were tested for specificity using several different DNA extracts. The tests were performed with the following species: Rice, rye, wheat, millet, lentil, white bean, mung bean, bitter lupin, teosinte, tomato, potato, sorghum, soybean, rape, oat, barley, spelt, linseed, buck wheat, sesame, man DNA, salmon, Neurospora, Bacillus subtilis, maize lines: Bt11,T25, MON810, CBH351, DBT418, GA21 and 20 different conventional maize lines.

The maize reference system (ZM1-F/ZM1-R/ZM1) reacted positive only with all conventional maize lines, teosinte, the different maize certified reference materials (Bt176, Bt11, MON810) and the other maize GMO lines. When the transgene specific system (Cry2-F/Cry2-R/BTSYN) was tested positive only with the DNAs extracted from the Bt176 maize materials. No cross-reactivity with conventional maize or with other GMO materials were observed. The limit of detection of both systems was 1 copy of target DNA (absolute) or at least 0.1% Bt176 maize (relative). Limit of quantification was 20 copies of target DNA in both detection systems (absolute, correspond to lowest concentration of calibration curve) or 0.1% Bt176 maize (relative).

**d) Plant chloroplast DNA**

For detection of plant chloroplast DNA in blood and tissue samples, Plant 2 primer pair was used. Plant 2 primer pair amplified at 199 bp as published before by Einspanier et al. (2001).

**Table 6.** Oligonucleotides primer, probes sequences and their target element

| Name | Sequence (5´ to 3´) | Target element and amplification size | Reference |
|---|---|---|---|
| Ivr1-F | CCG CTG TAT CAC AAG GGC TGG TAC C | Maize *invertase* gene (226 bp) | Ehlers et al. (1997) |
| Ivr1-R | GGA GCC CGT GTA GAG CAT GAC GAT C | | |
| Cry03 | CTC TCG CCG TTC ATG TCC GT | Transition site from the CDPK-promoter into the amino terminal sequence of synthetic *cry1A(b)* gene in Bt176 maize (211 bp) | Hupfer et al. (1998) |
| Cry04 | GGT CAG GCT CAG GCT GAT GT | | |
| MY-f | TTG TGC AAA TCC TGA GAC TCA T | Mammals and Poultry chromosomally encoded *myostatin* gene (97 bp) | Laube et al. (2003) |
| MY-r | ATA CCA GTG CCT GGG TTC AT | | |
| MY-probe | (FAM)-CCC ATG AAA GAC GGT ACA AGG TAT ACT G-(TAMRA) | | |
| ZM1-F | TTG GAC TAG AAA TCT CGT GCT GA | Detection of a sequence of the *High Mobility group* Gene (HMG) in maize genome (79 bp) | Newly established [1] |
| ZM1-R | GCT ACA TAG GGA GCC TTG TCC T | | |
| ZM1-Probe | (FAM)-CAA TCC ACA CAA ACG CAC GCG TA-(TAMRA) | | |
| Cry2-F | CCC ATC GAC ATC AGC CTG AGC | A part of *Cry1A(b)* gene in transgenic maize line Bt 176 (129 bp) | Newly established [1] |
| Cry2-R | CAG GAA GGC GTC CCA CTG GC | | |
| BTSYN-Probe | (FAM)-ATG TCC ACC AGG CCC AGC ACG-(TAMRA) | | |
| Plant 2 forward | GGA AGC TGT TCT AAC GAA TCG | Ubiquitous maize and other plants *chloroplast* gene (199 bp) | Einspanier et al. (2001) |
| Plant 2 reverse | CTC GAA AAC AAT GAA TTG AAG G | | |

1) GeneScan Analytics GmbH, Germany

### 3.4.7 PCR and real time PCR conditions

#### 3.4.7.1 Maize grains and diets

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 (25 µl total volume) contained 2.5 µl PCR buffer (10x concentrate, Perkin Elmer), 2 µl $MgCl_2$ solution (25 mM), 1µl dNTP solution 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.5 µl of each primer, 1 Unit AmpliTaq Gold polymerase (Perkin Elmer), 2 µl of template DNA and completed to 25 µl with sterile distilled water (Fluka, Germany). The amplification conditions for both primer pair Ivr1-F/Ivr1-R and Cry03/Cry04 were similar and consists of initial denaturation of DNA and activation of polymerase enzyme at 95°C for 12 min followed by 40 cycles of amplification, each consisting of denaturation 30 s at 95°C, annealing 30 s at 64°C and extension 30 s at 72°C. The final elongation applied for 10 min at 72°C.

The PCR products were stained by 2 µl xylenecyanol dye solution (1 mg xylenecyanol, 400 mg sucrose and completed to 1 ml with water) separated together with 50 bp DNA marker on 2% w/v agarose gel (LE, Roche) using 150 Volt and 100 mA for 45 min. The amplicons were made visible by ethidium bromide staining (0.01%) and documented using UV transillumination (254 nm) with the help of Phoretix workstation software (Biostep, Germany).

#### 3.4.7.2 Digesta, excreta, blood and tissue samples

A) For detection of maize specific fragments in digesta, excreta, blood and tissue samples, real-time PCR was performed on the ABI PRISM® 7700 Sequence Detector (Real-Time TaqMan™ PCR-Technology, Applied Biosystems), the plots generated by real-time PCR represented the standardized value of the reporter dye fluorescence (Figure 3). Each PCR reaction mix was performed in a final volume 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 for all primers used for analyses of these samples (maize specific, Bt176 specific, and animal tissue specific) were identical and consists 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 s followed by annealing temperature at 60°C for 1 min. An initial template concentration was determined on the basis of the crossing point, or cycle threshold ($C_T$) (Wittwer and Hermann, 1999).

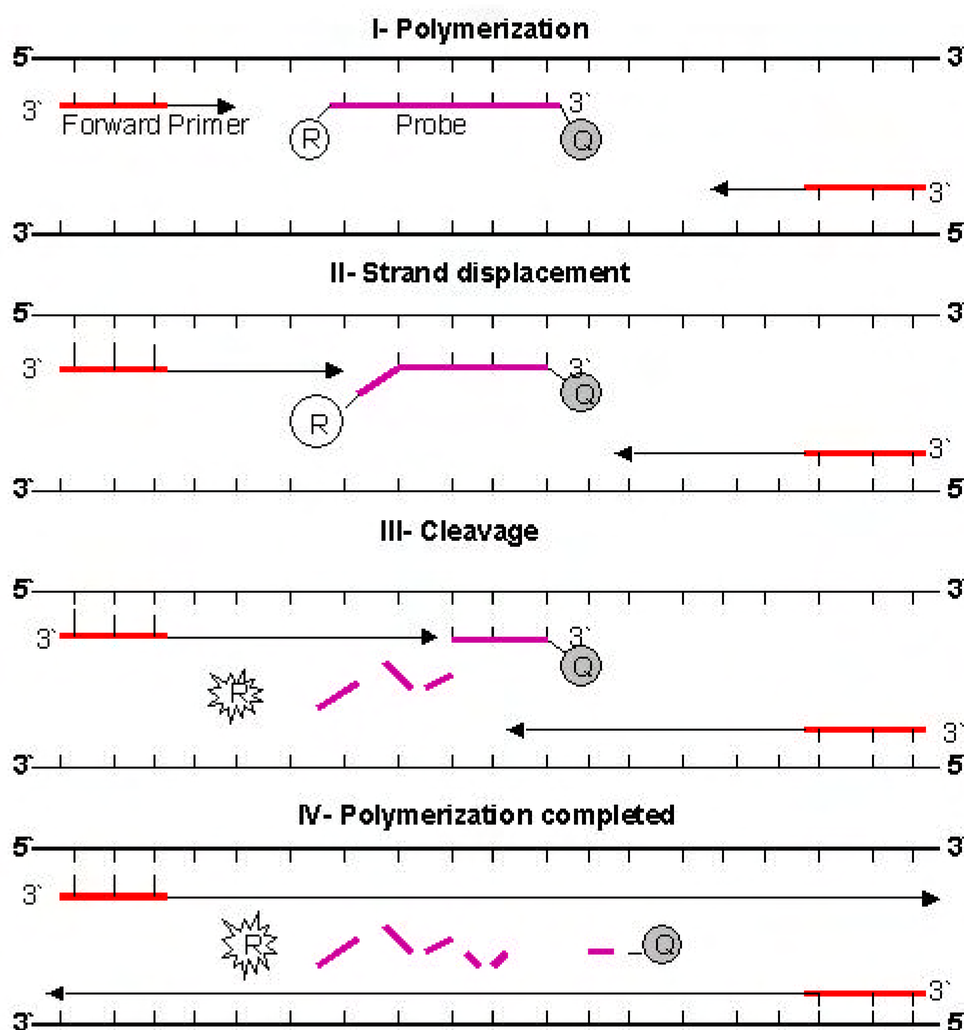


**Figure 3.** The forklike-structure-dependent, polymerization-associated, 5'-3' nuclease activity of AmpliTaq Gold DNA Polymerase during PCR (R= Reporter and Q= Quencher), (Applied Biosystems)

B) For detection of plant DNA in blood and tissue samples, plant 2 primer pair was used. PCR was carried out on a Gene Amp.-PCR system 2400 (Perkin Elmer, Germany) and the reaction mix was similar to that mentioned for detection of maize DNA in diets. The PCR conditions were, initial DNA denaturation and enzyme activation at 94°C for 10 min, followed by 40 cycles of 94°C for 30 s and annealing temperature at 60°C for 1 min. The final stage was 72°C for 3 min. The PCR products were treated by the same way discussed for detection of maize DNA in diets.

### 3.4.8 Statistical analyses

All data of the feeding experiments were statistically analyzed using SAS® software for personal computer (SAS, 1986). Means were compared by the Student-Newman-Keuls test.