**90-day feeding study of rats with Monsanto MON 810 maize**

**Study Plan**

**Study No:** 311957 - A / 13/ GLP

Rev 09/07/2013

| | |
|---|---|
| **Sponsor:** | EU Project GRACE |
| **Sponsor´s representative:** | Prof. Dr. Joachim Schiemann |
| **Test Facility:** | Slovak Medical University<br>Testing Laboratories Center<br>Laboratory of Toxicology<br>Limbová 14, 83303 Bratislava |
| **Test Facility Representative:** | [redacted]<br>Slovak Medical University<br>Limbová 14, 83303 Bratislava<br>[redacted] |
| **Study Director:** | Dagmar Zeljenková, MVD, PhD.<br>Department of Toxicology, head,<br>Slovak Medical University,<br>Limbová 12, 83303 Bratislava<br>E-mail: dagmar.zeljenkova@szu.sk<br>[redacted] |
| **Test Site 1:**<br>**Histopathology** | TOPALAB, s.r.o.<br>Lidické námestie 1<br>040 22 Košice |
| **Test Site Principal Investigator:** | [redacted] |
| **Test Site 2:**<br>**Diet analysis** | RIKILT – Institute of Food Safety<br>Wageningen University and Research Center Campus<br>Building 123, Akkermaalsbos 2<br>NL-6708WB Wageningen<br>Netherlands<br>[redacted]<br>Principal investigator: Dr G.A. Kleter (gijs.kleter@wur.nl) |
| **Test Site 3:**<br>**Diet preparation** | Mucedola s.r.l. [redacted] |

[redacted]

**Test Site 4:**
**Maize production, diet and transcriptomic analysis**

Center for Research in Agricultural Genomics (CRAG)
Campus UAB – CRAG building
Bellaterra, Cerdanyola del Vallès
08193 Barcelona
Spain
[redacted]
Principal investigator: Dr M. Pla de Sola Morales (maria.pla@udg.edu)

**Test Site 5:**
**Diet analysis, and immunological and metabolomic analyses**

Laboratoire d'Immuno-Allergie Alimentaire
Service de Pharmacologie et Immunologie (SPI)
CEA Saclay / Building 136
iBiTec-S
F-91191 Gif-Sur-Yvette cedex
France
[redacted]
Principal investigator: Prof J.-M. Wal (jean-michel.wal@cea.fr)

**Test Site 6:**
**Experimental procedures**

Institute of Veterinary Biochemistry
Free University of Berlin
Oertzenweg 19b
14163 Berlin
Germany
[redacted]
Principal investigator: Prof Dr R. Einspanier (einspani@zedat.fu-berlin.de)

## Approval of the Study plan

| | Name | Date | Signature |
|---|---|---|---|
| **Study Director** | Dagmar Zeljenková, VMD, PhD. | | |
| **Test Facility representative** | [redacted] | | |
| **Principal Investigator** Test Site 1 | [redacted] | | |
| **Principal Investigator** Test Site 2 | Dr Gijs A. Kleter | | |
| **Principal Investigator** Test Site 3 | [redacted] | | |
| **Principal Investigator** Test Site 4 | Dr Maria Pla de Sola Morales | | |
| **Principal Investigator** Test Site 5 | [redacted] Prof Jean-Michel Wal | | |
| **Principal Investigator** Test Site 6 | Ralf Einspanier, Prof., Dr.rer.nat. | | |

| **Monitor** | **Name** | **Date** | **Signature** |
|---|---|---|---|
| | [redacted] | | |

| **Sponsor** | **Name** | **Date** | **Signature** |
|---|---|---|---|
| | Prof. Dr. Joachim Schiemann | | |

**Confirmation of Study plan accordance with GLP**

This study plan meets the requirements for GLP compliance

| **Head of QAU** | **Name** | **Date** | **Signature** |
|---|---|---|---|
| | [redacted] | | |

| **Head of QAU** | **Name** | **Date** | **Signature** |
|---|---|---|---|
| **Test Site 1** | [redacted] | | |

**Confirmation of Study Plan accordance with ISO 17025**

| | **Name** | **Date** | **Signature** |
|---|---|---|---|
| **Head of QAU** | [redacted] | | |
| **Test Site 2** | | | |

| **Head of QAU** | **Name** | **Date** | **Signature** |
|---|---|---|---|
| **Test Site 3** | [redacted] | | |

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## Regulatory Test Guidelines

The study will be carried out in accordance with OECD Test Guideline 408 for Testing of Chemicals, adopted September 21st, 1998 and the EFSA Guidance on repeated-dose 90-day oral toxicity studies on whole food/feed in rodents, EFSA Scientific Opinion, 2011.

## Good Laboratory Practices

**Animal trials (SZU, Slovakia)**:
The study will be conducted in accordance with the OECD Principles of Good Laboratory Practice, as revised in 1997, ENV/MC/CHEM(98)17 and the EU Commission Directive 2004/10/EC of 11th February 2004 (Official Journal No L 50/44). The national GLP compliance programme in the Slovak Republic is based on Act No. 67/2010 Coll. and in compliance with Government Decree No. 320/2010 Coll. The toxicology laboratory of the Slovak Medical University (certificate No. G-036) and the histopathology laboratory TOPALAB (certificate No. G-037) have received a statement of GLP compliance from the Slovak National Accreditation Service. The laboratory of clinical chemistry of the Slovak Medical University holds an accreditation certificate (M-013) from Slovak National Accreditation Service and is subject to the national quality control programme for clinical biology and is controlled by the quality assurance unit (QAU) of the Slovak Medical University. All procedures executed by the toxicology laboratory and the histopathology laboratory are described in standard operating procedures (SOP), approved by the QAU.

**Analysis of feed materials**
Maize culture, harvesting and grain packaging will be performed in experimental and commercial fields not subjected to specific GLP. This will be supervised by CRAG-UdG. Sampling is performed according to EN ISO 24333:2009, based on "Cereals and cereal products".

Maize and diet samples collected at Mucedola srl. (Test Site 3, diet manufacturer) are to be sent to RIKILT (Test Site 2), where these samples will be registered through the sample registration system based on information provided in the Sample Information Form ("MIF") to be prepared and submitted to RIKILT's Sample Room ("Monsterkamer"). These samples will be assigned a Laboratory Information Management System (LIMS) number and divided into subsamples for dispatch towards the subcontractor Covance and the other Test Sites 2 and 4-6 for further analysis. Registration and processing of samples is done under the pertinent SOPs. The analyses of maize and diet samples for target constituents are to be carried out by various partners and a subcontractor, as follows:

- RIKILT (Test Site 2) is to analyse samples for mycotoxins, organic contaminants (dioxins, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), nitrosamines, and presence of genetically modified organisms (GMOs). Analyses will be carried out under ISO 17025:2005 on "General requirements for the competence of testing and calibration laboratories". Methods have been validated and accredited except for nitrosamines, which will be carried out under a SOP. More specifically, the following SOPs apply: dioxins, A0565; PAHs, combined A0824 / A0834, PCBs (included in aforementioned SOPs); and GMOs, A1033 and A1132. The subcontractor Covance will analyse maize and diets for key compounds according to the OECD consensus document (proximate composition, micronutrients including vitamins and minerals, fatty and amino acid profiles, anti-nutrients, secondary metabolites) as well as heavy metals, pesticide residues, and nitrate.
- Mucedola (Test Site 3) will test maize for the presence of mycotoxins, and maize and diets for microbiological quality and proximate composition, under ISO 17025. Manufacturing of

custom feeds is done under Good Manufacturing Practice. Coding of diets and samples is carried out according to instructions received from the contractor.

- INRA (Test Site 5) will test selected samples of maize and diets for the presence of the newly expressed Cry1Ab protein (known to be present in the genetically modified MON810 maize).

Additional research studies on the maize and diet materials, without providing direct inputs to the feeding experiment, are also to be carried out at test sites 2, 4-6.

**Supplementary analysis of diet and animal tissues at other Test Sites**
Omics analyses on animal tissues to be performed by FUB (Test Site 6) and CRAG/UdG (Test Site 4): omics labs are not subjected to GLP. However, the experimental procedures will be guided by the principles of GLP when applicable. Analyses performed at INRA (Test Site 5) on the feed materials will be done in respect of the quality reference system developed and used for research and experimentations at INRA in order to meet the objectives of INRA's quality policy, *i.e.* traceability of research activities and reliability of measurable results.

## Animal Welfare

The study will be conducted in accordance with EU Directive 2010/63/EU of the European Parliament and the Council of 22nd September 2010 on the protection of animals used for scientific purposes.
This study will be approved by the Veterinary State Administration, Slovak Republic (Statna veterinarna a potravinova sprava Slovenskej republiky) Ro-4372/12-221. Animal care will be in compliance with SOPs of the Department of Toxicology, Slovak Medical University Bratislava and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

## 1. OBJECTIVE

This study is based on the recommendations of EFSA on the conduct of 90-day feeding studies to further improve the health risk assessment of GM food/feed. The objective of this study is to determine the suitability and scientific value of 90-day studies in the health risk assessment of GM food/feed using Monsanto MON810 maize.

## 2. PROFESSIONAL AND SUPERVISORY STAFF

**Test facility SMU:**
CVs of all engaged scientists are deposited in Department of Toxicology, Slovak Medical University.

**Toxicology:**
Dagmar Zeljenková, VMD, PhD.
SMU, Department of Toxicology, Limbová 14, 833 03 Bratislava 37, Slovak Republic

**Veterinary and gross pathology:**
[redacted]
SMU, Department of Toxicology, Limbová 14, 833 03 Bratislava 37, Slovak Republic

**Clinical chemistry:**
[redacted]
Laboratory of Clinical and Experimental Biochemistry, Limbová 14, 833 03 Bratislava 37, Slovak Republic

**Haematology:**
[redacted]
Laboratory of Imunotoxicology
Limbová 14, 833 03 Bratislava 37, Slovak Republic

**Ophthalmology:**
[redacted]
Slovak Medical University and University Hospital
Anatolská 11, 85107 Bratislava, Slovak Republic

**Quality Assurance Manager:**
[redacted] SMU QA Unit, Limbová 14, 833 03 Bratislava 37, Slovak Republic

**Statistical Analysis:**
[redacted]
SMU, Department of Biophysics, Biostatistics and Informatics,
Limbová 14, 833 03 Bratislava 37, Slovak Republic

**Ethics Committee**:
[redacted]

Cancer Research Institute, Slovak Academy of Sciences, Vlárska7, 83391 Bratislava, Slovak Republic

**Test Site 1:**
**Histology preparation:** [redacted]

**Histology evaluation:** [redacted]

**Quality assurance manager:** [redacted]

**Test Site 2:**
Diet analysis
Dr Esther J. Kok, Dr Gijs A. Kleter

**Test Site 3:**
Diet preparation
[redacted]

**Test Site 4:**
Maize production and handling, diet analysis
Dr Maria Pla

**Test Site 5:**
Diet analysis, and immunological and metabolomic analyses
[redacted]
Prof Jean-Michel Wal

**Test Site 6:**
Experimental procedures:
[redacted]

## 3. TEST FACILITIES

**Test Facility:**
Testing Laboratories Center
Laboratory of Toxicology,
Slovak Medical University
Limbová 14,
83303 Bratislava 37

**Test Site 1:**
TOPALAB, s.r.o.
Lidické námestie 1
040 22 Košice

**Test Site 2:**
RIKILT – Institute of Food Safety
Wageningen University and Research Center Campus
Building 123, Akkermaalsbos 2
NL-6708WB Wageningen
Netherlands

**Test Site 3:**
Mucedola s.r.l. (licensed by Harlan)
Via Galileo Galilei 4
20019 Settimo Milanese (MI)
Italy

**Test Site 4:**
Center for Research in Agricultural Genomics
Campus UAB - CRAG building
Bellaterra
Cerdanyola del Vallès
08193 Barcelona
Spain

Maize growing, harvesting and drying:
Estació Experimental Mas Badia
17134 La Tallada d'Empordà, Girona, Spain.
[redacted]

**Test Site 5:**
Laboratoire d'Immuno-Allergie Alimentaire
iBiTec-S, Service de Pharmacologie et Immunologie (SPI), Building 136
CEA de Saclay
F-91191 Gif-Sur-Yvette cedex
France

**Test Site 6:**
Freie Universitaet Berlin
Institute for Veterinary Biochemistry
Oertzenweg 19b
14163 Berlin
Germany

## 4. TIME SCHEDULE

| | | |
|---|---|---|
| Test feeds arrival | | Planned date: March 18-20, 2013 |
| Arrival of animals | | March 27, 2013 |
| Starting of the treatment | males | April 2, 2013 |
| | females | April 4, 2013 |
| Last necropsy of the animals | | July 6, 2013 |
| Histology | Slides preparation | July 12–July 30, 2013 |
| | Histology evaluation | July 20 – September 30, 2013 |
| Final report – draft to Sponsor: | | November 30, 2013 |

## 5. TEST AND CONTROL CROPS

GM crop: Variety: DKC6667-YG (containing the MON810 event with insect-resistance trait based on expression of the newly expressed Cry1Ab protein)
Non-GM near-isogenic crop: Variety: DKC6666
Conventional crop 1: PR33W82
Conventional crop 2: SY-NEPAL

All crops: production during the 2012 season, all in a small area in the Empordà (NW of Catalonia, Spain) in the same conditions and according to the standard cultural practices in the region. No insecticides applied in any case. Herbicide and other treatments recorded. Monitoring of the date of sowing, flowering and harvesting, yield, grain humidity and relevant pathogen attacks, particularly corn borer incidence. Climatic data are available.
Crops are dried in a forced air oven at 60ºC and sampled (EN_ISO_24333) to prepare about 30, 90 or 100 kg (for conventional, GM and near-isogenic varieties, respectively) for preparation of the diets. Grains are packaged in autoclave plastic bags inside containers of 30-35 kg, each labeled with the full name of the variety and other details.

Batches and batch numbers: Maize kernels are packed in bags of approximately 11 kg each. Three bags with a particular maize variety are packed into a container, containing approximately 35 kg of maize kernels. Batch numbers include the name of the variety plus a lot number affixed to it (see example).

Example of container label:

Producto / Product: MAIZE GRAIN
Variedad / Variety: PR33W82
Masa (Kg) / Mass weight (Kg): 10 Kg
Lote nº / Batch nº: PR33W82-1 (code is variable between varieties)
Proyecto o Contrato / Project or Contract: GRACE
Fecha realización / Date: 19/11/2012
Lugar realización / Location: Centre for Research in Agrigenomics (CRAG)
Persona contacto / Contact person: [redacted]
Muestra para / Sample for: Rat Feed Compound

## 6. TEST SYSTEM

***Species and strain***
Rat Wistar Rcc Han /Specific Pathogen Free (SPF)

***Source***
Harlan Italy, reg. No 2-2914 – 15-06-1994

***Number of animals***
85 male and 85 female rats will be ordered. Only 80 males and 80 females will be used for the study. Females will be nulliparous and non-pregnant. Animals not assigned to the study will be deemed as sentinels.

***Approximate weight and age***
Upon arrival, the animals will weigh between 100-120g and will be 5 weeks old. The animals will be 6 weeks old at the start of the study and will weigh between 110-140g. Ideally, they should be born within 1-5 days of each other and be of uniform weight (± 20% of the mean).

***Identification***
Within the frame of treatment groups, each rat will be marked by code (Tattoo, or marked every 2 weeks with a permanent marker) on the tail base in accordance with SOP: ŠPP/TOX/V002 to identify the animal individually. Each cage will be marked with a colored cage card.

***Justification for the selection and number of animals***
This species (*Rattus norvegicus* ssp. *alba*) and strain (Wistar) of animal is generally recognized as appropriate for the conduct of sub-chronic toxicity studies. The Wistar rat is a widely used strain of rats for which significant control data are available. The toxicology laboratory of the Slovak Medical University has a record of the regular use of this strain of rats. The number of animals used in this study is considered appropriate to obtain meaningful food and feed safety data and allow proper interpretation of the study results. The number of animals was chosen based on a power analysis for ANOVA with 5 groups with equal size, power=0.8, $\alpha$=0.05.

***Animal housing***
All animals will be housed in rooms N$^{o}$ B 2/ 3 of the Specific Pathogen Free (SPF) experimental animal house equipped with a pressure climatic system at the Department of Toxicology of the Slovak Medical University. The temperature and relative humidity in the animal room will be recorded every 20 minutes by the PMICRO-LCD-THSYS, Dallas Semiconductor system and every week the computer readout for the past week will be evaluated. Mean temperature will be maintained at 22 $\pm$ 2°C and relative humidity at 40 -70%. The animals will be subjected to a 12-hour light/ 12-hour dark cycle.

Rats will be housed in TECNIPLAST cages Type 2145 F with an H-Temp™ (PSU) from the Tecniplast Company, Italy. The cages have a high density polypropylene body, measuring 480 x 265 x 210 mm - floor area 940 cm$^2$.

We will use sterilized bedding from JRS Lignocel®, Hygienic animal bedding, sterilized sawdust Charles River Germany. It will be stored in the clean, dry and cold store room on the second floor in the animal facility. One lot of sawdust bedding will be purchased and used for the entire study.

The cages will be cleaned twice a week outside of the animal room. The cages will be emptied and cleaned with water and detergent. After cleaning they will be dried and then immersed in disinfectant. The cages will then be brought into the animal house and placed in an additional Tecniplast disinfectant solution (produced). Then the cages will be placed into the SPF unit on a drying rack before use.

The cage racks will be cleaned in the SPF rooms every week manually with water and detergent.

Feed containers and any other containers or equipment being used in the SPF rooms will be cleaned the same way that the cages are cleaned.

Bottles will be exchanged and cleaned daily. They will be cleaned in a special automatic washing machine set aside for the bottles in this study. The cleaning solution will include detergent followed by disinfect.

***Diet formulation, sampling and analysis***

Diet formulation, sampling and shipping

- Maize harvested from the Catalonian production sites is shipped to the Italian production facility (Mucedola srl.) licensed by Harlan for the production of diets. Shipping can be done with the monthly truck service offered by the facility. Grains are to be packaged in autoclave plastic bags inside containers of 30-35 kg, each labelled with the full name of the variety and other details.
- Milling of maize kernels is done by this facility, as is the formulation, *i.e.* mixing with other ingredients, using a customized pelletizing process using a pasta press without the use of steam, which aids to prevent loss of heat-labile compounds.
- Formulation is carried out according to the diet composition recommended by the Harlan Company's nutritionist so as to achieve isoproteic and isocaloric diets with 11% and 33% transgenic variety and 22% and 33% for the near-isogenic maize inclusion levels as well as 33% any other conventional variety. Hence, each diet contained 33% maize *in toto*. The composition will include plant-based ingredients (hence no animal-derived ingredients). Samples for dispatch to the analytical laboratories for nutrition and contaminants, as well as for "omics" studies, are taken after milling and after pelletizing (before and after irradiation) according to instructions from the responsible GRACE scientist (company has been instructed to take multiple, i.e. at least five samples, at different spots from the batches prepared).
- A complete battery of tests for different GMOs will be performed on a sample of each variety at the RIKILT facilities (including a broad GMO screen and a quantitative event-specific PCR assay for MON810), while INRA will test for the presence of the Cry1Ab protein expressed by transgenic maize MON810.
- Diets are coded in a "double blind" fashion by the diet-producing company (Mucedola srl.). Samples of the diets are coded with different codes than the diets themselves. The coding scheme is shared with the study monitor and Gijs Kleter (the company's contact within the GRACE consortium). It is to be kept confidential and therefore not to be distributed further among

consortium members during the course of the animal experiments and analyses of samples derived from these experiments, and the code is to be broken after termination of these activities when analytical and experimental data have been recorded and stored.

Analysis of diet admixes

- The key parameters for the analysis of maize will include:
  - Macronutrients & fibre (ADF, NDF, dietary),
  - Minerals,
  - Vitamins (A, B, C, E), zeaxanthin
  - Amino acid composition (including tryptophan),
  - Fatty acid composition,
  - Antinutrients (phytic acid, trypsin inhibitor),
  - Secondary compounds (furfural, phenolics, sterols, and carbohydrates, e.g. raffinose, stachyose).
  - GMOs (DNA), pesticide residues, mycotoxins, heavy metals, other contaminants (e.g. dioxins, PAHs, PCBs, nitrate, nitrosamine), Cry1Ab protein
- The key parameters of the analysis of the diets will include:
  - Same parameters as for maize, plus:
    - Isoflavones
    - Lectins

Storage conditions

- Kernels and pellets will be kept at ambient temperature and measures will be taken to avoid build-up of moisture and fungal growth (e.g. transport of bags containing desiccant in closed boxes). The size of each bag is about 10 kg (autoclaved plastic bags). Every variety can be considered as a single batch as it all was cultured, harvested and dried as a single batch. The size of the batches depends on the variety (i.e. larger for near-isogenic, smaller for other conventional varieties). Drying prevents grains from fungal infections, while gamma-irradiation of the diets will be performed after milling and diet preparation.
- After receipt of the analytical samples, the receiving laboratories will keep them under controlled cool, dry and confined conditions to ensure the stability of the sample.

Spare samples from the irradiated diets after receipt at the animal testing facility will be taken and kept for later analysis.

Samples of diets will be sent to the analytical laboratories contracted for the analysis of the composition (macronutrients, micronutrients, anti-nutrients, toxins, secondary compounds) as well as for the presence of genetically modified organisms (GMOs; element screen and event-specific test for MON810), mycotoxins, residues of pesticides and contaminants (*e.g.* dioxins, PAHs, PCBs, nitrate, nitrosamines, heavy metals), and pathogens.

Storage of the test diet during the study: in closed rooms (cool and dry, controlled temperature and humidity), Laboratory of toxicology, SZU, Limbová 14, Bratislava, Slovak Republic. The test diets will be provided as single batches (containing portions of diets packed in separate vacuum, gamma-irradiated packs).

Water

The rats will be supplied water *ad libitum* during the acclimation and study periods. We will use tap water with a special filter to eliminate microorganisms. The bottles containing this water will be autoclaved before use. The water from the local mains will be monitored for quality by testing for the microbiological and chemical quality by Waterworks Bratislava quarterly. We will receive a certificate of quality.

## 7. EXPERIMENTAL DESIGN

***Animal receipt and acclimation***
Upon arrival, the animals will be placed in cages, 4 per cage. 48 hours after arrival, the animals will be weighed and kept in cages for the next 3 - 5 days prior to the start of the study to allow for acclimation to the laboratory conditions. These are identical to those defined for the experimental part of the study. During this period the animals will be monitored for their health status twice a day (see section 8. PERIODICAL HEALTH STATUS OBSERVATIONS below for a full description of the health status evaluation).

***Randomisation***
One day before the start of treatment, all animals will be housed in 2 separate rooms (1 for males, 1 for females) under standard SPF conditions and will be randomized using completely randomised designs (SOP: ŠPP/TOX/V001).

Tables with cage numbers and the random diet assignment will be prepared by the local statisticians. We will use the Random Number Generators (RNG) of SPSS software for male and female animals separately.
All animals will be numbered from 1 to 85. We will assign 2 animals into 1 cage, using RNG.
These animals will be excluded from next option and random choice will be repeated until all animals are randomly assigned to cages.

All animals will be purchased from Harlan and will be only a few days apart in age. Therefore, we will have the required number of test animals of uniform weight and age, and house them all under identical conditions.

2 animals will be placed in 1 cage. Animals will be randomly allocated to cages by dose group and sex. To minimise the chance of mistakes being made, cages of the same treatment groups will be clustered in vertically arranged groups, which will be rotated on a regular basis (once per week). Each vertical row of cages (within the same dose group) will be rotated from top to bottom. Racks will be rotated clockwise every two weeks within the original room configuration.

***Group allocation and dosing***
Prior to the start of treatment on study day 1, a detailed examination of all animals will be carried out to verify their health condition (see section 8. PERIODICAL HEALTH STATUS OBSERVATIONS for a full description).

***Route of administration***
The route of administration will be the oral route as this route is the most appropriate for the safety assessment of foods. The test item (maize) will be administered by incorporation into the diet since this mimics most human exposure to these foods. Attention will be paid that there will be no nutritional imbalances as a result of dietary incorporation of the test item.

Food will be supplied *ad libitum*. Feed consumption will be determined weekly for 90 days. At the beginning of each food consumption measurement, weighed full feeders with stainless steel lids will be placed in each cage. At feeder change-out (once weekly), the feeders will be weighed again, the

difference in weight is an estimate of total amount consumed by the 2 occupants of the cage. Feed consumption will be recorded, and will be reported as grams/animal/day.

Feed containers and scoops will also be colour coded. However, animal house staff will be "blind" with respect to the identity of the diets.

The different feeds will be coded and labelled by Mucedola company. The code will be given only to Gijs Kleter and the study monitor. All others will be blinded to the feeds.

**General experimental design with Monsanto MON810 maize, start April 2013**

| *Group* | *% (w/w) of daily dietary intake* | | | | *No of animals* | |
|---|---|---|---|---|---|---|
| *Unknown identity for the staff ** | *Reference diet* | *GM* | *Near-Isogenic non-GM* | *Conventional* | *Males* | *Females* |
| x* | 67 | 33 | 0 | 0 | 16 | 16 |
| x* | 67 | 11 | 22 | 0 | 16 | 16 |
| x* | 67 | 0 | 33 | 0 | 16 | 16 |
| x* | 67 | 0 | 0 | 33 | 16 | 16 |
| x* | 67 | 0 | 0 | 33 | 16 | 16 |
| Total | | | | | 80 | 80 |

| *Group/ colour coding by Mucedola (example)* | *No of animals Males* | *No of cages* | *No of animals Females* | *No of cages* |
|---|---|---|---|---|
| 1 blue | 16 | 8 | 16 | 8 |
| 2 red | 16 | 8 | 16 | 8 |
| 3 green | 16 | 8 | 16 | 8 |
| 4 yellow | 16 | 8 | 16 | 8 |
| 5 white | 16 | 8 | 16 | 8 |
| sentinels | 5 | 2 | 5 | 2 |
| Total | 85 | 42 | 85 | 42 |

## 8. PERIODICAL HEALTH STATUS OBSERVATIONS

***Morbidity, mortality***

Normally observations are done twice a day. However, in case of moribund animals, we will isolate them in the quarantine area to prevent cannibalism and will observe them carefully at least 4 times daily. If a study animal dies, we will subject it to necropsy as soon as possible after death. Any animal whose condition makes it unlikely that it will survive to the next observation period will be euthanized by ketamine/xylazine anaesthesia (SOP No. TOX/TS/004) and immediately necropsied.

***Clinical signs***

Cage side observations / uncovered cage

Rats will be inspected twice daily for evidence of reaction to treatment or ill-health which includes the following signs: changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions as well as activity level and change in behaviour.

Detailed physical examination

Once weekly, rats will be examined out of cage. Any deviations from normal will be recorded in terms of nature and severity, date and time of onset, duration and progress of the observed response. Signs noted will include changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity such as lacrimation, piloerection, pupil size, and unusual respiratory patterns as well as activity level and change in behaviour.

Functional assessment

Towards the end of the exposure period changes in gait, posture and response to handling as well as the presence of clonic or tonic movements or bizarre behaviour (self-mutilation, walking backwards) will also be recorded. Sensory reactivity to stimuli of different modalities (e. g. auditory, visual and proprioceptive stimuli), will be recorded. The outcome of this examination will be recorded for each animal, in accordance with SOP: ŠPP / TOX / V003 (Origin of score system: Ország A. et al. (1985): Veterinárnaortopédia a rontgenológia, Bratislava: Príroda, 243 s. (Veterinary orthopaedy and X-ray). The animals will also be assessed for gait disturbances using the AccuPacer treadmill equipment.

Ophthalmologic examination

**U**sing an ophthalmoscope, we will examine the eyes of all animals prior to the administration of the test feeds and at the termination of the study. This will be done by the chief of ophthamology who has expertise in this area.

Body weight

Each animal will be weighed at the following times: 1) 48 hours after arrival, 2) on the first day of feeding, 3) weekly during the study period, 4) at the termination of the study, 5) in the event of an early death or sacrifice in extremis. The General Linear Model (GLM) for Repeated Measures will be used for analysis of the body weight.

## 9. PROCEDURES FOR SAMPLE COLLECTION

Sample collection for the following analyses will be done: haematology, blood chemistry, omics, and pathology. Samples collected will include blood, tissues and organs. Blood samples will be divided for haematology, clinical chemistry and omics. Tissues and organs will be removed and evaluated by histology and omics.

Sample collection and tissue processing:

Personal disposition for forced progress (40 animals per day will be necropsied):
- Animals will be euthanized by person N$^{o}$ 1
- Blood taking will be done by person N$^{o}$ 2
- Decapitation and necropsy of the head by person N$^{o}$ 3
- Animal transport to the necropsy room on the same floor:
- Necropsy of thorax part body - person N$^{o}$ 4
- Necropsy of abdominal part body - person N$^{o}$ 5
- Weighing of organs in line with OECD guideline - person N$^{o}$ 6
- Weighing of selected organs for "omics" study and their preparation - person N$^{o}$ 7

All organs will be stored into formaline except omics samples which are immediately frozen in liquid nitrogen and then stored at -80°C.

***Haematology***

At the end of the study before sacrifice, blood samples from the tail vein will be taken from all animals for haematological examination after 12 hours fasting. EDTA will be used as anticoagulant. Blood samples will be stored under room temperature (17-25°C) maximum up to 4 hours until measurement. Haematological analysis will be performed in accordance with SOP: ŠPP/IMU/M002 using Haematological analyzer Sysmex K-4500, SYSMEX TOA Medical Electronics Co. LTD, Japan.
Parameters scheduled for examination are
- Erythrocyte Count (RBC)
- Haematocrit (HT)
- Haemoglobin (Hb)
- Mean Corpuscular Haemoglobin (MCH)
- Mean Corpuscular Haemoglobin Concentration (MCHC)
- Mean Cell Volume (MCV)
- Leukocyte Count (WBC)
- Differential Leukocyte Count
- Platelet Count (PLT)
- Activated Partial Thromboplastin Time
- Prothrombin Time (PT) from citrate-treated plasma.

Differential Leukocyte Count will be examined using light microscope. Blood smears will be stained by panoptic staining using May-Grunwald and Giemsa-Romanowski dyes. The percentage of lymphocytes, neutrophils, eosinophils, basophils and monocytes will be determined by examining of 100 cells.

***Clinical chemistry***

At the end of the study, before sacrifice, blood samples from the tail vein will be taken from all animals for blood chemistry examination after 12 hours fasting. Samples will be analysed using an Analyzer Vitros 250, Ortho-Clinical Diagnostics, No. 219037234, USA. Methodologies include colorimetric, potentiometric and rate tests using multi-layered Vitros Slides. In accordance with SOP: ŠPP/LEKB/M001. Blood samples will be stored at room temperature (17-25° C) for a maximum of 4 hours until measurement. Parameters will include total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), creatinine (CREA), urea nitrogen, fasting blood glucose, total bilirubin (TBIL), total cholesterol, triglycerides, Na, K, Ca, Cl, P.

***Metabolomics, Transcriptomics and Proteomics***

The procedure will be as follows:

- At day 91, blood and organs will be collected immediately after euthanasia.
- Blood will be collected in heparinised vials which will be centrifuged at 3000 g at 4°C for 15 min. following the preparation description. For every animal, after collecting blood in one heparinised vial, samples will be kept not more than 15 min at 4°C before starting centrifugation to separate red cells from plasma.
- Aliquots of plasma (ca. 500 µL) will be collected for the metabolomic and immunological analyses and kept at -80°C. Centrifuged plasma will be kept not more than 15 min at 4°C before preparation of aliquots and storage at -80°C. Plasma will be stored in 3-ml polypropylene vials with a cap or seemingly equivalent vials.
- Liver will be excised and weighed. The right lateral lobe will be collected and immediately snap frozen in liquid nitrogen. Liver samples will be stored in 1.5 ml Eppendorf vials or seemingly equivalent containers.
- Both kidneys will be dissected and weighed. The third to the half higher part of the right kidney will be cut and immediately snap-frozen in liquid nitrogen. Kidney samples will be stored in 1.5 ml Eppendorf vials. **The dissection of kidneys will be done by the same person to ensure consistency and reproducibility.**
- Intestinal and spleen samples will be dissected: Intestinal sections of mid-jejunum, ileum, ascending colon (1cm each minimum), mesenteric lymph nodes from ileum and ascending colon (3 lymph nodes each minimum). Intestinal and spleen samples will be dissected: Spleen, intestinal sections of mid-jejunum, mid-ileum, ascending colon (2cm each), superior mesenteric and ileocolic lymph nodes (2 ileocolic and 1 superior mesenteric lymph nodes). **This will be done by the same person to ensure consistency and reproducibility.**
- After preparation all samples will be frozen in liquid nitrogen and then stored at -80°C.
- Blood, plasma and tissue samples will be sent on dry ice to FUB (Test Site 6), INRA (Test Site 5), and CRAG (Test Site 4) for further immunological and “omics” analyses (according to the work plan for GRACE Work Package 2) with the list of samples every partner must receive and their identification (e.g. no of rats, type of sample, and day of collection) as well as the weight of the organs.

- Each sample must be clearly and unambiguously identified by the animal number/nature of the sample (*e.g.* liver, kidneys, plasma).
- Aliquots are to be set aside so that these can be used in case of problems with transport of samples shipped from the animal testing facility to other test sites.

## 10. PATHOLOGY

***Gross necropsy***
A complete necropsy will be performed on all animals at study termination on day 91. The weight of organs will be recorded in line with OECD guideline 408 and organs/tissues will be examined macroscopically for any deviations from normal (in accordance with ŠPP / TOX / V005).

The wet-weight of the following organs will be recorded: brain, lungs, heart, liver, kidneys, spleen, adrenal glands, pancreas, testes, uterus, ovaries, epididymides, and thymus. Histological evaluation of tissue specimens will be done in all animals.

The tissues will be preserved in the fixative medium (neutral buffered 10% formalin) for histopathological examination for gross lesions.

Tissue specimens include:
- brain (representative regions including cerebrum, cerebellum , medulla/pons and pituitary)
- spinal cord
- thyroid
- parathyroid
- thymus
- oesophagus
- aorta
- salivary glands
- stomach
- small intestine
- pancreas
- large intestines (including Peyer's patches)
- liver
- kidneys (L, R)
- adrenals
- spleen
- heart
- trachea and lungs (inflated with fixative and then immersed in formalin)
- gonads (testes, L, R; ovaries L, R)
- uterus
- female mammary glands
- prostate
- urinary bladder
- lymph nodes: submandibular and mesenteric
- peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle
- section of bone marrow and/or a fresh bone marrow aspirate

- skin from the back will be taken from the same area of each rat
- eyes (if changes were observed during ophthalmological examinations)
- additional tissues may need to be investigated based on clinical or any other findings. Also any organs/tissues that are likely to be considered as target organs based on the known toxicological properties of the test material should be preserved.

Parts of specific organs will be snap frozen in liquid nitrogen in order to allow for additional examinations.

***Histopathology***
Organs and tissues preserved in neutral buffered 10% formalin will be shipped to TOPALAB for histopathological evaluation in accordance with SOPs: **TPLB** 08 – Tissue processing; ŠPP **TPLB** 09 - Procedure for histopathological slides evaluation. Complete microscopic examination of the tissues listed above will be performed on 16 animals from each group in accordance with the OECD TG 408.

***Transcriptomics of tissue samples for FUB (Test Site 6)***
At the end of the 90-day trial, the following tissues should be collected for transcriptomics studies:
Intestinal sections of mid-jejunum, mid-ileum, ascending colon (2cm each), superior mesenteric and ileocolic lymph nodes (2 ileocolic and 1 superior mesenteric lymph nodes ) and spleen (identical anatomical section for each animal). For these specimens, it is important to have the same-sized pieces, weighed equivalents. Freshly sampled tissue sections must be quick-frozen in liquid nitrogen as soon as possible after necropsy to prevent degradation of RNA. Storage and Shipping: Long-term storage of frozen tissue samples is possible at -80°C and samples can be shipped on dry ice. Data on sample identification have to be included. Organs will then be stored at -80°C until further processing. For transcriptomics studies total RNA will be extracted from frozen tissue sections and global as well as specific mRNA expression will be studied by quantitative real-time PCR and microarray analyses.

***Metabolomic studies of plasma and tissue samples for INRA (Test Site 5)***
At the end of the 90-day trial, plasma coming from blood collected on heparinised vials and the following tissues, *i.e.* the r**ight lateral lobe of the liver** and the **third to the half higher part of the right kidney** will be collected for metabolomic studies. The technical conditions of dissection and collection of these matrices are described above.
Storage and Shipping: Long-term storage of frozen tissue samples is possible at - 80°C and samples will be shipped on dry ice. Data on sample identification have to be included. Organs will then be stored at -80°C until further processing. For metabolomic studies, plasma, liver and kidney samples will be extracted with organic solvents to discard insoluble macromolecules and non-polar analytes. The resulting hydrosoluble fractions will be fingerprinted by ultra high resolution mass spectrometry (plasma, liver and kidney extracts) or by nuclear magnetic resonance (liver extracts) to quantify the distribution of detectable analytes.

***Immunological studies of plasma for INRA (Test Site 5)***
For immunological studies, plasma will be tested for total and maize/cry1Ab specific antibodies (i.e. IgG, IgM and IgE) using specific immunoassays developed and validated in Test Site 5.

## 11. DATA EVALUATION AND STATISTICAL ANALYSIS

The statistical analysis will be done by local statistical team, using any high-level statistical packages (BMDP).

As a first step the data will be screened for any obvious errors and outliers. Outliers will be checked against the original paper records. Outliers which are not due to transcription or other obvious types of error will be retained, but noted. The statistical analysis will then be done with and without the outliers. If the conclusion depends on the presence of one or more outliers, then this will require further investigation on a case-by-case basis. If an outlier makes no difference to the conclusions, it will be retained.

Data from males and females will be analysed separately and together (ANOVA).

Summary statistics (e.g. “n”, means, standard deviations and/or medians and quartiles, as appropriate), will be tabulated based on the cage means (as the cage is considered the experimental unit in this study). A one-way analysis with planned or *post-hoc* comparisons will be used to evaluate statistical significance of each outcome (trait). In some cases more detailed statistical analysis including correlations between characters or even a multivariate analysis may be needed, but this should be decided on a case-by-case basis. Methods of analysing longitudinal data such as growth and food consumption will be decided on a case-by-case basis.

Tables of results (means, SDs and statistical significance; raw individual data) will be prepared and in some cases additional statistical analyses and graphical methods may also be used.
The raw data will be made publically available on the GRACE web site.

## 12. REFERENCES

BMDP Statistics Software, Inc. (1990). BMDP Statistical Software Manual. W.J. Dixon, Chief Ed. 1990 rev. or later. University of California Press, Berkeley, CA, USA.

European Committee for Standardization (2010) EN ISO 24333:2009 Cereals and cereal products – Sampling.

European Committee for Standardization (2010) General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025:2005).

EFSA Scientific Committee (2011) Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed. European Food Safety Authority (EFSA), Parma, ItalyJournal 2011;9:2438.

The European Parliament and the Council (2004) Directive 2004/10/EC. Official Journal of the European Union L 50: 44-45.

The European Parliament and the Council (2010) Directive 2010/63/EU. Official Journal of the European Union L 276: 33-79.

OECD (1998)Test No. 408 - Repeated Dose 90-day Oral Toxicity Study in Rodents. OECD Guidelines for the Testing of Chemicals, Section 4 Health Effects.

OECD (1998) Principles of Good Laboratory Practice, as revised in 1997- ENV/MC/CHEM(98)17. Series on Principles of Good Laboratory Practice and Compliance Monitoring No. 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris.

Slovak Republic, Act No 67/2010 on Conditions of Marketing of Chemical Substances and Chemical Mixtures and on amendment and supplement of other acts.

Slovak Republic , Government Decree No. 320/2010 Coll.

## 13. ARCHIVING

**Under the Code Number:** 311957 / A

The following will be archived until the year 2022 at the SMU, Department of Toxicology:

- Study plan
- Correspondence
- Final report
- Reports of quality inspection
- All histological samples
- All original documents/Primary documentation

## 14. REPORTING

The final report will include the reporting requirements as described in OECD TG 408 method:

The final report will be written in English language. The sponsor can revise the draft report for 14 days from its date of issue. Then the final report will be finalized.

The draft report will be made in two copies – one electronic copy for the sponsor and one paper copy for test facility. The study report will be made in four paper copies - two for the sponsor and two copies for the test facility and will include, but not limited to, the following:

- The name and address of the sponsor and the testing facility.
- The study schedule, the data of the start and the end of the study.
- The names of all personnel involved in the study, including the study director, other scientists and supervisory personnel.
- The item identification by code number. The appropriate properties of the item.
- The description of the test system, including species, strain, source, allocation, sex, age and method of identification.
- The description of the coded doses, dose regimen, route of administration and duration of the treatment period, the description of all methods used.
- Clinical signs and relevant raw data.
- The summary and description of all the toxic signs.
- Body weight data.
- Food consumption data.
- A description of all circumstances that may have affected the quality or integrity of the study.
- The authentification signed by study director.
- Test Facility Management Statement.
- The QAU Statement signed by QA Manager.
- The copy of the Certificate of GLP.
- The storage locations of study plan, all raw data, specimens and the reports.

## 15. DISTRIBUTION

This study plan will be distributed as follows:
1 Copy: Sponsor
1 Copy: Study Director
1 Copy: Study Monitor
1 Copy: QA Manager

## 16. ATTACHMENTS

***Attachment 1***

**90-DAY STUDY IN RATS ACCORDING TO OECD GUIDELINE 408 and EFSA**

### Time schedule

| | **March 2013** | **April 2013** | **May 2013** | **June 2013** | **July 2013** | **August 2013** | **Sep-tember 2013** | **Nov-ember 2013** |
|---|---|---|---|---|---|---|---|---|
| Quarantine | 5/7 days | | | | | | | |
| Randomi-sation | | April 1male April 3 female | | | | | | |
| Ophtalmology | | | | June 10-14 | | | | |
| Application males | | | | End June 30, July 1 | | | | |
| Application females | | | | End July 2,3 | | | | |
| Weighing of the feed | | Every 7 days | Every 7 days | Every 7 days | | | | |
| Weighing of animals | | Every 7 days | Every 7 days | Every 7 days | | | | |
| General clinical observations | | Everyday – Twice or more frequently | Everyday Twice or more frequently | Everyday Twice or more frequently | | | | |
| Detailed clinical observations | | Every 7 days | Every 7 days | Every 7 days | | | | |
| Sensory reactivity | | | | June 17-20 | | | | |
| Hematology males females | | | | 1,2,July<br>3,4, July | | | | |
| Clinical Chemistry males females | | | | 1,2,July<br>3,4, July | | | | |
| Gross necropsy males | | | | 1,2,July | | | | |
| Gross necropsy females | | | | 3,4, July | | | | |
| Slides preparation | | | | Start- July 12 | Until August 30 | | | |
| Histology evaluation | | | | | Start- July 20 | August | End - Sep. 30 | |
| Final report acceptance | | | | | | | | Nov.15 |
| Final report to sponsor | | | | | | | | Nov. 30 |

***Attachment 2***

LIST OF MATERIAL AND EQUIPMENT

**Equipment:**
***Laboratory of Toxicology:***

- Electronic balance Kern ABJ 220-4M, No. WB 0850106, range: 0.01-220g, precision: 0.0000g, Kern &Sohn GmbH, Germany, room No. B2-326
- Personal computers, office

Experimental animal rooms

- Temperature and humidity detector, PMICRO-LCD-THSYS, Dallas Semiconductor, rooms No. B2-609, B2-610.
- Personal computers, office
- Data backup system - 2 external hard drives and the eXtplorer system established by JKI
- Electronic balance Sartorius BP 1200, No. 6080646, range: 0-1000g, Sartorius AG, Germany, the operating room of Experimental animal rooms.
- Pressure air conditioning system VENTO, No. RMK 01.2, REMAK LTD., Czech Republic, Experimental animal rooms on the 3$^{th}$ floor at SMU.
- Personal computers, office
- Type of animal cages in TECNIPLAST Filter top cages Type 2145 F with an H-Temp™ (PSU) durable filter cover from the Tecniplast Company, Italy. The cages have a high density polypropylene body, measuring 480 x 265 x 210 mm - floor area 940 cm$^2$
- Ophthalmoscope Welch Allyn
- Apparatus for neurobehavioural testing: Accupacer treadmill

Laboratory of Immunotoxicology

- Haematological analyzer Sysmex K-4500, SYSMEX TOA Medical Electronics Co. LTD, Japan, No. VČ F-1466, room B2-212.
- Personal computers, office

Laboratory of Clinical and Experimental Biochemistry

- Analyzer Vitros 250, Ortho-Clinical Diagnostics, No. 219037234, USA, room B-048.
- Personal computers, office

Software for processing of the data

- Windows XP, program Office 2003
- Windows 2007, program Office 2010
- Software SPPS version 16.0.

Material

- Syringes, needles, tubes, Tubes microvette, tips, gloves, gauze, racks, paper, cartridge

Equipments Histology – TOPALAB

- Tissue processor AT 4 Medexport, USSR
- Equipment for embedding WD-4 Kunz International a/s., Koegevel 2006, DK 4000, Roskilde
- Leica-Reichert-Jung microtome Leica Instruments GmbHD, 6907 Nusslock, Germany
- Tissue heat regulator PTR – 02 fy BETIP, Vojtech Bilišič, Nové Zámky, SK
- Biological thermostat BT 120 Laboratornípřístroje Praha, CzechRepublic
- Hot - air heater CHIRANA Brno, CzechRepublic
- Hot - air sterilizer CHIRANA Brno, CzechRepublic
- NIKON microscope Nikon Corporation Japan
- PC Profi Energotel, Košice, SK
- HP printer Energotel, Košice, SK

**Chemicals**

- Parafine HISTOWAX Optoteam s.r.o. (Bratislava, SK)
- 70% alcohol Distilleries Leopoldov, SK
- 100% alcohol Distilleries Leopoldov, SK
- Chemicals used for tissues staining:
- Xylen $C_8H_{10}$ CHÉMIA (Ing. Sokol, Košice, SK)
- *Spiritus cum benzino* denaturovaný Distilleries Leopoldov, SK
- 70% alcohol Distilleries Leopoldov, SK
- 100% alcohol (benzínalcohol) (96%) Distilleries Leopoldov, SK
- $CuSO_4.5H_2O$ (pentahydrát p. a.) CHÉMIA (Ing. Sokol, Košice, SK)
- Mayer's hematoxylin MERCK Diagnostic, E. Merck, D-61 Darmstadt
- Canadabalsam LOBA FEINCHEMIE, A-2401 Fischamed

***Attachment 3***

List of records to be maintained for this study includes:

- Animal receipt records and quarantine records
- Randomization records
- Serology reports
- Feed log and analysis reports
- Water analysis reports
- Moribundity/mortality checks
- Rack/cage rotation
- Temperature/relative humidity/light intensity and cycle checks
- Dose analysis data
- Dose preparation and accountability records
- Dose administration
- Necropsy and histopathological findings
- Pathology specimens as specified
- Histology processing records

Records – primary documentation -will be kept in room B 2 – 209
All records during the study will be kept in computer room B 2 – 221
External backup will be kept in room B2 – 210
Second external backup will be kept in room B – 358 (QA)

***Attachment 4 - 6***

*(available from SZU)*

4. GLP CERTIFICATE SZU
5. GLP CERTIFICATE TOPALAB
6. ACCREDITATION CERTIFICATE Clinical chemistry lab