90-day feeding study of rats with Pioneer MON 810 maize

Study Plan

Study No: 311957 - B / 13/ GLP

Sponsor:	EU Project GRACE
Sponsor´s representative:	Prof. Dr. Joachim Schiemann
Test Facility:	Slovak MedicalUniversity Testing Laboratories Center Laboratory of Toxicology Limbová 14, 83303 Bratislava
Test Facility Representative:	[redacted] Slovak Medical University Limbová 14, 83303 Bratislava [redacted]
Study Director:	Dagmar Zeljenková, MVD, PhD. Department of Toxicology, head, Slovak Medical University, Limbová 12, 83303 Bratislava E-mail: dagmar.zeljenkova@szu.sk [redacted]
Test site 1: **Histopathology**	TOPALAB, s.r.o. Kameničná 7, 04015, Košice Laboratory of histopathology, Komenského 73, 041 81 Košice
Test site Principal Investigator:	[redacted]
Test site 2: **Diet analysis**	RIKILT – Institute of Food Safety Wageningen University and Research Center Campus Building 123, Akkermaalsbos 2 NL-6708WB Wageningen Netherlands [redacted] Principal investigator: Dr G.A. Kleter (gijs.kleter@wur.nl)
Test site 3: **Diet preparation**	Mucedola s.r.l. [redacted]

Test site 4: **Maize production, diet 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**	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: **Diet analysis**	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

Study Director

Name	Date	Signature
Dagmar Zeljenková, VMD, PhD.	2003-04-15	

Test facility representative

Name	Date	Signature
[redacted]	2003-04-15	

Principal Investigator Test Site 1

Name	Date	Signature
[redacted]	2003-04-15	

Principal Investigator Test Site 2

Name	Date	Signature
Dr Gijs A. Kleter		

Principal Investigator Test Site 3

Name	Date	Signature
[redacted]		

Principal Investigator Test Site 4

Name	Date	Signature
Dr Maria Pla de Sola Morales		

Principal Investigator Test Site 5

Name	Date	Signature
Prof Jean-Michel Wal		

Principal Investigator Test Site 6

Name	Date	Signature
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

	Name	Date	Signature
Headof QAU	[redacted]	2003-04-15	

	Name	Date	Signature
Headof QAU Test site 1	[redacted]		

	Name	Date	Signature
Head of QAU Test site 2	[redacted]		

	Name	Date	Signature
Head of QAU Test site 3	[redacted]		

	Name	Date	Signature
Head of QAU Test site 4	[redacted]		

	Name	Date	Signature
Head of QAU Test site 5	[redacted]		

	Name	Date	Signature
Headof QAU Test site 6	[redacted].		

TABLE OF CONTENTS

1. OBJECTIVE 9
2. PROFESSIONAL AND SUPERVISORY STAFF 10
Test site 1-6: 11
3. TEST FACILITIES 12
Test Facility 12
Test site 1-5 12
Test site 6: 12
4. TIME SCHEDULE 14
5. TEST AND CONTROL CROPS 15
6. TEST SYSTEM 16
Species and strain 16
Source 16
Number of animals 16
Approximate weight and age 16
Identification 16
Justification for the selection and number of animals 16
Animal housing 16
Diet formulation, sampling and analysis 17
7. EXPERIMENTAL DESIGN 19
Animal receipt and acclimation 19
Randomisation 19
Group allocation and dosing 19
Route of administration 19
8. PERIODICAL HEALTH STATUS OBSERVATIONS 21
Morbidity, mortality 21
Clinical signs 21
9. PROCEDURES FOR SAMPLE COLLECTION 22
Haematology 22
Clinical chemistry 22
Metabolomics 22
10. PATHOLOGY 24
Gross necropsy 24
Histopathology 25
Transcriptomics of tissue samples for FUB 25
11. DATA EVALUATION AND STATISTICAL ANALYSIS 26
12. REFERENCES 27
13. ARCHIVING 27
14. REPORTING 27
15. DISTRIBUTION 28
16. ATTACHMENTS 29
Attachment 1 29
Attachment 2 30

Attachment 3 32
Attachment 4 33
Attachment 5 33

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 Draft for Public Consultation, Scientific Opinion, 2011.

GoodLaboratoryPractices

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 11 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.

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 standard operating procedures (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. Methods have been validated and accredited except for nitrosamines, which will be carried out under a standard operating procedure (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 select 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 animal tissues and fluids for research purposes

The Institute for Veterinary Biochemistry (Test site 6) is a university research laboratory with a focus on basic molecular and cell biology and biochemistry. Accreditation of GLP compliance is not provided. However, the experimental procedures will be guided by the principles of GLP when applicable. Immunologic and metabolomic analyses on animals to be performed by INRA (Test site 5) in connection with the 90-day feeding trial, as well as analyses on the feed materials, will not be conducted under GLP. Analyses will be performed using 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.
CRAG and UDG's (Test site 4) omics labs are not subjected to GLP. However, the experimental procedures will be guided by the principles of GLP when applicable.

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, SR

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

Ophthalmology:
[redacted]
Limbová 14, 833 03 Bratislava 37, 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

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
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:
Experimental Agricultural Stationa Fundació Mas Badia
[redacted] supervision: Dr Maria Pla

Test site 5:
Laboratoire d'Immuno-Allergie Alimentaire
Service de Pharmacologie et Immunologie (SPI)
CEA Saclay / Building 136
iBiTec-S
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 arrive		Planned date: April 8-10, 2013
Arrival of animals		April 10, 2013
Starting of the treatment	males	April 16, 2013
	females	April 18, 2013
Last autopsy of the animals		July 18, 2013
Histology	Slides preparation	August 30 – September 30, 2013
	Histology evaluation	September 30 – December 15, 2013
Final report – draft to Sponsor:		January 2014

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 (UNE-EN_ISO_2433320101.pdf) 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
Eighty-five 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 assentinels.

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 age and 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 (*Rattusnorvegicusssp. 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, α=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 ± 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 cm^2 940.

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, in a total of 3 diets with 33% maize. 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 the company's contact within the GRACE consortium and 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. The most recent certificate is included as attachment 7.

7. EXPERIMENTAL DESIGN

Animal receipt and acclimation

Upon arrival, the animals will be placed in cages, 4 per cage. Forty-eight 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 (one for males, one for females) under standard SPF conditions and will be randomized using completely randomised designs (SOP: ŠPP/TOX/V001).

A completely randomised design rather than a randomised block design will be used, as blocking is unlikely to increase the statistical power of this study. We will use the Microsoft excel standard programme for randomising the rats to their groups. This approach is easier for the staff to manage the rats without the possibility of mixing up the animals or groups.

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 choose two animals into one 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.

Two animals will be placed in one 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, all animals will be examined 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 two 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 Pioneer MON810 maize, start April 2013

Group *Unknown identity for the staff **	*% (w/w) of daily dietary intake*				*No of animals*	
	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 (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	3	5	3
Total	85	43	85	43

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: Országh A. et al. (1985): Veterinárnaortopédia a rontgenológia, Bratislava: Príroda, 243 s. (Veterinary orthopaedy and X-ray). Scoring system is in attachment 9. The animals will also be assessed for gait disturbances using the Accupacer treadmill equipment.

Ophthalmologic examination

Using 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, metabolomics, pathology, and transcriptomics. Samples collected will include blood, tissues and organs. Blood samples will be divided for haematology, clinical chemistry and metabolomics. Tissues and organs will be removed and evaluated by histology and transcriptomics.

Sample collection and tissue processing.-personal disposition for forced progress (40 animals per day will be necropsied:)
1. - Animal will be euthanized by person No. 1
- Blood taking will be provided by person No. 2
- Decapitation and necropsy of the head by person No. 3

2. Animal transport to the Autopsy room on the same floor:
- Autopsy of thorax part body - person No. 4
- Autopsy of abdominal part body - person No. 5
- Weighting of selected organs for "omics" study and their preparation - person No. 6
- Weighting of rest organs - person No. 7

3. All organs will be stored into Formaline.

Haematology
At the end of the study on the day before sacrifice, blood samples from the tail vein will be taken from all animals for haematological examination 12 hours after fasting. EDTA will be used as anticoagulant and for some sample aliquots, citrate will be used for blood clotting analysis i.e. PT and APTT. Blood samples will be stored at room temperature (17-25° C) for a maximum of 4 hours until measurement. Haematology 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 will include Erythrocyte Count (RBC), Haematocrit (HT), Haemoglobin (Hb), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Mean Cell Volume (MCV), Leukocyte Count (WBC), Platelet Count (PLT).

Clinical chemistry
At the end of the study on the day before sacrifice, blood samples from the tail vein will be taken from all animals for blood chemistry examination 12 hours after 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-layeredVitros 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 bilirubine (TBIL), total cholesterol, Triglycerides, Na, K, Ca, Cl, P.

Metabolomics
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 RPM at 4°C for 15 min. preparation description

- Aliquots of plasma (ca. 100 µL) will be collected for the metabolomic analysis and kept at -80°C.
- Liver will be excised and weighed. The right lateral lobe will be collected and immediately snap frozen in liquid nitrogen.
- Both kidneys will be dissected and weighed. The third to the half higher part of the right kidney will be cut. This will be done by the same person to ensure consistency and reproducibility. The kidneys will be immediately snap-frozen in liquid nitrogen. Figures depicting the methodology (see attachment 10) will be available for the staff to use so that they are aware of how to perform the sample collection.
- Intestinal 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).
- After preparation all samples will be stored at -80°C.
- 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" analysis (according to the work plan for GRACE Work Package 2) with the list of samples and their identification (e.g. no of rat, type of sample, and day of collection) as well as the weight of the organs.
- Each sample must be clearly and unambiguously identified. It can be the animal number/nature of the sample (e.g. liver, kidney, plasma) in the case of the 90-day studies and in addition for the OMICS studies: the date of the sample collection (e.g. rat #/urine or plasma/dd-mm).
- **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 all organs harvested in the study will be recorded 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, kidney, spleen, adrenal gland, pancreas, testes, uterus, ovaries, epididymides, and thymus. Histological evaluation of tissue specimens will be done in 10 animals per group. The remaining tissues will be used in other assays. The remaining 6 animals will be used for metabolomics studies. Organs will be divided from the 10 mice for histology and transcriptomics.

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, trachea, salivary glands
- stomach
- small intestine and large intestines (including Peyer's patches)
- pancreas
- liver
- kidneys (L, R)
- adrenal
- spleen
- heart
- lungs (perfused to remove blood, and then inflate with fixative and then immerse in formalin)
- gonads (testes, L, R; ovaries L, R)
- uterus
- mammary gland
- 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.

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 10 animals from each group in accordance with the OECD TG 408.

Transcriptomics of tissue samples for FUB

At the end of the 90-day trials, the following tissues should be collected for transcriptomics studies: Intestinal sections of mid-jejunum, ileum, ascending colon (1cm each minimum), Mesenteric lymph nodes from ileum and ascending colon (3 lymph nodes each minimum), Spleen (identical anatomical section for each animal) description of how the organs will be handled. 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.

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 (a two-way ANOVA applied to males and females combined has been found to be inappropriate as variation can differ between genders).

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) will be prepared for presentation to a toxicologist. In some cases additional statistical analyses and graphical methods may also be used.
The raw data in a suitably annotated format will, at some point, be made publically available on the GRACE web site.

The following statistical methods will be used:

- Group data will be represented by mean, standard deviation and median
- Statistical analysis in case of data measured once during experiment (organ weight, haematology, biochemistry): one-way ANOVA and Dunnett post-hoc test between control and individual experimental groups on significance level alfa = 0,05 or Kruskal-Wallis test and adjusted Mann-Whitney U test for pair wise comparison between control and individual experimental groups on significance level alfa = 0,05.
- Statistical analysis in case of repeated data measurement (body weight, food intake data): Repeated measures ANOVA (procedure General Linear Model (GLM) for Repeated Measures in SPSS statistical software).

12. REFERENCES

OECD Guidelines for theTesting of Chemicals, Vol. 2, No. 408:
SUBCHRONIC ORAL TOXICITY- RODENT: 90 DAY STUDY IN RATS

Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed, EFSA ScientificCommittee, European Food Safety Authority (EFSA), Parma, Italy, EFSA Journal 2011;9(12):2438.

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.

13. ARCHIVING

Under the Code Number: 311957

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

16. ATTACHMENTS

Attachment 1

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

Time schedule

	April 2013	May 2013	June 2013	July 2013	August 2013	September 2013	November 2013
Quarantine							
Randomi-sation	April 15 male April 17 female						
Ophtalmology	april		June 10-14				
Application males	April 16,17 start		End July 15,16				
Application females	April 18,19 start		End July 17,18				
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 27-30				
Hematology males			15,16,July				
females			17,18 July				
Clinical Chemistry			15,16,July				
males							
females			17,18 July				
Gross necropsy males			15,16,July				
Gross necropsy females			17,18 July				
Slides preparation					Start-August 30	Until September 30	
Histology evaluation						Start-September	End december
Final report acceptance							January 15, 2014
Final report to sponsor							January 31, 2014

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 3th 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 cm^2 940
- 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

EquipmentsHistology – 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% alkohol — Distilleries Leopoldov, SK
- 100% alkohol (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 - 9 – separate sites

4. GLP CERTIFICATE SZU
5. GLP CERTIFICATE TOPALAB
6. ACCREDITATION CERTIFICATE Clinical chemistry lab
7. WATER cleannes
8. Scoring system - neurobehavioral changes, gait
9. Picture: Metabolomics – methodology