Bundesamt für
Verbraucherschutz und
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Position statement of the ZKBS
on application EFSA/GMO/UK/2005/19 from the company Syngenta
on approval of placing genetically modified maize "GA21" on the market
as genetically modified food and feed
according to Regulation (EG) No. 1829/2003

1. Subject of the application and purpose of placing on the market

The application EFSA/GMO/UK/2005/19 (BVL Ref. No. 6787-01-0019) was submitted in 2005 by Syngenta Seeds S.A.S. on behalf of Syngenta Crop Protection AG to the Competent Authority of the UK, who passed it on to the European Food Safety Authority (EFSA) on August 8, 2005. The subject of the application is placing on the market genetically modified maize (*Zea mays* L.) line GA21, which is tolerant against glyphosate-containing herbicides.

As listed below, the application covers all areas of use as food and feed:

- Genetically modified plants (GMP) for food and feed use
- Food and feed composed of or containing GMP
- Food and feed manufactured from GMP or containing components of GMP
- Import and processing according to Part C of the Directive 2001/18/EG

However, cultivation in the EU was not addressed in the application.

In addition, on June 29, 2007, the company Syngenta submitted an application (EFSA/GMO/RX/GA21) to the EFSA on renewing approval for the use of maize GA21 as a food supplement and feed according to Regulation (EG) No. 1829/2003. All the data necessary for a risk assessment of application EFSA/GMO/RX/GA21 are already available in the documents of application EFSA/GMO/2005/19. Therefore, the following position statement of the ZKBS, which includes all the anticipated areas of use for genetically modified maize GA21, applies to both applications made by the company Syngenta according to Regulation (EG) No. 1829/2003.

The company Monsanto has already made two applications for placing genetically modified maize GA21 on the market according to Directive 90/220/EG (C/ES/98/01 for cultivation [BVL Ref. No. 6788-02-31] and C/GB/97/M3/2 for import [BVL Ref. No. 6788-02-32]). The ZKBS gave its approval to each of these applications in the position statements from September 7, 1999 (C/ES/98/01) and February 7, 2000 (C/GB/97/M3/2). However, Monsanto has withdrawn both applications by now.

In a position statement from April 4, 2000, the ZKBS supported a further application by the company Monsanto for placing on the market food and food additives derived from genetically modified maize line GA21 as new food or food additives according to Regulation (EG) 258/97 (BVL Ref. No. 6789-02-02-4). This application has meanwhile received a positive decision from the European Commission (Authorization Resolution 2006/69/EG from January

13, 2006) according to which food and food additives made from GA21 maize are approved until January 12, 2016.

2. Description of the genetically modified organism

Using a particle gun transformation system, an isolated *Not* I restriction fragment from the plasmid pDPG434 was introduced into suspension culture cells of maize. The *Not* I fragment used for the transformation is 3.49 kb in size and contains the following expression cassette:

An *in vitro* modified variant (*mepsps*) of the endogenous *epsps* gene from maize coding for an enolpyruvylshikimate-3-phosphate synthase (EPSPS). The modification of the gene involved exchanging two out of the 445 amino acids of EPSPS (position 102: T → I and position 106: P → S), which results in 60,000-fold reduced sensitivity of the enzyme to the herbicide compound glyphosate. The *mepsps* gene is under the control of the promoter, plus first non-coding exon and intron of the actin gene from rice (*Oryza sativa*) (McElroy *et al.*, 1990). The termination signal is from the nopalin synthase gene (*nos*) from *Agrobacterium tumefaciens* (Bevan, 1984). The *mepsps* gene also has an optimized chloroplast transit peptide sequence at its 5'-terminal, derived from the chloroplast transit peptide sequence of ribulose-1.5-bisphosphate carboxylase from maize (*Zea mays*) and the sunflower (*Helianthus annuus*) (Lebrun *et al.*, 1996). The fusion with the optimized transit peptide results in an additional N-terminal methionine in the modified EPSPS (mEPSPS) protein.

As a pUC19 derivative, the backbone of the plasmid pDPG434 contains the ColE1 replication origin for replication in *E. coli* (Yanisch-Perron *et al.*, 1985), *lac* sequences (partial coding sequences under the control of the P*lac* promoter) (Yanisch-Perron *et al.*, 1985) and the bacterial ampicillin resistance gene *bla* under the control of its own promoter (Sutcliffe, 1978). Before transformation the plasmid was cleaved by the restriction endonuclease *Not* I to separate the vector backbone from the target fragment (*mepsps* expression cassette). From the Southern blot analyses described in application EFSA/GMO/UK/2005/19 and statements made by the applicant it is concluded that only the *Not* I fragment carrying the modified *epsps* gene, but not sequences of the vector backbone, is stably integrated into the genome of the GA21 transformant.

Several copies of the *Not* I fragment are integrated at one locus (six contiguous complete or truncated versions: fragments 1-6) in the genome of the maize line GA21. According to analyses by the applicant, the integrated DNA fragments comprise the following components:

I) Fragment 1:

Due to incomplete transfer this fragment contains a truncated *mepsps* cassette comprising a truncated rice actin promoter with a deletion of 696 bp at its 5'-end, the first exon and intron of the actin gene, the optimized transit peptide sequence, the *mepsps* coding region and the *nos* terminator.

The applicant assumes that the *mepsps* sequence in this truncated cassette is expressed in the maize plants, since it was previously shown that a truncated actin promoter retaining the actin intron is functional (McElroy *et al.*, 1990 and 1991). The expression driven by the truncated promoter will result in the same gene product as expression from the complete *Not* I fragment (see II).

II) Fragments 2, 3 and 4:

The fragments 2, 3 and 4 represent three complete copies of the 3.49 kb *Not* I restriction fragment with the *mepsps* expression cassette.

An mRNA of the expected length (ca. 1.8 kb) and a protein with the expected molecular weight (ca. 47.4 kDa) were detected in samples from leaf extracts from GA21 maize using Northern or Western blot analyses.

III) Fragment 5:

Due to incomplete transfer this fragment contains a truncated *mepsps* cassette that includes the complete rice actin promoter, the first exon and intron of the actin gene, the optimized transit peptide sequence and 288 bp of the *mepsps* gene, which ends with a stop codon.

Northern and Western blot analyses showed that the truncated *mepsps* gene localized on fragment 5 is not expressed, since neither a predicted transcript of about 0.7 kb nor an immuno-reactive EPSPS fragment in the range of 10 kDa or smaller could be detected.

IV) Fragment 6:

This is a truncated *Not* I fragment that contains only the rice actin promoter and a truncated first actin exon.

The 3'-end of the integrated DNA fragment and the directly adjacent genomic maize DNA was cloned by the applicant. Sequence analysis revealed two open reading frames (ORF) derived entirely from maize DNA that are coupled to the actin promoter found on fragment 6 and which could code for putative proteins with a length of 98 and 108 amino acids. Northern blots of poly(A+) RNA hybridized with maize DNA fragments adjacent to the actin promoter showed no specific hybridization signal in GA21 maize in comparison to non-genetically modified maize. The applicant concludes that in the absence of the intron the actin promoter is inactive or any transcript formed is rapidly degraded.

In addition, sequencing analyses revealed a single base pair exchange in the *nos* terminator in fragment 1 and 2 (nucleotide C instead of G, as in fragments 3 and 4). Furthermore, a single base pair deletion in the actin promoter of fragment 6 was found. However, the observed mutations are unimportant since they have no effect on the amino acid sequence of the new protein expressed.

The flanking regions of the inserts (ca. 4.2 kb for the 5' and 1 kb for the 3' flanking sequence) were isolated and analyzed. The sequence of the region flanking the 5'-end of the insert proved to be homologous to sequences of chloroplast DNA from maize. The integration of organelle DNA in plant nuclear DNA, either existing before the transformation or occurring through this event, is known in plants. A BLAST analysis of the 3'-flanking sequence showed homology to repetitive maize genome sequences and revealed no indications that the introduced DNA is inserted in a functional maize gene.

Possible ORFs that may have arisen as a result of the integration of the insert in maize GA21 were investigated by carrying out bioinformatics-based analyses. Amino acid sequence comparisons of possible expression products from four putative ORFs identified in the flanking regions (two ORFs in the 5'-region and two ORFs in the 3'-region [also see IV]) revealed no sequence homology to known toxins or allergens. The same applies to a putative ORF of 378 bp that starts within the genomic maize DNA flanking the 3'-end of the insert and terminates in the insert. Also a putative ORF identified within the insert at the transition between fragment 5 and 6 shows no homology to known toxins or allergens.

Expression of the introduced insert was investigated by using ELISA to determine the average mEPSPS concentrations over four growth phases (whorl, anthesis, seed maturity, and senescence) in leaves, roots and whole plants of maize GA21. The measured concentration lay between < 0.2 µg/g FW (fresh weight) and 15 µg/g FW (between < 0.3 and 70 µg/g DW [dry weight]). The average mEPSPS content measured in maize grain in the seed maturity and senescence phases ranged from ca. 4 to 7 µg/g FW (5 to 10 µg/g DW). The endogenous maize EPSPS protein was expressed at a significantly lower concentration than the genetically modified mEPSPS protein in maize GA21. An ELISA showed that mEPSPS accounted for about 96% of the total EPSPS protein in the leaves from GA21 maize.

Expression of the introduced *mepsps* gene gave the genetically modified plants tolerance to the herbicide compound glyphosate. This herbicide tolerance was inherited as a monogene trait.

3. Experience from previous field experiments

The application is based on numerous field experiments carried out over many vegetative periods and at various locations.

In the USA, field experiments were carried out at five locations in 1996, a further seven locations in 1997, and six locations in 2004 and 2005. In addition, field experiments were carried out at four locations in Italy and Spain in 1997. These experiments involved comparing the genetically modified plants with suitable non-genetically modified control plants, and served primarily as a basis for the compositional analyses (see 5.2.). There was no evidence of metabolic-related, phenotypic changes in the plants as confirmed by the comparative assessment of numerous compounds contained in the genetically modified plants. Any unintentional effects on the plant metabolism due to the genetic modification, in terms of a so-called positional effect or pleiotropic effects, could not be detected in the genetically modified plants based on the parameters investigated.

In further field trials carried out at various locations in the USA (1999 and 2004) and Brazil (2003), additional data was collected on agronomic parameters and the susceptibility of GA21 maize to plant pathogen infections (e.g. fungal infections). In addition, the efficacy and selectivity of herbicide treatment was investigated. There was no evidence of changes in morphological and phenotypic characteristics, or dispersal and effect on human health and the environment. There was no difference between the genetically modified and control plants with respect to various agronomic properties (e.g. plant development, time of flowering, morphology, grain yield, survival, or susceptibility to plant diseases). Therefore, there is no evidence for any change with respect to the ability to survive, reproduce and disperse.

Further controlled release experiments by the company Syngenta are being carried out or have been running since 2006 in Brazil, Spain, the Czech Republic, France and Rumania.

4. Granted approval for placing on the market outside of the EU

In the USA, Canada, Argentina and Japan, GA21 maize is already cultivated commercially and has received unrestricted approval for use in food and feed from the competent authorities.

5. Assessment of use as food and feed

A risk estimate concerning the use of GA21 maize as food and feed requires evaluation concerning the newly produced protein (mEPSPS), any possible change in the compounds and in the phenotype respectively, the genetically modified plant as a whole, as well as possible horizontal gene transfer to microorganisms of the gastrointestinal tract.

5.1. Assessment of the newly produced protein (mEPSPS)

The gene for a glyphosate tolerant EPSPS from maize in the genetically modified maize plants is expressed constitutively, controlled by the rice actin promoter (McElroy *et al.*, 1990) and the *nos* termination sequence from *Agrobacterium tumefaciens*. The presence of the first non-coding exon and intron of the actin gene from rice in the transcription unit has the purpose of increasing gene expression, probably due to the RNA processing step (Luehrsen & Walbot, 1991). The fusion of the optimized transit peptide mediates posttranslational import of the mEPSPS protein into the chloroplasts. The transit peptide is usually cleaved off during import (processing).

The *epsps* gene was modified by exchanging two of the 445 amino acids of EPSPS (position 102: T → I and position 106: P → S). The modified enzyme shows an amino acid sequence homology of > 99.3% with endogenous EPSPS from maize. The amino acid sequence homology between EPSPS and several other cultivated plant species (soy bean, tomato, rapeseed) is around 82 to 83%.

In chloroplasts, the endogenous EPSPS protein as well as the expressed mEPSPS resulting from the genetic modification in the maize plants catalyses the reaction of shikimate-3-phosphate with phosphoenol pyruvate to give 5-enolpyruvylshikimate-3-phosphate, an intermediate for the biosynthesis of aromatic amino acids and other aromatic substances of plant secondary metabolism. The mEPSPS protein additionally expressed in the genetically modified maize catalyses the same reaction as the corresponding enzyme naturally present in maize and other cultivated plants. Since the EPSPS protein is involved in the biosynthesis of aromatic amino acids, the applicant analyzed its relative concentration. Here, no significant change was measured in maize grains. In contrast to endogenous EPSPS protein, the modified EPSPS protein is not inhibited by glyphosate.

Since no detrimental health potential is known for the chloroplast transit peptide sequence of ribulose-1,5-biphosphate carboxylase from maize and sunflowers, nor for other currently known signal peptides, whether processed or unprocessed, it is assumed that this also applies to a complex comprising optimized transit peptide and an enzyme (here mEPSPS). There are no grounds for expecting a detrimental effect in the newly formed EPSPS protein.

5.1.1. Assessment of toxicity and allergenic potential

The assessment of toxic and allergenic properties of novel food or feed is mainly based on investigating the newly formed protein (mEPSPS). Such an assessment is possible because analyses of the composition of maize GA21 detected no biologically relevant differences compared to conventional maize in terms of substance content (see 5.2.) or agronomic and morphological parameters (see 3.).

For the assessment of the protein a broad spectrum of analyses was available (“weight of evidence approach”):

- Amino acid sequence homology comparison with known toxins and allergens
- Acute toxicity studies in rodents
- *In vitro* digestion studies
- Analysis of thermostability

A databank-based *in silico* comparison of the amino acid sequence of mEPSPS revealed no homology to known toxins and allergens. Similarities were only found to other known EPSPS enzymes and enolpyruvyl transferases. Thus, no evidence for any toxic or allergenic potential can be derived from the primary sequence of the protein. Also a comparison of all possible eight contiguous amino acids scanning along mEPSPS gave no evidence of similarities to epitopes of known allergens.

Due to the low concentration of the new protein synthesized in maize GA21, the test for acute toxicity and also studies on digestibility and thermostability were carried out using protein produced in bacteria. In this connection, the protein synthesized in bacteria was proven to be physicochemically, immunologically and functionally equivalent to the mEPSPS produced in plants. Acute toxicity was tested in albino mice (strain Alpk:AP$_f$CD-1) with a single oral administration of mEPSPS protein at a dosage of 2000 mg/kg body weight (BW). This resulted in no substance-related effects on mortality, body weight, food consumption, or in clinical observations, pathology, organ weight, haematology and clinical blood chemistry tests. This gives a NOEL (no observed effect level) = 2000 mg/kg BW and an LD_{50} > 2000mk/kg BW. Also a further acute toxicity study with CD-1 albino mice, already available to the ZKBS with application Ref. No. 6789-02-02-4 based on Regulation (EG) 258/97, showed no substance related effects with a single oral dosage of 3.7, 11.8 and 45.6 mg/kg BW. If one compares the maximal applied dose with the low exposure in human and animal diets due to the low protein expression (see 2.), the view of the ZKBS is that there is a sufficiently large safety factor even on the basis of the last named study.

The stability of the mEPSPS protein produced in GA21 maize and *E. coli* towards proteolytic enzymes was investigated in simulated mammalian gastrointestinal fluid (gastric and intestinal fluid). Results from SDS PAGE and Western blot analyses showed that after 1 minute of incubation in simulated gastric fluid (SGF) no intact mEPSPS protein (ca. 47.4 kDa) could be detected. After 1 minute, Western blot analysis revealed an immunoreactive fragment of about 6 kDa appearing with the sample isolated from plant material. This low molecular weight band represents a breakdown product and was no longer detectable after an incubation time of 5 min. Further *in vitro* analyses on stability towards simulated gastrointestinal digestive fluids already presented to the ZKBS with the application based on Regulation (EG) 258/97 (ref. No. 6789-02-02-4), showed that mEPSPS protein was no longer detectable after 15 sec in the SGF test and after 1 min in the SIF test (simulated intestinal fluid). Furthermore, there was no evidence for the appearance of stabile peptide fragments of > 2 kDa. Based on these results one can conclude that the mEPSPS protein is rapidly broken down in the gastrointestinal system. The results of these *in vitro* analyses give no reason to expect that the protein has a toxic or allergenic effect.

The thermostability of the mEPSPS protein was investigated by incubation at 25, 37, 65 and 95℃ over a period of 30 min. Up to a temperature of 37℃ no effect on the stability of the protein was observed. In contrast, no enzymatic activity was detected at a temperature of 65℃ and higher.

5.2. Assessment of possible changes in compound composition

Material from field trials carried out in 2004 and 2005 at six locations in the USA, were used to analyze the compound composition in genetically modified maize GA21 (with and without treatment with glyphosate) in comparison to a non-genetically modified maize variety with a comparable genetic background. The parameters investigated were: proximate (protein, fat, ash, moisture, total carbohydrate [calculated], starch), acid detergent fibre (ADF), neutral detergent fibre (NDF), 18 amino acids (including aromatic amino acids), fatty acids (palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid), vitamins and provitamins (ß-carotene, B_1, B_2, B_3, B_6, E, folic acid), minerals (Ca, Cu, Fe, Mg, Mn, P, K, Na, Zn, Se), anti-nutrients (phytic acid, raffinose, trypsin inhibitor) and secondary metabolites (ferulic acid, p-coumaric acid, inositol, furfural) in grain as well as proximate, ADF, NDF, Ca and P in forage.

The analyses showed statistically significant differences in some parameters compared to controls (ß-carotene, calcium, phosphorus, individual fatty acids). However, the listed parameters also vary in conventional varieties. Since the significant differences measured between the genetically modified and control plants lie within the range of this variability, these were attributed no biological relevance.

Based on the results of the comparative contents analyses it can be assumed that the composition of grain and forage from the maize line GA21 – except for the presence of the mEPSPS protein – is equivalent to conventional maize varieties that have no genetic modification. This applies to glyphosate treated GA21 maize as well as those only sprayed with conventional herbicides.

Also based on the results of the compositional analyses there is no evidence for an unintended effect on the plant's metabolism by the genetic modification in terms of a so-called positional effect or by pleiotropic effects.

Further compositional analyses based on material from field experiments carried out in 1996 and 1997 in the USA and Europe (Spain and Italy), and according to Regulation (EG) 258/97 already included with the application made available to the ZKBS (Ref. No. 6789-02-02-4), similarly proved the substantial equivalence of maize GA21 with non-genetically modified maize.

5.3. Assessment of the genetically modified plant as a whole

As presented in section 5.1., no detrimental effect of the mEPSPS protein or the transit peptide is expected. Since the enzymatic activity of the mEPSPS protein is clearly limited and also corresponds to the endogenous enzyme, it is assumed that besides the formation of mEPSPS and the transit peptide in GA21 maize plants, there is no further influence on the plant's metabolism. This assumption is also supported by the results of the compositional analyses, which proved the substantial equivalence of maize GA21 to conventional maize varieties (see 5.2.). Also evaluation of agronomical parameters as well as phenotypic characterization revealed no effect of the genetic modification on plant development and metabolism in the genetically modified plants (see 3.).

In addition, the non-detrimental nutritional physiology of GA21 maize was confirmed by carrying out a sub-chronic 90-day feeding study in rats. Groups each comprising twelve male and twelve female Wistar rats (strain Alpk:Ap_fSD) were fed test diets containing 10% or 41.5% (w/w) kernels from GA21 maize, compared to control groups whose diet contained 10% or 41.5% (w/w) of maize grains from a non-genetically modified maize variety with a compara-

ble genetic background. The study included testing the effect of two GA21 variants (with and without glyphosate treatment). The aim of the study was to monitor substance-dependant effects on mortality, body weight, organ weight, feed consumption, haematology, clinical blood chemistry and motor activity. In addition, various organs and tissues were analysed and ophthalmoscopy was carried out. A number of statistically significant differences were observed with some body and organ weights, as well as various parameters from haematology and clinical chemistry tests. However, these findings were generally not dose dependant, nor limited to one gender, and/or revealed no uniform pattern when taking herbicide treatment into account. Moreover, the findings did not correspond with histopathological changes in the corresponding organs or tissues, and are thus not considered biologically relevant. Taken together, the results of the 90-day rat feeding test gave no reasons to suppose that negative effects on animal or human health are to be expected with the use of GA21 maize for feed or food purposes.

To test the nutritional physiological equivalence of GA21 maize with conventional maize, a further 49-day feeding study was carried out with broiler chickens. In this study two test diets with GA21 maize grains (with and without glyphosate treatment) were compared with non-genetically modified control maize with a comparable genetic background as well as a conventional reference maize. Per test diet, 300 chickens (150 males and 150 females) were kept between hatching and 49 days old and fed three different age-related feed mixtures with 51% to 64% (w/w) of the chosen maize sort. Evaluation of the feeding study provided no evidence that the genetic modification had changed the nutritional physiological properties of the GA21 maize. No biologically relevant differences were determined with respect to mortality, feed consumption and nutritional value between chickens fed on feed containing genetically modified maize grains and chickens fed on feed containing kernels of the non-genetically modified, comparable maize or another conventional maize variety.

The application included an additional peer reviewed study (Sidhu *et al.*, 2000) where the feed value and safety of GA21 maize was investigated with chickens. This publication also provided no evidence of undesirable effects on the animals' health or the product quality.

5.4. Assessment of horizontal gene transfer in the digestive tract

Horizontal gene transfer to microorganisms in the digestive tract cannot be excluded. If such gene transfer is possible, it would involve a natural mechanism. Gene transfer would only be expected to have any effects if there is an advantageous selection pressure for the transferred gene. In the case discussed here, spread of the gene for modified glyphosate tolerant EPSPS from the genetically modified maize to microorganisms of the gastrointestinal tract is not expected, since there is no selective advantage for glyphosate. This supposition is supported by analyses where the DNA of phage M13 was administered orally to mice. Phage DNA could only be detected in faeces samples for up to a maximum of 7 h after feeding, providing no evidence of colonization of gut flora with foreign DNA-containing bacteria. Foreign DNA could be identified in the blood in very low amounts (<0.1%) over a short period of time (maximum 24 h) (Schubbert *et al.*, 1994). Other genes, e.g. antibiotic resistance genes, are not present in the genetically modified maize GA21 (compare 2.)

5.5. Effects due to using glyphosate-containing herbicides

Determining the amount of residual herbicide after application of glyphosate-containing herbicides to genetically modified maize plants is not the subject of the assessment of the appli-

cation presented here, but is addressed in the context of approval procedures for plant protection substances.

6. Risk evaluation in relation to the environment

The cultivation of genetically modified maize GA21 is not the subject of the application, so it is assumed that genetically modified maize plants derived from the starting line GA21 only release into the environment accidentally and thus in a small amount.

Maize is a domesticated form that can only survive through human intervention. In addition, maize plants are not winter resistant and therefore cannot become established under the climatic conditions in mid-Europe.

Cross-fertilization with native wild plants is not possible since maize has no cross-fertilization partner among mid-European flora.

It is not expected that the properties of maize discussed above are influenced by the genetic modification described in the application. In controlled release experiments with the genetically modified plants the applicant has carried out investigations into various contents components, vegetative development, formation of flowers, behaviour of the plants towards diseases as well as harvest yields. These investigations have confirmed that the genetically modified plants do not differ significantly from non-genetically modified maize varieties with respect to these properties. The possibility of survival, spread, becoming wild or pollen dispersal from unintentionally released genetically modified plants should be assessed no differently than for traditionally bred maize.

7. Recommendations of the ZKBS

The ZKBS welcomes the fact that the transformation only involved transfer of the expression cassette with the gene necessary for the desired change (glyphosate tolerance). The same applies to all maize lines derived from the original transformant GA21.

Upon weighing up the view points presented in the risk assessment, the ZKBS has come to the conclusion that based on the current state of knowledge, placing genetically modified maize GA21 on the market as genetically modified food or feed in the context of the operative area applied for (see 1.) is not expected to cause detrimental effects to the health of humans or animals or the environment.

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