An assessment of the risks related to the consumption of food products containing or consisting of genetically modified organisms

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PREAMBLE

The assessment and control of the risks associated with the use of Genetically Modified Organisms (GMOs) are of interest to society as a whole. As an introduction to the recommendations, of necessity highly technical, contained in this report, it is therefore worth recalling certain elements which place the question in its scientific and historical context. The use of GMOs covers a variety of different situations from the research carried out prior to any application, to the marketing of crops for mass consumption. Public attention is mainly focussed on the transgenic plant varieties intended for human or animal consumption, but the GMOs affecting the everyday life of all of us could also be micro-organisms (used in an industrial process or in the production of medicines) or, in the foreseeable future, farm animals. Furthermore, the difference between the GMOs themselves and the processed food products derived from them is essential in terms of risk assessment.

Context (in France)

- The first GMOs were obtained in a laboratory in the 1970s. These were transgenic micro-organisms (bacteria, yeasts, etc.), followed by animals (1980) and then plants (1983). A coherent set of regulations was produced early on, both for laboratory experiments and for the release of the GMOs, and a system of risk assessment and control was put in place which is still in operation today. The use of GMOs is now an everyday and ordinary occurrence in most biological and medical research laboratories but it is subject to assessment by a specialist scientific body, the Commission de Génie Génétique (CGG) [Genetic Engineering Committee].

- With the use in agriculture of plants obtained by transgenesis, most consumers are now in contact with GMOs or may consume food or industrial products produced from GMOs. A turning point was reached in the 1980s, with the first medicines obtained by transgenesis (human insulin and human growth hormone are now produced from GMOs which under these conditions provide specificity, quality and safety). During the same period, the fight against rabies used (with considerable success leading to the near eradication of the disease) vaccine baits produced from GMOs which were widely distributed in certain parts of France. There is now a demand for information (mainly in terms of product labelling) which has led to an intense debate on the safety of GMOs and the products produced from them.

- The deliberate release of GMOs is strictly controlled, where it is subject to authorisation, following consultation and evaluation by the Commission du Génie Biomoléculaire (CGBM) [Biomolecular Engineering Committee], within a well-defined EU regulatory framework. Public distrust, even hostility, is nonetheless clearly visible, especially as regards transgenic plants. In fact, while some are "legally" marketable in Europe, their cultivation is restricted to a few thousand hectares, and crops such as rape and sugarbeet are still subject to a strict moratorium. The contrast with North American (US and Canada) agriculture is striking, where transgenic crops cover tens of millions of hectares.

Risk type and control in terms of the food safety of transgenic plants

In the current conditions in which plant GMOs are obtained, the objective and potential hazards in terms of food safety may arise principally from the following facts:

1) a transgenic plant could synthesise a foreign protein which could produce acute or long-term toxic effects and/or allergenic effects;

2) the extinction of genes or the expression of silent sequences peculiar to the genome of the plant of origin may result in an unexpected effect. In the case of transgenic plants, insertion of the transgene may induce such phenomena in the transformed plant;

3) metabolic interactions, subtle or otherwise, may result in the occurrence of unpredictable and toxic metabolites.

Only the first potential hazard is specific to transgenic plants; hazards 2 and 3 also concern plants produced by the application of conventional methods of selection through cross-breeding, notably in inter-species cross-breeding.

These phenomena may or may not occur and therefore become objective or potential risks.

The objective risks (the toxic protein for example) and the potential risks (those mentioned in points 2 and 3 which can rarely be defined beforehand) require, to guarantee consumer safety, the implementation of a set of experimental assessment protocols, whatever the level of potential toxicity and/or allergenicity in the short and long term.

THE REFERRAL AND ITS CONTEXT

The French Food Safety Agency was asked on 22 October 1999 by the Ministers responsible for health, agriculture and consumer affairs to produce an opinion on the risks related to the consumption of food products containing or consisting of genetically modified organisms (GMOs).

This referral identified two questions to be answered:

Question 1: what are the key points for assessing health risks associated with the human and animal consumption of GMOs or products derived from GMOs and what are the relevant elements of this assessment?

Question 2: What are the risks associated with human and animal consumption of GMOs containing antibiotic-resistance genes?

This request for an opinion concerns all genetically modified food products of animal or plant origin and micro-organisms. As there are no genetically modified animals or micro-organisms currently on the market, even though discussions are underway within European and international institutions with the intention of determining assessment criteria for these organisms, the opinion will be restricted to plants and the products derived from them.

1 DEFINITIONS

Organism
Any biological entity capable of replication or of transferring genetic material (c.f. Directive 2001/18/EC of the European Parliament and of the Council) [3].

Genetically modified organism (GMO)
An organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination (c.f. Directive 2001/18/EC).
Within the terms of Directive 2001/18/EC:
(a) genetic modification occurs at least through the use of the techniques listed in Annex I A, part 1 of the Directive 2001/18/EC;
(b) the techniques listed in Annex I A, part 2 of the Directive 2001/18/EC, are not considered to result in genetic modification.

Product derived from GMOs
A food and food ingredient produced from genetically modified organisms within the meaning of Directive 2001/18/EC.

2 GMOs AUTHORISED IN FRANCE

To date, only four plant transformation events have received consent for use (cultivation, import and industrial processing):

- "ITB-1000-0X" tobacco, herbicide tolerant, but which has not yet been widely marketed;
- Bt-176 maize, resistant to the corn borer and herbicide tolerant;
- MON 810 maize, resistant to the corn borer;
- T25 maize, herbicide tolerant.

Two plant transformation events are authorised solely for import with a view to their industrial processing (their cultivation is therefore not authorised in Europe).

- 40-3-2 soya, herbicide tolerant;
- Bt-11 maize, resistant to the corn borer and herbicide tolerant.

As regards products derived from genetically modified plants, between 1997 and 1999, 11 food products or food ingredients for human consumption were placed on the market based on a simplified procedure as provided for in Regulation (EC) No. 258/97 of the European Parliament and of the Council [2]: rapeseed oils and ingredients (flour, semolina, starch, gluten, glucose, oil) obtained from maize[1].

3 REGULATIONS COVERING GMOs

The placing on the market of GMOs or products derived from GMOs is subject to **specific authorisation**, granted on a **case by case basis** at EU level.

When this involves living GMOs intended for cultivation (release) on French or European territory, whether for research or for the purposes of production for human or animal consumption, authorisation is granted on the basis of a technical dossier which enables assessment of the risks to public health and the environment associated with the release of said GMO. This authorisation is governed by Council Directive 90/220/EEC [1] which is to be repealed on 17 October 2002 and replaced by Directive 2001/18/EC of the European Parliament and of the Council.

When food or food ingredients containing or consisting of GMOs are involved (for example: soya lecithin), authorisation to place these products on the market (cultivated on European territory or imported into the Community) or to use them with a view to their industrial processing for human consumption is granted on the basis of a technical dossier enabling the public health risk to be assessed in accordance with Regulation (EC) No. 258/97 of the European Parliament and of the Council.

The placing on the market of GMOs intended for animal consumption is currently covered by Council Directive 90/220/EEC. The European Commission, in accordance with the undertakings made in the White Paper on Food Safety [4], has submitted to the Council and the European Parliament the draft of a new regulation which will cover the placing on the market of all GMOs or products derived from GMOs intended for human or animal consumption.

4 THE EUROPEAN AND INTERNATIONAL GUIDELINES FOR THE ASSESSMENT OF THE SAFETY OF GMOs AND PRODUCTS DERIVED FROM GMOs INTENDED FOR HUMAN OR ANIMAL CONSUMPTION BEFORE THEY ARE PLACED ON THE MARKET

Council Directive 90/220/EEC (2001/18/EC) and Regulation (EC) No. 258/97 of the European Parliament and of the Council define the elements which must be contained in a dossier requesting authorisation for placing on the market, as well as, in the case of the Regulation, a guide for the assessor's use [5].

[1] On its website: www.finances.gouv.fr/ogm/ "les OGM en questions" the Direction Générale de la concurrence, de la consommation et de la répression des fraudes [Directorate General for Competition, Consumer Affairs and Trading Standards] presents a list of authorised GMOs and products derived from GMOs.

The specialist Scientific Committees are proposing, at the request of the European Commission, guidelines for the use of notifiers which specify the technical elements to be provided in the dossiers to enable the evaluation of GMOs or products derived from GMOs for human or animal consumption.

At international level, the Codex Alimentarius has also prepared draft guidelines for the assessment of GMO dossiers (Guidelines for the conduct of safety assessment of foods derived from modified plants), which are based, notably, on the FAO/WHO consultation of international experts (Safety aspects of genetically modified foods of plant origin).

5 SUBSTANTIAL EQUIVALENCE

The OECD originally developed the concept of substantial equivalence for new foods, and it has been adopted by FAO/WHO and by the regulations covering new foods.

The **concept of substantial equivalence**[2] characterises an approach which is designed to compare the analytical characteristics of a new (food) product with those of a "conventional"[3] product (for a GMO plant, compared with the parental line of the same, non-modified plant). The application of this concept does not constitute definitive proof of the safety of a new product; the result of the comparison is one of the elements in the risk assessment of a product derived from or containing GMOs.

Three scenarios may be envisaged:

- substantial equivalence can be established; in such case, it is not necessary for any tests to be conducted other than those carried out on the protein introduced by genetic transformation;

- substantial equivalence can be established from the nutritional composition and any anti-nutritional factors except as regards the new element or elements introduced; the safety assessment must then focus on the characteristics of the new element or elements;

- substantial equivalence cannot be established; the new product must then be subjected to an in-depth safety assessment.

In the case of a GMO, because of the introduction, modification or deletion of a characteristic, the resulting organism is different at the outset; risk assessment on the sole basis of demonstrating substantial equivalence is therefore not sufficient and does not permit any exemption from the safety assessment of such products.

It should be noted that as this notion of substantial equivalence for GMOs is an extremely controversial one within the European Union, the draft of the new regulation relating to genetically modified foods intended for humans or animals provides for the deletion of the simplified procedure in Regulation (EC) No. 258/97.

[2] **Substantial equivalence**: the notion of substantial equivalence expresses the idea that existing organisms which are used as foods or sources of foods may be used as a basis for comparison during the safety assessment of a new or modified food. (Commission Recommendation relating to Regulation (EC) N°258/97).

[3] **Conventional counterpart**: a related plant variety that has a history of safe use for consumption as food. (Codex Document – Guideline for the conduct of safety assessments of food derived from modified plants).

METHODOLOGY

To satisfy the requirements of this referral, AFSSA consulted the working parties appointed from the Expert Committee on Biotechnology and sought advice from experts. A list of all the persons who contributed to the preparation or validation of this opinion appears in Annex 3.

THE QUESTIONS

Question 1: what are the key points for assessing health risks associated with human and animal consumption of GMOs or products derived from GMOs and what are the relevant elements of this assessment?

Any modification of a foodstuff is liable to have repercussions on consumer safety either directly through the foodstuff itself or indirectly through the products derived from animals which have ingested GMOs. For this reason, before a new food product containing or consisting of GMOs is placed on the market, its safety has to be ensured.

As there are no genetically modified animals[4] or micro-organisms currently on the market, the assessment elements discussed below will relate only to plants and the products made from them.

Given the very wide diversity of products and their applications which currently exist or which can be envisaged, the principle retained is that the safety of these products is assessed on a case by case basis. This assessment is based on:

- analysis of the genetic modification,

- analysis of the finished product through:
  - the physical, chemical and functional characterisation of the transgene expression products and a toxicological assessment,
  - the identification of any possible unintentional or unexpected effects resulting from the genetic modification and their repercussions on the safety of the product,
  - the chemical composition and nutritional value.

The scientific review conducted by the French Food Safety Agency focussed on the key points of the safety assessment of GMOs or products derived from GMOs intended for human or animal consumption, with regard to the regulatory provisions laid down by Council Directive 90/220/EEC (or 2001/18/EC) (Annex III) and Regulation (EC) No. 258/97 (Commission Recommendations of 29 July 1997) and the draft European or international guidelines (Codex Alimentarius).

In terms of the information which should be contained in a dossier prepared prior to the placing on the market of a new genetically modified plant or a product derived from it, intended for human or animal consumption, the information relating to **genetic modification**, on the one hand, and the **toxicological** information and information relating to the assessment of its **allergenic potential**, on the other hand, are essential for establishing the safety of a new food product at the outset. The value of having available information on the effects of the transgene products or the genetically modified plant in the digestive tract will be reiterated for the record.

For some of these key points, AFSSA has identified a certain amount of data which is currently lacking or is insufficiently documented and which could assist in refining the food safety assessment of GMOs and the products derived from them before they are placed on the market.

[4] As regards the risks likely to result from the consumption of animals or animal products derived from biotechnology, refer to the symposium organised by AFSSA on 29 September 1999 on "Biotechnology in animal reproduction and food safety".

The implementation of the tests referred to in this opinion requires documenting on a case by case basis, taking into account the genetic modification introduced, the nature of the protein expressed and any specificities proper to the GMO under consideration.

1 INFORMATION RELATING TO GENETIC MODIFICATION AND THE GENETICALLY MODIFIED PLANT

The placing on the market of a new genetically modified plant (GM plants) involves the constitution of a dossier containing certain information relating to the genetic modification and the genetically modified plant. This information concerns the description of the methods used to obtain the genetic modification, the nature and source of the vector used, the size and source of the DNA fragment inserted (insert), the description of the characteristic(s) introduced or modified and the DNA sequence actually inserted or deleted (number of copies, location in the plant's cells, expression).

It would seem that the data usually supplied in the dossiers describing the DNA sequences inserted or deleted should be supplemented by the transgene sequence[5] and the regions making the junction with the genome.

These additional data would enable:

- verification of whether the inserted fragment is genuinely identical with the one introduced into the transforming vector;

- examination of the environment of the inserted gene;

- localisation of the insert in the plant's genome.

2 INFORMATION RELATING TO THE ANALYSIS OF THE FINISHED PRODUCT

The dossier submitted to obtain consent for placing a new food product on the market, under the terms of Regulation (EC) No. 258/97 of the European Parliament and of the Council, must contain the following information: specification of the new food, possible effect of the process of production of the food, utilisation of the original organism, predicted level of consumption and types of use, nutritional and toxicological properties.

Regarding the possible toxicity more specifically, the standard methods of assessing the toxicity of medicines or chemicals are not, in principle, applicable to food, which *a priori* devoid of toxic potential.

However, the absence of toxicity in these products can be assured by the following measures:

- sub-chronic toxicity tests on laboratory animals with the gene products. These tests can provide information on the potentially toxic effects resulting from genetic modification;

- tolerance tests on laboratory animals and additional tolerance, feeding value and digestibility tests on target animals with the parts of the plant and its by-products intended for consumption. These tests could provide information on the potential consequences of repeated consumption of a product by humans or animals.

[5] In order to facilitate the comparison between the sequences supplied using databases, it would be desirable for the assessor to be given these sequences in computerised form.

In addition, any genetic modification can influence the synthesis of anti-nutritional factors or toxic substances naturally present in the plant. Tolerance studies in laboratory animals and on the target animals should reveal the presence of potentially harmful factors.

These two complementary approaches, tolerance tests on laboratory animals and food safety tests on target animals, however, both comprise technical constraints or problems: preparation of the material to be tested in sufficient quantities for animal experimentation over long periods, the judicious selection of samples, the statistical interpretation of the tolerance tests and the cost in terms of the benefit-risk balance.

2.1 The origin of genetically modified plants subjected to assessment

It is important that the safety assessment of a new genetically modified plant or a new product derived from it is always conducted in comparison with a plant, or its products, which constitutes an appropriate frame of reference (related isogenic product or conventional variety).

In view of the influence of environmental factors on plant growth, this assessment must cover plants cultivated for at least two seasons in different sites and which are representative of a variety of environments.

2.2 Sub-chronic toxicity testing of laboratory animals with the gene product

2.2.1 The origin of the gene product used for food safety assessment

When it is difficult or impossible to obtain a sufficient quantity of the gene product in the pure state from the plant itself, the gene must be cloned in a micro-organism which will express this gene product. However, the risk remains that the product thus obtained may not be strictly identical to the one synthesised by the plant.

For this reason, there must be an understanding and a systematic comparison of the **post-translational modifications** between the gene product originating from the plant and the micro-organism:

- the gene product extracted from the plant (from plants cultivated in a greenhouse) and that extracted from the micro-organism in which the gene has been cloned must be analysed and compared (molecular weight, amino acid sequence data, glycosylation, immunological equivalence, biological action and protease sensitivity in a digestive simulation environment).

- determination by western blot of the molecular weight of the product of the inserted gene(s) could be supplemented, where possible, by mass spectrometry which would enable the highlighting of any post-translational modifications.

2.2.2 Evaluating the sub-chronic toxicity of the gene product on laboratory animals (rats, mice, guinea pigs)

Limitations of the single dose acute toxicity tests

With very few exceptions, the only information available relating to toxicity has been obtained through single doses (acute toxicity) on laboratory animals, carried out with the purified protein.

From 1997-1999, as part of their discussions on the placing on the market of new foods for animal and human consumption, the experts at CIIAA[6] and the CSHPF[7] considered the importance of longer term

[6] CIIA: Commission interministérielle et interprofessionelle de l'alimentation animale [Interministerial and Intertrade Committee on Animal Feed]

[7] CSHPF: Comité supérieur d'hygiène publique de France [French Higher Public Health Committee]

toxicity tests on laboratory animals to demonstrate potential harmful effects on the body's systems, notably the immune and reproductive systems.

Problems with the conduct of a sub-chronic toxicity test for plants

For the medicines and enzymes used in human or animal food, sub-chronic toxicity testing can be envisaged on laboratory animals (rats, mice, guinea pigs). However, certain difficulties arise in the case of plants:

- the gene product is not consumed in the pure state but with other components of the plant,

- it can be difficult to obtain sufficient quantities of the gene product (see 2.2.1) and to ensure that the preparation tested after extraction is genuinely equivalent to the one which will actually be consumed,

- the administration of a sufficiently large dose must respect the animal's nutritional balance.

Elements on which the selection of the test period should be based

a) Repeated oral administration for 28 days

Some experts consider that the minimum period of exposure required to demonstrate effects on the sexual organs in rats is 28 days, mainly due to the characteristics of the gamete maturation cycle.

A 28-day toxicity study is generally considered to be a preliminary to a long term study. It is acceptable when the product already has the benefit of a history and therefore the bibliographical and/or drugs monitoring data which certify its tolerance. The corollary of this short exposure period is the use of strong doses, which can be achieved with medicines or chemicals but not with food. This generally constitutes more than 50% of the diet, if the animal's nutritional balance is to be strictly respected.

b) Repeated oral administration for 3 to 6 months

The fact that animal or human exposure to GMOs is chronic, means that experimental exposures also have to be on a chronic basis. An exposure period of three months seems a good compromise between a 28-day toxicity study and a six month study. It enables the revelation, with an improved probability, of the potential effects on the body's systems, notably the immune, hormonal and reproductive systems and of possible effects associated with the phenomena of accumulation. Furthermore, this is the exposure period recommended and habitually used for assessing the safety of the enzymes used as processing aids in human food or as additives in animal feed.

The protocol selected for assessing the sub-chronic toxicity of the gene product on laboratory animals (rats, mice, guinea pigs)

- **Exposure period: 90 days**

  This study is designed to demonstrate potential harmful effects on the development of the body's organs or systems during long term exposure. An exposure period of 90 days seems appropriate.

- **Determination of doses**

  In order to expose the animal to doses which will reflect what could be consumed by humans and to prevent any additional effects from deficiencies in the diet, the doses to be administered have to be determined in view of the animal's nutritional balance, with the highest dose unable to exceed total protein intake, or approximately 15% of the animal's diet.

  The gene product may be obtained from a micro-organism in which the gene has been cloned or from the plant. In either case, its purity and identity must be verified (see 2.2.1.).

- **Observations and type of examination to be conducted**
  For a list of the clinical observations, the organs to be examined and the histopathological tests to be performed, reference may be made to the protocols published as Test Method B.26 in Directive 67/548/EEC [6] or OECD guideline No. 408 [7] for the assessment of chemicals.

2.3 Evaluating the tolerance to the finished product obtained from the genetically modified plant in animals

The objective of these studies, carried out with the GMO product or the product derived from a GMO (parts of the plant or by-products intended for consumption), **firstly using laboratory animals, then secondly possibly using the target animals,** is to demonstrate the potential effects of the regular consumption of a product by humans or animals and any unexpected or unintentional toxic effects which had not been revealed during the acute or sub-chronic toxicity studies.

The tests on the target animals, meaning on at least one species of farm animal (single-stomached or ruminant) which usually consumes the designated products, seem necessary due to:

- the varying sensitivity of different species to certain anti-nutritional, hormonomimetic, etc. factors and the particularities of their digestive physiology;
- the fact that their diet can contain up to 90% of the genetically modified plant during the animal's full lifetime;
- the particular metabolism of certain animals and the risk of the storage of potentially toxic products in certain specific organs and tissues (the liver for example).

2.3.1 Toxicity/tolerance study on laboratory animals of the parts of the plant intended for consumption or their by-products

It is appropriate for these tests to be conducted, in accordance with the standard recommendations which apply to toxicity studies on medical substances, on laboratory animals, rodents in principle, for 90 days, with batches of young animals composed of 10 animals of each sex. The animals should be fed individually. The dose must be as high as possible whilst remaining compatible with the animal's nutritional balance.

The clinical observations and the list of organs to be examined are the same as those for the sub-chronic toxicity test. In the event of anomalies observed at post-mortem, a histopathological examination should be carried out. The animals' growth rate and food consumption should also be recorded as well as any mortality (cause identified at post-mortem).

Depending on the type of food being assessed, the test on laboratory animals could be replaced with a test on rabbits (test period: from weaning to slaughter) or chickens (test period: from day-old to slaughter).

2.3.2 Tolerance, feeding value and digestibility study on the target animals of the parts of the plant intended for consumption or their by-products

Since 1995, although this type of test is not included in the standard assessment dossier for placing GMOs on the market, several experimental studies have been carried out on farm animals (beef cattle, dairy cows, sheep, chickens, pigs) aimed at evaluating the safety of genetically modified products for animals and humans [30-33]. The following points have been considered:

- the chemical composition of these new foods: content of proteins and amino acids, fats and fatty acids, minerals, vitamins, ADF[8], NDF[9] and known anti-nutritional factors;

- what happens to genetically modified products in animal feed during processing (ensiling, heat treatment);

- the relative performances of animals fed on these new feeds and those fed on products made from non-genetically modified parental plants;

- the digestibility of the main constituents, dry matter, organic matter, energy, total nitrogen, NDF, ADF;

- demonstration of the absence of foreign or new substances due to genetic modification in milk, meat and eggs.

The results of these studies have shown, within the limits of the tests carried out, the similarity of performances between animals (dairy cows, chickens or pigs) given feed composed of genetically modified plants and those given feed composed of isogenic plants.

However, consideration must be given, when interpreting these results, to the concept of the statistical power[10] of a test, which increases the probability of demonstrating a given difference (see Annex 1). The statistical power of a test is linked to the number of animals used. The results of such tests may not be statistically significant due to the insufficient number of animals tested.

Determination of the number of animals per test

The number of animals necessary for the achievement of a given statistical power (see Annex 1) depends on several factors:

- the natural variability of the characteristic studied: the more naturally stable the characteristic is in a population, the easier it is to demonstrate a slight difference;

- the required statistical power, defined in advance;

- the difference which one wishes to be able to demonstrate: the slighter the difference, the more individuals required.

Selection of the target species and observation parameters[11]

The choice of the target animal depends on the plant to be tested.

In the case of a feed intended for a single-stomached animal, a food safety test on rats or chickens, as described above, will generally suffice. In certain cases (presence of substances of an oestrogenic nature, anti-nutritional factors) a test on pigs may prove more informative. The observation parameters would be weight gain, feed consumption and efficiency, and/or possibly digestibility of dry matter, organic matter, total nitrogen and energy.

In the case of a feed intended more specifically for poultry, laying hens, for example, or quail, the parameters to be observed are laying rate, egg weight, feed efficiency, egg quality (AFNOR standard). If the test is conducted with chickens or turkeys, the parameters to be observed are weight gain, feed consumption and efficiency, and/or possibly the digestibility of the ingredients of the diet.

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8 ADF: acid detergent fibre

9 NDF: neutral detergent fibre

10 The power of a statistical test corresponds to the probability of concluding a statistically significant difference, if one actually exists. This concept should not be confused with the α risk a value generally selected at 5%, corresponding to the probability of concluding a significant difference when there is no actual difference.

11 The observations obtained will have to be compared with those gathered within the framework of the surveillance plan implemented after authorisation for placing on the market is given, in accordance with the provisions laid down in Council Directive 2001/81/EC.

In the case of a feed intended more specifically for a ruminant, the test may be conducted on small ruminants, beef cattle or dairy cows. The observation parameters are growth performance and feed consumption (small ruminants and cattle) and total milk and fat corrected milk production, feed consumption and milk composition (dairy cows). In the case of tests on small ruminants, digestibility of dry matter, organic matter, energy, total nitrogen, ADF and NDF would be measured.

The number of animals, their physiological stage (lactation stage, age/weight at start of test, etc.), the parameters selected for monitoring and the test period[12] will be defined in such a way as to be able to demonstrate a possible difference of one standard deviation between the treatments with a power of at least 80% (see Annex 1).

2.4 Degradation in the digestive tract

The study of the degradation of the gene product in the digestive tract can be a decisive element of the assessment. The study should include an *in vitro* stage (simulation of gastric and intestinal digestion) and an *in vivo* stage. Given the fact that a foodstuff (or the gene product) can behave differently (be more stable *in vivo* than *in vitro*) in the animal's digestive tract, the sub-chronic toxicity study on laboratory animals and the tolerance, feeding value and digestibility tests of the major constituents or parts of GM plants on the target animals may usefully contribute to the study of the degradation of the new foodstuff in the digestive system.

2.5 Evaluation of allergenic potential

The Codex Alimentarius has, within the context of the draft guidelines for the assessment of GMO dossiers (Guideline for the conduct of safety assessment of foods derived from modified plants), produced some specific recommendations on the assessment of the allergenic potential of proteins, based on the conclusions of the FAO/WHO international expert consultation (Foods derived from biotechnology, allergenic potential of genetically modified foods, 22-25 January 2001) [8]. The FAO/WHO report proposes an evaluation process based on a decision tree approach (see diagram) and gives recommendations on the development of standardised procedures and for post-marketing surveillance.

After having identified whether or not the gene in question originates from an organism known to be allergenic, the initial stage of the assessment of a GMO's allergenic potential consists in comparing, in all cases, the sequence homology of the new protein with that of the sequences of known allergens listed in databases. If a sequence homology exists, the product is deemed to pose an allergenic risk.

If the new protein shows no sequence homology:

- **when the protein originates from a source known to be non-allergenic**, the protein should be tested on a panel of fifty samples of *targeted serum*, serum from allergic subjects selected on the basis of the origin of the protein responsible for the allergy (6 groups of source organisms are distinguished: yeasts/moulds, monocots and dicots, vertebrates and invertebrates, and others). If the protein presents no cross-reaction, an assessment is made of the protein's resistance to pepsin degradation based on a protocol established in accordance with Good Laboratory Practice. In principle, if the peptidic fragments obtained are smaller than 3.5 Kda, there is little likelihood of the protein being allergenic. The protein's sensitivity to pepsic digestion is, however, only one parameter for demonstrating an allergen.

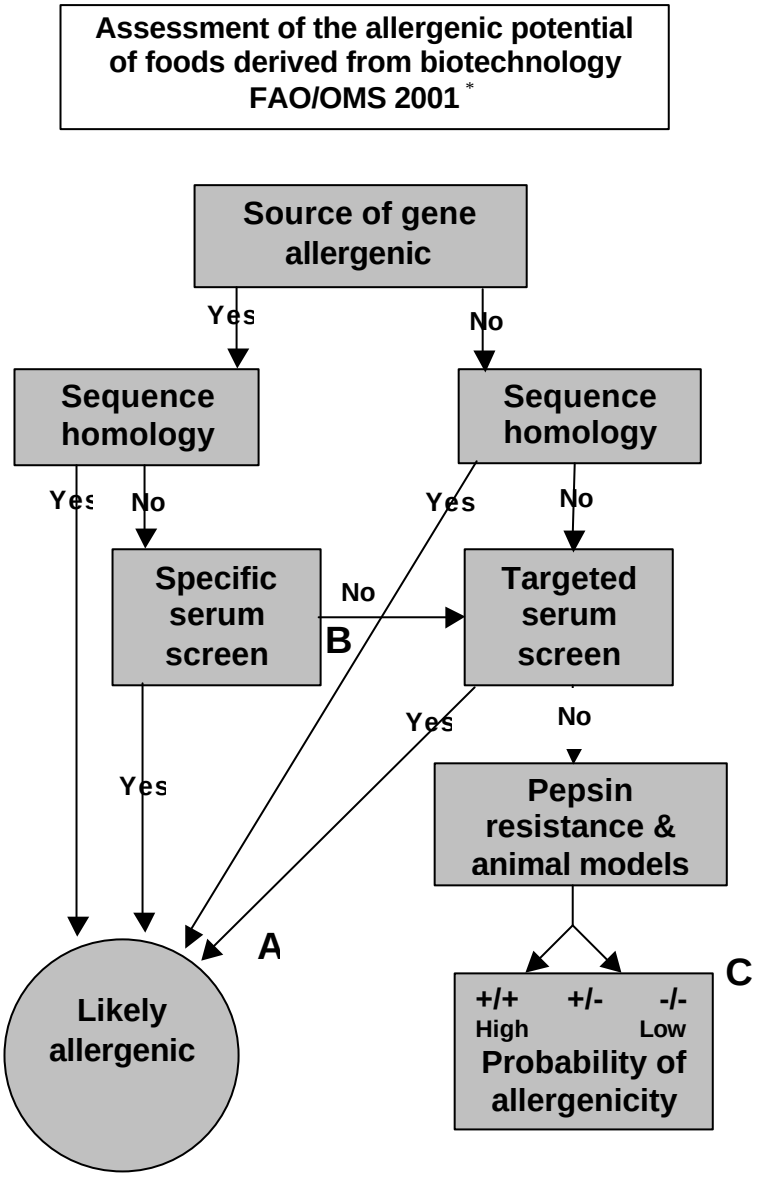
  The *in vitro* model does not claim to mimic the conditions of gastric digestion, which is why additional information on the protein's immunoallergenicity may be obtained from studies on animal models. However, these models also have limitations; they do not reflect all the aspects of IgE-mediated food allergies;

[12] Indicative test periods for pigs: 100 days, laying hens or quail: 2 months around peak of lay, chickens or turkeys: from day-old to slaughter, small ruminants: 3 months, dairy cow: 2 months.

- **when the protein originates from a source known to be allergenic**, the protein should be tested on *specific serums* from patients known to present allergies (the number and quality of such serums is discussed in FAO/WHO document 2001 [8]). If the protein does not show a cross-reaction with these specific serums, the absence of a cross-reaction with targeted serums is also verified. The resistance power of the protein to pepsin degradation is then assessed as well as its allergenic behaviour on animals.

  Finally, in special cases, to confirm or invalidate the results obtained in the preceding tests, clinical tests on patients allergic to certain foods may be conducted if necessary, subject to the agreement of the Ethics Committee.

All the tests conducted are required in order to eliminate a possible allergenicity. However, as in the case of a medicine in which the drug monitoring system may reveal unexpected effects in the medium or long term, the putting in place of a surveillance system after the product is placed on the market is absolutely essential. This is not without its problems, though, as because the GMO or the product containing or consisting of it is integrated into a complex diet which naturally contains a certain number of allergenic substances, the attribution of an allergic reaction to a GMO will be difficult to establish.



Notes

A Any positive results obtained from sequence homology comparisons to the sequences of known allergens in existing allergen databases or from serum screening protocols, both constructed in accordance with the guidelines defined in FAO/WHO document 2001*, indicate that the expressed protein is a likely allergenic.

B The degree of confidence in negative results obtained in the specific serum screen is enhanced by the examination of larger numbers of individual sera as explained in FAO/WHO document 2001*.Conducting the specific serum screen with small numbers of individual sera when larger numbers of such serums are readily available should be discouraged.

C When positive results are obtained in both the pepsin resistance and animal model protocols, the expressed protein has a high probability to become an allergen. When negative results are obtained in both protocols, the expressed protein is unlikely to become an allergen. When different results are obtained in the pepsin resistance and animal model protocols, the probability of allergenicity is intermediate, although rational explanations may be possible in some situations.

* Evaluation of the allergenicity of genetically modified foods. Report of a joint FAO/WHO expert consultation on the allergenicity of foods derived from biotechnology. 22-25 January 2001 [8]. The English version is the reference version.

3 TEST LIMITATIONS

However in-depth the initial assessment, designed, notably, to demonstrate unexpected potential effects and to predict any possible long term effects, it presents limitations on three different levels:

- limitations in terms of the use of new techniques still at the research stage,
- limitations in terms of the feasibility of certain tests,
- limitations in terms of the evaluation of long term effects in animals and effects in humans.

3.1 Limitations in terms of the use of new techniques still at the research stage

Certain aspects of risk assessment may improve in the future through the use of new analytical and statistical techniques or methods which are currently being developed. They may enable an overall view to be obtained of the changes caused by the genetic modification introduced on the synthesis of mRNA, of proteins (notably the creation of fusion proteins or the expression of other genes) and of metabolites (notably detecting an unexpected effect on other or between different metabolic pathways (amplification, deletion)). These new techniques or methods will enable:

- the identification or quantification of the mRNA transcribed in the plant cell, tissue or organ (transcriptome);
- characterisation of the proteins produced in the plant cell, tissue or organ (proteome).
- characterisation of the metabolites produced in the plant cell, tissue or organ (metabolome).

In terms more specifically of the characterisation of the proteins and metabolites produced by the genetically modified plant and its isogenic parent, both cultivated in several environments, the use of these new techniques will enable testing to:

1) approximate the influence of the genetic modification on protein expression (appearance and disappearance of proteins, extinction or modification of metabolic pathways),

2) identify the expression products resulting from the genetic modification introduced into a plant placed in contrasted and known environments,

3) analyse the adaptive responses of the GM plant genome and thereby to draw conclusions with greater understanding as to the consequences of genetic modification on composition and safety.

These techniques will be able to contribute to the interpretation of any possible differences in composition of the new products once a certain frame of reference is available based on an understanding of the natural variations in the various cellular components which exist in plants.

3.2 Limitations in terms of the feasibility of certain tests

Tolerance, feeding value and digestibility tests on target animals may provide information on the potential effects of chronic and massive consumption (up to 90% of the diet) of a product by animals or humans. They should also reveal the presence of potentially harmful factors or anti-nutritional factors.

These tests, however, comprise some constraints or practical difficulties in terms of:

- the need for a sufficiently large number of animals to be able to observe a possible effect,

- the preparation of material to be tested in sufficient quantities to expose the animals for long periods.

To ensure sufficient power in the statistical test, namely one which has a probability of demonstrating a statistically significant difference, it is necessary for an appropriate group of animals to be brought together. In addition, besides problems of cost, large quantities of genetically modified plants will be required to feed this large pool of animals throughout the test period. The products, subject to tests on the target animal within the context of the pre-marketing assessment, are only authorised as part of an experimental release, therefore only involving small plots of land. Since 1998, at least in Europe, experimental cultivation has been and still remains, very unpopular. This explains why most of the experiments involving large numbers of subjects have been conducted in the United States with genetically modified plants already authorised for cultivation and a limited number in France (1) and Germany (10) in 1998.

3.3 Limitations in terms of the evaluation of long term effects in animals and effects in humans

- ***Evaluating long term toxic effects on laboratory animals***

  Short term toxicity studies (90 days) on laboratory animals carried out to evaluate the modified protein or the transformed plant itself should reveal any toxic or harmful effects in relation to the introduced genetic modification.

  In the case of a GMO intended for food use, if the acute and sub-chronic toxicity studies have revealed a possible toxic effect, the GMO does not receive authorisation. It is also not proposed for an assessment by the notifier with a view to placing on the market.

  There may be some doubt, however, as to the necessity of prolonging (up to 2 years, for example) this type of testing on laboratory animals to look for other toxic or harmful effects which would not appear for a long time and require repeated dosing.

  In most cases, and up till now, the genetic modifications introduced into plants have not resulted in the creation of new chemical entities. It is for this reason that, as in the case of biological products product by living organisms (bacteria, yeasts, plants, animals) intended for use as medicines, with some exceptions, such long terms studies are not justified. However, in certain specific cases, if alerting elements arising from the data available in the dossier for consent for placing on the market suggested the existence of a particular hazard, it would then be necessary to expand the evaluation, on a case by case basis, through additional tests.

  Such tests present a number of difficulties in terms of their conduct:

  - prior to testing, the potentially toxic effect and its possible consequences are unknown;

  - this type of test necessitates, to be able to observe an effect, the administration of high doses, whilst respecting the animals' nutritional balance, while the potentially toxic element is generally synthesised in very small quantities, and the use of a large number of animals.

- **Evaluation of effects in humans (clinical trials)**

  The possibility of implementing study protocols in humans for GMOs or products derived from GMOs, based on the clinical trial model, which would involve "testing" them on a limited number of volunteers prior to a wider distribution to the whole of a population, constitutes a concept which on the face of it merits examination.

In the case of a GMO intended for food use and claiming a health benefit, the conduct of such tests would be fully justified in order to verify the claim being made. This aspect is not developed further in this opinion, since it is concerned only with risk assessment.[13]

As regards GMOs for which the sole effects sought do not involve health (crop improvement, environmental protection, etc.), such studies would be very hard to justify in terms of ethics. Moreover, the strengthening of studies on animals, as described in this opinion, would appear better suited to the assessment of risk. To implement clinical studies, a number of theoretical, ethical and practical difficulties would have to be overcome:

- in general terms, the demonstration of the long term effects of food on health depends more on the overall diet and the interaction of this food with the lifestyle as a whole than on a particular food;

- such trials would necessitate rigorous (randomised, double blind), long-term studies, all the more difficult to conduct given that the ingestion of the actual (and unique) GMO food, even in varied forms, could be impractical over long observation periods;

- when no health claim is being made, it would seem extremely difficult, the face of it, to identify the assessment criteria for such a study, the expected effect to be tested – even less the extent of this effect – and the number of subjects for inclusion in the study;

- it is difficult to conceive what type of study would enable the determination of the potential effects on all the physiological systems (cardiovascular, cognitive, reproductive, immunological, skeletal, etc.).

In fact, this review could be widened, beyond GMOs, to include nutriments such as vitamins or minerals sometimes consumed in large amounts in concentrated form.

3 RISK ASSESSMENT AFTER PLACING ON THE MARKET

However in-depth the risk assessment prior to placing on the market, surveillance of the products marketed is still required in order to be able to detect any occurrence of potential effects, both unexpected or cumulative in the long term, and the manifestation of any harmful effects which may be revealed in particularly sensitive subjects or populations. To that end, new provisions have been introduced in Directive 2001/18/EC of the European Parliament and of the Council and the draft regulation on foodstuffs containing or consisting of GMOs, which provide for the design and implementation of a surveillance plan for monitoring GMOs after their placing on the market. This surveillance forms part of the risk assessment and the data collected will be added to the data from the initial assessment.

[13] On the concept of evaluating the health benefits of GMOs, refer to the proceedings of the symposium organised by AFSSA "GMOs and food: can benefits for health be evaluated?" held on 17 and 18 December 2001, in Paris.

Question 2: What are the risks associated with human and animal consumption of GMOs containing antibiotic-resistance genes?

The emergence over more than a decade of pathogenic bacteria resistant to a number of antibiotics is a subject of concern in human and veterinary pathology. It is for this reason that public opinion and scientists are currently concerned, with good reason, by the appearance and use of transgenic plants carrying antibiotic-resistance genes used as selection markers in the preparation of these new plants.

Are the antibiotic-resistance genes introduced into the genome of a transgenic plant liable, in different ecological situations, to transfer to bacteria and result in an expansion of antibiotic resistance likely to be harmful to human health?

This question has already been examined by a group of experts as part of a joint seminar of the Genetic Engineering and Biomolecular Engineering Committees held in January 1999 which led to the signature in January 2000 of a joint opinion from these two scientific bodies [9, 10].

Insofar as the presence of antibiotic-resistance genes in environmental bacteria is an objective risk but that the probability of transfer of these genes from a plant to the soil bacteria is a potential risk, these two risks can be compared and evaluated through the examination of the following points:

A What are the conditions for using antibiotic-resistance genes in genetically modified plants?

B What are the conditions for the transfer of a gene from a plant to a bacterium? Although this transfer has never been observed, is it conceivable?

C What is the contribution of the natural pool of antibiotic-resistance genes to the development and/or the release of antibiotic-resistance genes?

D The massive use of antibiotics in human and veterinary medicine is creating a selection pressure in favour of bacteria which have acquired resistance genes. Should a possible widening of the resistance spectrum during successive transfers of genes be taken into consideration?

E What additional risk could plant transgenesis pose compared with the current situation in terms of the observed increase in antibiotic resistance?

Insofar as, at the present time, the only GMOs on the market are plants, the elements of evaluation discussed below will concern plants. However, although no genetically modified micro-organism is yet in use in human or animal feed, we will look at the risks associated with the use of antibiotic-resistance genes as selection markers in micro-organisms.

A What are the conditions for using antibiotic-resistance genes in genetically modified plants?

It is possible to differentiate between the use of antibiotic-resistance genes according to whether these genes are used: i) as markers at the point when the transforming plasmids are constructed, ampicillin being used in that case, or ii) for the selection of transformed plant cells, with kanamycin being the most often used.

Antibiotic-resistance genes are thus used as markers for the selection of:

- the vector for the genetic sequence of interest; in this case, the antibiotic-resistance gene (ampicillin) is under the control of a bacterial promoter,

- the transformed elements of the plant intended to generate the transgenic plants sought; in this second case, the antibiotic-resistance gene (kanamycin) is under the control of a plant eukaryote type promoter.

The risk assessment associated with the use of this type of marker gene now falls within a slightly different regulatory regime as the new Directive 2001/18/EC [3] provides in its provisions (Article 4-2) for the *" …phasing out of antibiotic resistance markers in GMOs which may have adverse effects on human health and the environment."* The text states that this phasing out must take place by 31 December 2004 in the case of GMOs placed on the market according to Part C and by 31 December 2008 in the case of GMOs authorised under Part B (deliberate release for any purpose other than placing on the market).

Regarding the markers used for selecting the vector for the genetic sequence of interest, other than Bt 176 maize (old construction) which contains a gene conferring resistance to ampicillin, it should be noted that the recent constructions authorised have been freed of the marker genes used for the construction of the transforming plasmid.

In terms of the selection markers for the transformed plant cells, the use in human medicine of kanamycin, as in veterinary medicine, is now very infrequent. It has been suggested that use of the gene conferring kanamycin resistance could result in the emergence of resistance to amikacin through isolated mutations of the NPT11 gene or increase its level of resistance. However, on the one hand, amikacin[14] is a poor substrate for the NPT11 inactivation enzyme (neomycin phosphotransferase) and on the other hand, these mutations have been obtained in *Escherichia coli* exclusively in a laboratory under extremely rigorous selection conditions [11-16]. Hygromycin, likely to be used in future constructions, is no longer used for treating humans (information from Dr P. Berche, U411-INSERM).

B What are the conditions for the transfer of a gene from a plant to a bacterium? Although this transfer has never been observed, is it conceivable?

The mobilisation of a genetic sequence conferring resistance to an antibiotic from GM plants to soil bacteria, a bacteria from the intestinal tracts of herbivores or humans, implies a cascade of interspecific horizontal transfer processes [17]. Furthermore, it would be necessary for the sequence to be integrated into the final host in conditions in which it could be expressed. These stages are as follows:

- release of the plant's DNA into the environment;

- persistence of DNA fragments in the environment;

- transformation of competent bacteria (physiological state at a given moment of certain bacteria which enables them to incorporate fragments of nucleic acids into their cytoplasm) by the DNA retained (the absorption of DNA in soil clay [18, 19] can favour the conservation of DNA fragments but renders them less available for transformation);

- integration (or maintenance) in the bacterial genome of this DNA originating from the transformed plant; this stage is limiting due to the existence of effective restriction systems (after passage into the plant, a DNA of bacterial origin is no longer recognised as a bacterial DNA), to the necessity for homologous sequences to enable recombination and generally to the initial absence of replication.

- expression/selection; the selection pressure which may exist in the soil microniches may play an important role.

[14] The resistance enzymes inactivating the amikacin are essentially acetylases and adenylases or nucleotidyl-transferases and not phosphotransferases (NPT). The genes coding for these inactivation enzymes are different and it seems highly unlikely that a truncated NPT11 gene, as present in certain constructions, could result in amikacin resistance.

The likely combination of these conditions makes the accomplishment of such a transfer highly improbable.

The calculations for estimating the transfer frequency of genetic sequences of prokaryotic origin from GM plants to bacteria are not very convi ncing. These transfers are theoretically possible, but to date no published study has demonstrated such a transfer. Moreover, there is no data to allow the supposition that genetic sequences of non-plant origin introduced into the plant's genome would behave differently from the other plant genetic sequences as regards the penetration and sensitivity to restriction enzymes.

C What is the contribution of the existing natural pool of antibiotic-resistance genes to the development and/or the release of these genes?

Antibiotics are substances synthesised by living organisms, certain fungi in particular, which have the property of halting the growth of micro-organisms. In order to protect themselves from the deleterious effects of these substances, certain micro-organisms have therefore developed resistance to these antibiotics. The soil bacteria naturally contain antibiotic-resistance genes which they have acquired due to selection pressure. These genes are most often carried by the bacterial cell's mobile genetic elements (transposons, plasmids).

Therefore, the existence of antibiotic resistance had been recognised prior to the use of most antibiotics [20].

A study [21] has shown that the digestive tracts of humans and animals already contain plenty of plasmids carrying these antibiotic-resistance genes, even in the new-born, two days after birth.

D The massive use of antibiotics in human and veterinary medicine is creating selection pressure in favour of bacteria which have acquired resistance genes. Should a possible widening of the resistance spectrum during successive transfers of genes be taken into consideration?

Antibiotics are widely used in human and veterinary medical treatment. They are also administered under veterinary supervision as a prophylactic treatment for animals at doses close to therapeutic doses. Finally, they have been extensively used as a growth factor in animal feed at much lower doses. This potential cause of resistance should reduce in the future as, of the eight antibiotics permitted as growth factors under the terms of Council Directive 70/254/EEC [22], only four are still permitted, of which two are ionophores.

All these uses are contributing to an increase in the selection of resistances. In the veterinary field, the growth in antibiotic resistance is relatively well-known [23, 24] and regularly monitored through surveillance plans conducted within the context of the control of veterinary medicines.

In the human field, although research into antibiotic resistance in adult humans has not been the subject of very extensive studies, a number of multi-centre studies with the aim of evaluating the antibiotic sensitivity of certain current strains prevalent in patients seen in general practice [25] or in hospital [26] have been conducted. These studies show, within the limits of the types of infections studied, the nature of the bacteria isolated and the antibiotics used, an increase in resistance of *E.coli* strains to certain β-lactamines, both in general practice and in hospitals, in particular in amoxycillin-clavulanic acid combinations; resistance to quinolones and aminosides seems more marked in strains of hospital origin due to a lower rate of use of quinolones in general practice. In addition, antibiotic resistance in hospitals is monitored in France by the Comités de Lutte contre les Infections Nosocomiales (CLIN) [Committees for the Combat of Hospital-borne Infections].

E What additional risk could plant transgenesis present compared with the current situation in terms of the observed increase in antibiotic resistance?

Based on the elements discussed above, it would appear that:

- the risk of transferring genetic sequences of prokaryotic origin from transgenic plants to soil micro-organisms is a theoretical risk but one which to date has not actually been reported. It has not been demonstrated in either natural or experimental conditions;

- the probability of transfer of an antibiotic resistance gene from plant to bacteria is very low, given the set of conditions required to enable such a transfer to occur;

- the natural prevalence of genes for resistance to kanamycin and ampicillin is very high in telluric bacteria and the digestive tracts of humans and animals;

- the use of antibiotics as a growth factor in animal nutrition and their use in human and veterinary medicine are recognised as a major source of the emergence and spread of antibiotic resistance, on an altogether different scale from the hypothetical risk associated with the presence of an antibiotic-resistance gene in a genetically modified plant.

As far as we know, the consumption by humans or animals of food products containing or consisting of genetically modified plants containing genes for resistance to kanamycin and/or ampicillin does not, in consequence, pose any more than a theoretical, and in any case, negligible, risk to human or animal health in terms of the presence of these antibiotic-resistance genes in the environmental bacteria. While the constructions using ampicillin as a selection marker for the transforming vector are now free of this type of marker, the substitute methods which would remove the need for a plant selection marker (kanamycin type) are still at the development stage and require validation.

For future constructions, as provided for in Directive 2001/81/EEC of the European Parliament and of the Council [3], the recommendation is that the presence should be avoided, in genetically modified plants intended for human and animal consumption, of antibiotic-resistance genes likely to have damaging effects on human and animal health.

The case of genetically modified micro-organisms

The same level of risk should not be assigned to the transfer of an antibiotic-resistance gene from plants, which is highly unlikely, as to that from micro-organisms which could pose a much greater risk, as the genetic barriers (such as eukaryote/prokaryote promoters) do not exist or are not as effective.

Although no genetically modified micro-organism intended for human or animal consumption has yet received consent for placing on the market in the European Union, there is a need to adopt a much more restrictive approach to the use of antibiotic-resistance genes as selection markers in micro-organisms[15] intended for human or animal consumption; the risk of transfer of these genes to the intestinal flora bacteria would then be considerable.

[15] Certain micro-organisms used in food production possess intrinsic antibiotic resistance. There is no reason to exclude such micro-organisms from being receiver organisms as long as it has been verified that these resistance genes are not carried by the organism's mobile genetic elements (plasmids, transposons and integrons).

CONCLUSIONS

QUESTION 1

What are the key points for assessing the health risks associated with human and animal consumption of GMOs or products derived from GMOs and what are the relevant elements of this assessment?

The scientific review carried out by the French Food Safety Agency (AFSSA) focussed on the key points in the safety assessment of GMOs or products derived from GMOs intended for human or animal consumption, with regard to the regulatory provisions laid down by Council Directive 90/220/EEC (or 2001/18/EC which will replace Council Directive 90/220/EEC in October 2002) and Regulation (EC) No. 258/97 (Commission Recommendations of 29 July 1997) and the planned reviews of European and international guidelines (Codex Alimentarius).

The information relating to genetic modification, on the one hand, and toxicological information on the other hand, is essentially to establish the *a priori* safety of a new food. For some of these key points, AFSSA has identified a certain amount of data which is currently lacking or insufficiently documented which might serve to refine the food safety assessment of GMOs or products derived from GMOs before they are placed on the market.

Information relating to genetic modification and the genetically modified plant

The data usually supplied in the dossiers describing the DNA sequences inserted or deleted should be supplemented by the transgene sequence[16] and the regions forming the junction with the genome. These additional data would have the purpose of:

- verifying whether the inserted fragment is genuinely identical to the one introduced into the transforming vector;
- examining the environment of the inserted gene;
- localising the insert in the plant's genome.

The origin of genetically modified plants

In view of the influence of environmental factors on plant growth, this assessment should be conducted on plants which have been in cultivation for at least two seasons in different sites representative of a variety of environments.

Evaluation of the sub-chronic toxicity of the gene product on laboratory animals

With very few exceptions, the only elements available relating to toxicity have been obtained by single doses (acute toxicity) on laboratory animals, carried out with the purified protein.

Because animal or human exposure to GMOs is chronic, exposures which are themselves also chronic must be carried out. These will enable the revelation, with improved probability, of the potential effects on the body's systems, particularly the immune, hormonal and reproductive systems, and possible effects associated with accumulation phenomena.

A 90-day sub-chronic toxicity test of the gene product on laboratory animals (rats, mice, guinea pigs) is aimed at demonstrating potential harmful effects on the development of the body's organs or systems when there is long term exposure.

[16] In order to facilitate the comparison of the sequences supplied with the databases, it would be desirable for these sequences to be supplied to the assessor in computerised form.

Evaluation on animals of tolerance of the finished product

The objective of these studies, carried out with the product containing or consisting of a GMO (parts of the plant or derived products intended for consumption) is to demonstrate the potential effects of the regular consumption of a product by humans or animals and the unexpected or unintentional toxic effects which would not be revealed in acute or sub-chronic toxicity studies.

Two types of study would enable such effects to be demonstrated:

- tolerance/toxicity studies on laboratory animals of the parts of the plant intended for consumption or their by-products;

- tolerance, feeding value and digestibility studies on the target animals of the parts of the plant intended for consumption or their by-products.

The results of such tests may not be statistically significant due to the numbers of animals tested being too low. The concept of the statistical power of a test therefore has to be taken into consideration (see Annex 1). The number of animals, their physiological stage (stage of lactation, age/weight at start of test, etc.) the parameters selected for monitoring and the test period must be defined so as to allow demonstration of a possible difference of one standard deviation between the treatments with a statistical power of at least 80%.

Test limitations

However in-depth the initial assessment which is intended, notably, to demonstrate any potential unexpected effects and to predict any long term effects, it has limitations on three different levels:

- ♦ limitations in terms of the use of new techniques still at the research stage,
- ♦ limitations in terms of the feasibility of certain tests,
- ♦ limitations in terms of the evaluation of long term effects in animals and effects in humans.

- **Limitations in terms of the use of new techniques**

Certain aspects of risk assessment may improve in the future through the use of new analytical and statistical techniques or methods which are currently being developed. They may enable an overall view to be obtained of the changes caused by the genetic modification introduced on the synthesis of mRNA, on proteins (notably the creation of fusion proteins or the expression of other genes) and metabolites (notably detecting an unexpected effect on other or between different metabolic pathways (amplification, deletion).

- **Limitations in terms of the feasibility of certain tests**

Tolerance, feeding value and digestibility tests on target animals comprise some constraints or practical difficulties in terms of:

- the necessity for a sufficiently large number of animals to enable the demonstration of a statistically significant difference;

- the preparation of material to be tested in sufficient quantities to expose the animals for long periods in view of the fact that these products, subject to tests within the context of the pre-marketing assessment, are only authorised as part of an experimental release, therefore only involving small plots of land.

- **Limitations regarding the evaluation of long term effects**

The evaluation of long term toxic effects on laboratory animals poses a number of problems of realisation:

- the potentially toxic element and its possible consequences are unknown beforehand;

- this type of test requires, in order to observe an effect, the administration of high doses, whilst respecting the animal's nutritional balance, while the potentially toxic element is generally synthesised in very small quantities as well as the use of a large number of animals.

The evaluation of effects in humans using study protocols of the clinical trial variety seems less appropriate to the assessment of risk than the strengthening of tests on animals as described in this opinion. Use of clinical trials would involve a number of theoretical, ethical and practical restrictions being overcome. However, in the case of a GMO intended for food use which claimed a health benefit, the conduct of such trials would be fully justified to demonstrate the claim being made[17] (an aspect not discussed in this opinion on risk assessment). This review could be extended, beyond GMOs, to include nutriments such as vitamins or minerals sometimes consumed in large quantities in concentrated form.

The design and implementation of a surveillance plan for monitoring GMOs after their placing on the market (c.f. Directive 2001/18/EC and the draft regulation on foodstuffs containing or consisting of GMOs) should enable the highlighting of any harmful effects which may be revealed in particularly sensitive subjects or populations.

QUESTION 2

What are the risks associated with human and animal consumption of GMOs containing antibiotic-resistance genes?

In other words, are the antibiotic-resistance genes introduced into the genome of a transgenic plant liable, in different ecological situations, to transfer to bacteria and result in an expansion of antibiotic resistance likely to be harmful to human health?

Antibiotics used as selection markers

When constructing GMOs, antibiotic-resistance genes are used as markers for the selection of:

- the vector for the genetic sequence of interest; in this case the antibiotic-resistance gene (ampicillin type) is under the control of a bacterial promoter;
- transformed elements of the plant intended to generate the transgenic plants being sought; in this second case, the antibiotic-resistance gene (kanamycin type) is under the control of a plant eukaryote promoter.

In order to answer this question, the AFSSA experts studied the following points[18]

What are the conditions for using antibiotic-resistance genes in genetically modified plants?

What are the conditions for the transfer of a gene from a plant to a bacterium? Although this transfer has never been observed, is it conceivable?

What is the contribution of the natural pool of antibiotic-resistance genes to the development and/or the release of antibiotic-resistance genes?

The massive use of antibiotics in human and veterinary medicine is creating a

[17] C.f. proceedings of the AFSSA symposium 17/18-12-01 "GMOs and food: can benefits for health be evaluated?"

[18] This question has already been examined by a group of experts within the context of a joint seminar of the Genetic Engineering and Biomolecular Engineering Committees in January 1999 and led to the signature in January 2000 of a joint opinion from these two scientific bodies.

selection pressure in favour of bacteria which have acquired genes for resistance. Should a possible widening of the resistance spectrum during successive transfers of genes be taken into consideration?

What additional risk could plant transgenesis present compared with the current situation in terms of the observed increase in antibiotic resistance?

The experts took the view that

- the risk of the transfer of genetic sequences of prokaryotic origin from transgenic plants to soil micro-organisms is a theoretical risk but one which has not yet been reported in reality. It has not been demonstrated either in natural or in experimental conditions;

- the probability of the transfer of an antibiotic-resistance gene from plant to bacteria is very low given all the conditions required to enable such a transfer to occur;

- the natural prevalence of genes for resistance to kanamycin and ampicillin is very high in the telluric bacteria and the digestive tracts of animals and humans;

- the use of antibiotics as a growth factor in animal nutrition and their use in human and veterinary medicine are recognised as a major source for the emergence and spread of antibiotic resistance.

As far as we know, the consumption by humans or animals of food products containing or consisting of genetically modified plants containing resistance genes to kanamycin and/or ampicillin does not, in consequence, pose any more than a theoretical, and in any case, negligible, risk to human or animal health in terms of the presence of these antibiotic resistance genes in the environmental bacteria. While the constructions using ampicillin as a selection marker for the transforming vector are now free of this type of marker, the substitute methods which would remove the need for a plant selection marker (kanamycin type) are still at the development stage and require validation.

For future constructions, as provided for in Directive 2001/81/EEC [3], the recommendation is that the presence should be avoided, in genetically modified plants intended for human and animal consumption, of antibiotic-resistance genes likely to have damaging effects on human and animal health.

The case of genetically modified micro-organisms

Although no genetically modified micro-organisms intended for human or animal consumption have yet received consent for placing on the market in the European Union, the use of antibiotic-resistance genes as selection markers could pose a considerable risk of transfer of these genes to the bacteria of the intestinal flora, as the genetic barriers (such as eukaryote/prokaryote promoters, the presence of a restriction system) would no longer exist or would not be as effective.

There is a need therefore, for much greater restrictions on the use of antibiotic-resistance genes as a selection markers in micro-organisms[19] intended for human or animal consumption.

[19] Certain micro-organisms used in food production possess intrinsic antibiotic resistance. There is no reason to exclude such micro-organisms from being receiver organisms as long as it has been verified that these resistance genes are not carried by the organism's mobile genetic elements (plasmids, transposons and integrons).

ANNEX 1

THE STATISTICAL POWER OF THE TESTS ON TARGET ANIMALS INCLUDED IN THE RISK ASSESSMENT OF THE CONSUMPTION OF GENETICALLY MODIFIED ORGANISMS

The objective of the tests on target animals is **to show that the incorporation of GMOs in the diet has no *harmful effect* on a certain number of zootechnical criteria** to be defined, namely, the **equivalence** of these criteria between the animals given feed based on new plant varieties and the animals given feed based on non-modified plant varieties. Now, "[standard] experiments are designed to demonstrate a **difference** between two or more treatments. It could be, however, that the experimenter's aim is to prove that there is no difference between these treatments. One knows that one cannot content oneself with conducting the usual test in the hope that it will be non-significant, as a non-significant result only means that one has not succeeded in proving that the difference existed. Moreover, to obtain a non-significant result, one simply has to work with very low sample size." [27].

In these tests, a sufficient number of animals must be used to ensure **the statistical power of the test**[20], meaning the probability of **demonstrating a given difference** which is sufficient and tested.

"The power of a test may be compared to that of a magnifying glass: if one perceives a sign, one can confirm its existence; if one does not perceive it, one cannot confirm that it does not exist as it might be perceptible with a more powerful magnifier." [28]

The number of animals required for the obtention of a given power depends on several factors:

- the natural variability of the studied characteristic: the more the characteristic is naturally stable in a population, the easier it is to demonstrate a slight difference;
- the power desired, defined beforehand (i.e. before the protocol is produced): the higher the desired power, the higher the number of animals required;
- the difference one wishes to demonstrate: the slighter the difference, the more individuals required;
- the type I error allowed (generally: α=5%);
- the one-tailed or two-tailed nature of the statistical test: in practice, one could use a **two-tailed** test if the harmful effect corresponded to a decrease **or** an increase in the parameter studied. One could use a **one-tailed** test if the harmful effect was expressed as a variation of the parameter in a single direction, known beforehand[21].

Statistical method enables the calculation of a minimum sample size based on this difference, the α risk allowed (generally 5%), the power required, the variability of the characteristic studied and the two-tailed or one-tailed character of the test conducted. An illustration of this calculation is given below.

To calculate the number of subjects to be studied, one must therefore define **the difference one wishes to be able to demonstrate** between a control group and a treated group. The slighter this "**biologically significant**" difference, the more animals required for a given power.

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[20] The power of a statistical test corresponds to the probability of concluding a statistically significant difference, if one genuinely exists. This concept should not be confused with the α risk, generally with a selected value of 5%, corresponding to the probability of concluding a significant difference in the absence of an actual difference.

[21] For example, if the parameter studied is Average Daily Weight Gain (ADWG), the harmful effect is a **decrease** in ADWG (an increase in ADWG is not, in principle, considered a harmful effect): one would use a one-tailed test. If the parameter is the secretion of a hormone, the harmful effect could be an increase **or** a decrease in the secretion: one would use a two-tailed test. NB: if the test objective is to ensure strict equivalence between GMOs and non-GMOs (no variation in the parameter desired, in any direction): one would always use a two-tailed test.

The difference to be demonstrated can be expressed either as a percentage of acceptable variation (1%, 2% difference, and so on), or as a value of acceptable variation (difference of 1 unit, 2 units, and so on). The expression of this difference as a number of standard deviations can enable one to be freed from the need to fix a difference for each parameter (c.f. illustration below).

In practice, one usually defines:

- the one-tailed or two-tailed character of the statistical test required, based on the parameter being studied:
  - if the harmful effect corresponds to a decrease in the parameter (an increase in the parameter is not considered harmful): one-tailed test;
  - if the harmful effect corresponds to an increase in the parameter (a decrease in the parameter is not considered harmful): one-tailed test;
  - if the harmful effect corresponds to an increase **or** a decrease in the parameter, or if the objective is strictly an objective of equivalence between GMOs and non-GMOs (no variation in the parameter desired, in either one direction or the other): two-tailed test;
- the principal biological indicators, devoted *F*, enabling the demonstration of a difference in physiological behaviour in the animals after ingestion of GMOs;
- for each of these criteria, a variation threshold, devoted r or d, (see below) considered **biologically** significant;
- the minimum statistical test power, devoted *x*, considered necessary for these tests;

The tests on target animals should show that no statistically significant difference exists at threshold α% for the factor *F* (one-tailed test/two-tailed test) between control animals and animals fed on GMOs, with the protocol being able to demonstrate a difference of r (%) (or d standard deviations[22]) with a power of *x*%.

Illustration of a calculation to determine the number of animals based on the choice of statistical parameters

Based on the results of a variety of experiments measuring a biological parameter, conducted on 60 chickens, the irreducible "natural" variability of this biological parameter is evaluated.

This variability can be estimated whatever the experimental protocol (Latin square, balanced complete block, unbalanced incomplete block, etc.), after taking into account the different effects tested by the experimenter (treatment effect or block effect, for example). In practice, if one considers that the variance of this parameter is the same in all groups (control and treated), the best estimate of this variability is the residual variance obtained from a variance analysis.

From the "control" groups (14 individuals), one estimates the mean of a population fed on a normal diet.

Using these two estimates, it is possible to calculate an order of magnitude for the number of subjects needed in each group (control and treated) to provide a given **power**, based on the percentage difference compared with the mean which one **hopes** to be able to demonstrate.

*Devoting s^2 the estimate of the variability of the characteristic studied (s is therefore the standard deviation), a = 5% the type I error allowed by the experimenter, b the type II error (1- b is therefore the power), r the percentage decrease (or increase) of the characteristic considered to be biologically significant and m the estimate of the mean, one obtains the number of subjects necessary n <u>in each group</u> (control and treated) for a **one-tailed test**:*

[22] See example

$$n = \frac{2\,s^2}{(r\ m)^2}\left(t_{2n-2,1-a} - t_{2n-2,b}\right)^2 \qquad (1);$$

in which $t_{2n-2,1-a}$ is the (1-a) percentile of a 2n-2 degrees of freedom Student distribution. For a ***two-tailed*** *test:*

$$n = \frac{2\,s^2}{(r\ m)^2}\left(t_{2n-2,1-a/2} - t_{2n-2,b}\right)^2 \qquad (1);$$

in which $t_{2n-2,1-a/2}$ is the (1-a/2) percentile of a 2n-2 degrees of freedom Student distribution. The condition of application of this formula is a normal distribution of the parameter, with identical variance in each group.

Results (example: one-tailed test, a = 5%):

m = 13.12 s = 0.31

		Power				
ρ	ρm	80%	85%	90%	95%	99%
-1%	-0.13	68	79	94	119	173
-2%	-0.26	18	21	25	31	45
-3%	-0.39	9	10	12	15	21
-4%	-0.52	6	6	7	9	13
-5%	-0.66	4	5	5	6	9

Table 1: number of animals required in each group to ensure a given power, based on the relative decrease in the parameter one wishes to demonstrate (one-tailed test)[23]

Interpretation

Statistical: to have a 90%[24] power in order to demonstrate a 1% **decrease** in the parameter compared with the mean (or a decrease of 0.13), a minimum of **94** chickens must be used per group. For a 99% power, **173** chickens are required per group.

Or, in more practical terms:

If the **decrease** in the parameter in the treated group is **actually** 1% (or - 0.13) one has at least a 90% chance of detecting it using an experiment involving **94** chickens per group. If, with such an experiment, the one-tailed test is not significant at the α threshold of 5%, one could then say without much chance of being wrong (less than 10%) that the actual decrease in the parameter associated with the consumption of GMOs is less than or equal to 1%.

Using 173 chickens per group, one has at least 99% chance of detecting a difference less than or equal to 1%.

It should be noted that the residual variability of this characteristic is very slight: therefore few animals are required to demonstrate, with a good power, a slight variation in this criterion.

[23] As with any evaluation of the number of subjects required for an experiment, these figures, produced from estimates, should only be considered as orders of magnitude.

[24] The values selected are only supplied to illustrate the example and do not in any way prejudge the biological significance of the difference thresholds proposed.

NB: the above calculation can only be carried out with iterations. A more direct calculation of n could be carried out by using an approximation of the Student distribution by the Normal law. The calculation is then straightforward:

- *one-tailed test:*

$$n = \frac{2\,s^2}{(rm)^2}\left(e_{1-a} - e_b\right)^2 \qquad (3)$$

in which e_{1-a} is the (1-a) percentile of a standard normal distribution (e_{1-a} = 1.64 if a = 5%)

- two-tailed test:

$$n = \frac{2\,s^2}{(rm)^2}\left(e_{1-a/2} - e_b\right)^2 \qquad (3)$$

in which e_{1-a} is the percentile (1-a/2) of a standard normal distribution ($e_{1-a/2}$ = 1.96 if a = 5%), and e_b is the b percentile of a standard normal distribution (e_b = -0.84 for a power of 80%, -1.28 for a power of 90%, -1.64 for a power of 95% and –2.33 for a power of 99%) [29]. The application condition of this formula is a normal distribution of the parameter in the two populations and n ≥30.

Generalisation

It is possible to express the difference one wishes to demonstrate, not in a relative variation compared with the mean (parameter ρ) but in number of standard deviations (which we will devote δ). If, in the formula (1) one replaces the parameter ρm with a parameter δs one then obtains the following formulas:

- one-tailed test:

$$n = \frac{2}{(d)^2}\left(t_{2n-2,1-a} - t_{2n-2,b}\right)^2 \qquad (5)$$

- two-tailed test:

$$n = \frac{2}{(d)^2}\left(t_{2n-2,1-a/2} - t_{2n-2,b}\right)^2 \qquad (5)$$

which no longer depend on the standard deviation of the parameter s. Parameter δ represents the number of standard deviations of difference which one wishes to be able to demonstrate. The following table is obtained:

	Power				
d	**80%**	**85%**	**90%**	**95%**	**99%**
¼	199	231	275	348	506
½	51	59	70	88	128
1	14	16	18	23	33
3/2	7	8	9	11	16
2	5	5	6	7	10

Table 2: number of animals required in each group to ensure a given power, based on the decrease in the parameter which one hopes to demonstrate (unit: number of standard deviations, a =5%, one-tailed test)

Interpretation
To demonstrate a difference of one standard deviation between the control group and the treated group with a power of 95%, 28 individual must be used in each group.

	Power				
d	**80%**	**85%**	**90%**	**95%**	**99%**
¼	253	289	338	417	590
½	64	73	86	106	149
1	17	19	23	28	39
3/2	9	10	11	13	18
2	6	6	7	8	11

Table 3: number of animals required in each group to ensure a given power, based on the decrease in the parameter which one likes to demonstrate (unit: number of standard deviations a =5%, two-tailed test)

Interpretation
To demonstrate a change (decrease or increase) of one standard deviation between the control group and the treated group with a power of 95%, 28 individuals should be used in each group.

These tables are in theory applicable to all normal distribution parameters in a population.

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ANNEX 3

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