

Comments of Consumers Union on Docket No. 2004D-0369,  
“Draft Guidance for Industry: Recommendations for the Early Food Safety  
Evaluation of New Non-Pesticidal Proteins Produced by New Plant Varieties  
Intended for Food Use”

January 17, 2005

**Introduction/Summary**

Consumers Union\*, the non-profit publisher of Consumer Reports magazine, appreciates the opportunity to comment on Docket No. 2004D-0369, “Draft Guidance for Industry: Recommendations for the Early Food Safety Evaluation of New Non-Pesticidal Proteins Produced by New Plant Varieties Intended for Food Use”

We commend the FDA for addressing the issue of early food safety evaluation before field testing of transgenic proteins produced by genetically engineered plants. However, we do not feel the agency’s proposal goes far enough. The present proposals are far too weak and could conceivably lead to a less safe, rather than more safe, food supply. The proposals do not recommend enough testing—only recommending testing for toxicity and allergenicity of the product of the inserted gene. The FDA proposals ignore the potential for nutritional changes in genetically engineered foods and the potential for unexpected and unintended effects, both of which could result in unsafe food. Furthermore, this inadequate safety assessment is entirely voluntary; and the FDA even lacks the authority to stop the company from marketing the plants even if found unsafe. Finally, FDA establishes no limit (or tolerance level) on the level of “inadvertent, intermittent” contamination with incompletely-tested or un-tested proteins that result from genetic engineering.

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We are concerned that companies, upon completing such a skimpy early food safety evaluation, might decide not to do a complete safety assessment. We are in case there are no limits on contamination, companies might even be more lax with the containment procedures during their field trials. If the FDA found their inserted engineered protein to be safe, why bother trying to contain the pollen during a field test, as any level of contamination would be permitted? As a result the U.S. food supply could become contaminated with in-completely tested or untested proteins. This could pose safety problems and could result in trade problems if other countries decide to ban such contaminated food items. The proposed level of testing is far lower than that proposed by FDA in their 2001 Pre-market Biotech Notification proposal (at <http://www.cfsan.fda.gov/~lrd/fr010118.html>)

For the reasons described below, we urge the FDA to require:

- **A full safety assessment before field testing of transgenic crops.** The full range of tests required in the food safety assessment should be at least as stringent as the range of tests laid out in the Codex Alimentarius “Guidelines for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants” (CAG/GL 45-2003) and as complete as in the 2001 PBN proposal (e.g. data should be required from each separate transformation event). For the allergenicity testing, we feel the companies should be required to follow the protocols laid out by the WHO/FAO Joint Expert Consultation on the Allergenicity of Foods Derived from Modern Biotechnology and as further elaborated by Gendel (1998a,b) and Kleter and Peijnenburg (2001).
- **Zero tolerance for any contamination of the food supply with any genetic material or gene products from a transgenic crop that is undergoing field testing, if the FDA is to allow field tests prior to a completed full safety assessment.** In addition, prior to field tests, the company should be required to submit complete sequence information for all the genetic material they plan to insert in the plant as well as simple tests (preferably PCR tests) that could be used to detect contamination coming from the field tested materials.

Because the FDA proposal is only for a partial safety assessment of genetically engineered crops going into field trials and because the FDA proposal permits any level of contamination of the food supply, this proposal poses a risk to public health.

## I. Introduction

A basic problem with this draft Guidance is that it is merely voluntary rather than mandatory: "FDA's guidance documents, included this guidance, do not establish legally enforceable responsibilities." Thus, FDA will not have the authority to ask the companies for any further data beyond what the companies deign to show FDA. And the companies can ignore any request from the FDA with no fear of reprisal. A study of the present "biotechnology consultations" procedure, which is also voluntary and based on a Guidance document (at <http://www.cfsan.fda.gov/~lrd/consulpr.html>), by the Center for Science in the Public Interest (CSPI) found that that in 6 of the 14 FDA "biotechnology consultation" files CSPI looked at, FDA requested further safety data; in 3 of these 6 cases, the company either ignored FDA's request or affirmatively declined to provide the requested information (Gurian-Sherman, 2002/3).

We are further concerned that even if the "early safety assessment" turned up safety problems, the FDA could stop the company from field testing the product; the Guidance only suggests that the companies "discuss" the issue with FDA. FDA indicates it would send a letter that states, "we [FDA] have completed our evaluation of you submission. Based on this submission, and as discussed in this letter, the submission raises questions about the food safety of your new protein. *You may wish to discuss the identified issues with us prior to engaging in any activity that might result in material from your plant inadvertently entering the food supply.*" Italics added

A third concern is that if companies now ignore 50% of the FDA's requests for further data during the "biotechnology consultations" (based on CSPI study), and they know that the "early safety consultation" requires even less data and that FDA cannot stop them from field testing plants with traits with identified safety concerns, why would the company bother to even go through the "biotechnology consultations" once they've completed an "early safety consultation"? FDA seems to realize that this may be a problem as they even have a question and answer that states that the FDA does: "recommend that you [the company] participate in FDA's biotechnology consultation process even if you have submitted to us and completed the early food safety evaluation of a new protein in your bioengineered plant."

This guidance gives the appearance of taking action to deal with the problem of contamination of the food supply with un-approved transgenic proteins, yet its voluntary nature makes it ineffectual.

**Recommendation: Early food safety assessment must be mandatory, not voluntary.**

## II Background

Although the FDA has taken a step forward by saying that there should be an early food safety assessment for introduced transgenic proteins, that step is far too small. The FDA proposes that the only food safety issues for this early assessment would be whether the intended transgenic protein has toxic or allergic properties: “FDA believes that any food safety concern related to such material entering the food supply would be limited to the potential that a new protein in food from the [bioengineered] plant variety could cause an allergic reaction in susceptible people or could be a toxin in people or animals.” We strongly disagree. The food safety concerns, even at the field trial stage, are far broader than just toxicity and allergenicity, including compositional analysis of key components (key nutrients and key anti-nutrients), evaluation of metabolites, food processing, nutritional modification and unintended effects.

For testing for toxicity and allergenicity, the FDA refers to companies to the approach laid out in the Codex Alimentarius “Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants” (CAC/GL 45-2003). This Codex document represents the global consensus on how safety assessments for transgenic plants should be performed. In addition, Codex Standards and Guidelines are referenced in the General Agreement on Tariffs and Trade (GATT) and therefore would be assumed to be “trade legal” in any World Trade Organization trade dispute. Thus, we feel that the FDA should require companies to submit the full range of data as laid out in this Codex document, in order to avoid trade disputes in the future. However, FDA only refers the companies to a small portion of the safety assessment section of the Codex Guideline—paragraphs 34-43—which covers testing *only* for toxicity and allergenicity. In fact, the rest of the Safety Assessment section of the Codex Guideline refers to compositional analysis of key components (key nutrients and key anti-nutrients) (paragraphs 44, 45), evaluation of metabolites (paragraph 46), food processing (paragraph 47), nutritional modification (paragraphs 48-53), all of which may have an impact on food safety.

The issue of impact of nutritional modification is particularly important for the present discussion. FDA notes that “scientific advances are expected to accelerate over the next decade, leading to the development and

commercialization of a greater number and diversity of bioengineered crops.” The first generation of genetically-engineered crops focused on single traits—herbicide tolerance, production of Bt toxins or virus tolerance. The second generation of genetically-engineered crops is expected to be far more complex with metabolic pathways being engineered in ways to “enhance” the nutritional profile of a food to make it more attractive to consumers. But such engineered plants may have dramatically altered levels of vitamins, nutrients, starches or levels of edible oils (in the case of plants engineered to change oil content). Since the FDA says they plan to only focus on the *protein(s)* produced by an introduced gene(s), this approach would completely miss all the nutritional changes. Thus, by inserting two genes that code for key protein enzymes in major metabolic pathways, the FDA approach would only have you look at the safety of the two enzymes themselves, rather than the potentially large nutritional changes that result from the introduction of these two enzymes (this doesn’t include the possibility of unexpected effects as well.). Given that the companies and others state that the new wave of genetically engineered crops will be focused on functional foods and/or nutritionally enhanced foods (refs to come), we would expect more changes in the non-protein profiles of such crops. Such changes need to be looked at as they can have health impacts. For example, large changes in the levels of vitamins and minerals in plants could have an adverse impact on health. Consumption of too much vitamin A via excessive use of supplements can cause liver and cell membrane damage (WCRF, 1997), while high levels during pregnancy can increase the risk of birth defects (McLaren et al., 1993). In addition, there can be unexpected effects associated with such nutritional modification. For example, when Monsanto engineered canola to produce beta-carotene and other carotenoids, the engineered plants had lower tocopherol (including vitamin E) levels and alterations to the fatty acid composition, which remain unexplained (Shrewmaker et al., 1999). When German researchers engineered potatoes with genes from yeast and a bacterium to reduce the sugar level and increase the starch content, the resultant transgenic potatoes had lower starch levels and many unexpected compounds produced as a result to the metabolic disruption in the potato (Gura, 2000). Thus, the early food safety assessment must focus not just on the protein produced by a transgene, but also on all the other nutritional parameters that may be affected, particularly in crops engineered to improve/change their nutritional profile.

FDA might argue that because the crop is in the experimental stage, it is not necessary to look at nutritional changes because only a small amount of the crop is being grown. However, with no limits on levels of contamination that

will be allowed, contamination could become pervasive and nutritional changes could be of concern.

In addition, this Codex Guideline also has detailed information about the proper Framework of the Food Safety Assessment for these rDNA plants. This framework includes: Description of the rDNA plant (para 22), Description of the host plant and its use as food (paragraphs 23-25), description of the donor organism (paragraph 26), description of the genetic modification (paragraphs 27-29), characterization of the genetic modification(s) (paragraphs 30-33). This framework, or background information, includes extensive molecular characterization/data on the genetically-engineered plant that is absolutely key toward identifying all the unintended (whether predictable or unexpected) effects that may occur. Consequently, FDA should require all these background data—especially the detailed molecular characterization data—as part of the early food safety assessment.

Finally, a section of the Codex Guideline is devoted to “Unintended Effects” (paragraphs 14-17). This is an important issue, because in the mid-1990s, when many genetically-engineered plants were being approved for use field testing and further use in the US, there was virtually no attention paid to the subject of unintended effects. As examples of such unintended effects started to be published in the scientific literature (see Kuiper et al. [?] for examples), the issue started to be discussed in international scientific circles. Unintended effects may occur if a promoter gene, due to the random nature of insertion, ends up near a gene for toxin production, for example, turning that gene on or silencing another gene that may be involved in limiting levels of a toxin. In the area of human gene therapy, there have been a couple of cases of leukemia in children who were receiving genes to overcome their lack of an immune system (these children had severe combined immune deficiency, [SCID]); research showed this resulted when the inserted genetic cassette inserted itself close to an oncogene which was then turned on (Sadelain, 2004)). It became such an important issue that 4 paragraphs in the Codex Guideline are devoted to it. The Guideline recognizes that such unintended effects can have adverse impacts on human health, states that they should be investigated as part of a proper safety assessment and even recommends methods to investigate such effects: “Safety assessment should include data and information to reduce the possibility that a food derived from a recombinant-DNA plant would have an unexpected, adverse effect on human health. Unintended effects can result from the random insertion of DNA sequences into the plant genome which may cause disruption or silencing of existing genes, activation of silent genes, or modifications in the

expression of existing genes. Unintended effects may also result in the formation of new or changed patterns of metabolites. . . . *The safety assessment of foods derived from recombinant-DNA plants involves methods to identify and detect such unintended effects and procedures to evaluate their biological relevance and potential impact on food safety*” (CAG/GL 45-2003: paras 14, 15, 17) italics added. We agree that unintended effects are very important and must be part of any early food safety assessment.

For all the reason articulated above, we feel that the FDA should require a full safety assessment, not just one limited to toxicity and allergenicity of the intended protein(s) produced by inserted gene(s). For the kinds of data that should be submitted in such a safety assessment, we recommend that FDA require all the data as laid out in the Codex Alimentarius “Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants” (CAC/GL 45-2003). The safety assessment should also be conducted on any newly introduced protein, whether its expression was intended or not (e.g. the result of an unintended effect). For the details of allergenicity testing, see the following section for more details.

**Recommendation: Require full safety assessment, not just one limited to toxicity and allergenicity (e.g. must also include compositional analysis of key components [nutrients and anti-nutrients], evaluation of metabolites, food processing, and nutritional modification). Also safety assessment should be for all newly introduced proteins—whether intended or unintended. That is, the assessment should pay attention to the problem of unintended effects (both predictable and unexpected)**

**Such a full safety assessment should ideally be done prior to field-testing the genetically-engineered plant. Since USDA is responsible for field-testing of genetically-engineered plants, they may allow field-testing prior to completion of a safety assessment. FDA should maintain a zero tolerance for any trait that has not gone through a mandatory safety assessment that includes all the data requirements laid out in the Codex Alimentarius “Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants” (CAC/GL 45-2003).**

**In addition, FDA should, prior to field-testing, require methods by which the bioengineered food could be detected. This would include a method for detecting the inserted DNA sequence as well as a method for detecting the introduced substance. Such a requirement would be very useful**

in traceability of the food, as well as serving to tell when there is unexpected gene flow. Since there is a market for non-transgenic or non-engineered food, and since organic foods cannot contain any genetically engineered ingredients, a test would be needed to determine when the foods destined for GE-free markets have been contaminated. In addition, many countries have not approved genetically engineered foods and have laws that state that unless such foods are explicitly approved, it is illegal for them to be on the market. Since the US has approved more engineered varieties than any other country, some of it may be illegal to ship to other countries. For example, not all the varieties of engineered corn approved in the U.S. have been approved in the European Union (EU). The result is that the US has lost a \$300 million corn export market to the EU. Shipments of food have already been rejected at foreign ports due to contamination with unapproved varieties, eg., the StarLink fiasco. So detection methods should absolutely be required. Furthermore, the detection methods should be available for the raw agricultural commodity as well as the representative finished product. We feel that the detection methods should include one for testing the presence of the inserted DNA as well as one for the expressed product. For the former, we suggest the use of a PCR (polymerase chain reaction) test as this is the most sensitive test to date. To facilitate such testing, the agency should require that the complete identity of the primer sequences be made available so that technically-proficient non-governmental laboratories can use them. The agency should require that the detection methods are adequate for detecting the presence of the inserted DNA and its expression products at the level at which it will appear in the food and that the test is of a reasonable cost. This requirement could be done along the lines of the detection method that is required when a new drug or pesticide is put on the market.

### III. Scope

In discussing the scope of their guidance, FDA states that “If a protein has been evaluated in an early food safety evaluation and no safety concerns are identified, we would not expect an additional early food safety evaluation to be submitted if the same protein is introduced into another plant species.” We disagree with this approach as it is a much weaker than the FDA’s proposed policy requiring a Premarket Biotech Notification (PBN). As part of the PBN policy, the FDA admitted that the process of genetic engineering is different from conventional breeding—due to the phenomenon of insertional mutagenesis—and so the FDA would require data from each separate transformation event:



“Because some rDNA-induced unintended changes are specific to a transformational event (e.g., those resulting from insertional mutagenesis), FDA believes that it needs to be provided with information about foods from all separate transformational events, even when the agency has been provided with information about foods from rDNA-modified plants with the same intended new trait and has had no questions about such foods. Similarly, the agency believes that it needs to be provided with information about foods from rDNA-modified plants whose intended change is the introduction of a pesticidal protein subject to oversight by the Environmental Protection Agency (EPA) rather than the FDA, because the transformational event that is used to introduce the pesticidal trait may also cause unintended changes to the food that would raise adulteration or misbranding questions subject to FDA jurisdiction” (FR 66(12), pg. 4711).

Since the FDA, as part of a "biotechnology consultation", wants data on each separate transformation event even if the intended new trait has been previously looked at and even if the host plant is the same, how can the FDA have a lower standard for an "early food safety consultation"? After all, if insertional mutagenesis can result in unintended effects with the same trait and the same crop species that the FDA deems worthy of study, then the same would be true for a trait (the protein going through the "early safety assessment") produced not in the same, but in different species. Thus, FDA must have the same standard for an "early food safety evaluation" as for a "biotechnology consultation." Otherwise, this guidance will seriously weaken the FDA's already lax oversight of the safety of genetically-engineered foods.

Even if we ignore unexpected effects and concentrate just on the protein which results from the introduced gene, we cannot assume that a transgenic protein produced in one plant will be identical to one produced in another plant. First, there is the phenomenon of post-translational processing, which consists of the modification of a protein after it has been translated from the genetic message. And such post-translational processing can have a significant impact on the structure and function of a gene. Furthermore, post-translational processing can differ between organisms, so that the same gene expressed in different genetic backgrounds may have the same amino acid sequence but may differ in structure and function. Examples of such processing includes glycosylation and acetylation.

Glycosylation consists of the addition of sugar groups (usually oligosaccharides) and can dramatically affect the three-dimensional structure

and thus, function of a protein. Indeed, glycosylation is thought to be connected to allergenic and immunogenic responses (Benjuoad et al., 1992). The different proteins produced from the same gene are called glycoforms. Research with recombinant human tissue plasminogen activator (rt-PA) revealed that different glycoforms were created depending on whether the rt-PA gene was expressed in human, Chinese hamster ovary, or mouse cells (Parekh et al., 1989a,b). Different glycoforms were even produced when different human cell lines were used (Parekh et al., 1989a). The activity (or behavior) of these glycoforms differed. Further work demonstrated that when the rt-PA gene was inserted into tobacco, although it was expressed and the protein had the normal amino acid sequence, it had no physiological activity whatsoever (ref. to come). Parekh et al. (1989b) argue that recombinant glycoproteins produced in plants could be allergenic as it is known that many allergens are glycoproteins.

But perhaps the most dramatic example of how glycosylation can affect the structure and function of proteins and have negative results occurs with the prion protein, which is thought to be the causative agent for transmissible spongiform encephalopathies (Scott et al, 1999). Prion proteins are normally found attached to the surface of cells in the nerve and immune system. Research has demonstrated that the prion proteins in people suffering nvCJD – a particularly severe form of Creutzfeldt-Jakob disease (CJD) that has been recently strongly linked to bovine spongiform encephalopathy (BSE) – have a glycosylation pattern that differs significantly from that of prion proteins from people suffering other forms of CJD and is identical to the glycosylation patterns of prion proteins from cows with BSE (Hill et al., 1997; Scott et al., 1999). This occurs despite the fact that the amino acid sequence from normal prion proteins and those suffering nvCJD is identical. In this case, the altered glycosylation pattern has had a catastrophic effect on the behavior of the prion protein.

**Given that glycosylation patterns can dramatically change the structure and function of proteins and may affect antigenicity and allergenicity, we feel that FDA should require information on the glycosylation patterns of all transgenes expressed in GE foods.**

Acetylation of proteins consists of the addition of acetyl groups to certain amino acids, thereby modifying their behavior. Although incompletely understood, acetylation of the amino acid lysine has been most studied in certain groups of proteins that bind with DNA – histones and high-mobility group proteins – and such acetylation appears to be involved with the regulation of interaction of these proteins with negatively charged DNA molecules (Csordas, 1990). However, it has been discovered that some the lysine residues in rbGH are acetylated, to form epsilon-*N*-acetyllysine when it is produced in *E. coli* .

Harbour et al. (1992) found this to occur at lysine residues 157, 167, 171 and 180 or rbGH, while Violand et al. (1994) found it at residues 144, 157, and 167. The creation of this mutant amino acid may be overlooked because “(T)he identification of this amino acid cannot be determined by simple amino acid analysis because the acetyl group is labile to the acidic or basic conditions normally used for hydrolysis” (Violand et al, 1994: 1089). The effect this has on the safety, structure and function of rbGH is not known as it hasn’t been actively studied.

The differences in glycosylation and acetylation that can happen when transgenes are expressed in plants or bacteria can possibly affect toxicity and therefore lend further support to the need for toxicity testing using the whole engineered food. At present, to test for acute toxicity of a given transgene, the companies invariably do not use the protein that is produced in the plant itself. Rather, in order to obtain large enough quantities of the protein for testing, the companies will put the transgene into a bacteria (invariable *E. coli*), isolate the expression product (i.e. the protein) and use that for the acute toxicity testing. However, the protein produced in the bacteria may be glycosylated differently than the same protein produced in the plant. Even if there are no differences in glycosylation, acetylation of lysine residue(s) could cause differences. The presence of such mutant lysine residues could easily be missed as routine amino acid analysis will remove the acetyl group; to find if there are mutant lysine residues, one must specifically look produce the transgene of interest (gene for herbicide tolerance or Bt endotoxin, for example). **Thus, whenever possible, FDA should require the companies to use material derived from the transgenic plants themselves in toxicity studies rather than bacterially-derived proteins.**

**Recommendation: FDA should require: i) separate early food safety evaluations for each separate transformation event in keeping with their 2001 proposed PBN policy, ii) information on the glycosylation patterns of all newly introduced proteins—whether intended or unintended—in genetically-engineered foods, and iii) companies to use material derived from the transgenic plants themselves in toxicity studies rather than bacterially-derived proteins.**

#### **IV. Early Food Safety Evaluation of New Proteins**

**What are the important considerations in the early food safety evaluation of a new protein?**

Although the FDA says that the company should only consider whether a new protein is an allergen or toxin, we feel that the companies should be required to do a full safety assessment—see discussion under **II. Background** above and, for details on the allergy testing and sequence similarity testing see **VI. Format for Submission of My Early Food Safety Evaluation Part II** below.

## **VI. Format for Submission of My Early Food Safety Evaluation Part II**

This section details the “safety data and information about the new protein.” The FDA lists 7 questions that the companies should answer as part of a early food safety evaluation. The first four questions—“1. The name, identity, and function of the new protein(s) in the new plant variety; 2. Data and information as to whether this protein has been safely consumed in foods; 3. A list of the identity (ies) and source(s) of the introduced genetic material; and 4. A description of the purpose or intended technical effect of the new protein”—address background information about the new genetically engineered plant. We feel that this section should be expanded and should be made consistent with the material laid out in paragraphs 20 to 31 of the Codex Alimentarius Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant DNA Plants (CAC/GL 45-2003). Paragraphs 20 to 31 in Section 4 cover General Considerations, Description of the New Variety (para 20), Description of the Host Plant and its Use as a Food (para 21-23), Description of the Donor Organism (para 24), Description of the Genetic modification(s) (paras 25-27) and Characterization of the Genetic Modification(s) (paras 28-31). By requiring these data and information, FDA could harmonize their requirements with those that are already agreed upon at a global level through Codex, thus reducing the potential for trade disputes. The U.S. data requirements should not in any case be less stringent than those for other countries, given that it has always been the goal of US food safety regulators to have the safest food supply in the world.

Question 5 calls for “an assessment of the amino acid similarity between the new protein and known allergens and toxins.” The FDA recommends looking at the Codex Plant Guideline, particularly the Codex Allergenicity Annex, Section 3.3. Although this annex does contain information on how to carry out such an assessment, there is some ambiguity about the exact nature of an amino acid similarity assessment. Since this annex was based on the report of the WHO/FAO Joint Expert Consultation on Allergenicity of Foods Derived from Modern Biotechnology, we recommend that the FDA suggest looking to this report for full details.

The FAO/WHO Expert Consultation developed a standardized methodology for determining sequence homology between and introduced protein and known allergens. FAO/WHO suggested using identity of 6 rather than 8 identical contiguous amino acids as a criterion for further concern and using local alignments rather than global alignments when comparing unrelated proteins. They also suggested additional criteria such as a 35% overall amino acid sequence homology as a cause for further concern and the development of databases and methods to test for discontinuous epitopes including those changed by glycosylation patterns. FAO/WHO recommended the following standardized methodology for determining sequence homology in Section 6.1:

*“Suggested procedure on how to determine the percent amino acid identity between the expressed protein and known allergens.*

***Step 1:*** *obtain the amino acid sequence of all allergens in the protein databases . . . in FASTA-format (using the amino acids from the mature protein only, disregarding the leader sequences, if any). Let this be data set (1).*

***Step2:*** *prepare a complete set of 80-amino acid length sequences derived from the expressed protein (again disregarding the leader sequence, if any). Let this be data set (2).*

***Step 3:*** *go to EMBL internet address: <http://www2.ebi.ac.uk> and compare each of the sequences of the data set (2) with all sequences of data set (1), using the FASTA program on the web site for alignment with the default settings for gap penalty and width.*

*Cross-reactivity between the expressed protein and a known allergen (as can be found in the protein databases) has to be considered where there is: 1) more than 35% identity in the amino acid sequence of the expressed protein (i.e. without the leader sequence, if any), using a window of 80 amino acids and a suitable gap penalty (using Clustal-type alignment programs or equivalent alignment programs) or: 2) identity of 6 contiguous amino acids.*

If any of the identity scores equals or exceeds 35%, this is considered to indicate significant homology within the context of this assessment approach. The use of amino acid sequence homologies to identify prospective cross-reacting allergens in genetically-modified foods has been discussed in more detail elsewhere (Gendel, 1998a, Gendel, 1998b).

Structural similarity with known allergens may still be important if significant amino acid identity is found, but it is below 35%. In this case

significant cross-reactivity is unlikely. However, some families of structurally related proteins are known to contain several allergens. Some examples are: lipocalins, non-specific lipid transfer proteins, napins (2S albumins from seeds), parvalbumins.

If the expressed protein belongs to such a family, it may be considered to have a higher probability to be an allergenic protein. . . . Since identity of 6 contiguous amino acids has an appreciable risk of occurring by chance, verification of potential crossreactivity is warranted when criterion (1) is negative, but criterion (2) is positive. In this situation suitable antibodies (from human or animal source) have to be tested to substantiate the potential for crossreactivity” (FAO/WHO, 2001: 10-11).

The report of the FAO/WHO Expert Consultation makes a reference to a pair of papers by Dr. Steven Gendel, chief of FDA’s Biotechnology Studies Branch. These papers discuss the various databases of allergens and how to use them to determine sequence similarity between an expressed protein and known allergen (Gendel, 1998a, b). Dr. Gendel argues persuasively for use of local algorithms rather than global algorithms when assessing allergenicity of novel proteins because most novel proteins are not evolutionarily related. As he points out, “sequence algorithms can be divided into global algorithms that optimize alignments across the entire length of the sequences involved and local algorithms that attempt to optimize alignments only with regions of high similarity. Global alignment algorithms are of greatest utility when the sequences involved are related. Allergenicity assessment involves sequence alignments between proteins that are not evolutionarily related. Therefore, it is likely that local alignment will be more useful” (Gendel, 1998b: 50). Dr. Gendel tests this assumption with known allergens and finds that the local alignment works best. Local alignment algorithms include the FASTA and BLAST program, which give similar results (Gendel, 1998b); FAO/WHO recommends use of the FASTA program.

Gendel also notes that “Although it is likely that immunological cross-reactivity requires extensive sequence similarity, absolute identity may not be necessary (for example, see Elsayed et al., 1982)” (Gendel, 1998b: 57). Other researchers have noticed the same phenomenon, pointing out “the IgE binding to peptides carrying linear IgE epitopes of the shrimp allergen Pen a 1 was not impaired and in some cases enhanced by various specific substitutions of amino acids within these peptides (Lehrer et al. 2002; Ayuso et al., 2002)” (Kleter and Peijnenburg 2002). Dr. Gendel then goes on to develop a “biochemical similarity

matrix” which “divides the amino acids into six classes based on biochemical characteristics (i.e., hydrophilic acid amino acids, hydrophilic basic amino acids, etc.). . . Alignment of members of the same class is scored as a mismatch. The realignment was confined to a region of 15 to 20 amino acids in each case to preserve the previously located identities” (Gendel, 1998b: 58).

Using this methodology, Gendel finds significant sequence homology between  $\beta$ -lactoglobulin (major milk allergen) and Cry3A (found in Bt potatoes) and between Cry1Ab or Cry1Ac and vitellogenin (egg allergen). He concludes, “although it is clear that some amino acid residues are critical for specific binding, some conservative substitutions may not affect allergenicity. Therefore, it may be prudent to treat sequence matches with a high degree of identity that occur within regions of similarity as significant even if the identity does not extend for eight or more amino acids. For example, the similarity between Cry1A(b) and vitellogenin might be sufficient to warrant additional evaluation” (Gendel, 1998b: 60).

On the issue of continuous epitopes within transgenic proteins, the WHO/FAO expert consultation recommended that the minimal degree of identity should be six contiguous amino acids, while the industry would like to use eight contiguous amino acids. WHO/FAO based their recommendation for using six contiguous amino acids, in part, on the fact that linear epitopes of peanut or cod fish only consist of 6 or 4 contiguous amino acid residues which are essential for IgE binding (Becker, 2001: 1). The Codex Plant Guideline annex on allergenicity notes that the “smaller the peptide sequence used in the stepwise comparison, the greater the likelihood of identifying false positives, inversely, the larger the peptide sequence used, the greater the likelihood of false negatives, thereby reducing the utility of the comparison.”

Although the use of 6 contiguous amino acids in a sequence comparison may lead to a lot of false positives, there are mechanisms to further select from these positives to weed out the false positives. Dutch scientists, in a paper titled “Screening of transgenic proteins expressed in transgenic food crops for the presence of short amino acid sequences identical to potential, IgE-binding linear epitopes of allergens,” used the WHO/FAO’s recommended protocol for sequence similarity and screened 33 transgenic proteins for identities of at least six contiguous amino acids (Kleter and Peijnenberg, 2001). To decrease the problem of false positives, any “positive” outcomes were further screened for the

presence of potential linear IgE-epitopes via two methods: 1) use of literature data on Ig-E epitopes, and 2) an antigenicity prediction algorithm.

Of 33 transgenic proteins investigated by the Dutch scientists, 22 were found to have identities of at least six contiguous amino acids with known allergenic protein; in fact, there were 83 sequences with identities to known allergens (Kleter and Peijnenberg, 2001). Use of the two further screens reduces this to “a limited number of identical stretches shared by transgenic proteins (papaya ringspot virus coat protein, acetolactate synthase GH50, and glyphosate oxoreductase) and allergenic proteins [that] could be identified as (part of) potential linear epitopes. . . . As shown in this study, identical stretches can be further screened for relevance by comparison with linear IgE-binding epitopes described in the literature. In the absence of literature data on epitopes, antigenicity prediction by computer aids to select potential antibody binding sites that will need verification of IgE binding by sera binding tests. *Finally, the positive outcomes of this approach warrant further clinical testing for potential allergenicity*” italics added (Kleter and Peijnenberg, 2001: 1).

In sum, we urge FDA to ask companies to follow the protocol laid out by FAO/WHO as modified by Dr. Gendel (e.g. allow chemically similar—rather than identical—amino acid residues to be used when determining short sequence similarity/identity for the contiguous amino acid sequences). We recommend that 6 similar (rather than 8) contiguous amino acids be used in the sequence similarity protocol to reduce the possibility of false negatives. To deal with the problem of false positives, we recommend the two further screens (use of epitope data base and the antigenicity prediction algorithm) as discussed by (Kleter and Peijnenberg, 2001). We note that both Gendel (1998) and Kleter and Peijnenberg (2001) found sequence similarity/identity between known human allergens and a number of transgenic proteins in foods approved for human consumption. In neither case have the suggestion of these authors about the need for further research on particular proteins been followed. These examples also reveal the flaw in the present approval systems of the FDA and EPA, which allowed crops containing these transgenic proteins onto the market.

We also agree with the FAO/WHO that developing databases and methods (such as monoclonal antibodies using animal and/or human materials) to test for conformational or discontinuous epitopes including those caused by changed glycolysation patterns is of key importance and urge FDA to try and encourage studies in these areas.



**Recommendation: Sequence similarity—use approach of WHO/FAO joint Expert Consultation as extended by Gendel (1998) and Kleter and Peijnenberg (2001).**

Question 6 deals with protein stability and resistance to enzymatic digestion. We suggest FDA require the protocols discussed below for assessing protein stability and resistance to enzymatic digestion which are an essential part of assessing allergenicity.

*Heat stability*

Both allergy scientists as well as the Environmental Protection Agency (EPA) consider stability of a protein to heat to be a characteristic property of food allergens (Sampson, 1999; EPA, 2001; Helm, 2001; and Taylor and Hefle, 2001). During the Bt crop reregistration process, EPA vaguely adopted heat stability as a criterion for potentially allergenicity for the Bt Cry endotoxins, stating that a characteristic “considered as an indication of possible relation to a food allergen are [is] a protein’s ability to withstand heat or the conditions of food processing” (EPA, 2001b: IIB2). However, EPA has neither strictly required nor even suggested a test protocol for such data. Indeed, for a couple of Bt crops—Novartis’ Bt corn (Cry1Ab) and Monsanto’s Bt cotton (hybrid Cry1Ac/Ab)—the EPA accepted data that processed corn or cottonseed meal were inactive in an insect bioassay. Monsanto submitted a more formal heat stability study for a relatively new Bt corn variety (containing Cry1F rather than the usual Cry1Ab), but the methodology was flawed. The study’s main methodological flaw consisted of the sole end-point (e.g., measure of degradation) being “growth inhibition of neonate tobacco budworm larvae” following “application of treated Cry1F to the surface of an insect diet” (EPA 2001b: 10). Such a study implicitly assumes that the insecticidal mode of action correlates with allergenicity and that loss of insecticidal action means no allergenicity. There is no scientific justification for such an assumption. Theoretically, a protein could be allergenic and have insecticidal activity; loss of that activity does not imply loss of allergenicity. As has been noted by a number of scientists, degraded proteins or protein fragments can still elicit an allergenic response even though the protein is functionally inactive; a perfect example is the major milk allergen  $\beta$ -lactoglobulin (Haddad et al., 1979).

In contrast to the EPA’s lack of a consistent protocol, Dr. Riki Helm has developed a science-based protocol as part of the paper on the topic that he wrote for the 2001 FAO/WHO Expert Consultation: “Heat Stability: The

definition of heat stability should be standardized using the following criteria. 1- Heat treatment of the novel protein, native and recombinant, should be for 5 minutes at 90°C. 2-Assessment of stability by a combination of molecular sieving using HPLC and standardized SDS-PAGE analysis (both native and denaturing/reducing gels). See SDS-PAGE protocol below” [see the section on digestive stability, above for this protocol] (Helm, 2001: 8-9).

**Recommendation: We urge that the FDA require data on heat stability and use the science-based protocol as outline by Dr. Helm (Helm, 2001). We would suggest the following additions/explanations to the protocol. The recombinant protein should be tested in both purified form and as part of the food in which it occurs. The purified form of the protein should be extracted from the engineered organism (usually plant) that will make up the food; the company should not be permitted to use a bacterial or other microbial source to produce the recombinant protein. Also, the engineered protein should be added to a food matrix/matrices, preferably to the matrix in which it will occur.**

#### *Enzymatic digestion*

A number of scientific and other sources—including the Environmental Protection Agency, FIFRA’s Science Advisory Panel (SAP), the International Life Sciences Institute (ILSI), the FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology and Codex Alimentarius’ Plant Guideline document—have agreed that digestive stability (or enzymatic digestion) of new protein produced in foods developed via bioengineering should be a criterion that is assessed. A number of these sources agree that in order for the criterion of digestive stability to be used, standardized methods need to be developed so that any laboratory can repeat them. As Drs. Steve Taylor and Samuel Lehrer pointed out in an early paper in this area, “Although the assessment of the resistance to hydrolysis of proteins could offer valuable information regarding the potential allergenicity of specific proteins, a rigorous protocol for such experiments has not been established. Ideally, this protocol would mimic digestive proteolysis and included tests on the isolated protein and the protein in the appropriate food matrix” (Taylor and Lehrer, 1996: ).

All sources quoted above agree that assessing digestive stability should involve simulating the environment of the human digestive system. One can either simulate the environment of the stomach, via simulated gastric fluid (SGF), or simulate the environment of the intestine, via simulated intestinal fluid (SIF).

Most of the authors prefer the use of SGF. However, some note that if significant amounts of the undegraded or protein fragments survive SGF, then SIF testing should ensue (Helms, 2001). There has also been debate about the protocol for developing SGF. One of the first studies that demonstrated a link between allergenicity of a protein and resistance to digestion used the United States Pharmacopoeia (USP) protocol for SGF (Astwood et al., 1996). However, the USP protocol for SGF has been criticized for not being sufficiently physiological in nature (Helms, 2001). Since the publication of the Astwood et al. paper in 1996, there have been a number of scientific meetings, symposia and papers that have further discussed protocols (or the need for them) for testing digestive stability; these are reviewed by Dr. Ricki Helm, of the Arkansas Children's Hospital Research Institute, in his paper "Stability of Known Allergens (Digestive and Heat Stability)" written for the FAO/WHO expert consultation. In this paper, Dr. Helms, after reviewing the scientific work in this area, makes recommendations for protocols for SGF and SIF.

The paper by Dr. Helm (Helm, 2001) served as a starting point for discussion of the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology. The final report of the Expert Consultation recommended a slightly modified version of Dr. Helm's protocol (for example, rather than test the protein at a range of pHs to simulate the stomach at various times after feeding, the FAO/WHO Expert Consultation recommends testing only at pH 2.0), but it contained far more specific details about what the protocol should contain. Their recommendation follows:

#### *"6.4. Pepsin Resistance*

Purified or enriched expressed protein (non-heated and non-processed) should be subjected to pepsin degradation conditions using Standard Operating Procedures and Good Laboratory Practices (SOP/GLP). In addition, the expressed protein should be assessed in its principle edible form under identical pepsin degradation conditions to those used to examine the expressed protein. Both known non-allergenic (soybean lipoxygenase, potato acid phosphatase or equivalent) and allergenic (milk beta lactoglobulin, soybean trypsin inhibitor or equivalent) food proteins should be included as comparators to determine the relative degree of the expressed pepsin resistance. The protein concentrations should be assessed using a colorimetric assay (e.g., Bicinchoninic acid assay (BCA), Bradford Protein Assay, or equivalent protein assay) with bovine serum albumin (BSA) as a

standard. Pepsin proteolytic activity should be assessed (Ryle). Enzyme/protein mixtures should be prepared using 500µg of protein in 200µL of 0.32% pepsin (w/v) in 30mM/L NaCl, pH 2.0, and maintained in a shaking 37°C water bath for 60 minutes. Individual 500 microgram aliquots of pepsin/protein solution should be exposed for periods of 0, 15, 30 seconds and 1, 2, 4, 8, 15, and 60 minutes, at which time each aliquot should be neutralized with an appropriate buffer. Neutralized protein solutions should be mixed with SDS-PAGE sample loading buffer with and without reducing agent (DTT or 2-ME) and heated for 5 minutes at 90°C. Samples containing 5µg/cm gel of protein should be evaluated using 10-20% gradient Tricine SDS-PAGE gels or equivalent gel system under both non-reducing and reducing electrophoretic conditions. Protein in the gels should be visualized by silver or colloidal gold staining procedures. Evidence of intact expressed protein and/or intact fragments greater than 3.5 kDa would suggest a potential allergenic protein. Evidence of protein fragments less than 3.5 kDa would not necessarily raise issues of protein allergenicity and the data should be taken into consideration with other decision tree criteria. For detection of expressed protein in an edible food source, a polyclonal IgG immunoblot analysis should be performed according to the laboratory procedures. The immunoblot analysis should be compared to the silver or colloidal gold stained SDS-PAGE gel and reflect the stained pattern of the expressed protein run under identical conditions” (FAO/WHO, 2001: 12-13).

One significant extension of Dr. Helm’s protocol that the FAO/WHO Expert Consultation included was the notion that “the expressed protein should be assessed in its principle edible form under identical pepsin degradation conditions to those used to examine the expressed protein” (FAO/WHO, 2001: 12). CU absolutely agrees that the expressed form of the protein should be assessed both in purified form and as part of the food that it occurs in. The reason for this is that the food matrix can act as a buffer allowing the expressed protein to survive digestion. There are many examples of this. For instance, a number of growth hormones in milk, such as insulin-like growth factor-1 (IGF-1) or epidermal growth factor (EGF), are protected from digestion by the presence of casein (Kimura et al., 1997; Playford et al., 1993; Xian et al., 1995). One study with IGF-1 found that 9% survived digestion when fed in pure form to rats; in the presence of casein, 67% survived digestion (Kimura et al., 1997). More recently, a study involving transgenic soy or corn DNA found that while 80% of the naked DNA was degraded in gastric simulations, none of the transgene DNA was digested when it was part of the food stuff: “The data showed that 80% of

the transgene in naked soya DNA was degraded in the gastric simulations, while no degradation of the transgene contained within GM soya and maize were observed in these acidic conditions” (Martin-Orue et al., 2002: 533). While we realize that DNA is not a protein, the general phenomenon—partial survival of substance when part of a food compared to testing the pure substance—we feel is applicable. Furthermore, as Dr. Helm pointed out in his paper for the FAO/WHO Expert Consultation, recent industry and scientific thinking in this area concur: “The working committee on the ‘Characteristics of Protein Food Allergens’ held by ISLI/HESI following the symposium established the following criteria be taken into consideration. . . . 3-Deliver: Consideration should be given to how the material will be introduced into the diet. Assessment of allergenicity should be based on the matrix/matrices that the novel protein would be introduced into the diet” (Helm, 2001: 6).

**Recommendation:** We urge that FDA require companies to follow the protocol for assessing digestive stability (e.g. pepsin resistance) as laid out in FAO/WHO Expert Consultation, which we described above. If there are to be deviations from this protocol, companies should be required to give a scientific justification for such deviations. In particular, we feel the FDA should not allow the companies to simply use USP protocol for SGF. Furthermore, FDA should not allow the companies to simply test the protein at pH 1.2 (as per the USP protocol). If a company wants to test the protein at pH 1.2, the FDA should also require higher pHs as well, including, at least, pH 2.0.

Second, we feel the FDA should require the company to test the protein in both the purified expressed form as well as in the form in which it occurs in food, e.g. as part of the food matrix. For the purified expressed form, we feel that the company should extract the protein from the transgenic material that is intended to be commercialized and not use a form of the protein that is extracted from a bacterial or other microbial source.

Finally, if a significant portion of the expressed protein does survive digestion in SGF, we recommend that it be tested further in SIF, using the protocol laid out by Dr. Helm (2001).

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