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Summary report of the ILSI Europe workshop on detection methods for novel foods derived from genetically modified organisms

H.A. Kuiper

State Institute for Quality Control of Agricultural Products (RIKILT-DLO), Bornsesteeg 45, 6708 PD Wageningen, The Netherlands

1. Introduction

ILSI Europe in collaboration with the ILSI International Food Biotechnology Committee invited several experts in the fields of biotechnology, chemical analysis, food toxicology and regulatory affairs from academia, governments, international bodies, industry, and consumer groups to discuss the state of the science of methods development and application for the detection of foods and food ingredients derived from genetically modified organisms (GMOs).

According to the European Commission (EC) Novel Food Regulation 258/7 and the recently issued Council Regulation 1139/98, novel foods and food ingredients that are considered to be "no longer equivalent" to conventional counterparts are subject to labelling. From these regulations, it was concluded that the presence of recombinant DNA (recDNA) or modified protein in the food products would classify those products as no longer equivalent if present at levels above a yet-to-be-defined threshold. The development and application of reliable and quantitative analytical detection methods are essential for the implementation of labelling rules.

The objectives of the workshop were to examine (1) appropriate sampling strategies and methods for sample preparation, (2) the state of the science of method development and application for both protein-based and recDNA-based methods to detect GMOs and their derivatives, (3) validation criteria and performance assessment of detection methods, (4) needs and criteria for manufacturing standard reference materials, and (5) other scientific issues related to the establishment of thresholds for GMOs and their derivatives in commodities destined for use by the food industry.

The workshop was chaired by Prof. M. Grasserbauer (EC Joint Research Centre, Geel, Belgium) and Dr. M.E. Knowles (Coca-Cola Greater Europe, Brussels, Belgium). Dr. H.A. Kuiper (State Institute for Quality Control of Agricultural Products [RIKILT-DLO], Wageningen, The Netherlands) acted as overall workshop rapporteur.

During the workshop, scientific presentations focused on sampling and detection strategies for GMOs, immunoassay detection methods for modified proteins, and qualitative and quantitative methods for the detection of recDNA. Following these presentations, four working groups reviewed strategies for sampling and producing reference materials and the current validation and compliance status for protein- and DNA-based detection methods. The conclusions and recommendations of each working group were presented and discussed.

The workshop was opened by Mr. O. Lluansi of the Cabinet of Mme. E. Cresson, Commissioner of Directorate General XII (Science, Research and Development), Brussels, Belgium. He emphasised that the European Commission considers the issue of labelling of novel foods derived from genetically modified organisms of high priority. It is of great importance to maintain and possibly strengthen European consumers' trust in the quality and origin of foods introduced into the market. In this context, European consumer organisations have expressed their views that quality assurance systems and detection methods must be developed to prove the authenticity of foods which have been produced using modern biotechnological methods. He indicated that there is a need for positive labelling of food, based on thresholds that are still to be defined and for the establishment of a "negative list" of products that do not contain GMOs. Within this framework, validated quantitative detection methods for GMOs should be developed that can be applied to raw materials and to derived products.

2. Detection strategies for GMO foods: sampling and reference materials (Co-rapporteur F. Gendre, Groupe Danone, France)

2.1. Detection strategies for food authenticity and GMOs

Prof. J. Lüthy (University of Bern, Switzerland) presented an overview of the applicability of polymerase

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chain reaction (PCR) methods for various purposes in the area of food production, food quality, and safety control. PCR methods have been applied, among other applications, in the field of microbiology for tracing bacterial contamination, for genotype characterisation, for identification of food authenticity, for characterisation of food-related toxicological problems, and for the detection of GMOs in food products. Examples of food authenticity applications are detection of meat products derived from different animal species, detection of soy in meat, and detection of wheat in non-wheat products.

As a first approach for the detection of GMOs in foods, PCR screening methods can be designed based on the presence of the cauliflower mosaic virus (CaMV) 35S promoter, the nopalin synthase (NOS) terminator, and the kanamycin-resistance marker gene (*nptII*) in many of the transgenic plants that have been developed. However, it should be noted that these sequences may occur naturally in plants and soil micro-organisms. A positive result will not necessarily confirm the presence of recDNA. Advantages of DNA detection methods versus protein detection methods are related to specificity and sensitivity, but there is still room for improvement. Parameters to be taken into account when detection methods for GMOs are developed include expenditure, applicability, specificity, sensitivity, quantification, and potential for automation.

2.2. Sampling of raw material and processed foods

Prof. J. Gilbert (Ministry of Agriculture, Fisheries and Food, United Kingdom) presented a paper on sampling strategies for GMOs. He emphasised that the development of sampling plans should be regarded as an integral part of method development. The quality of the outcome of analytical detection procedures is as good as the quality of the sampling. In the area of GMOs, sampling will be an issue mainly of testing of raw materials and ingredients where most problems of non-homogeneity are likely to exist. The extent of sampling is dependent on (1) the type of material to be sampled (raw materials, food ingredients, and finished processed foods), (2) the purpose of the analysis (producer acceptance or regulatory compliance), and (3) the degree of risk acceptable for the producer to reject a batch which conforms to desired specifications or for the consumer to accept a batch which does not conform to desired specifications.

Two different approaches for the development of sampling plans were discussed: (1) taking large samples and subsequent subsample analysis (high cost for sampling but low cost for analysis) and (2) taking multiple small samples (low cost for sampling but high analysis costs). Adequate sampling plans are needed in particular for raw materials where GMO content may be heterogeneously distributed or for indications of non-segregation or co-mingling. In the case of ingredients and processed food, a more uniform distribution can be expected. In designing proper sampling plans for raw materials, one could profit from experiences gained with the detection of mycotoxins. The importance of developing sampling strategies that take into account the specific situation in the food production chain was emphasised. Adequate sampling plans are needed by food producers to resolve disputed issues and to comply with (non-) labelling requirements.

2.3. Report of the working group on sampling standards and reference materials (Rapporteur Dr. D.J.G. Müller, Procter & Gamble, European Service GmbH, Germany)

2.3.1. Sampling plans

- Appropriate sampling is the precondition for the correct interpretation of the analyses of the samples taken. For effective testing by both industry and control authorities, only samples taken in accord with scientifically and statistically sound sampling plans can provide a basis for definitive decisions, especially when the results might be used in legal proceedings.
- To ensure that samples taken for analysis are accepted as representative, it is necessary for the European Commission to work towards the development of harmonised sampling plans. These plans should be co-ordinated on a world-wide basis (e.g., within Codex Alimentarius) to ensure acceptability of those GMO products traded internationally and should be based on both (1) solid scientific/statistical concepts and (2) practical constraints within the food industry, e.g., sample sizes and the cost of sampling and sample processing.
- The purpose of sampling GMO-derived materials is to ensure legal compliance, and is not related, as stated in Council Regulation 1139/98, to safety concerns. Thus, false positives must be avoided.
- The process for developing sampling plans needs to be accelerated because of the regulatory situation: the existing rules on labelling currently do not refer to specific and obligatory procedures. It would be desirable to co-ordinate related developments via a European or a supranational scientific organisation (e.g., International Life Science Institute, ILSI).
- A need exists for different sampling plans for various applications, e.g., different matrices (raw materials like soy beans and ingredients like soy flour), and for expected declarations such as "GMO-free" (non-detectable levels), "unlabelled" (below threshold), or "contains GMO". The same sampling plans must be used both by industry and by control authorities.
- It is desirable to develop a system of "quality control chain management" which shares the responsibility for appropriate controls along the production chain

and leaves the final food manufacturer with the obligation for verification in the context of "due diligence".

2.3.2. Reference materials

The availability of validated reference materials before the introduction of a new GMO and its derivatives into the European markets is essential for appropriate analytical evaluation by industry and authorities.

- Appropriate reference materials for positive and negative controls provide the basis for the validation of analytical methods and for assessing the performance of methods and laboratories. Reference materials should be independent of the analytical methods used and should be focused on raw materials/base ingredients rather than on finished foods.
- The provider of such reference materials has to guarantee consistent quality over a long period of time and must account for specific sample homogeneity, level of GMO content and stability. Each GMO needs specific reference materials. To ensure the validity of the reference materials, a guaranteed supply of the relevant GMO materials as well as GMO-free control materials, each at 100% purity, is needed in sufficient quantities (several hundred kilograms each year).
- To allow the development of reference materials, the "inventor" of the GMO must provide in a timely fashion a defined 100% pure GMO material and information or samples of relevant target sequences representative of the modifications.
- The production of the reference materials should be co-ordinated centrally by, or under the control of, the European Commission through the Institute for Reference Materials and Methods (IRMM), Geel, Belgium. When appropriate, samples may be distributed via commercial channels.

3. Detection methods for genetically modified crops through identification of modified protein (Co-rapporteur R. Havenaar, TNO Nutrition and Food Research, The Netherlands)

GMOs are characterised by an altered genome which may lead to the expression of new proteins. Detection of GMOs is therefore focused on either altered gene(s) or their protein product(s). One session during the workshop was devoted to the development and practical use of immunoassays for the detection of modified proteins in foods derived from GMOs. Application of immunoassays is worth examining, since these methods already have broad application in the fields of medicine and agriculture.

3.1. Detection methods for genetically modified crops

Dr. G. van Duijn (TNO Nutritional and Food Research Institute, The Netherlands) presented data on Western blot analysis of modified soy and soy-derived products, using monoclonal antibodies raised against synthetic peptides corresponding to antigenic and non-homologous parts of the *Agrobacterium* sp. strain CP4 5-enolpyruvylskikimate-3-phosphate synthase (EPSPS) protein. Analysis of raw transgenic soy beans and soy protein fractions revealed the presence of the modified protein with a sensitivity between 0.5% and 1%, but the protein could not be detected following further processing of soy products. The method is currently validated for half products and final products. Dr. van Duijn also presented data for a PCR method for the detection of CP4 EPSPS synthase (see Section 4.1.1).

3.2. Design and development of immunoassays for detection of proteins

Dr. M. Morgan (Institute of Food Research, United Kingdom) presented an overview of the design and development of immunoassays for the detection of proteins, and concluded that immunoassays are currently the methods of choice for the quantitative and semi-quantitative detection of many types of proteins in complex matrices. He discussed a number of problems associated with the immunological detection of proteins in foods, such as uniqueness of the target protein (sequence homology with other proteins), availability of relevant antibodies, and the behaviour of the protein during food production and processing. Both monoclonal and polyclonal antibodies may be used, depending on the amounts needed and the specificity of the detection system (e.g., antibodies to whole proteins or to specific peptide sequences). When antibodies have to be raised against specific peptide sequences, problems may arise with recognition of the peptide as part of the protein structure. With respect to the assay format, a two-site assay is preferable to a competitive assay. Assays can be semi-quantitative for reasons of ease and simplicity, and greater effort should be undertaken to design quantitative assays for food applications. Dr. Morgan pointed out that assay validation for food analysis is complex, given the large diversity of food matrices. In addition to normal validation procedures, test performance and experience in the laboratory become important. With respect to confirmation, different quantitative immunoassays based on different antibodies can be used. In the near future, improvements in immunoassays are expected to occur through the combination of advances in antibody technology and improved instrumentation (e.g., real-time biosensors).

3.3. Immunodiagnostic methods for detection of EPSPS in Roundup Ready soya beans

Mr. G. Rogan (Monsanto Company, St. Louis, MO, United States) presented data from Western blot and enzyme-linked immunosorbent assay (ELISA) analysis of CP4 EPSPS Roundup Ready™ (Monsanto Company) soy beans and soy-derived products. These methods have been validated and are of sufficient sensitivity for the detection of modified protein in processed soy bean fractions: detection limits with Western blotting varied between 0.25% for seeds and 1.0% for toasted meal, and with the ELISA method varied between 0.25% for seeds and 1.4% for toasted meal. Because Western blotting is a semi-quantitative method, application is most effective for determining whether a sample contains the target protein below or above a predetermined threshold level. The ELISA method appears to be appropriate for quantitative detection of the modified protein in processed fractions. However, optimisation of the protein extraction process is a key challenge for quantitative analysis. During soy bean processing, the biological activity and solubility of soy protein may be reduced and the proteins denatured. Further validation of the immune-based methods is required to determine the performance characteristics of the assays in different laboratories.

3.4. Detection of new or modified proteins in foods derived from GMOs: future needs

Dr. J.W. Stave (Strategic Diagnostics Inc., Newark, DE, United States) emphasised that immunoassays have the capacity to be widely implemented on a commercial scale for the quantitative detection of modified proteins in foods. Strong antibody binding to protein antigens is the basis for the high sensitivity of these assays, and antibody specificity minimises sample preparation. To enable immunoassay testing of foods for the presence of modified protein, companies developing GMOs containing *proprietary* proteins must provide validated methods or make antigens and antibodies available for test development. He pointed out that on the basis of typical concentrations of transgenic material in plant tissues (>10 μg/g tissue), the detection limits of protein immunoassays are, in general, sufficiently low to detect the presence of modified protein at detection limits in the range of 1% GMO. The performance characteristics of immunoassays are well known, and quality standards and validation guidelines have been published for many applications. It is important for *threshold assays* to consider the precision characteristics of the method, since variability will determine whether significant percentages of foods will be labelled incorrectly.

Matrix effects are an important aspect of method validation. Given the complexity of food matrices, a practical approach is to incorporate standard reference materials with known concentrations of GMO in the same matrix as the test samples as part of the test method performance evaluation.

Two strategies for determining GMOs in foods were discussed: the *quantitative* and the *threshold* approaches. Both were evaluated by a study of the detection of GMO in Roundup Ready™ soy beans using certified reference materials prepared by the IRMM in a commercial ELISA method.

Establishment of process controls and chain-of-custody procedures could limit the number of food fractions to be tested at critical control points. Labelling of final foodstuffs could then be based on test results of major food commodities and on the characteristics of the downstream production process. One of the challenges facing the implementation of systematic testing for GMOs in food is the development of standardised reference materials of major (processed) food fractions, which obviously cannot be done for every foodstuff.

3.5. Report of the working group on detection of modified proteins (Rapporteur Dr. R. Battaglia, Federation of Migros Cooperatives, Switzerland)

- Both recDNA and protein detection methods have their place because European legislation requires both. If the analysis for one is negative, the other has to be tested for. The method that is best suited for each foodstuff must be determined on a case-by-case basis.
- The merits and shortcomings of ELISA and other immune-based protein detection methods should be evaluated with respect to applicability for GMO detection. Immunoassays are applicable and robust under field conditions and are suitable for routine automated testing. The costs per analysis are generally low (less than 10 euros), and the time required per analysis is on the order of minutes. However, users and designers of immunochemical methods should incorporate the concepts of method validation as used in classical chemical analysis. A common understanding of performance characteristics such as reliability, specificity, and accuracy, and their estimation, must be sought.
- Because GMO detection is related to European labelling requirements and is not connected in any way with food safety, detection methods must have a very high degree of reliability and specificity. The possibility of false-positive findings has to be avoided by all means to prevent the recall or destruction of food because of alleged mislabelling. Therefore, in view of the quantitative thresholds that are to be established, the methods must conform to the highest standards of accuracy and precision.

- The state of the science in developing and validating immunochemical methods can fulfil requirements for method performance to a high degree. False-positive results do occur, but they do so with practically every analytical method. Expert groups should therefore be asked to collaboratively test ELISA methods supplied to them in case they should be used in arbitration.
- For screening purposes, methods and test kits that have been validated in accord with sound analytical principles and fulfil minimal performance requirements (which are yet to be defined) may be acceptable. Based on these prerequisites, ELISA methods will be the methods of choice for testing raw materials, semi-processed food, and ingredients for further processing. The rather low detection power of ELISA assays compared with PCR methods for testing finished food products consisting of many food components, will limit its applicability. This must also be taken into account when regulatory thresholds are established. If these thresholds, which presumably will be in the low percent range, are applied to finished foods, ELISA testing may be applicable. If a threshold applies to ingredients, ELISA will not be able to detect minor ingredients containing a fraction of the target proteins.

3.5.1. Conclusions and recommendations

- The definition of a series of performance standards for immunochemical methods (e.g., ELISA) needs to be established.
- It is essential to validate ELISA methods along sound analytical principles to reach defined performance standards.
- Because test methods may be subject to arbitration, collaborative testing of these methods should be conducted in accordance with Association of Official Analytical Chemists International (AOACI) protocols.
- The production of standard reference materials, such as pure GMO crop DNA, traditional crop DNA, gene products, or antibodies to those products, is essential to fulfil the requirements of method validation. It is obvious that reference materials can be obtained only in co-operation with the GMO-producing industry.
- Both ELISA and PCR should be regarded as complementary rather than exclusive of each other. In critical cases, one might be used for screening and the other for confirmation. Western blotting and other confirmatory techniques should also be considered. At present, ELISA appears to be the method of choice in screening for a particular GMO, provided the presence of proteins can be expected. Application is dependent on the degree and nature of the processing the material has undergone.

4. Detection methods for genetically modified crops through identification of recDNA

4.1. Qualitative analysis (Co-rapporteur H. Broll, Federal Institute for Health Protection of Consumers and Veterinary Medicine [BgVV], Germany)

4.1.1. Development and application of DNA analytical methods for the detection of GMOs in food

Dr. R. Meyer (Nestlé, Switzerland) presented an overview of the development and application of DNA analytical methods for GMOs in food. Several food ingredients, such as wheat, soy, maize, celery, etc., have been successfully investigated by the DNA analytical method based on PCR. A critical point in this method is sample preparation. Currently, two DNA isolation methods are used, the cetyltrimethylammonium bromide (CTAB) method based on incubating a food sample in the presence of the detergent CTAB and using DNA-binding silica resins (e.g., Wizard® Purification Systems, Promega Corp.) to extract and purify DNA from food samples. It was shown that a combination of the classical CTAB method and a subsequent DNA purification step with DNA-binding resins increased the yield of DNA. Various factors contribute to the degradation of DNA (e.g., heat treatment, nuclease activity, and low pH); data suggest that a critical minimum average DNA size for successful analysis is approximately 400 bp.

Inhibition of the DNA polymerase by co-purification of proteins, fats, polysaccharides, polyphenolics, and other compounds present in highly processed foods is a major problem in the preparation of DNA for PCR. Therefore, before conducting a GMO-specific or screening PCR assay, the presence of amplifiable DNA in food samples must be determined by using species-specific primers in a PCR assay. In general, no DNA is detectable in highly heat-treated food products, hydrolysed plant proteins, purified lecithin, starch derivatives, and refined oils derived from GMOs.

Product-specific PCR methods have been developed for a range of different GMOs (e.g., potatoes, microorganisms, tomatoes, soy, and different maize varieties). The product-specific PCR is based on the use of primer pairs that span the boundary of two adjacent genetic elements (e.g., promotors, target genes, and terminators) or that are specific for detection of the altered target gene sequence. Detection limits range from 20 pg to 10 ng of target DNA and from 0.0001% to 1% of the mass fraction of the GMO.

Most currently available GMOs contain three genetic elements (i.e., CaMV 35S promoter, NOS and *nptII*), and screening PCR methods can be performed to detect each of these. Only if the PCR assay gives a positive result should subsequent product-specific PCR assays be performed. Four different methods are available to

confirm the PCR results: (1) specific cleavage of the amplification product by restriction endonuclease digestion, (2) hybridisation with a DNA probe specific for the target sequence (as is used in all official methods listed in the German Food Act), (3) direct sequencing of the PCR product, and (4) nested PCR, in which two sets of primers bind specifically to the amplified target sequences.

Certain limitations of the PCR-based methods were described. Because CaMV 35S promoter, NOS, and *nptII* sequences may occur naturally in foods, their detection does not necessarily prove that recDNA is present. Additionally, false-positive results from accidental contamination of samples or reagents are often a major problem in routine diagnostic analytical applications. This includes cross-contamination between samples or carry-over from previous PCR analysis. In contrast, false-negative results are less common owing to the extremely high sensitivity of the PCR. Nevertheless, strict physical separation of all individual PCR steps is absolutely necessary to prevent carry-over contamination of extracted DNA and PCR products.

Owing to the number of tests that are expected to be performed to identify GMOs in food, it is necessary to improve the efficiency of routine analysis. Automation and the application of new techniques are required for fast and specific routine screening of GMOs. For the development and standardisation of methods based on PCR, certified reference material with different amounts of GMOs is essential for quantification and interlaboratory test comparisons. For example, the IRMM has done this with dried soy bean powder.

Dr. G. van Duijn (TNO Nutritional Food Research Institute, The Netherlands) reported a specific and sensitive nested PCR method that was developed for the detection of transgenic Roundup Ready™ soy bean DNA. Different primers were used for the specific detection of the *Agrobacterium* sp. CP4 EPSPS gene in combination with the NOS terminator as a unique DNA construct. Detection limits were between 0.01% and 0.1%, depending on the type of product examined, e.g., soy bean meal, protein, lecithin, oil, or a number of processed products.

4.1.2. Results of an interlaboratory assessment of a screening method of GMO in soya beans and maize

Dr. M. Lipp (EC Joint Research Centre, Ispra, Italy) presented the results of an interlaboratory study of a PCR screening method for GMO in Roundup Ready™ soy beans and Maximizer (Novartis–Agribusiness)™ maize co-ordinated by the EC Joint Research Centre. A screening method was applied for the detection of the CaMV 35S promotor and the NOS terminator gene from *Agrobacterium tumefaciens*. The method was applied to 26–28 transgenic plants in 1997. A total of 41 laboratories in 14 countries were invited to participate, and results from 29 laboratories were received.

The sensitivity and reproducibility of the PCR method depend on the reagents and apparatus used. Each participating laboratory was requested to perform its own optimisation of the PCR method using defined samples containing varying amounts of GMO that were prepared at the IRMM. For DNA extraction, no particular method was specified, but detailed descriptions of two DNA extraction methods were given. For PCR measurements, primers specific to the CaMV 35S promotor and the NOS terminator were prescribed. Verification of the amplification products was done by restriction endonuclease digestion. The results of this validation study demonstrated that the PCR screening method is suitable for the detection of GMO in raw material derived from soy beans and maize. Samples containing 2% transgenic soy beans or maize were unequivocally and correctly identified by all laboratories. Furthermore, correct classification was achieved by analysing the CaMV 35S promotor in samples containing 0.5% GMO soy beans. The method for the detection of the NOS terminator is less sensitive compared with that for the detection of the CaMV 35S promotor, and therefore a somewhat higher number of false-negative results was reported. Owing to the larger size of the genomic DNA of maize, this screening method is somewhat less sensitive for the detection of transgenic maize in raw material (i.e., there was a larger number of false negatives with respect to the false-positive rate for soy beans).

4.1.3. Report of the working group on qualitative recDNA analysis of GMOs (Rapporteur Dr. R. Fuchs, Monsanto Company, United States)

4.1.3.1. DNA detection methods. A tiered approach using qualitative PCR methods as an initial screen was recommended for the analysis of food products. If no GMO-specific DNA is detected by an appropriately validated qualitative method, the product(s) would need to be evaluated for the presence of protein. If no protein was detected, the product(s) would not need to be labelled. If the qualitative PCR showed a positive result, the manufacturer would have the opportunity to either label the resulting product(s) or to utilise an appropriately validated quantitative PCR method to assess whether the detected level of GMO was above or below an established threshold. If the quantitative PCR assay showed the level of GMO to be above the threshold, the product(s) would be labelled. If the quantitative PCR assay showed that the level of the GMO was below the threshold level, the product(s) would not need to be labelled. Both the qualitative and the quantitative PCR methods must be validated before their use for these analyses.

4.1.3.2. Threshold levels. The workshop supported the council's recommendation that a threshold level of GMOs that would trigger labelling of GMO-containing foods should be established. Definition of this threshold is critical to the development and validation of detection and enforcement methods for both DNA and protein. This threshold needs to clearly define both the numerical threshold and whether this threshold applies to the total amount of GMO in a food product or to the ratio of GMO in specific ingredients (e.g., 2% of GMO relative to the corresponding non-GMO). Moreover, different thresholds may need to be established, one for compulsory labelling as recommended by the European Union and one (possibly much lower) to guarantee the GMO-free status of the product. Threshold values should not be based on or influenced by the sensitivity of available detection methods. Instead, establishment of threshold levels should take into account the levels of co-mingling that occur during the typical growing, harvesting, collecting, shipping, and processing of a grain or food. These levels should assume that production is conducted under "good agricultural practices". Current Codex Alimentarius guidelines should be taken into account in establishing a threshold. For example, Codex has established guidelines for acceptable levels of co-mingling of soft wheat in durum wheat, waxy maize in commodity maize, and maize in soy bean. The working group agreed that the current detection methods would allow implementation of a threshold of 2%.

4.1.3.3. "Negative list" of products. The working group strongly supported the council's recommendation to establish a "negative list" of ingredients that would not need to be analysed for GMO content. This list should be based on the extensive knowledge of processing methods and composition of food ingredients and should be developed by food science experts. Minor amounts of DNA or protein should not preclude inclusion on the list. For example, highly refined oils, sugars, and processed starch or starch-derived products should be included on the negative list. Per the council's recommendation, a de minimis level of DNA or protein that may be detected in such fractions is not meaningful. Because the sensitivity of detection methods will continue to increase, the ability to detect extremely minute amounts of DNA or protein in some highly processed products may change with time. Such levels are not meaningful, and their inclusion in a negative list would save the time and resources required to develop, validate and continuously monitor such ingredients. Establishment of this negative list will also help resolve many issues for the development, validation, and implementation of detection methods by focusing these efforts on the more relevant food ingredients.

4.1.3.4. Samples to be analysed. The working group recommended that raw agricultural commodities and major processed food components should be analysed. Validated methods already exist for flour from Roundup Ready™ soy beans and for Maximizer™ maize. Qualitative PCR methods have also been developed for other processed food components from these products and for other genetically modified plant products. However, these methods need appropriate validation and need to be subjected to ring testing to establish their robustness.

4.2. Quantitative analysis

4.2.1. Quantitative competitive PCR for the detection of GMOs in food

A critical aspect of the analysis of GMOs in foods is quantification, because maximum limits of GMOs in foods are the basis for labelling in the European Union. Dr. P. Hübner (University of Bern, Switzerland) demonstrated that specific detection of GMOs involves a combination of several genetic elements such as the promoter, signal peptide gene, structural gene, and terminator. In Switzerland, the decision for labelling is based on the presence of the CaMV 35S promoter. Furthermore, he reported on the development of a quantitative competitive PCR (QC-PCR) system for the detection and quantification of Roundup Ready™ soy beans and Maximizer™ maize DNA in food samples. The principle of the method is the amplification of internal DNA standards together with target DNA and their subsequent quantification by photometric methods. DNA standards were constructed in a manner similar to the construction of the target DNA, but were distinct from the GMO by specific sequence insertions. The system was calibrated by co-amplification of mixtures of GMO DNA and corresponding amounts of conventional soy bean or maize DNA. Such standards are commercially available and contain known amounts of standard DNA. Determination of the so-called *equivalence point* is the basis for quantification. The system has been tested on commercial food samples (lecithin, flour, protein, and grist), with varying GMO contents ranging from less than 0.1% to more than 2%, using certified Roundup Ready™ flour mixtures for calibration. Detection of as little as 0.1% GMO DNA in samples is possible. Results of an interlaboratory study carried out among laboratories mainly from Switzerland showed that there are no problems with matrices containing more than 2% GMO (i.e., there were no false positives or false negatives) and that the semi-quantitative method is applicable for matrices containing more than 1% GMO. One of the advantages of QC-PCR methods relative to non-competitive PCR methods is that interference of PCR inhibitors will be readily evident because they will affect both internal standard and

target DNA amplification. QC-PCR methods provide excellent possibilities for the simple and reliable detection and quantification of GMOs in food samples.

4.2.2. Quantitative analysis of GMO in processed foods

Dr A. Wurz (Gene Scan Gmbh, Freiburg, Germany) further addressed issues related to the application of quantitative PCR methods. He explained the use of a double competitive PCR, which is well suited to determine thresholds of GMOs in food, and an on-line method for determining ratios of transgenic to non-modified DNA components. One of the challenges to be resolved is the discrimination between real GMO mixtures and inevitable traces of GMO resulting from contamination. It is important to characterise the DNA quality (modification, fragmentation) and purity (heterogeneity of DNA) when conducting such analyses.

The double competitive approach was demonstrated with DNA extracted from GMO soy bean reference material and the estimation of the lectin gene copy number. A second PCR, specific to Roundup Ready™ soy beans, is performed with different quantities of competitor DNA, yielding band intensities around a pre-set threshold level. The method is independent of DNA quality and of heterologous DNA content, and can distinguish between 0.5% and 2% GMO content. An advantage of this method is that no special equipment has to be purchased by laboratories already carrying out qualitative PCR.

A second quantitative approach used the TaqMan® ABI PRISM® 7700 Sequence Detection System (The Perkin–Elmer Corporation), a method that allows for the quantification of DNA by measuring the kinetics of PCR amplification instead of an endpoint measurement. Quantification is possible by the use of an internal standard. The TaqMan® method has been evaluated using GMO soy bean reference materials ranging from 100% to 0.1% GMO content. A 0.5% and 2% content could be clearly distinguished. The method is well suited for automation and high throughput of samples, and can be used for raw, processed, and even mixed products.

4.2.3. Report of the working group on quantitative recDNA analysis of GMOs (Rapporteur Dr. D.A. Neumann, ILSI Risk Science Institute, United States)

The working group limited its discussion to consideration of current methods employing PCR. The essence of this method is to extract DNA from the food of interest in a manner that will yield highly purified DNA that is not overly degraded and is free of impurities and/or contaminants that can inhibit the polymerase reaction. The recDNA of interest is replicated through an empirically determined number of amplifications using nucleic acid sequence-specific primers for the gene region of interest. Amplification may be performed using nested primers; an unnested protocol also may be used. Quantitation is achieved by using an internal or external calibration standard which is amplified, with unique primers, in conjunction with the sequence of interest.

Quantitative PCR (Q-PCR) likely will ultimately be the method of choice for routine determination of absolute and relative amounts of GMOs or their products in foods. Q-PCR methods are sufficiently robust to be effective for virtually all DNA-containing food matrices and have sufficient sensitivity to be suitable for quantification at any conceivable threshold concentration/level. The specificity of Q-PCR methods depends on the investigator's selection of the relevant genomic target sequence and the flanking primer sequences.

As is typical of all sophisticated laboratory methods, the adequacy, reliability, and reproducibility of Q-PCR analyses will reflect the training, experience, and knowledge of the investigator. Laboratory personnel conducting Q-PCR analyses will need rigorous training with regular proficiency testing. Analyses should be performed in a controlled and well-defined laboratory environment that promotes the maintenance of sample integrity, eliminates or minimises the potential for sample contamination, and is managed in a manner consistent with good laboratory practices. Indeed, approaches and procedures developed for clinical PCR facilities could serve as models for laboratories evaluating the GMO content of foods. All analyses should be conducted in a scientifically rigorous manner, including the use of appropriate control procedures and reagents to ensure the reliability and relevance of the results.

Q-PCR analyses are rapid and reliable, and the methods are suitable for routine analysis. Certain aspects of the analytical process may be amenable to automation and thus could reduce per-sample analysis costs. At present, per-sample costs are greater for Q-PCR than for qualitative PCR and for ELISA-based protein analysis. The greater costs associated with Q-PCR reflect, in large measure, the time and labour necessary to recover high-quality, inhibitor-free DNA suitable for amplification. Although a variety of DNA extraction procedures and reagents are available, the efficacy of each will be determined in part by the food matrix to which it is applied. The adequacy of the DNA extract can best be assessed by determining its amplifiability. The quantity of DNA recovered, which is difficult to establish, provides little insight into the adequacy of the preparation.

The sensitivity and specificity of Q-PCR methods and their ability to be tailored for application to various food matrices suggest that such methods are well suited for establishing the GMO content of foods. The greatest uncertainty, however, is the establishment of the basis for expressing GMO content. Recognising that not all products derived from, e.g., GMO soy beans (e.g., refined oil) contain DNA and that heating and other

processes associated with finished food production can degrade DNA, the degree of precision possible remains unclear. Similarly, if GMO content is to be expressed on a relative basis, i.e., as percent GMO, investigators will need to know whether, particularly in the context of finished foods, the estimate is to be based on total DNA from all sources or on, for example, the total soy bean DNA present if the soy beans are being analysed for GMO content. This latter approach, referred to as genomic equivalence, is a pragmatic and scientifically sound approach that has been shown to correlate with the results of studies where GMO content was expressed on a percent weight basis.

Because DNA extraction methods are so readily applied to raw or unprocessed food and food ingredients, the working group observed that Q-PCR might best be applied at early stages in the food production chain. By using the genome equivalence approach to assessing the GMO content of food ingredients and tracking ingredient use, it should be possible to calculate the GMO content of finished food products. Such an approach, based on establishing GMO content on the basis of genomic equivalence at the ingredient level, would be consistent with current food labelling regulations that focus on ingredients. In addition, this approach would be applicable to finished foods containing more than one GMO-derived ingredient.

The deliberations of the working group revolved around the concept of GMO content thresholds, i.e., the relative proportion of the food, food ingredient, or finished food product derived from a GMO. Because the establishment of such a threshold is an issue of policy rather than science, the working group observed that the high sensitivity and specificity of Q-PCR methods may require the establishment of two thresholds. One lower threshold would, by definition, distinguish between GMO-free and foods inadvertently containing small amounts of GMO material, and the other higher threshold would trigger mandatory labelling.

4.2.3.1. Conclusions and recommendations.

- Quantitative PCR is a sensitive, specific, and robust method for determining the GMO content of any food matrix.
- This method will be effective for any GMO content threshold likely to be set for the purposes of mandatory food labelling.
- For this method to be used effectively, laboratory personnel need to be well trained and tested for proficiency.
- Analyses need to be conducted in a scientifically rigorous manner to ensure test integrity.
- The critical rate-limiting step in quantitative PCR analysis is DNA extraction; the best measure of the adequacy of the extraction method is the amplifiability of the recovered DNA.
- The concept of genomic equivalence provides a scientifically sound approach to the quantification of GMO DNA in foods and food ingredients.
- Assessment of GMO content of raw and partially processed foods and food ingredients may afford the best approach to satisfying GMO labelling requirements.

5. Future requirements for detection methods for GMOs

Dr. G. Schreiber (EC Directorate General III, [Industry], Brussels, Belgium) identified a number of challenges to the development of methods to detect GMOs in foods. He referred to EC Council Regulation 1139/98 and noted that quantitative detection systems are needed. The reliability of the available methods for quantification of GMOs in foods remains to be determined and should be evaluated in interlaboratory trials. He referred in this respect to an ongoing research project supported by the European Union that is addressing the development of PCR-, RNA-or protein-based detection methods for GMO-containing foods and the establishment of a database of GMO foods introduced into the market. Furthermore, he emphasised that the efficiency of screening and confirmation strategies should be examined with respect to false-positive rates, disappearance of marker genes, increased use of specific regulator sequences, and the rapidly increasing number of genetically modified foods. The greatest challenge will be the development of multi-detection methods which are flexible and rapidly adjustable. New ideas are needed to tackle the problem of the detection of non-approved GMO foods where information on modified gene sequences is not available. Establishment of databases in this area is of great importance. Furthermore, the fast-paced developments related to GMO detection methods were noted and contrasted with the relatively slow procedures of the standardisation process as carried out in the framework of the European Committee for Standardisation (CEN). Preparation of European prestandards may be an appropriate approach in this rapidly developing field.

6. General discussion, conclusions and recommendations

Issues raised during the general discussions and working groups discussions confirm that though substantial progress has been made with respect to the identification and detection of genetically modified foods, a number of problems remain to be solved. There is an urgent need to tackle these problems, since EC Council Regulation 1139/98 concerning the compulsory labelling of GMO foodstuffs entered into force on 1 September 1 1998.

- Development of internationally accepted, harmonised sampling plans based on sound scientific and statistical principles is urgently needed, and initiatives should be undertaken quickly to ensure the acceptability of GMO products, which to a great extent are traded internationally. Expertise can be gained through co-operation with the Codex Alimentarius Committee on Methods of Analysis and Sampling and with other groups of experts (e.g., Food Chemist, Confederation of Food and Drink Industries of the EU [CIAA]). Possibilities for funding such activities should be explored, perhaps through the Fifth Framework Programme of the European Union.
- The production of appropriate reference materials as the basis for the validation of analytical detection methods and for the assessment of method performance should be regulated and *not* the methods for detection. Production of reference materials should be limited to key commodities such as raw materials and basic ingredients rather than finished foods. This may be difficult for primary products because methods of production could be different. Manufacturing and quality control of reference materials within the EU should be organised and co-ordinated at a single research centre, such as IRMM. GMO producers should provide information on the nature of gene sequences and provide test materials such as antigens and antibodies or the means for the co-ordinating centre to produce them.
- Both DNA and protein detection methods are available for the detection of GMO foods. Both methods have their place, because protein and DNA analyses are both currently required by EC Council Regulation 1139/98. ELISA methods appear to be the methods of choice for screening raw materials and basic ingredients when the presence of modified proteins can be expected. These assays are fast, robust, suitable for routine testing, and relatively inexpensive; and detection limits are of the order of 0.5–1%. However, their applicability is limited and dependent on the degree and nature of processing the food has undergone. Most ELISA methods should be considered research tools because none are yet commercially available.
- PCR methods are available for qualitative screening and quantitation of the recDNA of interest in virtually all DNA-containing food matrices. Quantitation is achieved by using internal or external calibration standards. The sensitivity of these methods is suitable for quantitation at very low levels (e.g., as low as 0.01%). As for all sophisticated laboratory methods, training, experience, and skills of laboratory personnel are essential for adequate performance of the tests. Quantitative PCR will ultimately be the preferred method of analysis of GMO foods.
- A tiered approach for the detection of GMO foods has been proposed with respect to regulatory requirements for labelling or for GMO-free declarations. If a qualitative PCR method indicates the absence of recDNA, the product has to be screened for the presence of protein. If no protein is detected, products need not be labelled. If the qualitative PCR shows positive results, the manufacturer may decide to label the food or apply to a validated quantitative PCR method to assess whether detected levels are below or above established threshold levels.
- The occurrence of false-positive findings with recDNA or protein detection methods may pose serious problems. Because GMO detection is relevant to European labelling requirements and is not related to safety concerns, a high degree of reliability and specificity of the detection methods should be expected. False-positive findings must be avoided by all means because they may result in the unnecessary destruction of foods.
- Available protein and recDNA detection methods need to be validated, and ongoing activities organised by the Joint Research Centre (Ispra) within the framework of European Union-sponsored research programmes are of great importance. There is an urgent need for internally validated detection methods that fulfil certain (but undetermined) performance criteria, rather than for standardised methods, given the relatively slow process of standardisation. Performance criteria should be established and internationally accepted. In this way, laboratories may adapt more quickly to rapid developments in the area of genetically modified food products. One of the challenges in this area is the development of multidetection methods that can be adjusted quickly according to the type of genetic modification.
- The establishment of thresholds for labelling and/or for GMO-free declarations is an urgent matter, particularly with respect to numerical values, basis of expression, and types of foods. There is still uncertainty whether the GMO content is to be expressed on a relative, percent basis of total DNA from all sources present in the food, or related to the recDNA and total corresponding non-recDNA (e.g., recDNA from soy beans relative to total DNA from soy beans). The latter, genomic equivalence approach renders it possible to establish the GMO content in finished food products and is consistent with current food-labelling regulations for food ingredients.
- There is great uncertainty with respect to the establishment of a "negative list" of ingredients that would not need to be analysed for GMO content. Establishment of such a list was strongly supported by the workshop participants. The list should be based on extensive knowledge of processing methods and of the composition of food ingredients, recognising that

not all products derived from GMO foods contain DNA and that food processing may substantially degrade DNA and protein. The presence of minor amounts of DNA or protein in a food should not preclude inclusion on the list (e.g., highly refined oils or processed starch products). With establishment of a negative list, method development and validation for the detection and monitoring of GMO foods can be focused on the more relevant food ingredients.

- Concerns have been expressed with respect to non-approved GMO foods and feed that may enter international markets. There is a need for data collection with respect to types of genetic modifications, target and marker gene sequences, target organisms, and traceability. International organisations like the International Service for the Acquisition of Agri-biotech Applications (ISAAA) and other scientific institutions may take initiatives in this area.
- Given the state of the science of detection and identification of genetically modified foods, further activities should be undertaken in this field. ILSI may play a leading role in organising expert meetings on the specific scientific problems identified during this workshop.