

# METHOD DEVELOPMENT IN RELATION TO REGULATORY REQUIREMENTS FOR THE DETECTION OF GMOs IN THE FOOD CHAIN



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SUMMARY REPORT OF A JOINT WORKSHOP HELD IN DECEMBER 2000

Prepared by the ILSI Europe Novel Food Task Force  
in collaboration with  
the European Commission's Joint Research Centre (JRC)  
and ILSI International Food Biotechnology Committee



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Summary Report on Method Development in Relation to Regulatory Requirements for the Detection of GMOs in the Food Chain

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REGULATORY REQUIREMENTS FOR THE  
DETECTION OF GMOs IN THE FOOD CHAIN***

SUMMARY REPORT OF A JOINT WORKSHOP HELD IN DECEMBER 2000 IN BRUSSELS, BELGIUM

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ORGANISED BY THE ILSI EUROPE NOVEL FOOD TASK FORCE IN COLLABORATION WITH  
THE EUROPEAN COMMISSION'S JOINT RESEARCH CENTRE (JRC)  
AND ILSI INTERNATIONAL FOOD BIOTECHNOLOGY COMMITTEE



March 2001



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## INTRODUCTION

**W**ith the introduction of foods derived from genetically modified organisms (GMO) – the first products entered the European Market in 1996 – there has been an increasing need for appropriate analytical methodology to facilitate the implementation of various directives and regulations of the European Union (EU) relative to the release and labelling of such foods. EU legislation addressing various aspects of GMO development, release, and food use are summarized in Table 1.

Agricultural GMOs have increasingly been the subject of controversy in public discussion. Consumers demand the right to choose between GMO and non-GMO products, and within the EU, food labels identifying foods derived from GMO crops are considered the most appropriate means of providing for consumer choice.

*Table 1: EU Legislation related to genetically modified organisms*

LEGISLATION	PURPOSE
Directive 90/219/EEC	Contained use
Directive 90/220/EEC (in revision)	Deliberate release into environment
Regulation 258/97/EEC	Novel Foods and ingredients
Regulation 50/2000/EEC	Additives and flavourings
Regulation 1139/98/EEC	Labelling of two GMOs (Roundup Ready® soybeans and BT-176 maize)
Regulation 49/2000/EEC (amendment of 1139/98/EEC)	1% threshold
In preparation	Novel feeds from GMOs
In preparation	Novel Seeds

The purpose of GMO detection is to assess whether a product contains material derived from genetically modified crops. Qualitative screening methods can be used for this purpose, yielding a positive or negative answer. If the result is positive, it is important to know whether the GMO is authorized within the EU. This leads to the need for suitable identification methods. This is necessary not only for raw agricultural products but also for processed and highly refined ingredients. Processing makes detection more difficult. If a product is found to contain one or more authorized GMO, the next step is to assess compliance with the content-based labelling threshold established by the EU. For this purpose, quantitative methods are required. New developments in biotechnology provide new challenges to the existing analytical techniques, and new approaches must be developed. To ensure comparable analytical results from different laboratories, analyses should be carried out with validated methods, or at least with methods meeting similar performance criteria with respect to precision, accuracy, sensitivity, specificity and robustness.

The aim of the workshop that is summarized in this report was to investigate progress made in the development of analytical methods since the previous workshop in June 1998.\* Scientific presentations (oral and posters) were given at the workshop on subjects such as sampling strategies, methods for detection and quantification, reference materials, analytical method performance criteria, and identity preservation. GMO regulations worldwide and the development of biotechnology-derived products were reviewed during the workshop. Each of these topics was discussed in greater detail afterwards when workshop participants met as working groups to address these issues. The conclusions and recommendations reached by the working groups were presented to and discussed by all participants during the final plenary session of the workshop. The invited presentations, the working groups' reports, and the conclusions and recommendations of the workshop are summarized below.

This workshop held in Brussels, Belgium, on 11–13 December was jointly organized by the European Commission's (EC) Joint Research Centre (JRC) and the International Life Sciences Institute (ILSI Europe and the ILSI International Food Biotechnology Committee). Experts in biotechnology, official food control, food toxicology, and related regulatory fields from national institutions (academia, governments) and from industry, were invited to discuss the state of the art of analytical methods for the detection, identification, and quantification of GMO, together with subjects such as worldwide identity preservation and legislative aspects. The workshop was co-chaired by Mr. B. McSweeney, director of the JRC, Institute for Health and Consumer Protection (Italy) and by Mr. M. Knowles of the Coca-Cola West Europe Group (Belgium).

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\* The information presented at the June 1998 meeting is available in two publications: International Life Sciences Institute, *Detection Methods for Novel Foods Derived from Genetically Modified Organisms* (a summary report of the meeting presentations and discussions). ILSI Europe, Brussels, 1998. 24 p. and M. Grasserbauer, M. Knowles, H.A. Kuiper, & F. Gendre. (eds). Special Issue: *Detection Methods for Novel Foods Derived from Genetically Modified Organisms*. *Food Control* **1999**;10:3339–414 (which contains the journal presentations).

## WORKSHOP INTRODUCTION

**M**r. McSweeney opened the workshop with a brief overview of JRC's activities. The JRC is a Directorate General (DG) of the European Commission. Its mission is to provide customer-driven scientific and technical support for the conception, development, implementation, and monitoring of EU policies. As a service of the EC, the JRC functions as a reference centre for science and technology for the EU. Close to the policy-making process, it serves the common interest of the member states while being independent of special interests, whether private or national.

Mr. McSweeney and Mr. Knowles summarized the previous workshop that focused primarily on sampling methods, reference materials and detection methods. Many changes have taken place within the field of GMO EU legislation, such as the labelling threshold regulation and planned legislation on novel seeds and novel animal feeds. In addition, more analytical methods have been validated since 1998, experience has been gained with respect to the development of reference materials, and there is a better understanding of considerations in the development of sampling strategies. The concept of traceability has become important in the context of EU legislation and the distribution of biotechnology-derived products in Europe. These developments, in part, provided the motivation for convening this workshop. The workshop summary and proceedings will be published in the journal of the *AOAC International* in 2001.

## INVITED PRESENTATIONS

### *Regulations*

Ms. H. Hoffmann (EC, DG Health and Consumer Protection, Belgium) discussed the Novel Foods Regulation (Regulation (EC) No. 258/97) as the legal basis for the mandatory labelling of foods and food ingredients that contain or consist of GMOs. The labelling requirements for foods derived from GMO but no longer containing GMO are based on the concept of equivalence. When a characteristic or property of the product, such as its composition, its nutritional value or nutritional effect, or its intended use, renders the food or food ingredient no longer equivalent to an existing counterpart, labelling is required and must indicate the method of its production, i.e., by genetic modification. Council Regulation (EC)1139/98 applies specifically to the labelling of products derived from a variety of maize with insecticidal properties conferred by an introduced gene from *Bacillus thuringiensis* (Bt) and of products derived from Roundup Ready® (RR) soybeans. These varieties were approved within the EU prior to the entering into force of the Novel Foods Regulation under Directive 90/220/EEC on the deliberate release into the environment of GMO. In both cases, labelling is predicated on the analytical detection of the introduced DNA or its protein product in the food or food ingredient. Council Regulation (EC) 1139/98 now serves as a model for the labelling of all GMO-derived foods and food ingredients. Early in 2000 the EC began harmonizing labelling requirements relative to GMO-derived additives and flavourings through Regulation (EC) 50/2000. Regulation (EC) 49/2000 introduces a *de minimis* threshold of 1% for the adventitious presence of genetically modified DNA or protein from EU-approved GMO varieties in conventional foodstuffs. Foodstuffs found by chemical analysis to contain modified DNA or its protein product at or above the threshold must be labelled as genetically modified. These policies reflect a commitment by the EC to provide consumers with information to enable them to choose between GMO and non-GMO foods. The EC currently is exploring approaches for assuring adequate traceability of products, how to meet public expectations for labelling, and the technical feasibility and need for establishing GMO-free labelling criteria. Some of these issues will result in new legislation.

International guidelines and safety and labelling regulations in various non-European countries were reviewed by Mr. M. Tabata (Food and Agriculture Organization of the United Nations, Italy). He described the findings and conclusions of the 1996 and 2000 Joint FAO/WHO expert consultations that provided guidance for the safety assessment of biotechnology-derived foods, and reviewed the work of the Codex Alimentarius Commission's Ad Hoc Task Force on Foods Derived from Biotechnology. He noted that the Codex Committee on Food Labelling is currently trying to reach agreement on whether biotechnology-derived foods should be labelled based on content or specific safety concerns, or be mandatory based on the method of production. Mr. Tabata reviewed U.S. policy regarding labelling, as articulated by the U.S. Food and Drug Administration's (FDA) 1992 policy statement. The FDA's ongoing review of its 1992 policy, which calls for labelling only when there are significant health concerns, when there have been changes in composition, or when information should be provided on the preparation of a food, was acknowledged. On April 1, 2001, Japan will adopt a mandatory labelling policy for products from biotechnology-derived maize, soybean, canola, potato, and cotton; most of the 23 specifically targeted products are derived from soybeans. Mandatory labelling will be required for products that are no longer equivalent and those that are substantially equivalent but have detectable introduced DNA or its protein product present; labelling will not be required if DNA or protein cannot be detected. In Australia and New Zealand all biotechnology-derived foods are subject to

safety assessment; of those reviewed so far, no specific safety concerns have been identified. Labelling is required for any product found not to be substantially equivalent to its conventional counterpart; highly processed foods, flavours, and food additives are exempt from labelling. Both countries will operate under a single labelling policy that will enter into effect in September 2001.

### ***Biotechnology-derived crops: development and analytical challenges***

The history of food crop improvement was described by Mr. D. Grothaus (Pioneer Hybrid International, United States), who noted that per hectare maize yields were constant from the 1860s through the 1920s, when the introduction of hybrid varieties resulted in increased yields. Almost all new varieties of maize introduced since then have been hybrids. As understanding of crop genetics and of the tools of molecular biology has improved, the possibility to further enhance productivity and to introduce other traits has become possible. He described the many steps required to bring a biotechnology-derived product to the marketplace, noting that DNA and protein detection methods were originally designed and intended to distinguish between plants expressing the desired traits and those that did not, and for evaluating products as they moved through the development stream. The methods used by developers were not designed to certify the absence of a gene or its protein product, and because they were designed for assessing plants and seeds they were not expected to be useful for application to other matrices, e.g. final food products. A survey of more than 3000 food products marketed in the United States indicated that more than 7000 maize derivatives are used in their production. He discussed the rationale for, and the role of marker genes and how “stacked genes” may be introduced by inserting a single gene construct containing multiple genes or by crossing varieties that have been independently transformed with different genes. Mr. Grothaus noted that globally, biotechnology-derived crops now account for 34% of soybean production, 16% of cotton production, and 7% of maize production.

Mr. G. van den Eede (EC, DG Joint Research Centre – Institute for Health and Consumer Protection, Italy) noted that biotechnology-derived varieties authorized for use in the EU typically are hybrids or varieties derived from one or more genetically transformed parental lines. Because of this, there can be uncertainty regarding the actual copy number of the inserted gene and the DNA sequence of the border region in each of the derived commercial varieties. He also noted that gene expression can vary between different parts of a seed; e.g. the endosperm and the embryo in a maize kernel have different patterns of gene expression. Observations such as these can lead to a variety of questions: What are the appropriate negative and positive reference samples? What part of the kernel should be tested, and what is the appropriate reference material? How should testing of different food matrices be addressed? and What is the most effective means of expressing DNA content? Mr. van den Eede pointed out the technical challenges posed when considering the testing of products that have introduced DNA from different sources, e.g. maize and soybean, and he noted that the difference in the size of the genome of each will confound DNA quantification. Although the intent of the 1% threshold is to recognize the potential for adventitious mixing, analytical methods cannot distinguish between adventitious and non-adventitious material. Nevertheless, the Novel Foods Regulation makes clear that mill and plant operators cannot intentionally mix a material with GMO content below the threshold with GMO content material exceeding the threshold in order to yield a product that would not require labelling. Operators must be able to document the absence of such mixing.

## *Sampling, sample preparation, and reference materials*

Mr. G. Brooks (PG Economics, United Kingdom) observed that the concept of identity preservation (IP) is not new, and may at times be used to refer to either separation (i.e. selecting for desired properties) or segregation (i.e. weeding out undesirable properties). Testing and tolerances are important components of IP systems, but testing alone may not be sufficient to verify claims. In the case of the current generation of biotechnology-derived products where the benefits tend to accrue to farmers, there is no commercial incentive for IP; however, at least some consumers are calling for the segregation of such materials. The costs of implementing an IP system for biotechnology-derived crop plants will vary depending on the range of products to be derived, the use/importance of the crop in the end products, the tolerances and specifications established, and the sophistication of the distribution system. Cost estimates for such a system are dynamic, reflecting the volume of material, operator experience, and the adoption of dedicated supply chains, factories, and machinery.

In the United States, the Department of Agriculture (USDA) is responsible for grading and assuring the quality of commodity crops, stated Mr. S. Tanner (USDA, Grain Inspection, Packers and Stockyards Administration (GIPSA, United States), and hence plays a key role in the international marketing of U.S. agricultural products. GIPSA has developed a set of comprehensive guidelines for grain sampling to test for the presence of biotechnology-derived products. These methods are consistent with those used for assuring grain quality with respect to fungus contamination and other parameters associated with quality assessment. Working with the U.S. National Institute for Standards and Technology (NIST), GIPSA has been instrumental in collaborating with a variety of partners to assess and validate methods, including commercially produced test kits, for detecting the presence of the protein product of introduced or modified genes. Mr. Tanner noted that the scale of agriculture production in the United States, and elsewhere is such that only protein immunoassay methods are practical for these applications because of their ability to quickly evaluate large numbers of samples. He discussed the relationship between sample size and test sensitivity, and noted that effective sampling methods are critical to both material characterization and compliance testing. GIPSA also is developing criteria for the certification of laboratories providing testing services, and although it is well known that certification alone does not guarantee the quality of the results, it is anticipated that reports from US certified laboratories would be accepted in Europe and elsewhere. Mr. Tanner referred workshop participants to the GIPSA website ([www.usda.gipsa.gov](http://www.usda.gipsa.gov)) for further information about the agency's programs and sampling and testing guidelines, including recommendations for grain sampling plans, sample size, and sampling methods.

Mr. A. Heissenberger (Federal Environment Agency, Austria) described the work of the European Normalisation Committee (CEN) in developing standard methods for the detection of biotechnology-derived products. Working groups of experts from Europe and elsewhere are developing standard methods for sampling, sample extraction, protein immunoassays, and qualitative and quantitative polymerase chain reaction (PCR) assays for DNA. The emerging guidelines are intended for application to commodity grains and early derivatives, e.g. flours and meals, and are unlikely to be broadly applicable across diverse food matrices. The CEN guidelines differ from those of GIPSA in calling for continuous sampling during the loading and unloading of bulk commodity grains; the GIPSA guidelines call for intermittent sampling. Because of the scale and complexities of agricultural production in the United States, GMO detection methods must be rapid, accurate, safe, and cost effective; protein methods are therefore most appropriate. In Europe, where testing is mandated by labelling regulations, the same criteria are applicable but are weighed differently, resulting in greater reliance on PCR-based methods. Mr. Heissenberger

distinguished between sample grouping errors and sampling errors, and noted that the error in analysing biotechnology-derived products is approximately 20%. The success of any method will be contingent on the availability of suitable reference materials and standards. The Institute for Reference Materials and Measurements (IRMM) of the EC's JRC has taken a leadership role in this area (see the summary below of Ms. Trapmann's presentation), and transatlantic discussions with NIST have been initiated to foster international harmonization in the development of reference materials. Some discussion of this collaboration has taken place within the context of the Consultative Committee on the Quality of Materials, but it will be critical to engage representatives of national competent authorities in this dialogue. The 11 ad hoc CEN working groups are expected to complete the majority of their work during 2001; a plenary meeting in March 2001 is expected to initiate a Europe-wide review and acceptance process that will probably culminate in the adoption of the standards by the International Organization for Standardization (ISO).

DNA and protein extraction methods were discussed by Ms. H. Parkes (Laboratory of the Government Chemist, United Kingdom), who noted that the quality of the extraction process underpins the validity of an analysis. For example, the quality and integrity of the extracted material and, in the case of DNA, the presence of inhibitors can influence the outcome of the analysis. Other factors influencing the analysis include the sample matrix, sample storage conditions, and the degree of processing of the material to be analysed. Although a variety of extraction methods have been described, the optimum methods need to be determined empirically. False-negative results tend to be common in analyses on samples containing target molecules at concentrations near the detection limits of the method. Conversely, false positives are more likely to be indicative of sample contamination or reflective of laboratory operating procedures. Analyses should include extraction negative controls and controls to determine whether inhibitors are present. Extraction methods should be validated with respect to yield, purity, quality and integrity, presence of inhibitors, and reproducibility. Many of these issues are being addressed through the CEN initiative described by Mr. Heissenberger (above).

Mr. M. van den Bulcke (Aventis, Belgium) discussed the relationship between gene constructs and plant breeding relative to the development of reference materials for methods used for detecting GMO products. Reference materials initially developed in this context are intended to be used to monitor the product as development proceeds from production of a gene construct to its introduction into the plant genome, its integration and stabilization through conventional breeding practices, its expression in various tissues and varieties, and its introduction into commercially important varieties. Such applications are distinct from the development of reference materials and methods for regulatory purposes and for confirming the absence of such material from a particular product. Introduced gene constructs are kept simple to minimize the introduction of extraneous DNA, are highly defined, and should integrate at a low copy number (preferably one) to ensure the stability of the introduced material. DNA analyses are performed to confirm the presence of the DNA in transformants and hybrids and to determine copy number, whereas protein assays provide quantitative estimates of the expression of the protein product of the introduced gene. Reference materials are important in the context of product development by providing information on product purity and phenotypic stability, and by allowing for the evaluation of processed fractions of the commodity crop. DNA detection methods may be trait specific or transformation event specific; the latter are more critical because they provide the ability to distinguish between authorized and unauthorized products bearing the same introduced trait, e.g. insect resistance. Importantly, reference materials are needed for both greenhouse – and field – produced product, and for stored materials, e.g. seeds, recognizing that storage conditions will be determined by the type of product under consideration.

The work of the Institute for Reference Materials and Measurements (IRMM) in developing certified reference materials (CRM) for use with methods to detect GMO products was described by Ms. S. Trapmann (DG JRC – IRMM, Belgium). Such materials are essential for evaluating the reliability and reproducibility of methods and for establishing calibration curves for quantitative methods. The IRMM has developed CRM for several GMO maize and soybean commodities authorized by the EU, and these are available commercially. Using such materials, enzyme-linked immunosorbent assays (ELISA) for RR soybeans were validated through a rigorous evaluation protocol and were found to be particularly reliable when the sample concentration was within the assay calibration range. Ms. Trapmann noted that DNA content can be only indirectly quantified and that DNA inhomogeneities can compromise validation efforts. Methods for producing CRM are similar to those associated with sample preparation and share similar technical considerations. For example, a variety of DNA extraction methods for Bt maize and RR soybeans have been described, but none appear to have universal applicability. For seeds and other types of products, the first step in the extraction process involves grinding the material into fine particles with particle size influencing the extractability of the protein or DNA of interest. Disparities between the size of particles used to produce reference materials and the size of particles derived from the material to be tested can affect the sensitivity and precision of the assay. Similarly, the quality of the extracted DNA, usually determined by evaluating its size and function, can influence the results of the analyses. Other methods for evaluating DNA quality, e.g. gas chromatography/mass spectrometry, can be used in research settings, and possibly during CRM development, but are less applicable to use during routine testing. However, such approaches may yield information that could contribute to improving extraction procedures. It has been suggested that specific methods may be applicable to certain types or classes of products, but further research will be needed to evaluate this possibility. IRMM and others are interested in developing CRM for degraded target protein and DNA from transformed food crops. Whether such laboratory-derived materials would reflect changes that protein and DNA molecules undergo during food processing remains to be determined. There also is interest in developing CRM for biotechnology-derived products that have not been authorized for release in Europe.

### *Detection methods*

Ms. I. Malmheden Yman (National Food Administration, Sweden) reviewed the molecular biochemical basis for antibody-antigen reactions that are critical to the use of ELISA and other immunoassays for detecting biotechnology-derived products. Proteins have both linear and conformational epitopes that may be recognized by specific antibody molecules, with the latter being subject to degradation when exposed to heat, acids, or other forms of processing. For this reason, a polyclonal antibody, i.e. a mixed population of antibody molecules recognizing different epitopes on a single protein molecule has the potential to be more versatile for these types of applications than does a monoclonal antibody that recognizes a single epitope. Ms. Yman described the theory and practice of performing ELISA, dipstick, lateral flow strip, and immunoblotting techniques, and presented data on detecting Bt protein and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, an enzyme responsible for herbicide tolerance) expression in GMO products using both qualitative and quantitative immunoassays. The potential for antibody cross-reactivity with both related and unrelated proteins, the influence of detergents and other agents on antibody-antigen interactions, and the potential effects of sampling and food processing on protein detection were discussed. Currently, there is little collective experience with applying immunoassays to complex food matrices; most available assays are designed for seeds or raw or partially processed products. The potential for developing standards or reference materials for partially degraded/denatured proteins is under

consideration. The next generation of immunoassays is expected to provide for greater automation and throughput, and could include biosensor technology. Because the next generation of GMO products might possess two or more introduced or altered proteins, an appropriate approach to determining whether the material is above or below the labelling threshold will need to be established. With the anticipated introduction of food crops containing multiple introduced proteins, access to reference materials and the development of antibody reagents might challenge the ability of analysts to develop and apply relevant immunoassays to detect biotechnology-derived products.

Protein detection methods currently focus on proteins associated with insect-protected (e.g. Bt) and herbicide-tolerant (e.g. EPSPS) maize, soybean, canola, and cotton according to Mr. J. Stave (Strategic Diagnostics, Inc., United States). Most work conducted to date has focused on the five transformation events that have been authorized for import into the EU. However, a variety of other transformation events are commercially available in other parts of the world, and appropriate immunoassays have been developed for these products as well. The development of ELISA-based test kits for Bt maize and RR soybeans began prior to the implementation of the Novel Foods Regulation in Europe. At that time the assays were designed to have a > 99% probability of detecting the target protein when present at or above a 2% (weight/weight) threshold, which was widely anticipated to become the EU standard. With the subsequent adoption of the 1% threshold, the assays have been appropriately modified for greater sensitivity and have been validated through international ring trials. Such assays are not intended to be quantitative in the sense of yielding specific measures of protein content; e.g. the introduced protein concentration is  $1\% \pm 0.01\%$ . As currently used, quantitative immunoassays are influenced by the performance characteristics of the method, variability of protein expression as affected by growing conditions, tissue-specific variability in target protein distribution, and sample preparation, e.g. protein denaturation (unfolding) and degradation (hydrolysis). The lateral flow strip assay was designed to be a rapid, reliable event-specific field test for controlling the distribution of biotechnology-derived products based on its ability to detect its protein target in a large sample; it was not anticipated to become a tool for regulatory compliance testing. A key factor in establishing the utility of an assay is to determine a maximum sample size, because this influences the ability of the assay to yield a positive (protein present) or negative outcome. The sensitivity of the assay is a function of the sample size and the number of samples tested. Hence, methods that provide statistical confidence that the protein content is below the threshold have particular application in identifying preservation systems where the goal for testing is to confirm that the product is above or below the threshold, rather than to determine the amount of GMO material present.

Mr. G. van Duijn (TNO Nutrition and Food Research Institute, Netherlands) discussed the various applications of protein and DNA detection methods, noting that each may be applied qualitatively or quantitatively. DNA methods potentially offer more distinct targets, e.g. the transgene itself, its promoter, and/or border region sequences, than do protein assays. Protein immunoassays are rapid and relatively inexpensive, but are not as sensitive as DNA assays. Both have applicability to seeds, meal, and flour, but the methods potentially become more limiting with further processing of these products. For example, lecithin and starch derived from maize generally contain DNA but not proteins, whereas DNA is occasionally found in maize-derived oils and syrups. The protein content of food products is determined on a per-weight basis, e.g. micrograms per gram, and without reliance on an internal standard. The ability to quantify the protein is a function of extraction efficiency and the reproducibility of the analytical method. DNA content cannot be determined on a per-weight basis and, hence, relies on the use of an internal standard when estimating the amount of introduced DNA present in a sample. Such estimates are

confounded by the number of copies of the introduced gene present, the presence of stacked genes, and differential degradation of the DNA. DNA extraction methods and sample contamination can impact on the reliability of the results. A variety of studies suggest that there can be good qualitative agreement between the results of protein and DNA analyses of biotechnology-derived products; however, the quantitative results from such analyses can differ markedly. The relationship between the two measures is important with respect to international trade, because protein-based methods are the methods of choice in commodity crop-producing countries, e.g. the United States, Canada, and Argentina, whereas DNA-based methods are used preferentially in the EU and elsewhere.

Mr. G. Wiseman (RHM Technology, United Kingdom) discussed some of the procedural details of using PCR methods to detect DNA introduced into biotechnology-derived products. PCR-based analyses can be adopted for qualitative, semi quantitative, or quantitative applications. Such analyses assume that the transgene and the host DNA are equally degraded during the sample collection, handling, and processing stages; that the amplicons used for the transgene and the endogenous reference gene are of the same size; and that there are sufficient numbers of copies of the gene of interest present to yield a meaningful signal. Because of the minute amount of DNA present in samples from biotechnology-derived products, the target DNA must be amplified over a number of temperature-driven polymerization cycles. The number of thermal cycles required is determined by the amount of amplifiable DNA present in the sample; this number may differ between the internal sample, e.g. a so-called housekeeping gene, and the target DNA, because the former may be constitutively present in greater amounts than the introduced gene. Consequently, it may be difficult to interpret results when large numbers (e.g. >40) of amplification cycles are used. These and other factors need to be considered when evaluating both the performance of and results from DNA analyses. Indeed, such information is likely to be required by the Food Standards Agency in the United Kingdom when reviewing submissions for biotechnology-derived products. Reference materials to be used in conjunction with DNA analyses should be derived from the same matrix (e.g. commodity, meal, or flour) as the test material.

A network of laboratories in Belgium capable of analysing commodities and other foods for the presence of biotechnology-derived products was described by Mr. W. Moens (Institute of Public Health, Belgium). During 2001, EU guidelines will mandate complete traceability of biotechnology-derived products from seed to the final food product. This will likely involve the use of taxon-specific probes, i.e., amplicons that recognize a genetically modified species, cultivar, and transformation event. Transgene-specific and gene construct-specific probes will also play an important role in authentication testing. The ability to detect the presence of unauthorized biotechnology-derived products is expected to pose a considerable challenge to these and other laboratories, and will depend on knowledge of the sequence of the introduced DNA or border sequences that may be markers of hot spots for genetic recombination. The availability of probes and reference materials for unauthorized transformation events also will influence the ability to detect unauthorized products.

Mr. H. Kuiper (State Institute for Quality Control and Agricultural Products – RIKILT, Netherlands) noted that although no safety concerns have emerged regarding the consumption of biotechnology-derived foods, the labelling provisions of the Novel Foods Regulation have resulted in extensive efforts to develop and validate appropriate protein and DNA detection methods. Although the current focus is on Bt maize and RR soybeans, the number of genetically transformed food crops and the number and diversity of transformants are expected to increase rapidly. Similarly, new promoters and terminator genes will be introduced into food plants. Given these expectations, DNA microarray technology may provide an efficient and viable means of

testing for the presence of biotechnology-derived products. Such methods are currently under development at RIKILT and elsewhere, and although not ready for routine use, this approach offers the potential for simultaneously assaying for and quantifying the presence of large numbers of gene sequences. Technical challenges to developing this technology include optimizing DNA labelling efficiency (which influences assay sensitivity), determining optimum hybridization conditions (e.g. probe length and buffer characteristics), and identifying and preparing appropriate reference materials.

According to Mr. Y. Bertheau (National Institute for Agronomic Research – INRA, France), the full spectrum of qualitative and quantitative protein and DNA detection methods for the detection of GMO products is under development or is already in use in France and elsewhere in Europe. These evaluations are intended to compare the various available methods, determine the comparability of results obtained with various methods, and establish criteria for accepting or rejecting results obtained with specific methods. There is considerable interest in establishing performance criteria related to precision, specificity, sensitivity, accuracy, and applicability of the various methods, rather than developing a prescribed procedure. Such criteria could factor in the costs associated with equipping and maintaining a laboratory. In the development of performance criteria, consideration must be given to factors such as statistical evaluation of the data; the absolute and relative limits of detection and the limits to quantification relative to sampling, sample matrix, and sample preparation; DNA and protein extraction procedures; analytical instrumentation and instrument calibration; and overall variability (i.e., both experimental and routine laboratory variability). This is particularly relevant for methods used to assess compliance with regulations and their enforcement. Ultimately, each method must be validated in-house as well as externally, and good laboratory practices will need to be developed to provide quality assurance. The relationships between the available methods and labelling designations such as GMO-free or produced from non-GMO products remain to be determined.

Mr. B. Popping (Eurofins Scientific, Germany) reviewed numerous inter-laboratory validation studies that have been conducted over the last several years. These studies have been sponsored by a variety of groups including the Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV, Germany), the American Association of Cereal Chemists, the Swiss government, the Food Standards Agency (United Kingdom), the EC's JRC, and others. These studies have evaluated protein immunoassay-based detection methods, as well as both qualitative and quantitative DNA detection methods. All evaluations have focused principally on Bt maize and RR soybeans, although for the DNA-based methods other products have been evaluated. These studies have underscored the importance of having appropriate CRM. In general, these studies have revealed good inter-laboratory reproducibility and most laboratories are consistently able to correctly identify biotechnology-derived specimens. When quantitative DNA methods are examined, most laboratories are successful when the introduced DNA content is at or above the 1% level as mandated by the Novel Foods Regulation. These methods can often accurately detect smaller amounts of the target DNA, but at the expense of increased variability. Overall, the validation studies suggest that a number of methods are available that can be used in conjunction with the labelling provisions of the Novel Foods Regulation, although there is residual uncertainty about the broader applicability and adaptability of the methods to other biotechnology-derived products and to other food matrices, the standardization of these methods through CEN or ISO, the need to establish laboratory certification or proficiency standards, and the use of such methods for regulatory and legal purposes.

## WORKING GROUP REPORTS

At the conclusion of the invited presentations (described above), seven working groups of approximately 18 workshop participants each were formed. Each group was provided with written questions or issues to guide their discussion. Topics addressed by the working groups included identity preservation, reference materials, sampling, methods performance criteria, and detection methods, the last of which was discussed by three groups. Following the deliberations of the working groups, each reported back in the final plenary session; the conclusions of the three groups that considered detection methods were combined into a single report. The findings of each group and the discussion surrounding the presentations of each group and the final general discussion are incorporated in the following summary.

### *Identity preservation*

The working group on identity preservation (IP) was chaired by Mr. B. Moseley (independent consultant, United Kingdom), and its report was presented by Ms. J. Hunt (Kraft Foods, Germany). The group defined IP as a tool for providing consumers with the opportunity to exercise choice in the selection of food products based on the presence or absence of specified characteristics, in this case GMO content. As is evident from current IP applications, e.g. organic content or kosher preparations, implementation of an IP system does not imply that there are safety concerns about the food, rather that the system serves to facilitate delivery of products that adhere to defined specifications. IP protocols may be used to include or exclude items with defined characteristics. Such systems need to provide a degree of tolerance for adventitious mixing, e.g. the 5% rule for organic products and the 97% purity standard for grains. The EU has established a 1% tolerance for biotechnology-derived food products approved for distribution within the EU. However, no tolerance currently exists for products approved by authoritative bodies outside the EU but not approved by the EU. The working group observed the need for establishing such a tolerance, noting that zero tolerance is not achievable and should thus not be based on the limits of detection.

The group considered it imprudent to prescribe a specific IP system for biotechnology-derived products, because such a system must reflect case-by-case situations, e.g. volume of material, its source, collection, distribution, and handling, and other factors that can vary in product-specific fashion. Food producers need IP to do business and demonstrate due diligence. The working group proposed that industry and government work together to develop a general procedure guideline for IP that would be global in scope and, hence, promote fair trade practices. Such a standard might identify quality analysis critical control points (QACCP) that would be used to assure the integrity of the IP protocol. Finally, the group saw little value in developing a negative list, i.e. a list of crops to be excluded from IP protocols and analytical testing\*. Because of the potential development of methods with increased sensitivity, inter-laboratory variability in specific method selection, variation in performance and sensitivity, and the potential need to analyse diverse matrices, products named to a negative list would likely be under constant review and reevaluation.

In France and perhaps elsewhere in Europe, the public tends to equate the use of IP protocols to the identification of products as being free of biotechnology-derived content (i.e. GMO-free)

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\* At zero tolerance levels.

because the products are derived from non-GMO sources. Indeed, it was suggested that EU law implies that IP protocols will be applied to non-GMO products. However, IP systems may be more appropriate when applied to GMO-derived products. This confusion will influence the further development of IP protocols for biotechnology-derived products. Although IP protocols exist for a variety of applications, the working group did not specifically examine the utility of these systems relative to biotechnology-derived foods; such considerations are more appropriately part of a process to develop an internationally agreed-upon general IP procedure guideline for such products. Any such effort would need to be synergized with similar initiatives by other international bodies. For example, such an initiative might build on the work of the Organisation for Economic Cooperation and Development (OECD) that are exploring similar issues related to seed purity.

### ***Reference materials***

Mr. M. Grasserbauer (DG JRC-IRMM, Belgium) chaired the working group on reference materials, and Mr. S. Bowadt (DG Research Trade and Development, Belgium) presented its report. The group identified a number of priority issues that need to be resolved promptly to facilitate the EC's resolution to proceed with the authorization and release of biotechnology-derived foods. These include the development of an international agreement (principally between the United States and the European Union) on common and mutually acceptable CRM for biotechnology-derived products. CRM supplies must be increased and they must be produced in accord with ISO guidelines and Good Manufacturing Practices to ensure batch-to-batch continuity, consistency, and quality. Within the EU the need to develop certified DNA reference materials should have the greatest priority, followed by the development of matrix-specific CRM for all EU-authorized biotechnology-derived products. The third priority is to develop appropriate protein CRM; there is some expectation that these materials will be supplied by the developers of biotechnology-derived products. A priority is to establish absolute zero and negative control standards. The development of CRM for processed foods is a lesser priority. Additional information is needed regarding the long-term stability of CRM and the appropriate conditions for their storage.

Mr. Bowadt described a proposal under which the developers of biotechnology-derived products would provide CRM to central repositories in Europe and the United States. Submission of protein and DNA CRM, including control (e.g. parental material) would be linked to the product authorization process. It was suggested that IRMM serve as the European repository and that the National Institute of Standards and Technology (NIST) serve a similar function in the United States. Although described in terms of seeds, similar approaches might be used with respect to other grains and commodities. As conceptualised, CRM for vegetatively propagated products such as potatoes would not be included in the repositories. Each DNA CRM would be accompanied by sufficient descriptive material to facilitate its appropriate use, because the materials deposited in the repository will be used as the standards for all other reference materials.

### ***Sampling***

The working group on sampling was chaired by Mr. J. Gilbert (Ministry of Agriculture, Fisheries and Food, United Kingdom), and Mr. R. Fuchs (Monsanto, Inc., United States) presented its report. The deliberations of the group focused on three key topics: (1) What is to be sampled (e.g. grain or seed, food ingredient, or final food product)? (2) Why are the samples collected (e.g. for

acceptance by a food producer, for regulatory or enforcement purposes, or for surveillance)? and (3) Where in the food supply chain will the sampling occur (e.g. early, at the commodity level, or later to confirm the integrity of the material)? The group recognized that the Novel Foods Regulation applies to foods rather than grains, but agreed that analysis of the latter could provide the basis for estimating the GMO content of subsequent derivatives used in foods.

When sampling is being conducted with regard to acceptance by producers, the seller and the buyer should agree to a specified sampling plan that accounts for the confidence levels, costs, and other factors associated with sampling and analysis and that reflects appropriate levels of risk to each party. In practice, because sampling plans are likely to be negotiated between various buyers and sellers, numerous sampling plans are expected to arise. The working group noted that sampling plans currently used to validate grain quality are consistent between the USDA, GIPSA, CEN, and ISO. At present, the JRC is evaluating the consistency of various plans proposed for sampling to determine the GMO content of commodity grains.

It is critically important to any sampling plan that the samples are random, that an upper limit be placed on sample size, and that the qualitative or quantitative analytical methods to be used are acceptable, recognizing that the sensitivity of the analytical method will determine, in part, the appropriate sample size. The group considered sample preparation to be more challenging than the sampling itself. For regulatory purposes it may be prudent to sample, analyse, and quantify GMO content at the ingredient level and to screen final food products. The group noted that although many regulatory sampling plans have been developed for various purposes, few are implemented because of the costs and complexity of sampling final food products. Sampling for surveillance is intended to determine the level of regulatory compliance through the development of statistically based sampling plans, recognizing that the costs associated with extensive sampling and analysis may become prohibitively expensive with little appreciable gain in the ability to detect non compliance.

Any sampling plan needs to recognize the inherent variability associated with each step in the process: sampling, sample preparation, and analysis. Although considerable effort has and likely will continue to be devoted to the development and validation of analytical methods, sampling is likely the greatest source of variability. Hence, regulatory endpoints that directly impact sampling should be scientifically meaningful and not predicated on the sensitivity of the method. For example, because there is no true zero standard, the limit of detection or the confidence limits for each step in the process should be defined in advance.

### *Performance criteria*

The performance criteria working group was chaired by Mr. U. Pauli (Federal Office of Public Health, Switzerland) and its report was presented by Mr. P. Hübner (Cantonal Laboratory of Zurich, Switzerland). The group's discussion focused only on the analyte, recognizing that although matrix effects and other factors are important in terms of defining performance criteria, such considerations exceeded the group's terms of reference. The group noted that protein detection methods are based on extraction methods that have maximum efficiency; whereas for DNA detection methods the efficiency of DNA extraction is less important than the quality of the extracted DNA. Each method, whether protein or DNA based, must be defined in terms of its specificity; i.e. which material(s) is suitable for analysis by a given method and under which conditions. The sensitivity of the assay must be carefully determined. Immunoassays typically are

sensitive to approximately  $10^{-12}$  mol/l. The sensitivity of PCR-based DNA assays generally have not been defined because CRM with low and very low GMO content have not been used to establish this parameter. Most studies to date have employed CRM in the 1% to 2% range, i.e. approximating the EU labelling threshold. The relevance of studies in which CRM with low GMO content have been prepared by diluting more concentrated reference materials is unclear.

The accuracy or “trueness” of the analysis should be defined at the EU threshold of 1%, and the RSD should be <30%. The reproducibility of an analytical method should be established by a ring trial involving at least 12 laboratories with consensus defined on the basis of the results from two replicates of each of two test specimens. Where reproducibility cannot be demonstrated, consideration should be given to differences in the equipment and instrumentation used in the participating laboratories. Validation of a method should involve studies of repeatability as well as reproducibility using a “gold standard.” The utility of a method may depend on the analytical equipment or apparatus used. A number of methods referred to during the course of the workshop have been validated through multi laboratory ring trials and will be annexed to the CEN documents noted in Mr. Heissenberger’s presentation. Finally, an operation curve should be constructed for each method.

### *Detection methods*

Three working groups independently considered the attributes, limitations, and scientific uncertainties associated with both protein and DNA detection methods. The chair and rapporteur, respectively, for each group were Mr. M. Morgan (University of Leeds, United Kingdom) and Mr. A. Wurz (Gene-Scan, Germany), Mr. G. van Duijn (TNO, Netherlands) and Mr. A. Knight (Leatherhead Food, United Kingdom), and Ms. M. Schauzu (BgVV, Germany) and Mr. R. Meyer (Nestlé, Switzerland). Mr. R. Battaglia (Swiss Quality Testing Service, Switzerland) reported on the deliberations of the three groups.

The groups attached little value to attempting to develop negative lists, i.e. lists of GMO crop derivatives, food ingredients, and final food products that would be excluded from testing. Such a list would be feasible only if sample processing and analytical methods were standardized and rigorous and were enforced and supervised closely, independently, and often. Moreover, items named on the list would be subject to continuing challenges as extraction methods improve and as analytical sensitivity increases, and would effectively lead to universal labelling (i.e. abandoning the labelling threshold), either now or in the future. However, universal labelling would compromise the intent of the Novel Foods Regulation, i.e. to provide consumers with the opportunity to choose between foods that are derived from biotechnology and those that are not.

The groups noted that the term “substantial equivalence” was defined and agreed to by the members of an expert consultation of the OECD. Although the definition has been further refined by other international scientific organizations, the basic concepts remain scientifically valid. Assessing for substantial equivalence is considered as the starting point in the safety assessment of a biotechnology-derived food, and detection methods play a role in this process by providing for the quantification of the molecules of interest in the context of assessing exposure. If a food is authorized, i.e. determined to be as safe as a comparable food already included in the human diet, then detection methods play a role in establishing biochemical differences between the new food and its conventional counterpart. Labelling of authorized, biotechnology-derived foods in the EU is predicated on the scientific demonstration of such a difference. The groups concluded that a variety of validated methods are currently available to implement the labelling provisions of the Novel Foods Regulation.

Protein and DNA detection methods exploit different target molecules, have different applications, and can be used qualitatively or quantitatively. The decision to use one or the other is subject to market and economic factors, specific test requirements, and the type of information being sought. Opportunities exist for comparative studies of protein and DNA detection methods and for establishing, if and where possible, correlations between test outcomes. Such correlations have been scientifically documented for the results of qualitative protein and DNA assays. However, there is some uncertainty regarding the ability to establish correlations between the results of quantitative analyses using protein- and DNA-based methods. In the United States, where protein detection methods predominate, weight/weight analyses are possible using CRM. In the EU, where DNA detection methods are preferred, quantification is expressed in terms of genomic equivalents, which are influenced by the number of copies of the gene present in the test material. In the future, as multiple genes are introduced into food crops through stacked-gene technology or by the crossing of GMO parental lines, the uncertainty associated with DNA quantification will be exacerbated. The situation is confounded by differential gene expression in various parts of the plant. For example, most commercially produced maize varieties are hybrids in which genes from the parental varieties are unequally distributed in the endosperm and embryo tissues that form the maize seed that is used in the production of foods and food products.

The groups recognized that the development, validation, and implementation of GMO detection methods have advanced considerably since 1998. Although methods are available to facilitate implementation of the labelling provisions of the Novel Foods Regulation, the need for further scientific development of the methods is apparent. Further work around DNA quantification, sample extraction, matrix effects, reference materials, and validation is needed. The work needed to improve and optimise these detection methods will be enhanced and facilitated by improved understanding of the genetics and molecular biology of plants.

## CONCLUSIONS AND RECOMMENDATIONS

**B**ased on the presentations, the working group discussions, and the plenary discussions following each working group report and the final plenary discussion, the workshop yielded the following conclusions and recommendations.

A variety of validated protein and DNA detection methods are currently available to implement the labelling provisions of the European Union's Novel Foods Regulation. The selection of any specific method is subject to market and economic factors, specific test requirements, and the type of information being sought. Many of these methods will be described in a report of the European Normalisation Committee (CEN) expected to be published in 2001. Acceptance of the report would effectively lead to the adoption and standardization of those methods within the European Community.

Protein and DNA detection methods have three distinct applications relative to biotechnology-derived foods. They may be used to (1) identify and characterize transformed plants during product development, (2) quantify the molecules of interest in the context of assessing exposure to the modified food, and (3) implement the labelling provisions of the Novel Foods Regulation by establishing biochemical differences between the new food and its conventional counterpart. Assays developed for one application may have limited utility relative to the other applications.

As new analytical methods are introduced they should be evaluated by ring trials for reliability, reproducibility, etc. International ring trials involving at least 12 laboratories are recommended, with consensus being defined on the basis of the results from at least two replicates of each of at least two test specimens.

Although the Novel Foods Regulation applies to foods rather than to grains, analysis of the latter could provide the basis for estimating the GMO content of subsequent derivatives used in foods. For regulatory purposes, it may be prudent to sample, analyse, and quantify GMO content at the ingredient level and to screen final food products.

Protein detection methods, which are preferred in the United States, are based on extraction methods that have maximum efficiency; whereas for DNA detection methods, which are preferred in Europe, the efficiency of DNA extraction is less important than is the quality of the extracted DNA. Each method, whether protein or DNA based, must be defined in terms of its specificity, i.e. which material(s) is suitable for analysis by a given method and under which conditions. The sensitivity of the assay must be carefully determined.

Quantitative results from protein assays are expressed on a weight/weight basis, whereas quantitative results from DNA assays are expressed in terms of genomic equivalents. Hence, DNA quantification is influenced by the number of copies of the gene present in the test material. In the future, as multiple genes are introduced into food crops through stacked-gene technology or by the crossing of GMO parental lines, greater uncertainty will be associated with DNA quantification.

With respect to GMO-free labelling, zero percent tolerance is not achievable because of the complexities of plant biology (e.g. pollen dissemination), agricultural practices, and international commodity trading. Tolerances should not be based on the limits of detection, but rather should reflect the unavoidable co-mingling of GMO and non-GMO varieties during transport, etc. Such a tolerance should be consistent with internationally agreed-upon tolerances for grains or organic foods. Tolerances also should be established both for products approved in the countries of consumption (e.g. establishment of the 1% labelling threshold by EU member states) and for products that have undergone a thorough safety assessment and have been approved in countries of production but are yet to be approved in countries of consumption. Any tolerance proposed to establish a GMO-free designation would reflect an arbitrary decision that could not be scientifically supported.

To facilitate implementation of the labelling provisions of the Novel Foods Regulation, the following priorities (in descending order) were identified with respect to reference materials: development of certified DNA reference materials for all EU-authorized biotechnology-derived products, development of matrix-specific DNA CRM for all such products, development of appropriate protein CRM for such products, establishment of absolute zero and negative control standards, and development of CRM for processed foods.

An international agreement (principally between the United States and the European Union) recognizing common and mutually acceptable CRM for biotechnology-derived products is needed.

International standardization of CRM might be facilitated if the developers of biotechnology-derived products simultaneously provided protein and DNA CRM to central repositories in Europe and the United States. Submission of protein and DNA CRM, including control (e.g. parental material) could be linked to the product authorization process. The Institute for Reference Materials and Methods might serve as the European repository, and the National Institute for Standards and Technology might serve a similar function in the United States.

The success of any detection method will be contingent on the sampling plan. It is critically important to any sampling plan that the samples are random, that an upper limit is placed on sample size, and that the qualitative or quantitative analytical methods to be used are acceptable, recognizing that the sensitivity of the analytical method will determine, in part, the appropriate sample size. Any sampling plan needs to recognize the inherent variability associated with each step in the process: sampling, sample preparation, and analysis.

When sampling is being conducted relative to acceptance by food producers, the seller and the buyer should agree to a specified sampling plan that accounts for the confidence levels, costs, and other factors associated with sampling and analysis. Sampling plans to facilitate trade will reflect the business risk as understood and negotiated by both parties. Such negotiated sampling plans may be distinct from sampling plans developed for regulatory purposes. Although many regulatory sampling plans have been developed for various purposes, few are implemented owing to the costs and complexity of sampling final food products.

Because precedents have been established globally for the sampling of grains and other commodities for quality assurance, it should be possible to readily develop internationally agreed-upon sampling methods for identifying the presence of biotechnology-derived products. The sampling and analytical procedures used by the U.S. Department of Agriculture's Grain Inspection, Packers, and Stockyards Administration provide a basis for discussions leading to such an agreement. Point-of-origin sampling and certification would facilitate the development of identity preservation systems to ensure traceability and minimize the need for reanalysis throughout the food supply chain.

Representatives of industry and government along with consumers, should work together to develop a general guideline for establishing an identity preservation system for biotechnology-derived products. Such an initiative should lead to the development of an internationally agreed-upon Identity Preservation guideline for such products. Any such effort would need to build on similar initiatives by international bodies such as the Organisation for Economic Co-operation and Development (OECD), which are addressing IP in the context of seed purity. Such programs should include the establishment of reasonable thresholds for commercial GMO products consistent with current capabilities and practices of modern farming, production, and manufacturing processes.

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