codex alimentarius commission

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

JOINT OFFICE: Viale delle Terme di Caracalla 00100 ROME Tel: 39 06 57051 www.codexalimentarius.net Email: codex@fao.org Facsimile: 39 06 5705 4593

Agenda Item 7b **CX/MAS 02/9**
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JOINT FAO/WHO FOOD STANDARDS PROGRAMME
CODEX COMMITTEE ON METHODS OF ANALYSIS AND SAMPLING

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CONSIDERATION OF METHODS FOR THE DETECTION AND IDENTIFICATION OF FOODS DERIVED FROM BIOTECHNOLOGY

GENERAL APPROACH AND CRITERIA FOR THE METHODS[1]

BACKGROUND

There is considerable interest in the methods of analysis for the detection and identification of foods derived from biotechnology. This is illustrated by the methods that have been collated by the *ad hoc* Intergovernmental Task Force on Food Derived from Biotechnology, and given in paper CX/MAS 02/8.

However, the analysis of such foods is problematic and an understanding of the principles involved is required before the methods can be accepted by Codex.

This paper is intended to aid the understanding of this complex area.

INTRODUCTION

Selective breeding of plant and animals has been practised for many centuries. This has led to increased productivity, disease resistance and improved quality of food. As knowledge of genetics has increased methods of breeding have become increasingly sophisticated.

Genetic modification has revolutionised selective breeding, allowing individual genes to be identified, copied and transferred. Two features of genetic manipulation set it apart from traditional methods of selective breeding. Firstly a particular gene within an organism can be modified to alter the way in which it is expressed. Secondly a gene can be transferred from one species to another. Much of the initial work involved Roundup Ready® Soybeans; the modifications involved here are therefore described below, and serve to be illustrative of the technology involved.

Roundup Ready® Soybeans

Roundup Ready® Soybeans have been modified to make them tolerant to glyphosate, the active ingredient in Roundup® herbicide. Glyphosate controls weeds by inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphatesynthase (EPSPS) which is used by plants for the biosynthesis of aromatic amino acids.

The modification of the soybean was accomplished by inserting a 'gene expression cassette' containing a glyphosate-tolerant EPSPS coding region isolated from *Agrobacterium sp.* strain CP4 into the genome of the soybean (see figure 1). This 'cassette' contains all the genetic elements necessary for the synthesis of EPSPS consisting of a Cauliflower mosaic virus (CaMV) 35S promoter (E35S), a chloroplast transit peptide (CTP), the EPSPS gene and a Nopaline synthase 3' transcription terminator (NOS). The artificial combination of the elements from different sources represents a unique characteristic of the result of gene technology.

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[1] Paper prepared by the United Kingdom and Germany

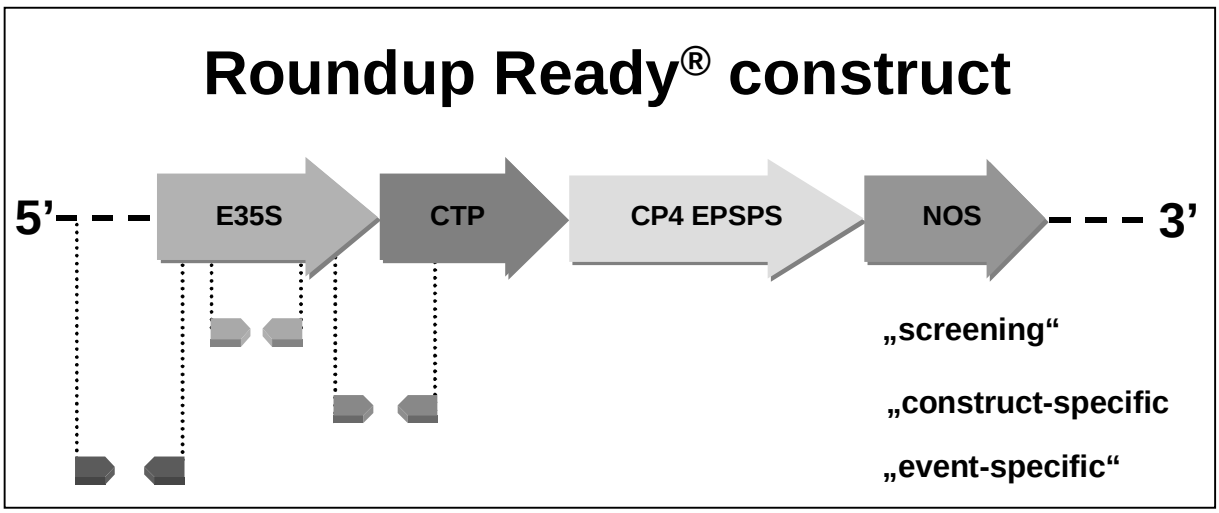


Figure 1: Schematic description of the genetic construct introduced into the soybean genome. Below the construct, primer binding sites are indicated by filled arrows used either for screening, construct specific or event specific detection of genetically modified organisms.

METHODS

General

The presence of a genetically modified organism or its derivatives can be accomplished by the detection of either dna sequences present as a result of recombination or the protein coded for by the inserted gene.

Protein based methods such as immunoassays are commercially available and offer high selectivity and sensitivity. However proteins are denatured during processing and therefore these techniques are only suitable for raw material analysis. If no protein is expressed from introduced DNA, this technique cannot be applied.

DNA is much more thermally stable than protein and can survive food processing. Methods for detection of DNA markers based on the Polymerase Chain Reaction (PCR) have been used in a variety of food analyses and are by far the most widely used for detection of GM derivatives in food. A typical method involves extraction and purification, amplification by PCR and detection / quantification.

Any review of potential methodology available for the quantitative determination of GM soya in processed products would probably conclude that Real Time PCR (Polymerase Chain Reaction) techniques offers the best probability of success for the determination of GM soya in foods.

DNA extraction and purification

The first step in the analysis is the extraction and purification of DNA from the test sample. Extraction methods can be either developed from common molecular biological protocols or commercially available kits can be used. Further purification may be required to remove substances that inhibit the PCR. There are two main principles used for extraction which can be illustrated by considering two commonly used procedures. The CTAB (Cethyl-trimethyl-ammonium-bromide) method involves the removal of impurities from a crude DNA extract and can be divided into 5 steps:

- Solubilisation of DNA by addition of CTAB buffer
- Denaturation of proteins
- First precipitation of DNA with a second CTAB solution
- Second degradation of proteins
- Second precipitation of purified DNA by addition an alcohol solution.

The Wizard extraction method involves the retention of the DNA on a resin and subsequent elution of purified DNA; this procedure has three steps:

- Solubilisation of DNA by addition of extraction buffer and proteinase K (enzyme that degrades proteins)
- Solution is transferred to a column containing the resin.
- Purified DNA is eluted from the column.

These methods respectively form the basis of official German and Swiss methods for detection of GMO's.

The Polymerase Chain Reaction

It is not possible to detect individual DNA sequences; however they can be amplified to produce a large number of copies using the Polymerase Chain Reaction (PCR). PCR was first described in 1985 by Kary Mullis *et. al.* and has since been utilised for a wide range of molecular biological applications.

PCR amplifies specific sequences of DNA known as the 'target' sequence. The first step of the reaction is the denaturation of the double stranded DNA (dsDNA) to produce two complimentary single strands (ssDNA). 'Primers' (short stretches of DNA) which have been designed to bind selectively to either side of the target sequence are then annealed to the two ssDNA sequences and extended by the action of the enzyme taq polymerase to produce two new fragments of the target DNA. This process is repeated with the newly created copies acting as templates for subsequent reactions. Thus for each original DNA target sequence two copies are produced in the first cycle, 4 in the second, 8 in the third etc. until after 20 cycles over 1 million copies of the original target are created. The steps in a single cycle of a PCR are illustrated in Figure 2.

PCR is therefore a unique methodology due to the above described amplification steps, where the enzyme DNA polymerase plays the key role. If no further changes are made PCR results only in a qualitative "yes" or "no" answer (Figure 4).

Quantification using Real Time PCR

Traditionally detection of specific DNA sequences has involved the separation of the PCR products by gel electrophoresis. After electrophoresis the gel is stained with ethidium bromide which intercalates with the DNA and fluoresces in UV light. Identification of the PCR product is by comparison with molecular weight standards run alongside the test samples. This analysis was done at the end of a fixed number of PCR cycles and is called the 'end-point' determination. With the exception of competitive PCR these methods are not quantitative.

In 1992/3 Higuchi *et al.* developed a system that followed the accumulation of PCR products in 'real-time'. This system used ethidium bromide and an adapted thermal cycler to irradiate the samples with UV light. The resulting fluorescence was monitored by a CCD camera. Thus by plotting fluorescence against cycle number an amplification plot could be constructed and a more complete picture of the PCR process was obtained.

This system has since been developed further and the accumulation of PCR products is now followed using fluorogenic 'probes'. These probes are oligonucleotides which have a 'reporter' and 'quencher' dye attached to either end. In the intact probe the proximity of the quencher greatly reduces the fluorescence emitted from the reporter dye (Förster resonance energy transfer). During PCR the probe anneals to the target sequence as shown in figure 3. As the primer extends along the target sequence it cleaves and fragments the probe and the reporter dye emits fluorescence as it is no longer in close proximity to the quencher.

Target choice

Essential for the identification of a GMO is an appropriate target for the primers. In principal, there are three different strategies as starting points for choosing an appropriated target (figure 1).

Genetic elements commonly used in GMOs ("screening")

It is possible to focus on PCR amplification of genetic elements which are commonly used in GMO's, such as the 35-S promoter of cauliflower mosaic virus (CaMV) or the *nos*-terminator of *Agrobacterium tumefaciens*. Here, if a positive response is obtained, the presence of GMOs may be suspected. However, these PCR methods will not discriminate between the elements occurring naturally in infected plants or their presence in genetic constructs of GMOs. This is particularly in the case of rapeseed and other *Brassica* members a positive result from a P35S screening may well be a false positive since these plants can be infected by the CaMV. However by performing a CaMV-specific PCR based on genes normally not present in GMOs, false positives, as a result of virus infected plants, can be detected.

Construct Specific

The gene construct consists of several elements derived from different sources. Therefore the junction sequences between two adjoining DNA segments could be the target for a specific detection of the genetic construct. It has to be considered that these two joint elements can be introduced into other organisms resulting in a different genetically modified organism containing the same genetic construct. Consequently this is not conclusive to detect a specific GMO event.

Event Specific

When the GMO is the result of a non-homologous recombination, the integration site is unique. Therefore this plant-construct junction fragment used as a target for detection will clearly detect a specific transformation event. Whenever the same gene construct is used to produce different GMOs, this will be the only strategy to distinguish between GMOs containing the same gene construct.

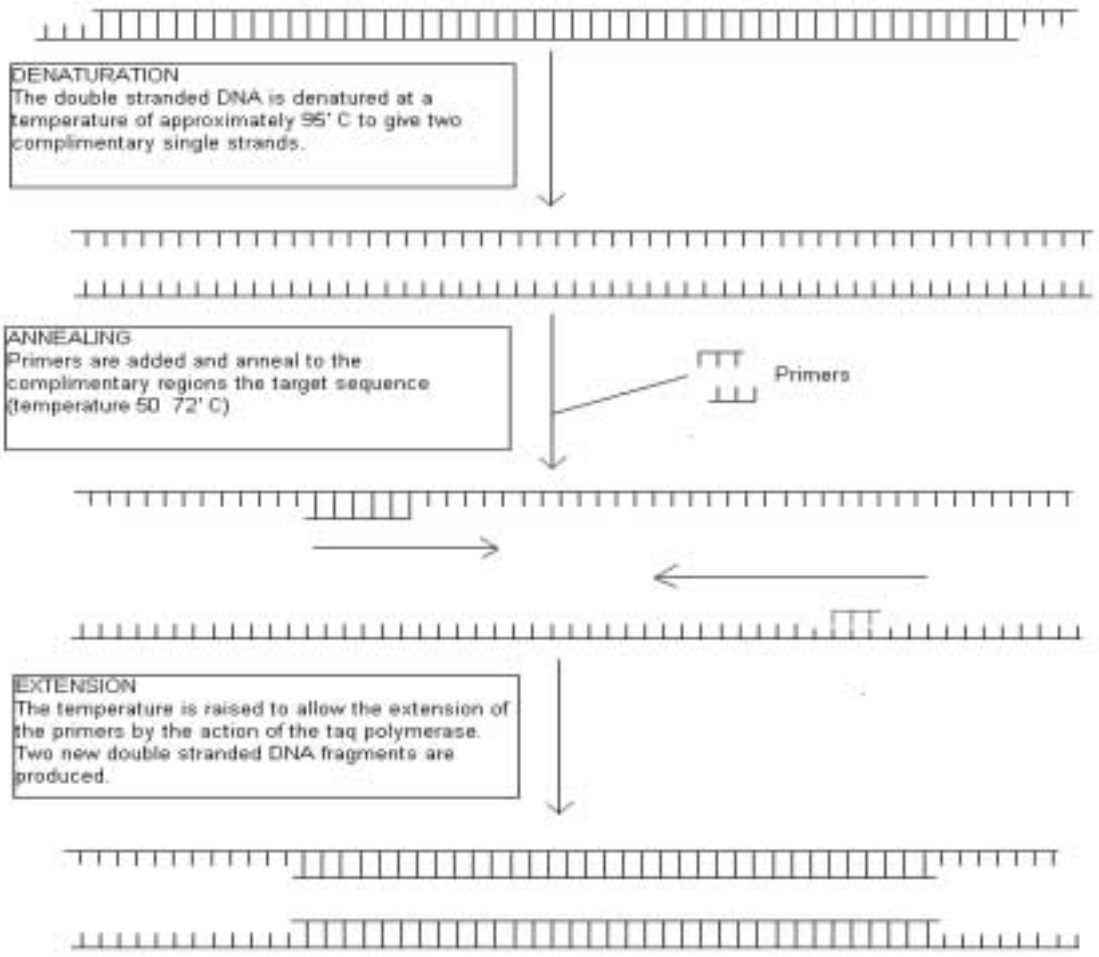
Notwithstanding which approach is to be applied, it is an absolute prerequisite for the development of the PCR that the DNA sequence integrated into the host genome is known.

Other Methods

ELISA was initially used as the method of analysis for the determination of GMOs in food ingredients (e.g. modified soya flour in flour) but was found to be less appropriate for the determination of GMOs in compound foods. If raw materials are analysed, protein-based methods could be an alternative to DNA-based PCR methods.

Although the use of Real-Time PCR for the determination of DNA is the current method of choice, there is considerable work being undertaken to assess the application of large molecule MS techniques (MALDI-TOF, orthogonal-TOF) for the determination of GMOs.

Figure 2. The Polymerase Chain Reaction (PCR)



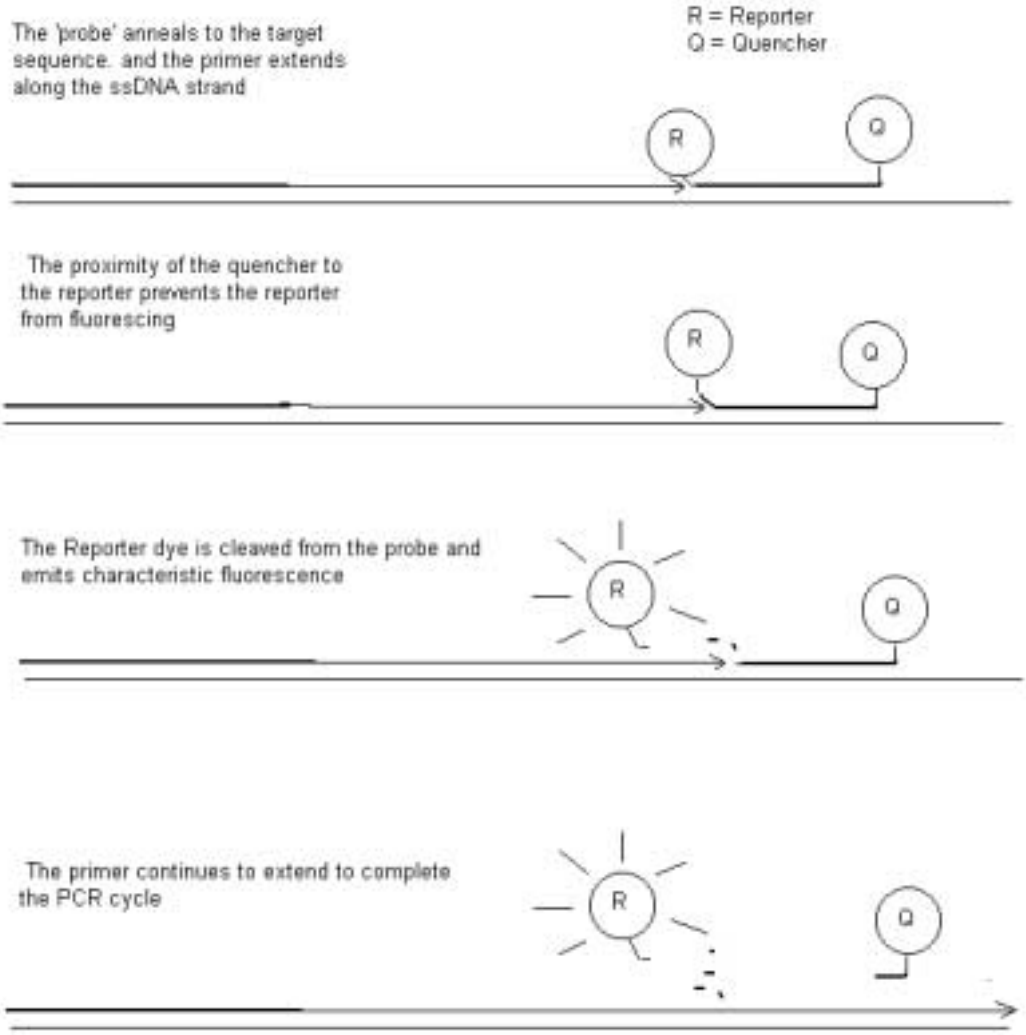


Figure 3: Use of fluorogenic probe for Real Time PCR

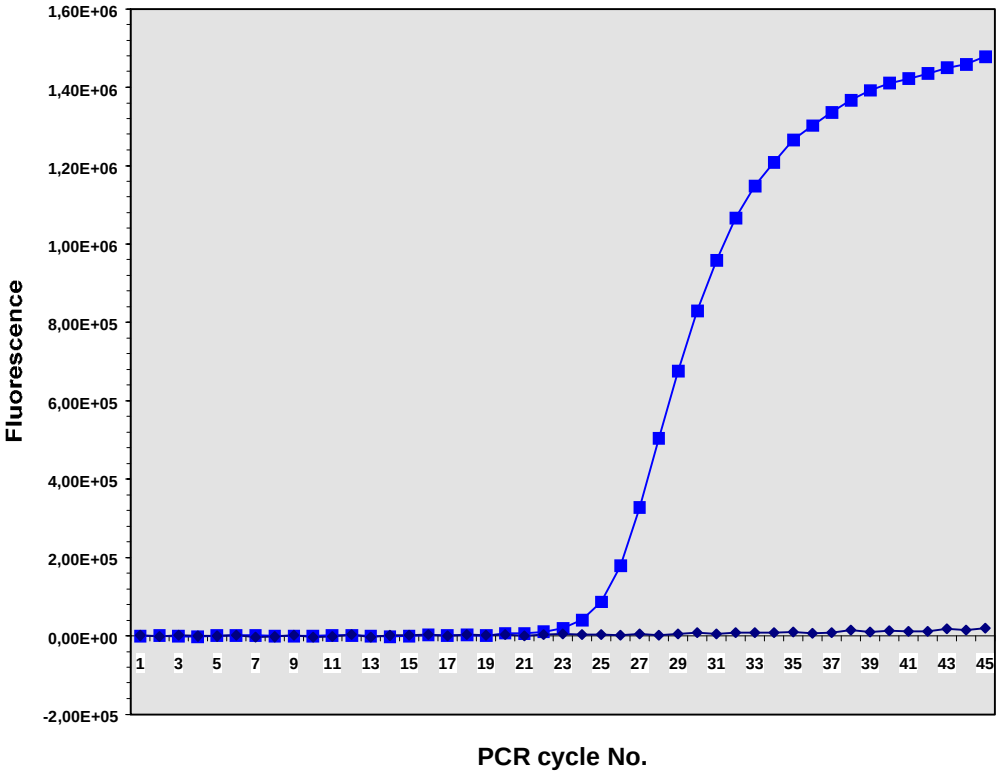


Figure 4: Accumulation of target molecules during the cycling process of PCR. *Depending from the initial amount of target molecules in the reaction, the detected signal leaves a threshold resulting in an exponential amplification until a plateau is reached. The plateau value doesn't correlate with the starting amount of DNA.*

PRECISION CHARACTERISTICS OF METHODS

Although a number of methods are described in CX/MAS 02/8, there have been some very recent collaborative trials which demonstrate that methods with "acceptable" precision may be forthcoming. As an example, a international collaborative trial co-ordinated by the Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), Germany, resulted in the following:

Sample	**Sample 1 0,1%**	**Sample 2 0,5%**	**Sample 3 1%**	**Sample 4 2%**	**Sample 5 5%**	**Sample 6 2% TVP**
Number of laboratories	14	14	14	14	14	14
Number of outliers according to Cochran and Grubbs	2	0	0	0	1	0
Number of laboratories retained after eliminating outliers	12	14	14	14	13	14
Mean value (%)	0,11	0,49	1,00	2,26	4,91	1,71
Recovery (%)	108,75	98,71	99,86	113,23	98,27	85,38
Repeatability standard deviation s_r	0,02	0,12	0,17	0,20	0,56	0,39

Repeatability relative standard deviation RSD_r (%)	14,17	25,04	16,80	8,63	11,33	22,64
Repeatability limit r ($r = 2.8 \times s_r$)	0,04	0,35	0,47	0,55	1,56	1,08
Reproducibility standard deviation s_R	0,02	0,12	0,27	0,60	0,95	0,48
Reproducibility relative standard deviation RSD_R (%)	17,78	25,04	26,82	26,54	19,34	27,98

As unknown samples Certified Reference Material (CRM) has been used. The material in the range from 0.1 - 5% Roundup Ready® soybean in soya flour was produced by the Institute for Reference Materials and Measurements (IRMM), Belgium. The quantitation method used is based on Real Time PCR and was carried out on ABI PRISM™ 7700 Sequence Detection Systems. Further description is given in CX/MAS 02/8 (p. 34). The mean values obtained are in close correlation to the true values of the CRMs.

RESULTS FROM PROFICIENCY TESTING EXERCISES

Notwithstanding the above, an indication of the difficultly of the analysis in this area may be obtained by the assessment of the results from proficiency test exercises. Two of these are described below:

A: Specialist Laboratories undertaking Analysis of IQC Test Materials

Here a number of specialist laboratories were asked to undertake the replicate analysis of two materials to be used for IQC test materials. The results are given below:

(a) Bread containing 4.5% GM soya of total soya m/m

Laboratory	Number of Replicates	Mean	Standard Deviation
1	11	2.2	0.54
2	10	3.1	1.13
3	10	4.8	1.57
4	14	1.6	1.62
5	10	2.6	0.22

(b) Cake containing 1.5% GM soya of total soya m/m

Laboratory	Number of Replicates	Mean	Standard Deviation
1	11	2.0	0.28
2	10	3.3	1.08
3	10	2.5	0.90
4	14	1.5	1.00
5	10	2.2	0.14

Evaluation of the IQC data

If the measurement uncertainty that each of these laboratories would quote was twice the standard deviation, then this value becomes unacceptably high in some cases.

The statistical analysis of the IQC samples indicates that each laboratory's data for each test material appears to belong to a different population and so must be treated on an individual basis. As the performance of each of the laboratory methods is different the minimum level measured in a sample that would indicate that GM soya is above the 1% threshold will also vary. The table below shows the MU added to 1% for each laboratory and each test material.

Laboratory no	Cake % 1%+MU	Bread % 1%+MU
1	1.6	2.1
2	3.2	3.3
3	2.8	4.1
4	3.0	4.2
5	1.3	1.4

Laboratory five appears to offer the best performance producing repeatable results with a low MU. Comparing the results with the actual added levels of GMO in the IQC materials would suggest that laboratory five under reports for the bread samples and very slightly over reports for the cake samples. However it should be noted that the mean of the IQC data for laboratory five is close to the overall mean for all 5 laboratories.

Comparison with other methods of analysis

It is difficult to compare the concentration of GM soya and method performance to other determinations as the threshold applies to the percentage of GM soya in total soya and not to the concentration of GM soya in the total product.

The percentage would be most usefully expressed for method comparative purposes as percentage of GM DNA in total DNA extracted, which for the bread is in the region of 0.015% (150 ppm) and for the cake in the region of 0.05% (500 ppm). These figures are calculated on a dry weight basis taking into account those ingredients that do not contain any DNA such as sugar and fat.

For proximate analysis such as the determination of nitrogen an MU calculated as 2 SD would be expected to be 0.08 at a concentration of 1% and 0.002 at 0.01% (100ppm), however these concentrations are well above the detection limits of the method. As the GM soya determinations on the IQC test materials are close to the detection limits of the methods a comparison with an MU estimation carried out near to a method detection limit may be more appropriate. For the contaminant 3 MCPD the method detection limit is 5ug/kg (ppb) and at a level of 16 ug/kg (ppb) the MU was determined as10 ug/kg (ppb) and is comparable to the GM soya data obtained in these experiments.

B: Results from Proficiency Testing

The results from a proficiency testing exercise, organised according to the International Protocol adopted by Codex, are given below:

Round No	Date of Report	Test Material	Number of participants	Number of quantitative results	% satisfactory
GMO1	Dec 1999	Flour + 2% GM soya	25 (32)	19	**42%** (8/19) 58% (11/19)
GMO2	Jan 2000	Flour + 0.5% GM soya	31 (34)	19	**89%** (17/19) 58% (11/19)
GMO3	Feb 2000	Flour + 0.1% GM Soya	34 (40)	21	**71%** (15/21) 38% (8/21)
GMO4	Apr 2000	Flour + 2% GM Soya	36 (41)	27	**56%** (15/27) 85% (23/27)
GMO5	June 2000	Biscuit + 1% GM Soya	33 (42)	28	**57%** (16/28)
GMO6	October 2000	S/B 0.75% S/B 1.5% DB 2.5% Bread 2.5%	39 (48)	33 33 32 24	**76%** (25/33) **82%** (27/33) **78%** (25/32) **46%** (11/24)
GMO7	March 2001	Flour + Maize 0% Flour + Maize 0.5% Flour + Maize 1.5%	29 (42)	 23 24	 **57%** (13/23) **67%** (16/24)
GMO8	July 2001	Flour + Maize MON810 0% Flour + Maize MON810 0.75% Flour + Maize MON810 2%	21 (24)	 13 14	 **54%** (7/13) **36%** (5/14)
GMO9	May 2002	Veg Puree + 0% GM Soya Veg Puree + 0.2% GM Soya Veg Puree + 2% GM Soya	36 (40)	 33 33	 **58%** (19/33) **94%** (31/33)

S/B: shortbread biscuit powder

D/B: Dutch biscuit powder

Bread; bread powder

These results show that there is some variability amongst the population of laboratories undertaking these analyses.

PERFORMANCE CRITERIA

Codex will accept the criteria approach for methods of analysis. In the case of the GMO area the use of conventional performance characteristics will have to be supplemented by others.

Several characteristics of the PCR methodology are unique and no comparable property is present in classical chemical analysis.

- PCR involves the amplification of a specific DNA target
- the amplification is done by an enzyme called DNA polymerase
- the efficiency of amplification is highly dependent from the target size (the efficiency of PCR is proportional increasing with the decrease of target size)

In addition, because of the nature of the analysis others, e.g. the Horrat values, will not be appropriate.

REFERENCE MATERIALS

It is international recognised that for the accredited, proficiency laboratory the role of reference materials is of prime importance.

In the GMO analysis area there have been a number of difficulties, and in particular:

- the status of ploidy degree of the chromosomes in cells and the number of integrated copies of the genetic modification
- the same "cassette" could be used in different varieties like corn and soybean
- the use of different "cassettes" in the same genetically modified organism (called as "stacked" genes) could lead to ambiguous results
- the access of GM free material from the parent line for the preparation of CRMs
- without reference material no method could be developed

Although such problems are being slowly addressed, doubts will remain until they have been resolved.

CONCLUSIONS AND RECOMMENDATIONS

PCR is widely used for the purpose of identification of GM material used for food production. Nevertheless, Real Time PCR used for quantitative analysis is a novel technique and has only recently been used outside of the research laboratory. The results of the above evaluations have highlighted the difficulties in obtaining accurate quantitative data when working with low levels of GM material in processed foods, which contain a low level of soya. Work is in progress on a worldwide basis to refine and improve the technique.

However, the data generated so far demonstrates that the technique could be used to distinguish ‘identity preserved’ non-GM soya from soya containing much larger proportions of GM soya in certain baked products. However, if a more accurate determination of the proportion of GM soya is required, a Real Time PCR analysis would need to be carried out on the ingredients themselves before incorporation in the processed food.

For Codex purposes, it is therefore stressed that notwithstanding which method is used, it is of prime importance that the laboratories undertaking the analysis participate in intercomparison exercises and uses appropriate reference materials wherever these are available.

In view of the difficulty with the practical application of methodology in this area, it is recommended that CCMAS discusses at its Twenty-fourth Session the formation of an ad hoc Working Group to develop recommendations both with respect to quality control measures that a laboratory offering GMO analyses should undertake, and specific criteria for methods of analysis to supplement the general criteria recommended in CX/MAS 02/05.