

"Detection of Genetically Modified Crops in Real-Time Practice: a State of the Art".

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Abstract

To sustain the commercialisation of genetically modified (GM) crops, informing the consumer for GMO presence through labelling of products, has been considered a milestone in the European Union. The detection, the identification and the quantification of the presence of GMOs and products thereof along the food/feed chain is essential to properly fulfil this requirement. Here, we present a real-time evaluation of the past experience/current practice/future perspective at the SBB-ISP with GMO detection in raw commodities, fruits, and food/feed processed products.

Introduction

Genetically modified (GM) crops have become a reality in agriculture and the food/feed market (James, 2004; Demont & Tollens, 2004). The recent worldwide increase of commercially available GM crops, especially as commodity products, has created a novel global market situation. In many countries (a.o. in the European Union), threshold levels for labelling products with GMO presence have been established as to guarantee consumer information on the application of biotechnology in the generation of seed/grain (derived) raw materials for food/feed purposes (EC, 2003b; 2004). The detection, the identification and the quantification of the GMO presence in certified seed lots and along the food/feed production chain is essential to properly fulfil downstream labelling and traceability requirements.

To date, the key technology applied in GMO detection, identification and quantification is the "Polymerase Chain reaction" (PCR) (Innis *et al.*, 1990). By choosing the appropriate conditions, generic or specific detection of GMO elements can be obtained (for a review see Holst-Jensen & Berdal, 2004). The major advantages of the PCR technology are the very high sensitivity of the method, the relative stability of the DNA molecule during processing, and the possibility to establish a precise standard GMO measure unit (e.g. GMO Haploid Genomes Content) (Terry *et al.*, 2002; Wiseman, 2002; EC Recommendation, 2004). The PCR methods have, however, also a number of disadvantages: i) a relatively high frequency of false positive/negative results, ii) elaborate, expensive and time-consuming analysis, iii) the need for highly skilled personnel, iv) complicated method validation procedures and requirements, and v) the purchase/installation of very expensive, sophisticated equipment and laboratory facilities (Berteau *et al.*, 2002; Anklam *et al.*, 2002).

Next to alterations at the DNA level, genetic modification also results in the presence of one or more novel recombinant proteins. Protein-based GMO detection methods (e.g. ELISA, Protein Strip Tests) have been developed for most introduced traits and are commercially available (Stave, 2002). These methods do not allow the specific identification of the GMO present and may not detect GMO presence in processed matrices due to the sensitivity of proteins to severe chemical or physical treatments (esp. solvents, heating). However, these methods (especially the Protein Strip Tests) are simple, fast, cheap and reliable, making them a complementary tool to the PCR GMO detection methods (Van Duijn *et al.*, 2002).

To support the vigilance on complying to the GMO labelling legal rules in European Union (EC, 2003b), the legislation on GM Food/Feed (Regulation (EC) 1829/2003) stipulated the establishment of the 'Community Reference Laboratory (CRL) for Genetically Modified Food and Feed'. The CRL has been attributed to the 'Biotechnology and GMOs Unit' at the 'Institute of Health and Consumer Protection' (IHCP, JRC-Ispira, Italy) (<http://gmo-crl.jrc.it>). Core tasks of the CRL with respect to experimental GMO detection methodology are: i) the international validation of event-specific detection and quantification methods for GM food and feed products destined for the market approval, and ii) the reception, preparation, storage, maintenance and distribution to national enforcement laboratories of the appropriate positive and negative control samples. The 'Institute of Reference Materials and Methods' (IRMM, JRC-Geel, Belgium) is responsible for the production of the necessary Reference Materials (<http://www.irmm.jrc.be>). The CRL co-ordinates the organisation of the pre-validation trials and consecutive broader ring trials for the GMO detection method assessment. For this purpose, a scientific platform to GMO detection in food/feed, the so-called 'European Network of GMO Laboratories' (ENGL) (<http://engl.jrc.it/>) has been established. Within this network, the national enforcement laboratories of all 25 Member States together with representatives of the European Commission are being integrated (including the GMOLAB at SBB-ISP, Belgium), allowing a co-ordinated approach towards the organisation of the GMO labelling compliance enforcement in the different Member States.

At the laboratory of the SBB-ISP, Belgium, we are currently running both DNA and protein based analysis for the presence of GMO derived materials. Here, we present an overview of the practical results obtained with the methods applied for detecting GMO presence in seeds, grain and food/feed matrices.

Materials and Methods

Materials:

Test Substances were obtained from the Technology provider Companies (BayerBioscience, Monsanto, Syngenta) or through the 'Central State Laboratory' (CSL, United Kingdom) organised GEMMA-Proficiency testing. Reference Materials were purchased from the Institute of Reference Materials and Methods (IRMM, JRC-Geel, Belgium) or obtained from the Technology Provider (BayerBioscience). Control materials were purchased locally (see table 1).

The analysed samples comprise seeds, grain, fruits, flour, starch, and various food/feed matrices. These samples were obtained during official enforcement controls or were purchased at local retailers (for a compilation of analysed samples see results).

Methods:

Protein detection by Protein Strip Test analysis

All Protein Strip Test (PST) analyses have been performed following the manufacturer's procedures (*in casu* Envirologix, Neogene, or Strategic Diagnostics). The following kits have been used: CP4 (Neogene); CP4, CryIAb, Cry9C, Cry3, PAT/*pat* (Envirologix); CP4, PAT/*bar* (SDI) (www.envirologix.com; www.sdix.com; www.neogen.com). The basic principle of the applied kits is a lateral flow detection, which allows on the one hand to determine the functioning of the strip and on the other hand to determine the presence of the respective recombinant proteins (Stave, 2002).

Table 1. List of Test Substances and Reference Materials (source indicated in italics)

Test Substances	Reference Materials
GM seed/grain <i>(Technology Provider)</i> : Corn: Event Bt176, Bt11 grain; Oilseed rape: Event MS8/RF3 seed; Soya: Event GTS-40-3-2 grain	GM seeds <i>(Technology provider)</i> Corn: Event CBH351
GEMMA matrices <i>(CSL)</i> : Corn: Matrices containing Events Bt176, Bt11, MON810, NK603, MON 863, or GA21. Soya: Event GTS-40-3-2 powder	Grain powders <i>(IRMM)</i> : Corn: Event Bt176, Bt11, MON810, GA21, NK603 and control powders Soya: Event GTS-40-3-2 and control powders
Control Material : Corn: <i>Zea mays</i> var. Anjou 245 seeds, Sweet corn seeds; Oilseed rape: <i>Brassica napus</i> var. Stego seeds; Papaya: Papaya fruits (Trademarks Bahia, Nino, tropical Sunset)	Leaf powder <i>(Technology Provider)</i> Oilseed Rape: Event MS8/RF3

Qualitative and quantitative GM detection by PCR analysis

From all matrices, a homogenised sample was prepared by crushing in a blender (type: Retz, Kika-Werke Type A10). DNA was extracted from homogenised material using a CTAB extraction (Hüpfner et al., 1998) or commercially available DNA extraction kits (Wizard® Magnetic DNA Purification System for Food from Promega, ChargeSwit with gDNA Plant kit from Invitrogen, GeneSpin Extraction Kit from GeneScan). The extraction was performed according to the manufacturer's recommendations. The extracted DNA concentration was determined spectrophotometrically (UV 260/280) or fluorimetrically (Picogreen).

For the detection of elements derived from the CP4-EPSPS (Barry *et al.*, 1992), the 35S *Cauliflower Mosaic Virus* promoter (Odell *et al.*, 1985) and the *Agrobacterium tumefaciens* NOS terminator (Depicker *et al.*, 1982), element-specific primers were used applying standard amplification and electrophoresis protocols (Berben & Dardenne, 2001; Lipp *et al.*, 2001). Generic plant primers and crop specific primers for soya (*lectin*) and corn (*zein*) markers were included in the analysis (Pietsch *et al.*, 1997; Berben & Dardenne, 2001; Studer *et al.*, 1997). Quantitative analyses were performed using event-specific Taqman primers/probe system within a Real-Time PCR DNA amplification set-up (ABIPrism 7000 system). Tested event-specific methods comprised the Roundup Ready (RR) soya event GTS-40-3-2, and the *Bacillus thuringiensis* (Bt) Maize Events Bt176, Bt11, and Mon 810 (Kuribara *et al.*, 2002).

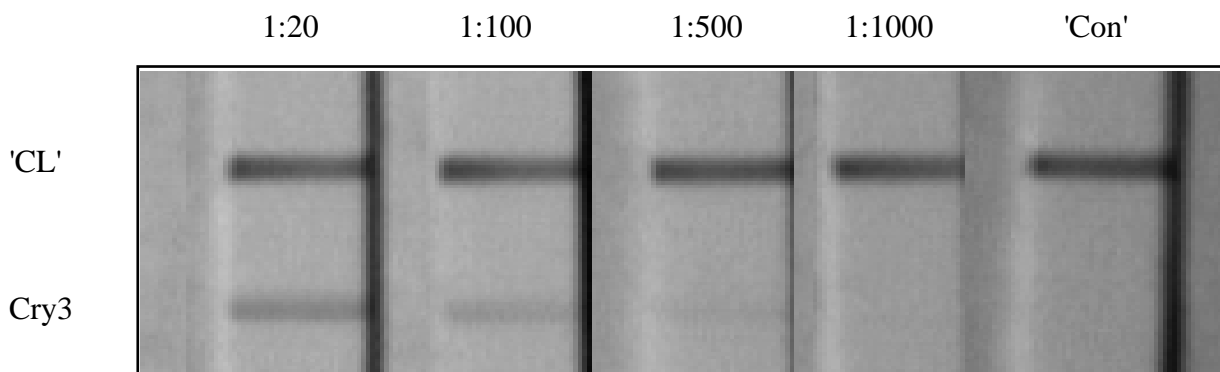
Results and Discussion

PST as tool for GMO detection in plant raw products: seeds, grain and powders

Next to holding a key reference position within the food/feed production chain, seeds and grain offer a number of additional advantages with respect to GMO detection: i) seeds/grain of the same species represent a fairly uniform matrix, ii) they have a well described composition, iii) their processing and compound extraction is very well documented, and iv) they can be relatively easily stored retaining viability and composition.

Here, the detection sensitivity by PST of each recombinant protein present in a number of available test substance and reference materials has been determined by v/v dilution of an extract of a stock sample (see table 2). An example of such dilution series using IRMM MON 863 corn reference powder, is shown in figure 1. The results of the analysis are summarised in table 2. (detailed results will be presented elsewhere: Van den Bulcke *et al.*, in preparation)

Figure 1: Determination of the detection sensitivity of the Cry3 protein in extracts from IRMM corn MON863 powder by dilution analysis using a Cry3 PST (indicated are the corresponding dilution ratio's).



note: 'CL': 'Control line' ; 'Con': non-transgenic corn powder

Table 2. Detectable rec protein levels in different GM plant (derived) test substances or reference materials by PST: the dilution approach

Trait	CP4	CryIAb	Cry9C	Cry3	PAT/ <i>pat</i>	PAT/ <i>bar</i>
Crop/Matrix (Event)						
a. Soya/IRMM (GTS-40-3-2)	1:10000	na	na	na	na	na
b. Corn/IRMM (Bt176)	na	nd	na	na	na	nd
c. Corn/IRMM (Bt11)	na	nd	na	na	na	nd
d. Corn/IRMM (MON810)	na	1:1000	na	na	na	na
e. Corn/IRMM (NK603)	1:1000	na	na	na	na	na
f. Corn/IRMM (MON863)	na	na	na	1:500	na	na
g. Soya/crush. mat. (GTS-40-3-2)	1:10000	na	na	na	na	na
h. Corn/crush.mat. (Bt11)	na	1:200	na	na	nd	na
i. Corn/crush.mat. (CBH351)	na	na	1:1000	na	na	1:5000
j. Corn/leaf (Bt11)	na	1:100	na	na	1:2	na
k. Corn/leaf (CBH351)	na	na	1:1000	na	na	1:10
l. Oilseed rape/leaf (MS8/RF3)	na	na	na	na	na	1:5000

note: 'nd': not detectable; 'na': not applicable; crush. mat. (crushed material)

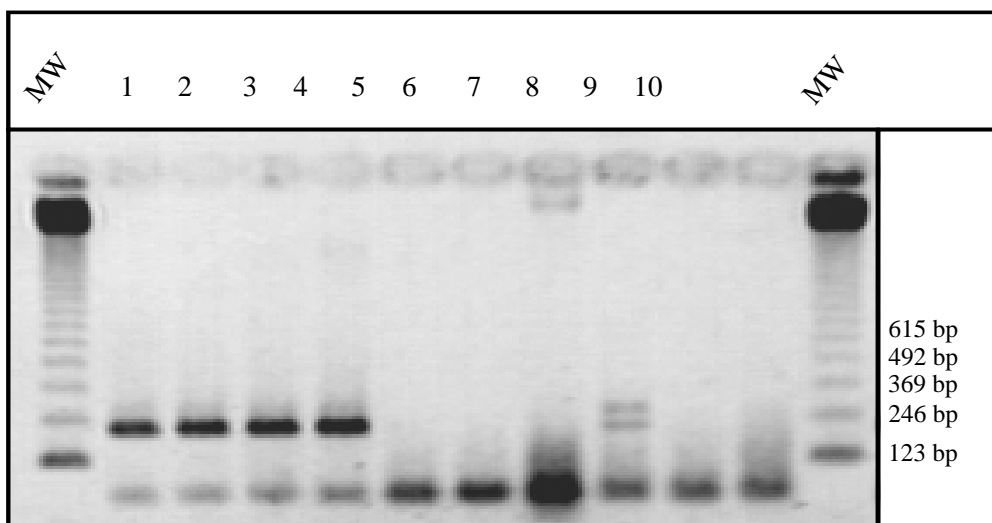
The detection sensitivity of the PST falls within the range of the legal labelling threshold limits (0.1-1.0% GMO). The obtained detectable dilution levels correlate well with the data on the recombinant protein concentration levels indicated in the official registration files for similar matrices (restricted information). PST as such can be a complementary method to estimate the GMO presence in a rapid and cheap way in a seed lot or in unprocessed matrices. The identification and quantification of each of the GM events present in the lot will, however, require additional analysis by PCR (see further discussion below).

GMO detection in fruits

Fruits and vegetables can be analysed as single units, which facilitates the labelling of a sample lot. A PCR method designed for the GM papaya Event 55-1 (APHIS Petition 96-051-01P) was evaluated (Wall *et al.*, 2004). As GM papaya positive reference material, a genomic DNA sample isolated from GM Event 55-1 positive papaya was used (GM papaya positive genomic DNA material was kindly provided by S. Pecoraro (BLGL, Oberschleißheim (Germany)))

The results of the analysis are shown in figure 2. The analytical method referred to a duplex PCR allowing the amplification of a 211 bp amplicon from *papain*, a papaya-specific gene, next to the amplification of a 273 bp amplicon from the introduced trait. Both amplicons could be amplified from the genomic DNA of the GM papaya Event 55-1 positive reference material (lane 8). Only the papain endogene was amplified from tested commercial papaya (lanes 1 to 4), demonstrating their non-GM nature. In all negative PCR test controls, no amplicons from any of the two targeted genes could be detected (lanes 5 and 6: extraction negative control; lane 7: IRMM GM maize MON810 (5%); lanes 9 and 10: PCR negative control (minus DNA)).

Figure 2: GM detection by PCR analysis of papaya seeds.



note: Lane 1 & 2: Papaya Bahia™ ; Lane 3: Papaya Nino™ ; Lane 4: Papaya Tropical Sunset™ ; Lane 5 & 6: extraction neg.control ; Lane 7: MON810 (5%) ; Lane 8: GM papaya Event 55-1; Lane 9 & 10: PCR neg. control (min DNA); External lanes: Molec. Weight (MW) marker (123 bp ladder).

GMO detection in food/feed matrices

Over the period 2000-2004, the SBB-ISP laboratory has performed GMO detection analyses on different samples for GMO presence by PCR analysis. At the SBB-ISP, a GMO detection accreditation platform has been established since 2003. The PCR analysis for GMO detection at the SBB-ISP has gone through an evolution paralleling the available DNA sequence information relevant for the detection of the GMO events, as provided by the notifiers on request of the Competent Authorities (especially of Belgium).

During the period 2000-2001, GMO detection was performed through a combination of i) generic screening for the presence of plant, maize *zein*, and soya *lectin* sequences and ii) for the

presence of CaMV promoter 35S and terminator NOS sequences as indication of GMO presence. From 2002-2004, the screening for the presence of corn Events Bt176, Bt11 and MON810 and the soya Event GTS-40-3-2 was included into the accreditation. To date, a constant updating of the scope of analysis is required paralleling the availability of validated methods and reference materials of (newly) authorised events. A representative summary of the obtained results of the analyses during this 4 years period, is shown in Table 3.

Table 3: Period 2000-2004: GMO detection analysis of food/feed samples at SBB-ISP by qualitative and quantitative PCR analysis

Year	Matrix Description	Sample Nr
2000	Soya Seeds, Schrimp Powders, Flours (Soya, Corn, Wheat), Bread (Soya, Prokorn, Wheat), Instant Soups (Tomato,...), Milk Powder (Soya)	17
2001	Flour (Soya, Corn, Wheat), (Modified) Starch (Corn), Soya Lecithin, Chips (Tortilla, Bangles, Taco, Dip Chips), Corn Flakes, Cookies (Biscuits, Waffles), Meat Products (Sausages, 'Cassolet', Beef Meat), Pasta (Spaghetti,...)	50
2002	Flour (Soya, Corn, Wheat), (Modified) Starch (Corn), Soya Products (Tofu, Lecithin, Soya Additives), Chips (Tortilla), Corn Flakes, Popcorn, Maltodextrin, Meat Products (Sausages, Hamburger Frankfurter), Cookies (Biscuits, Crackers, Nuts, 'Speculoos')	66
2003	Flour (Soya, Corn, Wheat), Instant Soups (Tomato, Chicken), Meat Products (Bouillon), Bread Adjuvants, (Modified) Starch (Corn), Grains (Soya, Corn, Oilseed Rape), Soya Sauce, Pet feed, Dried milk Powder, Milk Concentrate, Ketchup	50
2004	Flour (Soya, Corn, Wheat), Meat Products (Bioproducts), Bread Adjuvants, (Modified) Starch (Corn), Grains (Soya, Corn, Wheat), Corn Flakes, Popcorn, Chips (Tortilla, Baggles, Chips), Feed Products (Rabbit, Chicken, Cattle, 'Vollailles'), Pasta (Polenta), Papaya Fruits	95

Within the analysed samples, the majority of the GMO positive samples contained RR soya. To lesser extend, mainly during the last two years, also Bt maize material could be identified during these analyses.

Towards an integrated GMO detection approach

Based on our experience, we are currently working towards the application of the following analytical scheme, retaining a broad flexibility but a common methodology (Annex 1). A distinction is made between raw materials (seeds and grain), fruits and vegetables on the one hand and processed food/feed products on the other hand.

Seeds and grain in a lot can be visually determined and mechanically separated by sieving (in our case soya, corn and oilseed rape seeds). Applying the crop relevant PST, a primary screening for introduced trait presence is performed (CP4, CryIAb, Cry9C, Cry3, PAT/*pat*, PAT/*bar*). A focused event identification and quantification is then performed applying the appropriate event-specific PCR analysis. For fruits and vegetables, a direct event-specific PCR analysis is performed.

GMO detection in processed food/feed matrices is the most complicated. Up to recently, a generic screening for the presence of sequences derived from the 35S promoter and the NOS terminator allowed to score all events present in the commercial GM crops (corn and soya) (see above). Several new events (e.g. maize Event GA21), however, are not detectable in this way. Much in the sense applied for seeds/grain using the PST (see above), we are currently working on the full development of a trait-based PCR screening analysis. In this way, we hope to be able to narrow down substantially the required downstream detailed quantitative analysis for the presence of the specific events in a matrix.

Future perspectives in GMO detection tools tailored to the real-time Market

The complexity of the global GM crop market is rapidly increasing. The advent of new GM events exploring the use of plant-derived traits (e.g. maize Event GA21) and the crossing of different GM events (e.g. so-called "stacked hybrids", such as Event NK603xMON810) result in an exponential growth of the need of GMO detection methods. In parallel, the production of the Reference Materials for GMOs and the development of appropriate robust but cost-effective GMO detection tools represents a major challenge.

We have embarked on the further development and optimisation of target plasmids as key element in the establishment of a 'GM crop detection technology platform'. Previous studies have already demonstrated the potential of 'GMO-plasmids' as calibrants for the quantitative determination of GMO presence in complex matrices (Kukibara *et al.*, 2002, Taverniers *et al.*, 2001). We are currently building up a 'GMO plasmid' library comprising plasmids that contain relevant DNA sequences to the detection of the GMO events authorized for commercial use in the European Union. In this way, large quantities of plasmid Reference Material can be produced and made available at low cost and in a very suitable format for application in different DNA detection technologies (PCR, microarrays,...).

Linked to the 'GMO plasmid' library, we are setting the first steps towards the creation of a 'GMO Bio-informatics Platform', aiming at providing an optimised *in silico* virtual analytical tool to explore potential new synergies between current GMO detection approaches and novel technologies emerging from the extensive world-wide efforts within genome analysis in all areas of life sciences.

References

Anklam, E., Heinze, P., Kay, S., and Van den Eede, G. : "Validation Studies and proficiency testing." (2002) *Journal of AOAC Int.*, Vol. 85, Nr. 3, 809-815.

Barry, G., Kishore, G., Padgett, S., Taylor, M., Kolacz, K., Weldon, M., Re, D., Eichholtz, D., Fincher, K. & Hallas, L. : "Inhibitors of amino acid biosynthesis: strategies for imparting glyphosate tolerance to crop plants." (1992). In: *Biosynthesis and Molecular Regulation of Amino Acids in Plants*. Singh *et al.* (eds.). American Society of Plant Physiologists. p. 139-145.

Berben, G., Dardenne, P.: "Traçage et authentification des produits à base d'organismes génétiquement modifiés". (2001) In: Final report of contract NP/42/026

Berteau, Y., Dioloz, A., Kobilinsky, A., and Magin K. : "Detection Methods and performance Criteria for Genetically Modified organisms." (2002) *Journal of AOAC Int.*, Vol. 85, Nr. 3, 801-808.

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gosselé, V., Movva, N.R., Thompson, C., Van Montagu, M., and Leemans, J. : "Engineering herbicide resistance in plants by expression of a detoxifying enzyme." (1987) *EMBO J.*, 6: 2513-2518.

Demont, M., and Tollens, E. : "First Impact of Biotechnology in Europe: Bt maize adoption in Spain." (2004) *Ann. Appl. Biol.*, 145: 197-207.

Depicker, A. Stachel, S., Dhaese, P., Seurinck, J., Deboeck, F., De Greve, H., Lemmers, M., Van Montagu, M., Schell, J. : " Nopaline Synthase: transcript mapping and DNA sequence." (1982) *J. of Molecular and Applied Genetics*, 1, 561-573.

EC (2001) Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. *Official Journal of the European Communities* L106: p. 1-39.

EC (2003a) Regulation (EC) 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Official Journal of the European Communities* L 268: p. 1-23.

EC (2003b) Regulation (EC) 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. *Official Journal of the European Communities* L 268: p. 24–28.

EC (2004) Commission Recommendation 787/2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in the context of Regulation (EC) 1830/2003. *Official Journal of the European Communities* L348: p. 18-26.

Holst-Jensen, A.H. and Berdal, K.J. : "The Modular Analytical Procedure and Validation Approach and the Units of Measurement for Genetically Modified Materials in Foods and Feeds." (2004) *Journal of AOAC Int.* 87: 927-936.

Hüpfner, C., Hotzel, H., Sachse, K., and Engel, K.H. : "Detection of the genetic modification in heat-treated products by BT-maize polymerase chain reaction." (1998) *Zeitschrift für Lebensmittel-Untersuchung und –Forschung* A206, 203-207.

Innis, M.A., Gelfand, D.H., Sninsky, J. J., White, T. J. : "PCR Protocols: A Guide to Methods and Applications. " (1990) Academic Press, Inc., San Diego, California.

James, C. : "Global Status of Commercialized Transgenic Crops: 2004. " (2004) ISAAA Brief No 32, Ithaca, New York.

Kok, E., J., Aarts, H., J., M., van Hoef, A., and Kuiper, H. : "DNA Methods: Critical Review of innovative Approaches." (2002) *J. Of AOAC Int.*, Vol. 85, Nr. 3, 797-800.

Kozziel, M., G., Carozzi, N. B., Currier, T. C., Warren, G. W., and Evola, S. : The Insecticidal Crystal Proteins of *Bacillus Thuringiensis*: Past, Present and Future Uses. In: "Biotechnology and Genetic Engineering Reviews" (1993) Ed; Tombs M. P. Intercept Ltd, Andover, UK. Vol 11: 171-228.

Kuribara, H., Matsuoka, T., Takuba, K., Futo, S., Hirao, T., Akiyama, H., Goda, Y., Toyoda, M., and Hino, A. : " Novel Reference molecules for quantification of genetically Modified Maize and soybean." (2002) *Journal of AOAC Int.* , vol 85, nr. 5 : 1077-1089.

Lipp, M., Bluth, A., Eyquem, F., Kruse, L., Schimmel, H., Van denEede, G., Anklam, E.: "Validation of a method based on polymerase chain reaction for the detection of genetically modified organism in various processed foodstuffs" (2001) *Eur. Food Res Technol.* 212, 497-504.

Odell J.T., Nagy F., Chua N.H.: "Identification of DNA sequences required for the activity of the cauliflower mosaic virus 35S promoter." (1985) *Nature* 313: 810-812.

Pietsch, K., Waiblinger, H.U., Brodmann, P., Wurz, A.: "Sreeningverfahren zur Identifizierung "genetisch veränderter" pflanzlicher Lebensmittel." (1997) *Deutsche Lebensmittel-Rundschau* , 93 (2), 35-38.

Stave, J. : "Protein Immunoassays Methods for detection of Biotech Crops: Applications, Limitations and Practical Considerations." (2002) *Journal of AOAC Int.*, Vol 85, No 3: 780-786.

Studer, E., Dahinden, I., Lûthy, J. Hübner, P.: "Nachweis des genetisch veränderten "Maximizer"-Mais mittels der polymerase-Kettenreaktion (PCR)." (1997) " *Mitteilungen aus dem Gebiet der Lebensmittel und Hygiene*, 88, 515-524

Taverniers, I., Windels, P., Van Bockstaele, E., and De Loose, M. : "Use of cloned DNA fragments for event-specific quantification of genetically modified organisms in pure and mixed food products." (2001) *Eur. Food Res Technol.*, 213, 417-427.

Terry, C.F., Harris N., and Parkes, H.C. : "Detection of Genetically Modified Crops and their Derivatives: Critical steps in sample preparation and extraction." (2002) *Journal of AOAC Int.*, Vol 85, nr 3, 768-774.

Trappmann, S., Schimmel, H., Kramer, G.N., Van Den Eede, G., and Pauwels, J. : "Production of Certified Reference Materials for the Detection of Genetically Modified Organisms." (2002) *Journal of AOAC Int.*, Vol 85, No 3: 775-779.

Van Duijn, G., Van Biert, R., Bleeker-Marcelis, H., Van Boeijen, I., Adan, A.J., Jhacie, S., and Helsing, M. : "Detection of Genetically Modified Organisms in Foods by PCR- and DNA-based techniques: Bridging the Methods." (2002) *Journal of AOAC Int.*, Vol. 85, Nr. 3, 787-791.

Wall, E. M., Lawrence, T.R., Green, M.J., and Rott, M.E. : "Detection and identification of transgenic virus resistant papaya and squash by multiplex PCR." (2004) *Eur. Food Res. Tech.*, 219: 90-96.

Wiseman, G. : "State of the Art and limitations of Quantitative polymerase Chain reaction." (2002) *Journal of AOAC Int.*, Vol. 85, Nr. 3, 792-796.

Wohlleben, W., Arnold, W., Broer, I., Hillemann, D., Strauch, E., and Pühler, A. "Nucleotide sequence of the phosphinotricine N-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*." (1988) *Gene*, 70: 25-37.

Annex 1 : GMO detection approach for seed/grains (and derived powders), fruits, vegetables and GM food/feed derived or containing products (EU approved GM Food/Feed/novel Food events, December 2004)

LEVEL 1 Seeds, grain, fruits	Visual identification & mechanical separation (seeds/grains/fruits)									
	Subdivision according to species (weight percentage)									
LEVEL 2 All matrices	Screening by PST (seeds/grain) or PCR (fruits, procesed food/feed)									
	Soya*		Maize					Oilseed rape		
	CP4	CP4	CryIAb	Cry3°	Cry9C°	PAT/pat	PAT/bar	CP4	PAT/pat	PAT/bar
	GTS-40-3-2	NK603	Bt176 Bt11 MON810	-	-	T25	Bt11	GT73	Topas 19/2 HCN10	MS1/RF1/RF2 MS8/RF3
LEVEL 3	Identification/quantification of the GM events by Q-PCR									

*' : Maize Event GA21 is not included in the table as a plant derived EPSPS protein is expressed (PCR detectable only); '°' : Cry3 and Cry9C PST are used for screening for presence of unapproved events.