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Kernel Lot Distribution Assessment (KeLDA): a Comparative Study of Protein and DNA-Based Detection Methods for GMO Testing

Marco Mazzara · Claudia Paoletti · Philippe Corbisier · Emanuele Grazioli · Sara Larcher · Gilbert Berben · Marc De Loose · Imma Folch · Christine Henry · Norbert Hess · Lotte Hougs · Eric Janssen · Gillian Moran · Roberta Onori · Guy Van den Eede

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Abstract Monitoring of market products for detection of genetically modified organisms (GMO) is needed to comply with legislation in force in many regions of the world, to enforce traceability and to allow official control along the production and the distribution chains. This objective can be more easily achieved if reliable, time and cost-effective analytical methods are available. A GMO can be detected

using either DNA-based or protein-based methods; both present advantages and disadvantages. The objective of this work was to assess the performance of a protein-based (lateral flow strips—LFT) and of a DNA-based (polymerase chain reaction—PCR) detection method for GMO analysis. One thousand five hundred samples of soybean, deriving from the sampling of 15 independent bulk lots in large

M. Mazzara (✉) · E. Grazioli · S. Larcher · G. Van den Eede
European Commission, Joint Research Centre,
Institute for Health and Consumer Protection,
Via E. Fermi 2749,
21027 Ispra, Varese, Italy
e-mail: marco.mazzara@jrc.ec.europa.eu

C. Paoletti
European Food Safety Authority (EFSA),
Via Carlo Magno 1A,
43126 Parma, Italy

P. Corbisier
Reference Material Unit, European Commission, Joint Research
Centre—Institute for Reference Materials and Measurements (IRMM),
Retieseweg 111,
2440 Geel, Belgium

G. Berben · E. Janssen
Département Qualité des Productions Agricoles,
Centre wallon de Recherches Agronomiques,
Chaussée de Namur 24,
5030 Gembloux, Belgium

M. De Loose
Institute for Agricultural and Fisheries Research (ILVO), T&V,
Merelbeke, Belgium

M. De Loose
Department of Molecular Genetics, University Ghent,
Ghent, Belgium

I. Folch
Laboratori Agroalimentari, DAR de la Genetalitat de Catalunya,
Ctra. de Cabrils, s/n.,
08348 Cabrils Barcelona, Spain

C. Henry
The Food and Environment Research Agency (FERA),
Sand Hutton York YO41 1LZ, UK

N. Hess
Institut für Hygiene und Umwelt,
Haus 6, Raum 29, Marckmannstr. 129b,
D20539 Hamburg, Germany

L. Hougs
Laboratory for Diagnostics in Plants, Seed, and Feed,
Danish Plant Directorate,
Skovbrynet 20,
2800 Lyngby, Denmark

G. Moran
SASA,
Roddinglaw Road,
Edinburgh EH12 9FJ Scotland, UK

R. Onori
GMOs and Mycotoxins Unit, Department of Veterinary Public
Health and Food Safety, National Institute of Health,
Viale Regina Elena, 299,
00161 Rome, Italy

shipments, were analysed to assess and compare the performance of the analytical methods and evaluate their suitability for GMO testing. Several indicators were used to compare the performance of the methods, including the percentage correlation between the PCR and LFT results. The GMO content of the samples ranged from 0 up to 100 %, allowing a full assessment of both analytical approaches with respect to all possible GMO content scenarios. The study revealed a very similar performance of the two methodologies, with low false-negative and false-positive results, and a very satisfactory capacity of both methods in detecting low amounts of target. While determining the fitness for purpose of both analytical approaches, this study also underlines the importance of alternative method characteristics, like costs and time.

Keywords Genetically modified organisms · DNA-based detection · Protein-based detection · Limit of detection

Introduction

The availability of analytical methods to detect, identify and quantify genetically modified organisms (GMOs) and genetically modified (GM) derived ingredients in food and feed products as well as in seeds is necessary to comply with EU legislation labelling requirements, to enforce traceability of market products and to allow official control in trading along the production and the distribution chains (European Commission 2003a, b). This requirement to monitor the presence of GMOs in various market products generated an increasing demand for reliable, time and cost-effective analytical methods. As GMOs are characterised by the presence of one or more additional traits encoded by one or more genes, a GM plant or derived product can be distinguished from the wild-type counterpart by testing for either the presence of the introduced DNA sequence(s) (DNA-based methods) or by detecting, if any present, the expressed novel protein encoded by the integrated gene(s) (protein-based methods).

Currently, the majority of DNA-based methods use polymerase chain reaction (PCR) technology (Ahmed 2002; Holst-Jensen et al. 2003; Lipp et al. 2005) and they can be grouped into three main categories, according to the level of information desired for the selected target DNA sequence: screening, identification, and quantification.

- Screening methods are the broadest tests.
- Identification or “event-specific” methods, designed to reveal the number and identity of the GMOs present in a sample.
- Quantification methods, enabling the determination of the amount of one or more GMOs in a product. These

are used to verify compliance with threshold regulation requirements that are set at different levels in various countries (e.g. 5 % in Japan, 1 % in Australia and New Zealand and 0.9 % in the EU) or in any situation where estimates of the amount of GMO content are required.

Protein-based methods, relying upon immunoassay technologies, are designed to recognise the synthesised proteins encoded by the integrated gene(s) using either monoclonal (more specific) or polyclonal (more sensitive) antibodies (Grothaus et al. 2006). Double antibody sandwich methods like enzyme-linked immunosorbent assay (ELISA) are the most commonly used immunoassays in several applications including GMO detection (Stave 2002). The high level of specificity of these methods allows the detection of all the GM lines expressing the protein under investigation. As a result, double antibody sandwich methods provide trait specific results.

A variation of the ELISA format is the immunochromatographic (lateral flow) strip test (LFT) which operates in a similar way to a double antibody sandwich, where the target protein is linked simultaneously by two antibodies: one monoclonal linked to a solid support, the other, a polyclonal complex, with a detectable tag attached (Stave 2002). Compared to standard ELISA procedures, lateral flow strips present the advantage that the reaction takes place on a solid support exploiting the protein solution flux through an absorbent strip. As a consequence, results are obtained in few minutes and the method is very practical and cost-effective.

Both DNA- and protein-based methods are regularly employed at different stages of quality control along production and distribution chains, and both present advantages and disadvantages. Method selection depends on many factors, among which the most critical ones are the specific characteristics of the samples under investigation and the objective of the analysis. PCR technology has the advantage of being very flexible and sensitive, therefore applicable to a wide range of materials and matrices, including processed and medium-refined ingredients. Additionally, its flexibility in the design of primers ensures the highest possible specificity towards events when flanking regions are targeted. Conversely, PCR is rather expensive in terms of equipment needed, time-consuming and complex to execute (Holst-Jensen et al. 2003; Lipp et al. 2001 for a detailed overview).

Immunoassays methods are certainly easy to execute, time efficient, have a low unitary (per sample) cost and do not require highly sophisticated equipment and specialised operators. This is especially true for lateral flow strip tests. However, protein-based methods rely upon the expression level of target proteins, which is known to vary among tissues and according to physiological age (Grothaus et al. 2006), therefore potentially affecting reliability and comparability of results. Furthermore, the production and

availability of specific antibodies is a time-consuming process and most immunological methods provide qualitative rather than quantitative results. Finally, their application to heat-treated samples is generally more limited.

Although in depth discussion of advantages and drawbacks of DNA and protein-based detection strategies is well documented (Ahmed 2002; Anklam et al. 2002), so far no experimental data allowing a direct comparison of the performance of these techniques are available, where methods are compared when used on a very large set of real-world samples, not artificially spiked.

The Institute for Health and Consumer Protection (IHCP), Molecular Biology and Genomics Unit, of the European Commission (EC), Joint Research Centre (JRC), in collaboration with the Institute of Reference Materials and Measurements (EC, JRC, IRMM) and eight laboratories from six different member states, all members of the European Network GMO Laboratories, conducted a research project entitled “Kernel Lot Distribution Assessment” (*KeLDA*).

The KeLDA project had two objectives: firstly, to assess the distribution of GM material in soybean lots imported into the European Union (Paoletti et al. 2006); secondly, to compare the suitability of protein-based (lateral flow strips) and DNA-based (PCR technology) detection methods for GMO screening in bulk commodities. The project provided a unique, large series of samples from real scenarios on which to test the reliability of these methods.

Materials and Methods

Definitions

LOD—limit of detection	the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation. The presence of the analyte should be detected at least 95 % of the time at the LOD, ensuring ≤ 5 % false-negative results (European Union Reference Laboratory for GM Food and Feed and European Commission 2008).
LOQ—limit of quantification	the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy (European Union Reference Laboratory for GM Food and Feed and European Commission 2005).
False-positive sample	a sample with a GMO content below the LOD of the real-time PCR method

(at least 0.01 % or five copies) that tested positive in the qualitative analysis.

False-negative sample	a sample with a GMO content above the LOD of the real-time PCR method (at least 0.01 % or five copies) that tested negative in the qualitative analysis.
Grey zone sample	a sample with a GMO content above the LOD of the real-time PCR and below the minimum sensitivity required for the qualitative method ($0.01 < x < 0.03$).

Materials

Soybean was chosen as the most suitable experimental material for the *KeLDA* project because it was likely to demonstrate heterogeneous distribution patterns (Paoletti et al. 2006). Also, at the time of the project, only one GM soybean event was approved and being marketed in the world (Center for Environmental Risk Assessment), Monsanto’s GTS 40-3-2 (or Roundup Ready® (RR) soybean), and methods for the quantification of this GM line were readily accessible. Roundup Ready® (RR) soybean, was created by introducing the gene coding for the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), isolated from *Agrobacterium tumefaciens* strain CP4, into the commercial soybean variety “A5403” (Asgrow Seed Co.) The inserted transgene codes for a glyphosate tolerant form of EPSPS.

In the context of the KeLDA project, 15 independent soybean lots imported within the European Union were analysed. A total of 100 increment samples of approximately 500 g were collected from each lot (collection was equally spread in time during off-loading), resulting in 1,500 samples analysed in the context of the project. This mass represents a mean equivalent of approximately 3,000 kernels. The analysis of each lot comprised five steps: sampling of the lots (done by each participant in the study); grinding and homogenisation of the collected increment samples (done by JRC-IRMM and partially by CRA-W); qualitative analysis by LFT (done by JRC-IRMM and partially by CRA-W); PCR analyses (qualitative end-point with agarose detection, done by each participant to the study) and quantitative real-time PCR (done by JRC-IHCP).

Collection, Grinding and Homogenisation of the Soybean Samples (Steps 1 and 2)

Sampling, grinding and homogenisation of increments were performed as previously described (Paoletti et al. 2006).

Protein-Based Qualitative Analysis (Step 3)

The immunoassay was performed using the commercial kit Trait✓[™] RUR Test Kit, (hereafter referred to as LFT) for bulk grain testing from Strategic Diagnostics Inc. (Lateral Flow Test Kit, Part Number 700001)(Strategic Diagnostics Inc 2012). This kit is a variation of the ELISA that allows the qualitative detection (presence or absence) of the protein CP4 EPSPS produced by a gene derived from *Agrobacterium* sp. strain CP4. This gene was incorporated into herbicide-resistant crops, including Monsanto Roundup Ready brands (RUR). At the time of the collection and testing of the samples, the only commercialised soybean GM event expressing the CP4 EPSPS was the GTS 40-3-2; more recently (2007) a new soybean GM lines called MON89788 and expressing the same protein was introduced in USA and Canada (Center for Environmental Risk Assessment) and is now authorised in the EU (European Commission 2008).

Optimisation of the Protocol and Determination of the Limit of Detection of the LFT

In order to ensure optimal sample homogenisation, the grinding procedure applied to KeLDA materials was designed to produce soy flour particles characterised by an average particle size of 200 μm , where 95 % of the powder had a particle size below 710 μm , which is a much smaller size than the one normally obtained with laboratory-grade blenders routinely used for lateral flow strip testing. The advantage of the small flour particle size is that it permits higher protein extraction efficiency; the disadvantage is that it may cause pore-membrane occlusion in the strips and may lead to the alteration of protein–antibodies binding specificity and coprecipitation events of protein–antibodies complexes, resulting in unspecific bands on the strips.

Even if the manufacturer (User Guide Part Number 700001) (Trait✓[™] RUR Lateral Flow Test User Guide) only guarantees a LOD of 0.1 %, it was assessed that the presence of one GM kernel among 3,000 kernels (~0.033 % in weight) was still detectable. This improvement of the sensitivity was obtained by increasing the extraction volume and introducing a centrifugation step of the protein extract. One gram of flour was thoroughly mixed with 16 ml of water in a tube. One millilitre was taken from the obtained suspension and centrifuged at $10,000\times g$ for 20 s.

Samples containing 0, 0.033, 0.1, 0.33 and 1 % of RR soybean were prepared from non-GM and RR soybean kernels mixed in appropriate proportions, milled according to IRMM grinding protocol and screened with the Trait✓[™] RUR Test Kit. For each level of GM content, ten analyses

were performed; as usual water was used as the negative control. A lateral flow strip was introduced in the tube and the result was recorded after 15 min. Two independent tests were performed on each increment sample.

DNA-Based Qualitative Analysis (Step 4)

DNA Extraction

Total genomic DNA was extracted by the testing laboratories from soybean flour of the increments of the respective lots. A CTAB-based protocol (Murray and Thompson 1980), subsequently improved was used. The resulting protocol was validated in collaborative inter-laboratory trial for soybean matrix involving 25 laboratories and was adopted as ISO standard method (ISO, Standard No 21571, Annex A.3.1. Genève, Switzerland 2005). DNA extraction was performed in duplicate from each sample, and one extraction negative (buffer) control was added to each set of extraction. Approximately 200 mg of the test sample was weighed in a 2-ml tube; 400 μl of sterile deionised water were added and the sample mixed with a loop and left rehydrate for 5 min. A 1.3-ml pre-warmed (65 °C) CTAB extraction buffer (CTAB=20 g/l, NaCl=1.4 mol/l, Tris=0.1 mol/l and Na₂EDTA=0.02 mol/l) was added and the solution mixed with a loop. Ten microlitres of RNase A solution (10 mg/ml) was added and the sample mixed gently. The sample was incubated for 30 min at 65 °C, under gentle agitation. After addition of 10 μl of proteinase K (20 mg/ml), a second incubation was performed as above. The solution was then centrifuged at approximately $12,000\times g$ for 15 min. Eight hundred microlitres of the supernatant were transferred to a new 2-ml tube containing 1 volume of chloroform, thoroughly mixed for 30 s and centrifuged at approximately $12,000\times g$ for 15 min. The upper aqueous phase was transferred into a new tube containing 2 volumes of the CTAB precipitation buffer (CTAB=5 g/l, NaCl=0.04 mol/l). The mixture was incubated at room temperature for 60 min, then centrifuged at $12,000\times g$ for 15 min. The supernatant was discarded and the precipitated DNA was dissolved in 350 μl of NaCl 1.2 mol/l by gentle pipetting. Three hundred fifty microlitres of chloroform was added and the solution thoroughly mixed for 30 s. After centrifugation at $12,000\times g$ for 10 min, the aqueous phase was transferred into a new tube containing 0.6 volumes of isopropanol. The tubes were inverted gently, kept at room temperature for 20 min and centrifuged at $12,000\times g$ for 10 min. The supernatant was discarded and the pellet was dried and then re-dissolved into 100 μl of TE buffer: Tris=0.01 mol/l, Na₂-EDTA=0.001 mol/l, pH 8.0. This DNA solution was used as template for PCR assays.

Verification of the Quality of DNA After Extraction

After DNA extraction, a PCR test to check the amplification efficiency and thereby the DNA quality and quantity was carried out by the laboratories. For this purpose, the species-specific, single copy soybean lectin gene *le1*, obtained from gene data banks (GenBank accession no. K00821 M30884) was chosen as a target taxon-specific sequence (ISO, Standard No 21569, Annex A.1. Genève, Switzerland 2005). PCR was performed as previously described (ISO, Standard No 21569, Annex A.1. Genève, Switzerland 2005).

As a positive control, DNA extracted from the certified reference materials of Roundup Ready soybean (CRM, IRMM-410R 0.1 % RR soybean) was used. An extraction blank control and a reaction mix control (reagents mix + water) were included in each PCR. The PCR products were separated and visualised by gel electrophoresis on standard agarose gel (agarose 2.5 % w/v) with ethidium bromide.

Detection of 35S Promoter in Soybean

Samples were tested for the presence/absence of genetically modified (GM) soybean using a qualitative PCR method for the detection of the 35S promoter fragment present in GM soybean, which was previously validated by the Joint Research Centre, European Commission in an inter-laboratory collaborative trial (Lipp et al. 2001). Two amplification replicates were carried out on each extraction duplicate, providing four test results for each increment sample with the GM-specific PCR method. One negative DNA target control (CRM, IRMM-410R 0 % RR soybean), one positive DNA target control (CRM, IRMM-410R 0.1 % RR soy), one extraction blank control and one reaction mix control (reagents mix + water) were included in each PCR.

The PCR reaction volume of 25 μ l contained about 100 ng of genomic DNA, 0.6 μ M of each primer (p35S-cf3, 5'-ccacgtcttcaagcaagtgg-3; p35S-cr4, 5'-tctctccaatgaaatgaactcc-3'), 0.8 units of Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA, part number 4311816), 0.16 mM dNTP, 1.5 mM MgCl₂ and 10 \times AmpliTaq PCR buffer. The volume was adjusted to 25 μ l with DNase-free sterile water. The PCR thermal profile included an initial activation and denaturation step at 95 °C for 10 min followed by 50 cycles of 25-s denaturation at 95 °C, 30-s annealing at 62 °C and 45-s extension at 72 °C. A final extension for 7 min at 72 °C completed the amplification. The PCR products were separated and visualised by gel electrophoresis on standard agarose gel (agarose 2.5 % w/w).

Quantitative Analysis by Real-Time PCR (step 5)

All increment samples obtained from KeLDA lots were analysed by real-time PCR to assess the relative quantity of GM soybean. Quantification of GM material in each sample was based on the ratio between the quantity of GM DNA and the quantity of a chosen reference DNA sequence, according to standard real-time PCR procedures (ISO, Standard No 21570, Annex C.2. Genève, Switzerland 2005). Quantification of the GM content was carried out as described previously (Paoletti et al. 2006).

The method used was validated through a collaborative trial involving 19 laboratories: method precision, accuracy and LOQ were determined during the collaborative trial, while data on specificity, linearity and on the detection limit were previously determined (ISO, Standard No 21569, Annex A.1. Genève, Switzerland 2005). The LOQ of the method is ≤ 50 genome copies of soybean line GTS 40-3-2. The LOD of the method, calculated by measuring serial target DNA solutions, is five copies of the target sequence; this corresponds to a relative content of 0.01 % GM soybean when about 50 ng of total DNA is used in PCR (Arumuganathan and Earle 1991).

Results

Determination of the Sensitivity of LFT

In order to assess the sensitivity of the assay and the homogeneity of the ground material, LFT was performed on 10 independent extractions of the same powder containing 0.033 % of GM soybean; water was used as a negative control. All 10 extracts were positive and no false-negative results obtained. To exclude the possibility of false-positive results, 10 independent analyses of the 0 % GM soybean flour (CRM, IRMM-410R) were carried out. Water was used as a negative control, whereas a sample containing 5 % of GM soybean (CRM, IRMM-410R) was used as positive sample. Visible EPSPS-specific bands were present only on the positive sample; all other samples showed only the control line (Fig. 1).

Protein-specific test bands were present in all the samples containing RR soybean, thus including the sample containing 0.033 % RR soybean (Fig. 2), while they were absent in the 0 % GM sample (Fig. 1). This suggests that the LOD of the assay is ≤ 0.033 % and indicates that the homogeneity of the samples is suitable for detection at this GM level.

Table 1 Comparison of the analytical performance of PCR and lateral flow strips test (LFT) on all soybean lots samples

Lot	Mean GM% of the lot	% matching LFT-PCR	Lateral Flow Test (LFT)			PCR		
			No of false positive (FP)	No of false negative (FN)	No of correct results in grey zone (CGZ)	No of false positive (FP)	No of false negative (FN)	No of correct results in grey zone (CGZ)
1	3.9	96	0	1	0	1	0	2
2	76.9	100	0	0	0	0	0	0
3	78.9	100	0	0	0	0	0	0
4	65.1	100	0	0	0	0	0	0
5	0.01	100	0	13	0	0	13	0
6	0.02	63	0	1	18	0	19	0
7	0.15	78	0	4	1	1	13	5
8	0.22	84	0	17	0	0	3	0
9	89.6	100	0	0	0	0	0	0
10	90.6	100	0	0	0	0	0	0
11	92.8	100	0	0	0	0	0	0
12	96.1	100	0	0	0	0	0	0
13	92.1	100	0	0	0	0	0	0
14	0.01	79	0	15	0	6	4	2
15	0.02	74	1	5	13	5	6	16
	Mean % of matching		Total FP	Total FN	Total CGZ	Total FP	Total FN	Total CGZ
	91.6		1	56	32	13	58	25
	Proportion on total (%)		0.37	4.55	41.5	4.9	4.71	32.5

The column defined as “% matching PCR-LFT” relates to samples that gave identical results with both methods. For definition of false positive (FP), false negative (FN) and “grey zone” (CGZ) see “Materials and Methods”

Determination of the Sensitivity of Qualitative PCR

The sensitivity of the 35S promoter-specific PCR method used was investigated on soybean flours prepared from negative samples, 1 % sample (used as positive control) and 0.033 % sample. The assessment was performed on 10 replicates. A lectin gene-specific fragment was amplified by PCR and checked on a 2.5 % w/w agarose gel. The samples

prepared from non-GM soybean content gave negative results. The 1 % RR soybean samples showed a band with a length corresponding to the target sequence (123 bp). The same positive result was displayed by the samples containing 0.033 % of RR soybean (Fig. 3). Therefore, these estimated comparable performance characteristics (reliable detection of one kernel in 3,000) allow a direct comparison of the two approaches.

Fig. 1 Specificity of the Trait/RUR lateral flow test on flour samples of Roundup Ready soybean (Certified Reference Material, 0 % series 410R); *negative control*: water; *positive control*: 5 % RUR soybean



Fig. 2 Assessment of the sensitivity of the Trait✓ RUR lateral flow test on replicated samples prepared at 0.033 % (w/w) GM Roundup Ready soybean; the specific band is indicated by the arrow; *negative control*: water. For details on lateral flow strips detection procedure see “Materials and Methods”



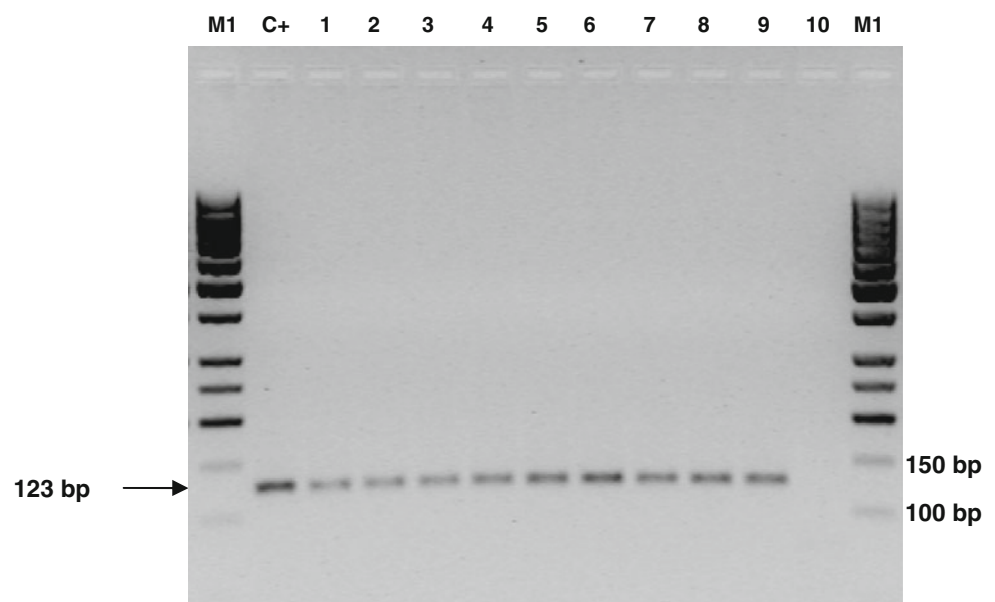
Analysis of the Lots

The comparison of the performance of the methods was based on a number of parameters. These included the main indicators routinely used to assess the performance of qualitative tests, number of false-positive (FP) and of false-negative (FN) results (Codex Alimentarius, CAC/GL 74–2010 2010), and two new indicators that we propose in this study: the “% of matching between PCR and LFT (lateral flow test)” and the “number of correct results in the grey zone” (CGZ). The first consists of the number of samples that gave identical results, regardless if correct or not. This indicator is therefore a direct measurement of agreement between the two methodologies. The second is defined as the number of correct results for those samples containing a quantity of GMO above the LOD of the real-

time PCR (0.01 %) but below the LOD of both qualitative methods (≤ 0.03 % for both PCR and LFT), as assessed in our laboratory in the conditions of this study. This indicator is therefore a direct measurement of the power and reliability of the two methodologies when operating at the limit of the range of their applicability.

The results of the analyses conducted on all increments by real-time PCR were taken as reference for classifying the samples as positive or negative, due to the higher sensitivity of this method (relative LOD 0.01 %) and to its specificity, experimentally assessed by the method developers prior to the collaborative trial conducted to validate the method (ISO, Standard No 21570, Annex C.2. Genève, Switzerland 2005). The overall mean percentage of correlation of results for the 15 lots between PCR and LFT is very high, equal to 91.6 %, (Table 1); for 9 out of

Fig. 3 Assessment of the sensitivity of the qualitative PCR method of detection of the CaMV 35S in replicated samples containing 0.033 % of Roundup Ready soybean. After PCR, 8 μ l of solution was separated on agarose gel (2.5 %) for 1 h at 100 V. *M1* marker 50 bp, *C+* positive control 1 % RUR soy; *Lanes 1–9* 0.033 % RUR soybean, *10* no template control (PCR reagents + water). Specific band size: 123 bp



the 15 lots the matching is absolute (100 %), while the lowest level of matching is 63 % for lot number 6 (63 correct results). This means that in more than 90 % of the cases (1,374 out of 1,500 samples analysed) the two methodologies led to the same qualitative result.

Comparing the percent of matching between LFT and PCR with the mean GM% content of the lots, it can be noted that there is a correlation between the two data: in fact the lots for which the lowest level of matching was observed (lots 6, 7, 8, 14 and 15) are those contaminated by low amounts of GM, in the range of 0.01–0.22 %. The only exception is represented by lot number 5 that showed a 100 % of matching while containing only a mean GM% of 0.01.

Remarkably, only one sample analysed by LFT gave a FP result (Table 1, no. of false positive for LFT correspond to the 0.37 % on the total number of negative samples). The PCR analysis generated a limited number of false-positive results (4.9 %) with 13. This low ratio of false-positive results of the PCR method is in line with the inter-laboratory validation study results published (Lipp et al. 2001) that reported an overall average of false-positive results of 3.9 %, obtained on foodstuffs containing either GM maize or soybean or both.

As far as the number of FN results is concerned, we observed almost identical results for LFT and PCR; the total number of FN was 56 for LFT and 58 for PCR (Table 1), corresponding to a proportion of 4.55 % for LFT and 4.71 % for PCR. For eight lots, none of the two methods showed FN, while for LFT the majority of FN were in lots 5, 8 and 14, while for PCR in lots 5, 6 and 7, and to a lower extent in lots 14 and 15 (Table 1).

A more in depth analysis of the occurrence of FN results is presented in Table 2, where we report the mean percent of GM of the samples (increments) that gave FN results, for the two methods and for the lots to which FN results belong. For significance of the data presented, the mean percent of GM of the samples that gave FN results for each lot was calculated only in the case of five or more samples classified as FN.

Results summarised in Table 2 demonstrate that in general, for both methods employed, FN results appeared

correlated to a rather low GM content in the sample, ranging from 0.05 to 0.15 %. No evident difference can be noted between the two methods: the LFT produced FN results on samples containing GM from 0.05 % (lot 15) to 0.13 % (lot 8), and the PCR produced FN results on samples containing GM from 0.05 % (lot 14) to 0.15 % (lot 7).

Concerning the number of correct results for those samples containing a very low amount of GM soybean (CGZ in Table 1), we observed a slightly improved performance of the LFT over the PCR; in fact, LFT gave a higher number of correct results (32 samples) for samples in the grey zone (CGZ, Table 1). This corresponds to a proportion of 41.5 % of the total in comparison to PCR analysis, which resulted in 25 correct results, corresponding to 32.5 % of the total. For LFT analysis, virtually all CGZ samples were detected in two lots (6 and 15), while for PCR CGZ samples were detected in 4 lots out of 15 (lots 1, 7, 14 and 15).

Discussion

The first step of an official control system for the presence of GMO in food, feed and seed production is the sampling at the import point, followed by the analytical testing in the laboratory. Depending on the specific legislation in place, this control aims at verifying the presence/absence of approved or unapproved GMO and at supporting the decision of rejecting or labelling the product, as mandatory in the EU (European Commission 2003b).

At this stage, the analyst is confronted with the decision on the choice of the most suitable and effective analytical approach for the testing of the laboratory samples deriving from the sampling plan. This choice should take into account the aim of the tests (e.g. qualitative screening for the presence/absence of a GMO, identification of the GM trait and identification of the GM event), the nature and composition of the samples, the availability of suitable methods (e.g. validated methods) and the availability of suitable resources, including expertise, quality management, infrastructure and financial means. All these elements will contribute to the best quality of the final analytical result (Van den Eede et al. 2002).

The testing of samples derived from imported bulk commodities, as a first analytical insight into the possible GMO presence in them, is a real challenge; at this stage, the sample is truly “unknown”, meaning that no information is available on the presence of one or more GMOs, (possibly belonging to different species) contaminating the lot. In addition, with the increasing number of GM events approved worldwide and in the pipeline for approval (Stein et al. 2009), the analysis is becoming more and more complex, challenging and time and money consuming.

Table 2 Mean percent of GM of the samples (increments) that gave false-negative (FN) results, for the two methods (lateral flow test and PCR) and for the lots to which the samples belong

Lot	Lateral flow test (LFT)	PCR
	Mean GM% of FN Samples (SD)	Mean GM% of FN Samples (SD)
5	0.09 (0.04)	0.09 (0.04)
6	–	0.06 (0.02)
7	–	0.15 (0.08)
8	0.13 (0.05)	–
14	0.06 (0.02)	0.05 (0.01)
15	0.05 (0.01)	0.06 (0.03)

In such a situation, the first analytical step is a general detection, also referred to as “screening”, with the objective of determining whether a product contains a GMO (Anklam et al. 2002). Current methodology for the event-specific identification of GMOs is limited, PCR being the method of choice (Holst-Jensen et al. 2003). However there is a vast availability of techniques that can be used for the screening steps (Anklam et al. 2002). This, while representing an advantage for the analyst, poses at the same time a certain degree of uncertainty on the choice of the most suitable method.

The study presented here focuses on this crucial step, with the purpose of providing sound data on the comparative performance of two methodologies currently employed in GMO detection: a LFT for the qualitative detection (presence/absence) of the protein CP4 EPSPS expressed by GM crops (including Monsanto Roundup Ready brands) and a screening qualitative PCR for the detection of the 35S promoter inserted in most GM lines. To do so, we tested a considerable number of samples (1,500) taken from several independent lots (15) of soybean grains imported into the EU. In order to assess the performance of the two methods, we considered the percentage of correlation between the results of LFT and PCR, the rate of FN and FP results and the number of correct results for samples containing a very low amount of the target GMO (CGZ).

As highlighted by the considerably large percentage of matching of results (Table 1), the two methods behaved overall in a very similar manner. A lower matching was always associated to a very low level of GMO and it was not dependent upon the method used; the number of false-negative results was very similar between LFT and PCR (55 and 59, respectively), although distributed in different lots. For example, for lot 6 the LFT gave only one sample as FN, while the PCR gave 18 FN results.

FN results were obtained, for both methods, on samples containing GM amounts in the range of 0.05–0.15 % (Table 2). Considering that both methods were experimentally assessed to be able to detect at least 0.033 % in optimal condition, the occurrence of FN scores in samples with a GMO content close to the LOD has to be considered normal. In fact, the amount of detectable target (DNA copies for PCR and concentration of GM protein for LFT) may be too low or absent in the laboratory test sample considering the real probability of including a sufficient number of DNA copies (Kay and Van den Eede 2001) or the probability of detecting the protein in the sample (Stave 2002).

Therefore, attention should also be given to the distinction between the LOD of the method, usually determined through testing of reference materials in standard optimal conditions, and the practical LOD (Holst-Jensen et al. 2003), which can only be determined from results of analyses of test samples or real samples, as in the case of this study. Many factors, difficult or impossible to control, may

lower the sensitivity of the method, including the biochemical composition of grains (e.g. fat, protein or polysaccharides contents) produced by different varieties in different environmental conditions (the soybean lots of this study originated from Brazil, Paraguay, USA and Canada). Nonetheless, taking into consideration this extensive potential source of variation, both methods performed excellently in terms of sensitivity, with an overall proportion of FN results equal to 4.55 % for the LFT and to 4.71 % for the PCR (Table 1).

Both methods were equally sensitive and sensitive enough to detect the presence of their respective targets in samples at GM content below the estimated LOD of the methods (“grey zone samples”, see “Materials and Methods” for definition and Table 1 for results). This should not to be considered unusual because the practical LOD of the methods was assessed experimentally as “at least” 0.033 % GM soybean, which does not exclude that the sensitivity might be higher under particular circumstances. In this regard, it should not be overlooked that the GM content of the samples used in this study was estimated by means of a validated real-time quantitative PCR method (ISO, Standard No 21569, Annex A.1. Genève, Switzerland 2005), and that the uncertainty of the quantitative measurement at very low levels of GM content may be high, in the range of 30–50 % of relative repeatability standard deviation.

A very important parameter for any detection method is the tendency to produce FP results in the absence of the specific target in the sample. FP results can arise as result of a lack of specificity to the target or as consequence of a cross-contamination of the sample during the processing steps. In our study we observed a very low number of FP results for the PCR (13 samples out of 1,500 tested, equal to 0.9 %) and, remarkably, one FP result for the LFT assay.

The fact that almost the totality of the FP results for PCR (11 out of 13) were observed in only 2 lots out of 15 (lot 14 and 15) both analysed by a single laboratory suggests that their presence may be due to cross-contamination during the analyses or to contamination by one or more GMO other than Roundup Ready® soybean line GTS 40-3-2. If a different GMO containing the common target sequence of the 35S promoter was present, the PCR assay would give a positive signal while the LFT used in this study would be negative, as would the Roundup Ready® soybean GTS 40-3-2 construct-specific real-time PCR test.

The results on the FP ratio show that a DNA-based method like PCR, although performing satisfactorily, may be subject to risks of cross-contamination across samples during their handling during the complex process of analysis, which includes various laborious steps, from DNA extraction, to reaction mix preparation and pipetting. Protein-based methods, and in particular a simplified version like the LFT used in this study, do not present this drawback, as the manipulations of the samples are very limited.

In this study, we demonstrated that in the case of the most cultivated GMO worldwide (75.4 million ha of Roundup Ready® soybean in 2011, corresponding to 47 % of the global biotech area) (James 2011), the two methods, representing the two main different analytical approaches (protein-based and DNA-based detection) gave comparable results in the conditions of the study (i.e. bulk commodity). In this situation, the choice of the most suitable method may be driven by other considerations, both scientific/technical and economical.

Both analytical approaches have advantages and disadvantages as thoroughly reviewed by other authors (Ahmed 2002; Anklam et al. 2002). In the case of bulk commodities, a PCR method capable of detecting common elements present in most GMOs (e.g. 35S promoter and t-nos terminator) offers the possibility of detecting the presence of GMO accidentally contaminating the lot, belonging to any plant species and being legal or illegal in the country of testing. This screening step is of utmost importance in providing a first insight on the composition of the lot and is the basis for further analytical steps aiming at identifying the single GM lines in the sample.

Protein-based methods are trait specific, thus lacking the characteristics of a broad screening test offered by PCR. For plant species commodities like soybean, for which one or few GMO are cultivated worldwide, a simple lateral flow test may provide almost complete information on the contamination by GMO. However, this becomes more complex for other crops such as maize, rapeseed and cotton, for which the number of different GMO expressing different traits is increasing (Center for Environmental Risk Assessment 2012). In these cases, the application of multiple tests for different traits may substitute for a screening PCR. With this regard, the availability of protein-based methods, particularly lateral flow strip tests, has increased steadily (www.sdix.com; <http://envirologix.com/>; <http://www.neogen.com/>) also with “combo” strips for detection of multiple targets in one test.

Besides the analytical performance characteristics of the methods, the costs associated to the testing should be evaluated. These costs include not only the actual price of the test but also investments in laboratory facilities, human resources, staff training, reporting, etc.

We have highlighted that lateral flow strip tests, unlike PCR, are easy and fast to perform (5/8 min per test, excluding sample preparation) and do not require specialised staff and costly laboratory setup. We estimate the cost of two qualitative tests compared in this study as follows: PCR = 4.2 €/sample (including DNA extraction), lateral flow strip test = 4.5 €/sample.

Consideration should be also given to the maximum number of tests one operator can conduct in one working day: for PCR this number is about 10. Considering a rather

practicable DNA extraction method, many more samples can be analysed by the same operator in one working day.

In conclusion, the data presented in this paper revealed a very similar performance of the two methods when taking into account the parameters listed above; both methods performed in a very efficient manner under the conditions of this study, with a high degree of matching of the results (100 % matching in the majority of the tests) and a proportion of FP and FN results always below 5 %.

Taking into account, besides the analytical performance, the unitary cost of the tests and other costs associated (e.g. laboratory facilities, staff), it clearly appeared that, in the conditions of the present study (testing bulk soybean grain lots with only one expected GM line), the lateral flow test is the most appropriate choice. However, an informed choice of the methodology must fully consider different scenarios in which the PCR technique has the potential to provide more reliable and broadly applicable results, e.g. in the case of commodities potentially contaminated by more than one GMO, e.g. belonging to different plant species represented in traces in the bulk.

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