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Quantitative Detection of Genetically Modified Soybean and Maize: Method Evaluation in a Swiss Ring Trial

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Introduction

Over 30 different genetically modified crops have been approved worldwide during the last years (1); some of them with identical traits, e.g. Roundup Ready maize, soybeans and sugarbeet all carrying the tolerance against the herbicide glyphosate. The use of GMO crops as food in Switzerland and Europe requires an

authorization. Once approved they have to be labelled if an ingredient contains more than 1 % of GMO (2–4). In the beginning the enforcement of these legal stipulations was mostly done with PCR based qualitative detection methods (5–8). More recently quantitative methods are used due to shortcomings of the qualitative approaches (9–14).

Although not all of these crops are commercially grown, there is the possibility that some of them may be present as adventitious minglings in our foodstuffs. The experiences in GMO analysis during the last years pointed to problems with respect to analytical methods: 1) quantitation of each individual crop (also in mixed foodstuffs) must be feasible, due to the legislation, which stipulates a threshold for labelling on the basis of each ingredient, 2) the distinction of different GMO varieties (specificity) and 3) non-authorized GMO varieties must be identified. Whereas the second and the third problem may be solved with appropriate qualitative methods, quantitation requires the development and evaluation of new detection methods. In order to perform quantitative tests, certified reference material has to be available for calibration purposes. Furthermore for qcPCR internal standards (competitor template DNA) also have to be available. In parallel with the development of quantitative detection methods for GMOs during the last two years, these certified reference substances became commercially available on the market.

However, knowledge about the performance of qcPCR and real-time PCR methods for detection of genetically modified crops in foodstuffs is very limited. To assess the analytical parameters such as accuracy, trueness, precision, repeatability and robustness/reproducibility a ring trial was conducted. Since soybean and maize are the most important commercially grown GM-plants today and since certified reference material was available, it was decided to perform the ring trial with RR-soybeans (RRS) and Bt176 maize. QcPCR methods were applied for screening (35S) and for GMO-specific detection (RRS, Bt176). Furthermore soybean samples were also examined with real-time PCR methods (35S screening, RRS-specific). The results of the ring trial were expected to indicate which of the tested methods are applicable for the enforcement of the legislative requirements of the 1 % labelling threshold. Additionally, the analysis of the results should give an estimation of the accuracy of the individual methods.

For this reason, several qcPCR and real-time PCR detection methods were included in the Swiss ring trial.

Materials and methods

Samples and reagents

Samples containing soybean and maize flours with known content of GMO were ordered from FLUKA Chemie AG (Switzerland) and were produced by the Institute of Reference Materials and Measurements (IRRM, Belgium). In order to cover GMO concentrations close to the threshold limit, 200 g soybean and maize

flour of each of the following mixtures were ordered: 0.7 % (sample D), 1 % (sample E), 1.4 % (sample A), 1.8 % (sample B) and 3 % (sample C) of GMO content. All samples were aliquoted in 5 g portions and labelled S(oybean)A-SE and M(aize)A-ME.

All participants of the ring trial received the following reagents:

- 5 g of each soybean sample: SA, SB, SC, SD, SE in a 50 ml conical tube
- 5 g of each maize sample: MA, MB, MC, MD, ME in a 50 ml conical tube
- 1 flask of each certified reference material (external standard) of Roundup Ready soybean: SB0.5 (0.5 % GMO; Fluka #85477), SB1 (1 % GMO; Fluka #17106), SB2 (2 % GMO; Fluka #85478), SB5 (5 % GMO; Fluka #17135)
- 1 flask of each each certified reference material (external standard) Bt176 maize: MZ0.5 (0.5 % GMO; Fluka #63197), MZ1 (1 % GMO; Fluka #17109), MZ2 (2 % GMO; Fluka #63198), MZ5 (5 % GMO; Fluka #17111)

for qcPCR

- internal PCR standards (competitor template DNA): soybean: cSL (Fluka #29249); RRS: cRRS (Fluka #29246); 35S: cP35S (Fluka #29247), maize: cHMG(maize) (Fluka #29251); Bt176: cCRY (Fluka #29248)
- primers: soybean: SL1/SL2; RRS: GM07/GM08; 35S: 35S-A/35S-B; maize: HM3/HM4; Bt176: CRYIA3/CRYIA4 (Microsynth, Switzerland; for sequences see table 1)

for real-time PCR

- primers: 35S system: 35S-F; 35S-R; 35S-TMP; RRS system: RRS-F; RRS-R; RRS-TMP; soybean system: Lectin-F; Lectin-R; Lectin-TMP (Perkin Elmer, Switzerland; for sequences see table 1)
- TaqMan Universal Master Mix (2×concentrated, Perkin Elmer, Switzerland).

Primers, internal standards and TaqMan probes were shipped in dry ice whereas the samples and the external standards were shipped without any cooling. All other required reagents had to be supplied by the participant laboratory.

Extraction and quantification of nucleic acids

All samples and the external standards were extracted with a modified Wizard™ procedure (Promega, Wisconsin, USA). Briefly, 100 mg of the homogenous sample material was taken and 200 µl H_2O were added. Then 860 µl extraction buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1 % SDS), 100 µl guanidinium-hydrochlorid (5 mol/l) and 40 µl proteinase K (20 mg/ml) were added. The solution was incubated at 56–60° C for at least 3 hours. After cooling, the samples were centrifuged for 10 min at 12'000–20'500 x g. 500 µl of the supernatant were transferred to a new Eppendorf tube and 5 µl RNase (10 mg/ml) were added. After 5 min at 56–60° C (hydrolysis of the RNA), 1 ml Wizard®-Resin was added and mixed by gentle inversion. Further processing of the samples was done according to the recommendations of the manufacturer. Finally the DNA was eluted with 50 µl of hot (70° C) elution buffer (10 mM Tris-HCl, pH 9.0). The concentration of the

Table 1
Primers and sequences

Primer	*Sequence*	*Detected Gene*	*Specificity*	*qc[1]/real-time PCR*	*Reference*
SL1	atg ggc ttg cct tct ttc tc	lectin	soybean	qcPCR	(5)
SL2	ccg atg tgt gga ttt ggt g	lectin	soybean	qcPCR	(5)
HM3	gaa atc cct gag cga gtc ggt a	high mobility group	maize	qcPCR	(5)
HM4	gcg atg gcc ttg ttg tac tcg a	high mobility group	maize	qcPCR	(5)
GM07	atc cca cta tcc ttc gca aga	EPSPS[2]	RoundupReady	qcPCR	(15)
GM08	tgg ggt tta tgg aaa ttg gaa	EPSPS	RoundupReady	qcPCR	(15)
CRYIA3	ccg cac cct gag cag cac	cryIA(b)[3]	Bt176	qcPCR	(6)
CRYIA4	ggt ggc acg ttg ttg ttc tga	cryIA(b)	Bt176	qcPCR	(6)
35S-A	aag ggt ctt gcg aag gat ag	35S promoter	RoundupReady, Bt176	qcPCR	(5)
35S-B	agt gga aaa gga agg tgg ct	35S promoter	RoundupReady, Bt176	qcPCR	(5)
RRS-F	ggc atg ttg tta att tgt gcc at	EPSPS	RoundupReady	real time PCR	(5)
RRS-R	gaa gtt cat ttc att tgg aga gga c	EPSPS	RoundupReady	real time PCR	(5)
RRS-TMP	*FAM*[4]-ctt gaa aga tct gct aga gtc agc ttg tca gcg-*TAMRA*[5]	EPSPS	RoundupReady	real time PCR	(5)
Lectin-F	tcc acc ccc atc cac att t	lectin	soybean	real time PCR	(5)
Lectin-R	ggc ata gaa ggt gaa gtt gaa gga	lectin	soybean	real time PCR	(5)
Lectin-TMP	*FAM*-aac cgg tag cgt tgc cag ctt cg-*TAMRA*	lectin	soybean	real time PCR	(5)
35S-F	gcc tct gcc gac agt ggt	35S promoter	RoundupReady	real time PCR	(5)
35S-R	aag acg tgg ttg gaa cgt ctt c	35S promoter	RoundupReady	real time PCR	(5)
35S-TMP	*FAM*-caa aga tgg acc ccc acc cac g-*TAMRA*	35S promoter	RoundupReady	real time PCR	(5)

[1] qcPCR = quantitative competitive PCR
[2] EPSPS = Enol-Pyruvyl-Shikimate-Phosphate-Synthase
[3] cryIA(b) = delta-endotoxin gene from *Bacillus thuringiensis*
[4] *FAM* = 6-carboxyfluorescein
[5] *TAMRA* = 6-carboxytetramethylrhodamine

DNA was determined at OD_{260} in 0.2 M NaOH in order to transform all DNA molecules into their single stranded conformation (5).

Primers and PCR reactions

All primers used in the ring trial were HPLC purified. Concentrations of reagents for qcPCR were: 200 ng DNA, 0.5 µM of each primer, 2.5 mM $MgCl_2$ in 100 µl reaction volume. Cycling profiles for qcPCR are indicated in table 2. The concentration of the internal standard (IS) was approximately adjusted by the manufacturer to correspond to 1 % GMO when using 1 µl per reaction. However, each ring trial participant had to recalibrate the amount of IS to 1 % before it could be used in the qcPCR experiments. The concentrations of PCR reagents for real-time PCR were: 900 nM (TaqMan) or 250 nM (LightCycler) of each primer and 200 nM (TaqMan) or 150 nM (LightCycler) of the probe(s). The reaction volume was 50 µl (TaqMan) and 20 µl (LightCycler).

Quantitation

Quantitation by visual inspection was done by comparing the intensity of the GMO-specific band to the band of the IS and to the known concentrations of the external standard (ES = certified reference material). In addition, the ring trial laboratories were asked also to analyse the gels with an image analysis software. However, only eight laboratories had such a software; calculation of the percentage of GMO was done with seven different software programms. Finally, only 10 laboratories had the opportunity to perform real-time PCR.

Results and discussion

General remarks

Upon the introduction of a threshold limit of 1 % for the labelling of foodstuffs containing GMOs, the evaluation of the accuracy, sensitivity and reproducibility of quantitative PCR-methods was needed. For this reason the Swiss Federal Office of Public Health started a ring trial in fall 1999 to compare the performance of several qcPCR and real-time PCR methods. It was decided to use only Roundup Ready soybean (RRS) and Bt176 maize as samples because ES and IS were only available for these GMO lines. Furthermore, the ring trial concentrated on low processed maize and soybean (flour) samples because the performance of the methods was the main subject, whereas the evaluation of the extraction methods was not part of the ring trial. The sample of 0.7 % was chosen to have a value below but close to the threshold limit of 1 %, which should indicate if a 30 % difference in percentage could be distinguished. The 1 % sample was chosen to represent the threshold value. 1.4 % and 1.8 % had a difference of 0.4 % and 0.8 % to the threshold value and a difference between themselves of 0.4 %, which should indicate if these values could be distinguished from each other and if they were identified to be above the thresh-

Table 2
PCR cycling profiles for qcPCR. The following profiles were used: for Lectin (A); high mobility group (B); RRS, Bt176 and 35S (C)

A *Step*		*B* *Step*		*C* *Step*	
Denaturation	3 min/95° C	Denaturation	5 min/95° C	Denaturation	3 min/95° C
Amplification	30 sec/95° C	Amplification	30 sec/95° C	Amplification	30 sec/95° C
	60 sec/64° C		60 sec/62° C		60 sec/60° C
	60 sec/72° C		60 sec/72° C		60 sec/72° C
Cycles	40	Cycles	40	Cycles	40
Final extension	3 min/72° C	Final extension	3 min/72° C	Final extension	3 min/72° C

old value. Finally, as a clear value above the threshold, the 3 % concentration was chosen.

During the initial experiments of the ring trial it turned out that the soybean ES was not consistent in itself, i.e. both the 1 % and the 2 % ES yielded the same results with the applied qcPCR methods. The investigations revealed that the diverging ES were manufactured using different procedures at different time points leading to different degrees of DNA degradation (data not shown). The ring trial participants were advised during the course of the ring trial to use the 2 % ES and dilute the DNA to get a 1 % ES for use in the quantitation. With the maize ES such problems did not occur although they were also produced at different times with different procedures (H. Schimmel, IRRM, personal communication). Since the samples SA-SE were mixtures of these divergent standards these samples could not be used for the evaluation of the trueness. However, the results of both the soybean and the maize samples could be compared with respect to the variation of the applied methods. The described problem illuminates the crucial influence of the ES for the entire analysis and indicates that strict quality control measures have to be applied in the production process of certified reference materials.

Quantitative competitive PCR

Semi-quantitative determination of GMO contents by visual inspection

In a first step the ring trial participants had to calibrate the amount of IS to a GMO concentration of 1 %. This is done by titrating the IS against the 1 % ES to the point where the IS- and the GMO-specific band are equal in intensity (9, 10). For all subsequent experiments this empirically determined amount of IS had to be applied for quantitation purposes. It is interesting to notice that the amount of IS used by the different laboratories varied in most cases by a factor of 5–10, in one case (RRS) by a factor of 100 (maize: 35S (0.2 to 1 ul), Bt176 [0.25 to 3 ul]; soybean: 35S [0.25 to 3 ul], RRS [0.05 to 5 ul]), indicating that already at this step the sensitivity of the same PCR methods was highly variable in the different ring trial laboratories. The most probable explanation for this discrepancy are differences in the DNA quantitation of the samples.

After the calibration of the IS the soybean and the maize samples were checked for the contents of soy-specific (lectin) and maize-specific (high mobility group) genes, in order to demonstrate that the amplification quality of the DNA was equal in all samples (data not shown). Then the two qcPCR methods (35S and GMO-specific) had to be applied for soybeans and maize.

The result of the visual assessment of the quantity by eye is displayed in figure 1. In all four experimental settings most ring trial participants correctly identified the samples A, B and C (1.4, 1.8 and 3 %, respectively) to be above 1 %. As described above, the 1 % and the 2 % ES yielded the same result, which means that using a diluted 2 % ES leads to an overestimation of the GMO-content if the measured

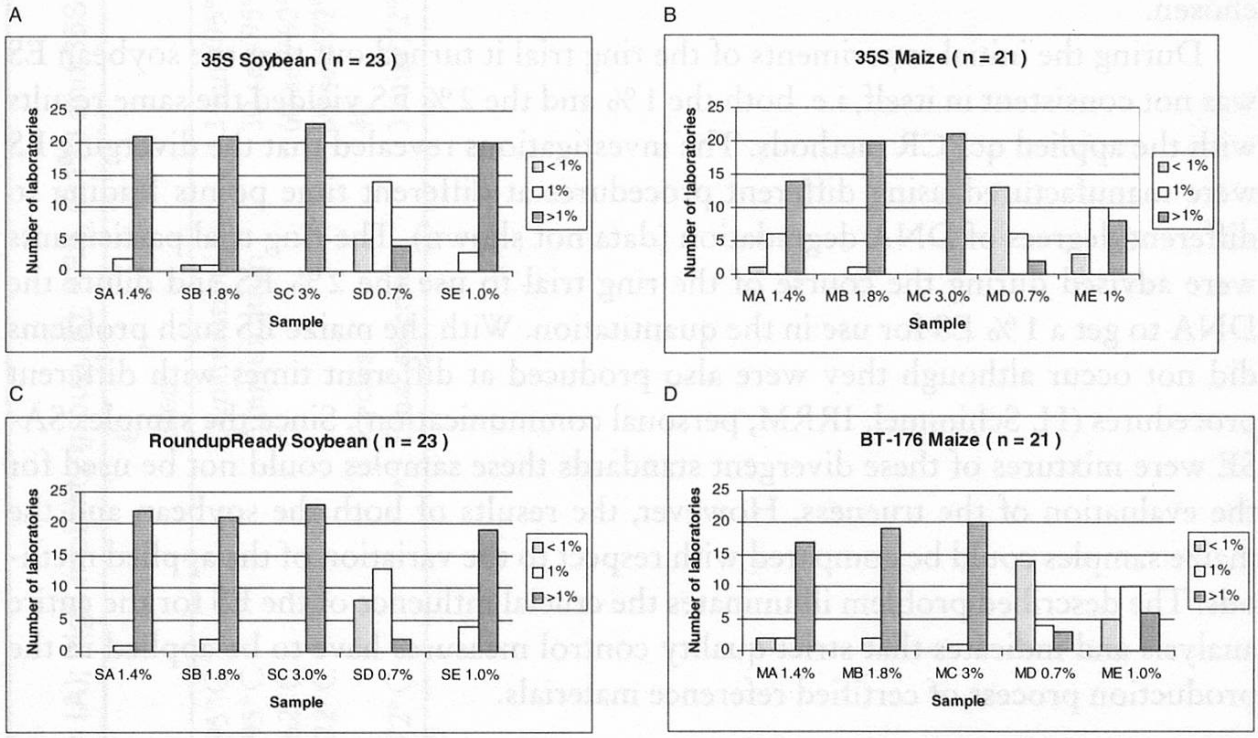

Figure 1 **Semi-quantitative determination of the GMO content by visual inspection.** Soybean samples (SA-SE) and maize samples (MA-ME) were analysed either with the screening (35S) method (A, B) or the GMO-specific method (C, D). *n* = number of ring trial laboratories from which the results were compiled

sample consists mainly of 1 % standard material. Probably due to this inconsistency the soy samples D and E (0.7 % and 1 %, respectively) were shifted to higher values. A clearer identification is observed for maize, where the samples D and E were better classified in the correct group. However, among both the soybean and the maize samples, it did not matter if the screening method (35S) or the GMO-specific method was used, indicating that these methods have similar performance characteristics.

Quantitative determination by image analysis software

The ring trial participants were asked to quantify the GMO content from the gel bands for the 35S- and the GMO-specific experiments using commercially available image analysis software. Quantitation includes scanning of the electronically stored gel picture and integration of the band signal intensities. However, only seven of the 26 ring trial participants had the possibility to perform this type of analysis (fig. 2). In order to evaluate the influence of the different software (six different software programs) used by these seven laboratories, all determinations were repeated with one software by the Federal Office of Public Health using the electronically pro-

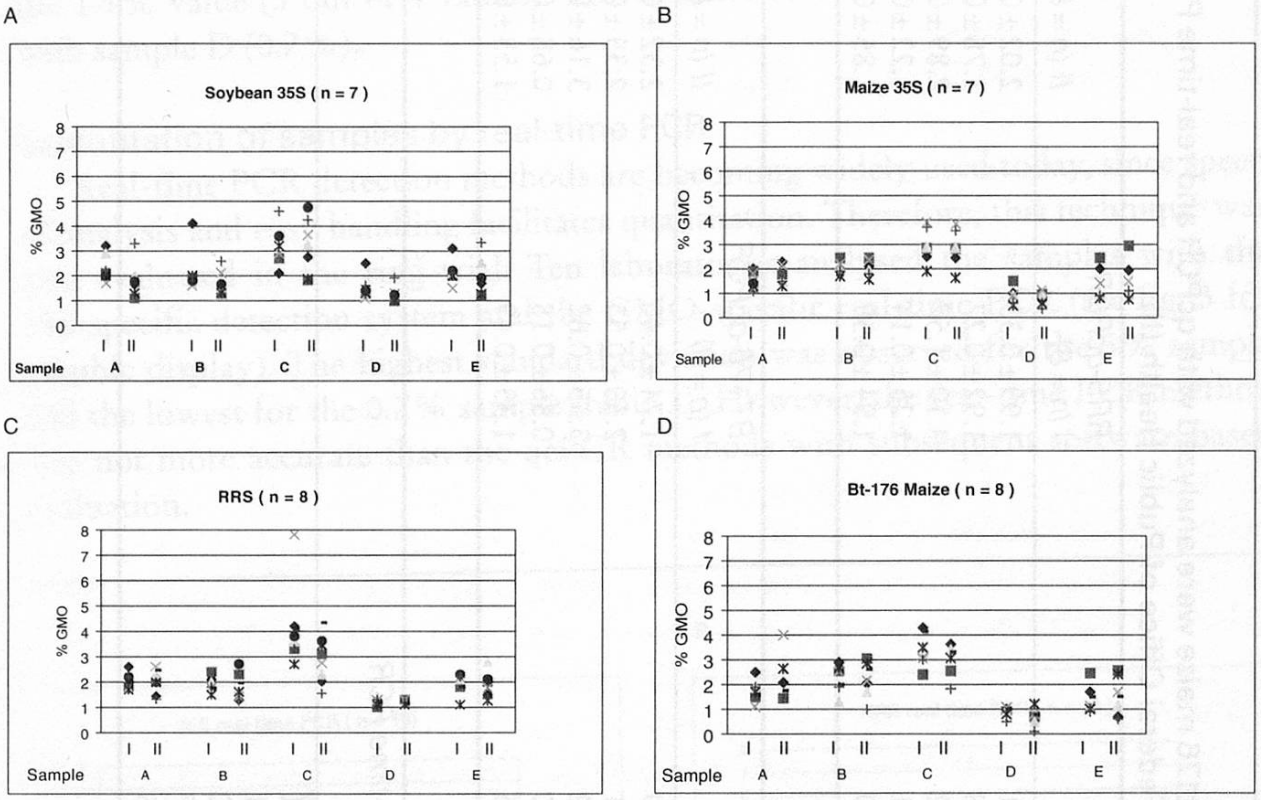

Figure 2 **Quantitative determination of the GMO content by image analysis software.** I and II = analysis by the ring trial laboratories and the Federal Office of Public Health, respectively. Samples A-E = 1.4, 1.8, 3, 0.7 and 1% GMO content, respectively. *n* = number of ring trial laboratories from which the results were compiled

vided gel images of the ring trial participants (fig. 2). With a few exceptions the distribution of the values was similar, although the individual values differed in some cases, indicating that the type of software or the application of the software to integrate the gel bands might yield diverging results. The mean values and the standard deviation were similar between the results provided by the ring trial participants and the results recalculated by the Federal Office of Public Health (table 3) and there was no case where the mean values differed significantly. From these data it can be concluded that the most consistent results were achieved for the 3 % sample which displayed in each set of results the highest value whereas sample D (0.7 %) displayed always the lowest obtained value. In the case of RRS the mean value of sample D was determined to be between 1.22 % and 1.49 % using the different systems. This high value might have arisen through the non-consistent external soybean standard, because for maize, the sample D value was between 0.68 % and 0.86 %, which is very close to the theoretical expected value. Whereas in the soybean samples the values of 1.4 % and 1.8 % could not be distinguished from each

Table 3
Comparison of results qcPCR and real-time PCR. RR-soybeans and Bt176 maize were analyzed with qcPCR and real-time PCR. Image analysis software performed by ring trial laboratories (I) and Federal Office of Public Health (II)

Sample RR-soybean	*35S-qcPCR I (n = 7)*	*II (n = 7)*	*RRS-qcPCR I (n = 8)*	*II (n = 8)*
A (1.4% GMO)	2.30 ± 0.54	1.84 ± 0.71	1.99 ± 0.33	2.02 ± 0.46
B (1.8% GMO)	2.17 ± 0.86	1.70 ± 0.48	1.92 ± 0.27	1.78 ± 0.51
C (3% GMO)	3.24 ± 0.80	3.19 ± 1.12	4.10 ± 1.57	2.89 ± 0.92
D (0.7% GMO)	1.49 ± 0.47	1.26 ± 0.31	1.25 ± 0.14	1.22 ± 0.08
E (1.0% GMO)	2.14 ± 0.48	1.89 ± 0.79	1.94 ± 0.39	1.85 ± 0.49

Sample Bt176 maize	*35S-qcPCR I (n = 7)*	*II (n = 7)*	*BT176-qcPCR I (n = 8)*	*II (n = 8)*
A (1.4% GMO)	1.56 ± 0.34	1.69 ± 0.39	1.74 ± 0.40	2.32 ± 0.85
B (1.8% GMO)	2.00 ± 0.25	2.02 ± 0.31	2.30 ± 0.53	2.35 ± 0.78
C (3% GMO)	2.98 ± 0.68	2.89 ± 0.72	3.35 ± 0.62	3.16 ± 0.75
D (0.7% GMO)	0.86 ± 0.34	0.72 ± 0.30	0.86 ± 0.18	0.68 ± 0.32
E (1.0% GMO)	1.39 ± 0.62	1.47 ± 0.75	1.35 ± 0.49	1.55 ± 0.73

Sample RR-soybean	*35S-real-time PCR (n = 10)*	*RRS-real-time PCR (n = 10)*
A (1.4% GMO)	1.62 ± 0.38	1.78 ± 0.71
B (1.8% GMO)	1.79 ± 0.43	1.80 ± 0.51
C (3% GMO)	4.02 ± 1.30	3.79 ± 1.30
D (0.7% GMO)	0.87 ± 0.42	0.86 ± 0.17
E (1.0% GMO)	1.66 ± 0.39	1.65 ± 0.56

other, in the maize sample the 1.8 % value showed a clear tendency to be higher than the 1.4 % value (3 out of 4 values). The smallest standard deviation was observed with sample D (0.7 %).

Quantitation of samples by real-time PCR

Real-time PCR detection methods are becoming widely used today, since speed of analysis and easy handling facilitates quantitation. Therefore, this technique was also evaluated in the ring trial. Ten laboratories analysed the samples with the 35S-specific detection system and the GMO-specific real-time PCR (see fig. 3 for graphic display). The highest standard deviation was observed for the 3 % sample and the lowest for the 0.7 % sample (table 3). However, the real-time PCR method was not more accurate than the qcPCR methods with subsequent software based evaluation.

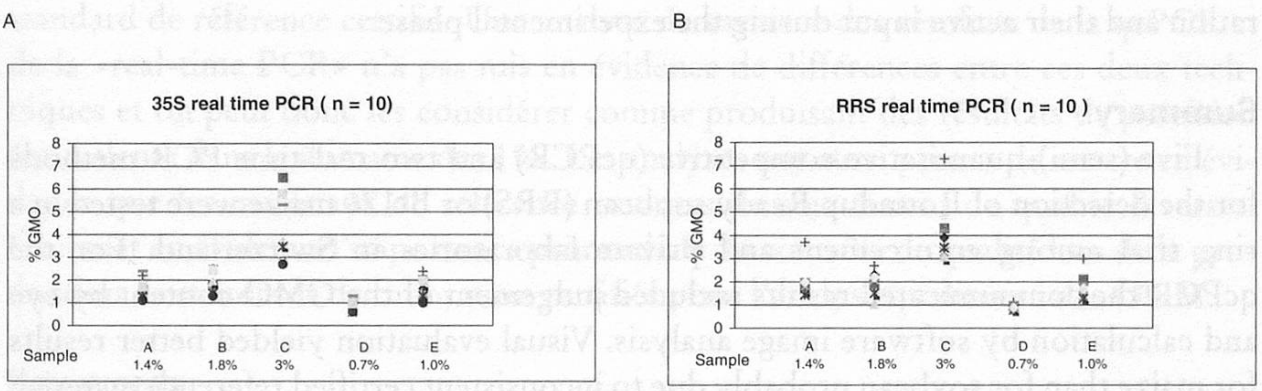

Figure 3 **Quantitative determination of the GMO content by real-time PCR.** For real-time PCR only soybean samples were analysed either with the screening method (A) or with the GMO-specific system (B). Samples A-E = 1.4, 1.8, 3, 0.7 and 1% GMO content, respectively. n: number of ring trial laboratories from which the results were compiled

Conclusion and outlook

This ring trial has evaluated several (semi)-quantitative PCR methods for the detection of genetically modified foodstuffs. As could be demonstrated all the applied methods yielded correct results. Although the judgement by visual inspection of qcPCR results showed a high percentage of correct results with respect to the 1 % threshold value, this procedure can only judge whether a value is above or below a certain threshold, whereas precise numerical determinations of the GMO content are not possible. Application of qcPCR combined with image analysis software and of real-time PCR led to standard deviations between the participating laboratories which are displayed in table 3. The results indicate that it will be difficult

to distinguish a value of 1 % from 1.4 % since the mean value was already 1.66 % instead of 1 % for real-time PCR. However, in the described experimental setting the standard deviation is smallest at values around 1 % of GMO. Interestingly, the 1 % sample (sample E) was determined in all cases to contain more than 1 % GMO, independent of the used method, suggesting that the obtained values are representing an overestimation of the GMO content.

The results show that qcPCR and real-time PCR methods can be applied for the detection of GMOs with similar accuracy. However, real-time analysis is less time consuming and thus might be preferred. It can be concluded that the described methods do not allow to distinguish a value of 1 % GMO from a value of 1.4 % GMO in a statistically significant manner. The future task will be to establish further performance parameters of the qcPCR and real-time PCR methods.

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Summary

Five (semi)-quantitative competitive (qcPCR) and two real-time PCR methods for the detection of Roundup Ready soybean (RRS) or Bt176 maize were tested in a ring trial among enforcement and private laboratories in Switzerland. For the qcPCR the communicated results included judgement of the GMO content by eye and calculation by software image analysis. Visual evaluation yielded better results for maize than for soybean probably due to inconsistent certified reference material. Comparison of standard deviations of the results obtained by software image analysis (qcPCR) with real-time PCR revealed no significant difference demonstrating that at the 1 % threshold the precision of qcPCR is comparable to real-time PCR. The ring trial also revealed that the provided external soybean standards were not consistent. Therefore quality control in the production of certified reference materials for GMO analysis is an essential prerequisite for the entire quantitation.

Zusammenfassung

Fünf (semi)-quantitative kompetitive (qcPCR) und zwei «real-time» PCR-Methoden zur Detektion von Roundup-Ready Soja oder Bt176 Mais wurden in einer schweizerischen Methodenprüfung von Privat- und Vollzugslaboratorien getestet. Verlangt wurde von den Laboratorien eine visuelle Auswertung sowie nach Möglichkeit eine Bestimmung des GVO-Gehaltes mittels einer Bildanalysen-Software. Die visuelle Auswertung führte bei Mais zu besseren Resultaten als bei Soja, was wahrscheinlich auf das in sich nicht konsistente zertifizierte Referenzmaterial zurückzuführen ist. Eine Analyse der Standardabweichungen führte zu keinem signifikanten Unterschied zwischen der Software- und der «real-time»-Auswertung und deutet darauf hin, dass im Bereich der 1 % Deklarationslimite die Resultate der

qcPCR mit denjenigen der «real-time» PCR vergleichbar sind. Die Methodenprüfung zeigte auch, dass die zur Verfügung gestellten externen Sojareferenzmaterialien in sich nicht konsistent sind. Dieses Resultat demonstriert, dass die Qualitätskontrolle bei der Produktion von Referenzmaterialien für die Analyse von GVO eine essentielle Rolle für die Quantifizierung spielt.

Résumé

Cinq méthodes de PCR (semi)-quantitative compétitive (qcPCR) et deux méthodes de «real-time PCR» pour la détection de soja roundup-ready (RRS) ou de maïs Bt176 ont été testées dans la Suisse au cours d'un essai collaboratif incluant des laboratoires privés et des laboratoires de contrôle officiels. Pour la qcPCR, il était demandé de fournir également une estimation visuelle de la quantité d'OGM et aussi, dans la mesure du possible, une détermination de la quantité d'OGM au moyen d'un logiciel de traitement d'images. L'estimation visuelle a produit des meilleurs résultats pour le maïs que pour le soja à cause de la qualité médiocre du standard de référence certifié. Une analyse de variance des résultats de la qcPCR et de la «real-time PCR» n'a pas mis en évidence de différences entre ces deux techniques et on peut donc les considérer comme produisant des résultats de précision équivalente dans le domaine des 1%. Les analyses ont aussi permis de mettre en évidence que les matériaux de référence externes de soja étaient de qualité diverses. Ceci met en évidence l'importance primordiale du contrôle de qualité lors de la production de standards de calibration certifiés pour l'analyse qualitative des OGM.

Key words

GMO, Validation study, Comparison of quantitation, Quantitative competitive PCR, Real-time PCR

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