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Single-laboratory validation of a method for detection of Roundup Ready soy in soybeans: application of new strategies for qualitative validation

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RESEARCH ARTICLE

Abstract

Considering expansion of genetically modified organisms and the basic principle of consumers' right to access information about products, legislations of several countries establish a limit for labelling transgenic food. Qualitative tests based on the polymerase chain reaction (PCR) have been recommended. However, validation of qualitative methods is still a critical point in the quality management of food analysis laboratories. A screening method for the detection of Roundup Ready (RR) soy in soybeans by nested PCR was validated by a novel qualitative approach, in a single-laboratory, considering all fundamental parameters for an adequate evaluation of fitness for purpose. Blank samples of soybeans and formulations containing 0.001 to 1% of RR soy were analysed. Agarose gel electrophoresis and fluorimetry techniques were compared in terms of the quantification of extracted DNA. False-positive rate obtained was 0%, with selectivity and reliability rates of 100.0% for both techniques. Sensitivity and reliability rates varied between 23.3 and 100.0% (agarose gel) and between 30.0 and 100.0% (fluorimetry), respectively. Levels above 0.030% presented 100.0% positive results. Unreliable regions were estimated by non-linear models, and the calculated detection limits were 0.0067 and 0.0047%, for agarose gel electrophoresis and fluorimetry, respectively. Accordance and concordance values of 1.0 were obtained for levels near the regulated limit. The method was considered fit for screening purposes. Analysis of commercial samples demonstrated the applicability of the method and the compliance with Brazilian legislation.

Keywords: genetically modified organisms, screening method, single-laboratory validation, Roundup Ready soy, labelling regulations

1. Introduction

Soybean (*Glycine max* L. Merr.) is one of the most important agricultural products. The 2014/2015 worldwide yield of soybean crops was estimated at 304.8 million tons, with Brazilian production corresponding to 91 million tons (USDA, 2014). From a nutritional point of view, soybeans are an important source of protein and also have functionality recognised by international regulation bodies (Bressani, 1975).

In Brazil, nature of genetically modified (GM) foods and food ingredients commercialised and that contain or are produced from genetically modified organisms (GMO), with a presence above the limit of 1%, must be indicated on product labels (Brasil, 2003). In the European Union, labelling is mandatory on products that present 0.9% or more of GMO (EU, 2003). In Japan, the limit is 5% and in the USA labelling is not mandatory (Thomson, 2003).

Methods based on the determination of DNA, such as the PCR, have been recommended by international regulatory and research bodies for the detection and quantification of GMO in food and feed due to the higher stability of these molecules compared to proteins (JRC, 2011). In this context, the use of qualitative methods for screening purposes

has increased, mainly because these methods provide objective and rapid results, with low costs, simplicity and minimisation of errors (Pulido *et al.*, 2003).

The confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled is defined as validation (ISO, 2005). Validation studies can be structured in single-laboratory (intra-laboratory) or inter-laboratory procedures. An intra-laboratory validation involves a single laboratory using one method to analyse the same or different test materials under different conditions over justified time intervals (EC, 2002). Single-laboratory validation is critical to the application of any method, to ensure method viability before the costly exercise of a formal inter-laboratory study and to provide evidence of the reliability of analytical methods if inter-laboratory data are not available (Thompson *et al.*, 2003).

Inter-laboratory study means organisation, performance and evaluation of tests on the same sample by two or more laboratories in accordance with predetermined conditions to determine testing performance (EC, 2002). The inter-laboratory validation is considered as a complete validation procedure. However, these studies may not be practicable or even necessary (Van der Voet *et al.*, 1999). In food analysis, even when there are situation where the inter-laboratory validation is practicable, it becomes impossible to cover all the combination of analytes, concentrations and matrices in which the method will be applied (Hill and Reynolds, 1999).

Despite the advantages and disadvantages presented by these different processes, they are complementary and do not exclude one to another. On one hand the inter-laboratory study cannot be conducted without previous single-laboratory validation, but on the other hand precision under reproducibility conditions cannot be evaluated through a single-laboratory study (Wood, 1999).

According to the Eurachem Guide to Method Validation, working in isolation inevitably reduces the amount of validation data and restricts the type of information on inter-laboratory comparability. However, this information is not always necessary and it may be feasible to estimate the comparability of measurement results of any method by measuring certified reference materials. Then, whether or not methods validated in a single-laboratory will be acceptable for regulatory purposes depends on a consensus in the area of measurement concerned (Magnusson and Örnemark, 2014).

The documents of Codex Alimentarius (Codex Alimentarius, 2010) and the European Network of GMO Laboratories (ENGL, 2015) are important references for the validation of methods for GMO detection in food. The Codex Alimentarius published a guideline on criteria for methods of detection, identification and quantification of specific DNA sequences in foods derived from modern biotechnology (Codex Alimentarius, 2010). The ENGL defined the acceptance criteria and performance requirements for DNA extraction and purification methods, PCR methods for the purpose of quantification and qualitative detection (ENGL, 2015).

The parameters stablished by the Codex Alimentarius for the single-laboratory validation of quantitative PCR methods are: accuracy, applicability, dynamic range, limit of detection, limit of quantification, practicability, repeatability, robustness, sensitivity, target specificity and trueness. And when performing an inter-laboratory validation the incorporation of the reproducibility study is suggested (Codex Alimentarius, 2010). For single-laboratory validation, the ENGL recommends the study of applicability, practicability, DNA concentration, DNA yield, DNA structural integrity, purity of DNA extracts, specificity, dynamic range, trueness, amplification efficiency, R^2 coefficient, repeatability, limit of detection, limit of quantification and robustness. In an inter-laboratory study, the reproducibility and trueness are included in the validation process (ENGL, 2015).

Concerning qualitative parameters, in the Codex Alimentarius guidelines the study of false-negative rate (FNR), false-positive rate (FPR), detection limit (DL) and robustness is recommended (Codex Alimentarius, 2010). In the ENGL document, the study of FNR, FPR and probability of detection is suggested (ENGL, 2015).

However, important parameters related to validation of qualitative methods, such as sensitivity rate (SNR), selectivity rate (SLR), reliability rate (RLR), unreliability region (UR), accordance (ACC) and concordance (CON) are not considered in both documents. The parameters FPR, FNR, SNR, SLR, RLR, DL, UR, ACC, CON, robustness and selectivity in relation to interferents are defined as fundamental in a qualitative method validation, as proposed in a protocol by Gondim *et al.* (2014). SNR, SLR and RLR can be easily estimated from false results rates. However, UR, ACC and CON, parameters related to uncertainty and precision of the method, had been frequently neglected in validation studies in this area.

Some qualitative methods have been published in the literature for detection of Roundup Ready (RR) soy in soy products, using conventional PCR (JRC, 2011; Kodama *et al.*, 2011; Lipp *et al.*, 1999, 2001; Meyer and Jaccaud, 1997; Sieradzki *et al.*, 2008; Taverniers *et al.*, 2001) and Real Time PCR (Bahrdt *et al.*, 2010; Leimanis *et al.*, 2008; Mano *et al.*, 2012). The validation of these methods reflects the validation guidelines for GMO analysis, resulting in the fact that most of them do not address all the important qualitative performance parameters.

In this study, new strategies for ensuring the reliability of qualitative methods were applied to GMO in food analysis. A screening method for the detection of RR soy in soybeans was single-laboratory validated for application in the Official Programs of the National Health Surveillance Agency (ANVISA). Techniques usually employed for the quantification of extracted DNA were also compared.

2. Materials and methods

Samples

Soybean blank samples and certified reference materials (CRMs) were used in the validation procedures. Soybean blank samples were provided by a certified Brazilian producer. CRMs containing 0.1, 1.0 and 10% RR soy (ERM-BF410k) were obtained from the Institute for Reference Materials and Measurements (IRMM) (Geel, Belgium). CRMs of GM maize, including Bt11 at 5% (ERM-BF412), GA21 at 5% (ERM-BF414), MON810 at 5% (ERM-BF413), NK603 at 5% (ERM-BF415), TC1507 at 10% (ERM-BF418) and Bt176 at 2% (ERM-BF411), were also purchased from the IRMM. CRMs were stored under refrigeration, at a maximum temperature of 4 °C.

To apply the method, 32 samples of commercial soybean were collected from markets in Minas Gerais State, Brazil, by ANVISA. Samples were prepared under strict conditions to avoid cross-contamination. Each sample was transferred to a plastic bag and manually homogenised. The samples were ground to 20/30 mesh (Marconi MA-090/CF, Piracicaba, Brazil) and collected in another plastic bag, which was properly sealed, labelled and stored at room temperature until the moment of analysis.

Chemicals and reagents

Cetyltrimethylammonium bromide (CTAB) and ethanol were obtained from Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (Tris HCl), ethylenediamine tetraacetic acid (EDTA), RNase, proteinase K and ethidium bromide were supplied by Sigma Aldrich Co. (St Louis, MO, USA). Chloroform and isopropanol were obtained from Vetec (Duque de Caxias, Brazil). Primers were provided by Integrated DNA Technologies Inc. (Coralville, IA, USA). Bacteriophage lambda was purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). All chemicals used were of adequate purity, and the water used was purified through a Milli-Q Purification System (Millipore, Billerica, MA, USA).

Analytical procedure

DNA extraction

A portion of 200 mg of samples was weighed in 1.5 ml tubes and moistened with 300 µl of ultrapure water. After that, 700 µl of CTAB buffer (20 g/l CTAB, 1.4 mol/l NaCl, 0.1 mol/l Tris HCl, 0.02 mol/l EDTA) pre-warmed to 65 °C was added. The mixture was vortexed, 10 µl of RNase solution (10 mg/ml) was added, vortexed again and incubated for 30 min at 65 °C. This last step was repeated but with 10 µl of Proteinase K (20 mg/ml). After incubation, samples were centrifuged for 10 min at 12,000×*g*. Supernatant was transferred to a new 1.5 ml tube containing 500 µl of chloroform, vortexed and centrifuged for 15 min at 12,000×*g* until phase separation occurred. Upper layer (aqueous phase) was transferred to a new 1.5 ml tube containing 500 µl of chloroform, vortexed and centrifuged for 5 min at 12,000×*g*. Upper layer was transferred, and 2 volumes of CTAB precipitation solution (5 g/l CTAB, 0.04 mol/l NaCl) were added. The solution was homogenized by inversion and incubated at room temperature for 60 min. The tubes were centrifuged for 5 min at 13,000×*g*, and supernatant was discarded. The precipitate was dissolved in 350 µl of a 1.2 mol/l NaCl solution, 350 µl of chloroform was added and the solution was vortexed for 30 sec. Samples were centrifuged for 10 min at 12,000×*g* until phase separation occurred. Upper layer was transferred to a new 1.5 ml tube, and a 0.6 volume of refrigerated isopropanol (2-10 °C) was added. The solution was homogenised by inversion, incubated at room temperature for 20 min and centrifuged for 10 min at 14,000×*g*. Supernatant was discarded, and pellet was washed with 500 µl of an ethanol:water (70+30) solution. The mixture was centrifuged for 10 min at 12,000 and supernatant was discarded. Pellet was air-dried, re-suspended in 50 µl Tris-EDTA buffer (1 mol/l Tris-HCl, 0.5 mol/l EDTA) and stored at -18 °C until the moment of use (JRC, 2005).

DNA quantification by agarose gel electrophoresis

DNA quantification of extracts was estimated by visual comparison of fluorescence intensity of undigested total DNA isolated with standardised amounts of undigested bacteriophage lambda DNA (concentrations of 50, 100 and 200 ng/µl) on 1% agarose gel. Gel was stained with ethidium bromide (0.5 mg/ml) and electrophoresis was performed at 80 V for 10 min and 100 V for 50 min. DNA bands were visualised and photographed by digital camera (Kodak Digital Science, Rochester, NY, USA).

DNA quantification by fluorimetry

A Qubit® 2.0 Fluorometer was used for the fluorimetric quantification of extracted DNA with a Qubit® dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA).

Oligonucleotide primers

Sets of primer pairs GMO5/GMO9 and GMO7/GMO8 were designed for the detection of RR soy by nested PCR as described by Meyer and Jaccaud (1997). Amplificability of DNA extracted was verified using plant-specific primer pair GMO3/GMO4 targeting lectin gene, which is specific for soybean (Table 1).

Qualitative polymerase chain reaction detection of Roundup Ready and lectin sequences

Regardless of technique used for estimate DNA concentration, all DNA extracts were diluted to a final concentration of 50 ng/µl. PCR reactions were carried out on a Mastercycler ep Realplex (Eppendorf, Hamburg, Germany). The first reaction of nested PCR was performed for a final volume of 25 µl containing 1 µl of diluted DNA extract (50 ng/µl) and 24 µl of a mixed solution (1× Taq Polymerase buffer, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTP, 0.24 µmol/l primers GMO9 and GMO5 and 1 U Taq DNA Polymerase). Amplification was performed with denaturation at 95 °C for 3 min, 25 cycles of amplification at 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 40 sec, followed by a final extension at 72 °C for 5 min. In the second reaction 1 µl of the first reaction product was diluted in 10 µl of ultrapure water. 1 µl of this solution was added to 24 µl of a mixed solution (prepared as the first reaction, but with primers GMO7 and GMO8). PCR program employed was the same as the first step but with 35 cycles. For the negative samples, a study of endogenous gene (lectin gene) was performed using 1 µl of the diluted DNA extract, 24 µl of the mixed solution (1× Taq Polymerase buffer, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTP, 0.24 µmol/l primers GMO3 and GMO4 and 1.5 U Taq DNA polymerase). Amplification was performed with denaturation at 95 °C for 3 min, 40 cycles of amplification at 95 °C for 30 sec, 63 °C for 30 sec and 72 °C for 30 sec, followed by final extension at 72 °C for 3 min. After that, PCR products were subjected to electrophoresis on a 2% agarose gel supplemented with 0.5 µg/ml ethidium bromide in Tris-borate-EDTA buffer and applied a voltage of 80 V for 10 min and 100 V for 1 h and 40 min. Visualisation was performed in a UV-transilluminator, and images were captured (Kodak Digital Science).

Table 1. List of primers utilised in polymerase chain reactions (Adapted from Querci *et al*., 2006).[1]

GMO 3	Forward	5' GCC CTC TAC TCC ACC CCC ATC C 3'
GMO 4	Reverse	5' GCC CAT CTG CAA GCC TTT TTG TG 3'
GMO 9	Forward	5' CATGAAGGACCGGTGGGAGAT 3'
GMO 5	Reverse	5' CCACTGACGTAAGGGATGACG 3'
GMO 7	Forward	5' ATCCCACTATCCTTCGCAAGA 3'
GMO 8	Reverse	5' TGGGGTTTATGGAAATTGGAA 3'

[1] GMO: genetically modified organisms.

Single-laboratory validation

The validation process was conducted in four stages as described in the procedure developed by Gondim *et al.* (2014).

Comparison of the DNA quantification techniques

In the validation study, for each analytical batch, DNA extracts were quantified using two techniques: agarose gel electrophoresis and fluorimetry. A Student's t-test for paired data was applied to FNR estimated at different concentration levels with a 5% of significance level to compare techniques for quantifying DNA extracted.

Preliminary tests

Soybean blank samples were added to CRMs of GM RR soy to obtain formulations at different concentration levels. Powders were mixed and co-extracted. Preliminary study involved blank samples and formulations covering RR soy levels of 0.001, 0.002, 0.003, 0.005, 0.010 and 0.030% in 10 replicates, which were prepared and analysed randomly. FPR was estimated for blank samples, FNR were estimated for each concentration level and these results were evaluated to verify the suitability of the selected concentration range. The concentration range was considered appropriate for UR estimation when FNR were between 0-20 and 80-100% (Gondim *et al.*, 2014).

Rates, accordance, concordance, limit of detection and unreliability region

In the second step of the validation process, blank samples (0.000%) and formulations at nine concentration levels were prepared and analysed randomly (0.001, 0.005, 0.010, 0.015, 0.020, 0.025, 0.030, 0.035 and 1.000% RR soy) in 30 replicates. Analyses were performed under repeatability and intermediate precision conditions which are related respectively to ACC and CON in the qualitative method validation. Samples were split into three analytical batches, 10 replicates for each concentration level (from 0.000 to 1.000%) in each batch, involving different analysts, reagents and equipment. Each replicate was analysed by two techniques for DNA quantification, resulting in 600 assays.

Contingency tables were used to evaluate rates and performance curves (probability of positive results versus analyte concentration) were plotted to determine the parameters UR and DL. Unreliability region limits were estimated by non-linear regression, considering 5 and 95% false-negative results. Detection limit was reported as the upper limit of the UR. These parameters were considered appropriate if they were below the regulated level, so the

method can be applied to evaluate samples at the legally relevant level with less than 5% of probability to obtain false-negative results (Gondim *et al.*, 2014).

ACC and CON were calculated by combinatory analysis and reported as acceptable when greater than or equal to 0.8, near the target or regulated concentration level (considering the possibility of one false-negative result in each analytical batch with ten replicates) (Gondim *et al.*, 2014).

Complementary evaluation of selectivity

For selectivity evaluation, different events of maize (Bt11, GA21, MON810, NK603, TC1507 and Bt176) were studied. Bt11 and GA21 were chosen for further investigation because Bt11 has promoter 35S and terminator T-NOS in common with RR soy, and GA21 has terminator T-NOS in common. Considering the difficulty in obtaining genuine GMO seeds of these events, CRM produced by IRMM were used. Formulations were elaborated to obtain two types of samples, in 10 independent replicates. One was formulated with soybean blank sample added to CRM of Bt11 maize, and the other was formulated with soybean blank sample added to CRM of RR soy and CRM of Bt11 maize. The same procedure was applied to GA21 maize. The concentration of RR soy selected was 0.030% (lowest level that presented 100% RLR in the second step of the validation) and concentration of interferents of 2%, considered relevant. DNA quantification was performed for each replicate by the two forms of quantification, yielding 80 assays. The RLR was estimated. To be considered as interferents, RLR obtained should be lower than 90% (one false-negative result was considered acceptable in 10 replicates of each formulation) (Gondim *et al.*, 2014).

Robustness evaluation

For robustness study, a complete factorial design was performed and included two factors. Taq DNA Polymerase (catalyst of primer extension) used: brand A and B; and target DNA concentration (proportion of primer/DNA template significantly affects PCR): 25, 50 and 75 ng/µl, resulting in six treatments.

Samples were formulated with RR soy at the lowest concentration, in which 100% RLR was obtained in the second validation step, with 10 independent replicates for each treatment. For each replicate DNA was quantified by agarose gel electrophoresis and fluorimetry, yielding 120 assays. The factors and levels were considered significant when the RLR was lower than 90% (Gondim *et al.*, 2014).

Applicability

The validated method was applied to the analysis of 32 commercial soybean samples collected by ANVISA.

3. Results and discussion

Preliminary tests

FNR varied from 0 to 80.0%, resulting in SNR and RLR values ranging from 100.0 to 20.0%. These results suggest that the concentration range was appropriate and could be maintained in the second step of the validation study.

Rates

Analysis of blank samples resulted in 0% FPR (SLR and RLR values of 100%), indicating selectivity of the method for the detection of RR soy in soybeans. FNR obtained for levels ranging from 0.001 to 0.025% varied from 6.7 to 76.7% when quantified by agarose gel electrophoresis and from 6.7 to 70.0% when quantified by fluorimetry. Thus, SNR and RLR varied from 93.3 to 23.3% and from 93.3 to 30.0%, respectively. For both techniques, 100.0% positive results were obtained for all levels above 0.030%. These results indicated sensitivity for detecting the target event, even at concentration levels significantly lower than those recommended by Brazilian and international legislation. Despite the adequate performance of both techniques, in general, fluorimetry presented better results (Table 2).

Some inter-laboratory studies evaluated and calculated the SNR and SLR of fragments (35S, T-NOS, specific sequence EPSPS, sequence CTP) present in the RR soy in different food matrices (JRC, 2011; Kodama *et al.*, 2011; Leimanis *et al.,* 2008; Lipp *et al.*, 1999, 2001). The SNR obtained in these studies varied from 92.8 to 100% and SLR obtained was higher than 90%. In all cases, lower numbers of concentration levels than that examined in this study were investigated, possibly due to the limitations encountered when organizing inter-laboratory studies, which include the preparation and evaluation of each material for homogeneity and stability. Additionally, concentration levels were higher than or concentrated at the upper limit of the concentration range covered by this study, which justifies lower variation in SNR. An explanation for why previous work was done at higher concentration levels is that these levels correspond to regulated thresholds.

In this study, it is important to consider that the method validated in a single laboratory presented an SNR of 100.0% for levels above 0.030% and no false-positive results. Similar performance was achieved by Sieradzki *et al.* (2008). In a single-laboratory study, these authors studied one concentration level (0.1% of RR soy) with 20 replicates, presenting an FNR of 0%. The number of analyses indicated in the qualitative validation may vary among authors, but there is an agreement that a significant number of randomised trials is fundamental to assess all performance parameters of the method (Cárdenas and Valcárcel, 2005; Gondim *et al.*, 2014; Pulido *et al.*, 2003; Ríos *et al.*, 2003).

Table 2. False results, sensitivity and selectivity rates, accordance and concordance values obtained under intermediary precision conditions for different concentration levels of Roundup Ready (RR) soy.[1]

Levels of RR soy (%) (n=30)	Validation parameters (%)											
	Agarose gel electrophoresis						Fluorimetry					
	FNR/ FPR	SNR/ SLR	ACC1	ACC2	ACC3	CON	FNR/ FPR	SNR/ SLR	ACC1	ACC2	ACC3	CON
0.000	0.0	100.0	1.0	1.0	1.0	1.0	0.0	100.0	1.0	1.0	1.0	1.0
0.001	76.7	23.3	0.6	0.8	0.5	0.6	70.0	30.0	0.6	0.5	0.5	0.6
0.005	26.7	73.3	0.5	0.5	0.8	0.6	16.7	83.3	0.5	1.0	0.8	0.7
0.010	16.7	83.3	0.5	0.5	1.0	0.7	13.3	86.7	0.6	1.0	0.8	0.8
0.015	3.3	96.7	1.0	0.6	1.0	0.9	0.0	100.0	1.0	0.8	1.0	0.9
0.020	6.7	93.3	0.6	1.0	1.0	0.9	3.3	96.7	0.8	1.0	1.0	0.9
0.025	6.7	93.3	0.8	0.8	1.0	0.9	6.7	93.3	0.6	1.0	1.0	0.9
0.030	0.0	100.0	1.0	1.0	1.0	1.0	0.0	100.0	1.0	1.0	1.0	1.0
0.035	0.0	100.0	1.0	1.0	1.0	1.0	0.0	100.0	1.0	1.0	1.0	1.0
1.000	0.0	100.0	1.0	1.0	1.0	1.0	0.0	100.0	1.0	1.0	1.0	1.0

[1] n: number of replicates in each concentration level; FNR: false-negative rate (for 0.001 to 1.000% level); SNR: sensitivity rate (for 0.001 to 1.000% level); FPR: false-positive rate (for 0.000% level); SLR: selectivity rate (for 0.000% level), ACC: accordance estimated in three analytical batches (1, 2 and 3); CON: concordance.

Some single-laboratory validation studies of qualitative methods for the detection of RR soy in food discussed sensitivity and selectivity but did not present estimations of rates (Bahrdt *et al.*, 2010; Mano *et al.*, 2012; Marcelino *et al.*, 2008; Taverniers *et al.*, 2001).

Unreliability region and detection limit

To construct performance curves and estimate UR and DL, only evenly spaced levels (0.000 to 0.035%) of RR soy were considered. Performance curves and estimated UR and DL are illustrated in Figure 1. For agarose gel electrophoresis, UR varied between -0.0004 and 0.0067% RR soy, considering positive result percentages of 5 and 95%, respectively. DL was estimated to be 0.0067%. Considering the results of fluorimetric quantification, UR was between -0.0002 and 0.0047% RR soy, with the latter corresponding to the DL. Negative lower limits of UR indicated that they were not different from zero. These limits were considered appropriate for detection of RR soy at levels regulated by Brazilian and international legislation.

The modelling approach was considered more appropriate, once it compiles all the experimental observations and not only the rates inspection in specific points of the contingency table that can be outliers. It is important to consider that exact rates of false-negative results (for example 5%) may not be obtained experimentally, which leads to the need for interpolation or approximation to

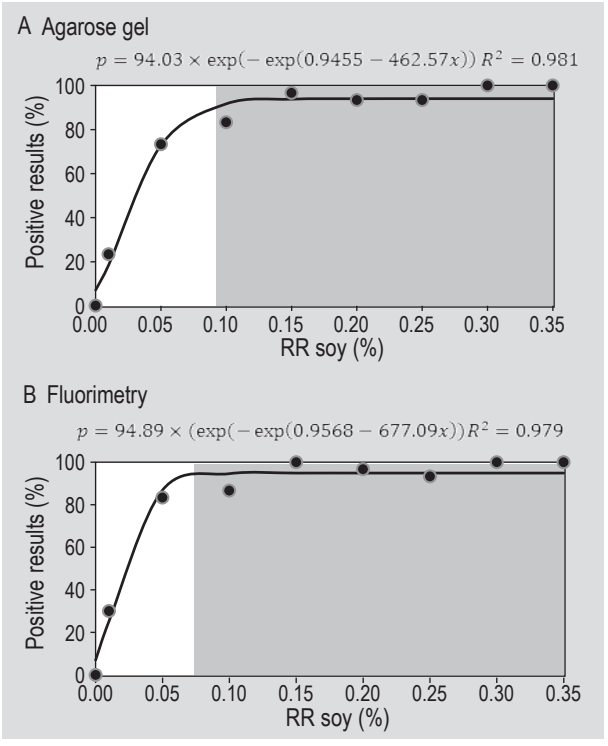

Figure 1. Experimental data (•), performance curves (-), equations and determination coefficients (R^2) obtained by Gompertz non-linear regression, highlighting levels outside the unreliability region (shaded area) – quantification by agarose gel electrophoresis (A) and fluorimetry (B).

estimate the corresponding concentration level, or the DL. In this sense, models enable DL estimation in a more reliable way (Cárdenas and Valcárcel, 2005; Gondim *et al.*, 2014; Ríos *et al.*, 2003).

Unreliability region estimation has not been reported in the literature encompassing validation studies of similar analytical scope (Bahrdt *et al.*, 2010; JRC, 2011; Kodama *et al.*, 2011; Leimanis *et al.*, 2008; Lipp *et al.*, 1999, 2001; Mano *et al.*, 2012; Marcelino *et al.*, 2008; Meyer and Jaccaud, 1997; Sieradzki *et al.*, 2008; Taverniers *et al.*, 2001). Lack of study may be explained because uncertainty estimation in qualitative analysis is an innovative approach.

Different techniques (serial dilutions and analysis of samples with different concentrations of RR soy), criteria (lowest concentration that produced a positive result, lowest concentration that amplified the DNA fragment to the expected size, lowest concentration level in which a SNR of 100% is obtained, and lowest concentration level in which a SNR of 95% is obtained) and DL values (ranging from 0.0001 to 0.5%) have been reported in the literature, considering the same analytical scope (Kodama *et al.*, 2011; Lipp *et al.*, 1999; Marcelino *et al.*, 2008; Meyer and Jaccaud, 1997; Sieradzki *et al.*, 2008; Taverniers *et al.*, 2001). Although, most of them presented DL values higher than those reported in this paper, it is not possible to establish a parameter for comparison because different numbers of replicates, experimental designs and criteria were used. Criterion adopted by Leimanis *et al.* (2008), Kodama *et al.* (2011) and Mano *et al.* (2012), based on an RLR and SNR of 95% was similar to that defined in this study.

Accordance and concordance

For agarose gel electrophoresis, the ACC values varied between 0.5 and 1.0 and the CON values ranged from 0.7 to 1.0, considering levels outside the UR (0.010, 0.015, 0.020, 0.025, 0.030, 0.035 and 1% of RR soy). For fluorimetry, ACC values varied between 0.6 and 1.0 and CON values varied from 0.8 to 1.0, outside the UR.

Differences between batches obtained for ACC values were reflected in the CON values. However, the maximum ACC and CON values, i.e. 1.0, were achieved for levels 0.000, 0.030, 0.035 and 1% for both DNA quantification techniques. These results, greater than or equal to 0.8, near the regulated concentration level, indicate that the method was sufficiently standardised (Table 2).

The expected behaviour of the ACC and CON values as a function of analyte concentration was observed, with values decreasing to an intermediate value of the UR and then, increasing to a maximum of 1.0 (Gondim *et al.*, 2014).

The study of ACC and CON parameters is often neglected in the literature. Kodama *et al.* (2011) studied concentrations of 0.05 and 0.1% RR soy and obtained values of 0.88 and 0.96 for ACC and values of 0.89 and 0.95 for CON, respectively.

These parameters indicate whether the procedure used is sufficiently standardised. ACC when the procedure is used by the same laboratory under repeatability conditions and CON when the procedure is applied in different analytical batches (for a single-laboratory study under intermediate precision conditions) or by different laboratories (for inter-laboratory studies) (Gondim *et al.*, 2014).

Selectivity

In the first round of the selectivity study, negative results were obtained for all six maize events (Figure 2). Subsequent evaluation of selectivity with Bt11 and GA21 maize indicated no suppressive or potentiator effect in detecting RR soy, for both forms of DNA quantification. No change was observed in FPR or in FNR, for blank soybean samples and formulations containing RR soy, respectively, in the presence of these potential interferents. All samples presented an RLR value of 100%, and the method was considered selective in detecting RR soy in the presence of Bt11 and GA21 (Table 3). It was assumed that the extraction of DNA with this method was similarly efficient from maize as it was from soybean.

Although SLR has often been considered in the literature, investigations of selectivity in relation to potential interferents are not commonly found. Leimanis *et al.* (2008) and Bahrdt *et al.* (2010) performed multiplex PCR assays

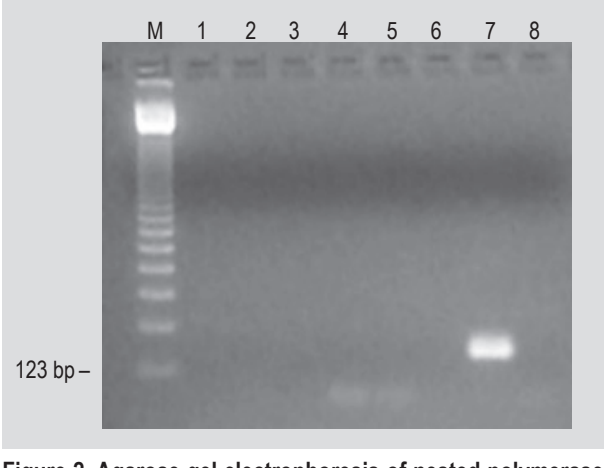

Figure 2. Agarose gel electrophoresis of nested polymerase chain reaction products obtained in the selectivity preliminary study with six events of maize. Lane M: DNA molecular size marker (123 bp); lane 1: Bt11 maize (5%); lane 2: MON810 maize (5%); lane 3: GA21 maize (5%); lane 4: NK603 maize (5%); lane 5: TC1507 maize (10%); lane 6: Bt176 maize (2%); lane 7: positive control of Roundup Ready soy (5%); lane 8: negative control.

Table 3. False-positive, false-negative and reliability rates obtained in the selectivity study, considering both techniques of DNA quantification.[1]

Formulation	Agarose gel electrophoresis			Fluorimetry		
	FPR	FNR	RLR	FPR	FNR	RLR
Blank soybean sample + Bt11 maize (2.0%)	0	–	100	0	–	100
Blank soybean sample + Bt11 maize (2.0%) + RR soy (0.03%)	–	0	100	–	0	100
Blank soybean sample + GA21 maize (2.0%)	0	–	100	0	–	100
Blank soybean sample + GA21 maize (2.0%) + RR soy (0.03%)	–	0	100	–	0	100

[1] FPR = false-positive rate; FNR = false-negative rate; RR soy: Roundup Ready soy; RLR = reliability rate; criterion adopted: RLR ≥90%.

in which inhibition and presence of competitive effects were verified. Leimanis *et al.* (2008) did not observe any interaction between amplicons in the hybridisation step, and Bahrdt *et al.* (2010) proved single-copy sensitivity for all GMO detection systems in the presence of 64 copies of all other GMO targets of each detection channel studied.

Robustness

In robustness assessment using agarose gel electrophoresis, brand B of Taq DNA Polymerase and DNA concentration of 25 ng/µl, 90% RLR was obtained due to one false-negative sample (Table 4). One sample had its concentration estimated as 200 ng/µl (agarose gel), whereas in fluorimetry, the same sample presented a concentration of 98 ng/µl. Meaning that the dilution used for agarose gel quantification was twice as high as the dilution used for fluorimetry (to obtain 25 ng/µl). Possibly due to higher dilution, the result obtained was a false-negative. Given this assumption, the false result was not related to the brand of Taq DNA Polymerase used but to the combination of DNA concentration and DNA quantification technique employed. Thus, agarose gel electrophoresis is limited because it is based on visual analysis, which can be subjective. Despite this one false-negative result, the method was considered robust for factors and levels studied, considering an RLR value of 90%.

Sieradzki *et al.* (2008) studied robustness of similar detection method in relation to three different lots of PCR reagents. There were no significant differences in PCR reaction, and no changes observed in SNR, SLR and DL.

Comparison of techniques

The *P*-value of 0.037 was obtained and significant difference ($P<0.05$) was observed when fluorimetry and agarose gel electrophoresis quantifications were compared.

After comparing quantification techniques in relation to the validation parameters, differences were observed in rates (FNR, SNR and RLR) as well as in UR, DL, ACC and CON. The techniques did not diverge in relation to potential interferents studied. In the robustness evaluation, limitation of agarose gel technique was evident, which could yield false-negative results, therefore compromising reliability.

Table 4. Reliability rates obtained in the robustness study, concerning the factors Taq DNA polymerase brand and target DNA concentration, considering both techniques of DNA quantification.[1]

Taq DNA polymerase brand	Target DNA concentration (ng/µl)	Agarose gel electrophoresis		Fluorimetry	
		FNR	RLR	FNR	RLR
A	25	0	100	0	100
B	25	10	90	0	100
A	50	0	100	0	100
B	50	0	100	0	100
A	75	0	100	0	100
B	75	0	100	0	100

[1] FNR = false-negative rate; RLR = reliability rate; criterion adopted: RLR ≥90%.

Frequencies of DNA concentrations were obtained for agarose gel electrophoresis and fluorimetry. Concerning agarose gel technique, 218 (72%) of 300 samples presented a DNA concentration in the range of 200-249 ng/µl. With respect to fluorimetry, the DNA concentration varied over the ranges of 100-149 (22%), 150-199 (40%) and 200-249 ng/µl (34%). Therefore, there are two plausible explanations for these results: quantification by agarose gel electrophoresis overestimates DNA concentration, increasing the number of false-negative results, due to the higher dilution. Another explanation is that fluorimetric quantification underestimates DNA concentration, and after the dilution, the final concentration will be higher than that obtained by agarose gel technique, thus fluorimetry can be more susceptible to give positive responses.

Applicability

Qualitative analysis of 32 commercial soybean samples employing the validated method indicated 20 positive samples. These samples were further analysed by a quantitative method to determine the percentage of RR soy contained in the samples. One of the positive samples presented concentration higher than 1%. Only 1 of 20 samples claimed to be a GMO-free product on its product label, whereas the others did not present such information. That sample was considered in disagreement with Brazilian legislation.

The method was applied to routine analysis of an ANVISA official laboratory during two years. Adequate performance was verified during this period through analysis of positive and negative control samples in all analytical batches. In practice, the number of confirmatory analysis was reduced. Instead of performing 100% of commercial samples analysis, only 62.5% of samples were submitted to further quantitative analysis. The importance of this qualitative strategy was evidenced by the reduction of confirmatory analysis as well as the cost and time for decision about products' adequacy.

4. Conclusions

A qualitative method based on nested PCR for the detection of RR soy in soybeans was single-laboratory validated and considered fit for screening purposes. Application of a complete validation process that involved all fundamental parameters for an adequate study of qualitative methods allowed for reliable decision making in food quality control.

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