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Bt11 event detection by real-time PCR: single-laboratory validation, comparison of DNA extraction and quantification techniques and application

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RESEARCH ARTICLE

Abstract

The detection of genetically modified organisms (GMO) by real-time polymerase chain reaction (PCR) is recommended due to its effectiveness in GMO analysis. A complete in-house validation method was applied to the detection of Bt11 events by real-time PCR. A full factorial design was used to compare DNA extraction (cetyltrimethyl ammonium bromide; CTAB, and NucleoSpin® Plant II Kit) and DNA quantification techniques (conventional GENESYS™ 10S UV-Vis spectrophotometer and confined drop-based NANOVUE™ Plus spectrophotometer). In the validation, various levels (0.0007 to 0.0315%) of Bt11 maize were formulated with blank maize and certified Bt11 reference material. A false-positive rate of 0% was obtained for blank samples, which corresponded to selectivity and reliability rates of 100%. The false-negative rate varied from 0 to 83.3%, consistent with sensitivity and reliability rates ranging from 16.7 to 100%. The Bt11 level that presented 100% positive results was 0.0315%, which indicated the sensitivity of the method. Non-linear models were used to estimate the region of unreliability and to calculate the detection limit of 0.014%. Accordance and concordance values of 1.0 were obtained for the 0.0315% level, which indicated method standardisation. Selectivity in the presence of interference was confirmed by the detection of Bt11 maize in the presence of other events. The method was considered robust for different DNA extraction and DNA quantification techniques. Higher DNA concentration values were obtained using CTAB. The absorbance ratio of A260/A230 was negatively influenced by quantification using a conventional spectrophotometer. Both DNA extraction techniques gave values of A260/A280 higher than 1.7, which indicated DNA of great purity. This validated method was applied to routine samples.

Keywords: qualitative method validation, genetically modified organisms, Bt11 maize, real-time PCR, DNA extraction, DNA quantification

1. Introduction

Maize (*Zea mays* L.) contains approximately 72% starch, 10% protein and 4% fat and provides vitamin B, essential minerals and fibre. However, it is poor in vitamins B12 and C, calcium, folate, and iron. It provides 15% of the total annual protein and 19% of the calories produced by food (Nuss and Tanumihardjo, 2010).

In low-income populations in Africa, America and Asia, maize is the main source of energy and protein. Africa has the highest consumption of maize, and its consumption is fundamental for the population. In the Americas, the highest consumption of maize occurs in Mexico. In Brazil, the intake is less high, but maize is the basis of energy for many people who live in the semi-arid region. In Asia, the Timor Leste region is the largest consumer of maize (Duarte, 2000; Ranum *et al.,* 2014).

The economic importance of maize in Brazil is a result of its diversity because it can be used in feed and food and in the high-tech industry. However, the most maize is used

for the production of feed for poultry, cattle and swine culture, which are responsible for a large economic share. It is estimated that 70% of all maize is used as animal feed (FAO, 1983).

The worldwide production of maize in 2016/2017 is estimated at 1.01 billion tons, with a harvested area of 178.8 million ha. The major producers are the United States, China and Brazil, with an estimated production in 2016/2017 of 366.5, 218.0 and 82.0 million tons, respectively (USDA, 2016). In 2016, the Brazilian crop area for maize will reach a total of 15.2 million hectares, of which 13.5 million hectares correspond to genetically modified (GM) maize (Celeres, 2016; CONAB, 2016).

In Brazil, 37 transgenic agricultural products are already approved for commercialisation, 19 of which are maize (CTNBio, 2014). The use of genetically modified organisms (GMO) in food and food-derived products are subjected to regulations, which differ from country to country. The threshold limit ranges from 0% in China, 0.9% in the European Union, 1.0% in Brazil, Australia, New Zealand and Saudi Arabia, 3% in South Korea to 5% in Japan and Taiwan (Anvisa, 2003; EU, 2003; Gruère and Rao, 2007). In the USA, labelling is not mandatory, but the government recommends the notification of regulatory bodies about a new product before its commercialisation (James, 2014; Thomson, 2003).

Bt11 maize has characteristics that confer resistance to insects and to the herbicide glufosinate ammonium, and it resists the main plagues of the Lepidoptera order that affect corn culture, such as *Spodoptera frugiperda*, and *Helicoverpa zea*. The genes introduced code for an incomplete form of the Bt insecticide protein, which is obtained from HD-1 strain of soil bacteria *Bacillus thuringiensis* var. *kurstaki*, and an enzyme (phosphinotricin-N-acetyl transferase) that confers tolerance to the herbicide glufosinate ammonium, which can also be obtained from a soil bacteria, *Streptomyces viridochromogenes* (CTNBio, 2008).

In GMO analysis, an effective extraction method is essential to guarantee the presence and quality of extracted deoxyribonucleic acid (DNA). An appropriate extraction method accounts for the relation of cost of analysis, the yield of DNA and the removal of inhibitory substances that could influence the polymerase chain reaction (PCR), as a function of the food matrix and the researchers' needs (Cankar *et al.*, 2006; Marmiroli *et al.*, 2008).

The cetyltrimethyl ammonium bromide (CTAB) protocol is suitable for the extraction and purification of DNA from plants and plant derived foodstuffs. It is effective for the removal of polysaccharides and polyphenolic compounds that could affect the DNA purity and, therefore, its quality. This technique has been extensively applied in molecular analysis of plants, and it is a flexible and validated protocol that has demonstrated its efficiency to detect GMO in different matrices (Querci *et al.,* 2006).

Commercial kits have been designed for the isolation of genomic DNA from plant tissue using lysis buffer systems, columns for filtration and packed columns, commonly with silica, for DNA purification. These kits offer optimised processing, suitable yields and DNA of sufficient quality for the most common plant species with the benefit of speeding up the extraction process. However, the yield of DNA produced from commercial kits is often low, and the cost could be an issue for small laboratories (Macherey-Nagel, 2014; Xin and Chen, 2012).

The spectrophotometric determination of the purity and concentration of DNA can be done directly in liquid solutions, which could be diluted or undiluted, followed by the measurement of absorption of ultraviolet or visible radiation. For pure samples, this measurement is simple and accurate (Querci *et al.,* 2006).

In conventional UV-Vis spectrometry, 10 mm optical path cuvettes are typically employed as absorption cells, although these types of cuvettes have some practical limitations, especially when dealing with scarce samples. The use of semi-micro cuvettes has allowed a reduction in sample volume while maintaining the optical path that improves sensitivity by increasing the ratio between the optical volume and the sample volume (Pena-Pereira *et al.*, 2011).

The amount of nucleic acid solution used for measuring absorbance depends on the capacity of the cuvette. An adequate cuvette should be chosen in terms of the sample concentration range, dilution factor and available sample volume (Querci *et al.,* 2006).

Advances have been made to improve and miniaturise UV-Vis spectrometry by using new materials and technologies, thus allowing the use of micro- or nano-volumes of samples with a sensitivity close to that provided by conventional UV-Vis spectrometers. One of these new technologies is confined drop-based systems that are centred on surface tension or hydrophobicity (Pena-Pereira *et al.*, 2011). These systems use a hydrophobic sample-plate coating to hold a sample micro drop in place during measurement. A low sample volume is pipetted directly onto the hydrophobic surface, without dilution or the use of cuvettes, which is ideal when dealing with highly concentrated samples or when the use of ultra-low volumes is required. Cleaning is also quick and easy, thus minimising the risk of cross-contamination. Nonetheless, the physical properties of the micro-volume sample, such as the boiling point and vapour pressure, should be considered because the micro drop is partially exposed to air during drop deposition and UV-Vis

spectrometric measurement, which could cause evaporation (GE Healthcare, 2010; Pena-Pereira *et al.*, 2011).

Regardless of the analytical technique, methods must be validated. Modern approaches for the validation of qualitative methods (Gondim *et al.*, 2014) include the use of fundamental parameters such as the sensitivity rate (SNR), selectivity rate (SLR), reliability rate (RLR), unreliability region (UR), accordance (ACC) and concordance (CON), which are not considered in important GMO validation guidelines (Codex Alimentarius, 2010; ENGL, 2015). The Codex Alimentarius guideline suggests using the false-negative rate (FNR), false-positive rate (FPR), detection limit (DL) and robustness (Codex Alimentarius, 2010). In the European Network of GMO Laboratories document, the studies of FNR, FPR and probability of detection are recommended (ENGL, 2015).

The present study includes the following: (1) a single-laboratory validation of a qualitative method for the detection of a Bt11 event by real-time PCR, including the FPR, FNR, SNR, SLR, RLR, UR, DL, ACC, CON, complementary study of selectivity, and robustness; (2) a comparison between DNA extraction (CTAB and commercial kit) and quantification (conventional UV-Vis spectrometry and confined drop-based system) techniques by full factorial design; and (3) the application of the validated method to the routine inspection samples of the National Agricultural Laboratory of the Brazilian Ministry of Agriculture, Livestock and Food Supply (LANAGRO/MG – MAPA).

2. Materials and methods

Samples

Validation

Maize blank samples provided by Dow AgroSciences (Indianapolis, IN, USA) and certified reference materials (CRMs) were used to formulate the samples for the validation process. Maize samples were ground in a Knife Mill Grindomix GM 200 (Retsch GmbH, Hann, Germany) under controlled conditions to avoid cross-contamination, sieved to 1 mm size on test sieves (Retsch GmbH, Hann, Germany), collected in a 250 ml sterile Corning bottle (Corning, NY, USA), and then properly sealed and labelled.

CRMs of Bt11 maize at 0.1 and 0.5% (ERM-BF412), of Roundup Ready soy at 10% (ERM-BF410) and of MON810 maize at 5% (ERM-BF413) were obtained from the Institute for Reference Materials and Measurements (IRMM) (Geel, Belgium). These samples were kept at a temperature <4 °C until the moment of analysis.

Application

Commercial animal feed samples were collected from industrial locations by the Livestock Input Inspection Service (SEFIP/MAPA), which inspects Brazilian livestock inputs. Samples were ground in a Knife Mill Grindomix GM 200 (Retsch GmbH), sieved to 1 mm on test sieves (Retsch GmbH), collected in a 50 ml sterile Falcon tube (Sarstedt, Nümbrecht, Germany), properly sealed and identified, and stored at a temperature <4 °C until analysis.

Chemicals and reagents

Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). CTAB, ethylenediamine tetraacetic acid (EDTA), chloroform and sodium chloride were supplied by Sigma Aldrich Co. (St. Louis, MI, USA). Tris base was obtained from Biotec Ludwig (Alvorada, Brazil), hydrochloric acid was obtained from Dinâmica Química Contemporânea (Diadema, Brazil), ethanol was supplied by Exodo Científica (Hortolândia, Brazil), isopropanol was purchased from Amresco (Solon, OH, USA), proteinase K was obtained from Applied Biosystems (Foster City, CA, USA) and RNAse was supplied by Macherey-Nagel (Duren, Germany). Primers for Bt11 were synthesised by Integrated DNA Technologies Inc. (Coralville, IA, USA), primers for SSIIb (maize endogenous starch synthase IIb) were synthesised by GBT Oligos Genbiotech (Buenos Aires, Argentina) and probes for Bt11 and SSIIb were synthesised by Applied Biosystems.

Analytical procedure

DNA extraction

CTAB extraction was carried out according the protocol described in ISO 21571 (ISO, 2005b). The NucleoSpin® Plant II (NSP II) kit (Macherey-Nagel, Duren, Germany) was used for genomic DNA extraction according to the flowchart represented in Figure 1.

DNA purity and concentration

The nucleic acid concentration was determined by measuring the absorbance at 260 nm against a blank. DNA purity was assessed by the A260/A280 ratio, in which pure DNA should have a ratio of 1.7 to 2.0. Higher values of absorbance at 230 nm indicated that organic compounds or chaotropic salts were present in the purified DNA. The A260/A230 ratio measured the level of salt carryover in the purified DNA. For pure samples, the A260/A230 ratio was best when it was greater than 1.5. The 320 nm reading indicates turbidity in the solution, which is another indication of potential contamination (Kheyrodin and Ghazvinian, 2012; Querci *et al.*, 2006).

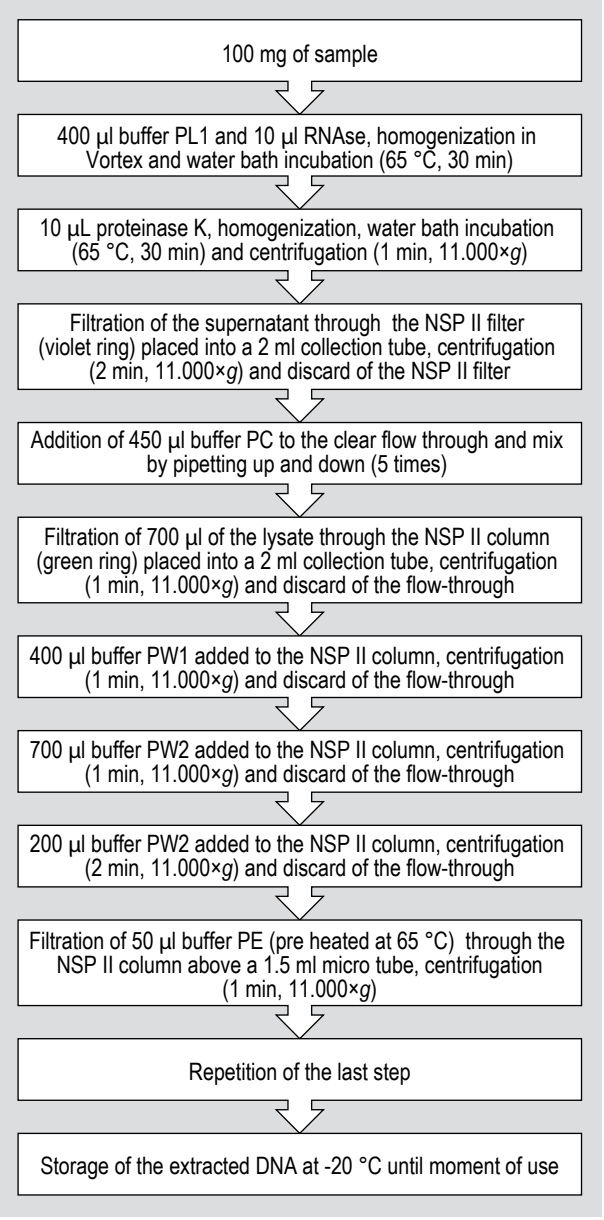

Figure 1. Flowchart of the DNA extraction method with the NucleoSpin® Plant II (NSP II) kit.

Conventional UV-Vis spectrometry

The extracted DNA was diluted in a solution composed of Tris dilution buffer (0.01 mol/l) and NaOH (0.2 mol/l) and analysed using a conventional GENESYS™ 10S UV-Vis (UV-VIS) spectrophotometer (Thermo Scientific, Bremen, Germany). First, 15 µl of DNA was diluted in 1,485 µl (dilution 1:100) of this solution and placed into a quartz cuvette of 10 mm. Absorbance data at 230, 260, 280 and 320 nm were collected. Each absorbance reading was repeated two times. Nucleic acids were quantified based on the reading at 260 nm and calculated as described in ISO 21571 (ISO, 2005b).

Confined drop-based system

The NANOVUE™ Plus (NANO) spectrophotometer (GE Healthcare, Piscataway, NJ, USA) was used to determine the extracted DNA concentration. First, 2 µl of DNA sample was pipetted onto the hydrophobic surface. The sampling head was closed on top of the sample, and a short path length of either 0.2 mm or 0.5 mm was generated. The absorbance value was normalised to reflect a standard path length of 10 mm; thus, the result was automatically multiplied by a factor of 50 (for DNA analysis). The concentration was calculated and generated by the equipment, and absorbance ratio data were recorded.

TaqMan probes and primers

The sequence of primers and TaqMan probes used in this study are presented in Table 1, according to ISO 21570 (ISO, 2005a).

Real-time PCR

After quantification, the extracted materials were diluted to a final concentration of 20 ng/µl and used as stock solutions for the following PCR analysis. The real-time PCR reactions were performed on a StepOnePlus™ Real Time PCR System (Applied Biosystems). First, 5 µl of DNA was amplified in a total volume of 20 µl containing 1X TaqMan Universal PCR Master Mix (Applied Biosystems), and the thermal cycling conditions were as follows: 2 min of Uracil-N-glycosylase incubation at 50 °C and 20 s of denaturation at 95 °C, followed by 40 cycles of a two-step programme (denaturation at 95 °C for 1 s and annealing/extension at 60 °C for 20 s). The fluorescence threshold and baseline settings were adjusted.

Qualitative PCR analysis

The results obtained from the real-time PCR were expressed as Cycle threshold (Ct) values. Because the approach of the method was qualitative, Ct values under 38 (based on previous laboratory experiments) for the GM amplification were determined to be positive results, and values above 38 or with no fluorescent signal were determined to be negative results.

Single-laboratory validation

A method based on the NSP II kit and conventional UV-VIS spectrometry was validated as recommended in the procedure published by Gondim *et al.* (2014).

Table 1. Primers and probes utilised in real-time PCR reactions (adapted from ISO, 2005a).

Target	Primers and probe name	Sequence	Concentration (nM)	Amplicon size (bp)
SSIIb	SSIIb 1 Forward	5'-CTC CCA ATC CTT TGA CAT CTG C-3'	500	151
	SSIIb 1 Reverse	5'-TCG ATT TCT CTC TTG GTG ACA GG-3'	500	
	SSIIb – Probe	5'-FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA-3'	200	
Bt11	Bt11 3 Forward	5'-AAA AGA CCA CAA CAA GCC GC-3'	500	127
	Bt11 3 Reverse	5'-CAA TGC GTT CTC CAC CAA GTA CT-3'	500	
	Bt11 – Probe	5'-FAM-CGA CCA TGG ACA ACA ACC CAA ACA TCA-TAMRA-3'	200	

Preliminary assays

In the preliminary evaluation, levels of 0.0015, 0.0030, 0.0045, 0.0060, 0.0075, 0.0090 and 0.0120% of Bt11 were prepared. Six replicates per level were prepared and analysed randomly. The results were evaluated to verify the suitability of the selected concentration range, which, to be considered appropriate for the UR estimation, should have an FNR between 0-20 and 80-100% (Gondim *et al.*, 2014).

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

The concentration range for the validation process was established. Formulations of fourteen concentration levels (0.0000, 0.0007, 0.0015, 0.0030, 0.0045, 0.0060, 0.0075, 0.0090, 0.0105, 0.0120, 0.0135, 0.0180, 0.0225, 0.0270 and 0.0315% Bt11 maize) were prepared in 30 replicates and randomly analysed. The experimental design covered the repeatability and intermediate precision conditions as demonstrated in Figure 2.

Contingency tables were used to evaluate the rates. Performance curves were plotted, and the limits of the UR were estimated by non-linear regression, considering 5 and 95% limits for false negatives. DL was defined as the upper limit of the UR. To be considered appropriate, the DL should be below the Brazilian regulated level for GMO. Combinatorial analysis was utilised to calculate ACC and CON, which should give values equal or greater than 0.8 near the regulated concentration level (regarding the possibility of one false negative result for each analytical batch) (Gondim *et al.*, 2014).

Complementary selectivity study

Two events were selected to be evaluated as potential interference: one event of soy (RR soy) and another of maize (MON810). RR soy has the 35S promoter and the T-NOS terminator in common with Bt11, and MON810 has the 35S promoter and the cry1A (b) gene sequence in common with Bt11.

The lowest level of Bt11 that presented 100% RLR in the second validation step was used in this study. The levels selected for the interference were 1.0% for MON810 maize and 2.0% for RR soy (levels were chosen based on the CRM available and the feasibility to formulate samples with CRM of Bt11 maize and blank maize). The experimental design of this validation step is outlined in Figure 3.

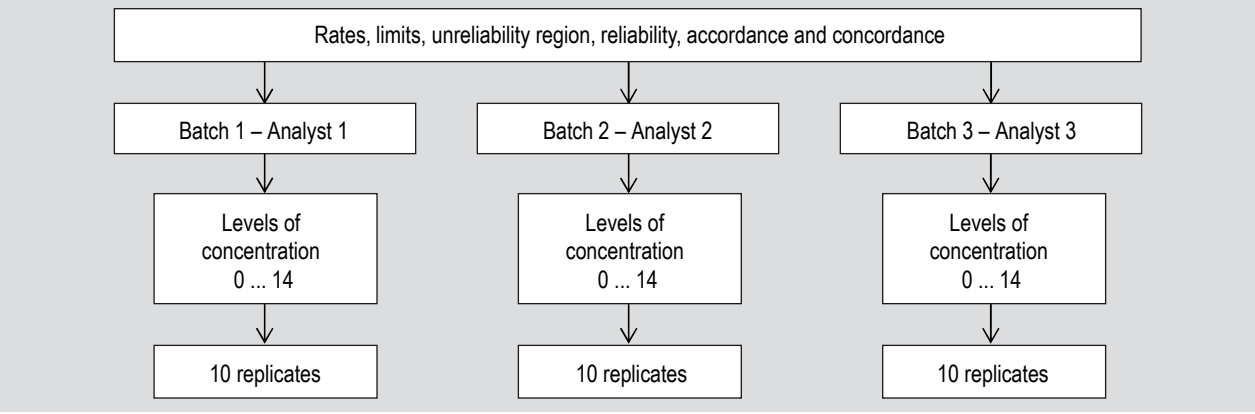

Figure 2. Schematic representation of the experimental design for evaluation of rates, limit of detection, unreliability region, accordance and concordance. Analytical batches varying analysts, time and reagents trades/batches; analyte concentration levels: 0.0000, 0.0007 to 0.0315% Bt11 maize (adapted from Gondim *et al.*, 2014).

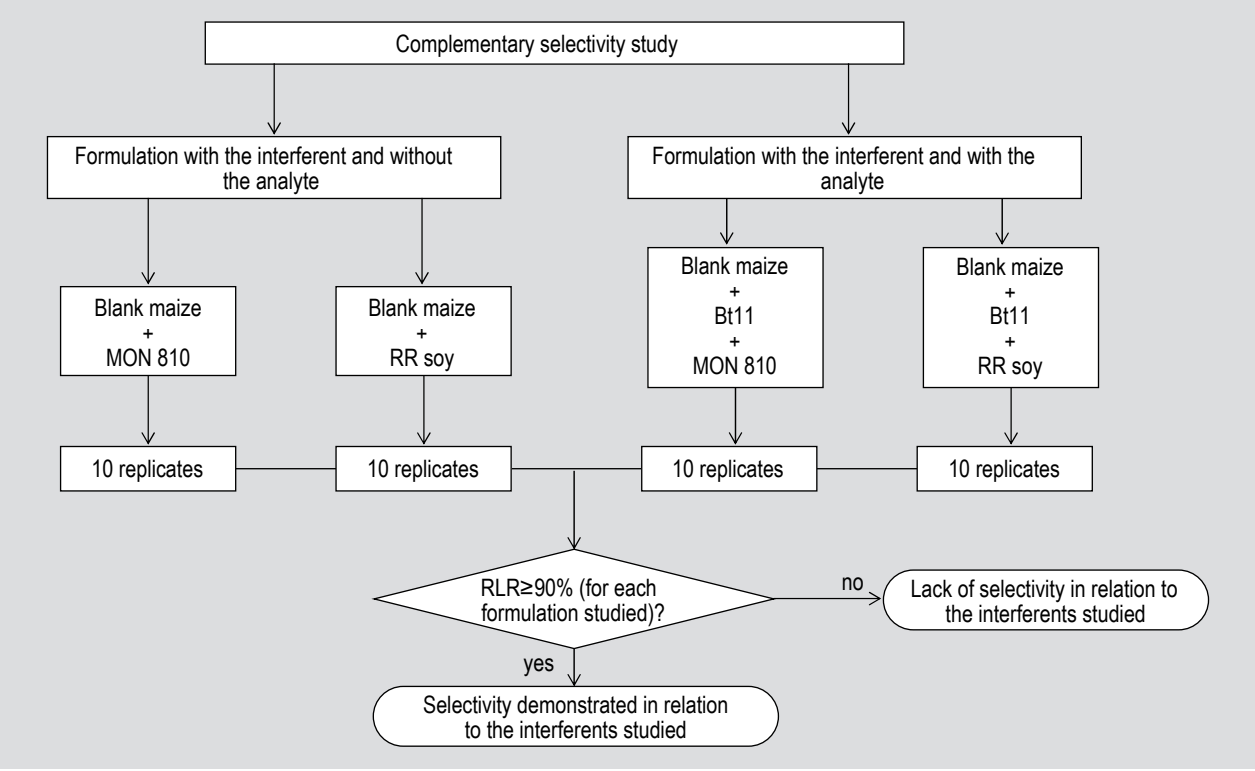

Figure 3. Schematic representation of the experimental design for the selectivity study in relation to interferents and criterion adopted. Analyte concentration level: lowest level of Bt11 that presented 100% RLR in the second validation step; interferents concentration levels: 1.0% for MON810 maize and 2.0% for RR soy; RLR: reliability rate (adapted from Gondim *et al.*, 2014).

Samples were analysed with Real time PCR. To consider the RR soy and MON810 maize as interferents, the RLR obtained should be lower than 90% (implying one false negative result per analytical batch of 10 replicates for each formulation) (Gondim *et al.*, 2014).

Robustness

A full factorial 2×2 experiment was designed (Figure 4) that used different DNA extraction (CTAB and NSP II kit) and quantification (conventional UV-VIS spectrophotometer and NANO spectrophotometer) techniques. Samples were formulated with Bt11 maize at the lowest concentration that exhibited 100% RLR in the second validation step, with 10 independent replicates for each treatment. The extracted DNA was analysed by Real-time PCR for each replicate obtained from each condition. The factors and levels were considered significant if the RLR was lower than 90% (Gondim *et al.*, 2014).

Comparison between different extraction and DNA quantification techniques

DNA concentration and absorbance ratios A260/A230 and A260/A280 were estimated for each replicate of the different treatments of the factorial design and were used to compare the studied techniques. One way ANOVA and Tukey's multiple comparison test (α=0.05) (Snedecor and Cochran, 1989) were used to analyse differences in the treatments. The results were graphically represented by Box Plot diagrams.

Application

The validated method was applied to inspect 15 animal feed samples that were collected by SEFIP/MAPA in 2014 and analysed by the Plant Diagnostic and GMO Laboratory of the LANAGRO/MG. The collected samples were from 5 of the 12 mesoregions of the state of Minas Gerais, including the mesoregion that was largest producer in the state. The samples represented feed for lactating cows, beef cattle, laying hens, broilers, started chicken, porcine concentrate and complete feed for dogs.

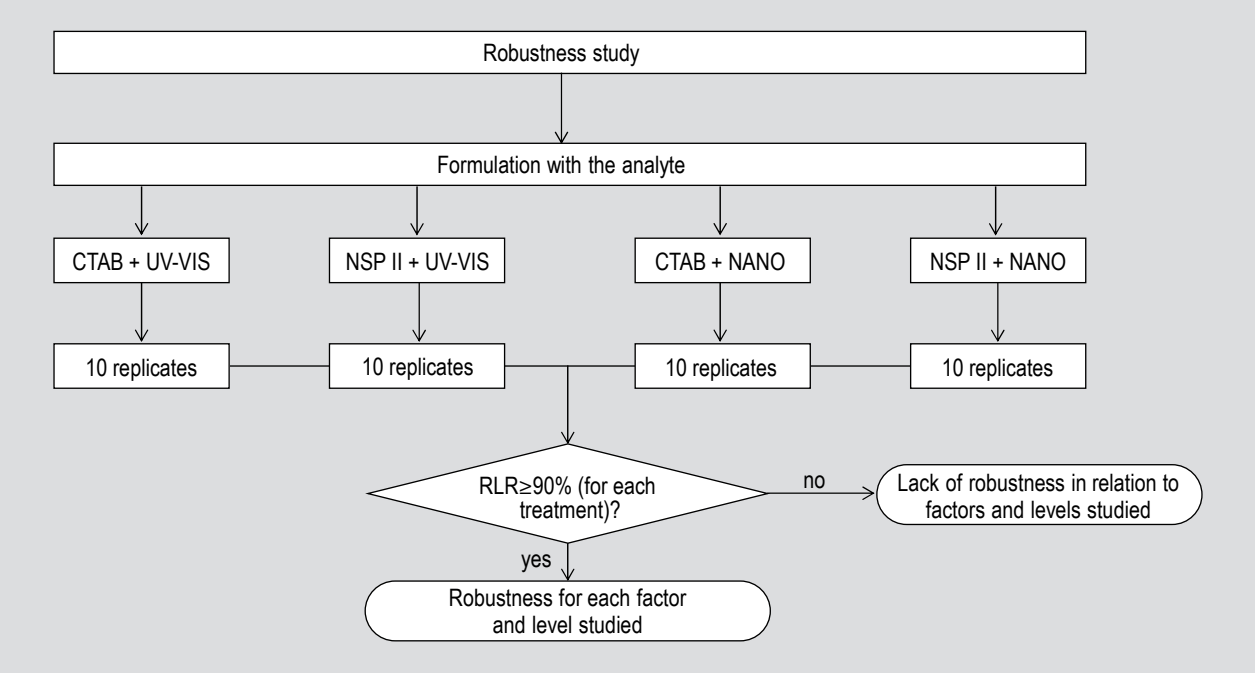

Figure 4. Schematic representation of the experimental design for the robustness study and criterion adopted. Analyte concentration level: lowest level of Bt11 that presented 100% RLR in the second validation step; NSP II = NucleoSpin® Plant II kit; NANO = NANOVUE™ Plus; RLR = reliability rate (adapted from Gondim *et al.*, 2014).

3. Results and discussion

Preliminary assays

The rates achieved in the preliminary study did not cover the range between 0-20 and 80-100%. Thus, the concentration levels needed to be expanded for the subsequent steps of the validation process.

Rates

Blank samples had a 0% FPR and 100% SLR and RLR, which means that the method was selective for the detection of Bt11 in maize. In Table 2, the values obtained for FNR, SNR and RLR for each studied level are reported.

For levels ranging from 0.0007 to 0.0270%, the FNR varied from 3.3 to 76.7%, and the corresponding SNR and RLR values varied from 96.7 to 23.3%. The 0.0315% level presented 100.0% positive results; this level is significantly lower compared to the regulated levels in Brazil, European Union, Japan and other countries, which indicates that the method can detect Bt11 events at lower concentration levels than is required.

Germini *et al.* (2004) developed and validated, by interlaboratory assays, a screening method for the simultaneous detection of soybean and maize events, including Bt11 at 0.5 and 2%. The DNA extracts were amplified in a multiplex PCR system, and the amplification products were analysed by agarose gel electrophoresis. False positive and negative results were reported, but these rates were not calculated. The selectivity was discussed based on the false positive response, but this rate was not calculated either.

In an interlaboratory study presented in the JRC Compendium (JRC, 2011), six samples containing Bt11 and Bt176 maize were examined through agarose gel electrophoresis. Two independent DNA extractions were analysed in duplicate. The reported FPR and FNR were 4.7 and 9.4%, respectively. The SLR and SNR obtained corresponded to 95 and 91%, respectively. The concentration levels of the events were not reported.

Kim *et al.* (2014) developed a method to detect four individual pentaplex PCR analysis systems for the event-specific identification of sixteen GM maize events in agarose gel electrophoresis. The method was intralaboratory validated by three researchers. The studied concentrations levels were 25, 2.5, 1.25, 0.75, 0.25, 0.125, and 0.025%; however, the authors reported that they only consistently detected events at levels higher than 0.25%. They discussed selectivity based on the absence of cross-reactivity. Rates were not reported.

A lower number of concentrations was tested in all cases, compared with the present study, which probably occurred because the preparation and evaluation of materials involve homogeneity and stability tests in interlaboratory studies.

Table 2. False-negative, sensitivity and reliability rates obtained under intermediary precision conditions for different concentration levels of Bt11 maize.[1]

Levels of Bt11 (%) (n=30)	Validation parameters (%)		
	FNR	SNR	RLR
0	–	–	100.00
0.0007	76.67	23.33	23.33
0.0015	83.33	16.67	16.67
0.0030	66.67	33.33	33.33
0.0045	66.67	33.33	33.33
0.0060	40.00	60.00	60.00
0.0075	23.33	76.67	76.67
0.0090	30.00	70.00	70.00
0.0105	23.33	76.67	76.67
0.0120	20.00	80.00	80.00
0.0135	26.67	73.33	73.33
0.0180	6.67	93.33	93.33
0.0225	3.33	96.67	96.67
0.0270	3.33	96.67	96.67
0.0315	0.00	100.00	100.00

[1] n = number of replicates in each concentration level; FNR = false-negative rate; SNR = sensitivity rate; RLR = reliability rate.

Additionally, in the present study, lower levels were investigated: 100% SNR was achieved for the 0.0315% level, and no false positive results were obtained. The studies of Germini *et al.* (2004) and Kim *et al.* (2014) did not report the number of replicates per concentration level. In the JRC study (JRC, 2011), only two independent replicates were analysed. It is important to highlight that a significant number of randomised assays is necessary to verify all performance parameters in the qualitative validation process (Cárdenas and Valcárcel, 2005; Gondim *et al.*, 2014).

Unreliability region and detection limit

Figure 5 shows the performance curve and equation obtained by non-linear regression after outlier treatment. Considering 5 and 95% limits for the positive results, the UR varied between -0.0013 and 0.014%, and the upper limit of the UR corresponded to the DL. A negative lower limit for the UR indicated that it was not different from zero. The DL was considered suitable for the detection of Bt11 because it was quite small compared to the level required by Brazilian and international legislations.

It is important to consider that the experimental DL and the DL estimated using a non-linear regression model were different because the experimental data could be influenced by one dispersed value, whereas the model

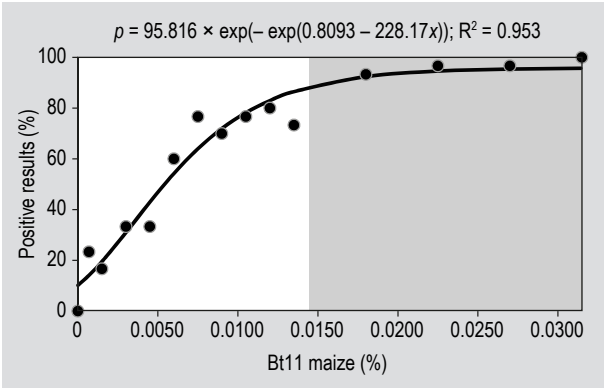

Figure 5. Experimental data (•), performance curve (solid line), equation and determination coefficient (R^2) obtained by Gompertz non-linear regression and levels outside the unreliability region are highlighted (shaded area).

compiles all experimental observations to provide an estimate (Gondim *et al.*, 2014). The uncertainty estimation is a recent parameter employed in qualitative validation studies, which is why there are few studies in the literature that have estimated the UR.

DL levels of 0.25% (Germini *et al.*, 2004; Kim *et al.,* 2014) and ≤0.1% (JRC, 2011) were reported by other authors. These levels were higher than those obtained in the present study. However, it is difficult to compare the data because the authors applied different experimental designs, numbers of replicates and criteria.

Accordance and concordance

Figure 6 presents the results obtained for ACC and CON. The ACC values calculated were between 0.87 and 1.0, and the CON values varied from 0.87 to 1.0, considering the levels outside the UR (0.0180, 0.0225, 0.0270 and 0.0315% of Bt11). The maximum ACC and CON values of 1.0 were obtained for the 0.0 and 0.0315% levels, which indicated that this method was sufficiently standardised at these levels. The profile of values decreased until an intermediate region and then increased until a maximum value of 1.0 was reached (Ellison and Fearn, 2005; Gondim *et al.*, 2014).

Germini *et al.* (2004) calculated the parameters of repeatability and reproducibility. Repeatability was measured as the ratio x/n (x is the number of correct results under repeatable conditions, and n is the total number of analyses) and was calculated to be equal to 1. Reproducibility was measured as the ratio y/n (y is the number of correct results under reproducible conditions) and was calculated to be equal to 0.9, which was considered by the authors to be satisfactory. The frequency of positive results was calculated to evaluate the standardisation of the method, which could be considered the study of ACC and CON.

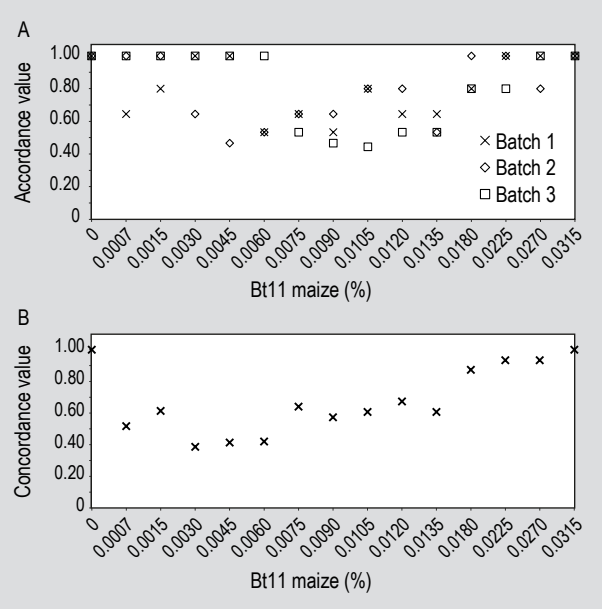

Figure 6. (A) Accordance values versus the concentration of Bt11 maize, and (B) concordance values versus the concentration of Bt11 maize, highlighting levels outside the unreliability region (shaded area).

Complementary study of selectivity

The FPR and FNR did not change for blank maize samples or for formulations containing Bt11 in the presence of RR soy and MON810 maize interference. All samples had a RLR of 100%, and this method was considered sufficiently selective to detect Bt11 in the presence of these events.

Germini *et al.* (2004) conducted a selectivity test containing 20% of each transgene that was amplified in 7 PCR processes using only one pair of primers initially starting from the shortest amplicon, followed by the addition of a second primer pair and so forth, until the last pair. To evaluate the correct primer pairs/target response, each transgene was tested individually in a PCR reaction containing all primer pairs. Two bands were simultaneously amplified for each event, one corresponding to the endogenous control (zein gene or lectin gene) and the other corresponding to the GMO specific amplicon (MON810, RR, Bt11, Bt176, GA21), thus confirming the specificity of the primer pairs chosen for each transgene.

In the selectivity step of the validation developed by Kim *et al.* (2014), one primer was designed to specifically target the transgenic insert, and the other was designed to target the flanking region of the host genome for each event. The selectivity of the designed primer pairs was individually assessed by a simplex PCR assay. As a result, only PCR products of the expected sizes were amplified from each target DNA, and no apparent non-specific signals were present in the other tested GMO.

The selectivity of a method can be better ensured when specific probes are used for the detection of amplicons, such as the real-time PCR technique adopted in the present study. In contrast, the use of agarose gel electrophoresis for multiplex PCR (Germini *et al.*; 2004; Kim *et al.,* 2014) is limited for use in routine analysis due to the presence of non-specific amplifications and the inability to discriminate slight length differences in amplicons (Hamels *et al.*, 2009).

Robustness

One false negative result was obtained from CTAB + UV-VIS, and one false negative result was obtained from CTAB + NANO. One false negative result for each analytical batch is considered acceptable, corresponding to a 90% RLR. Therefore, the method was considered robust for all of the factors and levels studied.

In the papers of Germini *et al.* (2004), JRC (2011) and Kim *et al.* (2014), the study of robustness was not included.

Comparison of different extraction and DNA quantification techniques

One-way ANOVA revealed significant differences in the DNA concentration ($P<0.001$), absorbance ratio A260/A230 ($P<0.001$) and A260/A280 ($P<0.05$) parameters.

DNA concentration

The result of the Tukey test, as shown in Figure 7A, indicated that all of the treatments were significantly different from each other, except for NSP II + NANO (c) and NSP II + UV-VIS (c), thus implying that there is no difference in quantifying the NSP II extracts with NANO or UV-VIS spectrophotometers. With respect to CTAB extraction, the quantification techniques differed from each other. Additionally, the treatment with the highest yield of DNA was CTAB + NANO (a).

One possible explanation for higher values of DNA concentration quantified using a NANO spectrophotometer is that the samples are not diluted, as they are when UV-VIS is used (100 times dilution). Thus, when the DNA is quantified using a UV-VIS spectrophotometer, the quantification is probably underestimated, giving lower concentrations than the NANO.

These results are in agreement with those reported by Marmiroli *et al.* (2008) that compared various DNA extraction methods for GMO analysis in maize and soybean. The DNA yield obtained from CTAB was higher than that obtained with the NSP II Kit.

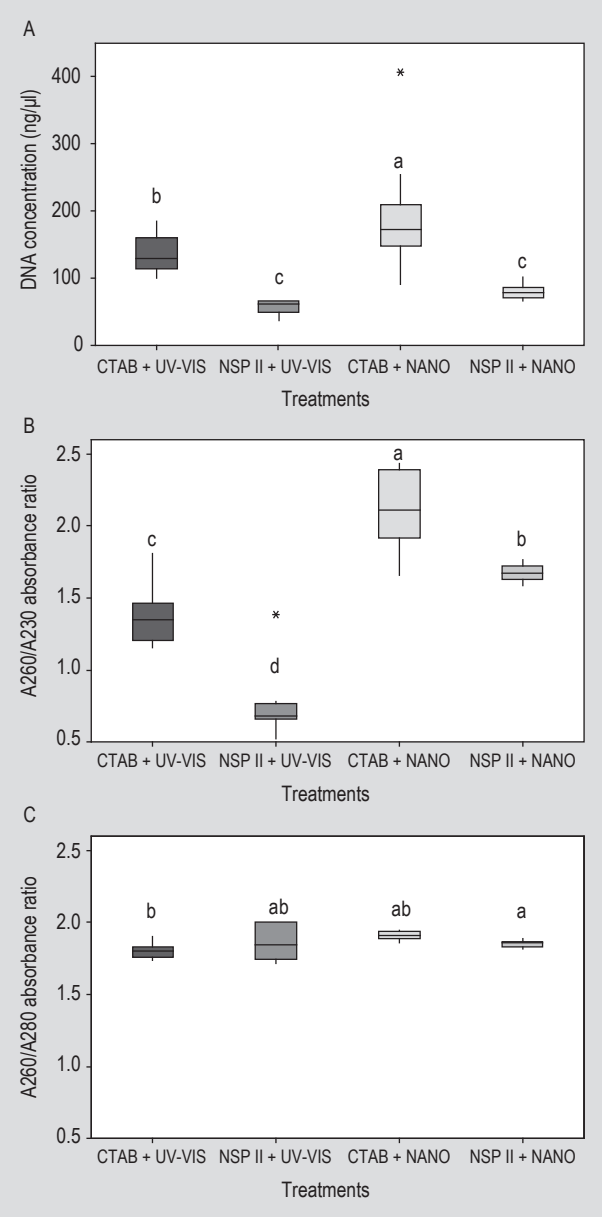

Figure 7. Boxplot diagram of each studied treatment (n=10) of (A) DNA concentration, (B) absorbance ratio A260/A230 and (C) A260/A280. Median values are indicated by the line within the box plot. The box extends from the 25th to 75th percentiles and whiskers indicate the minimum and maximum values. The asterisks represent the outliers. Different letters above the bars indicate significantly different means (*P*<0.05, Tukey HSD Test). NANO = NANOVUE™ Plus; NSP II = NucleoSpin® Plant II kit; UV-VIS = GENESYS™ 10S UV-VIS.

Absorbance ratios

The results of the Tukey test applied to the absorbance ratio A260/A230 (Figure 7B) indicated that all treatments were different from each other (α=0.05). The combinations CTAB + UV-VIS (c) and NSP II + UV-VIS (d) had the lowest values (A260/A230 lower than 1.5). Both treatments were quantified using a UV-VIS spectrophotometer, in which the samples were diluted in a solution composed of Tris and NaOH (0.2 mol/l). The presence of salt is known to decrease the ratio, which may have caused the lower results (Querci *et al.,* 2006).

Considering NANO quantification, the CTAB technique had higher A260/A230 values than did the NSP II kit. This low A260/A230 value could be explained by the presence of residual guanidine in the reagents of the kit (Macherey-Nagel, 2014). However, CTAB and NSP II gave ratios higher than 1.5, which indicated that both extraction techniques gave pure DNA extracts and had no contamination.

These results suggested that the A260/A230 absorbance ratio is influenced by the quantification technique and that when a UV-VIS spectrophotometer is used, the evaluation of this parameter isolated from the other parameters could lead to an underestimation of DNA purity.

The results obtained for the A260/A280 absorbance ratio (Figure 7C) indicated that CTAB + NANO (a) and CTAB + UV-VIS (b) treatments were significantly different from each other. Treatment with NSP II + UV-VIS (ab) or NSP II + NANO (ab) did not differ from the first two treatments. One possible reason is that the quantification technique interferes directly with the A260/A280 absorbance ratio for CTAB extraction, but not when the NSP II kit was used. Nevertheless, all treatments had values higher than 1.7, thus providing DNA of high purity.

Disregarding the bench costs required to produce the analytical solutions used to perform the CTAB extraction and comparing the costs of purchasing a NSP II kit and reagents used for CTAB extraction, the NSP II kit is almost 3 times more expensive than CTAB. Additionally, CTAB results in higher DNA concentration values, presenting the greatest cost benefit relation.

Application

To test the application of this method in practical samples, 15 animal feed products were analysed for the presence of Bt11 maize. Eleven samples had positive results for the presence of the event and underwent quantitative analysis to determine the percentage of Bt11. From the 11 samples analysed, 6 had concentrations higher than 1.0%, and 5 had concentrations lower than this limit.

This qualitative method is already in use as a screening method in the routine of the laboratory of Plant Diagnostic and GMO Laboratory. The main advantages are that the method provides rapid results for negative samples (speeding up the decision about the product's adequacy), the number of analyses is reduced (because only the positive samples proceed to quantitative analysis) and the

cost decreases (lower amounts of CRM and reagents are needed).

4. Conclusions

In this study, a complete qualitative validation approach was applied to the detection of Bt11 events in maize by Real-Time PCR. After evaluating the fundamental parameters, the method was considered fit for screening purposes. The CTAB and NSP II kit DNA extraction techniques using UV-VIS and NANO spectrophotometers for DNA quantification were considered robust. In terms of the DNA concentration, the CTAB technique provided higher values than the NSP II kit did. For the determination of A260/A230, the quantification with UV-VIS negatively influenced the results due to the presence of salt in the dilution solution, so higher values of this parameter were achieved with NANO spectrophotometer and CTAB extraction. The A260/A280 ratio was higher than 1.7 for both extraction and quantification techniques. Given the cost benefit relation and the results obtained in the study, CTAB extraction and quantification with NANO are the most suitable, but the NSP II kit and the UV-VIS spectrophotometer could be used as alternative techniques.

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