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Comparison of Three DNA Extraction Methods for Feed Products and Four Amplification Methods for the 5′-Junction Fragment of Roundup Ready Soybean

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ABSTRACT: Three methods of DNA extraction from feed products and four detection methods for the 5′-junction fragment of genetically modified (GM) Roundup Ready soybean (RRS) were compared and evaluated. The DNA extraction methods, including cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), and guanidine hydrochloride (Kit), were assessed for their yields and purity of DNA, extraction time, and reagent cost. The DNA yields of CTAB, SDS, and Kit were 52−694, 164−1750 and 23−105 ng/mg sample, and their extraction time was 2.5−3, 2−2.5, and 1.5−2 h with reagent cost about US dollar 0.24, 0.13, and 1.9 per extraction, respectively. The SDS method was generally well suited to all kinds of feed matrices tested. The limits of detection for the four amplification protocols, including loop-mediated isothermal amplification (LAMP), hyperbranched rolling circle amplification (HRCA), conventional polymerase chain reaction (PCR), and real-time PCR, were 48.5, 4.85, 485, and 9 copies of the pTLH10 plasmid, respectively. The ranked results of the four detection methods were based on multiattribute utility theory as follows (from best to worse): HRCA, LAMP, PCR, and real-time PCR. This comparative evaluation was specifically useful for selection of a highly efficient DNA extraction or amplification method for detecting different GM ingredients.

KEYWORDS: *comparison, genetically modified soybean, DNA extraction, detection, LAMP, HRCA, feed*

INTRODUCTION

The rapid development of genetic engineering techniques has led to genetically modified (GM) crops and their byproduct being widely distributed in the feed industry. Approximately 60−70% of feed raw materials are related to GM crops and their byproduct. Over 74 million tons of GM soybeans are used annually worldwide as a source of protein in feed. The glyphosate-resistant (Roundup Ready) soybean (RRS) is the most popular variety of GM soybeans.[1,2] European Commission Regulations (EC) No. 1829/2003 and (EC) No. 1830/2003 regulate the placement of food and feed products containing genetically modified organisms (GMOs) on the market, and they mandate the labeling of such products.[3,4] Many countries require the detection and monitoring of GMOs to enable safety assessments and enforce labeling, which leads to a high demand for reliable and easy to perform GMO detection and identification methods.

The quality and purity of the nucleic acids are two critical factors for detection analysis. To obtain high-quality template DNA that is free from inhibiting contaminants, suitable extraction methods should be applied. Extraction methods for GMO food products have already been compared by several researchers, although the validation of DNA extraction methods in different types of feed matrices is rarely discussed.[5−9]

Several GMO testing strategies using protein- or DNA-based technologies have been employed recently.[10−13] However, protein-based assays are not suitable for processed feed because of the loss of epitopes during processing. Thus, much attention has focused on the potential utility of DNA-based methods to amplify exogenous DNA sequences in raw ingredients and processed feed. In particular, polymerase chain reaction (PCR) and real-time PCR have been widely used to identify and quantify the GM contents in feed because of their simplicity, specificity, and sensitivity. Several PCR and real-time PCR methods for GM soybean, maize, and cotton in feed have been reported and evaluated in previous studies.[14−16] However, there were some disadvantages of sophisticated equipment requirements and time-consuming analysis for PCR products, which limited these methods being widely used for GMO detection in resource-poor settings.[17]

The recent application of loop-mediated isothermal amplification (LAMP) and rolling circle amplification (RCA) have no need for special instrumentation and provide new alternatives to detect GMOs. These methods are more sensitive and specific than the earlier methods of GMO detection.[18] The LAMP reaction requires a set of four specially designed inner and outer primers that recognize six distinct sequences on the target DNA. The LAMP reaction relies mainly on autocycling strand displacement DNA synthesis, which is similar to cascade RCA. The amplification of padlock probes (PLPs) by RCA is also a novel approach to nucleic acid amplification and was first reported by Demidov (2002).[19] This method amplifies a small fragment of DNA with high specificity and efficiency under isothermal conditions. The PLPs usually contain a ∼30-nucleotide (nt) 5′ target with a T_m of 68−70 °C and a ∼15-

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Table 1. Category and Country of Origin of the Feed Products in This Study

feed products	country of origin (number of samples)	details of sample category
soybean	Argentina (1), Belgium (2), Brazil (1), China (17), U.S. (1), Uruguay (1)	soybean seed: Brazil (1); Uruguay (1); U.S. (1); Argentina (1); China (5) soybean meal: China (10) soybean powder: China (2) the certified reference material (CRM) RRS: Belgium (2)
maize	China (11)	maize seed: the drought-resistant maize lines XZ17 (1), 4346-1-1 (1), and 78920B-2 (1); non-transgenic maize (1); transgenic PhyA maize (1); the insect-resistant Bt176 (1) and MON 810 (1); unknown samples (4)
cotton	China (7)	Bt-cottonseed meal: Zhumadian in Henan (1); Tacheng in Xinjiang (1); Weixian in Hebei (1); Sanhe in Hebei (1); Qingdao in Shandong (1); Weifang in Shandong (1); Beijing (1)
animal feed	China (13)	feed: concentrated feed (2); mixed feed for piglets (1); mixed feeds for broilers (3); mixed feed for hens (4); mixed feed for fish (2); "Four Seas" mixed feed (1)

nt 3′ target with a T_m of 40 °C. The lengths of the PLPs vary slightly because the target sequences must meet specific requirements.

In this study, to determine which DNA extraction and detection method is most appropriately used for detection of RRS in feed, we compared the yield and purity of three DNA extraction methods (applying cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), and guanidine hydrochloride (Kit)), and the specificity, limit of detection (LOD), ease of handling, interpretation of results, and batch testing of four detection methods (hyperbranched rolling circle amplification (HRCA), LAMP, conventional PCR, and real-time PCR).

MATERIALS AND METHODS

Samples. Fifty-four samples of feed products were collected in various markets (Table 1). Among them, Chinese soybean products including soybean seed, soybean meal, and soybean powder were collected from Shandong, Jiangsu, Guangdong, Hebei, and Guangxi provinces, respectively. The certified reference material (CRM) RRS (2 samples containing 2 g/kg (ERM-BF410e) and 0 g/kg (ERM-BF410a) RRS) was purchased from the EU Joint Research Centre, IRMM (Institute for Reference Materials and Measurements, Geel, Belgium). The drought-resistant maize lines XZ17, 4346-1-1, and 78920B-2 were provided by the National Maize Improvement Center, Beijing, China. The non-transgenic maize and transgenic PhyA maize were kindly supplied by the Chinese Academy of Inspection and Quarantine and Biotechnology Research Institute, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China. The insect-resistant Bt176 and MON 810 maizes were collected from the Beijing Seed Administration Station, Beijing, China. Four unknown maize samples were purchased from supermarkets and markets. Among 13 animal feed samples, concentrated feed samples were collected from Handan in Hebei and Shanghai, respectively. The mixed feed for piglets, broilers, hens, and fish was kindly supplied by the Feed Research Institute, CAAS, Beijing, China. The "Four Seas" mixed feed was purchased from a market in Beijing. Wild-type soybean (Heilongjiang, China) was collected as the conventional non-GM soybean. The Bt176, MON 810, and Bt-cottonseed meal (Shandong) were used to study the specificity of the designed primer pairs. A standard molecular plasmid, pTLH10, was constructed in our laboratory that contained 10 fragments: (1) the endogenous soybean *Lec1* gene (GenBank Accession No. K00821), (2) the *35S* promoter (GenBank Accession No. AJ308514.1), (3) the *NOS* terminator (GenBank Accession No. JN153032.1), (4) the *PAT* gene (GenBank Accession No. GQ497217), (5) the 5′-junction of soybean event RRS (GenBank Accession No. AJ308514.1), (6) the *Cry1A(c)* gene (GenBank Accession No. EU816953.1), (7) the endogenous cotton *Sad1* gene (GenBank Accession No. AJ132636), (8) the endogenous maize *Hmg* gene (GenBank Accession No. AJ131373.1), (9) the 3′-junction of maize event Bt176 (GenBank Accession No. AJ878607.1) and (10) the 3′-junction of maize event MON 810 (GenBank Accession No. AY326434).

Three DNA Extraction Methods for Feed Products. DNA extraction was performed by starting from 0.1 g of homogenized material using three different methods. DNA extraction methods for each sample were made in duplicate.

I. CTAB Method. The CTAB method was performed as described by Lipp et al. (1999) with some modifications.[20] Six volumes (w:v) of CTAB extraction buffer (2% CTAB; 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-base, pH 8.0), supplemented with ribonuclease A (at a final concentration of 10 μg/mL), was added, mixed, and incubated for 30 min at 65 °C with occasional stirring. The samples were then centrifuged for 15 min at 12000*g* at room temperature. A volume of the supernatant (500 μL) was transferred to a new 2 mL tube, extracted with 500 μL of hydroxybenzene−trichloromethane (v:v, 1:1) and centrifuged for 10 min at 12000*g* at 4 °C. The upper phase was mixed with the same volume of trichloromethane−isoamyl alcohol (v:v, 24:1) and centrifuged for 10 min at 12000*g* at 4 °C. The supernatant was mixed with an equal volume of chloroform and centrifuged for 10 min at 12000*g*. The aqueous phase was collected, and 0.6 volume of isopropanol was added, mixed, and incubated at −20 °C for 30 min. After centrifugation (15 min, 12000*g*, 4 °C), the pellet was washed twice with 600 μL of 75% ethanol and centrifuged for 2 min at 12000*g*. The pellet was dried for 10 min at room temperature, dissolved in 100 μL of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA), and stored at −20 °C until use.

II. SDS Method. The homogenized sample (0.1 g) was mixed with 1 mL of preheated (65 °C) SDS lysis buffer (100 mM NaAc, pH 4.8; 50 mM $EDTANa_2$, pH 8.0; 500 mM NaCl; 2% PVP; 1.4% SDS, pH 5.5) using an SDS method with some modifications.[21] The mixture was incubated for 30 min at 65 °C with occasional stirring. After centrifugation at 12000*g* for 10 min, the upper phase was transferred to a new tube, mixed with one-third its volume of 2.5 M KAc (pH 4.6), and incubated for 30 min on ice. After centrifugation at 12000*g* for 10 min, the upper phase was mixed with 0.6 volume of precooled (−20 °C) isopropyl alcohol and incubated for 20 min at room temperature. The mixture was centrifuged at 12000*g* for 10 min. The pellet was washed twice with 2 mL of ethanol solution (70%, v/v), dried, and dissolved in 100 μL of TE buffer.

III. Commercial Kit Method. The same samples were extracted with a DNA Extraction Kit for GMO Detection Ver. 2 (TaKaRa Biotechnology Co., Ltd., Dalian, China), following the manufacturer' instructions. Briefly, homogenized samples of up to 0.1 g were mixed with 850 μL of pretreatment buffer (10 mM Tris−HCl; 150 mM NaCl, 2 mM EDTA; 1% SDS, pH 8.0), 50 μL of proteinase K (20 mg/mL), and 200 μL of 5 M guanidinium hydrochloride. The samples were incubated for 1 h at 58 °C in a water bath. After centrifugation (10 min, 14000*g*), 300 μL of the supernatant was combined with 100 μL of solution I, 200 μL 0.2% SDS, and 1 mL of silica gel in a 2.0 mL microtube. The content was mixed by inversion and centrifuged for 30 s at 12000*g*. The pellet was washed with 300 μL of 1× wash solution three times. The DNA was eluted by the addition of 100 μL of Tris−EDTA buffer (10 mM Tris; 1 mM EDTA) at 70 °C, incubation for 5 min, and centrifugation (3 min, 14000*g*).

Evaluation of Extracted DNA and Statistical Analysis. The concentration of the extracted genomic DNA was determined by measuring the absorbance at 260 nm, and the DNA purity was

measured by calculating the ratio of the absorbances at 230, 260, and 280 nm with a Nano-Drop ND-1000 spectrophotometer (Saveen Werner, USA). The quality of the extracted DNA was analyzed by electrophoresis in a 1.0% agarose gel.

Statistical analyses were performed using ANOVA models in SAS 9.2 (Institute Inc., Cary, NC, USA) to compare the DNA yields and purities obtained by various protocols. Before conducting two-way ANOVA, normality of error terms was evaluated via Kolmogorov–Smirnov test for goodness of fit, and homoscedasticity was evaluated via Levene's test for equality of variances. The extraction methods and samples were treated as independent variables, and DNA yield and purity as the dependent variable separately. When treatment effects or interactions were significant, Duncan's multiple range test was used to compare the mean values among the treatments at 95% probability. This data also was tested using MANOVA procedure. Criteria for evaluating and dealing with violations to multivariate normality and homogeneous variance–covariance matrices were adopted from Tabachnick and Fidell (1996).[22] Furthermore, significance of MANOVA was determined with Pillai's trace, the most robust of the four test statistics used by SAS to calculate MANOVA *P*-values.[23] However, test statistics obtained with Wilk's lambda and Hotelling's trace produced the same results. Post hoc analyses on significant MANOVA were obtained via univariate ANOVA.

Oligonucleotide Primers. The primers/probes for LAMP, HRCA, PCR, and real-time PCR were designed on the basis of the 5′-transgene integration sequence of RRS with the programs Primer Explorer V4 (Fujitsu, System Solutions Ltd., Tokyo, Japan) and Primer 5.0. The T_m calculations of the PLP were performed using HYTHER (http://ozone3.chem.wayne.edu/), and significant secondary structures in the molecule were predicted by Mfold.[24] The TaqMan probe in real-time PCR harbored a reporter dye (FAM) at the 5′ end and a quencher dye (BHQ) at the 3′ end. All of the primers/probes were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China).

The LAMP primers contained four oligonucleotide primers based on six distinct regions of the event-specific 5′-junction of the RRS. The PLP was 97 nt in length, consisting of two adjacent target complementary sequences with a 59-nt linker region. To optimize binding to the target DNA, the probes were designed with minimal secondary structure and with a T_m of the 5′-end probe binding arm greater than the temperature used for probe ligation (62 °C; see below). To increase the specificity, the 3′-end binding arm of the PLP was designed to have a T_m (51–56 °C) below the ligation temperature.[25] The two primers used for HRCA (HRCA-F, HRCA-R) were designed to specifically bind to the linker region of the probes. The PLP contained a 5′ phosphate group to allow ligation. All of the above primer sequences are presented in Table 2.

Four Amplification Methods for Detecting the 5′-Junction Fragment of RRS. *I. LAMP Assay.* The betaine-free LAMP was performed according to the method described by Notomi (2000) with the *Bst* DNA polymerase large fragment (New England Biolabs Inc., NEB).[26] Briefly, the reaction mixture (25 μL) contained 50 ng of template DNA, 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3, 4 U of *Bst* DNA polymerase, 1.4 mM deoxynucleoside triphosphates (dNTPs), 2.5 μL of 10× *Bst* DNA buffer and 8 mM $MgSO_4$. The reaction mixture was incubated at 63 °C for 1 h and heated at 85 °C for 5 min to terminate the reaction. The LAMP products were subjected to electrophoresis on a 2% agarose gel in a Tris–acetic acid–EDTA (TAE) buffer.

II. HRCA Assay. (a) Ligation of PLP. The ligation was performed in a 10 μL reaction mixture containing 1× T4 ligase buffer, 100 pM PLP, 50 ng of the target DNA, and 2.5 U of T4 DNA ligase (NEB). The mixture was incubated at 37 °C for 30 min. *(b) Exonucleolysis.* Exonucleolysis was performed to remove the nonligated PLP and template DNA, thus reducing subsequent ligation independent amplification events. Exonucleolysis was performed in 20 μL volumes by adding 40 U of exonuclease I (NEB), 2 μL of 10× Exo I buffer, and 6 μL of ddH_2O to the ligation mixtures. The mixture was incubated at 37 °C for 30 min and at 90 °C for 1 min. *(c) HRCA Reaction.* After exonucleolysis of the now circularized probes, 4 μL of the digested

Table 2. The Primer Sequences and Amplification Conditions for the LAMP, HRCA, PCR and Real-Time PCR Methods[a]

application	primer names and sequence (5′–3′)	reaction conditions	amplified enzyme
LAMP	F3, GTGGAGATATCACATCAATCC; B3, ATAGGGAACCCAAATGGAA; FIP, GTGGTCCCAAAGATGGACCC-CTTGCTTTGAAGACGTGGT; BIP, GTCGGCAGAGGCATCTTCAA-AAGGAAGGTGGCTCCTAC	63 °C 1 h, 85 °C 5 min	*Bst* DNA polymerase large fragment (New England Biolabs Inc., NEB)
HRCA	PLP, AAGGTGGCTCCTACAAATGCCA**tgctggtgctgatgctggt**aagaagaaa gatcaaaaggatgacaaagtgccgagcaggACCCCAAATGGAAAAGG; HRCA-F, CCAGCATCAGCACCAGCA; HRCA-R, ACAAAGTCGCCGAGCAGG	37 °C 30 min; 37 °C 30 min, 90 °C 1 min; 63 °C 1 h, 85 °C 5 min	*Bst* DNA polymerase large fragment (NEB)
PCR	35SG-F, CAACGATGGCCTTTCCTTTAT; 35SG-R, CAAACCCTTCAATTTAACCGAT	94 °C 10 min; 94 °C 1 min, 58.2 °C 1 min, 72 °C 1 min (40 cycles); 72 °C 10 min	*Ex Taq* DNA polymerase (TaKaRa Bio Inc.)
real-time PCR	TaqMan-F, CGCAATGATGGCATTTGTAGG; TaqMan-R, GCATTTCATTCAAAATAAGATCATACAT; TaqMan-P, FAM-CCACCTTCCTTTTCCAATTTGGGTTCCC-BHQ	50 °C 2 min; 95 °C 10 min; 95 °C 15 s, 60 °C 1 min (45 cycles)	*Ex Taq* Hot Start (Takara, Bio Inc.)

[a]The 5′ and 3′ ends of the PLP that are complementary to the target sequence of the event-specific 5′-junction of RRS are underlined. The regions where the two padlock probe-specific primers (HRCA-F and HRCA-R) bind for rolling cycle amplification are in bold lowercase letters.

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ligation products was added into the HRCA reaction and incubated at 63 °C for 1 h in a 25 μL reaction volume containing 1× buffer, 0.5 mM each of the dNTPs, 0.2 μL of bovine serum albumin (BSA), 6 mM $MgSO_4$, 5 μM each of the primers HRCA-F and HRCA-R, and 4 U of *Bst* DNA polymerase. The HRCA products were heated at 85 °C for 5 min and subjected to 2% agarose gel.

III. PCR Assay. The PCR reaction was performed in a 25 μL volume using a TC-512 thermal cycler (Duxford, Cambridge, UK). The PCR mixture consisted of 0.5 mM of each dNTP, 50 mM Tris-HCl (pH 8.4), 50 mM KCl, 25 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 2.5 U of *Ex Taq* DNA polymerase (TaKaRa Bio Inc.), 0.5 μM of each primer (35SG-F/35SG-R), and 1 μL of template DNA. Amplification was followed by one cycle at 94 °C for 10 min for the initial denaturation and then 40 cycles of 94 °C for 1 min, 58.2 °C for 1 min, and 72 °C for 1 min. A final extension at 72 °C for 10 min followed the final cycle to complete the synthesis of the elongated DNA molecules. Samples (5 μL) of the PCR products were analyzed on 2% agarose gel.

IV. Real-Time PCR Assay. Real-time PCR was performed in 15 μL of a reaction mixture consisting of 7.5 μL of Premix Ex Taq Mix (Takara, China), 0.3 μL of ROX Dye II (50×), 200 nM of each primer, 250 nM of the TaqMan probe, and 1.5 μL of the DNA extracts (50 ng/μL). The reactions were run on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) using the following program: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The fluorescence signals were measured at the extension step for each cycle, and the data was then analyzed using the Detection System V2.03 (Applied Biosystems, USA). Each reaction was repeated three times, and each time with parallel triplicates.

The amplification conditions of these four methods are summarized in Table 2.

Evaluation of the Four Detection Methods. These four methods were compared for their ability to detect 5′-junction fragment of RRS.

I. Analytical Specificity of Primers in the Four Methods. The specificity of primers in the four detection methods was assessed by using 50 ng of template DNA extracted from eight different samples: soybean (Brazil), soybean (U.S.), soybean powder (Guangxi, China), soybean meal (Hebei, China), "Four Seas" (mixed feed), Bt176 maize, MON 810 maize, and Bt-cottonseed meal. The linear plasmid DNA pTLH10 digested with *Eco*RI was used as a template of the positive control, and DNA extracted from the wild-type soybean was used as the template of the negative control. The template DNA was replaced by ddH_2O in the blank control.

To further confirm the specificity of the LAMP and HRCA products, the amplified products were digested with several restriction endonucleases (*Ava*II and *Pfl*FI). *Ava*II cuts between forward outer primer 1 (F1) and backward outer primer 1 (B1) of the target sequence of the LAMP products, and *Ava*II and *Pfl*FI cut between the 59-nt linker region of the HRCA products. If amplification occurred as expected, then the amplified products would be fragmented into 130- and 80-bp fragments by *Ava*II digestion located in the F1−B1 region in LAMP; 80- and 17-bp fragments by *Ava*II digestion and 65- and 32-bp fragments by *Pfl*FI digestion in HRCA. Aliquots (2 μL) of the LAMP/HRCA products and 5 μL of the products digested with restriction enzymes were also subjected to electrophoresis on a 2.5% agarose gel.

These digestion products and PCR products were gel purified and subsequently sequenced by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China).

II. Analytical LOD of the Four Methods in Feed Detection. To determine the LOD of LAMP, HRCA, and PCR assays, 10-fold serial dilutions of the linearized pTLH10 plasmid DNA digested with *Eco*RI, ranging from 10^5 to 10^0 copies/μL (485000, 48500, 4850, 485, 48.5, and 4.85 copies/μL), were prepared as templates for the three amplification reactions. In addition, a gradient of 40, 20, 10, and 6 copies/μL of pTLH10 plasmid DNA was prepared to determine the LOD of the real-time PCR assay. All amplification reactions were performed in triplicate as previously described.

III. Ranking of the Four Methods in Feed Detection. Each test was evaluated by using the method of multiattribute evaluation described by Kehl et al. for the comparative study of diagnostic tests.[7,27−30] The tests were assigned a comparative score for each attribute: LOD, hands-on time, reagent cost, ease of handling, interpretation of products, batch testing and performance. Ease of handling and interpretation of the products were subjective evaluations based on the number of reagent steps and the ease with which decisions were made regarding the results of detection. The ability to test a large number of samples was also evaluated. Each attribute was ranked from 1 to 4, with 4 being the highest rank.

RESULTS

Three DNA Extraction Methods for Feed Products. DNA from 54 feed samples was extracted successfully using the SDS method. However, isolating DNA from soybean powder, Bt-cottonseed meal, and animal feed with the CTAB and Kit methods was very difficult (data not shown). Gel electrophoresis revealed that four samples (out of seven) extracted from the Bt-cottonseed meal by the CTAB protocol yielded no detectable DNA by gel electrophoresis. Better results with a higher quantity of DNA were obtained with SDS method in comparison to CTAB (Figures 1A and 1B). The genomic DNA

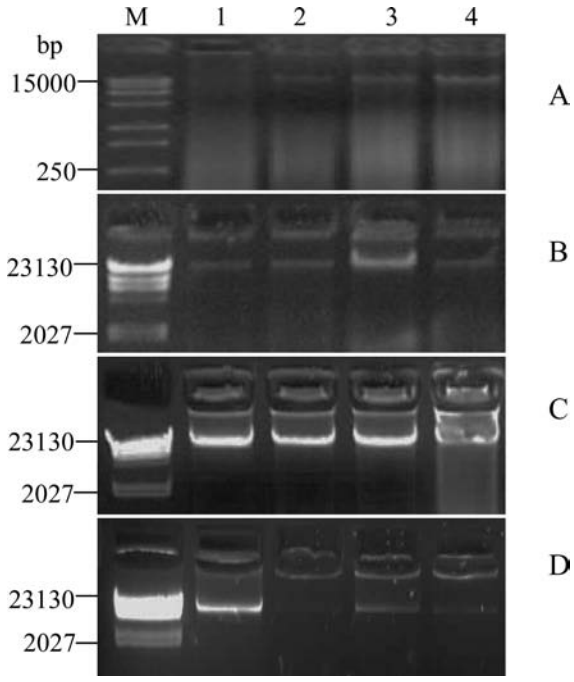

Figure 1. Agarose gels of DNA extracted from feed using different methods of extraction. Five microliters of extracted DNA was electrophoresed on a 1.0% agarose gel. A. The modified CTAB method. M, D15000 DNA marker (15000, 10000, 7500, 5000, 2500, 1000, 250 bp); lane 1, soybean powder (Guangxi, China); lanes 2 and 4, soybean meal (Hebei, China); lane 3, soybean seed (U.S.). B. The SDS method. M, λ/*Hin*dIII DNA marker (23130, 9416, 6557, 4361, 2322, 2027, 564 bp); lane 1, soybean powder (Guangxi, China); lanes 2 and 4, soybean meal (Hebei, China); lane 3, soybean seed (U.S.). C. The SDS method. M, λ/*Hin*dIII DNA marker (23130, 9416, 6557, 4361, 2322, 2027, 564 bp); lanes 1, 2, and 3, Bt-cottonseed meal (Shandong, China); lane 4, soybean seed (U.S.). D. The modified CTAB and Kit methods. M, λ/*Hin*dIII DNA marker (23130, 9416, 6557, 4361, 2322, 2027, 564 bp); lanes 1 and 2, DNA extraction from soybean meal (Hebei, China) with the CTAB method; lanes 3 and 4, DNA extraction from soybean meal (Hebei, China) with the Kit method.

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from soybean powder was only successfully extracted by the SDS method and not by the CTAB (Figures 1A and 1B, lane 1) and (guanidine hydrochloride) Kit protocols (data not shown). The CTAB method produced a significantly higher DNA content than the Kit (Figure 1D). The DNA extracted with the Kit method appeared as very weak bands on the gel. Moreover, the yield of DNA obtained from Bt-cottonseed meal was higher than the DNA obtained from the same amount of soybean meal using the SDS method (Figures 1B and 1C).

In order to get the best possible match between the different DNA extraction methods and the various types of feed samples, the analysis was carried out by comparing the yield and purity of DNA. As shown in Table 3, the SDS method gave not only the highest DNA yield ranging from 164 to 1750 ng/mg sample but also the best purity (A260/A280 and A260/A230 ratios of ≥1.7). In contrast, the Kit protocol produced the lowest yield of DNA in the range from 23 to 105 ng/mg sample and the lowest DNA purity. A moderate level of DNA yield in the range of 52 to 694 ng/mg sample and purity (≥1.3) were obtained using the CTAB method. The results from both ANOVA and MANOVA (F = 2.58, P = 0.02) indicated a significant difference among three methods (Table 4).

Apart from DNA extract yield and purity, operating time and reagent cost were taken into account to choose the optimal extraction method. The extraction time of CTAB, SDS and Kit was 2.5−3 h, 2−2.5 h and 1.5−2 h, respectively (Table 3). Moreover, Kit was more expensive than the two other extraction methods.

As shown in Table 5, the SDS method could be used in all types of feed matrices tested, including seed, meal, powder and the highly processed animal feed samples, but both CTAB and Kit methods could only be used in some unprocessed feed, such as soybean or maize seed. The genomic DNAs used in the following experiments were extracted with the SDS method.

Four Amplification Methods for the 5′-Junction Fragment of Roundup Ready Soybean. *I. Specificity of Primers in Four Detection Methods.* Four assays were carried out using DNA templates from eight different feed samples and wild-type soybean with the SDS method. Successful RRS event-special DNA amplifications with the pTLH10 plasmid, soybean, soybean powder, soybean meal, and "Four Seas" mixed feed were confirmed (Figure 3 and Table 6), which indicated the specificity of the primers of four detection methods, and demonstrated a characteristic ladder of multiple bands pattern when subjected to the LAMP and HRCA assays (Figures 3A, 3C). An approximately 214 bp band was also amplified successfully from positive samples by PCR (Figure 3E). In no case was a cross-reaction observed using DNA from Bt176, MON 810 maize, and Bt-cottonseed meal which contained nontarget genes. The negative samples also tested negative.

The specificity of the amplification of LAMP and HRCA was further confirmed by restriction endonuclease digestion. The fragment sizes of the LAMP products digested with the restriction enzyme *Bsr*FI were approximately 130 and 80 bp, in good agreement with the predicted sizes (Figure 3B). The sizes of the fragments in the HRCA reaction were approximately 80 and 17 bp for *Ava*II digestion (Figure 3D) and 66 and 31 bp for *Pfl*FI digestion (data not shown), which were consistent with the predicted sizes. These digestion products and the PCR products were further confirmed by sequencing.

II. LOD of Four Detection Methods. The analytical LOD of the four assays for the detection of RRS in feed was determined by testing a series of dilutions of the plasmid pTLH10. Our

Table 3. A Comparison of the DNA Yields (Mass of DNA/Mass of Sample) and Purities (A260/A280 and A260/A230) Obtained by the Three DNA Extraction Methods

			raw soybean and derivatives (23 samples)			raw maize (11 samples)			cottonseed meal (7 samples)			animal feed (13 samples)		
extraction method	extraction time (h)	reagent cost per extraction (US $)	yield (ng/mg)	A260/A280	A260/A230	yield (ng/mg)	A260/A280	A260/A230	yield (ng/mg)	A260/A280	A260/A230	yield (ng/mg)	A260/A280	A260/A230
CTAB	2.5−3	0.24	54−132	1.9−2.0	1.9−2.3	135−694	1.7−2.0	1.9−2.2	104−521	1.3−1.4	1.5−2.1	52−73	1.3−1.9	1.7−2.5
SDS	2−2.5	0.13	975−1025	1.8−2.0	1.7−2.2	340−989	2.0−2.1	1.8−2.3	785−1750	2.0−2.2	2.1−2.3	164−419	1.7−1.9	1.8−2.1
Kit	1.5−2	1.9	35−105	1.2−1.6	1.2−1.5	28−40	1.2−1.5	1.0−1.5	23−83	1.1−1.6	1.4−1.5	36−100	1.1−1.5	1.0−1.1

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Table 4. The Statistical Analysis on the DNA Yields (Mass of DNA/Mass of Sample) and Purities (A260/A280 and A260/A230) Obtained by the Three DNA Extraction Methods

variable	factors	DF	*F* value	*P*
yield	methods	2	66.75	<0.0001
	samples	5	2.24	0.06
	method × sample	9	1.79	0.09
A260/A280	methods	2	107.24	<0.0001
	samples	5	13.47	<0.0001
	method × sample	9	11.1	<0.0001
A260/A230	methods	2	34.66	<0.0001
	samples	5	1.07	<0.3911
	method × sample	9	1.62	<0.138

Table 5. Efficient Methods Used To Extract DNA from Different Feed Matrices

feed matrix	DNA extraction methods[a]		
	SDS	CTAB	Kit
soybean			
soybean seed	+++	++	+
soybean meal	+++	++	+
soybean powder	+++	−	−
maize seed	+++	++	+
cottonseed meal	+++	+	−
animal feed			
concentrated feed	+++	+	+
mixed feed for piglets (broilers, hens, fish)	+++	+	+
"Four Seas" mixed feed	+++	+	+

[a]+++, high efficiency; ++, moderate efficiency; +, low efficiency; −, non-efficiency.

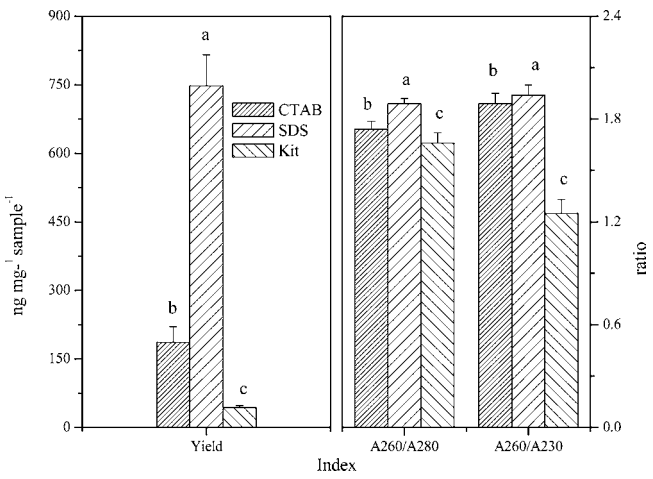

Figure 2. Mean differences among three methods in relation to DNA yield and purity (A260/A280 and A260/A230). The same and different lowercase letters represent no significant and significant (α = 0.05), respectively. Values are means ± SEM; different lowercase letters indicate significant differences among method groups.

results showed that the lowest number of targets detected by LAMP, HRCA, PCR, and real-time PCR assays were 48.5, 4.85, 485, and 9 copies, respectively (Figure 4 and Table 6).

III. Ranking of Feed Detection Methods. The LOD, hands-on time, reagent cost, ease of handling, interpretation of products, batch testing, and performance of the four detection systems are compared in Table 7. LAMP was easy to perform,

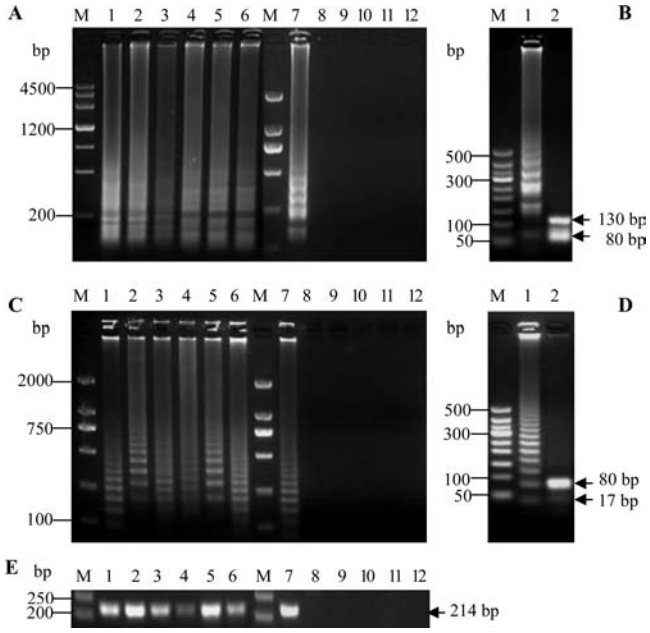

Figure 3. The specificity of primers in the LAMP-, HRCA-, PCR-amplified products by electrophoretic analysis. A. The specificity of the LAMP primers. M, DNA marker III (4500, 3000, 2000, 1200, 800, 500, 200 bp); A, C, E: lanes 1 and 7, plasmid molecules pTLH10 (positive control); lane 2, soybean seed (Brazil); lane 3, soybean seed (U.S.); lane 4, soybean powder (Guangxi, China); lane 5, soybean meal (Hebei, China); lane 6, "Four Seas" mixed feed (Beijing, China); lane 8, Bt176 maize seed ; lane 9, MON 810 maize seed; lane 10, Bt-cottonseed meal; lane 11, wild soybean seed (negative control); lane 12, no template (blank control). B. Restriction enzyme analysis of LAMP products with *Bsr*FI. M, 50-kb DNA ladder; lane 1, LAMP products; lane 2, *Bsr*FI-digested LAMP product (with two bands of 130 bp and 80 bp). C. The specificity of the HRCA primers. M, trans 2K DNA marker. D. Restriction enzyme analysis of HRCA products with *Ava*II. M, 50-kb DNA ladder; lane 1, HRCA products; lane 2, *Ava*II-digested HRCA product (with two bands of 80 bp and 17 bp). E. The specificity of the PCR primers (amplified a 214 bp product in length); M, 50-kb DNA ladder.

with this procedure judged to be slightly better because it had fewer reagent steps. Moreover, the LAMP was ranked slightly higher than HRCA and PCR because of the white precipitate of magnesium pyrophosphate, which is easily observed with the naked eyes during the interpretation of the products. The LAMP assay was only adaptable to single tests. The HRCA assay required more reagents and reaction procedures, thus requiring a greater cost and more hands-on time than LAMP. The HRCA assay was ranked lower for ease of handling. However, this assay was well suited for large-volume batch testing because the same procedure could be applied to analyze simultaneously many gene sequences in one tube. In comparison, the PCR assay required the most hands-on time because of cycling through the denaturation and annealing/extension cycles, and it was judged to be slightly more adaptable to batch testing. The real-time PCR was ranked highest for reagent cost and interpretation of products, but it was ranked the lowest for ease of handling.

DISCUSSION

Evaluation of Three DNA Extraction Methods. The detection or identification of GMOs by using PCR and other DNA-based methods depends on the ability to extract intact

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Table 6. The Specificity and LOD of the Real-Time PCR Method ($y = -3.449x + 37.655$, $R^2 = 0.999$)[a]

samples	mean ± SD Ct	signal ratio (positive signal/total reactions)
samples of feed		
S1	20.77 ± 0.29	9/9
S2	19.85 ± 0.11	9/9
S3	22.87 ± 0.09	9/9
S4	24.13 ± 0.15	9/9
S5	25.61 ± 0.09	9/9
S6	22.55 ± 0.17	9/9
S7	NA	0/9
S8	NA	0/9
S9	NA	0/9
S10	NA	0/9
S11	NA	0/9
plasmid pTLH10 DNA (copies)		
60	32.11 ± 0.27	9/9
30	33.95 ± 0.43	9/9
15	35.05 ± 0.49	9/9
9	36.24 ± 1.41	8/9
4.5	39.11	1/9

[a]The R^2 value obtained from the real-time PCR system was 0.999, and amplification efficiency was 95.0% according to the equation ($E = 10^{(-1/\text{slope})} - 1$), indicating a highly efficient reaction; S1, plasmid molecules pTLH10 (positive control); S2, soybean seed (Brazil); S3, soybean seed (U.S.); S4, soybean powder (Guangxi, China); S5, soybean meal (Hebei, China); S6, "Four Seas" mixed feed (Beijing, China); S7, Bt176 maize seed; S8, MON 810 maize seed; S9, Bt-cottonseed meal; S10, wild soybean seed (negative control); S11, no template (blank control); NA, no amplification. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold.

DNA from raw or processed feed. The extraction of DNA from raw materials is much easier when compared to processed feed in which the DNA is heavily degraded.[31] Protocols for extracting genomic DNA from feed have to contend with two major challenges: (1) deep processing leading to DNA degradation and (2) the complexity of the feed products due to different components with different properties. These challenges determine its extraction efficiency, which may alter during processing.[9]

A comparison of the three extraction methods in this study highlighted the different efficiencies in extraction and removing the inhibitors interfering in downstream detections. This may be due to the fact that three DNA extraction methods employed different DNA separation principles affecting DNA yield. The CTAB is a cationic detergent which binds DNA and favors the extraction of DNA−CTAB complex from proteins in lipid-phase extraction (chloroform−water). However, lysis of SDS is performed with an anionic detergent (SDS), and proteins are removed with salt precipitation. Unlike the above two principles, lysis of Kit is performed with proteinase K and guanidine hydrochloride, and binding of DNA to a resin.[6,8,33] Intact DNA was extracted successfully from soybean powder with the SDS assay, while no DNA from the other two methods was observed in the agarose gel (Figure 1). Of the various protocols tested in this study, the SDS method giving DNA yield more than 164 ng/mg sample was the most favorable method for extracting DNA from any type of feed samples (Tables 3 and 5 and Figure 2), and eliminated PCR inhibitors (such as hydroxybenzene−trichloromethane). The Kit protocol gave the lowest yield (less than 105 ng/mg sample) of DNA. The CTAB method comparably gave moderate yields (52−694 ng/mg sample) (Table 3 and Figure 2). These were inconsistent with previous studies, which have shown that the CTAB method was highly applicable for extracting total DNA from feed samples in comparison with the other methods. Nevertheless, previous DNA yields ranging from 19.7 to 174 ng/mg sample, obtained from feed sample, maize leaves, corn cob, and soybean seed, were generally lower than those obtained with the CTAB method in our study.[8,9,32−34]

The spectrophotometric analysis was also used to verify the quality of extracted DNA. The values of A260/A280 and A260/A230 ratios are shown in Table 3, which indicated that remarkable differences between purity data were obtained from the different extraction methods (Figure 2). The A260/A280 and A260/A230 values greater than 1.7 indicate that the DNA sample extracted by the SDS method gave a good DNA quality

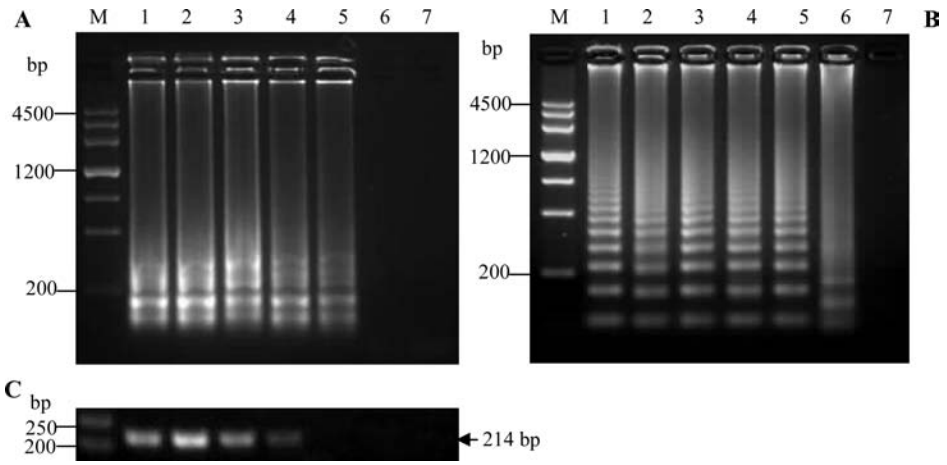

Figure 4. A comparison of the LODs of LAMP, HRCA and PCR. A. LOD of LAMP. B. LOD of HRCA. C. LOD of PCR (214 bp amplification products). M, DNA marker III (4500, 3000, 2000, 1200, 800, 500, 200 bp); lane 1, the linear plasmid molecules were diluted to 485,000 copies/μL; lane 2, 48,500 copies/μL of the linear plasmid molecules; lane 3, 4850 copies/μL of the linear plasmid molecules; lane 4, 485 copies/μL of the linear plasmid molecules; lane 5, 48.5 copies/μL of the linear plasmid molecules; lane 6, 4.85 copies/μL of the linear plasmid molecules; and lane 7, without DNA template in the reaction (blank control).

Table 7. The Ranking of the Four Amplification Methods for Detection of RRS[a]

method	LOD	hands-on time	reagent cost/run[b]	ease of handling	interpretation of products	batch testing	performance
LAMP	2	4	3	4	3	1	1
HRCA	4	3	2	2	2	4	4
PCR	1	1	4	3	1	3	2
real-time PCR	3	2	1	1	4	2	3

[a]The evaluation method is described in ref 28. The tests were ranked from 1 to 4 for each attribute, with 4 being the highest or best. [b]Calculated as follows: cost for equipment not included.

and should be suitable for amplification analysis (Table 3). A medium quality of the extracted DNA was obtained from the CTAB method (Figure 2). The DNA extracted with the Kit method had the lowest A260/A280 and A260/A230 ratios less than 1.6, and it is due either to the remaining buffer components from the lysis mixture or to some metabolites originating from the sample. The low purity may result from peptides and proteins, which adhere to the DNA, or too many phenol remnants, or contamination caused by the presence of carbohydrates, some solvents and salts. Results shown in Tables 4 and 5 and Figure 2 support the validity of the SDS method in the DNA extraction for many feed matrices. The Kit protocol was not able to eliminate contamination of chemical reagents. Results above demonstrate that the SDS method is highly applicable for extracting DNA from deeply processed feed samples, and that it is a quick, simple, and inexpensive method that does not utilize environmentally hazardous reagents for the isolation of genomic DNA from feed products.

Within the analyzed samples in Tables 3 and 4, the cottonseed meal sample produced the DNA yield of 104−521 ng/mg sample using the CTAB method. Joint Research Centre (2007) reported that the yield of DNA extracted with this method was 286 ng/mg cotton seed.[35] In our study, the highly processed animal feed sample had the poor DNA yields and quality, indicating high levels of DNA degradation. This finding agrees with the result of Bernardo et al. (2007), who showed that food manufacturing involves a number of processing steps that affect the integrity of genomic DNA in tissues or matrices of individual food ingredients.[33] In addition, the DNA yield of raw soybean or its derivatives and raw maize samples isolated by the SDS method ranged from 340 to 1025 ng/mg sample, which is much higher than the previous reports that ranged from 12.6 to 182.2 ng/mg sample.[6,33]

In addition, the operation time and the reagent cost of each methodology were also compared (Table 3). The Kit method would be most expensive and the fastest requiring 1.5−2 h, while the CTAB and SDS methods were cost-effective but may require more operative time (>2 h).

Evaluation of Four Amplification Methods. Several studies evaluating PCR, real-time PCR, LAMP, and HRCA for the detection of GMOs in feed have been published in recent years.[18,36−38] Each study used a single amplification method and different types of samples. In conventional PCR and real-time PCR, the LOD of RRS-specific detection was reported with values of 40 and 20.5 copies, respectively.[39] Guan et al. (2010) reported that the LOD by the established visual LAMP assay was approximately four copies of the haploid soybean genomic DNA.[17] To our knowledge, only one comparative LAMP and nested PCR study has been conducted for RRS, and in that study, the LODs of RRS by LAMP and nested PCR assays were 5 and 50 copies, respectively.[18] With the aim of finding the most efficient method for detecting RRS in feed, we compared four amplification methods and assessed their LODs and specificities. The LODs of HRCA, LAMP, and real-time PCR were 100, 10, and 53.9 times more sensitive than that of conventional PCR, which had its lowest LOD of 485 copies for the pTLH10 plasmid (Figure 4 and Table 6). The high sensitivity of HRCA, LAMP, and real-time PCR methods is particularly beneficial in the detection of RRS when low amounts of RRS are expected.

In addition, we evaluated the four detection methods for other criteria, such as cost, the ability to process large numbers of samples, and performance. Each method was compared by using a linear ranking scale (1 being the least desirable characteristic and 4 being the most desirable characteristic) (Table 7). This approach assumes that each attribute is of equal importance and that within each attribute the ranking scores are proportionally related on a linear scale. These assumptions may not be true for each laboratory and should not be generalized between laboratories. The multiattribute utility theory and the analytical hierarchy process can be applied to this type of decision-making in the laboratory. The application of multi-attribute utility theory or the analytical hierarchy process to the linearly ranked attributes allows individual laboratories to apply their own value or utility to these results.[28] If each attribute were equally desirable, then the ranking of the tests would be as follows (from best to worse): HRCA, LAMP, PCR, and real-time PCR.

Of the four techniques, the LAMP detection test was the most rapid and simple, providing an answer with the formation of a white precipitate that is observable by the naked eye within approximately 1 h (data not shown). However, this technique is modestly expensive and has a very high risk for contamination. Therefore, when handling LAMP-amplified products, the opening and closing of the reaction tube should be conducted in a different room from where the reagents and reaction mixtures are prepared. In contrast, the HRCA, PCR, and real-time PCR tests required several hours (2 to 3 h) to complete. The HRCA technique is the most sensitive, but it is moderately expensive and lengthy. This technique could easily be integrated into the work flow in the laboratory, and samples could be easily batched.[40−44] One of the characteristics of the HRCA method is the white precipitate of magnesium pyrophosphate that forms at the bottom of the reaction tube after centrifugation, which was an additional critical finding in this work. The amount of the precipitate was much less than that of the LAMP method. The greatest advantage of the HRCA method is that it is based on a target sequence spanning approximately 45 nucleotides (about 30 nt in the 5′ target and about 15 nt in the 3′ target of the PLP), compared to usually >100 nucleotides in an average PCR setting. This assay adapts well to the detection of small fragments of the target genes in deeply processed products in which some degree of DNA degradation has occurred, compared to the traditional PCR approach.[5,9,10,31] Another advantage of the HRCA technique is that its amplification primers in reaction are designed as

universal primers that can be used for many different targets. The primer sequences are present in the PLP backbone and not in the portion of the PLP that anneals to target, which is another advantage over the other three methodologies. The PCR assay was the least expensive and most versatile. However, the PCR assay has the lowest LOD and specificity of primers in the four detection methods tested in this study. Real-time PCR, which allows the monitoring of the accumulation of PCR product at any time point during the amplification reaction, was easy to interpret visually and was ranked highest for the interpretation of products. The real-time PCR is performed in a closed reaction vessel that requires no post-PCR processing, thereby minimizing the chances for cross contamination in the laboratory. But the real-time PCR method is the most expensive in terms of equipment, reagents, and time; now one set of regular equipment of real-time PCR costs about 100,000 US dollars; obviously this method would not be widely and quickly applied in practice for detection of products of GMO especially in developing countries, thus there is much room for improvement in the real-time PCR. On the another hand, there are more chances to open for other more suitable approaches. As newer techniques become available for the detection of GMOs, including the currently available HRCA and LAMP assays, comparative evaluations on the multiple attributes of the tests will assist us to make a decision in the rational choice of laboratory procedures for the detection of GMOs in feed or food.

In conclusion, the SDS method gave the highest yield and quality of DNA, and it could be used in different types of feed matrices. The DNA-based rapid amplification method (LAMP) was the most rapid and simple to run, taking only 1 h. The HRCA method was well suited to large-volume batch testing. The conventional PCR assay is the least expensive, and the real-time PCR method was ranked highest for its interpretation of products. In particular, LAMP/HRCA reactions can be conducted by using very simple equipment, such as a temperature controller (i.e., water bath) at a single point of temperature. The LAMP- and HRCA-based detection methods described and developed in this study may constitute a turning point in the detection of GM ingredients in feed. HRCA-based detection may be suitable for high-throughput analysis of complex mixtures in feed. Generally, the outcome of this analysis would be expected to help colleagues working in the laboratory to choose reasonably the most effective and appropriate DNA extraction and GM detection method that meets their needs.

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Notes

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