JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Loop-Mediated Isothermal Amplification (LAMP): Methods for Plant Species Identification in Food

Felix Focke, Ilka Haase,* and Markus Fischer

Hamburg School of Food Science, Institute of Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany

Supporting Information

ABSTRACT: Loop-mediated isothermal amplification (LAMP) is a DNA-based analytical method that can be used as an isothermal alternative to polymerase chain reaction (PCR). In comparison to PCR, the advantage of LAMP is the possibility to perform the isothermal reaction without any sophisticated technical equipment; only a water bath is needed, and naked eye detection is sufficient. Up to now, an application of LAMP methods for the detection of even closely related plant species in food or feed matrices has not been described, whereas a large number of PCR methods for that topic are cited in the literature. The aim of the study was the evaluation of LAMP-based methods for plant species identification with respect to method parameters such as R^2, LOD, and LOQ. An existing (real-time) PCR method (for the detection of spices) was used for comparison. It could be shown that the developed LAMP methods have potential as alternative strategies to PCR in DNA-based analysis.

KEYWORDS: *spice, mustard, celery, caraway, cumin, loop-mediated isothermal amplification, real-time*

INTRODUCTION

The loop-mediated isothermal amplification (LAMP) was first described by Notomi et al. in 2000.[1] Currently, numerous LAMP-based methods are described to detect pathogenic microorganisms, genetically modified ingredients, tumor tissues, and the sexing of bovine embryos.[2] With regard to the food and feed sector, mainly LAMP methods for the detection of foodborne pathogens[3−15] and genetically modified crops[16−25] are described. Only a few publications can be found for the detection of mycotoxigenic molds[26] and *Fusarium* species[27,28] or for the identification of meat species (pork, chicken, and beef).[29] LAMP methods for the detection of even closely related plant species in food or feed matrices are not described in the literature. Whereas the classical polymerase chain reaction (PCR) performance requires laboratory equipment such as a thermocycler and a detection unit (e.g., real-time detection, electrophoretic detection), LAMP methods have the potential to be performed independently of a laboratory because of the isothermal amplification conditions and the possibility of naked eye detection.[30] More precisely, thermal strand displacement is replaced by the high strand displacement activity of the polymerase, and a complex primer design enables a permanent regeneration of primer binding sites during the isothermal amplification process. For the amplification process, a set of four primers is needed (forward (F) and backward (B) outer primers and forward (FIP) and backward (BIP) inner primers). After an initial process, a central stem-loop DNA fragment is formed. This is the core structure for the ongoing LAMP reaction exhibiting single-stranded primer hybridization sites for FIP (H2) and BIP (H5). A distance of ∼40 bp between the two hybridization sites FIP-H1 and FIP-H2 of FIP and BIP-H4 and BIP-H5 of BIP, respectively, is recommended for an optimal LAMP process (Figure 1).[1] The LAMP reaction leads to a high formation of amplification products as well as magnesium pyrophosphate, which can both be used for a direct detection: (i) LAMP products can visually be detected using SYBR Green I and (ii) magnesium pyrophosphate formation leads to a visual detectable turbidity.[31,32] Alternatively, pyrophosphate can indirectly be detected using calcein and manganous ions.[30]

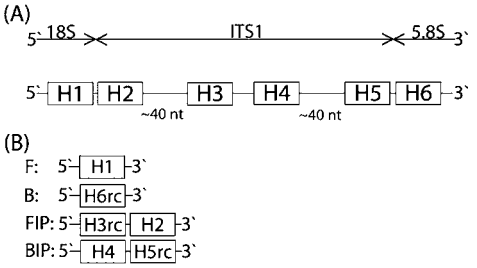

Figure 1. (A) Location of the LAMP primer binding sites within the rDNA. H1, hybridization site of F; H2 and H3, hybridization sites of FIP; H4 and H5, hybridization site of BIP; H6, hybridization site of B; rc, reverse and complement. (B) Schematic overview of the primer organization.

The aim of this study was the development of a LAMP-based method for the detection of plant species in food matrices. Prerequisites for the introduction of a new method like LAMP besides the commonly used PCR for food analysis are that the new LAMP assay (i) should be at least comparable to an existing PCR method in terms of specificity and sensitivity and (ii) should show some additional beneficial properties. This additional value of LAMP can be seen in its lower demands on laboratory equipment and the possibility of naked-eye detection.

Recently, a PCR method for the detection of even closely related spices such as caraway (*Carum carvi*) and cumin (*Cuminum cyminum*) or black and white mustard (*Brassica nigra* and *Sinapis alba*) was presented by the authors.[33]

Received: October 12, 2012
Revised: February 22, 2013
Accepted: February 23, 2013
Published: February 23, 2013

Table 1. LAMP Primers

Specific Amplifying Primer Sets (5′→3′)
FIP and BIP for caraway
CTACCTGGGGTTCGCCAACATGGG-TAAT-TCGAACCCTGCGATAGCAGAAC
CTACAAAATAATTCGGGCGTGGAATGCG-TTAT-GTTTGTGTTTTAGAATGACGCCACAG
FIP and BIP for cumin
ACCTGCCTTGGGTTTGCAAACAGGG-TAAT-TCGAATCCTGCAATAGCAGAATGAC
CAAAATCAACCGGGCGCTGACTGTGC-TTAT-ATTTGTGTTTCCAACTGACTTCGCTT
FIP and BIP for black mustard
CACGGAAATCGGCAGGCACGGAATC-TTTT-TCGTACCCTGGAAACAGAACGAC
AGACTTACTTAGGTCTCGGTCGGATCG-TTAA-GGCGAAAGCAGACTGTTTAGTTGC
FIP and BIP for white mustard
AACCACGGAATCGGCAGGCACAGAA-TTTT-TCGTATCCTGGAAACAGAACAACC
CCAGTACTTCGGTCTTGGTTGGGTCT-AATT-CGAACGCAGGCTACCTAGTTGAA
FIP and BIP for celery
CTACCAAAGGTTTGCAAACGGCGGACCA-ATAA-TTCGAATCCTGCGATAGCAGAATGAC
TTTGGTGGCCACCGGCCTACGAATCAT-ATTA-CCGCCGCCCCTAACGGGTT
F3 and B3 for caraway, cumin, black and white mustard, and celery
AACCTGCGGAAGGATCATTG
GATATCCGTTGTCGAGAGTC
Loop Primer Sets (5′→3′)
forward and reverse loop-primer for caraway
CCCGATGTTTTAACATGTTAGC
TGACCACTTTCCGATAGTCG
forward and reverse loop-primer for cumin
CTTGCCCATTGTTTTTACGTG
TTCGCTTCTCGTTCGCGC
forward and reverse loop primer for black mustard
GTGATGTTTCATCGTTCTCG
GGCACGAAAAGTGTCAAGG
forward and reverse loop primer for white mustard
AGAGTGATGTTTCATTGTTCTCG
GGCACGAAAAGTGTCAAGG
Universal Amplifying Primer Sets (5′→3′)
FIP and BIP for reference reactions of all five spices
TCAGGATTGGGTAATTTGCGCGCC-TATA-GGAGAGGGAGCCTGAGAAACGG
TGGTAATTGGAATGAGTACAATCTAAATCCC-TTAT-TATTGGAGCTGGAATTACCGCGG
F3 and B3 for universal amplification of all five spices
ACGGAGAATTAGGGTTCGATT
ACTGCAACAACTTAAATATACGC
forward and reverse loop primers for all spices
TGCTGCCTTCCTTGGATG
CAAGTCTGGTGCCAGCAG

Additionally, real-time PCR methods for the detection of even low milligram per kilogram levels of caraway, black and white mustard, and celery (*Apium graveolens*) in a bread mix were presented.[34] In this study, LAMP-based assays were developed for all four spices mentioned above, performed under the same conditions as described for the PCR studies, and were evaluated with respect to specificity, sensitivity, and reaction time. Real-time LAMP methods were analyzed by two different strategies: relative quantification using a reference sample with a defined amount of the relevant spice and absolute quantification using an external calibration curve.

MATERIALS AND METHODS

Samples. All materials (spices and white bread mix (wheat flour, table salt, dry yeast, malt extract, glucose, sodium stearoyl-2-lactylate, ascorbic acid, folic acid)) were purchased from the local market.

Powdered caraway and cumin, seeds of black and white mustard, and a powder of celery (made of freeze-dried fresh roots) were used. For quantitative studies, black mustard was mixed in different concentrations (12.5, 30.3, 48.3, 62.4, 74.5, and 85.6%, m/m) with caraway. Apart from that, the bread mix was spiked with 100000 mg/kg (10%) of each spice, respectively. These stock mixtures were homogenized accurately in a mill (M20 universal grinder, IKA-Werke GmbH & Co. KG, Staufen, Germany) for 5 min. A stepwise dilution series was made with pure baking mixture (100000, 50000, 10000, 2000, 400, 80, 16, 3.2, and 0.6 mg/kg). All mixtures were homogenized carefully by manual shaking with a tumbling mixer between each dilution step.

DNA Isolation and PCR Methods. These methods are performed as described previously.[33,34] Briefly, DNA was extracted from the spice matrix by a cetyltrimethylammonium bromide (CTAB) buffer and a mechanical grinding step. Lipophilic compounds were removed by a chloroform extraction step, and the DNA was purified using silica spin columns. Finally, the DNA was eluted with double-distilled, sterile water and set to 10 ng/μL.

LAMP Primer Design. Following Focke et al.,[33] the rDNA was used as the target sequence. The hybridization regions H2, H3, H4, and H5 of FIP and BIP are located in the variable sequence of the internal transcribed spacer 1 (ITS1) of the rDNA. H1 and H6 of the outer primers (F and B) are located at the end of the conserved 18S and at the beginning of the conserved 5.8S coding region (5′→3′) of the rDNA, respectively (Figure 1). Consequently, F3 and B3 are universally usable for the detection of all five spices. A universal amplifying LAMP primer set for all five spices was additionally designed for reference reactions. The binding region for the universal primer set used for normalization in relative quantification experiments is located in the 18S coding region of the rDNA showing high sequence homology for all spices. The primer sequences are given in Table 1. The hybridization sites for the spice primers can be seen in the alignment in Figure S1 of the Supporting Information.

Real-Time LAMP Assays. The reactions were performed in a total volume of 20 μL in a real-time thermocycler (IQ5 with IQ5 optical system software, version 2.0, Bio-Rad, München, Germany). For an initial denaturation step, 5 μL of isolated DNA (10 ng/μL), 1 μL of *Bst*-ThermoPol buffer (New England Biolabs, Frankfurt, Germany), 14% dimethyl sulfoxide (DMSO), FIP and BIP (4 μM each), F3 and B3 (0.5 μM each), and the loop primer (2 μM each, in case of loop-LAMP experiments) were incubated at 94 °C for 5 min in a total volume of 10 μL. After this initial denaturation step, 10 μL containing 30 U of *Bst* polymerase (New England Biolabs), 0.8 mM of each dNTP (Bioline, Luckenwalde, Germany), 1 μL of SYBR Green I (1:32000, Invitrogen, Karlsruhe, Germany), and 1 μL of *Bst*-ThermoPol were added to complete the reaction mix. The reaction was performed at 62 °C for about 2 h (reaction time was varied), and the polymerase was inactivated for 10 min at 80 °C.

In real-time LAMP experiments without initial denaturation, all components were added in a total volume of 20 μL.

Real-time LAMP experiments were also performed using *phi29* DNA polymerase (New England Biolabs), $Vent_R$ (exo-) DNA polymerase (New England Biolabs), or *Aac* polymerase (kindly provided by Mast Diagnostica, Reinfeld, Germany) with the corresponding buffer supplied by the manufacturer.

For quantitative analysis, two different strategies were pursued: for relative quantification, a reference sample (pure target spice) was used; for absolute quantification, calibration curves with six (black mustard in caraway) or nine (target spice in bread mix) standards were used.

LAMP Assays with Calcein Detection. The reactions were performed in a thermocycler (T3000, Biometra, Göttingen, Germany) as described above (without an initial denaturation step but with loop primers) in a total volume of 20 μL with the following differences: the amount of *Bst* polymerase was 8 U, and 1 μL of a calcein solution (Loopamp Fluorescent Detection Reagent, Mast Diagnostica GmbH) was added instead of SYBR Green I. Just as in real-time experiments, the reaction was performed at 62 °C for 2 h followed by a polymerase inactivation for 10 min at 80 °C.

Agarose Gel Electrophoresis (AGE). LAMP products were analyzed on 2% agarose gels using TAE buffer (40 mM Tris/acetate, 2 mM EDTA, pH 8.2) at 100 V (Powerpac 1000, Bio-Rad Laboratories Inc., Hercules, CA, USA). The DNA was stained using a 0.01% ethidium bromide solution and visualized under UV light (Biostep, Felix 1040, Biostep GmbH, Jahnsdorf, Germany).

RESULTS AND DISCUSSION

Primer Design. rDNA as template for a LAMP primer design had the advantage that the variable ITS1 region could be used for specific primer design (FIP and BIP), flanked by the highly conserved regions 18S and 5.8S suitable for the design of universal outer primers F and B (Figure 1). Additionally, the total length of the ITS1 region of the species under study varied in a range that was perfect for an optimal LAMP reaction (recommended length of 5′-H2→H5-3′ $\leq$ 300 bp[1]).

LAMP primers were designed on the basis of rDNA sequences, which were available from the National Center for Biotechnology Information (NCBI). In Figure S1 of the Supporting Information an alignment of representative sequences with highlighted primer binding sites of all five spices under study is shown. It is important to note that a successful amplification in the LAMP process is dependent on not only the presence of the hybridization sites of the LAMP-FIP and -BIP primers but also the distance between these regions enabling the formation of the essential loop structures. Positive hits for the four primer binding sites of each FIP/BIP system using the nucleotide Basic Local Alignment Search Tool (nBLAST) have therefore be manually evaluated with respect to their distances to each other. On the basis of these prerequisites, nBLAST was carried out with the complete sequence region starting with FIP and ending with BIP of each spice. For white mustard and caraway no in silico cross-reactivity with other food-relevant plant species could be observed. In the case of cumin, a second *Cuminum* species, *C. setifolium* ("white cumin") showed homologous rDNA sequences, but is not known to be used as a spice.[35] In the case of celery, only one (*A. sellowianum*) to four single-nucleotide polymorphisms (*A. prostratum*) are observed in the hybridization regions of eight *Apium* species, which may result in primer cross-reactivities. However, to our knowledge these *Apium* species have either no (*A. australe*, *A. chilense*, *A. commersonii*, *A. fernandezianum*, *A. panul*, *A. sellowianum*) or only regional (*A. insulare* = Flinder's Island celery, *A. prostratum* = sea celery) food relevance. Homologies were also found using the sequence of black mustard in nBLAST. For the two food-relevant *Brassica* species, *B. juncea* (brown mustard) and *B. carinata* (Ethiopian mustard), the LAMP primers show in silico cross-reactivities. Consequently, these primers cannot be defined as "specific" for black mustard, but as "selective" for all three mustards of the *Brassica* familiy. It should be noted that for a final evaluation of the primer specificity, experiments with further samples are mandatory for all spices. The focus on this study was the comparison of a new LAMP method with an existing PCR method.

Optimization of LAMP Conditions. LAMP reactions were optimized with respect to time and ingredient concentrations. The results were evaluated by (i) the presence of reaction products (AGE) and (ii) the reaction kinetic (real-time LAMP).

Table 2 represents an overview of the optimized parameters of the LAMP conditions and the results. Four DNA polymerases were tested, which were commercially available at the time of the study: *Bst* polymerase, *Aac* polymerase, *phi29* polymerase, and $Vent_R$ (exo-)polymerase. *Bst* polymerase is the most commonly used enzyme for LAMP reactions. Using this enzyme, the typical LAMP reaction pattern was observed in AGE (Figure 2). *Aac*

Table 2. LAMP Optimization Parameters

parameter	conditions	optimum
polymerase	*Aac*, *Bst*, phi29, Vent	*Bst*
polymerase activity	8−50 U	30 U
FIP/BIP concentration	1−5 μM	2 μM
Mg^{2+} concentration	2−8 mM	2 mM
enhancer	DMSO, betaine	7% DMSO[a]
temperature	60−65 °C	62 °C
initial denaturation	94 °C or pH 12	thermal[a]
loop primer		yes[b]

[a]Only for real-time LAMP. [b]Only for conventional LAMP.

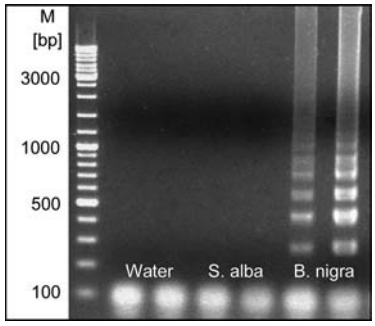

Figure 2. LAMP reaction pattern exemplarily shown from black mustard (*Brassica nigra*) with negative controls (water and white mustard (*Sinapsis alba*)). Each sample was run in duplicate.

polymerase is an enzyme with properties similar to those of *Bst* polymerase and is used for amplification processes that are related to LAMP (e.g., smart amplification process version 2).[36,37] In our studies it could be observed that when the *Aac* polymerase was used, longer reaction times were needed to reach the same DNA content in real-time experiments. For the *phi*29 enzyme, a very strong displacement activity is described. However, it is known that the mesophilic temperature optimum of this polymerase may not fit the primer melting temperatures.[38−40] In LAMP experiments with the *phi29* enzyme, no reaction products were observed, confirming the supposed problems caused by differences in primer melting temperatures (>60 °C) and polymerase temperature optimum (<40 °C). The advantage of the $Vent_R$ (exo-)polymerase is its heat resistance, which may enable a thermal denaturation without splitting the reaction mixes.[41] However, probably due to the reported lower strand displacement activity, no amplification could be observed using $Vent_R$ (exo-)polymerase in our studies. In summary, the *Bst* polymerase gave the best results and was chosen for further experiments.

In the next step, the *Bst* polymerase activity was varied between 8 and 50 U (Table 2). Amplification products could be observed with all enzyme activities, but a shorter reaction time was achieved with polymerase activities up to 30 U, whereas between 30 and 50 U, no improvement was observed. Consequently, 30 U per reaction was defined as the optimal activity of polymerase for the real-time LAMP (8 U for conventional LAMP).

In the same manner, the concentrations of FIP and BIP were optimized in a range of 1−5 μM. An optimum at 2 μM could be determined. With lower or higher concentrations, the reaction was slower or not reliably finalized.

The use of enhancers for the LAMP reaction has been well established as support for strand displacement.[42,43] Betaine is a commonly used enhancer for the LAMP reaction, and DMSO is one of the most used enhancers in PCR reactions. With an addition of DMSO, an acceleration of the reaction with an optimum at a concentration of 7% could be observed (Figure 3). In contrast, the addition of betaine to the reaction mix showed no comparable success.

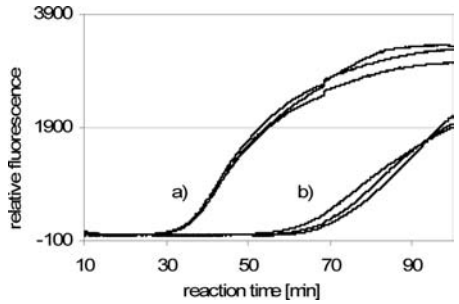

Figure 3. Reaction curves of real-time LAMP experiments (DNA isolate of caraway): (a) with DMSO (7%); (b) without DMSO.

An initial denaturation step as well as the use of loop primers shortens the reaction time, but both are described as not essential for the LAMP reaction.[44,45] The results of LAMP experiments with and without initial denaturation step and/or loop primers confirmed these observations, as can be seen in Figure 4. The use

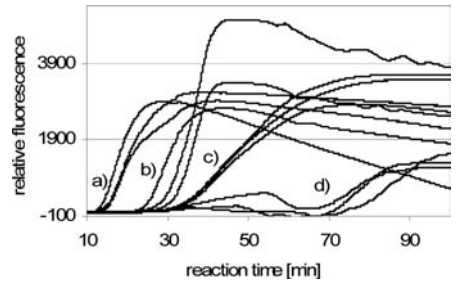

Figure 4. Reaction curves of real-time LAMP experiments (DNA isolate of caraway): (a) with initial denaturation/with loop primers; (b) without initial denaturation/with loop primers; (c) with initial denaturation/without loop primers; (d) without initial denaturation/without loop primers.

of loop primers accelerated the reaction but also increased the variation of the LAMP reaction, prohibiting a quantitative data analysis. Additionally, it could be observed that using loop primers, DMSO did not significantly accelerate the reaction (data not shown). Therefore, quantitative real-time experiments were performed with an initial denaturation step (+ DMSO) but without loop primers, whereas for conventional LAMP reactions, only loop primers (no DMSO, no denaturation step) were used. Moreover, in these conventional reactions, an activity of 8 U of *Bst* polymerase per reaction was sufficient.

As an alternative for the heat denaturation step, a chemical denaturation (pH 12, adjusted with potassium hydroxide) was tested. After 10 min of denaturation time, the reaction batches were neutralized before the reaction was started. However, after this chemical denaturation, no amplification could be observed.

Validation of LAMP Experiments. To validate the results, the data of the real-time LAMP were evaluated quantitatively. Two methods for the calculation were compared.

(i) The so-called ΔT_t method (T_t = threshold time) was derived on the basis of the ΔC_t method in real-time PCR.[46] In this case, for all PCR reactions an efficiency of $E = 1$ is assumed, allowing the simplification of eq 1:

$$C = \frac{N_0(S)}{N_0(R)} = \frac{(1 + E(S))^{C_t(S)}}{(1 + E(R))^{C_t(R)}} = \frac{2^{C_t(S)}}{2^{C_t(R)}} = 2^{(C_t(S) - C_t(R))} = 2^{\Delta C_t} \quad (1)$$

C is the relative content of the target, $N_0(S)$ is the amount of the target in the sample, $N_0(R)$ is the amount of the target in the reference sample, $C_t(S)$ is the C_t value of the target DNA in the sample, and $C_t(R)$ is the C_t value of the reference sample.

For LAMP reactions, such reaction efficiency cannot be defined, as amplification does not proceed in cycles. Nevertheless, if the same kinetics is assumed for the LAMP reaction in all samples, eq 1 can be adopted (eq 2):

$$C = 2^{(T_t(S) - T_t(R))} = 2^{\Delta T_t} \quad (2)$$

$T_t(S)$ is the T_t value of the target DNA in the sample, and $T_t(R)$ is the T_t value of a reference sample (e.g., pure spice standard).

Using this mathematical relationship and a pure black mustard sample as reference, six different caraway samples containing different amounts of black mustard were analyzed. In Figure S2A of the Supporting Information the recovery function of these LAMP experiments is shown, indicating a slight underestimation of the spice content.

(ii) In real-time PCR experiments, additional normalization of a data set is achieved by the use of an internal reference amplificate (RA), resulting in eq 3 ($\Delta\Delta C_t$ method):

$$C = 2^{[C_{t,RA}(S) - C_t(S)] - [C_{t,RA}(R) - C_t(R)]} = 2^{\Delta C_t(S) - \Delta C_t(R)} = 2^{\Delta\Delta C_t} \quad (3)$$

The same equation can be adopted to real-time LAMP experiments (eq 4):

$$C = 2^{[T_{t,RA}(S) - T_t(S)] - [T_{t,RA}(R) - T_t(R)]} = 2^{\Delta T_t(S) - \Delta T_t(R)} = 2^{\Delta\Delta T_t} \quad (4)$$

This $\Delta\Delta T_t$ method with internal reference amplification (using the universal primer set) was also used for quantification of black mustard contents in caraway but did not give results in the expected range (data not shown). This was probably caused by different "efficiencies" of the target and the internal reference LAMP reactions.

(iii) Additionally, an absolute quantification strategy was performed. The linear equation of PCR calibration experiments is given in eq 5

$$C_t = -\frac{1}{\log(1 + E)} \times \log N_0 + \frac{\log N_{C_t}}{\log(1 + E)} = b \times \log N_0 + a \quad (5)$$

where N_0 is the absolute content of the target, b is the slope, and a is the intercept of the linear equation. Assuming again that the reaction efficiency of the LAMP experiments is unknown but identical in all reactions, eq 3 can be adopted for LAMP calibration (eq 6) and used for the calculation of target amount $N_0(S)$ in unknown samples (eq 7):

$$T_t = b \times \log N_0 + a \quad (6)$$

$$N_0(S) = 10^{T_t(T) - a/b} \quad (7)$$

For evaluation of the absolute quantification strategy, the T_t values of the LAMP experiments with the six different caraway samples containing different amounts of black mustard were used for a linear calibration according to eq 6. With each T_t value of the concentration steps the concentration was calculated using eq 7 and compared with the real value, which is illustrated as recovery function in Figure S2B of the Supporting Information. Compared to the results of the relative quantification, the recovery rates are significantly better. This is due to the fact that matrix effects with influence on the reaction efficiency (slope b) are considered in this evaluation strategy.

Table 3 gives an overview of the results of the real-time LAMP experiments with absolute quantification of spices in a matrix of bread mix. Parameters (R^2, limit of detection (LOD), limit of quantification (LOQ)) of the accordant PCR experiments[34] are given for comparison. Contents from 16 to 100000 mg/kg of spices could be detected in the bread mix. In this range of 4 orders of magnitude, also quantification was possible. However, it should be noted that for a reliable quantitative analysis, adequate material of the target spice as well as of the matrix is necessary. It can be seen that real-time LAMP offers comparable results to previously shown results of the real-time PCR.[34] The calibration parameters (e.g., LOD/LOQ, R^2) of both methods are similar. These data indicate that LAMP has the same potential for food analysis as PCR.

Table 3. Results of the Real-Time LAMP in a Matrix of Bread Mix

actual content (mg/kg)	calculated content (mg/kg)	recovery (%)	positive reactions
Black Mustard			
0.6	–	–	0/6
3.2	–	-	2/6
16	13.9	86.7	6/6
80	95.9	119.9	6/6
400	370	92.5	6/6
2000	2200	112.4	6/6
10000	9900	98.9	6/6
50000	46800	93.6	6/6
100000	63200	63.2	6/6
R^2 = 0.998; LOD = 16 mg/kg; LOQ = 16 mg/kg			
comparable PCR data:[34] R^2 = 0.993; LOD = 3.2 mg/kg; LOQ = 16 mg/kg			
White Mustard			
0.6	–	–	0/6
3.2	–	–	0/6
16	20.7	129.6	6/6
80	49.8	62.3	6/6
400	403	100.7	6/6
2000	2300	113.2	6/6
10000	12900	128.8	6/6
50000	50700	101.4	6/6
100000	83200	83.3	6/6
R^2 = 0.993; LOD = 16 mg/kg; LOQ = 16 mg/kg			
comparable PCR data:[34] R^2 = 0.996; LOD = 16 mg/kg; LOQ = 16 mg/kg			
Celery			
0.6	–	–	0/6
3.2	–	–	1/6
16	20.2	126.4	6/6
80	49.3	61.6	6/6
400	472	118.0	6/6
2000	2100	104.9	6/6
10000	12000	120.0	6/6
50000	43400	86.8	6/6
100000	44300	44.3	6/6
R^2 = 0.998; LOD = 16 mg/kg; LOQ = 16 mg/kg			
comparable PCR data:[34] R^2 = 0.998; LOD = 16 mg/kg; LOQ = 80 mg/kg			

Detection of Conventional LAMP Experiments. The advantage of LAMP over PCR is the possibility of naked-eye detection of the turbidity caused by magnesium pyrophosphate or the color change using calcein.[30] To prove the possibility of naked-eye detection for the developed method, the LAMP reactions were carried out under conventional conditions with the addition of calcein. Figure S3 in the Supporting Information shows that LAMP could be easily detected by the naked eye. With calcein and UV light (Supporting Information, Figure S3a), positive reactions showed an intense green fluorescence. With white light (Supporting Information, Figure S3b), a colorimetric change from red to green could be observed in positive reactions (colors not shown). Additionally, without calcein, the positive reactions could be identified by turbidity (Figure S3c).

In conclusion, the detection of ingredients and contaminations via LAMP has great potential as a tool for food analysis. LAMP shows a similar performance compared to PCR with the advantage of possible naked-eye detection.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1. Alignment of the rDNA regions (end of 18S rDNA, ITS1, beginning of 5.8S rDNA) of cumin (U78362.2), caraway (AF077878.1), celery (FJ986043.1), white mustard (AF28106.1), and black mustard (GQ268057.1), which were used for the LAMP primer design. Figure S2. Quantification of black mustard in caraway using two different strategies: (A) ΔT_t method; (B) calibration curve (exemplary for black mustard in a mixture with caraway). Figure S3. Detection of LAMP experiments: (a) with calcein and UV light; (b) with calcein and white light; (c) without calcein under white light. From left to right: white mustard (positive/negative) and black mustard (positive/negative). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49-40-42838-4379. Fax: +49-40-42838-4342. E-mail: ilka.haase@chemie.uni-hamburg.de.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AGE, agarose gel electrophoresis; DMSO, dimethyl sulfoxide; F and B, forward and backward outer primers; FIP and BIP, forward and backward inner primers; ITS1, internal transcribed spacer 1; LAMP, loop-mediated isothermal amplification; LOD, limit of detection; LOQ, limit of quantification; nBLAST, nucleotide Basic Local Alignment Search Tool; NCBI, National Center for Biotechnology Information; PCR, polymerase chain reaction; rDNA, rDNA; T_t, threshold time

■ REFERENCES

(1) Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* **2000**, *28*, E63.

(2) Fu, S.; Qu, G.; Guo, S.; Ma, L.; Zhang, N.; Zhang, S.; Gao, S.; Shen, Z. Applications of loop-mediated isothermal DNA amplification. *Appl. Biochem. Biotechnol.* **2011**, *163*, 845−850.

(3) Jiang, K.; Lv, Q.; Zhang, D.; Gao, X.; Zhao, Y.; Li, M.; Liu, L.; Wu, J.; Lu, X.; Luo, C. A novel, sensitive, accurate multiplex loop-mediated isothermal amplification method for detection of *Salmonella* spp., *Shigella* spp. and *Staphylococcus aureus* in food. *J. Food Agric. Environ.* **2012**, *10*, 252−256.

(4) Li, C. H.; Ying, Q.; Su, X. R.; Li, T. W. Development and application of reverse transcription loop-mediated isothermal amplification for detecting live *Shewanella putrefaciens* in preserved fish sample. *J. Food Sci.* **2012**, *77*, M226−M230.

(5) Nagarajappa, S.; Thakur, M. S.; Manonmani, H. K. Detection of enterotoxigenic *Staphylococci* by loop-mediated isothermal amplification method. *J. Food Saf.* **2012**, *32*, 59−65.

(6) Ravan, H.; Yazdanparast, R. Development and evaluation of a loop-mediated isothermal amplification method in conjunction with an enzyme-linked immunosorbent assay for specific detection of *Salmonella* serogroup D. *Anal. Chim. Acta* **2012**, *733*, 64−70.

(7) Song, L. Y.; Li, J. T.; Hou, S. P.; Li, X. D.; Chen, S. Y. Establishment of loop-mediated isothermal amplification (LAMP) for rapid detection of *Brucella* spp. and application to milk and blood samples. *J. Microbiol. Methods* **2012**, *90*, 292−297.

(8) Sowmya, N.; Thakur, M. S.; Manonmani, H. K. Rapid and simple DNA extraction method for the detection of enterotoxigenic *Staphylococcus aureus* directly from food samples: comparison of PCR and LAMP methods. *J. Appl. Microbiol.* **2012**, *113*, 106−113.

(9) Techathuvanan, C.; D'Souza, D. H. Reverse-transcriptase loop-mediated isothermal amplification as a rapid screening/monitoring tool for *Salmonella enterica* detection in liquid whole eggs. *J. Food Sci.* **2012**, *77*, M200−M205.

(10) Wan, C. X.; Yang, Y. J.; Xu, H. Y.; Aguilar, Z. P.; Liu, C. M.; Lai, W. H.; Xiong, Y. H.; Xu, F.; Wei, H. Development of a propidium monoazide treatment combined with loop-mediated isothermal amplification (PMA-LAMP) assay for rapid detection of viable *Listeria monocytogenes*. *Int. J. Food Sci. Technol.* **2012**, *47*, 2460−2467.

(11) Wang, D. G.; Chen, J. L.; Xiao, F. G.; Zhang, G. P.; Huo, G. C. Rapid and sensitive detection of *Escherichia coli* 0157 in raw milk by loop-mediated isothermal amplification combined with chemosensor. *Milk Sci. Int.* **2012**, *67*, 385−388.

(12) Wang, F.; Jiang, L.; Yang, Q.; Prinyawiwatkul, W.; Ge, B. Rapid and specific detection of *Escherichia coli* serogroups O26, O45, O103, O111, O121, O145, and O157 in ground beef, beef trim, and produce by loop-mediated isothermal amplification. *Appl. Environ. Microbiol.* **2012**, *78*, 2727−2736.

(13) Xu, Z. B.; Li, L.; Chu, J.; Peters, B. M.; Harris, M. L.; Li, B.; Shi, L.; Shirtliff, M. E. Development and application of loop-mediated isothermal amplification assays on rapid detection of various types of *Staphylococci* strains. *Food Res. Int.* **2012**, *47*, 166−173.

(14) Zhong, Q.; Wang, L.; Wang, B.; Chen, H. Loop-mediated isothermal amplification method for rapid detection of *Shigella dysenteriae*. *Appl. Mech. Mater.* **2012**, *140*, 369−373.

(15) Ziros, P. G.; Kokkinos, P. A.; Papanotas, K.; Vantarakis, A. Loop-mediated isothermal amplification (LAMP) for the detection of *Salmonella* spp. isolated from different food types. *J. Microbiol. Biotechnol. Food Sci.* **2012**, *2*, 152−161.

(16) Chen, J.; Huang, C.; Zhang, X.; Yu, R.; Wu, Z. Detection of herbicide-resistant maize by using loop-mediated isothermal amplification of the pat selectable marker gene. *Afr. J. Biotechnol.* **2011**, *10*, 17055−17061.

(17) Chen, L.; Guo, J.; Wang, Q.; Kai, G.; Yang, L. Development of the visual loop-mediated isothermal amplification assays for seven genetically modified maize events and their application in practical samples analysis. *J. Agric. Food Chem.* **2011**, *59*, 5914−5918.

(18) Chen, X.; Wang, X.; Jin, N.; Zhou, Y.; Huang, S.; Miao, Q.; Zhu, Q.; Xu, J. Endpoint visual detection of three genetically modified rice events by loop-mediated isothermal amplification. *Int. J. Mol. Sci.* **2012**, *13*, 14421−14433.

(19) Fukuta, S.; Mizukami, Y.; Ishida, A.; Ueda, J.; Hasegawa, M.; Hayashi, I.; Hashimoto, M.; Kanbe, M. Real-time loop-mediated isothermal amplification for the CaMV-35S promoter as a screening method for genetically modified organisms. *Eur. Food Res. Technol.* **2004**, *218*, 496−500.

(20) Kiddle, G.; Hardinge, P.; Buttigieg, N.; Gandelman, O.; Pereira, C.; McElgunn, C. J.; Rizzoli, M.; Jackson, R.; Appleton, N.; Moore, C.;

Tisi, L. C.; Murray, J. A. GMO detection using a bioluminescent real time reporter (BART) of loop mediated isothermal amplification (LAMP) suitable for field use. *BMC Biotechnol.* **2012**, *12*, 15.
(21) Lee, D.; La Mura, M.; Allnutt, T.; Powell, W.; Greenland, A. Isothermal amplification of genetically modified DNA sequences directly from plant tissues lowers the barriers to high-throughput and field-based genotyping. *J. Agric. Food Chem.* **2009**, *57*, 9400−9402.
(22) Lee, D.; La Mura, M.; Allnutt, T. R.; Powell, W. Detection of genetically modified organisms (GMOs) using isothermal amplification of target DNA sequences. *BMC Biotechnol.* **2009**, *9*, 7.
(23) Liu, M.; Luo, Y.; Tao, R.; He, R.; Jiang, K. Y.; Wang, B. J.; Wang, L. Sensitive and rapid detection of genetic modified soybean (Roundup Ready) by loop-mediated isothermal amplification. *Biosci., Biotechnol., Biochem.* **2009**, *73*, 2365−2369.
(24) Wang, X.; Teng, D.; Tian, F.; Guan, Q.; Wang, J. Comparison of three DNA extraction methods for feed products and four amplification methods for the 5′-junction fragment of Roundup Ready soybean. *J. Agric. Food Chem.* **2012**, *60*, 4586−4595.
(25) Zhang, M.; Liu, Y.; Chen, L.; Quan, S.; Jiang, S.; Zhang, D.; Yang, L. One simple DNA extraction device and its combination with modified visual loop-mediated isothermal amplification for rapid on-field detection of genetically modified organisms. *Anal. Chem.* **2013**, *85*, 75−82.
(26) Luo, J.; Vogel, R. F.; Niessen, L. Development and application of a loop-mediated isothermal amplification assay for rapid identification of aflatoxigenic molds and their detection in food samples. *Int. J. Food Microbiol.* **2012**, *159*, 214−224.
(27) Denschlag, C.; Vogel, R. F.; Niessen, L. Hyd5 gene-based detection of the major gushing-inducing *Fusarium* spp. in a loop-mediated isothermal amplification (LAMP) assay. *Int. J. Food Microbiol.* **2012**, *156*, 189−196.
(28) Niessen, L.; Grafenhan, T.; Vogel, R. F. ATP citrate lyase 1 (acl1) gene-based loop-mediated amplification assay for the detection of the *Fusarium tricinctum* species complex in pure cultures and in cereal samples. *Int. J. Food Microbiol.* **2012**, *158*, 171−185.
(29) Minhaz, U. A.; Quamrul, H.; M. Mosharraf, H.; Masato, S.; Eiichi, T. Meat species identification based on the loop mediated isothermal amplification and electrochemical DNA sensor. *Food Control* **2010**, *21*, 599−605.
(30) Tomita, N.; Mori, Y.; Kanda, H.; Notomi, T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat. Protoc.* **2008**, *3*, 877−882.
(31) Mori, Y.; Kitao, M.; Tomita, N.; Notomi, T. Real-time turbidimetry of LAMP reaction for quantifying template DNA. *J. Biochem. Biophys. Methods* **2004**, *59*, 145−157.
(32) Mori, Y.; Nagamine, K.; Tomita, N.; Notomi, T. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem. Biophys. Res. Commun.* **2001**, *289*, 150−154.
(33) Focke, F.; Haase, I.; Fischer, M. DNA-based identification of spices: DNA isolation, whole genome amplification, and polymerase chain reaction. *J. Agric. Food Chem.* **2011**, *59*, 513−520.
(34) Focke, F.; Haase, I.; Fischer, M. Determination of plant contaminants in food using real-time PCR. *Dtsch. Lebensmittel. Rundsch.* **2011**, *107*, 221−224.
(35) Khosravinasab, Z.; Safarnejad, A. Comparison of biochemical characterization in *Cuminum cyminum* and *Cuminum setifolium* seeds. *Int. J. Sci. Nat.* **2011**, *2*, 874−877.
(36) Araki, T.; Shimizu, K.; Nakamura, K.; Nakamura, T.; Mitani, Y.; Obayashi, K.; Fujita, Y.; Kakegawa, S.; Miyamae, Y.; Kaira, K.; Ishidao, T.; Lezhava, A.; Hayashizaki, Y.; Takeyoshi, I.; Yamamoto, K. Usefulness of peptide nucleic acid (PNA)-clamp smart amplification process version 2 (SmartAmp2) for clinical diagnosis of KRAS codon 12 mutations in lung adenocarcinoma: comparison of PNA-Clamp SmartAmp2 and PCR-related methods. *J. Mol. Diagn.* **2010**, *12*, 118−124.
(37) Mitani, Y.; Lezhava, A.; Kawai, Y.; Kikuchi, T.; Oguchi-Katayama, A.; Kogo, Y.; Itoh, M.; Miyagi, T.; Takakura, H.; Hoshi, K.; Kato, C.; Arakawa, T.; Shibata, K.; Fukui, K.; Masui, R.; Kuramitsu, S.; Kiyotani, K.; Chalk, A.; Tsunekawa, K.; Murakami, M.; Kamataki, T.; Oka, T.; Shimada, H.; Cizdziel, P. E.; Hayashizaki, Y. Rapid SNP diagnostics using asymmetric isothermal amplification and a new mismatch-suppression technology. *Nat. Methods* **2007**, *4*, 257−262.
(38) Blanco, L.; Bernad, A.; Lazaro, J. M.; Martin, G.; Garmendia, C.; Salas, M. Highly efficient DNA-synthesis by the phage Phi-29 DNA-polymerase − symmetrical mode of DNA-replication. *J. Biol. Chem.* **1989**, *264*, 8935−8940.
(39) Garmendia, C.; Bernad, A.; Esteban, J. A.; Blanco, L.; Salas, M. The bacteriophage-Phi-29 DNA-polymerase, a proofreading enzyme. *J. Biol. Chem.* **1992**, *267*, 2594−2599.
(40) Kim, J.; Easley, C. J. Isothermal DNA amplification in bioanalysis: strategies and applications. *Bioanalysis* **2011**, *3*, 227−239.
(41) Kong, H. M.; Kucera, R. B.; Jack, W. E. Characterization of a DNA-polymerase from the hyperthermophile Archaea *Thermococcus litoralis* - Vent DNA-polymerase, steady-state kinetics, thermal-stability, processivity, strand displacement, and exonuclease activities. *J. Biol. Chem.* **1993**, *268*, 1965−1975.
(42) Henke, W.; Herdel, K.; Jung, K.; Schnorr, D.; Loening, S. A. Betaine improves the PCR amplification of GC-rich DNA sequences. *Nucleic Acids Res.* **1997**, *25*, 3957−3958.
(43) Ralser, M.; Querfurth, R.; Warnatz, H. J.; Lehrach, H.; Yaspo, M. L.; Krobitsch, S. An efficient and economic enhancer mix for PCR. *Biochem. Biophys. Res. Commun.* **2006**, *347*, 747−751.
(44) Nagamine, K.; Hase, T.; Notomi, T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol. Cell. Probes* **2002**, *16*, 223−229.
(45) Nagamine, K.; Watanabe, K.; Ohtsuka, K.; Hase, T.; Notomi, T. Loop-mediated isothermal amplification reaction using a nondenatured template. *Clin. Chem.* **2001**, *47*, 1742−1743.
(46) Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45.