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Point-of-care suitable identification of the adulterants *Carthamus tinctorius* and *Curcuma longa* in *Crocus sativus* based on loop-mediated isothermal amplification (LAMP) and lateral-flow-assay (LFA)

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ABSTRACT

Saffron (*Crocus sativus*) is one of the most valuable spices and therefore exceptionally vulnerable to fraudulent practices. Established methods for authentication depend on laboratory infrastructure and qualified personnel. To circumvent this dependency, a cost-effective test must be developed that allows the detection of foreign plant material rapidly and in a manner suitable for in-field analysis.

The LAMP reaction is widely used for point-of-care diagnostic because of its robustness, reaction speed, and the independence from laboratory environment. Safflower (*Carthamus tinctorius*) as well as turmeric (*Curcuma longa*) is commonly used to stretch saffron. To detect adulteration, a primer set selective for the internal transcribed spacer sequence of safflower was designed in the present study and used in combination with a primerset selective for turmeric published by Sheu et al., in 2021.

Here we present a LAMP-based rapid test system for detection of the adulterants turmeric and safflower in saffron samples. The developed rapid test system can be performed within 25 min and consists of an isolation protocol, a LAMP-assay, and visualization of the test result with a lateral-flow-assay.

1. Introduction

Saffron, the dried stigma of the plant *Crocus sativus*, is traditionally used as a spice in several dishes and drinks and is valued for its flavor and coloring properties. Furthermore, it is used in traditional Chinese medicine and Ayurveda for the treatment of various diseases and ailments. The health-promoting properties have been investigated and could be partly attributed to the antioxidant and radical scavenging properties of the secondary metabolites crocin, picocrocin and safranal, which quantities are also a quality criterion for saffron (Kumar et al., 2011; Zhao et al., 2016). Herbs and spices have historically been traded as a valuable commodity, saffron being one of the most expensive examples. The price for high grade saffron can easily exceed 2000 USD per kilogram, due to the labor and time intensive culturing process and therefore limited production. To produce 1 kg of saffron, the stigmata of approximately 150,000 flowers are harvested and processed manually (Kumar et al., 2011; Shahnoushi, Abolhassani, Kavakebi, Reed, & Saghaian, 2020).

Financially motivated crime in the food sector is known as food fraud and harms both retailers and consumers economically and in terms of health. Herbs and spices are especially vulnerable to food fraud due to their long and complex supply chain, as each intermediary increases the risk of adulteration (Maquet et al., 2021; Sasikumar, Swetha, Parvathy, & Sheeja, 2016). Since it is a low volume, high price commodity, high profit margin can be achieved by adulterating saffron with a cheaper surrogate.

According to the 2021 published Technical Report of the Joint Research Centre (JRC), 11% of the 141 analyzed saffron samples were found suspicious of adulteration although no trend could be observed at which point of the supply chain adulteration predominantly happened. The majority of suspicious samples contained non-declared plant material, mainly parts of the plant safflower (*Carthamus tinctorius*), which is about 80–100 times cheaper than saffron (Ma, Zhu, Li, Dong, & Tsim, 2001; Maquet et al., 2021). Turmeric (*Curcuma longa*) was also reported as a common adulterant in saffron. Turmeric is the powdered rhizome of the *Curcuma longa* plant and sometimes misleadingly trademarked as “Indian Saffron” (Kumari, Jaiswal, & Tripathy, 2021; Marieschi, Torelli, & Bruni, 2012; Petrakis, Cagliani, Polissiou, & Consonni, 2015; Sasikumar et al., 2016; Zhao et al., 2016).

Various methods are currently available for the authentication of saffron and the detection of adulteration (Kumari et al., 2021). These

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Abbreviations

6-Fam/FITC	Fluorescein/Fluorescein-5-isothiocyanate
EDTA	Ethylenediaminetetraacetic acid
F3 and B3	Forward and backward outer primers
FIP and BIP	Forward and backward inner primers
HPLC	High performance liquid chromatography
ISO	International Organization for Standardization
ITS	Internal transcribed spacer
LAMP	Loop-mediated isothermal amplification
LFA	Lateral-flow-assay
loopF and loopB	Loop forward and loop backward primers
NCBI	National Center for Biotechnology Information
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
rbcL	Ribulose bisphosphate carboxylase large chain
Tris	Tris(hydroxymethyl)aminomethan
ttp	Time-to-positive
UV	Ultraviolet
NIR	Near-Infrared
SCAR	Sequence Characterized Amplified Region

methods include but are not limited to ^{1}H NMR metabolite fingerprinting (Petrakis et al., 2015), HPLC analysis of the quality markers safranal, crocin and picocrocin (Lage & Cantrell, 2009), NIR-spectroscopy (Zalacain et al., 2005) and SCAR based PCR-assays (Babaei, Talebi, & Bahar, 2014; Marieschi et al., 2012; Torelli, Marieschi, & Bruni, 2014). At present, the method for authentication recommended by ISO is based on UV/Vis spectrophotometry (ISO/TS 3632, 2011).

Depending on the type of suspected adulteration (lower quality saffron, different origin, different plant material, inorganic fillers, unauthorized dye, or combination of the former) certain methods are preferable to others. For the detection of foreign plant material, biomolecular technology is superior since it does not depend on the morphological state of the processed spice and is highly specific and sensitive (Sasikumar et al., 2016).

For point-of-care diagnostic, isothermal amplification methods are predestined, because they do not need the utilization of biolaboratory infrastructure and can be conducted with e.g. a heating block. Especially the LAMP reaction (loop-mediated isothermal amplification) offers a high potential due to its robustness against inhibitors, specificity, sensitivity and possible combination with point-of-care suitable detection methods (T. Notomi et al., 2000; Tsugunori Notomi, Mori, Tomita, & Kanda, 2015). For the LAMP reaction four to six primers with six to eight primer binding sites on a 300 basepairs long target sequence making the LAMP highly specific (Focke, Haase, & Fischer, 2013; Nagamine, Hase, & Notomi, 2002; T. Notomi et al., 2000). Several LAMP assays have been published in conjunction with point-of-care suitable extraction protocols, e.g. for the detection of genetically modified organisms, demonstrating the robustness of the LAMP reaction (Lee, La Mura, Allnutt, Powell, & Greenland, 2009; Zhang et al., 2013). Although specific LAMP primersets already exist for the detection of saffron (Zhao et al., 2016, 2019) and turmeric (Sheu, Wu, Lien, & Lee, 2021), no method exists for the detection of safflower.

A characteristic of the LAMP reaction is the substantial amount of product formed. This and the occurrence of various by-products lead to a variety of point-of-care suitable detection methods, for example measuring the turbidity of the reaction medium, caused by insoluble magnesium pyrophosphate (Mori, Nagamine, Tomita, & Notomi, 2001), colorimetric monitoring of pH change or the use of the fluorescent dye calcein (Tomita, Mori, Kanda, & Notomi, 2008). Detection via lateral-flow-assay (LFA) is the most user-friendly method because of the direct readout format. By labeling two of the LAMP primers with Biotin and 6-FAM, the reaction is modified to allow detection of the reaction product by LFA. The doubly-labeled reaction product binds to the immobilized 6-FAM/FITC antibodies and simultaneously to the gold nanoparticles labeled with biotin antibodies, resulting in a visible colored stripe on the LFA (Zasada et al., 2020). This format is widely used for point-of-care testing in every field of research from medicine to food chemistry (Frohnmeyer et al., 2019). The most recent example, which also demonstrates the user-friendliness of the method, is the use of LFAs in antigen-based Covid-19 rapid tests (Zhou, Wu, Ding, Huang, & Xiong, 2021).

Isolation of quality plant DNA is generally perceived as difficult compared to isolation from bacteria or animal tissue (Varma, Padh, & Shrivastava, 2007), especially as limiting conditions exist *in field*. However, some examples can be found in the literature where minimal effort resulted in PCR-amplifiable isolate. For instance, Berthomieu and Meyer successfully amplified the first intron of the two homologous nitrate reductase genes in tobacco by adding a piece of leaf tissue directly to the reaction mixture (Berthomieu & Meyer, 1991). Another example is the use of a microneedle patch for the sampling process, which can generate amplifiable isolate from leaf material without any purification steps (Paul et al., 2019).

In this study, we aimed to develop a rapid and user-friendly DNA-based method to detect the adulterants safflower and turmeric in saffron. The key steps for the development of the testing protocol were (i) the isolation of amplifiable DNA from dried and processed plant material without lab infrastructure, (ii) the specific amplification of the desired sequence, whereby the method of choice should be feasible with a simple heat source and therefore must be isothermal, and (iii) visualization of the result, i.e., clear distinction between a positive and a negative reaction.

2. Materials and methods

2.1. Acquisition of plant material

The plant material used in this study was kindly provided by HELA GmbH (Ahrensburg, Germany), Husarich GmbH (Hamburg, Germany), Gewürzmühle Brecht GmbH (Eggenstein, Germany) (saffron, turmeric, safflower, calendula, oregano, majorana, cassia and ceylon cinnamon, black pepper) and the Botanical Garden of Hamburg (thyme, safflower, oregano). Fresh ginger, carrot, broccoli, sage, garlic, onion and basil samples were purchased at a local marketplace. Fresh samples were ground to a powder using liquid nitrogen and mortar and pestle.

Mixtures of dried and ground saffron and the adulterants turmeric and safflower with different content (w/w) of adulterant (90%, 80%, 70%, 60%, 50%, 25%, 10%, 5%, 2.5%, 1% and 0.1%) were prepared.

2.2. Extraction of DNA for selectivity evaluation and assay optimization

The DNeasy Plant Pro Kit (Qiagen GmbH, Hilden, Germany) was used accordingly to the manufacturers' instructions for the extraction of genomic DNA from 50 mg of saffron, turmeric and safflower samples as well as mixtures of the former. The DNA was eluted with 50 µL Millipore water. The DNA was further cleaned with the Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Inc., Frankfurt am Main, Germany), eluted with 10 µL Millipore water and the concentration determined via photometric measurement with a Quantus™ Fluorometer and QuantiFluor® dsDNA System (Promega GmbH, Walldorf, Germany). Quality was ensured by UV/VIS-measurement of the 260/280 and 260/230 ratios with a Nanodrop™ one/oneC (Thermo Fischer Scientific Inc., Waltham, USA). The isolate was diluted to a concentration of 1 ng/µL and used immediately or stored at −20 °C until use.

DNA isolation from species used for cross-reactivity testing was performed according to the protocol based on adsorption on silica columns published by Focke et al. (Focke, Haase, & Fischer, 2011).

2.3. Protocol for point-of care extraction A

4 mg of plant material and 400 µL of Millipore water were added to a 1.5 mL tube and incubated at room temperature for 5 min, swirling occasionally. The isolate was freed from excess plant material by centrifugation (10.000 g, 1 min). The isolate was used immediately or stored at −20 °C until use.

2.4. Protocol for point-of-care extraction B (modified following Zhang et al., 2013)

A previously published extraction protocol (Zhang et al., 2013) was slightly modified and adapted for easier conduction in laboratory environment.

20 mg tissue and 1 mL extraction buffer (2.5 M guanidine thiocyanate, 50 mM Tris, 20 mM EDTA, 21.3 mM Triton X-100, pH 6.4) were vortexed and incubated for 5 min. The suspension was centrifuged, and the supernatant added to a silica spin column (EconoSpin™ by Epoch Life Science Inc., Missouri City, USA). After centrifugation, 400 µL of wash buffer I (2.5 M guanidine thiocyanate, 50 mM Tris, pH 6.4) and 200 µL wash buffer II (10 mM Tris, 100 mM NaCl, pH 8.0) were subsequently added. DNA was eluted with 50 µL Millipore water and used immediately or stored at −20 °C until use.

All centrifugation steps were conducted at 12.000 g for 1 min.

2.5. PCR for evaluation of amplifiability

A universal rDNA primerpair (universal-1) and PCR protocol, published by Focke et al. was used for evaluation of amplifiability of the generated isolates (Focke et al., 2011). Primers were synthetized by IDT DNA Inc. (Coralville, USA).

0.5 U of Taq-Polymerase (Biozym Scientific GmbH, Hessisch Oldendorf, Germany), 2 µL of 10X reaction buffer (Biozym Scientific GmbH, Hessisch Oldendorf, Germany), each Primer in a final concentration of 0.5 µM and each dNTP (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in a final concentration of 0.2 nM were combined in a reaction tube and filled to 20 µL with Millipore water and varying amounts (1 µL–10 µL) of sample. The negative control was filled up to 20 µL with Millipore water.

For initial denaturation the reacting temperature was set to 94 °C for 5 min, followed by 35 cycles at 94 °C for 15 s, 58 °C for 25 s, and 72 °C for 25 s. The reaction tubes were heated to 72 °C for 5 min for terminal elongation. Presence of desired amplicon was checked via agarose gel electrophoresis.

2.6. Primerdesign

A LAMP primerset selective for *Curcuma longa* has been published for the demarcation of turmeric against commonly mixed up species (Sheu et al., 2021). This primerset was adapted to detect turmeric in mixtures with saffron in this study.

Different LAMP primersets selective for the sequence of plastidary internal transcribed spacer 1 and 2 of *Carthamus tinctorius* were designed with the open access software PrimerexplorerV5. In silico cross-reactivity with other species, especially saffron, was excluded by blast search. The consensus sequence of 32 NCBI deposited sequence files of the internal transcribed spacer 1 and 2 was used for the final design (Table S1, supp. inf.). Table 1 provides the sequence information of the developed primerset as the labeled primers for species detection with lateral-flow-assay.

2.7. LAMP reaction

Bst 3.0 Polymerase, $MgSO_4$ solution and 10x isothermal reaction buffer were purchased from New England Biolabs GmbH (Frankfurt am Main, Germany). Primers were ordered from IDT DNA Inc. (Coralville, USA). Primerstocks (25x) containing 40 µM FIP, 40 µM BIP, 5 µM F3, 5 µM B3, 10 µM LoopF and 10 µM LoopB were prepared. Fluorescent dye Syto™9 was purchased from invitrogen™ by Thermo Fisher Scientific (Waltham, USA) and used in a final concentration of $2{\cdot}10^{-3}$ mM for real-time measurements. All reactions were prepared on ice to prevent an early start of amplification. LAMP reactions were conducted accordingly to the proposed standard reaction protocol from NEB prior to optimization. A total volume of 25 µL and 1 ng purified DNA or 2 µL point-of-care isolate (protocol A or B) was used per reaction. All reactions were carried out in triplicates when not explicitly stated otherwise and continued for 60 min reaction time followed by inactivation at 80 °C for 5 min.

The time when the amplification curve intersects the threshold-line is referred to as time-to-positive and abbreviated with ttp in the course of this work. The parameters $MgSO_4$, dNTP and polymerase concentration as well as the reaction temperature were optimized by Design of Experiments with a D-optimal custom design to maximize the gap between ttp and the start of unspecific amplification. JMP software version 16.2 from SAS Institute Inc. (Cary, USA) was used for this purpose. The concentrations in the standard and optimized protocol as well as the range of optimization are shown in Table 2.

Table 1
Sequence information of the developed safflower specific LAMP primerset and the turmeric specific primerset published by Sheu et al., in 2021 as well as the labeled primers for species detection with lateral-flow-assay.

Name	Sequence 5'→ 3' (Safflower)
F3	GCCTTAGCCCTACGATGCT
B3	TTCATCGATGCGTGAGCC
FIP (F2+F1C)	CGGGGTTTGTTTTTGTGCCGAC-CATGCGTGCAAGGTGCTT
BIP (B2+B1C)	GGTTCGTCTCGTGTTGCCCC-CGTTGCCGAGAGTCGTTTA
LoopF	GACGTCCACGATGCCTAGAGAT
LoopB	TTGCGGTGTGCACACGG
6-Fam/LoopF	6-Fam/GACGTCCACGATGCCTAGAGAT
Biotin/FIP	Biotin/CGGGGTTTGTTTTTGTGCCGAC-CATGCGTGCAAGGTGCTT
Name	**Sequence 5'→ 3' (Turmeric, Sheu et al., 2021)**
F3	GTCGCGAGCGAGAAC
B3	GGCTGATCCCGGTTCACT
FIP (F2+F1C)	AATGATTGACGCGGCGCTTTC-GTTTTGGGATGAGCCCTCAA
BIP (B2+B1C)	AGACCACCCGCCGAGTTTAAG-GCCGTTACTAGGGGAATC
LoopF	CATCAATCACACAGGGTCTCTTTA
LoopB	AAATAAGCGGAGGAGGAGAAACTTA
6-Fam/LoopF	6-Fam/CATCAATCACACAGGGTCTCTTTA
Biotin/FIP	Biotin/AATGATTGACGCGGCGCTTTC-GTTTTGGGATGAGCCCTCAA

2.8. Species detection with lateral-flow-assay

5′- Biotin labeled FIP-primer and 5′-6-Fam labeled LoopF-primer (Table 1) were ordered from IDT DNA Inc. and used for preparing the primermix. The HybriDetect Universal Lateral-Flow-Assay Kit was purchased from Milenia Biotec GmbH (Gießen, Germany).

The LAMP assay was conducted as described for 5–17.5 min with optimized parameters and without the inactivation period. Subsequently 7 µL of the reaction mixture were added to the sample pad of the lateral-flow strips and the assay carried out accordingly to the manufacturer's instructions. Readouts were confirmed by agarose gel electrophoresis.

Table 2
Reaction parameters used prior to optimization, the range for optimization and the optimized parameters for the turmeric and safflower specific LAMP assay.

Parameter	Standard	Range	Optimized
Temperature [°C]	65	62–72	72
Each dNTPs [mM/µL]	1.4	0.8–1.4	1.2
Bst 3.0 [U/µL]	0.32	0.32–0.04	0.32
$MgSO_4$ [mM/µL]	6	4–10	4

3. Results and discussion

3.1. Development and optimization of turmeric and safflower specific LAMP assays

Three LAMP primersets for the detection of *Carthamus tinctorius* were designed based on the sequence of internal transcribed spacer 1 and 2, which is localized on the rDNA of the genome and the rbcL gene, which is localized in the plastid genome. The designed primersets were specific to *Carthamus tinctorius* in the scope of the NCBI database. Since an experimental verification of the primersets against all existing species is not feasible, the term selectivity rather than specificity can be used in the case of LAMP primersets and is defined as the absence of cross-reactivity. Advantages of the ITS regions for the planned application are the high copy number of the rDNA, which can lower the detection limit by a factor of 100–1000, as well as the high biodiversity of this non-coding region between species (Chiou, Yen, Fang, Chen, & Lin, 2007; Rogers & Bendich, 1987). The plastid genome is also present in a high copy number per cell.

The initial primer screening was conducted at different temperatures in a range from 62 °C to 72 °C to assess reaction kinetics. Evaluation of selectivity was conducted according to the NEB standard protocol for LAMP assays with Bst. 3.0 polymerase at 65 °C for 60 min. The best performing primerset was selected based on the absence of cross-reactivity and reaction kinetic. No cross-reactivity was observed for the chosen primerset, which has its binding sites in the ITS region of the rDNA.

A selective LAMP primerset for *Curcuma longa* was already published in 2021 by Sheu et al. for the demarcation of *Curcuma longa* against closely related species. The primerset was checked for selectivity against various spices and herbs and no cross-reactivity was found (Table 3).

Despite the advantages of the LAMP reaction, one potential pitfall must be considered when developing an assay for a rapid test system with an endpoint detection method. The LAMP reaction leads to non-specific amplification even in the absence of template DNA. The onset of nonspecific amplification depends primarily on the primers and reaction conditions used. One possibility for distinguishing a positive and negative reaction is the difference in the onset of product formation which can be monitored with real-time fluorescence measurements or the use of a labeled probe. Furthermore, specific and non-specific amplicons can be distinguished by agarose gel electrophoresis, since the characteristic ladder-like pattern is not present in the case of non-specific amplification. However, real-time reaction monitoring and agarose gel electrophoresis are not suitable for in-field analysis. Thus, the reaction time of the assay is of critical importance for the test result when working with detection methods that can only detect the occurrence of amplification but cannot distinguish between specific and non-specific.

Table 3
Species used for evaluation of selectivity of the designed safflower specific primerset and the turmeric specific primerset, published by Sheu et al..

Species tested for cross-reactivity	Turmeric (Sheu et al.)	Safflower
Onion (*Allium cepa*)	–	–
Garlic (*Allium sativum*)	–	–
Broccoli (*Brassica oleracea* var. *italica*)	–	–
Marigold (*Calendula officinalis*)	–	–
Safflower (*Carthamus tinctorius*)	–	+
Cassia Cinnamon (*Cinnamomum cassia*)	–	–
Ceylon Cinnamon (*Cinnamomum verum*)	–	–
Saffron (*Crocus sativus*)	–	–
Turmeric (*Curcuma longa*)	+	–
Carrot (*Daucus carota*)	–	–
Basil (*Ocimum basilicum*)	–	–
Olive (*Olea europaea*)	–	–
Marjoram (*Origanum majorana*)	–	–
Greek Oregano (*Origanum onites*)	–	–
Mediterranean Oregano (*Origanum vulgare*)	–	–
Black Pepper (*Piper nigrum*)	–	–
Sage (*Salvia officinalis*)	–	–
Thyme (*Thymus vulgaris*)	–	–
Ginger (*Zingiber officinale*)	–	–

The optimization of the LAMP assay with Design of Experiments (DoE) was carried out based on the turmeric-specific primerset (Sheu et al., 2021). The parameters listed in Table 2 were screened for their effect on the time-gap between ttp and nonspecific amplification and the reaction speed. A timeframe of 60 min was observed and the difference between the ttp and the time at which the negative control's (saffron) unspecific amplification curve intersects the threshold line were used as input. It was found that reducing the $MgSO_4$ concentration to 4 mM and increasing the temperature to 72 °C suppressed unspecific amplification completely in the observed timeframe (Fig. 1A). Reducing the dNTP concentration to 1.2 μM/μL did not change the reaction speed significantly. The dNTP and Bst 3.0 concentration can optionally be reduced for cost efficiency at the expense of reaction speed.

The optimized reaction conditions were successfully transferred to the safflower specific assay. Fig. 1 shows the median real-time fluorescence amplification curve for the turmeric (A) and safflower (B) specific assay with standard (left) and optimized (right) reaction conditions.

3.2. Evaluation of point-of-care DNA-isolation protocols

The point-of-care extractions methods protocol A and protocol B were performed on saffron, turmeric and safflower samples and isolates quantity and quality were compared to the DNeasy Plant Pro Kit (Table 4). Protocol A can be conducted within 5 min and protocol B in less than 10 min.

The generally lower DNA concentration in the turmeric isolates can be explained by the high processing degree of the ground rhizome, the overall lower DNA content of rhizomes compared to petals (safflower) and stigma (saffron) as well as the high content of secondary metabolites like polyphenols and polysaccharides, which can hamper the isolation process (Syamkumar, Lowarence, & Sasikumar, 2003; Varma et al., 2007). Considering the different sample amount deployed in the extraction protocols, the absolute amount of DNA obtained from point-of-care protocols is higher or comparable to the laboratory method for safflower and saffron. The quality of the isolates, characterized by a strong deviation of the 260/280 and 260/230 ratios from the targeted ideal values was considerably lower for protocol B in comparison to the laboratory method. However, since the DNA concentrations are below the 20 ng/μl required to reliably determine these ratios, their accuracy is limited, and they can only be used for guidance. The absence of purification steps leading to high levels of secondary metabolites and the even lower DNA concentration in the isolate obtained with method A make quality determination obsolete.

Nevertheless, the single important criterion for evaluating the suitability of isolation methods was its capability of producing amplifiable DNA with as little time and resource requirement as possible. Therefore, the isolates obtained from point-of-care methods were used in LAMP reactions with optimized conditions. All isolates showed amplifiability. Isolates obtained with protocol B showed similar ttp and relative fluorescence to the isolates obtained with the DNeasy Plant Pro Kit whereas isolates obtained with method A showed longer ttp and lower relative fluorescence. The effect was more pronounced in the case of turmeric, even though the absolute amount of DNA used per reaction was higher than with protocol B (Fig. 2).

3.3. Combination of LAMP assay with point-of-care isolation protocol – Turmeric

Real-time monitored LAMP reactions were performed on saffron/turmeric mixtures extracted with isolation protocol A. The amplification curves were characterized by a flattened slope and little reproducibility of the time to-positive values for turmeric contents of 60% and less.

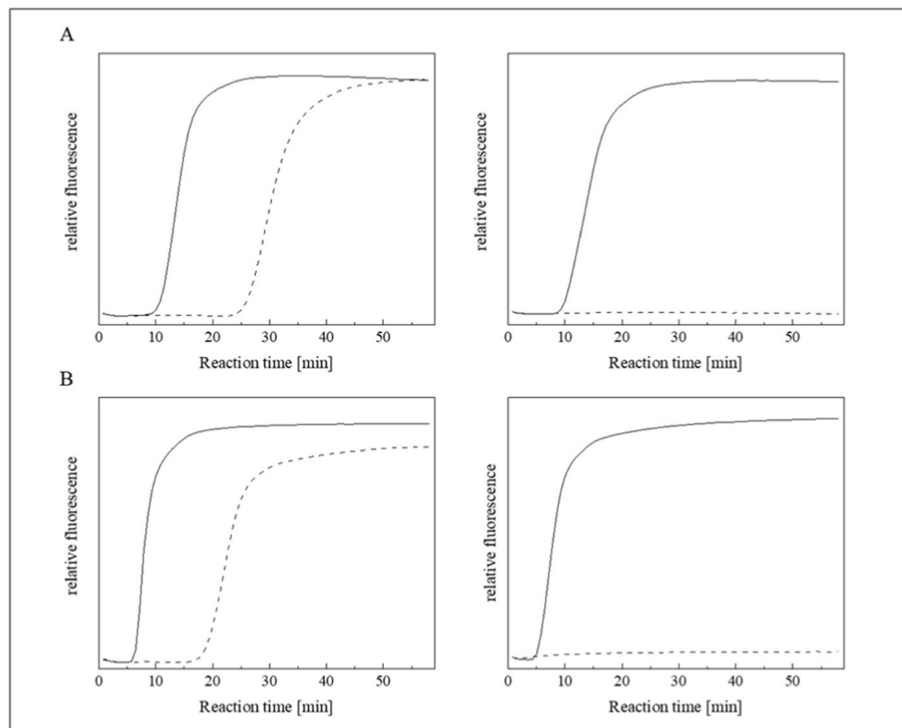

Fig. 1. Amplification curve of LAMP reaction before (left) and after (right) optimization for (A) turmeric and (B) safflower specific primersets. Negative control (unspecific amplification) indicated as dashed line.

Table 4
Concentration and absolute amount of DNA determined with fluorometric measurement and quality criteria obtained with photometric measurement for saffron, safflower and turmeric isolated with protocol A, B and the DNeasy Plant Pro Kit combined with the Monarch® PCR & DNA Cleanup Kit.

	Saffron				Safflower				Turmeric			
	Conc. [ng/μL]	DNA [ng]	260/280	260/230	Conc. [ng/μL]	DNA [ng]	260/280	260/230	Conc. [ng/μL]	DNA [ng]	260/280	260/230
A	0.63	252	–	–	0.69	276	–	–	0.30	120	–	–
B	8.32	416	2.17	0.05	4.27	214	1.96	0.01	0.21	11	2.99	0.01
DNeasy	72.7	727	1.83	2.22	51.0	510	1.84	2.09	17.0	170	1.90	1.52

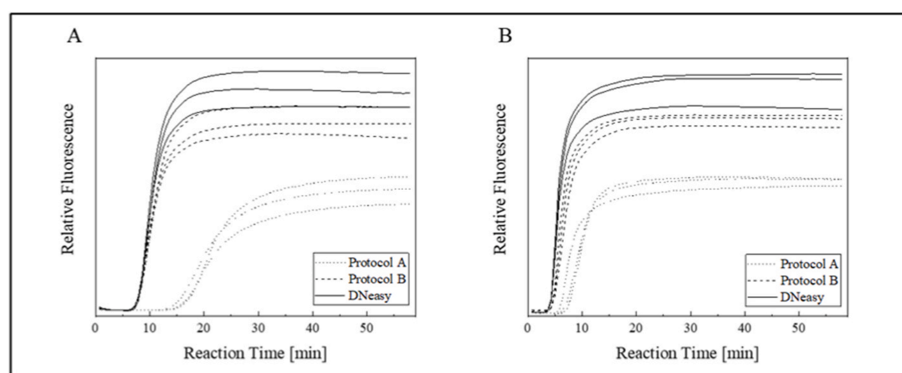

Fig. 2. Amplification curves of LAMP reaction in triplicates for protocol A, protocol B and the DNeasy Plant Pro Kit for the (A) turmeric and the (B) safflower specific primerset.

Additionally, a false negative rate of 9.2% for samples with a turmeric content of 25% and less was documented. The issue persisted when using different amounts of isolate in the LAMP reaction or sample in the isolation process.

The possible cause of inhibition by saffron-derived metabolites could be excluded, since amplification of safflower DNA from saffron mixtures was possible under identical conditions (see section 3.4). Furthermore, the saffron isolate obtained by protocol A proved to be PCR amplifiable. Hence, the reason for the poor amplifiability is most likely the low concentration of target-DNA obtained with isolation protocol A and the high content of inhibitors in turmeric. This falls in line with the previously described high content of polyphenols and inhibitors found in turmeric, as well as the high processing grade and the generally low DNA-content in rhizomes, causing problems in the isolation and

amplification process (Syamkumar et al., 2003; Varma et al., 2007). Therefore, protocol A was not suitable to detect turmeric as an adulterant in saffron.

LAMP reactions were performed on saffron/turmeric mixtures extracted with isolation protocol B and the DNeasy Plant Pro Kit and carried out as a eightfold determination. The mean ttp and its standard deviation were used to evaluate the reaction performance, since short ttp and a low standard deviation correlate with reaction speed and reproducability (Fig. 3).

A slight elevation of ttp with reduction of the turmeric content was observed for both extraction protocols, as well as a jump in the ttp and its standard deviation upon decrease of the turmeric content from 1% to 0.1%. Even though the reaction time for 0.1% mixtures was considerably longer than for mixtures with a turmeric content of 1% or more, the results were still correctly identified as positive.

3.4. Combination of LAMP assay with point-of-care isolation protocol – Safflower

The procedure was conducted analogous with saffron/safflower mixtures and the safflower specific primerset (Fig. 4).

As already documented for the turmeric assays, an elevation of the ttp was observed with deminishing safflower content. In contrast, no increase in the standard deviation was monitored and the mixture containing 0.1% safflower was detected in comparable reaction time. The mixtures extracted with protocol A showed overall higher ttp values than the other methods. Moreover, even mixtures with a content of 0.1% safflower were correctly identified as positive. The fluorescence curves (A) showed a flattened slope with diminishing safflower content, but were still uniform in shape. The reaction product showed the ladder like pattern with similar intensity as the other mixtures in agarose gel electrophoresis.

Isolation method B is very well suited for the purpose of combining it with the developed LAMP assay and the used primersets for both turmeric and safflower. The performance of the LAMP reaction can be considered comparable to the DNeasy Plant Pro Kit in terms of ttp and its standard deviation. Protocol A works for safflower but not for turmeric since the quality of the isolates, which most likely depends on the characteristics of the plant part used (rhizome or petal), plays an integral role for amplification success. Protocol A leads reliably to identification of a 0.1% adulteration with safflower in combination with the developed LAMP and is in the case of safflower preferable over method B because of its simplicity and low cost.

3.5. Endpoint detection – Lateral-flow-assay

Since the LAMP assay and isolation method were designed for point-of-care testing, the chosen detection method should meet the demands of in-field diagnostic as well. Visualizing the result of the LAMP assay is possible with various methods, of which the lateral-flow-assay or dip-stick is the most user-friendly.

It has been shown that labeling two of the six LAMP primers with Biotin and 6-Fam respectively works in combination with the HybriDetect Universal Lateral-flow-assay Kit from Milenia Biotec GmbH. Biotin and 6-FAM/FITC antibodys on the dip-stick will bind to the obtained doubly labeled LAMP template. Lateral-flow-assays do not allow quantitative detection, but semi-quantitative information can be obtained based on the intensity of the bands (Allgöwer, Hartmann, & Holzhauser, 2020; Zasada et al., 2020).

Different reaction times between 10 and 17.5 min were tested in 2.5-min steps to find the shortest possible reaction time. Fig. 5 shows the LFAs for reaction times of 10, 12.5 and 15 min. The shortest possible reaction time to detect 1% turmeric was 12.5 min under the given extraction and reaction conditions. As expected, the reaction time required for the pure turmeric isolate was shorter and already showed a positive signal after 10 min.

Turmeric is a popular spice that is often traded in finely ground form, with particles being 0.2–0.25 mm in size (Parvathy, Swetha, Sheeja, & Sasikumar, 2015). This circumstance promotes contamination of workspace in the turmeric and spice processing industry and could lead to false positives if the test is too sensitive, hence a detection limit of 1% is acceptable.

For the safflower specific assay different reaction times between 5 and 15 min were tested in 2.5-min steps for 0.1% safflower extracted with isolation protocol A and B and 100% safflower extracted with isolation protocol B as positive control. Fig. 6 shows the LFAs for reaction times of 5, 7.5 and 10 min. For the safflower specific assay 7.5 min was the shortest possible reaction time to detect 0.1% safflower in saffron extracted with protocol A. If extracted with protocol B, a weak positive signal was visible after 5 min. However, since a strong positive signal is preferable, 7.5 min was chosen as optimal reaction time as well. Pure safflower extracted with protocol B is already distinctly positive after 5 min (see Fig. 6).

4. Conclusion

The LAMP-based rapid test systems presented in this study consist of a point-of-care suitable DNA-isolation protocol, a specific LAMP assay and detection of the result with a lateral-flow-assay. PCR and LAMP amplifiable isolate could be generated from turmeric and safflower by incubation of plant material in water. In this study, it was shown that contamination of saffron with 0.1% safflower or 1% turmeric can be detected in less than 25 min without the need for biochemical laboratory infrastructure.

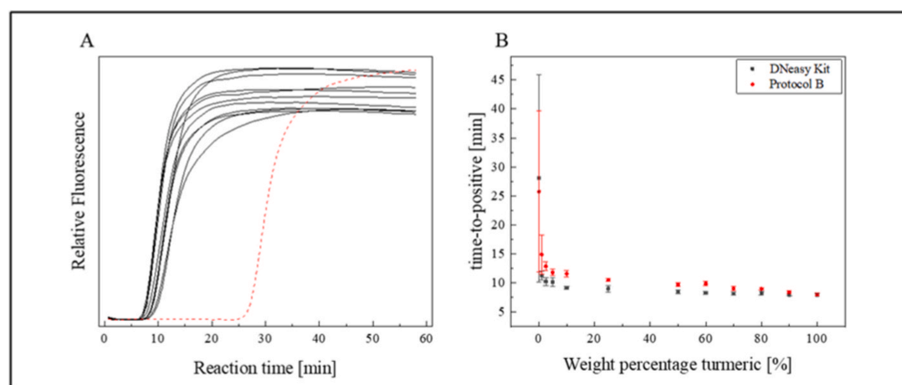

Fig. 3. (A) Amplification curve of LAMP with isolates obtained by protocol B from mixture with a turmeric content between 100% and 0.1%. Mean amplification curve of eightfold determination is depicted. Mixture with 0.1% turmeric content is indicated as red dashed line. (B) Average ttp value and its standard deviation calculated from eightfold determination for DNeasy Plant Pro Kit and extraction protocol B.

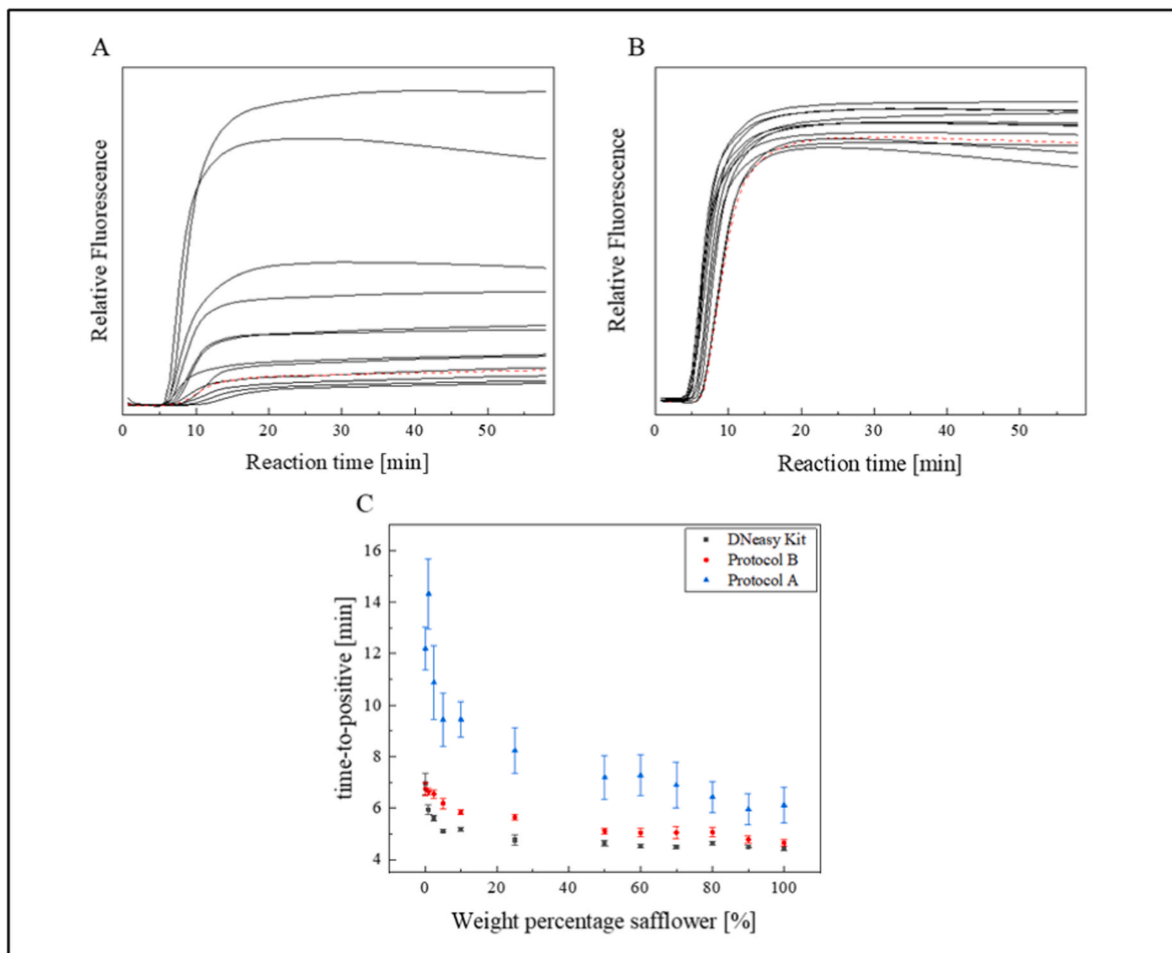

Fig. 4. Amplification curve of LAMP with isolates obtained by (A) protocol A and (B) protocol B from mixtures with a safflower content between 100% and 0.1%. Mean amplification curve of eightfold determination is depicted. Mixture with 0.1% safflower content is indicated as red dashed line. (C) Average ttp value and its standard deviation calculated from eightfold determination for DNeasy Plant Pro Kit, isolation protocol A and protocol B.

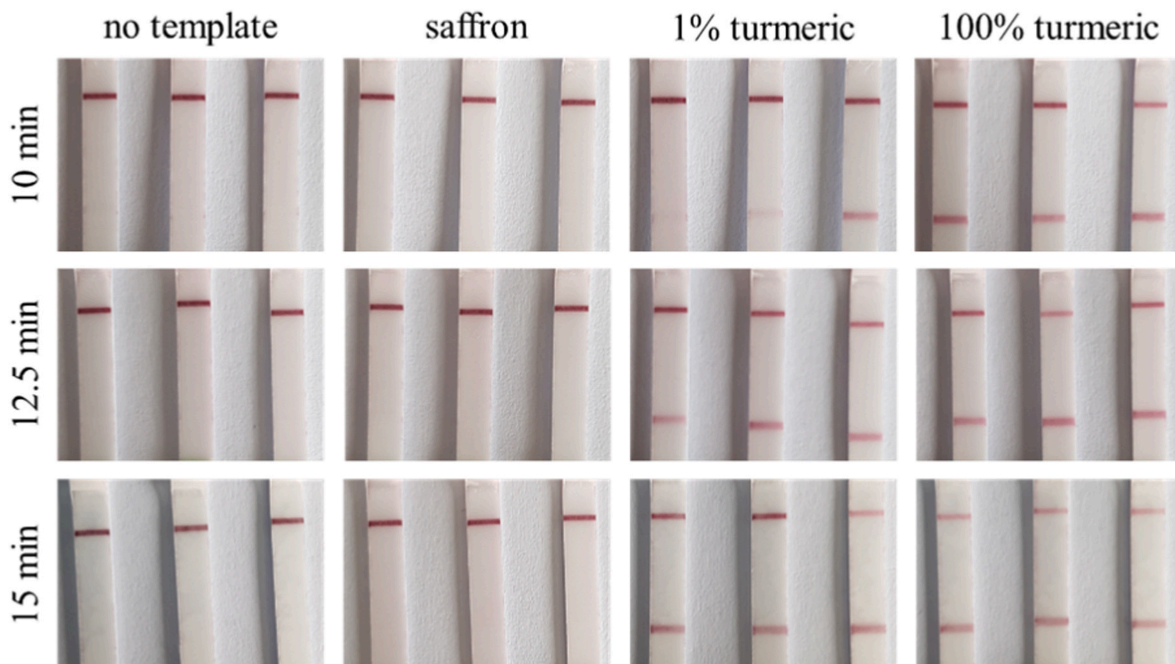

Fig. 5. LFAs with 1% mixture isolate method B in triplicates with no template and negative control (saffron) after 10, 12.5, and 15 min.

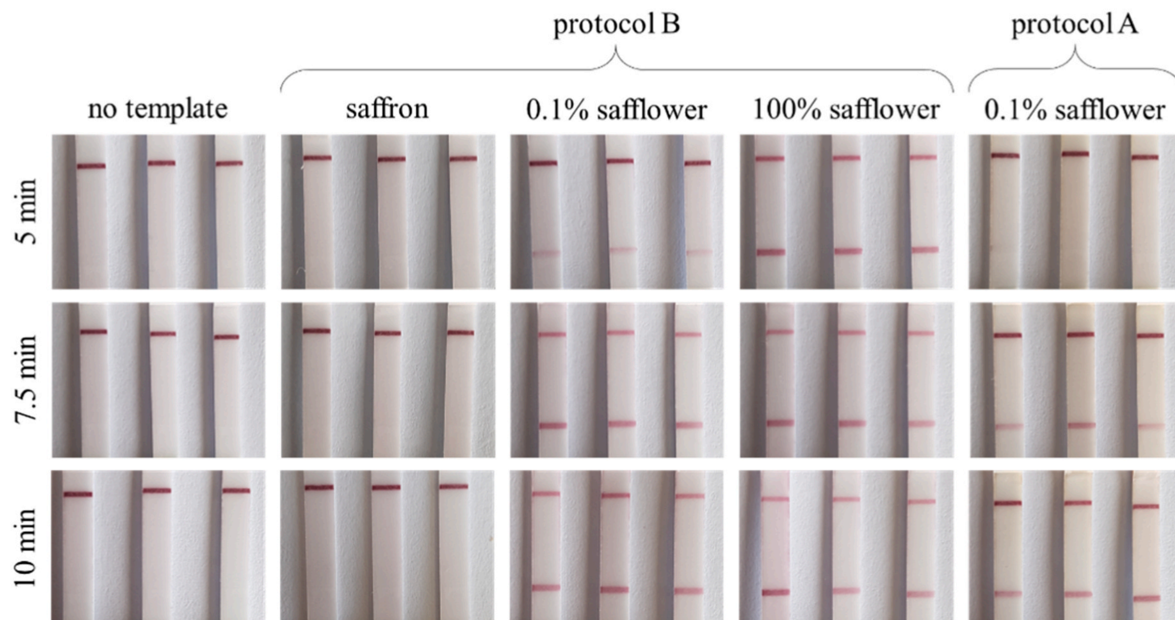

Fig. 6. LFAs after 5-, 7.5- and 10-min reaction time for the no template and negative control (saffron), 0.1% and 100% safflower extracted with protocol B and 0.1% safflower extracted with protocol A (from left to right). The yellowish staining on the LFAs on the right is caused by the intensive coloring of the isolate obtained with protocol A.

The 25 min of performance time include the time needed for preparing a single sample with protocol B, the 12.5 min reaction time for the turmeric assay (or 7.5 min for the safflower assay) and 5 min run time for the detection of the result with an LFA. To enable feasibility in 25 min, the reaction mix for the LAMP reaction must already be prepared and the heat source preheated to 72 °C.

The method developed has the potential to become a useful tool for combating food fraud, as it enables rapid and on-side identification of the plant contaminants in saffron.

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CRediT authorship contribution statement

Nathalie Holz: Conceptualization, Investigation, Visualization, Methodology, Writing – original draft. **Boris Illarionov:** Conceptualization, Methodology, Writing – original draft. **Nils Wax:** Conceptualization, Investigation, Writing – review & editing. **Celina Schmidt:** Investigation, Writing – review & editing. **Markus Fischer:** Conceptualization, Supervision, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2023.109637.

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