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# DISSERTATION

## Development and Application of Rapid Isothermal Amplification Techniques for the Analysis of Food Products

ausgeführt zum Zwecke der Erlangung des akademischen Grades der Naturwissenschaften unter der Leitung von

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*“The chemistry must be respected.”*

*Walter H. White*

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## Glossary

| | |
|---|---|
| Ct | cycle threshold value |
| DNA | desoxyribonucleic acid |
| ELISA | enzyme-linked immunosorbent assay |
| GC | gas chromatography |
| gDNA | genomic desoxyribonucleic acid |
| GMO | genetically modified organism |
| HDA | helicase-dependent amplification |
| IR | infrared spectroscopy |
| LAMP | loop-mediated isothermal amplification |
| LC-MS | liquid chromatograpy-mass spectrometry |
| LOD | limit of detection |
| MS | mass spectrometry |
| mtDNA | mitochondrial desoxyribonucleic acid |
| NEAR | nicking enzyme amplification reaction |
| NTC | no template control |
| PCR | polymerase chain reaction |
| RCA | rolling circle amplification |
| SDA | strand displacement amplification |
| TAE buffer | tris-acetate buffer |
| TE buffer | tris-ethylenediaminetetraacetic acid buffer |

## Abstract

The main objective of this thesis was the development of DNA-based isothermal amplification techniques for the rapid analysis of unwanted compounds in food products with focus on allergens, GMOs and animal species identification. Although tests for said analytes are available, many of these methods are laborious, time-consuming and difficult to transfer into a more field-applicable approach. Core of this thesis was the development of three isothermal amplification assays for the detection of the food allergen celery, transgenic maize and horse meat to show a methodical transfer of these techniques to matters of food safety. Although a DNA-based approach for the detection of these targets is recommended, conventional PCR has several drawbacks that make it unsuitable for the rapid on-site analysis of foods. The application of isothermal amplification techniques eliminates many of these drawbacks and smoothens the way for a new generation of DNA-based assays.

Since 2005, celery and celery products have to be labeled according to Directive 2003/89/EC due to their allergenic potential. In order to provide a DNA-based, rapid and simple detection method, a loop-mediated isothermal amplification (LAMP) assay for the detection of celery (*Apium graveolens*) was developed. The assay was tested for specificity for celery since closely related species also hold food relevance. The limit of detection (LOD) for spiked food samples was found to be as low as 7.8 mg of dry celery powder per kilogram food product. An evaluation of different amplification and detection platforms was performed to show reliable detection independent from the instrument used for amplification (thermal cycler or heating block) and detection mechanisms (real-time fluorescence detection, agarose gel electrophoresis or nucleic acid staining). The analysis of 10 commercial food samples representing diverse and complex food matrices, and a false-negative rate of 0 % for approximately 24 target copies or 0.08 ng celery DNA for three selected food matrices show that LAMP has the potential to be used as an alternative strategy for the detection of the food allergen celery. The performance of the developed LAMP assay ist the first specific LAMP assay for the detection of celery and turned out to be equal or superior to the best available PCR assay for the detection of celery in food products. The article was published in the journal *Analytical & Bioanalytical Chemistry* (publication #1).

In 2003 the European Commission introduced a 0.9 % threshold for food and feed products containing genetically modified organism (GMO)-derived components. For commodities containing GMO contents higher than this threshold, labelling is mandatory. To provide a DNA-based rapid and simple detection method suitable for the screening of GMOs, several isothermal amplification approaches for the P35S promoter, which is responsible for the transcription of the inserted gene, were tested: strand displacement amplification, nicking-enzyme amplification reaction, rolling circle amplification, loop-mediated isothermal

amplification (LAMP) and helicase-dependent amplification (HDA). The LAMP method was developed by the author of this thesis, the HDA method was developed by Claudia Kolm. To compare said methods, this publication was written under co-authorship and both authors contributed equally. The developed assays were tested for specificity in order to distinguish between samples containing genetically modified (GM) maize and non-GM maize. For those assays capable of this discrimination, tests were performed to determine the lower limit of detection for the GM content. A false-negative rate was determined to rule out whether GMO-positive samples were incorrectly classified as GMO-negative. A robustness test was performed to show reliable detection independent from the instrument used for amplification. The analysis of three GM maize lines showed that only LAMP and HDA were able to differentiate between the GMOs MON810, NK603, and Bt11 and non-GM maize. Furthermore, with the HDA assay it was possible to realise a detection limit as low as 0.5 %. A false-negative rate of only 5 % for 1 % GM maize for all three maize lines shows that HDA has the potential to be used as an alternative strategy for the detection of transgenic maize. All results obtained with the LAMP and HDA assays were compared with the results obtained with a previously reported real- time PCR assay for the 35S promoter in transgenic maize. This study presents two new screening assays for detection of the 35S promoter in transgenic maize by applying the isothermal amplification approaches HDA and LAMP. This study was published in the journal *Analytical & Bioanalytical Chemistry* (publication #2).

In the third study the development of a simple and rapid high-throughput method for the detection of horse meat in processed food products is described. Specific LAMP primers were designed to target the mitochondrial genome of horse (*Equus caballus*). No cross-reactions were observed for beef, pork and chicken. Sensitivity tests showed reliable detection of 0.1 ng of extracted horse DNA. Spiking experiments were performed to show that the assay is capable of detecting 0.1% horse meat in prepared model sausages, independent from their cooking time. Additionally, five different commercial horse meat products were analysed to ensure the robustness of the assay when applied to varying food matrices. All experiments were performed on a heating block followed by visual detection using an intercalating dye. Results were confirmed by real-time fluorescence monitoring using a thermal cycler and compared to a previously published real-time PCR assay. The presented assay is the first LAMP assay for the detection of horse meat in food-products. A revised version was submitted to the journal *Food Analytical Methods* (publication #3).

The assays presented in this thesis represent a new and promising alternative to conventional test systems for food products that can only be performed within a laboratory and extenive labour costs. All investigated analytes (celery, GMOs, horse meat) require a DNA-based approach due to the fact, that the specific detection without co-detection of other constituentsis is only possible based on the specific DNA sequence. Isothermal amplification

techniques provide the capability to detect these analytes on-site and in shorter time in order to perform rapid and simple but substantial controls with high sample numbers in the future.

## Zusammenfassung

Ziel dieser Arbeit war die Entwicklung DNA-basierter isothermaler Amplifikationstechniken für die rasche Analyse unerwünschter Bestandteile in Lebensmitteln in den Bereichen Allergen- und GVO-Nachweise sowie Lebensmittelauthentifizierung. Obwohl durchaus Testsysteme für diese Zielbereiche existieren, sind viele der dabei angewandten Methoden komplex in der Durchführung, zeitintensiv und lassen sich kaum als vor-Ort-Analysen einsetzen. Kernthema dieser Studie war die Entwicklung dreier isothermaler Amplifikations-Assays für die Analyten Sellerie, GVO-Mais und Pferdefleisch, um diese neuen Amplifikationstechniken in den Bereich Lebensmittelsicherheit zu transferien. Obwohl DNA-basierte Methoden für den Nachweis dieser Targets empfehlenswert sind, hat die konventionelle PCR mehrere Nachteile, die diese Methode ungeeignet für schnelle vor-Ort-Analysen machen. Die Anwendung isothermaler Amplifikationstechnologien kann viele dieser Nachteile eliminieren und ebnet den Weg für eine neue Generation DNA-basierter Test-Methoden.

Seit 2005 wird die Etikettierung von Sellerie und Sellerieprodukten aufgrund des allergenen Potentials durch die Richtlinie 2003/89/EC der Europäischen Kommission vorgeschrieben. Im Zuge der Entwicklung von DNA-basierten und einfach durchzuführenden Test-Methoden, welche eine rasche Analyse erlauben, wurde ein Loop-mediated Isothermal Amplification (LAMP) Assay für den Nachweis von Sellerie (*Apium graveolens*) entwickelt. Dieser Test wurde auf seine Spezifität für Sellerie untersucht, da eine Vielzahl von eng verwandten Arten ebenso große Bedeutung in der Lebensmittelindustrie besitzt. Die Nachweisgrenze für mit Sellerie versetzten Proben lag bei 7.8 mg Selleriepulver pro Kilogramm. Eine Evaluierung verschiedener Amplifikations- und Detektionsplattformen konnte die zuverlässige Detektion, unabhängig von verwendeten Geräten (Thermocycler oder Heizblock) und Nachweismechanismen (real-time Fluoreszenzmessung, Agarosegelelektrophorese oder Nukleinsäure-Färbung), zeigen. Die Analyse zehn kommerzieller Lebensmittelprodukte mit möglichst komplexen Matrices ergab eine Falsch-Negativ-Rate von 0% für 24 Target-Kopien bzw. 0.08 ng Sellerie-DNA in drei ausgewählten Produkten und zeigt, daß LAMP das Potenzial als alternative Nachweis-Strategie für die Detektion von allergenem Sellerie besitzt. Die Effizienz des entwickelten LAMP-Assays ist wenigstens als gleichwertig anzusehen im Vergleich zu früher entwickelten PCR-Assays für Sellerie in Lebensmitteln. Der entwickelte Assay ist der erste LAMP-Assay zum spezifischen Nachweis von Sellerie in Lebensmitteln. Diese Studie wurde im Journal A*nalytical & Bioanalytical Chemistry* publiziert (Publikation #1).

Im Jahre 2003 wurde von der Europäischen Kommission eine Grenze von 0.9 % für gentechnisch veränderte Organismen (GVOs) und daraus abgeleitete Produkte in Lebens-

und Futtermitteln eingeführt. Für Produkte, die diesen Grenzgehalt überschreiten, ist die entsprechende Etikettierung verpflichtend. Im Zuge der Entwicklung von DNA-basierten und einfach durchzuführenden Testmethoden, welche eine rasche und unkomplizierte Analyse erlauben, wurden verschiedene Isothermale Amplifikationstechniken für den Nachweis des P35S-Promoters untersucht: Strand Displacement Amplification, Nicking-Enzyme Amplification Reaction, Rolling Circle Amplification, Loop-mediated Isothermal Amplification (LAMP) und Helicase-dependent Amplification (HDA). Die entwickelten Assays wurden auf ihre Selektivität hinsichtlich der Unterscheidung zwischen gentechnisch verändertem und nicht verändertem Mais untersucht. Für diejenigen Techniken, die eine entsprechenden Diskriminierung zwischen GVO-Mais und Nicht-GVO-Mais erlauben, wurde eine Nachweisgrenze ermittelt. Zusätzlich wurde die Falsch-Negativ-Rate ermittelt, um eine Deklaration von GVO-Mais als nicht GVO-Mais auszuschließen. Ebenso wurde Tests durchgeführt um einen zuverlässigen Nachweis, unabhängig von verwendeten Instrumenten, zu gewährleisten. Die Analyse von drei verschiedenen GVO-Mais-Sorten zeigte, daß lediglich LAMP und HDA in der Lage waren, zwischen den gentechnisch veränderten Mais-Sorten MON810, NK603 und Bt11 und Nicht-GVO-Mais zu unterscheiden. Für die HDA konnte außerdem eine Nachweisgrenze von 0.5 % realisierte werden, sowie eine Falsch-Negativ-Rate von 5 % für alle drei Sorten 1%-GVO-Mais. Dies zeigt, daß die HDA als alternative Nachweis-Strategie für GVO-Mais eingesetzt werden kann. Alle Ergebnisse für LAMP und HDA wurden mit einem früher publizierten real-time PCR-Assay zum Nachweis des P35S-Promoters in gentechnisch verändertem Mais verglichen. Diese Studie erläutert die Entwicklung zwei neuer Screening-Assays für den P35S-Promoter in GVO-Mais mittels isothermaler Amplifikationstechniken. Diese Studie wurde im Journal *Analytical & Bioanalytical Chemistry* publiziert (Publikation #2).

In der dritten Studie wird die Entwicklung einer simplen und schnellen Nachweis-Technik für Pferdefleisch in prozessierten Lebensmitteln beschrieben. Ziel-Molekül der spezifisch entwickelten LAMP-Primer ist das mitochondriale Genom der Spezies Pferd (*Equus caballus*). Für Rind-, Schweine- und Hühnerfleisch konnten keine Kreuzreaktionen beobachtet werden, eine zuverlässige Nachweisgrenze von 0.1 ng extrahierte Pferde-DNA wurde ermittelt. Die Analyse von Modell-Würsten mit definiertem Pferdefleischgehalt konnte zeigen, daß der entwickelte Assay Gehalte von 0.1 % Pferdefleisch, unabhängig von Kochzeiten, zuverlässig nachweisen kann. Zusätzlich wurden fünf verschiedene, kommerziell erhältliche Pferdefleischprodukte untersucht, um außerdem eine Unabhängigkeit von Lebensmittelmatrices zu zeigen. Alle Experimente wurden auf einem Heizblock durchgeführt, die Detektion erfolgte visuell mittels eines interkalierenden Farbstoffes. Die Ergebnisse wurden mittels real-time Fluoreszenzmessung mit einem zuvor publizierten PCR-Assay für den Nachweis von Pferdefleisch verglichen. Der entiwckelte Assay ist der erste publizierte

LAMP-Assay für den Nachweis von Pferdefleisch in Lebensmitteln. Eine überarbeitete Version wurde beim Journal *Food Analytical Methods* eingereicht (Publikation #3).

Die in dieser Arbeit entwickelten Methoden stellen eine neue und vielversprechende Alternative zu konventionellen Testsystemen für Lebensmittelprodukte dar, die bislang nur im Labor durchführbar und mit entsprechendem Zeit- und Personalaufwand verbunden sind. Für alle untersuchten Analyten (Sellerie, GVOs, Pferdefleisch) ist ein DNA-basiertes Testsystem notwendig, da ein spezifischer Nachweis ohne Co-Detektion andere Inhaltsstoffe nur über die entsprechende DNA-Sequenz möglich ist. Isothermale Amplifikationsmethoden bieten die Möglichkeit, diese Analyten ohne besonderen Geräteaufand in wesentlich kürzerer Zeit vor Ort nachzuweisen und werden in Zukunft dazu beitragen, umfangreiche Kontrollen mit einer großen Anzahl an Proben rasch und einfach durchzuführen.

# List of Publications and Presentations

## International Reviewed Publications (SCI Ranked)

**Zahradnik C**, Martzy R, Mach RL, Krska R, Farnleitner AH, Brunner K (2014) Detection of the food allergen celery via loop-mediated isothermal amplification technique. Anal Bioanal Chem 406(27):6827-6833

**Zahradnik C**, Kolm C, Martzy R, Mach RL, Krska R, Farnleitner AH, Brunner K (2014) Detection of the P35S promotor in transgenic maize via various isothermal amplification techniques: a practical approach. Anal Bioanal Chem 406(27):6835-6842

**Zahradnik C**, Martzy R, Mach RL, Krska R, Farnleitner AH, Brunner K (2014) Loop-mediated Isothermal Amplification (LAMP) for the Detection of Horse Meat in Processed Foods. Food Analytical Methods [Submitted & Revised]

## Other Publications

**Celine Zahradnik**, Kurt Brunner (2014) Vertrauen ist gut, Kontrolle ist besser - Isothermale Amplifikation in der Lebensmittelanalytik. Labor & More 6.14: 48-50

**Celine Zahradnik**, Claudia Kolm (2014) Response to the Letter to the Editor regarding "Detection of the P35S promotor in transgenic maize via various isothermal amplification techniques: a practical approach." Anal Bioanal Chem 406(30):8061-8062

## Posters Presented at Scientific Conferences

**Celine Zahradnik**, Roland Martzy, Robert Mach, Rudolf Krska, Andreas Farnleitner & Kurt Brunner. Detection of Celery (Apium graveolens L.) in food via Loop-mediated Isothermal Amplification technique. 6$^{th}$ International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic. 5$^{th}$ - 8$^{th}$ November 2013.

**Celine Zahradnik**, Claudia Kolm, Roland Martzy, Robert Mach, Rudolf Krska, Andreas Farnleitner & Kurt Brunner. Detection of the P35S-Promotor in GM maize via Loop-mediated Isothermal Amplification and Helicase-dependent Amplification. 9$^{th}$ Conference on Rapid Methods Europe, Noordwijkerhout, Netherlands.31$^{st}$ March - 2$^{nd}$ April 2014

**Celine Zahradnik**, Roland Martzy, Robert Mach, Rudolf Krska, Andreas Farnleitner & Kurt Brunner. A Simple Method for the Detection of Horse Meat in Food. 10th ASAC JunganalytikerInnenforum, Tulln, Austria. 13th - 14th June 2014.

# Introduction

This introduction aims to provide basic information about legal systems of the European Union regarding the food supply chain and food safety in general. With focus on allergens, genetically modified organisms (GMOs) and meat species identification the most important and recent developments that led to the introduction of new directives, guidelines or recommendations are summarised. Commonly used methods for the analysis of food products with respect to the target of interest are briefly described. Furthermore, the necessity to develop rapid and simple on-site methods in order to sustain an efficient "tracing" and "tracking" system throughout Europe to ensure the safety of consumers is underlined. Recent advancements in molecular biology led to the development of several new DNA-based techniques that can be subsumed under the term "isothermal amplification". This new field of DNA amplification techniques is very promising in terms of efficiency and simplicity and might significantly facilitate controls for unwanted compounds in food products in the future.

# 1 Food Safety

## 1.1 General Aspects

Throughout history, people have occansionally suffered from foodborne diseases or food-poisoning. One of the earliest incidents of foodborne disease was the intoxication with ergotalkaloids produced by the plant-parasitic fungi *Claviceps purpurea* which preferredly infests grain [1]. The first documented epidemic occured 857 AD in Xanten, Germany. 65 years later, around 40.000 people fell victim to another epidemic in France and Spain. Around 1780 AD the correlation between the ingestion of flour polluted with ergot and the recurring epidemics was drawn for the first time, resulting in certain legislative measures [2]. In 1985 several vineyards in Austria and Germany were discovered to admix diethylene glycol to choice wines [3]. Although there have been no documented cases of intoxication, this incident caused great economic damage to wineries throughout Europe and and several winemakers were sentenced for adulteration. Since 1990 incidents of food-poisoning due to contamination with *Salmonella* sp. have been reduced to 25% in 2005. However, a lot of cases are still reported every year, mostly due to non-compliance with hygienic standards or through the use of expired or infected ingredients like eggs or poultry [4]. Another hazardous

microbiological contaminant is the bacterium *Clostridium botulinum*, which primarily infects meat and meat products as well as tinned food. Similar to *Salmonella Clostridium* infections are notifiable and can lead to severe health issues and death. Curing and heat sterilisation are important measures to avoid a contamination of food products with this agent [5]. Bovine spongiform encephalopathy (BSE) is another hazardous agents of public interest. In 1992 37.000 people worldwide developed Creutzfeldt-Jakob-disease, most probably through the consumption of affected beef. The disease is characterized by the abnormal folding of proteins in the brain, the so called prion proteins, which trigger several biochemical processes that lead to the accumulation of amyloids and subsequent degeneration of brain matter. The cases of illness were contained by many emergency slaughters and legislation reacted to the scandal by introducing new laws that prohibit the use of meat and bone meal as animal feed stuff [6]. 2009 33 people developed listeriosis after the consumption of certain cheeses. The disease is caused by the bacterium Listeria monocytogenes and has a fatality rate of around 33%. The contamination was caused by disregarded hygienical standards and the use of deteriorated enzymes needed for cheese production [7,8]. In 2011 the public was warned to consume raw tomatoes, cucumbers and salad because of the HUS-outbreak, caused by the enterohemorrhagic *Escherchia coli* bacterium. Several products were banned from import into certain countries which caused great economic losses [9]. It is still unclear how the bacterium was distributed or where it originated and ingnited discussion throughout Europe about the inadequate traceability of food ingredients which was even further sparked by the horsemeat scandal in 2013 [10].

Nevertheless, the European Union nowadays has the highest food safety standards in the world. Regulation (EC) 178, published in 2002, discusses general principles and requirements of food law, and provides procedures for the installation of traceability systems in Europe. Furthermore, the European Food Safety Authority (EFSA) was installed to support the authorities in their desicions regarding the risk managment of food-related issues throughout Europe. Since 2005, this EU regulation legally binds all food and feed business operators to have sufficient traceability systems in place. Although many companies already comply with the General Food Law, many smaller companies still have to follow up to reach European standards. Traceability covers all stages from manufacturing, packaging and distribution, which involves ingredients, processes, test results, environment, transport methods etc. Articles 19 and 20 of Regulation (EC) 178 (2002) demand operators to immediately initiate procedures to withdraw the food or feed from the market and inform competent authorities if any part of the supply chain is suspected to have occurred without compliance with the law [11,12].

Directive 2000/13/EC outlined the approximation of laws relating to the labelling, presentation and advertising of foodstuffs. Manufacturers are required to declare all ingredients present in

pre-packaged foods sold in the European Union [13]. This new legislation also concerns the labelling of allergens and genetically modified organisms (GMOs) in food and feed products as well as food authenticity and related issues. Directive 2003/89/EC [14] introduced Annex IIIa, which contains a list of allergens that must always be labelled when present in a product. In later amendments several potential allergenic ingredients were added, with Directive 2007/68/EC [15] being the most recent amendment of Annex IIIa. The latest regulation, Regulation (EC) 1830/2003 concerning GMOs, declared labelling mandatory for products that consist of GMOs or contain GMOs and for products derived from GMOs if proteins or DNA still are present from the genetic modification [16]. Recently, an initiative of the European Parliament (EP) discovered that a number of foods, including olive oil, fish, dairy products, honey and meat are frequent targets of fraudulent activities. With the use of appropriate test systems, falsified or mislabelled products and ingredients can be traced to the source of origin and legitimate actions to withdraw the product in question from the market can be initiated.

A complete summary of all facets regarding the highly complex matters of food safety would go beyond the scope of this thesis. Therefore, this work ocncentrates on specific topcis such as allergen analysis, GMO analysis and animal species authentication, where advances in the development of rapid on-site test systems have been achieved.

## 1.2 Allergens

Food allergies are a serious concern in industrialised countries since almost 2% of the adult population are affected. For sensitised individuals the ingestion of allergenic food can result in a wide array of diverse symptoms, ranging from skin reactions or digestive disorders to more severe reactions such as respiratory symptoms [17,18]. In some cases, only minor ingested amounts of the allergen can inherently provoke a life-threatening anaphylactic shock [19]. Food allergies are characterised by abnormal immunological responses to certain foods or food components. Allergic reactions are referred to as acute hypersensitive reactions with the immediate onset of symptoms mediated by the allergen-specific immunoglobulin E (IgE) [20].

Due to the fact that a long-term treatment for food allergy does not exist at the time, the only way for an affected person is to avoid the allergenic food itself. Unfortunately, avoidance of the allergy-causing food is not always sufficient, since contamination of food with potential allergens during shipping, storage or processing is a valid concern. Affected individuals can only rely on the correct execution of the labelling requirements introduced by the European Commission through Directive 2000/13/EC [13]. As mentioned above, Directive 2007/68/EC is the most recent amendment of Annex IIIa, which contains a list of foods and food

components that are considered potential allergens and includes the following: celery, cereals, crustaceans, eggs, milk and dairy products, fish, mustard, nuts, peanuts, sesame seeds, soybeans and sulphites [15]. Although various other food components like a multitude of fruits can also cause allergic reactions, Annex IIIa is limited to those allergens, that cause 90% of all food allergies and frequently contaminate food products during shipping, storage and processing [21,22].

## 1.3 Genetically Modified Organisms (GMOs)

In 2007, the International Service for the Acquisition of Agri-biotech Applications (ISAAA) estimated the acreage of GM crop cultivation to be around 114 million hectares, distributed across 23 countries. The most frequently cultivated GM crops are canola, maize, cotton and soybean and the interest in GM crops is continuing to rise, especially in developing countries [23]. However, the position of the European Union is still precautious since the debate about the safety of GM crops for animal and human health as well as the ecological impact is ongoing [24]. Over the last decade various genetically modified organisms (GMOs) have been introduced into food and feed products. In the course of Directive 2000/13/EC [13] adequate labelling of GM products and their derivatives is required, and the European Commission introduced a threshold of 0.9% for the mandatory labelling of GMOs based on the Directives (EC) 1139/98 and 49/2000 [25,26]. GM contents exceeding this threshold have to be labelled adequately. Regardless, GM crops are in demand due to their features of improved product quality, pest resistance and agronomic benefits [27].

Improved shelf life is one of many properties desired in GM plants and the first genetically modified plant to enter commerce was the Flavr savr™ tomato in 1994. Inhibition of the polygalacturonase (PG) gene decreases the production of PG, an enzyme degrading pectin, which is a main component of the cell wall. Thereby, fruits are prevented from becoming soft and shelf-life is increased drastically [28]. Apart from improved product qualities such as durability or firmness, delayed fruit ripening is also a desired feature in GM plants. Nevertheless, pest resistance and herbicide tolerance play a more important role in agriculture and therefore have been pursued further over the last two decades. The genome of the soybean Roundup Ready™ contains an inserted gene from bacterium *Agrobacterium tumefaciens*, that produces the enzyme 3-enol-pyruvyl.shikimate-5-phosphatase-synthase. The bacterial enzyme is not inhibited by the herbicide glyphosate and therefore makes the plant resistant to Roundup Ready™[29]. Many maize varieties contain the cryIA(b) gene from *Bacillus thuringiensis* which expresses the cryIA(b)-protein, an insecticidal toxin, that induces pest resistance [30].

However, GM components in food and feed are still considered a contaminant in many countries. Thus, the demand for analytical methods for detection, identification and quantification is given in order to obtain information about the DNA or proteins of a GM crop at a farm, processor and/or retailer level. There is yet a considerable amount of unresolved issues concerning the development of appropriate test systems, such as sampling strategies, extraction methods, reference materials, multiplexing, new targets, quality endurance and accreditation, harmonisation needs and the economic impact of testing [24,31].

## 1.4 Food Authenticity

In 2012/2013 controls, performed by the European Commission, revealed the addition of horse meat in pre-packaged food products labelled as 100% beef [32]. The scandal unsettled consumers throughout Europe and raised awareness of the origin, composition and processing of food products. Sadly, adulteration, mislabelling and intentional falsification of food compounds is not an individual case and still occurs frequently in a wide range of foodstuffs. Thus, the issue of food authenticity is currently a hot topic of global interest [33].

The term “authentic” is basically defined as “reliable, genuine, trustworthy, of undoubted origin” [27]. However, food authenticity questions a wide array of potential liabilities that have to be addressed with a similar extensive band width of analytical methods, each perfectly adapted to its purpose [33,34]. The identification of the origin of a food compound is one example of the complexity of this endeavour and includes not only the geographic origin, but also questions about organic versus conventional farming, and especially for meat, slaughter age, sex, feed intake and meat cuts. Furthermore, potential substitutions in terms of species and tissue, vegetable and animal fat and/or proteins have to be evaluated. Processing treatments such as irradiation, fresh versus thawed foods and preparations (cooking, baking, frying) have to be taken into account as well. Another major issue is the addition of colorants, aromas, preservatives or water [33,35]. In terms of traceability and trackability, as defined by the EC regulation 178/2002, food- and feed-producing components, animals or ingredients have to be traceable through all stages of production and distribution [11].

Due to the increased controlling efforts undertaken by the European Union over the last years, it became evident, that a multitude of commodities are frequent targets of adulteration. Coffee for example is an important agricultural product with an annual turnover of around 35 billion US dollars [36]. Although 66 *Coffea* species have been identified so far, two varities are of particular importance: *Coffea arabica* L. (arabica coffee) and *Coffea canephora* Pierre (robusta coffee) [37]. Arabica coffee has a different chemical composition and is considered a higher quality product than robusta coffee which is reflected in its higher commercial value

price. A consequence is the frequent blending of both varities in order to sell an inferior product for the prize of a product of higher value. A similar situation has been discovered for Basmati rice, an aromatic variety of rice of higher quality, which is sold at a high prize on international markets [38]. Due to the potential gain, this crop is also heavily adulterated with non-fragrant varities from different growing areas [39]. Nonetheless, it has been discovered that the situation is even worse for mislabelled fish species like snapper and tuna. Snapper is a highly demanded variety that comes with a commensurate prize. Apparently, from 186 analysed samples labelled as snapper, only 25 samples are correctly labelled. In the case of tuna only 47 out of 116 samples happen to be actual tuna [40].

Apart from fraudulent intentions to sell an inferior commodity for the prize of a higher quality product, the falsification of food components might also pose a potential health risk, as is the case for the unlabelled addition of horse meat to meat products. In the course of the Commission recommendation 2013/99/EU the veterinary drug phenylbutazone is a widely applied drug and animals treated with this drug cannot enter the food chain by all means [41].

For these reasons, the European Commission highly prioritises strategies to prevent illegal actions in food production in order to protect consumers and facilitate trade in- and outside of Europe. Furthermore, the installed regulations and directives aim to protect products and brands from unfair competition. The integration of research institutes, technology providers and industries aims to develop standardised analytical methods to assure a harmonised and structured food supply [12,35].

## 2 Analytical Methods

In many cases, routine techniques like the microbiological survey, the examination of colour, odour and pH value are not sufficient to evaluate the quality and origin of a commodity. Mostly, the utilization of (bio)analytical methods involving a certain effort cannot be avoided in order to ensure the product is indeed labelled accordingly. Due to the diverse nature of food contaminants or unwanted and/or non-authentic compounds in food products, analytical methods have to be equally diverse and precisely adapted to serve their purposes. Although many groups of components can be approached differently in a methodical sense, in some cases a very specific approach is recommended to detect the presence of specific compounds [34,35,42]. Furthermore, some components can be masked to avoid detection and standard analytical methods have to be bypassed, as is the case for the addition of water to meat, which affects the weight and therefore the price. Usually, the protein/water ratio can be determined to obtain the actual water content in meat. Nevertheless, a higher

water content can be concealed through the addition of protein and phosphate to increase water binding, and resulting in a protein/water ratio close to the natural ratio [33,43]. Also, processing and heat treatment can further complicate analyses and practical performance has to be evaluated carefully in order to provide reliable results [42]. Another parameter that has to be taken into account is the potential sample throughput achievable with a certain technique, since it directly affects the costs to perform an analysis. Thus, the development of rapid high-throughput methods with as little instrumental requirements as possible is required to keep up with the challenges of the growing industry to further provide protection to consumers and markets.

## 2.1 Chromatographic Techniques

Chromatographic methods are capable of separating an analyte by distributing its compenents between a stationary and a mobile phase [44]. With the use of liquid chromatography (LC) or gas chromatography (GC), which is limited to volatile compounds, a wide array of substances such as proteins, amino acis, carbohydrates and phenolic compounds can be identified, if coupled with a detection device such as a mass spectrometer [45]. Several chromatographic techniques have successfully been applied for the identification of origin of certain food products by profiling their chemical composition [34].

An adulteration of olive oil can be detected by comparing the fatty acid composition using GC. The inclusion of chemometric analyses to GC analyses allows the determination of varietal and geographical authenticity due to the varying compound profiles present in different oils. [46,47]. The combination of GC with chemometric analyses has also been applied for the differentiation of coffee and fruit varities in order to detect the application of heat-treatments and the blending of products with ingredients of inferior quality [47-50]. HPLC was used to the detect the adulteration of bovine milk in ovine and caprine cheeses, and also wines of different areas of origin [51]. HPLC was also used the analysis of triglyceride and tocopherol compositions in different coffee varieties and olive oils and detects the adulteration of olive oils with hazelnut oils [52,53]. Another relevant application is the detection of marker proteins in durum wheat for the production of high-quality pasta [54]. Although the time for analysis can be greatly reduced by running a GC-MS system, the acquisitions cost are still to high to use GC-MS as routine technique for the authentication of food [34].

## 2.2 Spectroscopic Techniques

Based on the separation by chromatographic techniques, several spectroscopic

methods like spectrophotometry (UV-vis), fluorescence detection (FLD) and mass spectrometry can be applied to detect and identify the composition of an analyte. The principle of mass spectrometry is based on the separation of individual molecules with different mass-to-charge ratios (*m/z*) by utilizing electromagnetic fields [55] in order to identify peptide sequences or to detect trace elements, pollutants and drugs in a sample. MS is frequently used for the detection, identification and quantification of contaminants in food products which are either brought in during production, e.g. pesticides, or are produced during storage or processing, as is the case for most mycotoxins [56]. Many of these compounds are toxic and/or carcinogenic, therefore the analysis method has to be sensitive enough to detect trace amounts, but also selective to clearly identify the critical components independent from matrix interferences. Generally, gas chromatography-MS (GC-MS) and high performance liquid chromatography-MS (HPLC-MS) are preferred due to the fast sample preparation and high selectivity and sensitivity, although GC-MS is limited to volatile compounds [42].

Mass-spectrometric techniques are also used in allergen analysis in order to examine the structural elucidation of epitope regions with regard to molecular weight as well as primary, secondary and tertiary structures. Epitopes can still be active, even if proteins were modified or degraded during processing or heat and enzymatic treatments [57,58]. Although this analysis techniques are necessary to understand allergen reactions and it may be possible to reduce allergenicity by altering the epitopes, MS only plays a minor role in routine allergen screenings [42].

Stable isotope analysis is commonly used to determine the origin of a food product [35]. Ratios of the stable isotopes of oxygen ($^{16}O/^{18}O$) and hydrogen ($^{2}H/^{1}H$) are good indicators of environmental conditions and allow traceability back to the origin of a food product containing animal material [59]. Trace element analysis is also used to determine the geographic origin of soils, plants and fruits due to the intake of different isotopic “signatures” among different geographical locations by organic tissue [60]. This has been demonstrated for the adulteration of sugar in orange juices and for the geographic origin of wines, milk and cheese products [61-64]. Among the multitude of spectroscopic methods infrared spectroscopy (IR) plays a special role. It can provide substantial structural information via the vibrational behaviour of molecular bonds present in samples and is a powerful analytical tool, and can further be combined with chemometric analysis [34]. The great advantage of near IR spectroscopy in particular is that it is a rapid, non-destructive technique, that requires only little sample preparation and technical competence once extensive calibration models have been established [65]. IR spectroscopic methods have been successfully applied for the determination of fungal contaminants in cereal grains based on alterations in the carbohydrate or protein content of the crops [65]. IR has also been performed for the

analysis of fruit purees and juices, maple syrup, honey, *Echinacea* root, milk powder and fishmeal, either based on specific markers such as carbohydrate, protein or lipid contents or simply based on comparison between NIR spectra of "true" and "false" samples [66-72] .

## 2.3 Immunological Techniques

Based on the principle of the antibody-antigen-binding, several immunological techniques for food analysis are available. Lateral flow devices (LFDs) can be described as immunochromatographic technique based on a antigen-antibody-reaction on a nitrocellulose membrane and visualisation by the formation of a coloured band due to attached gold beads [73]. A more recent approach is the application of electrochemical immunosensors which provide potential on-site analysis, low instrument costs and higher sensitivity, compared to the frequently used enzyme-linked immunosorbent assays (ELISA) [74]. However, the majority of immunological methods in food analysis rely on the use of enzyme-linked immunosorbent assays (ELISA). This technique enables to the detection and quantification of proteins of interest due to the binding of antibodies or antisera to the immobilised antigen. This method provides high sensitivity and selectivity as well as the simple visual detection by a colour change of the reaction [34]. ELISA assays have been successfully applied for the authentication of meat and fish species [75-78], of milk and dairy products [79] and fruit juices [80]. Especially in allergen analysis, ELISA provides a well-suited method for the detection of allergens in trace amounts in food products [81]. However, the detection of some foods is more difficult than others, as it is the case for celery. Due to the close relationship of celery to other members of the Apiaceae family, a co-detection of parsley, carrot and other herbs and spices is commonly oberserved [82]. Therefore, ELISA is not the method of choice for the detection of celery. This analyte requires another approach, for example the detection via DNA-based techniques. Similarly, the detection of GMOs with immunoassays is delicate since these techniques rely on the binding of antibodies to proteins. If a genetically modified plant expresses a specific protein, it can be detected via ELISA as long as the protein is present in the investigated tissue and is not degraded. Screening assays, for example for the P35S promotor, which is present in many GMOs, cannot be performed using ELISA. One highly interesting approach is the detection of irradiated food products via immunological techniques. When food is treated with irradiation, the base thymidin is transformed into dihydrothymidin which can be detected within a range of 0.5-2 kGy [81].

Over the last years, a wide range of commercially available ELISA kits has been developed for various targets. However, all immunoassays rely on the availability of appropriate antibodies. In many cases the production costs are high and, as mentioned above, adequate antibodies are not available for all analytes [81].

## 2.4 DNA-based Techniques

Especially for processed foods, a DNA-based approach is recommedable due to the higher thermostability of nucleic acids compared to proteins. One methods is the hybridisation of labelled DNA probes to genomic DNA which is covalently bound to a membrane [83]. This technique is quite time-consuming and tedious, which sparked the advancement of biosensors and DNA microarrays and the so called "Lab-on-a-chip" format. DNA probes are immobilised onto a transducer surface and the duplex formation is detected via an appropriate hybridisation indicator [84]. Howver, most DNA-based techniques are based on the principle of the polymerase chain reaction (PCR), that was developed by Kary B. Mullis in 1983. This method enables the quick amplification of DNA fragments of interest for the subsequent analysis via electrophoretic or fluorimetric techniques. Nowadays PCR is a standard analytical tool of significant relevance for various kinds of analyses with organisms as targets.

PCR basically consists of recurring changes of temperatures, which are also described as cycles, in which a single DNA molecule can be amplified exponentially, resulting in $10^9$ copies of the specific, desired molecule. One cycle typically involves three steps: a denaturation step, where the double stranded molecule is melted into two separated DNA strands by heating up the reaction to 95°C; an annealing step at lower temperatures around 50 - 70°C, where short oligo nucleotides (primers) bind to one of the separated strands, and an extension step at around 70-80°C. During this step the DNA polymerase binds to the primer-template-complex and starts synthesising a new strand by adding complementary desoxyribonucleotides in a 5'→3' direction. With each extension step the concentration of the DNA target is doubled. To check whether the PCR generated the desired amplicon, the n products can be size-separated by agarose gel electrophoresis and compared with a molecular weight marker [85,86]. A more advanced approach is the real-time PCR, which enables the amplification and simultaneous detection of a DNA target as the reaction progresses. The two prevalent methods of detection are the use of unspecific fluorescent dyes that are intercalated into a DNA double strand, or the usage of sequence-specific oligonucleotides, so called probes, which are labelled with a fluorescent reporter that can only emit fluorescence after degradation of the probe by the 5'➔3' exonuclease activity of the polymerase [87].

DNA-based techniques are widely used for the analysis of food products and provide high specificity and sensitivity for detection and quantification. Although allergen analysis is typically performed via ELISA tests, in some cases only PCR is capable of specifically detect a certain analyte, e.g. celery. The difficulties of the specific detection of celery were described in chapter 2.3. Also, most GMOs are analysed via DNA-based techniques, especially PCR or real-time PCR due to the high selectivity and sensitivity. According to the

guidelines from the European Network of GMO laboratories, the specific inserted gene present in a transgenic commodity or plant has to be determined apart from comprehensive screening assays for certain elements such as the P35S promoter from the cauliflower mosaic virus or the nopaline synthase terminator from *Agrobacterium tumefaciens*, which are present in the multitude of transgenic plants [88].

Due to the capability of DNA-based methods to detect even minute differences like single nucleotide polymorphisms (SNPs) in the genetic sequences of two organisms, these methods can be used to reliably differentiate between closely related species or varities [89-91]. In food analysis PCR- or DNA-based techniques have been used for a broad spectrum of targets of interest. A major field of application is the detection of fungal contaminants in food products like the detection of aflatoxigenic species of *Aspergillus* and patulin-producing *Aspergillus* and *Penicillium* species [92,93]. Similar approaches have been developed for the detection of microbial contaminants like *Staphylococcus* and *Bacillus* species [94,95]. PCR-based techniques have also been applied to the detection of allergens that are likely to cause cross-reactivity to other species, like celery, hazelnut and lupin [96-98]. Another field of application for PCR-based techniques is food authentication, like the authentication of cattle and buffalo milk, cheese products and fish species [99-101].

# 3 Isothermal Amplification

## 3.1 General Aspects

PCR and real-time PCR are frequently used techniques if a DNA-based approach is recommended. However, due to the repeated heating and cooling cycles, a reaction takes at least 70 minutes. Besides, a thermal cycler is needed to perform the reaction and apart from high acquisition costs, trained personnel is required to operate and maintain the instrument. The discovery of DNA polymerases with strand displacement activity, such as the *phi29* polymerase [102] and the *Bst* polymerase [103], led to the development of various isothermal amplification approaches over the last two decades. Due to their capability of synthesising a new DNA strand by primer hybridisation and simultaneous separation of a double-stranded DNA product, a denaturation step like in conventional PCR is not needed. The amplification reaction can be performed at a constant temperature with a simple heating block. Figure 1 shows the temperature profile for standard PCR and isothermal amplification in comparison. The temperature profiles needed to perform a PCR assay prolong the reaction time and increase energy consumption. With isothermal amplification conditions, the reactions can be performed at a constant temperature, eliminating the need for a denaturation at 95°C. This

not only saves valuable time but also reduces the complexity of the required instruments. Assays based on isothermal amplification are, once developed, easy-to-perform and can perfectly be combined with visual detection techniques.

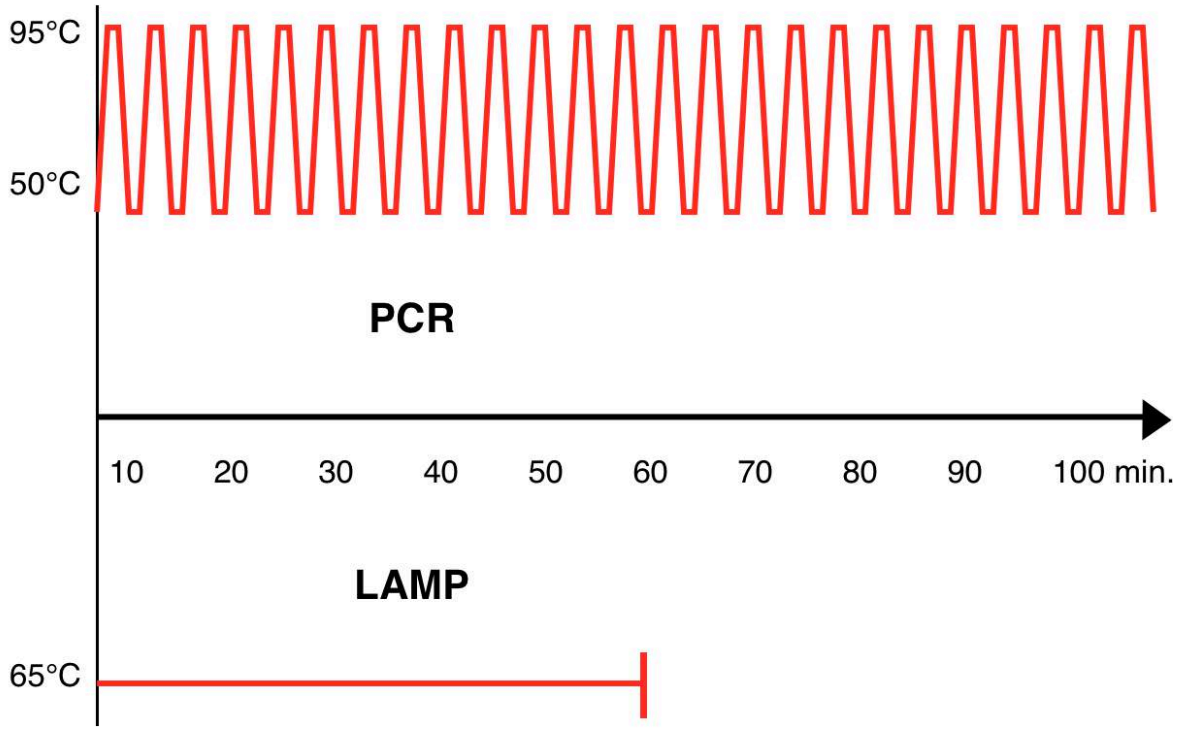


Fig. 1. Comparison of temperature profiles/run methods for conventional PCR (15 sec. at 95°C - 1 min. at 62°C) and isothermal amplification (20-60 min. at 65°C).

Due to the simplicity and effectiveness of these newly developed amplification methods, more and more isothermal amplification assays for various targets have been published. Especially in medicine and clinical diagnostics an increase of publications focusing on isothermal amplification approaches can be observed, as demonstrated in Figure 2. In 2013 the yearly output of research papers concerning isothermal amplification reached a number of 278 publications [104]. Due to the rising number of fraudulent activities regarding the composition and origin of food products as well as tightening food laws, rapid test systems that can be performed without trained personnel and sophisticated equipment start to play a major role in food safety.

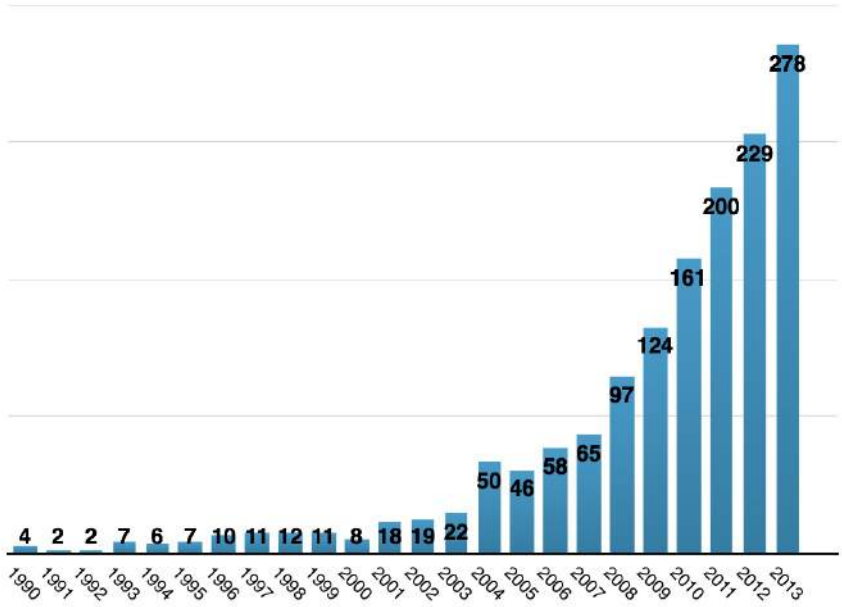


Fig. 2. Number of studies found using the search criterion "isothermal amplification" at the NCBI PubMed database.

## 3.2 Loop-mediated Isothermal Amplification

The principle of the loop-mediated isothermal amplification technique is based on the hybridisation of two different primer sets, the inner and outer primers, onto three distinct regions of the target sequence. The inner primers are particularly designed to form a loop, once they new strand has been elongated. The resulting dumbbell-like structure serves as starting structure for the actual LAMP cycling and enables the continuous hybridisation of the inner primers and the release of each newly synthesised strand by the outer primers that function as displacement primers (Figure 3). Thus, the DNA polymerase produces long DNA products with alternately inverted target repeats (Figure 4).

Although this DNA amplification technique is simple and quickly to perform, the primer design is a highly complex and crucial step in developing a loop-mediated isothermal amplification assay. Factors that strongly influence the efficiency of a LAMP assay are the guanine/cytosine content, the distance between the primer binding regions, the melting temperature of the oligonucleotides and the stability of the primer ends which has an important impact on the formation of loop structures. LAMP primer sets are ideally designed using the software Primer explorer V4, provided by Eiken Chemical Co., Ltd.; [105] which has been specifically designed for LAMP primer calculation.

The first LAMP assay which was developed by Notomi et al. in 2000 [106], detected the hepatitis B virus within a reaction time of 45 min. The amplification products were visualised via agarose gel electrophoresis. Two years later, Nagamine et al. [107] recommended the

additional use of another primer set, the so called loop primers, to further enhance the assay. Through the addition of loop primers more starting points for the amplification reaction are available and the LAMP reaction can be significantly accelerated and the reaction time can be shortened by more than 50%. In this publication the LAMP products were detected via real-time fluorescence monitoring using ethidium bromide. In 2003, Iwamoto et al. [108] developed a LAMP assay targeting *Mycobacterium tuberculosis* and performed a naked eye detection via staining with SYBR Green I. If the amplified product is present, a colour change from orange to green is observed.

Although the assay sensitivity is influenced by genome size and the particular target sequence, the precise primer design is the most crucial step for developing a highly sensitive and specific assay. Detection limits reach from 1 picogram of DNA to less than five copy numbers depending on the target. Due to the use of at least four primers, high specificity is provided for most assays, and only a few base mismatches in the loop region would lead to the inhibition of the amplification process [106].

One drawback of this method is that it cannot be used for quantification since reliable quantification is only possible at ranges from $1 \times 10^4$ - $1 \times 10^{10}$ target copy numbers, but not at lower concentrations [109-111]. Another disadvantage is the length of the target DNA needed for primer design. A typical LAMP amplicon consists of around 140 to 220 basepairs. Short target sequences complicate the primer design and make it difficult to design sensitive and efficient reactions.

Although LAMP is more or less limited to qualitative assays and cannot be used for short targets of less than 50 basepairs, this technique has various benefits that make it highly suitable for simple and rapid on-site testing. LAMP is much less susceptible to co-isolated substances such as carbohydrates that can inhibit conventional PCR reactions. The reaction time is much shorter and the assay can be performed on a simple heating block without an initial denaturation phase. Another advantage is the simple visual detection of LAMP products by staining the produced DNA with SYBR Green I. All these features make LAMP an interesting approach for future rapid DNA testing.


Fig. 3. Schematic drawing of the early steps of a typical LAMP reaction leading to the starting structure used for the actual amplification reaction. (1) The forward inner primer (FIP) binds to the complementary sequence on the target strand (2) the FIP is elongated by the Bst polymerase and synthesises a new complementary strand in 5'→3' direction (3) - (5) the forward outer primer (F3) binds to the DNA target and releases the prior synthesised strand (6) The F1c region binds to the self-complementary region on the same strand forming a loop structure; simultaneously the backward inner primer (BIP) binds to the target, synthesises a new strand in 5'→3' direction which is again replaced by the backward outer primer (B3) (7) - (8) The B1c region binds to the self-complementary region on the same strand, forming a second loop structure which results in a dumbbell-like structure which acts again as a starting structure for amplification. Eiken Chemical Ltd.

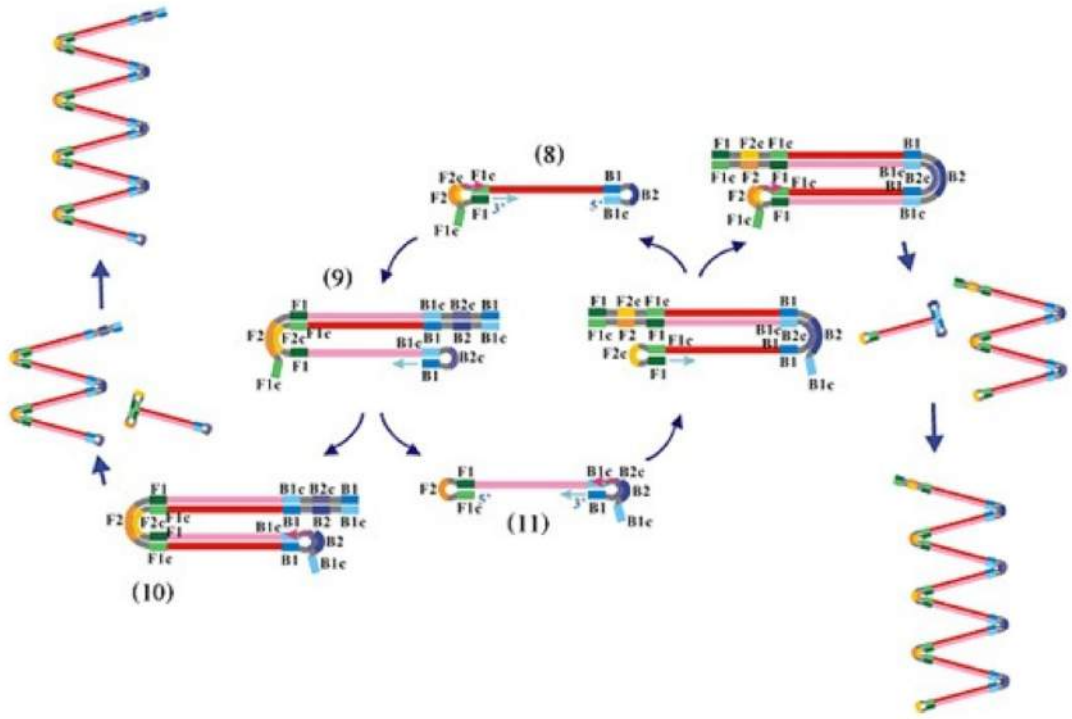


Fig. 4. Basic principle of later stages of a LAMP amplification. (8) At the F1 region the strand is elongated by self-priming, dissolving the loop structure on the 5' end; simultaneously, the FIP hybridises to the F2c region on the loop structure on the 3' end and releases the previously synthesized strand (9) The newly formed loop structure again initiates a self-priming reaction, the elongated strand releases the FIP-replaced strand (10) - (11) Similarly, the BIP induces a self-priming reaction which leads to the same antisense structures as (10). Continuous cycling on both ends leads to various-sized products with alternately inverted repeats of the target sequence. Eiken Chemical Ltd.

### 3.3 Other Isothermal Amplification Techniques

With the discovery of thermostable DNA polymerase with strand displacement activity, several novel amplification techniques have been developed, some of them more promising than others. Each method offers different benefits but also comes with certain drawbacks. The balance between complexity and application spectrum has to be determined for each problem and highly depends on the target of interest.

Rolling Circle Amplification (RCA) was originally developed by Fire & Xu [112] and later modified by Lizardi et al. [113] and proposes an interesting approach due to its very high efficiency without the requirements of a long known target sequence for the design. An open circle probe that consists of two target-complementary regions at the 3' and 5' ends in a juxtaposed position including a generic spacer region in between is circularized by hybridizing to its complementary DNA target and is subsequently ligated. Ligation can only

work when all nucleotides of the open circle probe are complementary to the DNA target. In case of a mismatch, the DNA ligase cannot advance and the ligation process is aborted. If the matching target is present, the ligated DNA circle can subsequently be amplified by hybridization of one or more primers producing a long strand of DNA consisting of numerous repeats of the target sequence. Although this technique can be used for quantification purposes, the reaction set-up itself is more laborious than with other isothermal techniques since it has to be divided into two steps, the ligation step and the amplification step. Furthermore, an initial denaturation step preliminary to the ligation is required for successful hybridisation of the open circle probe to the target. The reported issue of non-specific background amplification can only be avoided by restriction digestion to remove any unligated probes [114,115].

Helicase Dependent Amplification (HDA) reaction uses the unwinding activity of a helicase in order to separate a double-stranded DNA sequence [116]. The helicase is unwinding and displacing the strands simultaneously during the amplification readtion. Therefore, heat denaturation and thermal cycling as in conventional PCR are not required. The reaction uses two target-specific primers, a helicase and a DNA polymerase with strand displacement activity. This technique offers simple primer design, high sensitivity and the possibility of multiplexing for short and long target sequences [117].

Another technique is the Strand Displacement Amplification (SDA), an isothermal amplification method initially described by Walker et al. [118]. DNA amplification is achieved by the use of two primer sets (S1, S2, B1 and B2), with S1 and S2 containing a recognition site for the restriction enzyme at the 5’ end, the restriction enzyme itself with the ability to nick an unmodified strand of hemi-modified DNA and an exonuclease-deficient polymerase. Primers B1 and B2 function as displacement primers at the early stage of the reaction to release the newly synthesized strand. All four primers hybridise to the target DNA and are extended by the polymerase. The initially produced strand is displaced during the elongation of the displacement primer B1 and B2. Short double-stranded target sequences are produced due to repeated annealing, elongation and displacement. These generated amplicons are flanked by recognition sites for the restriction enzyme used in SDA. After the target generation phase, these sites are nicked by the restriction enzyme and elongated by the DNA polymerase, which thus displaces the downstream strand. Exponential amplification is achieved by cyclic nicking, extension and displacement [119,120].

Nicking-enzyme amplification reaction (NEAR) is based on the principle of SDA. It is distinguished only by the use of nicking endonucleases, capable of hydrolysing only one strand of the double-helix. In contrast, the nicking step in SDA is accomplished by the addition of cost-extensive phosphorothioate nucleotides, which are incorporated into the newly synthesised DNA strand to protect the recognition site against full cleavage. Similarly

to SDA, the reaction further requires target-specific primers and an exonuclease-deficient polymerase capable of inducing the nicking step and subsequent strand displacement [121,122].

Due to the possibility to perform the amplification reactions at a constant temperature, these techniques are highly suitable for the development of rapid on-site test systems for food analysis. However, the costs and benefits for each method have to be evaluated. Some techniques require an intial denaturation step at 95°C, which again complicates the fundamental simplicty of isothermal amplification reactions. Furthermore, if expensive reagents, like the phosphorothioate nucleotides for SDA, are required, the value of this technique is questionable. One benefit of some techniques is, that the known target sequences, necessary for the primer design, can be shorter than 50 basepairs, which is especially suitable for GMO analysis. In general, each method has to be carefully evaluated and adapted to the specific analyte in order to assure the optimal utilization and efficiency and the least laboratory and instrumental effort.

## Aims and Structure of this Thesis

The presented thesis was performed at the Center for Analytical Chemistry (IFA Tulln) under the supervision of Dr. Kurt Brunner. The aim of this work group is the development of DNA-based techniques for the detection of undesired compounds in food and feed products including mycotoxin-producing fungi, allergens and genetically modified organisms (GMOs) as well as food authentication.

The thesis at hand is a **cumulative work** and consists of three chapters. The **introduction** gives an outline on current legislation within the European Union, including regulations, directives and recommendations regarding the issues of food safety with special focus on allergens, GMOs and food authenticity. A short summary of frequently used methods for the detection of such is given, including protein- and DNA-based methods as well as spectroscopic approaches. Additionally, the advantageous use of DNA-based techniques for the detection of some analytes, like closely related species, is emphasized. Concluding, an overview of recently developed DNA-based methods, that enable field-applicable, rapid and easy-to-perform tests, is given. The **conclusion** points out the relevant achievements and highlights of the original works. Subsequently following are three major **Science Citation Index (SCI) publications** selected for this thesis. Prior to each publication a short summary is given.

Due to recent incidents as well as trends in applied sciences the development of simple and rapid high-throughput test systems is required. Whenever a DNA-based approach is recommended, PCR is used for analyses as state-of-the-art technique. However, this method is laborious and costly in terms of equipment and operating personnel and cannot be described as rapid method, capable of analysing a large number of samples in short time. With the development of isothermal amplification techniques a new generation of DNA amplification procedures is available that has the potential to overall facilitate testing of various analytes. This new approach provides the possibility of on-site testing in the future, without the need of trained operators and costly instruments.

The main objective of this thesis was the development of DNA-based rapid methods for the detection of certain unwanted compounds in food. The first aim was to develop a **loop-mediated isothermal amplification assay** for the detection of **the potential food allergen celery** in various food matrices (Publication #1). The second aim was an investigation on **various isothermal amplification techniques** for the detection of **transgenic maize** (Publication #2). The third aim was the development of a **loop-mediated isothermal amplification assay** for the detection of **horse meat** in model sausages and commercial products (Publication #3). All of the performed experiments intended to facilitate testing for certain analyses in the future, enabling rapid easy-to-perform tests with visual detection modes.

# Original Works

## List of Selected Publications

**Publication #1:** Zahradnik Celine, Martzy Roland, Mach Robert, Krska Rudolf, Farnleitner Andreas, Brunner Kurt (2014) Detection of the food allergen celery via loop-mediated isothermal amplification technique. Anal Bioanal Chem 406(27):6827-6833

**Publication #2:** Zahradnik Celine, Kolm Claudia, Martzy Roland, Mach Robert, Krska Rudolf, Farnleitner Andreas, Brunner Kurt (2014) Detection of the P35S promotor in transgenic maize via various isothermal amplification techniques: a practical approach. Anal Bioanal Chem 406(27):6835-6842

**Publication #3:** Zahradnik Celine, Martzy Roland, Mach Robert, Krska Rudolf, Farnleitner Andreas, Brunner Kurt (2014) Detection of the food allergen celery via loop-mediated isothermal amplification technique. Food Analytical Methods [Submitted & Revised]

## Publication #1

# Detection of the food allergen celery via loop-mediated isothermal amplification technique

**Celine Zahradnik**, Roland Martzy, Robert Mach, Rudolf Krska, Andreas Farnleitner, Kurt Brunner

**Published in:** Analytical and Bioanatical Chemistry (2014)

**Abstract** Since 2005, celery and celery products have to be labeled according to Directive 2003/89/EC due to their allergenic potential. In order to provide a DNA-based, rapid and simple detection method suitable for high-throughput analysis, a loop-mediated isothermal amplification (LAMP) assay for the detection of celery (*Apium graveolens*) was developed. The assay was tested for specificity for celery since closely related species also hold food relevance. The limit of detection (LOD) for spiked food samples was found to be as low as 7.8 mg of dry celery powder per kilogram. An evaluation of different amplification and detection platforms was performed to show reliable detection independent from the instrument used for amplification (thermal cycler or heating block) and detection mechanisms (real-time fluorescence detection, agarose gel electrophoresis or nucleic acid staining). The analysis of 10 commercial food samples representing diverse and complex food matrices, and a false-negative rate of 0 % for approximately 24 target copies or 0.08 ng celery DNA for three selected food matrices show that LAMP has the potential to be used as an alternative strategy for the detection of allergenic celery. The performance of the developed LAMP assay turned out to be equal or superior to the best available PCR assay for the detection of celery in food products.

**Zusammenfassung** Seit 2005 wird die Etikettierung von Sellerie und Sellerieprodukten aufgrund des allergenen Potentials durch die Richtlinie 2003/89/EC der Europäischen Kommission vorgeschrieben. Im Zuge der Entwicklung von DNA-basierten und einfach durchzuführenden Test-Methoden, welche einen hohen Proben-Durchfluss erlauben, wurde ein Loop-mediated Isothermal Amplification (LAMP) Assay für den Nachweis von Sellerie (*Apium graveolens*) entwickelt. Dieser Test wurde auf seine Spezifität für Sellerie untersucht, da eine Vielzahl von eng verwandten Arten ebenso große Bedeutung in der Lebensmittelindustrie besitzt. Die Nachweisgrenze für mit Sellerie versetzten Proben lag bei 7.8 mg Selleriepulver pro Kilogramm. Eine Evaluierung verschiedener Amplifikations- und Detektionsplattformen konnte die zuverlässige Detektion, unabhängig von verwendeten

Geräten (Thermocycler oder Heizblock) und Nachweismechanismen (real-time Fluoreszenzmessung, Agarosegelelektrophorese oder Nukleinsäure-Färbung), zeigen. Die Analyse zehn kommerzieller Lebensmittelprodukte mit möglichst komplexen Matrices ergab eine Falsch-Negativ-Rate von 0% für 24 Target-Kopien bzw. 0.08 ng Sellerie-DNA in drei ausgewählten Produkten und zeigt, daß LAMP das Potenzial als alternative Nachweis-Strategie für die Detektion von allergenem Sellerie besitzt. Die Effizienz des entwickelten LAMP-Assays ist wenigstens als gleichwertig anzusehen im Vergleich zu früher entwickelten PCR-Assays zum Nachweis von Sellerie in Lebensmitteln.

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RESEARCH PAPER

# Detection of the food allergen celery via loop-mediated isothermal amplification technique

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**Abstract** Since 2005, celery and celery products have to be labeled according to Directive 2003/89/EC due to their allergenic potential. In order to provide a DNA-based, rapid and simple detection method suitable for high-throughput analysis, a loop-mediated isothermal amplification (LAMP) assay for the detection of celery (*Apium graveolens*) was developed. The assay was tested for specificity for celery since closely related species also hold food relevance. The limit of detection (LOD) for spiked food samples was found to be as low as 7.8 mg of dry celery powder per kilogram. An evaluation of different amplification and detection platforms was performed to show reliable detection independent from the instrument used for amplification (thermal cycler or heating block) and detection mechanisms (real-time fluorescence detection, agarose gel electrophoresis or nucleic acid staining). The analysis of 10 commercial food samples representing diverse and complex food matrices, and a false-negative rate of 0 % for approximately 24 target copies or 0.08 ng celery DNA for three selected food matrices show that LAMP has the potential to be used as an alternative strategy for the detection of allergenic celery. The performance of the developed LAMP assay turned out to be equal or superior to the best available PCR assay for the detection of celery in food products.

**Keywords** Loop-mediated isothermal amplification · Celery · Food allergen · Isothermal amplification · Visual detection

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## Introduction

Celery (*Apium graveolens* L.) is a widely used ingredient in seasonings, sauces, bouillons, and instant meals. Allergy to celery is one of the most common food allergies in Europe causing digestive disorders, respiratory distress, and skin reactions when ingested [1, 2]. In severe cases, exposure can even induce anaphylaxis of the affected individual. Doses triggering such a response typically are around 0.7 g of fresh celery; however, lower amounts may also elicit a reaction [3]. Celery is highly associated with an allergization to birch and mugwort, termed birch-mugwort-celery syndrome [4, 5]. Known allergens of celery are the 16 kD heat-labile protein Api g1, which is homologous to the profilin Api g4 and the glycoprotein Api g5, and the protein Bet v1 from birch. Several studies have proven Api g1 to be the most relevant allergen in celery causing allergic reactions [6–8].

Avoiding the allergen-containing food is the only option to prevent an allergic reaction which may often be difficult due to the contamination of food products with allergenic ingredients. Contaminants can be introduced through food-processing machines, storage, and shipping or are added deliberately for flavoring purposes. Directive 2003/89/EC constituted by the European Union states the mandatory labeling

of 12 groups of ingredients with known allergenic potential, including celery and celery products [9].

This new legislation rises the demand for specific and sensitive methods for the detection of allergens in food products. Although the characterization of celery allergens via mass spectrometry (MS) has been reported [10], currently, the two most prominent analytical techniques for allergen detection in food are protein-based enzyme-linked immunosorbent assays (ELISA) and nucleic acid-based polymerase chain reaction (PCR) assays. At present, no ELISA assay is available for the specific detection of celery due to cross-reactions with parsley (*Petroselinum crispum* (Mill.) Nyman & A.W. Hill) [11], carrot (*Daucus carota* (Hoffm.) Schübl. & G. Martens), and other closely related members of the Apiaceae family. So far, several real-time PCR assays have been developed targeting the gene encoding sequence for Api g 1. However, due to the high risk of cross-reactions, targeting the celery-specific mannitol dehydrogenase is recommended [12, 13]. Although two quantitative real-time PCR (qPCR) assays for the detection of celery have been developed [13, 14], real-time PCR is still a laborious procedure, requiring sophisticated equipment such as thermal cyclers and trained personnel making it unsuitable for a simple on-site high-throughput detection method for celery in food matrices.

An alternative DNA amplification method termed loop-mediated isothermal amplification (LAMP) was developed in 2000 [15]. The use of three primer sets binding six distinct regions of the target sequence and a *Bst* polymerase with strand displacement activity [16] initially forms a dumbbell-like structure which then undergoes cyclic amplification producing various sized DNA strands with alternately inverted repeats of the target sequence [15, 17]. Based on the lack of a denaturation step, the assay can be carried out under isothermal conditions eliminating the need for a thermal cycler and thus making this method highly cost- and time-efficient as well as field-applicable and easy-to-perform. The high efficiency and sensitivity of a LAMP assay allows detection with the naked eye through the use of an intercalating dye such as SYBR Green I [18].

A recently published assay for the detection of celery based on LAMP [19] uses a primer set showing high-sequence homologies for parsley, dill *(Anethum graveolens*), and fennel (*Foeniculum vulgare*) in nBLAST (National Center for Biotechnology Information NCBI), and for carrot and parsley as experimentally proven by the authors of this study (see Electronic Supplementary Material, Fig. S1 and Table S1). Since all of these plants are widely used, this assay is not a suitable tool for the detection of celery in food-related products.

Here, we report the development of a highly specific LAMP-based assay for the detection of celery in food products targeting a diagnostic fragment of the celery mannitol dehydrogenase gene, which was demonstrated to be specific in an earlier publication [20]. We evaluated species-specificity and sensitivity by testing several different primer sets and comparing it to conventional real-time PCR. To investigate the potential of the assay for the rapid analysis of celery in food, ten different commercially available products were tested with LAMP and PCR. Celery-negative samples were spiked with celery powder to ensure reliable detection despite the potential of inhibition due to complex food matrices. The LAMP amplification products were detected by real-time fluorescence monitoring, via agarose gel electrophoresis and by staining LAMP products with SYBR Green I for visual detection and similarly compared with a previously published real-time PCR assay for the detection of celery in food [13].

## Materials and methods

### Plant material and samples

Celery root (*A. graveolens* var. *rapaceum*), parsley (*P. crispum* (Mill.) Nyman & A.W. Hill), carrot (*D. carota* (Hoffm.) Schübl. & G. Martens), anise (*Pimpinella anisum* L.), dill (*A. graveolens* L.), fennel (*F. vulgare* Mill.), coriander (*Coriandrum sativum* L.), caraway (*Carum carvi* L.), cumin (*Cuminum cyminum* L.), lovage (*Levisticum officinale* W.D.J. Koch), parsnip (*Pastinaca sativa* L.), Kotanyi steak seasoning, Billa mushroom instant soup, Wasa sesame crisp bread, Bad Ischler 7 herbs salt, Maggi vegetable bouillon cube, Inzersdorfer wellness turkey spread, Kotanyi herbal mix, Hipp vegetable baby food, Rio Mare tuna pastry and Lorenz Naturals potato chips with garlic, and herbs were obtained from local Austrian supermarkets. All samples except for the commercial food products were extracted from fresh material.

### DNA extraction

For specificity tests and for generating a calibration curve, fresh celery root as well as all other plant materials were lyophilized for 48 h and then ground to a fine powder using a mortar and pestle. Extraction of nucleic acids was performed using a CTAB extraction protocol as previously described [21]. Briefly, 100 mg of plant material were used for extraction using a 10 % CTAB buffer and chloroform/isoamylalcohol (24:1) solution. Subsequent to an ethanol precipitation, the obtained DNA pellet was washed twice with 70 % ethanol and then resuspended in 10 mM Tris buffer. Sensitivity tests, false-negative rate, and the analysis of commercial food samples were performed using DNA extracted with the SureFood® PREP Allergen Kit (Congen, Berlin, Germany) according to the manufacturer's instructions. DNA concentration and purity were determined photometrically using NanoVue Plus (VWR, Radnor, USA).

**Table 1** List of LAMP primers and their sequences targeting the celery mannitol dehydrogenase gene from celery (GenBank acc. no. AF067082)

| LAMP primer | Sequence 5′–3′ |
|---|---|
| FIP | GCTGCAAAATCAAGCATTTCTTGTGCAGGGAGA AAGCTTCTTGG |
| BIP | AACAGCTGATGTTGAAGTTATTCCTAAATCTGT ATCGAACATCTGAC |
| F3 | CATTGTTATACTAAATGTGAGTCAC |
| B3 | CGTATTTGCAATGTCGATGAC |
| LoopF | CTTTATCCCACCATTAATAGTGCCT |
| LoopB | ATGGACTATGTGAACACCGCA |

## Primers and probes

All oligonucleotides were synthesized by Eurofins MWG (Ebersberg, Germany). Real-time PCR primers and probe were used as published by Hupfer et al. [13]. LAMP primers were designed using the Primer Explorer V4 software (Eiken Chemical Co., Ltd.; Tokyo, Japan) on basis of the sequence for celery mannitol dehydrogenase, GenBank acc. no. AF067082. The LAMP primer sequences are given in Table 1.

## Real-time PCR reaction

DNA extract (2 μL) either undiluted or diluted 1:4 was added to 13 μL of reaction mix containing Kapa™ Probe® Fast (Peqlab, Erlangen, Germany), primers and probe (in a final concentration of 0.3 μmol/L each). Amplification reactions were performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, New York, USA) according to the following thermal cycling protocol: initial steps of 2 min at 95 °C, followed by 60 cycles of 15 s at 95 °C and 1 min at 60 °C. Unless noted otherwise, amplification reactions were carried out in five replicates.

## LAMP reaction

LAMP reactions were carried out in a total reaction volume of 25 μL containing 1.4 μM each FIP and BIP, 0.2 μM each F3 and B3, 0.8 μM each LoopF and LoopB, 1.4 μM of each dNTP (Peqlab, Erlangen, Germany), 0.8 M betaine solution (Sigma-Aldrich, St. Louis, USA), 8 U *Bst* DNA polymerase (New England Biolabs, Ipswich, USA), 20 mM Tris-HCl (pH 8.5), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 6 mM $MgSO_4$, 0.1 % Triton X-100 (Sigma-Aldrich, St. Louis, USA), 1 μL Syto® 9 Green Fluorescent Nucleic Acid Stain (Life Technologies, Carlsbad, USA) [22], and 2 μL undiluted DNA extract. The reaction mixture was incubated for 60 min at 65 °C. Amplification reactions were carried out in five replicates. Aliquots of 10 μL of LAMP products were used for agarose gel electrophoresis. For the detection with the naked eye 1× SYBR® Green I Nucleic Acid Stain (Life Technologies) was added to aliquots of 15 μL of LAMP products and viewed under UV light.

# Results

## Design and specificity of LAMP primers

To ensure efficient and specific amplification of celery DNA, five primer sets were designed and tested. All subsequent experiments were performed using the primer set given in Table 1, which showed the highest fluorescence intensity and the lowest Ct value without amplifying DNA other than

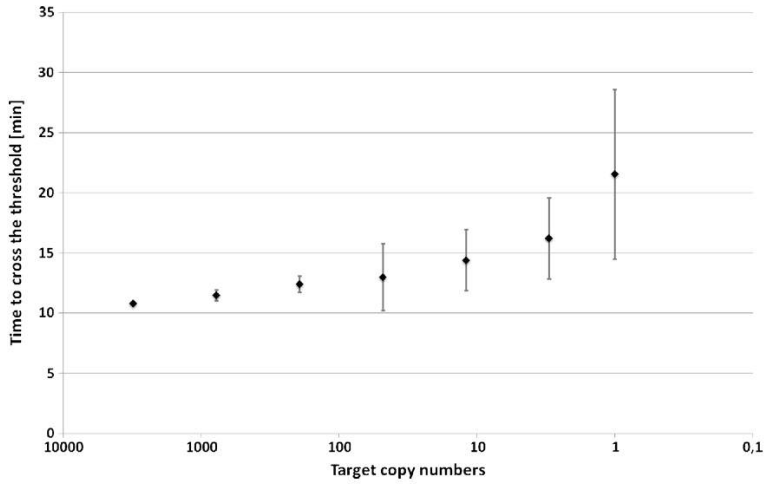


**Fig. 1** Calibration curve shows standard deviation (SD) increasing from 0.01 % for 3,093 target copies to 55 % for three target copies. The increasing error for decreasing target copies clearly demonstrates that this assay cannot be used for a reliable quantification of trace amounts of celery DNA

celery and lacks relevant homologies with other closely related species as checked by NCBI Blast search. To rule out cross-reactive detection of other members of the Apiaceae family, genomic DNA from herbs and spices commonly used as food ingredients (see plant material & samples) was extracted and analyzed. All tested species did not produce an amplicon in LAMP which demonstrated the specificity of the assay (data not shown).

## Sensitivity and limit of detection

To evaluate the potential of the assay for quantitative analysis, DNA was extracted from fresh celery roots, nucleic acid concentrations were determined photometrically in order to prepare serial dilutions ranging from 10 to 0.0039 ng/μL. Based on the estimation that celery contains 3.0 pg DNA per haploid nucleus [23] and one base pair has a molecular weight of 650 Da, the target copy number can be calculated. Therefore, 10 ng/μL celery DNA correspond to a dilution of 3,093 target copies. Each concentration was analyzed in 20 replicates. A calibration curve was generated by plotting the average threshold time against the corresponding DNA amounts (Fig. 1). The European Network of GMO Laboratories requires a correlation coefficient higher than $R^2 \geq 0.98$ for the real-time PCR quantification of genetically modified organisms [24]. The assay failed to meet this requirement due to its deviating Ct values, even though 20 out of 20 replicates gave a positive signal for an estimated concentration of three target copies. Although development of quantitative real-time LAMP assays for several targets using intercalating dyes has been reported, it is pointed out that the quantitative capability relates to ranges of $1 \times 10^4$–$1 \times 10^{10}$ target copies [25–27]. This clearly shows that the designed celery LAMP assay is not suitable for the accurate quantification of trace amounts of DNA.

Two food samples, previously tested negative for celery DNA, were spiked with defined amounts of dry celery powder as given in Table 2 and were then extracted two times each. Tuna pastry and crisp bread were both selected due to their high contents of salts and carbohydrates or proteins which can act as inhibitors of amplification reactions [28]. Each sample was analyzed by PCR and LAMP in five replicates. Table 2 shows that 7.81 mg celery/kg sample still could be detected with 100 % positive replicates in tuna pastry whereas 500 mg/kg are required for five positive replicates for crisp bread. This variation between the two food matrices might be due to the high amount of carbohydrates typical for crisp bread which can compromise the accurate amplification of DNA. However, both samples representing both matrices could still be identified as positive even if a low amount of 1.95 mg celery powder was spiked to 1 kg food.

**Table 2** Results of spiking experiments with LAMP for two food matrices. One hundred grams of food sample were spiked with 5 g of celery powder and then diluted with the same homogenized sample matrix to obtain low concentrations of celery. Both matrices were extracted twice and analyzed in five replicates per extraction

| Celery content (mg celery/kg) | Wasa sesame crisp bread positive reactions | | Rio Mare tuna pastry positive reactions | |
|---|---|---|---|---|
| | Extraction 1 | Extraction 2 | Extraction 1 | Extraction 2 |
| 1.95 | 2/5 | 2/5 | 4/5 | 3/5 |
| 7.81 | 2/5 | 2/5 | 5/5 | 5/5 |
| 31.25 | 2/5 | 2/5 | 5/5 | 5/5 |
| 125 | 3/5 | 2/5 | 5/5 | 5/5 |
| 500 | 5/5 | 5/5 | 5/5 | 5/5 |
| 1.000 | 5/5 | 5/5 | 5/5 | 5/5 |
| 5.000 | 5/5 | 5/5 | 5/5 | 5/5 |
| 10.000 | 5/5 | 5/5 | 5/5 | 5/5 |
| 50.000 | 4/5 | 5/5 | 5/5 | 5/5 |

## False-negative rate

The determination of a false-negative rate is used to identify the lowest concentration at which the assay detects celery positive samples correctly. Below this concentration, a certain percentage of celery DNA containing replicates appear to be negative. DNA isolated from crisp bread, turkey spread, and baby food, previously tested negative for the presence of celery, was spiked with a decreasing number of celery DNA target copies. Figure 2 shows that decreasing the estimated target copy number below 24, leads to false negative replicates. If 24 or more copies per assay are present, the false-negative rate drops down to 0 % for all three food matrices. These results indicate that LAMP is quite insensitive to co-isolated amplification inhibitors and enables analysis of

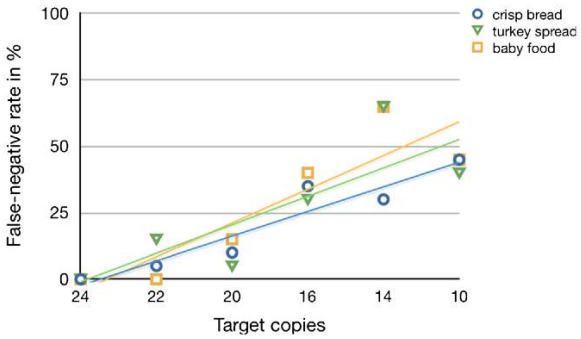


**Fig. 2** DNA extracted from celery-free food samples was spiked with different amounts of celery DNA. The false-negative rate rate was 0 % for 24 or more target copies for all tested matrices and increased to 50 % when analyzing concentrations lower than 16 copies. Number of replicates $n=20$

**Table 3** Ten food samples from the supermarket were analyzed for celery DNA with PCR and LAMP

| Sample ID | Product | Declaration | extraction 1 | | extraction 2 | | extraction 3 | |
|---|---|---|---|---|---|---|---|---|
| | | | PCR | LAMP | PCR | LAMP | PCR | LAMP |
| 1 | Steak seasoning | (+) | +++++ | +++++ | +++++ | +++++ | +++++ | +++++ |
| 2 | Mushroom soup | (+) | ++−−− | +−−−− | +++−− | +++−− | ++−−− | ++++− |
| 3 | Crisp bread | − | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− |
| 4 | Herbs salt | + | +++++ | +++++ | +++++ | +++++ | +++++ | +++++ |
| 5 | Bouillon cube | + | +−−−− | +++−− | −−−−− | −−−−− | ++−−− | ++++− |
| 6 | Turkey spread | + | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− |
| 7 | Herbal mix | – | −−−−− | +−−−− | −−−−− | −−−−− | −−−−− | +−−−− |
| 8 | Baby food | – | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− |
| 9 | Tuna pastry | – | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− |
| 10 | Potato chips | – | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− | −−−−− |

Labeling on the food packages: + contains celery, (+) may contain traces of celery, − contains no celery or not declared

samples without extensive clean-up procedures before introducing isolated DNA into the amplification reaction [29, 30].

## Analysis of commercial food samples

Ten food samples obtained from local supermarkets were selected with regard to a broad spectrum of matrices rich in carbohydrates, proteins, fats and other components known to inhibit amplification reactions [26, 27]. Three different batches of each product were used for the extraction of DNA. For each extraction, both, PCR and LAMP reactions were performed in five replicates (Table 3). The analyte could be detected in four samples that were declared to contain celery. Interestingly, celery DNA was found in the herbal mix which was not declared to contain celery, while turkey spread, positively declared, could not be confirmed to contain celery neither with PCR nor with LAMP. This might be due to DNA fragmentation caused by extensive food processing like heat treatment or harsh chemical conditions during the production. The remaining four samples crisp bread, baby food, tuna pastry and potato chips did not indicate the presence of celery in compliance with their declaration. Figure 3 shows that the numbers of positive and negative signals for the analysis of these samples correlate closely between PCR and LAMP. For mushroom soup and bouillon cube, the LAMP reactions showed more positive replicates than PCR. LAMP resulted in a positive amplification signal for the herbal mix, whereas with PCR no signal could be detected.

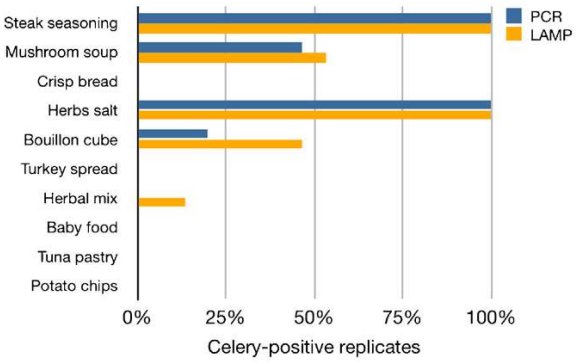


**Fig. 3** Both methods, PCR (*blue*) and LAMP (*orange*), give similar numbers of positive replicates. In some cases LAMP shows to be even more sensitive than the corresponding PCR assay

## Evaluation of different amplification and detection platforms

Several tests were performed to show the independence of this method from experimental conditions. Table 4 shows that at least 95 % of replicates containing approximately 40 target copies (0.13 ng/μL) of celery DNA can be detected when

**Table 4** Comparison of positive amplification signals obtained with one thermal cycler (Applied Biosystems 7500 Fast Real-time PCR) and two different heating blocks (Eppendorf Thermomixer plus and Peqlab Digital Block Heater HX-1), resulting in at least 19 of 20 positive replicates. Three different methods for the detection of the amplified DNA were chosen—Syto® 9 in a real-time PCR cycler, agarose gel electrophoresis and staining with SYBR Green I, 40 target copies were applied per reaction and the number of replicates was 20

| Detection method amplification instrument | Real-time fluorescence detection | Agarose gel electrophoresis | SYBR Green I staining |
|---|---|---|---|
| Applied Biosystems 7500 Fast Real-Time PCR System | 20/20 | 20/20 | 20/20 |
| Heating block Eppendorf | Not possible | 19/20 | 19/20 |
| Heating block Peqlab | Not possible | 19/20 | 19/20 |

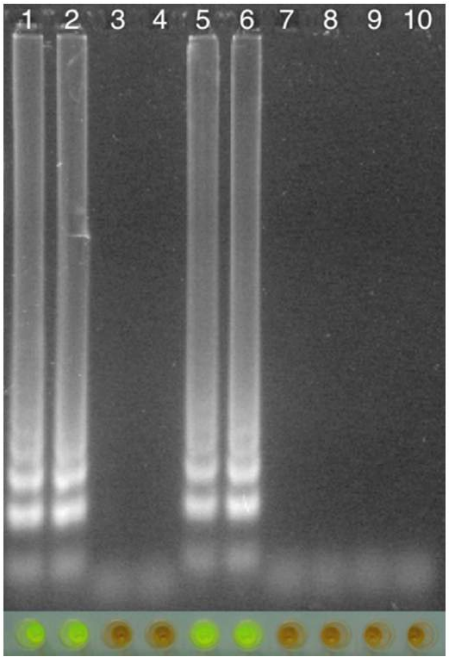


**Fig. 4** *Top* agarose gel electrophoresis, *bottom* staining with SYBR Green I (*green* target present, *orange* no target present); *1*, *2*, *5*, and *6* celery-positive sample (1 ng celery DNA/μL); *3* and *4* celery-negative sample, *7–10* no template control

changing the instrument for amplification (thermal cycler with real-time detection and a simple heating block) and subsequent detection techniques (agarose gel electrophoresis and nucleic acid staining). Figure 4 shows four celery-positive samples resulting in a typical LAMP gel electrophoresis pattern with various-sized amplification products. Staining with SYBR Green I results in a clearly distinguishable color change from orange to green in accordance with the results from the agarose gel electrophoresis.

## Conclusion

The loop-mediated isothermal amplification assay was shown to be a highly specific and sensitive method for the detection of celery in food products. Although it is not suitable for the quantification of low amounts of celery DNA, it represents an accurate and robust technique that can be performed in less than 1 h without special equipment and therefore is a valuable tool for rapid and high-throughput analyses of food samples. Furthermore, the naked eye detection using SYBR Green I eliminates the need for a gel documentation system and allows the on-site visualization of the amplified DNA. This work contributes to a future approach on food analysis by describing a novel assay which meets the current demands for analytical methods to be rapid, easy-to-perform and to have the potential for on-site applications.

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## References

1. Etesamifar M, Wüthrich B (1998) IgE-vermittelte Nahrungsmittelallergie bei 383 Patienten unter Berücksichtigung des oralen Allergie-Syndroms. Allergologie 21:451–457
2. André F, André C, Colin L, Cacaraci F, Cavagna S (1994) Role of new allergens consumption in the increased incidence of food sensitization in France. Toxicology 92:77–83
3. Ballmer-Weber BK, Vieths S, Lüttkopf D, Heuschmann P, Wüthrich B (2000) Celery allergy confirmed by double-blind, placebo-controlled food challenge: a clinical study in 32 subjects with a history of adverse reactions to celery root. J Allergy Clin Immunol 106:373–378
4. Wüthrich B, Stäger J, Johansson SGO (1990) Celery allergy associated with birch and mugwort pollinosis. Allergy 45:566–571
5. Jankiewicz A, Aulepp H, Baltes W, Bögl KW, Dehne LI, Zuberbier T (1996) Allergic sensitisation to native and heated celery root in pollen-sensitive patients investigated by skin test and IgE binding. Int Arch Allergy Immunol 111:268–278
6. Breiteneder H, Hoffmann-Sommergruber K, O'Riordain G, Susani M, Ahorn H, Ebner C, Kraft D, Scheiner O (1995) Molecular characterization of Api g 1, the major allergen of celery (*Apium graveolens*), and its immunological and structural relationships to a group of 17-kDa tree pollen allergens. Eur J Biochem 233:484–489
7. Hoffmann-Sommergruber K, Demoly P, Crameri R, Breiteneder H, Ebner C, Laimer Camara da Machao M, Blaser K, Ismail C, Scheiner O, Bousquet J, Günther M (1999) IgE reactivity to Api g 1, a major celery allergen, in a Central European population is based on primary sensitization by Bet v 1. J Allergy Clin Immunol 107:478–484
8. Bublin M, Radauer C, Wilson IB, Kraft D, Scheiner O, Breiteneder H, Hoffmann-Sommergruber K (2003) Cross-reactive *N*-glycans of Api g 5, a high molecular weight glycoprotein allergen from celery, are required for immunoglobulin E binding and activation of effector cells from allergic patients. FASEB J 17:1697–1699
9. European Commission (2003) Off J Eur Union 46:15
10. Bollen MA, Garcia A, Cordewener JHG, Wichers HJ, Helsper JPFG, Savelkoul HFJ, van Boekel MAJS (2007) Purification and characterization of natural Bet v 1 from birch pollen and related allergens from carrot and celery. Mol Nutr Food Res 51:1527–1536
11. Demmel A, Hahn A (2010) In: Busch U (ed) Molekularbiologische Methoden in der Lebensmittelanalytik, 1st edn. Berlin, Springer
12. Stephan O, Weisz N, Vieths S (2004) Protein quantification, sandwich ELISA, and real-time PCR used to monitor industrial cleaning procedures for contamination with peanut and celery allergens. J AOAC Int 87(6):1448–1457
13. Hupfer C, Waiblinger HU, Busch U (2007) Development and validation of a real-time PCR detection method for celery in food. Eur Food Res Technol 225:329–335
14. Mustorp S, Engdahl-Axelsson C, Svensson U, Holck A (2008) Detection of celery (*Apium gaveolens*), mustard (*Sinapis alba*, *Brassica juncea*, *Brassica nigra*) and sesame (*Sesamum indicum*) in food by real-time PCR. Eur Food Res Technol 226:771–778
15. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28(12):I–VII

16. Aliotta JM, Pelletier JJ, Ware JL, Moran LS, Brenner JS, Kong H (1996) Thermostable Bst DNA polymerase I lacks a 3′→5′ proofreading exonuclease activity. Genet Anal Biomol Eng 12(5–6):185–195
17. Nagamine K, Hase T, Notomi T (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 16:223–229
18. Iwamoto T, Sonobe T, Hayashi K (2003) Loop-mediated isothermal amplification for direct detection *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J Clin Microbiol 41(6):2616–2622
19. Focke F, Haase I, Fischer M (2013) Loop-mediated isothermal amplification (LAMP): methods for plant species identification in food. J Agric Food Chem 61:2943–2949
20. Dovičovičová L, Olexová L, Pangallo D, Siekel P, Kuchta T (2004) Polymerase chain reaction (PCR) for the detection of celery (*Apium gaveolens*) in food. Eur Food Res Technol 218:493–495
21. Brunner K, Paris MPK, Paolino G, Bürstmayr H, Lemmens M, Berthiller F, Schuhmacher R, Krska R, Mach RL (2009) A reference-gene-based quantitative PCR method as a tool to determine *Fusarium* resistance in wheat. Anal Bioanal Chem 395:1385–1394
22. Monis PT, Giglio S, Saint CP (2005) Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concetration on amplification and DNA melting curve analysis. Anal Biochem 340(1):24–34
23. Yang X, Quiros CF (1995) Characterizing the celery genome with DNA-based genetic markers. J Amer Soc Hortic Sci 120(5):747–751
24. European Network of GMO Laboratories (2008) Definition of minimum performance requirements for analytical methods of GMO testing. http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf
25. Han F, Ge B (2010) Quantitative detection of *Vibrio vulnificus* in raw oysters by real-time loop-mediated isothermal amplification. Int J Food Microbiol 142:60–66
26. Aoi Y, Hosogai M, Tsuneda S (2006) Real-time quantitative LAMP (loop-mediated isothermal amplification of DNA) as a simple method for monitoring ammonia-oxidizing bacteria. J Biotechnol 125:484–491
27. Mori Y, Kitao M, Tomita M, Notomi T (2004) Real-time turbidimetry of LAMP reaction for quantifying template DNA. J Biochem Biophys Methods 59:145–157
28. Cankar K, Štebih D, Dreo T, Žel J, Gruden K (2006) Critical points of DNA quantification by real-time PCR effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. BMC Biotechnol 6:37
29. Kaneko H, Kawana T, Fukushima E, Suzutani T (2007) Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J Biochem Biophys Methods 70:499–501
30. Lee D, La Mura M, Allnutt TR, Powell W (2009) Detection of genetically modified organisms (GMOs) using isothermal amplification of target DNA sequences. BMC Biotechnol 9:7

Analytical and Bioanalytical chemistry

Electronic Supplementary Material

# Detection of the food allergen celery via loop-mediated isothermal amplification technique

**C. Zahradnik, R. Martzy, R.L. Mach, R. Krska, A.H. Farnleitner, K. Brunner**

According to Focke et al., a celery-specific LAMP assay has been developed in 2013 [19]. A BLAST search with the NCBI database using the specified primer sequences given in this publication showed that these oligonucleotides are not specific for celery (Apium graveolens) and cross-detection for fennel, dill and parsely can be expected due to their high sequence identity (see Fig. S1).

```
GQ148795.1      ----------------------------------TCGAATCCTGCGATAGCAGAATGACC
HQ377212.1      CTTCTCGTAGGGTGAACCTGCGGAAGGATCATTGTCGAATCCTGCGATAGCAGAATGACC
GQ148800.1      ----------------------------------TCGAATCCTGCGATAGCAGAATGACC
FJ986043.1      ----------------------------------TCGAATCCTGCGATAGCAGAATGACC
                                                  **************************

GQ148795.1      CGCTAACACGTAAACACATTGGGCAAGCTTCAGAGGGCTTCGGTCCCCTGTTTGCAAACC
HQ377212.1      CGCTAACACGTAAACACATCGGGCAAGCGTCAGAGGGCTTCGGTCCCCTGATTGCAAACC
GQ148800.1      CGCTAACACGTAAACACATTGGGCAAGCGTCAGAGGGCTTCGGTCCCCTGTTTGCGAACC
FJ986043.1      CGCTAACACGTAAACACATTGGGCAAGCGTCGGTGGGCTTTGGTCCGCCGTTTGCAAACC
                ****************** ******** ** * ****** ***** * * **** ****

GQ148795.1      CTTGGTAGGTGTCCCCCTCTATGGTGGTCACCGGCCTACGAAATCATCCGGGCGCGGAAT
HQ377212.1      CTTGGTAGGTGTCCCC-TCTATGGTGGCCACCGGCCTACGAAATCATCCGGGCGCGGAAT
GQ148800.1      CTTGGTAGGTGGCCCC-TCTGTAGTGGCCATCGGCCTACAAAATCATCCGGGCGCGGAAT
FJ986043.1      TTTGGTAGGTGGCCCC-TCTTTGGTGGCCACCGGCCTACGAA-TCATCCGGGCGCGGAAT
                 ********** **** *** * **** ** ******** ** ****************

GQ148795.1      GCGCCAAGGAACTTAAAATTGAATTGTACGTTCGCATCCCGTTAGCGGGCATCGAACGTC
HQ377212.1      GCGCCAAGGAACTTGAAATTGAATTGTACGTTCGCATCCCGTTAGCGGGCATCGAACGTC
GQ148800.1      GCGCCAAGGAACTTTAAATTGAATTGTACATTCGCTTCCCGTTAGCGGGCAGC-AATGTC
FJ986043.1      GCGCCAAGGAACTTAAAATTGAATTGTACGTTCGCAACCCGTTAG-GGGCGGC-GGCGTC
                ************** ************** *****  ******** ****  *    ***

GQ148795.1      ATTCCAAAACACAACGACTCTCGACAACGGATATCTCGGCTCTCGCATCGATGAAGAACG
HQ377212.1      ATTCCAAAACACAACGACTCTCGACAACGGATATCTCGGCTCTCGCATCGATGAAGAACG
GQ148800.1      ATTCCAAAACACAACGACTCTCGACAACGGATATCTCGGCTCTCGCATCGATGAAGAACG
FJ986043.1      ATTCCAAAACACAACGACTCTCGACAACGGATATCTCGGCTCTCGCATCGATGAAGAACG
                ************************************************************
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**Fig. S1.** Alignment for primer sequences for celery (FJ986043.1) as published by [17], fennel (HQ377212.1), dill (GQ148795.1) and parsley (GQ148800.1)

Following the recommended reaction conditions and using this primer set a real-time LAMP assay was performed, resulting in the detection of celery, carrot and parsely, as shown in Figure S1. Therefore, this assay cannot be described as celery-specific, especially with regard to the relevance of carrot and parsley as frequent ingredient in food products.

**Table S1.** Comparison of data demonstrating the specificity of primers used in this study and the primers as published by [19] for celery, carrot, parsley and no template control, n=5

| | **celery** | **carrot** | **parsley** | **no template control** |
|---|---|---|---|---|
| **This study** | + + + + + | - - - - - | - - - - - | - - - - - |
| **Focke et al. (2013)** | + + + + + | + + + + - | + + + + + | - - - - - |

## Publication #2

# Detection of the P35S promotor in transgenic maize via various isothermal amplification techniques: a practical approach

**Celine Zahradnik,** Claudia Kolm, Roland Martzy, Robert Mach, Rudolf Krska, Andreas Farnleitner, Kurt Brunner

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**Abstract** In 2003 the European Commission introduced a 0.9 % threshold for food and feed products containing genetically modified organism (GMO)-derived components. For commodities containing GMO contents higher than this threshold, labelling is mandatory. To provide a DNA-based rapid and simple detection method suitable for high-throughput screening of GMOs, several isothermal amplification approaches for the 35S promoter were tested: strand displacement amplification, nicking-enzyme amplification reaction, rolling circle amplification, loop-mediated isothermal amplification (LAMP) and helicase-dependent amplification (HDA). The assays developed were tested for specificity in order to distinguish between samples containing genetically modified (GM) maize and non-GM maize. For those assays capable of this discrimination, tests were performed to determine the lower limit of detection. A false-negative rate was determined to rule out whether GMO-positive samples were incorrectly classified as GMO-negative. A robustness test was performed to show reliable detection independent from the instrument used for amplification. The analysis of three GM maize lines showed that only LAMP and HDA were able to differentiate between the GMOs MON810, NK603, and Bt11 and non-GM maize. Furthermore, with the HDA assay it was possible to realise a detection limit as low as 0.5 %. A false-negative rate of only 5 % for 1 % GM maize for all three maize lines shows that HDA has the potential to be used as an alternative strategy for the detection of transgenic maize. All results obtained with the LAMP and HDA assays were compared with the results obtained with a previously reported real- time PCR assay for the 35S promoter in transgenic maize. This study presents two new screening assays for detection of the 35S promoter in transgenic maize by applying the isothermal amplification approaches HDA and LAMP.

**Zusammenfassung** Im Jahre 2003 wurde von der Europäischen Kommission eine Grenze von 0.9 % für gentechnisch veränderte Organismen (GVOs) und Aaraus abgeleitete Produkte in Lebens- und Futtermitteln eingeführt. Für Produkte, die diesen Grenzgehalt überschreiten ist die entsprechende Etikettierung verpflichtend. Im Zuge der Entwicklung von DNA-basierten und einfach durchzuführenden Test-Methoden, welche einen hohen Proben-Durchfluss erlauben, wurde verschiedene Isothermale Amplifikationstechniken für den Nachweis der P35S-Promoters untersucht: Strand Displacement Amplification, Nicking-Enzyme Amplification Reaction, Rolling Circle Amplification, Loop-mediated Isothermal Amplification (LAMP) und Helicase-dependent Amplification (HDA). Die entwickelten Assays wurden auf ihre Selektivität hinsichtlich der Unterscheidung zwischen gentechnisch verändertem und unverändertem Mais untersucht. Für diejenigen Techniken mit einer entsprechenden Diskriminierung zwischen GVO-Mais und Nicht-GVO-Mais wurde eine Nachweisgrenze ermittelt. Zusätzlich wurde die Falsch-Negativ-Rate ermittelt, um eine Deklaration von GVO-Mais als nicht GVO-Mais auszuschließen. Ebenso wurde Tests durchgeführt um einen zuverlässigen Nachweis, unabhängig von verwendeten Instrumenten, zu gewährleisten. Die Analyse von drei verschiedenen GVO-Mais-Sorten zeigte, daß lediglich LAMP und HDA in der Lage waren, zwischen den gentechnisch veränderten Mais-Sorten MON810, NK603 und Bt11 und Nicht-GVO-Mais zu unterscheiden. Für die HDA konnte außerdem eine Nachweisgrenze von 0.5 % realisierte werden, sowie eine Falsch-Negativ-Rate von 5 % für alle drei Sorten 1%-GVO-Mais. Dies zeigt, daß die HDA als alternative Nachweis-Strategie für GVO-Mais eingesetzt werden kann. Alle Ergebnisse für LAMP und HDA wurden mit einem früher publizierten real-time PCR-Assay zum Nachweis des P35S-Promoters in gentechnisch verändertem Mais verglichen. Diese Studie erläutert die Entwicklung zwei neuer Screening-Assays für den P35S-Promoter in GVO-Mais mittels isothermaler Amplifikationstechniken.

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RESEARCH PAPER

# Detection of the 35S promoter in transgenic maize via various isothermal amplification techniques: a practical approach

**Celine Zahradnik · Claudia Kolm · Roland Martzy · Robert L. Mach · Rudolf Krska · Andreas H. Farnleitner · Kurt Brunner**

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**Abstract** In 2003 the European Commission introduced a 0.9 % threshold for food and feed products containing genetically modified organism (GMO)-derived components. For commodities containing GMO contents higher than this threshold, labelling is mandatory. To provide a DNA-based rapid and simple detection method suitable for high-throughput screening of GMOs, several isothermal amplification approaches for the 35S promoter were tested: strand displacement amplification, nicking-enzyme amplification reaction, rolling circle amplification, loop-mediated isothermal amplification (LAMP) and helicase-dependent amplification (HDA). The assays developed were tested for specificity in order to distinguish between samples containing genetically modified (GM) maize and non-GM maize. For those assays capable of this discrimination, tests were performed to determine the lower limit of detection. A false-negative rate was determined to rule out whether GMO-positive samples were incorrectly classified as GMO-negative. A robustness test was performed to show reliable detection independent from the instrument used for amplification. The analysis of three GM maize lines showed that only LAMP and HDA were able to differentiate between the GMOs MON810, NK603, and Bt11 and non-GM maize. Furthermore, with the HDA assay it was possible to realize a detection limit as low as 0.5 %. A false-negative rate of only 5 % for 1 % GM maize for all three maize lines shows that HDA has the potential to be used as an alternative strategy for the detection of transgenic maize. All results obtained with the LAMP and HDA assays were compared with the results obtained with a previously reported real-time PCR assay for the 35S promoter in transgenic maize. This study presents two new screening assays for detection of the 35S promoter in transgenic maize by applying the isothermal amplification approaches HDA and LAMP.

**Keywords** 35S promoter · Genetically modified · Transgenic · Isothermal amplification · Screening · Maize · Loop-mediated isothermal amplification · Helicase-dependent amplification

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## Introduction

Over the last decade, various genetically modified organisms (GMOs) have been introduced in food and feed products. Since consumers demand adequate labelling of genetically modified (GM) products and their derivatives, the European Commission introduced a threshold of 0.9 % for the mandatory labelling of GMOs based on Council Regulation 1139/98 and Commission Regulation 49/2000 [1, 2]. GMO contents exceeding this threshold have to be labelled adequately. Although many validated polymerase chain reaction (PCR) assays for the event-, construct- or element-specific detection

of GMOs are available [3], the rising number of GM crops as well as the increasing variety of elements used for the design of such pose a major challenge for the development of assays suitable for analysis. The least common denominator still widely used in many GM plants is the *Cauliflower mosaic virus* 35S promoter. It was first characterized by Odell et al. [4] and was proven to be highly active when inserted into the genome of transgenic plants and therefore enhances gene expression [5, 6]. PCR assays targeting the 35S promoter sequence are able to detect a wide range of GMOs, including many events for maize, rice, canola, soy and cotton [7]. However, depending on the event, the 35S promoter is not always inserted evenly, partially intended or coincidental [8]. The differing lengths of inserted promoter regions further complicate the design of nucleic acid based assays, which have to be adapted precisely in order to detect a wide range of transgenic plants [9].

Although real-time PCR is the gold standard in modern DNA analysis and provides the highest sensitivity and efficiency when compared with many other amplification methods, it still has a few drawbacks which prevent it from being considered for the rapid on-site screening analysis of GMOs in food and feed. According to guidelines from the European Network of GMO Laboratories [10], GMO-containing samples have to be analysed using event-specific detection methods. Applying screening methods first could help to narrow down sample numbers for subsequently performed event-specific PCR assays as GMO-negative samples can be identified this way. Recently, several amplification methods have been designed for isothermal reaction conditions, all of them using DNA polymerases with strand displacement activity [11]. In some methods, the essential denaturation step of conventional PCR assays becomes obsolete and is replaced by the direct amplification of a double-stranded DNA target. Furthermore, the actual amplification reaction can be performed at one particular temperature. Since a multistaged temperature profile is not needed to perform isothermal amplification reactions, the assays can be conducted without the use of a thermal cycler but with a simple heating block instead. As for PCR, the isothermal assays can be prepared as a ready-to-use plate with immobilized reagents including positive and negative controls. Additionally, amplification products can be detected via simple agarose gel electrophoresis or even more simply by nucleic acid staining with SYBR Green I, which intercalates the double-stranded products and causes a colour change from orange to green when the target is present [12]. The combination of ready-to-use kits, a simple detection method such as nucleic acid staining and the fact that isothermal reactions can be performed on a heating block eliminates the need for personnel trained in the use of instruments and emphasizes the capability of isothermal amplification methods to be performed without laboratory equipment. So far, several isothermal amplification methods using different approaches have been developed, all of them having advantages and drawbacks.

Strand displacement amplification (SDA) is an isothermal amplification method initially described by Walker et al. [13]. Copies of the target sequence are generated by using a set of two primer pairs (S1, S2, B1 and B2), a restriction enzyme able to nick an unmodified strand of hemimodified DNA and an exonuclease-deficient polymerase. Primers S1 and S2 both contain recognition sites for the restriction enzyme at the 5′ end. Primers B1 and B2 are displacement primers needed at the early stage of the reaction to release the newly synthesized strand.

Firstly, all four primers anneal to the target DNA and are elongated by the DNA polymerase. Since the polymerase has strand displacement activity, the elongation of the two outer primers displaces the initially produced strand. Owing to repeated annealing, elongation and displacement steps, short double-stranded DNA target molecules are generated. These amplicons are flanked by recognition sites for the restriction enzyme used in SDA. After this so-called target generation phase, these sites are then nicked by the restriction enzyme and extended by the DNA polymerase, which thus displaces the downstream strand. Exponential amplification is achieved by repeated SDA cycles (nicking, extending, displacing) [14, 15].

Nicking-enzyme amplification reaction (NEAR) is based on SDA. It differs mainly in the use of altered restriction enzymes (‘nicking endonucleases’) capable of hydrolysing only one strand of the duplex. In contrast, the nicking step in SDA is accomplished by addition of costly phosphorothioate nucleotides, which are incorporated into the newly synthesized DNA strands to protect the recognition site against full cleavage. Similarly to SDA, the reaction further requires target-specific primers and an exonuclease-deficient polymerase capable of initiating a nick and strand displacement [16, 17].

Rolling circle amplification (RCA), originally developed by Fire and Xu [18] and later modified by Lizardi et al. [19], is an interesting approach owing to its very high efficiency without the requirement of a long known sequence for the design. An open circle probe that consists of two target-complementary regions at the 3′ and 5′ ends in a juxtaposed position including a generic spacer region in between is circularized by hybridising it to its complementary DNA target and subsequent ligation. The ligated DNA circle is amplified by hybridization of one or more primers, producing a long strand of DNA consisting of numerous repeats of the target sequence.

Loop-mediated isothermal amplification (LAMP) was developed in 2000 [20]. The use of three primer sets binding six distinct regions of the target sequence and a *Bst* polymerase [11] with strand displacement activity initially forms a dumbbell-like structure which then undergoes continuous

amplification, producing DNA strands of various sizes with alternately inverted repeats of the target sequence [21]. One main advantage is the sensitive detection of amplification products using the intercalating dye SYBR Green I, making possible visual on-site detection of samples. One drawback of this method is the large amplicon size produced in this reaction. Long target sequences are needed to design an efficient primer set, a requirement that cannot always be met.

Helicase-dependent amplification (HDA) uses the unwinding activity of a helicase to separate double-stranded DNA [22]. Instead of heat denaturation steps and thermal cycling, a helicase unwinds and displaces DNA strands alongside the exponential amplification reaction. The main components of the reaction are two target-specific primers, a helicase and a DNA polymerase. Besides the simplicity of the method, it offers all the advantages of PCR such as high sensitivity, simple primer design and the possibility of multiplexing [23].

The aim of this study was a comparative evaluation of the isothermal amplification methods mentioned above with regard to the feasibility of a screening assay targeting the 35S promoter in GM maize. For this purpose, various assays based on SDA, NEAR, RCA, HDA and LAMP for the detection of the 35S promoter were developed and tested for their capability to distinguish between GM and non-GM maize samples. Furthermore, all methods suitable for the detection of the 35S promoter were tested for sensitivity by analysing DNA serial dilutions of MON810. The detection limits for NK603 and Bt11 were established by analysing reference materials in concentrations of 5 %, 1 %, 0.5 %, 0.1 % and 0 %. All the results were compared with the results obtained with a validated real-time PCR [24] assay for the detection of the 35S promoter in GM maize. A previously reported LAMP assay [25] suggested the detection of MON810 and Bt11 using oligonucleotides originally reported by Lee et al. [26] for the detection of the 35S promoter in Roundup Ready® soy. However, this primer set targets a part of the promoter sequence not occurring in MON810 and Bt11 [27]. One of the primers (F3) lies upstream of the actual target sequence for the 35S promoter in these two GM maize lines and therefore this assay is not able to amplify the target, as theoretically and experimentally proven by the authors (see Figs. S1, S2). This study presents the development of two new isothermal amplification approaches for the detection of the 35S promoter in transgenic maize.

## Materials and methods

### Plant materials and DNA extraction

For this study, non-GM maize powder spiked with GM maize powder of MON810, NK603 and Bt11 in concentrations of 5 %, 1 %, 0.5 %, 0.1 % and 0 % was obtained from the Joint Research Centre, Institute for Reference Materials and Measurements (Geel, Belgium). DNA extraction was performed using an optimized cetyltrimethylammonium bromide protocol [28]. DNA concentration and purity were determined photometrically using a NanoVue Plus spectrophotometer (GE Healthcare, Little Chalfont, UK).

### Primers and probes

All oligonucleotides were obtained from MWG Eurofins (Ebersberg, Germany). The PCR primer/probe system was used as reported and validated previously [24]. LAMP primers were designed using Primer Explorer V4 (Eiken Chemical, Tokyo, Japan) on the basis of the sequence of the 35S promoter (GenBank accession number JX139718) and are given in Table 1. RCA padlock probes and primers, and NEAR and SDA oligonucleotides were designed using the Web-based tool Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). HDA primer sets were used according to PCR primers designed and reported previously [29, 30].

### SDA reactions

SDA was performed in 25-μL reaction mixtures containing 40 mM $K_iPO_4$ buffer (pH 7.6), 50 mM KCl, 6 mM $MgCl_2$, 0.4 mM dGTP, 0.4 mM dCTP, 0.4 mM dTTP and 0.4 mM dATPαS (BioLog, Bremen, Germany), 0.5 μM primers S1 and S2, 0.05 μM primers B1 and B2, 1 μL DNA (concentration 40 ng/μL), BSA at 0.1 mg/mL, 5 U *Bst* polymerase large fragment and 40 U *Brs*I (New England BioLabs, Ipswich, MA, USA). Prior to the addition of enzymes (*Bst* polymerase and *Brs*I), SDA reaction mixtures were overlaid with mineral oil and heated at 95 °C for 4 min to denature the target DNA. After equilibration at 60 °C for 3 min and addition of enzymes, reactions proceeded at 60 °C for 30 min and were then stopped by incubation at 95 °C for 10 min. Amplification products were detected by loading 4-μL aliquots of the sample

**Table 1** The primer sequences used for the loop-mediated isothermal amplification (LAMP) assay based on the sequence of *Zea mays* transgenic strain MON810 genetically modified (GM) cassette (GenBank accession number JX139718)

| Primer region | Sequence 5′→3′ |
|---|---|
| F3 | AAGATGCCTCTGCCGACA |
| B3 | CAGCGTGTCCTCTCCAAAT |
| FIP | ACGTGGTTGGAACGTCTTCTT-CCCA AAGATGGACCCCCA |
| BIP | ATCTCCACTGACGTAAGGGATG-ATAG AGGAAGGGTCTTGCGA |
| LoopF | TCCACGATGCTCCTCGTG |
| LoopB | ACGCACAATCCCACTATCCT |

mixed with loading dye onto a 2.6 % agarose gel stained with SYBR Gold. Designed oligonucleotides are given in Table S1.

Nicking-enzyme amplification reactions

NEARs were performed in a 30-μL volume containing 40 mM tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 8.0), 25 mM KCl, 8 mM $MgCl_2$, each dNTP (Peqlab, Erlangen, Germany) at 0.4 mM, 0.5 μM primers S1 and S2, 0.05 μM primers B1 and B2, 1 μL DNA (concentration 40 ng/μL), BSA (Sigma-Aldrich, St Louis, MI, USA) at 0.1 mg/mL, 10 U *Bst* polymerase large fragment and 20 U Nt.*Bst*NBI (New England BioLabs, Ipswich, MA, USA). Mixtures for which reactions were incomplete (26 μL, enzymes excluded) were overlaid with mineral oil (Sigma-Aldrich, St Louis, MI, USA) and heated at 95 °C for 4 min to denature the target DNA, followed by 3 min at 55 °C for primer annealing. Subsequently, 4 μL of enzyme mix (*Bst* polymerase and Nt.*Bst*NBI) was added, and the reaction proceeded at 55 °C for 30 min on an Eppendorf Thermomixer Plus (Eppendorf, Hamburg, Germany). After the reaction had been stopped at 95 °C for 10 min, 1-μL aliquots from each sample were mixed with loading dye (Peqlab, Erlangen, Germany) and analysed by 2.6 % agarose gel electrophoresis with SYBR Gold (Invitrogen, Carlsbad, CA, USA) staining. Designed oligonucleotides are given in Table S1.

RCA reactions

Ligation reactions were performed in a total reaction volume of 10 μL containing 1 U Ampligase (Epicentre Biotechnologies, Madison, WI, USA), 10 pM padlock probe, 1× Ampligase reaction buffer (20 mM Tris–HCl pH 8.3, 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD, 0.1 % Triton X-100) and 1 μL genomic DNA (concentration 40 ng/μL). Reaction mixtures were incubated at 95 °C for 2 min, followed by the addition of Ampligase and another incubation step at 65 °C for 30 min. Standard RCA reactions were performed in a total volume of 25 μL containing 1 μL ligation product, 0.4 mM dNTP mix, 8 U *Bst* polymerase large fragment (New England BioLabs, Ipswich, MA, USA), the two primers each at 1 μM, 1× ThermoPol buffer containing 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$ and 0.1 % Triton X-100 and 1× SYTO® 9 [31]. Reaction mixtures were incubated at 65 °C for 60 min. In total, 12 different padlock/primer sets were tested, and reaction mixtures were modified as to buffer concentrations, Ampligase concentrations, and amplification temperatures (data not shown). Padlock probe and primers used for RCA feasibility tests are given in Table S1.

LAMP reactions

Standard LAMP reactions as optimized by the authors were performed in a total reaction volume of 25 μL containing 0.8 μM FIP, 0.8 μM BIP, 0.4 μM F3, 0.4 μM B3, 0.2 μM LoopF, 0.2 μM LoopB, each dNTP (Peqlab, Erlangen, Germany) at 0.4 mM, 0.5 M betaine solution, 32 U *Bst* DNA polymerase (New England BioLabs, Ipswich, MA, USA), 20 mM Tris–HCl (pH 8.5), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 0.1 % Triton X-100 (Sigma-Aldrich, St Louis, MI, USA) and 1 μL DNA sample (concentration 40 ng/μL). One slight modification was made by using 1 μL

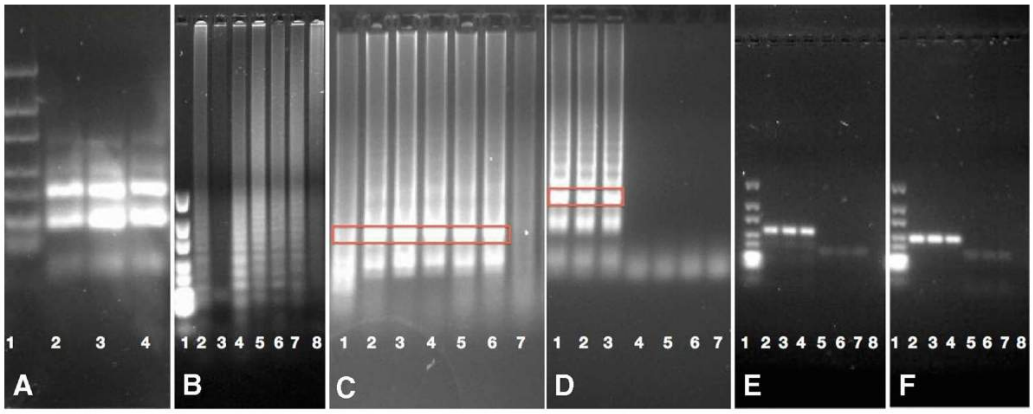


**Fig. 1** Specificity tests performed with agarose gel electrophoresis for **a** strand displacement amplification, **b** nicking-enzyme amplification reaction, **c** rolling circle amplification, **d** loop-mediated isothermal amplification (LAMP), **e** helicase-dependent amplification and **f** polymerase chain reaction. For **a**, *1* ultra-low-range ladder (0.01–0.3–kb) (Peqlab, Erlangen, Germany), *2* MON810, *3* genetically modified organism (GMO)-free 1, *4* no-template control. For **b**, *1* ultra-low-range ladder (0.01–0.3 kb), *2* MON810, *3* NK603, *4* Bt11, *5* GMO-free 1, *6* GMO-free 2, *7* GMO-free 3, *8* no-template control. For **c**, *1* MON810, *2* NK603, *3* Bt11, *4* GMO-free 1, *5* GMO-free 2, *6* GMO-free 3, *7* no-template control, *rectangle* 450 bp. For **d**, *1* MON810, *2* NK603, *3* Bt11, *4* GMO-free 1, *5* GMO-free 2, *6* GMO-free 3, *7* no-template control, *rectangle* 350 bp; all GM targets show the typical pattern for LAMP amplicons of various sizes. For **e**, *1* ultra-low-range ladder (0.01–0.3 kb), *2* MON810, *3* NK603, *4* Bt11, *5* GMO-free 1, *6* GMO-free 2, *7* GMO-free 3, *8* no-template control. For **f**, *1* ultra-low-range ladder (0.01–0.3 kb), *2* MON810, *3* NK603, *4* Bt11, *5* GMO-free 1, *6* GMO-free 2, *7* GMO-free 3, *8* no-template control

**Table 2** Sensitivity of helicase-dependent amplification (*HDA*), LAMP and polymerase chain reaction (*PCR*) for DNA extracts of MON810, diluted with a non-GM maize background in concentrations of 100 %, 10 %, 5 %, 1 %, 0.5 %, 0.1 % and 0 %. HDA and PCR are able to detect 0.5 % reliably in five out of five replicates. LAMP gives one positive replicate out of five for 1 %

| Concentration (%) | HDA | LAMP | PCR |
|---|---|---|---|
| 100 | 5/5 | 5/5 | 5/5 |
| 10 | 5/5 | 5/5 | 5/5 |
| 5 | 5/5 | 2/5 | 5/5 |
| 1 | 5/5 | 1/5 | 5/5 |
| 0.5 | 5/5 | 0/5 | 5/5 |
| 0.1 | 2/5 | 0/5 | 2/5 |
| 0 | 0/5 | 0/5 | 0/5 |

SYTO® 9 green fluorescent nucleic acid stain (Life Technologies, Carlsbad, CA, USA) [31] for real-time fluorescence monitoring of each reaction. The reaction mixture was incubated at 65 °C for 90 min. Amplification reactions were performed in five replicates. LAMP products (10-μL aliquots) were used for agarose gel electrophoresis, and the remaining 10 μL of the reaction volume was used for visual detection by nucleic acid staining with SYBR Green I. Reaction mixtures as well as run protocols were modified according to [25, 26].

### HDA reactions

All HDA reactions were performed with an IsoAmp® II Universal tHDA kit (New England BioLabs, Ipswich, MA, USA) in a final reaction volume of 25 μL. Following a two-step protocol, 12.5 μL of mix A containing 1x annealing buffer II, 0.12 mM primers and 1 μL DNA (concentration 40 ng/μL) was overlaid with mineral oil and heated at 95 °C for 4 min for initial target DNA denaturation. After subsequent equilibration at 65 °C for 3 min, 12.5 μL of mix B containing 1× annealing buffer II, 8 mM $MgSO_4$, 80 mM NaCl, 3.5 μL IsoAmp® dNTP solution and 3.5 μL IsoAmp® enzyme mix was added. Reaction mixtures were then incubated at 65 °C for 90 min. HDA products (10-μL aliquots) were analysed in 2 % agarose gels stained with SYBR Gold. Primers for HDA were used as reported in [29, 30] for PCR.

### Real-time PCR

One microlitre of DNA extract (concentration 40 ng/μL), diluted accordingly, was added to 14 μL of reaction mix containing Kapa™ Probe® Fast (Peqlab, Erlangen, Germany), primers, probe and water (each in a final concentration of 0.3 μM). Amplification reactions were run on a 7500 fast real-time PCR system (Applied Biosystems, Grand Island NY, USA) according to the following thermal cycling protocol: initial steps of 2 min at 95 °C, followed by 60 cycles of 15 s at 95 °C and 1 min at 60 °C. Unless noted otherwise, amplification reactions were performed in five replicates.

## Results and discussion

### Feasibility of isothermal methods to distinguish between GM and non-GM maize

Feasibility tests were performed with HDA and LAMP oligonucleotides for three GM maize lines (MON810, NK603 and Bt11) as well as for three non-GM maize varieties. HDA primers were previously reported and tested in a PCR assay, resulting in the specific detection of six different GM maize strains (MON810, Bt11, MON802, Event176, T25, DLL25) [30]. The tested primer set was capable of differentiating between the three tested GM maize strains (MON810, NK603, Bt11) and three non-GM maize varieties even if the reaction was performed under isothermal conditions.

Each sample (40 ng of extracted DNA per reaction) was tested in five replicates, resulting in the detection of all GM showing high accordance for HDA and PCR results. LAMP was not able to detect five out of five replicates for 5 % MON810

**Table 3** Detection of spiked maize samples in concentrations of 5 %, 1 %, 0.5 %, 0.1 % and 0 % diluted with non-GM maize powder for HDA, LAMP and PCR for transgenic maize MON810, NK603 and Bt11,

| Dilution (%) | HDA | | | LAMP | | | PCR | | |
|---|---|---|---|---|---|---|---|---|---|
| | MON810 | NK603 | Bt11 | MON810 | NK603 | Bt11 | MON810 | NK603 | Bt11 |
| 5 | 5/5 | 5/5 | 5/5 | 2/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| 1 | 5/5 | 4/5 | 5/5 | 1/5 | 3/5 | 2/5 | 5/5 | 5/5 | 5/5 |
| 0.5 | 5/5 | 4/5 | 4/5 | 0/5 | 2/5 | 1/5 | 5/5 | 5/5 | 5/5 |
| 0.1 | 2/5 | 3/5 | 3/5 | 0/5 | 0/5 | 0/5 | 4/5 | 3/5 | 4/5 |
| 0 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1/5 | 0/5 |

**Table 4** False-negative rates (%) for MON810, NK603 and Bt11 for HDA, LAMP and PCR in concentrations of 5 %, 1 %, 0.5 % and 0.1 %. HDA meets the requirements of 5 % or less of undetected positive samples, but LAMP failed owing to the lack of sensitivity

| Concentration of reference material (%) | MON810 | | | NK603 | | | Bt11 | | |
|---|---|---|---|---|---|---|---|---|---|
| | HDA | LAMP | PCR | HDA | LAMP | PCR | HDA | LAMP | PCR |
| 5 | 0 | 60 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 90 | 0 | 5 | 45 | 0 | 5 | 80 | 0 |
| 0.5 | 0 | 95 | 0 | 15 | 90 | 0 | 25 | 100 | 0 |
| 0.1 | 50 | 95 | 30 | 35 | 95 | 15 | 70 | 100 | 0 |

lines for both HDA and LAMP primers. No signals were detected for the non-GM targets, which were then used as negative background DNA in later experiments. All results were confirmed by real-time PCR. Similar experiments were conducted for the isothermal methods SDA, NEAR and RCA. Careful and thorough attempts were undertaken to eliminate the reported issue of non-specific background amplification [32, 33]. For this purpose a variety of sequence variations and buffer concentrations were tested. Furthermore, different enzyme concentrations as well as their reaction optima were investigated (see Tables S2, S3, S4). All efforts to make possible differentiation between GMO-positive and GMO-negative samples were unsuccessful, and thus the SDA, NEAR and RCA methods were not included in subsequent experiments to determine sensitivity, false-negative rate and robustness. Figure 1 shows the results of agarose gel electrophoresis for every method tested, indicating that only HDA, LAMP and PCR were able to specifically detect the target. In SDA, NEAR and RCA, the observed strong background amplification resulted in positive signals also for non-GM maize and negative template controls. Therefore, these methods cannot be used for the reliable differentiation of GM and non-GM maize.

### Sensitivity tests with DNA dilution series

To establish the limit of detection for transgenic maize MON810, DNA dilution series (40 ng/μL) in concentrations of 100 %, 10 %, 1 %, 0.5 %, 0.1 % and 0 % (diluted with GMO-free maize background DNA) were analysed with HDA, LAMP and PCR in five replicates each. Table 2 shows that HDA and PCR achieve similar results in detecting 0.5 % MON810 in a non-GMO background. Although LAMP has the ability to specifically detect MON810, the sensitivity does not suffice for detection below the European threshold of 0.9 %, but it is capable of detecting 1 % GMOs in at least one replicate.

### Analysis of reference materials

To establish a detection limit for the reference materials used in this study, maize powder samples, diluted with non-GM maize powder, resulting in the specified concentrations, were analysed. All samples were extracted using the same extraction protocol. A comparison of maize lines and methods is given in Table 3, showing that, in accordance with the PCR results, the HDA method is able to detect all three GM maize lines below the threshold of 0.9 %. LAMP, in contrast, was able to detect NK603 and Bt11 in a concentration of 5 %. However, similar sensitivity for MON810 in comparison with NK603 and Bt11 could not be achieved.

### False-negative rate

To investigate at what concentration 100 % of positive samples are still clearly detectable, a false-negative rate was determined. Table 4 shows the false-negative rates for reference material samples of MON810, NK603 and Bt11 in concentrations of 5 %, 1 %, 0.5 % and 0.1 %, tested in 20 replicates. Additionally GM-free maize was also tested, representing a 0 % sample, which resulted in no detection signals in all cases; however, these data were not included in the graphs. HDA has a false-negative rate of 5 % for 1 % NK603 and 1 % Bt11 and 0 % for MON810 for 0.5 % GMO content. LAMP has severely higher false-negative rates and does not fulfil the European requirement for GMO testing of a false-negative rate of 5 % or lower [32] for all three GM maize targets.

**Table 5** Robustness tests performed on three different heating blocks for HDA and LAMP. All positive sample are detected via HDA, independently of the instrument used. For no-template controls (*NTC*), no signals were detected

| Method | Ditabis HLC Heating-ThermoMixer | Eppendorf Thermomixer plus | Peqlab heating block HX-1 |
|---|---|---|---|
| HDA (MON810, 0.5 %) | Positive 20/20, NTC 0/20 | Positive 20/20, NTC 0/20 | Positive 20/20, NTC 0/20 |
| LAMP (NK603, 5 %) | Positive 20/20, NTC 0/20 | Positive 20/20, NTC 0/20 | Positive 0/20, NTC 0/20 |

Robustness tests

To determine whether the instruments used for amplification affect the results, robustness tests were performed for HDA and LAMP by performing them on various heating blocks from different manufacturers and evaluating the results via agarose gel electrophoresis. Robustness tests were performed using concentrations where five out of five replicates could still be detected. Additionally, no template controls were amplified, and all reactions were performed in 20 replicates. Table 5 shows that the HDA results are independent of the heating block used for amplification: 100 % of positive samples can be detected reliably. For LAMP, 100 % of positive samples for the Ditabis (Pforzheim, Germany) and Eppendorf (Hamburg, Germany) heating blocks were detected, whereas the Peqlab heating block did not amplify any samples. For LAMP, this experiment was repeated four times, with the same results. To investigate possible causes, the temperature of the Peqlab heating block was remeasured using a thermometer, resulting in temperature fluctuations of about 5 °C, which were not shown on the temperature display. This factor may cause the collapse of the LAMP reaction since the temperature optimum was tested and it was shown that there is no amplification at temperatures above 66 °C.

## Conclusion

The data clearly show that the designed HDA assay has the capability to reliably detect concentrations of transgenic maize lines MON810, NK603 and Bt11 as low as 0.5 %. The LAMP assay is also able to detect GM maize and allows precise differentiation from non-GM maize. A drawback of this technique is that the limit of detection does not suffice to detect concentrations below the required 0.9 %, and therefore this assay can be considered as not suitable for GMO screening of maize. HDA, on the other hand, is able to achieve sensitivities comparable to PCR, and fulfils the European requirements for GMO testing in regard to false-negative rate, robustness and limit of detection. The detection of HDA products is still laborious, and reactions can only be detected via agarose gel electrophoresis. Owing to the formation of one smaller by-product which can easily be differentiated via gel electrophoresis, the detection via SYBR Green I is not possible as this leads to incorrectly classified samples. However, these results indicate both HDA and LAMP are suitable for GMO testing but still need further improvements to perform as reliably as PCR, but with simpler run conditions in a shorter time. Both techniques can be bought as commercial kits for the detection of bacterial and viral targets, with ready-to-use oligonucleotides and buffer solutions. The pipetting effort for both methods is comparable to that for PCR, but both methods can be performed using a heating block instead of a thermal cycler, which drastically decreases the costs of performing an assay.

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## References

1. European Council (1998) Council Regulation (EC) No. 1139/98 concerning the compulsory indication of the labelling of certain foodstuffs produced from genetically modified organisms of particulars other than those provided for in Directive 79/112/EEC. Off J Eur Communities L 159:4–7
2. European Commission (2000) Commission Regulation (EC) No 49/2000 of 10 January 2000 amending Council Regulation (EC) No 1139/98 concerning the compulsory indication on the labelling of certain foodstuffs produced from genetically modified organisms of particulars other than those provided for in Directive 79/112/EEC. Off J Eur Communities L 6:13–14
3. GMOMETHODS: EU database of reference methods for GMO analysis () Joint Research Centre, European Union Reference Laboratory for GM Food and Feed. http://gmo-crl.jrc.ec.europa.eu/gmomethods/
4. Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810–812
5. Nagy F, Odell JT, Morelli G, Chua NH (1985) Properties of expression of the 35S promoter from CaMV in transgenic tobacco plants. In: Zaitlin M, Day P, Hollaender A, Wilson CM (eds) Biotechnology in plant science: Relevance to agriculture in the eighties. Academic, Orlando, pp 227–235
6. Benfey PN, Ren L, Chua NH (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J 8(8):2195–2202
7. Scholtens I, Laurensse E, Molenaar B, Zaaijer S, Gaballo H, Boleij P, Bak A, Kok E (2013) Practical experiences with an extended screening strategy for genetically modified organisms (GMOs) in real-life samples. J Agric Food Chem 61:9097–9109
8. Paszkowski J, Baur M, Bogucki A, Potrykus I (1988) Gene targeting in plants. EMBO J 7(13):4021–4026
9. Scientific Panel on Genetically Modified Organisms (2009) Applications (EFSA-GMO-RX-MON810) for the renewal of authorisation for the continued marketing of (1) existing food and food ingredients produced from genetically modified insect resistant maize MON810; (2) feed consisting of and/or containing maize MON810, including the use of seed for cultivation; and of (3) food and feed additives, and feed materials produced from maize MON810, all under Regulation (EC) No 1829/2003 from Monsanto. EFSA J 1149:1–84
10. European Network of GMO Laboratories (2008) Definition of minimum performance requirements for analytical methods of GMO testing. Community Reference Laboratory for GM Food and Feed, Ispra
11. Aliotta JM, Pelletier JJ, Ware JL, Moran LS, Brenner JS, Kong H (1996) Thermostable Bst DNA polymerase I lacks a 3'→5' proofreading exonuclease activity. Genet Anal 12(5–6):185–195
12. Iwamoto T, Sonobe T, Hayashi K (2003) Loop-mediated isothermal amplification for direct detection Mycobacterium tuberculosis complex, M. avium, and M. intracellulare in sputum samples. J Clin Microbiol 41(6):2616–2622

13. Walker GT, Little MC, Nadeau JG, Shank DD (1992) Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. Proc Natl Acad Sci U S A 89:392–396
14. Walker GT, Fraiser MS, Schram JL, Little MC, Nadeau JG, Malinowski DP (1992) Strand displacement amplification—an isothermal, in vitro DNA amplification technique. Nucleic Acids Res 20:1691–1696
15. Spargo CA, Fraiser MS, Van Cleve M, Wrigth DJ, Nycz CM, Spears PA, Walker GT (1996) Detection of M. tuberculosis DNA using thermophilic strand displacement amplification. Mol Cell Probes 10:247–256
16. Ehses S, Ackermann J, McCaskill JS (2005) Optimization and design of oligonucleotide setup for strand displacement amplification. J Biochem Biophys Methods 63:170–186
17. Joneja A, Huang X (2011) Linear nicking endonuclease-mediated strand-displacement DNA amplification. Anal Biochem 414:58–69
18. Fire A, Xu S (1995) Rolling circle replication of short DNA circles. Proc Natl Acad Sci U S A 92(10):4641–4645
19. Lizardi P, Huang X, Zhu Z, Bray-Ward P, Thomas D, Ward D (1998) Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat Genet 19:225–232
20. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28(12):I–VII
21. Nagamine K, Hase T, Notomi T (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 16:223–229
22. Vincent M, Xu Y, Kong H (2004) Helicase-dependent isothermal DNA amplification. EMBO Rep 5(8):795–800
23. Kim J, Easley CJ (2011) Isothermal DNA amplification in bioanalysis: strategies and applications. Bioanalysis 3(2):227–239
24. Waiblinger HU, Ernst B, Anderson A, Pietsch K (2008) Validation and collaborative study of a P35S and T-nos duplex real-time PCR screening method to detect genetically modified organisms in food products. Eur Food Res Technol 226:1221–1228
25. Kiddle G, Hardinge P, Buttigieg N, Gandelman O, Pereira C, McElgunn CJ, Rizzoli M, Jackson R, Appleton N, Moore C, Tisi LC, Murray JAH (2012) GMO detection using a bioluminescent real time reporter (BART) of loop mediated isothermal amplification (LAMP) suitable for field use. BMC Biotechnol 12:15
26. Lee D, La Mura M, Allnutt TR, Powell W (2009) Detection of genetically modified organisms (GMOs) using isothermal amplification of target DNA sequences. BMC Biotechnol 9:7
27. Shrestha HK, Hwu KK, Wang SJ, Liu LF, Chang MC (2008) Simultaneous detection of eight genetically modified maize lines using a combination of event- and construct-specific multiplex-PCR technique. J Agric Food Chem 56:8962–8968
28. Joint Research Centre (2007) Maize seeds sampling and DNA extraction. Report on the validation of a DNA extraction method from maize seeds and grains. Community Reference Laboratory for GM Food and Feed, Ispra, http://gmo-crl.jrc.ec.europa.eu/summaries/3272_DNAExtrReport.pdf. Accessed 18 Apr 2007
29. Kuribara H, Shindo H, Matsuoka T, Takubo K, Futo S, Aoki N, Hirao T, Akiyama H, Goda Y, Toyoda M (2002) Novel reference molecules for quantitation of genetically modified maize and soybean. J AOAC Int 85(5):1077–1089
30. Matsuoka T, Kuribara H, Takubo K, Akiyama H, Miura H, Goda Y, Kusakabe Y, Isshiki K, Toyoda M, Hino A (2002) Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). J Agric Food Chem 50:2100–2109
31. Monis PT, Giglio S, Saint CP (2005) Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal Biochem 340(1):24–34
32. Pang S, Qureshi F, Shanahan D, Harris N (2007) Investigation of the use of rolling circle amplification for the detection of GM food. Eur Food Res Technol 225:59–66
33. Hadersdorfer J (2012) Development of an isothermal nucleic acid amplification protocol for high-throughput monitoring of *Plum pox* virus infection in stone fruit production. Doctoral thesis, Technische Universität München, Munich

Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

# Detection of the P35S promoter in transgenic maize via various isothermal amplification techniques: a practical approach

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```
pRR21_EU760495.1    CATGGAGTCAAAGATTCAAATAGAGGACCTAACAGAACTCGCCGTAAAGACTGGCGAACA
MON810_JX139718     ------------------------------------------------------------

pRR21_EU760495.1    GTTCATACAGAGTCTCTTACGACTCAATGACAAGAAGAAAATCTTCGTCAACATGGTGGA
MON810_JX139718     ------------------------------------------------------------

pRR21_EU760495.1    GCACGACACACTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAGGGC
MON810_JX139718     ------------------------------------------------------------

pRR21_EU760495.1    AATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGATTCCATTGCCCAGC
MON810_JX139718     ------------------------------------------------------------

pRR21_EU760495.1    TATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCA
MON810_JX139718     ------------------------------------------------------------

pRR21_EU760495.1    TTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGG
MON810_JX139718     ----------------------GTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGG
                                          ************************************

pRR21_EU760495.1    ACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCA
MON810_JX139718     ACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCA
                    ************************************************************

pRR21_EU760495.1    AGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTC
MON810_JX139718     AGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTC
                    ************************************************************

pRR21_EU760495.1    GCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGAACACGGGGGACT--
MON810_JX139718     GCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGCTGACAAGC
                    ******************************************** *****  *

pRR21_EU760495.1    ----------------------------------------------
MON810_JX139718     TGACTCTAGCAGATCATAAAGGAAAGGCCATCGCGCAAGACCCTTCCT
```

**Fig.S1.** Alignment of the P35S promoter sequences for RoundUp Ready™ soy (EU760495.1) and transgenic maize MON810 (JX13971). Forward outer primer (F3) and backward outer primer (B3) as published by [26] in red and as designed by the authors in green. The alignment shows that the primer set designed for RoundUp Ready™ soy cannot be used for amplification of the P35S promotor in transgenic maize, since this sequence is shorter and the F3 primer lies outside of the target sequence. Therefore, efficient amplification cannot be expected, since LAMP relies on the hybridization of all primers to the target in order to amplify the desired DNA sequence (Fig. S2)

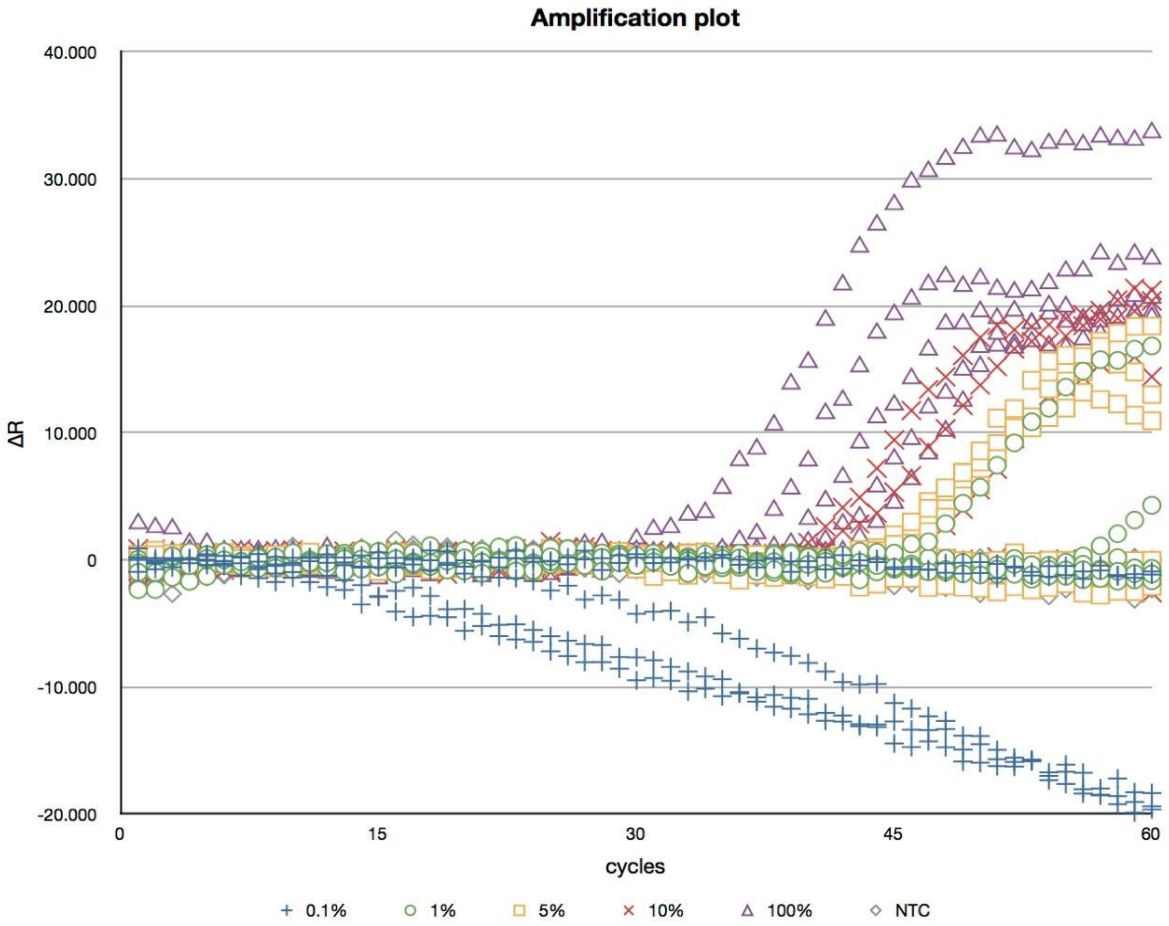


**Fig.S2.** Amplification plot with primers and reaction conditions as previously published and performed [26, 25] for GM maize, resulting in poor amplification efficiency as well as low fluorescence intensity. n = 5

**Table.S1.** Various primer sets for NEAR, SDA and RCA were tested, including different nicking and restriction enzymes (Nt.*Bst*NBI, Nt.*Bsp*QI, *Hinc*II, *Bsr*I). *Primer and oligo nucleotide sequences for NEAR (A1-8), SDA (B1-4) and RCA (C1-7) used in feasibility tests as shown in figure 1. Recognition sites of restriction enzymes are underlined.

| Method | Primer | Sequence 5' □3' |
|---|---|---|
| **NEAR*** | S1 | CAAAAAAAAAAAAGC*GAGTC*TAGCACTCCACTGACGTAAGGGATG |
| | S2 | CAAAAAAAAAAAAGC*GAGTC*TAGCAAAGGGTCTTGCGAAGGATAG |
| | B1 | ACGTCTTCAAAGCAAGTGG |
| | B2 | TCCAAATGAAATGAACTTCC |
| **NEAR_ Nt.*Bst*NBI** | S1 | CAAAAAAAAAAAAGC*GAGTC*TAGCATCTCCACTGACGTAAGGGA |
| | S2 | CAAAAAAAAAAAAGC*GAGTC*TAGCAGAAGGGTCTTGCGAAGGAT |
| | B1 | ACGTCTTCAAAGCAAGTGG |
| | B2 | TCCAAATGAAATGAACTTCC |
| **NEAR_ Nt.*Bst*NBI** | S1 | CAAAAAAAAAAAAGC*GAGTC*TAGCACACGTCTTCAAAGCAAGTGG |
| | S2 | CAAAAAAAAAAAAGC*GAGTC*TAGCAAGGATAGTGGGATTGTGCG |
| | B1 | CGAGGAGCATCGTGGA |
| | B2 | GTCCTCTCCAAATGAAATGAAC |
| **NEAR_ Nt.*Bst*NBI** | S1 | CAAAGTCT*GAGTC*TGGGACACGTCTTCAAAGCAAGTGG |
| | S2 | CAAAGTCT*GAGTC*TGGGAAGGATAGTGGGATTGTGCG |
| | B1 | CGAGGAGCATCGTGGA |
| | B2 | GTCCTCTCCAAATGAAATGAAC |
| **NEAR_** | S1 | CAAAAAAAAAAAAGC*GAGTC*TAGCACTCCACTGACGTAAGGGATG |

| **Nt.*Bst*NBI** | | |
|---|---|---|
| | S2 | CAAAAAAAAAAAAGC*GAGTC*TAGCAAAGGGTCTTGCGAAGGATAG |
| | B1 | ACGTCTTCAAAGCAAGTGG |
| | B2 | TCCAAATGAAATGAACTTCC |
| **NEAR_ Nt.*Bst*NBI** | S1 | CAAAAAAAAAAAAGC*GAGTC*TAGCAACGTAAGGGATGACGCA |
| | S2 | CAAAAAAAAAAAAGC*GAGTC*TAGCAGAGGAAGGGTCTTGCGA |
| | B1 | ACGTCTTCAAAGCAAGTGG |
| | B2 | TCCAAATGAAATGAACTTCC |
| **NEAR_ Nt.*Bst*NBI** | S1 | CAAAAAAAAAAAAGC*GAGTC*TAGCACCACTGACGTAAGGGATGA |
| | S2 | CAAAAAAAAAAAAGC*GAGTC*TAGCAGGGTCTTGCGAAGGATAGT |
| | B1 | ACGTCTTCAAAGCAAGTGG |
| | B2 | TCCAAATGAAATGAACTTCC |
| **NEAR_ Nt.*Bsp*QI** | S1 | CAAAAAAAAAAAAAA*GCTCTTC*TCCAACCACGTCTTC |
| | S2 | CAAAAAAAAAAAAAA*GCTCTTC*TGGGATTGTGCGTC |
| | B1 | CCACGAGGAGCATCG |
| | B2 | GAAGGGTCTTGCGA |
| **SDA_*Bsr*I** | S1 | CCGAGACTTAGGATTCACTCTACAA*CCAGT*GGATGACGCACAATCCCAC |
| | S2 | CCGAGACTTAGGATTCACTCTACAA*CCAGT*AGAGGAAGGGTCTTGCGA |
| | B1 | GCCGACAGTGGTCCCAAAGAT |
| | B2 | AGCGTGTCCTCTCCAAATGAAATG |
| **SDA_*Hin*cII** | S1 | CAAAAAAAAAAAA*GTTGAC*CGTCTTCAAAGC |
| | S2 | CAAAAAAAAAAAAGTTGACTGGGATTGTGC |
| | B1 | TCGTGGAAAAAGAA |
| | B2 | GTCTTGCGAAGG |
| **SDA_*Hin*cII** | S1 | CAAAAAAAAAAA*GTTGAC*AAGGGATGACG |
| | S2 | CAAAAAAAAAAA*GTTGAC*GAGGAAGGGTC |
| | B1 | ACCACGTCTTCA |
| | B2 | CCAAATGAAATGAAC |
| **SDA*** | S1 | TAGGATGAGCATTCTGCGGTTG*CCAGT*CGTTCCAACCACGTCTTCAAAGCAAGT |
| | S2 | TAGGATGAGCATTCTGCGGTTG*CCAGT*AGGAAGGGTCTTGCGAAGGATAGTGGG |
| | B1 | GCCGACAGTGGTCCCAAAGAT |
| | B2 | AGCGTGTCCTCTCCAAATGAAATG |
| **RCA*** | hybridization region | TTTTCCACTATCTTCACAATAAAGTTTTGATGCCGTATGCCTAGCACGGAATTAACTTGCTAG CCGTCCAGGTTAGCCACCTTCC |
| | Primer1 | GCCGTATGCCTAGCA |
| | Primer2 | TTGCTAGCCGTCCAG |
| | DNA backbone | TTTGATGCCGTATGCCTAGCACGGAATTAACTTGCTAGCCGTCCAGGTT |
| **RCA2** | hybridization region | GCCTCTGCCGACAGTGTTTGATGCCGTATGCCTAGCACGGAATTAACTTGCTAGCCGTCCA GGTTATCTTCAACG ATCATTGCGATAAAGGAAAGGCCATCGTTGAAGAT |
| | Primer1 | GCTAGGCATACGGCATCAAA |
| | Primer2 | AACTTGCTAGCCGTCCAGGTT |
| | DNA backbone | TCACTTAGGACGTAGTGAAGCAGGAAACACCTATGCC |
| **RCA3** | hybridization region | TCTATATAAGGAAGTTCATTTCATTTTTGATGCCGTATGCCTAGCACGGAATTAACTTGCTAG CCGTCCAGGTTCCTTCCCAGAACGCACAATCCCACTATCCTTCGCAAGACCCTTCC |
| | Primer1 | TGC TAG GCA TAC GGC ATC AAA |
| | Primer2 | AAC TTG CTA GCC GTC CAG GTT |
| | DNA backbone | TTTGATGCCGTATGCCTAGCACGGAATTAACTTGCTAGCCGTCCAGGTT |
| **RCA4** | hybridization region | GAATCCGAGGAGGTTTCCGTTTGATGCCGTATGCCTAGCACGGAATTAACTTGCTAGCCGT CCAGGTTCTGGGCAATG |
| | Primer1 | AAACGGAAACCTCCTCGGATTCCATT |
| | Primer2 | TTGATGCCGTATGCCTAGCACGGA |
| | DNA backbone | TTTGATGCCGTATGCCTAGCACGGAATTAACTTGCTAGCCGTCCAGGTT |
| **RCA5** | hybridization region | TTTTCCACTATCTTCACAATAAAGTTTTGATGCCGTATGCCTAGCACGGAATTAACTTGCTAG CCGTCCAGGTTAGCCACCTTCC |
| | Primer1 | GCCGTATGCCTAGCA |
| | Primer2 | TTGCTAGCCGTCCAG |
| | DNA backbone | TTTGATGCCGTATGCCTAGCACGGAATTAACTTGCTAGCCGTCCAGGTT |
| **RCA6** | hybridization | AGTGGAAAAGGAAGGTGGCTCTTTGATGTAGTATGCCTAGTACTGAATTAACTTGCTAGCCG |

| | | |
|---|---|---|
| | region | TCCAGGTTATCTTCACATATCTGTCACTTTATTGTGAAGAT |
| | Primer1 | ACCTTCCTTTTCCACTATC |
| | Primer2 | TAGTATGCCTAGTACTGAATTAACTT |
| | DNA backbone | TCACTTAGGACGTAGTGAAGCAGGAAACACCTATGCC |
| **RCA7** | hybridization region | ATCTTCAACGATGGCCTTTCTGAACGACGAATCTGTACCATGCTAATGCGGCGTGATGTATTATGCGTATGACGGCAGAGGC |
| | Primer1 | TGCTAATGCGGCGTGATGT |
| | Primer2 | GGAATCCGAGTGAACGACGA |
| | DNA backbone | TGAACGACGAATCTGTACCATGCTAATGCGGCGTGATGTATTATGCGTATGGA |

**Table S2.** Reaction conditions and buffer concentrations tested for Rolling Circle Amplification, resulting in a total number of tested combinations of 3481

| **Ligation** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Denaturation step at 95°** | 3 min. at 95°C | none | | | | | |
| **Ligation reaction time** | 30 min. | 60 min. | 90 min. | 120 min. | 180 min. | 240 min. | |
| **Ligases used** | Ampligase at 65°C | T4 DNA ligase at 37°C | | | | | |
| **padlock probe IDs melting temperatures** | RCA | RCA2 | RCA3 | RCA4 | RCA5 | RCA6 | RCA7 |
| **Tm of padlock probe 5'arms** | 70°C | 68.3°C | 63.9°C | 69.5°C | 50°C | 70°C | 70.2°C |
| **Tm pf padlock probe 3'arms** | 50°C | 77.7°C | 68.3°C | 32.6°C | 70°C | 61.1°C | 49.6°C |
| **padlock probe concentrations** | 100pM | 10pM | 1pM | | | | |
| **DNA-concentration applied to ligation reaction** | 10 ng/µL | 1 ng/µL | 0.1 ng/µL | | | | |
| **Exonuclease I concentration** | 1 Unit | 10 Units | 20 Units | | | | |
| **Exonuclease III concentration** | 1 Unit | 10 Units | 20 Units | | | | |
| **Incubation time of exonucleases** | 10 min. | 30 min. | 120 min. | | | | |
| **Amplification** | | | | | | | |
| **Denaturation step** | 3 min. at 95°C | none | | | | | |
| **Polymerases tested** | Bst polymerase (65°C) | phi29 polymerase (37°C) | | | | | |
| **Divalent cations concentrations** | 6 mM | 4 mM | 2 mM | 1 mM | | | |
| **buffer pH** | 7 | 7.5 | 8 | 8.5 | 9 | | |

**Table S3.** Reaction conditions and buffer concentrations tested for Nicking Enzyme Amplification Reaction

| **Tm of primer target region** | 37°C | 40° C | 45°C | 55°C | 60° C | 65° C |
|---|---|---|---|---|---|---|
| **Tm of primer 5' end** | 37°C | 40° C | 45°C | 55° C | 60° C | 65° C |
| **Nicking Enzymes (recognition site)** | Temp: 37°C | | Temp: 55°C | | | |
| | Nt.BbvCI | Nb.BtsI | Nt.BstNBI | Nt.BspQI | | |
| **Assay time** | 15 min | 30 min | 60 min | | | |
| **Buffer pH** | 7 | 7.5 | 8 | 8.5 | | |
| **Buffer concentration** | 20 mM | 40 mM | 80 mM | | | |
| **KCl concentration** | 25 mM | 50 mM | 100 mM | | | |
| **$MgCl_2$ concentration** | 2.5 mM | 5 mM | 10 mM | | | |
| **dNTPs** | 0.2 mM | 0.4 mM | 0.8 mM | | | |
| **Additives** | Formamid (5%) | Betain (1M) | DMSO (5%) | Et.SSB (4 ng/µL) | Glycerin (5%) | BSA 100 µg/µL |
| **Nicking Enzyme concentration** | 2.5 units | 5 units | 10 units | 20 units | | |
| **Polymerase** | Bst polymerase (55-65°C) | Phi29 polymerase (37°C) | | | | |
| **Polymerase conc.** | 2.5 units | 5 units | 10 units | | | |

**Table S4.** Reaction conditions and buffer concentrations tested for Strand Displacement Amplification.

| | | | | | | |
|---|---|---|---|---|---|---|
| **Tm of primer target region** | 37°C | 40° C | 45°C | 60° C | 65° C | |
| **Tm of primer 5' end** | 37°C | 40° C | 45°C | 60° C | 65° C | 70°C |
| **Restriction Enzymes (recognition site)** | Temp: 65°C | | Temp: 37°C | | | |
| | BsrI | BsmI | HincII | | | |
| **Assay time** | 30 min | 60 min | 90 min | | | |
| **Buffer pH** | 7 | 7.5 | 8 | 8.5 | | |
| **Buffer concentration** | 20 mM | 40 mM | 80 mM | | | |
| **KCl concentration** | 25 mM | 50 mM | 100 mM | | | |
| **$MgCl_2$ concentration** | 2.5 mM | 5 mM | 10 mM | | | |
| **Protection dNTP** | dATP$\alpha$S | dCTP$\alpha$S | | | | |
| **dNTPs concentration** | 0.2 mM | 0.4 mM | 0.8 mM | | | |
| **Restriction Enzyme concentration** | 2.5 units | 5 units | 10 units | 20 units | | |
| **Polymerase** | Bst polymerase (55-65°C) | $Exo^-$ Klenow (37°C) | | | | |
| **Polymerase conc.** | 2.5 units | 5 units | 10 units | | | |

## Publication #3

# Loop-mediated Isothermal Amplification (LAMP) for the Detection of Horse Meat in Processed Foods

**Celine Zahradnik,** Roland Martzy, Robert Mach, Rudolf Krska, Andreas Farnleitner, Kurt Brunner

**Submitted and revised in:** Food Analytical Methods (2014)

**Abstract** In this study a simple and rapid high-throughput method for the detection of horsemeat in processed food products is described. Specific LAMP primers were designed to target the mitochondrial genome of horse (*Equus caballus*). No cross-reactions were observed for beef, pork, chicken, goat, mutton, turkey, boar, roe, rabbit and deer. Sensitivity tests showed reliable detection of 0.1 ng of extracted horse DNA. Spiking experiments were performed to show that the assay is capable of detecting 0.1% horsemeat in prepared model sausages, independent from their cooking time. Additionally, five different commercial horsemeat products were analyzed to ensure the robustness of the assay when applied to varying food matrices. All experiments were performed on a heating block followed by visual detection using an intercalating dye. Results were confirmed by real-time fluorescence monitoring using a thermal cycler and compared to a previously published real-time PCR assay. In conclusion, this method is a good candidate for the simple and efficient testing of horsemeat in food-products in the future.

**Zusammenfassung** In dieser Studie wird die Entwicklung einer simplen und schnellen Nachweis-Technik für Pferdefleisch in prozessierten Lebensmitteln beschrieben. Ziel-Molekül der spezifisch entwickelten LAMP-Primer ist das mitochondriale Genom der Spezies Pferd (*Equus caballus*). Für Rind, Schwein, Huhn, Ziege, Lamm, Truthahn, Wildschwein, Reh, Hase und Hirsch konnten keine Kreuz-Reaktionen beobachtet werden, eine zuverlässige Nachweisgrenze von 0.1 ng extrahierte Pferde-DNA wurde ermittelt. Die Analyse von Modell-Würsten mit definiertem Pferdefleischgehalt konnte zeigen, daß der entwickelte Assay Gehalte von 0.1 % Pferdefleisch, unabhängig von Kochzeiten, zuverlässig nachweisen kann. Zusätzlich wurde fünf verschiedene kommerziell erhältliche Pferdefleischprodukte untersucht, um außerdem eine Unabhängigkeit von Lebensmittel-Matrices zu zeigen. Alle Experimente wurden auf einem Heizblock und darauffolgender visueller Detektion mittels

eines interkalierenden Farbstoffes durchgeführt. Die Ergebnisse wurden mittels real-time Fluoreszenzmessung und Vergleich mit einem zuvor publizierten PCR-Assay für den Nachweis von Pferdefleisch verglichen. Diese Methode stellt eine einfach und effiziente Alternative für den Nachweis von Pferdefleisch in der Zukunft dar.

# Loop-mediated Isothermal Amplification (LAMP) for the Detection of Horse Meat in Meat and Processed Meat Products

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**Abstract** In this study a simple and rapid high-throughput method for the detection of horse meat in processed food products is described. Specific LAMP primers were designed to target the mitochondrial genome of horse (*Equus caballus*). No cross-reactions were observed for beef, pork and chicken. Sensitivity tests showed reliable detection of 0.1 ng of extracted horse DNA. Spiking experiments were performed to show that the assay is capable of detecting 0.1% horse meat in prepared model sausages, independent from their cooking time. Additionally, five different commercial horse meat products were analyzed to ensure the robustness of the assay when applied to varying food matrices. All experiments were performed on a heating block followed by visual detection using an intercalating dye. Results were confirmed by real-time fluorescence monitoring using a thermal cycler and compared to a previously published real-time PCR assay. In conclusion, this method is a good candidate for the simple and efficient testing of horse meat in food-products in the future.

**Keywords** loop-mediated isothermal amplification, horse meat, visual detection, sausages, meat species identification

## Introduction

In 2012 and 2013 the European Commission performed official controls in several European Union member states, revealing the addition of horse meat in pre-packaged food products labelled as 100% beef. Followed by these discoveries the meat adulteration scandal was sparked in January 2013, especially in the United Kingdom, Ireland and Germany, where most adulterated products were found (European Commission, 2013). Although horse meat is considered a delicacy in some countries, the European Commission raised concerns about these findings. Since labelling requirements were violated, the fraudulent nature of this matter can be assumed. Apart from that, the consumption of non-declared horse meat might also pose a health risk to consumers. Phenylbutazone is widely used veterinary drug whose use is only allowed on non-food producing animals. The use of phenylbutazone has to be excluded clearly for any meat species going into the food chain (European Commission, 2010). Although several protein-based (enzyme-linked immunosorbent assay, ELISA) and DNA-based (polymerase chain reaction, PCR) test systems for meat species identification exist, these methods are time-consuming and require costly equipment, e.g. thermal cyclers, and trained personnel to perform these reactions (Walker et al., 2013). Rapid high-throughput methods are needed to facilitate controls to ensure proper labelling in the future and in compliance with the law.

In 2000 an alternative DNA amplification method termed Loop-mediated Isothermal Amplification (LAMP) was developed (Notomi et al., 2000). Three primer sets bind to six distinct regions of the target sequence. Through the use of a *Bst* polymerase with strand displacement activity (Aliotta et al., 1996) a dumbbell-like structure is formed which then undergoes cyclic amplification producing various sized DNA strands with alternately inverted repeats of the target sequence (Notomi et al., 2000; Nagamine et al., 2002). Since a denaturation step is not required, the assay can be carried out under isothermal conditions. A thermal cycler is not needed to perform the amplification reaction, thus making this method highly cost- and time-efficient as well as field-applicable and easy-to-perform. LAMP products can be detected with the naked eye through the use of an intercalating dye such as SYBR Green I (Iwamoto et al., 2003). Although the first developed LAMP assay by Notomi et al. (2000) targeted the HB virus, LAMP as well as isothermal methods in general are becoming more and more interesting for food analysis, e.g. for the detection of allergens, genetically modified organisms (GMOs) and for meat species identification (Ahmed et al., 2010; Zahradnik et al., 2014a; 2014b).

In this study we report the development of a Loop-mediated Isothermal Amplification assay for the detection of horse meat in meat products targeting part of the mitochondrial genome which was demonstrated to be specific for horse in earlier publications (Köppel et al., 2008; Köppel et al., 2009). Several LAMP primer sets were evaluated according to their specificity and sensitivity and compared with the results of a previously published real-time PCR assay. To test the capability of the assay to detect horse meat in highly complex food matrices, model sausages with defined horse meat contents were prepared and analyzed. Additionally, five different commercial food products, containing horse meat, were tested. The LAMP assay performed similar to PCR regarding the detection of horse meat in food-products but can be performed much faster with an analysis time of around 30 minutes. The use of a simple heating block and the visual detection using SYBR Green I make this assay much more time- and cost-efficient compared to PCR and could prove to be a valuable tool for the detection of horse meat in pre-packaged food products.

**Material and Methods**

Meat material and samples

Horse meat and five horse meat products (two differently spiced hard cured sausages, meat loaf, blood pudding and knackwurst) were obtained from a local butcher, specialized on the commerce of horse meat. Other meat species (beef, pork, chicken) and ingredients for the production of model sausages were obtained in local supermarkets.

Preparation of model sausages

For the preparation of model sausages a meat mixture containing beef (35%), pork (35%), bacon (20%), salt (2%), pepper (0.7%), worcester sauce (0.7%), vinegar (0.7%), garlic (0.7%) as well as various herbs and spices (5,2%). Each ingredient was added in (w/w). The mixture was homogenized thoroughly with fixed portions of horse meat in concentrations of 10%, 5%, 1%, 0.5%, 0.1% and 0% using a meat cutter and filled into sheep gut. The sausages were then cooked 40 minutes.

DNA extraction

All samples were extracted using a previously published, slightly modified DNA extraction protocol for plant tissue (Amani et al., 2011). Each sample was extracted three times. 200 milligrams of each sample were incubated with extraction buffer, followed by addition of 500 µL chloroform:isoamyl alcohol (24:1). After centrifugation (15000 g) the supernatant was added to 400 µL isopropanol (precooled at -20°C) and incubated at -20°C for 30 minutes. After another centrifugation step the liquid phase was removed with a pipette and the pellet was dissolved in 100 µL 1X TE buffer (10 mM Tris, 1 mM EDTA, pH 8). DNA concentration and purity were measured photometrically using the NanoVue Plus (VWR, Radnor, USA). Extracted DNA samples were stored at -20°C until further use.

Primers and probes

All oligonucleotides were synthesized by Eurofins MWG (Ebersberg, Germany). Real-time PCR primers and probe were used as published by Köppel et al. (2008) and Köppel et al. (2009). Seven LAMP primer sets were designed using the Primer Explorer V4 software with standard settings (Eiken Chemical Co., Ltd.; Tokyo, Japan) on basis of the complete sequence of the *Equus caballus* mitochondrial DNA, GenBank acc. no. X79547. The LAMP primer sequences, used for the final assay, target the NADH dehydrogenase at the position [3353-3565bp] and are given in Table 1.

Real-time PCR reaction

1 µL diluted DNA extracts (1:10) were added to 14 µL of reaction mix containing Kapa™ Probe® Fast (Peqlab, Erlangen, Germany), primers and probe (in a final concentration of 0.3 µmol/L each). Amplification reactions were performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, New York, USA) according to the following thermal cycling protocol: initial steps of 2 min at 95°C, followed by 60 cycles of 15 sec at 95°C and 1 min at 62°C.

Table 1 List of LAMP primers (ID132) used for experiments in this study and their sequences targeting the NADH dehydrogenase gene from horse (GenBank acc. no. X79547).

| **LAMP Primer** | **Sequence 5' → 3'** |
|---|---|
| **FIP** | CTGCTAGGAAGAATAGAGCAAATGG-AGAAGGAGAATCAGAACTCGT |
| **BIP** | ACATCTTCACAACAACCCTATTTCT-ATTAATTGAGTAGAGTTCTGGC |
| **F3** | CGAGCTCCATTTGACCTAAC |
| **B3** | AAGGAGAGCTTTAATGGTGA |
| **Loop F** | TGCGTATTCAACGTTGAATCCAG |
| **Loop B** | AGGAGCATTTCACAACCCCT |

Amplification reactions were carried out in five replicates. To prove the presence of amplifyable DNA, all DNA extracts were amplified using a previously published 12S rRNA universal primerset (Kitano et al., 2007) (Supporting Information, Table S1). 1 µL diluted DNA extracts (1:10) were added to 14 µL of reaction mix containing Kapa™ SYBR® (Peqlab, Erlangen, Germany), and primers (in a final concentration of 0.3 µmol/L each).

LAMP Reactions

LAMP reactions were carried out in a total reaction volume of 25 µL containing 0.8 µM each FIP and BIP, 0.2 µM each F3 and B3, 0.4 µM each LoopF and LoopB, 1.4 µM of each dNTP (Peqlab, Erlangen, Germany), 0.8 M betaine solution (Sigma-Aldrich, St. Louis, USA), 8 U *Bst* DNA polymerase (New England Biolabs, Ipswich, USA), 20 mM Tris-HCl (pH 8.5), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 3 mM $MgSO_4$, 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, USA), 1 µL diluted (1:10) DNA extract and 1 µL Syto® 9 Green Fluorescent Nucleic Acid Stain (Life Technologies, Carlsbad, USA) (Monis et al., 2005) for detection via real-time fluorescence monitoring. Reactions were run in the thermal cycler at 67°C for 90 minutes. Amplification reactions were carried out in five replicates. Alternatively, reactions were incubated on an Eppendorf Thermomixer Plus (Eppendorf, Hamburg, Germany) at 67°C for 90 minutes. For visual detection 1 µL 1X SYBR Green® I was added to each reaction tube after incubation. In order to prove, that the signals measured via LAMP are a product of DNA amplification, a 1,5% agarose gel electrophoresis with 1X TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8,2) was performed (Supporting Information, Figure S1).

Analytical Sensitivity and Specificity of the Horse Meat LAMP Assay

To investigate the analysis sensitivity of the presented assay, horse meat DNA in a concentration of 10 ng/µL was serially diluted (1:10) with beef DNA in a constant concentration of 10 ng/µL and analyzed with PCR, using a previously published primer set for the detection of horse meat (Köppel et al., 2009), and LAMP via real-time fluorescence monitoring via Syto® 9 Green Fluorescent Nucleic Acid Stain using the same conditions as mentioned above.

To ensure the specific detection of horse meat, DNA extracts of horse, beef, pork, chicken, goat, mutton, deer, roe, rabbit, boar, turkey and donkey with a concentration of 40 ng/µL, respectively, were introduced as template into real-time LAMP reactions, under the same reaction conditions.

Limit of Detection (LOD) for Horse Meat

Model sausages with fixed concentrations of horse meat (10%, 5%, 1%, 0.5%, 0.1%, 0%, respectively) were analyzed with real-time PCR and LAMP in five replicates each. LAMP reactions were performed on two different amplification platforms, real-time fluorescence monitoring via Syto® 9 using a thermal cycler and amplification on a heating block with subsequent detection via SYBR Green® I, using the same conditions as mentioned above. Positive (PPV) and negative predicted values (NPV) were calculated as follows: PPV = (number of true positives)/(number of true positives + number of false positives) x 100; NPV = (number of true negatives)/(number of true negatives + number of false negatives) x 100.

Analysis of Commercial Horse Meat Products

To evaluate the performance of the LAMP assay when analyzing commercial horse meat products containing high amounts of fat, salt and spices, DNA from five different meat products was extracted and introduced as template (diluted 1:4, except for blood pudding which was used undiluted) into PCR and LAMP reactions, using the same conditions as mentioned above, and subsequent staining of the LAMP products via SYBR Green® I.

**Results**

Analytical Sensitivity and Specificity

The detection probability was 100% for 0.1 ng/µL horse meat in 10 ng/µL beef DNA background with a detection time of 21 minutes (±1.6 min). Non-beef samples and no template controls did not result in any positive amplification signals (Table 2). Specificity tests for horse, donkey, pork, chicken, beef, mutton, goat, deer, roe, boar, rabbit and turkey resulted in positive signals for ten out of ten replicates. All other tested meat species did not result in positive amplification signals (Table 3).

Table 2 Analytical sensitivity for the detection of horse meat with PCR and LAMP was found to be 0.01 ng/µL for PCR and 0.1 ng/µL for LAMP. No template controls (NTC) were negative, n = 5.

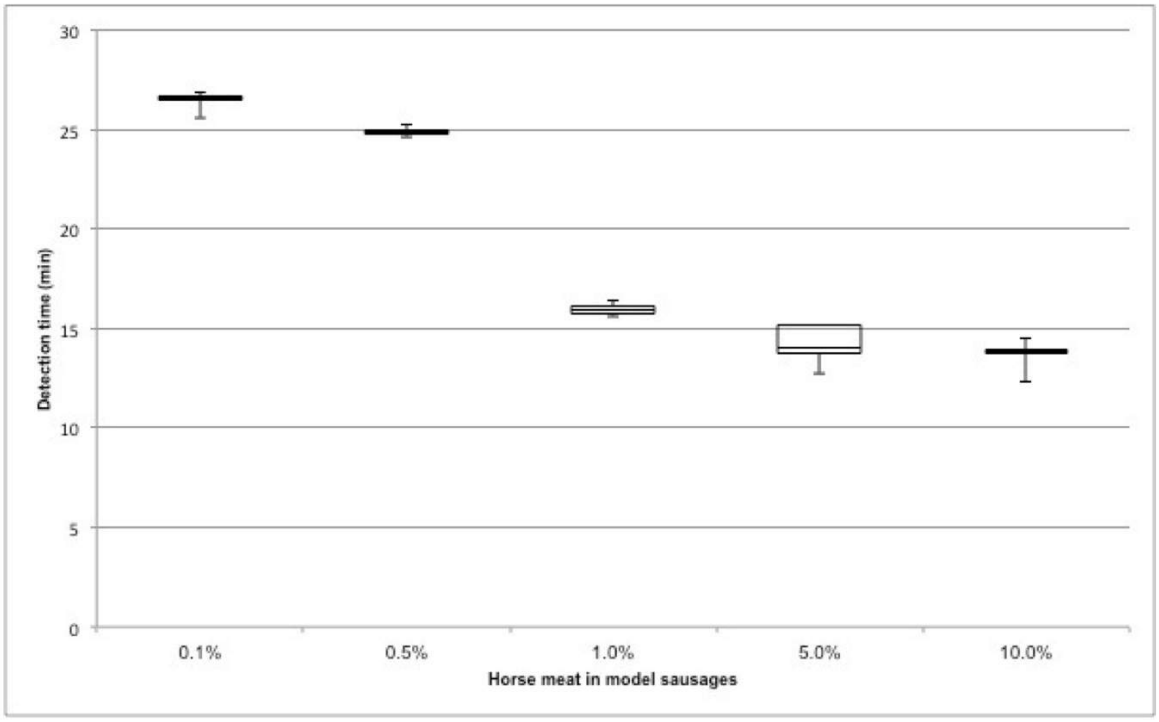
| Nanogramme horse meat DNA with beef background (10 ng/µL) | Positive Reactions | |
|---|---|---|
| | PCR | LAMP |
| 10 | 5/5 | 5/5 |
| 1 | 5/5 | 5/5 |
| 0.1 | 5/5 | 5/5 |
| 0.01 | 5/5 | 2/5 |
| 0.001 | 1/5 | 0/5 |
| 0.0001 | 0/5 | 0/5 |
| 0 | 0/5 | 0/5 |
| NTC | 0/5 | 0/5 |

Table 3 Specificity of the LAMP assay. Positive amplification signals were only obtained for horse and donkey DNA. All other meat species and the no template controls (NTC) were negative, n = 10.

| DNA extracts [40ng/µL] | LAMP (positive reactions) |
|---|---|
| Horse | 10/10 |
| Donkey | 10/10 |
| Pork | 0/10 |
| Chicken | 0/10 |
| Beef | 0/10 |
| Mutton | 0/10 |
| Goat | 0/10 |
| Deer | 0/10 |
| Roe | 0/10 |
| Boar | 0/10 |
| Rabbit | 0/10 |
| Turkey | 0/10 |
| No template control (NTC) | 0/10 |

Limit of Detection (LOD) of the LAMP Assay

In order to establish a limit of detection (LOD), based on real food matrices, the prepared model sausages with different concentration of horse meat (10%, 5%, 1%, 0.5%, 0.1%, 0%, respectively) were analysed via LAMP. The LAMP reactions were performed on two different amplification platforms, real-time fluorescence monitoring via Syto® 9 using a thermal cycler (n = 5), and amplification on a heating block with subsequent detection via SYBR Green® I (n = 2). The time of detection reached from 26.4 minutes (± 0.5 min) for 0.1% to 13.7 minutes (± 0.8 min) for 10%, respectively (Figure 1). No amplification signals were observed for the model sausages containing no




horse meat at all, and for the non-template controls. The visual detection via SYBR Green® I gave similar results (Figure 2).

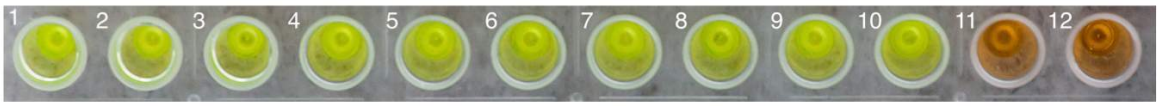



Figure 1 Detection time of the LAMP assay for the horse meat concentrations (10%, 5%, 1%, 0.5% and 0.1%, respectively) in model sausages, n = 5.

Figure 2 Analysis of model sausages detected via SYBR Green I staining. 1, 2 - 10% horse meat; 3, 4 - 5% horse meat; 5, 6 - 1% horse meat, 7, 8 - 0.5% horse meat; 9, 10 - 0.1% horse meat; 11, 12 - 0% horse meat, n = 2.

Analysis of Commercial Products

To evaluate the performance of the LAMP assay when analyzing commercial horse meat products containing high amounts of fat, salt and spices, DNA from five different meat products was extracted and introduced as template into PCR and LAMP reactions. Table 4 shows DNA concentrations in extracts for each of the products and the results obtained from real-time PCR and real-time LAMP experiments. All samples were analysed in five replicates, and reliably detected, except for blood pudding. Similar to the analysis of the model sausages, performing the LAMP assay on a thermal cycler and on a heating block, gave the same results. Figure 3 shows the results for the visual detection via staining with SYBR Green I.

| Products | Amount of extracted DNA (ng/µL) | Positive Reactions | |
|---|---|---|---|
| | | PCR | LAMP |
| **Meat loaf** | 471 | 5/5 | 5/5 |
| **Knackwurst** | 601 | 5/5 | 5/5 |
| **Blood pudding** | 4 | 0/5 | 0/5 |
| **Hard cured sausage with garlic** | 785 | 5/5 | 5/5 |
| **Hard cured sausage with pepper** | 879 | 5/5 | 5/5 |
| **NTC** | - | 0/5 | 0/5 |

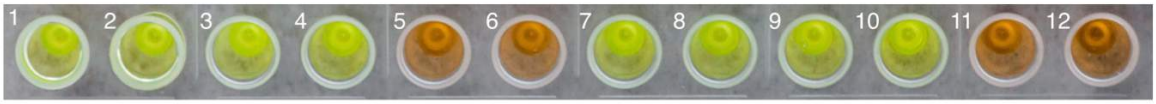



Table 4 DNA extraction results, PCR and LAMP analysis of commercial horse meat products. Except for blood pudding, all products were reliably detected. The analysis of blood pudding did not result in any amplification, n = 5.

Figure 3 Analysis of commercial horse meat products via SYBR Green I staining. 1, 2 - meat loaf; 3, 4 - knackwurst; 5, 6 - blood pudding; 7, 8 - hard cured sausage with garlic, 9, 10 - hard cured sausage with pepper; 11, 12 - no template control, n = 2.

**Discussion**

The 2013 meat adulteration scandal drastically raised the awareness of the authorities and the public regarding the authenticity of meat and meat products. The increasing demand for versatile and high-quality products poses a challenge not only for controlling agencies but also for food industry and retail companies. Due to increased analysis efforts and and increasing sample numbers, rapid test system, that allow a simple on-site detection of specific analytes, are required.

The authentication of meat species is more or less limited to DNA-based test systems due to the high specificity provided by PCR-based methods. However, conventional PCR methods are laborious and time-consuming and cannot be transferred into a more field-applicable approach. Since LAMP is capable of amplifying a DNA target under isothermal conditions and the amplification products can be visually detected, this method offers a great advantage for the rapid on-site testing of food products and has been successfully applied for the detection of allergens, genetically modified organisms (GMOs) and food-borne pathogens (Zahradnik et al., 2014a; Lee D et al., 2009; Yamazaki et al., 2008). However, to date only a few publications have utilised the LAMP method for meat species authentication, like the detection of ostrich meat (Abdulmawjood et al., 2014) and the detection of meat species via an electrochemical DNA sensor (Ahmed et al., 2010).

The here presented LAMP assay for the detection of horse meat is capable of reliably detecting 0.1% horse meat in prepared model sausages in less than 20 minutes. The calculated PPV and NPV were 100% for the analysed model sausages. These results underline the findings of previous publications that LAMP is much less succeptible to known PCR inhibitors such as fats and salts (Kaneko et al., 2007). Specificity tests were able to show that positive amplifications signals are obtained only for horse (*Equus caballus*) and donkey (*Equus asinus*), which were both found in food products during the 2013 meat adulteration scandal (Walker et al., 2013), but no other more or less frequently consumed meat species in Europe (turkey, chicken, pork, beef, deer, roe, boar, rabbit, mutton and goat). The high specificity is provided by the use of three primer sets, all of which have to bind perfectly in order to amplify the target sequence. The analytical sensitivity of the assay was found to be 100 pg per µL with a detection probability of 100%. Although lower sensitivities for LAMP assays for the detection of meat have been reported (Abdulmawjood et al., 2014), the addition of horse meat to meat products is an issue of food adulteration and not of trace analysis. Therefore, the analytical sensitivity obtained in this publication is sufficient in order to detect horse meat in meat products when admixed by a butcher or producer to increase the financial gain. The analysis of commercial horse meat products resulted in the reliable detection of all products except for blood pudding. Although three DNA extractions were performed, the photometrically obtained yields were <5 ng/µL, no horse meat was detected in the blood pudding sample with PCR or LAMP. This result might have been caused by the fact that blood pudding mainly consists of coagulated blood which on its part consists of >90% erythrocytes which do not contain nuclei or mitochondria. Hence, extracted DNA will originate from thrombocytes and leucocytes which represent only a minor part of the composition of mammalian blood (Bowen, 1963), and of genomic DNA extracted from spices and bacon, which are part of the typical Austrian recipe for blood pudding. All other commercial horse meat products (meat loaf, knackwurst, two different types of hard cured sausages) were detected with 100% probability, in less than 25 minutes.

**Conclusion**

The Loop-mediated Isothermal Amplification assay was shown to be a specific and sensitive method for the detection of horse meat in food products. Although it is not suitable for the quantification of trace levels, it represents an accurate and robust technique that can be performed in less than one hour without special equipment. With a reaction time of less than 30 minutes it is a valuable tool for the rapid control of food samples for horse meat. Furthermore, the naked eye visualization using SYBR Green I eliminates the need for a UV transilluminator and allows the on-site analysis of food samples. This work contributes to a future approach on food analysis by meeting the demands for quick and easy-to-perform analytical methods.

**Acknowledgements**

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**Compliance with Ethics Requirements**

Celine Zahradnik declares that she has no conflict of interest. Roland Martzy declares that he has no conflict of interest. Robert L. Mach declares that he has no conflict of interest. Rudolf Krska declares that he has no conflict of interest. Andreas H. Farnleitner declares that he has no conflict of interest. Kurt Brunner declares that he has no conflict of interest.

This article does not contain any studies with human or animal subjects.

**References**

Abdulmawjood A, Grabowski N, Fohler S, Kittler S, Nagengast H, Klein G (2014) Development of Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid and Sensitive Identification of Ostrich Meat. PloS one 9(6):1-6

Ahmed MU, Hasan Q, Hossain MM, Saito M, Tamiya E (2010) Meat species identification based on the loop mediated isothermal amplification and electrochemical DNA sensor. Food Control 21(5):599-605

Aliotta JM, Pelletier JJ, Ware JL, Moran LS, Brenner JS, Kong H (1996) Thermostable Bst DNA polymerase I lacks a 3'→5' proofreading exonuclease activity. Genetic Analysis: Biomolecular Engineering 12(5-6):185-195

Amani J, Kazemi R, Abbasi AR, Salmanian AH (2011) A simple and rapid leaf genomic DNA extraction method for polymerase chain reaction analysis. Iranian Journal of Biotechnology 9(1): 69-71

Bowen HJM (1963) The elementary composition of mammalian blood. Wantage, Berkshire, United Kingdom

European Commission (2010) Official Journal of the European Union 15:1

European Commission (2013) Official Journal of the European Union 48:1

Iwamoto T, Sonobe T, Hayashi K (2003) Loop-mediated Isothermal Amplification for Direct Detection *Mycobacterium tuberculosis* Complex, *M. avium*, and *M. intracellulare* in Sputum Samples. J Clin Microbiol 41(6):2616-2622

Kaneko H, Kawana T, Fukushima E, Suzutani T (2007) Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J Biochem Biophys Methods 70:499-501

Kitano T, Umetsu K, Tian W, Osawa M (2007) Two universal primer sets for species identification among vertebrates. Int J Legal Med 121:423-427

Köppel R, Ruf J, Zimmerli F, Breitenmoser A (2008) Multiple real-time PCR for the detection and quantification of DNA from beef, pork, chicken and turkey. Eur Food Res Technol 227:1199-1203

Köppel R, Zimmerli F, Breitenmoser A (2009) Heptaplex real-time PCR for the identification and quantification of DNA from beef, pork, chicken, turkey, horse meat, sheep (mutton) and goat. Eur Food Res Technol 230:125-133

Lee D, La Mura M, Allnutt TR, Powell W (2009) Detection of genetically modified organisms (GMOs) using isothermal amplification of target DNA sequences. BMC Biotechnol 9:7

Monis PT, Giglio S, Saint CP (2005) Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concetration on amplification and DNA melting curve analysis. Anal Biochem 340(1):24-34

Nagamine K, Hase T, Notomi T (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. Molecular and Cellular Probes 16:223-229

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 28(12):I-VII

Walker MJ, Burns M, Burns DT (2013) Horse Meat in Beef Products - Species Substitution 2013. Journal of the Association of Public Analysts 41:67-106

Yamazaki W, Ishibashi M, Kawahara R, Inoue K (2008) Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of Vibrio parahaemolyticus. BMC Microbiology 8:163.

Zahradnik C, Martzy R, Mach RL, Krska R, Farnleitner AH, Brunner K (2014a) Detection of the food allergen celery via loop-mediated isothermal amplification technique. Anal Bioanal Chem 406(27):6827-6833

Zahradnik C, Kolm C, Martzy R, Mach RL, Krska R, Farnleitner AH, Brunner K (2014b) Detection of the P35S promotor in transgenic maize via various isothermal amplification techniques: a practical approach. Anal Bioanal Chem [Epub ahead of print]

## Supporting Information

| DNA extracts [40ng/µL] | PCR (positive reactions) |
|---|---|
| **Horse** | 10/10 |
| **Donkey** | 10/10 |
| **Pork** | 10/10 |
| **Chicken** | 10/10 |
| **Beef** | 10/10 |
| **Mutton** | 10/10 |
| **Goat** | 10/10 |
| **Deer** | 10/10 |
| **Roe** | 10/10 |
| **Boar** | 10/10 |
| **Rabbit** | 10/10 |
| **Turkey** | 10/10 |
| **No template control (NTC)** | 0/10 |

Table S1 The presence of amplifyable DNA was proven via real-time PCR using a universal primer set for vertebrates targeting parts of the 12S rRNA, as previously published (Kitano et al., 2007), n = 10.

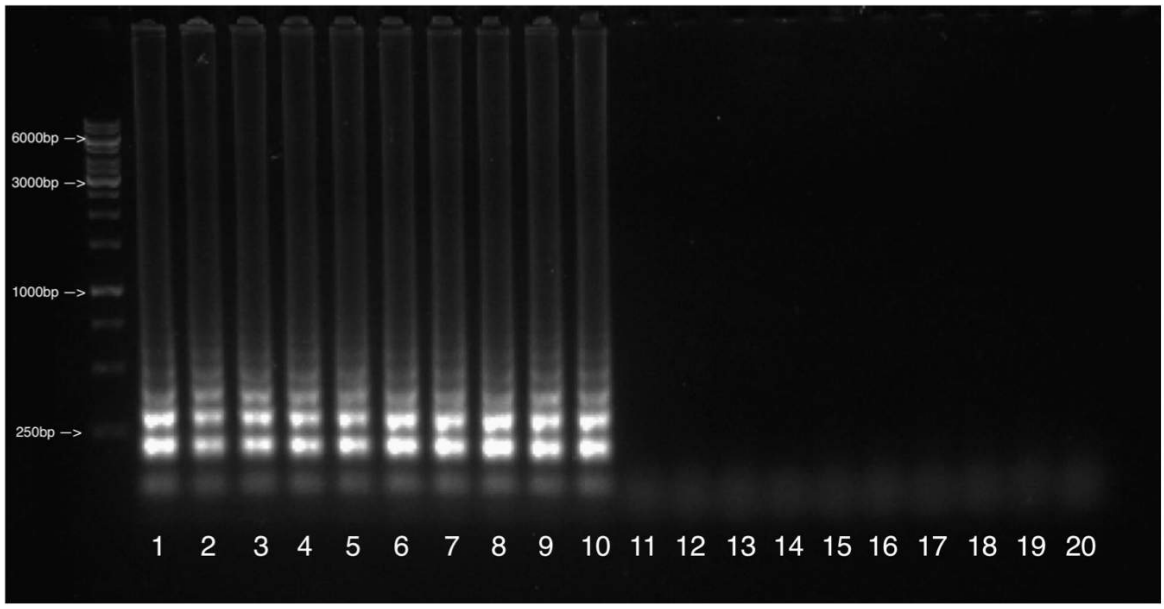


Figure S1 Agarose gel electrophoresis of the amplification products, resulting in a pattern typical for the various-sized LAMP products. Lane 1-10 - horse meat [10ng/µL], lanes 11-15 - beef [10ng/µL], lanes 16-20 - non-template control (NTC).

## Conclusion

Although test systems for the here described analytes (detection of celery, GMOs and horse meat) already exist, they cannot provide the practicability to be used for on-site analysis. Several PCR- and real-time PCR assays for the detection of celery have been published previously [97,82,123]. However, some of them result in cross-reactions with closely related plant species and all of them are time-extensive and limited to the use with a thermal cycler in order to produce amplification products. Furthermore, conventional PCR assays without real-time fluorsecence monitoring require agarose gel electrophoresis for visualisation. Therefore, these assays do not fulfill the demands for rapid on-site test systems. Similarly, various singleplex and multiplex PCR assays have been published for the detection of GMOs and the authentication of meat species [124-126], but with the same outcome that these assays cannot be performed without a laboratory and sophisticated equipment. In 2010 a test system based on LAMP and visualisation via an electrochemical sensor has been developed for the authentication of several meat species [127]. However, this was three years before the horse meat scandal which further raised the concern of the authorities and of the public regarding the authenticity of food products. Similarly, the development of LAMP assays for the rapid detection of allergens has not been properly adressed yet. Since immunologial test systems for the detection of celery do not exist, but the labelling of celery in food products is mandatory, the developed LAMP assay for celery detection in processed food is an important contribution to the field of rapid detection methods in food safety.

The aim of this thesis was to investigate the feasibility of isothermal amplification assays for the detection of celery, transgenic maize and horse meat in food products. For all three targets of interest a DNA-based approach is recommended, since the assays have to be highly specific in order to distinguish the targets from closely related species or sequences that can also be present in the examined samples. These new test systems were designed to facilitate the presented assays to become independent from sophisticated lab equipment as far as possible.

In case of celery, which has to be labelled accordingly due to its allergenic potential, the detection without the co-determination of closely related species has to be assured, as many members of the Apiaceae family are frequently used in food processing for flavouring purposes of instant meals, sauces and spice blends, such as carrot, parsley and lovage. For celery, ELISA is not the method of choice due to the probable cross-detection of these non-target species. In publication #1 the successful development of a LAMP assay for the detection of celery in processed food products is described. The assay has been developed

and optimised to specifically detect low concentrations of celery in various processed food products and was confirmed to perform equally to PCR. Also, the impact of complex food matrices on the performance of the assay was evaluated. Due to the simple nature of an optimised LAMP assay, reactions can be performed quickly and without any sophisticated equipment. The simple visual detection via intercalating dyes further facilitates the analysis, thus the developed LAMP assay is a good futurecandidate for detection of celery in foods directly on a sample-site.

In case of transgenic maize, the feasibility of five different isothermal amplification techniques was investigated under co-authorship and equal contribution with Claudia Kolm. Publication #2 pointed out that only HDA is a suitable candidate for the detection of the P35S promoter in transgenic maize lines MON810, Bt11 and NK603. For the isothermal amplification techniques NEAR, SDA and RCA the reported issues of non-specific background amplification could not be eliminated, therefore it was not possible to differentiate between GM- and nonGM-samples, and these three techniques were excluded for the further development of an assay for the detection of the P35S promotor. Although LAMP was able to reliably detect the target without co-detecting nonGM-samples, it could not be further optimised and substantially lacked in sensitivity. The major handicap for further optimisation is the fact, that the sequence, used for calculating LAMP primer sequences, is too short. Although GM maize lines MON810, Bt11 and NK603 share the same sequences for the P35S promotor, the individual lengths of the sequences differ significantly. In order to detect all three maize lines, the shortest promoter sequence has to be used for assay design, which has around 230 basepairs. A range to optimise primer sets by up- or downstream shifts does not exist, therefore the possibilities for approveable primer design are limited. However, HDA was capable of detecting all three GM targets with a sensitivity of less than 0.9%, as instructed in the Guidelines for GMO testing [64]. The only drawback of HDA is the formation of non-specific byproducts that can easily be differentiated using agarose gel electrophoresis for detection. In contrast to LAMP, a visualisation of amplification products via staining with intercalating dyes is not possible, therefore the detection of HDA products requires slightly more time. However, it is suggested that this technique could probably be transferred to lateral flow devices in the future what further facilitates analysis.

In publication #3 a LAMP assay for the detection of horse meat in sausages was developed. Since all commonly used meat species share a major part of their common DNA, a DNA-based approach, that is able to distinguish different species based only on a few deviating basepairs, is recommended. For the here presented assay, a clear differentiation between horse meat, beef, pork and chicken, which are the most frequently used meat species in Europe, was possible. In an experiment to detect horse meat in prepared model

sausages, it could be shown that the assay was able to detect horse meat concentrations as low as 0.1% in cooked sausage matrices. The analysis of commercial horse meat products showed that the assay was able to reliably detect horse meat in all samples except for blood pudding, where the amount of extracted DNA was too low to expect positive amplification. However, the developed assay can specifically detect horse meat in concentrations of 0.1% in less than 30 minutes via simple visual detection with SYBR Green I and therefore it is a perfect candidate for the analysis of potentially horse meat-contaminated meat products.

All presented publications and/or assays aim to facilitate testing for the above mentioned targets celery, GM maize and horse meat and could significantly narrow down sample numbers that subsequently have to be tested with more laborious techniques such as conventional PCR. Furthermore, a DNA-based approach for all these targets can provide the specific detection of targets in matrices with closely related plant and animal species or very similar sequences. Especially isothermal amplification techniques are highly suitable to fulfil this purpose due to their simple workflow. Furthermore, LAMP provides high specificity due to the use of two or three primer sets, that all have to hybridise to the target DNA in order to successfully amplify the target. Since recurring denaturation steps during amplification are not necessary, reaction times can significantly be shortened and reactions can be performed on a heating block. Nevertheless, it has to be mentioned that not every isothermal amplification technique is appropriate for every target. Since each method has its benefits and drawbacks, the used method has to be carefully adapted to the requirements each target poses. LAMP is definitely a suitable method for many targets, but should not be used when options for primer design are limited due to the issue of very short sequences, as is the case for the P35S promoter in GM targets. Another handicap is that some isothermal techniques recommend an initial denaturation phase at 95°C for a few minutes that further complicates the assay.

In general, it can be stated that only LAMP and HDA can comply with the requirements for rapid on-site testing in food analysis. Although assay design tends to be complicated, the workflow for already developed assays is very simple and can be performed by untrained personnel without impractical instruments. HDA has already been applied to the detection via lateral flow devices which further facilitates the analysis. Although the available heating blocks can be transported to the site of analysis, the future development of instruments that are perfectly adapted for transport and might include electrochemical DNA sensors for visualisation will further facilitate the transfer of these amplification platforms to rapid and simple on-site detection systems. Both HDA and LAMP bring their benefits and drawbacks, and while HDA is much more suitable for the detection of GMOs due to the short sequences

available for assay design, a LAMP reaction is more robust because a helicase to unwind the double-helix is not required [128]. What further supports the turnround from laboratory-dependent analyses to on-site analyses is the development of rapid DNA extraction methods. Until recently, DNA extraction has always been the bottleneck in the sample-to-result process due to the fact, that most extraction protocols require several centrifugations steps. Apart from that, it mostly involves the use of several organic solvents and reagents. The centrifuge as well as the reagents are impractical or simply too heavy to be transported to the site of analysis. The development of rapid DNA extraction methods using ionic liquids adresses an important issue in rapid on-site analysis and will clear the way for future applications concerning the matter of food safety [129]. Once the technological know-how of DNA extracion, amplification technique and subsequent visualisation has been established, it can easily be adapted to new targets and analytes in less time.

Isothermal amplification techniques are on the rise, and while most applications have been developed in the field of clinical diagnostics, a gradual integration in food analysis can be observed. The simplicity of these methods together with the cost-efficient and time-saving properties make isothermal amplification a perfect future candidate for food analysis and eventually could be applied to a multitude of targets, that have to be tested categorically throughout Europe.

## References

[1] Merhoff CG, Porter JM (1974) Ergot Intoxication: Historical Review and Description of Unusual Clinical Manifestations. Ann Surg 180(5):773-779

[2] Maja P, Rasic D (2013) The Impact of Mycotoxicoses On Human History. Archives of Industrial Hygiene and Toxicology 63(4):513-518

[3] Holmberg L (2010) Wine fraud. International Journal of Wine Research 2:105-113

[4] Glynn JR, Palmer SR (1992) Incubation Period, Severity of Disease, and Infecting Dose: Evidence from a Salmonella Outbreak. Am J Epidemiol 136:1369-1377

[5] Sakaguchi G (1982) *Clostridium botulinum* toxins. Pharmacology & Therapeutics 19(2):165-194

[6] Kimberlin RH, Wilesmith JW (1994) Bovine Spongiform Encephalopathy. Slow Infections of the Central Nervous System: The Legacy of Dr Björn Sigurdsson 724:210-220

[7] Schlech WF 3rd, Lavigne PM, Bortolussi RA, Allen AC, Haldane EV, Wort AJ, Hightower AW, Johnson SE, King SH, Nicholls ES, Broome CV (1993) Epidemic listeriosis - evidence for transmission by food. The New England Journal of Medicine 308(4):203-206

[8] Fretz R, Sagel U, Ruppitsch W, Pietzka AT, Stöger A, Huhulescu S, Heuberger S, Pichler J, Much P, Pfaff G, Stark K, Prager R, Flieger A, Feenstra O, Allerberger F (2010) Listeriosis outbreak caused by acid curd cheese 'Quargel', Austria and Germany 2009. EuroSurveillance 15(5):1-2

[9] Wieler LH, Semmler T, Eichhorn I, Antao EM, Kinnemann B, Geue L, Karch H, Guenther S, Bethe A (2011) No evidence of the Shiga toxin-producing *E. coli* O104:H4 outbreak strain or enteroaggregative *E. coli* (EAEC) found in cattle faeces in northern Germany, the hotspot of the 2011 HUS outbreak area. Gut Pathogens 3:1-17

[10] Premanandh J (2013) Horse meat scandal - A wake-up call for regulatory authorities. Food Control 34(2):568-569

[11] Regulation (EC) 178/2002 of the European Parliament and of the Council (2002) Official Journal of the European Communities L31:1-24

[12] Regattieri A, Gamberi M, Manzini R (2007) Traceability of food products: General framework and experimental evidence. Journal of Food Engineering 81:347-356

[13] Directive 2000/13/EC of the European Parliament and of the Council (2000) Official Journal of the European Communities L109:29-42

[14] Directive 2003/89/EC of the European Parliament and of the Council (2003) Official Journal of the European Union L308:15-18

[15] Directive 2007/68/EC of the European Parliament and of the Council (2007) Official Journal of the European Union L310:11-14

[16] Regulation (EC) 1830/2003 of the European Parliament and of the Council (2003) Official Journal of the European Union L268:24-28

[17] Etesamifar M, Wüthrich B (1998) IgE-vermittelte Nahrungsmittelallergie bei 383 Patienten unter Berücksichtigung des oralen Allergie-Syndroms. Allergologie 21:451-457

[18] André F, André C, Colin L, Cacaraci F, Cavagna S (1994) Role of new allergens consumption in the increased incidence of food sensitization in France. Toxicology 92:77-83

[19] Ballmer-Weber BK, Vieths S, Lüttkopf D, Heuschmann P, Wüthrich B (2000) Celery allergy confirmed by double-blind, placebo-controlled food challenge: a clinical study in 32 subjects with a history of adverse reactions to celery root. J Allergy Clin Immunol 106:373-378

[20] Taylor SL (1987) Allergic and sensitivity reactions to food components. Nutritional Toxicology 2:173-197

[21] Sicherer SH (2001) Clinical implications of cross-reactive food allergens. Journal of Allergy and Clinical Immunology 108:881-890

[22] Poms RE, Klein CL, Anklam E (2004) Methods for allergen analysis in food: a review. Food additives and contaminants 21(1):1-31

[23] International Service for the Acquisition of Agri-biotech Applications (ISAAA) (2004) US to strengthen biotech regulation for GMOs. CropBiotech Update, 4 Feb 2004. http://www.isaaa.org/kc/CBTNews/2004_Issues/Feb/CBT_Feb_6.htm - us. Accessed 30 April 2008

[24] Marmiroli N, Maestri E, Gulli M, Malcevschi A, Peano C, Bordoni R, De Bellis G (2008) Methods for detection of GMOs in food and feed. Analytical and bioanalytical chemistry 392(3):369-384

[25] Council Regulation (EC) No. 1139/98 (1998) Official Journal of the European Communities L159:4-7

[26] Commission Regulation (EC) No 49/2000 (2000) Official Journal of the European Communities L6:13-14

[27] Lüthy J (1999) Detection strategies for food authenticity and genetically modified foods. Food Control 10:359-361

[28] Kramer MG RK (1994) Commercialization of a tomato with an antisense polygalacturonase gene: The FLAVR SAVR™ tomato story. Euphytica 79(3):293-297

[29] Njiti VN, Myers O, Schroeder D, Lightfoot DA (2003) Roundup Ready Soybean. Agronomy Journal 95(5):1140-1145

[30] Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merlin E, Rhodes R, Warren GW, Wright M, Evola SV (1993) Field Performance of Elite Transgenic Maize Plants Expressing an Insecitcidal Protein Derived from Bacillus thuringiensis. Nature Biotechnology 11:194-200

[31] Joint Research Center European Commission (2008) Book of abstracts, 1st global conference on GMO analysis, Villa Erba, Como, Italy, 24–27 June 2008

[32] European Commission Recommendation 2013/99/EU (2013) Official Journal of the European Union 48:1

[33] Ballin NZ (2010) Authentication of meat and meat products. Meat Science 86:577-587

[34] Reid LM, O'Donnell CP, Downey G (2006) Recent technological advances for the determination of food authenticity. Trends in Food Science & Technology 17:344-353

[35] Schwägele F (2005) Traceability from a European perspective. Meat Science 71:164-173

[36] Risticevic S, Carasek E, Pawliszyn J (2008) Headspace solid-phase microextraction–gas chromatographic–time-of-flight mass spectrometric methodology for geographical origin verification of coffee. Anal Chim Acta 617(1-2):72-84

[37] Esteban-Díez I, González-Sáiz JM, Pizarro C (2004) An evaluation of orthogonal signal correction methods for the characterisation of *arabica* and *robusta* coffee varieties by NIRS. Anal Chim Acta 514(1):57-67

[38] Ganopoulos I, Argiriou A, Tsaftaris A (2011) Adulterations in Basmati rice detected quantitatively by combined use of microsatellite and fragrance typing with High Resolution Melting (HRM) analysis. Food Chemistry 129(2):652–659

[39] Lopez S (2008) TaqMan based real time PCR method for quantitative detection of Basmati rice adulteration with non-basmati rice. European Food Res Technol 227:619-622

[40] Rapid Methods 2014 Conference Noordweijkerhout, Niederlande

[41] European Commission Regulation No 37/2010 (2010) Official Journal of the European Union 15:1

[42] Müller A, Steinhart H, (2007) Recent developments in instrumental analysis for food quality. Food Chemistry 101:1136-1144

[43] V E (2010) Chicken fillets 'secretly pumped up with water' to increase weight. http://www.timesonline.co.uk/tol/life_and_style/food_and_drink/article1980529.ece.

[44] Ettre LS (1993) Nomenclature for chromatography (IUPAC Recommendation 1993). Pure Appl Chem 65:819-872

[45] Guillarme D, Veuthey JL (2013) State-of-the-art of (UHP)LC-MS(-MS) techniques and their practical application J Chromatogr A 1292:1

[46] Ceraci L, Rodriguez-Estrada MT, Lercker G (2003) Solid-phase extraction thin-layer chromatography-gas-chromatography method for the detection of hazelnut oil in olive oils by determination of esterified sterols. Journal of Chromatography A 985:211-220

[47] Ollivier D, Artaud J, Pinatel C, Durbec JP, Guerere M (2003) Triacylglycerol and fatty acid compositions of French virgin olive oils. Characterization by chemometrics. Journal of Agricultural and Food Chemistry 51:5723-5731

[48] Alves MR, Casal S, Oliveira MBPP, Ferreira MA (2003) Contribution of FA profile obtained by high-resolution GC/chemometric techniques to the authenticity of green and roasted coffee varieties. Journal of the American Oil Chemists Society 80:511–517

[49] Del Castillo MLR, Caja MM, Blanch GP, Herraiz M (2003) Enantiomeric distribution of chiral compounds in orange juices according to their geographical origins. Journal of Food Protection 66:1448–1454

[50] Reid LM, O'Donnell CP, Downey G (2004) Potential of SPME-GC and chemometrics to detect adulteration of soft fruit purees. Journal of Agricultural and Food Chemistry 52:421–427

[51] Reid LM, O'Donnell CP, Kelly J, Daniel J, Downey G (2004) Preliminary studies for the differentiation of apple juice samples on the basis of heat-treatment using chemometric analysis of chromatographic data. Journal of Agricultural and Food Chemistry 52:6891–6896

[52] Ferreira I, Cacote H (2003) Detection and quantification of bovine, ovine and caprine milk percentages in protected denomination of origin cheeses by reversed-phase high-performance liquid chromatography of beta-lactoglobulins. Journal of Chromatography A 1015:111–118

[53] Rodriguez-Delgado MA, Gonzalez-Hernandez G, Conde-Gonzalez J E, Perez-Trujillo JP (2002) Principal component analysis of the polyphenol content in young red wines. Food Chemistry 78:523–532

[54] Bonetti A, Marotti I, Catizone P, Dinelli G, Maietti A, Tedeschi P, et al. (2004) Compared use of HPLC and FZCE for cluster analysis of *Triticum* spp. and for the identification of *T. durum* adulteration. Journal of Agricultural and Food Chemistry 52:4080–4089

[55] Murray KK, Boyd RK, Eberlin MN, Langley GJ, Li GJ, Naito Y (2013) Definitions of terms relating to mass spectrometry (IUPAC Recommendation). Pure Appl Chem 85:1515-1609

[56] Dabrowski WM, Sikorski ZE (2004) Toxins in food. Cambridge: Woodhead Publishing

[57] Meno K, Thorsted PB, Ipsen H, Kristensen O, Larsen J, Spangfort M (2005) The crystal structure of recombinant proDer p 1, a major house dust mite proteolytic allergen. Journal of Immunology 175(6):3835-3845

[58] Pastorello EA, Varin E, Farioli L, Pravettoni V, Ortolani C, Trambaioli C (2001) The major allergen of sesame seeds (*Sesamum indicum*) is a 2S albumin. Journal of Chromatography, B: Biomedical Sciences and Applications 756(1-2):85-93

[59] Ziegler H, Osmond CB, Stichler W, Trimborn P (1976) Hydrogen isotope discrimination in higher plants: correlations with photosynthetic pathway and environment. Planta 128:85-92

[60] Anderson KA, Magnuson BA, Tschirgi ML, Smith B (1999) Determining the geographic origin of potatoes with trace element analysis using statistical and neural network classifiers. Journal of Agricultural Food Chemistry 47:1568-1574

[61] Kosir IJ, Kocjancic M, Ogrinc N, Kidric J (2001) Use of SNIF-NMR and IRMS in combination with chemometric methods for the determination of chaptalisation and geographical origin of wines (the example of Slovenian wines). Analytica Chimica Acta 429:195-206

[62] Antolovich M, Li X, Robards K (2001) Detection of adulteration in Australian orange juices by stable carbon isotope ratio analysis (SCIRA). Journal of Agricultural and Food Chemistry 49:2623-2626

[63] Brescia MA, Caldarola V, Buccolieri G, Dell'Atti A, Sacco A (2003) Chemometric determination of the geographical origin of cow milk using ICP-OES data and isotopic ratios: A preliminary study. Italian Journal of Food Science 15:329-336

[64] Manca G, Camin F, Coloru GC, Del Caro A, Depentori D, Franco MA (2001) Characterization of the geographical origin of pecorino sardo cheese by casein stable isotope (C-13/C-12 and N-15/N-14) ratios and free amino acid ratios. Journal of Agricultural and Food Chemistry 49:1404-1409

[65] McMullin D, Mizaikoff B, Krska R (2014) Advancements in IR spectroscopic approaches for the determination of fungal derived contaminants on food crops. Analytical and bioanalytical chemistry [Open Access]

[66] Contal L, Leon V, Downey G (2002) Detection and quantification of apple adulteration in strawberry and raspberry purees using visible and near infrared spectroscopy. Journal of near Infrared Spectroscopy 10:289-299

[67] Rodriguez-Saona LE, Fry FS, McLaughlin MA, Calvey EM (2001) Rapid analysis of sugars in fruit juices by FT-NIR spectroscopy. Carbohydrate Research 336:63-74

[68] Paradkar MM, Sakhamuri S, Irudayaraj J (2002) Comparison of FTIR, FT-Raman, and NIR spectroscopy in a maple syrup adulteration study. Journal of Food Science 67:2009-2015

[69] Downey G, Fouratier V, Kelly JD (2004) Detection of honey adulteration by addition of fructose and glucose using near- infrared spectroscopy. Journal of Near Infrared Spectroscopy 11:447-456

[70] Laasonen M, Harmia-Pulkkinen T, Simard CL, Michiels E, Rasanen M, Vuorela H (2002) Fast identification of Echinacea purpurea dried roots using near-infrared spectroscopy. Analytical Chemistry 74:2493-2499

[71] Maraboli A, Cattaneo TMP, Giangiacomo R (2002) Detection of vegetable proteins from soy, pea and wheat isolates in milk powder by near infrared spectroscopy. Journal of Near Infrared Spectroscopy 10:63-69

[72] Murray I, Aucott AS, Pike I (2001) Use of discriminant analysis on visible and near infrared reflectance spectra to detect adulteration of fishmeal with meat and bone meal. Journal of Near Infrared Spectroscopy 9:297-311

[73] Sharma SK, Eblen BS, Bull RL, Burr DH, Whiting RC (2005). Applied and Environmental Microbiology 71(7):3935-3941

[74] Ricci F, Volpe G, Micheli L, Palleschi G (2007) A review on novel developments and applications of immunosensors in food analysis. Anal Chim Acta 605(2):111-129

[75] Jha VK, Kumar A, Mandokhot UV (2003) Indirect enzyme-linked immunosorbent assay in detection and differentiation of cooked and raw pork from meats of other species. Journal of Food Science and Technology-Mysore 40:254-256

[76] Taylor WJ, Jones JL (1992) An immunoassay for verifying the identity of canned sardines. Food and Agricultural Immunology 4:169-175

[77] Céspedes A, García T, Carrera E, González I, Fernández A, Asensio L (1999) Indirect ELISA for the identification of sole (*Solea solea*), European plaice (*Pleuronectes platessa*), flounder (*Platichthys flesus*), and Greenland halibut (*Reinhardtius hippoglossoides*). Journal of Food Protection 62:1178-1182

[78] Fernández A, García T, Asensio L, Rodríguez MA, González I, Lobo E (2002) Identification of the clam species *Ruditapes decussatus* (grooved carpet shell), *Venerupis romboides* (yellow carpet shell) and *Venerupis pullastra* (pullet carpet shell) by ELISA. Food and Agricultural Immunology 14:65-71

[79] Hurley IP, Coleman RC, Ireland HE, Williams JHH (2004) Measurement of bovine IgG by indirect competitive ELISA as a means of detecting milk adulteration. Journal of Dairy Science 87:215-221

[80] Sass-Kiss A, Sass M (2000) Immunoanalytical method for quality control of orange juice products. Journal of Agricultural and Food Chemistry 48:4027-4031

[81] Asensio L, González I, García T, Martín R (2008) Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). Food Control 19:1-8

[82] Stephan O, Weisz N, Vieths S (2004) Protein Quantification, Sandwich ELISA, and Real-Time PCR Used to Monitor Industrial Cleaning Procedures for Contamination with Peanut and Celery Allergens. Journal of AOAC International 87(6):1448-1457

[83] Lockley AK, Bardsley RG (2000) DNA-based methods for food authentication. Trends in Food Science & Technology 11:67-77

[84] Wang J (2000) From DNA biosensors to gene chips. Nucleic acids research 28(16):3011-3016

[85] Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalysed chain reaction. Methods Enzymol 155:335-350

[86] Schochetmann G, Ou CY, Jones WK (1988) Polymerase Chain Reaction. The Journal of Infectious Diseases 158(6):1154-1157

[87] Wilhelm J, Pingoud A (2003) Real-Time Polymerase Chain Reaction. ChemBioChem 4(11):1120-1128

[88] European Network of GMO Laboratories (2008) Definition of minimum performance requirements for analytical methods of GMO testing. Community Reference Laboratory for GM Food and Feed, Ispra

[89] Vignal A, Milan D, SanCristobal M, Eggen A (2002) A review on SNP and other types of molecular markers and their use in animal genetics. Genet Sel Evol 34:275-305

[90] Calvo JH, Osta R, Zaragoza P (2002) Quantitative PCR detection of pork in raw and heated ground beef and pate. Journal of Agricultural and Food Chemistry 50:5265-5267

[91] Comesana AS, Abella P, Sanjuan A (2003) Molecular identification of five commercial flatfish species by PCR-RFLP analysis of a 12S rRNA gene fragment. Journal of the Science of Food and Agriculture 83:752-759

[92] Kim DM, Chung SH, Chun HS (2011) Multiplex PCR assay for the detection of aflatoxigenic and non-aflatoxigenic fungi in meju, a Korean fermented soybean food starter. Food Microbiology 28(7):1402-1408

[93] Lukue MI, Rodríguez A, Andrade MJ, Gordillo R, Rodríguez M, Córdoba JJ (2011) Development of a PCR protocol to detect patulin producing moulds in food products. Food Control 22(12):1831-1838

[94] Trnčíková T, Piskernik, S, Kaclikova E, Smole-Možina S, Kuchta T, Jeršek B (2010) Characterization of Staphylococcus aureus strains isolated from food produced in Slovakia and Slovenia with regard to the presence of genes encoding for enterotoxins. Journal of Food and Nutrition Research 49(4):215-220

[95] Wehrle E, Didier A, Moravek M, Dietrich R, Märtlbauer E (2010) Detection of *Bacillus cereus* with enteropathogenic potential by multiplex real-time PCR based on SYBR green I. Molecular and Cellular Probes 24(3):124-130

[96] Arlorio M, Cereti E, Coïsson JD, Travaglia F, Martelli A (2007) Detection of hazelnut (Corylus spp.) in processed foods using real-time PCR. Food Control 18(2):140-148

[97] Hupfer C, Waiblinger HU, Busch U (2007) Development and validation of a real-time PCR detection method for celery in food. Eur Food Res Technol 225:329-335

[98] Galan AMG, Brohée M, de Andrade Silva E, van Hengel AJ, Chassaigne H (2011) Development of a real-time PCR method for the simultaneous detection of soya and lupin mitochondrial DNA as markers for the presence of allergens in processed food. Food Chemistry 127(2):834-841

[99] De S, Brahma B, Polley S, Mukherjee A, Banerjee D, Gohaina M, Singh KP, Singh R, Datta RK, Goswami SL (2011) Simplex and duplex PCR assays for species specific identification of cattle and buffalo milk and cheese. Food Control 22(5):690-696

[100] Zhang CL, Fowler MR, Scott NW, Lawson G, Slater A (2007) A TaqMan real-time PCR system for the identification and quantification of bovine DNA in meats, milks and cheeses. Food Control 18(9):1149-1158

[101] Gil LA (2007) PCR-based methods for fish and fishery products authentication. Trends in Food Science & Technology 18(11):558-566

[102] Blanco L, Bernad A, Lázaro JM, Martín G, Garmendia C, Salas M (1989) Highly efficient DNA synthesis by the phage phi 29 polymerase. Symmetrical mode of DNA replication. The Journal of Biological Chemistry 264:8935-8940

[103] Aliotta JM, Pelletier JJ, Ware JL, Moran LS, Brenner JS, Kong H (1996) Thermostable *Bst* DNA polymerase I lacks a 3′ →5′ proof-reading exonuclease activity. Genet Anal Biomol Eng 12(5-6):185– 195

[104] National Center for Biotechnology Information (NCBI) PubMed database http://www.ncbi.nlm.nih.gov/pubmed/

[105] Eiken Chemical Co. Ltd. http://loopamp.eiken.co.jp/e/

[106] Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic acids research 28(12):E63

[107] Nagamine K, Hase T, Notomi T (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 16:223–229

[108] Iwamoto T, Sonobe T, Hayashi K (2003) Loop-mediated isothermal amplification for direct detection *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J Clin Microbiol 41(6):2616–2622

[109] Han F, Ge B (2010) Quantitative detection of *Vibrio vulnificus* in raw oysters by real-time loop-mediated isothermal amplification. Int J Food Microbiol 142:60-66

[110] Aoi Y, Hosogai M, Tsuneda S (2006) Real-time quantitative LAMP (loop-mediated isothermal amplification of DNA) as a simple method for monitoring ammonia-oxidizing bacteria. J Biotechnol 125:484–491

[111] Mori Y, Kitao M, Tomita M, Notomi T Real-time turbidimetry of LAMP reaction for quantifying template DNA. J Biochem Biophys Methods 59:145–157

[112] Fire A, Xu S (1995) Rolling circle replication of short DNA circles. Proc Natl Acad Sci USA 92(10):4641–4645

[113] Lizardi P, Huang X, Zhu Z, Bray-Ward P, Thomas D, Ward D (1998) Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat Genet 19:225–232

[114] Pang S, Qureshi F, Shanahan D, Harris N (2007) Investigation of the use of rolling circle amplification for the detection of GM food. Eur Food Res Technol 225:59–66

[115] Hadersdorfer J (2012) Development of an isothermal nucleic acid amplification protocol for high-throughput monitoring of Plum pox virus infection in stone fruit production. Doctoral thesis, Technische Universität München, Munich

[116] Vincent M, Xu Y, Kong H (2004) Helicase-dependent isothermal DNA amplification. MBO Rep 5(8):795–800

[117] Kim J, Easley CJ Isothermal DNA amplification in bioanalysis: strategies and applications. Bioanalysis 3(2):227– 239

[118] Walker GT, Little MC, Nadeau JG, Shank DD (1992) Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. Proc Natl Acad Sci USA 89:392–396

[119] Walker GT. FM, Schram JL, Little MC, Nadeau JG, Malinowski DP (1992) Strand displacement amplification - an isothermal, in vitro DNA amplification technique. Nucleic acids research 20:1691–1696

[120] Spargo CA, Fraiser MS, Van Cleve M, Wrigth DJ, Nycz CM, Spears PA, Walker GT (1996) Detection of *M. tuberculosis* DNA using thermophilic strand displacement amplification. Mol Cell Probes 10:247–256

[121] Ehses S, Ackermann J, McCaskill JS (2005) Optimization and design of oligonucleotide setup for strand displacement amplification. J Biochem Biophys Methods 63:170–186

[122] Joneja A, Huang X (2011) Linear nicking endonuclease-mediated strand-displacement DNA amplification. Anal Biochem 414:58–69

[123] Dovicovicová L, Olexová L, Pangallo D, Siekel P, Kuchta T (2004) Polymerase chain reaction (PCR) for the detection of celery (*Apium graveolens*) in food. Eur Food Res Technol 218:493-495

[124] Waiblinger H-U, Ernst B, Anderson A, Pietsch K (2007) Validation and collaborative study of a P35S and T-nos duplex real-time PCR screening method to detect genetically modified organisms in food products. European Food Research and Technology 226(5):1221-1228

[125] Scholtens I, Laurensse E, Molenaar B, Zaaijer S, Gaballo H, Boleij P, Bak A, Kok E (2013) Practical experiences with an extended screen- ing strategy for genetically modified organisms (GMOs) in real-life samples. J Agric Food Chem 61:9097–9109

[126] Köppel R, Zimmerli F, Breitenmoser A (2009) Heptaplex real-time PCR for the identification and quantification of DNA from beef, pork, chicken, turkey, horse meat, sheep (mutton) and goat. Eur Food Res Technol 230(1):125-133

[127] Ahmed MU, Hasan Q, Hossain MM, Saito M, Tamiya E (2010) Meat species identification based on the loop mediated isothermal amplification and electrochemical DNA sensor. Food Control 21(5):599-605

[128] Kaneko H, Kawana T, Fukushima E, Suzutani T (2007) Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J Biochem Biophys Methods 70:499-501

[129] Gonzalez Garia E, Ressman AK, Gaertner P, Zirbs R, Mach RL, Krska R, Bica K, Brunner K (2014) Direct extraction of genomic DNA from maize with aqueous ionic liquid buffer systems for applications in GMO analysis. Anal Bioanal Chem [Epub ahead of print]

# Curriculum Vitae

## Celine Zahradnik

### Personal Data

Date and place of birth: September 6th 1984 in La Seyne sur Mer/France

Nationality: Austrian

### Education

| | |
|---|---|
| May 2011 - recent | Doctoral degree study at the Center for Analytical Chemistry of the Department for Agriobiotechnology (IFA Tulln) of the Vienna University of Technology (TU Wien) |
| March 2014 - July 2014 | Lecturer at FH Campus Wien, lecture “Wissenschaftliche Grundlagen der Ökologie” |
| November 2010 - March 2011 | Research assistant at the Department of Botany and Biodiversity Research, University Vienna; FFG project “Cultivation, identification & bioactivity of fungal endophytes in *Vitis vinifera* for pest control” |
| October 2010 | Degree with distinction at the Department of Botany and Biodiversity Research, University Vienna (academic degree: Mag. rer. nat.) |
| June 2008 - October 2010 | Research assistant at the Department of Botany and Biodiversity Research, University Vienna; FFG project “Cultivation, identification & bioactivity of fungal endophytes in Zingiberaceae” |
| May 2007 - February 2008 | Research assistant at the Department of Botany and Biodiversity Research, University Vienna; FWF project “DNA-Sequenzierung von Großpilzen in Forst- und naturnahen Wäldern” |
| October 2002 | Diploma degree study Biology at Vienna University, Faculty of Life Sciences |
| September 1994 - June 2002 | Grammar School BG and BRG Hollabrunn |