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D I P L O M A R B E I T

Optimierung und Evaluierung eines Loop-mediated Isothermal Amplification Assays zum Nachweis des Lebensmittelallergens Sellerie

Ausgeführt am

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Abstract

Food allergies are widely distributed and pose a major health risk to affected people, which is why the European Union introduced labelling directives for packaged and unpackaged foods with regard to allergenic ingredients. There are already many immuno-based and DNA-based assays for the detection of the most important food allergens available, but in order to provide a rapid and on-site applicable method, an existing loop-mediated isothermal amplification (LAMP) assay for the detection of Apium graveolens in foods and products thereof was optimized and evaluated. The assay was shown to be highly specific for celery, and the limit of detection (LOD) was found to be as low as 7.8 mg celery powder per kilogram spiked food sample. The analysis of ten commercially available food products and a determination of the false-negative rate for the DNA extracted from three food matrices spiked with celery DNA allowed a comparison of the LAMP assay to the selected reference real-time PCR assay. The LAMP reaction was also performed on different heating blocks to demonstrate a reliable detection of the analyte independent from the instrument used. Furthermore, the amplification products were visualized via agarose gel electrophoresis and fluorescence staining in order to compare the results to those obtained from real-time fluorescence monitoring. These outcomes show that the LAMP assay for the detection of celery is highly specific, easy-to-perform and on-site applicable, and in combination with a simple visualization technique, it has the potential to become a promising tool for commercial uses.

Zusammenfassung

Lebensmittelallergien sind weitverbreitet und stellen für die Betroffenen ein großes Gesundheitsrisiko dar. Aus diesem Grund hat die Europäische Union Gesetze zur Kennzeichnungspflicht allergener Inhaltsstoffe erlassen, die sowohl verpackte als auch unverpackte Lebensmittel betreffen. Es sind bereits zahlreiche auf Antikörper bzw. auf DNA basierende Assays zum Nachweis der wichtigsten Lebensmittelallergene erhältlich, aber um auf eine schnelle und vor Ort durchführbare Methode zurückgreifen zu können, wurde ein bereits entwickelter Loop-mediated Isothermal Amplification (LAMP) Assay zum Nachweis von *Apium graveolens* in Lebensmitteln und prozessierten Produkten optimiert und evaluiert. Dieser Assay stellte sich als hochspezifisch für Sellerie heraus - mit einem Detektionslimit von 7,8 mg Selleriepulver pro Kilogramm gespiketer Lebensmittelprobe. Die Analyse von zehn kommerziell erhältlichen Lebensmittelprodukten und die Bestimmung einer Falsch-Negativ-Rate für extrahierte DNA aus drei verschiedenen Lebensmittelmatrizes, die mit DNA aus Sellerie gespiket wurde, erlaubten einen Vergleich des LAMP-Assays mit dem ausgewählten Referenz-PCR-Assay. Die LAMP-Reaktion wurde auch auf Heizblöcken durchgeführt, um den verlässlichen Nachweis des Analyten unabhängig vom verwendeten Gerät zu demonstrieren. Weiters wurden die Amplifikationsprodukte mittels Agarose-Gelelektrophorese und Fluoreszenzfärbung visualisiert, um die Ergebnisse mit jenen zu vergleichen, die durch das Echtzeit-Fluoreszenz-Monitoring produziert wurden. Diese Resultate zeigen, dass der LAMP-Assay zum Nachweis von Sellerie hochspezifisch sowie einfach und vor Ort durchführbar ist und dass er in Kombination mit einer einfachen Visualisierungsmethode das Potential hat, zu einem vielversprechenden Analyseinstrument für kommerzielle Anwendungen zu werden.

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1 Introduction

This work addresses the optimization and evaluation of a DNA-based rapid detection method for the food allergen *Apium graveolens*, commonly known as celery. The following introductory pages will discuss food allergy with a special emphasis on celery as well as the corresponding legal regulations for food labelling in the European Union. There is also an overview on approved analytical methods and a detailled description of the applied method, the loop-mediated isothermal amplification (LAMP) technique.

1.1 Food Allergy

Food allergy is defined as an adverse immune response to foods that poses a major health risk to all age groups. It is estimated that about 4% of the global population are affected, though the situation is even more serious in infants and children with prevalence levels up to 8% [1]. Besides the age, nutritional habits, location, sociocultural background, and also the season are factors for the varying frequency and diversity of food allergies [2, 3].

Most allergenic foods may cause two different types of abnormal immune responses: immediate hypersensitivity and delayed hypersensitivity reactions [4]. Immediate hypersensitivity is associated with the production of immunoglobulin E (IgE) antibodies, called sensitization, which is evoked by the consumption of allergen-containing foods. The symptoms are then elicited by re-exposure to those food proteins that are able to bind to their specific IgE antibodies and trigger the release of mediators, e.g. histamine [3], which in most cases occurs within minutes to hours of consuming a food [2]. Contrarily, delayed hypersensitivity involves T-cell-mediated immune reactions, and the symptoms mostly occur not until 24 hours or more following consumption of the offending food. However, the role of delayed hypersensitivity in food allergic reactions remains poorly understood [4].

Generally, adverse reactions to foods may involve multiple target organs [2], resulting in acute and chronic disease which can be severe and potentially fatal. Typical food-elicited disorders are asthma, rhinitis, atopic dermatitis, enterocolitis, proctitis, or temporary itching. The severest case of a reaction to food allergens is a potentially life-threatening clinical syndrome called *anaphylaxis*. Food allergies hereof account for one third to a quarter of cases seen [5].
Severe allergic reactions are usually treated by prompt responses, such as the use of epinephrine in case of an anaphylactic shock, but still, the most effective treatment is to prevent the consumption of those foods and food products containing allergens known to elicit a reaction.

1.1.1 Common Allergenic Foods in Europe

The most recent study from Nwaru et al., 2014, which is part of the *EAACI Guideline for Food Allergy and Anaphylaxis*[a], summarizes several previous studies with regard to the occurrence of food allergies, and presents the prevalence of the 8 most common ones in Europe, which are also called "The Big Eight", namely **egg**, **cow's milk**, **fish**, **wheat**, **shellfish**, **tree nuts**, **peanuts**, and **soy** (Figure 1.1.1[b]). Although celery does not belong to this group, it still poses a major threat to affected people and therefore it is inevitable to have it declared amongst the ingredients (see *1.3. The EU Labelling Directive for Allergenic Foods*).

Figure 1.1.1: The 8 most common allergenic foods in Europe, also termed "The Big Eight": egg, cow's milk, fish, wheat, shellfish, tree nuts, peanuts, and soy

There is a big discrepancy between the self-reported allergies and all assessment methods of those studies taken into account. Together with the heterogeneity between the studies themselves, it is impossible to give a clear statement about the distribution or a breakdown of the percentages of affected people. In their conclusion, they report up to 15-fold differences between self-reported and challenge-verified prevalences of food allergy. In other cases the discrepancy may partly be due to non-IgE-mediated food allergies, which was not acquired [6]. Thus, the analysis can only provide summaries with ranges and pooled results.

1.1.2 The Allergenic Food Celery

Celery (*Apium graveolens*) belongs to the *Apiaceae* family and is cultivated as a vegetable in North America and Europe. The most commonly available variety in Europe is celeriac (*Apium graveolens* var. *rapaceum*) from which mainly the root is taken for culinary uses. The other major varieties are

[a] http://www.eaaci.org/resources/food-allergy-and-anapyhlaxis-guidelines.html

[b] Figure taken from: https://dcheal.files.wordpress.com/2012/10/big-8-allergens-for-allertrain-on-lone-allergy-training-for-restaurants-275x300.jpeg *(modified)*

pascal celery (*Apium graveolens* var. *dulce*), from which the stalks are used, and cutting or Chinese celery (*Apium graveolens* var. *secalinum*), from which the leaves are used, but in some countries celery is also grown for its seeds (Figure 1.1.2[a]) [7].

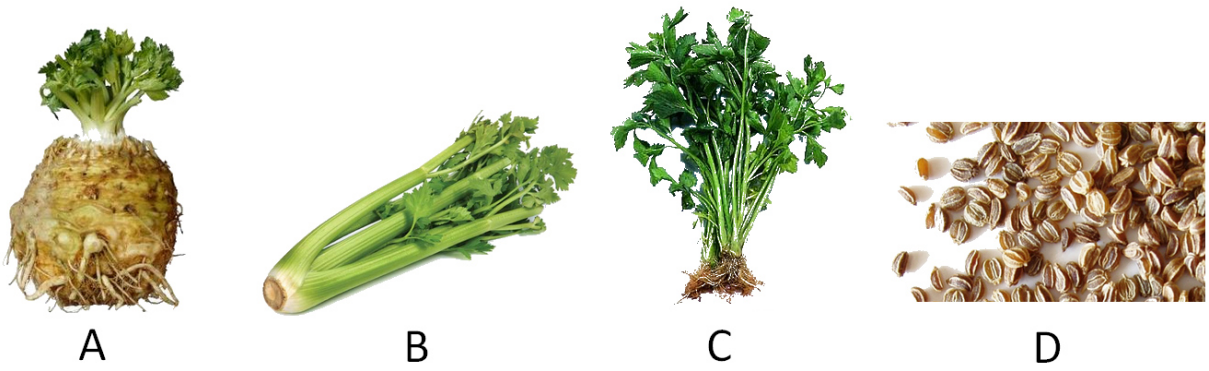

Figure 1.1.2: The most common varieties or used parts of celery: (A) *Apium graveolens* var. *rapaceum* (celeriac); (B) *Apium graveolens* var. *dulce* (pascal celery); (C) *Apium graveolens* var. *secalinum* (cutting or Chinese celery); (D) celery seeds

The different parts of all those sorts of celery are not only consumed in salads and as cooked vegetables in different cuisines, but especially dried celery powder is widely used in the food industry as a cheap ingredient for flavoring purposes, e.g. in seasonings, herbal mixtures, sauces, bouillons, pastries, or instant meals [8].

The Allergenic Potential of Celery

Celery allergy is one of the most frequent pollen-related food allergies in parts of Europe (e.g. Switzerland, France, or Germany) [9]. In 1985, Pauli et al. studied 20 persons known to suffer from this very allergy, and reported specific symptoms such as attacks of urticaria and angio oedema respiratory complaints, and even systemic anaphylaxis with vascular collapse [10]. The rare but severe case of an anaphylactic reaction to celery was also reported in several other publications [11–14].
In 2000, the allergy could be confirmed for the first time by double-blind placebo-controlled food challenges (DBPCFC) as well as by the identification of the allergens reacting with IgE antibodies from affected subjects. In course of that clinical study, 32 patients were tested and the symptoms and the according reaction-triggering doses of raw celery tuber were documented. It has been shown that 22 of the 32 individuals responded to the celery ingestion with itches of the palate, lips, throat, tongue, and ears, as well as with nausea, flatulence or abdominal cramps. The lowest dose of raw celery to elicit a reaction was hereby found to be 0.7 g [15], and as for celery spice, the threshold was 0.16 g [16].

[a] Figures taken from: http://www.wiveswithknives.net/wp-content/uploads/2011/10/celeriac-root-with-tops-intact.jpg (A), https://becomehealthyorextinct.files.wordpress.com/2012/05/celery.jpg (B), http://database.prota.org/PROTAhtml/Photfile%20Images%5CApium%20graveolens%20pp402.jpg (C) and http://www.myhealthyeatingfacts.com/wp-content/uploads/2014/10/Celery-seeds.jpg (D)

Allergens in Celery

The term *allergen* refers to a protein that may elicit a food-allergic reaction. In celery, there are 5 known (groups of) allergens:

- **Api g 1**[a] is a protein consisting of 154 amino acids that is considered to be the major allergen from celery [17–19]. An NCBI protein BLAST alignment[b] revealed 62% structure similarity and 42% structure identity to the major birch pollen allergen Bet v 1[c], both belonging to pathogenesis-related proteins in plants. Certain members of this class can induce allergen-specific IgE-synthesis, and therefore it can be speculated that celery allergy may arise due to prior sensitization by inhalation of cross-reacting pollen allergens [17]. This hypothesis fits the findings by Ebner et al., 1995 [20] where they observed allergic reactions elicited by Bet v 1 homologous proteins in fruits and vegetables in areas where birch trees grow. This could also explain why celery allergy is prevalent in Central Europe where birch and mugwort are common [18].

- **Non-specific lipid transfer proteins (nsLTPs)** belong to the superfamily of prolamins and are represented in celery by the **Api g 2** allergen that was characterized in 2011 [21]. Its binding to IgE antibodies is not influenced by thermal denaturing, and it is highly resistant to simulated gastrointestinal digestion. Cross-reactions occured with nsLTPs from peach and mugwort pollen.

- **Api g 3** is chlorophyll a/b binding protein of which only the mRNA sequence is known[d]. However, the characterization of the protein was not published (submission in 1996 by Hoffmann-Sommergruber et al.), but there is nothing known about allergy-eliciting doses or potential cross-reactions [22].

- **Profilins** are a family of cross-reactive allergens in grass and tree pollen, vegetables, and also in fruits. These 12-15 kDa proteins are widely distributed in the plant kingdom, which is why they are often regarded as pan-allergens [23]. In celery, the heat-stable **Api g 4** profilin[e] is considered a minor allergen that is cross-reacting with the birch pollen profilin Bet v 2 [24].

- **Cross-reactive carbohydrate determinants (CCDs)** are complex N-glycans produced in every seed-bearing plant. These oligosaccharides are binding to IgE and can be found in different amounts in pollen as well as in vegetables and fruits, which is why they also hold food allergy relevance [25]. In 2003, Bublin et al. purified the high molecular weight (HMW)

[a] RCSB Protein Data Bank ID: P49372

[b] https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=blasttab

[c] RCSB Protein Data Bank ID: P15494

[d] GenBank accession number: Z75663

[e] RCSB Protein Data Bank ID: Q9XF37

glycoprotein **Api g 5**[a] from celery and described its cross-reactive N-glycans [26]. The N-glycans carried by Api g 5 contain α1,3-fucosyl and β1,2-xylosyl residues which make it capable of binding and activating basophils, and it has been shown by the authors that deglycosylation of Api g 5 disrupts its IgE binding epitopes. The protein is presented as the first HMW glycoprotein allergen that contributes to the birch-mugwort-celery-spice syndrome.

Effects of Industrial Processing on the Celery Allergens

Celery does not only undergo thermal processing when used for commercial food products, but apart from the conventional heating of ready-to-serve meals and the electromagnetic radiation in microwaves there are also modern technologies for the preservation of shelf-stable products or for higher yields in juice production, e.g. high voltage impulse treatment, ultra high pressure, γ-irradiation, drying, or pickling [27]. The results of the *in vitro* study by Jankiewicz et al. [8] indicate that thermal processing of celery only clearly affected the antibody-binding properties of the allergen Api g 1, but left other groups of allergens only weakly affected. All non-thermal technologies showed a slight or no effect on any group of celery allergens.

The first *in vivo* study focussing on the allergenicity of cooked celery by DBPCFC was carried out by Ballmer-Weber et al. in 2002 [28] and could confirm the results of the *in vitro* experiments by showing that even extensive heat treatment of celery ($>$75 min at 100°C) could not abrogate its potential to elicit allergic reactions in four out of five patients.

1.1.3 The EU Labelling Directive for Allergenic Foods

Labelling Directive 2000/13/EC from 20 March 2000 is an EU legislation on labelling, presentation and advertising of foods which *"requires manufacturers to declare all ingredients present in pre-packaged foods sold in the EU with very few exceptions"*[b], where 2 important amendments regarding allergens have been made:

1. **Directive 2003/89/EC** came into force on 25 November 2005 and introduced Annex IIIa, where all those allergenic foods are listed that must always be labelled when present in pre-packaged food products. However, there are no threshold levels for allergens in foods or food products determined, and the analytical requirements with regard to immunological or DNA-based methods are not defined.

2. **Directive 2007/68/EC** from 27 November 2007 represents the most recently updated version of Annex IIIa. Altogether, the list includes 14 groups of foods and products thereof, with one of them being celery[c]. Officially, this term is used *"to refer to stick celery and celery root tuber (also often known as celeriac). However, the term refers to any part of the celery plant and*

[a] UniProt accession number: P81943
[b] http://www.foodallergens.info/Legal/Labelling/Labelling.html
[c] http://www.foodallergens.info/Legal/Labelling/FoodList.html

other forms that originate from it, such as celery leaf, celery root, celery seeds, celery oil, celery salt, celery spice, celery seed oil and celery seed oleoresin (an oil/resin extract from celery)"[a].

There is also a labelling guideline available that provides examples on how allergenic substances in foods and food products have to be declared[b]. For instance, a food packaging could be labelled with "contains celery" if celery was added deliberately, or "may contain" if celery is possibly present in traces due to product contaminations by processing, packaging, or shipping.

The latest legislation, which is entitled **EU Food Information for Consumers Regulation 1169/2011** (available as *The Food Information Regulations 2014*[c]) came into effect on 13 December 2014 and *"will require food businesses to provide allergy information on food sold unpackaged, in for example catering outlets, deli counters, bakeries and sandwich bars"*[d]. In this case, *The Codex Alimentarius Commission Committee on Food Labelling* recommends to provide the information on allergenic ingredients in written form. There is an official one-letter code representing the 14 groups of allergens which should be used for the labelling of meals, e.g. in menus. Celery and products thereof are represented by the letter **L**.

In addition, summarizing allergen ingredient information is no longer allowed, instead allergens have to be highlighted amongst the list of ingredients on packaged foods[e] (Figure 1.1.3[f]).

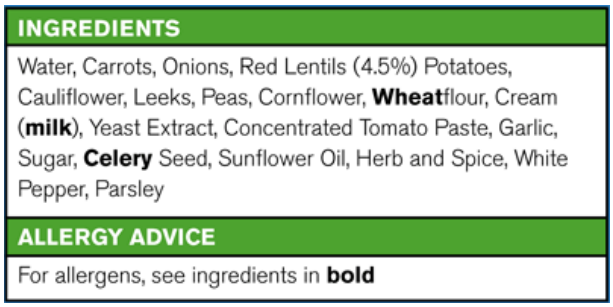

Figure 1.1.3: Example on how packaged foods have to be labelled since 13 December 2014. There is no longer a separate summary of allergens, instead they have to be highlighted in the list of ingredients

[a] http://www.food.gov.uk/sites/default/files/multimedia/pdfs/guidance/allergen-labelling-technical-guidance.pdf
[b] http://ec.europa.eu/food/food/labellingnutrition/foodlabelling/guidelines_6_10.pdf
[c] http://www.legislation.gov.uk/uksi/2014/1855/pdfs/uksi_20141855_en.pdf
[d] http://www.food.gov.uk/business-industry/allergy-guide
[e] http://www.gdalabel.org.uk/gda/product-packaging.aspx
[f] Figure taken from: http://www.foodlabel.org.uk/styling/gda/images//Food-Labelling-Section3-1.gif

1.2 Current Methods for the Detection of Celery in Food

Certain allergenic foods, including celery, do not necessarily have to be added to a product deliberately to be present in the final product. This poses a major challenge for the sensitivity of analytical methods, since the food allergens may be present only in traces. There are a lot of factors that can lead to the contamination with a particular food, e.g. contaminated ingredients, ineffective cleaning of equipment, improper packaging and/or storage, contact with other products during shipping, etc. [29].

Basically, there are two different analytical methods for the detection of food allergens: immunoassays and DNA-based technologies. Those practices are not only directly applied to foods or products thereof, but they may also serve for the verification of successful cleaning procedures after industrial processes [30]. Although immunoassays have been the method of choice so far, the lack of specific antibodies for certain allergens (e.g. for celery) and the need for confirmatory methods have led to the acceptance of DNA-based techniques in the last few years. However, mass spectrometry is more and more gaining in importance for the characterization and especially for the quantification of food allergens in complex food matrices [31].

1.2.1 Enzyme-linked immunosorbent Assay (ELISA)

ELISAs represent a diagnostic tool for the detection of complex analytical targets like bacteria, funga, viruses, proteins, and many low-molecular-weight substances in complex matrices, e.g. in serum, milk, or urine, and they are also used for the identification and quantification of allergens in foods. The mechanism of enzyme-linked immunosorbent assays is based on antibody-antigen interactions, followed by a color reaction for the visualisation of a successful reaction and the quantification of the target analyte.

Due to its specificity, sensitivity and existing automatized high-throughput systems, it is probably the most commonly used method by the food industry and by official food control agencies [1].
There is a multitude of commercially available ELISA-based test kits, e.g. for the detection of tree nuts, fish and crustaceae, milk and egg proteins. Dip-stick immunoassays represent ELISAs in a portable format, where either antigen or antibody are coated on a nitrocellulose membrane that is pasted to a small plastic housing. Those tests are not only inexpensive but rapid and easy-to-perform, and therefore a number of dip-stick tests for several allergens, including egg proteins [32] or peanut and hazelnut [33], have been developed.

As for celery, there are no specific antibodies available that do not cross-react with other non-allergenic proteins from closely-related species, especially carrot and parsley, but also potato and parsnip [22]. Furthermore, if allergens are targeted that are sensitive to thermal processing, e.g. Api g 1, another detection method for the denatured protein would have to be designed.

However, in their publication from 2011, Wang et al. claim to be the first to have developed a highly specific sandwich-ELISA to detect and quantify celery soluble proteins in processed foods, but their assay has also shown a slight cross-reactivity with carrot [34]. Besides, the protein content in celery is fairly low, which constitutes another reason why it is preferable to rely on DNA-based methods for its detection [1].

1.2.2 DNA-based Methods

Due to the lack of celery-specific antibodies, the only reliable option for the detection of *Apium graveolens* is on a DNA basis by means of conventional or real-time PCR. The specificity of these methods cannot only exclude the risk of cross-reactions, but they are also highly sensitive and therefore are suited for the analysis of trace amounts of celery. Furthermore, the development of PCR assays is easier and they can be rapidly adapted to new regulatory requirements [29]. Another advantage of DNA-based methods in general is that the level of DNA in plants is very stable, whereas the concentration of proteins depends on its variety and the growth conditions.

A controversial aspect of those methods is the fact that they only detect the allergen-containing species but not the allergenic substances themselves [1]. This could be an issue when it comes to food declarations since the presence of traces of the species' DNA does not necessarily have to correspond to the presence of allergy-eliciting compounds or to triggering doses thereof. Furthermore, those methods are very dependent on the DNA isolation method, concerning the concentration, purity and fragmentation of the template DNA, and they are also reliant upon the matrix from which the DNA is extracted - whether the commodities are rich in carbohydrates, proteins, or lipids, and if reaction-inhibiting substances are co-isolated. In addition, processing like heating, acidification, or fermentation, and also the release of DNases due to cell rupture may lead to a subsequent degredation of the DNA of interest. Food products may also be formulated by protein isolates or protein fractions without any food-specific DNA present, e.g. aromatic extracts, such as celery oleoresin [29].

The first PCR assay for the detection of celery in food was published by Dovičovičová et al. in 2004 [35]. The primers target the region of the gene encoding mannitol dehydrogenase[a] (MDH), a sequence to be exclusively found in celery varieties. This could be shown by the authors both theoretically by a BLAST search and experimentally by specificity tests. The detection limit was found to be 490-1530 pg DNA, corresponding to 10^2 target copies.
Later in 2004, Stephan et al. [30] published the first real-time PCR detection method for celery in food. The primers and probe were oriented to the sequence of the Api g 1 gene[b], but it has been found by Hupfer et al. that this approach delivers unspecific results when applied to complex food matrices due to cross-reactivities with other plants (e.g. carrot). In their novel real-time PCR assay of 2007, the primers and probe are localized on the target region of the mannitol dehydrogenase

[a] GenBank acc. no. AF067082
[b] GenBank acc. no. not listed

gene from celery. The specificity test includes several herbs and spices of the *Apiaceae* family as well as many other organisms frequently used in food products, and proved the detection system to be highly specific. Furthermore, a false-negative rate of 0% for 10 target copies was found, which is a drastical improvement in sensitivity compared to the conventional PCR from Dovičovičová et al. In addition, spiking experiments with sausages and ground celery seeds were performed. The lowest concentration to be reliably detected was 5-10 mg of celery seed per kg of food [36].

Another real-time PCR detection system for celery was developed by Mustorp et al. in 2008, also targeting the mannitol dehydrogenase gene, but with slight variations in the regions for the primers and the probe. However, they did not perform any sensitivity tests and the detection limit for celery in spiked food products was higher than that of Hupfer et al.
In 2010, a tetraplex real-time PCR for the detection of hazelnut, peanut, soy and celery was published by Köppel et al. [37]. Again, the celery mannitol dehydrogenase gene was targeted by another slightly different pair of primers and a newly designed probe. A second publication from 2010 describes a SYBR Green real-time PCR method where two fragments of the celery mannitol transporter (MaT3) gene[a] were targeted with 2 simultaneously applied primer pairs [38], but the region was reported to show cross-reactivities with many spices [39].
The most recently developed real-time PCR method for the detection of *Apium graveolens* targets the celery-specific NADPH-dependent mannose-6-phosphate reductase (M6PR) mRNA[b] [39]. However, there are no obvious improvements compared to the real-time PCR assay from Hupfer et al.

Table 1.2.1 gives an overview on the available (real-time) PCR detection methods with the corresponding target regions.

Table 1.2.1: Overview on different DNA-based detection methods for celery in food

(MDH) celery mannitol dehydrogenase gene; (MaT3) celery mannitol transporter gene; (M6PR) celery NADPH-dependent mannose-6-phosphate reductase mRNA

Year	Method	Target region	Reference
2004	PCR	MDH	Dovičovičová [35]
2004	real-time PCR	Api g 1	Stephan [30]
2007	real-time PCR	MDH	Hupfer [36]
2008	real-time PCR	MDH	Mustorp [40]
2010	SYBR Green real-time PCR	MaT3	Wu [38]
2010	Tetraplex real-time PCR	MDH	Köppel [37]
2012	real-time PCR	M6PR	Fuchs [39]

[a] GenBank acc. no. EU262657
[b] GenBank acc. no. U83687

1.3 Isothermal Amplification of DNA

1.3.1 General Overview on isothermal DNA Amplification Methods

Allergens often only occur in trace amounts, which is why the quantity of the corresponding DNA may be extremely low. The specific DNA regions are therefore required to be multiplied. In principle, isothermal DNA amplification methods are polymerase chain reactions without a changing temperature profile, i.e. *isothermal* indicates that the temperature stays constant. Therefore, the reactions can be performed on a heating block or in a water bath, which eliminates the need for sophisticated equipment such as a thermal cycler. Not only are those kinds of devices much more expensive, but the thereby executed heating and cooling within seconds requires much power. This makes it difficult to run them battery-operated, thus on-site applications are not an option. Another advantage of isothermal amplification methods is their rapidity of providing analysis results, because there is no time needed for the denaturation steps in the beginning or in between, but the polymerase is able to steadily generate target copies without being interrupted.
Since there is no controllable sequence of cycles, a major disadvantage of those methods is that the results are not quantifiable. Only the order of magnitude of the initial target copy number can be estimated, which is why simply a yes-or-no-answer with regard to the presence of the analyte in the sample can be provided, depending on the sensitivity of the assay.

Due to the complex primer design, the development of an isothermal amplification method for a certain target is more complicated than that of a PCR assay. On the other hand, these primers are highly specific, and once such a technique is designed, it is rapid, cheap, on-site applicable, and easy-to-perform.

The following techniques are the most important isothermal DNA amplification methods:

- **Loop-mediated isothermal amplification (LAMP)** [41] was used for this work and therefore is described in detail in Chapter 1.3.2.
- **Rolling circle amplification (RCA)** [42, 43]
- **Strand-displacement amplification (SDA)** [44]
- **Helicase-dependent amplification (HDA)** [45]
- **Nicking enzyme amplification reaction (NEAR)** [46, 47]

1.3.2 Loop-mediated isothermal Amplification (LAMP)

Method and Applications: Loop-mediated isothermal amplification was developed by Notomi et al. in 2000 [41] and represents a DNA amplification method that performs at a constant temperature of around 65°C. The technique relies on auto-cycling DNA synthesis, where a polymerase with high strand displacement activity and two specifically designed primer pairs are necessary to compensate the need for a cycling profile as it is common in (real-time) PCR. It was experimentally shown by Nagamine et al. that no denatured DNA template is required for a successful reaction, making even the initial denaturation step unnecessary [48].

Special softwares, e.g. *PrimerExplorer*[a] or *LAMP Designer*[b] help with the design of the primers for a LAMP assay. The two interdependent and therefore simultaneously applied primer pairs bind to six distinct regions on the DNA, and therefore make the LAMP reaction highly specific. Furthermore, the method has a very high amplification efficiency with about 10^9-10^{10} copies in only 15-60 minutes [41, 49], depending on the concentration and the purity of the isolated template DNA.
The technique is not only very cost-effective and easy-to-perform, since the only device that is needed is a simple heating block, but it also has the advantage of being rapid, providing results within a maximum 60 minutes. Furthermore, LAMP yields large quantities of DNA with more than 500 µg/ml, which inspired Nagamine et al. to develop a method for the isolation of ssDNA fragments after the LAMP reaction. This may be an interesting approach for the generation of strand-specific DNA for hybridization techniques, e.g. DNA micro-arrays [50].

There is already a multitude of widespread applications for the LAMP method, e.g. as a rapid, accurate, and cost-effective diagnostic tool for infectious diseases [51], or for the detection of genetically modified organisms (GMOs) [52]. When combined with a reverse transcription reaction, LAMP can also be used for the amplification of target RNA molecules (RT-LAMP), inter alia, making it useful for the detection of viruses with RNA genomes, e.g. human immunodeficiency virus (HIV) [53] or influenza A virus (H5N1) [54]. As for multiple targets, a multiplex LAMP (mLAMP) method for the simultaneous detection of bovine *Babesia* parasites was developed by Iseki et al. in 2007 [55], differentiating between 2 targets with both high specificity and high sensitivity.

The first LAMP assay for the detection of plant species in food (including celery) was published by Focke et al. in 2013 [56]. However, Zahradnik et al. reported that the primer sets show high sequence homology for parsley, dill, and fennel in nBLAST[c], and for carrot and parsley the cross-reactivity was also experimentally proven [57]. Their LAMP assay for the detection of the food allergen celery is oriented towards the real-time PCR method from Hupfer et al. [36], thus targeting the mannitol dehydrogenase gene and therefore being highly specific for *Apium graveolens*.

[a] http://primerexplorer.jp/elamp4.0.0/index.html (free)
[b] http://www.premierbiosoft.com/isothermal/lamp.html (fee required)
[c] National Center for Biotechnology Information NCBI

Polymerase: As previously mentioned, a DNA polymerase with high strand-displacement activity is needed to carry out a successful LAMP reaction.
DNA polymerases from the thermophilic bacterium *Bacillus stearothermophilus* (*Bst*) were purified for the first time in 1972 [58]. Kaboev et al. [59] later improved the purification method and described *Bst* DNA polymerase I as a 76 kDA enzyme with both 5'→3' and 3'→5' exonuclease activities, and with an optimum enzymatic activity at 60°C, pH 8-9, 0.25 M KCl, and 0.02 M $MgSO_4$. Furthermore, it was observed that the *Bst* polymerase is insensitive to sulfhydryl blocking agents. Some of these findings were corrected in 1996, when Aliotta et al. showed by both a sequence alignment with *E. coli* DNA polymerase and exonuclease activity assays that *Bst* polymerase I lacks any detectable 3'→5' exonuclease activity. In addition, an SDS-PAGE revealed that the protein has a size of about 97 kDa [60].

The enzyme's high strand-displacement activity makes it useful for LAMP reactions [61, 62], where that portion of the *Bst* polymerase is used, which contains the 5´→3´ polymerase activity, but lacks the 5´→3´ exonuclease domain, termed *large fragment* [60, 63].

Primers: Contrary to PCR, a set of 4 primers is necessary for a LAMP reaction, termed forward/backward inner primers (FIP/BIP) and forward/backward outer primers (F3/B3), respectively. Both FIP and BIP consist of 2 regions that are connected by some intermediate DNA which forms loop structures in single-stranded LAMP products (see paragraph *LAMP Reaction*). Although there are softwares to help with the design of the primers, it is convenient to keep some key points in mind [64]:

1. The inner primers should not consist of more than 40-60 basepairs
2. The amplified DNA region that is covered by the inner primers should not be longer than 200 basepairs
3. Both ends of the inner primers should not be rich in AT basepairs
4. The T_m value for each domain should be around 55-65°C

LAMP Reaction: Figure 1.3.1 shows the starting situation on the double-stranded (genomic) DNA with the targeted region (e.g. part of the mannitol dehydrogenase gene in celery), as well as the corresponding binding sites for the forward primers - F1(c), F2(c), F3(c) - and the backward primers - B1(c), B2(c), B3(c) -, respectively.

Figure 1.3.1: Starting situation for the LAMP reaction on the (genomic) dsDNA with the target region and the corresponding primer binding sites (*blue* forward primers; *red* backward primers) [65]

Initiation and Displacement Step I: The *Bst* polymerase has an optimum efficiency temperature of 60-65°C, where DNA is at a dynamic equilibrium between single- and double-strand conformation. This eliminates the need for a denaturation step at 95°C, and the **B2 region** of the **backward inner primer (BIP)** is able to directly anneal to the **B2c region** on the lagging strand of the target dsDNA (Figure 1.3.2). The subsequent synthesis of the complementary strand results in a double-stranded product, lacking the (genomic) DNA upstream of the newly synthesized strand.
At the same time, the **backward primer (B3)** anneals to the **B3c region** on the DNA and the polymerase is able to synthesize the complementary strand by simultaneously displacing the strand that was generated in the initiation step.

Figure 1.3.2: Initiation and displacement step I on the lagging strand. The backward inner primer (BIP) anneals to its binding site and the complementary strand is synthesized while the original strand is simultaneously displaced (not shown) [65]

The reverse events take place on the leading strand with the binding of the **F2 region** of the **forward inner primer (FIP)** to the **F2c region**, as well as with the binding of **forward primer (F3)** to the **F3c region**.

Initiation/Displacement Step II: Figure 1.3.3 shows the double-stranded product and the displaced strand after displacement step I. The **F1c region** (introduced with the FIP primer) on the displaced strand is complementary to the **F1 region** on the primer itself, leading to the formation of a loop structure on the 5' end.

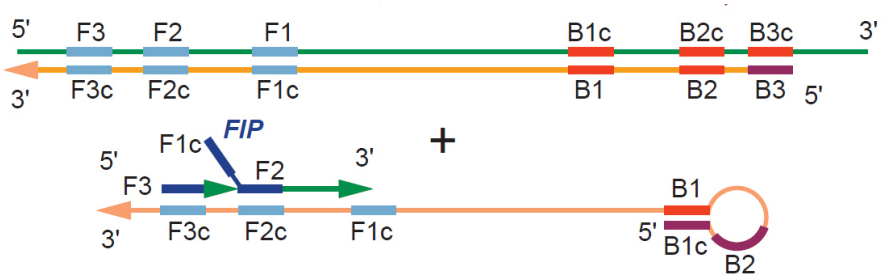

Figure 1.3.3: Double-stranded product and displaced strand with the formation of the first loop structure after displacement step I of the LAMP reaction. The single strand is again primed by FIP and B3 [65]

The displaced strand undergoes another initiation/displacement step by the binding of FIP and F3, with a subsequent synthesis of the complementary strands (Figure 1.3.3 bottom). Due to the complementary sequences of the F1 and F1c regions as well as the B1 and B1c regions, the displaced single strand forms a dumbbell-like structure with loops on both ends (Figure 1.3.4, **1a**). This structure then serves as a starting template for the LAMP amplification cycles.

The reverse event takes place on the other strand with the formation of the first loop by the hybridization of the F1c to the F1 region, followed by the binding of BIP and B3 (not shown), resulting in another dumbbell-like structure which serves as a second template for the subsequent amplification reaction steps (Figure 1.3.5, **1b**).

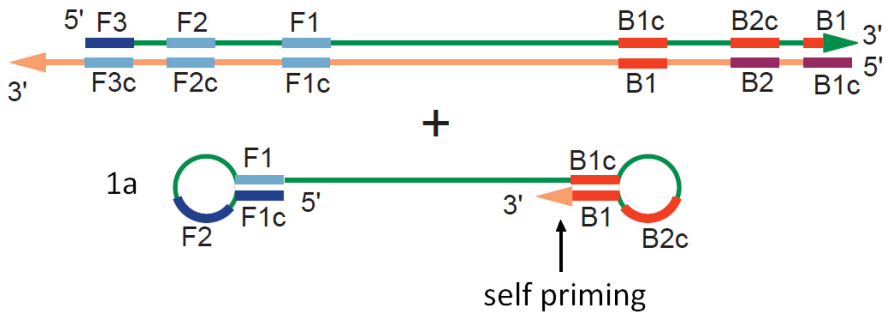

Figure 1.3.4: Double-stranded product and displaced strand with the formation of the first loop structure after displacement step I of the LAMP reaction. The single strand is again primed by BIP and B3 [65]

Cycling Amplification Steps: Self priming at the 3' ends of both dumbbell-like starting structures (1a/b) leads to an elongation throughout the F1c and B1c region, respectively, and in this way resolving the loop structures on the 5' ends. Simultaneously, the inner primers anneal to their corresponding regions on the opposite loops, which displaces the previously synthesized strands (2a/b) and generates dumbbell-like structures and templates for the elongation steps over and over again (Figure 1.3.5).

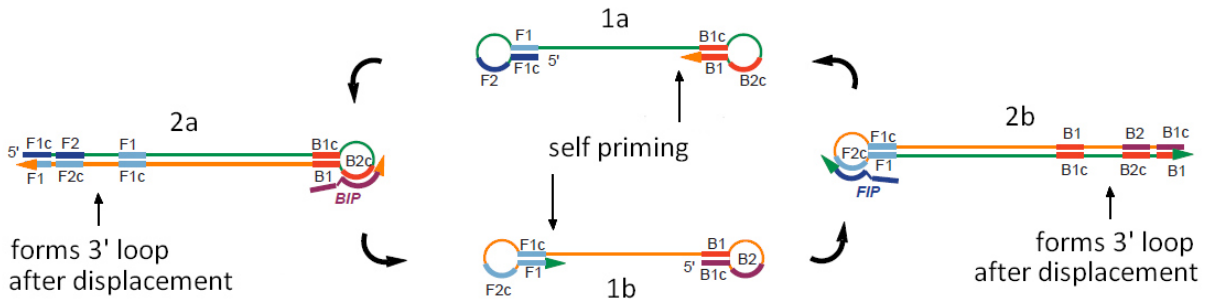

Figure 1.3.5: Self-priming at the 3' ends of the dumbbell-like ssDNA starting structures (1a/b) and simultaneous FIP/BIP-priming of the opposite loops (2a/b) leads to a cycling amplification reaction, generating both starting templates and dsDNA with loop structures for the elongation steps over and over again [65]. The orange arrows indicate the direction of synthesis

Elongation Steps: Figure 1.3.6 shows the first elongation steps, starting with structure **2a**. The single-stranded loop structure at center of the dsDNA (**2a**) is primed by BIP, and the ongoing synthesis displaces the strand with the open 3' end, forming another loop structure which serves as a primer for the subsequent synthesis of the complementary strand (resulting in dsDNA structure **3a**) with a simultaneous displacement of the previously BIP-primed strand as a consequence thereof (**2b**). This displaced strand can now enter the cycling amplification steps again.

Structure **3a** represents the first concatemer, consisting of 2 copies of the target DNA sequence in opposite directions (inverted repeats), and features a single-stranded loop that can be primed by FIP, resulting in structure **4a**. As with 2a, the strand with the open 3' end is displaced and primes its own single-strand by forming a new loop. The previously FIP-primed strand is then displaced (**5a**) and the new dsDNA product, now consisting of 4 inverted repeats of the target sequence, can undergo another elongation step by the binding of FIP to the single-stranded loop. This procedure also happens in inverse direction, starting with structure **2b** (not shown), and continues in both ways until there are DNA concatemers with more than 10 kbp and several hundred inverted repeats of the sequence of interest.

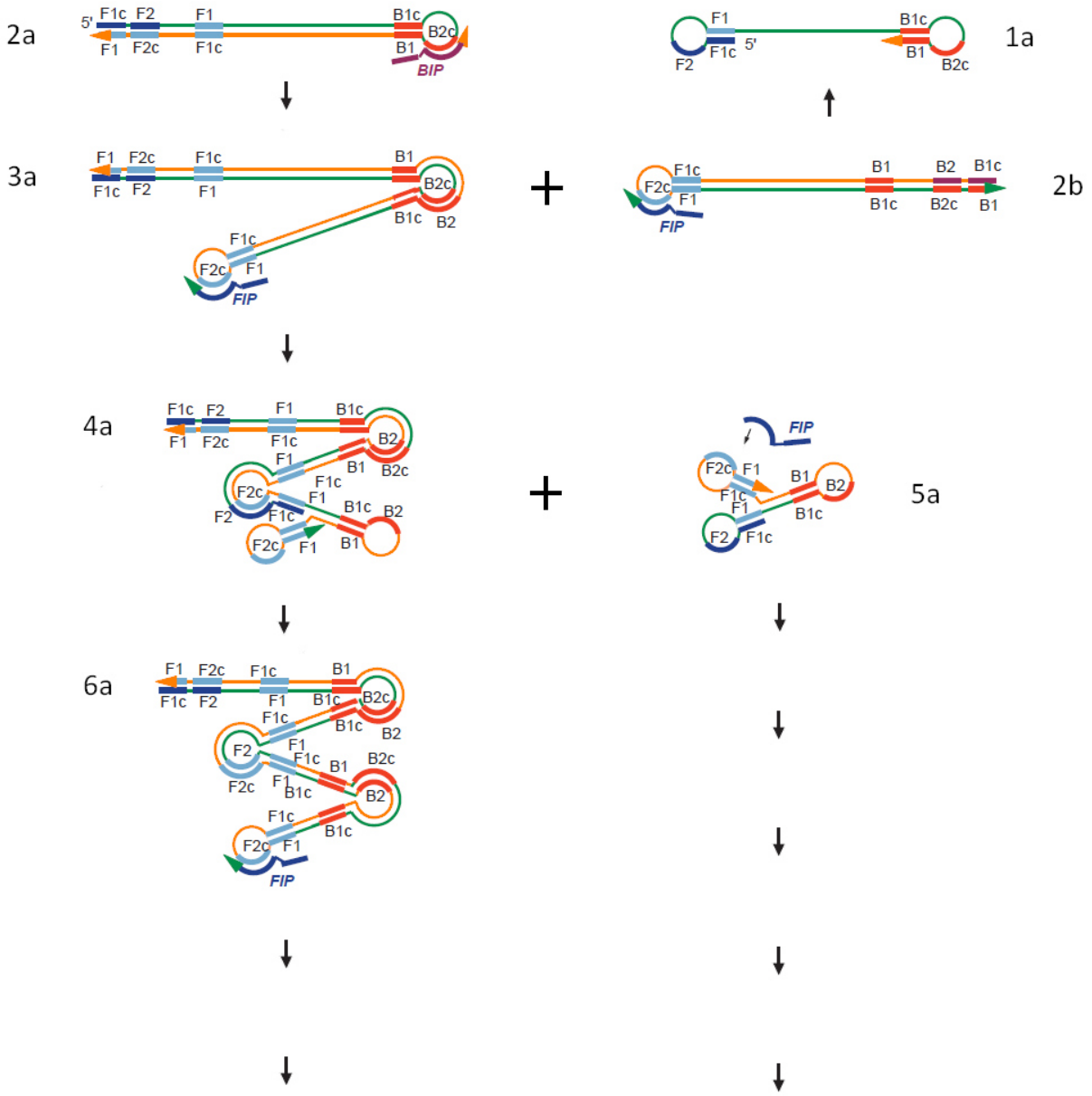

Figure 1.3.6: Elongation steps of the LAMP reaction that finally lead to the formation of concatemers with different quantities of inverted repeats [65]

Application of Loop Primers: In 2002, Nagamine et al. suggested the use of loop primers to accelerate the LAMP reaction. The loop forward (loop F) and loop backward (loop B) primers hybridize to the stem-loops other than those that are hybridized by the forward and backward inner primers, thus being positioned in the regions F2-F1 and B2-B1 in the directions of F1-F2 and B1-B2, respectively [65].

Figure 1.3.7 shows structures 4a and 5a as examples on where the loop primers are able to bind. This approach leads to an accelerated amplification procedure by introducing more starting points for the *Bst* polymerase, which in this way dramatically decreases the running time for a LAMP assay.

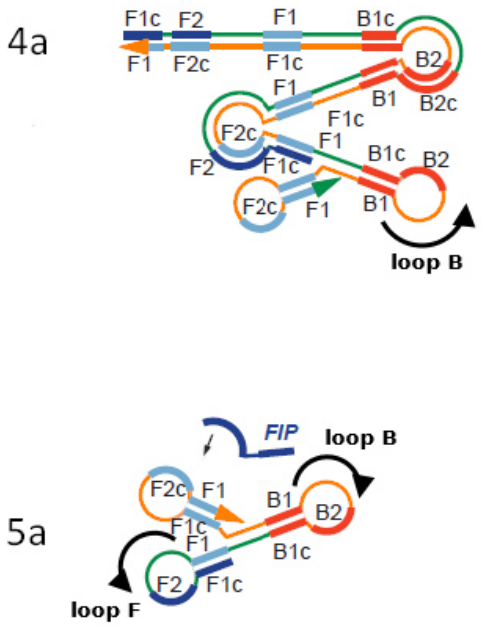

Figure 1.3.7: Illustration of structures 4a and 5a with regard to the binding sites of the loop forward and loop backward primers, respectively [65]

1.3.3 Potential Techniques for the Visualisation of Amplification Products

Since the LAMP reaction is meant to be performed on a heating block or in a water bath, real-time monitoring is not possible. Basically, there are four options for the identification of amplification products (see Figure 1.3.8[a]).

- **Agarose gel electrophoresis** is an established method, inter alia, to separate DNA fragments or to determine their sizes. The different quantities of inverted repeats in LAMP amplification products lead to a characteristic ladder-like pattern on an agarose gel. The major disadvantages of this technique are that expensive instruments are needed, and it takes about 1 hour to get a result.
- **Turbidimetry** is based on the turbidity derived from the formation of the white precipitation of magnesium pyrophosphate ($Mg_2P_2O_3$) during a LAMP reaction. At the end, the mixture's absorbance is measured spectrophotometrically at 400 nm, which requires a relatively large amount of reaction mix (75 µl) and a costly spectrophotometer [66]. Additionally, the signal in this detection system is reported to be rather weak [64].
- **Calcein as metal indicator** yields strong fluorescence in a complex with divalent metallic ions, e.g. Mg^{2+}, which is present in the LAMP reaction buffer. The addition of Mn^{2+} quenches this reaction by complexing calcein, but with the proceeding amplification more and more pyrophosphate is released by the incorporation of dNTPs, which then forms an insoluble salt with the manganous ion. The colorimetric change of metal indicator can be monitored when irridiated with a UV lamp, but also a yes-or-no answer by naked eye detection is conceivable [64].
- **Intercalating fluorescence dyes** such as SYBR® Green I are used to visualise DNA in agarose gels, but they can also be directly applied to a reaction tube after an amplification reaction. The differentiation between a positive and a negative result is possible by exposing the sample to UV light, and if the dye is used in higher concentrations, the detection is also possible with the naked eye.

  Zhang et al. showed the application of microcristalline wax-encapsulated detection beads that are directly placed in the reaction tube. The beads are embedded with SYBR Green fluorescent dye and can be melted after the LAMP reaction [67].

In 2002, Izadi et al. compared SYBR Green staining to turbidimetry methods for LAMP product detection, and they showed that those visualisation techniques correlate with one another, but that the application of SYBR Green is easier and more cost-effective [68].

[a] Figures taken from: http://loopamp.eiken.co.jp/e/tech/img/detect_index_01.jpg (B), http://loopamp.eiken.co.jp/e/tech/img/detect_index_02.jpg (C) and http://openi.nlm.nih.gov/imgs/512/341/3525532/3525532_pone.0052486.g001.png (D, modified)

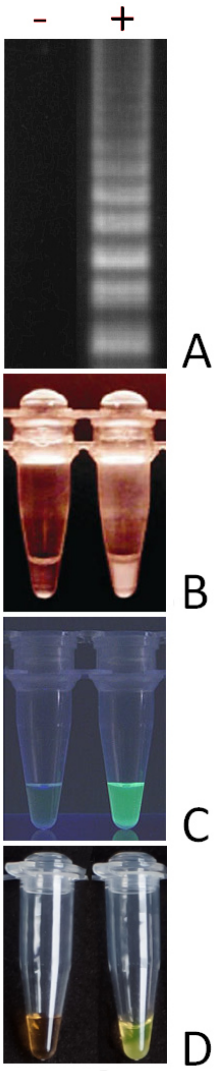

Figure 1.3.8: Visualised products after LAMP reactions - *left* no template control, *right* positive amplification: (A) agarose gel electrophoresis; (B) turbidimetry; (C) fluorescing metal indicators; (D) intercalating fluorescence dyes

2 Aim of the Work

The aim of this work was to evaluate the LAMP assay for the detection of *Apium graveolens* in foods, which was developed by Mag.Celine Zahradnik in course of her PhD project, i.e. the basic setup and the starting conditions for a successful amplification reaction were already established.

The real-time PCR assay from Hupfer et al. [36] was chosen as reference method for the different evaluation experiments, and this work's task was to test the LAMP assay with regard to the following qualities:

- **Specificity:** The application of the LAMP assay to different other members of the *Apiaceae* family should exclude the possibility of cross-reactions with closely related species.

- **Sensitivity:** The determination of the detectable minimum number of celery DNA copies in foreign background DNA should allow a comparison of the sensitivity of the LAMP assay to that of the reference real-time PCR method.

- **Analysis of commercial food products:** The application of the LAMP assay to processed foods should allow a comparison to the reference real-time PCR method with regard to the performance with complex food matrices.

- **False-negative rate:** The determination of the minimum number of celery DNA copies in foreign background DNA that can be detected in at least 95% of cases should allow a reliable statement with regard to the presence of celery at the lowest possible concentration in a food sample.

- **Limit of Detection:** Spiking of a carbohydrate-rich as well a protein-rich food sample with decreasing amounts of celery powder should provide a limit of detection in mg celery per kg food sample and reveal a possibly diverging performance of the LAMP assay with different food matrices.

- **Robustness:** The performance of the LAMP reaction on various heating blocks from different manufacturers should demonstrate the stability of the assay independent from the applied device.

3 Materials and Methods

3.1 Food and Plant Materials

For the evaluation experiments of the LAMP assay several food samples and various plant material was acquired. For this purpose, processed foods with different food matrices (carbohydrate-, protein-, or lipid-rich) as well as plant species which are closely related to celery had to be adduced as sample material. Table 3.1.1 shows the food samples which were acquired from local Austrian supermarkets and Table 3.1.2 shows the plants which were taken from own growings or markets and which were exclusively used in their unprocessed and uncooked state.

Table 3.1.1: Commercially available food products used for the evaluation of the LAMP assay and the corresponding labelling with regard to their celery content

Labelling on the food packages: + contains celery, **(+)** may contain traces of celery, - contains no celery or not declared

ID	Food sample	Labelling
1	Kotanyi steak seasoning	(+)
2	Billa mushroom instant soup	(+)
3	Wasa sesame crisp bread	-
4	Bad Ischler 7 herbs salt	+
5	Maggi vegetable bouillon cube	+
6	Inzersdorfer wellness turkey spread	+
7	Kotanyi herbal mix	-
8	Hipp vegetable baby food	-
9	Rio Mare tuny pastry	-
10	Lorenz Naturals potato chips with garlic and herbs	-

Table 3.1.2: Plants used for the specificity evaluation of the LAMP assay

Plant species	Common name
Anethum graveolens L.	Dill
Apium graveolens var. *rapaceum*	Celery
Carum carvi L.	Caraway
Coriandrum sativum L.	Coriander
Daucus carota (Hoffm.) Schübl. & G. Martens	Carrot
Foeniculum vulgare Mill.	Fennel
Levisticum officinale W.D.J. Koch	Lovage
Pastinaca sativa L.	Parsnip
Petroselinum crispum (Mill.) Nyman & A.W. Hill	Parsley
Pimpinella anisum L.	Anise

3.1.1 Sample Preparation

Since some food samples possibly only contained traces of the analyte, it was especially important to have them homogenized as diligently as possible for reasons of reproducability. Liquid N_2 was used to quick-freeze each of the materials which were then ground with mortar and pestle until a thoroughly mixed homogenizate was obtained. It was essential for the fresh plant material to be quickly processed due to the release of nucleases by the breaking of the plant cell walls.

The prepared samples were stored in 50 ml centrifuge tubes at -20°C.

3.1.2 DNA Extraction

For the DNA extraction of the food products and plant material the SureFood® PREP Allergen Kit (Congen, Berlin, Germany) was used.

Extraction procedure in brief:
100 mg of each ground sample were taken and incubated with the provided lysis buffer and proteinase K at 65°C for 1 hour. The lysate was then centrifuged and the supernatant was transferred onto a spin column. Several washing steps with the pre-wash buffer and the wash buffer included in the kit were followed by the elution of the column-bound DNA with 50 µl of the provided elution buffer. All DNA samples were stored at -20°C.

In order to determine the concentration of the extracted DNA and its purity regarding contaminations with proteins (A260/A280 ratio) and carbohydrates (A260/A230 ratio), the eluted DNA was measured spectrophotometrically using NanoVue Plus^{TM} (VWR, Radnor, USA).

3.2 DNA Amplification

3.2.1 Preparation of Reagents

Nuclease-free water: For the inactivation of nucleases it was necessary to prepare H_2O treated with 0.1% diethylpyrocarbonate (DEPC) (Sigma-Aldrich, St. Louis, USA). A solution of 0.1 ml DEPC in 100 ml H_2O was incubated overnight at room temperature and was then autoclaved and aliquoted in 1.5 ml Eppendorf tubes.

Deoxynucleotidetriphosphates (dNTPs): All 4 dNTPs (Peqlab, Erlangen, Germany) with a concentration of 100 mM each were mixed in equal amounts and were subsequently diluted 1:10 with a Tris-EDTA buffer solution (Sigma-Aldrich, St. Louis, USA). The resulting mix consisted of 2.5 mM of each dNTP and 10 mM of total dNTPs, respectively, and was used for the LAMP reactions.

3.2.2 Real-time PCR

Primers: The real-time PCR assay for the detection of celery targets a diagnostic fragment of the 6947 basepairs long gene encoding for the celery mannitol dehydrogenase (MDH)[a]. This enzyme is exclusively found in *Apium graveolens* and is therefore suited for the distinction between celery and closely related species, i.e. carrot or parsley.

The primers and probe were used as published by Hupfer et al. (Table 3.2.1 and Table 3.2.2) [36] and lead to the amplification of the region 2700 to 2800 of the gene (Figure 3.2.1), resulting in a 101 basepairs long amplicon. All oligonucleotides were synthesized by Eurofins MWG (Ebersberg, Germany).

Table 3.2.1: PCR primers and probe for the detection of a 101 bp diagnostic fragment of the celery mannitol dehydrogenase gene [36]

Oligonucleotide	Sequence (5' → 3')
Forward primer	CGATGAGCGTGTACTGAGTC (20)
Reverse primer	AATAGGAACTAACATTAATCATACCAAAC (29)
Probe	AACAGATAACGCTGACTCATCACACCG (27)

Table 3.2.2: Properties of the primers and probe for the real-time PCR reaction

c: concentration; **Tm:** melting temperature; **MW:** molecular weight

Oligo	c [pmol/µl]	Tm [°C]	MW [g/mol]	GC content
Forward primer	100	59.4	6173	55.0%
Backward primer	100	58.2	8854	27.6%
Probe	100	65.0	9898	48.1%

```
2641 tgtttcccgt acgagatata tttttgtctg gtttgagata tatattacat gctgagtcac
       forward primer                                        probe
2701 gatgagcgtg tactgagtca gtgttatgtt tggattacgg tgtgatgagt cagcgttatc
                          reverse primer
2761 tgtttttata tgtttggtat gattaatgtt agttcctatt acctaaccgg ttgcatcacc

2821 ctcactatat ataccctgtc aaacccatct ctttaacaca tcaaaaacca atctctctcc
```

Figure 3.2.1: Position of the PCR primers and probe in the target region

Binding regions: *Cel-MDH-iF* forward primer; *Cel-MDH-pr* probe; *Cel-MDH-iR* reverse primer

[a] GenBank acc. no. AF067082

Reagents and Reaction Setup: 2 µl of the undiluted DNA extract were added to 13 µl reaction mix consisting of Kapa™ Probe® Fast (Peqlab, Erlangen, Germany), primers and probe in a concentration of 0.3 µM each, and nuclease-free H_2O. The reagents and the respective amounts for a single PCR reaction mix and the settings for the reaction setup are listed in Table 3.2.3 and Table 3.2.4, respectively.

Amplification reactions were performed on a 7500 Fast Real-time PCR system (Applied Biosystems, New York, USA).

Table 3.2.3: Real-time PCR reaction setup

Reagent	**Reagent concentration**	**Amount for 1 reaction**	**Concentration in the mix**
Forward primer	6.25 µM	0.72 µl	0.3 µM
Reverse primer	6.25 µM	0.72 µl	0.3 µM
Probe	6.25 µM	0.72 µl	0.3 µM
Kapa™ Probe® Fast	2x	7.5 µl	1x
H_2O_{DEPC}		3.34 µl	
DNA extract		2 µl	
Total		15 µl	

Table 3.2.4: Real-time PCR temperature profile

	Holding step	**Cycling steps**	
Temperature [°C]	95	95	62
Duration [min]	02:00	00:15	01:00
Repeats	1	45	

3.2.3 Real-time LAMP

Primers: A diagnostic fragment of the celery-specific mannitol dehydrogenase gene is also the target for the 3 primer pairs which are involved in the LAMP reaction (Table 3.2.5 and Table 3.2.6). The complexity of the design of the LAMP primers limits the possibilities for suitable primer sets, which led to a target region that is about 1800 basepairs downstream from the region for the PCR reaction (Figure 3.2.2). The single-copy LAMP amplicon has a length of 222 basepairs and all other LAMP products are a concatemer thereof, which can go up to several thousand inverted repeats resulting in a characteristic ladder-like pattern on an agarose gel.

The LAMP primers were designed by Mag. Celine Zahradnik using PrimerExplorer V4 software[a] (Eiken Chemical Co., Ltd.; Tokyo, Japan) [57]. All oligonucleotides were synthesized by Eurofins MWG (Ebersberg, Germany).

Table 3.2.5: LAMP primers for the detection of a 222 bp diagnostic fragment of the celery mannitol dehydrogenase gene

FIP: forward inner primer; **BIP:** backward inner primer; **F3:** forward outer primer; **B3:** backward outer primer; **LoopF:** forward loop primer; **LoopB:** backward loop primer

Primer	Sequence (5' → 3')
FIP	**GCTGCAAAATCAAGCATTTCTTGTG** - **CAGGGAGAAAGCTTCTTGG** (44)
BIP	**AACAGCTGATGTTGAAGTTATTCCT** - **AAATCTGTATCGAACATCTGAC** (47)
F3	**CATTGTTATACTAAATGTGAGTCAC** (25)
B3	**CGTATTTGCAATGTCGATGAC** (21)
LoopF	**CTTTATCCCACCATTAATAGTGCCT** (25)
LoopB	**ATGGACTATGTGAACACCGCA** (21)

Table 3.2.6: Properties of the primers for the (real-time) LAMP reaction

c: concentration; **Tm:** melting temperature; **MW:** molecular weight

Primer	c [pmol/µl]	Tm [°C]	MW [g/mol]	GC content
FIP	100	73.2	13610	45.5%
BIP	100	70.3	14435	36.2%
F3	100	56.4	7654	32.0%
B3	100	55.9	6436	42.9%
LoopF	100	59.7	7526	40.0%
LoopB	100	57.9	6439	47.6%

[a] http://primerexplorer.jp/elamp4.0.0/index.html

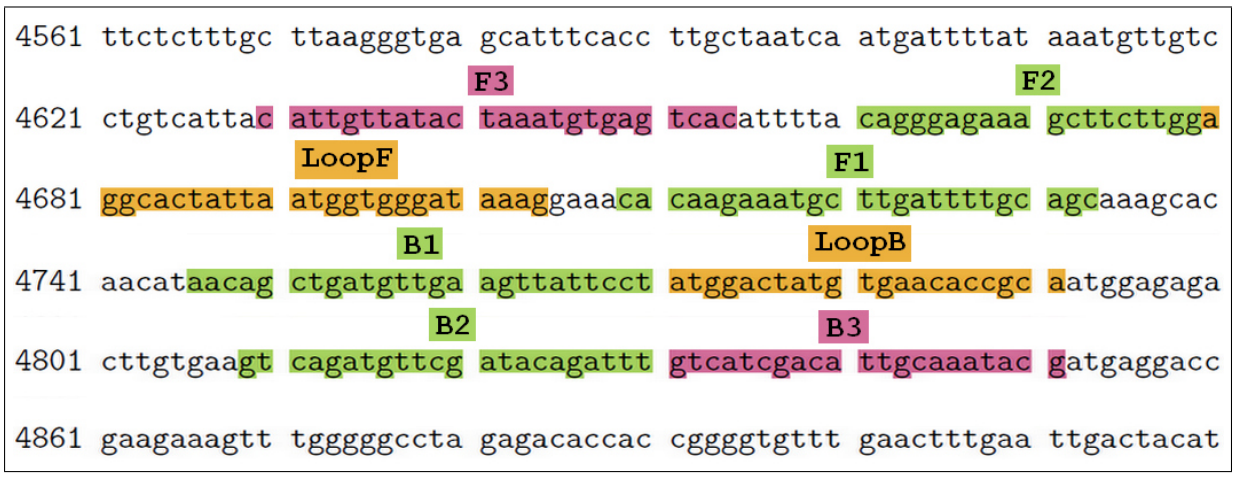

Figure 3.2.2: Positions of the binding sites of the LAMP primers for the detection of a diagnostic fragment of the mannitol dehydrogenase gene in celery [57]

Reagents and Reaction Setup: The addition of betaine (Sigma-Aldrich, St. Louis, USA) at a final concentration of 0.8-1.6 M reduces the formation of secondary structures in GC-rich regions, which eliminates the base pair composition dependence of DNA melting and thus improves the amplification of DNA.

SYTO® 9 Green Fluorescent Nucleic Acid Stain (Life Technologies, Carlsbad, USA) is an intercalating dye with a high affinity for DNA that exhibits enhanced fluorescence upon binding with an excitation maximum at 483 nm and fluorescence emission maximum at 503 nm (Figure 3.2.3[a]).

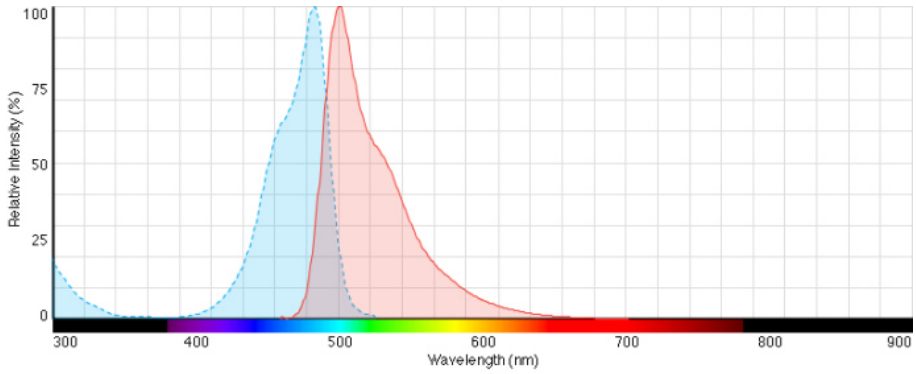

Figure 3.2.3: Absorbance spectrum of SYTO® 9, unbound *(blue)* and bound to DNA *(red)*

[a] Figure taken from: https://www.lifetechnologies.com/content/dam/LifeTech/Documents/spectra/images/34854dna.jpg

The reagents with their initial concentrations, respective amounts and end concentrations for a single LAMP reaction, and the composition of the LAMP buffer are listed in Table 3.2.7 and Table 3.2.8, respectively. Amplification reactions were performed on a 7500 Fast Real-time PCR system (Applied Biosystems, New York, USA).

Table 3.2.7: Reaction setup for the real-time LAMP

Reagent	**Reagent concentration**	**Amount for 1 reaction**	**Concentration in the mix**
F3	10 µM	0.5 µl	0.2 µM
B3	10 µM	0.5 µl	0.2 µM
FIP	100 µM	0.4 µl	1.6 µM
BIP	100 µM	0.4 µl	1.6 µM
LoopF	10 µM	2 µl	0.8 µM
LoopB	10 µM	2 µl	0.8 µM
dNTPs (total)	10 mM	4 µl	1.6 mM
LAMP buffer	10x	2.525 µl	1x
Betaine	5 M	4 µl	0.8 M
SYTO® 9 (1:500)	10 µM	1 µl	0.4 µM
Bst polymerase*	8 U/µl	1 µl	0.32 U/µl
H_2O_{DEPC}		4,675 µl	
DNA extract		2 µl	
Total		25 µl	

**Bst* polymerase was obtained from New England Biolabs, Ipswich, USA

Note: All reagents can be mixed and stored at -20°C without any observed efficiency losses or unwanted side reactions.

Table 3.2.8: LAMP buffer composition

Reagent	**Reagent concentration**	**Amount for 1 reaction**	**Concentration in the reaction mix**
Tris-HCl (pH 8.5)	500 mM	1 µl	20 mM
KCl	500 mM	0.5 µl	10 mM
$MgSO_4$	300 mM	0.5 µl	6 mM
$(NH_4)_2SO_4$	500 mM	0.5 µl	10 mM
Triton™ X-100	lab. grade	0.025 µl	1:1000
Total		2.525 µl	

All reagents for the LAMP buffer were obtained from Sigma-Aldrich, St. Louis, USA

Unlike the PCR reaction LAMP needs no conventional cycling profile but instead works at a constant temperature of 64°C for 60 minutes. It is necessary to set multiple data collecting points for the real-time reaction which is why there are 60 stage repeats with a respective duration of 1 minute, where each "cycle" represents 1 minute and vice versa (Table 3.2.9). For the real-time fluorescence-monitoring an Applied Biosystems 7500 Fast Real-time PCR cycler was used.

Table 3.2.9: Real-time LAMP temperature profile

	Cycling stage
Temperature [°C]	64
Duration [min]	01:00
Repeats	60

3.2.4 LAMP on a Heating Block

For amplification on a heating block, 2 different devices were used: Eppendorf Thermomixer plus and Peqlab Digital Block Heater HX-1. The reaction setup and the according reagents (*3.2.3. Real-time LAMP*, Table 3.2.7) only differ insofar as SYTO® 9 was replaced by H_2O_{DEPC} for the reaction on the heating block.

Each reaction was performed in a separate 1.5 ml Eppendorf tube, where a drop of mineral oil (Sigma-Aldrich, St. Louis, USA) was added on top of the reaction mix to prevent its evaporation. The amplification products were subsequently visualised via agarose gel electrophoresis or fluorescence staining.

3.3 Visualisation of the Amplification Products

3.3.1 Gel Electrophoresis

For the visualisation of the characteristic ladder-like pattern of the amplified product, the samples (including a no-template control) were applied on a 1.5% agarose gel (peqGOLD Universal-Agarose from Peqlab, Erlangen, Germany), which was stained with SYBR® Safe DNA Gel Stain (Invitrogen™, California, USA). All reagents for the Tris-acetate-EDTA (TAE) buffer were obtained from Sigma-Aldrich, St. Louis, USA. Table 3.3.1 and Table 3.3.2 show the composition of the TAE buffer and the agarose gel, respectively.

Table 3.3.1: Composition of a 50x stock solution of Tris-acetate-EDTA buffer for agarose gel electrophoresis

Reagent	Concentration	Amount for 1 l
EDTA	0.5 M, pH 8.0	100 ml
Tris base	pure	242.28 g
Glacial acetic acid	pure	57.1 ml
dH_2O		up to 1 l

Table 3.3.2: Composition of a 1.5% agarose gel for the visualisation of LAMP products

Reagent	Amount
Agarose	1.5 g
TAE buffer 1x*	100 ml
SYBR® Safe 10.000x	10 µl

*The 50x TAE stock solution is diluted 1:50 with deionized water for gel electrophoresis. The final solute concentrations in the 1x buffer are 40 mM Tris and 1 mM EDTA

5 µl of each sample were mixed with 1 µl 6x DNA Loading Dye (Thermo Scientific, Waltham, USA) prior to the application on the gel, and 5 µl GeneRuler™ 1 kb DNA Ladder (Thermo Scientific, Waltham, USA) served as reference. Table 3.3.3 shows the settings for the electrophoresis run.

Table 3.3.3: Settings for the agarose gel electrophoresis run

Voltage [V]	Current [mA]	Run time [min]
80	400	50

3.3.2 Fluorescence Staining

SYBR® Green I Nucleic Acid Stain (Life Technologies, Carlsbad, USA) is a cyanine dye used for nucleic acid staining to visualise DNA products after agarose gel electrophoresis or for the detection after an amplification reaction directly in the reaction tube. It preferentially binds to double-stranded

DNA, which makes it suitable for the naked eye distinction between a positive and a negative amplification reaction.

For the staining procedure, SYBR® Green I 10.000x (diluted 1:1000) was applied to aliquots of LAMP products (Table 3.3.4), which were then mixed for 5 s at 1400 rpm, incubated for 5 minutes at room temperature, and viewed under UV light.

Table 3.3.4: Fluorescence staining of LAMP products

Reagent	**Reagent concentration**	**Amount for 1 reaction**	**Concentration in the mix**
SYBR® Green I	10x	1.5 µl	1x
LAMP product		13.5 µl	
Total		15 µl	

4 Results and Discussion

The following sections are arranged according to the order in which the experiments were performed, and they address the results for all evaluation approaches listed in *2. Aim of the Work*. These outcomes will also be discussed and interpreted, and the LAMP assay will be compared to the reference real-time PCR method with regard to each evaluated quality.

4.1 Specificity Test with the previously developed LAMP Assay

Prior to any experiments, the chosen primer set was checked by an NCBI Nucleotide Blast Search[a] to theoretically rule out any relevant homologies with species that are closely related to *Apium graveolens*. Following the reference real-time PCR for the detection of celery in food [36], the DNA from several herbs and spices that also hold food-relevance, i.a. species from the *Apiaceae* family, was subsequently extracted and analysed with PCR and LAMP in order to confirm the specificity of the LAMP primers in practice.

4.1.1 DNA Extraction of the Plant Material

Those 10 plants listed in Table 3.1.2 in section *3.1. Food and Plant Materials* were treated according to *3.1.1. Sample Preparation* and *3.1.2. DNA Extraction*. Table 4.1.1 shows the concentrations and yields of the isolated plant genomic DNA and the corresponding A260/A280 and A260/A230 ratios, respectively.

Table 4.1.1: DNA concentrations and yields with the corresponding A260/A280 and A260/A230 ratios of the extracted plant materials used for the LAMP specificity test

Plant	c [ng/µl]	Yield [ng/mg]	A260/A280	A260/A230
Anise	802	401.0	1.905	1.493
Caraway	1067	533.5	1.922	0.670
Carrot	173	86.5	2.012	1.765
Celery	365	182.5	1.889	1.997
Coriander	1014	507.0	1.900	1.420
Dill	2057	1028.5	2.011	2.013
Fennel	455	227.5	1.959	1.659
Lovage	661	330.5	1.866	0.696
Parsley	430	215.0	1.982	1.995
Parsnip	238	119.0	2.052	0.671

Although all plants were processed as fresh material and only leaves were taken for the isolation of the genomic DNA, there were significant differences in DNA yields. This may be due to different plant-specific cell sizes and a therefore varying amount of cells per gram plant material from which the DNA is extracted. It is also conceivable that some genomes are easier degradable by nucleases than

[a] http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch &LINK_LOC=blasthome

others during sample preparation or that the extraction procedure is less efficient in some organisms, e.g. due to the inhibition of the proteinase caused by plant-specific substances.
The A260/A280 values for all plants are higher than 1.8, which is a good indicator that the extract does not contain protein residuals. By contrast, most of the A260/A230 values are considerably below 2.0, suggesting a carry-over of carbohydrates which is often a problem with plants. Since a column-based extraction kit was used, residuals from the potentially guanidine-containing binding buffer could also be an issue [69]. Possible inhibitors may cause adverse effects to subsequent PCR or LAMP reactions, which might be equalised by diluting the DNA extract.

4.1.2 Analysis via Real-time PCR and Real-time LAMP

In order to minimize inhibitory effects caused by potentially co-extracted carbohydrates, proteins or other substances, the DNA samples were uniformly diluted with H_2O_{DEPC} to a concentration of 40 ng/µl. This amount of DNA should also facilitate the binding of one or more primers if there were a high sequence similarity to the targeted diagnostic fragment. The amplification reactions were carried out according to *3.2.2. Real-time PCR* and *3.2.3. Real-time LAMP*, and the samples were analysed in 5 replicates each.

Table 4.1.2 shows the results of the specificity tests for the reference PCR and the LAMP assay.

Table 4.1.2: Analysis results of the herbs and spices for the specificity tests with the reference PCR and LAMP. Number of replicates *n=5*

Plant	**PCR**	**LAMP**
Anise	- - - - -	- - - - -
Caraway	- - - - -	- - - - -
Carrot	- - - - -	- - - - -
Celery	+++++	+++++
Coriander	- - - - -	- - - - -
Dill	- - - - -	- - - - -
Fennel	- - - - -	- - - - -
Lovage	- - - - -	- - - - -
Parsley	- - - - -	- - - - -
Parsnip	- - - - -	- - - - -

\+ amplification signal, - no signal

Neither PCR nor LAMP showed amplification signals when analysing celery-related species or herbs and spices that are commonly used in different cuisines. Therefore, the primer set has been proven in practice to be highly specific for the target sequence which is exclusively found in *Apium graveolens*. These findings confirm the the in silico results from the NCBI Nucleotide Blast Search and they are also in accordance with the specificity tests from the two PCR assays that also target part of the mannitol dehydrogenase gene in celery [36].

4.2 Sensitivity Test - Spiking of Soy DNA with Celery DNA

4.2.1 Dilution Series of Celery DNA and Analysis via Real-time LAMP

Celery has a genome size of approximately 3 Gb and contains 3.0 pg DNA per haploid nucleus [70]. Based on the estimation that one base pair has a molecular weight of 650 Da, it is possible to calculate the target copy number corresponding to a given DNA concentration, e.g. using the DNA copy number calculation tool from Thermo Scientific[a].

To assess the assay's potential for quantitative analysis, the DNA from celery leaves was extracted and its concentration was determined photometrically for the preparation of a dilution series ranging from approximately 10.000 to 1 target copies in a background matrix of 5 ng/µl soy DNA. The dilutions were prepared twice in independent working steps. The amplification reactions were carried out according to *3.2.2. Real-time PCR* and *3.2.3. Real-time LAMP*, and the samples were analysed for both dilution series in 5 replicates each.

Table 4.2.1 shows the dilution series of the celery DNA with the concentrations and target copy numbers per reaction, and the results for the LAMP analysis.

Table 4.2.1: Dilution series of the celery DNA starting at 32.4 ng per reaction, with corresponding target copy numbers and LAMP analysis results

DNA concentration	Target copy number [1 per reaction]	PCR	LAMP
32.4 ng/reaction	10.000	10/10	10/10
3.24 ng/reaction	1000	10/10	10/10
324 pg/reaction	100	10/10	10/10
32.4 pg/reaction	10	10/10	6/10
3.24 pg/reaction	1	6/10	2/10

Figure 4.2.1 shows an amplification plot of a real-time LAMP reaction of the DNA dilution series. There is a wide variation in the time where the amplification signals of the replicates with 100, 10, and 1 target copies cross the threshold, i.e. those amounts cannot be differentiated, which is why this technique is suitable for qualitative analysis only.

Figure 4.2.2 shows a calibration curve based on the DNA dilution series given in Table 4.2.1. The error bars clearly demonstrate that this LAMP assay cannot be used for any target copy quantification for trace amounts of the target analyte, but for a semiquantitative estimation at the most.

[a] http://www.thermoscientificbio.com/webtools/copynumber/

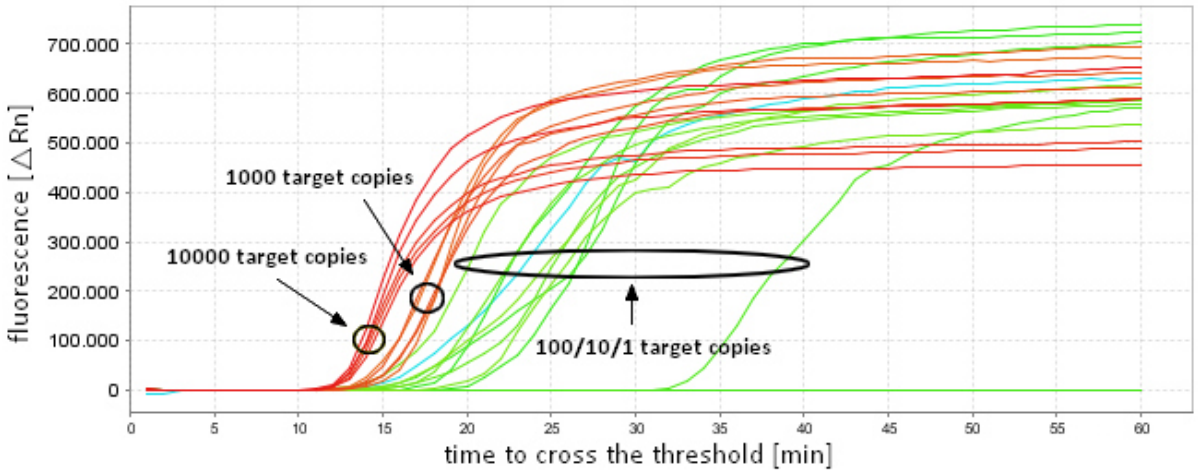

Figure 4.2.1: Amplification plot of one of the two dilution series with celery DNA to approximately 10.000, 1.000, 100, 10, and 1 target copies, analysed via real-time LAMP. Number of replicates *n=5*

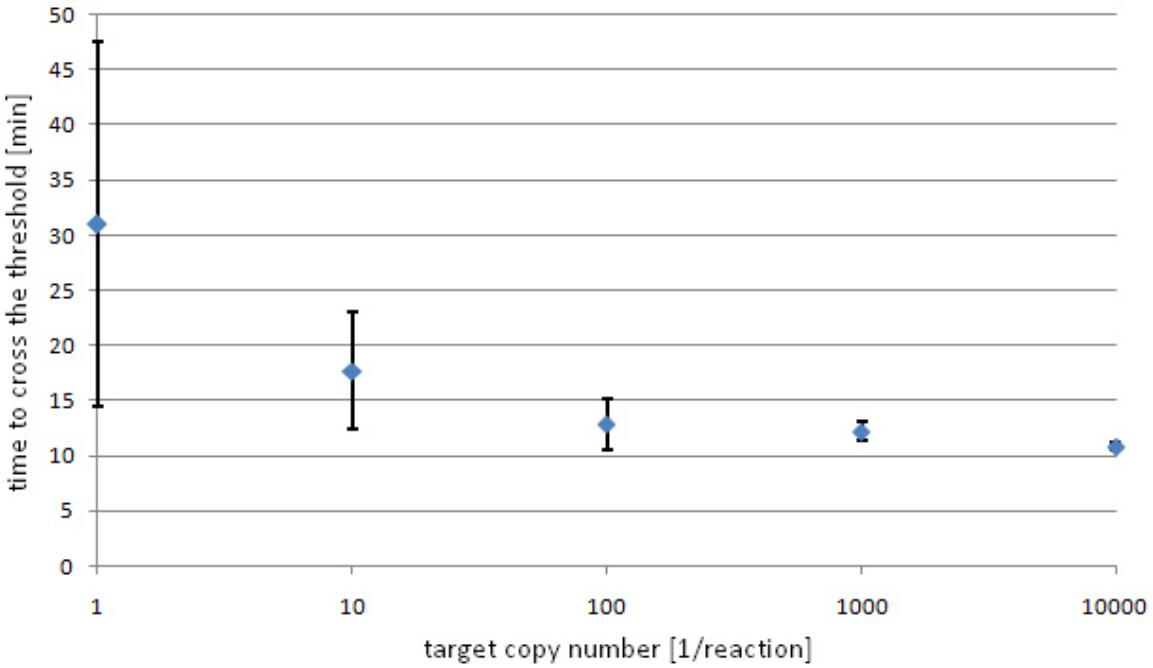

Figure 4.2.2: Calibration curve with standard deviations for the LAMP sensitivity test

The increasing error for decreasing target copy numbers shows that the LAMP assay is unsuitable for a reliable quantification of celery DNA in trace amounts. Number of replicates *n=10*

4.3 Analysis of commercial Food Products via LAMP and Comparison to its Reference-PCR Method

To demonstrate the stability of the LAMP assay when applied to complex and potentially inhomogenous matrices, ten commercially available food samples covering a broad spectrum of different kinds of ingredients were obtained from local supermarkets. Those ingredients are ranging from vegetable products to processed meat, spices and pastries as well as foods generally rich in proteins, carbohydrates, or lipids. Multiple independent extractions of the samples combined with a parallel analysis using the reference PCR assay should also show the reproducibility of results with the LAMP method.

4.3.1 DNA Extraction of the Food Products

The 10 food products listed in detail in Table 3.1.1 were treated according to *3.1.1. Sample Preparation* and *3.1.2. DNA Extraction*. Each sample was extracted three times in independent extraction procedures to also include the error caused by sample preparation. Table 4.3.1 shows the concentrations of the isolated DNA and the corresponding A260/A280 and A260/A230 values, respectively.

Table 4.3.1: DNA concentrations with the corresponding A260/A280 and A260/A230 ratios of the extracted commercial food products

Sample IDs (listed in detail in Table 3.1.1 in section *3.1. Food and Plant Materials*): **1** seasoning **2** instant 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; **E1**, **E2** and **E3** refer to the three independent DNA extractions

	c [ng/µl]			A260/A280			A260/A230		
Sample ID	**E1**	**E2**	**E3**	**E1**	**E2**	**E3**	**E1**	**E2**	**E3**
1	84	82	152	1.876	1.929	1.877	1.120	1.426	1.267
2	102	280.5	537.5	1.925	1.934	1.930	1.172	0.921	1.664
3	252	241.5	338.5	1.909	1.917	1.923	1.010	0.703	0.614
4	142.5	60	141.5	1.875	1.967	1.979	1.007	1.200	1.803
5	35.5	31	22	1.868	1.824	1.938	0.835	0.697	0.370
6	111	128.5	67	1.805	1.725	1.763	1.276	1.152	0.464
7	209	222.5	176.5	1.779	1.926	1.810	0.724	1.413	0.330
8	170	161	136.5	1.965	1.952	1.845	1.504	1.928	0.817
9	258.5	156	174	1.866	1.891	1.933	1.086	0.841	1.208
10	234.5	453	353.5	1.987	2.004	1.992	0.619	1.440	1.400

There are obvious discrepancies within each sample regarding the DNA concentrations yielded from the three independent extractions. This may be due to partially inhomogenous food matrices which consist of too many ingredients to be homogenized diligently enough to gain uniform mixtures of which only 100 mg are taken for the extractions. It is also possible that the raw materials for the production of the foods have not been homogenous themselves, potentially varying in source, age, or previous treatment in harvesting, storage, and shipping. Additionally, the breaking of cell walls and a thereby executed release of nucleases during processing or sample preparation may severely affect

the DNA content. It is also conceivable that aggregates or inclusions of specific substances may be formed in samples with high moisture contents like baby food, tuna pastry or turkey spread, which then contingently influence the efficiency of the extraction procedure.

The A260/A280 ratios are consistently above 1.8 or only slightly below, which indicates that the DNA extracts have a high purity with regard to co-extracted proteins. In contrast, none of the A260/A230 ratios of any of those samples are near the favored value of 2.0 or above, which suggests that the extracts contain residuals of co-extracted carbohydrates.

The lowest ratios were gained at extraction 3 of samples 5 (vegetable bouillon cube) and 7 (herbal mix) with values of 0.370 and 0.330, respectively. This can be explained by the high share of plant raw materials in those food products, which is a known issue regarding the co-extraction of carbohydrates. The other samples do not also contain certain percentages of vegetables, but the glycogen content of meat products may as well be reflected in the low A260/A230 ratios of the extracted DNA. However, those impurities did not affect the subsequent amplification reactions, which could be demonstrated by dilutions of the isolates.

4.3.2 Analysis via Real-time PCR and Real-time LAMP

Since there may only be trace amounts of celery present in the food samples, it was important to utilise the isolated DNA as concentrated as possible. Several samples were diluted 1:4 to exclude inhibitory effects by residuals from the extraction procedure, but no such effects could be observed, which is why the extracts were not diluted for the subsequent analysis. The amplification reactions were carried out according to *3.2.2. Real-time PCR* and *3.2.3. Real-time LAMP*, and each sample was analysed in 5 replicates for every extraction.

Table 4.3.2 shows the results of the analysis of the commercial food samples for the reference PCR and the LAMP assay.

Figure 4.3.1 shows an illustrated comparison between the PCR and LAMP results, where all replicates from the three extractions are pooled, i.e. the number of replicates is $n=15$. In summary, it was demonstrated that the LAMP assay delivers reproducible and consistent results and that it performs not only as well as the PCR, but that it is even superior to its reference method with the samples 1, 2, and 5. This means that this LAMP assay is more sensitive than its PCR equivalent in some cases.

Table 4.3.2: Packaging declarations on the commercial food products and the corresponding analysis results for PCR and LAMP

		Extraction 1		Extraction 2		Extraction 3	
ID*	**Declaration**	**PCR**	**LAMP**	**PCR**	**LAMP**	**PCR**	**LAMP**
1	(+)	+++++	+++++	+++++	+++++	+++++	+++++
2	(+)	++ - - -	+ - - - -	+++ - -	+++ - -	++ - - -	++++ -
3	-	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
4	+	+++++	+++++	+++++	+++++	+++++	+++++
5	+	+ - - - -	+++ - -	- - - - -	- - - - -	++ - - -	++++ -
6	+	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
7	-	- - - - -	+ - - - -	- - - - -	- - - - -	- - - - -	+ - - - -
8	-	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
9	-	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
10	-	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -

*The sample IDs refer to the food samples listed in Table 3.1.1 in section *3.1. Food and Plant Materials*. Labelling on the food packages: + contains celery, **(+)** may contain traces of celery, - contains no celery or not declared

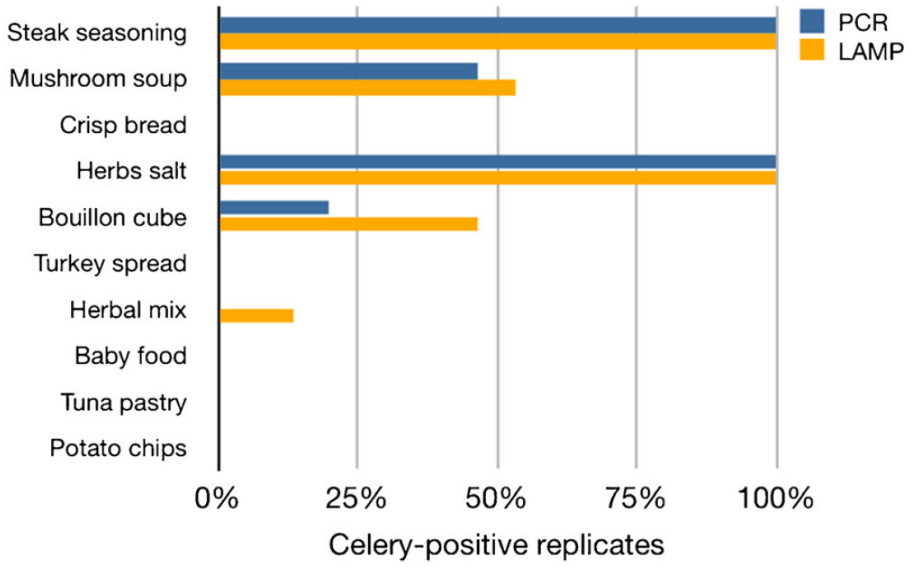

Figure 4.3.1: PCR (*blue*) and LAMP (*orange*) show similar results when analysing commercial food samples [57]. Number of replicates *n=15*

Congruency between Declaration and Analysis Results

An interesting issue with the analysis of commercial food products were potential divergences in the declaration of celery and its actual detection. In five of ten foods, celery was not declared to be an ingredient or that the food may possibly contain this allergenic substance: **3** crisp bread, **7** herbal mix, **8** baby food, **9** tuna pastry, and **10** potato chips. However, only four of those five products could actually confirmed to be celery-negative, which were **3** crisp bread, **8** baby food, **9** tuna pastry, and **10** potato chips. As for sample **7** (herbal mix), 2/15 replicates were positive with LAMP only.

Since no celery DNA could be detected with PCR and only 1/5 replicate of 2 extractions gave a positive amplification signal with LAMP, this may be due to very low levels of product contamination. This herbal mix originates from a company that processes many different spices, i.a. celery, and therefore one can assume that such contaminations may arise from insufficient cleaning of grinders, band conveyors or other instruments used for spice production. Another reason might be the storage or shipping of spices or mixtures together with celery.
Those false-negative declarations could generally be problematic for persons who are allergic to celery, since one is not warned about the consumption of the regarding food. It may not exactly be safe to say that those products actually contain allergy-eliciting compounds (see *1.1.2. The Allergenic Food Celery)* or triggering doses thereof, but due to the lack of specific antibody-based detection methods, allergic people would have to rely on the fact that the presence of allergens is possible.

The eventuality that the allergenic food is declared, but can not be detected, was also observed. While samples **1** (seasoning), **2** (instant soup), **4** (herbs salt), and **5** (bouillon cube) could be confirmed to contain celery, sample **6** (turkey spread) gave 0/15 positive results with both PCR and LAMP. As discussed in *1.2.2. DNA-based methods*, there are several possibilities that DNA is degraded during food processing. The cooking and mashing of the turkey meat could have led to a degradation of the DNA of the added celery. The analysis via mass spectrometry could reveal the possible presence of the corresponding allergens or other celery-related proteins, e.g. the enzyme mannitol dehydrogenase.
Generally, false-positive declarations do not pose a problem for allergic persons, but it would be in a company's interest to not exclude those people as customers by warning them about allergenic ingredients that are not actually present.

4.4 Evaluation of the false-negative Rate - Spiking of the DNA extracted from different Food Matrices with Celery DNA

False-negative results in food analysis can have a severe impact, especially if the missing of a certain ingredient in foods may pose a health risk to people with intolerances thereof. This means that the detection limit should be on the one hand as low as possible, but on the other hand the assay must not fail in detecting the analyte at this very limit or above. For this reason, the minimum number of celery DNA target copies at which the method still detects 95% (equals to a false-negative rate of 5%) of replicates was determined.

4.4.1 DNA Spiking and Analysis via Real-time LAMP

The LAMP sensitivity test has already shown that the assay is able to reliably detect 100% of replicates if celery DNA is present in at least 100 copies per reaction, whereas only 60% of the replicates were positive with 10 copies per reaction.

In order to narrow down the range for a calculable amplification of 100% of replicates, the amount of DNA was gradually increased, starting with a total of 10 copies per reaction. Furthermore, not soy DNA was spiked with celery DNA, but the DNA of three of those commercial food samples, which were proven to be celery-negative, served as background matrix with a concentration of 5 ng/µl each (**3** crisp bread, **6** turkey spread, **8** baby food). The food products were chosen to cover different matrices and also for reasons of importance as it is the case with baby food.

Each concentration for each background matrix was analysed in 20 replicates according to *3.2.1. Real-time PCR* and *3.2.3. Real-time LAMP*. Table 4.4.1 shows the false-negative rates for LAMP with increasing target copy number of celery DNA in the three different background matrices. Figure 4.4.1 is an illustration of the results shown in Table 4.4.1.

Table 4.4.1: False-negative rates for the LAMP assay tested with three celery-negative food samples

The false-negative rates decrease with increasing target copy numbers and reach 0% for 24 copies in all three matrices. Number of replicates *n=20*

Target copy number of celery DNA (total)	Concentration [pg/µl]	False-negative rates		
		Crisp bread	Turkey spread	Baby food
10	16.3	45%	40%	45%
14	22.8	30%	65%	65%
16	26.0	35%	30%	40%
20	32.5	10%	5%	15%
22	35.8	5%	15%	0%
24	39.0	0%	0%	0%

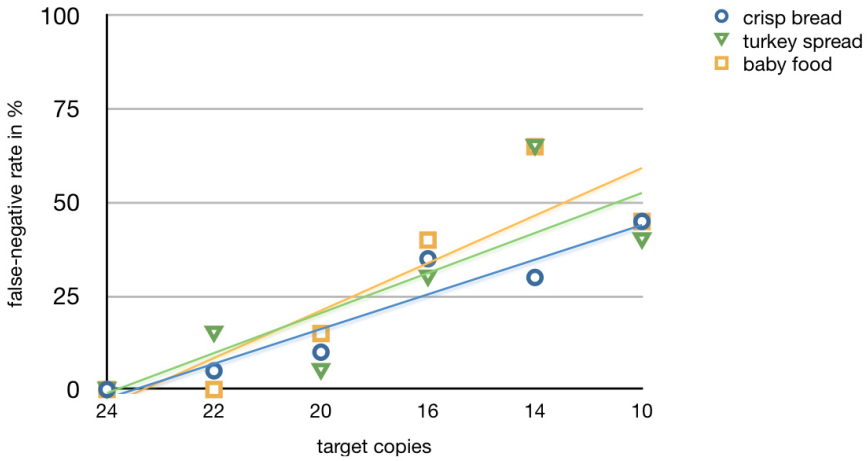

Figure 4.4.1: False-negative rates of the LAMP assay with DNA extracted from celery-free food samples spiked with decreasing target copy numbers of celery DNA [57]. All tested matrices showed a false-negative rate of 0% for 24 or more target copies and increased to 50% when analyzing amounts lower than 16 copies. Number of replicates *n=20*

The results show a false-negative rate of 0% for a total of 24 target copies in all three DNA background matrices. However, 10 copies of celery DNA could still be detected with at least 12 out of 20 replicates. The reference PCR method was reported to reliably detect 10 target copies per reaction when spiked to two different seasoning samples. This could also be confirmed for the three food matrices used for the determination of the false-negative rate for the LAMP assay, which means that in this case PCR is slightly more sensitive.

This is a contradiction to the results found in *4.3. Analysis of commercial Food Products*, where LAMP was found to be the more sensitive method. However, here the DNA extract of the food samples was adjusted to a concentration of 5 ng/µl whereas the pure extract was used for the DNA amplification of the extracted foods. This discrepancy could be explained by the observed insensitivity of the LAMP assay to impure DNA, i.e. the *Bst* polymerase seems to have less problems with carry-overs of carbohydrates or potential residues from the extraction procedure.

Table 4.4.2: Mean threshold minutes and mean standard deviations for the three food matrices spiked with 24 target copies in the false-negativity tests.

The values demonstrate that the LAMP assay might be stopped after a maximum of 30 minutes where a false-negative rate of 0% for a total of 24 target copies would still be obtained in the tested food matrices. Number of replicates *n=20*

	24 target copies	
Food sample	**Min$_{t\,\mathbf{MEAN}}$**	**Min$_{t\,\mathbf{SD}}$**
Crisp bread	16.5	3.58
Turkey spread	17.9	7.97
Baby food	16.0	2.85

Table 4.4.2 shows the mean threshold minutes and mean standard deviations for the analysis with 24 target copies. The least consistent results were obtained with the DNA extract from turkey spread, having a mean min$_t$ of 17.9$\pm$7.97. Nevertheless, this means that the LAMP assay could be stopped after a maximum of 30 minutes, where 100% of replicates would still be detected with an unchanged lower limit of 24 target copies.

4.5 Assessment of the Limit of Detection - Spiking of Food Matrices with Celery Powder

The applicability of a detection method to real foods is particularly important, but there are no commercial reference materials for celery available [36]. For this reason, two samples representing two different matrices that were previously confirmed to be celery-negative (**3** crisp bread and **9** tuna pastry, see *4.3. Analysis of commercial Food Products*) were spiked with celery powder in decreasing concentrations in order to determine a limit of detection in mg celery per kg food.

4.5.1 Spiking Procedure and DNA Extraction

Sample **3** (crisp bread) was spiked with 5% w/w celery powder (corresponding to a concentration of 50.000 mg/kg) and subsequently diluted with ground crisp bread to obtain the following concentrations: 10.000 mg/kg; 5.000 mg/kg; 1.000 mg/kg; 500 mg/kg; 125 mg/kg; 31.25 mg/kg; 7.81 mg/kg; and 1.95 mg/kg.

Sample **9** (tuna pastry) was also spiked with 5% w/w celery powder, but since it was a moist and sticky food matrix, it was mixed with an adequate amount of lysis buffer provided with the extraction kit to ensure a thorough homogenisation. This mix was then diluted with tuna pastry in lysis buffer to the same concentrations as it was done with sample 3.

The DNA was isolated twice according to *3.1.2. DNA Extraction*, where 100 mg of the dilutions of sample 3 and 1.2 ml of the dilutions of sample 9 were extracted. Tables 4.5.1 and 4.5.2 show the concentrations, A260/A280 and A260/A230 ratios for both extraction procedures of samples 3 and 9, respectively.

Table 4.5.1: DNA concentrations with the corresponding A260/A280 and A260/A230 ratios of the extracted crisp bread spiked with decreasing concentrations of celery powder

Wasa sesame crisp bread

Celery content	c [ng/µl]		A260/A280		A260/A230	
[mg/kg sample]	E1	E2	E1	E2	E1	E2
50.000	272.0	300.5	1.899	1.914	0.644	0.726
10.000	302.5	330.5	1.938	1.917	0.610	0.670
5.000	298.5	255.0	1.940	1.905	0.679	0.689
1.000	263.0	288.5	1.946	1.890	0.722	0.700
500	263.5	265.0	1.928	1.910	0.599	0.623
125	257.5	268.5	1.931	1.933	0.684	0.676
31.25	299.0	314.0	1.942	1.930	0.664	0.711
7.81	221.5	242.0	1.900	1.925	0.615	0.665
1.95	287.0	278.0	1.910	1.917	0.755	0.670

Table 4.5.2: DNA concentrations with the corresponding A260/A280 and A260/A230 ratios of the extracted tuna pastry spiked with decreasing concentrations of celery powder

	Rio Mare tuna pastry					
Celery content	**c [ng/µl]**		**A260/A280**		**A260/A230**	
[mg/kg sample]	**E1**	**E2**	**E1**	**E2**	**E1**	**E2**
50.000	172.0	185.5	1.902	1.914	1.076	0.991
10.000	202.5	205.0	1.923	1.899	1.114	1.003
5.000	198.5	190.5	1.890	1.790	0.919	0.927
1.000	189.5	200.0	1.898	1.995	0.936	0.879
500	210.0	209.0	1.868	1.877	1.221	1.193
125	215.5	217.0	1.891	1.850	1.200	1.180
31.25	199.5	200.5	1.842	1.912	0.874	0.968
7.81	203.0	205.0	1.905	1.900	0.999	1.054
1.95	201.5	198.0	1.877	1.895	1.157	1.205

4.5.2 Analysis via Real-time PCR and Real-time LAMP

The DNA extracts were analysed by PCR and LAMP according to *3.2.2. Real-time PCR* and *3.2.3. Real-time LAMP* in 5 replicates each. Table 4.5.3 shows the results for the LAMP analysis.

Table 4.5.3: LAMP analysis results for the two different celery-negative food matrices spiked with decreasing amounts of celery powder

Celery content	**Wasa sesame crisp bread**		**Rio Mare tuna pastry**	
[mg/kg sample]	**Extraction 1**	**Extraction 2**	**Extraction 1**	**Extraction 2**
50.000	5/5	5/5	5/5	5/5
10.000	5/5	5/5	5/5	5/5
5.000	5/5	5/5	5/5	5/5
1.000	5/5	5/5	5/5	5/5
500	5/5	5/5	5/5	5/5
125	3/5	2/5	5/5	5/5
31.25	2/5	2/5	5/5	5/5
7.81	2/5	2/5	5/5	5/5
1.95	2/5	2/5	4/5	3/5

The dashed lines indicate the limits at which 100% of replicates were detectable

These results show that there is a considerable variation of the detection limits in different food matrices. The lowest amount of celery in crisp bread that could be detected in 10/10 replicates was 500 mg/kg, whereas in tuna pastry it was as low as 7.81 mg/kg. This could be due to interactions with the high amount of carbohydrates in crisp bread, which may either influence the extraction procedure by itself or which may be co-extracted and inhibit the amplification reaction in some way. When the DNA of the same samples was spiked with celery DNA, no significant difference in the

detectable amount of target copy numbers could be observed, which means that the dilution of the background DNA to a concentration of 5 ng/µl obviously equalized those inhibitory effects. However, in both samples celery could still be detected at a concentration of 1.95 mg/kg in 4/10 and 7/10 replicates, respectively.

Table 4.5.4 shows the mean durations (since there are no cycles in a LAMP reaction, Ct values are represented by threshold minutes [min_t]) and mean standard deviations for the crisp bread and the tuna pastry spiked with the concentrations at which 100% of replicates were still positive. It is evident that all signals crossed the threshold distinctly before cycle 30, and since one cycle in LAMP represents one minute, the assay might be stopped after 30 minutes with no change in the results. This corresponds also with the findings from the false-negativity tests (see Table 4.4.2). On the other hand, some more replicates of the lower concentrations, of which less than 100% are detected, would be missed.

Table 4.5.4: Mean threshold minutes and mean standard deviations for crisp bread and tuna pastry spiked with different concentrations of celery powder

The values demonstrate that the LAMP assay might be stopped after a maximum of 30 minutes where 100% of replicates within the limit of detection would still be obtained in the tested food matrices. Number of replicates *n=10*

Wasa sesame crisp bread		
Celery content [mg/kg sample]	**Min_{tMEAN}**	**Min_{tSD}**
50.000	12.1	0.31
10.000	13.0	0.53
5.000	14.1	0.28
1.000	16.5	1.07
500	17.2	2.82
Rio Mare tuna pastry		
Celery content [mg/kg sample]	**Min_{tMEAN}**	**Min_{tSD}**
50.000	13.3	0.14
10.000	13.1	0.29
5.000	13.4	0.53
1.000	14.5	0.49
500	15.8	2.11
125	14.6	0.92
31.25	16.6	3.83
7.81	16.0	2.25

4.6 Robustness Test - Performance of the LAMP Assay on different Instruments

The aim of this work was to perform the LAMP assay on a heating block or in a water bath, both of which are on-site applicable and considerably less expensive than a thermal cycler. However, there were two challenges to overcome for this purpose, that is to say the robustness or stability of the assay with regard to possible temperature variations, and a cheap and fast visualisation of the products after the amplification reactions.

As a template, 5 ng/µl soy DNA were spiked with 64.8 pg/µl celery DNA, corresponding to a total of approximately 40 target copies per reaction. The tests were performed on two different heating blocks (Eppendorf Thermomixer plus and Peqlab Digital Block Heater HX-1) and additionally with one thermal cycler (Applied Biosystems 7500 Fast Real-time PCR) for checking purposes. The reactions were carried out in 20 replicates according to *3.2.3. Real-time LAMP* and *3.2.4. LAMP on a Heating Block*, respectively.

Table 4.6.1 shows the amplification and visualisation results of the LAMP robustness test. It was demonstrated that at least 95% of the celery DNA-containing replicates gave a positive result independent from the instrument used. In addition, agarose gel electrophoresis and fluorescence staining turned out to be perfectly in accordance.

Table 4.6.1: Amplification and visualisation results of the LAMP robustness test

At least 95% of the celery DNA-containing replicates were positive, and the visualisation procedures via agarose gel electrophoresis and SYBR Green I staining were in accordance. Number of replicates *n=20*

Amplification instrument	**Real-time fluorescence detection**	**Agarose gel electrophoresis**	**SYBR Green I staining**
Thermal cycler	20/20	20/20	20/20
Heating block Eppendorf	not possible	19/20	19/20
Heating block Peqlab	not possible	19/20	19/20

4.6.1 Visualisation of the Amplification Products

Agarose gel electrophoresis and fluorescence staining were carried out according to *3.3. Visualisation*. Figure 4.6.1 shows an exemplary agarose gel and, underneath, a section of a PCR well plate with the stained LAMP products where the green and orange wells represent positive and negative amplification reactions, respectively. Here, the SYBR Green I concentration was increased 100x to eliminate the need for a UV lamp for the naked-eye detection.

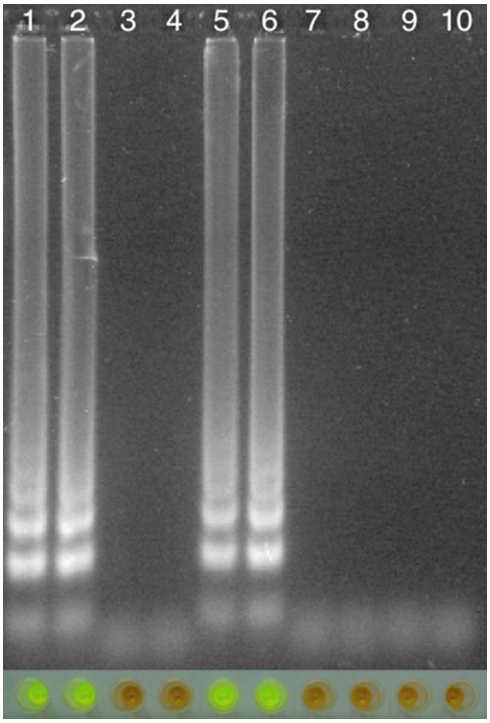

Figure 4.6.1: Agarose gel electrophoresis and SYBR Green I staining after a heating block LAMP reaction [57]

1-2; 5-6 samples containing celery; **3-4; 7-10** celery-free samples (soy DNA only)
green: positive staining reaction; **orange:** negative staining reaction

5 Conclusion

This LAMP technique is a promising tool for the rapid detection of *Apium graveolens* in foods and products thereof. The assay was shown to be highly specific, also excluding species that are closely related to celery, e.g. carrot or parsley. Although it is not suitable for quantitative analysis, the LAMP method has proven to be even slightly more sensitive than the reference PCR method when it comes to the analysis of complex matrices such as processed and spiced foods. The spiking experiments of commercial food samples with celery powder revealed a varying limit of detection, depending on the matrix from which the DNA is extracted. However, LAMP was still able to detect the analyte in each food in concentrations down to 1.95 mg per kg sample. Additionally, the transfer of the assay to different heating blocks showed no deviations in the results compared to the performance in a real-time monitored thermal cycler. Due to its stability, together with the confirmed reliability of product visualisation via fluorescence staining, LAMP was shown to have the potential for on-site applications.

The advantage of inexpensive and easily transportable instruments like heating blocks or water baths are accompanied by the fact that LAMP consumes far less time than an equivalent PCR analysis. That is to say, the PCR assay for the detection of celery in food spans 45 cycles at 1:15 minutes each, resulting in a net run time of 68:15 minutes, including 10 minutes equilibration at 50°C and 2 minutes denaturation at 95°C at the start [36]. In reality, a PCR run has a duration of approximately 100 minutes, which arises from the extra time needed for the thermal cycling between 95°C and 62°C. In contrast, the LAMP assay does not only go without the initial equilibration and denaturation steps, but it performs continuously at 64°C, thus resulting in a total run time of 60 minutes. Added to this, the findings in section *4.4. False-negative Rate* and *4.5. Limit of Detection* indicate that the whole amplification process might be shortened to 30 minutes while still delivering the same reliable results.

Rapidly and inexpensively detecting the allergenic food celery in trace amounts might become a standard for every food-manufacturing company that either processes celery or may handle contaminated raw materials. The LAMP technique could not only be applied to the analysis of surface residuals in grinders, blenders or on band conveyors, but especially the detection of celery in the food products themselves is a major issue for declaration purposes. With regard to the latest EU legislation, where also unpackaged foods will have to be labelled, the list of potential users may be continued with food control organs or operators of restaurants and confectioneries.

The next step in future perspectives would be a broadlier based validation study including several different varieties of celery, carrot, parsley, and other related species. In addition, the development of a lateral flow device for the visualisation of amplification products could further improve the handling of the whole analytical process.

This work is part of the paper **Detection of the food allergen celery via loop-mediated isothermal amplification technique**, published in *Analytical and Bioanalytical Chemistry*, 2014 [57].

6 List of Abbreviations

B3 Backward outer primer

BIP Backward inner primer

bp basepairs

Bst Bacillus stearothermophilus

Cel Celery

CCD Cross-reactive carbohydrate determinant

DBPCFC Double-blind placebo-controlled food challenge

DEPC Diethylpyrocarbonate

DNA Deoxyribonucleic acid

dNTP Deoxynucleotidetriphosphate

EDTA Ethylenediaminetetraacetic acid

ELISA Enzyme-linked immunosorbent assay

F3 Forward outer primer

FIP Forward inner primer

HDA Helicase-dependent amplification

HMW High molecular weight

kb Kilo-basepairs

LAMP Loop-mediated isothermal amplification

LoopB Backward loop primer

LoopF Forward loop primer

LOD Limit of detection

M6PR NADPH-dependent mannose-6-phosphate reductase

MaT3 Mannitol transporter

MDH Mannitol dehydrogenase

MS Mass spectrometry

NEAR Nicking enzyme amplification reaction

NTC No template control

PCR Polymerase chain reaction

RCA Rolling circle amplification

RT-LAMP Reverse transcriptase loop-mediated isothermal amplification

SDA Strand displacement amplification

TAE Tris-acetate-EDTA

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