Development of Novel Nucleic acid-based Reagents for Specific Allergen Detection

CUMULATIVE DISSERTATION

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Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen „Satzung der Justus-Liebig-Universitat Gießen zur Sicherung guter wissenschaftlicher Praxis“ in carrying out the investigations described in the dissertation.

Date, Place

Signature

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Abstract

Food allergies remain a significant global health concern, affecting up to 10% of the global population, with an upward trend. The most effective method of preventing an allergic reaction is the strict avoidance of allergens. The basic prerequisite for avoiding contact with food allergens is knowledge of the presence of allergens in food. According to European Regulation No. 1169/2011, allergens must be labelled if they are used as an ingredient in food. In contrast, there is currently no legal regulation for unintentional 'hidden' allergens that have been transferred, for example from an allergen-containing food to an 'allergen-free' food during the production. Food manufacturers have the option of applying a 'precautionary allergen labelling' (PAL) claim, such as 'may contain traces of (...)', to raise awareness of potential allergen risks. However, this is not subject to any regulatory defined threshold values.

Detection methods for allergens are therefore essential to ensure unambiguous allergen labelling on foods in the case of allergen ingredients and thus give consumers the opportunity to strictly avoid allergenic foods. Furthermore, sensitive and specific detection methods can be used to detect minor amounts of so-called 'hidden' allergens and thus contribute to the useful and reliable use of PAL claims. In the field of medicinal products, detection methods can be crucial in the assessment of the quality of diagnostic and therapeutic allergen preparations with regard to relevant allergen components. They can also be used to analyze, for example, study material used in double-blind, placebo-controlled food studies to verify allergen-containing and allergen-free (placebo) samples. Animal-derived antibodies, especially polyclonal antisera, play an important role in *in vitro* diagnostic detection systems, but can lead to quality problems due to natural variations and thus to inconsistencies from batch to batch. Animal testing is also associated with ethical concerns. In accordance with the 3R principle (Replacement, Reduction, Refinement) and the requirements of European Directive 2010/63/EU, procedures involving living animals should be replaced as soon as scientific alternatives are available.

In order to meet these requirements, the aim of this research project was to develop nucleic acid-based alternatives to replace antibodies in allergen detection methods. Two approaches were pursued. Firstly, the potential of single stranded DNA aptamers, which can bind a large number of molecules due to their sequence-dependent tertiary structure, was investigated. The SELEX process (Systematic Evolution of Ligands by Exponential Enrichment) was applied to generate specific aptamers, thereby achieving a selective enrichment of aptamers against the peanut allergen Ara h 1 (7S globulin). Subsequent next generation sequencing (NGS) was used to identify the underlying nucleic acid sequences. Additionally, the aptamers were characterized using surface plasmon resonance (SPR) with regard to their dissociation constants (K_D) and possible cross-reactivities. Three aptamers (LS_Arah1_367,

LS_Arah1_535, LS_Arah1_725) showed the strongest binding to the target protein Ara h 1 with K_D values between 88 and 988 nM, and no measurable cross-reactivity to the other peanut allergens Ara h 2, 3 and 6. The most suitable aptamers were then successfully applied in different assays such as an ELISA-like method for the detection of Ara h 1. By using the aptamer LS_Arah1_367 in an indirect assay, a sensitivity of 10 ng/mL Ara h 1 was achieved, which is comparable to published antibody-based ELISA systems. In addition, peanut flours and peanut-containing cookies as an example of a thermally processed food matrix were successfully analyzed, so that antibody-free methods for the detection of Ara h 1 were developed that meet the objectives of the European Directive 2010/63/EU.

The second approach involved the development of a LAMP primer set targeting the hazelnut-specific multicopy gene *internal transcribed spacer 2* (ITS2). After *in silico* generation of specific primers with subsequent empirical testing, a specific LAMP-based method was developed that can detect at or below 10 mg hazelnut per kg of food and showed no cross-reactivity to 22 other foods. In combination with a recently developed, efficient DNA extraction protocol and colorimetric visualization of results, the method can act as a fast and simple screening tool (1 min sample lysis, 20 min DNA purification, 45 min amplification and visual detection). Validation work has confirmed its suitability for the specific and sensitive detection of hazelnut in complex food matrices. The developed LAMP method thus allows the evaluation of the presence of hazelnut as an allergenic food and promotes risk-based, preventive labelling of hazelnut in compound foods. This method can also be used for the general authentication of hazelnut through the indirect detection of a hazelnut-specific DNA region, for example in standardized, clinical-diagnostic and future therapeutic approaches.

Both the development of aptamer-based methods and LAMP-based methods show the great potential of nucleic acid-based reagents as direct (aptamers) and indirect (LAMP primers) alternatives to antibodies (**Figure 1**). In contrast to polyclonal antibodies, nucleic acids can be chemically synthesized in a reproducible manner, so that the risk of batch-dependent variations is reduced to a minimum and standardization is possible. In addition, no animal testing is required for the synthesis of aptamers or LAMP primers, thus eliminating ethical concerns (neither for the animals nor for the people carrying out the animal testing).

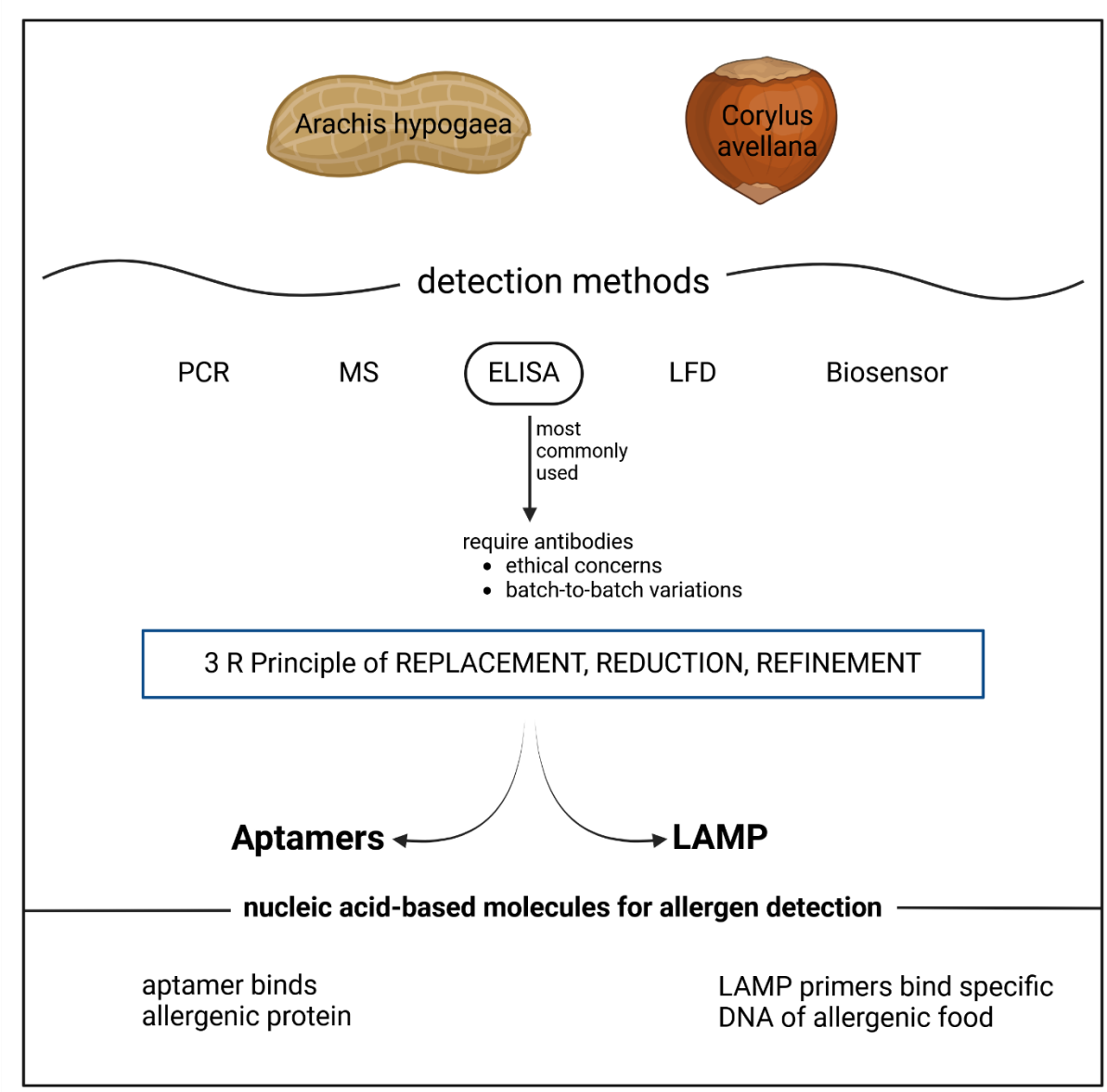

Figure 1. Analytical methods for the detection of peanut and hazelnut as food allergens are polymerase chain reaction (PCR), mass spectrometry (MS), enzyme-linked immunosorbent assay (ELISA), lateral flow device (LFD) and biosensors. The most commonly used method is the antibody-based ELISA. Polyclonal antibodies can raise ethical concerns and potential batch-to-batch variations. Aptamers and LAMP methods could solve these issues and represent nucleic acid-based alternatives to antibody-based test systems according to the 3R principle of replacement, reduction and refinement. Figure was created using BioRender.com.

Zusammenfassung

Nahrungsmittelallergien sind ein weltweites Problem und betreffen inzwischen bis zu 10% aller Menschen mit steigender Tendenz. Das strikte Vermeiden von Allergenen verbleibt bis heute die wirksamste Methode, um eine allergische Reaktion zu verhindern. Die Grundvoraussetzung, um den Kontakt mit Lebensmittelallergenen zu vermeiden, ist das Wissen über das Vorhandensein von Allergenen in Lebensmitteln. Gemäß der europäischen Verordnung Nr. 1169/2011 müssen Allergene gekennzeichnet werden, sofern sie als Zutat in Lebensmitteln verwendet werden. Allerdings existiert bislang kein gesetzlicher Rahmen für unbeabsichtigte „versteckte“ Allergenen, welche beispielsweise während der Produktion von einem allergenhaltigen in ein „allergenfreies“ Lebensmittel gelangt sind. Lebensmittelhersteller haben die Möglichkeit einen Warnhinweis beispielweise in Form von „kann Spuren von (…) enthalten“ („precautional allergen labeling“ (PAL)) anzubringen, um auf potenzielle Gefahren hinsichtlich Allergene aufmerksam zu machen. Dies unterliegt allerdings keinen regulatorisch festgelegten Grenzwerten.

Methoden zum Nachweis von Allergenen sind daher unerlässlich, um eine verpflichtende Allergenkennzeichnung auf Lebensmitteln im Falle von allergenen Zutaten überprüfen zu können und damit Verbrauchern die Chance des strikten Vermeidens allergener Nahrungsmittel zu ermöglichen. Zum anderen können sensitive und spezifische Nachweismethoden dazu dienen, geringe Mengen an „versteckten“ Allergenen nachzuweisen und somit einen Beitrag für eine sinnvolle und unterstützende Verwendung von PAL-Angaben zu leisten. Im Bereich der Arzneimittel können Nachweismethoden eine entscheidende Rolle bei der Qualitätsbewertung von diagnostischen und therapeutischen Allergenpräparaten bezüglich relevanter Allergenkomponenten sein. Darüber hinaus können diese bei der Untersuchung von Provokationsmaterial genutzt werden, welches in doppelblind, placebokontrollierten Studien zur Allergiediagnostik eingesetzt werden, beispielsweise zur Verifizierung von allergenenthaltenden und allergenfreien (Placebo) Proben. Tierische Antikörper, insbesondere polyklonale Antiseren, spielen eine wichtige Rolle bei *in-vitro*-diagnostischen Nachweissystemen, können allerdings herstellungsbedingt zu Qualitätsproblemen durch natürliche Schwankungen, resultierend in Chargeninkonsistenz, führen. Darüber hinaus sind Tierversuche mit ethischen Bedenken verbunden. Gemäß des 3R-Prinzips „Vermeidung, Verringerung, Verbesserung“ (Replacement, Reduction, Refinement) und den Forderungen der Europäischen Richtlinie 2010/63/EU sollen Verfahren mit lebenden Tieren ersetzt werden, sobald wissenschaftliche Alternativen dies ermöglichen.

Um diesen Anforderungen Rechnung zu tragen, war Ziel dieser Promotionsarbeit, nukleinsäurebasierte alternative Methoden zum Ersatz von antikörperbasierten Nachweismethoden zu entwickelt. Dafür wurden zwei Ansätze verfolgt. Zum einen wurde das Potenzial von

synthetischen einzelsträngigen DNA-Aptameren untersucht, die aufgrund ihrer sequenzabhängigen Tertiärstruktur an eine Vielzahl von Molekülen binden können. Zur Generierung spezifischer Aptamere wurde das SELEX-Verfahren (Systematic Evolution of Ligands by Exponential Enrichment) eingesetzt und damit eine selektive Anreichung von Aptameren gegen das Erdnussallergen Ara h 1 (7S-Globulin) erzielt. Durch anschließendes Next-Generation-Sequencing (NGS) wurden die zugrundeliegenden Nukleinsäuresequenzen identifiziert und die Aptamere mittels Surface-Plasmon-Resonance (SPR) bezüglich ihrer Dissoziationskonstanten (K_D) und möglichen Kreuzreaktivitäten charakterisiert. Drei Aptamere (LS_Arah1_367, LS_Arah1_535, LS_Arah1_725) wiesen die stärksten Bindungen gegenüber dem Zielprotein Ara h 1 mit K_D-Werten zwischen 88 und 988 nM auf und zeigten keine messbaren Kreuzreaktivitäten zu den Erdnussallergenen Ara h 2, 3 und 6. Anschließend wurden die charakterisierten Aptamere erfolgreich in unterschiedlichen Assays wie beispielsweise ELISA-ähnlichen Verfahren zum Nachweis von Ara h 1 eingesetzt. Durch den Einsatz des Aptamers LS_Arah1_367 in einen indirekten Assay wurde eine Sensitivität von 10 ng/mL Ara h 1 erreicht, welche mit publizierten antikörperbasierten ELISA Systemen vergleichbar ist. Darüber hinaus konnten sowohl Erdnussmehle als auch erdnusshaltige Kekse als Beispiel einer thermisch prozessierten Lebensmittelmatrix erfolgreich analysiert werden, sodass durch die Entwicklung von Aptameren und dessen Einsatz antikörperfreie Methoden zum Nachweis von Ara h 1 geschaffen wurden, die den Zielen der europäischen Richtlinie 2010/63/EU entsprechen.

Der zweite Ansatz umfasste die Entwicklung eines LAMP-Primersets, welches das haselnussspezifische Multikopie-Gen *internal transcribed spacer* 2 (ITS2) adressierte. Nach *in-silico* Generierung von spezifischen Primern mit anschließender empirischer Testung wurde eine spezifische LAMP-basierte Methode entwickelt, welche 10 mg Haselnuss oder weniger pro kg Lebensmittel nachweisen kann und keinerlei Kreuzreaktivitäten zu 22 anderen Lebensmitteln zeigte. In Kombination mit einer kürzlich selbst entwickelten, effizienten DNA-Extraktion und zusätzlicher kolorimetrischer Ergebnisvisualisierung kann die Methode als schnelles und einfaches Screening-Tool fungieren (1 min Probenlyse, 20 min DNA-Aufreinigung, 45 min Amplifikation und visuelle Detektion). Durch Validierungsarbeiten wurde die Eignung für den spezifischen und sensitiven Nachweis von Haselnüssen in komplexen Lebensmittelmatrizes bestätigt. Damit erlaubt das entwickelte LAMP-Verfahren die Evaluierung des Vorhandenseins von Haselnuss als potenziell allergenes Lebensmittel und fördert eine risikobasierte, präventive Kennzeichnung von Haselnuss in zusammengesetzten Lebensmitteln. Zudem kann die Methode eine generelle Authentifizierung von Haselnuss durch den indirekten Nachweis eines haselnussspezifischen DNA-Abschnitts bewerkstelligen, beispielsweise in standardisierten, klinisch-diagnostischen sowie künftigen therapeutischen Präparaten. Sowohl die Entwicklung aptamerbasierter Methoden als auch LAMP-basierter Verfahren für den

Allergennachweis verdeutlichen das große Potential von nukleinsäurebasierten Reagenzien als direkte (Aptamere) und indirekte (LAMP-Primer) Alternativen zu Antikörpern. Im Gegensatz zu polyklonalen Antikörpern können Nukleinsäuren reproduzierbar synthetisiert werden, sodass das Risiko von chargenabhängigen Schwankungen auf ein Minimum reduziert ist und eine Standardisierung möglich wird. Darüber hinaus sind für die Synthese von Aptameren oder LAMP-Primern keine Tierversuche erforderlich, so dass ethische Bedenken (weder für die Tiere noch für die Personen, die die Tierversuche durchführen) entfallen.

Publications and Conference contributions

Parts of this work have been published and presented elsewhere:

List of publications

1. **Schäfer, L**., Allgöwer, S., Holzhauser, T. (**2023**) Rapid DNA extraction and colorimetric amplicon visualisation speed up LAMP-based detection of soybean allergen in foods. *European Food Research and Technology*, 249, 2875–2886*

2. **Schäfer, L.**, Miskey, C., Hein, S., Völker, E., Reuter, A., Beyer, K., Ahrens, B., Mayer, G., Holzhauser, T. (**2024**) DNA Meets Protein – Development, Characterization, and Application of Aptamers against Peanut Allergen, *Journal of Agricultural and Food Chemistry*, 72 (32), 18225-18233

3. **Schäfer, L.**, Völker, E., Eule, M., Ahrens, B., Beyer, K., Holzhauser, T., (**2024**) Development and Validation of a Loop-Mediated Isothermal Amplification (LAMP) Method for the Rapid, Sensitive, and Specific Detection of Hazelnut as an Allergen in Food Matrix, *Journal of Agricultural and Food Chemistry*, 72 (43), 24093-24100

4. **Schäfer, L**., Miskey, C., Hein, S., Völker, E., Reuter, A., Beyer, K., Ahrens, B., Mayer, G., Holzhauser, T. (**2025**) Entwicklung, Charakterisierung und Anwendung von Aptameren zum direkten Nachweis von Erdnuss und seinem Hauptallergen Ara h 1, *Lebensmittelchemie*, 79 (2), 49–54

5. Ahrens, B., Meixner, L., Sturmfels, M., Kalb, B., Fischl, A., Schwendicke, F., Blumchen, K., Fickenscher, A., **Schäfer, L.**, Holzhauser, T., Schnadt, S., Beyer, K. Pre-chewing for weaning - A traditional method for allergy prevention? Rationale, study design and methods of the open-label trail Solidys-by-Kiss (**accepted** to the journal *Pediatric Allergy and Immunology*, 07/2025)

* The experimental part of this publication was done during the master's thesis "*Optimierung von DNA Extraktion und Detektion für den Schnellnachweis von Sojabohne als Allergen mittels loop-mediated isothermal amplification (LAMP)*", Justus-Liebig-Universität Giessen, 2019.

List of conference contributions

Presentation

- **Conference of the Paul-Ehrlich-Institut (29.-30.09.2022, online)**
  Schäfer, L., Miskey, C., Hein, S., Völker, E., Mayer, G., Holzhauser, T., Development of aptamers for the specific detection of allergens

- **International Symposium on Molecular Allergology and the European Rhinallergy Meeting (ISMA-RHINA) (04.-05.11.2022, online)**
  Schäfer, L., Miskey, C., Hein, S., Völker, E., Mayer, G., Holzhauser, T., Development of aptamers for the specific detection of peanut allergen

- **Regionalverbandstagung der Lebensmittelchemischen Gesellschaft LChG (28.-29.03.2023, Karlsruhe, Germany)**
  Schäfer, L., Miskey, C., Hein, S., Völker, E., Mayer, G., Holzhauser, T., Entwicklung von neuen nukleinsäurebasierten Reagenzien zum spezifischen Nachweis von Allergenen

- **Retreat of the Paul-Ehrlich-Institut (06.-08.12.2023, Ronneburg, Germany)**
  Schäfer, L., Miskey, C., Hein, S., Völker, E., Reuter, A., Mayer, G., Holzhauser, T., Development, characterization and application of aptamers for the specific detection of peanut and its major allergen Ara h 1

- **Frühjahrstagung der Deutschen Gesellschaft für Allergologie und klinische Immunologie e.V. (DGAKI), Mainzer Allergie Workshop (15.-16.03.2024, Mainz, Germany)**
  Schäfer, L., Miskey, C., Hein, S., Völker, E., Reuter, A., Mayer, G., Holzhauser, T., Development, characterization and application of aptamers for the detection of the major peanut allergen Ara h 1

Poster

- **Deutsche Lebensmittelchemietage der Gesellschaft Deutscher Chemiker GDCh (21.-23.08.2023, Bonn, Germany)**
  Schäfer, L., Eule, M., Völker, E., Holzhauser, T., Rapid, specific, and sensitive detection of hazelnut allergen in food using colorimetric loop-mediated isothermal nucleic acid amplification (LAMP)

- **Congress of the European Academy of Allergy and Clinical Immunology (EACCI) (31.05.-03.06.2024, Valencia, Spain)**
  Schäfer, L., Miskey, C., Hein, S., Völker, E., Reuter, A., Mayer, G., Holzhauser, T., Development, characterization and application of aptamers for the detection and quantification of the major peanut allergen Ara h 1

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List of abbreviations

ALS	Arbeitskreis Lebensmittelchemischer Sachverständiger der Länder und des Bundesamtes für Verbraucherschutz und Lebensmittelsicherheit
ALTS	Arbeitskreis der auf dem Gebiet der Lebensmittelhygiene und der Lebensmittel tierischer Herkunft tätigen Sachverständigen
BIP	Backward Inner Primer
BMEL	Bundesministerium für Ernährung und Landwirtschaft (Federal Ministry of Food and Agriculture)
ED_{01} / ED_{05}	Eliciting dose, that leads to reactions in 1% (ED_{01}) / 5% (ED_{05}) of the allergic population
ELISA	Enzyme-linked Immunosorbent Assay
EMA	European Medicines Agency
ITS	Internal Transcribed Spacer
FAO	Food and Agriculture Organization of the United Nations
FDA	United States Food and Drug Administration
FITC	Fluorescein Isothiocyanate
FIP	Forward Inner Primer
K_D	Dissociation Constant
LAMP	Loop-mediated Isothermal Amplification
LC	Liquid Chromatography
LFD	Lateral Flow Device
LFGB	Lebensmittel- und Futtermittelgesetzbuch (German Food and Feed Code)
LOD	Limit of Detection
LoopB	Backward Loop Primer
LoopF	Forward Loop Primer
LOQ	Limit of Quantification
MS	Mass Spectrometry
MST	Microscale Thermophoresis
NGS	Next Generation Sequencing
PAL	Precautionary Allergen Labelling
PCR	Polymerase Chain Reaction
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
ssDNA	single stranded Desoxyribonucleic Acid
SPR	Surface Plasmon Resonance
3R principle	Principle of Replacement, Reduction and Refinement
WHO	World Health Organization

Chapter I – Allergenic Food Detection using Nucleic acid-based Reagents

Food Allergies and European Labelling Regulations

Food allergies are prevalent worldwide, significantly affecting many individuals' daily lives, often for their entire lifetime. Food allergy is defined as a non-toxic, immune-mediated intolerance that is predominantly mediated by Immunglobuline E (IgE).[1] Symptoms of this abnormal reaction of the body, caused by normally harmless food ingredients, can range from mild ones, such as skin erythema, to a severe anaphylactic shock.[2] Currently, the estimated prevalence is 3-10% in adults and 8% in children worldwide and the incidence of food allergies is increasing.[3] 75% of all allergic reactions in children are attributable to the foods egg, peanut, milk, fish, and various nuts.[4] While some allergies diminish over time, others, such as allergies to peanut and tree nuts allergies can persist throughout life.[3] Apart from the first approved immunotherapeutic product for peanut allergy, with partly strong adverse reactions, there are no fully causative treatment options. Strict avoidance of allergenic foods is still the most effective way for allergy sufferers to prevent from an allergic reaction.[5]

The basic requirement to avoid contact with food allergens is knowledge about the presence of allergens in food. According to the European Regulation No. 1169/2011, fourteen allergens (groups) must be labeled if they are used as an ingredient in food.[6] This legal labeling requirement for allergenic ingredients is intended to inform allergy sufferers, but it only applies to ingredients that are intentionally used, and not to process-related cross-contacts. Cross-contacts may be present during the manufacturing process, e.g. on shared equipment when a small amount of peanut from a previous production passes into a peanut-free follow-up product.[7,8] Allergens that unintentionally enter a food in this way are also referred to as "hidden" allergens. In addition to the mandatory labeling for allergenic ingredients, numerous products may present voluntary precautionary allergen labeling (PAL) such as "may contain traces of..." or "may contain...".[9] Such PAL statements may not necessarily be concluded from risk-based allergen assessment, such as a risk-based comparison of amount of allergen present with clinically obtained eliciting dose for allergens above which labeling is recommended. The so-called ED_{05} and ED_{01} eliciting doses were statistically derived from clinical data, indicating the amounts of allergens at which either 5% or 1% of all allergy sufferers would still react.[10] PAL remains a precautionary measure on the part of the food manufacturer.[11] However, consumers do not fully benefit from this supposed protection. On the one hand, many foods with PAL do not contain allergens from cross-contact and, on the other hand, there are many foods without PAL besides potentially high levels of "hidden" allergens. As a result, the credibility of PAL among consumers is decreasing. Thus, some allergy sufferers are restricted in their diet

because they need to avoid a large number of foods. However, the risk of an allergic reaction due to hidden allergens is still evident.[11] Also, allergy sufferers may not take PAL seriously and so face the risk of an allergic reaction. A uniform regulation of the use of PAL statements which takes into account the clinical thresholds for the elicitation of allergic reactions would be desirable, but is not in place in most countries.[11,12]

Accordingly, sensitive and specific detection methods are needed to assess the presence of allergens and, thus, the allergenic potential of a food sample. On the one hand, detection methods are indispensable for detecting allergenic ingredients and, on the other hand, for identifying unintentional 'hidden' allergens. Many analytical requirements must be met for an unambiguous detection, such as appropriate sensitivity, specificity and matrix compatibility. The aim should be to provide sensitive methods that take into account the currently established eliciting doses.[10] The working groups of the ALS (Arbeitskreis Lebensmittelchemischer Sachverständiger der Länder und des Bundesamtes für Verbraucherschutz und Lebensmittelsicherheit) and ALTS (Arbeitskreis der auf dem Gebiet der Lebensmittelhygiene und der Lebensmittel tierischer Herkunft tätigen Sachverständigen) expert committees formulated a guideline on assessment values for unlabeled allergens for official monitoring authorities in Germany[13] and refer mostly to the ED_{05} values of the report on risk assessment of FAO and WHO in 2022, taking into account a portion of 100 g.[14] For peanut, for example, a value of 2.0 mg protein applies, so that the reference dose is 20 mg peanut protein per kg food (for a consumption of 100 g of food). For portions over 100 g, the method needs to be correspondingly more sensitive and for smaller usual consumption portions, a less sensitive method is required. Additionally, FAO and WHO suggest that the limits of quantification (LoQ) of appropriate analytical methods should be about three times lower than the proposed action level. Therefore, 6.66 mg of peanut protein per kg of food should be detectable by an appropriate method (for the assumption of a 100 g portion of a food).

Beside the sensitivity, potential matrix effects, the impact of food processing on detectability, and potential cross-reactivities need to be considered. In addition to the need for detection methods for foods, in the field of medicinal products, procedures for the determination of allergens are important for quality assessment. According to Allergen Products Monograph 1063, *in vivo* diagnostic and therapeutic allergen preparations may require quality assessment at the allergen component level.[15]

Allergen Detection Methods and the Potential of Novel Nucleic acid-based Reagents

Detection methods for allergens can be broadly divided into protein-based and DNA-based techniques. The protein-based methods include enzyme-linked immunosorbent assay (ELISA)

and mass spectrometry (MS). The most common DNA-based method represents polymerase chain reaction (PCR). Lateral flow devices (LFD) could be based on the detection of proteins or nucleic acids corresponding to the allergens. The most common detection tests like ELISA or LFD require the use of antibodies. Antibodies as strongly binding molecules have proven their worth for decades for the sensitive and specific detection of a large number of targets. Polyclonal antibodies are currently the least expensive antibodies and widely used.[16] Despite their well-proven functionality, they also have some disadvantages. In order to produce antibodies, a very complex manufacturing process with animals has to take place beforehand. In 2018, around two million animals were utilized for testing purposes in Germany. Of these, 23% were employed for regulatory purposes and routine production, including antibody production.[17,18] This is accompanied by ethical concerns and enormous costs for the breeding and keeping of the animals.[19] To protect animals used for scientific purposes, the European Directive 2010/63/EU was adopted, which is based on the principle of replacement, reduction and refinement (3R principle) and stipulates that procedures involving animals must be replaced as soon as scientific possibilities allow.[20] In addition to the ethical sense of duty towards the animals, the mental well-being of the researchers and caretakers also suffers to an extent that the responsibility for animals and their deaths can lead to depression.[19,21] In addition to the ethical aspects, there is also the analytical disadvantage that polyclonal antibodies are subject to natural variations due to batch-to-batch variations. Consistent quality is limited to one batch, because of the natural variations between animals. Monoclonal antibodies are less susceptible to batch-to-batch variations, though not entirely exempt.[22] They recognize only a single epitope, a limitation that can constrain their applicability in certain contexts. During food processing, protein structures and consequently epitopes can undergo significant changes. Therefore, polyclonal antibodies are frequently used in food allergen detection, as they recognize multiple epitopes and are thus more robust against such changes. Therefore, a certain degree of variation must be accepted by using more than one antibody batch.[16,23] For long term projects with a high number of measurements, the demand for antibodies may be so high that it cannot be covered by a single batch. Furthermore, batch-to-batch inconsistency plays an important role, particularly in scenarios where measurements must be repeated at a later point in time or if additional antibodies are required that cannot be planned in advance. Problematic, non-reproducible results are the consequences. It is assumed that 20% of all non-reproducible results are due to the variability of standard antibody reagents.[16,23,24] This problem is reinforced by limited or non-existent access to supplier information, so that no transparency regarding the product's availability period is guaranteed. Replacing an old batch with a new one without prior notification increases the difficulty of working with antibodies. In addition, limited validation scopes require researchers to generate this data themselves in advance (without knowing whether there are suitable products among

them). This can result in waste of time, money and sample material[23] as well as frustration on the part of users and led Bradburg and Plückthun, along with over 100 researchers, to start an initiative to transition to "renewable" affinity reagents in 2015.[22]

Identifying these bottlenecks and recognizing the need for novel reagents as antibody alternatives, represent the starting point for determining key prerequisites for the development of new molecules for use in detection methods. The primary disadvantages of animal testing-related ethical concerns and analytically impaired batch-to-batch variations, as well as secondary disadvantages like thermally limited instability and cost, must be addressed in aiming to find alternative binding molecules for the use in detection methods. In addition, ideal detection methods must be sufficiently sensitive to allow for the detection of allergen levels at clinically relevant doses, as previously described, while also being specific enough to avoid false-positive results due to cross-linking with non-targets. Furthermore, the requirements of robustness and reproducibility have to be met to ensure valid results, independent of operator, time and space.

Nucleic acid-based reagents have the potential to solve these issues while fulfilling the named key criteria such as sensitivity and specificity. In contrast to antibodies, nucleic acids can be synthesized with 100% reproducibility, which eliminates the risk of considerable batch-to-batch variations. Once the DNA sequence is known, any researcher can use this information to synthesize the binding molecule themselves, making work in the scientific community much easier and more transparent compared to the use of antibodies. In addition, no animal experiments are necessary, eliminating ethical concerns (neither for the animals nor for the people carrying out the animal experiments).

The aim of this research work was the development of nucleic acid-based reagents for the detection of two highly important allergenic foods, peanut and hazelnut, to overcome the antibody-related issues. Two strategies were pursued to achieve this objective. The first approach was aimed at developing aptamers for the detection of allergenic peanut proteins. Aptamers are short and single stranded nucleic acids, which can bind specifically to proteins via their tertiary structures.[25] The second approach was focused on the development of primers for the detection of hazelnut-specific DNA via loop-mediated isothermal amplification (LAMP). These groups of molecules (aptamers and LAMP primers) represent potential alternative reagents for the use in antibody-free methods. Detailed explanations of the background and suitability of these two approaches are presented in the following two chapters.

The Use of Aptamers for Allergen Detection

Since the first aptamers were developed in 1990[26,27], there has been a considerable surge in interest in these specific binding nucleic acids. The annual publication rate of aptamer-related research has increased by a factor of around 100 in the last 35 years. Several researchers are talking about the next generation of affinity reagents.[24,28] In the field of clinical diagnostics, the development of aptamer research is already so far advanced that there are currently 28 studies using the term "aptamer" (www.clinicaltrials.gov) for the treatment of diseases such as cancer, pneumonia or COVID-19.[29] These very exciting potential applications utilize aptamers that have similar or even better properties than antibodies and benefit from their *in vitro* synthesis providing standardized quality without animal requirements, higher stability, lower costs and easy modification options. So, the questions are: “Are aptamers the better antibodies?”, and especially in terms of food safety and food control “Could the Ara h 1 aptamers, developed in this project, take a step forward to reduce or replace antibodies in peanut detection methods according to the 3 R principle?”

The term "aptamer" originates from the Latin "aptus," meaning "to fit," and the Greek "meros," meaning "part"[26] and describes short and single stranded nucleic acids (RNA or single-stranded DNA (ssDNA)), which can bind specifically to target molecules. These 15 to 100 nucleotide bases are typically 1 to 2 nm in size and 7.5 to 32 kDa in weight.[30,31] The binding between aptamer and target occurs through their sequence-dependent tertiary structures e.g. hairpins, loops, stems, quadruplexes via an induced adaptation mechanism.[32,25] The interaction takes place through combined effects of molecular shape compatibility via van der Waals forces, hydrogen bonds, hydrophobic, electrostatic interactions and other weak interactions.[33] This enables specific binding of various targets such as proteins, peptides, carbohydrates or even ions (**Figure 2**). Today, aptamers against a large number of different target proteins like bisphenol A[34], pesticides[35], histamine[36] or SARS-COV-2 spike protein[37] have been selected and identified.

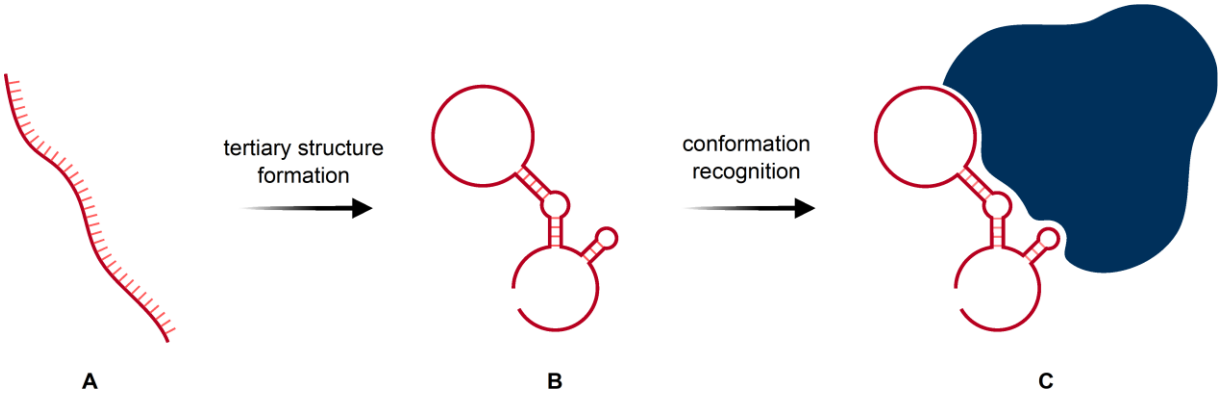

Figure 2. Identified, known nucleic acid sequences (**A**) lead to functional aptamers (**B**) with the ability to bind specifically to various target molecules such as proteins (**C**). Figure was created using Inkscape.

Aptamers are traditionally obtained using the method of systematic evolution of ligands by exponential enrichment (SELEX, **Figure 3**). In this repetitive screening process, specific individual sequences are enriched from a large nucleic acid library, while weaker binding sequences are removed from the pool during the entire SELEX process.[38–40] A library, consisting of 10^{12} to 10^{15} random unique oligonucleotide sequences, is used as a starting point for SELEX, which includes the repetitive cycles of incubation of library with target, the separation of unbound sequences (usually via several washing steps during immobilized target), the elution of bound sequences (e.g. via heat treatment), the amplification of the selected sequences using polymerase chain reaction (PCR), double-strand digestion (e.g. via lambda exonuclease) and the use of the generated single-stranded nucleic acids for the next SELEX cycle.[41,42] In addition, sequence purification steps can also be carried out and, in the case of RNA aptamers, reverse transcription must be included in each selection cycle. The entire SELEX process usually comprises 8 to 15 selection cycles.[25]

As this process follows the law of Darwinian evolution "survival of the fittest", selection stringency is increased throughout the entire selection procedure. This decisive step separates the strongest binding aptamers from the weaker ones. Such an increase in stringency can be achieved through various approaches, such as shortening the incubation time with the target molecule or increasing the number of washing steps after binding of potential specific nucleotide strands (potential aptamers). Furthermore, unspecificities can be counteracted early in the selection process by bringing the sequences into contact with possible cross-reactive surfaces. If the target molecules are immobilized on magnetic beads to perform the incubation and separation steps (between target and library), the cross-reactivity of potential aptamers with the bead surface must be excluded. This could be achieved by incubating the sequences with uncoupled beads followed by discarding the binding sequences. Furthermore, structurally similar compounds can also be introduced at this stage in order to generate more specific aptamers. Sequences that interact with the target and additionally with a structurally similar

compound are already removed during the process and are therefore no longer available as a potential aptamer in the selection pool. These strategies are commonly known as “counter SELEX” or “negative SELEX”.[42]

After nearly 30 years of optimization and modification of the conventional SELEX method, there are a variety of screening approaches depending on the target, the desired aptamer properties and their areas of application. The screening approaches take into account practical aspects such as efficiency, economy and universality of the method. There is a variety of interacting surfaces that include, for example, capillary electrophoresis-SELEX, cell-SELEX, microarray-SELEX or graphene oxide-SELEX.[38,31,43] Regardless of the SELEX variant, after the empirical selection process in the laboratory, the enriched pool must be sequenced to obtain information about the defined nucleotide compositions. The identification of the most dominant sequences was originally carried out using Sanger sequencing. Nowadays, next generation sequencing (NGS) methods, such as Illumina sequencing, are increasingly being used. A key benefit of round-by-round NGS sequencing over endpoint Sanger sequencing is its capacity to generate a comprehensive representation of the accumulation or depletion of millions of potential aptamers.[44] In Sanger sequencing, in contrast, the enriched nucleic acid pool is usually cloned, and subsequently 30 to 100 clones are sequenced, so that only a minor part of all bound sequences can be deciphered. By contrast, NGS provides maximum information on the enrichment of aptamer candidates and shed light on the darkness of the SELEX process for a better understanding of the systematic evolution.[44–46] By identifying the sequences that dominate the SELEX pool the most after stringency increase, information about the nucleic acid compositions of the precisely defined molecules are obtained. The unveiled sequence identity enables the animal-free and highly reproducible production of the resulting aptamers by chemical synthesis.

"From Random to Specific – From Library to Aptamer"

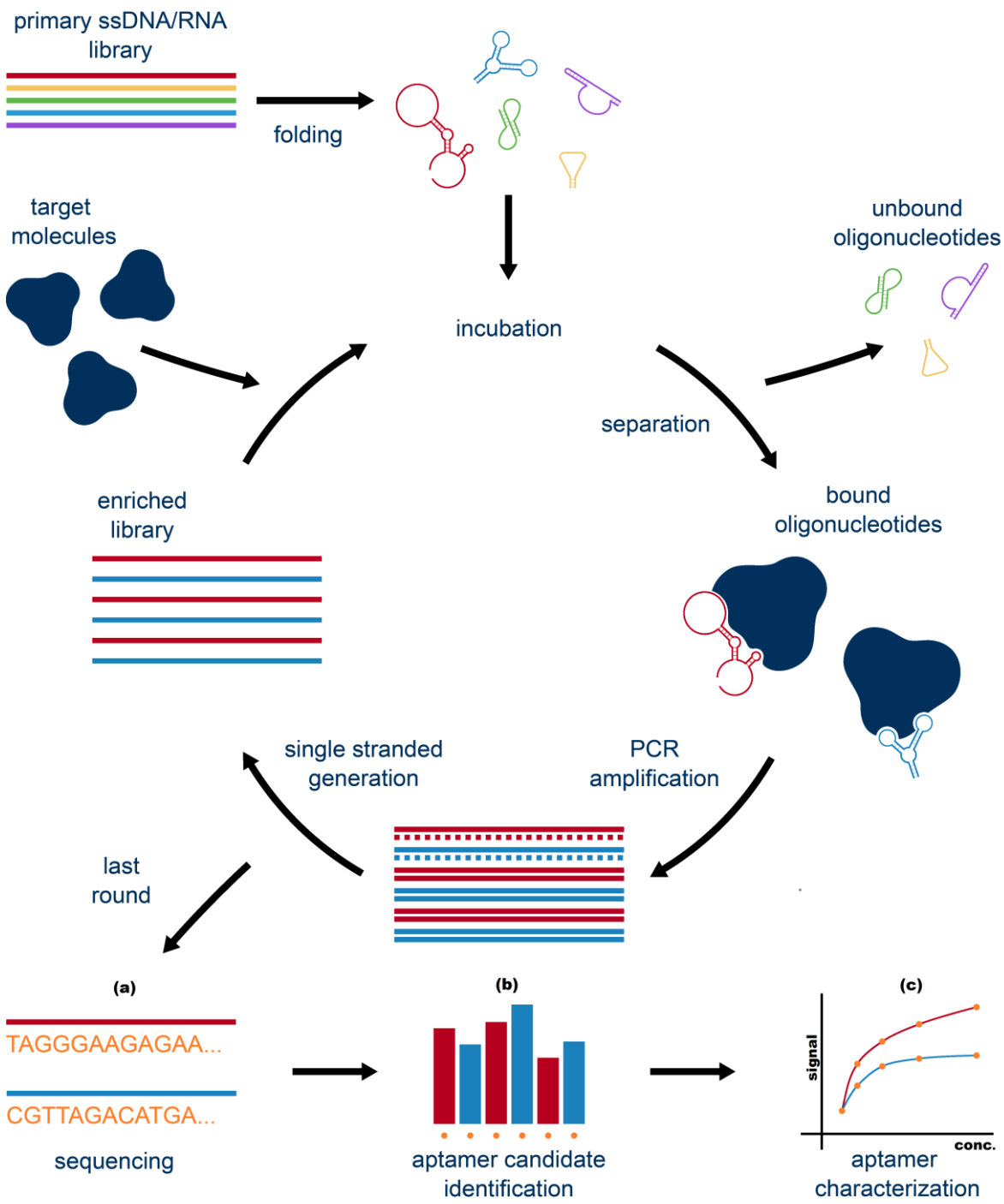

Figure 3. Illustration of the systematic evolution by exponential enrichment (SELEX) for the generation of specific aptamers. The primary library, comprising of billions of different single-stranded nucleic acid sequences, is heated and subsequently cooled, causing the sequences to transform into their characteristic tertiary structures. After incubation of the sequences with the target molecules, the unbound sequences are separated from the bound ones. The bound sequences are eluted from the target molecules and amplified using PCR. Single-stranded sequences are generated from the double-stranded PCR products e.g. using lambda exonuclease and the resulting single-stranded sequences are then incubated once more with the target molecules. This process is repeated 8 to 15 times. After the final sequencing of the enriched sequences (**a**), suitable aptamer candidates can be identified (**b**) and characterized (**c**). Adapted and modified from Komarova *et al.*[45] Figure was created using Inkscape.

As already mentioned, aptamers have a high potential to replace antibodies in detection methods and to benefit ethically and analytically from their chemical synthesis capability. Therefore, aptamer candidates identified by SELEX are subsequently characterized for their binding kinetics, using different methods such as surface plasmon resonance (SPR) or microscale thermophoresis (MST), for the use in various methodologies for diagnostic bio-analytical and therapeutics applications.[47,31]

The application spectrum of aptamers in analytical methods is extensive, as demonstrated by numerous publications on different detection methods. Aptamers can be found in immuno-histochemistry, flow cytometry, chromatography, capillary electrophoresis, mass spectrometry, biosensors, enzyme-linked aptamer assays and lateral flow assays.[24,36,48–56]

In addition to traditional food analysis methods such as immunoassays[31], numerous biosensors have been developed which also utilize the advantages of aptamers as bio-recognition elements. Different detection strategies such as fluorescence, colorimetry, chemiluminescence or surface enhancement of raman scattering[31] are available for the detection of different targets like potentially hazardous substances in food, including toxins, pesticides, veterinary drug residues, pathogenic microorganisms or heavy metals.[57,41,31] Regarding aptamer-based food allergen detection with biosensors, aptamers specific for the allergens peanut Ara h 1[58,59], hen's egg lysozyme[60,61], shrimp tropomyosin[62,63], lupine β-conglutin[64], shellfish arginine kinase[65] and cereal gliadin[66,67] have been developed and applied in biosensor technologies. In 2013, Tran *et al.* developed the first peanut Ara h 1 specific aptamer, which was used in a home-built fibre optic surface plasmon biosensor platform in combination with a secondary antibody, enabling the detection of Ara h 1 with a limit of detection (LOD) of 75 nM (corresponds by calculation approx. 5 mg/kg Ara h 1 or 31-41 mg/kg peanut protein (assumption of an average Ara h 1 content in peanuts of 12-16%[68])). Nine years later, Stidham *et al.* developed a microchip-based device that detected 12.5 mg/kg peanut protein in real food samples[59]. The feasibility of using Ara h 1 specific aptamers in biosensors instead of antibodies to detect peanut in food was confirmed by the aptamers developed in this project.[69] Here, Ara h 1 in peanut flour and powdered peanut butter at a dilution of 1:32 was unambiguously detected using an SPR-based sensor (Figure S6 in Schäfer *et al.* 2024a[69]). Today, there are further Ara h 1 biosensors, using the published aptamer sequences, with more recent enhancements, making it possible to completely replace antibody-based biosensor detection systems without compromising sensitivity and specificity.[59,70–74]

The immunoassay is currently the gold standard for the qualitative and quantitative protein-based detection of allergenic foods. Similar to the enzyme-linked immunosorbent assay (ELISA), an aptamer can be used as detection molecule instead of a conventional antibody to perform an enzyme-linked aptamer assay (ELAA). Other assay designations such as ELASA

(enzyme-linked aptamer sorbent assay) or ELONA (enzyme-linked oligonucleotide assay) are also commonly used for these aptamer-based test systems. Drohlet *et al.* developed the first sandwich assay containing a fluorescein-labelled RNA aptamer in addition to an antibody for the detection of vascular endothelial growth factor (VEGF).[75] The results in terms of precision, accuracy, interference and specificity were comparable to a conventional antibody-based sandwich assay, which demonstrated the potential of the small functional oligonucleotides, acting as suitable binding reagents in immunoassays, for the first time. Since then, numerous aptamer-based assays have been published, e.g. for the detection of aflatoxin B1[76], tetracycline[77] and biomarkers for cancer cells[78], not least due to the simplicity, affordability and time-efficiency of immunoassays compared to other allergen detection techniques. In addition to the general advantages of aptamers, a decisive argument especially for their use in ELAA is that they can be modified more easily to a detection agent (enzyme reporters e.g. horseradish peroxidase, fluorogenic reporters compared to antibodies. In addition, the modification is carried out at a specific position, e.g. at the 3' end with partial spacer molecules, in order to avoid interference of the binding sites.[79] Two examples of the application of aptamers for food analysis are the detection of zearalenone as a contaminant in cereals or agricultural products[80] and neomycin in milk, as an antibiotic residue[81]. The competitive ELAA developed by Sun *et al.* for the quantification of the mycotoxin zearalenone has a linear range of 0.1 to 160 ng/mL (LOD of 0.08 ng/mL) and shows no cross-reactivity with other mycotoxins. In comparative studies with a commercial ELISA, the ELAA results were evaluated as consistent.[80] Furthermore, Truong Giang *et al.* developed a neomycin-specific kit, using the ELAA technique, with a linear range of 0.1 to 100 ng/mL (LOD of 1.11 ng/mL, LOQ of 2.65 ng/mL) and demonstrated that they obtained the same quantitative results as with LC-MS/MS.[81] Despite the proven applicability and sensitivity comparable to antibody-based assays, the development of ELISA-like aptamer assays for the detection of food allergens is still limited. As far as is known, only ELISA-like assays against lupine ß-conglutin (colorimetric competitive assay)[82], hen's egg lysozyme (colorimetric competitive assay)[60] and shrimp tropomyosin (fluorescence binding assay)[83] have been published to date. In this project, identified peanut Ara h 1-specific aptamers were used in indirect and sandwich ELAA assays and thus represents the first microplate aptamer assay against one of the major peanut allergens (Figure 4 in Schäfer *et al.* 2024a[69]). The indirect ELAA, using the aptamer LS_Arah1_367 enabled the antibody-free detection of 10 ng/mL Ara h 1 which is comparable with published antibody-based ELISA systems.[84–86] In addition to the sensitivity, the specificity of the three aptamers LS_Arah1_367, LS_Arah1_535 and LS_Arah_725 was also tested against other major peanut allergens Ara h 2, 3 and 6. No or negligible cross-reactivities were measurable using the methods SPR and indirect ELAA. Furthermore, Ara h 1 in four different peanut flours could be detected and show an increased OD value between approximately 500

and 100 000 ng/mL total protein concentration. The only so far FDA- and EMA-approved immunotherapeutic product for the treatment of peanut allergy consists of a flour from partially defatted roasted peanuts.[87] The successful aptamer-based analysis of four peanut flours has demonstrated that an Ara h 1 investigation of this immunotherapeutic is in principle conceivable. Moreover, an aptamer-based assay was used for the first time to analyze baked peanut-containing cookies. In the published aptamer-based tests to date, the foods analyzed were either not real food samples, commercial foods with no known peanut content or a food such as a chocolate bar spiked with Ara h 1 protein.[58,59,70–72] In this study, approx. 1-10% peanut (~10^3–10^4 mg of peanut protein/kg) could be detected in the Ara h 1 incurred cookie matrix using the aptamer LS_Arah1_367-based assay (Figure 5b in Schäfer *et al.* 2024a[69]). Although this sensitivity is not yet sufficient to verify the eliciting doses assessed by the WHO and FAO[14], which are 2.1 mg peanut protein (ED_{05})[10], the assay provides the starting point for further optimization to achieve or exceed the method requirements in terms of sensitivity. This is comparable to the assays against zearalenone and neomycin, which have also been optimized and modified several times until they have reached a sensitivity and specificity that can keep up with the currently established ELISA tests. The development and publication of the first microplate-based Ara h 1 aptamer assay is a step forward to reduce the present antibody-requiring ELISA tests in the future and thus to improve allergen detection ethically and analytically.

Western blot analyses, which are indispensable for the detection of specific proteins in research as well as for regulatory investigations (e.g. batch comparability of allergen products[88]), are typically performed with antibodies. The alternative use of aptamers instead of antibodies is also possible in this method, with equal performance, as some published studies show, e.g. for *Escherichia coli*[89] or concanavalin A[90]. The aptamer-based western blot developed in this project for the detection of Ara h 1 thus represents the first time an antibody-free variant for the protein analysis of peanut using western blot technology (Figure 3 in Schäfer *et al.* 2024a[69]). Potential future applications could be the comparison of Ara h 1 in various peanut extracts in allergen research or the testing of Ara h 1 in official experimental batch control of approved immuntherapeutics for peanut allergy treatment.

When rapid qualitative detection of allergens is required, the lateral flow device (LFD) is the method of choice. LFDs have the great advantage of being simple and easy to use, requiring no sophisticated laboratory equipment, and can be performed in a very short time. For this purpose, antibodies generally act as targeting ligands. In particular, the superior thermal stability of aptamers compared to antibodies is a key feature for LFD usage, as these point-of-care rapid tests are rarely used in air-conditioned laboratories. Lateral flow aptamer devices have been developed for several target proteins including cholera toxin[91], thrombin[92] and

progesterone in environmental samples[93]. As part of this research project, experiments were carried out to apply the developed Ara h 1 aptamers in a LFD strip test. Preliminary tests in plate format (sandwich ELAA) were promising, as two different aptamers (LS_Arah1_367 and LS_Arah1_725, Tab. 1 in Schäfer *et al.* 2024a[69]) acted as capture or detection molecule, resulting in an Ara h 1 concentration-dependent increase in OD value. A combination of a polyclonal antiserum (rabbit anti-peanut) for Ara h 1 capture and an aptamer (LS_Arah1_367) for detection was also tested, which led to an increase in sensitivity by a factor of approx. 5 (10 to 2 ng/µL before optimization of the assay system) (data not shown). However, this approach (aptamer - target protein - aptamer) transferred to a strip test was not successful, i.e. no positive detection of peanut could be achieved. These observations were confirmed by SPR analysis, as the generation of a stable sandwich complex consisting of Ara h 1 between two aptamers was neither possible (data not shown). The conditions in ELAA proved suitable for a stable sandwich-type binding of the two aptamers to Ara h 1 in contrast to the test conditions in LFD or SPR. This implies that the transfer forces, to which the complex is subjected when passing the test strip or flowing over the SPR microplate, are too strong to retain a sufficient amount of aptamer-target-aptamer complex.[94] The published Ara h 1 aptamers by Stidham *et al.*[59] and Tran *et al.*[58] were also tested in strip test format but also did not lead to successful results, as a positive detection of Ara h 1 in solution was not possible. Intensive testing was carried out with the own and previously published aptamers. As part of these tests, different parameters such as incubation times, buffers, aptamer and Ara h 1 concentrations were varied to evaluate whether the binding of aptamer - Ara h 1 - aptamer in LFDs is possible by adjusting these parameters. Unfortunately, this was not successful, because Ara h 1 could not be detected positive. The limited number of published LFDs in relation to the widespread use and popularity of antibodies used in food allergen detection also indicate that some more research work is required to obtain valid and robust aptamer-based LFDs for allergen detection.[95] Moreover, Majdinasab *et al*. summarized the applications of aptamers in LFDs for clinical diagnostics and concluded that the use of aptamers in LFD strips is not ideal for samples with complex matrix such as blood or solid samples, due to limited sensitivity and sophisticated sample pretreatment.[96] Foods usually represent samples of complex composition, which further underpins this hypothesis.[96] In summary, aptamers in different detection methods could be a suitable and useful alternative to antibodies. In some areas, more research is needed before antibody-based systems can be replaced.

The Use of LAMP-based Methods for Allergen Detection

Besides the direct detection of allergenic proteins, DNA-based detection has become established, which is based on the identification of a section of DNA that is specific for the target allergenic food. Comparative studies between protein-based and DNA-based methods have shown that such indirect detection offers comparable results regarding the assessment of the presence or absence of allergenic food in compound food.[97–100] ELISA, the currently most widely used allergen detection method, bears the risk of cross-reactivity to other phylogenetically related proteins[101] as has been shown for example in the analytical differentiation of hazelnut with other foods such as walnut, cashew, or pecan nut[101–103] or almond with apricot kernel[104] or celery with carrot[105,106]. Furthermore, the utilization of MS as a reliable allergen detection methodology requires the availability of substantial laboratory equipment, a dedicated operating space, and personnel with the requisite training. A rapid test based on DNA detection on the other hand offers several advantages, making the technique suitable as an antibody-free alternative to existing ELISA methods. It also provides better opportunities for standardization, as primer sequences can be chemically produced without significant batch-to-batch differences. While traditional PCR relies on a thermocycler to maintain different temperatures in a repeated cyclic sequence, the LAMP amplification method runs under single isothermal conditions. This makes it suitable for point-of-care methods in combination with portable detection devices and so represents a potential on-site method.[107,108] For example, the isothermal DNA amplification can be carried out in a simple heating block or a water bath. In addition, a qualitative result can be obtained visually by using a pH indicator dye, such as phenol red or hydroxynapthol blue, without the need for an additional detection device. Furthermore, the LAMP method is inexpensive, simple, provides results rapidly and enables a high throughput.[109]

The key factor for a successful LAMP method is an adequate primer set. The principle of loop-mediated isothermal amplification is based on the use of four to six primers, recognizing six to eight distinct regions on the target DNA[110–112], which accounts for a potentially high method specificity. By using a strand-displacing DNA polymerase, the reaction can be performed continuously without the need for temperature cycling (**Figure 4**). Typically, a LAMP reaction takes place at 60 – 65 °C[108]. The qualitative assessment is usually carried out after 30 to 60 min[113], which gives the method the potential for a rapid test. Furthermore, the two major ethical and analytical problems associated with the use of antibody-based methods are omitted, because nucleic acid-consisting primers are chemically synthesized in a reproducible manner without the need for animals. Nowadays, there are numerous existing LAMP methods for the detection of allergens in foods (**Table 1**).

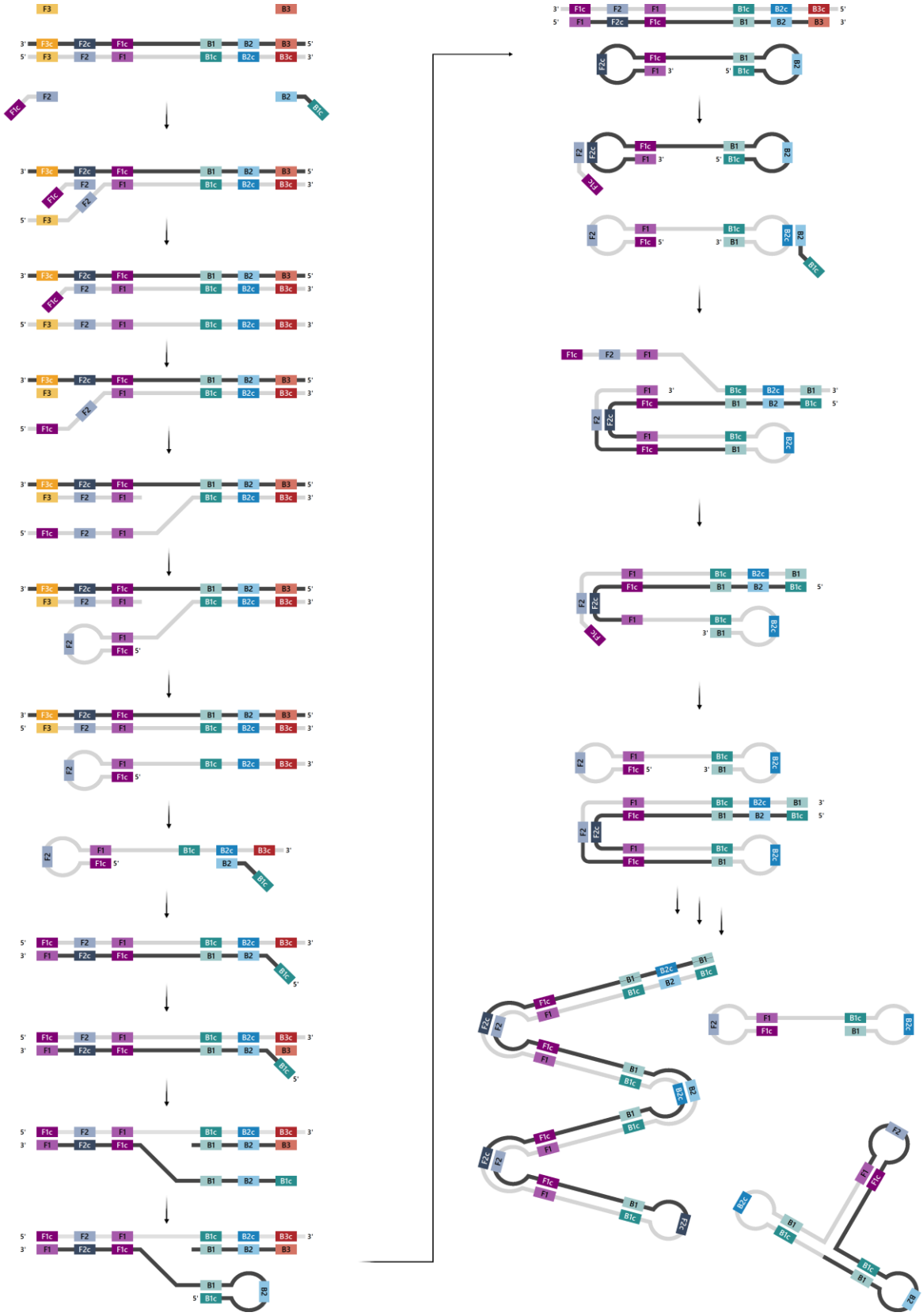

Figure 4. Schematic illustration of the reaction mechanism of loop-mediated isothermal amplification (LAMP). F2 of the forward inner primer (light grey) binds to the template DNA allowing the strand to be extended by the DNA polymerase. During the amplification process, the double-stranded start DNA is simultaneously separated into two single strands by the strand displacement activity of the polymerase. By hybridizing a second primer (F3 of the forward inner primer, light yellow) a few bases upstream and subsequently synthesizing the complementary strand, the single strand synthesized in the first step is separated from the start DNA again. Some bases of the forward inner primer (F1c, dark purple) are complementary to bases within the same strand (F1, light purple), resulting in

hybridization of this region and the formation of a single-stranded loop. In the next step, B2 of the backward inner primer (light blue) is annealed and the reaction proceeds analogously at the 5'-end of the strand with B3 (light red), B1c (dark green) and B1 (light green). The result is a dumbbell-shaped structure. This central LAMP structure contains primer binding sites on the outer loop structures so that the primers can anneal again, and new complementary strands are generated. These are the starting elements for many different annealing possibilities of the primers. As a result, products of different sizes with complex structures and characteristic loop structures are formed. Adapted and modified from Notomi *et al.*[110] Figure was created using Inkscape.

In this project, an easy-to-use rapid method based on LAMP was developed, targeting the gene of the hazelnut-specific *internal transcribed spacer 2* (ITS2). Hazelnut DNA can be detected at a dilution of $1:10^6$ by a visually distinguishable color change from pink to yellow (Figure 1 in Schäfer *et al.* 2024b[114]). The method showed a LOD at or below 10 mg/kg hazelnut in various food matrices. Considering the protein amount of 16.3% of the used hazelnut, a detection of 1.6 mg/kg hazelnut protein is possible. The current eliciting doses ED_{05} for hazelnut is 3.5 mg hazelnut protein. Assuming a consumption dose of hazelnut-containing foods between 100 and 500 g and the requirement according to the 2022 risk assessment of food allergens meeting report of the WHO and FAO, that the sensitivity of the method is 3-fold lower than the proposed level to be verified, a sensitivity of approx. 11.6 to 2.3 mg/kg protein would be necessary. Thus, the sensitivity of the developed hazelnut-specific colorimetric LAMP method is suitable for the verification of foods for relevant hazelnut quantities.

An overview of selected published LAMP developments is presented in Table 1 and provides a comprehensive cross-section. This overview demonstrates that the LAMP developed in this research project exhibits comparable performance in terms of sensitivity and specificity. Some published LAMP studies assess their sensitivity only as a theoretical consideration based on target DNA still detectable in buffer (peanut: Sheu *et al*., Yuan *et al*.; soybean: Yuan *et al*.; shrimp: Sheu *et al*.; sesame: Yuan *et al*.), for example 1 pg peanut DNA in buffer is still detectable. A more practice-oriented assessment is the evaluation of sensitivity in food. This was done by Zhang *et al.* (peanut, walnut), Garrido-Maestu *et al.* (gluten), Allgöwer *et al.* (soybean), Zahnradnik *et al*. (celery), Mao *et al.* (pistachio) and Wang *et al.* (cow's milk). In order to allow a direct comparison, only sensitivities of published methods with the same reference values (DNA or food) can be considered. The method developed in this project represents a sensitivity at or below 10 mg/kg hazelnut per kg food and is therefore one of the most sensitive allergen-specific LAMP methods currently available compared to the other published LAMP systems (7.8 mg/kg: Zahradnik *et al.* (celery)[115]; 10 mg/kg: Allgöwer *et al.* (soybean)[116], Mao *et al*. (pistachio)[117]; 50 mg/kg: Garrido-Maestu *et al.* (gluten)[118]; 100 mg/kg: Zhang *et al.* (hazelnut and peanut)[119], Wang *et al.* (cow's milk)[120]; 10,000 mg/kg: Zhang *et al.* (walnut)[119]). The specificity of the currently available methods is defined by the absence of cross-reactivity to other foods (which were tested as part of the method validation). Data on 2 to 15 foods, tested for cross-reactivity, are commonly presented in the referenced literature (**Table 1**). The scope of the validation and application measurements of the hazelnut-specific

LAMP developed in this project is remarkably more extensive (except for the soy LAMP according to Allgöwer *et al.* 2020[116,121]) than for the existing methods. Intensive testing of a newly developed method is important, as otherwise, possible cross-reactivities are not identified or possible application restrictions remain undiscovered.

The detections of the currently available methods occur via real time fluorescence, agarose gel electrophoresis, colorimetric or via LFD. Agarose gel electrophoresis is time-consuming and requires a lot of equipment. Real time fluorescence detection offers the advantage of quantitative evaluation but also requires a considerable amount of equipment. LAMP in combination with a time-efficient and simple detection system is suitable as a qualitative rapid method. Therefore, colorimetry and LFD are very promising options for rapid and simple detection as they allow for a qualitative result without (colorimetric) or within a few minutes (LFD) of additional detection time. The developed hazelnut-specific LAMP was combined with a colorimetric detection and the optimized DNA extraction method according to Schäfer *et al.* 2023[122] to use it as a rapid test system. In summary, the developed method benefits from a high sensitivity and specificity, and is suitable as a fast point-of-care method, due to the easiness and fast detection of hazelnut-specific DNA.

Table 1. Overview of developed LAMP methods for the detection of various food allergens.

TARGET ALLERGEN	TARGET GENE (GENBANK NO.*, PRIMER BINDING REGION* 5'-3')	SENSITIVITY (LOWEST DETECTABLE AND INVESTIGATED CONCENTRATION)	SPECIFICITY (FOOD TESTED NEGATIVE FOR CROSS-REACTIVITIES)	APPLICABILITY (SCOPE OF TESTED SAMPLES)	DETECTION METHOD
Hazelnut (Schäfer *et al.* 2024)[114]	ITS2 (HQ442260.1, 458 to 730 bp)	at or below 10 mg/kg hazelnut in food	22 food components e.g. peanut, almond, pistachio, cashew, pecan, macadamia, soybean	15 commercial foods, 16 proficiency test samples	colorimetric or LFD
Hazelnut (Zhang et al. 2022)[119]	Cor a 14 (FJ358504.1, 170 to 389 bp)	100 mg/kg hazelnut in food	walnut, peanut	none	colorimetric
Peanut (Sheu *et al.* 2018)[123]	ITS1 (AY615267.1, 116 to 321 bp)	1 pg DNA	walnut, hazelnut, almond, cashew, macadamia nut	13 commercial foods	agarose gel electrophorese
	Ara h 1 (AF432231.1, 1216 to 1410 bp)	100 pg DNA			
Peanut (Zhang *et al.* 2022)[119]	ITS1 (AY615267.1, 116 to 321 bp)	100 mg/kg peanut in food	walnut, hazelnut	none	colorimetric
Peanut (Yuan *et al*. 2018)[124]	Ara h 6 (EF609643.1, 720 to 920 bp)	2,000 pg DNA	soybean, sesame, pistachio, almond, hazelnut, walnut, mustard	16 commercial foods	colorimetric
Walnut (Zhang *et al*. 2022)[119]	Jug r 1 (U66866.1, 151 to 327 bp)	10,000 mg/kg walnut in food	peanut, hazelnut	none	colorimetric
Gluten (Garrido-Maestu *et al.* 2018)[118]	α2-gliadin (AJ133612.1, 559 to 847 bp)	50 mg/kg wheat in food	wheat, spelt, rice, corn, rice	5 commercial foods	real time fluorescence
Soybean (Allgöwer *et al.* 2020)[116,121]	ORF160b (JX463295.1, 3488077 to 349159 bp)	10 mg/kg soy in food	33 different food components, e.g. peanut, chickpeas, kidney pinto bean, lentil, pea, lupine	12 commercial foods	real time fluorescence, agarose gel electro-phoresis or LFD

TARGET ALLERGEN	TARGET GENE (GENBANK NO.*, PRIMER BINDING REGION* 5'-3')	SENSITIVITY (LOWEST DETECTABLE AND INVESTIGATED CONCENTRATION)	SPECIFICITY (FOOD TESTED NEGATIVE FOR CROSS-REACTIVITIES)	APPLICABILITY (SCOPE OF TESTED SAMPLES)	DETECTION METHOD
Soybean (Yuan *et al.* 2018)[124]	Gly m Bd 28 K (EU493458.1, 811 to 1031 bp)	2,000 pg DNA	peanut, soybean, pistachio, almond, hazelnut, walnut, mustard	16 commercial foods	colorimetric
Celery (Zahradnik *et al.* 2014)[115]	Mannitol dehydrogenase (AF067082, 4629 to 4851 bp)	80 pg DNA, 7.8 mg/kg celery in foods	parsley, carrot, anise, dill, fennel, caraway, coriander, cumin, lovage, parsnip	10 commercial foods	Colorimetric, real time fluorescence or agarose gel electro-phoresis
Shrimp (Sheu *et al.* 2020)[125]	16S rRNA (KT596762, 44 to 261 bp)	10 pg DNA	crab species, lobster	14 commercial foods	agarose gel electrophoresis
Pistachio (Mao *et al.* 2020)[117]	Pis v 1 (DQ631675.1, 328 to 534 bp)	10 pg DNA, 10 mg/kg pistachio in food	cashew, mango, walnut, almond, macadamia nut, hazelnut, peanut, soybean	10 commercial foods	colorimetric or agarose gel electrophoresis
Cow's Milk (Wang *et al.* 2022)[120]	cytochrome b (OR777285.1, 14964 to 15158)	0.010 pg DNA, 100 mg/kg cow's milk in food	goat, pork, chicken, duck, dog	4 commercial foods	colorimetric or agarose gel electrophoresis
Sesame (Yuan *et al.* 2018)[124]	2 S albumin (AF240005.1, 327 to 522 bp)	2,000 pg DNA	peanut, soybean, pistachio, almond, hazelnut, walnut, mustard	16 commercial foods	colorimetric

*NCBI Database GenBank (www.ncbi.nlm.nih.gov)

The possibility of simple and rapid on-site detection is important for food manufacturers to identify allergen carry-over and to contribute significantly to food safety through optimized allergen management. Rapid methods are on the one hand valuable detection methods for manufacturers and on the other hand useful screening methods for high throughput testing for monitoring by food authorities. In case of a positive qualitative result obtained during the screening process, a detailed quantitative verification can be carried out. This strategy saves time, material and effort compared to analyzing all samples in detail directly.

The most important aspects of screening methods are time efficiency, simplicity and cost. To date, the LFD is the most frequently used screening tool, due to the short performance time of several minutes and the simple application without complicated devices.[126] LFD combine thin-layer chromatography with immuno-detection and are often used in form of strip tests. The solute target molecule is deposited at the end of the strip, where it comes into contact with two capture molecules. These capture molecules are often antibodies with modifications like biotin and bind to the target molecule. The solution containing the complex of target and capture molecules passes through the test strip due to capillary forces until it encounters an immobilized molecule (e.g. avidin) that specifically binds the capture molecule via its label. As a result, the complex is focused on the test line. The modification of the second capture molecule is applied to detection. Therefore, gold nanoparticles can be used which produce a visual band by enriching these products on the test line.[127]

To the best of my knowledge, there are only three published tests that were newly developed for the detection of hazelnut in food using the antibody-based LFD.[128–130] The general performance of these three hazelnut LFDs does not differ fundamentally, but the validation work of the LFD of Civera *et al.* is more comprehensive than of those of Ross *et al.* and Anfossi *et al*. In order to allow an assessment of the developed LAMP test as an alternative method in the context of currently existing LFDs, an overview of the colorimetric DNA-based test, developed in this project, and the antibody-based method according to Civera *et al.* is therefore shown in Table 2. Like almost all analytical methods, both types of test methods have advantages and disadvantages. Protein extraction is faster than DNA extraction which results in shorter analysis time using the LFD method. However, potential cross-reactivity of the antibodies remains to be a drawback compared to nucleic acid-based detection methods such as LAMP. In principle, both methods are simple to perform, although the risk of cross-contamination from previous analysis is generally higher for DNA detection than for protein detection methods. The determined sensitivities of both tests are in a similar range, whereby the LAMP method has the major advantages of better standardization possibilities and ethical superiority.

Table 2. Overview of key criteria of the colorimetric LAMP-based method according to Schäfer *et al.* 2024b and the antibody-based LFD method according to Civera *et al.* 2023.

KEY TEST CRITERIA	COLORIMETRIC HAZELNUT LAMP METHOD Schäfer *et al.* 2024b[114]	ANTIBODY-BASED HAZELNUT LFD METHOD Civera *et al.* 2023[129]
Detection target	hazelnut-specific ITS2 region	hazelnut allergen Cor a 9
Detection method	colorimetric tube reaction or lateral flow device (visual evaluation)	lateral flow device (visual evaluation)
Sensitivity	1.63 mg/kg hazelnut protein[1] (colorimetric detection) and 0.16 mg/kg hazelnut protein[1] (LFD detection) in baked muffins	0.6 mg/kg hazelnut protein in baked cookies
Specificity	cross-reactivity study - negative to 22 foods - positive to 0 foods, including walnut and pecan nut	cross-reactivity study - negative to 72 foods - positive to 2 foods (walnut, pecan nut)
Applicability	successfully tested on complex food matrices	successfully tested on complex food matrices
Execution time	under one hour	within 15 minutes
Implementation complexity	simple	very simple
Ethical concerns	No, no animal experiments necessary	Yes, due to animal-derived antibodies being used
Possible standardization	Yes, primer production by chemical synthesis	Limited due to polyclonal antibody batch differences

[1]hazelnut in a muffin matrix was positively detected via the hazelnut-specific DNA region and calculated into hazelnut protein based on the hazelnut protein content of 16.3%

Commercial hazelnut rapid tests are available from various suppliers such as bioavid Lateral Flow Hazelnut (R-Biopharm AG, LOD: 10 mg/kg), AgraStrip® Pro Allergen Hazelnut (Romer Labs, LOD: 5 mg/kg), Reveal® 3-D for Hazelnut (Neogen, LOD: 5-10 mg/kg) or AllerTrace Hazelnut LFD (Biofront Technologies, LOD: 1 mg/kg). The LODs are in a typical sensitivity range of approx. 1 to 10 mg/kg, making the developed colorimetric LAMP method competitive with established rapid tests on the market. In 2009, Röder *et al.* tested three such hazelnut specific LFDs in an independent study regarding decisive criteria such as sensitivity, specificity or applicability.[131] The sensitivities as declared by the manufacturer have been confirmed. However, in all three LFDs, false-positive results were obtained with walnut using polyclonal antibodies as binding molecules. This phenomenon has also been reported in other research studies.[102,103] As part of the specificity testing of the colorimetric LAMP, no false-positive results from phylogenetically related foods such as walnut were obtained. Among 22 food components, hazelnut as the target allergenic food led to a true-positive result, whereas all other investigated foods tested true-negative (SI Fig. 2 in Schäfer *et al*. 2024b[114]). As a

conclusion, the colorimetric LAMP developed in this study offers an antibody-free alternative to the reliable antibody-based LFDs for the analysis of raw materials, intermediary or final products or to check routine purification steps on-site. Moreover, the method can also be used as a simple screening technique to identify those positive samples which potentially require quantitative verification with suitable methods. Using qPCR for subsequent quantification, the same extracted DNA can be used to speed up the combined analysis of screening and quantitative evaluation of positive samples. The developed method thus represents a significant advancement in the field of allergen analysis, which in turn will contribute to an enhancement of food safety.

Are Nucleic acid-based Reagents the Future for Allergen Detection Methods?

Antibodies represent the most commonly utilized class of reagents for detection methods.[22] Antibody-based methods have a high sensitivity and specificity, but are associated with ethical concerns due to the need for test animals. In addition, the analytical disadvantage of batch-dependent variations is often also critisized.[16,21–23,28] By contrast, nucleic acid-based tests represent alternative methods without these ethical and analytical disadvantages (**Table 3**). Nucleic acid based-reagents provide the benefits of a) *in vitro* synthesis (batch-to-batch consistency and high purity with low costs could be guaranteed), b) stability and suitability for flexible use in various point-of-care environments (nucleic acids are generally more stable than protein animal-derived antibodies, which can be denatured under certain circumstances), c) reduced use of animals (screening of aptamers or development of LAMP primers does not require animals), d) transparency and standardizability (once developed, nucleic acid reagents with publicly available sequences can be easily identified, produced and used by any researcher) and e) cost effectiveness due to simple chemical synthesis (production of monoclonal antibodies requires more complex biological systems and hence higher costs).[79,95,132]

Table 3. Comparison between the use of antibodies and nucleic acid-based reagents in detection systems with regard to the the characteristics of "composition", "target molecules", "generation and synthesis", and "stability".

	ANTIBODIES	**NUCLEIC ACID-BASED REAGENTS**
Composition	proteins	nucleic acids
Target molecules	usually proteins	any type of target if binding to nucleic acids is possible (aptamer) and a corresponding amount and information of target (LAMP primers) are available
Generation and Synthesis	*in vivo* selection, animals required	*in vitro* selection, no animals required
	biosynthesis in animals or by recombinant technology	chemically synthesized
	batch-to-batch variations in animal-derived preparations	batch-to-batch consistency
Stability	storage and transportation may require special cooling conditions	storage and transportation without special cooling conditions

In this research project it has been demonstrated that it is generally possible to replace antibodies in detection methods by using alternative nucleic acid molecules (**Figure 5**). The potential of aptamers as a substitute for antibodies was illustrated in an ELISA-like assay system (**Figure 5a**) and a Western blot (**Figure 5b**). In both instances, the detection of the

peanut allergen Ara h 1 was unambiguously possible. Furthermore, allergenic food detection can be achieved through the utilization of nucleic acid-based LAMP primers (**Figure 5c**), thereby reducing the necessity for antibodies. The reduction of antibody-based LFD methods is also feasible through the implementation of a LAMP reaction (LAMP-LFD) (**Figure 5d**). The detailed assessment with regard to relevant parameters such as sensitivity and specificity is provided in Chapters II and III. The overview clearly illustrates the availability and possibilities of antibody alternatives.

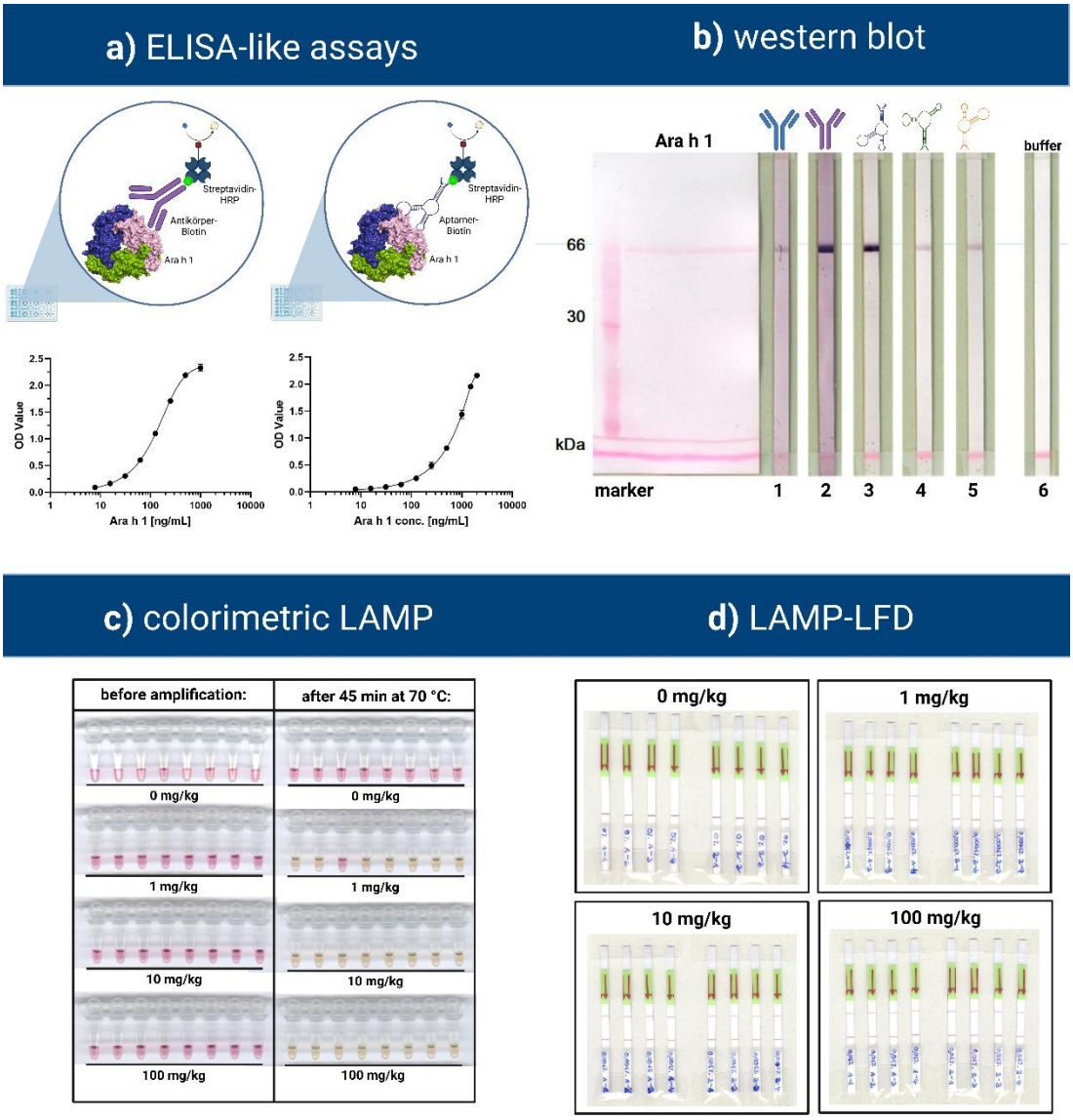

Figure 5. Replacement of antibodies by nucleic acid-based reagents in different allergen detection methods. **a**) Antibody-based ELISA vs. Aptamer-based ELAA for the detection of Ara h 1. **b**) Ara h 1 western blot analysis using monoclonal antibodies (lines 1 and 2) and aptamers (lines 3, 4 and 5) as well as buffer as negative control (line 6). **c**) Colorimetric LAMP for the detection of four different concentrations of hazelnuts in muffins (0, 1, 10 and 100 mg/kg) before (left) and after (right) amplification. **d**) LAMP-LFD for the detection of four different concentrations of hazelnuts in muffins (0, 1, 10 and 100 mg/kg).

The list of benefits of using aptamers as antibody alternatives is long, the protocols are very similar to those of antibody use and the equipment is the same and already available in laboratories. Despite all the benefits, these nucleic acid-based techniques are not yet the method of choice or the current gold standard, especially in the field of allergen detection. The reasons being discussed among scientists that hamper the gradual replacement or supplementation of antibodies by aptamers are the lack of awareness, familiarity of antibodies and high amount of money that has been invested in antibody research.[24,133] The key points and current challenges in the use of aptamers for allergen detection are a) the availability of data regarding practical applications (published aptamer developments often have a lack of comprehensive validation data comparable to ELISA methods)[134,135], b) the integration into commonly used detection platforms such as ELISA-like assays, existing workflows and infrastructures[136] and c) the regulatory acceptance, especially in the medicinal field (antibody-based methods are well-established and accepted in the regulatory field. New methods such as aptamer-based methods require regulatory review in order to be widely accepted in the future)[137]. Furthermore, confidence in alternative methods only grows through experience and routines. Finally, it should be mentioned that monoclonal antibodies have taken around 40 years to successfully establish themselves on the commercial market.[24,137] Transferred to the duration of aptamer research, it could simply take some more years of research, validation and increase of awareness in order to achieve a comparable establishment.[24]

The utilization of LAMP-based methodologies in the commercial and regulatory context is already at an advanced stage. Current challenges for LAMP-based methods are the need to avoid contamination with LAMP amplicons from previous assays through appropriate safety measures, and the more complex sample preparation for DNA isolation compared to protein extraction for protein-based methods. Despite these potential challenges compared to existing antibody-based methods, the many advantages of the specific and sensitive LAMP technology are so convincing that LAMP-based tests are available on the commercial market (for example commercial LAMP kits from providers like New England Biolabs or ThermoFisher Scientific). Moreover, in Germany, PCR methods, which target DNA, have been applied for many years in the Official Collection of Methods of Analysis (ASU) according to §64 LFGB for a series of allergenic foods.[99,138] LAMP-based methods are not yet represented.

Both, aptamer and LAMP primers, offer significant advantages as nucleic acid-based alternative molecules in detection methods. However, before these molecules can become a new standard in allergen detection, some research and technological improvements are needed to ensure reliable and regulatory acceptable results.

Prospects and Concluding Remarks

This work demonstrated that aptamers can be suitable synthetic binders for the specific detection of allergenic proteins in complex food matrices as demonstrated in the first published microplate-based aptamer assay for the major peanut allergen Ara h 1. Further work should refine the conditions to improve the sensitivity of the developed aptamer microplate assay to meet those levels as required by WHO and FAO.

The developed hazelnut LAMP demonstrated high sensitivity, specificity, and compatibility with complex food matrices. It could serve as an easy-to-use screening tool for cut-off-based identification of only those positive samples that would require further quantitative evaluation with more elaborate techniques such as qPCR, ELISA or MS.

In addition to the intensive development on the antibody-free detection of peanut and hazelnut described in this thesis, further work was carried out regarding aptamer development against hen's egg and cow's milk. A peanut-specific LAMP method was also developed. These data will be evaluated, assessed and possible publication approaches discussed in order to provide transparent and standardizable methods for allergen detection.

Finally, the results of this research project, in conjunction with those of numerous other researchers, demonstrate that both types of nucleic acid reagents possess considerable potential that is worth to be explored in more detail to finally reduce and replace animal derived reagents in allergen detection as soon as possible.

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DNA Meets Protein—Development, Characterization, and Application of Aptamers against Peanut Allergen

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ABSTRACT: Allergen detection methods support food labeling and quality assessment at the allergen component level of allergen preparations used for allergy diagnosis and immunotherapy (AIT). Commonly applied enzyme-linked immunosorbent assay (ELISA) requires animal antibodies but potentially shows batch variations. We developed synthetic aptamers as alternative binders in allergen detection to meet the replacement, reduction, and refinement (3R) principle on animal protection in science. ssDNA aptamers were specifically selected against the major peanut allergen Ara h 1 and identified by next-generation sequencing. Application in various detection systems (ELISA-like assays, western blot, and surface plasmon resonance) was demonstrated. The ELISA-like assay comprised a sensitivity of 10 ng/mL Ara h 1, comparable to published antibody-based ELISA, and allowed Ara h 1 detection in various peanut flours, similar to those used in peanut AIT as well as in processed food. This ELISA-like aptamer-based assay proofs antibody-free allergen detection for food labeling or quality assessment of diagnostic and therapeutic allergen products.

KEYWORDS: *ssDNA aptamer, peanut allergen, Ara h 1, systematic evolution of ligands by exponential enrichment (SELEX), enzyme-linked aptamer assay (ELAA)*

INTRODUCTION

Peanut allergies are a health risk that affects children and adults worldwide. In Europe, 0.1−1.8% of all people suffer from peanut (*Arachis hypogaea*) allergy.[1] While some food allergies are lost over time, peanut allergies can persist for a lifetime.[2] Furthermore, most severe and life-threatening anaphylaxis is observed in peanut-allergic patients.[1] Currently, there is only one United States Food and Drug Administration (FDA)-approved and European Medicines Agency (EMA)-approved immunotherapeutic medicinal product available for the treatment of peanut allergy in children and adolescents. This causative therapy can still lead to adverse reactions during the course of therapy and afterward, because a lifelong supply of a maintenance dose is required. Moreover, a complete cure of the disease does not seem achievable, but an increase of the level of tolerance seems achievable, ideally supporting peanut allergic subjects to avoid allergic reactions to very low amounts of peanuts in food. Independently, a strict avoidance of peanut as the allergenic food remains necessary to prevent allergic episodes.[3,4] This requires allergen identification by allergic consumers, which makes allergen labeling of foods essential. Allergen detection methods are substantial to ensure unambiguous allergen labeling of food according the European Regulation (EU) 1169/2011.[5] In addition, analytical methods are indispensable in the quality assessment of medicinal products. Diagnostic and therapeutic allergen preparations may require quality assessment at the allergen component level according to the *European Pharmacopoeia* (Allergen Products, Monograph 1063).[6]

A variety of allergen detection methods are based on an antibody−antigen reaction, such as the enzyme-linked immunosorbent assay (ELISA) or lateral flow device (LFD). However, the antibodies that have been applied for decades also have some disadvantages. Two major drawbacks are their ethically questionable *in vivo* generation and possible batch-to-batch variations, because antibodies generated in animals are subject to biological variations. European Directive 2010/63/EU requires the replacement of procedures using living animals as soon as alternatives are available according to the internationally recognized principle of replacement, reduction, and refinement (3R principle).[7]

The use of synthetic aptamers is a promising approach to replace or complement antibodies in detection systems for allergens. Aptamers are oligonucleotides (single-stranded DNA or RNA) that can bind specific target molecules and are routinely selected by the process of systematic evolution of ligands by exponential enrichment (SELEX).[8,9] Once the specific aptamer sequences were identified, they could be produced *in vitro* by chemical synthesis. This eliminates the need for animal experiments and enables standardization, because batch-to-batch variations can be reduced to a minimum.

In recent years, the development of specific aptamers for the detection of proteins in assay systems has progressed.[10−14] Also, aptamers against the peanut allergen Ara h 1 have been

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described,[15,16] which have been used in various biosensor-based systems.[15−20] A recent development by Stidham et al. describes the detection of 12.5 mg/kg of peanut protein using a microchip-based device.[16] These Ara h 1 aptamer-based detection methods have been designed as biosensors to date. However, biosensor systems, which are partly complex methods, require the use of expensive microchip equipment along with specialized expertise. In allergen analysis, ELISA has been the most widely used test method to date.[21] The aim of this study was to combine the advantages of aptamers, such as ethics and potential for standardization, with the simple and widespread performance of ELISA-like techniques. Furthermore, the broader application of our newly developed aptamers is demonstrated by the use of western blot and surface plasmon resonance (SPR) methods in addition to ELISA-like techniques of various designs.

MATERIALS AND METHODS

Aptamer Selection Process. The single-stranded DNA (ssDNA) library, containing a randomized region of 40 nucleotides flanked by two constant primer-binding regions of 23 nucleotides each (5′-TAGGGAAGAGAAGGACATAT-N_{40}-TTGACTAGTACATGACCACTTGA-3′, TriLink Biotechnologies via tebu-bio, Offenbach am Main, Germany), was used for separate aptamer selection against natural purified allergen, i.e., against either Ara h 1, Ara h 2, or Ara h 3 (sections M1 and M2 of the Supporting Information). Selection buffer consisted of phosphate-buffered saline (PBS) at pH 7.1 with 1 mM $CaCl_2$ (Merck, Darmstadt, Germany), 1 mM $MgCl_2$ (Sigma-Aldrich via Merck), and 1 mg/mL bovine serum albumin (BSA, Sigma-Aldrich) at pH 7.1. For the initial SELEX round, 500 pmol of ssDNA library in 100 μL of selection buffer was used, heated at 95 °C for 5 min, and subsequently cooled to room temperature (RT). A total of 50 μL of the prepared magnetic beads (section M3 of the Supporting Information) was washed twice with 250 μL of PBS and 3 times with 250 μL of selection buffer before incubation with the library. Incubation took place in a total volume of 200 μL of selection buffer at room temperature. Sedimentation of the magnetic beads was prevented by regular manual resuspension. Subsequent removal of unbound sequences was performed by washing 3 times for 5 min with a selection buffer. Elution of the bound sequences was carried out with 100 μL of nuclease-free, sterile, diethylpyrocarbonate (DEPC)-treated, autoclaved water (Carl Roth, Karlsruhe, Germany) at 95 °C for 5 min. The ssDNA libraries were incubated with non-coupled magnetic beads from round 3 for 30 min (counter SELEX). The selection pressure was increased over 10 SELEX rounds by reducing the incubation time from 30 to 5 min and the amount of target protein-coupled magnetic beads from 50 to 7.5 μL. From round 2 onward, only 10 pmol of ssDNA from the previous SELEX round was used (Table S1 of the Supporting Information). Amplification of the enriched sequences was performed by real-time polymerase chain reaction (PCR) using the AriaMx real-time PCR thermocycler (Agilent Technologies, Waldbronn, Germany). One reaction consisted of 10 μL of Luna Universal qPCR Master Mix (New England BioLabs, Frankfurt am Main, Germany), 0.5 μL each of biotinylated forward primer 5′-biotin-TAGGGAAGAGAAGGACATATGAT (tebu-bio) and phosphorylated reverse primer 5′-phos-TCAAGTGGTCATGTACTAGTCAA (biomers, Ulm, Germany), 4 μL of nuclease-free, sterile, DEPC-treated, autoclaved water (Carl Roth), and 5 μL of the eluted ssDNA sequences. The PCR program included a single initial denaturation at 95 °C for 60 s, denaturation at 95 °C for 15 s, and extension at 60 °C for 30 s. Amplification was stopped as late as possible and as early as necessary to avoid unwanted byproducts (Table S1 of the Supporting Information). PCR products were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. The concentration was measured with a NP80 NanoPhotometer (Implen, Munich, Germany). To generate ssDNA, the phosphorylated antisense strand was digested by lambda exonuclease (Thermo Fisher Scientific, Frankfurt am Main, Germany) at 37 °C for 30 min, followed by an inactivation step at 80 °C for 15 min using a SimpliAmp thermocycler (Thermo Fisher Scientifc). The ssDNA was then purified using NucleoSpin Gel and PCR Clean-up with the ssDNA-compatible NTC buffer (Macherey-Nagel) according to the instructions of the manufacturer. Finally, the ssDNA concentration was determined with a NP80 NanoPhotometer (Implen). After 10 selection rounds, the sequences were identified by NGS. The experimental details are given in section M4 of the Supporting Information.[22]

Protein Extraction of Food Samples. A 2.0 g portion of the homogeneous food sample (sections M5 and M6 of the Supporting Information) was weighed into 50 mL tubes (Greiner bio-one, Frickenhausen, Germany) and mixed with 40 mL of extraction buffer. Selection buffer (for analysis via SPR) and PBS including 500 mM NaCl (for assay applications) were used as extraction buffers. Extraction was performed in a water bath with frequent mixing at 45 °C for 10 min for western blot and SPR analysis and at 99 °C for 1 min for all other assay experiments (the buffer was preheated in any cases). Because Ara h 1 is stable to heat, the shortest possible extraction time of 1 min at 99 °C was chosen without impairment of ELAA sensitivity compared to lower temperatures (45−70 °C) at extended extraction times (10−20 min) (details not shown). The sample was subsequently placed on ice. Next, extracted samples were centrifuged at 4000*g* for 12 min at 4 °C. The obtained supernatant was filtered through a 5 and 0.45 μm Minisart NML hydrophilic syringe filter (Satorius, Göttingen, Germany), and finally, its protein concentration was determined using ROTI Nanoquant (Carl Roth) following the instructions of the manufacturer.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Aptamer-Based Detection via Western Blot. Ara h 1 (2 μg of protein/cm of gel lane; sections M1 and M2 of the Supporting Information) was run in 12% SDS−PAGE under reducing conditions using polyacrylamide gel 30 (Carl Roth) according to the instructions of the manufacturer and semidry blotted onto a nitrocellulose membrane (0.2 μm pore size, Cytiva, Uppsala, Sweden). Low-molecular-weight marker (Amersham GE Healthcare) and 5× sample buffer including dithiothreitol (DTT)[23] were used. The electrophoresis was performed at 150 V for 90 min, and the blotting was performed at 0.8 mA/cm^2 for 1 h. Transferred proteins were stained for 10 min using 0.1% Ponceau S in 5% acetic acid solution. The 0.3 cm wide membrane strips were blocked with 0.3% Tween 20 in tris-buffered saline (TBS) for 90 min after Ponceau S staining with several washes in TBS buffer. The bound 5′-biotinylated aptamers (custom synthesized by biomers.net) were detected with alkaline phosphatase-labeled streptavidin (Thermo Fisher Scientific) at a dilution of 1:100 000 in selection buffer and visualized by the addition of nitro blue tetrazolium (NBT, Sigma-Aldrich) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma-Aldrich) in TBS−alkaline phosphatase (AP) buffer. The reaction and, thus, band development were stopped after 10 min by washing the strips with ultrapure water.

Aptamer-Based Assays. Unless otherwise specified, Maxisorp Nunc-Immuno plates (Thermo Fisher Scientific) were used and washing steps were performed with 300 μL of selection buffer/well 4 times. All experiments were performed at least in technical duplicates and at room temperature. Aptamer sequences were labeled with a 5′-biotin modification, custom synthesized by biomers.net with a purification via high-performance liquid chromatography (HPLC). The software GraphPad Prism (version 9.5.0), BioRender.com, mfold, and the *R* environment (versions 3.5 and 4.2) were used to analyze or visualize the results (section M7 of the Supporting Information).

Indirect Enzyme-Linked Aptamer Assay (ELAA). A 100 μL portion of the target protein (Ara h 1 or extracts of food samples; sections M1, M2, M5, and M6 of the Supporting Information) diluted in PBS at pH 7.1 was coated on a microtiter plate for 1 h. Afterward, the wells were washed with PBS and 0.05% Tween 20 (PBST) and blocked with 1.5% BSA in PBS for 1 h. Then, 100 μL of 100 nM biotinylated aptamer diluted in selection buffer was added. Following 1 h of incubation, the plate was washed to remove unbound aptamers.

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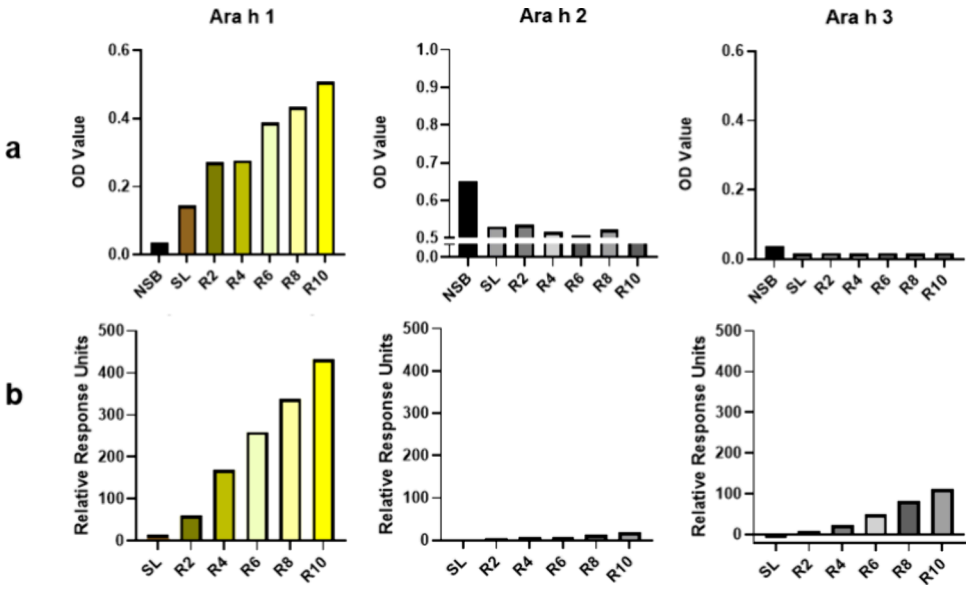

Figure 1. Avidity measurements of different SELEX rounds (SL, start library; R, round) for Ara h 1, 2, and 3 using (a) indirect ELONA (NSB, non-specific binding of the enzyme−conjugate using PBS buffer instead of the ssDNA library or reamplified sequences) and (b) SPR.

Subsequently, 100 μL of streptavidin−horseradish peroxidase (HRP) conjugate (Southern Biotech via Biozol, Eching, Germany), prediluted 1:50 000 in selection buffer, was added to each well and incubated for 1 h. Instead of the streptavidin−HRP conjugate, also, 100 nM HRP-labeled probe (HRP-5′-TCAAGTGGTCATGTA-CTAGTCAA-3′), which consists of 23 nucleotides and binds at the 3′ end of the aptamer, was investigated for aptamer detection. The plate was washed again and afterward incubated with 100 μL of substrate solution (1 mM TMB and 3 mM H_2O_2 in citrate buffer at pH 3.9) for 1 h. After the color development was stopped with 100 μL of sulfuric acid (3 M, Merck) after 10 min, the optical density (OD) was measured at 450 and 630 nm as background correction using SpectraMax ABS using the evaluation software SoftMax Pro 7.1 (Molecular Devices, Munich, Germany).

Sandwich Enzyme-Linked Aptamer Assay (Sandwich ELAA). For the analysis of Ara h 1 in a sandwich-type ELAA, 100 μL of 5 μg/mL concanavalin A (Thermo Fisher Scientific) in PBS was coated for 2.5 h in a 96-microwell plate. After washing, blocking was performed with 1.5% BSA in PBS overnight to avoid non-specific binding. The plate was washed again. Subsequently, 100 μL of Ara h 1 was added at concentrations ranging from 2000 to 7.81 ng/mL (diluted in a selection buffer) and incubated for 2 h. Unbound Ara h 1 was removed by washing. Then, 100 μL of the biotinylated detector aptamer LS_Arah1_367 (100 nM) was added to each well. The subsequent washing steps, incubation of the conjugate as well as stopping, and OD measurement were carried out as described for the indirect ELAA (see above). The enzymatic reaction was stopped after 15 min.

RESULTS AND DISCUSSION

Major Peanut Allergens Ara h 1, 2, 3, and 6 Were Purified and Verified. The purity of Coomassie-stained natural peanut allergens is displayed after a SDS−PAGE separation of 1 μg per lane under reducing conditions (Figure S1 of the Supporting Information). Identity and potential impurities of allergen preparations were further analyzed using mass spectrometry. The presence and identity of the allergens Ara h 1, 2, 3, and 6 were confirmed beyond a doubt in the main bands of the gel. The main mass spectrometric key figures, protein score (S, between 635 and 17 779), number of peptides detected (P, 6−83), sequence coverage (SC, 21.5−61.4%), mean precursor mass error (PE, 0.5−7.3 ppm), and mean fragment mass error (FE, 5.3−12.0 ppm) clearly showed this. Details of detected proteins are shown in Table S2 of the Supporting Information along with all mass spectrometric key parameters. For Ara h 1 and Ara h 3, the presence of multiple sequence variants or isoforms was confirmed. Because the elucidation of sequence variants is not the main focus of this study, we refrain from a detailed presentation of these results. Only the detected sequences are briefly mentioned here. For Ara h 1, the sequences with UniProt accession numbers P43238 and P43237 were detected. For Ara h 3, PEI009 (rAra h 3 wild type, on the basis of NCBI GenBank accession numbers AAC63045 and AAD47382), O82580 (Ara h 3.0101), Q9SQH7 (Ara h 3.0201), and Q647H2 (Arachin) were detected.

Additional weaker bands included Ara h 1-related sequence hits in the Ara h 1 preparation, Ara h 3-related sequence hits in the Ara h 3 preparation, and Ara h 6-related sequence hits in the Ara h 6 preparation, respectively. The purified allergens occasionally contained traces of other allergens. For example, Ara h 1 could also be detected within the Ara h 2 preparation in weak bands below 20 kDa. In comparison of the intensity of these bands in the SDS−PAGE gel to the main band of Ara h 1 and also, e.g., the number of Ara h 1 peptides detected therein (83 peptides in band 1 versus 1−2 peptides in weak bands below band 2.1), it can be assumed that the content of Ara h 1 in the Ara h 2 preparation is at least 2 orders of magnitude lower than the content of Ara h 2. A similar assumption applies to other occasionally detectable proteins, such as human keratin. In summary, mass spectrometry analysis verified the identity and purity of purified natural peanut allergens to allow for the assessment and confirmation of specificity of aptamer binding to these allergens.

ssDNA Aptamers against Peanut Allergen Were Selected. For the selection of affine aptamers, the target proteins Ara h 1, 2, and 3 were separately coupled to magnetic beads. During the coupling reaction, 56 μg of Ara h 1, 61 μg of Ara h 2, and 107 μg of Ara h 3 per milliliter of magnetic beads were immobilized via a covalent bond. The magnetic beads were coupled with either Ara h 1, Ara h 2, or Ara h 3 and

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separately incubated with a ssDNA library, consisting of approximately 10^{15} unique sequences, to generate specific aptamers within 10 SELEX rounds. To enrich the strong binding sequences, the stringency was increased by reducing the target amount and incubation time during the entire SELEX process. In addition, counter SELEX was introduced from round 3 to avoid non-specific binding (Table S1 of the Supporting Information). After completion of the SELEX process, the increase in avidity of the ssDNA pools after different SELEX rounds compared to the start library was examined by indirect enzyme-linked oligonucleotide assay (ELONA) and SPR (experimental details are given in sections M8−M10 of the Supporting Information). The sequences of the ssDNA pools included a biotin modification as a result of a biotin-labeled forward primer in the PCR during the SELEX process and the reamplification reaction. Thus, a streptavidin−HRP conjugate served for detection of the specific protein sequence binding. Non-specific binding (NSB) of the enzyme−conjugate in ELONA was controlled using PBS buffer instead of the ssDNA library or reamplified sequences. An increase in OD values from the start library to the ssDNA pool of the final SELEX round was measured, which is directly related to the increasing avidity of the ssDNA pools targeting Ara h 1 during the entire SELEX process. There was no detectable increase in OD between the start library and the individual ssDNA pools targeting Ara h 2 and 3 (Figure 1a). Verification of the enrichment of specific sequences by SPR showed similar results. The binding avidity of the Ara h 1-specific ssDNA pools increased, as indicated by higher response units from the starting point to the last round of selection. There was no signal increase for all ssDNA pools for Ara h 2, and only a slight trend was seen for Ara h 3 ssDNA pools (Figure 1b). An increase in avidity indicated the enrichment of strongly binding sequences. According to Famulok and Mayer, the statistical success rate in selecting specific aptamers against proteins is less than 30%.[24] This rate is in agreement with our findings, because one of the three proteins resulted in successful aptamer selection. Therefore, the ssDNA pools against Ara h 1 were further sequenced by NGS to identify the most affine aptamers.

Specific Aptamers against Ara h 1 Were Identified and Further Characterized. An in-house NGS method was developed for sequencing the enriched ssDNA pools. We made use of semi-quantitative nested PCR reactions to amplify and barcode the bound oligonucleotide fractions of the consecutive SELEX rounds. Non-specific PCR products and PCR artifacts (e.g., primer dimers) were selected against using agarose gel purifications between the nested PCR reactions. These procedures have been shown to increase sensitivity and reduce biases in candidate sequence identification. This lowers the error rate of bias in the sequence distribution of the SELEX ssDNA pools.[25] The introduction of different barcode sequences at both the 3′ and 5′ end of each ssDNA pool sequence (double indexing) allowed parallel sequencing of ssDNA pools from all SELEX rounds simultaneously. Double indexing also increased the assurance of the correct assignment of the samples.[26] After normalization of the numbers of sequences, an increase of individual sequence families was observed from SELEX rounds 6−9 (Figure 2). A sequence family represents identical sequences with a maximum of six mismatches. Figure S2 of the Supporting Information illustrates the sequence homologues within a sequence family of the 10 or 5 most enriched sequences (top 10 or top 5),

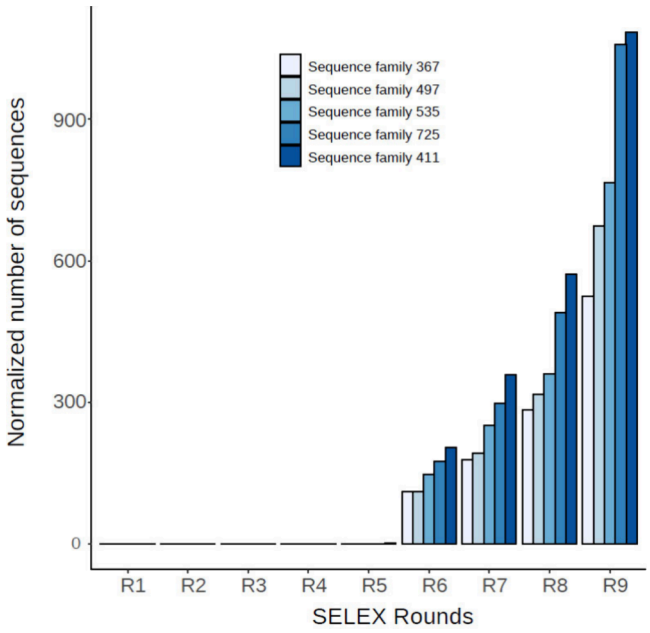

Figure 2. Enrichment in the normalized number to the total number of sequences of the top 5 sequence families against Ara h 1, allowing six mismatches, from SELEX round 1 (R1) to round 9 (R9).

showing the nucleotides at the different positions and, therefore, the possible locations of the mismatches. This allows for conclusions to be drawn concerning which aptamer binding region is most likely to be responsible for the strong binding to Ara h 1.

After identification of the sequences, the 10 most highly enriched sequences (most abundant in terms of copy numbers) were purchased from a commercial provider (Table S3 of the Supporting Information) and tested in indirect ELAA (data not shown). The top three aptamers LS_Arah1_367, LS_Arah1_535, and LS_Arah1_725 showed the highest OD value results and were further characterized. For determination of dissociation constants (K_D) and analysis of specificity regarding further peanut allergens, aptamers were immobilized via their 5′-biotin modification onto a neutravidin-coupled chip to measure their interactions with Ara h 1, 2, 3, and 6 using SPR (section M11 of the Supporting Information). On the basis of the binding kinetics between aptamer and Ara h 1, dissociation constants from 88 to 988 nM were calculated. Furthermore, no or negligible cross-reactivities to Ara h 2, 3, and 6 in SPR as well as in indirect enzyme-linked aptamer assay (ELAA) were observed (Table 1 and Figures S3 and S4 of the Supporting Information).

The predicted most stable secondary structures and corresponding values of Gibbs free energy (ΔG) of the three characterized aptamers were determined using mfold (Figure S5 of the Supporting Information).[27] Secondary structure prediction shows that the constant regions are partially involved in the hairpin structures. Furthermore, a shortened version of the aptamer LS_Arah1_367 (only 40 nt long random region) did not show better binding in the assay system (data not shown). Therefore, the full-length sequence was selected for the application experiments. Furthermore, a scrambled version of the aptamer LS_Arah1_367 was used, which shows no significant binding to Ara h 1, 2, 3, and 6 in indirect ELAA, which underlines the specific binding between

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Table 1. Identified Aptamer Sequences and Their Properties, Such as the Normalized Number of Sequences (Read Outs), Dissociation Constants (K_D), Cross-Reactivities to Ara h 2, 3, and 6, and the Calculated Gibbs Free Energy (ΔG) Value for the Secondary Structure Using Mfold[27]

designation	aptamer sequence (5′−3′)	read outs in SELEX round 9	K_D (nM)	cross-reactivities to Ara h 2, 3, and 6	ΔG (kcal/mol)
LS_Arah1_367	TAGGGAAGAGAAGGACATATGATATTCGGGTTGGCTTTGGGTTGGCGTCCTCAGACTCTATACTTGACTAGTACATGACCACTTGA	367	88 ± 1	non	−5.05
LS_Arah1_535	TAGGGAAGAGAAGGACATATGATTGCGCGAGACCTTCTCCTTCGGCTTAGTCCCCCAAACACCTTGACTAGTACATGACCACTTGA	535	538 ± 3	non	−9.93
LS_Arah1_725	TAGGGAAGAGAAGGACATATGATTGCGCGAGTCCTGCGACTTTATCTTTGCGAATAGCCGGTATTGACTAGTACATGACCACTTGA	725	988 ± 1	non	−7.95

this novel aptamer LS_Arah1_367 and Ara h 1 (Table S3 and Figure S4 of the Supporting Information).

Characterized Aptamers Detect Ara h 1 in Different Test Systems. First, the characterized aptamers were applied for the detection of Ara h 1 in western blot analysis. All three aptamers were able to bind Ara h 1 after electrophoretic separation and transfer to a nitrocellulose membrane, revealing a distinct band at approximately 64 kDa (Figure 3). The

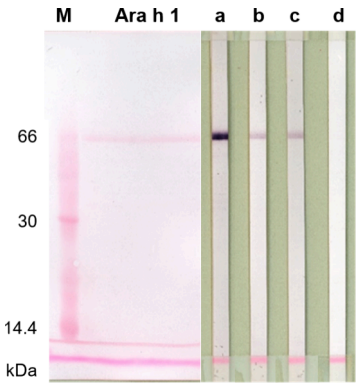

Figure 3. Western blot analysis. Ponceau S-stained Ara h 1 protein membrane (Ara h 1) with low-molecular-weight (LMW) marker (M) and aptamer-based detection with LS_Arah1_367 (a), LS_Arah1_535 (b), LS_Arah1_725 (c), and buffer (d).

target−aptamer binding was not negatively affected by the denaturation of the target protein that occurs during SDS−PAGE under reducing conditions, so that 0.4 μg of Ara h 1/strip was unambiguously detected (Figure 3). Furthermore, the intensity of the bands of the three aptamers correlates with the dissociation constants obtained by SPR. Western blot is a standard biochemical method for specific protein detection on a membrane after electrophoretic separation, commonly utilizing an antibody−antigen reaction.[28] Here, the developed aptamers demonstrated potential as an alternative detection reagent. To the best of our knowledge, this is the first proof of concept to use an aptamer for specific Ara h 1 detection in western blot analysis.

The aptamer LS _Arah1_367 was selected for the development of an indirect ELAA. Thus, Ara h 1 was coupled onto the surface of a microtiter plate. Before, the aptamer was modified with biotin to allow for detection by streptavidin−HRP via enzymatic staining with TMB. Accordingly, an increase in the OD value with an increase in the concentration of Ara h 1 was visible between approximately 10 and 2000 ng/mL (Figure 4a). Moreover, instead of a streptavidin conjugate, an aptamer-specific probe coupled with HRP was studied. The applied probe consists of a sequence that is complementary to a constant region of the aptamer. This approach likewise allows for the detection of Ara h 1 in the same concentration range without the need for aptamer modification (label-free) (Figure 4b). For the development of a sandwich assay, LS_Arah1_367 in combination with concanavalin A (Con A), was used as a detection or capture molecule, respectively. Con A, a jack bean protein, was used for the purification of Ara h 1 from peanut total protein and also for the development of the sandwich-like assay to detect Ara h 1 in peanut, because it binds to reactive polysaccharides and, therefore, also to the glycosylation of Ara h 1.[29] With this sandwich-like assay, approximately 50−2000 ng/mL Ara h 1 could be detected (Figure 4c). Sandwich-type

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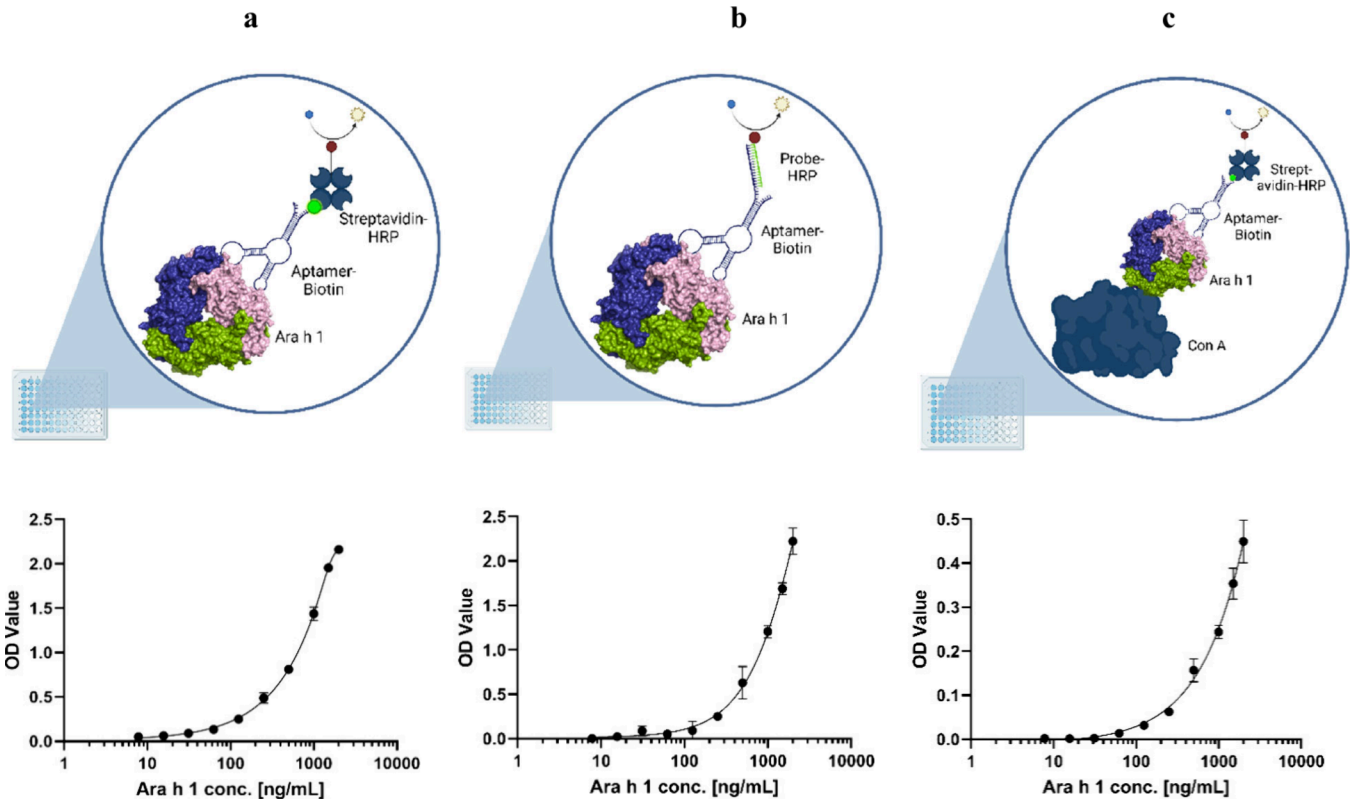

Figure 4. Application of aptamer LS_Arah1_367 in ELAAs for Ara h 1 detection. (a) Indirect ELAA with the streptavidin−HRP conjugate, (b) indirect ELAA with the probe−HRP conjugate, and (c) sandwich ELAA with Con A as the capture molecule and aptamer LS_Arah1_367 as the detection molecule with the use of the streptavidin−HRP conjugate (created with BioRender.com). Ara h 1 was visualized using the Protein Data Bank (PDB) 3SMH structure.

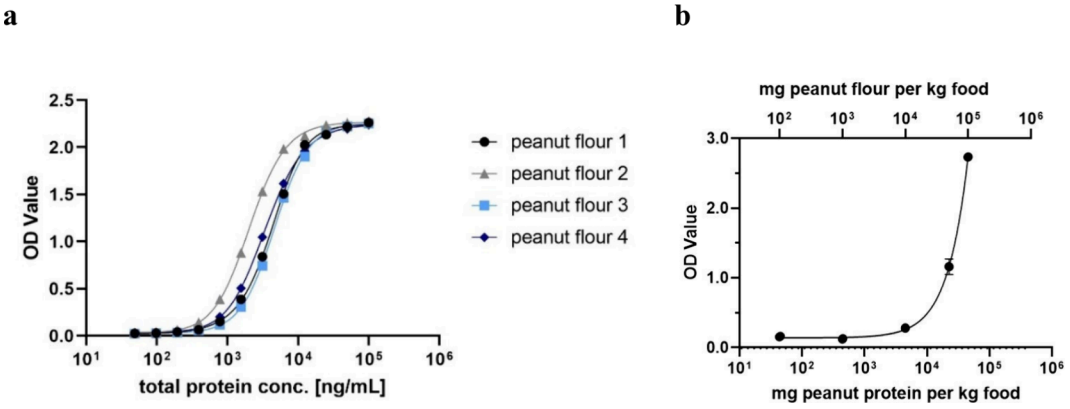

Figure 5. Indirect ELAA with the aptamer LS_Arah1_367−biotin and streptavidin−HRP conjugate for the analysis of Ara h 1 in (a) four different peanut flours and (b) peanut-containing cookies.

assays bear the advantage of potentially higher specificity as a result of two specific binding sites. Subsequent work should determine whether the design of the Con A−Ara h 1−aptamer sandwich can lead to an improvement in specificity (Figure 4c).

In general, there are a limited number of proven aptamers for the detection of peanuts in food. An Ara h 1-specific aptamer was identified in 2012 by capillary electrophoresis (CE)−SELEX with a K_D of 353 ± 82 nM (obtained using SPR) and inserted into a fiber optic surface plasmon resonance biosensor in combination with a secondary antibody.[15] In 2022, Stidham et al. used graphene oxide (GO)−SELEX to select aptamers against Ara h 1 with K_D of 54 ± 5.5 nM using fluorescence polarization.[16] These aptamers obtained by CE− and GO−SELEX were used in various biosensor systems. In our work, the SPR-based system was also used as an additional method to test the wider applicability of the newly developed aptamers on real food samples. The immobilization of biotinylated aptamer LS_Arah1_367 on a neutravidin-coupled sensor chip allows for unambiguous Ara h 1 detection of peanut flour and powdered peanut butter at a dilution of 1:32 (Figure S6 and section M12 of the Supporting Information). This dilution corresponds to approximately 3.1% peanut flour, 1.4% peanut protein, and 0.2% Ara h 1 (assumption of 12−16% Ara h 1 in an average peanut[30]). Although, one disadvantage of biosensor systems is that the direct transfer

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of such a method in practical application is limited. In official food monitoring in Germany, ELISA- and PCR-based methods are the main established methods for ensuring the safety of food in terms of allergenic ingredients and their labeling.[21,31] Biosensors are not commonly used in the field of allergen analytics and food and feed monitoring.[31] To our knowledge, the previously published Ara h 1 aptamers have only been used in sensor chip systems. Accordingly, the development of simple, rapidly transferable, ELISA-like assays is a major benefit to enable the application of aptamers in the area of allergen detection. Published antibody-based ELISAs have lower limits of detection (LLODs) of 31 ng/mL[32] or show Ara h 1 standard curves, ranging from 4 to 2000 ng/mL.[33] Peng et al. developed an Ara h 1 ELISA with a limit of detection (LOD) of 0.34 ng/mL calculated by adding 3 times the standard deviation to the mean value obtained for the blank sample.[34] On the basis of this calculation, the LOD of the developed indirect aptamer-based ELAA would be below 7.8 ng/mL (Figure 4). Accordingly, the method developed in this study demonstrates comparable sensitivity without the need for animal-derived antibodies.

The indirect ELAA with the streptavidin−HRP conjugate (Figure 4a) presented the most sensitive aptamer-based Ara h 1 detection on the microplate and was selected for further investigations. To learn whether the developed aptamer-based systems are also suitable in real food matrices, four commercial peanut flours were tested. In all four peanut flours, an increase in OD values between approximately 500 and 100 000 ng/mL total protein concentration was detectable (Figure 5a). The relative position of curves allows for assessing the relative potency with regard to the Ara h 1 content. Thus, peanut flour 2 contained less detectable Ara h 1/total protein (ng/mL), whereas the other three peanut flours showed comparable levels of Ara h 1/total protein (ng/mL). More quantitative assessment can be done once full validation of ELAA is accomplished, which can be done in a follow-up work of this proof-of-concept approach. Furthermore, cookies with defined peanut flour contents (0−10%) were baked, extracted, and analyzed to test the applicability of the assay on real-life composed and processed food samples. With the aptamer-based method, a peanut content between 1 and 10% was detectable in thermally treated cookies (Figure 5b).

A promising observation of the aptamer-based test systems in this exploratory approach is that Ara h 1 is detectable as a pure protein in not only buffer but also peanut flour and baked peanut-containing cookies. In the previously published aptamer-based tests, either no real food samples, commercial food without precisely known peanut content, or a food like a candy bar spiked with Ara h 1 protein was used.[15−17,19,20] Spiked foods exclude effects of processing on the analyte, like heat-induced chemical changes, such as Maillard reactions or other protein−carbohydrate interactions, which can lead to significant differences in their detectability.[35] In this study, cookies were chosen as a model test matrix because they represent a potentially common peanut-rich matrix and contain high amounts of sugars and fats that could cause potential interference in allergen analysis. Despite this, peanut contents from approximately 1% are detectable with the indirect ELAA. According to the 2022 risk assessment of food allergens meeting report of the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO),[36] an eliciting dose ED_{05} (eliciting dose predicted to provoke reactions in 5% of the allergic population, discrete dosing schema) of 2.1 mg of peanut protein was reported for peanut. Considering the amount of food consumed (portion size), action levels in "milligrams of total peanut protein per kilogram of food" can be derived. An assumed upper level of 2 mg of peanut protein in a small portion size of 10 g corresponds to a concentration of 200 mg of peanut protein/kg of food. The meeting report also suggests that limits of quantification (LoQ) of suitable analytical methods should be around 3-fold lower than the proposed action level. Hence, the LoQ of a method to verify 200 mg of total peanut protein/kg of food in a portion size of 10 g should be around 66 mg of total peanut protein/kg of food or 6.6 mg of total peanut protein/kg of food in a portion size of 100 g.[36] As summarized elsewhere,[21] most peanut allergen detection methods, including ELISA, would allow for verification of peanut protein action levels for food portions around 10−100 g. As seen in Figure 5b, our peanut-specific ELAA would allow for detection at a level of 10^3−10^4 mg of peanut protein/kg of food, which is about 100−1000-fold higher. Hence, we do not consider our ELAA at its current "proof-of-concept" state to be suitable for the verification of peanut action levels or a fully fledged replacement of peanut allergen detection methods for verification of action levels and, in turn, allergen labeling of foods. Thus, further work should focus on optimized protocols to improve sensitivity. As always, the applicability of a test depends upon the purpose and the stage of development. Here, we present a proof-of-concept study that our selected aptamer against Ara h 1 major peanut allergen allows for allergen-specific detection, even in a complex composed and processed food matrix. In the field of medicinal products, diagnostic and therapeutic allergen preparations may require quality assessment at the allergen component level, i.e., for specific allergens, such as Ara h 1.[6] The newly developed Ara h 1-specific aptamer-based assays potentially provide such an antibody-free alternative for the detection and quantification of Ara h 1. Currently, there is only one FDA- and EMA-approved medicinal product for peanut-specific allergen immunotherapy, called "Palforzia". Palforzia, a flour from partially defatted roasted peanuts, is used to desensitize individuals with peanut allergy by gradually improving their ability to tolerate increasing amounts of peanuts.[37] In our work, we have shown that peanut flours with similar characteristics can be investigated using our aptamer-based systems (Figure 5a). Furthermore, it was shown that the application of the developed method is not limited to simply heated peanut products, such as peanut flour. It is also possible to analyze a thermally treated cookie with regard to its peanut content (Figure 5b). If peanut was advertised in a food, e.g., as a quality ingredient, according to Article 22 and Annex VIII of Regulation (EU) No. 1169/2011, the manufacturer must indicate the quantitative content.[5] In most cases, the peanut content is in the one- to two-digit range, so that declarations of the manufacturer can potentially be verified by such an aptamer-based test, as demonstrated here (Figure 5). The results highlight the successful use of aptamers as novel diagnostic binders for peanut major allergen Ara h 1, which offer an ethical advantage over animal-derived antibodies because *in vitro* aptamer selection and chemical synthesis do not require the use of animals. In addition, aptamers possess a distinct quality benefit, because they are not subject to the issue of batch-to-batch variation. In the future, elucidation of the aptamer-specific binding site of Ara h 1 would allow for improvement of our general understanding of aptamer binding

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and potentially contribute to enhance *in silico* approaches for aptamer identification. In conclusion, the first ELISA-like aptamer-based assays for the detection of Ara h 1 have been presented here, which also fulfill the objectives of European Directive 2010/63/EU according to the 3R principle.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c03948.

Additional experimental details, materials, and methods, including photographs of the experimental setup (PDF)

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Author Contributions

The manuscript was written by Laura Schäfer, through contributions by Thomas Holzhauser (protein purification) and Andreas Reuter (mass spectrometry). Further contributions: conceptualization, Thomas Holzhauser, Laura Schäfer, Birgit Ahrens, and Kirsten Beyer; methodology, Thomas Holzhauser, Laura Schäfer, and Günter Mayer; project administration, Thomas Holzhauser, Birgit Ahrens, and Kirsten Beyer; funding acquisition, Thomas Holzhauser, Birgit Ahrens, and Kirsten Beyer; resources, Thomas Holzhauser; supervision, Thomas Holzhauser; validation, Laura Schäfer; formal analysis, Laura Schäfer; investigation, Laura Schäfer; data curation, Laura Schäfer; NGS data analysis, curation, and visualization, Csaba Miskey; SPR performance, data analysis, and curation, Sascha Hein; protein preparation, Elke Völker; and mass spectrometry data analysis, curation, and presentation, Andreas Reuter. All authors have reviewed and given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AIT, allergen-specific immunotherapy; Ara h, (*Arachis hypogaea*); ELISA, enzyme-linked immunosorbent assay; SELEX, systematic evolution of ligands by exponential enrichment; NGS, next-generation sequencing; SPR, surface plasmon resonance; ELAA, enzyme-linked aptamer assay; LFD, lateral flow device; ssDNA, single-stranded DNA; BSA, bovine serum albumin; RT, room temperature; PCR, polymerase chain reaction; DTT, dithiothreitol; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; *S*, protein score; *P*, number of peptides detected; SC, sequence coverage; PE, mean precursor mass error; FE, fragment mass error; ELONA, enzyme-linked oligonucleotide assay; SL, start library; *R*, SELEX rounds; K_D, dissociation constant; ΔG, Gibbs free energy; M, low-molecular-weight (LMW) marker; CE, capillary electrophoresis; GO, graphene oxide; Con A, concanavalin A

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Supporting Information

DNA meets Protein – Development, characterization and application of aptamers against peanut allergen

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Materials and Methods

M1 Purification of peanut allergens Ara h 1, 2, 3, and 6

Allergen components were purified from commercial roasted peanuts ("Arachides Jumbo", Seeberger peanuts jumbo giant), purchased from a local retailer. After deshelling and dehulling, peanut seeds were ground to a powder under liquid nitrogen using a cryogenic mill (CryoMill, Retsch, Haan, Germany). Depending on allergen components, different types of extraction and fast protein liquid chromatography (FPLC) were carried out. In general, enriched allergen component fractions with the least portions of additional components were identified according to molecular mass using SDS-PAGE[1] and Coomassie staining, pooled per allergen, and further purified if necessary.

Ara h 1 was extracted and purified from defatted peanut flour as previously described[2], with several modifications. One volume of peanut flour was defatted with five volumes of n-hexane, clarified through filter paper (Whatman 595, Fisher Scientific, Schwerte, Germany), and the defatting procedure was repeated twice. Air-dried defatted peanut flour was extracted for 1 h under permanent stirring at ambient temperature and 1/5 (w/v) ratio in unbuffered aqueous solution containing 6.2 mM NaN_3 and protease inhibitor cocktail (cOmplete Tablets, Mini, EDTA-free, Roche Diagnostics via Sigma-Aldrich, Taufkirchen, Germany). After centrifugation for 40 min at 20,000 *g* and 10 °C, supernatant was filtered through membranes of 5 µm nitrocellulose (Millipore via Merck, Darmstadt, Germany) and subsequently 0.22 µm polyethersulfone (Nalgene Rapid-Flow Sterile Disposable Filtration Devices with PES membrane via Fisher Scientific). Solid ammonium sulphate was added to reach 70 % saturation and precipitation allowed to proceed for 1 h in an ice bath. After centrifugation for 40 min at 20,000 *g* and 2 °C, supernatant was passed through 5 µm nitrocellulose and 0.22 µm PES membranes as described

above, and filtrate equilibrated against Tris buffer (20 mM Tris, 500 mM NaCl, 3 mM NaN_3, pH 7.5) using a dialysis cassette (Slide-A-Lyzer G2, 10K MWCO, Fisher Scientific). Purification of the glycoprotein Ara h 1 was done using FPLC (ÄKTApurifier System, Cytiva Europe, Freiburg, Germany) on a concanavalin A column (HiTrap Con A-4B, Cytiva Europe via Fisher Scientific). After equilibration with 5 column volumes (CV) of Tris buffer at 4 mL/min flow rate, sample (sterile filtered through 0.22 µm PES membrane) was loaded (2 mL/min), and column was washed with 2 CV of equilibration buffer at 4 mL/min. Ara h 1 was eluted at 4 mL/min flow rate of 5 CV of 0.4 M α-methyl-D-mannopyranoside in equilibration buffer. Pooled fractions containing purified Ara h 1 were dialyzed (Slide-A-Lyzer G2, 10K MWCO, Fisher Scientific) against equilibration buffer without sodium azide, and fourfold concentrated using spin columns (Vivaspin 20, 30 kDa cutoff, Sartorius via Fisher Scientific).

Ara h 6, Ara h 2, and Ara h 3 were extracted from ground peanut flour without defatting procedure. Protein extraction and purification of Ara h 2 was done as described elsewhere[3,4], with several modifications. Fractions of Ara h 6 and Ara h 3 were obtained from the same anion exchange chromatography (AEC) runs as Ara h 2, but further adaptions were necessary. Briefly, total protein was extracted from peanut flour for 2 h under permanent stirring at ambient temperature and 1/20 (w/v) ratio in 20 mM Tris-HCl (pH 8.2). Extract was centrifuged for 5 min at 5.000 *g* and 4 °C, and aqueous supernatant further centrifuged for 30 min at 25,000 *g* and 4 °C, and final supernatant clarified through filter paper (Whatman 595, Fisher Scientific). Prior to AEC, extract was sterile filtered through 0.22 µm PES membrane (Nalgene via Fisher Scientific). Using HiPrep Q HP 16/10 column (Cytiva Europe via Fisher Scientific), AEC was done on an ÄKTApurifier FPLC (Cytiva Europe, Freiburg, Germany). After equilibration with 5 CV of 20 mM Tris-HCl (pH 8) at 3 mL/min flow rate, sterile filtered protein extract was added to a 50 mL superloop at 1.5 mL/min, and the column was washed with 2 CV equilibration buffer at 1.5 mL/min. Proteins were eluted at 1.5 mL/min flow rate using a linear gradient over 10 CV of 0 M to 0.5 M NaCl in equilibration buffer, and elution continued for additional 5 CV at 3 mL/min flow rate of 0.5 to 1 M NaCl in equilibration buffer, and elution was continued for additional 5 CV with 1 M NaCl in equilibration buffer and 3 mL/min. Ara h 6 and Ara h 2 fractions eluted within the first 10 CV, followed by Ara h 1 and Ara h 3 fractions. Enriched allergen component fractions with the least portions of additional components were identified by SDS-PAGE analysis, and pooled per allergen. Pool of Ara h 3 fractions was depleted from residual Ara h 1 using Concanavalin A FPLC, as described above. Flow through contained purified Ara h 3. Pools of Ara h 2 or Ara h 6 fractions were separately dialyzed (Slide-A-Lyzer G2 Dialysis Cassette, 3.5 K MWCO, Fisher Scientific) against 0.1 M sodium phosphate buffer (pH 7.0), and additionally adjusted to 1.5 M ammonium sulphate (equilibration buffer) prior to hydrophobic interaction chromatography (HIC). HiTrap Phenyl HP column (Cytiva Europe via Fisher Scientific) was equilibrated with 5 CV of equilibration buffer at 1 mL/min, and pools of Ara h 2 or Ara h 6, sterile filtered through 0.22 µm PES membrane, were loaded to a 50 mL superloop. After washing with 2 CV of equilibration buffer, proteins were eluted at 1 mL/min flow rate using a linear

gradient of 20 CV to reach 0.1 M sodium phosphate buffer (pH 7.0, 0 M ammonium sulphate), and elution was continued for additional 5 CV. Purified fractions were pooled and dialyzed (Slide-A-Lyzer G2 Dialysis Cassette, 3.5 K MWCO, Fisher Scientific) against 0.1 M sodium phosphate buffer (pH 7.0). Protein concentration of purified peanut allergens was determined at 280 nm using UV absorption photometry (Nanophotometer NP80, Implen, Munich, Germany) based on computed (https://web.expasy.org/protparam/) sequence specific absorption coefficients and molecular weight as previously described for pea major allergen.[5]

M2 Confirmation of peanut allergens by mass spectrometry

The identity of the purified peanut allergens and that of the secondary components were examined after SDS-PAGE and trypsin digestion with UPLC-MS^E. The experiments were performed as described by Wangorsch *et al*.[6] This was varied in a few details as described below. Database searches were performed using an internal database. This database contained the UniProt database from July 2023 restricted to reviewed entries of all species and manually added sequences. Sequences from internal system suitability standards (Mix2, Waters, Milford UK), sequences from WHO/IUIS allergen nomenclature database (https://allergen.org) confirming allergen sequences if not included in UniProt, and internal sequences derived from e.g. cDNA sequencing were added.

M3 Immobilization of target proteins on magnetic beads

The Dynabeads™ Antibody Coupling Kit (Invitrogen via Fisher Scientific) and DynaMag™-2 magnet (Invitrogen via Fisher Scientific) were used to immobilize the target peanut protein on magnetic beads. Coupling was performed according to the coupling protocols version "June 2012" of the manufacturer. For this, 300 µg Ara h 1, 2 or 3 were incubated with 10 mg beads at 37 °C overnight in a rotary shaker. Similarly, 10 mg magnetic beads were incubated without protein to generate non-coated beads for counter SELEX. To verify the coupling event, qualitative SDS-PAGE and quantitative protein determination using ROTI® Nanoquant (Carl Roth, Karlsruhe, Germany) following the manufacturer's instructions was performed.

M4 Next generation sequencing (NGS)

The start library and the enriched dsDNA pools from each SELEX round were sequenced by NGS using NextSeq Illumina 550 System (Illumina, Munich, Germany). Therefore, the sequences were modified in a two-step PCR with barcode and adapter sequences. In the first step, the dsDNA pools were diluted 1:100 with nuclease-free, sterile, DEPC-treated, autoclaved water (Carl Roth) and 5 µL was used per reaction. The master mix consisted of 25 µL NEBNext Ultra II Q5 Master Mix (New England Biolabs, Frankfurt am Main, Germany), 2.5 µL forward primer "NNSRselexP5" (gctcttccgatctctTAGGGAAGAGAAggaCATATGAT), 2.5 µL reverse primer "NNSRselexP7" (gctcttccgatctgaTCAAGTGGTCATGTACTAGTCAA), 1 µL 50x SYBR Green (Invitrogen via Fisher Scientifc) and 14 µL nuclease-free, sterile, DEPC-treated, autoclaved water (Carl Roth) per reaction.

The following cycling conditions were used: 1 cycle of 98 °C 30 s; 12-20 cycles of 98 °C 5 s, 63 °C 10 s, 72 °C 5 s, depending on reaching the plateau of the amplification curve, as monitored in real-time.

The PCR products were then separated in an agarose gel to eliminate by-products by selectively isolating the desired DNA product from the gel. Agarose gel electrophoresis was performed using Rapid Agarose Gel Electrophoresis System (RAGE system, Cascade Biologics, Portland, USA). 15 µL PCR product was mixed with 3 µL 6xTriTrack DNA loading dye (Fisher Scientific) and loaded onto a 4% high resolution agarose gel (Carl Roth) in parallel with GeneRuler Low Range DNA Ladder, ready-to-use (Fisher Scientific). Gel electrophoresis run with TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA), pH 8.0, at 100 V for 60 min. After electrophoresis, the gel was stained with SYBR Safe DNA Gel Stain (Fisher Scientific) for 30 min. The desired DNA was isolated and purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Freiburg im Breigau, Germany) according to the manufacturer's instructions. Briefly, the DNA-containing gel piece was incubated with three times the volume of agarose dissolving buffer at 50 °C for 7 min and purified using spin columns. Elution took place with 20 µL nuclease-free, sterile, DEPC-treated, autoclaved water (Carl Roth). The resulting DNA was further diluted 1:10 and 5 µL per reaction was added to the second PCR. The PCR conditions (mastermix contribution and temperature program) were the same as for the first PCR. Seven different forward primers and 19 different reverse primers were used to uniquely barcode the PCR products of each SELEX round (**Table S4**). The resulting libraries - compatible with Illumina sequencing - were isolated and purified with agarose gel electrophoresis and Zymoclean™ Gel DNA Recovery Kit (Zymo Research), as described above. The libraries were sequenced on an Illumina NextSeq 550 instrument with 2x 77bp setting, using a custom sequencing protocol, which involved two initial “dark cycles” in the beginning of the reads. The raw sequencing reads were filtered for the ubiquities flanking sequences and were paired using Seqkit (PMID: 2770621). The paired reads were adapter trimmed, merged, quality- (phred score 30) and length-filtered using fastp (PMID: 30423086). After counting the reoccurring sequences with the *rmdup SeqKit* function, the number identified candidate sequences were normalized with the the corresponding library sizes. The candidates were further analyzed for sequence composition and visualized using the *R* environment (version 3.5 and 4.2).

Based on the sequencing results, the most enriched aptamers were identified and custom synthesized by biomers.net (Ulm, Germany) with a purification via high-performance liquid chromatography (HPLC).

M5 Commercial food items

The following commercial food samples were purchased: Powdered peanut butter "PB2" (Bell Plantation, Tifton, United States), peanut flour 1: "Mr. Brown" (GB-Foods, Schillingsfürst, Germany), peanut flour 2: roasted peanuts “Giant”, ground using a CryoMill (Globus, Sankt Wendel, Germany), peanut flour 3: “Dr. Almond Premium Selection” (Charrak Nutrition, Ramstein-Miesenbach, Germany), and peanut flour 4: “Düsseldorfer Ölmanufaktur” (Micheal Karlos, Goch, Germany).

M6 Production of peanut incurred cookies

For the production of cookies with final amounts of 0%, 0.01%, 0.1%, 1.0%, 5.0% and 10% peanut flour, a dough was made using 367 g of wheat flour type 550 (Bauck, Rosche, Germany), 180 g of refined sugar (Suedzucker, Mannheim, Germany), 250 g of butter (REWE Markt, Köln, Germany). A portion of approx. 80 g of this basic dough was taken, the appropriate amount of "Mr. Brown" peanut flour (GB-Foods) was incorporated and then baked in a convection oven at 175 °C for 15 min. The starting materials (wheat flour, refined sugar, butter) as well as the cookies without incurred peanut flour were tested using the commercial ELISA RIDASCREEN® Peanut (R-Biopharm, Pfungstadt, Germany) and no peanut was detected at or above the limit of quantification of 0.75 mg/kg. The cookies were ground using a CryoMill (Retsch, Haan, Germany) and stored at -80 °C until further use. The extracted cookie samples were analyzed in indirect ELAA in a 1:8 dilution in PBS pH 7.1.

M7 Software

NGS data analysis and visualization was performed using the R environment (version 3.5 and 4.2). The secondary structure of ssDNA aptamers was predicted using mfold software.[7] The parameters were selected in consideration of the conditions during the SELEX process (20 °C, $[Na^+]$ = 154.2 mM, $[Mg^{++}]$ = 1 mM). Data were visualized by GraphPad Prism (version 9.5.0, Graphpad Software, Inc.) and graphical illustrations were created using BioRender.com (Standard Academic License).

M8 Avidity measurements – general comments

Aptamer sequences were labeled with a 5'-TEG-biotin modification, custom synthesized by biomers.net. Unless otherwise specified, Maxisorp Nunc-Immuno plates (Fisher Scientific) were used, washing steps were performed four times with 300 µL/well selection buffer (PBS with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/mL BSA, pH 7.1) and all experiments were performed at ambient temperature and at least in technical duplicates.

M9 Avidity measurements via enyzme-linked oligonucleotide assay (ELONA)

To monitor the enrichment of sequences against the target proteins Ara h 1, 2, or 3, the avidity of the DNA pools from each SELEX round was determined using ELONA. For this purpose, the ssDNA from SELEX rounds 2, 4, 6, 8, and 10 as well as the start library were reamplified. Therefore, the start library and DNA pools were diluted to approx. 10^5-10^6 copies/5 µL, amplified and purified under the same conditions as applied for the aptamer selection process. Subsequently, the antisense strand was digested with lambda exonuclease and the resulting ssDNA was purified again as described for aptamer selection (see aptamer selection process). After measuring the concentration using a nanophotometer, the ssDNA sequences were used in ELONA.

The 96-well microtiter plates were coated with 100 µL Ara h 1, 2, or 3 (10 ng/µL) in carbonate buffer (0.1 M $NaHCO_3$ (Merck), 0.5% glycerol (Carl Roth), pH 9.6) for 1 h. After washing with PBST (PBS, 0.05% Tween® 20), the free binding sites were blocked with 100 µL of 1.5% BSA in PBS pH 7.1 for

1 h and then washed again. The reamplified ssDNA pools (50 nM) from each SELEX round were initially heated at 95 °C for 5 min, then cooled to ambient temperature, and 100 µL per well was added. After incubation for 1 h, the wells were washed to remove the unbound sequences. Negative controls were performed by adding selection buffer or start library under the same conditions. Afterwards, 100 µL of streptavidin-HRP conjugate (Southern Biotech via Biozol, Eching, Germany), diluted 1:50,000 in selection buffer, was added to each well and incubated for 1 h. After washing, 100 µL substrate solution (1 mM TMB, 3 mM H_2O_2 in citrate buffer, pH 3.9) was added to each well and then incubated in the dark. The enzymatic reaction was stopped after 20 min with 100 µL of sulfuric acid (3 M, Merck) and the optical density (OD) measured at 450 nm wavelength (with subtraction of background OD at 630 nm as reference wavelength) were on SpectraMax® ABS using the evaluation software SoftMax Pro 7.1 (Molecular Devices, Munich, Germany).

M10 Avidity measurements via surface plasmon resonance (SPR)

To analyze the enrichment of affine ssDNA sequences, the binding of ssDNA pools from different SELEX round was measured by SPR using the Biacore T200 system (Cytiva). Ara h 1, 2, and 3 were used in a concentration of 40 µg/mL in immobilization buffer (10 mM sodium acetate buffer, pH 4.5 for Ara h 1 and 2; pH 5.0 for Ara h 3) for amine based coupling onto a CM5 sensor chip (Cytiva). The immobilization was performed with the amine coupling kit (Cytiva). Final immobilization levels of 12595 response units (RU) for Ara h 1, 16239 RU for Ara h 2 and 2882 RU for Ara h 3 were obtained. In addition, an ethanolamine-blocked flow cell served as a reference for background signal subtraction. ssDNA pools from SELEX round 2, 4, 6, 8, and 10 were diluted in running buffer (PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/mL BSA, 0.05% Tween® 20, pH 7.1), with a final concentration of 20 nM. The prepared samples were applied with a contact time of 450 s and a dissociation time of 500 s at a flow rate of 5 µL/min. Glycine-HCl pH 2.5 was used as regeneration buffer.

M11 Dissociation constants (K_D) and cross-reactivity measurements

For the K_D determination of Ara h 1-specific aptamers and the analysis of potential cross-reactivities to peanut allergens Ara h 2, 3, and 6, biotinylated aptamers were coated onto the surface of a Series S Sensor Chip NA (Cytiva). The coupling of the aptamers with the neutravidin surface of the chip was performed according to the manufacturer's instructions. Briefly, the chip was conditioned twice with conditioning solution 1 (Cytiva) for 60 s at a flow rate of 10 µL/min, conditioning solution 2 (Cytiva) for 60 s at a flow rate of 10 µL/min. Coupling of the biotinylated aptamers was performed in immobilization buffer (15 mM Na_2HPO_4, 600 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4) with a flow rate of 5 µL/min and multiple injections to achieve the required immobilization level. Final response levels of 172.3 RU for LS_Arah1_367, 77.1 RU for LS_Arah1_535 and 82.4 RU for LS_Arah1_725 were achieved. Analogously, one flow cell served as a reference cell for background correction. For interaction analysis of Ara h 1 and the immobilized aptamers, a dilution series of Ara h 1 from 750 to 1.5 nM in running buffer (PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/mL BSA, 0.05%

Tween® 20, pH 7.1) was used. The contact time was set to 180 s, followed by 600 s dissociation and a constant flow rate of 50 µL/min. To investigate possible cross-binding, 3 µM Ara h 2, 3, and 6 were applied in running buffer with a contact time of 180 s. 50 mM NaOH was used to regenerate the flow cells.

M12 Ara h 1 detection in peanut flour and peanut butter via aptamer-based sensor chip

The coupling of the chip was performed in analogy to the K_D determination. Final immobilization levels of 344.0 RU (LS_Arah1_367), 391.7 RU (LS_Arah1_535) and 371.7 RU (LS_Arah1_725) were achieved. Peanut flour and peanut butter extract were diluted in running buffer (PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% Tween® 20, pH 7.1) in a dilution range of 1:1 to 1:32 and analyzed under the same conditions as for K_D determination using the aptamer-based sensor chip.

Supporting Information Tables

Table S1. SELEX conditions during the selection of aptamers against Ara h 1, 2, and 3 (input ssDNA, volume of magnetic particles, incubation time, washing steps and counter SELEX) and PCR cycles of the amplification step with the approx. Δfluorescence and the measured concentrations of the purified ds- and ssDNA.

SELEX round	Input ssDNA	Magnetic particle volume (amount)	Incubation time	Washing steps	Counter SELEX	Target protein	PCR Cycles	Approx. ΔFluorescence	Conc. dsDNA [ng/µL]	Conc. ssDNA [ng/µL]
1	500 pmol	50 µL (0.5 mg)	30 min	3 x 5 min	non	Ara h 1:	6	1900	25.0	10.9
						Ara h 2:	6	2000	17.2	7.7
						Ara h 3:	6	2100	25.0	11.4
2	10 pmol	50 µL (0.5 mg)	30 min	3 x 5 min	non	Ara h 1:	15	2000	18.8	7.8
						Ara h 2:	11	2000	14.2	5.6
						Ara h 3:	11	2000	18.4	5.8
3	10 pmol	50 µL (0.5 mg)	30 min	3 x 5 min	yes	Ara h 1:	17	2200	28.9	21.1
						Ara h 2:	13	2000	8.4	3.8
						Ara h 3:	13	2300	8.0	2.2
4	10 pmol	50 µL (0.5 mg)	20 min	3 x 5 min	yes	Ara h 1:	15	2200	19.5	9.9
						Ara h 2:	12	2300	16.5	8.5
						Ara h 3:	12	2100	17.3	6.8
5	10 pmol	50 µL (0.5 mg)	15 min	3 x 5 min	yes	Ara h 1:	12	2000	9.4	9.4
						Ara h 2:	12	2100	8.0	8.0
						Ara h 3:	15	2100	9.5	9.5
6	10 pmol	30 µL (0.3 mg)	15 min	3 x 5 min	yes	Ara h 1:	16	2100	22.9	10.2
						Ara h 2:	13	2100	20.2	19.0
						Ara h 3:	13	2100	17.9	18.7
7	10 pmol	15 µL (0.15 mg)	15 min	3 x 5 min	yes	Ara h 1:	15	2000	20.2	7.9
						Ara h 2:	11	2100	21.9	10.5
						Ara h 3:	11	2100	23.0	13.4
8	10 pmol	7.5 µL (0.075 mg)	15 min	3 x 5 min	yes	Ara h 1:	12	2200	23.6	10.5
						Ara h 2:	12	2100	22.5	8.3
						Ara h 3:	13	2200	24.4	10.8
9	10 pmol	7.5 µL (0.075 mg)	10 min	3 x 5 min	yes	Ara h 1:	13	2100	25.0	14.1
						Ara h 2:	14	2100	23.6	9.0
						Ara h 3:	13	2000	21.1	8.7
10	10 pmol	7.5 µL (0.075 mg)	5 min	3 x 5 min	yes	Ara h 1:	13	2300	25.7	12.4
						Ara h 2:	13	2300	20.6	9.4
						Ara h 3:	12	2300	24.5	9.9

Table S2. Main mass spectrometric key figures, as obtained from the analysis of the purified peanut allergens depicted in Figure S1 (Acc. No., usually refers to UniProt database or internal database entries "PEI").

Main Protein Band (*)	Acc. No.	Description	S	P	SC	PE	F	FE
1	P43238	Allergen Ara h 1, clone P41B	14795	83	51.6	3.8	1762	9.2
	P43237	Allergen Ara h 1, clone P17	15698	63	52.4	3.2	1755	9.2
2.2	PEI264	Ara h 2.0101 AAK96887.1 allergen II_ partial	2911	8	24.4	0.5	132	5.3
2.1	PEI264	Ara h 2.0101 AAK96887.1 allergen II_ partial	1373	8	24.4	0.8	126	6.8
3.3	PEI009	recArah3 wild type Arah34 from *Arachis Hypogaea*	12050	41	47.4	4.4	709	9.8
	O82580	Glycinin (Ara h 3)	5317	11	28.0	7.3	257	8.9
3.2	PEI009	recArah3 wild type Arah34 from *Arachis Hypogaea*	12224	37	46.1	4.3	556	9.2
	O82580	Glycinin (Ara h 3)	6825	13	26.6	3.0	296	8.2
3.1	PEI009	recArah3 wild type Arah34 from *Arachis Hypogaea*	17779	52	38.1	3.5	803	8.8
	Q9SQH7	Glycinin	11221	40	32.5	3.0	628	8.7
	O82580	Glycinin (Fragment)	8239	34	28.2	2.8	694	8.9
	Q647H2	Arachin Ahy-3	635	6	21.5	2.2	116	12.0
6	Q647G9	Conglutin (Ara h 6)	12280	56	61.4	2.6	726	8.9

S: PLGS protein score; SC: sequence coverage [%]; PE: mean peptide mass error [ppm]; P: number of tryptic peptides identified; F: Number of fragments detected, FE: mean fragment mass error [ppm]

Table S3. Identified aptamer sequences of the top 10 most enriched sequences (most abundant in terms of normalized copy numbers) in SELEX round 9 using NGS as well as the sequence of the scrambled version of LS_Arah1_367.

Ranking	Designation	Aptamer sequence (5‘ to 3‘)
1	LS_ Arah1_725	TAG GGA AGA GAA GGA CAT ATG AT TGCGCGAGTCCTGCGACTTTATCTTTGCGAATAGCCGGTA TTG ACT AGT ACA TGA CCA CTT GA
2	LS_ Arah1_535	TAG GGA AGA GAA GGA CAT ATG AT TGCGCGAGACCTTCTCCTTCGGCTTAGTCCCCCAAACACC TTG ACT AGT ACA TGA CCA CTT GA
3	LS_ Arah1_497	TAG GGA AGA GAA GGA CAT ATG AT TGCGCGAGTACTTCTCTCCCTCCAATTCGTGCCACCCACA TTG ACT AGT ACA TGA CCA CTT GA
4	LS_ Arah1_411	TAG GGA AGA GAA GGA CAT ATG AT TGCGCGAGTCCTTCACACCCCCCGATTTGGACAACACACC TTG ACT AGT ACA TGA CCA CTT GA
5	LS_ Arah1_367	TAG GGA AGA GAA GGA CAT ATG AT ATTCGGGTTGGCTTTGGGTTGGCGTCCTCAGACTCTATAC TTG ACT AGT ACA TGA CCA CTT GA
6	LS_ Arah1_349	TAG GGA AGA GAA GGA CAT ATG AT TGCGCGAGTACTTCTATATCCTACGGTTCTACCTGCCAT TTG ACT AGT ACA TGA CCA CTT GA
7	LS_ Arah1_340	TAG GGA AGA GAA GGA CAT ATG AT TGCGCGAGAACTCCTCTTCCTTACAGCCGTTCACTTATCT TTG ACT AGT ACA TGA CCA CTT GA
8	LS_ Arah1_334	TAG GGA AGA GAA GGA CAT ATG AT TGCGCGAGTACTTCTCTCCCTCCAATTCGTGCCACCCACG TTG ACT AGT ACA TGA CCA CTT GA
9	LS_ Arah1_328	TAG GGA AGA GAA GGA CAT ATG AT TGCGCGAGTCCTGATTGCTGTTTATGATAACCTTTACTTT TTG ACT AGT ACA TGA CCA CTT GA
10	LS_ Arah1_278	TAG GGA AGA GAA GGA CAT ATG AT TGCGCGAGTCCTGTTCCCGGCTTTTCACCGATTCCGTCT TTG ACT AGT ACA TGA CCA CTT GA
Scrambled version of LS_Arah1_367		ATGAGTACATTGCGGCGTCTAGTATGTGAACTCACGGCGCTAG GATTAGTAATTGTTGCGATCCTAAGAAAGTA ATCCTGCTGGGT

Table S4. Table of the forward and reverse primers used for the double-index barcoding of the Illumina libraries. The sequences in bold depict the eight-, or six-nucleotide long index sequences, * stands for phosphorothioate modification.

NNSR_Ill_8_1	AATGATACGGCGACCACCGAGATCTACAC**TATAGCCT**acac TCTTTCCCTACACGACGCTCTTCCGATCTC*T
NNSR_Ill_8_2	AATGATACGGCGACCACCGAGATCTACAC**ATAGAGGC**acac TCTTTCCCTACACGACGCTCTTCCGATCTC*T
NNSR_Ill_8_3	AATGATACGGCGACCACCGAGATCTACAC**CCTATCCT**acac TCTTTCCCTACACGACGCTCTTCCGATCTC*T
NNSR_Ill_8_4	AATGATACGGCGACCACCGAGATCTACAC**GGCTCTGA**acac TCTTTCCCTACACGACGCTCTTCCGATCTC*T
NNSR_Ill_8_5	AATGATACGGCGACCACCGAGATCTACAC**AGGCGAAG**acac TCTTTCCCTACACGACGCTCTTCCGATCTC*T
NNSR_Ill_8_6	AATGATACGGCGACCACCGAGATCTACAC**TAATCTTA**acac TCTTTCCCTACACGACGCTCTTCCGATCTC*T
NNSR_Ill_8_7	AATGATACGGCGACCACCGAGATCTACAC**CAGGACGT**acac TCTTTCCCTACACGACGCTCTTCCGATCTC*T
NNSRnestind1	CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd2	CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd3	CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd4	CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA

NNSRnestInd5	CAAGCAGAAGACGGCATACGAGAT**CACTGT**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd6	CAAGCAGAAGACGGCATACGAGAT**ATTGGC**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd7	CAAGCAGAAGACGGCATACGAGAT**GATCTG**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd8	CAAGCAGAAGACGGCATACGAGAT**TCAAGT**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd9	CAAGCAGAAGACGGCATACGAGAT**CTGATC**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd10	CAAGCAGAAGACGGCATACGAGAT**AAGCTA**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd11	CAAGCAGAAGACGGCATACGAGAT**GTAGCC**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd12	CAAGCAGAAGACGGCATACGAGAT**TACAAG**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd13	CAAGCAGAAGACGGCATACGAGAT**TTGACT**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd14	CAAGCAGAAGACGGCATACGAGAT**GGAACT**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd15	CAAGCAGAAGACGGCATACGAGAT**TGACAT**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd16	CAAGCAGAAGACGGCATACGAGAT**GGACGG**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd17	CAAGCAGAAGACGGCATACGAGAT**CTCTAC**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd18	CAAGCAGAAGACGGCATACGAGAT**GCGGAC**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA
NNSRnestInd19	CAAGCAGAAGACGGCATACGAGAT**TTTCAC**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCTGA

Supporting Information Figures

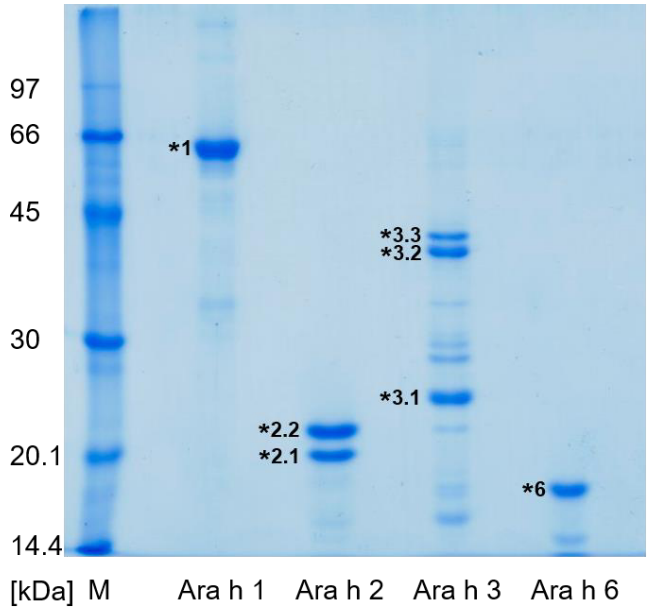

Figure S1. Coomassie-stained purified peanut allergens, each at 1 µg/lane, after separation in 12% SDS-PAGE. (M: molecular weight marker; * major protein band with numbers from 1 to 6 refer to entries in Table S2).

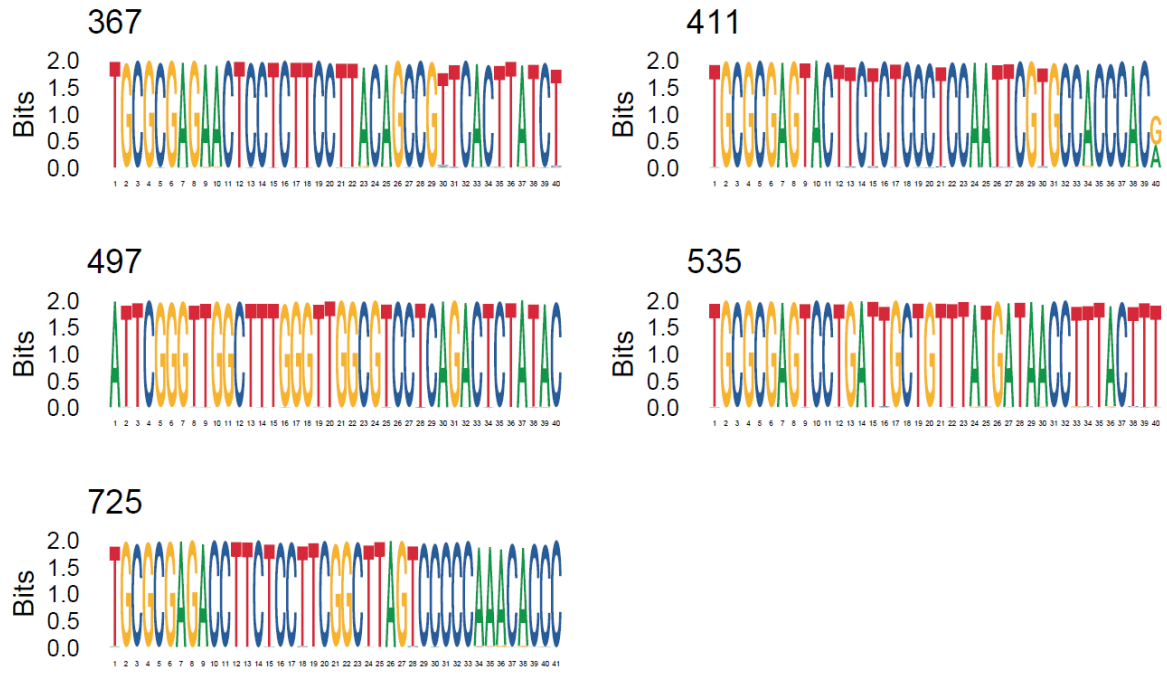

Figure S1. Sequence logo of the random region of the five most enriched sequence families 367, 497, 725, 411, 535, allowing up to six mismatches, in SELEX round 9 against Ara h 1.

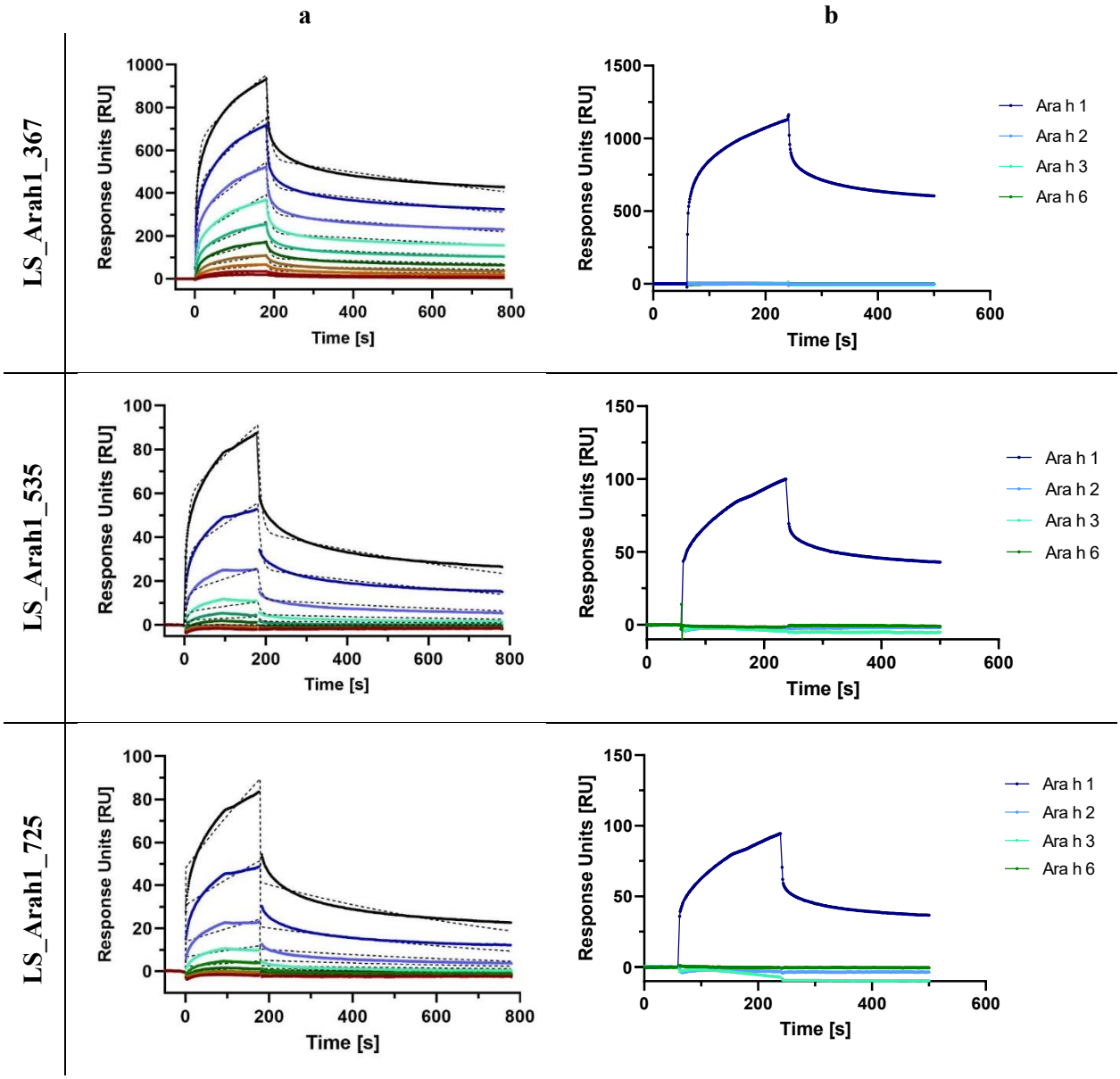

Figure S2. SPR Sensorgram of the three aptamers LS_Arah1_367, 535 and 725 **a**) to measure the binding kinetics as well as the determination of the K_D (1:2 dilution series, starting at 750 nM, fitted curves are depicted as black dotted lines) and **b**) for the measurement of potential cross-reactivities to Ara h 2, 3, and 6.

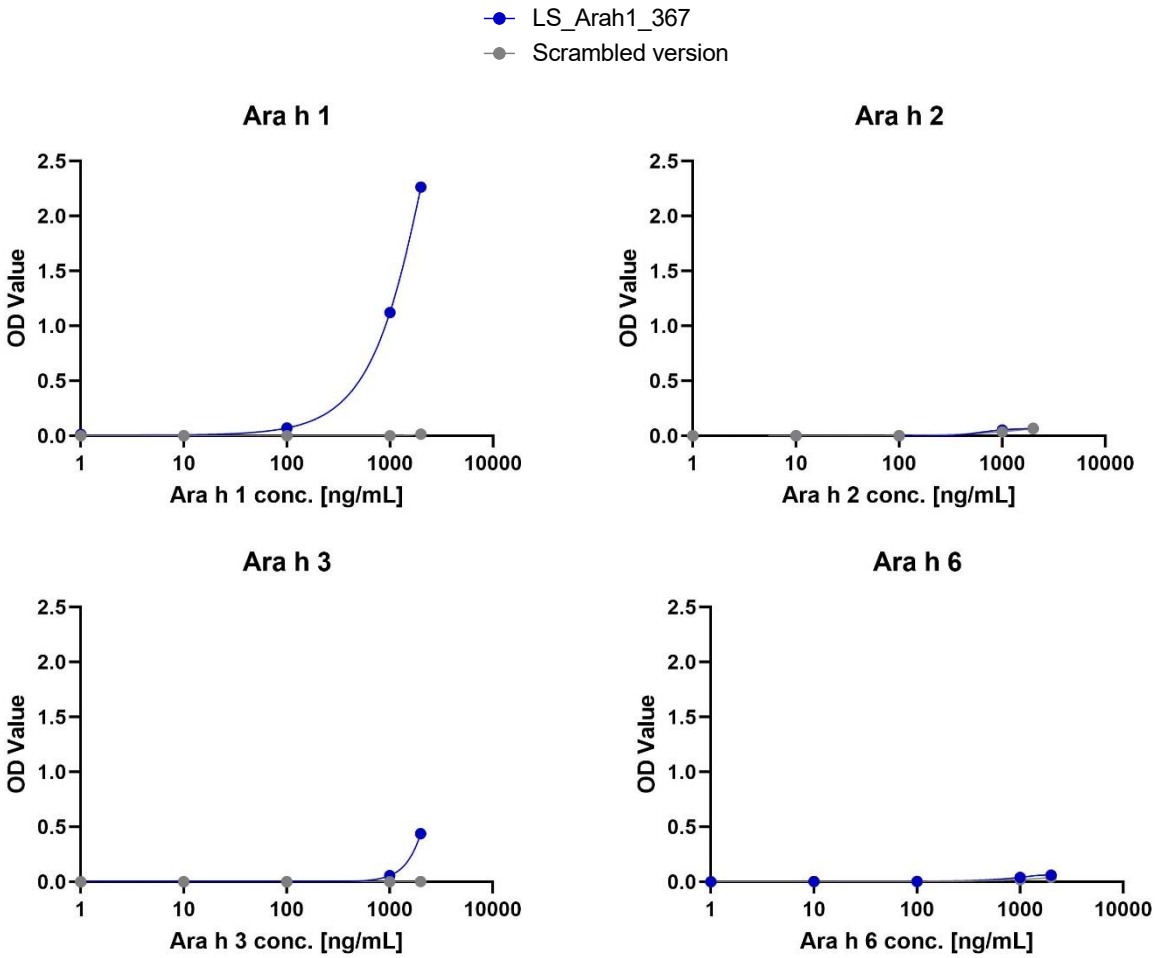

Figure S3. Cross-reactivity measurements of aptamer LS_Arah1_367 (blue dots) and the scrambled version (gray dots) to Ara h 1, 2, 3, and 6 using indirect enzyme-linked aptamer assay (ELAA) with streptavidin-HRP conjugate.

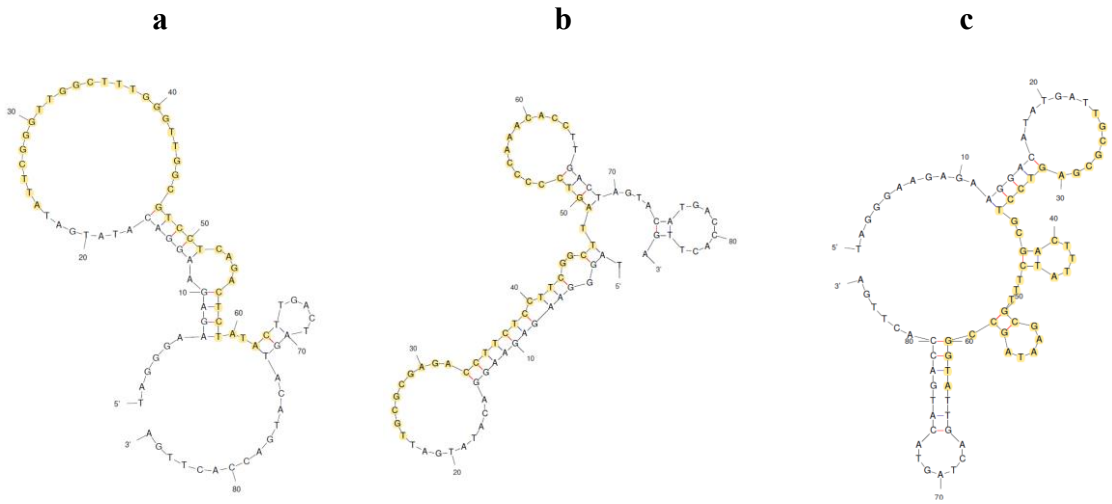

Figure S4. Predicted secondary structures of the most stable version of **a**) LS _Arah1_367 (ΔG = –5.05), **b**) LS_Arah1_535 (ΔG = –9.93) and **c**) LS_Arah1_725 (ΔG = –7.95) using mfold, the oligonucleotides highlighted in yellow represent the random region.

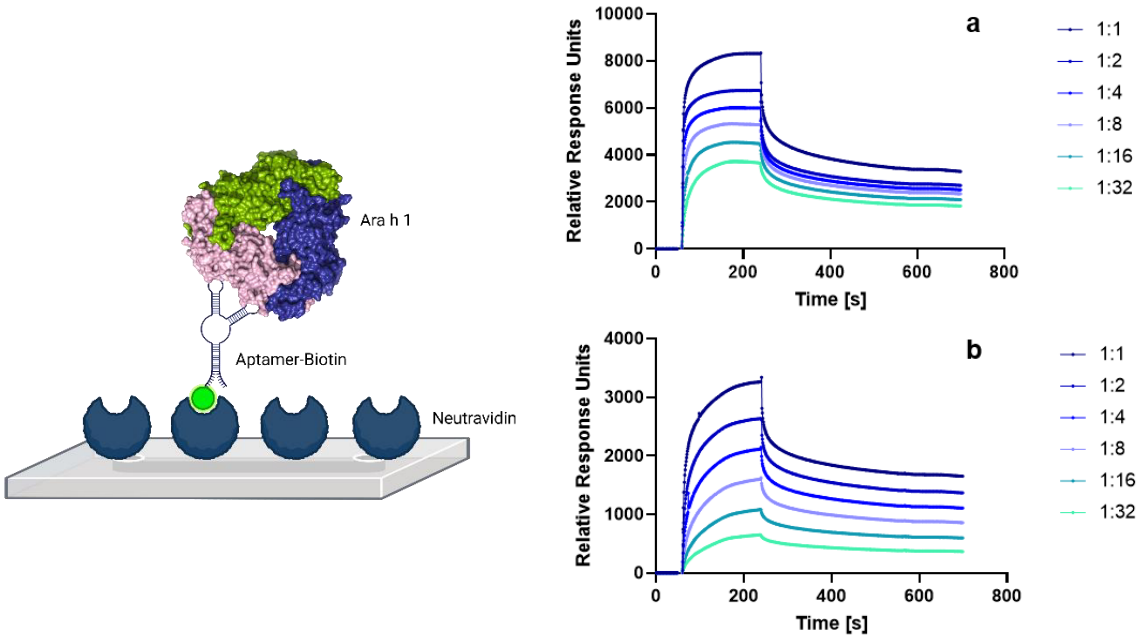

Figure S5. Aptamer-based sensor chip for the detection of Ara h 1 in **a**) peanut flour 1 and **b**) peanut butter with the aptamer LS_Arah1_367 (Image was created with BioRender.com). Ara h 1 was visualized using the PDB 3SMH structure.

References

(1) Seutter von Loetzen, C.; Reuter, A.; Spiric, J.; Schulenborg, T.; Bellinghausen, I.; Völker, E.; Vogel, L.; Rösch, P.; Schiller, D., Quality and potency profile of eight recombinant isoallergens, largely mimicking total Bet v 1-specific IgE binding of birch pollen. *Clin. Exp. Allergy* **2019**.

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Article

Development and Validation of a Loop-Mediated Isothermal Amplification (LAMP) Method for the Rapid, Sensitive, and Specific Detection of Hazelnut as an Allergen in Food Matrix

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ABSTRACT: Analytical verification of hazelnut in food supports risk-based approaches for proper allergen labeling to prevent unwanted allergic reactions or to quality control diagnostic or therapeutic allergen preparations for allergic subjects. We present the development and validation of a loop-mediated isothermal amplification (LAMP) assay for rapid detection of hazelnut by targeting the internal transcribed spacer 2 gene. The qualitative method requires neither sophisticated analytical equipment nor antibodies, allowing an easy-to-use application with no ethical concern related to the use of animals. It demonstrated a limit of detection at or below 10 mg/kg hazelnut in various food matrices, making it also suitable for verifying hazelnut at levels of clinically relevant eliciting doses. Validation against proficiency test samples and testing of applicability with commercial food items confirmed its usefulness in processed foods. The simplicity of the method, including visual colorimetric detection, combined with high specificity and sensitivity, represents an advancement over existing qualitative methods.

KEYWORDS: *loop-mediated isothermal amplification, LAMP, hazelnut, allergen, ITS2, DNA detection, colorimetric detection*

1. INTRODUCTION

Food allergies are hypersensitive reactions of the immune system and affect children and adults worldwide. Hazelnut (*Corylus avellana*) as a common food allergen may lead to allergic reactions, including anaphylaxis, in 2.2% of Europeans, as estimated from clinical history and food challenge studies.[1] Hazelnut allergy can persist a lifetime and is the most prevalent type of nut allergy in Europe.[2,3] For the treatment of food allergy against hazelnuts, there are no causative immunotherapeutic treatments available to date. Hence, a hazelnut-free diet and, after an allergic episode, the symptomatic treatment are the only means to manage the food allergy against hazelnut.[4] In Europe, the labeling of hazelnut ingredients in foods is mandatory according to Regulation (EU) No. 116/2011.[5] However, so-called "hidden allergens" can result from the cross-contact of food with allergens into an allergen-free food due to shared equipment for the production, storage, and/or transport.[6,7] In addition, there are many nonanalytically substantiated precautionary allergen labeling declarations (PAL). PAL on food is intended to inform allergic consumers that the presence of the allergen in this product cannot be ruled out and therefore warns of a possible allergic reaction. However, randomly applied PAL would weaken consumer confidence and complicate the management of food allergies due to possible anxieties. Therefore, detection methods are essential for the correct consumer-supporting labeling and serve as one important tool for risk-based allergen management. In addition, analytical methods represent indispensable tools to validate and ensure the quality of diagnostic foods, such as for the use in double-blind placebo-controlled food challenges (DBPCFC) in order to elucidate clinical reaction thresholds before and after allergen immunotherapy (AIT).

The enzyme-linked immunosorbent assay (ELISA) is still the most widely used allergen detection method in foods.[8] This well-established and easy-to-use antibody-based method for specific protein detection bears the risk of cross-reactivity to other phylogenetically related proteins, as has been shown, for example, in the analytical differentiation of hazelnut with other foods such as walnut, cashew, or pecan nut.[9−11] Besides the use of quantitative analysis via ELISA, an antibody-based lateral flow device (LFD) is the method for obtaining qualitative results within a few minutes.[12] However, both ELISA and LFD are based on the use of antibodies obtained by the immunization of suitable animals. Due to ethical concerns, a reduction of animal experiments should be sought as soon as possible.[13] Alternative common methods without the use of antibodies include mass spectrometry (MS) or polymerase chain reaction (PCR). PCR allows for indirect detection of the presence of the allergenic source by amplification of a specific DNA segment. Both proven methods are associated with analytical equipment, dedicated operating space, and trained personnel. A rapid method for the detection of hazelnut without the need for antibodies or high equipment standards would potentially ease the application of allergen-analytical tools for food manufacturers and food control agencies alike.

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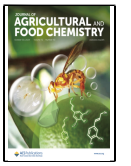

Table 1. Optimized LAMP Primer Set, Targeting the Gene Internal Transcribed Spacer 2 (ITS2), Consisting of Forward and Reverse Primers (F3 and B3), Forward and Reverse Inner Primers (FIP and BIP), and Forward and Reverse Loop Primers (LoopF and LoopB)[a]

LAMP primer	sequence (5′ → 3′)
F3	GCCCGAAGCCATCTGGT
B3	GGTCACAGAGCACAAGACG
FIP	AATGTCCGCCCCGCAGACCCGCATCGTTGCCCCCA
BIP	CGGCTGGCCTAAAAGCGAGTCGGGACACGAGGGTTTGTCAA
LoopF	CTTGGAGAGGCGATGGG
LoopB	CGAGCGCCACGACAATC

[a]BioRender.com (Standard Academic License) was used for the graphical labeling of the photographic images (Figures 1 and 2, SI Figures 2−7).

Loop-mediated isothermal amplification (LAMP) can serve as an alternative to DNA-based PCR detection because of several advantages, i.e., high specificity and sensitivity, rapid, and easy-to-use methodology with potential use as in field application. A strand displacement polymerase in combination with four to six primers, which recognize six to eight target DNA sequences, is used to enable an amplification at one temperature.[14,15] In addition to the high specificity and sensitivity of this amplification strategy, the generation of LAMP amplicons can be visually detected through colorimetric changes based on dyes such as pH-sensitive phenol red. Since neither a thermal cycler nor a detection device is needed, a colorimetric LAMP serves as a fast and simple detection method in permanently closed reaction tubes, further minimizing the risk of unwanted artificial contamination in subsequent analyses. LAMP technology, for its ease of use, has entered the fields of GMO analysis, pathogen detection, and food authentication.[16−21]

In previous work, a soy-specific LAMP was developed, and the steps of DNA extraction and detection were optimized so that a complete rapid detection method from the food to the qualitative result, regarding the presence or absence of soy as an allergen, is available.[22−24] Regarding hazelnut allergens, to the best of our knowledge, only one LAMP method has been reported so far.[25] This LAMP method is based on the amplification of the single-copy gene for the storage protein 2S albumin and detected hazelnut spiked at levels of 100 mg/kg in rice samples, which corresponds to 15 mg/kg total hazelnut protein per rice sample (assuming an average total hazelnut protein content of 15%).[26] The currently calculated predicted eliciting dose (ED) for total hazelnut protein is 3.5 mg (discrete ED_{05}), at which 95% of hazelnut-allergic subjects would not react.[27] A suitable analytical method should therefore be able to verify such an eliciting dose level in a relevant portion size of food. The 2022 risk assessment of food allergens meeting report of the WHO and FAO[28] applies rounded figures, i.e., 3 mg of total hazelnut protein as the ED_{05} reference dose, and additionally requires that the sensitivity of suitable analytical methods should be 3-fold lower than the proposed level to be verified. Translated into food portion sizes of 100 and 500 g, the method sensitivity should be around 10 and 2 mg/kg total hazelnut protein, respectively. The method according to Zhang et al. would allow detection of hazelnut equivalent to 15 mg/kg hazelnut protein in a rice sample.[25] This means that the method is limited to food portions of less than 100 g.

Thus, the aim of this study was to develop a more sensitive, specific, rapid, and easy-to-use closed-tube LAMP method for hazelnut detection in foods. Hence, different primer sets against four different genes were designed, generated, and specificity tested *in silico* and empirically in comparison with those of other relevant food species. Subsequently, the most suitable primer set was optimized so that the final method fulfills the sensitivity requirements in terms of established clinical data-based eliciting doses, is highly specific for hazelnuts without cross-reactivity to other tree nuts as reported for antibody methods, is rapid which makes it very interesting in comparison to PCR, and is easy to perform because of its simplicity. With this colorimetric hazelnut LAMP, we aim to present a novel method that fulfills the above-stated characteristics and so provides an alternative to existing antibody-based rapid tests such as LFD.

2. MATERIALS AND METHODS

2.1. Primer Design and Software. For target gene selection, *in silico* alignments, and the final primer design, the following databases and software were utilized. Potential target genes were selected from the National Center for Biotechnology Information (NCBI, ncbi.nlm.nih.gov). Alignment analyses between hazelnut and other relevant food species, for example, walnut or peanut (for more details, see SI Figure 2 and Section 2.2.1) were done with Basic Local Alignment Search Tool (BLAST+ 2.13.0 (NCBI, Bethesda)) and BioEdit 7.2 (Thomas Hall, Abot Park). Primer sets were designed using Primer Explorer 5.0 (EIKEN Chemical Co. Ltd., Tokyo, Japan) and LAMP-Designer 1.15 (Premier Biosoft, Palo Alta). In addition, verification and assessment of the primers occur via the online program NetPrimer (Premier Biosoft, Palo Alto), OligoAnalyzer (Integrated DNA Technologies, Coralville), and/or OligoEvaluator (Sigma-Aldrich, St. Louis). Finally, the designed primers were checked regarding homologies to nonhazelnut-specific sequences using BLAST and were modified manually if necessary. LAMP primer sets that were designed and tested in this study are listed in SI Table 1. The sequences of the finally selected primer set, targeting the internal described spacer 2 (ITS2, set 4), consisting of forward and reverse primers (F3 and B3), forward and reverse inner primers (FIP and BIP), and forward and reverse loop primers (LoopF and LoopB) are displayed in Table 1. All primers were custom synthesized by biomers (Ulm, Germany). For the validation experiments, ordered primers were additionally purified via high-performance liquid chromatography (HPLC). In addition, the BIP primers and LoopB primers were provided with biotin and fluorescein isothiocyanate (FITC) labels, respectively.

2.2. Study Food Items. *2.2.1. Single-Component Foods and Commercial Food Items.* Hazelnuts (Naturix24 hazelnuts, Taraxis Handels, Dransfeld, Germany) were deshelled, kernels ground using a CryoMill (Retsch, Haan, Germany), and stored at −80 °C until further use. For testing of potential cross-reactivities, 22 single-component foods (almond, pistachio, cashew, pecan nut, peanut, walnut, brazil nut, macadamia nut, chest nut, wheat, sesame, mustard, soy, celery, lupine, cherry, apple, cow's milk, hen's egg, tuna, squid, shrimp) were purchased at local retailers or were donated by German seed breeding companies, as described elsewhere.[22] The 15 analyzed food items (F1: muffin, F2: roll fondant white, F3: crunchy mixture cereal, F4: confectionery cereal, F5: white chocolate, F6: noisette

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chocolate, F7: butter cookie, F8: butter pastry, F9: shortbread cookie, F10: peanut flips, F11: whey cream, F12: porridge, F13: keto chocolate, F14: granola bar, F15: spelt cookie) were purchased from local supermarkets or online. Detailed information on these foods and ingredients can be found in SI Table 2.

2.2.2. Proficiency Test Samples. Proficiency test food samples (for comparative laboratory testing programs) were purchased from DLA Proficiency Tests (Sievershütten, Germany) and used as a material for validation of LAMP: Proficiency test sample 1 (DLA ptALS1 (2020)): "allergen screening I": cashew, hazelnut, macadamia, almond, brazil nut, pecan nut, pistachio, walnut. Proficiency test sample 2 (DLA 16-2018 ALM-Verification): "hazelnut in chocolate matrix": five samples with different amounts of roasted hazelnut. Proficiency test sample 3 (DLA 14-2018 Response PT hazelnut): "hazelnut processed samples": hazelnut (not roasted), nut crocant, nut butter (roasted), nut spread with cocoa, and nut nougat. Detailed compositions of all three proficiency test samples are shown in SI Tables 3−5.

2.2.3. Muffins with Known Amount of Hazelnuts. For the in-house production of muffins with known amounts of 0, 1, 10, 100, 1000, 10,000, and 100,000 mg/kg (0, 0.0001, 0.001, 0.01, 0.1, 1, and 10%) hazelnut (ground hazelnut kernels, Edeka, Hamburg, Germany), a dough was made using wheat flour type 550 (Edeka), hen's egg (Edeka), backing powder (Edeka), vanilla sugar (Edeka), butter (Edeka), powdered sugar (Südzucker, Mannheim, Germany), and 1.5% low-fat cow's milk (Edeka). A part of the remaining dough was spiked with an appropriate amount of dough, serving as a blank sample without the addition of ground hazelnuts. A part of the hazelnuts was homogenized and gradually diluted with muffin dough, resulting in six doughs with different hazelnut contents. Before use, the ingredients were tested negative for the presence of hazelnuts using a commercial ELISA Kit (RIDASCREEN Hazelnut, R-Biopharm, Pfungstadt, Germany). All muffins were baked in a convection oven at 175 °C for 15 min. The muffins were ground using a CryoMill (Retsch, Haan, Germany) and stored at −80 °C until further use.

2.3. DNA Extraction. DNA from food samples was extracted according to Schäfer et al.[24] Briefly, DNA from 100 mg of sample, homogenized by grinding via CryoMill (Retsch), was isolated by adding 580 μL of lysis buffer and 20 μL of proteinase K (SureFood Prep Advanced kit, R-Biopharm) as well as beads and sea sand in a high-speed benchtop homogenizer at 6.5 m/s for 60 s (FastPrep-24, MP Biomedicals, Eschwege, Germany) and subsequently purified via SureFood Prep Advanced kit according to protocol 1 following the manufacturer's instructions, version "June 2022" (R-Biopharm). The concentration of the extracted hazelnut DNA was 165 ng/μL, as measured using an NP80 NanoPhotometer (Implen, Munich, Germany). For DNA extraction of food samples based on a potato powder matrix, double the volume of extraction buffer must be used (as also mentioned in the manufacturer's instructions for strongly swelling samples).

2.4. Colorimetric LAMP. The basic components of the colorimetric master mix were described in Schäfer et al.,[24] apart from the hazelnut-specific primers as described in this study. In brief, 5 μL of extracted DNA was added to 20 μL of master mix (1.4 mM each of dATP, dGTP, dTTP, dCTP (Carl Roth, Karlsruhe, Germany), 10 mM $(NH_4)_2SO_4$ (Merck, Darmstadt, Germany), 8 mM $MgSO_4$ (New England Biolabs, Frankfurt am Main, Germany), 50 mM KCl (Carl Roth), 0.1% v/v Tween 20 (VWR, Darmstadt, Germany), 100 μM phenol red (Carl Roth), 0.2 μM of each F3 and B3 primer, 0.8 μM of each BIP and biotin-BIP primer, 1.6 μM FIP primer, 0.4 μM LoopF primer, 0.1 μM FITC-LoopB primer, 0.3 μM LoopB primer, and 0.32 U/μL Bst 2.0 WarmStart DNA polymerase (120 U/μL, New England Biolabs)). DNA from proficiency test samples 1 and 3 was used undiluted. DNA from proficiency test sample 2 was diluted in 1:2 before use. All dilution steps were done with elution buffer E of the SureFood Prep Advanced kit (R-Biopharm), which also served as a negative control (instead of extracted sample DNA). Before adding the DNA to the master mix, the pH value was verified and potentially adjusted to be at the color inversion point of phenol red (approximately pH 8.0) to ensure optimal colorimetric visualization of positive versus negative reactions. Isothermal amplification was conducted at 70 °C for 45 min in a thermocycler (SimpliAmp Thermocycler, Applied Biosystem, Waltham).

2.5. Additional Detection Methods of LAMP Products.

2.5.1. LAMP-LFD. For the optional detection of LAMP products via a lateral flow device (LFD), the primers BIP and LoopB were labeled with biotin and FITC (Table 1), respectively, leading to double-labeled LAMP products in isothermal amplification. After running LAMP, 2 μL of the reaction solution was diluted in 100 μL of TBS buffer pH 7.4 incl. 1% Tween 20 and detected within 5 min of incubation using LFD (HybriDetect Kit, Milenia Biotec, Gießen, Germany) as previously described.[22]

2.5.2. Agarose Gel Electrophoresis. LAMP products (5 μL/lane) were separated within 20 min in 3% high-resolution agarose (Carl Roth) gels (RAGE system, Cascade Biologics, Portland, OR), using "O'Gene Ruler 1kb Plus DNA Ladder" and "Low Range Marker" (Fermentas, Waltham), Loading Dye TriTrack (Invitrogen, Waltham), followed by staining with SYBR Safe DNA Gel Stain (Invitrogen) and UV-detection (Blue LED Transilluminator 470 nm (Intas Science Imaging Instruments GmbH, Göttingen, Germany)).

2.5.3. Real-Time LAMP. For the initial screening of the various primer sets with regard to sensitivity and specificity, DNA amplification was detected in real time using SYBR Green (Roche, Basel, Switzerland) and fluorescence recording at the FAM channel within each minute. The composition of the master mix was analogous to Allgöwer et al.,[22] "2.7. final ORF160b LAMP Protocol" (total reaction volume of 25 μL including 0.15 μM of each F3 and B3, 0.6 μM of each FIP and Biotin-FIP, 1.2 μM BIP, 0.075 μM FITC-LoopF, 0.225 μM LoopF, 0.3 μM LoopB, 1.4 mM of each dATP, dGTP, dTTP, dCTP (Carl Roth), 0.8 M DMSO (Carl Roth), 0.25 mg/mL BSA solution (Sigma-Aldrich), 8 U Bst 2.0WarmStart DNA Polymerase (New England BioLabs), 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 6 mM $MgSO_4$, 0.1% Tween 20, 1:60,000 diluted SYBR Green I Nucleic Acid Gel Stain (Roche), and 5 μL of template DNA). The amplification took place at 62 °C for at least 50 min in a real-time cycler (AriaMx Real-time PCR System, Agilent Technologies, California).

3. RESULTS

3.1. Primer Set Development and Assessment. In principle, any DNA sequence that allows specific detection of hazelnut can be selected.[29] Different genes (heat-shock protein 1 (HSP1, GenBank acc. No. AF021807.1), NADH dehydrogenase subunits J, K, and 3 (NADH-KJ3, GenBank acc. No. KX822768.2), internal transcribed spacer 2 (ITS2, GenBank accession no. HQ442260.1), and Cor a 14 (GenBank acc. No. FJ358504.1)) were selected according to certain criteria as outlined in detail in Allgöwer et al.[22] Based on these DNA sequences, various primer sets were derived (SI Table 1). The corresponding *in silico* analysis is displayed in SI Figure 1, with the position of the primers of ITS2 set 4 as an example. Key aspects such as the optimum length and primer binding position as well as 3′ end stability, GC content, melting temperature, and secondary structure (avoiding self-priming in the form of hairpins, self-dimers, cross-dimers) were considered. In subsequent alignment studies, possible cross-reactivities to other relevant food species were determined. Primer sets that indicated *in silico* target gene specificity were experimentally tested for sensitivity and specificity. A dilution series ranging from 1:10 to $1:10^7$ of hazelnut DNA in buffer simulated levels between 0.1 and 100,000 mg/kg hazelnut in food. In addition, 22 other foods, such as walnut and peanut, were analyzed with the various primer sets to potentially identify sequence-related cross-reactivities at an early stage.

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The tests were carried out in real-time LAMP to obtain more precise information about the amplification behavior of the respective primer sets. Further, amplicon patterns resulting from hazelnut and unintended cross-amplification of nonhazelnut foods were compared in RAGE. In order to keep the focus on the finally developed colorimetric LAMP method, detailed presentations of real-time LAMP for primer screening have been omitted at this point. Primer set 4, which targets ITS2 (Table 1 and SI Figure 1), showed the highest sensitivity and specificity compared to the other tested primer sets, so these designed primer sequences of ITS2 set 4 were chosen and further optimized (data not shown).

3.2. Assay Optimization. Assay optimization included the parameter primer sequence and temperature optimization, the adaption toward a colorimetric master mix, and primer labeling for optional detection using LFD. During specificity optimization of the LAMP reaction, the primer binding positions were adapted, and the amplification temperature was increased. The selected, optimized ITS2 targeting primer set was integrated into the colorimetric master mix according to Schäfer et al.,[24] enabling visual qualitative detection in real time. Previous work successfully demonstrated that colorimetric detection in LAMP provides concordant qualitative results with PCR or LAMP-LFD.[24,30] In a rapid colorimetric LAMP method, the reaction time should be as short as possible but still provide sufficient amplicon formation to allow for a distinct color change and subsequent clear interpretation of results. Amplification of $1{:}10^6$ diluted hazelnut DNA resulted in such a color change from pink to yellow after 35 min of thermal incubation (data not shown). In subsequent experiments, it was observed that a slightly longer reaction time has a decisive influence on the sensitive detectability of hazelnut in complex foods. To compensate for less amplification efficiency in DNA of complex food matrices, an incubation time of 45 min was finally chosen. In addition to colorimetric detection, the formation of hazelnut-specific amplicons can be visualized using LFD. Thus, the backward inner primer (BIP) and backward loop primer (LoopB) were labeled with biotin and fluorescence isothiocyanate (FITC), respectively, to obtain biotin- and FITC-labeled LAMP amplicons during isothermal amplification. The optional LFD-like detection allows to verify the result of colorimetric detection in case of doubt but remains time-efficient and easy to use.

3.3. Specificity. All analyses (from initial tests with hazelnuts to final validation with various food matrices) were performed with DNA extracted by the rapid extraction method according to Schäfer et al.[24] Twenty-two single-component foods besides hazelnut were tested to assess the specificity of the colorimetric LAMP method. The extracted DNA was analyzed in both 1:10 and 1:100 dilutions (simulating portions of 10 or 1% in a food, respectively). These single-component foods covered allergens requiring labeling according to Regulation (EU) No. 1169/2011 and foods with some phylogenetic relationship to hazelnut. The experimental analysis confirmed the previous *in silico* findings, i.e., the developed method exclusively detected hazelnut. Samples other than hazelnut showed no amplification and therefore no color change during the reaction (SI Figure 2). The specificity of the developed rapid method was further tested on proficiency test sample 1, which serves to validate analytical methods. This proficiency test sample (samples A-D) consisted of potato powder (approximately 75%) and maltodextrin (approximately 25%), which was spiked with various nuts (cashew, hazelnut, macadamia, almond, brazil nut, pecan nut, pistachio, walnut; Table 2). The accuracy of the quantities of allergenic food is guaranteed by the provider through multiple, extensive analyses using ELISA and PCR techniques.

Table 2. LAMP Analysis and Results of Proficiency Test Sample 1 in Comparison with the Presence of Allergenic Ingredients According to the Manufacturer[a]

ingredients in potato powder	sample A	sample B	sample C	sample D
cashew	−	+	−	−
hazelnut	−	+	−	+
macadamia	−	−	−	+
almond	+	+	−	−
brazil nut	+	+	−	−
pecan nut	−	+	+	−
pistachio	+	−	+	−
walnut	−	−	−	+
colorimetric LAMP result	negative	positive	negative	positive

[a]+ = contain, − = does not contain.

Samples B and D with hazelnut ingredients were detected as true-positive, and samples with no hazelnut ingredients (A and C) were detected as true-negative (SI Figure 3). These results underline the specificity of the method in the food matrix based on potato powder and various incurred nut allergens.

3.4. Sensitivity. DNA from native hazelnuts was used and serially diluted to determine the lowest detectable DNA concentration. Hazelnut DNA diluted down to 1:1,000,000 was still clearly detectable (four out of four reactions), while the limit of detection (LOD) ranged between $1{:}10^6$ and $1{:}10^7$ diluted hazelnut DNA (Figure 1). This result was reproduced by several measurements on five different days with at least technical duplicates (details not shown).

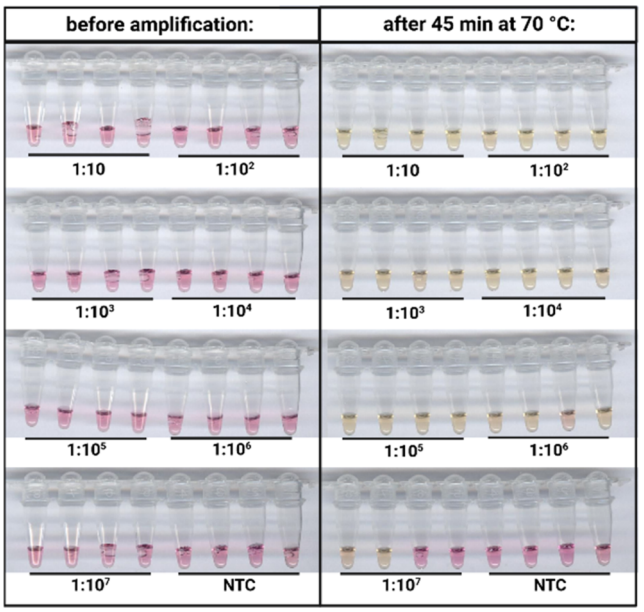

Figure 1. LAMP analysis of hazelnut DNA, extracted according to Schäfer et al.,[24] at $1{:}10-1{:}10^7$ dilution in the elution buffer, and colorimetric detection before (left) and after isothermal amplification (right). Elution buffer served as the non template control (NTC).

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To investigate the method sensitivity on real food samples, proficiency test samples in the form of dark chocolate with hazelnut levels of 0, 0.5, 2.5, 5.0, 12.5, and 25.0 mg/kg (proficiency test sample 2) were analyzed. Samples with 5.0 mg of hazelnut per kg dark chocolate or above tested positive in four out of four replicates (SI Figure 4). Potential inhibitors in dark chocolate, such as polyphenols, did not interfere with the detectability. Furthermore, muffins with defined levels of incurred hazelnuts were analyzed with the developed hazelnut LAMP method. Thus, a muffin dough was prepared from ingredients that were tested negative prior to use (below the limit of determination, 2.5 mg/kg) using a commercial ELISA Kit, spiked with 10% (100,000 mg/kg) of ground hazelnuts, gradually diluted with hazelnut-free muffin dough, and baked. Subsequently, all levels of 0, 1, 10, 100, 1000, 10,000, and 100,000 mg/kg incurred hazelnut were investigated with hazelnut LAMP. Furthermore, the gravimetrically calculated amounts of hazelnuts were confirmed through the analysis using a commercial ELISA Kit. All samples with levels of 10 mg/kg hazelnut in muffin matrix and above tested positive in eight out of eight replicates. The muffin with 1 mg/kg incurred hazelnut tested positive in seven out of eight replicates, which indicated a limit of detection between 1 and 10 mg/kg hazelnut in muffins (Figure 2). Sensitive and specific detection was possible in dark chocolate as well as in muffins, which confirms the applicability of the method.

3.5. Applicability. Moreover, possible limitations in detectability due to food processing are commonly known. As there are various processed hazelnuts available on the market, we investigated the suitability of the method in this context. Therefore, potato powder spiked with differently processed hazelnuts (proficiency test sample 3) was examined. Unroasted and roasted hazelnut, as well as hazelnut in the form of nut croquant, nut spread with cocoa, or nut nougat was detected unequivocally (SI Figure 5). Finally, to demonstrate the applicability of the developed rapid LAMP method in real-life foods, 15 commercial food items (e.g., cereals, cookies, cream, porridge) with different compositions of major and minor food components were examined. In the case of samples with the quantitative declaration of the hazelnut content in the ingredient list, amounts between 2 and 30% were specified (SI Table 2). DNA from the food samples was extracted, diluted in 1:10 and 1:100, and analyzed. All foods with hazelnut as an ingredient tested positive with a clear change in color, while hazelnut was not detected in any item without a declared hazelnut ingredient (SI Figure 6). Thus, the correct labeling of hazelnut ingredients was confirmed analytically using the developed hazelnut-specific LAMP.

The colorimetric LAMP reactions of all samples that were analyzed during this study were subsequently examined using additional LFD detection with 300 reactions in total (SI Tables 6−9). Each result obtained by colorimetric detection was confirmed in the LFD analyses with one exception. Using additional LFD detection, eight out of eight reactions of the muffin with 1 mg/kg hazelnut ingredient were tested positive (SI Figure 7) while colorimetric LAMP resulted in seven of eight positive reactions (Figure 2). Thus, in the case of additional verification, LFD detection serves as a sensitive and robust tool.

4. DISCUSSION

The sensitivity, specificity, and applicability of the developed method were demonstrated on a large number of food samples,

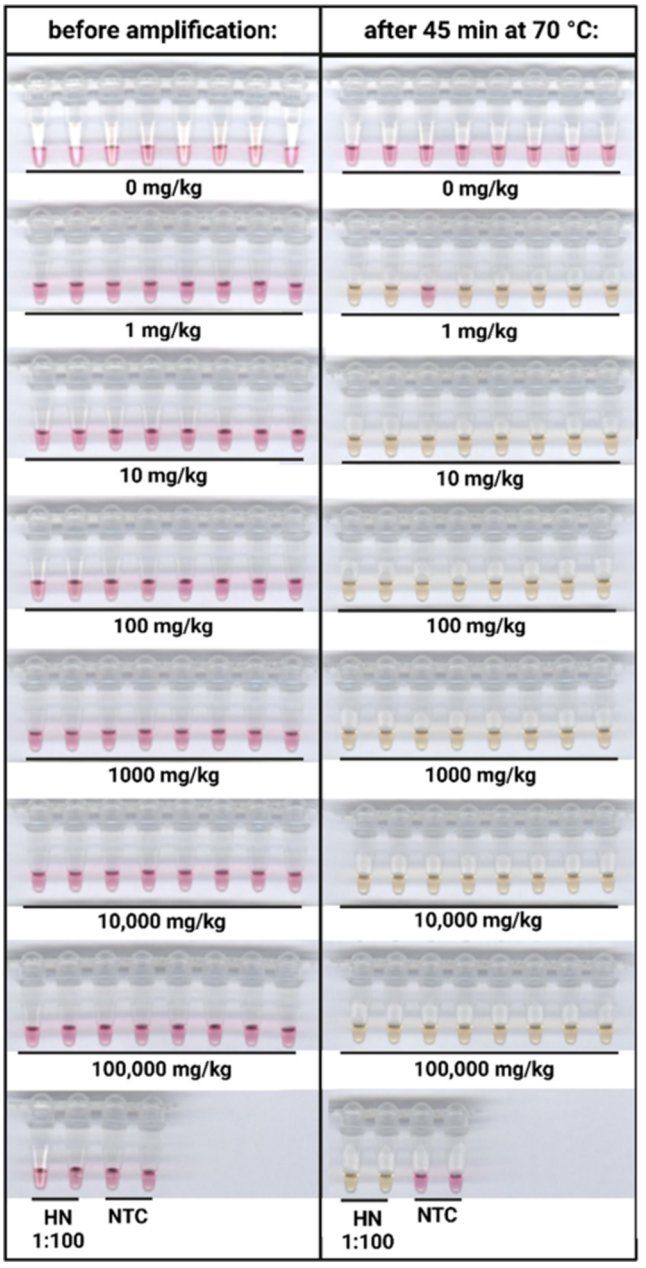

Figure 2. Colorimetric LAMP analysis of muffins with various levels (0−100,000 mg/kg) (0−10%) of incurred hazelnut before (left) and after isothermal amplification at 70 °C for 45 min (right). DNA was extracted using rapid DNA extraction according to Schäfer et al.[24] Elution buffer served as no template control (NTC), and hazelnut DNA (HN) diluted in 1:100 as a positive control.

considering parameters such as matrix dependencies, possible cross-reactivities, or different processing treatments. The specificity of a detection method is crucial to preventing false-positive results. In this study, no cross-reactions to other commonly used foods were observed (SI Figure 2), further underpinning the potential of nucleic acid−based allergen detection for high specificity.[8,31] Furthermore, the risk of cross-reactivity is particularly high among other nuts in cases of phylogenetic similarity. By testing nut-containing foods, it was shown that there are no sequence-dependent false-positive results within these different nuts in a simulated food matrix environment (SI Figure 3 and Table 2). In contrast, detection of hazelnut allergens based on antibodies could lead to false-

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positive results due to cross-reactivity, which has been reported in various works for both ELISA and LFD methods for hazelnuts.[9,32,33]

Assuming the need to verify hazelnut presence in a large food portion such as 500 g, a suitable detection method should allow verification of a level of 6.0 mg/kg of hazelnut protein in food.[28] Considering the requirement of a method sensitivity that is at least 3-fold lower than the allergen level to be verified,[28] the sensitivity should be at a level of 2 mg/kg hazelnut protein or 12.5 mg/kg hazelnut based on the source material investigated in our incurred muffin matrix. In our study, the developed colorimetric LAMP method demonstrated detection sensitivity at or below 10 mg/kg hazelnut in a muffin (Figure 2). This result is based on eight replicates per analyzed level of hazelnut in the muffin. In order to determine an even more accurate LOD value, a validation study should be conducted using hazelnut levels between 1 and 10 mg/kg in smaller increments and ideally in a collaborative trial, thus providing statistical power. Considering a protein content of 16.3% of the incurred hazelnuts, the detection of hazelnut at a level that corresponds to 1.6 mg/kg hazelnut protein is therefore possible. Consequently, the sensitivity of the developed colorimetric LAMP method is sufficient in light of recent approaches in predicting eliciting doses in hazelnut-allergic patients. As was demonstrated in the muffin matrix, the optional subsequent use of LFD-based detection (SI Figure 7) may improve sensitivity down to 1 mg/kg hazelnut in food, equivalent to 0.16 mg/kg hazelnut protein. Such sensitivity would allow for verification of hazelnut that is equivalent to 0.5 mg/kg hazelnut protein, i.e., the ED_{05} reference dose of total hazelnut protein in a theoretical food portion of 6000 g. However, in most analytical scenarios, very high sensitivity is not required. Moreover, special care needs to be taken to avoid false-positive results because of the very high sensitivity and risk of contamination of subsequent analysis due to open manipulation with amplified DNA.

With an increasing number of food processing technologies and different food products, the associated potential negative impact on the functionality of detection methods should be ruled out at least in characteristic food matrices. Previous studies have shown substantial differences in the quantitative detectability of allergens in foods depending, for example, on process-related treatments (e.g., pressure or thermal processing) or the impact of different matrix components such as fat or polyphenols.[29,34] Such potential limitations were addressed during the development of hazelnut-specific LAMP by testing suitable samples. It was successfully demonstrated that the method is not impaired by matrix-related inhibitors or technological processing in the studied food matrices (SI Figures 4 and 5 and Figure 2).

The applicability of a detection method generally depends on the sample material and must be assessed. Accordingly, high food diversity may require slight adjustments in the sample treatment. For the potato powder samples, it was necessary to use the double volume of extraction buffer, whereas the DNA of the dark chocolate samples had to be diluted in 1:2 prior to amplification. However, this is a general issue of analytical methods, and a universal approach that leads to ideal results for every type of food matrix has yet to be demonstrated, if possible at all.

As summarized elsewhere for hazelnut protein-specific methods, limits of detection usually range between 0.1 and 1 mg of hazelnut protein per kg food for published ELISA methods (commercial and noncommerical methods) and approximately 0.1−5 mg of hazelnut protein per kg for MS.[8] Hazelnut-specific PCR was reported to detect approximately 0.01−200 mg/kg hazelnut protein, depending on the method. Compared to previously published sensitive PCR methods, the advantage of the LAMP method developed in this study consists of the combination of high sensitivity and specificity with an easy-to-use and rapid performance without the need for sophisticated laboratory equipment. Compared to antibody-based methods like ELISA and LFD, no such hook effects, originating from high-target protein levels,[12,35] have been observed in this LAMP method. Further, no ethical issues in animal experiments, such as the generation of antibodies or antisera, arise with the use of LAMP methods, which is in accordance with Directive 2010/63/EU.[36] In summary, the developed DNA-based hazelnut-specific LAMP method represents a powerful alternative or further option as an analytical screening tool to current state-of-the-art methods for the detection of hazelnut. Moreover, the developed method addresses limited sensitivity of the previously published hazelnut-specific LAMP method according to Zhang et al., because the method in this study is more sensitive (10 mg/kg hazelnut in muffin vs 100 mg/kg hazelnut in rice sample) and proven more specific (testing of walnut and peanut plus 20 other tested food components vs testing of walnut and peanut).

In addition, the extensive testing of the developed LAMP method extends its applicability to a wide range of foods or food products, which may also serve as medicinal products in the form of allergen diagnostic or therapeutic preparations. Thus, the method may support (a) risk-based precautionary labeling of noningredient hazelnut, (b) quality verification of the hazelnut presence and calculable content in allergen preparations intended for the use in allergen-specific immunotherapy (AIT), and (c) in clinical diagnosis before and after AIT.

In conclusion, we developed and investigated several primer sets based on different hazelnut genes. Through *in silico* analysis, subsequent experimental testing, and optimization, a set of six primers addressing a segment of the hazelnut-specific *ITS2* gene was chosen. The resulting LAMP was successfully tested as a rapid method with DNA extraction and colorimetric detection by the naked eye in less than 1 h, making it predestined as a simple monitoring or verification tool. As demonstrated, the robust and straightforward method is sensitive and specific, which makes it suitable to identify low levels of hazelnuts in various foods. The method qualifies as a screening method not only to improve food control and food safety, potentially also in field-like applications, but also to support allergen analysis as a quality instrument in the field of allergen diagnostic and therapeutic medicinal products.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c07076.

This includes additional experimental details, materials, and methods, and supporting results (PDF)

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Author Contributions

The manuscript was written by L.S. Further contributions: L.S. (conceptualization, methodology, validation, formal analysis, investigation, data curation), T.H. (conceptualization, methodology, project administration, funding acquisition, resources, supervision), E.V. (assistance in the implementation, formal analysis and investigation of the final primer set), M.E. (formal analysis and investigation at an early stage of LAMP development), B.A. (conceptualization, project administration, funding acquisition), and K.B. (conceptualization, project administration, funding acquisition). All authors have reviewed and given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Compliance with Ethical Standards: No animal experiments were performed and no material derived from humans was used.

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ABBREVIATIONS

(AIT), allergen immunotherapy; (DBPCFC), double-blind placebo-controlled food challenges; (ELISA), enzyme-linked immunosorbent assay; (FITC), fluorescein isothiocyanate; (HSP1), heat-shock protein 1; (HPLC), high-performance liquid chromatography; (ITS), internal transcribed spacer; (LFD), lateral flow device; (LOD), limit of detection; (LAMP), loop-mediated isothermal amplification; (MS), mass spectrometry; (PCR), polymerase chain reaction; (PAL), precautionary allergen labeling; (LoQ), limits of quantification

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Supporting Information

Development and validation of a loop-mediated isothermal amplification (LAMP) method for the rapid, sensitive and specific detection of hazelnut as an allergen in food matrix

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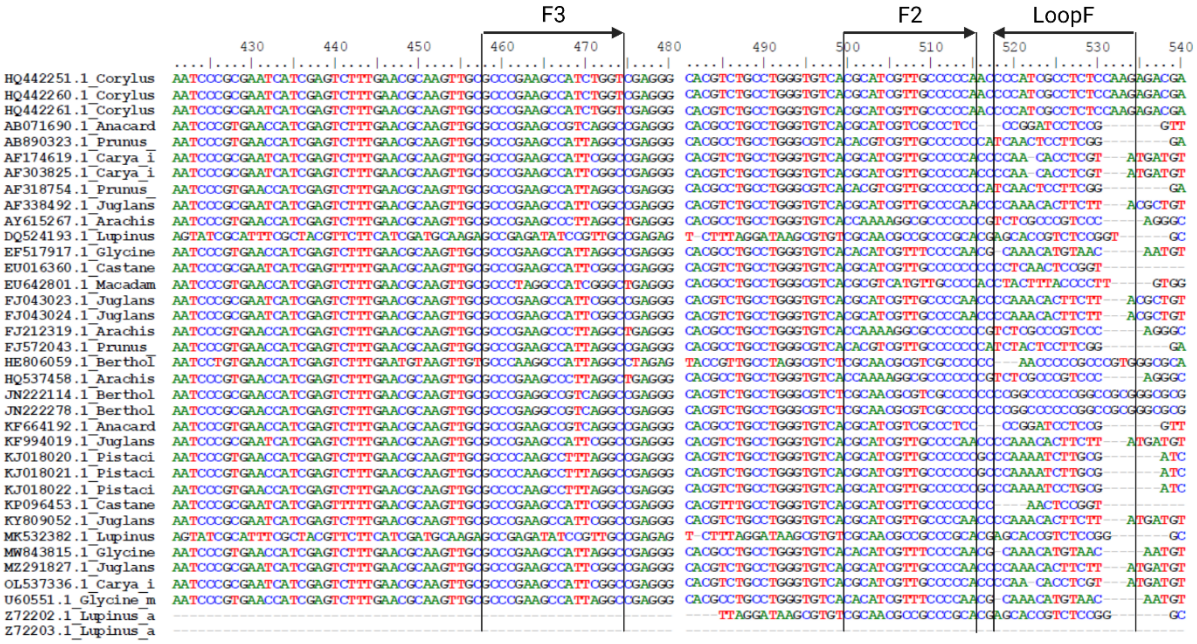
SI Figures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F2
LoopF
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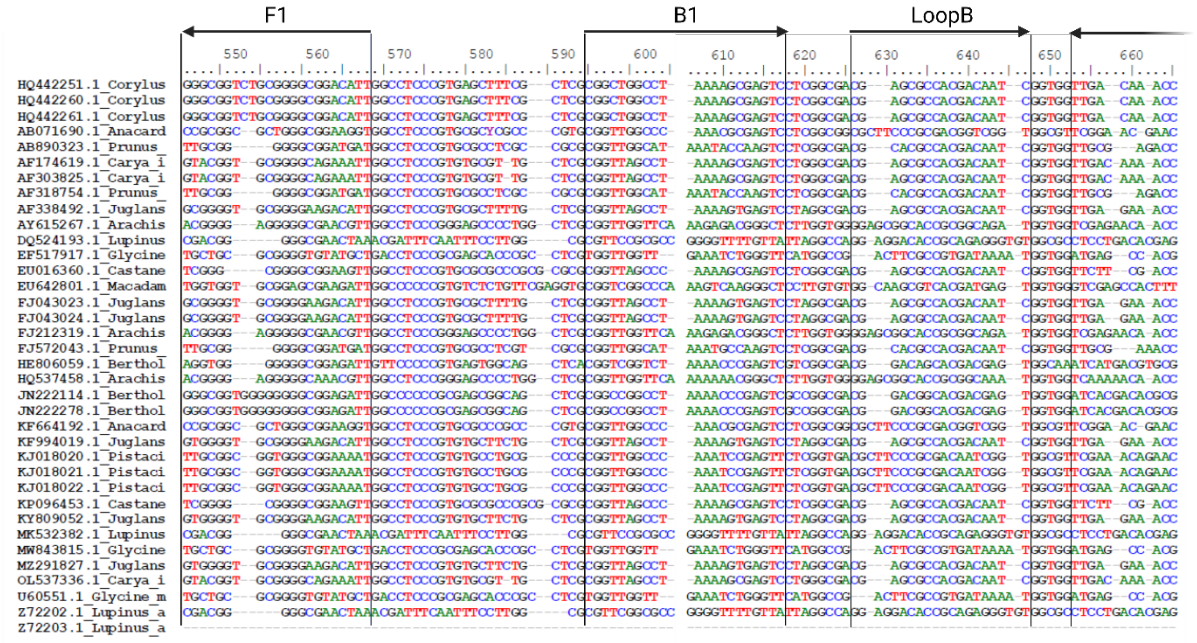
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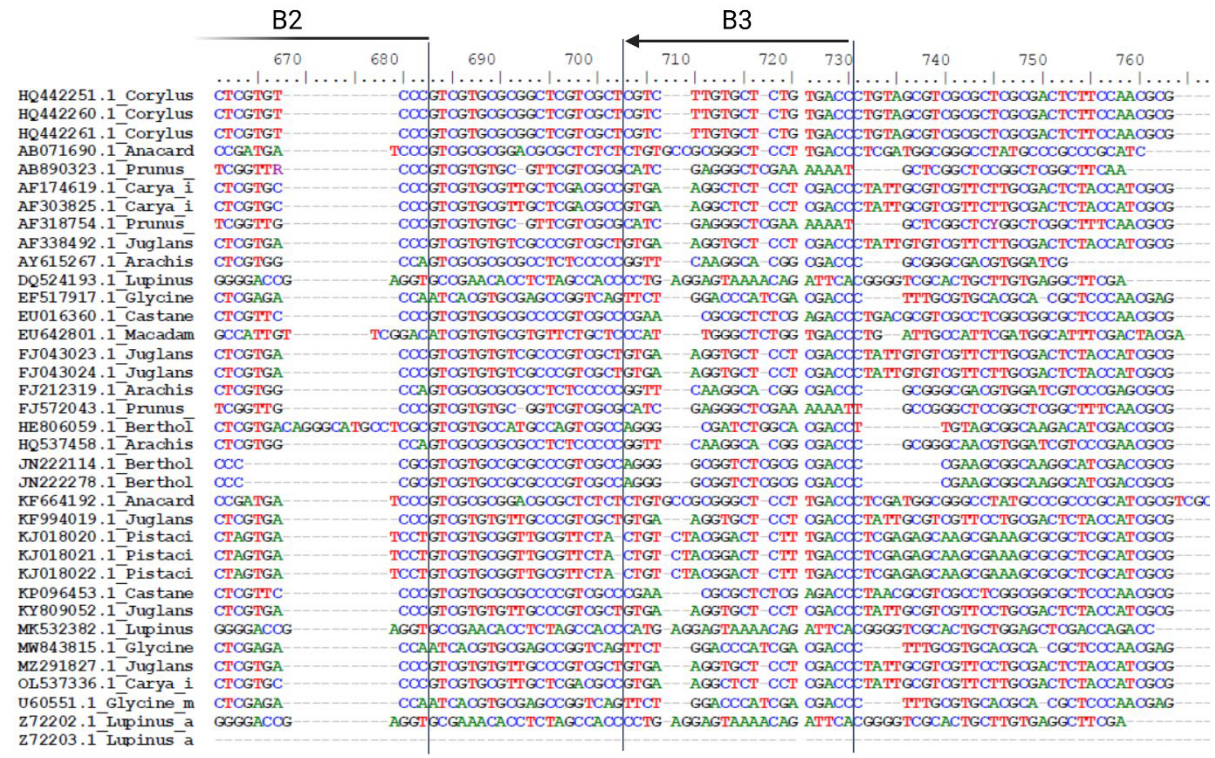
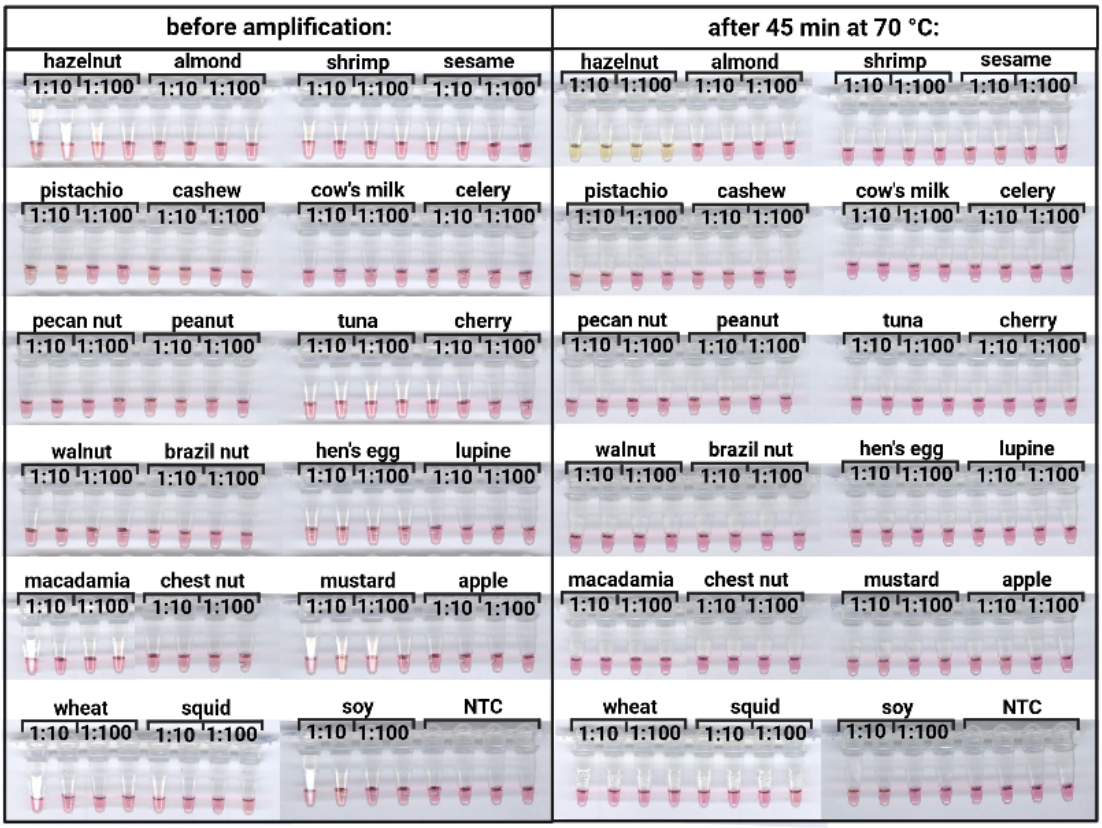
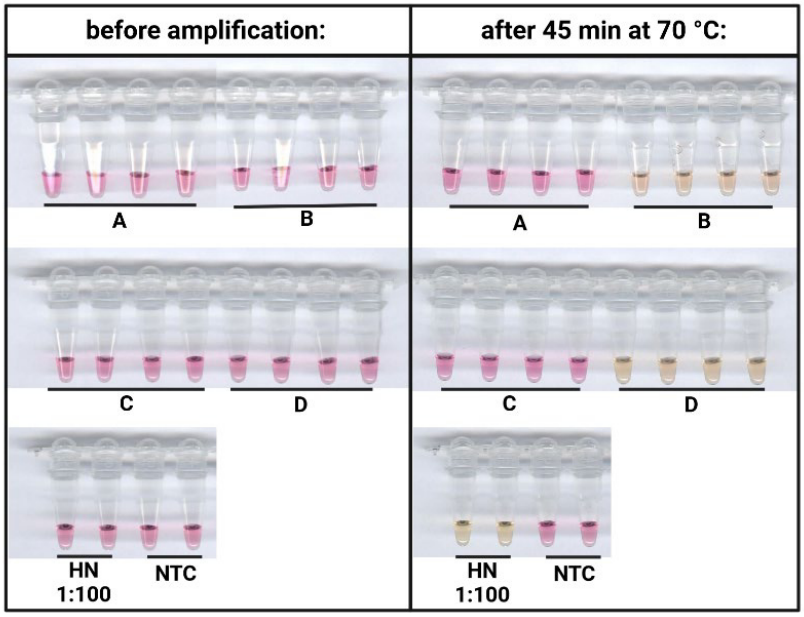


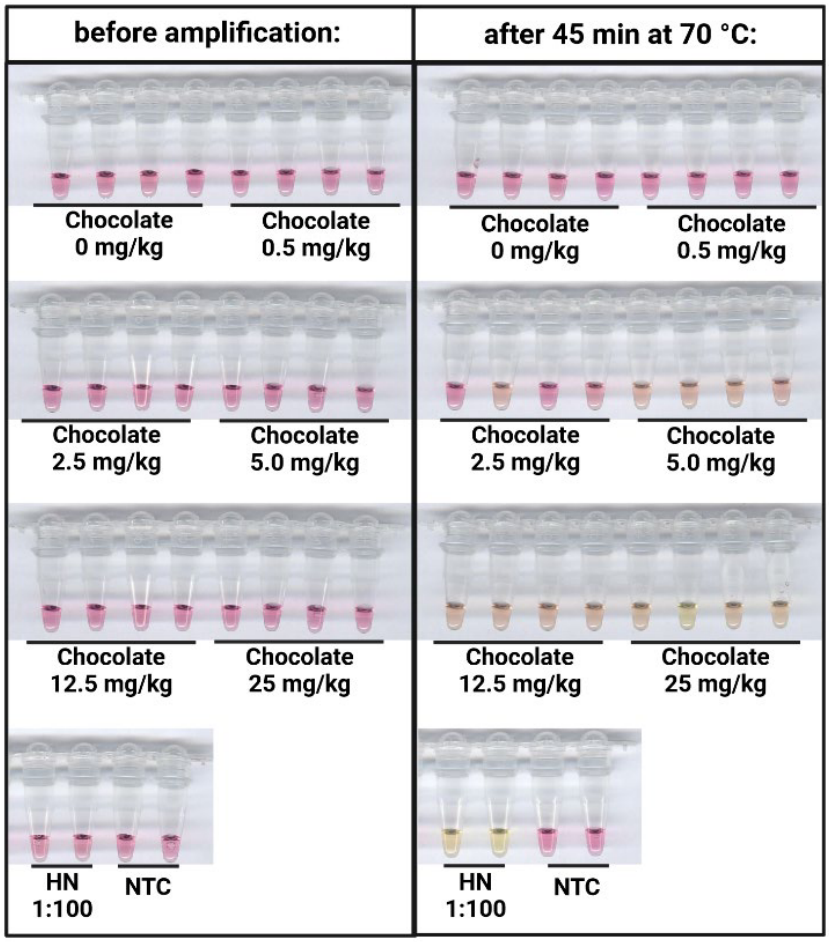
SI Fig. 1. Sequence alignment of the ITS2 gene of hazelnut (GenBank acc. No. HQ442260.1) and homologous food species and location of the binding sequences of the used primer set no. 4 including forward and reverse primers (F3 and B3), forward inner primers (FIP: F1 and F2), reverse inner primers (BIP: B1 and B2), forward and reverse loop primers (LoopF and LoopB).



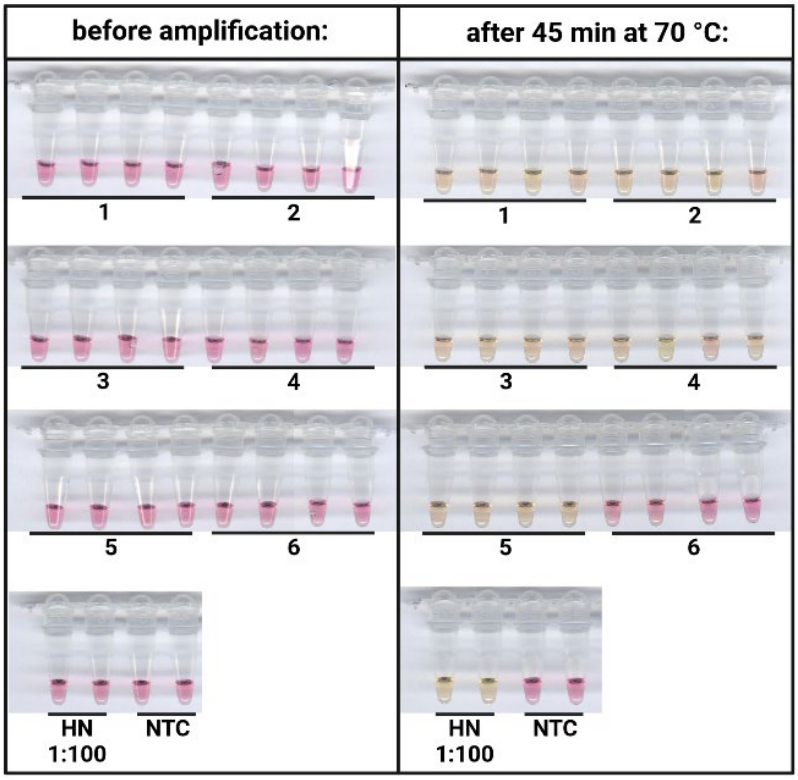
SI Fig. 2. Specificity testing of the colorimetric hazelnut LAMP method (ITS2, set 4) in 22 foods (diluted 1:10 and 1:100). DNA was extracted according to Schäfer *et al.* (2023). Elution buffer served as no template control (NTC).



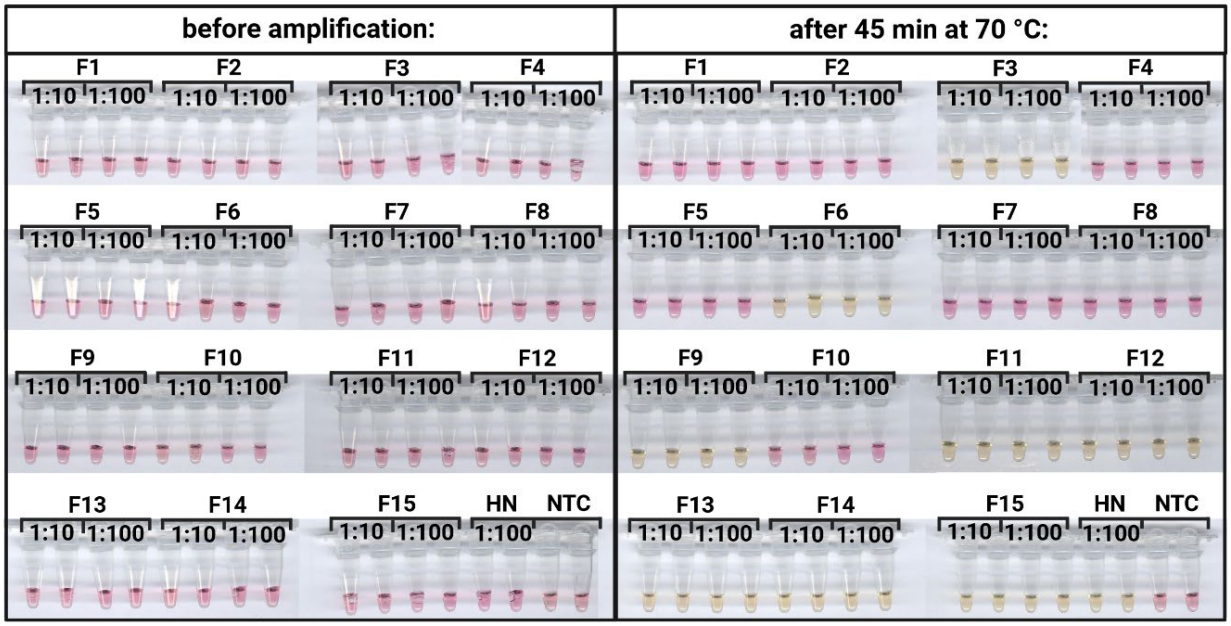
SI Fig. 3. LAMP analysis (ITS2, set 4) of the proficiency test sample 1 (details in SI Tab. 3) "Allergen Screening: cashew, hazelnut, macadamia, almond, brazil nut, pecan nut, pistachio and walnut". **A** and **C** does not contain hazelnut, **B** and **D** contains hazelnut (25-75 mg/kg and 50-150 mg/kg according to manufacturer), HN: hazelnut DNA, NTC: no template control (elution buffer).



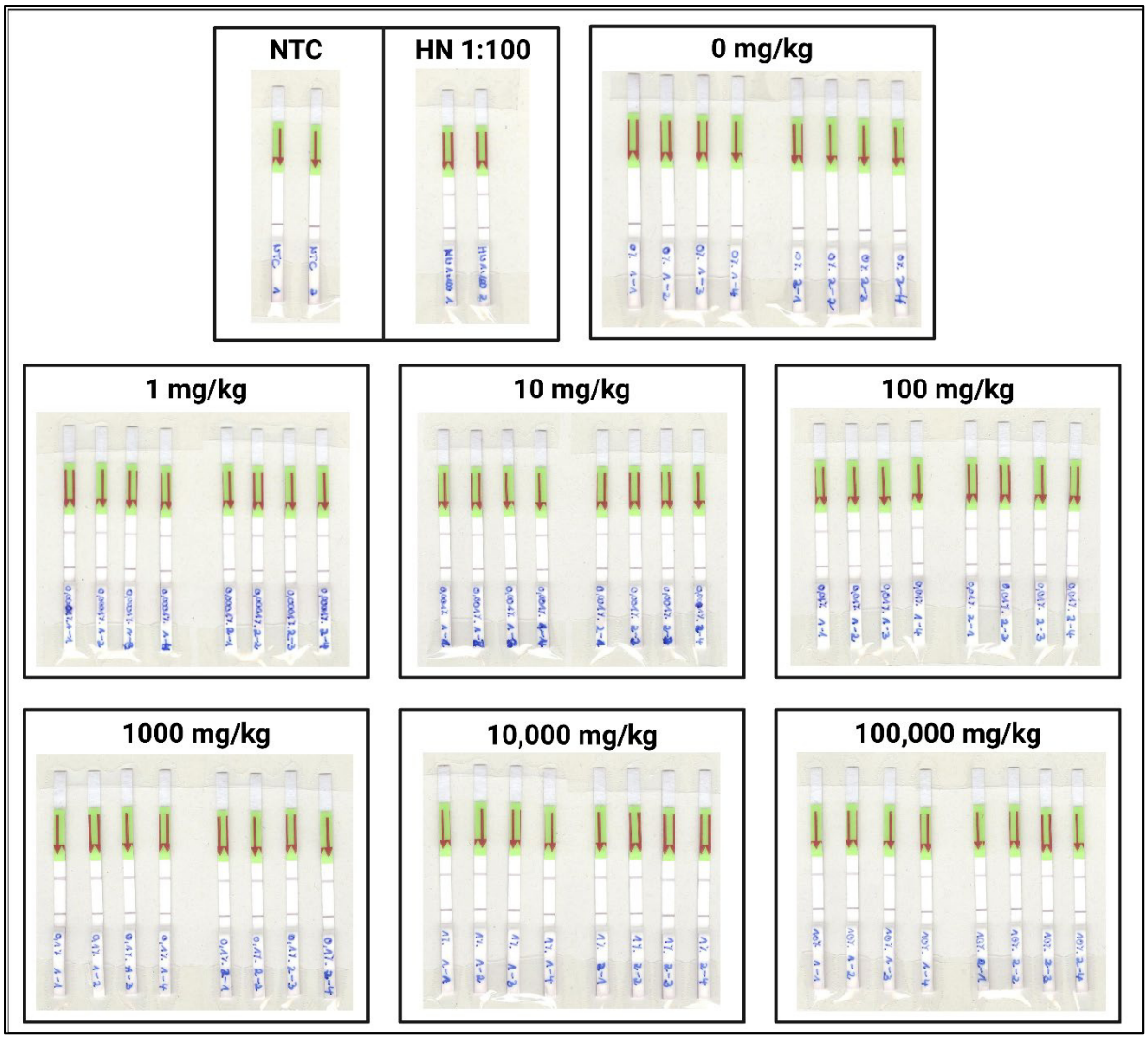
SI Fig. 4. LAMP analysis (ITS2, set 4) of the proficiency test sample 2 (details in SI Tab. 4): "hazelnut in chocolate matrix, 5 samples with roasted hazelnut" between 0.5 and 25 mg/kg, HN: hazelnut DNA, NTC: no template control (elution buffer). The extracted DNA was diluted 1:2 with elution buffer before use.



SI Fig. 5. LAMP analysis (ITS2, set 4) of the proficiency test sample 3 (details in SI Tab. 5): processed hazelnuts: **1**) hazelnut (not roasted), **2**) nut butter (roasted), **3**) nut crocant, **4**) nut spread with cocoa, **5**) nut nougat and **6**) blank sample without hazelnut. Each sample (except blank) contains approx. 50 mg/kg of processed hazelnut. HN: hazelnut DNA, NTC: no template control (elution buffer).



SI Fig. 6. LAMP analysis (ITS2, set 4) of 15 food items (details in SI Tab. 2, F1-F15, 1:10 and 1:100 diluted), before (left) and after isothermal amplification at 70 °C for 45 min (right), HN: hazelnut DNA (positive control), NTC: no template control (elution buffer). All positive samples (F3, F6, F9, F11, F12, F13, F14, F15) contain hazelnut as an ingredient. All other food items have no hazelnut ingredient declared. F2, F4, F5, F8, F10 have a PAL declaration with regard to “nut product”, “nut(s)”, or “hazelnuts”. F1 and F7 has neither an ingredient declaration nor a PAL declaration for hazelnut.



SI Fig. 7. LAMP-LFD analysis (ITS2, set 4) of the muffins (0 to 100,00 mg/kg), NTC: no template control (elution buffer), HN: 1:100 diluted hazelnut DNA (positive control).

SI Tables

SI Tab. 1. Tested primer sets for the genes heat-shock protein 1 (HSP1), NADH dehydrogenase subunit K, J and 3 (NADH-KJ3), Cor a 14 and internal transcribed spacer 2 (ITS2), in addition to the final primer set (ITS2 set 4, table 1).

HSP1		
sequences 5‘→ 3‘		
primer set 1	F3	CAGGACCCATTCAGCGATT
	B3	GGACCTTGAACCTGCTCAC
	FIP	TCTCGGGGTCTCCCTCCAGTTCCACGCTCTTCCCTCAC
	BIP	TCTCTTCCGGGGTTCGTGGACACTCTCGACGCTCACCT
	LoopF	CCTGGTGTTCACCGAGCT
	LoopB	CCAAGACGACCGAGTGCT
NADH-KJ3		
sequences 5‘ → 3‘		
primer set 1	F3	AGTCTCTATTCCTTGGTAATCG
	B3	TCGAATTAGGTTTCAGCAGG
	FIP	AGAGTTCAGTCTCTTCCCACGAAACTAGCCCATGTTTGACG
	BIP	ATCCCCGAAGTAGATGGTGGTGGCGAAGTATTCATACTGGAA
	LoopF	CAGGGTCGTTTGTCTGCT
	LoopB	TGATAGAGTAATCCTCGGTCAT
primer set 2	F3	CTCTATTCCTTGGTAATCGAAT
	B3	CGAATTAGGTTTCAGCAGGG
	FIP	GTCTCTTCCCACGAATTGGAACTAGCCCATGTTTGACG
	BIP	ATCCCCGAAGTAGATGGTGGTTTTCATATTGGGCGAAGTATT
	LoopF	GTCGTTTGTCTGCTTGGC
	LoopB	GATAGAGTAATCCTCGGTCATG
Cor a 14		
sequences 5‘ → 3‘		
primer set 1	F3	TTACGATGGCAGCAACCAG
	B3	TGAGGCGGCATTGATTTGG
	FIP	CAACCCCTCACATCGGCACCCAGCAGCAGCAGGAACTT
	BIP	GGCAGGCGGTAATGCAGCAGCAAGTCCCTAGCAGTCTCCA
	LoopF	CAGCTGTTGGCAGCACTG
	LoopB	GTGAGATGCGAGGTGAGGAAAT
primer set 2	F3	TTACGATGGCAGCAACCAG
	B3	CTTGCGGAGCGAATCTCAC
	FIP	TCAACCCCTCACATCGGCACCAGCAGCAGCAGGAACTT
	BIP	CAGGCGGTAATGCAGCAGCAGCAAGTCCCTAGCAGTCT
	LoopF	CAGCTGTTGGCAGCACTG
	LoopB	TGAGGAAATGAGGGAGGTTATGG
ITS2		
sequences 5‘ → 3‘		
primer set 1	F3	GCTCGCGGCTGGCCTAAA
	B3	TTAAATTCAGCGGGTAGT
	FIP	GACGGGACACGAGGGTTTGTGAGTCCTCGGCGACGA
	BIP	GCGCGGCTCGTCGCTCGTCTTGCCTGGGGTCGCGTTGGAAG
	LoopF	ACCACCGATTGTCGTGGCGC
	LoopB	GACCCTGTAGCGTCGCGCT
primer set 2	F3	CCCGAAGCCATCTGGTC
	B3	GGTCACAGAGCACAAGACG
	FIP	AATGTCCGCCCCGCAGACCCGCATCGTTGCCCCCA

	BIP	GCGGCTGGCCTAAAAGCGGTTTGTCAACCACCGATTGT
	LoopF	CTTGGAGAGGCGATGGG
	LoopB	GAGTCCTCGGCGACGA
primer set 3	F3	CCCGAAGCCATCTGGTC
	B3	GGTCACAGAGCACAAGACG
	FIP	AATGTCCGCCCCGCAGACCCGCATCGTTGCCCCCA
	BIP	CGGCTGGCCTAAAAGCGAGTCGGGACACGAGGGTTTGTCAA
	LoopF	CTTGGAGAGGCGATGGG
	LoopB	CGAGCGCCACGACAATC

SI Tab. 2. Tested commercial food items, ingredient list and precautionary allergen labelling (PAL) regarding hazelnut.

	food item	**list of ingredients** (labelled hazelnuts highlighted in bold)	**PAL**
F1	Mini muffins black and white (pastry)	wheat flour 28%, sugar, vegetable oils (soy, sunflower; in variable proportions by weight), eggs, glucose syrup, humectants: E422, E420i; low-fat cocoa powder 2%, raising agents: E450i, E500ii; salt, preservative: E200; aroma	-
F2	Roll fondant white (fondant)	sugar, glucose syrup, vegetable fats (shea, coconut), humectant glycerine, thickener (xanthan gum, E 464), emulsifier mono- and diglycerides of fatty acids, acidifier citric acid, preservative potassium sorbate, natural aroma	may contain: cereals containing gluten and cereal products containing gluten. nuts and nut products. milk and milk products (including lactose)
F3	Crunchy mixture (muesli)	wholemeal flakes (oats, wheat), nut kernel mix (**HAZELNUTS**, peanuts, almonds, cashews), barley flakes, sunflower seeds, hull-less pumpkin seeds, sesame seeds	may contain traces of nuts of all kind.
F4	Confectionery (muesli)	wholemeal oat flakes, wholemeal wheat flakes, raisins unsulphured, barley flakes, chocolate flakes (cane sugar, cocoa mass, cocoa powder de-oiled, emulsifier rapeseed lecithin, cocoa butter, milk powder), cornflakes (maize, barley malt, sea salt), wholemeal cocoa spelt balls* (wholemeal spelt*, cocoa powder de-oiled, grape juice concentrate)	may contain traces of soy, dairy products, peanuts and nuts of all kinds
F5	White chocolate	sugar, cocoa butter, skimmed milk powder, sweet whey powder (milk), clarified butter, emulsifier (soy lecithins), aroma	may contain: nuts
F6	Noisette chocolate	sugar, cocoa butter, **HAZELNUTS (13%)**, skimmed milk powder, cocoa mass, sweet whey powder (milk), clarified butter, emulsifier (soy lecithin), aroma	may contain other nuts
F7	Original butter cookie	wheat flour (70%), sugar, butter (12%), invert sugar syrup, raising agents: sodium carbonates, diphosphates; whey product (milk), whole milk powder, salt, emulsifier: lecithins; acidifier: citric acid; aromas (milk), whole egg powder	**-**
F8	Butter leaves	wheat flour, butter (25 %), sugar, glucose syrup, whey product (milk), salt, raising agents: sodium carbonates, diphosphates; starch (wheat)	may contain: hazelnuts
F9	Kipferl (cookies)	wheat flour, vegetable fats (palm), sugar, ground **HAZELNUTS (12%),** whole milk powder, whole egg powder, roasted barley whole grain flour, dextrose, starch (wheat), emulsifier: lecithins; raising agents: diphosphates, sodium carbonates; aroma, anti-caking agent: magnesium salts of fatty acids; acidifier: citric acid; salt, spice	-
F10	Peanut flippies	maize semolina, peanuts (32%), sunflower oil, table salt, sugar, natural aroma, emulsifier (mono- and diglycerides of fatty acids), yeast extract (contains barley)	may contain traces of nuts
F11	Protein cream: hazelnut and sweet whey cream	rapeseed oil, sweetener (maltitol), whey proteins (from milk, 25%), sweet whey powder (from milk, 7%), cocoa butter, **HAZELNUTS (6.5%),** low-fat cocoa powder (3.2%), emulsifier (lecithins), natural vanilla aroma	may contain nuts
F12	Chocolate spelt porridge with hazelnuts	41% whole wheat flakes, 26% whole oat flakes, 9% dried dates, 7% spelt wheat puffed, 7% banana flakes (95% banana puree, rice flour), 3.6% cocoa powder, **HAZELNUTS (2.2%)** chopped and roasted, ground tigernuts, popped amaranth	may contain traces of soy, sesame, almonds, cashew nuts, kernels and shells
F13	Dr. Almond choketo low carb & keto chocolate	cocoa butter, **HAZELNUTS (30%),** cocoa powder (lightly de-oiled), cream powder, erythritol, bourbon vanilla, sweeteners sodium cylamate and sodium saccharin	may contain traces of other nuts
F14	Bar with salty chocolate Nut flavor	glucose syrup, peanuts 15.7%, soy protein crispies 14.8%, almonds 7.9%, soy protein crispies 6.9% (soy protein, tapioca starch, salt), **HAZELNUTS (6.9%)**, maltodextrin, pea protein, chocolate drops 5% (sugar, cocoa mass, cocoa butter, emulsifier (soy lecithins), flavorings), humectant (glycerine), sunflower oil, cocoa butter, sea salt, emulsifier (lecithins)	may contain traces of milk, egg, gluten, sesame and nuts
F15	Wholemeal spelt chocolate cookies	wholemeal spelt flour (43%), beet sugar, palm oil, dark chocolate (9% cocoa mass, raw cane sugar, cocoa butter, emulsifier: lecithin), **HAZELNUTS (6%)**, low-fat cocoa, sea salt, raising agent: sodium carbonate, coriander, cinnamon	may contain traces of other nuts, milk and sesame

Proficiency test sample 1

The proficiency test sample 1 consists of 72-76% potato powder (ingredients: potato, E471, E304, E223, E100), 24-26% maltodextrin and 0.28-0.58% allergen premixes ingredients (maltodextrin (75% - 90%), sodium sulphate (6.1-14%), silicon dioxide (3.5-10%), nut butters (1.1-1.7% each)). The composition of the four samples tested (samples A - D) regarding containing nuts is shown in **SI Tab. 3**. Cashew (18.4% protein) and hazelnut (15.9% protein) were used as nut butters, macadamia (9.4% protein), brazil nut (14.8% protein), pecan nuts (12.2% protein), pistachio (25.6% protein) and walnut (13.9% protein) were used as crushed nuts.

SI Tab. 3. Composition of proficiency test sample 1.

nut ingredient	sample A	sample B	sample C	sample D
cashew	negative	positive (25-75 mg whole nut per kg sample)	negative	negative
hazelnut	**negative**	**positive** (25-75 mg whole nut per kg sample)	**negative**	**positive** (50-150 mg whole nut per kg sample)
macadamia	negative	negative	negative	positive (50-150 mg whole nut per kg sample)
almond	positive (25-75 mg whole nut per kg sample)	positive (50-150 mg whole nut per kg sample)	negative	negative
brazil nut	positive (25-75 mg whole nut per kg sample)	positive (25-75 mg whole nut per kg sample)	negative	negative
pecan nut	negative	positive (50-150 mg whole nut per kg sample)	positive (25-75 mg whole nut per kg sample)	negative
pistachio	positive (25-75 mg whole nut per kg sample))	negative	positive (25-75 mg whole nut per kg sample)	negative
walnut	negative	negative	negative	positive (25-75 mg whole nut per kg sample)

Proficiency test sample 2

Proficiency test sample 2, dark chocolate, consists of cocoa mass, sugar, cacao butter, emulsifier, soy lecithin, vanilla extract, maltodextrin, sodium sulfate and silicon dioxide. The hazelnut content of the 6 samples are shown in **SI Tab. 4**. The protein content according to laboratory analysis of the raw material is 14.1 ± 0.15%, n=5 (total nitrogen according to Kjeldahl with F=5.30 for hazelnut protein) according to the manufacturer.

SI Tab. 4. Composition of proficiency test sample 2.

	Level 0	Level 1	Level 2	Level 3	Level 4	Level 5
hazelnut content [mg/kg]	0	0.504	2.53	5.04	12.5	25.2

Proficiency test sample 3

Proficiency test sample 3 consists of 75% potato powder (Ingredients: potato, E471, E304, E223, E100) and 25% maltodextrin, sodium sulfate and silicon dioxide. The hazelnut content of the 6 samples are shown in **SI Tab. 5**.

SI Tab. 5. Composition of proficiency test sample 3.

	sample 1 hazelnut, unroasted	**sample 2 hazelnut, roasted**	**sample 3 nut crocant**	**sample 4 nut spread with cocoa**	**sample 5 nut nougat**	**sample 6 "blank"**
hazelnut content [mg/kg]	50.4	53.7	53.6	51.0	53.2	0

SI Tab. 6. Analysis of hazelnut (HN) DNA dilutions 1:10 to $1:10^7$ using LAMP-LFD, NTC: no template control.

Sensitivity	
HN dilution	positive LFDs
1:10	4/4
$1:10^2$	4/4
$1:10^3$	4/4
$1:10^4$	4/4
$1:10^5$	4/4
$1:10^6$	4/4
$1:10^7$	2/4
NTC	0/4

SI Tab. 7. Analysis of hazelnut (HN) and 22 other food species using LAMP-LFD, NTC: no template control.

Specificity					
food	**dilution**	**positive LFDs**	**food**	**dilution**	**positive LFDs**
almond	1:10	0/2	sesame	1:10	0/2
	1:100	0/2		1:100	0/2
pistachio	1:10	0/2	cow‘s milk	1:10	0/2
	1:100	0/2		1:100	0/2
cashew	1:10	0/2	celery	1:10	0/2
	1:100	0/2		1:100	0/2
pecan nut	1:10	0/2	tuna	1:10	0/2
	1:100	0/2		1:100	0/2
peanut	1:10	0/2	cherry	1:10	0/2
	1:100	0/2		1:100	0/2
walnut	1:10	0/2	hen‘s egg	1:10	0/2
	1:100	0/2		1:100	0/2
brazil nut	1:10	0/2	lupine	1:10	0/2
	1:100	0/2		1:100	0/2
macadamia	1:10	0/2	mustard	1:10	0/2
	1:100	0/2		1:100	0/2
chestnut	1:10	0/2	apple	1:10	0/2
	1:100	0/2		1:100	0/2
wheat	1:10	0/2	soy	1:10	0/2
	1:100	0/2		1:100	0/2
squid	1:10	0/2	HN	1:10	2/2
	1:100	0/2		1:100	2/2
shrimp	1:10	0/2	NTC	1:1	0/4
	1:100	0/2			

SI Tab. 8. Analysis of proficiency test samples 1, 2 and 3 using LAMP-LFD, HN: hazelnut, NTC: no template control.

Proficiency test sample 1		Proficiency test sample 2		Proficiency test sample 3	
sample	**positive LFDs**	**sample**	**positive LFDs**	**sample**	**positive LFDs**
A (without HN)	0/4	1 (unroasted HN)	4/4	Chocolate 0 mg/kg HN	0/4
B (with HN)	4/4	2 (roasted HN)	4/4	Chocolate 0.5 mg/kg HN	0/4
C (without HN)	0/4	3 (HN crocant)	4/4	Chocolate 2.5 mg/kg HN	2/4
D (with HN)	4/4	4 (HN spread)	4/4	Chocolate 5.0 mg/kg HN	4/4
HN 1:100	2/2	5 (HN nougat)	4/4	Chocolate 12.5 mg/kg HN	4/4
NTC	0/2	6 (blank)	0/4	Chocolate 25.0 mg/kg HN	4/4
		HN 1:100	2/2	HN 1:100	2/2
		NTC	0/2	NTC	0/2

SI Tab. 9. Analysis of 15 commercial food items using LAMP-LFD, HN: hazelnut DNA, NTC: no template control. Details about the commercial food items are shown in **SI Tab. 2**. (-) means no declaration of hazelnut as an ingredient, (+) means declaration of hazelnut as an ingredient.

Commercial food items					
	positive LFDs			**positive LFDs**	
	1:10	1:100		1:10	1:100
F1 (-)	0/2	0/2	F9 (+)	2/2	2/2
F2 (-)	0/2	0/2	F10 (-)	0/2	0/2
F3 (+)	2/2	2/2	F11 (+)	2/2	2/2
F4 (-)	0/2	0/2	F12 (+)	2/2	2/2
F5 (-)	0/2	0/2	F13 (+)	2/2	2/2
F6 (+)	2/2	2/2	F14 (+)	2/2	2/2
F7 (-)	0/2	0/2	F15 (+)	2/2	2/2
F8 (-)	0/2	0/2	HN 1:100	-	2/2
			NTC	0/4	

// Development, characterization and application of aptamers for the detection and quantification of the major peanut allergen Ara h 1 //

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Background and Aim

- Allergen-specific immunotherapies (AIT) are merely lacking in the field of food allergy
- Allergen detection methods support unambiguous allergen labelling of foods for improved allergen avoidance by allergic consumers
- In the field of medicinal products, allergen preparations used in *in vivo* allergy diagnosis and AIT may require quality assessment at the allergen component level
- Antibodies from animals have played a fundamental role in allergen-specific *in vitro* assays, but also bear potential quality issues e.g. natural variations and thus, batch-to-batch consistency
- Further, animal experiments raise ethical concerns and, following the 3R principle (replace, reduce, refine) (European Directive 2010/63/EU), procedures using live animals should be replaced once scientific alternatives allow for

Aim: Development of chemically synthetic aptamers (single-stranded nucleic acids) as an alternative to antibodies for the use in allergen detection systems

Methods and Results

- Ara h 1-specific aptamers were selected via Systematic Evolution of Ligands by Exponential Enrichment (**SELEX**)
- Enriched aptamer sequences were identified by Next Generation Sequencing (**NGS**) and finally obtained by chemical synthesis
- Binding characteristics were measured and the characterized aptamers were applied in: Western blot (Fig. 1), Surface Plasmon Resonance (**SPR**) (Fig. 2), indirect and sandwich Enzyme-linked Aptamer Assay (**ELAA**) (Fig. 3)
- The use of aptamers in Western blot showed comparable binding to monoclonal antibodies (Fig. 1)
- The strongest aptamer had an Ara h 1-specific dissociation constant of 88 nM as well as no cross-reactivity to the purified natural peanut allergens Ara h 2, 3 and 6 (Fig. 2)
- With the ELAA method, sensitivities of approx. 10 ng/mL Ara h 1 were achieved, which are comparable to published antibody-based assays (Fig. 3)
- Ara h 1 could be detected and quantified in various peanut flours, which are comparable to those already used in peanut AIT, as well as in a complex and processed food matrix (Fig. 4)

Western blot Analysis

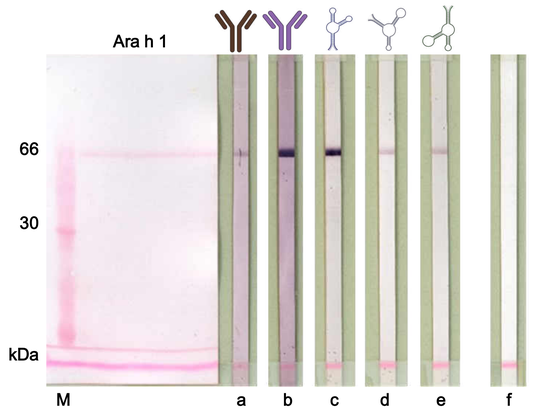

Fig. 1. Ponceau S stained Ara h 1 protein membrane (Ara h 1), marker (**M**). Ara h 1 detection with two different monoclonal antibodies (**a, b**) and three developed aptamers (**c, d, e**), buffer (**f**).

Indirect ELISA vs. Indirect ELAA

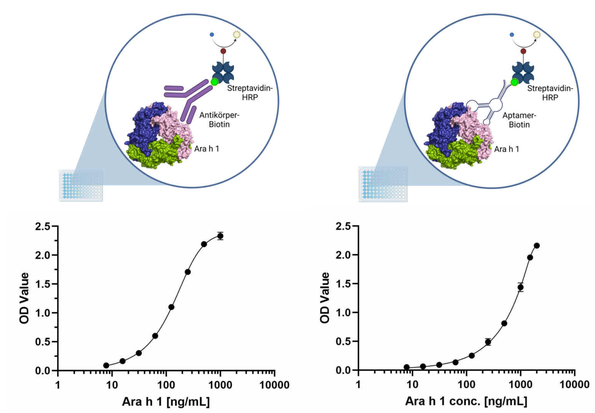

Fig. 3. Detection of Ara h 1 with an indirect antibody-based ELISA (left) vs. an aptamer-based ELAA (enzyme-linked aptamer assay) (right).

Aptamer Characterization via SPR

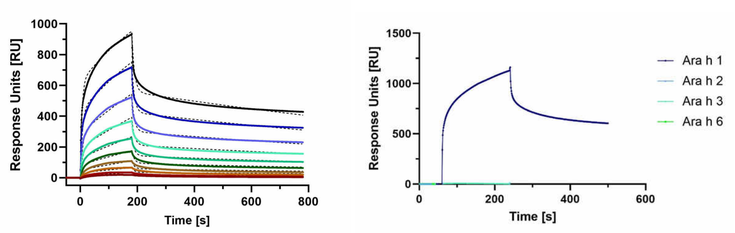

Fig. 2. Aptamer SPR sensorgram to measure the binding kinetics as well as the determination of the K_D (left) (1:2 dilution series, starting at 750 nM, fitted curves are depicted as black dotted lines) and for the measurement of potential cross-reactivities to Ara h 2, 3, and 6 (right).

Analysis of Food Items with an Aptamer Assay

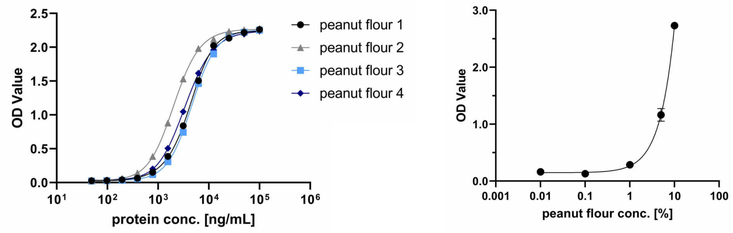

Fig. 4. Indirect enzyme-linked aptamer assay (ELAA) with aptamer-biotin as capture molecule and streptavidin-HRP conjugate for the analysis of Ara h 1 in four different peanut flours (left) and in peanut-containing cookies (right).

Conclusion

- Successful development, characterization and application of Ara h 1 aptamers in different detection methods
- First use of an aptamer-based Ara h 1 specific ELISA-like assay for the analysis of peanut flours and processed foods
- First proof-of-concept for potential use as antibody-free allergen detection for food labelling requirements or the quality assessment of allergen products for *in vivo* diagnosis and AIT

Secondary structure of ssDNA aptamers was predicted using mfold software (Zuker, M., Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 2003). Graphical illustrations were created using BioRender.com.

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// Rapid, specific, and sensitive detection of hazelnut allergen in food using colorimetric *loop-mediated isothermal nucleic acid amplification* (LAMP) //

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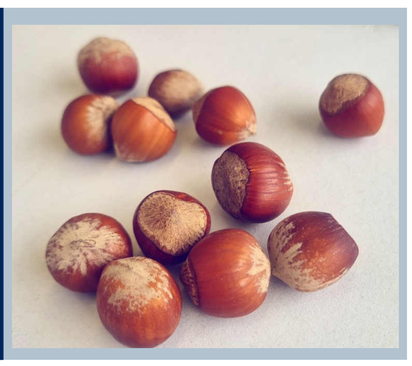
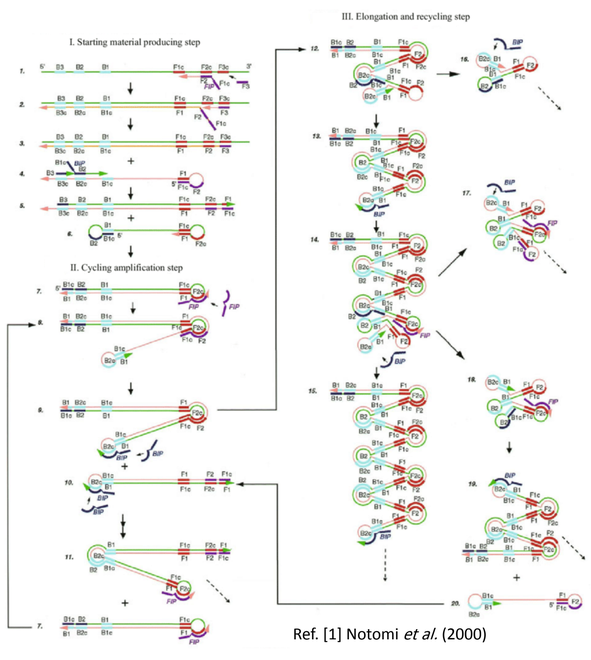


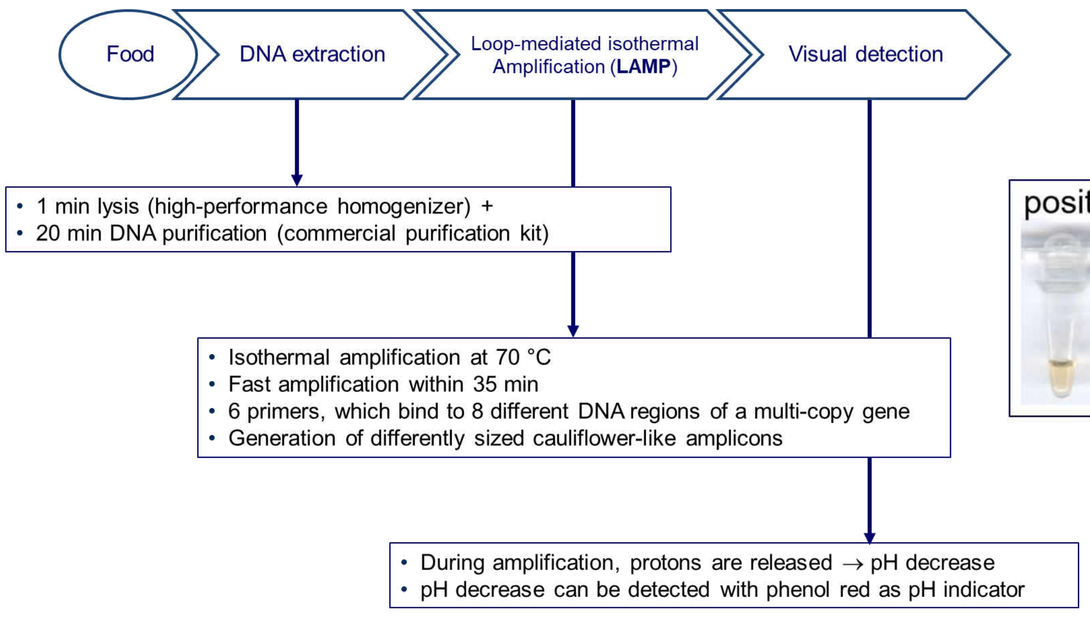
Background: Food allergy is a common immunological disease with potentially strong impact on health and life. A lack of causitive immunotherapies makes food allergen avoidance indispensable. However, this relies mainly on the identification of allergens in foods and, thus, on the correct labeling of the allergens present. Appropriate analytical methods are essential to support reliable allergen labeling.

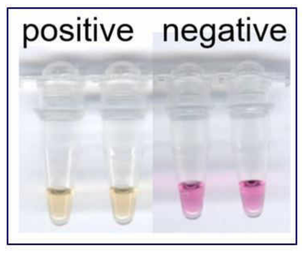
Aim: Development of a rapid method for the specific and sensitive detection of hazelnut in foods, allowing shorter analysis time and requiring less sophisticated laboratory equipment, suitable as a screening test.

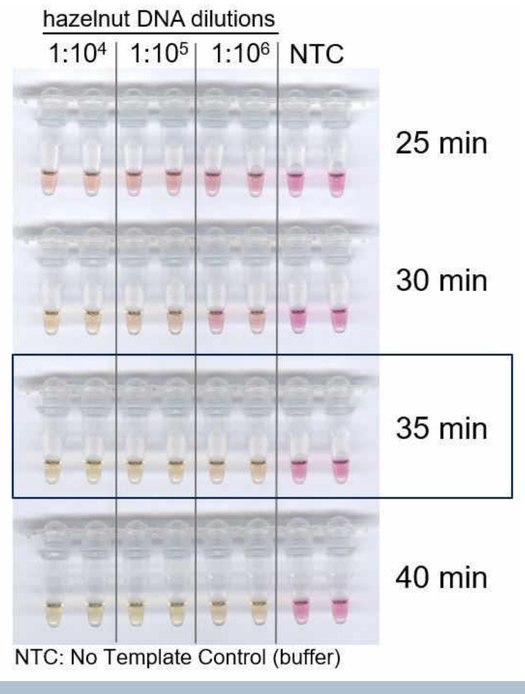
Principle of LAMP-based rapid test for hazelnut



Ref. [1] Notomi *et al.* (2000)





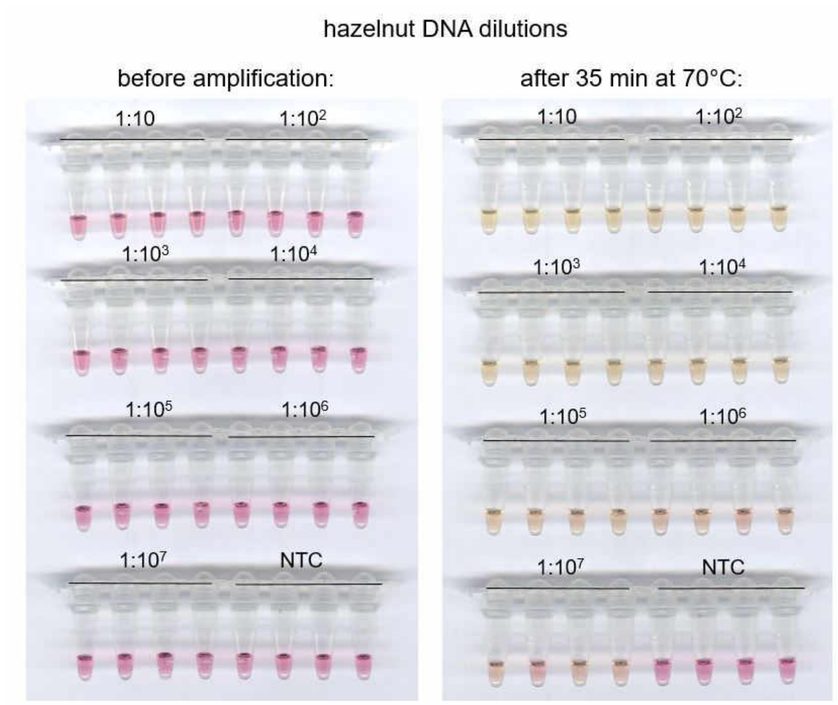


NTC: No Template Control (buffer)

Results

High Sensitivity

- Dilutions of hazelnut DNA down to $1:10^5$ are unambiguously detectable.
- Limit of detection is between $1:10^6$ or $1:10^7$ diluted hazelnut DNA.



NTC: No Template Control (buffer)

High Specificity

- No cross-reactivity found to 22 characteristic food ingredients, incl. tree nuts e. g. almond, walnut, pecan nut, brazil nut, pistachio and macadamia nut.

NTC: No Template Control (buffer)

Applicability in real-life food items 1

- Differently processed hazelnuts (Ref. [2]) are detected:
  Roasted and unroasted, crocant, hazelnut spread with cocoa, and hazelnut nougat (each 50 mg hazelnut/ kg matrix).

Applicability in real-life food items 2

- Ten commercially purchased food items with and without hazelnut as an ingredient were tested:
  All foods with hazelnut ingredient were detected positive, all others negative.

Conclusions

- This novel LAMP-based method is highly sensitive and specific for hazelnut.
- Easy-to-use sample preparation, isothermal DNA amplification and visual detection allow for rapid hazelnut screening within 1 hour.
- Rapid verification of the presence of potantially allergenic hazelnut ingredient supports a risk-based precautionary labeling of non-ingredient hazelnut in compound foods.

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References:
[1] Notomi *et al.* (2000), Nucleic Acids Res. 28 (12):I-VII
[2] DLA - Proficiency Tests GmbH (http://www.dla-lvu.de/)

>> www.pei.de